Chapter 5: Study the phenotypic changes induced by natural hemozoin (Hz) in human monocytes.
5.1. Introduction

Malaria is an endemic disease affecting 212 million individuals and causing 4, 29,000 deaths in 2015 (WHO report 2015). Plasmodium is the etiological agent of malaria. Of all the Plasmodium species, falciparum causes the most severe form of malaria with several manifestations including cerebral malaria, pulmonary edema, acute renal failure or severe anemia (219–222). Previously, falciparum malaria has been associated with immunosuppression, which leads to reduced immunity to malaria and increase of secondary infections (206–209). The parasite and host factors, both are involved in the development of severe malaria.

Hemozoin (Hz), is frequently reported to alter immune responses during malaria. Hz is the insoluble bio-crystal formed by a parasite in its food vacuole while utilizing host hemoglobin in infected RBC and protecting itself from toxic heme metabolites (143,223). Free Hz enclosed in food vacuole is released into the bloodstream during schizont rupture at the end of every erythrocytic cycle of 48h. The released pigment is taken up by phagocytic cells including monocytes (141). The ingested Hz is not digested and accumulates in the cells, where it alters several functions of monocytes such as repeated phagocytosis (149), bactericidal abilities (150), oxidative burst, MHC Class II expression, antigen presentation (151) and maturation to dendritic cells (152). The ingestion of Hz stimulates the production of several cytokines and chemokines in monocytes, suggesting its ability to influence immune responses. For in vitro and in vivo studies, Hz has been isolated from parasitic cultures by different methods. Some methods, involve isolation of purified Hz, wherein the chemicals are used to remove most of the parasite-associated lipids and proteins. The other methods involve isolation of natural Hz to maintain its physiological phagocytic form. There may be differences in results obtained with natural and purified Hz due to the absence of parasite components in the latter. Throughout this study, natural Hz was used for all experiments. Previously, natural Hz has been reported to induce secretion of pro- and anti-inflammatory cytokines -TNF α, IL-1β, IL-1RA, IL-10, IL-8 and chemokines CCL2, CCL3 CCL4, CXCL1, CXCL2, CXCL3 and CXCL5 (159,160). Moreover, NF-κB and p38-MAPK are involved in IL-1β, TNF-α and CCL3 production (162). Purified Hz, on the other hand, stimulates TLR9 signaling and induces production of cytokines -TNF-α, IL-6, IL-12p40, CCL2 in MYD88 dependent manner in murine spleen and dendritic cells (165). In murine MQ cell line- B10R, NF-κB has been demonstrated to be involved in the production of chemokines- CCL2, CCL3, CCL4 and
CXCL2 induced by Hz (168). Hz- induced activation of NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome and IL-1β production is mediated through Src kinase Lyn and the tyrosine kinase Syk pathways in mice (140,141).

Monocytes (MO) are phagocytic cells of blood mononuclear system. They play an important role in innate immune response including defense to pathogens, inflammation, resolution, and repair (224). Monocytes are plastic in nature and differentiate into M1 and M2 phenotypes in the presence of specific micro-environmental stimuli. The M1 phenotype is pro-inflammatory and driven by the presence of LPS and IFNγ. On the contrary, the M2 phenotype is anti-inflammatory and driven by TH2 cytokines, IL-4, IL-13, IL-10 and TGF-β. The M1 and M2 phenotypes are characterized by the production of different cytokines, chemokines and effector molecules with opposing functionalities. M1 and M2 monocytes have an indirect role in activation of Th1 and Th2 responses respectively (64). IL-12- producing M1 phenotype is associated with anti-parasitic and tumor resistance capabilities and IL-10- producing M2 phenotype is related to wound healing, immune-regulation and resolution of inflammation (64,101,225). The M2 phenotype is characterized by expression of mannose-binding receptor- CD206, high arginase activity and low NO production (78,97,106,226). The M2 type monocytes are divided into M2a, M2b and M2c subtypes that produce specific chemokines CCL17, CCL1 and CXCL13 respectively (54,64,227). Frequently, MO/MQ phenotypes do not fall into any one specific category but may show spectra related to a particular phenotype (60).

Multiple signaling pathways including JAK/STAT, PKC/ERK, and PI3K/AKT/mTOR are involved in M2 polarization of monocytes The pathways may act individually or in combination to drive M2 polarization of monocytes (88,101).The signaling pathways, p38-MAPK and PI3K-Akt pathway are involved in IL-4 - induced M2a polarization in mice model (228) . Notch1 signaling via NF-κB, p38-MAPK and AKT pathways play a role in M2b polarization of murine lupus mouse model (229). Recent studies have enhanced our understanding and importance of phenotypic changes in MO/MQ by modulating immune responses in several infectious diseases(108,226,230,231) and highlighted their potential for development of new therapeutics. Phenotypic changes in MO/MQ are reported in parasitic diseases such as leishmaniasis, filariasis and trypanosomiasis (104,107,230).
The clinical and experimental data reported to date supports the role of Hz in alteration of immune responses, however, the role of Hz in activation of monocytes towards specific phenotype has not been explored. Hence, a systematic study to investigate the role of natural Hz in driving monocytes to M2 phenotype was undertaken. The drugs chloroquine (CHQ) and artemisinin (ART) possess pleiotropic effects. Along with anti-parasitic effect, these conventional antimalarials are immunomodulatory and have shown therapeutic effect in viral, other parasitic disease and cancer (191,232). Even though the immunomodulatory effects of these drugs are reported in other diseases, it is not explored sufficiently in relation to malaria immunity. Hence, the immune-modulatory effect of widely used anti-malarial drugs, chloroquine (CHQ) and artemisinin (ART) was examined in monocytes fed with Hz.

5.2. Results

5.2.1. Purity and characterization of human monocytes

The monocytes isolated from peripheral blood by CD14 positive magnetic associated cell sorting (MACS) was 85-90 % CD14 positive (Fig 5-1). The viability of monocytes was >95% as assessed by trypan blue staining. Monocytes were subjected to adherence to further enrich the monocytes before phagocytosis. These adherent monocytes were characterized by screening for expression of several cell surface leucocyte markers by flow cytometry (depicted in Table 5-1). Monocytes were positive for CD14, CD86, HLA-DR, CD54, CD11c, CD11b and negative for CD80, CD3, CD1a, CD40 (Fig 5-2).

Table 5-1. Leucocyte markers assessed in adherent peripheral blood derived monocytes

<table>
<thead>
<tr>
<th>Surface molecule</th>
<th>Function</th>
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<tbody>
<tr>
<td>CD14</td>
<td>Monocyte specific marker</td>
</tr>
<tr>
<td>CD86</td>
<td>Ag presentation and co-stimulation</td>
</tr>
<tr>
<td>CD40</td>
<td>Ag presentation and co-stimulation</td>
</tr>
<tr>
<td>CD54</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>CD11c</td>
<td>Dendritic and monocylic marker</td>
</tr>
<tr>
<td>CD11b</td>
<td>Adhesion molecule and macrophage</td>
</tr>
<tr>
<td>HLA DR</td>
<td>Ag presentation and co-stimulation</td>
</tr>
<tr>
<td>CD80</td>
<td>Ag presentation and co-stimulation</td>
</tr>
<tr>
<td>CD1a</td>
<td>Ag presentation by dendritic cells and M2 macrophage</td>
</tr>
<tr>
<td>CD3</td>
<td>T cell marker</td>
</tr>
<tr>
<td>CD95</td>
<td>Ag presentation</td>
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</table>
Figure 5-1 Monocytes purity. The dot plot is a representative figure of monocytes purity, isolated by CD14+ magnetic associated cell sorting (MACS) from human PBMC.

Figure 5-2. Characterization of peripheral blood derived adherent monocytes Expression of leucocyte surface markers CD14, CD86, CD40, HLA DR, CD54, CD95, CD11b, CD11c, CD80, CD1a, CD3 on adherent human monocytes as detected by flow cytometry. Representative overlays depict percentage positive monocytes Unstained are indicated by black color and stained cells are indicated by pink color.
5.2.2. Phagocytosis of Hz and its effect on cytotoxicity to adherent human monocytes

The phagocytosis of Hz in human monocytes was visualized by microscopy. The monocytes were exposed to Hz for 2h followed by media washes to remove unphagocytosed pigment. The uptake of Hz by monocytes was observed under microscope (Fig 5-3A). The cytotoxic effect of Hz on monocytes was studied by exposing them to different doses of the malarial pigment and viability was assessed by MTT. The Hz dose assessed in the range from 25-200µg was well tolerated by monocytes and did not affect viability up to 48h (Fig 5-3B). The percent phagocytosis in human monocytes was calculated with 25µg/ml and 50µg/ml of Hz (Table 5-2).

Table 5-2. Percent phagocytosis of Hz in adherent peripheral blood derived monocytes

<table>
<thead>
<tr>
<th>Phagocytic dose (µg/ml)</th>
<th>Hz phagocytosis in monocytes (%)</th>
<th>No of Hz pigments/monocyte</th>
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<tbody>
<tr>
<td>25</td>
<td>65.59 ± 8.8*</td>
<td>8.34 ± 2.87*</td>
</tr>
<tr>
<td>50</td>
<td>84.34 ± 6.8*</td>
<td>15.16 ± 2.82*</td>
</tr>
</tbody>
</table>

Percent phagocytosis was calculated from more than 1000 cells and pigment per monocytes were enumerated from 100 cells. The data is mean ± SD from 3 independent experiments (P<0.05).
5.2.3. Expression profile of M1-M2 cytokine and chemokine markers in Hz-exposed monocytes

Monocytes producing high levels of cytokine IL-10 and low IL-12 have been characterized as M2 monocytes (64,101,106,225). The effect of Hz on monocytes in terms of expression of M2-related molecules was studied. Elevated transcript levels of IL-10 and but not IL-12 were observed in Hz-fed monocytes by qPCR (Fig.4A). The M2 monocytes are further divided into three subtypes i.e. M2a, M2b and M2c characterized by expression of CCL17, CCL1 and CXCL13 respectively (64,226). RNA isolated from Hz-fed monocytes after 12h was analyzed for expression of CCL17 (M2a), CCL1 (M2b) and CXCL13 (M2c) using genes specific primer by qPCR. Significant amounts of CCL1 and CCL17 transcripts were detected in Hz- fed monocytes suggesting induction of M2a and M2b like phenotype respectively. The levels of CXCL13 were undetectable suggesting the absence of M2c type (Fig 5-4A). In contrast to other M2 subtypes, M2b monocytes produces high amounts of inflammatory cytokines. Hence, culture supernatants collected from Hz-fed monocytes were assessed for inflammatory cytokines –IL-6, TNFα and IL-1β. As depicted in (Fig 5-4A), high amounts of inflammatory cytokines IL-6, IL-1β and TNFα were detected by qPCR in Hz-fed monocytes The transcript levels of all cytokines and chemokines correlated with the secreted levels as detected in culture supernatants by ELISA in Hz-fed monocytes (Fig 5-4B). The latex- fed monocytes did not show significant production of cytokines and chemokines at transcript or protein levels suggesting that the effect seen is specifically due to Hz. Collectively, these results suggest that Hz primes monocytes towards M2a and M2b like phenotype.

![Relative fold change for IL-10 and IL-12](image)
5.2.4. Hz induces IL-10 but not IL-12 production in human monocytes

Hz upregulated anti-inflammatory cytokine IL-10 (associated with M2) at transcript and secreted levels in monocytes. The intracellular induction of IL-10 was demonstrated by dual staining with anti-human monocytes specific CD14 and intracellular IL-10 (Fig 5-5A). To confirm that Hz does not stimulate production of IL-12 in monocytes a positive control with monocytes pre-treated with IFNγ for 2h followed by LPS stimulation for 12h was used. The stimulation of monocytes with the pro-inflammatory treatment of LPS and IFNγ led to the robust expression of IL-12 at the transcript and secreted levels after 12 and 24h respectively (Fig 5-5 B, C). The results from these experiments indicating the inability of Hz to induce IL12 production but secrete high IL-10 level suggests that Hz drives the monocytes towards M2-like phenotype.
Figure 5.5. Expression of IL-10 (intracellular) and IL-12 (transcript and secreted) levels in Hz-exposed monocytes (A) Expression of monocyte-specific CD14 and intracellular IL-10 was confirmed by dual staining. Scale, 10µm. (B) Monocytes exposed to LPS (1µg/ml) and IFNγ (20ng/ml), Hz and latex were assessed for cytokine IL-12p70 (M1 associated marker) at the transcript level (12h) as detected by qPCR (C) and secreted level (24h) in culture supernatants by ELISA. ***P < 0.001 in comparison with the controls.

5.2.5. Expression profile of M1and M2- related surface markers in Hz-fed monocytes

Monocytes fed with Hz were analyzed for M1 and M2 phenotypes using surface molecule expression. Monocytes were immune typed for CD14+HLADR+, and CD14+CD206+ as it corresponds to inflammatory subset M1 and anti-inflammatory M2 respectively (Mantovani et al.,2004). The Hz-fed monocytes showed a decrease in CD14+HLADR+ monocytes and increase in CD14+CD206+ monocytes as assessed by flow cytometry (Fig 5-6). Altogether, the data suggest the role of Hz in activation of monocytes towards M2-like phenotype.
Figure 5-6. **Expression of HLA-DR and CD206 in Hz-fed monocytes** Untreated, Hz and latex-fed monocytes (24h) were stained with FITC labeled anti-human HLA-DR, APC labeled anti-human CD206, PE-labeled anti-human CD14 and analyzed by flow cytometry. Representative overlays depict percent cells positive for CD206 and HLA-DR. The unstained indicated by the blue line and stained is indicated by red line under given conditions. The data is represented as mean ± SEM from at least 3 individuals. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001 in comparison with the controls.

### 5.2.6. Hz induces M2-like phenotypic characters at doses 25-75µg/ml in monocytes

The Hz amounts detected during *Plasmodium falciparum* infection and previous *in vitro* and *in vivo* studies are in the range of 25-75µg/ml (141,158,160). Hence, we assessed the expression of M2-phenotypic markers in monocytes exposed to different doses of Hz. The M2-related cytokine IL-10 and chemokines CCL17 and CCL1 were significantly upregulated in monocytes fed with Hz in dose range 25-75µg/ml (**Fig 5-7A**). M2-associated marker CD206 showed a similar trend in monocytes fed with different doses of Hz (**Fig 5-7B**). These data suggest that Hz can induce M2-like phenotypic characters in monocytes exposed to Hz concentrations relevant *in vitro* and *in vivo* conditions in malaria.
Objective 2

5.2.7. Effect of Hz on lymphocyte proliferation by ³(H) thymidine incorporation assay.

Monocyte polarization is dependent on micro-environmental stimuli which is responsible for activation of specific phenotypic features. One of the defining properties of M2 phenotype is their capacity to downregulate inflammatory responses. This function can be measured through the ability of M2 to decrease lymphocyte proliferation (75). Hz-fed monocytes were tested for their ability to inhibit autologous T cell proliferation by (³H) thymidine incorporation assay. Hz reduced the proliferation to 50% in comparison with control cells, latex had no effect on monocytes (Fig 5-8). This suggests the ability of Hz to inhibit lymphocyte proliferation and induce M2-like functionality in monocytes.

Figure 5-7. Expression of M2-related phenotypic markers in (25-75μg/ml) Hz-fed monocytes. IL-10, CCL17 and CCL1 as assessed in culture supernatants of Hz (25-75μg/ml) fed monocytes by ELISA (A) and surface expression of CD206 on monocytes by flow cytometry (B). Representative overlays depicting treated cells with percentage positive monocytes The unstained indicated by the blue line and stained is indicated by red line under given conditions. Significance levels: *P<0.05 in comparison with the control and latex.
**Figure 5-8. Hz-fed monocytes suppresses mitogen-stimulated lymphocyte proliferation.** Lymphocyte proliferation was determined by exposing monocytes to Hz and Latex for 2h, followed by washing and further co-culture with lymphocytes (1:4-1:8) and PHA (5μg/ml) for 72 h. Cells were pulsed with 1 μCi/well [methyl-3H] Thymidine for 18 h before harvesting. The CPM counts were recorded in liquid beta-scintillation counter. (3H) Thymidine incorporation was measured as CPM and compared with untreated and latex exposed cells. Significance levels: *P<0.001 in comparison with the control.

**5.2.8. Effect of Hz on Nitric oxide production and arginase activity in monocytes**

Monocytes phenotypes are bestowed with specific biochemical and functional properties. M1 monocytes is characterized by increased NO production, whereas M2 monocytes possesses elevated arginase activity. The iNOS and arginase enzyme act on the same substrate arginine to produce NO and polyamines respectively (233). The NO produced by M1 monocytes enables the killing of pathogens, which is counteracted by M2 monocytes Hz-fed monocytes robustly elevated arginase activity (Fig 5-9A) and significantly reduced the NO (Fig 5-9B) compared to untreated and latex- ingested monocytes controls. Thus, this data supports the ability of Hz to drive M2-like phenotype in monocytes.
Figure 5-9. **Effect of Hz on arginase activity and NO production in monocytes.** (A) Arginase (M2-associated character) activity in cell lysates from Hz-fed monocytes (24h) as determined by colorimetric assay. (B) NO (M1-related type) release in culture supernatants from Hz-fed monocytes (24h) as quantified by Griess assay. Significance levels: **P < 0.01, ***P < 0.001 in comparison with the controls.

**5.2.9. Effect of Hz on reactive oxygen intermediates (ROS) production in monocytes**

In contrast to M1 monocytes, M2 monocytes are unable to produce reactive oxygen intermediates-ROS, essential for the killing of pathogens (234). Hz-fed monocytes significantly reduced the ROS levels compared to untreated and latex-ingested monocytes as detected by DCFDA staining (**Fig 5-10**). The decreased production of ROS in Hz-ingested monocytes suggests M2-like activation in monocytes on Hz phagocytosis.
Figure 5-10. **Hz suppresses ROS production in monocytes** Intracellular ROS (M1-like activity) monitored by flow cytometry as a measure of DCF fluorescence. The relative MFI of DCFDA and representative overlays with percentage positive monocytes for ROS are shown. The unstained indicated by the blue line and stained is indicated by red line under given conditions. Results are mean ± SEM from at least three independent donors. Significance levels: ***P < 0.001 in comparison with the controls.

### 5.2.10. Kinetics of M2 markers - IL-10, CCL1 and CCL17 after Hz phagocytosis in monocytes

To determine the time course of immunosuppressive M2 phenotype induced after Hz phagocytosis in monocytes, time kinetics of M2a and M2b markers i.e. chemokines CCL1, CCL17 and cytokine IL-10 was done. The CCL1 and CCL17 production increased till 48h. IL-10 production decreased after 36h of Hz phagocytosis but was still significantly higher than control monocytes (Fig 5-11). IL-10, a prime marker of M2 phenotype is produced during acute phase after Hz phagocytosis resulting in their immunosuppressive nature. This suggests the existence of M2 phenotype up to 48h in Hz-fed monocytes.

Figure 5-11. **Hz-driven M2-like activation is sustained till 48h.** Human monocytes were fed with 50µg/ml Hz for 2hrs, followed by wash and incubation at different time points. The chemokines, CCL17, CCL1 and cytokine IL-10 were detected in cell-free supernatants using ELISA. Results are mean ± SEM from at least three independent donors. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001 in comparison with the controls.
5.2.11. Signaling pathways involved in Hz-induced IL-10 production in monocytes

IL-10 is an anti-inflammatory cytokine and a M2 marker, which is majorly produced by cells of innate immunity including monocytes. IL-10 production is mediated by ERK-dependent and independent pathways - through IL-10R, p38-MAPK, PI3K/AKT, STAT3 and NF-κB p65 (235). IL-10 is highly upregulated after Hz phagocytosis resulting in immunosuppressive M2 phenotype. The pharmacological signaling inhibitors of above pathways were used to inhibit specific pathways before Hz addition to assess their involvement in IL-10 production in monocytes. The human monocytes were pretreated with LY294002 (PI3K/Akt pathway inhibitor), SB203580 (MAPK p38 pathway inhibitor), parthenolide (NF-κB pathway inhibitor), rapamycin (mTOR pathway inhibitor), GW9662 (PPAR gamma pathway inhibitor), SP600125 (JNK2 pathway inhibitor), and stattic (STAT3 pathway inhibitor) and exposed to Hz for phagocytosis. IL-10 was significantly inhibited in monocytes treated with SB203580, parthenolide, LY294002, stattic, GW9662, rapamycin, but not SP600125. Most of the inhibitors showed significant downregulation of IL-10 at varying levels in culture supernatants as detected by ELISA after 24h of Hz phagocytosis (Fig 5-12). The pathways p38-MAPK (SB203580), NF-κB (parthenolide) and PI3K/AKT (LY294002) were considered to be of importance as they showed maximal IL-10 inhibition in comparison with Hz-ingested monocytes. Thus, the p38-MAPK, NF-κB and PI3K/AKT pathways may be involved in the Hz-mediated IL-10 production.
The pathways involved in Hz-mediated IL-10 production in monocytes. Human monocytes were pretreated with pharmacological inhibitors, 10µM LY294002 (LY), 10µM SB203580 (SB), 10µM Parthenolide (PAR), 50ng/ml Rapamycin (RAP), 10µM GW9662 (GW), 10µM SP600125 (SP), and 10µM stattic (ST) 1h, followed by Hz phagocytosis for 2h. After 24h, IL-10 was measured in culture supernatants using ELISA. Results are mean ± SEM from at least three independent donors. Significance levels: **P < 0.01, ***P < 0.001 in comparison with the Hz, # P<0.05 in comparison with control.

5.2.12. Effect of Hz on activation of NF-κB, p38-MAPK and PI3K/AKT kinase pathways in monocytes

The p38-MAPK, PI3K/AKT and NF-κB pathways have been implicated in M2 polarization. In this study, the pharmacological inhibitors of p38-MAPK, AKT and NF-κB significantly decreased IL-10 production in Hz-ingested monocytes The exposure to Hz led to a significant increase in the phosphorylation of p38-MAPK (pP38- Y182), AKT (pAKT- S473) and NF-κB (p P65-S276-) in monocytes indicating the activation of these pathways. Moreover, the decrease in IκBα further confirmed the activation of NF-κB (Fig 5-13). These data together implicate the involvement of these p38-MAPK, AKT and NF-κB pathways in Hz-induced monocyte activation.
5.2.13. Effect of NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors on Hz-induced M2 cytokine and chemokine markers

The next aim was to investigate the involvement of NF-κB, p38-MAPK and PI3K/AKT kinase pathways in Hz-mediated expression of M2-related cytokines and chemokines in monocytes. For this purpose, the effect of SB203580 (p38-MAPK inhibitor), parthenolide (NF-κB inhibitor) and LY294002 (PI3K/AKT inhibitor) was assessed on the expression of M2a and M2b related markers. The cytokines (IL-10, TNF alpha) and chemokine markers (CCL1 and CCL17) decreased at transcript levels with parthenolide, SB203580 and LY294002. Unlike parthenolide and SB203580, PI3K/AKT inhibitor LY294002 showed further increase in IL-6 and IL-1β levels (Fig 5-14A). The secreted levels of all above markers in supernatants of monocytes exposed to Hz with or without inhibitors, correlated with their respective transcript levels (Fig 5-14B). LY294002 can act on other pathways along with PI3K/AKT kinases that may be responsible for the regulation of IL-6 and IL-1β (236). To confirm the role of PI3K/AKT pathway, another PI3K/AKT inhibitor wortmannin was used in similar experiments. Wortmannin effectively reduced the level of IL-1β, IL-6 and IL-10 in supernatants of monocytes fed with Hz signifying the role of PI3K/AKT pathway in inhibiting M2-like phenotype (Fig 5-14C). This suggests the involvement of NF-κB, p38-MAPK and PI3K/AKT kinase pathways in Hz-mediated expression of M2-related cytokines and chemokines in monocytes.
Figure 5-14. The effect of pharmacological inhibitors SB203580 (SB), parthenolide (PAR), LY294002 (LY) and wortmannin (wort) on Hz-induced M2-related M2a and M2b markers.
in monocytes (A) IL-10, CCL17, CCL1, TNF-α, IL-1β and IL-6 transcript (12h) in Hz -fed monocytes pretreated with inhibitors, 10µM SB, 10µM PAR and 10µM LY. GAPDH was used as an endogenous control. (B) Secreted IL-10, CCL17, CCL1, TNF-α, IL-1β and IL-6 in culture supernatants (24h) of Hz- fed monocytes pretreated with inhibitors 10µM SB, 10µM PAR and 10µM LY were estimated by ELISA. (C) Secreted IL-1β, IL-6 and IL-10 in supernatants of monocytes pretreated with wort (50ng/ml) followed by Hz exposure as determined by ELISA (24h). The data represented is mean ± SEM from at least 3 individuals. Significance levels: **P < 0.01, ***P < 0.001 in comparison with the control.

5.2.14. NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors compromise M2-related CD206 expression on Hz-ingested monocytes

The decrease in M2-related cytokines and chemokines in Hz-fed monocytes in the presence of inhibitors of NF-κB, p38-MAPK and PI3K/AKT pathways necessitated the evaluation of these inhibitors on M2-related mannose-binding receptor expression. As expected, there was a decrease in Hz-induced surface expression of CD206 in the presence of parthenolide, SB203580 and LY294002 with a reduction in percent positive cells and MFI (Fig 5-15). This corroborates with the results of chemokine profiles confirming attenuation of M2-like phenotype by the inhibitors. The results suggest the involvement of NF-κB, p38 MAPK and PI3K/AKT kinase pathways in Hz-mediated M2-like activation.
Figure 5-15. The effect of pathway inhibitors SB203580 (SB), parthenolide (PAR), and LY294002 (LY) and wortmannin (wort) on Hz- induced CD206 expression in monocytes. Hz was fed to human monocytes pretreated with 10µM SB, 10µM PAR, and 10µM LY. The relative MFI of CD206 (M2) of Hz-fed monocytes in presence of inhibitors was determined by flow cytometry and a representative overlay of treated cells with percentage positive monocytes are shown. The unstained is indicated by blue line and stained is indicated by red line under given conditions. Results are mean ± SEM from at least 3 independent experiments. Significance levels: **P < 0.01, ***P < 0.001 in comparison with the Hz.

5.2.15. NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors prevent Hz- mediated inhibition of mitogen-stimulated lymphocyte proliferation in monocytes

The decrease of M2-related phenotypic markers by NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors in monocytes exposed to Hz, inferred the possible role of these pathways in M2 phenotype related functional properties. Hz alters the functionality in terms of inhibition of mitogen-stimulated lymphocyte proliferation in monocytes.
Hence, the effect of SB203580, parthenolide, and LY29402 on the functionality of Hz-mediated suppression of lymphocyte proliferation was assessed. Lymphocyte proliferation was significantly reversed by all three inhibitors (Fig 5-16). This suggests the involvement of NF-κB, MAPK P38 and PI3K/AKT kinase pathways in the Hz-mediated suppression of mitogen-stimulated lymphocyte proliferation in monocytes.

5.2.16. Signaling inhibitors of NF-κB, p38-MAPK and PI3K/AKT kinase pathway prevent Hz- mediated stimulation of arginase activity and inhibition of NO production in monocytes

The decrease in M2-related phenotypic markers and inhibition of mitogen-stimulated lymphocyte proliferation by NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors in Hz-exposed monocytes inferred the possible role of these pathways in M2- phenotype related biochemical properties. Hz alters NO production and increases arginase activity in monocytes. Hence, the effect of SB203580, parthenolide, and LY29402 on the Hz-mediated regulation of NO production and arginase activity was assessed. Elevated arginase activity was significantly reduced by all the inhibitors tested (Fig 5-17A). In line with this, the inhibitors also effectively prevented Hz- mediated inhibition of NO production (Fig 5-17B). These results suggest the involvement of NF-κB, p38-MAPK and
PI3K/AKT kinase pathways in Hz-mediated M2-related arginase activity and NO inhibition in Hz-ingested monocytes.

5.2.17. Effect of NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors on ROS production in Hz-fed monocytes

The decrease of M2-related phenotypic markers and restoration of functional and biochemical properties by NF-κB, p38-MAPK and PI3K/AKT kinase pathway inhibitors in Hz-exposed monocytes, inferred the possible role of these pathways in M2 phenotype related ROS reduction. Hence, the effect of SB203580, parthenolide, and LY29402 on the Hz-mediated inhibition of ROS production was assessed. The p38-MAPK and NF-κB inhibitors but not PI3K/AKT inhibitor effectively blocked Hz-mediated inhibition of ROS production. (Fig 5-18). This suggests the involvement of NF-κB, p38-MAPK but not PI3K/AKT kinase pathways in Hz-mediated reduction of ROS production. Collectively, these findings on M2-related phenotypic, functional and biochemical properties in the presence of pathway inhibitors suggest the involvement of NF-κB, p38-MAPK and PI3K/AKT kinase pathways in Hz-driven M2-like phenotype.
Figure 5-18. Effect of Hz on the ROS production in monocytes - pretreated with pathway inhibitors. ROS production after 24 h of phagocytosis with Hz in monocytes pretreated with 10µM SB203580 (SB), 10µM parthenolide (PAR) and 10µM LY294002 (LY) signaling inhibitors as monitored by FACS analysis as an increase in DCF fluorescence. The unstained is indicated by blue line and stained is indicated by red line under given conditions. Results are mean ± SEM from at least three independent donors. Significance levels: ***P < 0.001 in comparison with the controls.

5.2.18. Cytotoxicity of artemisinin (ART) in monocytes

ART is a well-known and effective anti-malarial drug. Along with anti-parasitic activity, ART exhibits pleiotropic effects including immune-modulation. The immune-modulatory property of ART has extended its use in other parasitic diseases and cancer. However, the immune-modulatory property of ART is not much explored with respect to malaria immunity. Here, the effect of ART on Hz-mediated M2-like phenotype was investigated. ART concentration used for treating monocytes was based on previous studies (237). To ensure that the concentration used for studying immunomodulatory effect is not toxic to monocytes, cytotoxicity was assessed by MTT. ART concentrations in the range of 5-400µM did not significantly affect the viability of monocytes (Fig 5-19).
Figure 5-19. **Effect of artemisinin (ART) on cytotoxicity of Monocytes.** Monocytes were treated with ART concentrations of 5-400µM for 48h and percentage of viable cell count was assessed by MTT assay. 20% DMSO was used as positive control. Viable count of untreated cells was assumed as 100%. The graphs represent % viable cell count +/- SEM of three similar experiments performed in triplicates.

**5.2.19. Effect of ART on Hz-induced M2-like cytokines and chemokines.**

Our data, so far indicated that malarial pigment Hz induces M2a and M2b phenotype in monocytes. The drugs which reverse the Hz-induced M2 phenotype may serve as an adjunctive treatment for malaria. To address this issue, we assessed the effect of ART on Hz-induced M2 phenotype on account of its reported immunomodulatory behavior. The chemokines CCL17 (M2a), CCL1 (M2b) and cytokines IL-10 (M2), IL-6, TNF-α, IL-1β (M2b) induced by Hz were significantly decreased with ART (Fig 5-20) suggesting the ability of ART to reverse the expression of Hz-induced M2-like cytokines and chemokines.
Figure 5-20. Effect of ART on M2-related cytokine and chemokine production in Hz-ingested monocytes. Hz-fed monocytes were post-treated with 20µM ART and assessed for expression of M2-related phenotypic markers – secreted IL-10 (M2), CCL17 (M2a), CCL1, TNF-α, IL-1β and IL-6 (M2b) (24h) using ELISA. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001 in comparison with the controls and Hz-ingested monocytes.

5.2.20. ART reversed the M2-related CD206 expression in Hz-ingested monocytes

ART significantly reversed Hz-induced expression of M2-related cytokine markers. In line with this, the effect of ART on M2-related mannose receptor i.e. CD206 was analyzed. The post-treatment of monocytes exposed to Hz with ART resulted in dramatic reduction of CD206 surface expression in terms of percent positive cells and MFI (Fig 5-21). This suggests the ability of ART to reverse Hz-induced M2-like phenotypic characters.

Figure 5-21. Effect of ART on CD206 expression in Hz-fed Monocytes. The relative MFI of CD206 (M2) in Hz-fed monocytes post-treated with 20µM ART as determined by flow
cytometry. Representative plots with an overlay of treated cells with percentage positive monocytes are shown. The unstained is indicated by the blue line and stained is indicated by red line under given conditions. Results are mean ± SEM from at least 3 independent experiments. Significance levels: **P < 0.01, in comparison with the Hz or untreated monocytes.

5.2.21. ART reversed the Hz-ingested monocytes mediated inhibition of mitogen-stimulated lymphocyte proliferation.

To study the ability of ART to reverse Hz-mediated M2-like phenotype, the mitogen-stimulated lymphocyte proliferation was studied in the presence of ART. ART significantly reversed the Hz-mediated suppression of lymphocyte proliferation (Fig 5-22). This suggests the ability of ART to reverse Hz-mediated suppression of mitogen-stimulated lymphocyte proliferation. ART-mediated reversal of phenotypic markers and suppression of mitogen-stimulated lymphocyte proliferation suggests that ART may reverse Hz-induced M2-like phenotype.

![Figure 5-22](image)

Figure 5-22. Effect of ART on Hz-mediated inhibition of (PHA) mitogen-stimulated lymphocyte proliferation. Lymphocyte proliferation was determined by post-treating monocytes exposed to Hz with 20µM ART, followed by washing and further co-culture with lymphocytes (1:4-1:8) and (5μg/ml) PHA stimulation for 72 hr. Cells were pulsed with 1 μCi/well [methyl-3H] Thymidine for 18 h before harvesting. The CPM counts were recorded and (3H) thymidine incorporation was measured as CPM and compared with untreated cells. Significance levels: **P < 0.01, ***P<0.001 in comparison with the Hz or untreated Monocytes.

5.2.22. Effect of ART on Hz-mediated arginase activity and NO regulation.

To further confirm the ability of ART to reverse Hz-induced M2 phenotype, the effect of ART on Hz-mediated arginase activity and NO regulation was assessed. Hz-fed monocytes produced significantly elevated arginase activity and less nitric oxide production supporting M2-like phenotype in Hz-exposed monocytes. However, the levels of arginase activity and NO were not influenced by post-treatment of ART in Hz-ingested monocytes.
(Fig. 5-23). Altogether, this data suggests that ART partially reverses Hz-induced M2-like monocyte activation.

![Graph](image)

**Figure 5-23.** Effect of ART on Hz-induced arginase activity and decreased NO production in monocytes. (A) Arginase (M2) activity in cell lysates from Hz-fed monocytes post-treated with 20µM ART (24h) as determined by colorimetric assay. (B) NO (M1) release in culture supernatants from Hz-fed monocytes (24h) as quantified by Griess assay. Significance levels: *P < 0.05, ***P < 0.001 in comparison with the controls.

### 5.2.23. The Hz-induced ROS levels are unaffected by ART treatment in monocytes

To further confirm the ability of ART to reverse Hz-induced M2-like phenotype, the effect of ART on Hz-mediated decreased ROS production was assessed. Hz significantly decreased ROS production in monocytes in comparison with untreated monocytes. However, post-treatment with ART did not reverse the ROS inhibition (Fig. 5-24).
The post-treatment of ART in Hz-fed monocytes reversed the M2-related phenotypic markers and suppression of lymphocyte proliferation. However, the levels of arginase activity, NO and ROS were not influenced by post-treatment of ART in Hz-ingested monocytes. Altogether, this data suggests that ART partially reverses Hz-induced M2-like monocyte activation.

5.2.24. Effect of (5-20µM) ART on Hz-induced M2-like phenotypic markers in Monocytes.

ART post-treatment in Hz-exposed monocytes significantly reversed M2-like phenotypic characters than biochemical properties, suggesting partial restoration of the M2-like phenotype. To confirm the ability of ART to significantly reverse Hz-driven M2-like phenotypic markers, Hz-fed monocytes were exposed to different doses of ART concentration i.e. 5-20µM and their effect on M2-related phenotypic markers was assessed. 5-20µM ART decreased secretion of phenotypic markers IL-10, CCL17, CCL1 and CD206 surface expression in Hz-fed monocytes (Fig 5-25 A, B). This data suggests the ability of ART to reverse Hz driven M2-like phenotypic properties at different concentrations.
5.2.25. ART reduces Hz-driven M2-related phenotypic markers by NF-κB inhibition.

The next aim was to identify the pathway that may be involved in reversion of Hz-induced M2-related phenotypic markers. The signaling data with p38-MAPK, NF-κB and AKT inhibition revealed that NF-κB inhibition was most effective in attenuating Hz-induced M2-like phenotype. This encouraged us to determine the effect that ART might have on
NF-κB activation in Hz-ingested monocytes. The Hz-induced NF-κB activation was inhibited by ART as indicated by decreased p65 phosphorylation and increased IκBα in monocytes (Fig 5-26). These results suggest the involvement of NF-κB in inhibition of M2 phenotypic markers by ART.

![Western blot analysis of whole-cell lysates from control and Hz-fed monocytes in the presence or absence of 20µM ART (2h) for total and phosphorylated levels of NF-κB p65 and IκBα. Fold change (indicated in blots) were obtained from densitometric analysis of phospho-p65-NF-κB, normalized with GAPDH.](image)

**5.2.26. Cytotoxicity of chloroquine (CHQ) in monocytes**

CHQ is a well-known, effective and safe anti-malarial. Along with anti-parasitic activity, CHQ exhibits pleiotropic effects such as immune-modulation. The immune-modulatory property of CHQ has extended its use to other parasitic diseases and cancer. However, the immune-modulatory property of CHQ is not much explored with respect to malaria immunity. We, therefore, examined the effect of CHQ on Hz-mediated M2-like phenotype. The CHQ concentration used for treating monocytes was based on previous studies (185). To ensure that the concentration used for studying immunomodulatory effect is not toxic to monocytes, cytotoxicity was assessed by MTT. The CHQ concentrations in the range of 5-50µM did not affect the viability of monocytes (Fig 5-27).
5.2.27. Effect of CHQ on Hz-induced M2-like cytokines and chemokines in monocytes

Further studies were conducted to examine the effect of CHQ on Hz-induced M2-like phenotype on account of its immunomodulatory role. The chemokines CCL17 (M2a), CCL1 (M2b) and cytokines IL-10 (M2), IL-6, TNF-α, IL-1β (M2b) induced by Hz were significantly decreased with CHQ (Fig 5-28). This suggests that CHQ possess the ability to reverse expression of M2-like phenotypic markers in Hz-fed monocytes.
5.2.28. CHQ reversed the M2-related CD206 expression in Hz-ingested monocytes

CHQ significantly reversed Hz-induced expression of M2-related cytokine markers. In line with this, the effect of CHQ on M2-related mannose receptor i.e. CD206 was analyzed. The post-treatment of monocytes exposed to Hz with CHQ resulted in dramatic reduction of CD206 surface expression in terms of percent positive cells and MFI (Fig 5-29). Altogether these findings suggest the ability of CHQ to reverse Hz-induced M2-like phenotypic characters.
Objective 2

Figure 5-29. Effect of CHQ on CD206 expression in Hz-fed monocytes. The relative MFI of CD206 (M2) of Hz-fed monocytes in presence of 20µM CHQ as determined by flow cytometry. A representative overlay plot of treated cells with percentage positive monocytes are shown. The unstained is indicated by blue line and stained is indicated by red line under given conditions. Results are mean ± SEM from at least 3 independent experiments. Significance levels: ***P < 0.001, in comparison with the Hz or untreated monocytes

5.2.29. CHQ reversed Hz-mediated inhibition of mitogen-stimulated lymphocyte proliferation.

To study the ability of CHQ to reverse Hz-mediated M2-like phenotype, the mitogen-stimulated lymphocyte proliferation was studied in the presence of CHQ. CHQ significantly reversed the Hz-mediated suppression of lymphocyte proliferation (Fig 5-30).

Figure 5-30. Effect of CHQ on Hz-mediated inhibition of (PHA) mitogen-stimulated lymphocyte proliferation. Lymphocyte proliferation was determined by treating Hz-exposed monocytes with 20µM CHQ, followed by washing and further co-culture with lymphocytes (1:4-
1:8) and (5μg/ml) PHA stimulation for 72 h. Cells were pulsed with 1 μCi/well [methyl-3H] Thymidine for 18 h before harvesting. The CPM counts were recorded and (3H) thymidine incorporation was measured as CPM and compared with untreated and latex exposed cells. Results are mean ± SEM from at least 3 independent experiments. Significance levels: *P<0.05, **P < 0.001, in comparison with the Hz or untreated monocytes.

This suggests the ability of CHQ to reverse Hz-mediated suppression of mitogen-stimulated lymphocyte proliferation. The CHQ mediated reversal of phenotypic markers and suppression of mitogen-stimulated lymphocyte proliferation suggests that CHQ may reverse Hz-induced M2-like phenotype.

5.2.30. Effect of CHQ on Hz- mediated arginase activity and NO regulation.

The treatment of CHQ in Hz-fed monocytes reversed the M2-related phenotypic markers and suppression of lymphocyte proliferation. To further confirm the ability of CHQ to reverse Hz-induced M2 phenotype, the effect of CHQ on Hz- mediated arginase activity and NO regulation was assessed. Hz- fed monocytes produced significantly elevated arginase activity and reduced nitric oxide production supporting M2-like phenotype in Hz exposed monocytes However, the levels of arginase activity and NO were not influenced by post-treatment of CHQ in Hz-ingested monocytes (Fig 5-31). Altogether, this suggests that CHQ partially reverses Hz-induced M2-like monocyte activation.

![Figure 5-31. Effect of CHQ on Hz-induced arginase activity and NO inhibition in monocytes.](image)

Figure 5-31. Effect of CHQ on Hz-induced arginase activity and NO inhibition in monocytes. (A) Arginase (M2) activity in cell lysates from Hz-fed monocytes post-treated with 20μM CHQ (24h) was determined by colorimetric assay. (B) NO (M1) release in culture supernatants from Hz-fed monocytes (24h) as quantified by Griess assay. Significance levels: *P < 0.05, ***P < 0.001 in comparison with controls.
5.2.31. Hz-induced ROS levels are unaffected by CHQ treatment in monocytes

The post-treatment of CHQ in Hz-fed monocytes reversed the M2-related phenotypic markers and suppression of lymphocyte proliferation. However, the unaffected levels of arginase activity and NO by post-treatment of CHQ suggests partial reversal of M2-like phenotype in Hz-ingested monocytes. To further confirm the ability of CHQ to reverse Hz-induced M2 phenotype, the effect of CHQ on Hz-mediated decreased ROS production was assessed. Hz significantly decreased ROS production in monocytes in comparison with untreated monocytes. However, post-treatment with CHQ did not reverse the ROS inhibition (Fig 5-32). Together, the levels of arginase activity, NO and ROS were not influenced by post-treatment of CHQ in Hz-ingested monocytes suggesting that CHQ only partially reverses Hz-induced M2-like monocyte activation.

![Graph showing Reactive Oxygen Species (ROS) generation in Hz-fed monocytes post-treated with CHQ](image)

**Figure 5-32. Reactive Oxygen Species (ROS) generation in Hz-fed monocytes post-treated with CHQ.** Hz-fed monocytes post-treated with 20µM CHQ (24h) were stained with PE anti-human CD14 and DCF-DA. The histoplots represent percentage positive for ROS in CD14 positive cells and MFI compared to control cells. The unstained is indicated by blue line and stained is indicated by red line under given conditions. The data is mean ± SEM from at least 3 independent donors. Significances levels: **P>0.01, in comparison with control.

5.2.32. Effect of (5-20µM) CHQ on Hz-induced M2-like phenotype in monocytes

The CHQ-treatment in Hz-exposed monocytes reversed M2-like phenotypic characters than biochemical properties, suggesting partial restoration of the M2-like phenotype. To further confirm the ability of CHQ to significantly reverse Hz-driven M2-like phenotypic markers, Hz-fed monocytes were exposed to different doses of CHQ concentration i.e. 5-
20μM and their effect on M2-related phenotypic markers was assessed. 5-20μM CHQ decreased secretion of phenotypic markers IL-10, CCL17, CCL1 and CD206 surface expression in Hz-fed monocytes (Fig 5-33 A, B). The data suggest the ability of CHQ to reverse Hz-driven M2-like phenotypic properties at different concentrations.

Figure 5-33. **Effect of (5-20μM) CHQ on Hz-driven M2-related phenotypic markers.** The secretion of cytokines- IL-10, CCL17, CCL1 as detected by ELISA in culture supernatants (A) and surface expression of CD206 by flow cytometry (B) in CHQ (5-20μM) post-treated Hz-fed monocytes Representative plots with an overlay of treated cells with percentage positive monocytes are shown The unstained is indicated by blue line and stained is indicated by red line under given conditions. The data represented is mean ± SEM from at least 3 individuals. Significance levels: *P<0.05 in comparison with the Hz-ingested monocytes, #P<0.05 in comparison with control.

**5.2.33. CHQ reduced Hz-driven M2-related phenotypic markers by NF-κB inhibition.**

The next aim was to identify the pathway that may be involved in reversion of Hz-induced M2-related phenotypic markers. The signaling data with p38-MAPK, NF-κB and AKT inhibition revealed that NF-κB inhibition was most effective in attenuating Hz-induced
M2-like phenotype. This encouraged us to determine the NF-κB activation in CHQ-treated Hz-ingested monocytes. The Hz-induced NF-κB activation was inhibited by CHQ as indicated by decreased p65 phosphorylation and increased IκBα in monocytes (Fig 5-34). These results suggest the contribution of NF-κB in inhibition of M2 phenotypic markers by CHQ.

![Western blot analysis](image)

Figure 5-34. **CHQ reduced NF-κB activation in Hz-fed monocytes.** Western blot analysis of whole-cell lysates from control and Hz-fed monocytes in the presence or absence of 20µM CHQ (2h) for total and phosphorylated levels of NF-κB p65 and IκBα. Fold change obtained from densitometric analysis of phospho-p65-NF-κB, normalized with GAPDH are indicated above blots.

### 5.3. Discussion

*Plasmodium falciparum* causes the most severe form of malaria with variable manifestations including uncomplicated, mild, severe and cerebral malaria. The controlled immune response causes immunoprotection, whereas excessive immune responses against pathogens may cause immunopathology. The constant battle between immunoprotection and immunopathology results in variable susceptibility to parasites in hosts. The interplay amongst anti- and pro-inflammatory responses decides the outcome of the disease. Since immune responses are greatly influenced by micro-environmental stimuli, the study of host and parasite component’s interactions becomes essential. Hz is one of the parasite components, which is an interesting target to study these interactions. The natural Hz is released during schizont rupture at the end of every intra-erythrocytic cycle (48h) into the bloodstream, which is taken up by patrolling phagocytic cells including monocytes. The undigested Hz gets accumulated in monocytes and alters their several functions, which in turn may contribute to sub-optimal immune responses seen in malaria. While many studies...
have documented the role of Hz in immunosuppression and various mechanisms involved have been identified, there are no reports so far on the effect of Hz in the polarization of monocytes. The present study aimed to unravel the role of Hz in driving the monocytes towards specific phenotype. The findings revealed the ability of natural Hz to induce M2-like phenotype mediated by PI3K/AKT, NF-κB and p38-MAPK signaling pathways. Also antimalarial drugs- CHQ and ART partially reversed Hz-induced M2-like phenotype supporting their role in immunomodulation.

Monocytes are heterogeneous and possess the ability to polarize into M1 or M2 phenotype depending on different environmental stimuli. Hz-induced M2-like phenotype in monocytes, characterized by expression of a panel of phenotypic markers such as elevated IL-10, CCL17, CCL1 and CD206 surface expression and downregulated HLA-DR. Hz showed no significant alteration in IL-12 levels. The Hz-inhibited M1-phenotype related ROS and NO production, whereas increased M2-related arginase activity. Hz-induced M2-like phenotype consists of two subtypes i.e. M2a and M2b subtypes based on expression of CCL17 and CCL1 respectively. Since chemokines, CCL17 and CCL1 function as agonists for CCR4 and CCR8 receptors respectively (64), it is likely that Hz might facilitate recruitment of CCR4 and CCR8-bearing TH2 cells and regulatory T cells of TH2 immunity which contribute to immunosuppression. It is noteworthy in this context that regulatory T cells suppress T-cell responses in malaria (238). In contrast with other M2 subtypes, M2b secreted high amounts of IL-10 along with inflammatory cytokines- TNF-α, IL-1β and IL-6. Hz-fed monocytes secreted a large amount of these cytokines corroborating with the reports demonstrating the presence of inflammatory cytokines- IL-1β, TNF-α, IL-6 along with high level of anti-inflammatory IL-10 in plasma of malaria patients (203,239).

The M1 and M2 phenotypes are also characterized on the basis of NO production and arginase activity. The M1 monocytes produce large amounts of NO and ROS which are essential for the killing of pathogens. The iNOS enzyme producing NO and arginase acts on same substrate arginine. The arginase converts arginine to ornithine and urea causing reduction of NO. The M2-type monocytes possess high arginase activity and low production of NO and ROS (60,101,240). In malaria, depletion of NO results in low NO bioavailability that affects adherence of parasites to endothelium and is associated with severity of the disease. High arginase activity and an inverse relation between disease severity and NO production are recently reported in malaria (203). In this context, Hz-ingested monocytes exhibited elevated arginase activity in comparison with untreated and
latex fed monocytes These results, along with previous reports suggest that arginase released from Hz-fed monocytes and Plasmodium parasite (241) and lysed infected erythrocytes (242) may together lead to M2-like activity in malaria. In the present study, natural Hz decreased NO and ROS production in monocytes In contrast, some studies have reported elevated production of NO (243) and ROS (168) in monocytes exposed to purified Hz. The differences in the results may be due to the nature of Hz used; while the earlier studies were performed with delipidized Hz and β-hematin, our experiments were conducted with natural Hz. The purified Hz may lack some parasitic factors leading to differences seen with physiologically more relevant natural Hz in monocytes Importantly, from the functional point, the Hz- ingested monocytes suppressed mitogen-stimulated lymphocyte proliferation. This observation is in line with the study that malaria patients have suppressed T and B cell functions (244).

IL-10 is an important anti-inflammatory cytokine and a predominant M2 marker of monocytes IL-10 production and signaling are mediated through complex overlapping pathways. Hence, to decipher the pathway involved in Hz-mediated M2 phenotype in monocyte, we screened a panel of inhibitors at concentrations known to inhibit specific pathways involved in IL-10 signaling and production. In monocytes, IL-10 is known to signal through MAPKs, which involves ERK1 and ERK2, JNK2 and p38-MAPK (245). NF-κB1 deficient macrophages were found to produce lower levels of IL-10 (246). STAT3 is involved in the IL-10 production (247). We assessed the involvement of PPARγ as it is found to be positively correlating with M2 marker CD206 in atherosclerotic lesions (80,248). We also looked at mTOR pathway as it increased CD86, CCR7, IL-6, and TNF-α, IL-1β release but decreased CD206 and IL-10 in human macrophages (249). Although, inhibition of STAT3, PPARγ and mTOR pathways showed a significant reduction in Hz-mediated IL-10 levels, the inhibition of p38 MAPK, PI3K/AKT and NF-κB pathways decreased the IL-10 levels robustly suggesting that these pathways may be involved in M2 phenotype. However, the possibility of partial involvement of STAT3, PPARγ and mTOR pathways in downstream signaling of the Hz-induced M2 phenotype cannot be denied. We also showed that JNK2 and ERK1/2 pathways are not involved in Hz-mediated IL-10 signaling.

Studies on IL-10 have established the role of several pathways in its production including p38-MAPK, PI3K-AKT and NF-κB (235). These pathways have also been implicated in M2 polarization (229,250). In agreement with this study, Hz-induced M2-like phenotype
was accompanied by activation of p38-MAPK, PI3K-AKT and NF-κB pathways in monocytes. The inhibition of these pathways using pharmacological inhibitors attenuated the Hz-induced M2-like phenotypic and functional properties. While these findings implicate the role of p38-MAPK, PI3K/AKT and NF-κB pathways, we cannot rule out the possibility of other signaling pathways in Hz-driven M2-like activation of monocytes. Hz stimulates activation of monocytes to M2 (M2a and M2b)-like phenotype mediated by PI3K/AKT, p38-MAPK and NF-κB pathways.

The next aim was to identify strategies that could aid in reversing the effect of Hz in driving the monocytes to an M2-like phenotype. In this regard, experiments were conducted with chloroquine (CHQ) and artemisinin (ART) for three reasons; firstly, they are reported to inhibit pathways crucial for polarization to M2 type, secondly, they are widely used for the treatment of malaria and thirdly, they possess immunomodulatory properties. Artemisinins have been reported to decrease IL-10, IL-1β, IL-6, TNF-α levels (190,191) through MAPK (187), NF-κB (192,237) and PI3K/AKT (193) pathways. CHQ has also been demonstrated to act through MAPK (182,251) and NF-κB (181,184) pathways and decrease IL-1β, IL-6 and TNF-α in mononuclear phagocytes in different diseases (181,185). In another study, CHQ has been shown to be an effective anticancer drug in mice by inhibiting tumor resistant MQ (M2 MQ) and decreasing TGF-β and IL-10 reduction. The effect was accompanied by decreased myeloid-derived suppressor cells (MDSC), Tregs and increasing CD8+ T cells in tumor milieu (186). The ability of these drugs to inhibit crucial pathways and production of cytokines/chemokines that are signature for M2 type MO/MQ has extended their use for other parasitic diseases, rheumatic diseases and cancer. In our study, the drugs were found to be more effective in reducing the expression of phenotypic markers - cytokines, chemokines and surface marker- CD206 associated with M2-like phenotype than in reversing the altered biochemical and functional properties. ART did not reverse arginase activity in Hz-fed monocytes. This finding is supported by an earlier report that ART did not significantly alter arginase activity in Leishmania donovani-infected MQ though it enhanced protective immune responses (189). These observations are not surprising as CHQ and ART are known for their antimalarial action through multiple modes on their target but their mechanism of action as immunomodulators is not clearly elucidated. The M2 phenotype is previously related to abnormal autophagy and angiogenesis (252). We speculate that CHQ, a known autophagy inhibitor may attenuate Hz-induced M2-like monocytes activation by inhibiting autophagy. Previously, falciparum
malaria pathogenesis has been related to upregulated angiogenic factors VEGF, Ang-2 and sFLT-1 along with dysregulated and excessive immune responses (253,254). The anti-parasitic action of ART is accompanied by disruption of parasite proteins, alteration of mitochondrial functions and angiogenesis (191,194). In relation to the ability of M2 phenotype to stimulate angiogenesis, ART may attenuate Hz-induced M2-like phenotype by altering the release of angiogenic factors. In conclusion, our findings suggest that though anti-malarial resistance against this drugs is reported, they may still be explored as immune-modulators for adjunctive treatment in malaria.