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Malaria pigment hemozoin drives M1 pro-inflammatory macrophage polarization in vitro

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Abstract

Severe malaria, a high burden parasitic disease, is characterized by hyperproduction of proinflammatory cytokines, most likely generated by M1-polarized macrophages. Malaria pigment or hemozoin (HZ), a byproduct of heme detoxification in intra-erythrocytic parasites, is internalized by circulating monocytes and tissue macrophages, modulating their functions. Although the immunomodulatory properties of HZ have been described, its specific role in M1/M2 macrophage polarization remains unclear. This study aims to fill this gap by elucidating whether HZ modulates M1/M2 polarization, contributing to the strong inflammatory response in severe malaria.

Primary human monocyte derived macrophages (MDM) and THP-1 cells differentiated into macrophages (dTHP-1) were stimulated with M1 or M2 signals in the presence of native HZ. Gene expression and protein secretion of TNF-α, IL-1β, CXCL-8, IL-6, IL-10 and PPARG were evaluated by Real-Time PCR and ELISA, respectively. STAT6 phosphorylation was evaluated by western blot analysis.

MDM and dTHP-1 showed a different polarisation response to classical M1/M2 stimuli and to HZ treatment. In both non-polarized (M0) MDM and dTHP-1, HZ induced an M1/pro-inflammatory phenotype, increasing gene expression and protein secretion of CXCL8, TNF-α, and IL-1β. In the presence of M1- or M2-polarizing stimuli, HZ further increased CXCL-8 and IL-1β in MDM but not in dTHP-1, where TNF-α secretion was even reduced. HZ did not affect M2 markers (PPARG and IL10 expression, STAT6 phosphorylation) in any condition.

This is the first in vitro study investigating the effect of HZ on macrophage polarization, showing its ability to promote M1 pro-inflammatory differentiation. Results vary across experimental models, emphasizing the importance of considering model-specific effects. Clarifying HZ's role remains crucial for understanding malaria pathogenesis and developing new immunomodulatory therapies.

Keywords: malaria, hemozoin (HZ), malaria pigment, innate immunity, macrophage polarization

1. Introduction

Malaria is a vector-borne parasitic disease, caused by protozoan parasites of the genus *Plasmodium*, accounting for 263 million cases and 597,000 deaths in 2023, with most fatalities in children under five years of age caused by *P. falciparum* [1]. Malaria manifestations vary widely, ranging from mild symptoms in partially immune adults to severe complications [2,3]. Key pathological features of severe malaria include capillary obstruction due to sequestration of parasitized red blood cells, endothelial activation, and systemic, macrophage-driven, pro-inflammatory response [4]. Both parasite burden and parasite products, including hemozoin (HZ), contribute to disease severity.

HZ or malaria pigment is an insoluble, iron-containing waste product of hemoglobin degradation during the *Plasmodium* intraerythrocytic stage in the human host [5]. HZ is released into circulation during asexual reproduction and accumulates in multiple organs as the infection progresses, contributing to severe malaria manifestations [6]. Evidence suggests that HZ interferes with the host immune response contributing to the development of malarial complications [7].HZ is internalized by circulating phagocytic cells and tissue-resident-macrophages, where it persists for extended periods without being degraded [8]. The interaction of HZ with the host immune system is complex, exhibiting both pro-inflammatory and anti-inflammatory effects [7,9]. HZ interacts with various cell types, including monocytes, macrophages, dendritic cells, and endothelial cells, modulating inflammatory and immune responses [10–13]. The majority of the described effects are pro-inflammatory [14–17], although anti-inflammatory effects have also been described [18]. This discrepancy may be due to factors including the type of HZ used (native vs. synthetic), the method of native HZ extraction, the experimental set-up (e.g., duration of cell exposure to HZ), the simultaneous presence of other stimuli, and the tissue origin of the cells [6,19,20].

As mentioned above, macrophages internalize HZ but fail to degrade it, resulting in prolonged persistence and alterations of macrophage functions. Macrophages demonstrate remarkable plasticity, allowing them to differentiate into distinct populations with specific functions in response to various stimuli. Depending on the nature of these stimuli, macrophages can polarize into M1

macrophages, which promote inflammation, or M2 macrophages, which exhibit anti-inflammatory properties [21]. M1 macrophages are induced by interferon-γ (IFN-γ) and other pro-inflammatory stimuli and are characterized by the production of cytokines such as TNF-α, IL-1β, IL-6 and IL-12 as well as the upregulation of major histocompatibility complex (MHC) class II antigens. In contrast, M2 macrophages, typically induced by IL-4 or other anti-inflammatory signals, secrete anti-inflammatory cytokines like IL-10 and are characterized by PPAR-γ activation [22]. Between these two extremes, macrophage polarization exists on a spectrum of intermediate phenotypes [23].

In severe malaria, both beneficial or deleterious roles of M1 and M2 macrophages have been described, depending on the anatomical localization (blood, lungs, or placenta) [24–26]. However, the available data remain scarce. It has been described that HZ stimulates inflammatory cytokines production by human monocytes through activation of p38 MAPK and NF-κB pathways [27]. In murine macrophages HZ induces chemokines expression through a mechanism that is ERK1/2 dependent and involves NF-κB activation [28]. Macrophage polarization is influenced by different signaling pathways including JAK/STAT, NF-κB, and MAPK pathways [29,30]). It has been shown that HZ increased the phosphorylation levels of p38 MAPK, PI3K-AKT, and NF-κB in monocytes, suggesting activation of these signaling pathways in macrophages polarization [18]. The latter study is the only one that investigates the effect of HZ on the polarization of human monocytes examining the signaling pathways involved in this process.

Thus the role of HZ in macrophage polarization remains unclear and needs further investigation. This study aims to fill this gap by exploring the direct effect of native HZ, extracted from *P. falciparum* cultures, on primary human monocyte-derived macrophages (MDM) and on the monocytic THP-1 cell line, differentiated into macrophage-like cells (dTHP-1) by phorbol esters. The immunomodulatory effect of HZ was evaluated in the presence of classical M1 and M2 stimuli.A deeper investigation into these interactions could be crucial to clarify the mechanisms driving immune pathology in malaria and may assist in the design of new adjunctive therapies able to modulate macrophage-mediated inflammation.

2. Results

2.1 MDM and dTHP-1 M1/M2 polarization induced by pro- or anti-inflammatory stimuli

The effect of polarizing stimuli on MDM and dTHP-1 was assessed 24 hours post-stimulation. The expression levels of M1-associated cytokines (CXCL8, TNF-α, IL-1β, and IL-6) were quantified at the RNA level using real-time PCR and at the protein level in culture supernatants via ELISA. Gene expression of the nuclear receptor PPAR-γ and the anti-inflammatory cytokine IL-10 along with phosphorylation of the transcription factor STAT-6, evaluated by Western blotting, were adopted as M2 markers.

Unpolarized MDM and dTHP-1 constitutively produced elevated levels of CXCL8 and low levels of TNF-α. Low levels of IL-1β and IL-6 were produced only by dTHP-1. IL-10 levels were below the detection limits (**Supplementary Table 1**). When MDM were stimulated with M1 inductive signals, IFN-γ and TNF-α, the following results were obtained. *TNFA* gene expression was significantly increased (**Figure 1A**), whereas protein secretion was not investigated, since cells had been stimulated with this cytokine. *CXCL8* expression did not change, while the protein secretion was decreased (**Figure 1B**), indicating post-transcriptional regulation. *IL1B* expression was significantly decreased (**Figure 1A**); however, the IL-1β levels were below the detection limit. IL6 gene expression was not significantly modulated: it was decreased in MDM from 3 out of four donors. The protein levels were below the detection limit. The expression of the M2-associated *PPARG* gene was significantly decreased. Consistently, IL10 gene expression was decreased whereas protein levels were below the detection limits (**Figure 1A** and **B**). When MDM were stimulated with the M2 inductive signal IL-4, *CXCL8* gene expression and protein secretion were significantly inhibited compared to untreated controls (**Figure 1A** and **B**). *TNFA* was not modulated. *IL1B* gene expression was decreased whereas protein levels were below the detection limits. *IL6* gene expression was not significantly modulated since it was increased in MDM from 3 out of four donors, while the protein levels were below the detection limit. The expression of *PPARG* was increased, whereas gene expression of the anti-inflammatory cytokine IL-10 was unchanged (**Figure 1A**); IL-10 levels in the supernatants remained below the detection limit. When dTHP-1

were stimulated with M1 inductive signals, IFN-γ and LPS, the following results were obtained. A significant increase of CXCL-8, TNF-α, IL-1β and IL-6 was observed both in terms of gene expression and protein secretion (**Figure 1C** and **D**). *PPARG* expression was unchanged, whereas *IL10* gene expression was increased, though not significantly (**Figure 1C**). To verify if the differences in cytokines expression and secretion between MDM and dTHP-1 were due to cell type or the different M1 stimuli used, dTHP-1 cells were stimulated with the same M1 inductive signals used for MDM (IFN-γ plus TNF-α). Comparable results were observed with these alternative M1 stimuli (**Supplementary Figure 1**).

When dTHP-1 were stimulated with the M2 inductive signals, IL-4 and IL-13, M1 markers were unchanged. A significant increase in the expression of *PPARG* (**Figure 1C**) was observed, whereas *IL10* gene expression was unchanged and protein levels in the supernatants were below the detection limit.

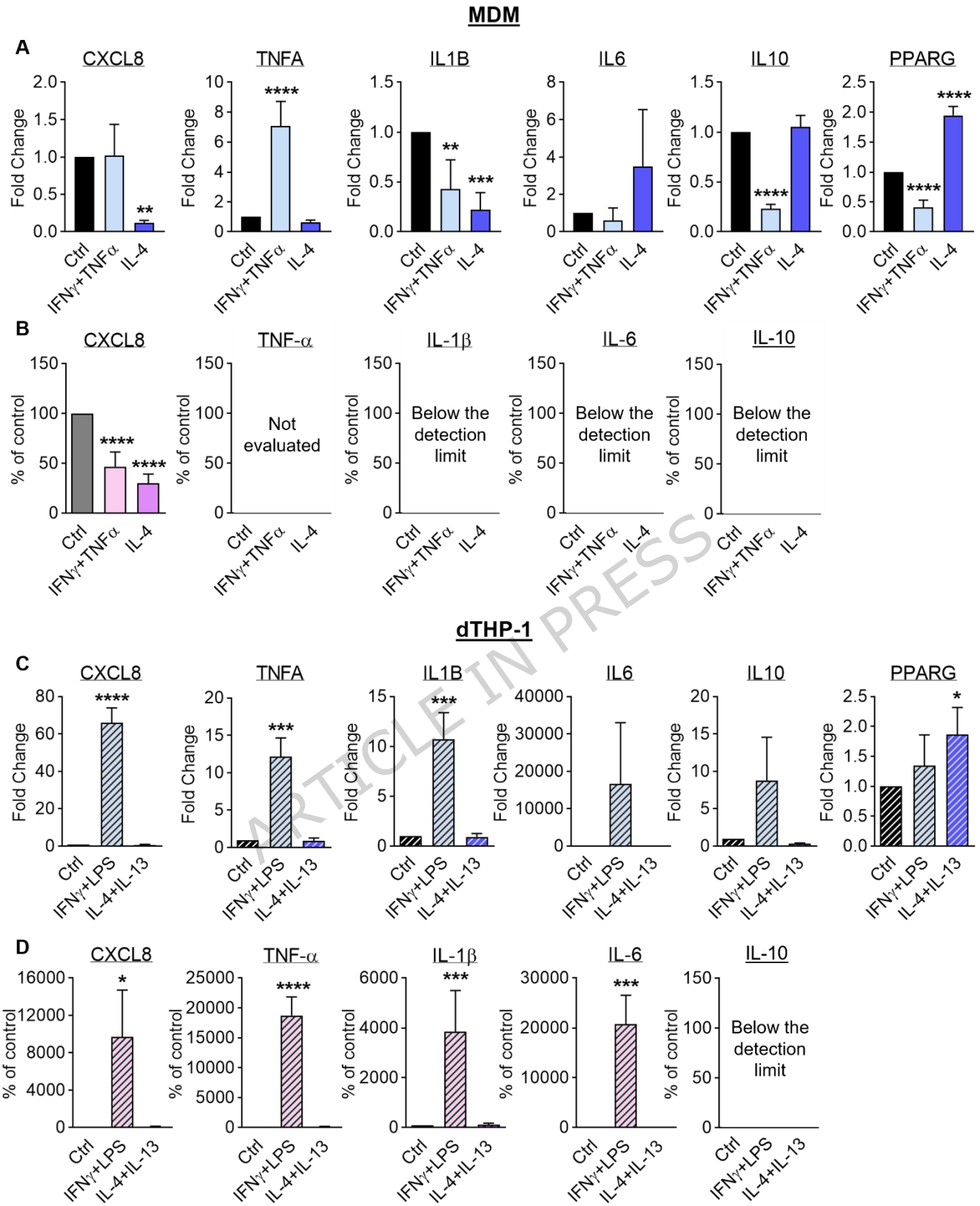

Figure 1. Macrophage polarization. *Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (filled columns – panels A and B) and dTHP-1 cells (striped columns – panels C and D) after 24 h of incubation with polarizing*

*stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test, comparing each gene or protein against M0 (untreated macrophages). Statistical significance is denoted as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

Expression of STAT-6 in both MDM and dTHP-1 was unaffected by the different experimental conditions, whereas its phosphorylation was not induced in M1-macrophages (**Figure 2A** and **Figure 2C**, lane 3 vs.1) but was increased, as expected, by M2 stimuli (**Figure 2A** and **Figure 2C,** lane 5 vs. 1). The original full uncropped Blots images are shown in **Supplementary Figure 2**. Figure 2 also shows the effect of HZ on STAT6 phosphorylation (**Figure 2B** and **2D**), which will be described in the subsequent sections.

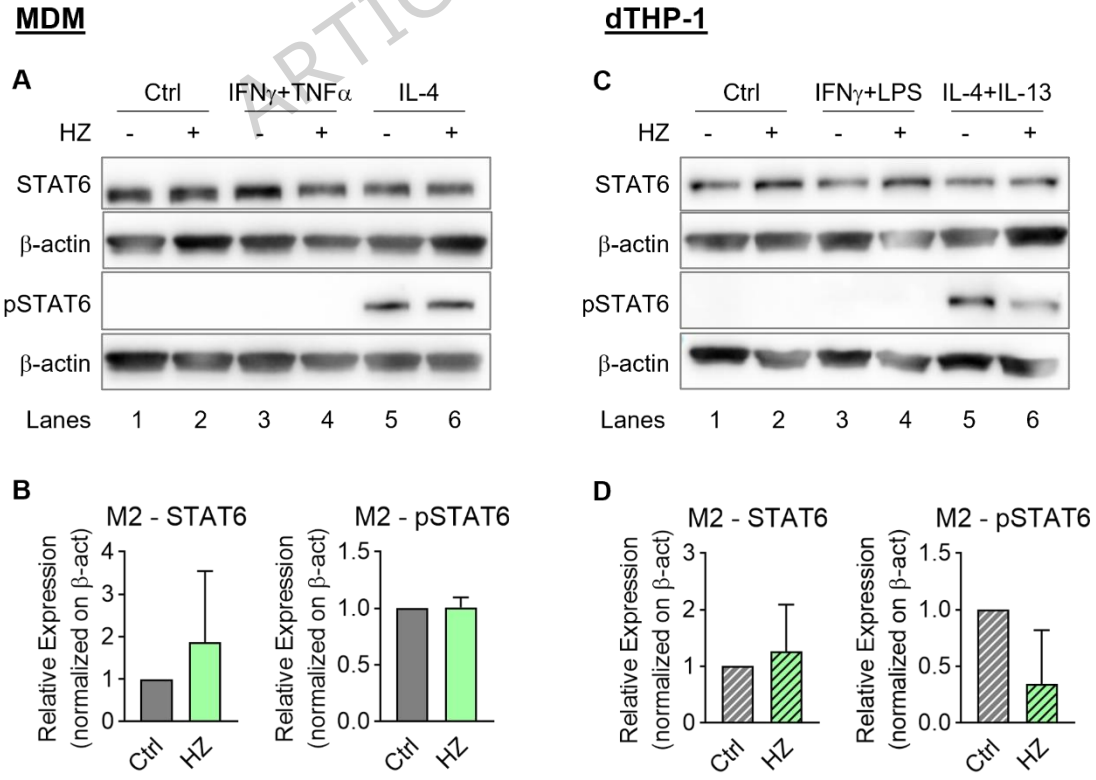

***Figure 2. STAT6 phosphorylation**. Western Blot analysis of STAT6 phosphorylation in primary MDM (A) and dTHP-1 cells (C) in control cells or cells treated with hemozoin (HZ). Relative expressions of total STAT6 and pSTAT6 normalized on β-actin in MDM (B) and dTHP-1 cells (D) were assessed in M2-like macrophages. Data are the mean ± SD from three different donors for MDM and from three independent experiments for dTHP-1. Statistical analysis was performed using an unpaired two-tailed t-test for each transcription factor and comparing the data versus the control M2-macrophages not incubated with HZ.*

2.2 Hemozoin phagocytosis by polarized macrophages

Optical microscopy was performed to verify whether the polarization state of macrophages could affect their ability to phagocytize HZ. As shown in **Figure 3**, both MDM (**panel A**) and dTHP-1 cells (**panel B**) phagocytized HZ with comparable efficiency, independently of the presence of polarizing stimuli. The percentage of HZ-engulfed MDM was 38±15, 40±12, 43±14 in M0, M1, M2 macrophages, respectively.

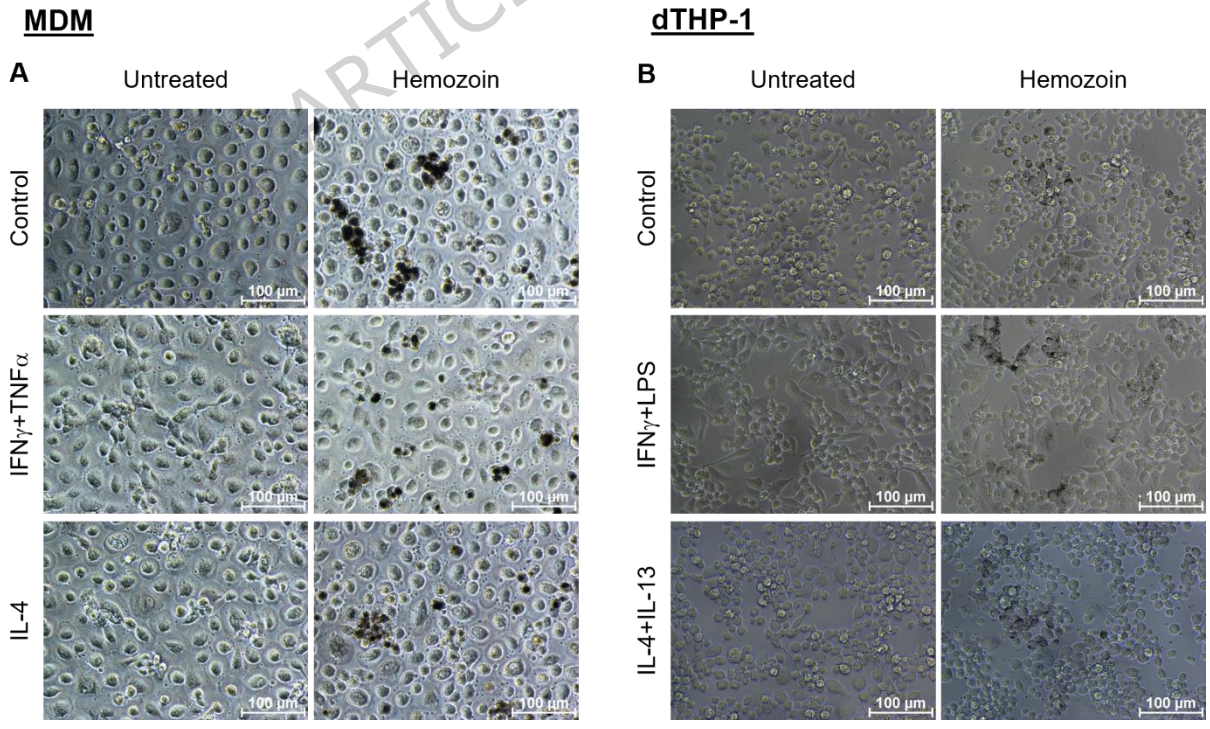

***Figure 3**. **HZ phagocytosis by M0, M1, M2 macrophages**. Representative images of MDM (A) or dTHP-1 (B) after 24h of incubation with polarizing stimuli in the presence or absence of HZ. Images were captured by*

using a Nikon Eclipse Ti-Series with 20x objective and the digital camera Nikon Digital Sight. Scale bar: 100 µm.

2.3 HZ induces a M1-like phenotype in unpolarized macrophages

HZ significantly increased *CXCL8* and *IL1B* gene expression in both MDM and dTHP-1 cells; an increase of *TNFA* expression was observed in MDM, but not in dTHP-1 cells, whereas *IL6* was not significantly modulated in either cell types (**Figure 4A** and **C).** Consistently with gene expression, TNF-α and CXCL8 levels in cell supernatants were significantly higher in HZ-treated MDM compared to control cells, whereas IL-1β and IL-6 levels remained below the detection limit (**Figure 4B**). In dTHP-1, secreted levels of CXCL8 and IL-1β were significantly higher following HZ stimulation, while the increase of TNF-α production did not reach statistical significance. IL-6 production was not modulated (**Figure 4D**). Conversely, HZ did not alter the gene expression or protein phosphorylation of anti-inflammatory M2 markers in both unpolarized (M0) MDM and dTHP-1 cells (**Figure 4A** and **C**; **Figure 2A and B**, lanes 1 vs. 2).

Overall, these findings suggest that HZ directly induced an M1-like phenotype.

In some experiments using dTHP-1 cells, latex beads were used as control to exclude that the observed immunomodulation was due to non-specific effects of phagocytosis (**Supplementary Figure 3**). Differently from HZ, latex beads failed to modulate CXCL8, TNF-α and IL-1β production.

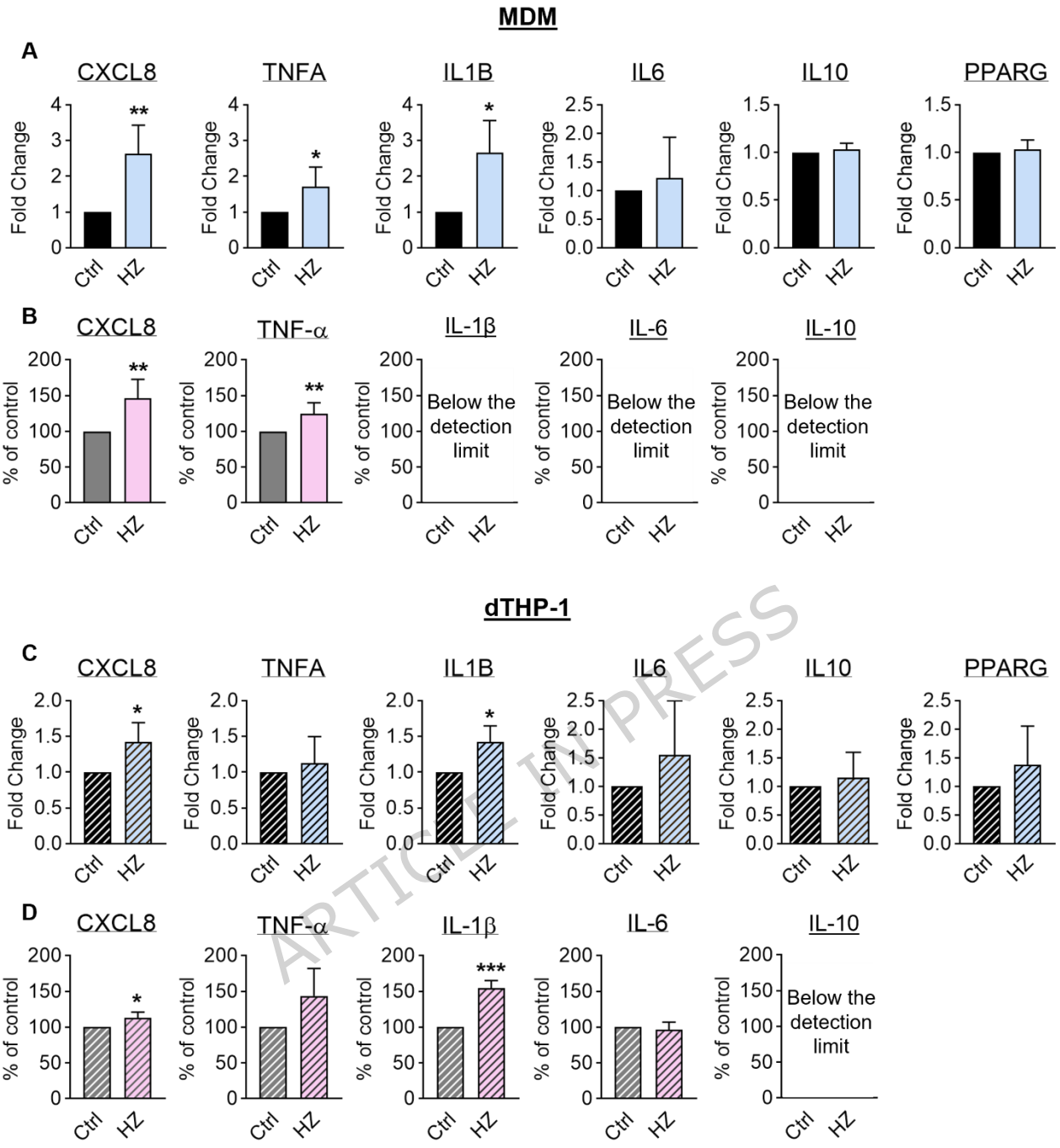

Figure 4. Effect of HZ on M0 macrophages. *Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (full columns – panels A, B) and dTHP-1 cells (striped columns – panels C, D) after 24 h incubation with HZ alone in the absence of other polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at*

*least three independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein in comparison to control (M0 - unpolarized macrophages). Statistical significance is denoted as follows: *p < 0.05; ** p < 0.01; ***p < 0.001.*

2.4 Effects of HZ on M1 macrophages

Both MDM and dTHP-1 cells were stimulated with M1-polarizing stimuli in the presence or absence of HZ for 24 h. In MDM, HZ increased the expression of *CXCL8* and *IL1B* genes but did not affect *TNFA* and *IL6* expression (**Figure 5A**). The expression of *IL6* was highly variable, being decreased in HZ-treated MDM from 3 out of 4 donors and increased (3.8 fold) in 1 out of 4. Consistently to gene expression, CXCL8 secretion was significantly enhanced by HZ (**Figure 5B**), while IL-1β was below the limit of detection. In contrast, in dTHP-1 cells, HZ did not affect *CXCL8, TNFA*, or *IL1B* gene expression (**Figure 5C**), whereas *IL6* was significantly decreased. Protein secretion was consistent with gene expression for all these cytokines except for TNF-α, which was decreased by HZ (**Figure 5D**). As observed in unpolarized macrophages (**Figure 4**), HZ failed to modulate gene expression of anti-inflammatory M2 determinants (*PPARG* and *IL10*) in both cell types (**Figure 5A** and **C**). Thus, HZ seems to potentiate the effect of classical M1-polarizing stimuli in MDM, but not in dTHP-1.

Of note is that the M1 stimuli (IFN-γ and LPS) for dTHP-1 cells are different from those used for MDM (IFN-γ and TNF-α). To verify whether these differences were due to the cell type or to the specific polarizing stimuli, dTHP-1 cells were stimulated with the same M1 cytokines used for MDM (IFN-γ and TNF-α). In these conditions, HZ enhanced both *CXCL8* gene expression (**Figure 6A**) and protein secretion (**Figure 6B**) and did not alter IL6 expression and secretion, suggesting that the observed discrepancy between MDM and dTHP-1 could be attributed to the different M1 stimuli used. Conversely, IL-1β was not modulated by HZ, indicating a different susceptibility to HZ between the two cell models.

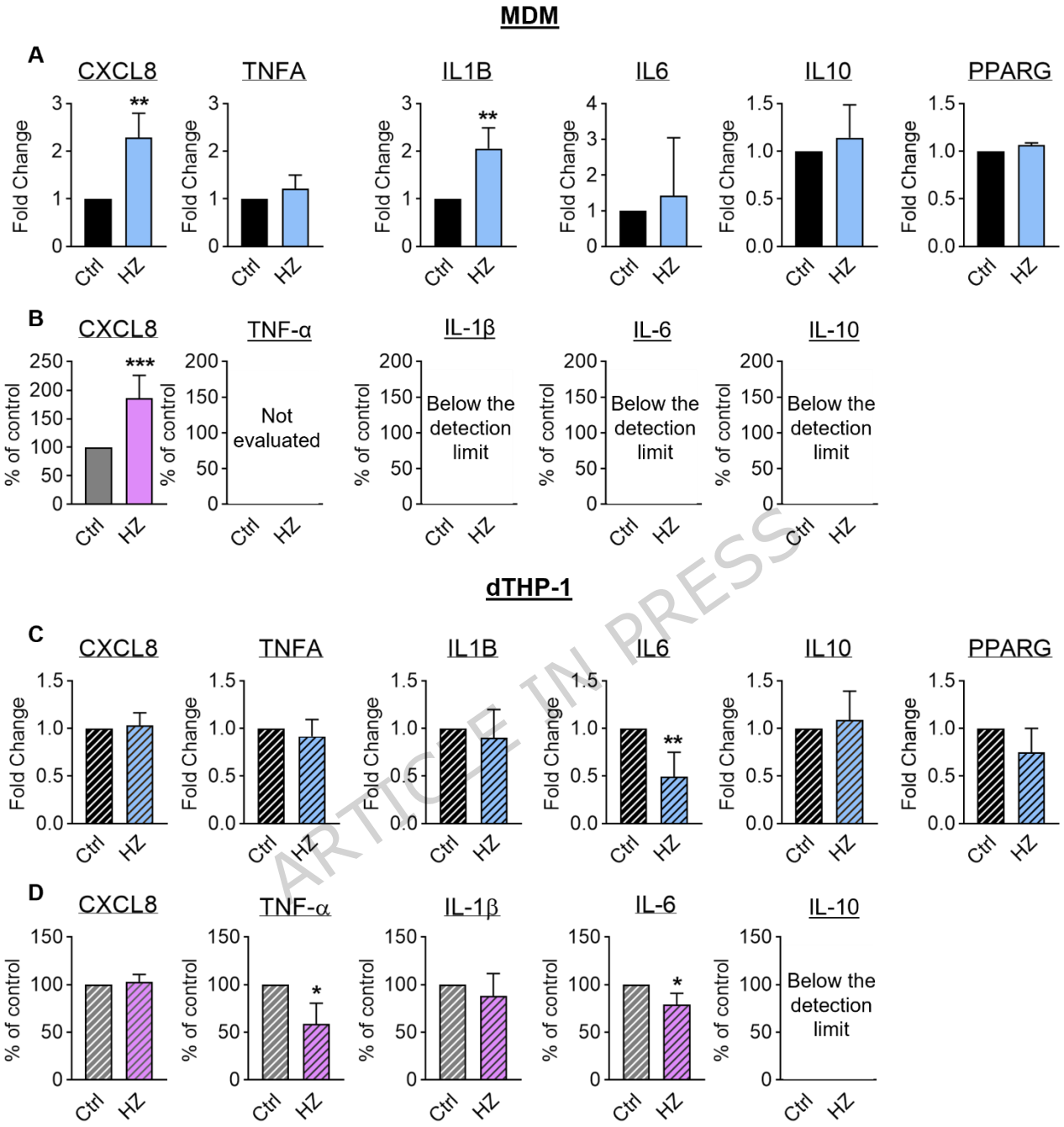

Figure 5. Effect of HZ on M1 macrophages. *Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (filled columns – panels A, B) and dTHP-1 cells (striped columns – panels C, D) after 24 h incubation with HZ and M1-polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured*

*by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein, comparing the data versus the control (M1-stimulated macrophages). Statistical significance is denoted as follows: *p < 0.05; **p < 0.01; ***p < 0.001.*

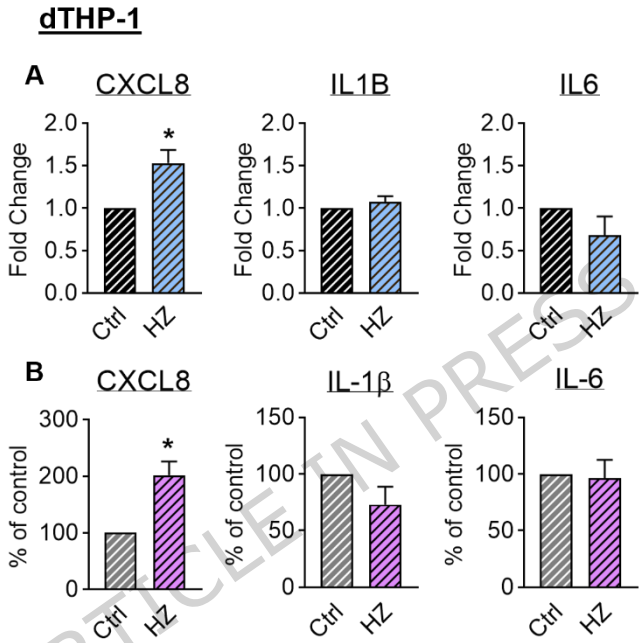

***Figure 6**. **HZ effect on dTHP-1 polarized with M1 stimuli used for MDM**. M1 macrophage polarization markers assessed as mRNA expression levels (panel A) and protein secretion levels in cell supernatants (panel B) in dTHP-1 macrophages after 24 h incubation with HZ and M1-polarizing stimuli used for MDM (IFN-γ +TNF-α). Gene expression of CXCL8, IL1B and IL6 was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from three independent experiments for dTHP-1. CXCL-8, IL-1β and IL-6 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein, comparing the data to the control (M1-stimulated macrophages). Statistical significance is denoted as follows: *p < 0.05.*

252 **2.5 Hemozoin increased the expression of M1 markers in M2-polarized MDM**

253 Finally, the effect of HZ was evaluated in the presence of M2-polarizing stimuli. In MDM, HZ
254 increased the expression of *CXCL8* and *IL1B* genes, whereas it induced a nonsignificant increase
255 of *TNFA* gene expression (**Figure 7A**). The expression of *IL6* was highly variable, being decreased
256 in HZ-treated MDM from 3 out of 4 donors and increased (2.2 fold) in 1 out of 4. In MDM, none of
257 the tested cytokines were significantly modulated (**Figure 7B**). In contrast, in dTHP-1 cells, gene
258 expression of these mediators was not significantly modulated by HZ (**Figure 7C**), although an
259 increase in CXCL8 and a decrease in TNF-α levels were observed in the supernatants (**Figure**
260 **7D**), suggesting a post-transcriptional regulation of these cytokines. As previously observed,
261 neither in primary MDM nor in dTHP-1 cells, modifications in M2 gene expression markers
262 (*PPARG* and *IL10*) were observed in the presence of HZ (**Figure 7A** and **C**). Moreover,
263 phosphorylation of STAT6 remained unaffected (**Figure 2A** and **C**, lane 6 vs. 5; **Figure 2B** and
264 **2D**). Therefore, it appears that HZ can partially restore M1 cytokine expression in M2-polarized
265 MDM.

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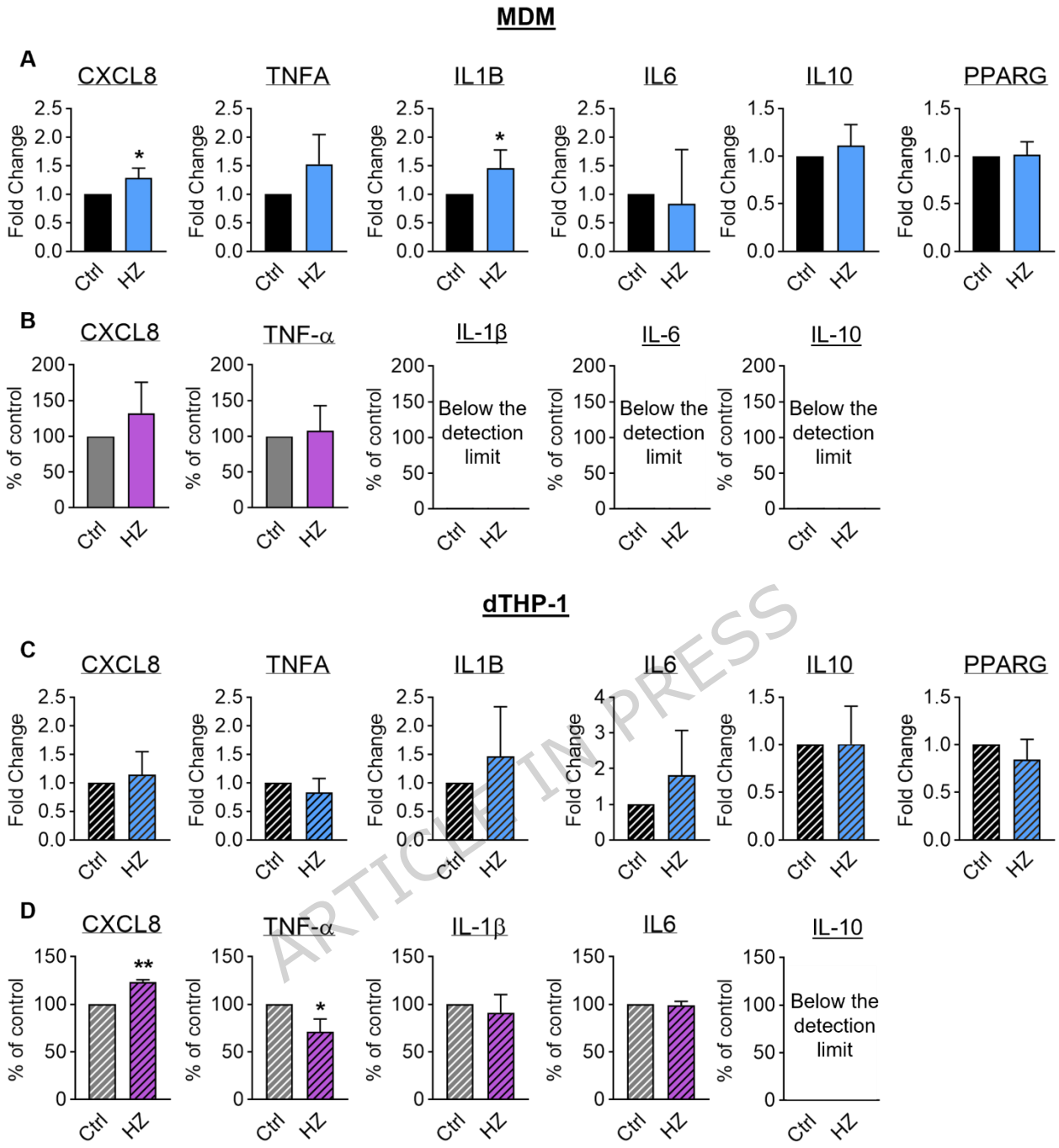

Figure 7. Effect of HZ on M2 macrophages. *Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (filled columns – panels A, B) and dTHP-1 cells (striped columns – panels C, D) after 24 h of incubation with HZ and M2-polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three*

*independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein and comparing the data versus the control (M2-stimulated macrophages). Statistical significance is denoted as follows: *p < 0.05; **p < 0.01.*

3. Discussion

Clinical manifestations of malaria are attributed to proinflammatory cytokines released in response to malaria parasites and their byproducts, including the malaria pigment HZ [31]. Elevated levels of inflammatory cytokines have been associated with severe malaria, especially in African children [32,33]. HZ is released during the rupture of red blood cells when parasites in the schizont stage release new merozoites into circulation. Notably, HZ has been detected in post-mortem samples from various organs of patients with severe malaria [7].

While the immunomodulatory effect of HZ has been extensively studied in human monocytes and murine macrophages [16,34,35], this study is among the first to investigate the effect of malaria pigment on human macrophages. Beyond the study by Bobade et al., which describes the M2-polarizing effect of HZ on monocytes [18], our work provides the first comprehensive analysis of HZ in human macrophages polarization and its modulatory effects in response to classical M1/M2 stimuli. Since macrophages are the main source of cytokines in malaria patients [36], classical or alternative polarization of these cells is relevant for malaria pathogenesis. However, few studies have been conducted, most of them referring to the in vivo analysis of the macrophage phenotype, but little is known on the signals that drive M1/M2 polarization in malaria.

In our study, primary monocyte-derived macrophages (MDM) and THP-1 monocytic cell line differentiated into macrophage-like cells (dTHP-1) were used for investigating the effect of HZ on macrophage polarization. THP-1 cells can be maintained in culture for extended periods with consistent results, albeit with the limitation of being tumor-derived. Moreover, THP-1 differentiation is obtained using chemical inducers, such as phorbol esters, which may induce a pro-inflammatory

phenotype themselves [37]. Conversely, MDM offers the advantage of being primary cells, although they derive from different donors and may exhibit variability in their responses. In this study, MDM from 4 donors were used and all the analyzed markers, except for IL-6, showed the same trend of modulation after stimulation with either classical M1/M2 stimuli and HZ.

Differences in cytokine production and polarization between MDM and dTHP-1 have been previously described [38,39]. Consistent with these observations, our study revealed differences in polarization between the two experimental models. In dTHP-1 cells, M1 stimuli increased gene expression and protein production of CXCL-8, IL-1β and IL-6. In contrast, MDM exposed to M1 stimuli decreased *IL1B* expression and CXCL-8 production, inducing no changes in *CXCL8* and *IL6* expression. It is noteworthy that the pro-inflammatory M1 stimuli differ between the two experimental models (IFN-γ +TNF-α for MDM and IFN-γ +LPS for dTHP-1 cells). Nevertheless, when the M1 polarizing stimuli used in this study for MDM were employed on dTHP-1 cells, the modulation of polarization markers was the same, confirming the different susceptibility of cell types.

In MDM, a discrepancy between gene expression and protein secretion of CXCL-8 was observed. This may be due to the specific molecular pathways controlling its transcription and to complex post-transcriptional regulation, which includes the presence of regulatory miRNA, the instability of CXCL-8 mRNA and the complex pathways of its stabilization [40]. In our study, IL-4 failed to induce IL-10 by MDM or dTHP-1. It is well established that IL-4 stimulates the production of IL-10 by T helper lymphocytes [41], however IL-4 inhibits IL-10 production in other immune cells, such as dendritic cells (Yao et al., 2005).

To evaluate the effect of HZ on macrophage polarization, native HZ, a crystal of ferriprotoporphyrin-IX bound to host and parasite lipids, DNA, and proteins [6], was used. It has been shown that these different components of HZ modulate immune responses through different mechanisms [6]. Thus, native HZ closely mimics physiological conditions. The dose of HZ chosen for this study is biologically relevant and calculated based on the iron content of trophozoites [43].

Macrophages are phagocytic cells, and previous studies have reported that M2 macrophages exhibit greater phagocytic capacity than M1 macrophages [44]. However, our results indicate that HZ is phagocytosed to a similar extent by M0, M1, and M2 macrophages. This may be explained by the fact that HZ crystals are internalized as inert material, like asbestos fibres, as shown by electron microscopy images from Olliaro et al., which demonstrate that HZ crystals within macrophages are not enclosed by a membrane [45].

When HZ was applied on unpolarized M0 macrophages, a clear pro-inflammatory effect was observed in both MDM and dTHP-1 cells. Gene expression and protein production of CXCL-8, TNF-α and IL-1β were significantly enhanced after incubation with HZ. These results are consistent with previous *in vitro* studies reporting that both native HZ and synthetic HZ induce the production of inflammatory cytokines and chemokines by human and murine monocytes/macrophages [16,31,46,47]. However, other studies have demonstrated that HZ can inhibit several monocyte functions such as the production of inflammatory mediators or the expression of major histocompatibility complex class II antigen [48]. Since HZ is known to induce oxidative stress, these contrasting effects have been attributed to differences in the antioxidant capacities of various cell types [20,35,49,50].

In this study, HZ, either alone or in the presence of M1 or M2 polarizing stimuli, failed to modulate M2-associated markers such as PPAR-γ or IL-10. This finding differs from a previous study by Bobade and colleagues reporting that HZ induces different M2 markers including IL-10 [18]. This observed discrepancy may be due to the higher HZ concentration used (50 µg/ml compared to 10 µg/ml in the present study), as well as differences in MDM isolation and culture conditions. Specifically, in this study, 5% human serum was added alongside 10% FBS, and MDM were incubated for six days to ensure complete differentiation into macrophages. In another study, HZ suppressed PPAR-γ expression in dendritic cells, thereby inhibiting their maturation [51]. This inhibition was attributed to 15(S)-hydroxyeicosatetraenoic acid (15(S)HETE), which is produced by close contact of unsaturated fatty acids with HZ via nonenzymatic heme catalysis [51]. The different effects of HZ on dendritic cells and macrophages may be due to variations in their fatty acid composition, as dendritic cells have a higher proportion of polyunsaturated fatty acids than macrophages [52].

In MDM, HZ consistently induces a pro-inflammatory response, regardless of the presence of additional pro- (M1) or anti-inflammatory (M2) stimuli. In contrast, the effects of HZ on dTHP-1 cells depended upon the presence of a particular combination of polarizing stimuli. Differences in cytokine production and polarization between MDM and dTHP-1 have been previously reported [38,39]. Consistent with these observations, our study revealed distinct polarization responses between the two experimental models. In the MDM model, HZ exhibited an additive effect on *CXCL8* and *IL1B* expression and on CXCL-8 production in the presence of M1-polarizing stimuli (**Figure 5**). In contrast, this additive effect was absent in dTHP-1 cells, where HZ instead inhibited TNF-α secretion, as previously demonstrated in murine macrophages treated with LPS and synthetic HZ [53].

Some discrepancies in M1 polarization between dTHP-1 and MDM may be due to differences in the stimuli used. When dTHP-1 cells were treated with the same M1 stimuli as MDM (IFN-γ + TNF-α), HZ increased the expression and production of CXCL-8 but not of IL-1β (**Figure 6**). LPS may exert a stronger stimulatory effect than TNF-α, potentially masking HZ effect on CXCL-8. THP-1 cells are known to respond differently to various polarizing stimuli, leading to distinct marker profiles [54].

On the contrary, IL-1β modulation by HZ in the presence of pro-inflammatory stimuli appeared to be cell type-dependent. IL-1β plays a role in the pathogenesis of malaria, and elevated plasma levels of IL-1β have been reported in patients with severe malaria [55]. This cytokine is typically produced by macrophages following NLRP3 inflammasome activation in response to microbial infection or danger signals, which triggers caspase-1-mediated processing and the subsequent release of mature IL-1β [56]. HZ is known to activate the inflammasome, thereby inducing IL-1β secretion [15]. In this study, HZ stimulated *IL1B* expression in unpolarized M0 MDM and dTHP-1 cells. However, in the presence of M1 stimuli, HZ increased *IL1B* gene expression in MDM but not in dTHP-1 cells. Notably, while dTHP-1 cells secreted mature IL-1β into the supernatants, IL-1β levels were undetectable in MDM supernatants. Higher production of IL-1β in dTHP-1 cells compared to MDM has been previously reported, although under different stimulatory conditions

383 [38,54]. Variations in inflammasome activation in different cell types were indeed reviewed by Cornut
384 and colleagues [57].

385 Notably, HZ retained its pro-inflammatory activity even in a M2-like environment. However, this
386 was not observed in dTHP-1 cells, where the production of TNF-α was significantly reduced
387 following HZ treatment. This reduction in TNF-α protein levels was also observed in M1-polarized
388 dTHP-1 cells, suggesting that HZ exerts an inhibitory effect on the release of mature TNF-α,
389 regardless of polarization environment (**Figure 7**). TNF-α is synthesized as a membrane-bound
390 precursor protein and released through proteolytic cleavage mediated by the metalloproteinase
391 TNF-α-converting enzyme (TACE). HZ is known to modulate both the expression and the activity
392 of other metalloproteinases, such as MMP-9 [58]. Therefore, the observed reduction in TNF-α levels
393 in the supernatants of HZ-treated dTHP-1 cells may be attributed to post-transcriptional regulation
394 [36].

395 Results have been summarized in **Figure 8,** which shows the modulation of M1/M2 markers in all
396 the tested conditions.

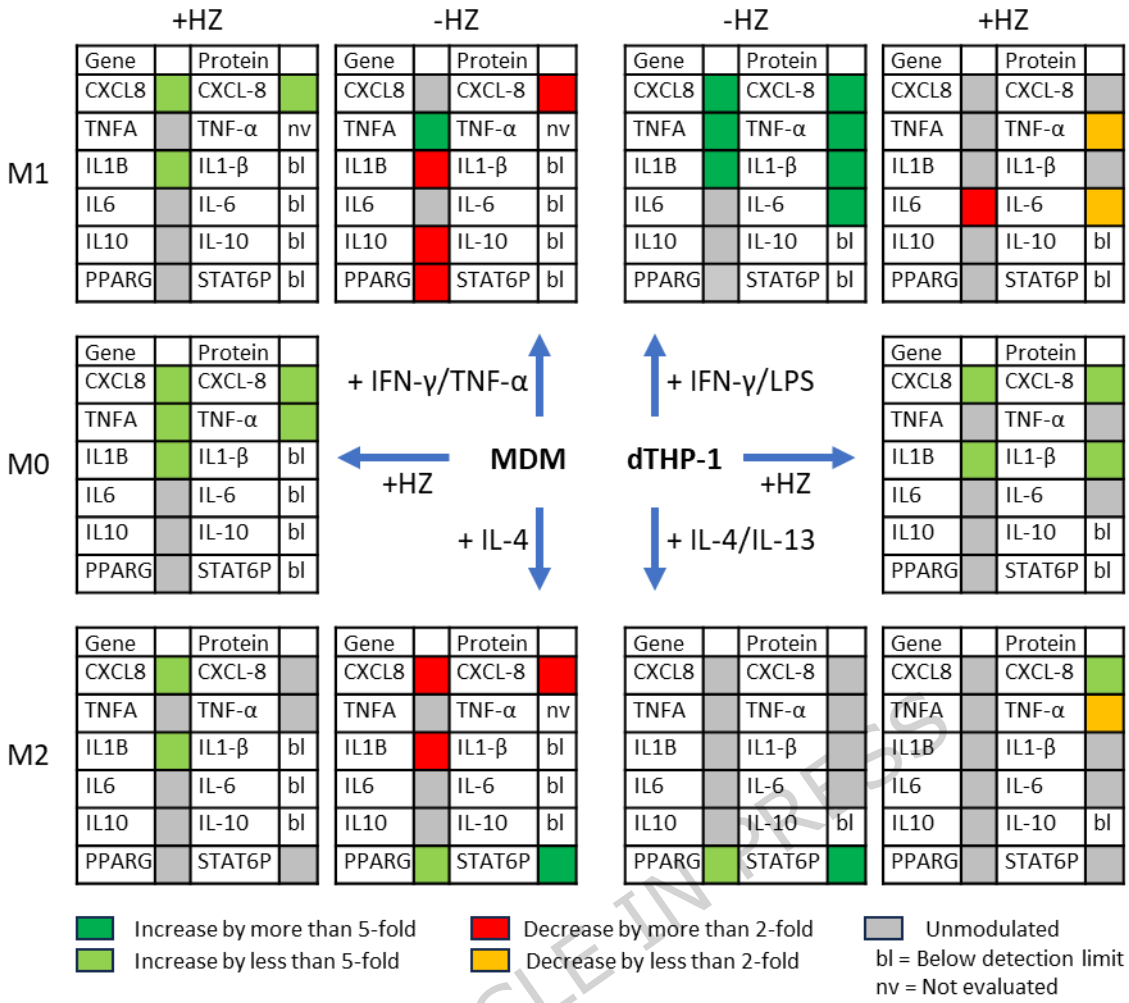

Figure 8. Schematic representation of the results. *Macrophage polarization markers evaluated as mRNA expression levels (gene) and protein secretion in the supernatants (protein) in M0/M1/M2 macrophages (MDM or dTHP-1 cells) in the presence of hemozoin.*

Although conflicting results have been reported in the literature, likely due to variations in cell models and HZ preparation, this *in vitro* study highlights the pro-inflammatory effects of HZ in human primary macrophages contributing to malaria pathogenesis. HZ can induce inflammatory effects through different mechanisms. Native HZ, associated with proteins and parasitic DNA, can bind TLR9 and TLR4, leading to NF-κB activation and subsequent transcription of pro-inflammatory mediators. Monocytes treated with HZ undergo intense oxidative stress, leading to the release of substantial quantities of peroxidation derivatives, resulting in excessive production of pro-inflammatory cytokines (Giribaldi 2010).

This aspect is clinically relevant and aligns with studies in patients. M1 macrophages have been shown to predominate in lung injury including acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) in patients with severe malaria, where the activation of lung macrophages and the subsequent release of inflammatory cytokines contribute to lung damage [24]. HZ-laden macrophages have been observed in the septal areas and alveolar spaces of ARDS patients [59] and HZ presence has also been associated with ARDS development in animal models [60].

Consistently, in an experimental murine model of cerebral malaria, treatment with IL-33, which plays an important role in Th2-associated immune responses, prevented the development of the disease by reducing pro-inflammatory cytokines and chemokines production while increasing type 2 cytokines that polarize macrophages towards the M2 phenotype [61]. However, other studies indicate that an M2-like phenotype of monocytes correlates with the severity of the disease in children [26] and pregnant women [62].

Macrophage polarization in malaria remains poorly studied. From a clinical perspective, elucidating the impact of hemozoin (HZ) on macrophage polarization enhances our understanding of how HZ modulates immune responses in malaria. These findings underscore HZ's potential role in driving inflammatory processes and shaping macrophage plasticity. Such insights may be valuable for the development of immunomodulatory adjunctive therapies.

This is a pilot study aimed at providing proof of concept regarding the importance of HZ in macrophage polarization, achieved by utilizing and comparing two commonly used cellular models. The results obtained will pave the way for future studies investigating the molecular mechanisms and signaling pathways, as well as the individual components of hemozoin involved in macrophage polarization.

In conclusion, these findings highlight the importance of using relevant models, such as primary cells and native hemozoin, to obtain a more accurate representation of *in vivo* conditions in malaria patients. Furthermore, this study may help to correlate the different clinical manifestations of malaria, ranging from uncomplicated to severe disease, with dysregulation of phagocyte functions and promote better therapeutic strategies to counteract the effects of hemozoin accumulation.

436

437 **4. Methods**

438 **4.1 Human macrophage models and *P. falciparum* cultures**

439 Primary human monocytes-derived macrophages (MDM) were obtained from healthy blood donors
440 as previously described [63]. Peripheral blood mononuclear cells (PBMC) were isolated from the
441 buffy coats using Ficoll-Hypaque density gradient centrifugation. The cells were then washed and
442 resuspended in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM)
443 supplemented with penicillin/streptomycin (1%), L-glutamine (1%), heat-inactivated bovine serum
444 (FBS, 10%), and heat-inactivated normal human serum (NHS, 5%). The suspension was seeded
445 into 75-cm^2 flasks (Falcon; BD Biosciences Labware) at a density of $8x10^6$ cells/ml. After 2 h of
446 incubation at 37°C in a humidified atmosphere containing 5% CO2, non-adherent cells, primarily T
447 lymphocytes, were removed by gentle pipette aspiration. An equal volume of fresh complete
448 medium was then added to each flask. After 24 h of culture, adherent cells were washed twice with
449 PBS, detached by scraping with a rubber policeman, and counted using trypan blue dye exclusion
450 [63]. The use of human peripheral blood cells derived from buffy coats for experimentation has been
451 approved by the Territorial Ethical Committee 1 of the regional Authority of the Lombardy region,
452 Milan, Italy; volunteer blood donors (>18 Years old) signed informed consent that material not of
453 medical use could be utilized for non-commercial research purposes. All methods were carried out
454 in accordance with relevant guidelines and regulations approved by the aforementioned Ethical
455 Committee.

456 The *mycoplasma*-free human monocytic leukaemia cell line, THP-1, was maintained in culture in
457 RPMI 1640 medium supplemented with 2 mM L-glutamine, 20 mM HEPES buffer pH 7.4, 10 µM
458 sodium pyruvate, 50 µM β-mercaptoethanol and 10% heat-inactivated FBS (Euroclone).
459 Differentiation into macrophages (dTHP-1) was obtained by incubating the cells with phorbol 12-
460 myristate 13-acetate (PMA, 10 ng/mL) for 48h at 37°C and 5% CO_2.

461 *Mycoplasma*-free *P. falciparum* parasites (D10 and W2 strains) were cultured as previously
462 described [64]. Cultures were maintained at 5% haematocrit using human type A red blood cells

(RBCs) in RPMI 1640 medium supplemented with 5% heat-inactivated A+ human plasma, 0.5% AlbuMax (Invitrogen), 20 mM HEPES buffer pH 7.4 and 0.01% hypoxanthine. The parasites were grown in a microaerophilic gas mixture comprising 1% O_2, 5% CO_2 and 94% N_2 at 37°C.

4.2 Hemozoin isolation and quantification

Parasitized RBCs at 4-8% parasitaemia were washed twice with serum-free medium and resuspended to 25% haematocrit. After fractionation on a discontinuous Percoll/4% sorbitol (wt/vol) gradient at 0%, 40% and 90% by centrifugation at 1075 x *g*, HZ was collected from the 0-40% interphase and repeatedly washed with PBS [65]. HZ concentration was quantified spectrophotometrically based on its haem content. Specifically, an aliquot of HZ was dissolved in 1M NaOH, and the optical density at a wavelength of 405 nm was interpolated against a standard curve of haemin. For all experiments, malaria pigment was used at the biologically relevant concentration, 10 µg/mL, consistent with levels easily reached in vivo, calculated considering the iron content of trophozoites as previously [43,64].

4.3 Polarization of MDM and THP-1 cells and stimulation with HZ

Primary human monocytes were seeded at a density of $2.5x10^5$ cells/well in 48-wells tissue culture plates (Corning®) in 500 µL of complete medium and incubated for 6 days to achieve differentiation into MDM. After differentiation, MDM were left unpolarized (M0) or polarized into M1 macrophages using 20 ng/ml IFN-γ and 2 ng/mL TNF-α, or into M2 macrophages using 20 ng/mL IL-4 [66] in the presence or not of 10 µg/mL HZ for 24h at 37°C and 5% CO_2.

Similarly, THP-1 cells were seeded at $5x10^5$ cells/well in 24-wells tissue culture plates (Corning®) and differentiated into macrophages (dTHP-1) as described in section 4.1. THP-1 were left unpolarized (M0) or polarized into M1 macrophages using 20 ng/mL IFN-γ and 10 ng/mL lipopolysaccharide (LPS) [67] or into M2 macrophages using IL-4 and IL-13 (20 ng/mL each) [63,68] in the presence or not of 10 µg/mL HZ for 24h at 37°C and 5% CO_2.

At the end of the incubation period, images were captured with an inverted microscope (Nikon Eclipse Ti-Series) at 200x magnification with a digital Nikon Digital Sight camera. The percentage

of HZ-engulfed MDM was determined by light microscopy. Data are the mean ± standard deviation of three different fields from 4 donors.

4.4 RNA extraction and quantification

MDM and dTHP-1 (0.5 – 1.5 x10^6) were washed with DPBS, lysed using 700 µL QIAzol Lysis Reagent (Qiagen), and RNA was extracted using the QIAamp® RNA Blood Mini Kit (Qiagen) following the manufacturer's instructions. The concentrations and quality of RNA were assessed using the NanoPhotometer® NP80 (Implen). Absorbance ratios A260/A280 and A260/A230 were evaluated to ensure sample purity.

4.5 Reverse transcription and Real-Time PCR

The isolated RNA (500 ng) was purified from genomic DNA and reverse transcribed into complementary DNA (cDNA) using the QuantiTect® Reverse Transcription kit (Qiagen). The expression of *CXCL8, TNFA, IL1B, IL6, IL10* and *PPARG* genes in M0/M1/M2 polarized MDM and dTHP-1 cells, with or without HZ treatment, was assessed by Real-Time PCR. Amplification was performed using the QuantiNova® SYBR® Green PCR Kit (Qiagen) on the Rotor-Gene Q 5plex (Qiagen). Gene expression was normalized on β-actin (*BACT*) as the reference gene. The reaction mix for each sample consisted of 10 µL of QuantiNova Master Mix (2x), 0.14 µL each of forward and reverse primers at 0.7 µM concentration, 7.72 µL of nuclease-free water, and 2 µL of cDNA, for a final volume of 20 µL. The sequences of forward and reverse primers are listed in Table 1.

Table 1*. Primer sequences for SYBR® Green Real-Time PCR.*

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
BACT	TGAGAGGGAAATCGTGCGTGAC	GCTCGTTGCCAATAGTGATGACC
CXCL8	CCACCGGAAGGAACCATCTC	GGGGTGGAAAGGTTTGGAGT
TNFA	CCACTTCGAAACCTGGGATTC	TTAGTGGTTGCCAGCACTTCA
IL1B	ATGCACCTGTACGATCACTGA	ACAAAGGACATGGAGAACACC
IL6	GTAGCCGCCCCACACAGACAGCC	GCCATCTTTGGAAGGTTC
IL10	AGAACCTGAAGACCCTCAGGC	CCACGGCCTTGCTCTTGTT

PPARG	TTGTCACGGAACACGTGCA	GGAGCGGGTGAAGACTCATG

The thermal profile consisted of an initial hold phase at 95°C for 10 minutes, followed by 40 amplification cycles, each comprising three stages: 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 20 seconds.

Gene expression analysis was conducted using the Fold Change method, calculated as follows:

$\Delta Ct = Ct_{gene\ of\ interest} - Ct_{BACT}$

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{control}$

$Fold\ Change = 2^{(-\Delta\Delta Ct)}$

4.6 Cytokines and chemokines quantification in the supernatants

Cell culture supernatants were collected after 24h of incubation with polarizing stimuli and HZ. The concentrations of CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 in the supernatants from both MDM and dTHP-1 cells were quantified using commercial ELISA kits according to the manufacturer's protocols: Human IL-8/CXCL8 DuoSet ELISA, Human TNF-alpha DuoSet ELISA, Human IL-1 beta/IL-1F2 DuoSet ELISA, Human IL-6 DuoSet ELISA, and Human IL-10 DuoSet ELISA (R&D Systems).

4.7 STAT6 and pSTAT6 protein levels

Western blot analysis was performed to evaluate STAT6 and phospho-STAT6 (pSTAT6) protein levels in polarized MDM and dTHP-1 cells. Proteins were extracted from $2x10^6$ macrophages using a lysis mix containing 3% (v/v) Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific) and 1% (v/v) 0.5 M EDTA solution (100x) (Thermo Fisher Scientific) in Pierce™ RIPA buffer (Thermo Fisher Scientific). After 30 min of centrifugation at 18,500 x g at 4°C, the debris was discharged, and proteins were quantified using the Pierce™ Bradford Plus Protein Assay reagent (Thermo Fisher Scientific) with the NanoPhotometer® NP80 (Implen). Total protein extracts (30 µg) were subjected to SDS-PAGE and separated proteins were transferred onto a nitrocellulose membrane

overnight at 45 V at 4°C. Membranes were blocked for 1h at room temperature with a solution containing 5% nonfat dried milk powder (EuroClone) in TBS-T (0.01% Tween 20 in TBS), and subsequently incubated overnight at 4°C with primary antibodies (Ab) diluted in the same blocking solution. The primary Abs used included total STAT6 (1:1,000 dilution; Cell Signaling Technology, #9362) and phospho-STAT6 (Tyr641) (1:500 dilution; Cell Signaling Technology, #9361). β-actin was used as a loading control, using the anti-β-actin monoclonal Ab (mAb) 13E5 (1:1,000 dilution; Cell Signaling Technology, #4970) prepared in 5% nonfat dried milk powder in TBS-T. After three washes with TBS-T, membranes were incubated for 1h at room temperature with a horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary Ab (1:2,000 dilution; Cell Signaling Technology, #7074) diluted in 5% nonfat dried milk powder in TBS-T. Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham GE Healthcare, Amersham, UK), performed according to the manufacturer's instructions.

4.8 Statistical Analysis

Comparisons between two groups were performed using an unpaired two-tailed *t*-test. Differences among more than two groups were analysed by one-way ANOVA analysis and post-hoc multiple comparisons tests (Dunnett), using GraphPad Prism 8 software. Data are representative of at least three independent experiments run in triplicate.

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Authors' contributions

Conceptualization: SDA, NB, FP; Data curation: FP; Formal analysis: FP; Funding acquisition: SDA, NB, SD; Investigation: FP, SP, MD, ECA, SG; Methodology: FP, MD, SG; Supervision: SD,

SDA, NB, SP, GP, EV; Roles/Writing - original draft; FP, NB, SDA; and Writing - review & editing: ECA, SP, GP, EV. All authors read and approved the final version of the manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials

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Figure legends

Figure 1. Macrophage polarization*. Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (filled columns – panels A and B) and dTHP-1 cells (striped columns – panels C and D) after 24 h of incubation with polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed*

*using one-way ANOVA followed by Dunnett's post-hoc test, comparing each gene or protein against M0 (untreated macrophages). Statistical significance is denoted as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

***Figure 2. STAT6 phosphorylation**. Western Blot analysis of STAT6 phosphorylation in primary MDM (A) and dTHP-1 cells (C) in control cells or cells treated with hemozoin (HZ). Relative expressions of total STAT6 and pSTAT6 normalized on β-actin in MDM (B) and dTHP-1 cells (D) were assessed in M2-like macrophages. Data are the mean ± SD from three different donors for MDM and from three independent experiments for dTHP-1. Statistical analysis was performed using an unpaired two-tailed t-test for each transcription factor and comparing the data versus the control M2-macrophages not incubated with HZ.*

***Figure 3. HZ phagocytosis by M0, M1, M2 macrophages**. Representative images of MDM (A) or dTHP-1 (B) after 24h of incubation with polarizing stimuli in the presence or absence of HZ. Images were captured by using a Nikon Eclipse Ti-Series with 20x objective and the digital camera Nikon Digital Sight. Scale bar: 100 µm.*

***Figure 4. Effect of HZ on M0 macrophages.** Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (full columns – panels A, B) and dTHP-1 cells (striped columns – panels C, D) after 24 h incubation with HZ alone in the absence of other polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein in comparison to control (M0 - unpolarized macrophages). Statistical significance is denoted as follows: *p < 0.05; ** p < 0.01; ***p < 0.001.*

***Figure 5. Effect of HZ on M1 macrophages.** Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (filled columns – panels A, B) and dTHP-1 cells (striped columns – panels C, D) after 24 h incubation with HZ and M1-polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three*

*independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein, comparing the data versus the control (M1-stimulated macrophages). Statistical significance is denoted as follows: *p < 0.05; **p < 0.01; ***p < 0.001.*

***Figure 6**. **HZ effect on dTHP-1 polarized with M1 stimuli used for MDM**. M1 macrophage polarization markers assessed as mRNA expression levels (panel A) and protein secretion levels in cell supernatants (panel B) in dTHP-1 macrophages after 24 h incubation with HZ and M1-polarizing stimuli used for MDM (IFN-γ +TNF-α). Gene expression of CXCL8, IL1B and IL6 was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from three independent experiments for dTHP-1. CXCL-8, IL-1β and IL-6 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein, comparing the data to the control (M1-stimulated macrophages). Statistical significance is denoted as follows: *p < 0.05.*

***Figure 7**. **Effect of HZ on M2 macrophages.** Macrophage polarization markers evaluated as mRNA expression levels (panels A, C) and protein secretion in the supernatants (panels B, D) in MDM (filled columns – panels A, B) and dTHP-1 cells (striped columns – panels C, D) after 24 h of incubation with HZ and M2-polarizing stimuli. Gene expression of CXCL8, TNFA, IL1B, IL6, IL10 and PPARG was evaluated by Real-Time PCR analysis. Results are expressed as fold change calculated by the ΔΔCt method. Data are presented as mean ± standard deviation from four different donors for MDM and from at least three independent experiments for dTHP-1. CXCL-8, TNF-α, IL-1β, IL-6 and IL-10 protein levels were measured by ELISA and expressed as % of the control, with data represented as the mean ± standard deviation. Statistical analysis was performed using an unpaired two-tailed t-test for each gene or protein and comparing the data versus the control (M2-stimulated macrophages). Statistical significance is denoted as follows: *p < 0.05; **p < 0.01.*

***Figure 8. Schematic representation of the results.** Macrophage polarization markers evaluated as mRNA expression levels (gene) and protein secretion in the supernatants (protein) in M0/M1/M2 macrophages (MDM or dTHP-1 cells) in the presence of hemozoin.*