Current Clinical Microbiology Reports (2021) 8:87–102
https://doi.org/10.1007/s40588-021-00166-8

PARASITOLOGY (M BELEN CASSERA, SECTION EDITOR)

Hemozoin: a Complex Molecule with Complex Activities

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Accepted: 23 March 2021 / Published online: 11 April 2021

Abstract

Purpose of Review Malaria is a disease caused by parasites that reside in host red blood cells and use hemoglobin as a nutrient source. Heme released by hemoglobin catabolism is modified by the parasite to produce hemozoin (HZ), which has toxic effects on the host. Experimentation aiming to elucidate how HZ contributes to malaria pathogenesis has utilized different preparations of this molecule, complicating interpretation and comparison of findings. We examine natural synthesis and isolation of HZ and highlight studies that have used multiple preparations, including synthetic forms, in a comparative fashion.

Recent Findings Recent work utilizing sophisticated imaging and detection techniques reveals important molecular characteristics of HZ synthesis and biochemistry. Other recent studies further refine understanding of contributions of HZ to malaria pathogenesis yet highlight the continuing need to characterize HZ preparations and contextualize experimental conditions in the in vivo infection milieu.

Summary This review highlights the necessity of collectively determining what is physiologically relevant HZ. Characterization of isolated natural HZ and use of multiple preparations in each study are recommended with application of in vivo studies whenever possible. Adoption of such practices is expected to improve reproducibility of results and elucidate the myriad of ways that HZ participates in malaria pathogenesis.

Keywords Malaria · Hemozoin · Beta-hematin · Pathogenesis · Methods · Comparative

Introduction

Malaria accounted for >400,000 deaths globally in 2019, primarily in tropical and subtropical regions, specifically sub-Saharan Africa and India [1]. The majority of these deaths were in children under the age of five [1]. Malaria is caused by obligate intracellular *Plasmodium* parasites. Of the six known species of human-infective *Plasmodium*, with *P. simium* being the most recently described [2], *P. falciparum* accounts for most cases in these regions and is considered the most virulent [1, 3].

The earliest symptom of malaria is fever, and additional nonspecific symptoms include chills, sweating, headaches, myalgia, and nausea [3]. Clinical manifestations of infection include anemia due to hemolysis and reduced erythropoiesis [4], renal failure, pulmonary edema, hypoglycemia, disseminated intravascular coagulation, acidemia/acidosis, and jaundice [4, 5]. Infection can progress to organ-specific syndromes such as placental malaria [6] and cerebral malaria [5].

Hemozoin (HZ) is an insoluble, iron-containing waste product of *Plasmodium* produced during the parasite's intraerythrocytic digestion of hemoglobin. As infection progresses, HZ can be observed within several organs and has been correlated with disease severity (reviewed by [7]). While extensive evidence indicates an active role for HZ in infection via interaction with host immunologic receptors [8, 9, 10••], studies have produced varying and sometimes conflicting results. Reasons for lack of reproducibility include the following:

1. Inconsistency of methods used to isolate natural HZ (nHZ). A number of protocols describe isolation of the material from in vitro-cultivated parasites and tissues of

This article is part of the Topical Collection on *Parasitology*

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infected mice with widely varying extraction and washing procedures (Table 1). Many manipulate HZ in ways that could disturb its biochemical nature.
2. Use of synthetic hemozoin (beta-hematin; sHZ) as a proxy for nHZ. The latter is covalently bonded to biomolecules [27•] and contains inorganic ions on its surface which may contribute to its pathogenic properties [28•]. Lack of these molecules on sHZ may contribute to altered interaction with host molecules.
3. Use of membrane-bound HZ in the form of digestive vacuoles (DVs), or residual bodies, that are released upon egress of parasites from the infected red blood cell (iRBC) and are molecularly more complex than free nHZ and sHZ.
4. Use of varying concentrations of HZ preparations that, based on estimates of in vivo concentrations, are often supraphysiological.

Intracellular Lifestyle of *Plasmodium*

Plasmodium spp. are apicomplexan, obligate intracellular parasites that are transmitted from the salivary glands of female *Anopheles* mosquitoes into the human bloodstream during blood meals. Parasites initially travel to the liver and develop into merozoites. Upon release from hepatocytes, *P. falciparum* merozoites infect RBCs of all ages [31]. While entering RBCs, merozoites are enclosed within the cellular plasma membrane, creating a parasitophorous vacuole that evades host defenses [32]. Merozoites develop into trophozoites, which are metabolically active and digest hemoglobin for nutrients [32].

Hemoglobin comprises approximately 95% of the cytosolic proteins within an RBC, of which a trophozoite can degrade 60 to 80% [32]. The uptake of hemoglobin into the PV is mediated by cytostomes, double-membrane invaginations of the PV, and parasite plasma membranes [33] that are trafficked to and lysed within the *P. falciparum* DV. The DV contains unique aspartic and cysteine proteases and supports an acidic pH [33–35]. The digestion of hemoglobin allows anabolism and increases the intraerythrocytic volume available to the parasite. Each trophozoite undergoes schizogony, or asexual reproduction, producing approximately 20 merozoites capable of infecting new RBCs [36].

Natural Synthesis of HZ

Understanding the natural synthesis of HZ is key to exploring the complexity of its interactions with the host upon release from iRBCs. As hemoglobin is digested within the DV, ferric heme is released. Human cells degrade heme using heme oxygenase; however, the *P. falciparum* genome does not encode heme oxygenase [37]. Instead, the parasite dimerizes heme by coordinating the central iron within the porphyrin group of one heme to an oxygen atom in the propionate carboxylate group of a neighboring heme molecule [12]. Dimers are polymerized through hydrogen bonding, producing HZ. Approximately 75% of the heme after hemoglobin digestion is processed into HZ [38]. This suggests that in addition to release of HZ into the host following schizogony, free heme is released. Consistent with this, a number of reports indicated elevated heme in malaria patients in association with severe disease outcomes [39, 40].

Despite a basic understanding of how heme is converted to HZ, the biochemical basis for transformation is still emerging [41••]. Early hypotheses focused on enzymatic and spontaneous synthesis. Slater and Cerami reported that a heme polymerase localized in *P. falciparum* trophozoites produced HZ and that the polymerase was inhibited by antimalarial drugs such as chloroquine and quinine [42]. However, Egan et al. found spontaneous formation of sHZ when hemin was placed in pH 5 at 37°C, conditions similar to those within the DV [43]. Because some antimalarials inhibit sHZ formation, Egan et al. concluded that heme itself was the drug target, not heme polymerase [43]. HZ/drug interactions have become a highly active area of investigation, but discussion thereto is outside the scope of this review.

Subsequent work has supported that HZ production is an active process, requiring the presence of lipids and proteins as catalysts and dimerization scaffolds [44–46]. Jani et al. isolated heme detoxification protein (HDP) from *P. falciparum* and found the gene to be conserved across the genus and recalcitrant to mutation [38]. HDP was shown to traffick to the DV with hemoglobin and to be up to 2000 times more efficient at production of sHZ relative to histidine-rich protein and lipid-mediated production of sHZ [38]. Using X-ray photoelectron spectroscopy (XPS) and MALDI-ToF mass spectrometry, Guerra et al. studied nHZ derived from saponin-lysed RBCs and reported adsorption of peptides. Peptides remained present on nHZ after washing suggesting covalent bonding via carboxylate groups; these peptides may be from HDP and other proteins involved in the biosynthesis of HZ [27•].

New information generated by synchrotron cryo-soft X-ray tomography and atomic element X-ray fluorescence shows that nHZ assembles at the DV membrane in a highly ordered fashion, with some crystals separating and floating free in an aqueous environment, rather than in a lipid droplet, in the organellar lumen. This ordered, membrane-associated assembly produces a square-shaped molecule that relies on HDP-generated hematin dimers that join the growing membrane-bound structure. The square shape differs from the flatter "lath" shape observed with sHZ [41••]. Evidence that lipid droplets may not be required for nHZ formation is interesting, since bioactive lipids are commonly reported to be associated with nHZ [15, 47]. Further work will be required to establish

where such lipids originate. Indeed, given the heterogeneous distribution of HZ in the intraerythrocytic parasite DV, it is tempting to speculate that HZ released into the host upon parasite egress from the iRBC may be present in free and membrane-bound forms, resulting in molecularly distinct nHZ molecules with differing capacities to interact with host receptors.

Release of HZ Upon Parasite Egress From iRBCs

It has been widely assumed that the DV is ruptured during iRBC lysis, and HZ is released as a free crystal. However, seminal findings from Dasari et al. suggested that lysis of each iRBC releases merozoites and one intact DV [18], also referred to as a residual body [7]. BCEF-AM, a fluorescent membrane-permeable intracellular pH indicator, was readily entrapped in DVs from naturally lysed cultures; stability of the staining indicated vacuolar membrane stability, suggesting that free HZ is not initially present after iRBC rupture [19]. The post-release fate of the DV in vivo remains to be fully investigated. Given its suggested role as a "decoy" in malaria pathogenesis that activates the immune system while allowing the parasites to evade the immune response [18], further understanding of the DV is a pressing issue for researchers to address.

Biology and Pathogenesis of HZ

As recently expertly reviewed, the interaction between HZ and the human host is complex, and an extensive literature outlines numerous physiological sites in which HZ sequestration has been observed [7]. Organs with heavy HZ accumulation include the spleen, liver, bone marrow, lungs, brain, and placenta; HZ has also been reported in the kidneys and eyes [48]. The integral presence of iron results in HZ being visualized as a "brown pigment." Ultimately, HZ is engulfed by circulating and tissue resident phagocytic cells and becomes trapped in tissues [7].

Mouse models have been instrumental in defining the impact of malaria on the liver [24]. C57BL/6 mice infected with murine-infective *P. chabaudi chabaudi* AS developed hepatomegaly and exhibited elevated serum levels of glutamate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase, indicative of tissue injury. Positive correlations between concentration of hepatic HZ, which increased steadily during infection, and enzyme levels and gene expression of inflammatory mediators were found. Hepatic inflammation was observed as massive mononuclear infiltrates. Mice directly injected with *P. falciparum*-derived HZ recapitulated dose-dependent upregulation of hepatic inflammatory mediators. The correlation between hepatic HZ concentration and inflammation during infection suggests that long-term persistence of hepatic HZ post-infection may contribute to persistent pathology [24]. HZ sequestration in the spleen and liver following resolution of murine infection was also characterized [49]. Mice sacrificed 180 and 270 days after clearance of parasites showed significantly increased splenic HZ concentrations compared to those in spleens assessed immediately upon resolution of infection (day 0). Interestingly, *P. chabaudi*-infected mice showed splenic concentrations 8 times greater on day 270 relative to day 0, whereas hepatic concentrations decreased by 70%. This suggested that HZ was redistributed from the liver to the spleen following resolution of parasitemia. Long-term persistence of splenic HZ, with movement from the liver over time, was reproduced in mice after infection with *P. berghei* NK65 [50]. Collectively, these results led researchers to speculate that a cycle of liberated and phagocytosed HZ leads to persistence and migration of HZ, the attachment of HZ to different biomolecules, and varying spatiotemporal biological effects [51]. Accordingly, in autopsies of children who died from cerebral malaria (CM), HZ was dispersed in liver lobules in the presence of parasitemia but concentrated in portal areas after parasitemia resolved [52].

HZ plays a role in the development of malaria-associated anemia. Post-mortem inspection of bone marrow biopsies from children who succumbed to malaria showed abundant myeloid and erythroid cells with HZ and abnormal erythroid precursors [14]. The proportion of circulating HZ-laden monocytes and neutrophils, which correlated with the severity of anemia, was directly related to reticulocyte suppression. Growth of CD34+ hematopoietic progenitor cells exposed to HZ derived from *P. falciparum* cultures was inhibited by approximately 50% following exposure to 1 to 10 μg/mL HZ, concentrations that were considered physiologically relevant based on measurements of HZ in plasma from patients with severe malaria [21]. HZ-mediated inhibition of erythropoiesis was attributed to HZ specifically as ablation of the proinflammatory cytokine tumor necrosis factor (TNF)-α did not restore erythropoiesis [14]. In other in vitro assays, HZ induced apoptosis in hematopoietic progenitor cells [53]. A potential mechanism is via HZ-driven generation of reactive oxygen species (ROS), yielding lipid peroxides including 4-hydroxynonenal (4-HNE). Erythroid progenitor cells treated with HZ were reduced in number as ROS levels increased, with the effect mitigated by exposure to antioxidative vitamin E [15].

Lung dysfunction and pathology are commonly observed during malaria in humans [54] and mice (reviewed in [55]) as acute respiratory distress syndrome. A common feature of the pathology is the presence of HZ-bearing phagocytic cells (monocytes and neutrophils) in the lungs. Inflammation, particularly that mediated by CD8+ T cells, is implicated in vascular disruption as part of the pathogenic process and may be directly related to HZ activation of the endothelium [56]. Endothelial disruption in the lung was associated with reduced

endothelial protein C receptor and thrombomodulin expression as well as apoptosis in pneumocytes [57] that was attributable to HZ and interleukin (IL)-1β [58]. Ongoing research is attempting to discern the role not only of HZ in malaria-induced respiratory distress but also of free heme which, like HZ, is a potent pro-oxidant molecule (reviewed in [55]).

The pathogenesis of CM involves sequestration of iRBCs, leukocytes, and platelets in the cerebral vasculature, cytokine-mediated inflammation, dysregulation of coagulation, disruption of endothelial cells, and cerebral edema (reviewed in [59, 60]). Recent studies suggest a direct role for HZ in CM pathogenesis by interacting with neurons and glial cells. Cocultures of glial cells and neurons stimulated with sHZ revealed responses that could contribute to neuroinflammation and neurotoxicity [61, 62]. Additionally, HZ may drive inflammation in CM by engaging the C-type lectin receptor CLEC12A, largely expressed by dendritic cells (DCs), driving effector functions of CD8+ T cells [10••]. HZ was recently found in the choroid plexus of mice infected with *P. berghei* ANKA and may play a role in breaching the blood-cerebrospinal fluid barrier [63]. As in the lung, elevated concentrations of heme during infection may also contribute to pathogenesis by inducing apoptosis of endothelial cells in the brain microvasculature [64].

Tissue accumulation of HZ in humans has been extensively characterized in the placenta. As the organ is easily accessed following parturition, many histological studies have reported HZ accumulation in maternal phagocytic cells and fibrin deposits within the maternal blood space and in the fetal syncytiotrophoblast which faces maternal blood [6]. Accumulation of inflammatory cells, including those bearing HZ, may contribute to poor birth outcomes such as low birth weight [65]. HZ is also associated with coagulation, as indicated by increased placental D-dimer levels, which drives poor birth outcomes in humans and mice [66]. Recent work indirectly connected HZ to inflammasome activation in placental malaria in an NLRP3-, AIM2-, and caspase 1-dependent manner [67], consistent with in vivo and in vitro mouse studies implicating this pathway in immune response to HZ [68, 69]. As with lung pathology, there is increasing evidence that free heme contributes to placental pathology during malaria [70], particularly when present in excess [71]. Interestingly, expression of heme oxygenase-1 positively correlated with HZ load in placentas of *P. chabaudi chabaudi* AS-infected outbred mice [72].

HZ Isolation and Preparation: Impact on Molecular and Functional Characteristics

As briefly summarized above, an extensive literature illustrates that HZ plays critical roles in malaria pathogenesis. To understand how HZ directly affects organs where it sequesters, in vitro studies are required. Toward this end, it is of foremost importance to use HZ that is physiologically representative and accurately simulates interactions with the host in vivo. Use of different preparations of HZ might be appropriate to advance understanding of its role in malaria pathogenesis. However, extreme care in HZ preparation, its use, and data interpretation are essential. Table 1 summarizes published methods for isolating nHZ, which include mechanical separation with sonication, washing, or magnetic-activated cell sorting and/or chemical separation with organic solvents and detergents, proteinases, and nucleases.

sHZ, also referred to as β-hematin, has been used in in vitro studies as an alternative to nHZ. The latter is typically isolated from in vitro-propagated iRBC cultures of *P. falciparum* or from organs, such as the liver and spleen, of infected mice, and is considerably more complicated that production of sHZ. Indeed, use of sHZ is preferred by some groups since it can be obtained commercially or synthesized in the laboratory free of biological or experimentally introduced "contaminants." Although nHZ and sHZ are similar in elemental composition, differences in chemical properties between the two molecules exist, including solubility in basic and aprotic solvents, varying intermolecular hydrogen bonding, different spin states [12], and shape [41••]. Despite the limited molecular complexity of sHZ, its biological effects remain poorly understood as studies report conflicting results. This may be due to slight modifications in the method used to make sHZ (based largely on the methods in [12, 43]), use of variable concentrations of the molecule, and use in differing in vitro and animal models.

Recent comparative studies of nHZ and sHZ illustrate that several molecules adsorb to the surface of HZ due to interactions with parasite and RBC membranes during rupture, natural biomineralization, and/or the process of isolation. Guerra et al. isolated nHZ from *P. falciparum* cultures and processed samples with either proteinase K ("partially" washed; pwHZ) or additionally DNase I and RNase A ("extensively" washed; ewHZ) [27•]. X-ray photoelectron spectroscopy (XPS) revealed that regardless of preparation method, sHZ, pwHZ, and ewHZ all lacked magnesium, phosphorus, and zinc, indicating a lack of nucleic acids. This is important because previous studies reported that nucleic acids associated with nHZ were responsible for immunologic activation of DCs [8]. pwHZ contained silicon and calcium, two elements that could be of plasmodial origin since silicon was found on the surface of dead *Plasmodium* parasites and calcium is necessary for parasite functions. Oxygen and nitrogen spectral analysis suggested biomolecule adsorption on the surface of pwHZ. To investigate what these biomolecules are, sHZ was incubated with parasite schizont lysates, RBC membranes, or selected amino acids or extensively processed as done for ewHZ. All four treatments yielded signals similar to those of pwHZ and ewHZ, suggesting parasites, RBC membranes, amino acids,

or the HZ isolation process itself could all be sources of adsorbed biomolecules [27•].

In subsequent research, Guerra et al. showed with XPS and scanning electron microscopy that an organic coating surrounds the HZ after mild washing with organic solvents. This coating was absent in HZ washed additionally with enzymes and detergents as with the preparation of ewHZ [28•]. In accordance with the previous study [27•], inorganic ions, including sodium, chloride, phosphorus, silicon, and calcium, were present on the surface of HZ samples. As sodium, chloride, and phosphorus were not present on the surface of pwHZ, it was hypothesized that these elements were adsorbed upon HZ emerging from the DV. Since *P. falciparum* debris also contained sodium and chloride, these elements may have originated from the parasite or the solvents used during isolation. In contrast, silicon and calcium were strongly adsorbed to the surface, suggesting they were introduced during biomineralization [28•].

These studies indicate that there are chemical properties that differ between sHZ and nHZ; the HZ isolation process may affect the presence or absence of adsorbed biomolecules that are introduced from parasites, RBC membranes, and/or processing reagents; and inorganic elements present on the surface of nHZ are from biomolecules that likely originate from the parasite. Some groups are interested in preserving the biomolecules adsorbed on the crystalline surface of HZ, while others consider these contaminants. Altogether, what is considered biologically relevant HZ is inconsistent between researchers and the experimental questions being pursued. Critical unresolved questions include the precise nature of how HZ is presented to the host following release from iRBCs during parasite egress, the temporal and molecular dynamics of HZ release from the DV, and the long-term influence HZ has as it circulates and accumulates in host cells and tissues. With regard to DVs, encapsulated contents including HZ, protein and lipids, undigested hemoglobin, and byproducts of hemoglobin degradation including heme are released into the host upon parasite egress from the iRBC. It remains unclear how long the DV remains intact in vivo. The DV membrane attracts complement, facilitating opsonic uptake by neutrophils [18, 19], yet deposition of the complement membrane attack complex presumably mediates disruption of the DV membrane and release of the DV contents into the host.

Consideration of Concentration

There is general acceptance that physiological effects resulting from receptor/ligand interactions is dependent on density of both as well as other factors such as on/off rates. Given this, it can be argued that studies using in vitro simulations of in vivo HZ/host interactions should employ use of HZ at physiologically relevant levels. Context of course is important: the concentration of HZ in a heavily laden phagocyte will certainly far exceed the concentration of "free" HZ in the blood. Schwarzer et al. found that approximately 10 mature trophozoite-bearing iRBCs were phagocytosed by human monocytes in vitro. Assuming 2 fmol of heme, and therefore HZ, per RBC and a monocyte volume of 1 fL, it was estimated that each monocyte, after phagocytosing 10 trophozoite-bearing iRBCs, accumulates 20 fmol of HZ, for a final concentration of 20 M [13, 15]. Toward understanding blood levels of HZ in patients, Keller et al. estimated concentrations in children at various severities of infection by multiplying the concentration of HZ per iRBC (47 fg, based on [73]) and the level of parasitemia [21]. Children with mild malaria were estimated to have 1.90 μg of HZ/mL of blood, and those with severe malaria, 12.90 μg of HZ/mL of blood. Consequently, the concentrations used in in vitro studies with peripheral blood mononuclear cells from children were 10, 1.0, and 0.1 μg/mL of medium. In a similar analysis, Corbett et al. assumed that each trophozoite contains 519 fg of heme (based on [73]); details on the different interpretations of Egan et al. [73] by Keller et al. (estimating HZ concentration per iRBC [21]) and Corbett et al. (estimating heme per iRBC [74]) are not evident. The latter estimated that with a hematocrit of 42% and 1% parasitemia, the resulting HZ concentration in a patient's blood would be 50 μM [74]. Sherry et al. estimated using "standard hematological measurements" that up to 200 μmol of HZ could be released in a 70-kg adult with 1% parasitemia [75]. Using this estimation and the quantitation that 25 μg of sHZ equals 26 nmol of heme, Jaramillo et al. used 25 to 75 μg/mL of sHZ in in vitro experiments with a murine macrophage cell line and 0.2 to 1.5 mg per mouse for in vivo experiments [16, 76]. Notably, estimates used by Sherry et al. and Jaramillo et al., assuming 5 L of blood in a 70 kg adult, yield a concentration of nHZ in blood approximately four times higher (38.46 μg/mL of blood) for 1% parasitemia compared to Keller et al.'s estimation (12.90 μg/mL of blood) for severe malaria in a child [21, 75, 76]. This point illustrates that reported immune effects of sHZ have been observed when notably higher concentrations were used relative to studies that used nHZ at estimated physiologically relevant concentrations. Therefore, reporting concentration of HZ, regardless of preparation, as both a function of culture volume and cellular surface area is recommended, and justification of concentrations should consider in vivo estimates.

Experimental Comparisons of Natural and Synthetic HZ Pathophysiological Effects: a Cautionary Tale

As depicted in Table 1, a number of protocols for nHZ isolation have been reported over the past several decades in studies seeking to understand host-parasite interactions. These

Table 1 Summary of published methods for nHZ isolation

Reference	iRBC source[a] and handling	Parasite disruption/HZ isolation	Endotoxin tested (Y/N)	Purification	Drying/storage
[11]	Lysis with anti-duck erythrocyte serum	French press, centrifuged at 100,000×*g*, sonicated, layered over 1.7 M sucrose, centrifuged at 70,000×*g*, extracted in 0.9 N acetic acid	N/A	Sonicated, centrifuged at 70,000×*g* for 1 h over 1.7 M sucrose solution, pellet extracted with 0.9 N acetic acid	
[12]	Lysis with saponin	Crude HZ made by [11]	N/A	Sonicated, centrifuged at 25,000×*g* for 30 min at 4°C, extracted twice for 3 h in 50 mM Tris-HCl buffer with 2% SDS, washed three times with buffer, digested overnight in 1 mg/mL proteinase E, extracted in 6 M urea for 3 h at 4°C	Centrifuged; washed with dH_2O; lyophilized; dried with phosphorous pentoxide
[13] Used by [14]	iRBCs isolated by Percoll-sorbitol gradient; lysis with 25 volumes of dH_2O for 5 min at 4°C	N/A	N	Centrifuged at 200×*g* for 10 min at room temperature, washed three times with dH_2O	Stored in PBS at −20°C
[15]	Synchronized cultures	HZ isolated using Percoll-mannitol gradient, centrifuged at 5000×*g*, collected at 0/40% interface	N	Washed with 10 mM phosphate buffer/10 mM mannitol	Washed and sonicated in PBS; one volume of human AB+ serum added immediately to suspension in PBS (50% weight/volume); incubated for 30 min at 37°C
[16, 17]	Mild saponin lysis, washed, and purified on 5% BSA cushion	Lyophilized, extracted in chloroform- methanol (2:1 volume/volume), chloroform-methanol-H_2O (10:10:3 volume/volume)	N [17] Y [16]	Dried, resuspended in 100 mM Tris-HCl, 1 mM CaCl2 (pH 7.5), digested with Pronase, extracted in 50 mM sodium phosphate (pH 7.2), 4 M guanidine hydrochloride, 0.5% Triton X-100, stirred overnight at 4°C, centrifuged and washed three times with H_2O and once with 80% 1-propanol, dried	Resuspended in endotoxin free PBS, stored at −20°C
[9]	Lysis with saponin	Sonicated and washed seven to eight times in 2% SDS	Y	Digested with 2 mg/mL proteinase K overnight at 37°C, washed three times with 2% SDS, extracted in 6 M urea for 3 h at room temperature, washed three to five times in 2% SDS, washed with dH_2O	Resuspended in dH_2O; sonicated
[8]	iRBCs isolated with LS column/MACS separator	N/A	Y (not directly measured)	LS column washed with PBS; HZ eluted after removal of magnetic field	Frozen
[18, 19]	Synchronized culture supernatants	Centrifuged at 400×*g* for 5 min to pellet DVs	N	Purification details for DV not available; nHZ retrieved from sonicated DVs followed by purification over Percoll (details not provided)	DVs stored in 50% glycerol at −20°C
[20]	Parasites grown in decomplemented plasma	HZ isolated from culture using Percoll-mannitol gradient,	Y	Washed with 10 mM phosphate buffer	One volume of fresh or decomplemented human

Table 1 (continued)

Reference	iRBC source[a] and handling	Parasite disruption/HZ isolation	Endotoxin tested (Y/N)	Purification	Drying/storage
		collected at 10/40% interface			serum for 30 min at 37°C
[21, 22] Used by [23]	iRBCs treated with 2 mL of saponin and 40 mL of 0.01 M PBS for 10 min	N/A	Y	Washed in PBS and centrifuged at 14,000 rpm for 15 min, repeated four to seven times	Dried, resuspended in filter-sterilized H_2O, sonicated
[24]	*Pf*HZ isolated following *P. berghei* NK65-infected mouse blood method reported in [25]	Homogenized in 50 mM Tris/HCl (pH 8), 5 mM $CaCl_2$, 50 mM NaCl and 1% Triton X-100	Y	Digested with proteinase K overnight at 37°C, sonicated for 1 min, centrifuged at 11,000×*g* for 45 min, washed three times in 100 mM $NaHCO_3$ (pH 9.0) and 2% SDS, sonicated, centrifuged for 30 min, sonicated in 100 mM NaOH, 2% SDS and 3 mM EDTA, centrifuged	
[26]	iRBCs isolated with LS column/MACS separator	N/A	Y	LS column washed with PBS, centrifuged, resuspended in PBS, dispersed with 1 mL syringe, washed with PBS/hypotonic solution	Stored at 4°C
[27•]	Lysis with 0.05% saponin, washed in PBS	Washed in PBS, extracted in CHAPS buffer, suspended in PBS	N/A	*Extensive washing (ewHz):* Extraction with chloroform, methanol, and H_2O, treated with 2mg/mL proteinase K for 18 h at 37°C, washed in 2% SDS, 10 mM Tris-HCl (pH 8); some HZ treated with 100 U/mL DNase I and 1 mg/mL RNase A, washed three times with 2% SDS and three times with purified H_2O *Partial washing (pwHz):* Treated with 2mg/mL proteinase K for 18 h at 37°C, washed three times in 2% SDS, 10 mM Tris-HCl (pH 8), washed three times with purified H_2O	Dried at room temperature, stored in vacuum
[28•]	Cultures processed as in [27•] *OR* splenic macrophages from *P. chabaudi* AS-infected mice	*Pf* HZ processed as in [27•]; macrophages lysed with 0.1% saponin, HZ pelleted	N/A	*Culture lysates:* Washed in PBS *Macrophage lysates:* Washed in PBS, sonicated for 20 s in endotoxin-free PBS with 2% SDS, pelleted, incubated overnight in 2 mg/mL proteinase K, washed three times with 2% SDS, washed with PBS, treated with 50 U/mL DNase I for 1 h at 37°C, heated to 95°C for 10 min, washed twice in PBS, pelleted at 15,000 rpm for 10 min *Both:* Washed with hexane, then dichloromethane, then	Dried at room temperature, stored in vacuum

Table 1 (continued)

Reference	iRBC source[a] and handling	Parasite disruption/HZ isolation	Endotoxin tested (Y/N)	Purification	Drying/storage
				hexane	
[29•]	Culture supernatant	DV purified over step Percoll-6% mannitol gradient, collected at 10/40%, passed through 27-gauge needle	N	Washed in 2 mM $MgSO_4$ (pH 7.4), 100 mM KCl, 25 mM Hepes, 25 mM $NaHCO_3$, 5 mM Na_3PO_4	
[30••]	Liver and spleen from mice with resolved *P. yoelii* 17XNL infection homogenized in dH_2O *OR* lysed in 0.2% IGPAL; *Pf*HZ from synchronized cultures incubated with 5 U/mL DNase for 12–15 h	*Py*HZ 100 micron filtered, isolated on 40/80% Percoll gradient at 5000×*g* for 20 min at 4°C, collected from 40/80% interphase and pellet, washed twice with dH_2O, suspended in saline; *Pf*HZ isolated on discontinuous Percoll gradient, collected at 25/40% interface	Y	Some *Py*HZ washed three to four times in 2% SDS, incubated overnight in 10 mM Tris-HCl (pH 8.0), 0.5% SDS, 1 mM $CaCl_2$, and 2 mg/mL proteinase K at 37°C, washed in 2% SDS, incubated for 3 h at 37°C in 6 M urea, washed in PBS; some *Py*HZ further incubated in 100 U/mL DNase at 37°C, 1mM $CaCl_2$, 2 mM $MgCl_2$ for 2 h, washed twice in 2 mM mannitol-containing phosphate buffer; *Pf*HZ incubated with 40 U/mL DNase, 2 mM $MgCl_2$, 1 mM $CaCl_2$ in PBS for 1 h at 37°C, washed several times in 10 mM phosphate buffer (pH 8.0) containing 2 mM mannitol	Suspended in sterile PBS *OR* saline

[a] All parasites derived from in vitro cultures of *P. falciparum* unless otherwise noted

Abbreviations: *N/A* not applicable, *dH_2O* distilled water, *PBS* phosphate-buffered saline, *SDS* sodium dodecyl sulfate, *s* seconds, *min* minutes, *h* hours

methodological variations combined with the use of various preparations of sHZ have yielded contradictory interpretations of the impact HZ has on host cellular functions. Perhaps the most significant outstanding questions are 1) the extent to which HZ itself, either devoid of, or independent of associated parasite and/or host DNA, protein, or lipids, induces or influences host responses, and 2) the role of these associated molecules in mediating host responses. In this section, we discuss several examples that directly or indirectly address these questions. Table 2 summarizes methodological details and findings from these works. The reader is directed to other recent reviews that discuss key studies of host-HZ interactions that we are unable to cover here [7, 78].

DNA and HZ: to Be or Not to Be

A classic example typifying how preparation of HZ can influence experimental results is the characterization of HZ-mediated activation of TLR9, a Toll-like receptor with specificity for unmethylated CpG motifs in DNA [79]. Coban et al. found that DCs exposed to DNase-treated nHZ dose-dependently produced cytokines and chemokines comparable to levels produced by DCs stimulated by positive control CpG oligodeoxynucleotide [9]. Supporting the hypothesis that HZ signals through TLR9, murine-derived $TLR9^{-/-}$ and $MyD88^{-/-}$ DCs exposed to nHZ exhibited a decreased secretory response. In vitro findings were extended to an in vivo investigation in mice treated with nHZ or sHZ intraperitoneally. Wild-type mice showed elevated cytokine and chemokine levels, while $TLR9^{-/-}$ and $MyD88^{-/-}$ mice did not. nHZ preparations were deemed pure as ethidium bromide-stained agarose gels did not show detectable DNA or RNA. In vivo results were recapitulated with sHZ, further suggesting that HZ itself was the activating ligand of TLR9. Moreover, isolated *P. falciparum* DNA and RNA did not induce cytokine production [9].

Subsequent work using comparable experimental approaches by Parroche et al. suggested the contrary: HZ itself was inert and instead facilitated phagocytosis that allowed adsorbed DNA to interact with intracellular TLR9 [8]. When

Table 2 Summary of discussed comparative studies of HZ/host interactions

Reference	HZ preparation method	Concentration	Reason for concentration	Cell/model	Major findings relevant to this review
[13]	nHZ: In Table 1	115 μg nHZ/well Corresponding to 30 nmol heme/well 50,000 monocytes/well	Not specified	Human PBMC	- Monocytes that phagocytosed nHZ initially exhibited prolonged oxidative burst and then impaired PMA-elicited oxidative burst - Stimulation with iron-stripped nHZ exhibited PMA-elicited oxidative burst, suggesting iron may be responsible for impairment
[15]	nHZ: In Table 1	100 fmol heme/monocyte Corresponding to 50 RBC/monocyte	Not specified	Human PBMC	nHZ induced production of esterified monohydroxy derivatives of polyenoic acids in a complex isomeric pattern, suggesting nonenzymatic oxidation
[17]	sHZ: [43] nHZ: In Table 1	10–50 μg/mL	Not specified	Murine MΦ cell line B10R	nHZ and sHZ alone did not lead to NO generation, while the presence of IFN-γ led to inducible nitrate oxide synthase and NO production
[16]	sHZ: [43] nHZ: In Table 1	10–75 μg/mL	Specified	Murine MΦ cell line B10R	nHZ and sHZ induced ROS generation, predominantly superoxide anion, and chemokines
[9]	sHZ: [43] nHZ: In Table 1	In vitro: 30 or 100 μM In vivo: 1500 μg HZ	Specified	Murine Flt3 ligand–induced BMDCs; Wild-type (WT) C57BL/6 or mutant mice ($MyD88^{-/-}$, $TRIF^{-/-}$, $TLR2^{-/-}$, $TLR4^{-/-}$, $TLR7^{-/-}$, and $TLR9^{-/-}$)	- nHZ increased levels of chemokines and cytokines, while $MyD88^{-/-}$ and $TLR9^{-/-}$ DCs showed decreased production - WT mice injected with nHZ or sHZ exhibited increased MCP-1 and IL-6, while no increases were seen with $MyD88^{-/-}$ and $TLR9^{-/-}$ mice
[8]	sHZ: [43] nHZ: In Table 1	50 μM nHZ 150 μM sHZ	Not specified	Murine Flt3 ligand–induced BMDC	- Crude bovine sHZ but not pure sHZ induced TNF-α secretion - DNase and nuclease treatment abolished previously observed immune effects, suggesting DNA, not HZ, mediated effects
[18]	DV: In Table 1	5×10^9 DVs	Not specified	Male Sprague-Dawley outbred rats	- DV membrane, but not merozoites or nHZ, supported the alternative complement pathway - Injected DVs induced complement consumption in vivo and accumulated within splenic phagocytes and within phagocytes in the vasculature of the lung
[19]	DV: In Table 1	*Phagocytosis*: 3-5 DVs/neutrophil *ROS generation and bacterial killing assay*: 2-3 DVs/5 neutrophils	Not specified	Healthy human neutrophils, in some experiments + *Staphylococcus aureus*	- Complement opsonized DVs, but not iRBCs, merozoites, or free nHZ, were readily phagocytosed by neutrophils and rapidly drove oxidative burst - Subsequent responses to *S. aureus* were blunted with reduced oxidative burst and bacterial killing
[20]	sHZ: [12] nHZ: In Table 1	100 fmol heme/monocyte Corresponding to 50 RBC/monocyte	Not specified	Human PBMC	- nHZ and sHZ bound to fibrinogen - Fibrinogen-HZ complexes interacted with TLR4 and CD11b/CD18, leading to ROS production
[23]	sHZ: [12] nHZ: In Table 1	10 μg/mL	Specified	Primary human ST	nHZ, but not sHZ, increased ERK1/2 phosphorylation, chemokine, and cytokine secretion, increased ICAM-1 expression, and monocyte migration
[26]		25-100 μM	Specified	WT C57/B6 BDMCs	

Table 2 (continued)

Reference	HZ preparation method	Concentration	Reason for concentration	Cell/model	Major findings relevant to this review
	nHZ: In Table 1				- Lower concentrations of HZ in the presence of IFN-γ led to NO production, while higher concentrations led to dose-dependent decrease - Decreased NO production was reversed by replenishing culture medium or supplementing with L-arginine
[61]	sHZ: [12, 75]	0.5, 0.8, 2.5, 4.0, 10, 12.5 and 20 nmol/mL	Specified	Human fetal neurons and astrocytes	Astrocytes and neurons phagocytosed nHZ and exhibited signs of apoptosis
[62]	sHZ: purchased from InvivoGen	200 and 400 μg/mL	Not specified	BV-2 murine microglial cell line ReNcell VM cell line	sHZ caused significant increases in proinflammatory cytokines, caspase activity, NF-κB and NLRP3 pathway activity, microglial-induced neurotoxicity, and direct neurotoxicity
[29•]	DV: In Table 1	Not specified	Not specified	Human neutrophils	NETosis is stimulated by heme but not DVs
[10••]	sHZ purchased from InvivoGen	*ROS*: 100 μg/ml *DC-T cell coculture assay*: 100, 50, 20 μg/ml	Not specified	CLEC12A$^{-/-}$ and WT C57/B6 BDMCs; CLEC12A$^{-/-}$ and WT C57L/B6 mice; CD8+ T cells from OT-I transgenic mice	- sHZ caused CLEC12A$^{-/-}$ BMDCs to produce less ROS, suggesting CLEC12A has a role in signaling for ROS production - Culturing CD8+ T cells with CLEC12A$^{-/-}$ BMDCs stimulated with sHZ abrogated effector functions - CLEC12A$^{-/-}$ mice that developed CM had higher survival rates
[77]	sHZ purchased from InvivoGen	50 and 100 ug/mL	Not specified	Human neutrophils	- sHZ was readily phagocytosed by neutrophils and induced intracellular morphological changes, but NETosis was not induced - Fibrinogen impeded sHZ uptake whereas platelets facilitated phagocytosis
[30••]	nHZ: In Table 1	In vitro: *P. yoelii* nHZ, 30 ug/mL In vivo: *P. yoelii* nHZ, 1 mg, *P. falciparum* nHZ, 1-1.5 mg	Not specified	In vitro: Murine splenic phagocytes + *L. monocytogenes* In vivo: Mice treated with *Py* nHZ then orally challenged with *L. monocytogenes* after 7 days; mice treated with *Pf* nHZ then intranasally challenged with *S. pneumoniae* after 7 days	- *Py* nHZ impaired phagocytic clearance of *L. monocytogenes* - As with natural infection, *Py* and *Pf* nHz impaired splenic clearance of *L. monocytogenes* but not *S. pneumoniae* - Effect of nHZ was independent of DNA but dependent on nHZ-associated proteins and/or lipids

treated with nHZ, wild-type murine DCs produced IL-12p40, and, similar to Coban et al. [9], TLR9$^{-/-}$ and MyD88$^{-/-}$ DCs exhibited reduced responses. However, treatment with DNase I, the same enzyme used in Coban et al. [9], abolished stimulatory activity, suggesting nHZ was not intrinsically activating. Parroche et al. further used two sources of hemin to produce sHZ. The first preparation was from bovine hemin that was considered "crude" as it consisted of only 65% hemin according to LC-MS analysis. Stimulation of DCs with this sHZ induced a TNF-α response. The same cultures treated with HPLC-purified sHZ, devoid of detectable contaminants on LC-MS, did not. Thus, stimulatory activity from the first sHZ preparation was concluded to be from contaminating DNA, highlighting the need to analyze sHZ with highly sensitive techniques. Finally, although these authors observed that isolated *P. falciparum* DNA did not stimulate DCs, DNA mixed with DOTAP, a transfection agent, did [8].

HZ and Innate Immune Function: Oxidative Burst

nHZ is readily phagocytosed by human monocytes [13, 26]. Schwarzer et al. observed that phagocytosis of nHZ caused a

robust, long-lasting oxidative burst, although subsequent phorbol myristate acetate (PMA)-elicited burst was impaired, suggesting cell exhaustion [13]. Uptake of nHZ increased intracellular lipid peroxide levels [13], including potentially cytotoxic 4-HNE [80] and esterified monohydroxy derivatives of polyenoic acids [15]. These phenomena were hypothesized to be driven by iron from HZ as conditions mimicking those of macrophage phagolysosomes led to iron release [13]. Removal of labile iron from nHZ mitigated suppression of subsequent PMA-elicited oxidative burst [13]. Jaramillo et al. similarly found that both nHZ and sHZ phagocytosed by murine macrophages generated ROS, predominantly superoxide anion, and induced chemokine gene expression [16]. Thus, molecules associated with nHZ may not be required to drive oxidative burst in host monocytes and macrophages following phagocytosis.

Recent evidence points to participation of HZ in additional mechanisms of ROS production and in cell types other than monocytes and macrophages. Raulf et al. reported that CLEC12A, expressed by DCs and a receptor of uric acid, interacted with sHZ and acted as an inhibitory receptor [10••]. As in monocytes [13], DCs stimulated with sHZ and later PMA exhibited impaired ROS production. This impairment was more pronounced in CLEC12A$^{-/-}$ DCs, suggesting a role for CLEC12A in ROS production induced by the naked HZ crystal [10••].

While nHZ and sHZ alone did not lead to nitric oxide (NO) production, Jaramillo et al. found that the presence of interferon (IFN)-γ elicited increased inducible nitric oxide synthase levels and NO production by murine macrophages via the ERK1/2 and NF-κβ pathways [17]. However, in contrast to the interpretation of HZ-driven ROS production by Schwarzer et al. [13], these authors did not attribute observations to iron in HZ, as the presence of deferoxamine, an iron chelator, did not abrogate NO production [17]. More recently, Corbett et al. found that IFN-γ-primed murine bone marrow-derived macrophages readily phagocytosed nHZ and led to NO production [26]. However, NO production decreased dose-dependently with higher concentrations of nHZ (50 and 100 μM), which was reversed through addition of fresh culture medium or L-arginine, the precursor to NO. The authors concluded that HZ may act as a sink for this amino acid, thereby reducing availability for synthesis of NO [26].

Importantly, Barrera et al. reported that HZ can participate in ROS production prior to phagocytic uptake by monocytes [20]. The mechanistic basis for this was attributed to rapid and stable association of nHZ (and sHZ) with fibrinogen. The nHZ-fibrinogen complex interacted with TLR4 and CD11b/CD18, leading to robust production of ROS in monocytes. Lipoperoxidation products including 15(S, R)-hydroxy-6,8,11,13-eicosatetraenoic acid and 4-HNE were produced. As isolated fibrinogen did not induce immune effects, it was hypothesized that nHZ promoted conformational changes to fibrinogen to mediate its interaction with the cell-activating receptors [20]. The ability of a sHZ-fibrinogen complex to drive cell activation was not tested in this work; thus, the extent to which biomolecules inherent to nHZ potentiate cell surface receptor-mediated responses remains unknown.

Impact of HZ on respiratory burst has also been explored in neutrophils. Dasari et al. approached this problem using DVs isolated from *P. falciparum*. The DV membrane attracted complement [18], facilitating phagocytic uptake by neutrophils [19]. nHZ released from sonicated DVs, mature parasite-containing iRBCs, and merozoites were not taken up by neutrophils, but addition of immune serum from malaria patients enhanced phagocytosis of merozoites. While DV uptake induced rapid ROS production, it blunted subsequent oxidative burst induced by *Staphylococcus aureus* and ability of the neutrophils to kill the bacteria [19]. *In vivo* studies with rats intravenously injected with DVs provided preliminary evidence that uptake by splenic macrophages and circulating neutrophils within the lung vasculature occurred within 4 to 6 h [18], but addition studies are needed to confirm this and provide details on the in vivo fate of DVs. More recently, Harding et al. assessed the impact of nHZ on splenic phagocytes, confirming induction of oxidative burst and exhaustion with reduced ability to control bacterial growth both in vitro and in vivo ([30••]; see additional discussion below). In contrast to Dasari et al., who found that nHZ released from sonicated DVs and then purified by Percoll gradient was not phagocytosed by neutrophils [19], these authors showed nHZ within neutrophils ex vivo 2 h following intravenous injection [30••]. Further experiments with head-to-head comparison of DVs and nHZ will be required to discern the key molecules associated with nHZ that drive respiratory burst in neutrophils.

HZ and Innate Immune Function: NETosis

HZ accumulation in leukocytes, particularly neutrophils, is linked to malaria severity and poor outcomes in children [81] and pregnant women [82]. Neutrophils are capable of killing parasites in vitro [83, 84], and a role for neutrophil extracellular traps (NETs), which contain decondensed chromatin and vesicular contents such as neutrophil elastase, in malaria is also emerging. While parasite killing and NETosis may be protective for the host, evidence from mouse models and human studies implicates NETosis as an important contributor to pathogenesis [85–87]. Aiming to understand the molecular basis for parasite-driven NETosis, Knackstedt et al. assessed the conditions under which TNF-α-primed, healthy human neutrophils would undergo NETosis [29•]. Interestingly, exposure to heme and plasma from severe malaria patients with elevated heme content elicited NET production by healthy human neutrophils, whereas DVs, iRBCs, and free merozoites did not. Oxidative burst induced by DVs, as

observed by Dasari et al. [19], was not reported, but NETosis induced by heme was related to this response. The extent to which the heme was internalized in the cells was not shown. A key difference in this work compared to Dasari et al. [19] was the brief pre-exposure of the cells to TNF-α; differences in ratio of DVs to cells cannot be assessed as this was not specified. In a related study, Lautenschlager et al. showed that while readily ingested by human neutrophils, sHZ did not promote NETosis [77]. sHZ concentration used was as high as 100 μg/mL, which may be supraphysiologic based on estimates of in vivo concentrations [21, 75]. Under these conditions, intracellular morphological changes including heterochromatin changes and nuclear enlargement were induced. Interestingly, exposure of sHZ to plasma proteins, including fibrinogen, inhibited uptake and morphological changes in these cells. This is in contrast to the observation that sHZ, nHZ, and DVs readily associated with fibrinogen in plasma, facilitating pre-phagocytic activation of monocytes [20].

HZ in the Pathophysiology of Placental Malaria and Cerebral Malaria

nHZ elicited distinct immune responses in primary syncytiotrophoblast, the outermost monolayer of the fetal placenta that is exposed to parasites and parasitic materials in placental malaria [23]. Lucchi et al. compared the responses of syncytiotrophoblast when stimulated with physiological concentrations of nHZ and sHZ [21, 22]. Only nHZ stimulation resulted in increased ERK1/2 phosphorylation, whereas both nHZ and sHZ induced activated JNK1/2. Additionally, only nHZ stimulated cytokine and chemokine secretion, release of soluble intercellular adhesion molecule-1, activation of conditioned medium-primed primary human monocytes, and chemotaxis of peripheral blood mononuclear cells toward syncytium [23]. The direct comparison between nHZ and sHZ with all other experimental conditions held constant illustrates that the variability in immune responses in syncytiotrophoblast is explained by differing properties of the HZ preparations themselves.

Expanded understanding of the role of HZ in CM pathogenesis has been recently achieved using in vitro astrocytic, neuronal, and microglial models exposed to sHZ. Astrocytes and neurons phagocytosed sHZ in a dose-dependent and time-dependent manner, and cocultures of these cells with sHZ showed morphological changes such as blebbing and ultimately apoptosis [61]. In similar work, microglial cells exposed to sHZ produced significantly elevated levels of proinflammatory cytokines, including pro-IL-1β/IL-1β, and nitrite and showed elevated caspase-1 activity and NF-κB p65 phosphorylation. Inhibition of the NF-κB and NLRP3 inflammasome pathways resulted in decreased levels of neuroinflammation. Neurons cocultured with sHZ-stimulated microglial cells or directly exposed to sHZ had reduced viability as well as increased caspase-6 activity and ROS generation [62]. Both of these studies used sHZ exclusively, and arguably supraphysiological concentrations of sHZ were employed. The extent to which nHZ or DVs mediates or alters responses of these cells remains to be tested.

Approaching CM from the perspective of DC function and CD8+ T cell activation, Raulf et al. studied the interaction between the C-type lectin receptor, CLEC12A, on DCs and sHZ [10••]. DCs exposed to sHZ exhibited impaired PMA-elicited ROS production, an effect exacerbated in CLEC12A$^{-/-}$ DCs, but pro-inflammatory cytokine and activation marker expression were not affected by the presence or absence of CLEC12A. Given the oft-cited role for activated CD8+ T cells in driving experimental CM [88–90], activation of CD8+ T cells exposed to intact and CLEC12A$^{-/-}$ sHZ-stimulated DCs was assessed. Lack of CLEC12A on DCs attenuated proinflammatory and cytotoxic molecule secretion by CD8+ T cells. To test whether this effect could be mediated by natural infection and nHZ, wildtype and CLEC12A$^{-/-}$ mice were infected with *P. berghei* ANKA. While both groups of mice produced similar systemic levels of cytokines, developed comparable parasitemia, and showed equivalent T cell sequestration in the brain, CLEC12A$^{-/-}$ mice had significantly improved survival and reduced granzyme B-expressing T cells compared to wild-type mice. Overall, the study provided compelling evidence that HZ, regardless of associated molecules, can impact CD8+ T cell activity via interaction with CLEC12A-expressing DCs, thereby driving the development of experimental CM [10••].

A Role for HZ in Immune Protection Against Co-infection

Particularly in children, bacterial co-infection in malaria is an important cause of morbidity and mortality [91]. Although understanding of the underlying mechanisms of how malaria infection predisposes patients to more severe bacterial disease is advancing [92, 93], questions remain. Recent work by Harding et al. established that nHZ mediated immune suppression even after resolution of parasitemia, facilitating growth, and dissemination of non-Typhoid *Salmonella, Listeria monocytogenes*, and *Streptococcus pneumoniae* in *P. yoelii* 17XNL-infected mice [30••]. Importantly, isolated nHZ from the spleen and liver of *P. yoelii*-infected mice, as well as nHZ isolated from cultured *P. falciparum*, recapitulated infection-associated facilitation of bacterial growth, while in vivo exposure to sHZ had no impact on subsequent exposure to *L. monocytogenes.* The nHZ isolation protocol was based on that reported by Barrera et al., which preserves HZ-associated proteins and lipids [20]. Preparation of nHZ in the presence of DNase confirmed that the effect was independent of DNA contamination, but treatment with detergents to dissolve lipids and proteases abrogated the immunosuppressive effect. These

authors concluded that lipids associated with nHZ mediated the immunosuppressive effect by suppressing neutrophil oxidative burst; changes in splenic macrophage populations could not explain the phenotype [30••].

Conclusions

Accumulating evidence suggests that rather than being a simple inert crystal, nHZ becomes associated with inorganic elements and organic molecules such as proteins and lipids during its biogenesis as well as during release from the iRBC. The precise timing of these associations and the conditions that influence them are key questions for future work to address. While we await clarification on persistent unknowns such as the dynamics of HZ release from the DV in vivo and biochemical state of HZ upon this emergence, it is important for researchers to contextualize their work within the extensive and growing literature that points to numerous molecular interactions between HZ, and its associated components, and host cells. This is especially critical given that responsiveness of different cell types to HZ is not universal. At best, researchers aiming to strengthen interpretation of findings and further elucidate how HZ influences the host should conduct comparative studies using various preparations of HZ, including sHZ, nHZ and DVs, with well-rationalized culture conditions such as HZ concentrations, and couple in vitro and ex vivo work with in vivo studies.

Declarations

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest The authors declare that they have no competing conflicts of interests.

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28.• Guerra ED, et al. Inorganic ions on hemozoin surface provide a glimpse into Plasmodium biology. J Inorg Biochem. 2019;200:110808 **X-ray photoelectron spectroscopy, scanning electron microscopy, and energy-dispersive X-ray spectroscopy are used to characterize elemental components on the surface of HZ from cultured P. falciparum and macrophages from P. chabaudi-infected mice. Ca and Si adhere strongly to the surface of HZ and may be introduced during crystal formation. Na, Cl, and P adhere weakly, and may be introduced during exit of HZ from the iRBC.**
29.• Knackstedt SL, et al. Neutrophil extracellular traps drive inflammatory pathogenesis in malaria. Sci Immunol. 2019;4(40):eaaw0336 **This work proposes that it is heme and not DVs, iRBCs or merozoites that drive NETosis in malaria. Also demonstrated are NET-driven intercellular adhesion molecule-1 endothelial upregulation and production of granulocyte colony-stimulating factor as mediators of inflammation.**
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