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

Federica Perego[1], Silvia Parapini[2], Estefanía Calvo-Alvarez[3], Maria Dolci[1], Serena Delbue[1], Silvia Ghezzi[4], Elisa Vicenzi[4], Guido Poli[5,6], Nicoletta Basilico[1]✉ & Sarah D'Alessandro[3]

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, CXCL8, 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 CXCL8 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

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

[1]Parvirlab, DipartimentDepartment of Biomedical, Surgical and Dental Sciences, University of Milan, MilanVia Pascal 36, 20133, Italy. [2]Department of Biomedical Sciences for Health, University of Milan, Via Pascal 36, 20133 Milan, Italy. [3]Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", University of Milan, Via Pascal 36, 20133 Milan, Italy. [4]Viral Pathogenesis and Biosafety Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. [5]Human Immuno-Virology (H.I.V.) Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy. [6]Vita-Salute San Raffaele University School of Medicine, Via Olgettina 58, 20132 Milan, Italy. ✉email: nicoletta.basilico@unimi.it

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.

Results

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 h 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 (Fig. 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 (Fig. 1B), indicating post-transcriptional regulation. *IL1B* expression was significantly decreased (Fig. 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 (Fig. 1A,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 (Fig. 1A,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 (Fig. 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 CXCL8, TNF-α, IL-1β and IL-6 was observed both in terms of gene expression and protein secretion (Fig. 1C,D). *PPARG* expression was unchanged, whereas *IL10* gene expression was increased, though not significantly (Fig. 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 Fig. 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* (Fig. 1C) was observed, whereas *IL10* gene expression was unchanged and protein levels in the supernatants were below the detection limit.

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 (Fig. 2A,C, lane 3 vs.1) but was increased,

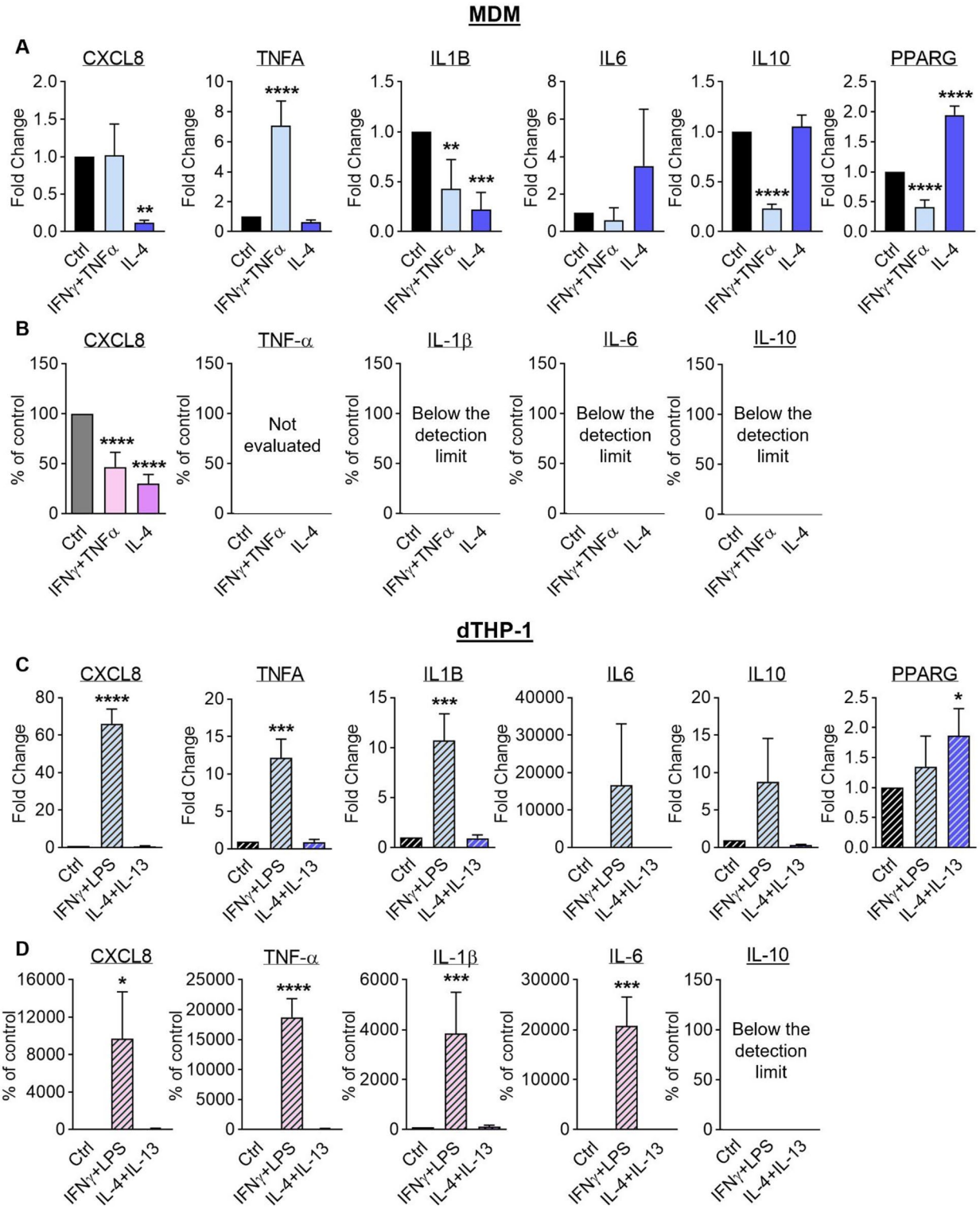

Fig. 1. Macrophage polarization. Macrophage polarization markers evaluated as mRNA expression levels (panels **A**,**C**) and protein secretion in the supernatants (panels **B**,**D**) in MDM (filled columns—panels **A**,**B**) and dTHP-1 cells (striped columns—panels **C**,**D**) after 24 h of incubation 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. CXCL8, 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: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

as expected, by M2 stimuli (Fig. 2A,C, lane 5 vs. 1). The original full uncropped Blots images are shown in Supplementary Fig. 2. Figure 2 also shows the effect of HZ on STAT6 phosphorylation (Fig. 2B,D), which will be described in the subsequent sections.

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 Fig. 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.

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 (Fig. 4A,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 (Fig. 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 (Fig. 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 (Figs. 4A,C; 2A,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 Fig. 3). Differently from HZ, latex beads failed to modulate CXCL8, TNF-α and IL-1β production.

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 (Fig. 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

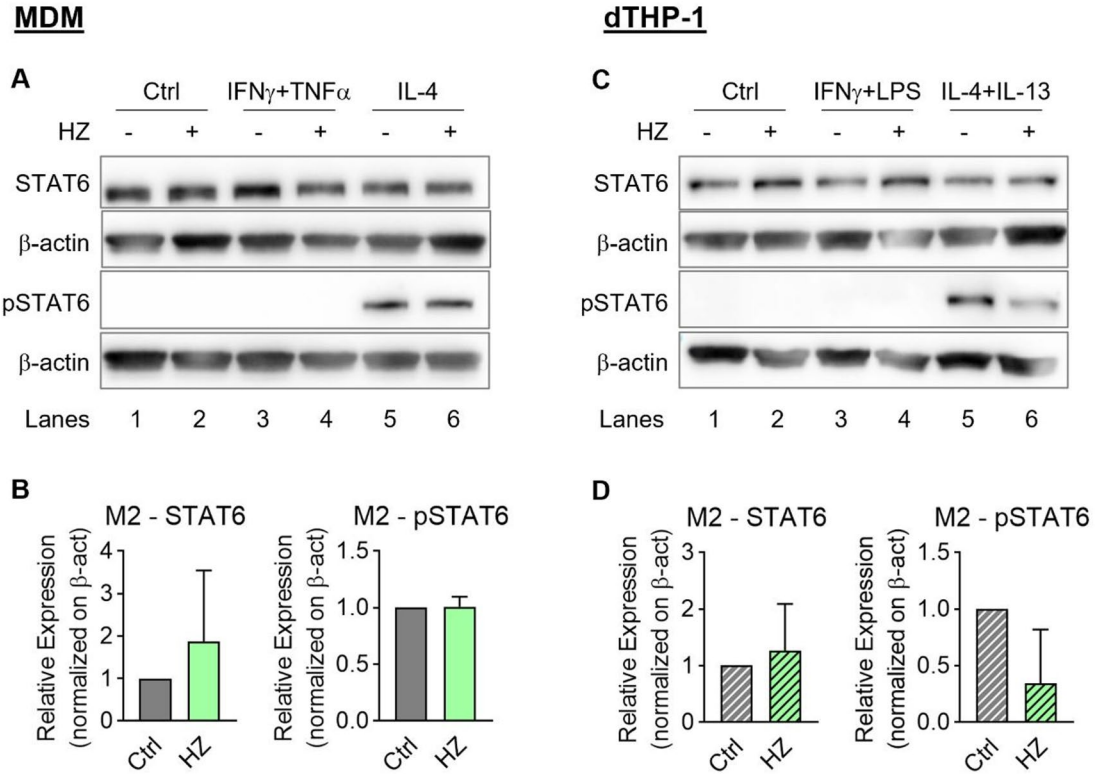

Fig. 2. STAT6 phosphorylation. Western Blot analysis of STAT6 phosphorylation in primary MDM (**A**) and dTHP-1 cells (**C**) in control cells or cells treated with hemozoin (HZ). Relative expressions of total STAT6 and pSTAT6 normalized on β-actin in MDM (**B**) and dTHP-1 cells (**D**) were assessed 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.

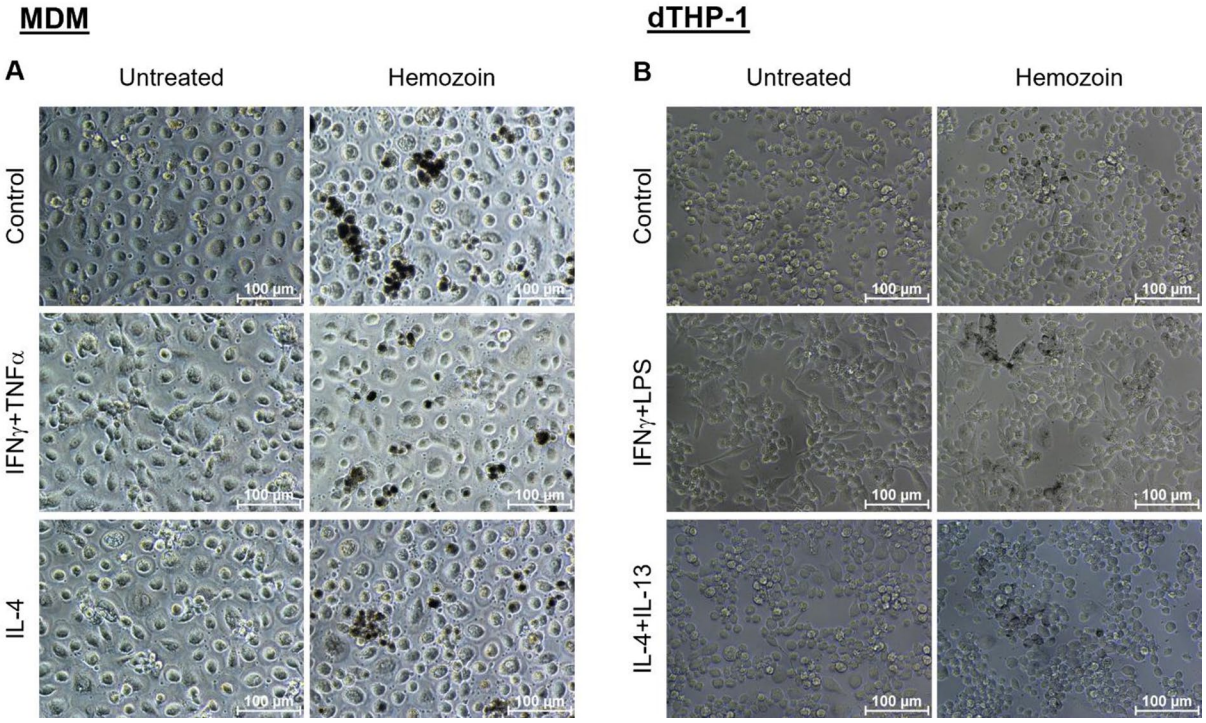

Fig. 3. HZ phagocytosis by M0, M1, M2 macrophages. Representative images of MDM (**A**) or dTHP-1 (**B**) after 24 h of incubation with polarizing stimuli in the presence or absence of HZ. Images were captured by using a Nikon Eclipse Ti-Series with 20× objective and the digital camera Nikon Digital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

enhanced by HZ (Fig. 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 (Fig. 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 (Fig. 5D). As observed in unpolarized M0 macrophages (Fig. 4), HZ failed to modulate gene expression of anti-inflammatory M2 markers (*PPARG* and *IL10*) in both cell types (Fig. 5A,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 (Fig. 6A) and protein secretion (Fig. 6B) and did not alter IL-6 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.

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

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

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].

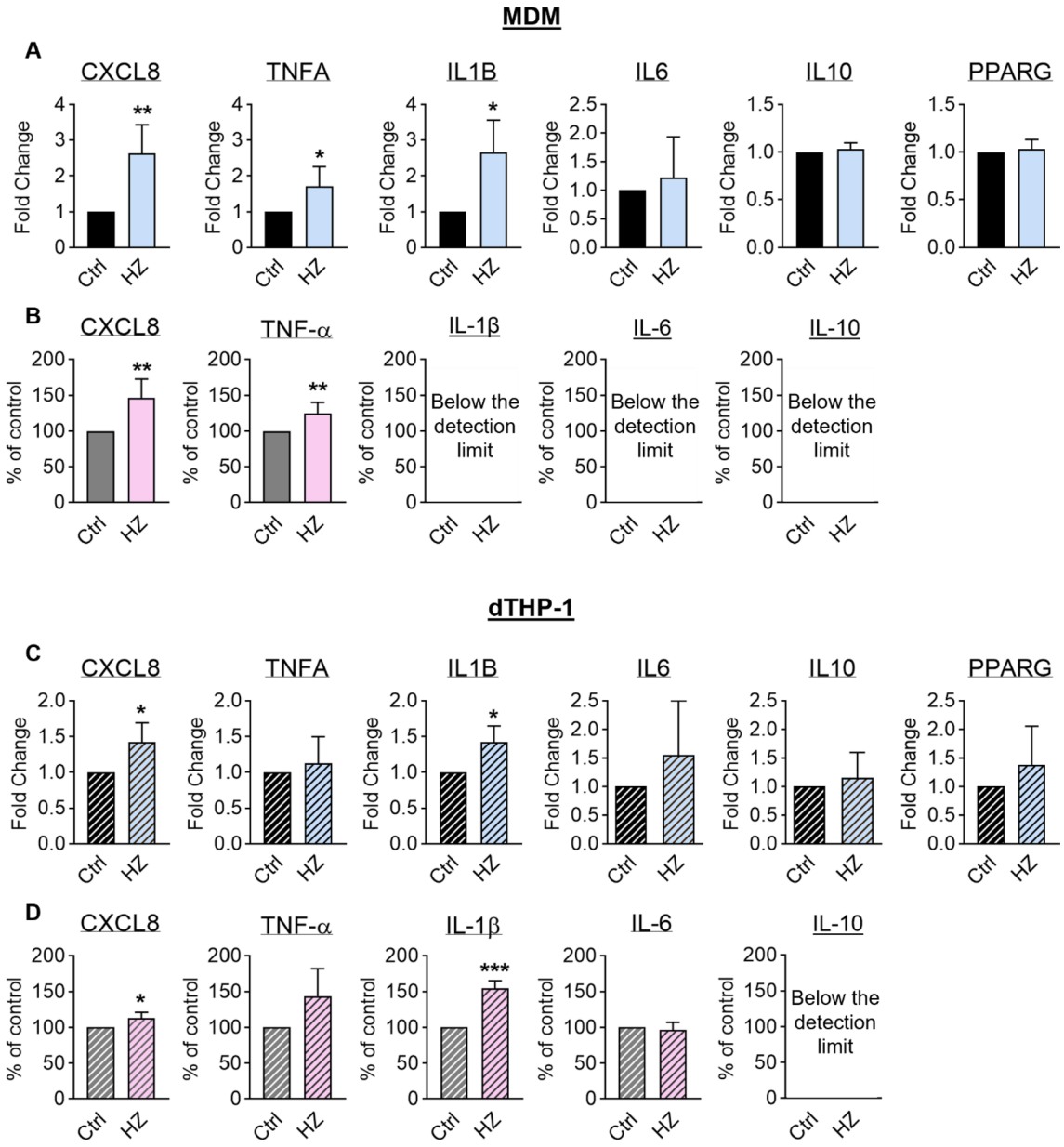

Fig. 4. Effect of HZ on M0 macrophages. Macrophage polarization markers evaluated as mRNA expression levels (panels **A**,**C**) and protein secretion in the supernatants (panels B, D) in MDM (full columns—panels **A**,**B**) and dTHP-1 cells (striped columns—panels **C**,**D**) after 24 h incubation 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. CXCL8, 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: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

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.

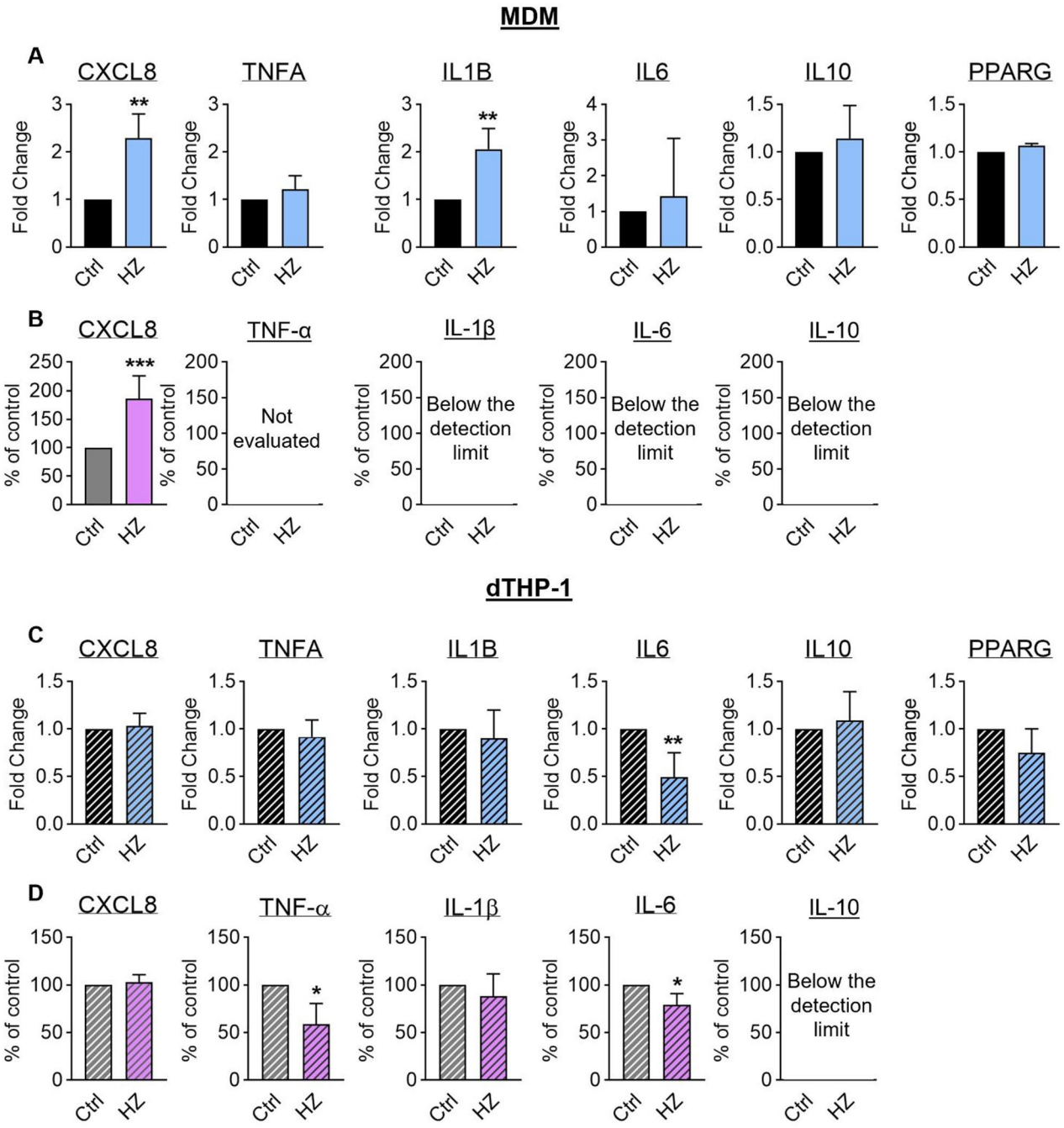

Fig. 5. Effect of HZ on M1 macrophages. Macrophage polarization markers evaluated as mRNA expression levels (panels **A**,**C**) and protein secretion in the supernatants (panels **B**,**D**) in MDM (filled columns—panels **A**,**B**) and dTHP-1 cells (striped columns—panels **C**,**D**) after 24 h incubation 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. CXCL8, 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: $*p<0.05$; $**p<0.01$; $***p<0.001$.

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

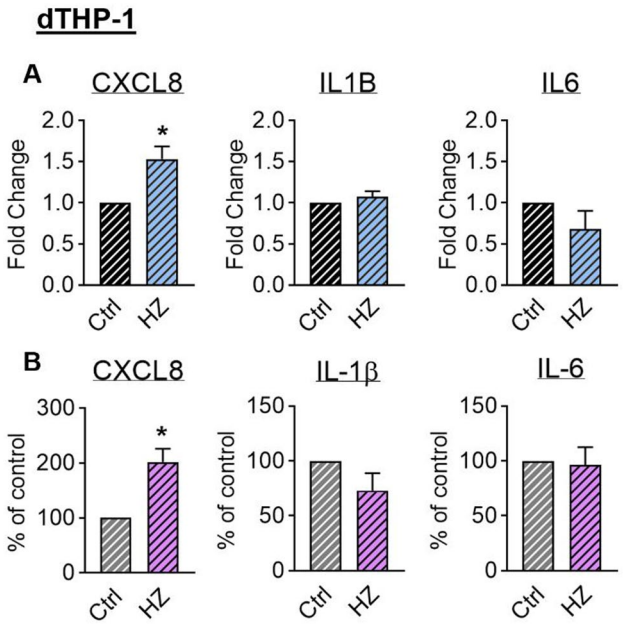

Fig. 6. HZ effect on dTHP-1 polarized with M1 stimuli used for MDM. M1 macrophage polarization markers assessed as mRNA expression levels (**A**) and protein secretion levels in cell supernatants (**B**) in dTHP-1 macrophages after 24 h incubation with HZ and M1-polarizing stimuli used for MDM (IFN-γ + 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. CXCL8, 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: $^{*}p < 0.05$.

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 CXCL8, IL-1β and IL-6. In contrast, MDM exposed to M1 stimuli decreased *IL1B* expression and CXCL8 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 CXCL8 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 CXCL8 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 [42].

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 CXCL8, 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 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

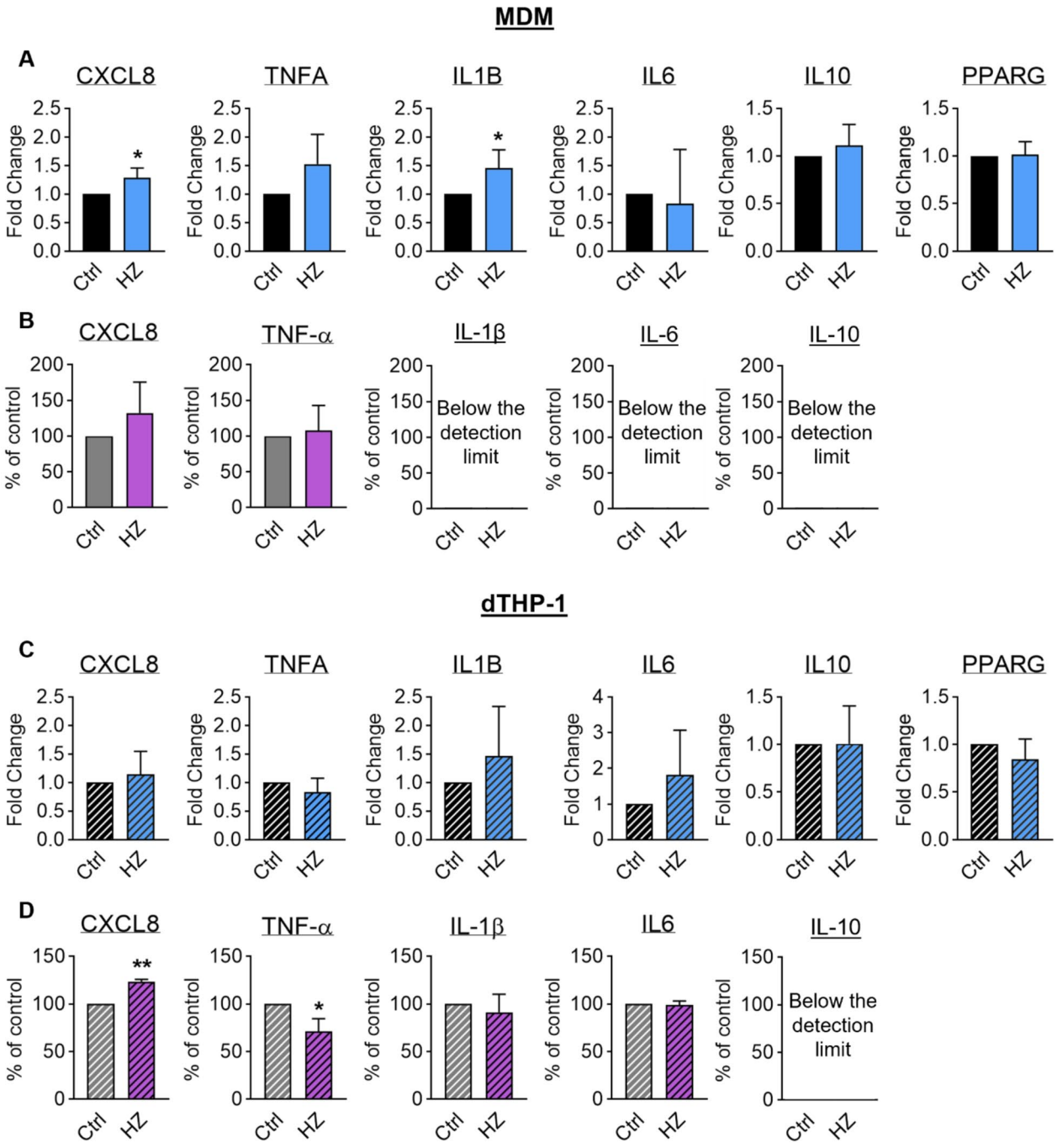

Fig. 7. Effect of HZ on M2 macrophages. Macrophage polarization markers evaluated as mRNA expression levels (panels **A**,**C**) and protein secretion in the supernatants (panels **B**,**D**) in MDM (filled columns—panels **A**,**B**) and dTHP-1 cells (striped columns—panels **C**,**D**) after 24 h of 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. CXCL8, 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: $^{*}p < 0.05$; $^{**}p < 0.01$.

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 induced 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 CXCL8 production in the presence of M1-polarizing stimuli (Fig. 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 CXCL8 but not of IL-1β (Fig. 6). LPS may exert a stronger stimulatory effect than TNF-α, potentially masking HZ effect on CXCL8. 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[38,54]. Variations in inflammasome activation in different cell types were indeed reviewed by Cornut and colleagues[57].

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

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

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 [46].

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 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, prevents 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

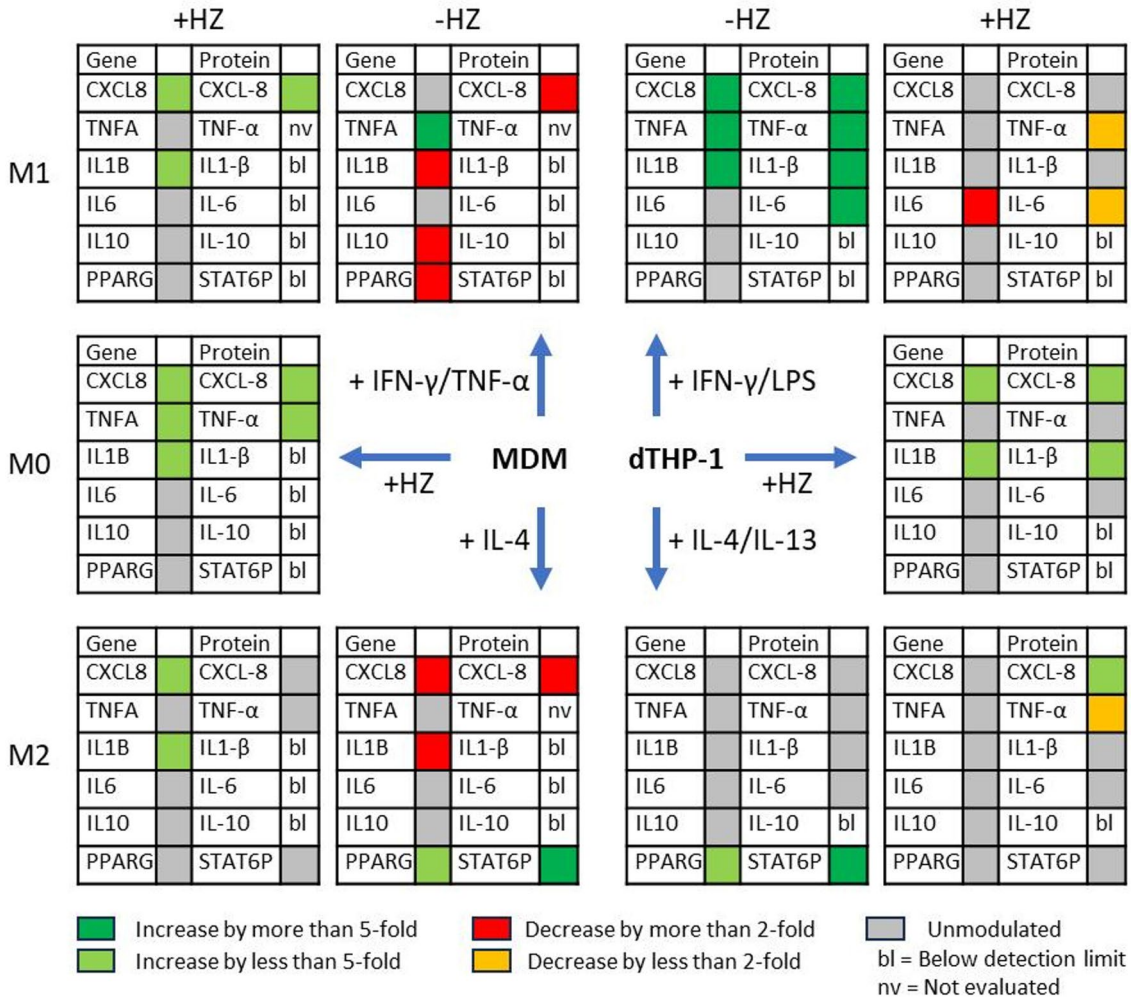

Fig. 8. Schematic representation of the results. Macrophage polarization markers evaluated as mRNA expression levels (gene) and protein secretion in the supernatants (protein) in M0/M1/M2 macrophages (MDM or dTHP-1 cells) in the presence of hemozoin.

pave the way for future studies investigating the molecular mechanisms and signaling pathways, as well as the individual components of HZ involved in macrophage polarization.

In conclusion, these findings highlight the importance of using relevant models, such as primary cells and native HZ, 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 HZ accumulation.

Methods

Human macrophage models and *P. falciparum* cultures

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

purposes. All methods were carried out in accordance with relevant guidelines and regulations approved by the aforementioned Ethical Committee.

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

Mycoplasma-free *P. falciparum* parasites (D10 and W2 strains) were cultured as previously 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.

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 × *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 1 M 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].

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

Primary human monocytes were seeded at a density of 2.5×10^5 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 24 h at 37 °C and 5% CO_2.

Similarly, THP-1 cells were seeded at 5×10^5 cells/well in 24-wells tissue culture plates (Corning®) and differentiated into macrophages (dTHP-1) as described in "Human macrophage models and P. falciparum cultures". 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 24 h 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.

RNA extraction and quantification

MDM and dTHP-1 ($0.5–1.5 \times 10^6$) 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.

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.

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

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

$$\Delta\mathrm{Ct} = \mathrm{Ct}_{\text{gene of interest}} - \mathrm{Ct}_{\mathrm{BACT}}$$

$$\Delta\Delta\mathrm{Ct} = \Delta\mathrm{Ct}_{\text{sample}} - \Delta\mathrm{Ct}_{\text{control}}$$

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

Cytokines and chemokines quantification in the supernatants

Cell culture supernatants were collected after 24 h of incubation with polarizing stimuli and HZ. The concentrations of CXCL8, 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/

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

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

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).

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 2×10^6 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 1 h 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 (1:1000 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 (1:1000 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 1 h at room temperature with a horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary Ab (1:2000 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.

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.

Data availability

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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Author contributions

Conceptualization: S.D.A., N.B., F.P.; data curation: F.P.; formal analysis: F.P.; funding acquisition: S.D.A., N.B., S.D.; investigation: F.P., S.P., M.D., E.C.A., S.G.; methodology: F.P., M.D., S.G.; supervision: S.D., S.D.A., N.B., S.P., G.P., E.V.; roles/writing-original draft; F.P., N.B., S.D.A.; and writing-review and editing: E.C.A., S.P., G.P., E.V. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to N.B.

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