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# Malaria-derived hemozoin exerts early modulatory effects on the phenotype and maturation of human dendritic cells

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## Summary

***Plasmodium falciparum* (*P. falciparum*)-induced effects on the phenotype of human dendritic cells (DC) could contribute to poor induction of long-lasting protective immunity against malaria. DC ability to present antigens to naïve T cells, thus initiating adaptive immune responses depends on complex switches in chemokine receptors, production of soluble mediators and expression of molecules enabling antigen-presentation and maturation. To examine the cellular basis of these processes in the context of malaria, we performed detailed analysis of early events following exposure of human monocyte-derived DC to natural hemozoin (nHZ) and the synthetic analog of its heme core, β-hematin. DC exposed to either molecule produced high levels of the inflammatory chemokine MCP-1, showed continuous high expression of the inflammatory chemokine receptor CCR5, no upregulation of the lymphoid homing receptor CCR7 and no cytoskeletal actin redistribution with loss of podosomes. DC partially matured as indicated by increased expression of major histocompatibility complex (MHC) class II and CD86 following nHZ and β-hematin exposure, however there was a lack in expression of the maturation marker CD83 following nHZ but not β-hematin exposure. Overall our data demonstrate that exposure to nHZ partially impairs the capacity of DC to mature, an effect in part differential to β-hematin.**

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## Introduction

To date, *Plasmodium falciparum* (*P. falciparum*) malaria is still one of the main global causes of death from infectious disease. Immunity to malaria is complex, develops only after repeated exposure, is not long-lasting (Langhorne *et al.*, 2008) and is both species and stage-specific (Todryk and Urban, 2008). The innate immune response to malaria is of pivotal importance. It is the first line of defence in combating the parasite, and it shapes the parasite-specific adaptive immunity (Olivier *et al.*, 2014). Dendritic cells (DC) are professional antigen-presenting cells and fundamental components of the innate immune response. One of their most important functions is to process and present antigens to naïve T cells thus initiating the adaptive immune response. DC must migrate to sites of inflammation and infection, where antigen sampling takes place. The migration to infected sites is governed by a gradient of various chemokines and the expression of C–C chemokine receptors (CCR) as well as the switch between various CCR (Sallusto *et al.*, 1999). Immature DC produce large amounts of inflammatory chemokines, such as monocyte chemoattractant protein (MCP)-1, in response to microbial stimuli. The chemokine production is rapidly induced and regulates further recruitment of more immature DC and other immune cells to the infected site (Lebre *et al.*, 2005). CCR5 is highly expressed by immature DC and regulates their trafficking. Upon maturation, DC down-regulate the CCR5 expression and instead up-regulate the expression of the lymphoid-homing CCR7. CCR7 expression enables maturing DC to migrate to secondary lymphoid tissues where adaptive immune responses can be initiated (Banchereau *et al.*, 2000).

The maturation process of DC is accompanied by morphological changes where one example is the loss of podosomes (Burns *et al.*, 2004). Podosomes are multifunctional actin-rich cylindrical adhesion structures that can accumulate at the leading edge of motile cells. Podosomes are important in cell migration, invasion and passage through endothelium and epithelium (Linder and Wiesner, 2015; Skorokhod *et al.*, 2014) and have been shown to facilitate antigen sampling by DC (Baranov *et al.*, 2014). Proper antigen presentation by DC is a requirement for potent priming of naïve T cells in secondary lymphoid tissues. This includes expression of high levels of major histocompatibility complex (MHC) class II molecules on the

cell surface, up-regulation of co-stimulatory molecules, such as CD86, and up-regulation of molecules associated with maturation such as CD83 (Banchereau *et al.*, 2000).

Natural hemozoin (nHZ), also known as malaria pigment, is an insoluble crystalline product of the haemoglobin degradation in the parasite-digestive vacuole. nHZ is produced by the *Plasmodium* parasite as a strategy to detoxify free heme, which is harmful for the parasite (Shio *et al.*, 2010). nHZ is released into the circulation during the lysis of mature parasite-infected erythrocytes and contains besides crystalline heme, lipids, bioactive lipoperoxidation products and proteins of both parasite and host origin. DC and other antigen-presenting cells such as monocytes have been shown to phagocytose nHZ (Schwarzer *et al.*, 1993; Coban *et al.*, 2002; Kremsner *et al.*, 2009). β-hematin, also known as synthetic hemozoin, can be considered as an analogue for nHZ, although β-hematin consists exclusively of the crystalline polyheme core (Arese *et al.*, 2014). Despite structural similarities between nHZ and β-hematin, the crystal size, shape and chemical composition are not identical, which can lead to differences in the immunological response induced by the two molecules (Coronado *et al.*, 2014). There are contradictory results from various studies concerning the modulatory effect of hemozoin, both natural and synthetic, on DC differentiation, maturation and functionality (Coban *et al.*, 2002; Skorokhod *et al.*, 2004; Millington *et al.*, 2006; Giusti *et al.*, 2011; Pinzon-Charry *et al.*, 2013). Less is known about how the early effects of nHZ manifests themselves in the maturation of DC and how DC respond to unmodified nHZ, because many studies addressing the effect of hemozoin use highly purified hemozoin molecules.

In the present study, we report that exposure to nHZ induces early effects at a transcriptional level of important genes for chemokines and co-stimulatory molecules, affects cell surface receptor expression of chemokine receptors and molecules important in antigen presentation, induces chemokine protein secretion and overall impairs the capacity of DC to mature and to activate T cells. The study also aimed at comparing the response of nHZ with that of β-hematin and lipopolysaccharide (LPS), a well-known DC activator found in the outer membrane of gram-negative bacteria. Our results demonstrate that DC exposed to nHZ display a partial impairment in their capacity to mature.

## Results

### *Natural hemozoin and β-hematin exposure leads to mRNA induction and protein secretion of monocyte chemoattractant protein-1*

MCP-1 is a chemokine of great importance for the chemotaxis of immature DC to affected sites for antigen sampling. We investigated the ability of DC to induce MCP-1 in the early response to nHZ and β-hematin at different time points, from 2 up to 24 h of exposure. Firstly, the transcriptional effects of nHZ and β-hematin were investigated. DC treated with nHZ showed a significant increase in MCP-1-specific mRNA levels compared with unstimulated control at 2 h, as did β-hematin. However, β-hematin exposure tended to be a more potent inducer of MCP-1 mRNA compared with nHZ ($P = 0.06$). After 4 h, both nHZ and β-hematin exposure induced significant increases in MCP-1 mRNA levels compared with unstimulated control (Fig. 1A). After 8 h and onwards, there were no detectable increases in MCP-1 mRNA levels following exposure to either nHZ or β-hematin (data not shown). LPS, used as a positive control, induced significant increases in MCP-1 mRNA levels after 2 h compared with unstimulated control (Fig. 1A). There was no further induction following LPS stimulation

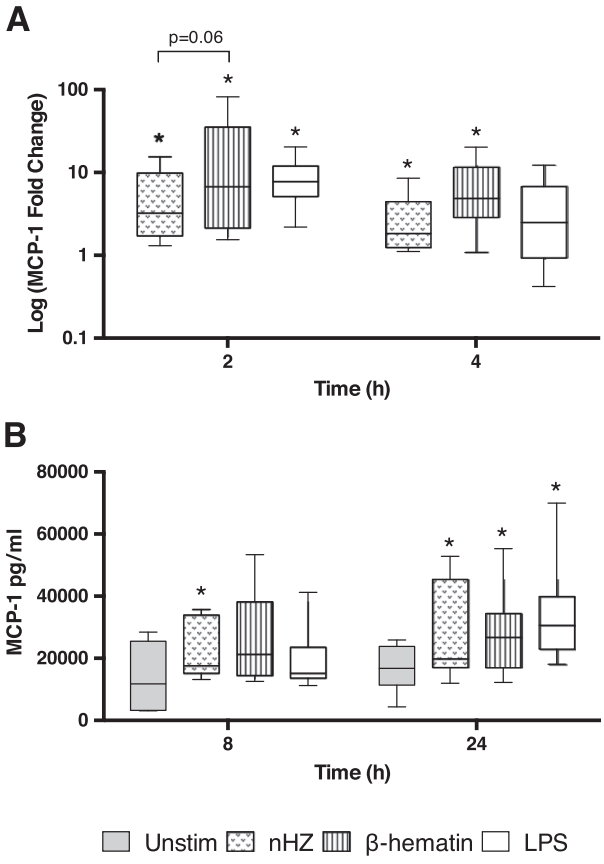


**Fig. 1.** MCP-1 induction after nHZ and β-hematin exposure. Human monocyte-derived DC were either exposed to nHZ or β-hematin, stimulated with LPS or maintained in complete medium (unstimulated) for 2–24 h as described in experimental procedures.
A. mRNA levels of MCP-1 were determined by qPCR ($n = 6$).
B. MCP-1 secretion in cell culture supernatants was determined by ELISA ($n = 5$–7). The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10–90 percentile. Asterisks indicate significant differences in corresponding time points versus unstimulated control (*$P < 0.05$).

at any of the time points measured (data not shown). To assess if the increase in mRNA levels of MCP-1 was translated into an increase in MCP-1 production, the secreted amounts of MCP-1 were measured in culture supernatants. nHZ exposure induced a significant increase of secreted MCP-1 8 and 24 h after exposure compared with unstimulated control (Fig. 1B). DC treated with β-hematin secreted significantly higher amounts of MCP-1 24 h after treatment, as did DC stimulated with LPS.

These data conclude that nHZ and β-hematin exposure induces expression of the chemokine MCP-1, an inflammatory chemokine with the potential to attract immature DC to sites of inflammation or infection. The effect was observed at both mRNA and protein level.

### *Natural hemozoin and β-hematin-exposed dendritic cells show a continuously high percentage of CCR5$^+$ cells but no up-regulation of CCR5 or CCR7-specific mRNA levels*

Next, we investigated whether DC were able to progress from immature stages characterized by high expression of inflammatory chemokine receptors, such as CCR5, to more mature stages characterized by high expression of CCR7 in response to nHZ and β-hematin. DC were assessed for CCR5 and CCR7 mRNA expression and percentage of CCR5$^+$ and CCR7$^+$ cells. There were no detectable differences in mRNA levels of CCR5 between the different stimuli at any of the time points measured (Fig. 2A). Treatment with nHZ did not affect the percentage of CCR5$^+$ cells, which continued to be high at 4, 8 and 24 h after exposure (Fig. 2B). Exposure to β-hematin showed similar results as nHZ, although β-hematin tended to be more potent in down-regulating the percentage of CCR5$^+$ cells 24 h after treatment as compared with nHZ and unstimulated control ($P = 0.06$). Stimulation with LPS gradually reduced the percentage of DC expressing CCR5, an effect initiated early on during stimulation (Fig. 2B). The continuous high levels in the percentage of CCR5$^+$ cells observed in nHZ and β-hematin-exposed DC are probably not coupled to transcriptional changes of CCR5 mRNA expression.

A significant increase in CCR7 mRNA levels following stimulation with LPS was detectable at 4 h and stable for up to 24 h after stimulation compared with unstimulated control. This effect was not observed in DC exposed to nHZ or β-hematin (Fig. 2C). The transcriptional response was however not reflected in the percentage of CCR7$^+$ cells, which did not differ between the different stimuli at any of the time points measured (Fig. S1).

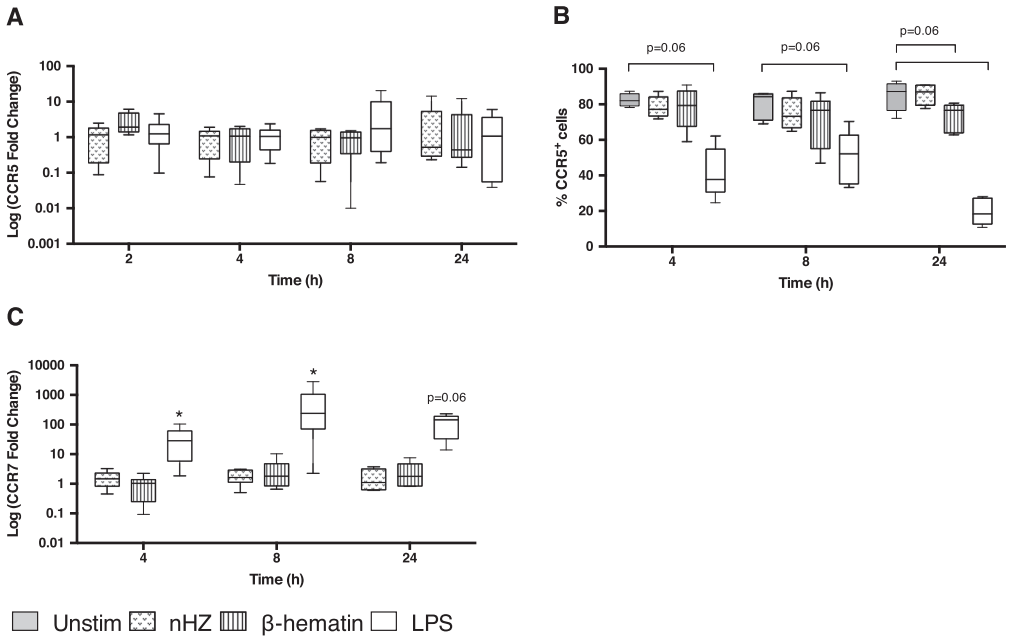


**Fig. 2.** Continuous high percentage of CCR5$^+$ cells and no up-regulation of CCR7 expression following nHZ and β-hematin exposure. DC were either exposed to nHZ or β-hematin, stimulated with LPS or maintained in complete medium (unstimulated) for 2–24 h as described in experimental procedures.
A. mRNA levels of CCR5 were determined by qPCR ($n = 6$).
B. The percentage of CCR5$^+$ cells was determined by flow cytometry ($n = 5$).
C. mRNA levels of CCR7 were determined by qPCR ($n = 6$). The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10–90 percentile. Asterisks indicate significant differences in corresponding time points versus unstimulated control (*$P < 0.05$).

Taken together, these data indicate that DC exposed to nHZ and β-hematin remain immature in the context of chemotactic properties.

### *Natural hemozoin and β-hematin-exposed dendritic cells have a high abundance of podosomes*

Podosomes have the potential to render cells more adhesive to the underlying extracellular matrix, and the loss of podosomes is a hallmark morphological marker of DC maturation. To assess the effect of nHZ on DC podosome dynamics, F-actin filaments were stained and analysed for the presence of podosomes. Before analysing podosome turnover, the uptake of nHZ by DC and its intracellular localization was analysed. A representative picture depicts nHZ localization in exposed DC (Fig. 3A). nHZ and β-hematin-exposed DC were consistently characterized by the presence of podosomes unlike DC stimulated with LPS, where the podosomes were lost. Unstimulated cells were also characterized by the presence of podosomes (Fig. 3B). After 24 h, DC exposed to nHZ and β-hematin had a high percentage of podosomes, comparable with unstimulated cells and unlike cells stimulated with LPS (Fig. 3C).

These data show that exposure to nHZ and β-hematin is not accompanied by morphological changes known to be associated with DC maturation, such as loss of podosomes.

### *Up-regulation of major histocompatibility complex class II and CD86 after natural hemozoin and β-hematin exposure*

One of the most important functions of DC is to present antigens to naïve T cells, which they do in the context of MHC class II molecules and co-stimulatory molecules such as CD86.

Neither nHZ nor β-hematin or LPS showed any significant effects on the MHC class II β-subunit mRNA levels over the course of 24 h compared with unstimulated control (Fig. S2) in accordance with previous studies where it was shown to be constitutively expressed (Choi *et al.*, 2011). However, exposure to nHZ and β-hematin led to significant up-regulation of the cell surface expression of MHC class II, an effect initiated already 4 h after exposure and stable for up to 24 h compared with unstimulated control (Fig. 4A). Stimulation with LPS led to significant up-regulation of MHC class II cell surface expression at all indicated time points compared with unstimulated control (Fig. 4A).

Next, we investigated CD86-specific mRNA levels. Higher CD86 mRNA levels were seen 2–4 h after β-hematin exposure while only at 4 h after nHZ exposure. β-hematin exposure was a more potent inducer of CD86 mRNA levels 4 h after exposure compared with nHZ. Stimulation with LPS led to significant induction of CD86 mRNA levels at all measured time points with the exception of 4 h (Fig. 4B). When we compared the percentage of $CD86^+$ cells in nHZ

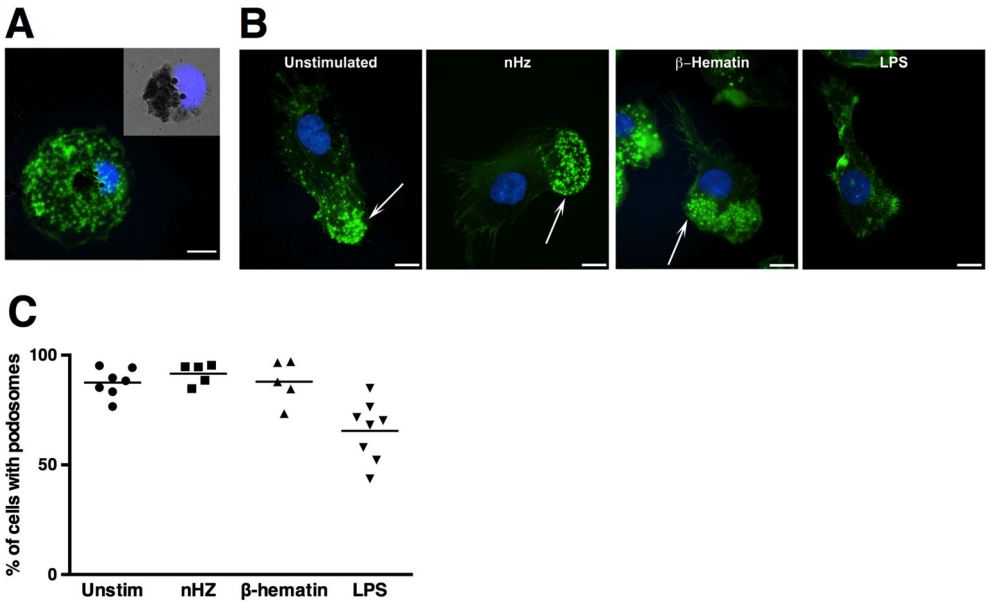


**Fig. 3.** High podosome abundance after nHZ and β-hematin exposure. DC were exposed to nHZ or β-hematin for 24 h. LPS was used as a positive control, and unstimulated cells were used as a negative control. DC were stained with Alexa Fluor 488 phalloidin to detect F-actin as described in experimental procedures.
A. A representative micrograph depicts nHZ phagocytosis and localization. Scale bar = 10 μm.
B. Actin condensation in podosome structures is indicated by arrows in the representative micrographs. Scale bar = 10 μm.
C. The graph depicts the percentage of cells with podosomes for each condition related to the total cell population. For each condition, a total of 80 cells were analysed from five to eight different donors. The horizontal line represents the mean value.

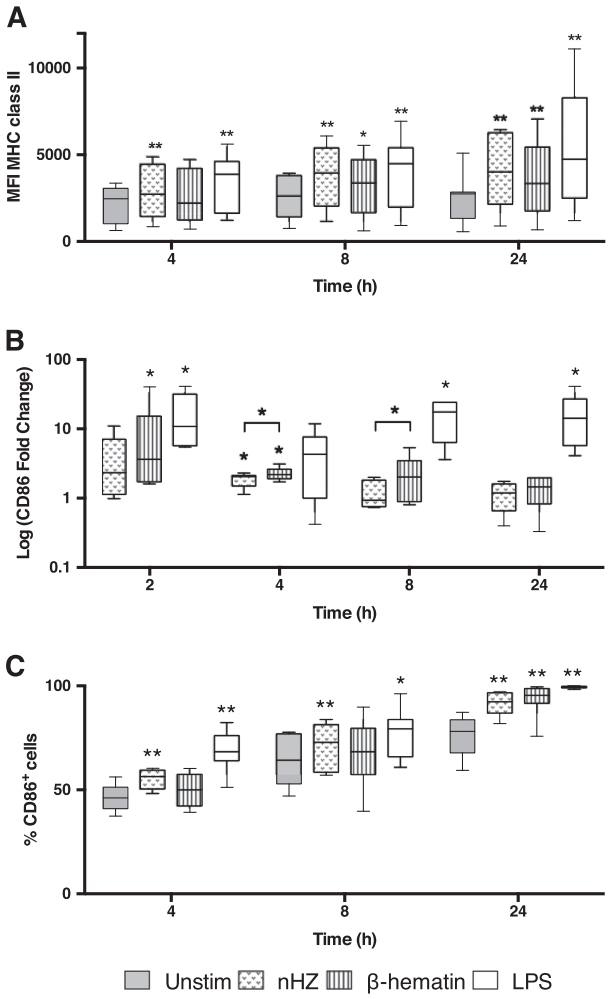


**Fig. 4.** Differential up-regulation of MHC class II and CD86 following nHZ and β-hematin exposure. DC were either exposed to nHZ or β-hematin, stimulated with LPS or maintained in complete medium (unstimulated) for 2–24 h as described in experimental procedures.
A. The mean fluorescence intensity (MFI) of MHC class II was determined by flow cytometry ($n = 9$).
B. mRNA levels of CD86 were determined by qPCR ($n = 6$).
C. The percentage of $CD86^+$ cells was determined by flow cytometry ($n = 8$).
The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10–90 percentile. Asterisks indicate significant differences in corresponding time points versus unstimulated control and between nHZ and β-hematin ($^*P < 0.05$ and $^{**}P < 0.01$).

and β-hematin-exposed DC with unstimulated control, only nHZ led to a significant increase 4 and 8 h after exposure. 24 h after treatment, there was an increase in $CD86^+$ cells following both nHZ and β-hematin exposure compared with unstimulated control (Fig. 4C). Stimulation with LPS led to significant increases in the percentage of $CD86^+$ cells at all indicated time points.

These data indicate a partial activation of DC in the context of molecules important in antigen presentation following exposure to nHZ and β-hematin. This was in contrast to what was observed after LPS stimulation.

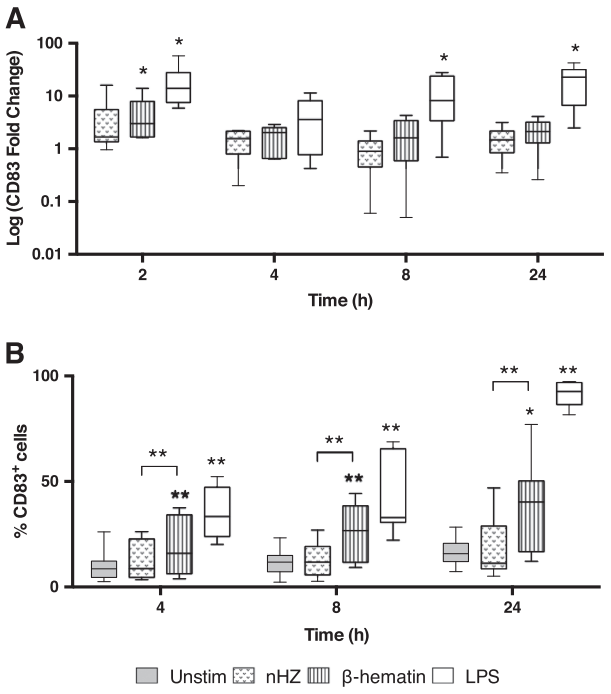


**Fig. 5.** Natural hemozoin-exposed DC have an immature phenotype. DC were either exposed to nHZ or β-hematin, stimulated with LPS or maintained in complete medium (unstimulated) for 2–24 h as described in experimental procedures.
A. mRNA levels of CD83 were determined by qPCR ($n = 6$).
B. The percentage of $CD83^+$ cells was determined by flow cytometry ($n = 9$).
The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10–90 percentile. Asterisks indicate significant differences in corresponding time points versus unstimulated control and between nHZ and β-hematin ($^*P < 0.05$ and $^{**}P < 0.01$).

### *Natural hemozoin exposure restrains dendritic cells from acquiring a mature CD83 expressing phenotype*

During the DC maturation process, high expression of CD83 is necessary for potent activation of naïve T cells. There was no induction of CD83 following exposure to nHZ at any of the indicated time points either at the transcriptional or protein level (Fig. 5A and B). Following treatment with β-hematin, a significant induction of CD83 mRNA was observed after 2 h. Stimulation with LPS led to significant and stable increase of CD83 mRNA levels over time (Fig. 5A). Unlike after nHZ exposure, β-hematin led to significant increases in the percentage of $CD83^+$ cells compared with unstimulated control 4 and 8 h after exposure. Furthermore, β-hematin was significantly more potent in inducing increased percentages of $CD83^+$ cells at all indicated time points compared with nHZ. DC stimulated with LPS showed a significant and stable increase in the percentage of $CD83^+$ cells over time (Fig. 5B).

From these data, we conclude that exposure to nHZ neither lead to transcriptional activation of CD83 nor an

increase in the percentage of CD83$^+$ cells; thus, the nHZ-exposed DC do not acquire a mature phenotype. The opposite is observed for β-hematin-exposed DC where they acquire a mature phenotype.

*Natural hemozoin exposure inhibits the loss of podosomes and impairs the up-regulation of CD83 induced by lipopolysaccharide stimulation*

To elucidate whether the inability of nHZ to fully activate DC is because nHZ has the potential to actively inhibit important features of DC maturation, we investigated the ability of nHZ to interfere with LPS-induced DC maturation. nHZ exposure of DC prior to LPS stimulation inhibited the loss of podosomes as compared with DC that had not been exposed to nHZ (Fig. 6A). DC exposed to nHZ prior to LPS stimulation were significantly impaired in their ability to up-regulate CD83 on the cell surface compared with DC that had not been exposed to nHZ (Fig. 6B). No differences were observed for the percentage of MHC class II$^+$, CD86$^+$, CCR5$^+$ and CCR7$^+$ DC or the secreted amounts of MCP-1 for DC matured with LPS in the presence or absence of nHZ (data not shown).

We conclude that nHZ seemed to selectively inhibit the loss of podosomes and significantly inhibited the up-regulation of CD83 in DC stimulated with LPS, a potent activator of DC maturation.

*Natural hemozoin exposure of dendritic cells does not lead to activation of T cells*

Whether nHZ-induced impairment of DC maturation could subsequently prevent T-cell activation was investigated in co-cultures of DC exposed or not to nHZ and autologous T cells. T-cell activation was assessed by IL-2 and IFN-$\gamma$ production in cell culture supernatants. DC exposed to nHZ and subsequent co-cultured with T cells and stimulated with the artificial superantigen CytoStim yielded similar production of IL-2 (Fig. 7A) and IFN-$\gamma$ (Fig. 7B) as DC that had not been exposed to nHZ. In regard to IL-2, there was greater variation, but there was no statistical difference between DC exposed or not to nHZ in CytoStim-stimulated co-cultures ($P = 0.16$). When the co-cultures were stimulated with CD3/CD28 beads, nHZ-exposed DC inhibited the production of IL-2 to the same extent as DC that had not been exposed to nHZ (Fig. 7A), while nHZ exposure led to significant inhibition of IFN-$\gamma$ production compared with DC that had not been exposed to nHZ (Fig. 7B). Thus, we conclude that DC exposed to nHZ do not activate T cells.

## Discussion

In this study, we demonstrate that human monocyte-derived DC early on during exposure to nHZ kept their characteristic features of immature DC, such as high expression of CCR5, and did not acquire key features associated with a mature phenotype, such as high expression of CD83.

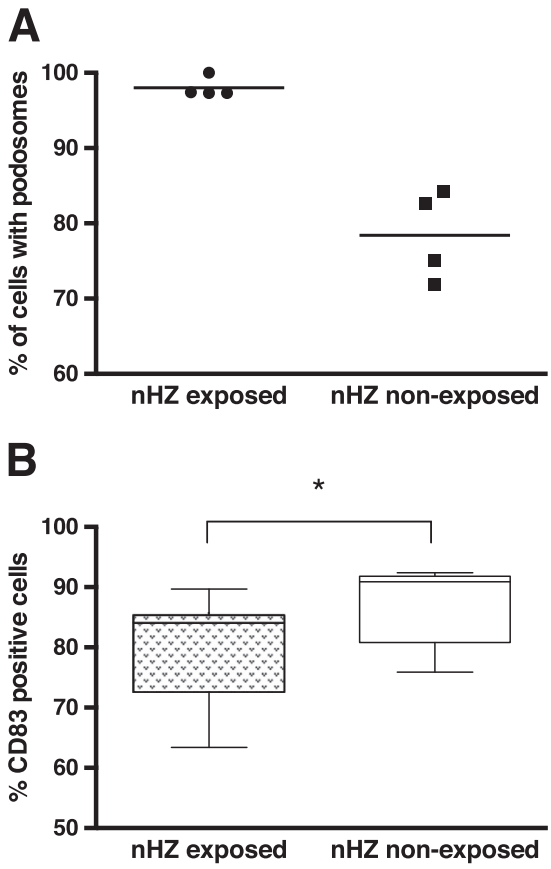


**Fig. 6.** Natural hemozoin inhibits the loss of podosomes and impairs the up-regulation of CD83 upon DC maturation. DC were incubated with or without nHZ for 4 h before maturation was induced by addition of LPS. The cells were incubated for an additional 24 h (A) and 18 h (B).
A. The percentage of cells with podosomes was investigated by staining with Alexa Fluor 488 phalloidin to detect F-actin as described in experimental procedures. For either condition, a total of 40 cells were analysed from four different donors. The horizontal line represents the mean value.
B. The percentage of CD83$^+$ cells was determined by flow cytometry ($n = 7$). The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10–90 percentile. Asterisks indicate significant differences ($^*P < 0.05$).

It is known that DC present at the site of infection or inflammation constitute an important source of inflammatory cytokines, such as MCP-1 (Sozzani, 2005). We show that nHZ and β-hematin exposure had an activating effect on MCP-1 levels. Similar effect on mRNA levels of MCP-1 has been shown in murine macrophages following nHZ and β-hematin exposure (Jaramillo *et al.*, 2005). Sallusto *et al.* (1998) showed that immature DC express chemokine receptors specific for inflammatory chemokines, such as CCR2 whose ligand is MCP-1. Taken together, our data show that DC rapidly after nHZ and β-hematin exposure produce MCP-1, a chemokine

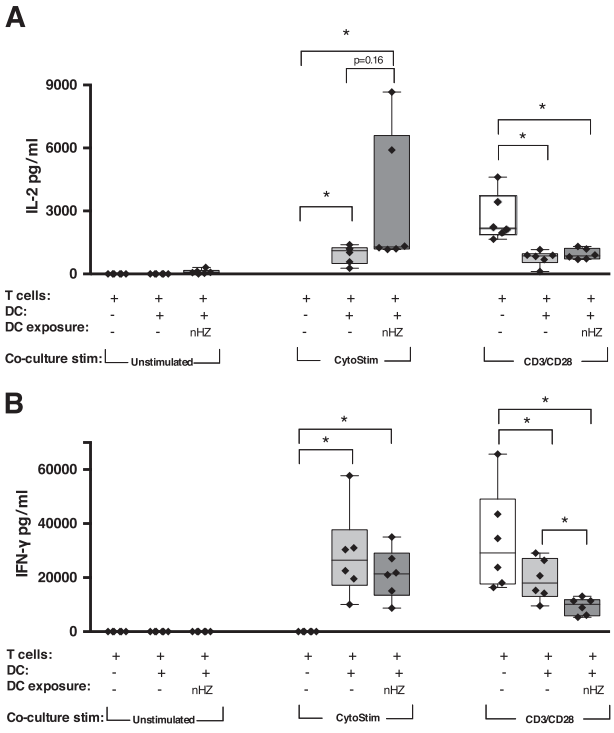


**Fig. 7.** Natural hemozoin exposure of DC does not lead to activation of T cells. DC were incubated with or without nHZ for 24 h before they were co-cultured with autologous $CD3^+$ T cells.
The co-cultures were stimulated with CD3/CD28 beads, CytoStim or left unstimulated for another 24 h after which cell culture supernatants were collected.
A. Analysis of IL-2 production by ELISA ($n = 6$).
B. Analysis of IFN-$\gamma$ production by ELISA ($n = 6$).
The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the minimum and the maximum.
Asterisks indicate significant differences ($^*P < 0.05$).

secreted by immature DC with the potential to attract more immature DC and other cells to the affected site.

During the maturation process of DC, there is a switch in the expression of CCR5, characteristic of immature DC, to the expression of CCR7, a hallmark marker for mature DC. This switch is not evident in DC after exposure to nHZ as we clearly demonstrate that DC continue to have a high and stable expression of $CCR5^+$ cells in the early response against nHZ unlike DC stimulated with LPS. The high percentage of $CCR5^+$ cells observed after nHZ exposure was not the result of transcriptional changes. Exposure to β-hematin appeared to more potently down-regulate the percentage of cells expressing CCR5 compared with nHZ and unstimulated control after 24 h. β-hematin has been shown to significantly decrease the percentage of $CCR5^+$ cells after 48 h of exposure (Diou *et al.*, 2010). The difference in response between nHZ and β-hematin suggests that the down-regulation of CCR5 positive cells is not due to the polyheme core itself. nHZ and β-hematin differ substantially in their chemical composition that explains differences in the elicited immunological response. While β-hematin composes exclusively of a polyheme crystal, nHZ is considered to be the natural uptake for phagocytes *in vivo* and was obtained after the expulsion of the digestive vacuole during schizogony (Barrera *et al.*, 2011). This nHZ is composed of a β-hematin core that is enveloped in membrane structures and carries firmly adherent bioactive molecules of parasite and host origin. These molecules have been shown to modify several cellular immune functions by binding to receptors or modifying functionally relevant intracellular target molecules during and after phagocytosis as reviewed in several papers (Olivier *et al.*, 2014; Tyberghein *et al.*, 2014). In our study, no differences between nHZ, β-hematin and unstimulated cells were observed with regard to in CCR7 mRNA expression and the percentage of $CCR7^+$ cells. This was in contrast to LPS stimulation where a clear transcriptional induction occurs. This supports our hypothesis that especially nHZ maintains DC in an immature state. Furthermore, various studies of patients with acute malaria infection have shown that the frequency of myeloid and plasmacytoid DC in peripheral blood was reduced compared with healthy controls (Pinzon-Charry *et al.*, 2013; Pichyangkul *et al.*, 2004). Our findings of high induction of MCP-1 production and continuous high percentage of $CCR5^+$ cells following nHZ exposure could in part help to explain the reduction of DC seen in peripheral blood of malaria-infected patients.

Maturation stimuli, such as LPS, induce a loss of podosomes thus reducing cell-matrix adhesion and enhancing fast migration (van Helden *et al.*, 2006). Podosomes have been shown to be critical components of immature DC. Our data demonstrate a high abundance of podosomes in DC exposed to nHZ in contrast to those stimulated with LPS, suggesting that nHZ exposure renders the DC immature and more adhesive to the underlying extracellular matrix. nHZ exposure not only leads to high abundance of podosomes but also selectively inhibits the loss of podosomes upon DC maturation. Taken together, we propose that the high MCP-1 production and the high percentage of $CCR5^+$ cells seen in DC exposed to nHZ can facilitate migration and recruitment of other immature DC to peripheral tissues for antigen sampling. However, the lack of CCR7 expression and the high abundance of podosomes suggest that the further migration to secondary lymphoid tissues for antigen presentation is impaired.

High expression of MHC class II molecules on the cell surface is important in antigen presentation to naïve T cells. A further key feature of potent antigen presentation is the high cell surface expression of the co-stimulatory molecule CD86. nHZ and β-hematin exposure did early on, and stably over time, increase the expression of MHC class II molecules. Furthermore, the present data demonstrate that DC exposed to nHZ and β-hematin up-regulate CD86, both transcriptionally and translationally,

where the translational effect was seen at earlier time points following nHZ exposure compared with β-hematin. Conflicting data have been reported on the expression of MHC class II (Millington *et al*., 2007; Bettiol *et al*., 2010) and CD86 (Coban *et al*., 2002) and impairment of MHC class II increase (Skorokhod *et al*., 2004; Skorokhod *et al*., 2005) and CD86 (Millington *et al*., 2006) following hemozoin exposure. Discrepancies between our data and previous data could, in part, be explained by the experimental set-up, where we have focused on the very early effects of nHZ and β-hematin. Our data suggest an activation of DC in the context of MHC class II molecules and CD86 following nHZ and β-hematin exposure. However, previous studies have shown that uptake of β-hematin by DC modulates their ability to potently interact with T cells despite normal levels of MHC class II on their cell surface (Millington *et al*., 2007). The effect of MHC class II and CD86 is important in the immunological response to *P. falciparum* infection as shown by previous findings that *P. falciparum*-infected patients have impaired cell surface expression of MHC class II and CD86 compared with healthy controls (Pinzon-Charry *et al*., 2013).

CD83 is a hallmark marker of mature DC and is of great importance in the immunological response, although the function of CD83 is still debated as reviewed in Prazma and Tedder (2008). For example, $CD83^{-/-}$ mice have been shown to be defective in their T-cell development (Fujimoto *et al*., 2002), and blocking CD83 expression in DC led to impaired allogenic T-cell proliferation (Aerts-Toegaert *et al*., 2007). We clearly demonstrate that nHZ did not up-regulate the mRNA expression of CD83 or increase the percentage of $CD83^{+}$ cells compared with unstimulated control. Others have shown an up-regulation of CD83 following exposure to purified hemozoin (Coban *et al*., 2002). Discrepancies could partly be due to the preparation of the hemozoin itself. Our findings demonstrate that nHZ does not induce proper DC maturation. That CD83 expression is important *in vivo* in the context of malaria is shown by the decreased levels of CD83 expression in patients with acute *P. falciparum* infection compared with healthy controls (Pinzon-Charry *et al*., 2013). In contrast to nHZ, β-hematin exposure up-regulates both CD83 mRNA expression and increases the percentage of $CD83^{+}$ cells. Why nHZ and β-hematin differ in their ability to modulate DC responses, as shown in the response of CCR5 and CD83, is not yet fully understood. One explanation could be that non-heme compounds, such as 15-hydroxyeicosatetraenoic acid and 4-hydroxynonenal (4-HNE), characteristic for nHZ, are absent in β-hematin (Skorokhod *et al*., 2005; Schwarzer *et al*., 2003). It has been shown that when nHZ-exposed human monocytes where differentiated and matured into DC *in vitro*, the DC exhibited impaired cell surface expression of CD83. In addition, nHZ exposure of human monocytes led to impairment of actin polymerization. Both effects were attributed to 4-HNE (Skorokhod *et al*., 2014; Skorokhod *et al*., 2005). Taken together, one can hypothesize that the bare crystal (i.e. β-hematin) or the lipid/protein-enveloped crystal (i.e. nHZ) could modulate DC via different signalling pathways.

As LPS induced CD83 expression both on the transcriptional level and on the cell surface, we investigated the effect of nHZ on LPS activation. We clearly observed that nHZ selectively impaired the CD83 up-regulation induced by LPS. Thus, one could speculate that nHZ triggered interference with CD83 expression and thus DC maturation could arguably be both an important and effective strategy used by *Plasmodium* parasites in order to evade or favourably alter an immunological response. This is a strategy used by other microbial pathogens. For example, infection with protozon parasites such as *Trypanosoma cruzi*-derived glycoinositolphospholipids interferes with CD83 expression (Brodskyn *et al*., 2002). This mechanism is not only restricted to protozoan parasites, as DC infected with herpes virus simplex type 1 down-regulated CD83 expression, whereas the expression of CD86 was unaffected (Kruse *et al*., 2000). The functional relevance of the inability of nHZ to induce CD83 expression and subsequently DC maturation was investigated in autologous DC and T-cell co-cultures, where we could show that nHZ exposure of DC does not induce activation of T cells.

Taken together, our data demonstrate that nHZ is a potent modulator of DC responses, an effect apparent early in the interaction between nHZ and DC. The effect of nHZ is not due to the phagocytosis process itself, as it has been established in our and various other studies that the response to latex beads did not differ significantly to the response seen in unstimulated cells (Skorokhod *et al*., 2014; Skorokhod *et al*., 2004). Importantly, the effect of nHZ differs in part to that of β-hematin as seen for CD83. Thus, our data on the nHZ-induced modulation of DC phenotype and maturation could in part help to explain the underlying reasons for the poor induction of long-lasting protective immunity that is characteristic for *P. falciparum* infection.

## Experimental procedures

### *Dendritic cell cultures*

Peripheral blood mononuclear cells (PBMC) from healthy anonymous blood donors (Karolinska Hospital, Stockholm, Sweden) were isolated by Ficoll–Paque density centrifugation (GE Healthcare, Uppsala, Sweden). $CD14^{+}$ monocytes were isolated using EasySep monocyte enrichment kit according to manufacturer's instructions (StemCell Technologies, Grenoble, France). The purity was 90% ± 1%, as evaluated by a FACSVerse flow cytometer (Becton Dickinson, Mountain View, CA). DC were

generated by culturing purified monocytes at a concentration of $1 \times 10^6$ cells/ml in RPMI-1640 Medium supplemented with L-glutamine, penicillin/streptomycin, sodium pyruvate, non-essential amino acids (all from HyClone Laboratories, Inc, South Logan, UT) and 5% inactivated (v/v) human AB serum (Sigma-Aldrich, St. Louis, USA) supplemented with 35 ng/ml IL-4 and 50 ng/ml granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MO). Fresh cytokines were added after 3 days in culture. On day 5, the cells were phenotyped using flow cytometry and treated with the different stimuli.

The regional Ethics Committee, Stockholm, Sweden, approved protocols involving human cells for research purposes.

### *Preparation of natural hemozoin and β-hematin*

Natural hemozoin was prepared as described elsewhere (Skorokhod *et al*., 2004; Barrera *et al*., 2011). Briefly, nHZ was isolated from the supernatant of synchronized FCR3 parasite cultures 12 h after schizogony. nHZ was collected from the 10%/40% interphase of a 6% mannitol-containing Percoll gradient to eliminate red blood cell remnants and washed repeatedly with 10 mM phosphate buffer (pH 8.0) and phosphate-buffered saline (PBS) (pH 7.4). nHZ was opsonized with equal volume of human AB serum for 30 min at 37°C. nHZ was quantified according to its heme content after complete solubilization as described elsewhere (Schwarzer *et al*., 1994). The heme content in the used nHZ preparation was 4 nmol/μl. β-Hematin was prepared as described by Giusti *et al*. (2011). Briefly, endotoxin-free β-hematin was produced from porcine hemin (Sigma-Aldrich, St. Louis, MO, USA) at high temperature in acetate buffer. The resulting pellet was dried, weighed and resuspended in PBS to a final concentration of 10 mg/ml.

### *Cell treatment*

Monocyte-derived DC ($1 \times 10^6$ cells/ml) were exposed to the various stimuli in 24-well or 96-well plates for four different time points as indicated and incubated at 37°C and 5% $CO_2$. The plates were centrifuged at 700 r.p.m. for 20 s prior to incubation in order to initiate contact between cells and nHZ (5 μl/24-well and 2.5 μl/96-well) or β-hematin (20 μg/ml). Prior to use, nHZ and β-hematin were resuspended by passage through a needle (0.4 mm diameter) fitted to a 1 ml syringe. LPS (50 ng/ml) was used as a positive control, and cells maintained in cell culture medium (unstimulated) were used as a negative control. Opsonized latex beads (0.114 μm) were initially used as a control for phagocytosis, and consistently the response to latex beads did not differ significantly to that seen in unstimulated cells. Therefore, unstimulated cells have been used as a negative control throughout the study.

### *Immunocytochemistry*

Monocyte-derived DC were cultured on poly-L-lysine-coated (Sigma-Aldrich, St. Louis, MO, USA) glass coverslips in the presence of nHZ or β-hematin. LPS was used as a positive control and unstimulated cells as a negative control. Fixation, visualization and mounting were performed as previously described (Weidner *et al*., 2013). Coverslips were assessed by epifluorescence microscopy (Leica DMRB) equipped with a charge-coupled device camera (Retiga EXi, Qimaging). Scoring of cell morphology was performed by monitoring phalloidin-stained DC using micrographs (Openlab version 5.0.2) of 10–15 randomly chosen fields of view. An average of 80 cells from each individual donor was graded and verified independently by two microscopists. The DC were evaluated based on their presence of podosome structures. The images were processed using Adobe Photoshop 7.0.

### *RNA extraction and real-time quantitative polymerase chain reaction*

Total RNA was extracted from unstimulated and stimulated DC using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The RNA was subjected to DNase I treatment for 30 min in 37°C and quantified by a ND-100 nanodrop spectrophotometer. cDNA was synthesized from 500 ng of total RNA using oligo$(dT)_{20}$ primers and the SuperScript III reverse transcriptase kit (Invitrogen). cDNA from each sample was used for real-time polymerase chain reaction amplification of target sequences using the SYBR Green Chemistry (Kapa) on a rotor 3000 (Corbett Research). Primer sequences can be found in Supporting Information Table 3 (S3). Acidic ribosomal phosphoprotein P0 was used as housekeeping gene. Data analysis was performed using ROTOR-GENE 6000 series software 1.7 (Corbett Research), and calculations were made using the $2^{-\Delta\Delta Ct}$ method.

### *Flow cytometry*

Following conjugated monoclonal antibodies were used for labelling of DC according to standard procedures; CD86-FITC (clone: FUN-1), CD14-PE (clone: MφP9), HLA-DR-PERCP (clone: L243), CD11c-APC (clone: B-ly6), CD83-PE-Cy7 (clone: HB15e), CD195-APC-Cy7 (clone: 2D7/CCR5) (all from BD Biosciences, San Diego, CA) and CD197-BV-421 (clone: G043H7) (Biolegend, San Diego, CA, USA). Corresponding isotype-matched antibodies or unstained cells were used as negative controls. The cells were fixated with 4% paraformaldehyde after surface labelling. Acquisition was performed using FACSVerse flow cytometer (Becton Dickinson, Mountain View, CA) and analysed by FLOWJO software 10.0 (TreeStar, Ashland, OR, USA). Gates were set according to forward/side scatter properties, and analysis was performed on $CD14^{low}$, $HLA\text{-}DR^+$ and $CD11c^+$ cells.

### *Dendritic cell and T-cell co-cultures*

DC were obtained as described previously. From each donor, PBMC were cryopreserved in liquid nitrogen. The DC (50 000 cells) were exposed to nHZ or maintained in cell culture media for 24 h in 96-well round bottom plates as described previously. PBMC were thawed and washed three times, and $CD3^+$ T cells were isolated using EasySep human T-cell enrichment kit according to manufacturer's instructions (StemCell Technologies, Grenoble, France) and resuspended at a concentration of

$1 \times 10^6$ cells/ml in cell culture medium. After 24 h, DC were washed three times before autologous $CD3^+$ T cells were added to each well at DC : T-cell ratio of 1:4, and the cells were co-cultured for 24 h. Dynabeads human T-activator CD3/CD28 beads were used as a positive control for T-cell activation at a cell : bead ratio of 4:1 (Gibco, Life Technologies, Carlsbad, CA, USA). Human CytoStim, an artificial superantigen, was used to enable crosslinking of the T-cell receptor to MHC molecules on the surface of the DC (Miltenyi Biotec, Bergish Gladbach, Germany). After 24 h, the plates were centrifuged, and the cell culture supernatant was collected and stored in −70°C until analysis was performed.

### *Cytokine detection*

Levels of MCP-1/CCL2 (R&D Systems, Minneapolis, MO), IL-2 and IFN-$\gamma$ (Mabtech, Nacka, Sweden) in cell culture supernatants were measured by sandwich ELISA according to manufacturer's guidelines. All samples were tested in duplicates, and the optical density was measured using an ELISA plate reader (VmaxTM Kinetic Microplate Reader, Menlo Park, CA).

### *Statistical analyses*

Statistical analyses were made using the Wilcoxon rank-sum test. All analyses were performed using GraphPad Prism v.6 (GraphPad Software, La Jolla, CA). Statistical significance was assumed when $P < 0.05$. Symbols are as follows: $^*P < 0.05$ and $^{**}P < 0.01$.

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## Conflict of interest

The authors have no conflict of interest to declare.

## Author Contributions

Conceived/designed the experiments: I. B., A. Ö. F. and M. T.-B. Performed the experiments: I. B. and J. W. Data analysis: I. B., O. S. and J. O. P. Contributed with essential non-commercial material and methods: E. S. and O. S. Wrote and discussed the manuscript: I. B., A. Ö. F., M. T.-B., O. S. and E. S.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Percentage of CCR7+ cells. DC were exposed to either nHZ or β- hematin, stimulated with LPS or maintained in complete medium (unstimulated) for 2-24h as described in experimental procedures. At indicated time points the percentage of CCR7+ cells were determined by flow cytometry (n=5). The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10-90 percentile.

**Fig. S2.** mRNA expression of MHC class II . DC were exposed to either nHZ or β-hematin, stimulated with LPS or maintained in complete medium (unstimulated) for 2-24h as described in experimental procedures. At indicated time points the mRNA levels of the β-subunit of the MHC class II complex were determined by qPCR (n=6). The box plots illustrate the median and the 25th and 75th quartile, while the whiskers represent the 10-90 percentile.

**Fig. S3.** Primer sequences for real-time quantitative PCR.