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Impact of heme on specific antibody production in mice: promotive, inhibitive or null outcome is determined by its concentration

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Abstract

Free heme is an endogenous danger signal that provokes innate immunity. Active innate immunity provides a precondition of an effective adaptive immune response. However, heme catabolites, CO, biliverdin and bilirubin trigger immunosuppression. Furthermore, free heme induces expression of heme oxygenase-1 to increase production of CO, biliverdin and bilirubin. As such, free heme can play a paradoxical role in adaptive immunity. What is the outcome of the animal immune response to an antigen in the presence of free heme? This question remains to be explored. Here, we report the immunization results of rats and mice after intraperitoneal injection of formulations containing BSA and heme. When the heme concentrations were below 1 μM, between 1 μM and 5 μM and above 5 μM, production of anti-BSA IgG and IgM was unaffected, enhanced and suppressed, respectively. The results suggest that heme can influence adaptive immunity by double concentration-thresholds. If the heme concentrations are less than the first threshold, there is no effect on adaptive immunity; if the concentrations are more than the first but less than the second threshold, there is promotion effect; and if the concentrations are more than the second threshold, there is an inhibitory effect. A hypothesis is also presented here to explain the mechanism.

1. Introduction

Heme is a type of iron porphyrin that plays versatile roles in organisms. First, many proteins need heme as their prosthetic group and are thus called hemoproteins [1, 2]. Hemoproteins can function as electron transporters (e.g., cytochromes in electron transport chains) [3], oxidation/reduction enzymes (e.g., peroxidase) [4], gas carriers (e.g., hemoglobin for O_2) or gas sensors (e.g., soluble guanylate cyclase for NO) [5]. Second, free heme is a regulator of heme-responsive proteins [2, 6]. Heme-responsive proteins can be protein kinases [7, 8], transcription factors [9, 10], ion channels [11, 12], or microRNA processing factors [13, 14]. Third, free heme produces reactive oxygen species (ROS) [15] through NADPH oxidases [16] and the Fenton reaction [17]. ROS regulates a diverse array of signaling pathways by controlling the thiol/disulfide redox states of proteins [18]. In a nutshell, via hemoproteins, heme-responsive proteins and ROS, heme controls a biomolecule network covering signal transduction, gene expression and metabolism.

In the heme-controlled network, there are two antagonistic mechanisms related to immunity [19]. On the one hand, free heme activates toll-like receptor 4 (TLR4)-dependent [20, 21, 22] and ROS-dependent signaling pathways [23, 24, 25]. The activation of these signaling pathways promotes leukocyte maturation/migration [26, 27, 28], anti-apoptosis [29, 30], pro-inflammatory cytokine secretion [31], adhesion molecule expression [32, 33] and ROS production [27, 34], which construct a vigorous innate immune response or inflammation [35, 36]. The active innate immunity provides a precondition of an effective adaptive immune response [37, 38]. On the other hand, there have been many reports about the negative effect of heme on innate immunity or inflammation, such as promoting cell apoptosis [39, 40] and anti-inflammatory cytokine secretion [41]. The suppressive mechanism depends on heme oxygenases (HO), including inducible HO-1 and constitutive HO-2. HO is the key rate-limiting enzyme to convert free heme into Fe^{2+}, CO and biliverdin [19]. Biliverdin is subsequently transformed to bilirubin by biliverdin reductase. CO, biliverdin and bilirubin can trigger anti-inflammatory/immunosuppressive signaling pathways [42, 43, 44]. Furthermore, free heme induces the expression of HO-1 to reinforce the anti-inflammatory/immunosuppressive signaling pathways [45]. HO-1 can be induced by many other stressors and becomes an important target for various anti-inflammatory or immunosuppressive therapies [46, 47].

The two antagonistic mechanisms raise a question. What is the outcome of animal immune responses to an antigen in the presence of free heme? Is the role promotion, inhibition or no influence? The question remains to be explored. We

report the effect of free heme on the production of anti-BSA antibodies in rats and mice.

2. Materials and methods

2.1. Animals

All animal experimental procedures were approved by the Animal Care and Use Committee of Sun Yat-Sen University. Sprague Dawley (SD) rats and BALB/c mice, including males and females, were obtained from the animal center of Sun Yat-sen University. All rats and mice were housed in individual cages with free access to sterile water and irradiated food in a specific pathogen-free facility.

2.2. Chemicals and reagents

Limulus amebocyte lysate (LAL) test reagents were purchased from Associates of CAPE COD. Bovine serum albumin (BSA), egg phosphatidylcholine (PC), Complete Freund's adjuvant (CFA) and heme (hemin, HPLC grade purity > 98.0%) were purchased from Sigma-Aldrich. The LAL test showed that the lipopolysaccharide (LPS) activity of 250 μM heme solution was less than 0.01 EU/ml. HRP labeled ant-rat IgG (H/L), ant-rat IgM (μ), ant-mouse IgG (H/L), anti-mouse IgM (μ) and other ELISA reagents were purchased from AbD Serotec. All other AR or higher grade chemicals were purchased from local chemical suppliers.

2.3. Sterile measures

The water used in all experiments was double distilled. All solutions or samples were sterilized by autoclaving or filtrating through 0.22 μm PVDF Syringe Filters. Solution or sample subpackage and mixing were performed in a laminar flow cabinet.

2.4. Preparation of erythrocyte lipids

Rat blood was diluted with normal saline and centrifuged at 4000 rpm for 3 min to collect erythrocytes. The collected cells were washed 3 times with normal saline by repeated suspension and centrifugation. The washed erythrocytes were suspended in normal saline and slowly dropped into a 10-fold volume of 0.2% acetic solution. The mixture was held at 4 °C for h to completely lyse erythrocytes and then centrifuged at 4000 rpm for 10 min. The pellet was washed 3 times with 0.2% acetic solution by repeated suspension and centrifugation to obtain white erythrocyte ghosts. A 20-fold volume of absolute ethanol was added into the erythrocyte ghosts. The mixture was shaken at intervals and held at 50 °C in a water bath for 2 h. Finally, erythrocyte lipid was obtained by centrifuging the mixture and recovering and drying the supernatant. The preparation of human

erythrocyte lipid was similar to that of rat erythrocyte lipid. Human erythrocyte suspension was purchased from local Guangzhou Blood Center, an official non-profit organization of Guangzhou City in Guangdong Province that collects blood from voluntary donors and provides erythrocyte suspension to hospitals. The center also provides erythrocyte suspension to scientific researchers with an official permission document of Department of Health of Guangdong Province. Human erythrocyte, rat erythrocyte, and hemolytic rat erythrocyte lipids are, respectively, abbreviated as HEcL, REcL and LREcL. It was noted that the erythrocyte ghosts from severe hemolytic rat blood were slightly brown such that LREcL had a deeper color than HEcL and REcL.

2.5. Preparation and quantification of hemozoin

Hemozoin was prepared after a previously published method with some modifications [48]. Briefly, 300 mg of heme was dissolved in 60 ml of 0.1 M NaOH by stirring for 30 min. Glacial acetic acid was slowly dropped into the heme solution to adjust the pH to approximately 4. The heme solution was heated at 70 °C for 18 h and then cooled down at room temperature to obtain hemozoin crystals. After cooling, the separated hemozoin crystals were washed three times with 0.1 M NaHCO$_3$ for 3 h and another three times by alternation of methanol and ddH$_2$O. Finally, the solid hemozoin was dried in a drying oven overnight at 70 °C. The quantification of hemozoin was conducted by dissolving it in 0.1 M NaOH and by the colorimetric method at 400 nm with purity heme as the standard.

2.6. Preparation of immunization formulations

Phosphate buffered saline (PBS 1 ×) at a pH of 7.4 was the basal solvent for all experiments and blank control. Heme solution was prepared by dissolving heme in 0.1 M NaOH to the concentration of 1 mM for storage and diluted by PBS to a designed concentration for use. BSA (test antigen) solution was prepared by dissolving it in PBS to the concentration of 10 mg/ml for storage and diluted by PBS to a designed concentration for use or negative control. BSA+CFA denotes the mixture of BSA solution and CFA, which was used as a positive control. BSA +Heme denotes the mixture of BSA solution and heme solution. BSA+HEcL, BSA+REcL, BSA+LREcL, BSA+Heme+PC, BSA+Heme+REcL and BSA +Hemozoin+PC were all the form of liposome suspension and prepared briefly as follows. Lipid (0.1 g) was completely dissolved in a 5-ml mixture of ethanol and ether (1:1) in a 50-ml round bottom flask. In a fume hood, the flask was rotated slowly by hand while a nitrogen stream blew on the inner wall until a thin lipid film was formed and the solvent completely evaporated. BSA solution (2 ml, 0.5 mg/ml) with or without heme or hemozoin (in designed concentrations) was added into the flask. The flask was shaken to hydrate the lipid film to form a multilamellar liposome suspension. Then, the multilamellar liposome suspension was transferred

into a 10-ml conical flask to make small unilamellar liposome suspension by bath sonication. In most cases, the heme-contained formulations were liposome suspension because of the two reasons. First, the water solubility of heme is poor at the pH of 7.4. The application of lipid can make the formulations more sTable Second, the positive control CFA contains oil components. Meanwhile, hemolytic rat erythrocyte lipid (LREcL) is another positive control.

2.7. Immunizations and sera preparation

Male and female SD rats or BALB/c mice were obtained one week before immunizations and fed in a SPF environment. Rats or mice were randomly grouped, and the number of each group was more than eight in case of accidental death by operations. Immunizations were conducted by intraperitoneal injection of 100-µl formulations containing 50 µg of BSA for rats (42 days old) or 30 µl of formulations containing 15 µg of BSA for mice (42 days old). Blood was collected by cardiac or jugular venous puncture on the planned dates after immunizations. After blood clotting, the clear amber sera were collected by centrifugation at 4000 rpm for 10 min and kept in 4 °C for further testing.

2.8. Measurements of anti-BSA IgG and IgM

The levels of anti-BSA IgG and IgM were measured by indirect ELISA according to general guidelines. Briefly, 96-well plates were coated with 10 µg/ml BSA overnight at 4 °C. The coated plates were washed 3 times in wash buffer (PBS containing 0.05% Tween-20, 0.1 ml/well). The plates were blocked with gelatin in PBS and incubated on a shaker for 2 h at room temperature and then washed 3 times in wash buffer. Subsequently, a series of variously diluted rat or mouse sera was added as the first antibodies to the plates and incubated on a shaker for 1 h at room temperature. After 3 washes, HRP-labeled anti-rat IgG (H/L), anti-rat IgM (µ), anti-mouse IgG (H/L) or anti-mouse IgM (µ) (diluted according to the reagent manual) was added as the secondary antibody to the plates and incubated on a shaker for 1 h at room temperature. The plates were washed 3 times and TMB solution was added for color development. After the reaction was stopped with 0.5 M H_2SO_4, the optical density was measured at 450 nm (OD_{450}) using an auto-plate reader. Each plate was read twice. The antibody standard control was the part of the protocol of the ELISA kit. The anti-BSA IgG or IgM level of each rat is indicated by the ELISA OD_{450} of the 100-fold diluted serum which lies in the range of a nearly linear relationship between the OD_{450} and dilution factor.

## 2.9. Spectroscopic measurements of BSA+Heme+PC

The preparation of BSA+Heme+PC was the same as in the "*Preparation of Immunization Formulations*", except that the heme concentration of 40 µM was

much higher than the concentrations used in immunizations. If the concentration of 40 μM does not cause the conversion of heme into hemozoin, it is more unlikely that those in immunizations result in the development of hemozoin. To simultaneously measure the samples (liposome suspensions) that were held at 37 °C for different days to make the errors as small as possible, the second sample was prepared 7 days following the first and so on. On the thirty-fifth day, the seventh sample was collected and all samples were treated to disassemble liposomes and make the solutions clear by adding 2% SDS and shaking. The treated samples were centrifuged at 10000 rpm for 10 min to check whether there were pellets (hemozoin crystals) in the tube bottoms. The supernatants were transferred for UV–vis scanning from 300 nm to 900 nm.

3. Results

3.1. Hemolytic rat erythrocyte lipid enhances rat anti-BSA antibody production

The sera of Sprague Dawley (SD) rats can agglutinate human red blood cells in medium intensity. The IgM antibodies separated from SD rat sera could strongly interact with ABO blood group antigens (data not shown). This finding indicates that SD rats may be used as model animals to test whether human erythrocyte lipid (HEcL) with A or B antigen has an adjuvant effect through the mediation of Fc receptors in the antigen-presenting cells of individuals with anti-A or B antibody [49]. In the experiments for testing this possibility, the positive control was Complete Freund's adjuvant (CFA), the negative control was rat erythrocyte lipid (REcL), the formulations were BSA-contained liposome suspension, and the administration was intraperitoneal injection. HEcL or REcL was prepared as the routine method; red cell ghosts were first made from fresh blood to separate cell membrane from hemoglobin and then the lipid was extracted from the ghosts. However, because of the mistake of substituting buffer for anticoagulant in drawing rat blood, the pooled rat blood was severely hemolytic and substantial heme interfused in the cell membrane. Therefore, hemolytic rat erythrocyte lipid (LREcL) had a deeper color (slight brown) than HEcL. To our astonishment, the levels of anti-BSA IgG and IgM in the sera of the negative control group (immunized by BSA+LREcL liposome suspension) were approximated to those of the positive control group (immunized by BSA+CFA emulsion) on the 16th day after immunizations (Fig. 1A–B). After repeating the experiments, including the extraction of LREcL, as well as studying related literatures, we deduced the following. First, the results were not caused by LPS or other contaminations because HEcL did not increase the level of anti-BSA IgG or IgM under the same experimental conditions (Fig. 1A–B). Second, free heme may be an amazing actor because it is a damage-associated molecular pattern (DAMP) that arouses innate immunity [35, 36] and innate immunity modulates adaptive immunity [37, 38].
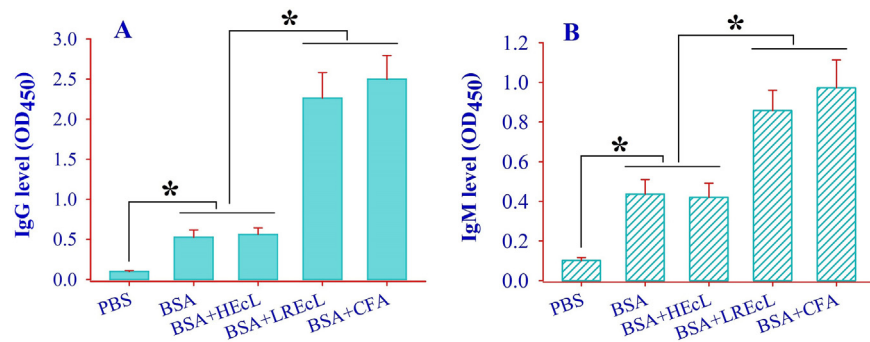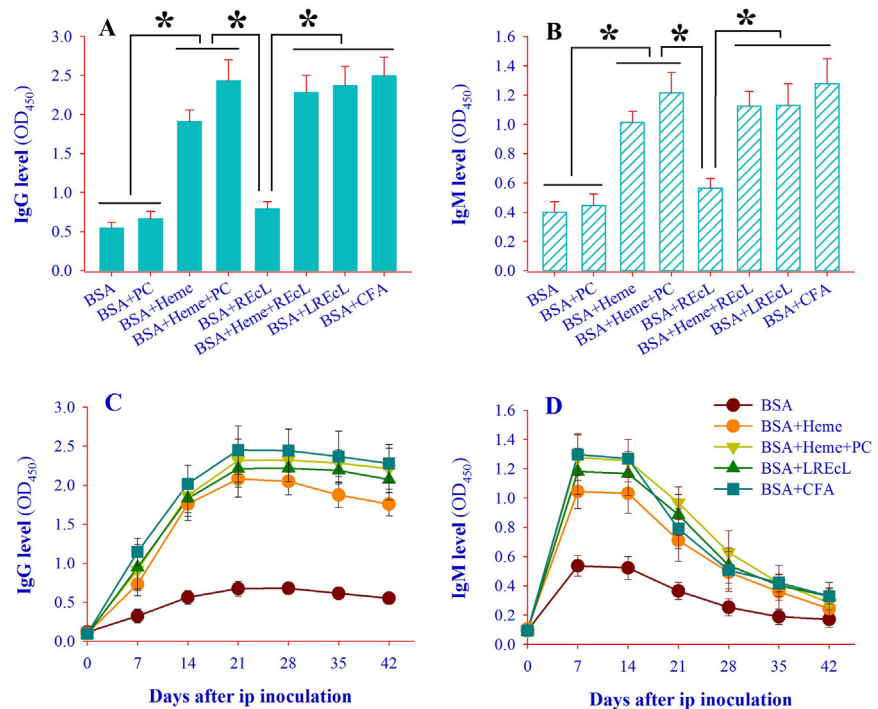
Fig. 1. Hemolytic rat erythrocyte lipid enhances rat anti-BSA antibody production. Levels of anti-BSA IgG and IgM on the 16th day after immunizations are indicated by the OD_{450} values of 100-fold diluted sera which are linearly related with the dilution factor. Immunizations were ip injection of 100 μl of formulations containing 50 μg BSA. Difference between each group (eight rats) is analyzed by t-test with two-tailed P-value. *, $p < 0.001$. BSA, bovine serum albumin. CFA, Complete Freund's adjuvant. PBS, phosphate buffered saline. HEcL, human erythrocyte lipid. LREcL, hemolytic rat erythrocyte lipid.

Finally, the heme concentration that caused the results should be very low. It should be noted that the concentration instead of the amount/body weight is used for the dose of heme or BSA in this paper because the immune response to intraperitoneal injection is a local effect instead of a whole body effect, such as the immune response to intravenous injection (the injectant will spread throughout the body).

3.2. Heme at several μM enhances rat anti-BSA antibody production

To confirm whether heme is the enhancer in LREcL for producing anti-BSA IgG and IgM in rats, commercial heme with HPLC grade > 98% purity was used. The limulus amebocyte lysate (LAL) test showed that the LPS activity was less than 0.01 EU/ml in 250 μM heme solution. In the experiments, heme-contained formulations were liposome suspension in most cases because of the two considerations. First, LREcL became a positive control in the subsequent experiments. Meanwhile, CFA, the positive control, also contains oil components. Second, the water solubility of heme is poor. The application of lipid (PC) can make the formulations more stable. The heme concentrations in all heme-contained formulations were adjusted by colorimetry to approximate that in LREcL (approximately 2.3 μM). On the fifteenth day after immunizations, compared to the formulations without heme, all with heme significantly raised the levels of anti-BSA IgG and IgM in rat sera (Fig. 2A–B). Furthermore, except heme alone, the promotion ability of heme+PC or heme+REcL was approximated to that of CFA or LREcL. In another independent experiment, the time kinetics of the production of anti-BSA IgG and IgM in the presence of heme paralleled that in the presence of

Fig. 2. Heme at several μM enhances rat anti-BSA antibody production. (A-B) Levels of anti-BSA IgG and IgM on the 15th day after immunizations. (C-D) Time kinetics of the production of anti-BSA IgG and IgM. Anti-BSA levels are indicated by the OD_{450} values of 100-fold diluted sera which are linearly related with the dilution factor. Immunizations were ip injection of 100 μl of formulations containing 50 μg BSA. The heme concentrations in all heme-contained formulations were about 2.3 μM adjusted by colorimetric comparison with LREcL sample. Difference between each group (eight rats) is analyzed by t-test with two-tailed P-value. *, $p < 0.001$. BSA, bovine serum albumin. CFA, Complete Freund's adjuvant. PBS, phosphate buffered saline. PC, phosphatidylcholine. REcL, rat erythrocyte lipid. LREcL, hemolytic rat erythrocyte lipid.

CFA or LREcL (Fig. 2C–D). The results demonstrate that heme, at least in very low concentrations and by intraperitoneal injection, can increase the production of specific antibodies in rats.

3.3. Different Heme concentrations show different effects on anti-BSA antibody production

The above results encouraged us to reproduce the experimental results in mice. However, to our astonishment, when the mice were immunized by the formulation (BSA+Heme+PC) containing a high heme concentration, the levels of anti-BSA IgG and IgM were below those in the mice immunized by BSA+PC or BSA alone. This demonstrates that high heme concentrations can exert a suppressive effect on the production of anti-BSA antibodies. To find the concentration at which heme exerts different effects on the production of anti-BSA antibodies, a series of heme concentrations was designed from 0.1 to 15 μM. Compared to BSA+PC, the

formulations with the heme concentrations below 1 μM did not raise the levels of anti-BSA IgG and IgM. However, when the heme concentrations were more than 5 μM, the levels of anti-BSA IgG and IgM decreased below the levels of the group given BSA alone. Only between 1 μM and 5 μM were the levels of anti-BSA IgG and IgM significantly increased (Fig. 3A–B). The results suggest that heme can affect adaptive immunity (at least humoral immunity) by double concentration-thresholds. If heme concentrations are below the first threshold, the effect of heme on adaptive immunity is zero; if the concentrations are between the first and the second thresholds, the effect is promotive; and if the concentrations are greater than the second threshold, the effect is inhibitive.
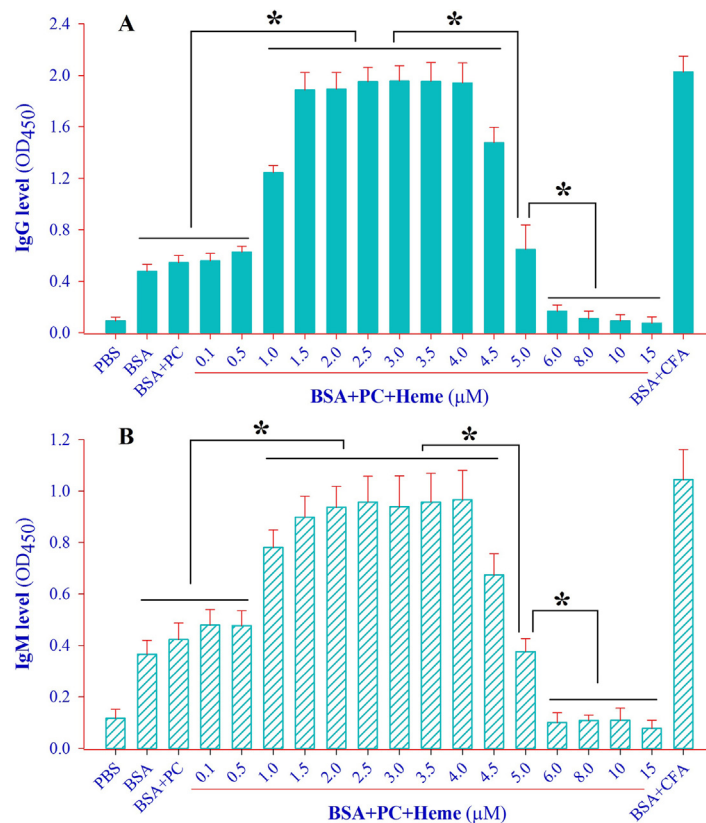
Fig. 3. Different heme concentrations show different effects on anti-BSA antibody production. Levels of anti-BSA IgG and IgM in mice on the 15th day after immunizations are indicated by the OD_{450} values of 100-fold diluted sera which are linearly related with the dilution factor. Immunizations were ip injection of 30 μl of formulations containing 15 μg BSA. Difference between each group (eight mice) is analyzed by t-test with two-tailed P-value. *, p < 0.001. BSA, bovine serum albumin. CFA, Complete Freund's adjuvant. PBS, phosphate buffered saline. PC, phosphatidylcholine.
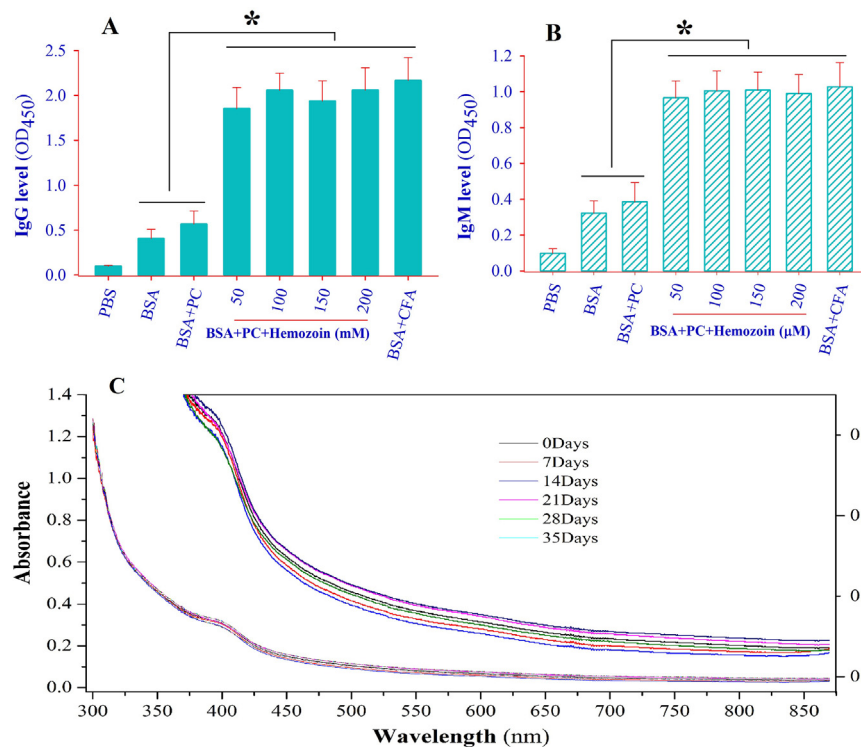
3.4. Enhanced anti-BSA antibody production is not due to Heme-derived hemozoin during immunizations

Hemozoin consists of heme-derived insoluble crystals, which causes innate immunity/inflammation and has been applied as an adjuvant [48, 50]. Therefore, it must be considered that the enhanced production of anti-BSA antibodies may be due to heme-derived hemozoin during immunizations. The pH and heme concentration for hemozoin development are less than 5 and more than 100 μM, respectively [48]. Although the pH (7.4) and heme concentrations (0.1–15 μM) in our experiments were theoretically not suitable for hemozoin occurrence, *in vivo* and *in vitro* experiments were performed to verify whether the conversion of heme into hemozoin occurred during immunization. In *in vivo* experiments, the concentrations of hemozoin (insoluble nanoparticles) used in the immunization formulations were equivalent to the concentrations of heme from 50 to 200 μM. If the transformation of heme into hemozoin occurred during the immunizations, as for heme at concentrations over 6 μM (Fig. 3A–B), hemozoin in the concentrations over 50 μM should suppress the production of anti-BSA antibodies. However, hemozoin in the concentrations over 50 μM enhanced the production of anti-BSA IgG and IgM (Fig. 4A–B). In *in vitro* experiments, the BSA+PC+Heme (40 μM) formulations were held at 37 °C for different days. If heme at concentrations from 0.1 to 15 μM can transform into hemozoin during immunizations, heme at a concentration of 40 μM should more preferentially form hemozoin. However, regardless of how many days the samples were held, there were no pellets (hemozoin crystals) in the tube bottoms after centrifugation. Furthermore, the spectra of the supernatants were almost the same, which meant there was no hemozoin occurrence (Fig. 4C). The results of the *in vivo* and *in vitro* experiments demonstrate that heme did not transform into hemozoin during immunizations, which means the production of anti-BSA IgG and IgM in our experiments was enhanced by heme and not by hemozoin.

4. Discussion

4.1. Question about LPS contamination

LPS is a TLR4 activator that is usually used as an adjuvant ingredient [51]. Free heme can synergize with low concentrations of LPS [25]. Therefore, there are two points to clarify. One is that the enhancer to produce anti-BSA antibodies may be LPS contamination and not heme. The other one is that, even if the LPS contamination is too low to have an effect, the enhancer may be the synergy of heme with LPS instead of heme alone. The two possibilities can be excluded by the following observation. First, the heme used in all experiments had high purity (>98%) and qualified with the LAL test. Second, all samples were prepared under sterile conditions to prevent them from LPS contamination. Third, if the enhancer

Fig. 4. Enhanced anti-BSA antibody production is not due to heme-derived hemozoin during immunizations. (A-B) Levels of anti-BSA IgG and IgM on the 15th day after immunizations. Anti-BSA Levels are indicated by the OD_{450} values of 100-fold diluted sera which are linearly related with the dilution factor. Immunizations were ip injection of 30 μl of formulations containing 15 μg BSA. Being insoluble, hemozoin concentration is indicated by the equivalent of heme. Difference between each group (eight mice) is analyzed by t-test with two-tailed P-value. *, $p < 0.001$. BSA, bovine serum albumin. CFA, Complete Freund's adjuvant. PBS, phosphate buffered saline. PC, phosphatidylcholine. (C) Spectra of heme in BSA+heme+ PC samples that were held at 37 °C for different days. The heme concentration was 40 μM.

is LPS contamination, under the same experimental conditions, all samples should have an equal chance of contamination by LPS and show the same promotive effect. However, the enhanced production of anti-BSA antibodies only occurs in the rats or mice that were immunized by formulations containing heme at proper concentrations (Figs. 1–4). Finally, if the enhancer is the synergy of heme with low concentrations of LPS contamination, the higher heme concentrations should at least have the same effect as the lower heme concentrations. However, unlike lower heme concentrations (1–5 μM) that promote the production of anti-BSA antibodies, the higher heme concentrations (>5 μM) suppressed the production of anti-BSA antibodies (Fig. 3).

## 4.2. Questions about the binding of Heme to BSA

Its unique chemical structure makes heme easily bind to proteins through diverse ways, including coordination of the central iron ion with N, S or O atom on the side

chain of His, Lys, Cys, Met or Tyr; hydrophobic interaction of the porphyrin ring, methyl and vinyl groups with non-polar amino acid residues; electrostatic interaction of propionate groups with positively charged amino acid residues and covalent bond of vinyl groups with Cys residues. A question arises. Did the binding of heme to BSA, but not free heme, enhance the antigenicity of BSA? This question conflicts with the following observations. First, it was reported that free heme, but not protein-bound heme, can stimulate the immune system [52]. Second, heme can easily be dissolved in lipid (or insert into membrane), which will impair the binding of heme to BSA. Therefore, if the assumption is true, the antibody levels induced by BSA+Heme should not be markedly lower than those induced by BSA+heme+Lipid at the same concentrations of heme and BSA. However, the results were the opposite (Fig. 2A–B & Fig. 3). Finally, higher heme concentrations will promote the binding of heme to BSA. Therefore, if the assumption is true, the higher heme concentrations should at least show the same effect as the lower heme concentrations. However, unlike lower heme concentrations (1–5 μM) that promote the production of anti-BSA antibodies, higher heme concentrations (>5 μM) suppress the production of anti-BSA antibodies (Fig. 3).

4.3. Hypothesis that Heme affects the adaptive immune response

Our experiments showed that the production of anti-BSA IgG and IgM in mice could be unaffected, increased and suppressed by heme with concentrations below 1 μM, between 1 μM and 5 μM and above 5 μM, respectively. The results suggest that heme can influence the adaptive immune response via double concentration-thresholds. When the concentrations of free heme are below the first threshold, heme has no effect on the adaptive immune response; when the concentrations are between the first and second thresholds, heme has a promotion effect; and when they are over the second threshold, heme exerts an inhibitive effect. Physiological release of heme may be one of the intrinsic mechanism for animals to maintain adequate immunity and pathological bleeding may result in animal immunosuppression.

Why does heme work this way? An essential reason is that heme drives two antagonistic immune mechanisms, the *heme effect* and *HO effect*. The heme effect runs the promotive process from innate to adaptive immunity (Fig. 5). Free heme evokes innate immunity by TLR4- and ROS-dependent mechanisms and their crosstalk. First, through TLR4 on monocytes, macrophages and dendritic cells [21], extracellular free heme activates the signaling pathways from MyD88/TRAM to the transcription factors NF-κB and AP-1 [53, 54]. Active NF-κB and AP-1 promote the expression of proinflammatory cytokines, such as IL-6, IL-12, TNFα, pro-IL-1β and pro-IL-18. Second, ROS-dependent mechanisms include the following: (i) extracellular free heme produces ROS by the Fenton reaction and activates NADPH oxidases, releasing ROS at both sides of the cell membrane [55];
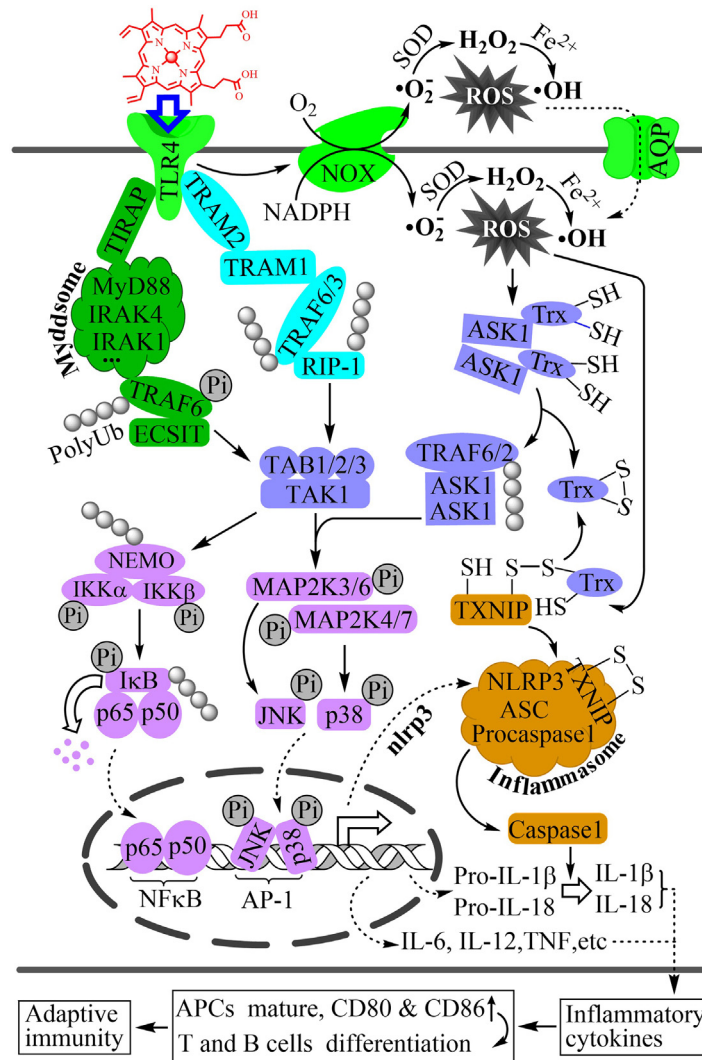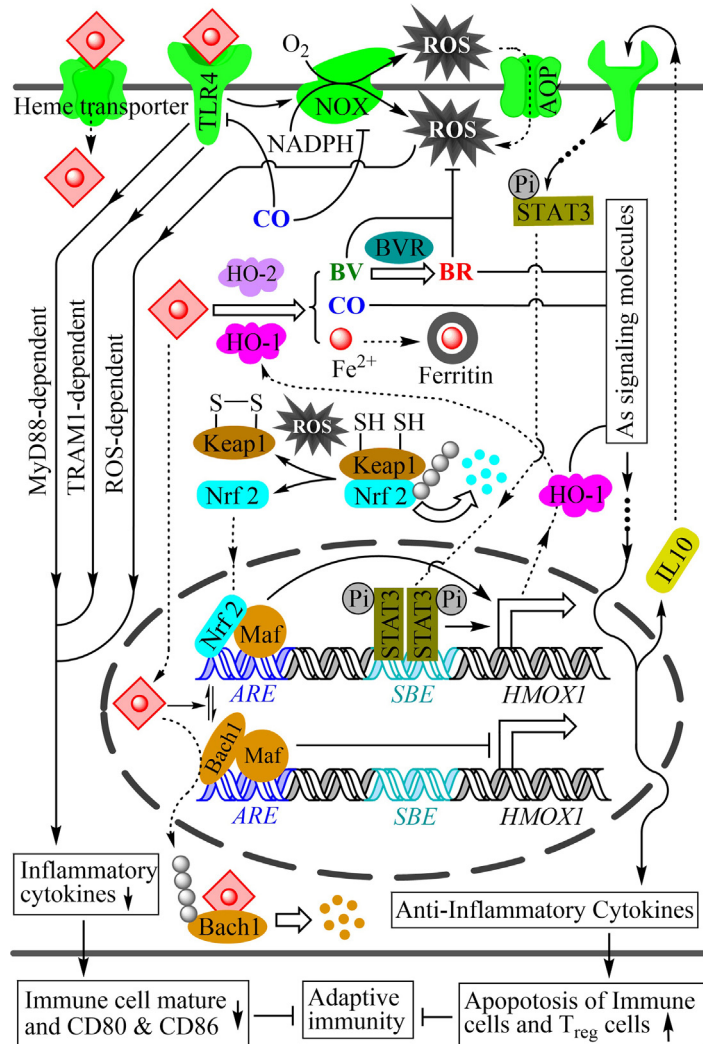
Fig. 5. Diagram of heme effect. →, promotion. —|, inhibition. ↑, rise. ↓, fall. ⇨, degradation or transformation. ⇢, transport or translocation. AP-1, activator protein 1. APCs, antigen-presenting cells. AQP, aquaporin. ASC, apoptosis-associated speck-like protein containing a CARD. ASK1, apoptosis signal-regulating kinase 1. CD, cluster of differentiation. ECSIT, evolutionarily conserved signaling intermediate in Toll pathway. IκB, inhibitor of NF-κB. IKK, IκB kinase. IL, interleukin. IRAK, interleukin-1 receptor-associated kinase. JNK, c-Jun N-terminal kinase. MAP2 K, mitogen-activated protein kinase kinase. MyD88, myeloid differentiation primary response gene 88. NEMO, NFκB essential modulator. NFκB, nuclear factor κ-light-chain-enhancer of activated B cells. NLRP3, NACHT, LRR and PYD domains-containing protein 3. NOX, NADPH oxidase. RIP-1, receptor-interacting serine/threonine-protein kinase 1. ROS, reactive oxygen species. SOD, superoxide dismutase. TAB, TAK1 binding protein. TAK1, TGFβ activated Kinase 1. TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein. TNF, tumor necrosis factor. TRAM, toll-like receptor adaptor molecule. TRAF, TNF receptor-associated factor. TRL4, toll-like receptor 4. Trx, thioredoxin. TXNIP, Thioredoxin-interacting protein.

(ii) extracellular ROS enters the cells by passing through water channels and diffusing across the cell membrane [55]; and (iii) through ASK1, intracellular ROS

not only activates NF-κB and AP-1 [56], it promotes the formation of NLRP3 inflammasomes [57]. Inflammasomes release activated caspase 1 to activate cytokine precursors, such as pro-IL-1β and pro-IL-18, into active forms of IL-1β and IL-18. Third, the TLR4- and ROS-dependent signaling pathways are mutually promoted by their crosstalk [58, 59]. In summary, heme induces innate immune cells to secrete diverse inflammatory cytokines, which return to stimulate innate immune cells to proliferate, differentiate and mature [60]. The enhanced innate immunity results in higher levels of activated antigen-presenting cells (APCs), costimulatory molecules on APCs, effective autophages for MHCI/MHCII to present antigens [61], and related cytokines to stimulate T and B cells [60]. These are the required initial conditions to achieve an effective adaptive immune response [37]. The HO effect controls the inhibitory process from innate to adaptive immunity (Fig. 6). First, HO, including inducible HO-1 and constitutive HO-2, depletes the activator of TLR4- and ROS-dependent signaling pathways by degrading free heme. Meanwhile, the degradation products, CO, biliverdin and bilirubin, can suppress TLR4- and ROS-dependent signaling pathways [42, 43, 44] to downregulate the secretion of pro-inflammatory cytokines and the differentiation of antigen-presenting cells, helper and effector T cells. Second, CO, biliverdin, bilirubin and HO-1 can act as signaling molecules [62, 63] to upregulate the secretion of anti-inflammatory cytokines, apoptosis of antigen-presenting cells, helper and effector T cells and differentiation of regulatory T cells and anti-inflammatory M2 macrophages [45, 63]. Finally, and more importantly, the anti-inflammatory cytokine IL-10 and free heme induce the production of HO-1 to reinforce the first and second outcomes [45, 63]. HO-1 is a highly inducible enzyme because its gene, HMOX1, is controlled by diverse negative and positive transcription factors that bind to the upstream regulatory region [64]. For example, the binding of Bach1 to antioxidant responsive element (ARE) prohibits the expression of HMOX1, while the binding of Nrf2 to ARE or STAT3 to STAT-binding element (SBE) promotes the expression of HMOX1 [45]. Bach1 and Nrf2 are mutually competitive for binding to ARE. Under non-stress conditions, cytosol protein Keap1 binds to Nrf2 to give Bach1 a great advantage of binding to ARE to suppress the expression of HMOX1 [45]. Under stress conditions, stressors activate the expression of HMOX1 by inducing ROS, leading to the liberation of Nrf2 and degradation of Keap1, and/or by directly binding to Bach1 to dissociate Bach1 from ARE [45]. Free heme is exactly the stressor that not only binds to Bach1, it produces ROS and efficiently induces the expression of HMOX1 [45, 62, 63, 64].

Based on the antagonism and crosstalk between the heme effect and HO effect, a hypothesis can be developed to explain the mode of double concentration-thresholds (Fig. 7). When the concentrations of free heme outside immunocytes are below the first threshold, the concentrations of free heme inside immunocytes will

Fig. 6. Diagram of HO effect. →, promotion. —|, inhibition. ↑, rise. ↓, fall. ⇨, degradation or transformation. ⇢, transport or translocation. AQP, aquaporin. ARE, antioxidant-response element. Bach1, BTB and CNC homology 1. BR, bilirubin. BV, biliverdin. BVR, BV reductase. CD, cluster of differentiation. HMOX1, heme oxygenase 1 gene. HO, heme oxygenase. IL, interleukin. Keap1, Kelch-like ECH-associated protein 1. Maf, transcription factor small Maf. NOX, NADPH oxidase. Nrf2, nuclear factor (erythroid-derived 2)-like 2. ROS, reactive oxygen species. SBE, STAT-binding element. STAT3, signal transducer and activator of transcription 3. TRL4, toll-like receptor 4.

not be over the normal capability of HO-2. Under this condition, the heme effect and HO-2 effect are balanced such that adaptive immunity is unaffected. When the extracellular concentrations of free heme are between the first and second thresholds, the intracellular concentration of free heme will be more than the normal capability of HO-2, but it cannot induce the expression of HO-1. In this case, the heme effect exceeds the HO-2 effect such that adaptive immunity is promoted. When the extracellular concentrations of free heme are more than the
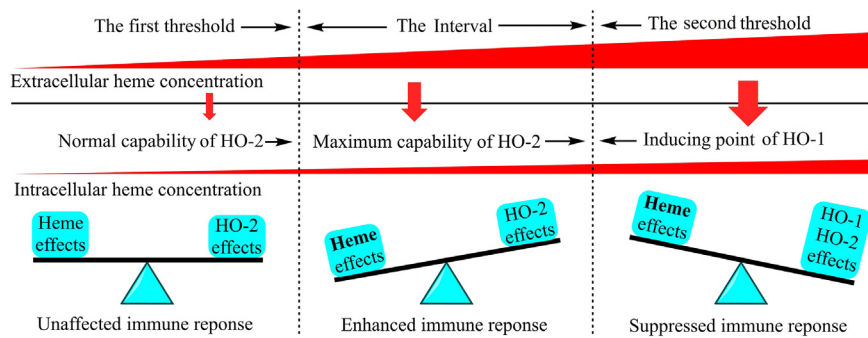
Fig. 7. Possible mechanism of the mode of double concentration-thresholds.

second threshold, the intracellular concentrations of free heme will be able to induce the expression of HO-1. The sum of the HO-1 and HO-2 effects surpasses the heme effect such that adaptive immunity is suppressed.

The way that heme affects adaptive immunity may generally run outside the circulatory system based on the following observations. Free heme can harm cells through directly wedging into biological membranes and modifying proteins, DNAs and lipids by producing reactive oxygen species (ROS) [15]. Therefore, most heme is in the protein-bound form and the concentration of free heme is strictly controlled under physiological conditions [65]. Red blood cells and muscle cells contain the most heme in an animal and tend to increase the level of free heme due to cell renewal or injury. To constrain the level of free heme, beyond balancing its synthesis and degradation, animals evolutionarily develop a buffer system consisting of haptoglobin, hemopexin and some other serum proteins [65]. Haptoglobin, a serum protein primarily produced from the liver, binds hemoglobin or myoglobin to form a high-affinity complex that will then be removed by the reticuloendothelial system. Hemopexin, another serum protein synthesized by the liver, has the highest affinity for heme, and it binds and delivers heme to the liver for further catabolism. Other serum proteins, such as serum albumin, function like hemopexin.

4.4. Immunological significance of this finding

The animaĺs immune system can recognize both exogenous and endogenous danger signals, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [66]. Both PAMPs and DAMPs can activate pattern recognition receptor-dependent signaling pathways to provoke innate immunity and further regulate adaptive immunity [37, 38]. Accordingly, many PAMPs or their analogues have been applied in the design of immunoadjuvants and vaccines [51]. However, there have thus so far been no reports about the effect of a DAMP on the adaptive immune response to an antigen. Free heme is an endogenous danger signal, a DAMP that provokes innate

immunity [35, 36]. To the best of our knowledge, this is the first report that different heme concentrations have different effects on the production of anti-BSA antibodies in rats and mice. This finding not only suggests that free heme can affect adaptive immune responses to an antigen by a mode of double concentration-thresholds, it indicates that DAMPs, such as heme, may be used as a basic ingredient of adjuvants in a similar way as with PAMPs.

Declarations

Author contribution statement

Guofu Li: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Haiyan Xue, Zeng Fan, Yun Bai: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

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