

The Malarial Metabolite Hemozoin and Its Potential Use as a Vaccine Adjuvant

Cevayir Coban^{1,2}, Masanori Yagi³, Keiichi Ohata^{1,4}, Yoshikatsu Igari⁴, Toshihiro Tsukui⁴, Toshihiro Horii³, Ken J Ishii^{1,3} and Shizuo Akira¹

ABSTRACT

Hemozoin, a bio-crystalline substance, is a hemin detoxification by-product of malaria parasites. The role of hemozoin crystals in host immune system modulation by malaria parasites, and how they interact with the immune system has been enigmatic. Here, we summarize recent progress in our understanding of how hemozoin might be interacting with the host immune system. In particular, the potential application of hemozoin crystals as an adjuvant may provide insights into the molecular mechanisms involved in immune responses to malarial infection and provide a rationale for the design of vaccines against malaria as well as other immunological disorders such as allergies.

KEY WORDS

adjuvant, allergy, hemozoin, innate and adaptive immunity, malaria, NLRP3, TLR9

INTRODUCTION

Vaccination has been used effectively for protection from, and treatment of, not only infectious diseases, but also non-infectious diseases such as cancer and allergies. A vaccine is comprised of two components: an antigen and an adjuvant. Antigens are usually pathogen-derived and mostly protein nature and successful vaccines usually elicit adequate humoral and/or cellular immune responses. An adjuvant is a compound that can either promote, or modulate, vaccine immunogenicity.¹ Without an adjuvant, it is not possible to induce optimal immune responses, referred to as the 'immunologist's dirty little secret' by Charles Janeway Jr.²

Although little is known about how adjuvants work, every successful vaccine contains an adjuvant, whether externally introduced, or endogenously "built-in". Recent advances in immunology research have shown that innate immune system receptors recognize molecular patterns associated with pathogens (PAMPs), which are often used as vaccine adjuvants.³ These receptors are localized in, or on, the

surface of many immune cells as well as non-immune cells. Toll-like receptors (TLRs) are located on the cell membrane or in the endosome, whereas RIG-I like receptors (RLRs) and NOD-like receptors (NLRs) are expressed in the cytosol. Many ligands, such as modified lipid-A (the TLR4 ligand), poly-IC (synthetic dsRNA, the TLR3 ligand), CpG oligodeoxynucleotides (synthetic ssDNA, the TLR9 ligand), and peptidoglycan (the NOD-like Receptor ligand), are known to act as potent adjuvants. These vaccine adjuvants can directly target antigen presenting cells (APCs), such as dendritic cells (DCs), thereby leading to the subsequent triggering of adaptive immune responses.⁴

Despite the need for better adjuvants, there are very few clinically approved adjuvants for human use (e.g. aluminum salts or monophosphoryl lipid A [MPL]) due to safety concerns, such as unwanted side-effects. Because the evaluation of new adjuvants is both time and energy consuming, basic research should focus on finding better, safer and more potent adjuvants for clinical use. In this review article, we focus on hemozoin, a malarial metabolite, as a novel

¹Laboratory of Host Defense, ²Laboratory of Malaria Immunology, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University, ³Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Osaka and ⁴ZENOAG, Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan.

Correspondence: Cevayir Coban, Laboratory of Malaria Immunol-

ogy, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.

Email: ccoban@biken.osaka-u.ac.jp

Received 18 February 2010.

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vaccine adjuvant. We discuss how it was found to be an adjuvant, and its underlying mechanism of action. We also discuss recent progress in the preclinical development of this hemozoin adjuvant for use in an anti-mite allergy vaccine against canine atopic dermatitis.

HEMOZOIN: A MALARIAL METABOLITE

Malaria is a disease caused by *Plasmodium* parasites, and has a history as old as that of the human race. According to the 2008 report of the World Health Organization (WHO), half of the world's population is still at risk of malaria; in 2006, 250 million cases were encountered with approximately 1 million deaths (WHO, World Malaria Report, 2008 at <http://apps.who.int/malaria/wmr2008/>). It has been almost 130 years since the microscopic identification of the malaria parasite and its complex life cycle through transmission by the *Anopheles* mosquito. A dark-brown pigment called hemozoin was the first noticed in the *Plasmodium*-infected blood of both patients and mosquitoes.⁵ As the parasite multiplies in the erythrocytes of its vertebrate host, hemozoin is continuously produced and released together with the merozoites, and engulfed by macrophages, monocytes, neutrophils and other immune cells such as DCs.⁶ Hemozoin is also carried into mosquitoes through the mosquito blood meal, either inside infected erythrocytes, or in leukocytes, (Fig. 1).⁷

Hemozoin is a metabolic byproduct of heme molecules, which are digested by the *Plasmodium* parasites. During its life in erythrocytes, the parasite uses the host-hemoglobin for its vital needs, such as amino acids and iron in which the free-heme (Fe²⁺-protoporphyrin IX) is detoxified by converting it into insoluble crystals. The conversion process from heme to hemozoin crystals is thought to be a biocrystallization process that takes place in the digestive vacuole of the parasites (pH < 6) residing within the erythrocytes (Fig. 2).⁸ The hemozoin bio-crystals are identical to synthetic β -hematin crystals (sHZ), which can be chemically synthesized from hemin chloride under acidic conditions. β -hematin crystals are composed of cyclic heme dimers integrated into each other through hydrogen bonds in which the dimeric heme consists of two heme molecules covalently linked through a reciprocal ferric iron (Fe³⁺) (Fig. 2b).⁹ Both synthetic (β -hematin) and natural hemozoin crystals have a brick-like shape, with approximate sizes of 50-500 nm (Fig. 2a, 2c). They also have similar infrared spectra and X-ray powder diffraction patterns.^{10,11} It should be noted, however, that the purification protocols (possibly using different solvents and temperatures) used to synthesize β -hematin crystals under laboratory conditions can affect the size, shape and faces of the crystals (Table 1).^{10,12} Not surprisingly, several β -hematin preparation protocols have been used to make different sizes

and shapes of crystals.¹³⁻¹⁵ Contrary to popular belief, this is a very important difference between synthetic and natural hemozoin; that the size and shapes of synthesized hemozoin crystals may not be identical, depending on the purification method (Table 1). While natural hemozoin is composed of smaller, evenly distributed crystals between 50-500 nm in size, β -hematin crystals range from 50 nm to 20 μ m (Table 1 and¹³). Moreover, β -hematin crystals in an aqueous medium tend to aggregate even within minutes of vigorous sonication (unpublished observations). This is an important point to consider as different sized hemozoin crystals may possess variable immunomodulatory properties.

INVOLVEMENT OF THE INNATE IMMUNE SYSTEM IN RESPONSES TO HEMOZOIN

During the repetitive life cycle of the malaria parasites in the blood, hemozoin continuously ruptures from erythrocytes, together with the merozoites and other parasite products, and accumulates in the reticuloendothelial system (i.e. macrophages, leukocytes and tissues such as the liver and spleen) (Fig. 1). It has been shown in both mouse and human malaria, that the accumulation of hemozoin in leukocytes and organs such as the spleen, liver or brain is well correlated with disease severity.¹⁶⁻¹⁸ Furthermore, it has been suggested that free hemozoin leads to the continuous targeting of the host innate immune system, leading to both pro- and anti-inflammatory responses. Accordingly, hemozoin continuously activates macrophages and DCs to produce pro-inflammatory cytokines and chemokines such as IL6, TNF α , IL12, MCP-1 and IL8, and certain anti-inflammatory cytokines and chemokines such as IL-10 and MIF.¹⁹ Of note, similar to observations in mammalian cells, hemozoin was found to induce NO synthase expression in mosquito tissues and cells via multiple signaling pathways (Fig. 1).⁷

Recently, many researchers have analyzed molecules from *Plasmodium* parasites that may activate the innate immune system. Both TLR2 and TLR9 have been shown to mediate innate immune system activation by GPI and hemozoin derived from *P. falciparum*.²⁰⁻²³ Also it was recently suggested that uric acid is released during malarial infections, presumably activating the innate immune system via NLRs.²⁴ This finding is supported by very recent reports that have shown that synthetic hemozoin (β -hematin) crystals directly stimulate both mice bone marrow-derived macrophages and human monocyte cell lines through the NLRP3 (also known as NALP3) inflammasome complex, leading to caspase-1 activation.²⁵⁻²⁷

TLR9 OR NLRP3?

Is hemozoin a ligand for TLR9, NLRP3 or both? Hemozoin purified from *P. falciparum* (or its synthetic version, β -hematin) has been reported to be a

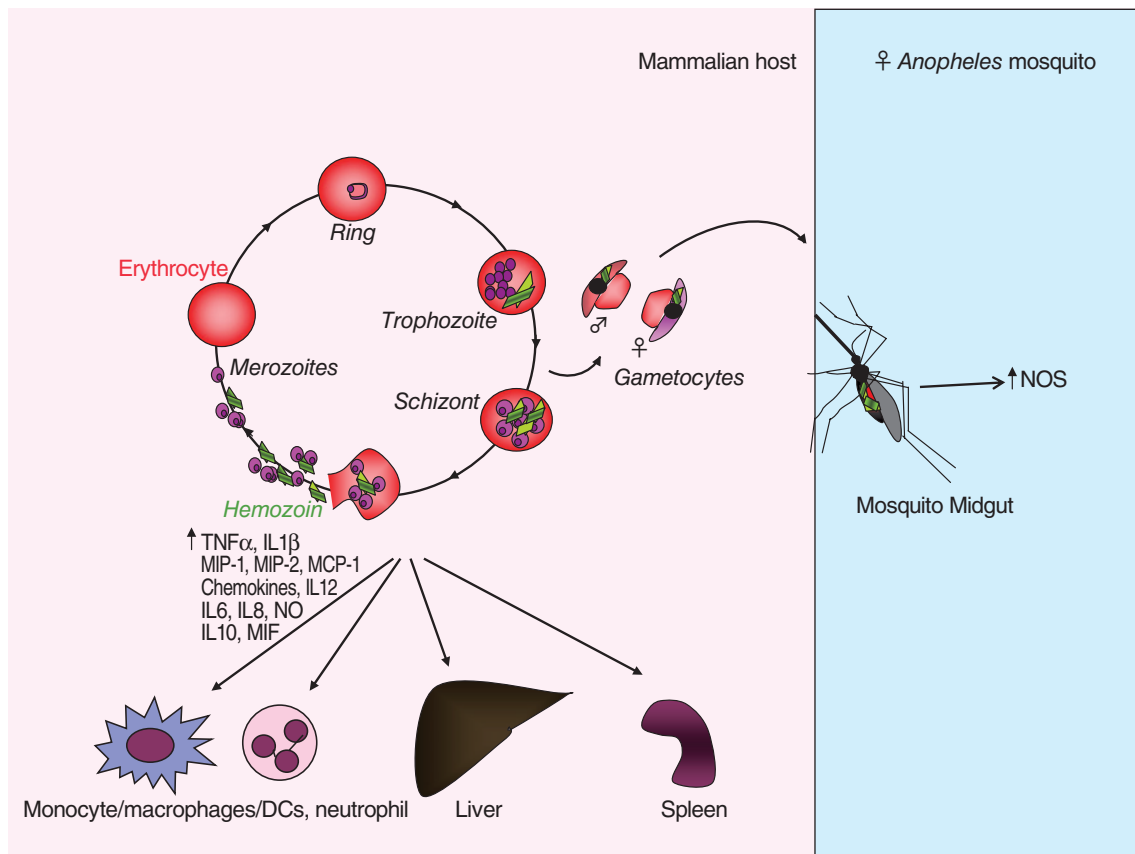


Fig. 1 Hemozoin in mammalian host circulation and in blood meal of mosquitoes. Infected female *Anopheles* mosquitoes transmit *Plasmodium* parasites into a vertebrate host through their bites during a blood meal. The repetitive life cycle within the erythrocytes of the mammalian host (erythrocytic blood-stage life cycle of *Plasmodium*) is responsible for the clinical symptoms of malaria, such as fever attacks. Hemozoin is produced during the repetitive life cycle of parasites in the erythrocytic blood stage (mostly trophozoites and schizonts). Accumulated hemozoin and parasite products are released into the blood stream on erythrocyte rupture and are readily captured by monocyte/macrophages/APCs and neutrophils and the reticulo endothelium system. Phagocytosis of hemozoin by immune cells triggers pro- and anti-inflammatory cytokines and chemokines (TNF α , IL1 β , MIP-1, MIP-2, MCP-1, IL12, IL6, IL8, NO, IL10, and MIF). Some of the merozoites differentiate into their sexual stages, and form both female and male gametocytes, which are central to malaria transmission. Gametocytes (also containing hemozoin) and many other parasite products are taken up during the mosquito blood-meal and trigger mosquito cells to produce NOS. Within 10 days, sporozoites are transferred in the salivary glands of mosquitoes, ready to be transmitted to humans.

non-DNA TLR9 ligand.²² It was shown that *P. falciparum*-purified hemozoin itself can activate innate immune responses both *in vivo* and *in vitro*, leading to the production of cytokines and chemokines, and the up-regulation of co-stimulatory molecules in a TLR9-dependent manner.²² Interestingly, the anti-malarial drug chloroquine abrogates hemozoin-induced cytokine production.²² However, it was questioned later by Parroche *et al.* that *Plasmodium* DNA, and not hemozoin, is the TLR9 ligand, and that hemozoin itself is not even immunogenic, but simply facilitates the entry of *Plasmodium* DNA into immune cells to target TLR9.²³ This controversial claim was challenged by many groups who confirmed that pure

hemozoin is an inflammatory molecule.^{13,15,25-27} Both Jaramillo *et al.* and our own group showed very clearly using agarose gel and SDS-page experiments that synthetic hemozoin is very pure and does not contain any DNA or protein contaminants (^{13,15,22} and unpublished observations). Furthermore, Jaramillo *et al.* elegantly showed that *Plasmodium* hemozoin, either while residing in the erythrocytes or after merozoite release into the bloodstream following schizont rupture, does not co-localize with *Plasmodium* DNA.¹⁵ However, there is a very likely possibility that parasite DNA may enter immune cells together with hemozoin and other parasite proteins, leading to immunomodulation during the natural in-

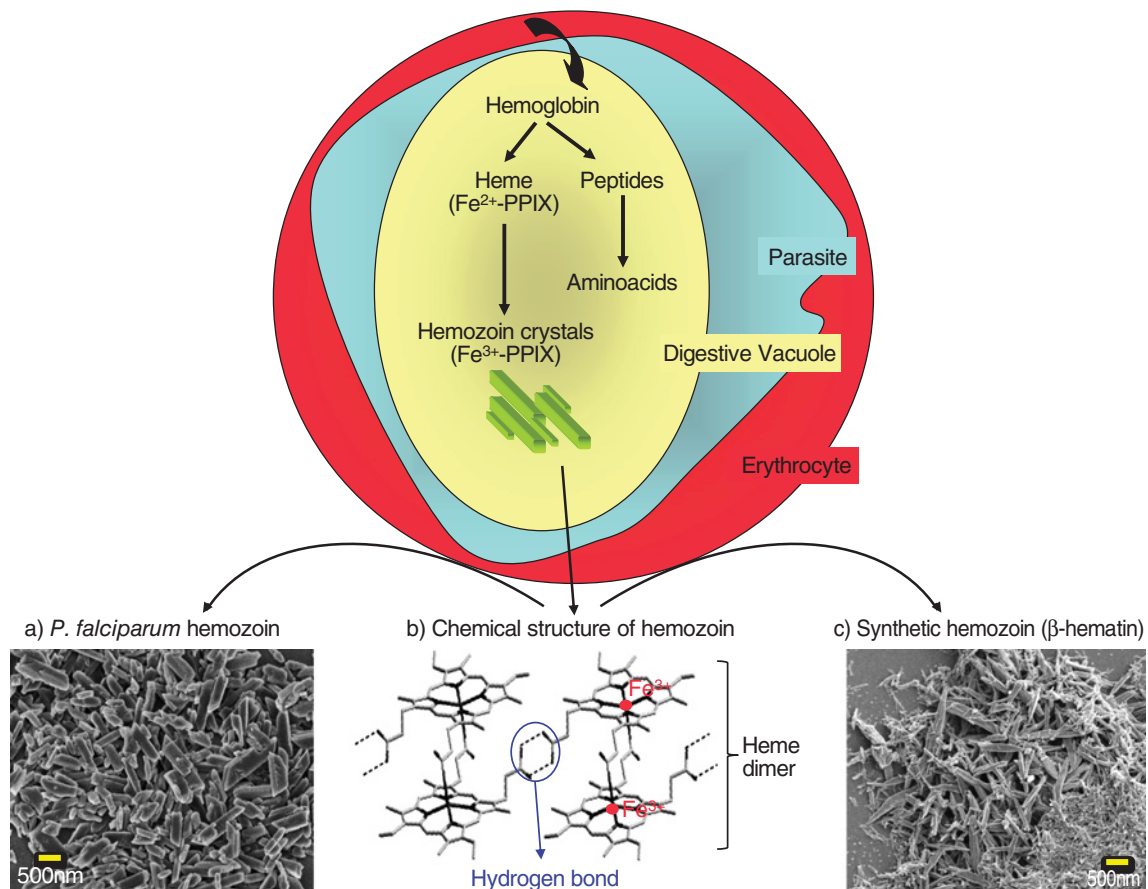


Fig. 2 Hemozoin formation takes place inside the digestive vacuoles of *Plasmodium* parasites in erythrocytes. *Plasmodium* parasites, while living in erythrocytes, form a digestive vacuole, an acidic compartment important for parasite metabolism and survival. The parasites use host-hemoglobin for their vital needs, such as amino acids and iron in which the free-heme (Fe^{2+} -protophyrin IX) is detoxified by converting it into insoluble crystals called hemozoin (Fe^{3+} -protophyrin IX). (a) SEM picture of purified natural hemozoin from *P. falciparum*. (b) Chemical structure of β -hematin crystals. Crystals are composed of cyclic heme dimers integrated into each other through hydrogen bonds to form large crystal structures (modified from Egan¹⁰). Dimeric heme consists of two heme molecules covalently linked through a central ferric iron (Fe^{3+}). (c) SEM picture of synthetic β -hematin crystals purified from ultrapure hemin chloride.

Table 1 Methods used to synthesize synthetic hemozoin crystals and their different sizes and adjuvant properties.

Synthetic Hemozoin purification methods	Crystal size	Adjuvant effect
1. Acetic acid method	50-200 nm	High
	2-20 μm	Medium
2. Organic base method	2-60 μm	Very low
Control Hemin chloride	<50 nm	None

fection process. In this context, further investigation is needed to understand how hemozoin interacts with other parasite components after it is taken up by immune cells. Nevertheless, this does not exclude immunostimulation by hemozoin itself, since synthetic hemozoin itself possesses powerful inflammatory

properties without any contaminant from host or parasite such as LPS or DNA.

A ligand is defined as a molecule that directly binds to a receptor with a certain affinity and specificity. Based on this definition, there are several ways to investigate ligand-receptor interactions. Circular dichroism (CD) spectrum analysis measures the absorption of circularly polarized light resulting from structural asymmetry, and is one of the most sensitive ways to study conformational changes in proteins. This strategy was used by Latz *et al.*, who showed that a recombinant TLR9 protein changed its conformation upon ligation by CpG DNA, i.e. a canonical TLR9 ligand.²⁸ We successfully reproduced their data and further investigated whether TLR9, and its non-DNA ligand hemozoin, directly interacted.¹³ Remarkable changes in the CD spectra of the TLR9 protein were

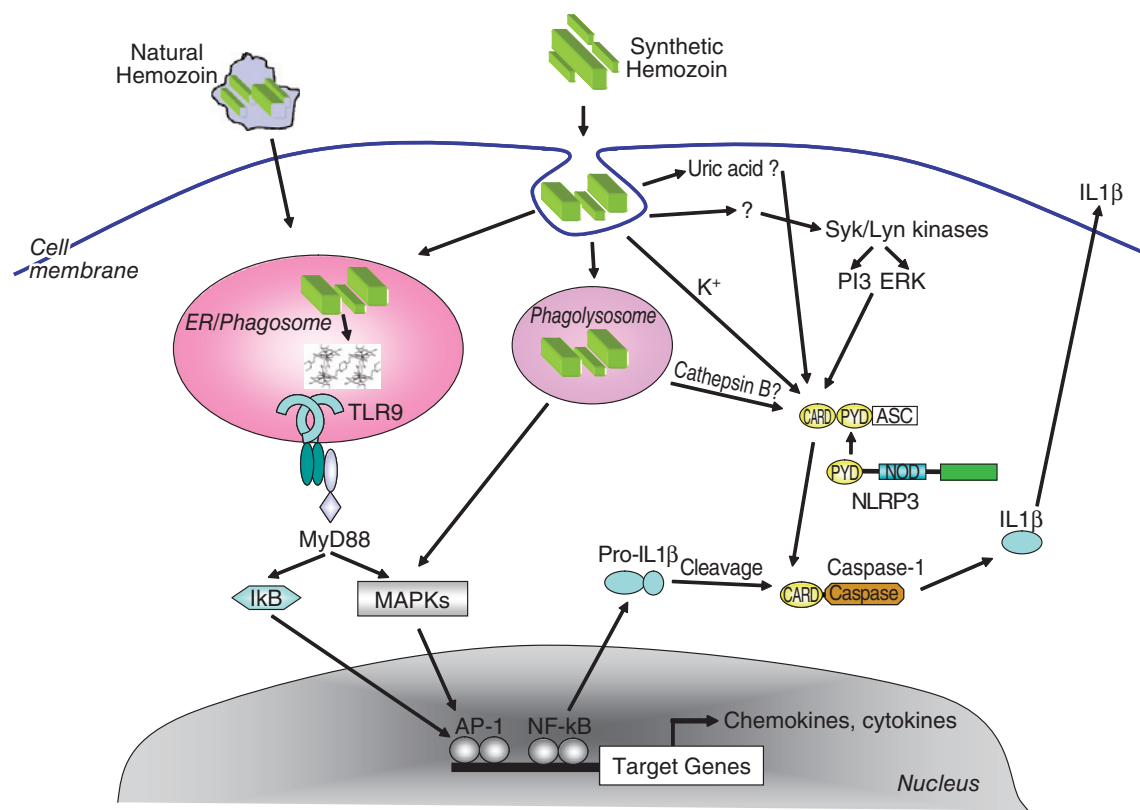


Fig. 3 Proposed models for innate immune system recognition of natural and synthetic hemozoin. While natural hemozoin is recognized extensively by TLR9, innate immune system recognition of synthetic hemozoin might be different. Synthetic hemozoin crystals may activate the NLRP3 inflammasome, although the mechanism is still not clear. One possibility is that uric acid might be induced by sHZ, and that uric acid is the ligand for NLRP3. Another possibility is the involvement of cathepsin B-related lysosomal destabilization. In addition, sHZ may activate the Syk/Lyn-kinase-mediated intracellular signaling pathway upstream of NLRP3 or ASC, indicating indirect activation of NLRP3 and the existence of other hemozoin receptor(s). Moreover, PI3-kinases downstream of Syk, may also be involved in the regulation of hemozoin-induced IL1 β production.

observed not only with CpG DNA, but also with synthetic hemozoin (β -hematin) crystals, whereas none were seen with dsRNA or monosodium urate (MSU) crystals.¹³ We also analyzed the nature of the interaction between TLR9 and hemozoin using short peptides containing unique sequences of the TLR9 extracellular domain. Using this approach, several unique CysXXCys, or Cys motifs, were identified that are responsible for binding TLR9 to CpG DNA, as well as hemin (the single unit of hemozoin).¹³ The direct interaction of these TLR9 motifs with CpG DNA, or hemozoin were of similar affinity, but their mode of binding was quite distinct at the atomic level (confirmed by NMR titration). These *in vitro* receptor-ligand binding studies suggest that hemozoin binds to a recombinant TLR9 protein with a certain affinity and specificity, thus fulfilling the definition of a ligand. It will be of interest to see if a single (or dimeric) TLR9 molecule can recognize these distinct ligands based on their structure as well as their chemistry. This may be clarified using more detailed

methods such as crystal structural analysis.²⁹

It has recently been shown that particulated nanomolecules, such as aluminum hydroxyl gel and MSU, asbestos or silica are recognized by NLRP3, and form large cytosolic complexes known as inflammasomes.^{30,31} Inflammasomes lead to the proteolytic activation of pro-inflammatory cytokines such as IL-1 β and IL-18.³² Similarly, recent reports have suggested that the immune recognition of, and inflammatory response to (IL-1 β secretion), sHZ is mediated by NLRP3 *in vitro*.^{25-27,33} Although all these reports show that IL-1 β production by macrophages in response to sHZ was reduced in mice lacking NLRP3, the precise mechanism of NLRP3-inflammasome activation by hemozoin is still unclear (Fig. 3).

It has been proposed that activation of the NLRP3 inflammasome is not due to specific ligation, but might occur via indirect NLRP3 activation by intermediary ATP molecules, or uric acid crystals, released from damaged cells.³⁴ In support of this idea, Griffith *et al.* suggested that uric acid is induced by sHZ, and

that uric acid is a NLRP3 ligand.²⁷ However, Dostert *et al.* completely disagree with the idea that uric acid or ATP are involved in NLRP3 activation by sHZ, and suggest that NLRP3 activation by sHZ is through ROS production and potassium efflux, similar to the mechanism they claim occurs with asbestos and MSU crystals.²⁵ Hornung *et al.* recently proposed that crystal structures such as silica, MSU and alum can destabilize lysosomes and cause the release of cathepsin-B into the cytosol, and that this activates the NLRP3 inflammasome.³⁵ However, by using cells from cathepsin-B-deficient mice, Dostert *et al.* showed that this was not the case for sHZ-induced IL1 β responses. In turn, Tiemi-Shio *et al.* suggested that cathepsin-B is activated during sHZ stimulation, but that lysosomal damage does not occur.²⁶ They also showed that sHZ activates Syk/Lyn-kinase-mediated intracellular signaling pathways upstream of NLRP3 or ASC, indicating the indirect activation of NLRP3 and the existence of other hemozoin receptor(s) such as Dectin1, TREM family members, Siglec or DAP12.²⁶ Accordingly, PI3-kinases, which are downstream of Syk, are also involved in the regulation of hemozoin-induced IL1 β production. Of note, PI3 kinases are the main regulators of CpG DNA (a known TLR9 ligand)-induced activation.³⁶ Currently, it is not clear why such different signaling mechanisms have been proposed by different groups.

ADAPTIVE IMMUNE RESPONSES TO HEMOZOIN: A NOVEL ADJUVANT

Successful vaccines contain an adjuvant component that triggers innate immune system activation for optimal immunogenicity.^{37,38} We recently found that whole malaria parasite vaccines using *P. falciparum* (Pf)-crude extracts derived from *in vitro* blood-stage cultures contain a “built-in” adjuvant, which is mediated by TLR9.¹³ Careful analysis of the components in the Pf-crude extract that may be responsible for this TLR9-dependent adjuvant effect showed that *Plasmodium* DNA is not involved. Another candidate component could be hemozoin, as reported earlier.³⁹ In support of this idea, synthetic hemozoin shows a potent adjuvant effect with several model antigens, as well as malaria antigens, when immunized subcutaneously, intraperitoneally or intranasally to mice. Antigen-specific IgG responses (mainly IgG1 followed by IgG2b and IgG2c) were increased by sHZ. Substantial amounts of IL-13 and IL-5 were produced by spleen cells, but no IFN γ or IL-17 was detected.¹³

These data suggest that hemozoin is the “built-in” adjuvant for whole-parasite vaccination using the *P. falciparum* (Pf)-crude extract. On the other hand, one can still argue that there may be a possibility that the Pf-crude extract may contain a non-DNA, non-hemozoin TLR9 ligand. It seems technically very difficult to remove hemozoin from the Pf-crude extract, or to prove that hemozoin is an essential TLR9 ligand

within the extract. However, as mentioned above, we have shown that both natural and synthetic hemozoin specifically bind to TLR9 and change its conformation, thereby confirming that hemozoin fulfills the definition of a TLR9 ligand.¹³

In contrast to the TLR9-dependency of the adjuvant effect of Pf-crude extract vaccination, the adjuvant effect of sHZ was not mediated by TLR9, but required MyD88.¹³ We postulated that NLRP3 might be involved, as hemozoin-induced IL-1 β secretion in macrophages is mediated by the inflammasome NLRP3/IL-1 β pathway.^{25-27,33} However, *in vivo* NLRP3 deficiency does not alter the adjuvant effect of sHZ. Thus, more studies are required to elucidate the mechanism by which hemozoin exerts its adjuvant activity in the innate immune response. This will involve detailed examination of whether TLR9 and NLRP3 have different roles to play, or whether other molecules are involved.⁴⁰

Alum (a well known particulate adjuvant), along with the type of cells recruited in response to alum and their role in its adjuvant effect, have been analyzed extensively.⁴¹ Neutrophils, monocytes, macrophages and eosinophils are recruited into the injection site after immunization with alum, but are not necessary for its adjuvant effects. Similarly, we found that synthetic hemozoin can recruit neutrophils to the peritoneum, and that this neutrophil recruitment is regulated entirely by MyD88, and not by TLR9 or NLRP3.¹³ Although these results contradict those of others who propose NLRP3- or ASC-dependent neutrophil recruitment into the peritoneum of mice after sHZ injection,²⁵⁻²⁷ our data nevertheless suggest that the adjuvant effect of hemozoin is closely correlated with neutrophil recruitment. Further studies are needed to understand this correlation, although the reason for the different results published by many groups is unclear.

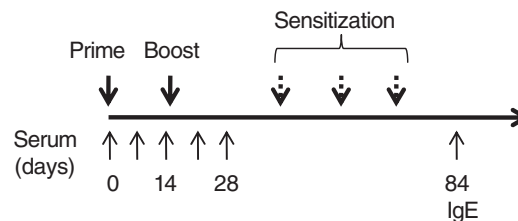
Nonetheless, we want to stress an important point needed to understand the adjuvant effect of synthetic hemozoin. Although the chemical properties of synthetic hemozoin produced using any of the several different purification protocols are similar, the variable sizes and appearance of the crystals may have a profound effect on their immunogenicity.^{13,15} Synthetic hemozoin crystals between 50 nm and 200 nm have optimal adjuvant effects when compared with larger sHZ molecules (2-20 μ m; Table 1). Given the fact that the size of other particle adjuvants taken up by antigen-presenting cells via receptor-mediated endocytosis is around 50-200 nm, it is reasonable to find ‘optimal size’ for the adjuvant effect of synthetic hemozoin crystals and their signaling pathways.^{42,43} It is conceivable that cellular uptake and internalization (i.e. modification of the integrity of the phagosomal and/or endosomal membrane) of both natural and synthetic hemozoin may differ due to their different crystal sizes. Clearly, further investigation is needed

a) Beagle dog house dust mite allergen (Derf2) sensitization

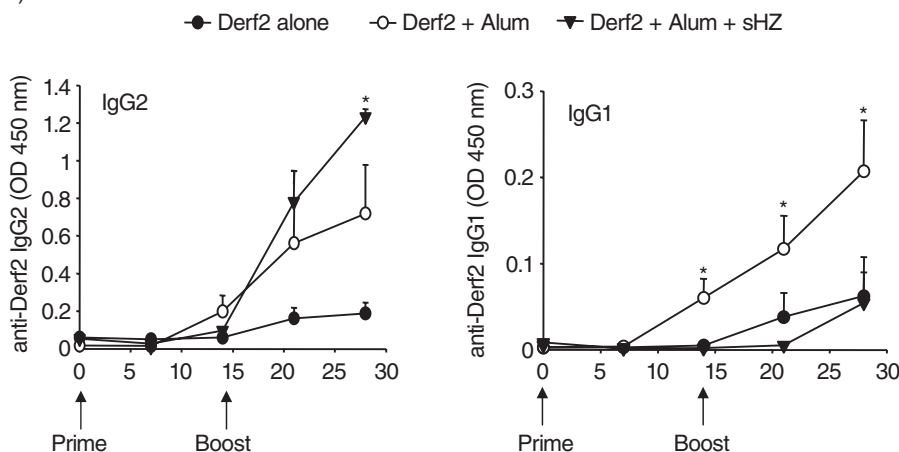


1. Derf2 (5 µg/ml)
2. Derf2 (10 µg/ml)
3. Saline
4. Histamine

b) Immunization protocol



c)



d)

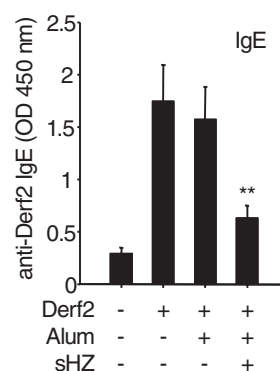


Fig. 4 Hemozoin is a potent adjuvant in dog allergy vaccines. (a) Successful sensitization of Beagle dogs with the Derf2 house dust mite allergen. Dogs were sensitized intradermally three times with a Derf2/alum mixture, before a skin test was carried out. Saline was used as negative control, while histamine was used as a positive control. Serum IgE responses correlated with the skin test reaction. (b) Immunization and sensitization protocol. (c) IgG2 and IgG1 responses 2 weeks after the booster immunizations (* $p < 0.05$). (d) IgE responses after sensitization with antigen (** $p < 0.01$). (b) and (d) were reproduced from Co-ban *et al.* under copyright permission.¹³

to uncover possible receptors(s), or to understand the co-ordination between receptors and signaling pathways in response to hemozoin. Currently, studies are underway to examine whether the different sizes of synthetic and natural hemozoin crystals require distinct delivery systems have different mechanisms of interaction with innate immune cells and their receptors.

In fact, the physiological relevance of TLRs and NLRs during adaptive immune responses to malaria infections, and the role of hemozoin *in vivo*, is the subject of much debate. Some have suggested, by using different mouse models and various *Plasmodium* species, that the role of NLRP3 in the pathogenesis of malaria infections is limited.^{25-27,33} The involvement of TLR9 during malaria infection in both in human⁴⁴⁻⁴⁷ and mouse studies⁴⁸⁻⁵² has gained much attention recently, with some reports suggesting that TLR9 has no role to play.⁵³⁻⁵⁵

HEMOZOIN IS A POTENT ADJUVANT AGAINST DOG ALLERGY

The discovery and evaluation of new adjuvants for use in humans is a time and energy consuming process. The final product must be safe, potent and cheap to distribute to those who need it most. The evaluation of new products in higher animals may lead to the use of some of these products as pet vaccines. So, can synthetic hemozoin be a good adjuvant in higher animals or, ultimately, in humans? To address this question, the adjuvant effects of synthetic hemozoin were studied in a dog allergy model. Atopic dermatitis is a common allergic skin disorder in both dogs and humans, with closely related symptoms and immunological mechanisms.⁵⁶ It is associated with the production of high levels of IgE antibodies against allergens such as *Dermatophagoides farinae*, the house dust mite. Beagle dogs sensitized by intradermal in-

jections of Derf2 (one of the major allergens of house dust mites⁵⁷) and alum develop allergic skin reactions that can be evaluated by a skin test (Fig. 4a). This skin test reaction has been found to be well correlated with serum IgE responses to given allergens.⁵⁸ Using this model, Beagle dogs were immunized with Derf2, together with alum and synthetic hemozoin, and boosted 2 weeks later (see Fig. 4b for immunization and sensitization protocol). After the immunizations, significantly elevated levels of IgG2, but not IgG1, antibodies were found in the sHZ-treated group, resembling Th1-like immune responses in dogs (Fig. 4c).⁵⁹ Finally, immunized dogs were sensitized with the Derf2 allergen and the Derf2-specific IgE responses were analyzed. The Derf2-specific IgE responses were significantly reduced in the sHZ co-treated dogs after allergen sensitization (Fig. 4d). These data suggested that, although it acts like a Th2-dominant adjuvant in a murine model, sHZ may serve as a potent Th1-like adjuvant in a canine model. Finally, canine allergies to house dust mites closely resembles those in humans, so these experiments warrant further attention, at least in terms of preventing allergic reactions to house dust mites in both dogs and humans.

CONCLUSIONS

Recent studies have improved our understanding of the mechanism by which hemozoin crystals may activate the innate immune system. DNA-free hemozoin, both in natural and synthetic form, can directly bind to and induce conformational changes in TLR9, leading to the activation of innate immune responses. Both forms of hemozoin possess adjuvant properties, but use different innate immune receptors. These studies suggest that one should be careful when producing synthetic hemozoin, since the adjuvant effect depends on its method of synthesis and particle size. Studies are underway to make a GMP-lot synthetic hemozoin as a safe and cheap adjuvant for the universal use in both animal and human vaccines.

ACKNOWLEDGEMENTS

We sincerely apologize to the authors whose valuable studies could not be cited due to limited space. We thank the members of both Akira's and Horii's Laboratories for their valuable comments and help. These studies were supported by JST as well as by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan, and from the RT Fund for Technology Development and CREST, JST, Japan. The authors declare that CC, TT, KJI, SA filed a patent application related to the method and usage of hemozoin as an adjuvant; YI, KO and TT are employees for Nippon Zenyaku Kogyo Co. Ltd., which develops GMP lot of sHZ, and were funded by JST.

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