

Hemozoin Enhances Maturation of Murine Bone Marrow Derived Macrophages and Myeloid Dendritic Cells

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ABSTRACT

Background: Falciparum malaria is a severe health burden worldwide. Antigen presenting cells are reported to be affected by erythrocytic stage of the parasite. Malarial hemozoin (HZ), a metabolite of malaria parasite, has adjuvant properties and may play a role in the induction of immune response against the parasite. **Objective:** To determine the immunological impact of hemozoin on the capacity of innate immune cells maturation. **Methods:** *Plasmodium falciparum* (F32 strain) was cultured in O⁺ blood group up to 18% parasitemia. Natural hemozoin was extracted from infected red blood cells. Murine bone marrow derived macrophages and myeloid dendritic cells were stimulated with 4 µg/mL or 40 µg/mL of synthetic hemozoin (β-hematin) or natural hemozoin. We assessed the immunomodulatory role of synthetic or natural hemozoin *in vitro* by flowcytometric analysis. **Results:** The maturation markers MHC-II, CD80 and CD86 were significantly upregulated (p<0.05) on the surface of murine bone marrow derived macrophages or myeloid dendritic cells. Data confirmed the potential of macrophages or myeloid dendritic cells, through hemozoin activation, to establish an innate immune response against malaria parasites. **Conclusion:** Both synthetic and natural hemozoin are potent inducers of cellular immunity against malaria infection. However, natural hemozoin is a stronger inducer as compared to synthetic hemozoin.

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INTRODUCTION

Malaria is a severe health burden worldwide (1,2). Antigen presenting cells (APC) orchestrate innate immune response against hemozoin (HZ), a malaria pigment (3). HZ based innate immune response is facilitated by distinct subsets of dendritic cells (DC) named as plasmacytoid DC (pDC) and myeloid DC (mDC). The pDC secrete type 1 interferons (IFN) and mDC secrete interleukin (IL)-12 against invading pathogens (4). The role of mDC is reported in viral infections, while in malaria infections its role needs more investigation. However, it is already established by *in vitro* experiments that mDC are affected by erythrocytic stage of *Plasmodium falciparum* (*P. falciparum*). This effect is characterized by elevated secretion of IFN- α (5).

Being a ligand for Toll-like receptor 9 (TLR9), HZ is reported as a potential adjuvant to enhance immune effect (6). The capacity of HZ to initiate an antibody response makes it a better option using it as an adjuvant of anti-malarial vaccine candidates (7). Functional disability of HZ-filled macrophage is another enigma to undermine protective immune response against malaria infection (8).

Immune response elicited by parasite derived HZ was reported scarcely. However, data describing the effect of synthetic HZ on APC have been reported as a debatable subject, which need further investigations. Skorokhod described the inhibitory effect of HZ on the functional maturity of DC (9,10) while Coban described that HZ upregulates the maturation markers on the surface of DC (11). Similar data were reported in murine models (12). Although HZ based immune response has been assessed previously, the current study was specifically designed to investigate an *in vitro* effect of parasite derived HZ on the maturation of murine bone marrow derived APC. The study would help to unravel HZ-based functional dichotomy of APC.

MATERIALS AND METHODS

***Plasmodium falciparum* culture.** *P. falciparum* (F32 strain) was cultured by a candle-jar method as described previously (13). Parasite culture was performed using malaria culture medium (MCM) (10.43 g RPMI 1640 powder medium, 0.5% albumax, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 7.5% sodium bicarbonate (Gibco, Invitrogen, Paisley Scotland), 50 mg/L gentamycin and 200 mM hypoxanthine (Sigma, Hamburg, Germany). The study was approved by bio-ethical committee (BEC-FBS-QAU-10), Quaid-i-Azam University.

Synchronization. Ring stage parasites were synchronized with 5% sorbitol (Merck, Darmstadt, Germany) treatment as described previously (14). Parasitemia of culture was quantified by acridine orange as described previously (15).

Hemozoin Extraction. Parasite cultures showing over 10% parasitemia and schizont stage were harvested and subjected to magnetic separation as described previously (16). After three freeze-thaw (-80°C -water bath at 37°C) cycles, lysate was passed through pre-wet (with washing buffer: 2% BSA, 30 min) MACS column (Miltenyi Biotec, Gumpersbach, Germany). Lysate stayed for 20 min in the MACS column and eluted three times with 50 mL washing buffer. Column was dislodged from magnet and retained HZ was eluted with 50 mL washing buffer. Elute was stored in weighed eppendorf tubes and dried at 37°C for three days. Dried pellet was weighed and resuspended in 1 mL sterile distilled water and stored at 4°C .

Cell Culture. Bone marrow was extracted from female BALB/c mice (Taconic Europe, Denmark) of 8-10 weeks old ($n=3/\text{group}$). Macrophages and mDC were generated from bone marrow in 12-well cell culture plates (Corning, NY, USA) as described previously (17). Briefly, 1×10^5 cells were seeded per well in respective media. For the generation of macrophages, complete DMEM (500 mL, supplemented with HEPES 10 mL, sodium pyruvate 5 mL, glutamine 5 mL, PEST 5 mL (Penicillin and Streptomycin, 1:1) was supplemented with 10% fetal calf serum (FCS) and 20% culture supernatant of L929 (a murine aneuploid fibrosarcoma cell line). The mDC were generated in RPMI media, which was supplemented with granulocyte macrophage colony stimulating factor (GM-CSF) (4 ng/mL) and IL-4 (4 ng/mL). Culture plates were incubated at 37°C and 5% CO₂. Media was changed on every third day. Confluence was monitored microscopically. Macrophages and mDC were ready with in 7 and 10 days of culture, respectively.

Stimulation. Macrophages or mDC were sub-cultured (5×10^5 cells/well) and stimulated with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ of synthetic HZ (β -hematin) (sHZ) (Sigma-Aldrich, Steinheim, Netherland) or natural HZ (nHZ). Stimulated culture was maintained at 37°C and 5% CO₂ for 20 h.

Immunophenotyping and Flowcytometric Analysis. Stimulated cells (1×10^5 / tube) were washed twice with PBS and labelled with fluorescent antibodies against maturation markers (MHC-II-PE, CD80-PE, CD86-PE) or identification markers (CD11b-FITC and CD11c-APC). Respective isotype controls were also used (BD Biosciences, Erembodegem, Belgium). Data were acquired and analysed using FACSCalibur (BD, CA, USA) and CellQuest™ Pro (ver. 5.2.1) (BD, CA, USA), respectively. Percentage of positive cells and corresponding mean fluorescence intensity (MFI) were recorded.

Statistical Analysis. Data were analysed by GraphPad Prism (v.5). Two-way ANOVA test was applied and data were considered significant where $p \leq 0.05$.

RESULTS

Parasitemia. Over 15% parasitemia was recorded after 6 days of subculture (Figure 1). Viability was monitored over 98% upto day 6 (data not shown).

Purification of nHZ. After magnetic purification, a total 41.56 mg nHZ was collected from six batches. The nHZ was resuspended as 20 mg/mL and 21.56 mg/mL fractions.

Immunophenotyping of mDC. Bone marrow derived mDC were characterized as CD11b⁺ and CD11c⁺ cells, which were stimulated with nHZ or sHZ (data not shown). A dose dependent maturation was observed in mDC population. Over 50% or 80% mDC expressing MHC-II, CD80 and CD86 were observed after stimulation with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ nHZ, respectively (Figure 2a). Similar trend of expression level of MHC-II, CD80 and CD86 was observed in sHZ-stimulated mDC. Nevertheless, MCH-II molecules were expressed more as compared to CD80 or CD86 molecules on the surface of mDC either stimulated with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ nHZ (Figure 2b). Around 1.5-fold increase was determined in mature cells or expression level of maturation markers on the surface of mDC when stimulated with higher dose (40 $\mu\text{g/mL}$ nHZ) as compared with lower dose (4 $\mu\text{g/mL}$ nHZ).

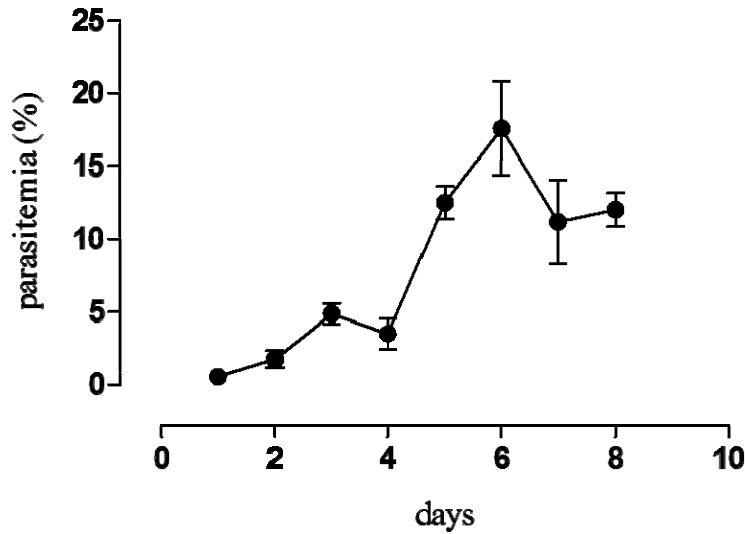


Figure 1. Parasitemia of synchronised subculture. Each data point represents an average parasitemia of six batches of synchronised subcultures of *P. falciparum* (F32 strain) used to extract HZ. Each batch contained 120 mL subculture. Data were calculated as mean \pm SE.

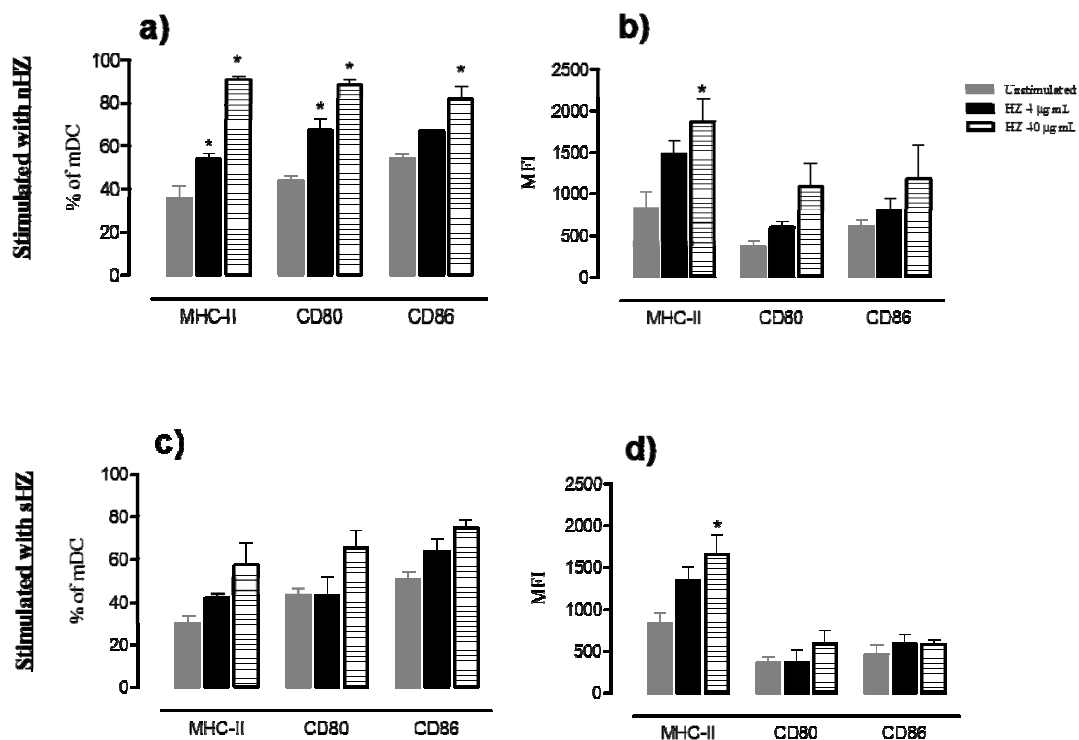


Figure 2. Percentage of positive mDC and corresponding expression level of maturation markers. (a) Percentage of positive mDC and (b) corresponding MFI representing level of expression for MHC-II, CD80 and CD86 after stimulation with 4 µg/mL or 40 µg/mL of nHZ. (c) Percentage of positive mDC and (d) corresponding MFI representing level of expression for MHC-II, CD80 and CD86 after stimulation with 4 µg/mL or 40 µg/mL of sHZ. Data were calculated as mean \pm SE. Steric (*) depicts significance of data where $p \leq 0.05$.

Alternatively, maturation of mDCs was determined after stimulation with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ sHZ (Figure 2c, d). Markedly, sHZ produced moderate effect on the maturation of mDCs. Dose dependency was also observed in this case. Over 55% positive cells for MHC-II, CD80 and CD86 were determined after stimulation with 40 $\mu\text{g/mL}$ sHZ (Figure 2c). However, expression of CD80 and CD86 was less as compared to MHC-II, on the surface of mDC. There was no apparent increase in the expression of CD80 and CD86 after stimulation either with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ sHZ (Figure 2d).

Immunophenotyping of Macrophages. MHC-II expressing macrophages were selected and stimulated with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ of nHZ or sHZ. To assess the maturation, percentage of positive population of macrophages for MHC-II, CD80 and CD86 were determined by flow cytometer. Over 70% macrophages were positive for all maturation markers after stimulation with 40 $\mu\text{g/mL}$ of nHZ (Figure 3a). A corresponding increase in expression of MHC-II, CD80 and CD86 was also observed (Figure 3b). There was no significant increase in percentage of positive population of macrophages or in the expression level of CD80 and CD86 after stimulation with 4 $\mu\text{g/mL}$ of nHZ (Figure 3a, b).

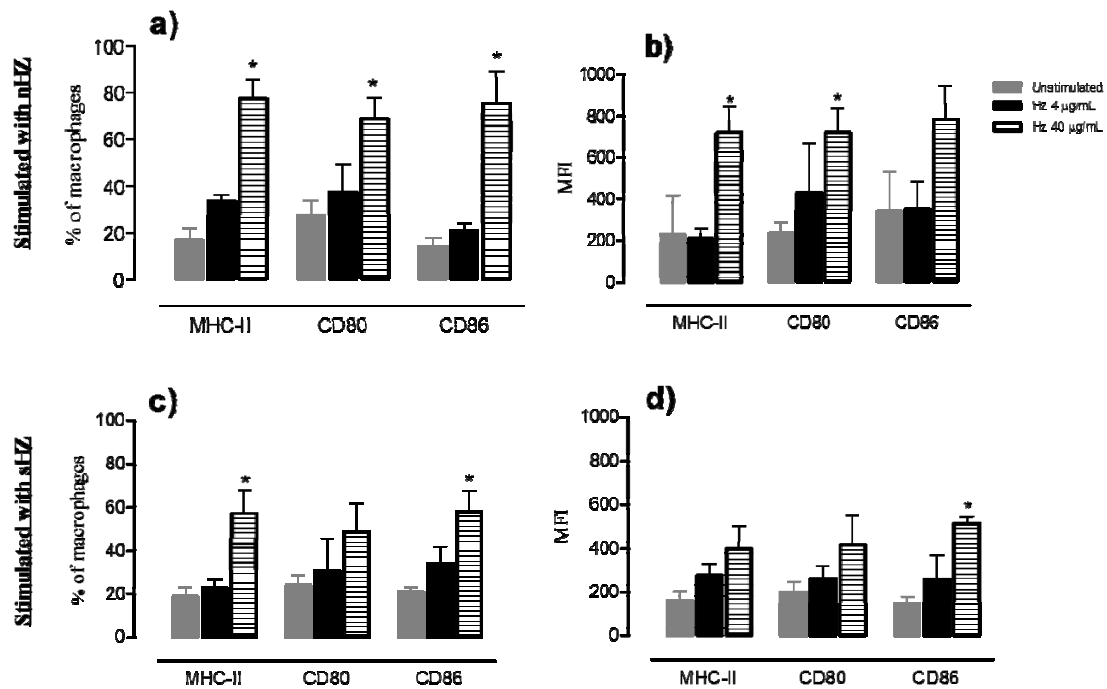


Figure 3. Percentage of positive macrophages and corresponding expression level of maturation markers. (a) Percentage of positive macrophages and (b) corresponding MFI representing level of expression for MHC-II, CD80 and CD86 after stimulation with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ of nHZ. (c) Percentage of positive macrophages and (b) corresponding MFI representing level of expression for MHC-II, CD80 and CD86 after stimulation with 4 $\mu\text{g/mL}$ or 40 $\mu\text{g/mL}$ of sHZ. Data were calculated as mean \pm SE. Steric (*) depicts significance of data where $p \leq 0.05$.

However, percentage of positive macrophages was also increased with stimulation of 40 $\mu\text{g/mL}$ of sHZ and a corresponding increase in expression of all maturation markers was also evident (Figure 3c, d).

DISCUSSION

It is reported that APCs recognize malaria parasite or its components and deliver innate immune response against malaria infection (16). Nevertheless, functional assessment of APC after exposure to malaria parasite or its components is an inconclusive area of malariology. The present study was designed where APCs (mDC and macrophages) were generated from bone marrow of BABL/c mice and subsequently the effect of both sHZ and nHZ was investigated on mDC and macrophages. The model deemed suitable, as it was not reported earlier, to answer the unsettled functionality of APC against *P. falciparum* or its components.

Previously reported data have shown that malaria parasite infected red blood cells (iRBC) or parasite components suppress the immune function of DC. Subsequently, DCs become unable to bridge the adaptive immune response. Nevertheless, the data shown previously are found inconsistent with present findings. Elevated expression of MHC-II and co-stimulatory molecules (CD80 and CD86) on the surface of DC confirmed the retention of antigen presenting capacity and potential to train naïve T cells, respectively. Expression of co-stimulatory molecules on the surface of DC indicated its contact potential with iRBC.

Parasitemia level ensured the contact potential of iRBC with mDC. It ensured the downstream protective immune response against malaria parasite. In the current study, parasitemia level was corresponding to the number of schizonts (after synchronization), which in turn produced large amount of HZ in iRBC. Capacity of HZ to modulate immune response might be a better option to understand its synergistic effect with potential anti-malarial vaccine candidates.

The role of HZ as a pathological agent to establish severe malaria, as reported elsewhere(18), was ruled out by our findings. Previous reports demonstrate that HZ-filled macrophages become dysfunctional (8). However, percentages of positive macrophages and corresponding expression of maturation markers demonstrated their intact function.

Intensity of innate immune response depends on the type of parasite strain. Different malaria parasite strains can opt for a variety of receptors to invade RBC. It corresponds to differential immune response (19). F32 parasite strain used in present study might be the cause of an elevated immune response in this case. F32 parasite produced large quantity of HZ inside food vacuole. Moreover, F32 iRBC might have better contact potential with corresponding innate immune cells.

Number of iRBC is critical to enhance or suppress the immune response. Lower doses of parasites (i.e. iRBC) enhance the immune response against malaria infection. Higher parasitemia conditions suppress the downstream immune response (20). Our data supported these findings. Maturation of mDC or macrophages by HZ was a result of lower parasitemic RBC. As over activation of mDC by TLR renders it dysfunctional.

In conclusion, current data demonstrated that malaria pigment (HZ) induces the maturation of macrophages and mDC. HZ acts as a potent inducer of cellular immune response against *falciparum* malaria. Immunomodulatory function of HZ warrants

further investigations in its role as an agent of protective immunity against malaria infection.

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