Malaria hemozoin modulates susceptibility of immature monocyte-derived dendritic cells to HIV-1 infection by inducing a mature-like phenotype

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Summary

Together, Plasmodium falciparum (P. falciparum) and HIV-1 infections cause more than four million deaths a year. There is still limited information about the putative impact of the malaria pigment hemozoin (HZ) on the dissemination of HIV-1. As so, we propose a premise where HZ present in human dendritic cells (DCs) could modulate HIV-1 transfer to CD4+ T cells. We report here that HZ promotes transmission of HIV-1 by immature monocyte-derived DCs (iMDDCs). Moreover, we noted that in the presence of HZ, iMDDCs were less permissive to productive HIV-1 infection. The HZ-dependent modulation of the interaction between iMDDCs and HIV-1 seems to be partly due to a decreased expression of CCR5 and also to the induction of a more mature phenotype as proven by microscopy and flow cytometry analyses. Therefore, exposure of iMDDCs to malaria pigments provokes their maturation rendering them more potent to trans-infect CD4+ T cells with HIV-1.

Introduction

Current understanding of the immune response to malaria and HIV-1 leads scientists to believe that both infections could influence the clinical course of the other. Indeed, it is well established that several co-infections in patients carrying HIV-1 result in a transient increase in viral load. Hence, it is logical to expect malaria to do the same and potentially accelerate HIV-1 disease progression (Whitworth and Hewitt, 2005). In a previous study, it was shown that HIV-1 viral load levels nearly doubled during co-infection with Plasmodium falciparum (P. falciparum) (Kublin et al., 2005). The study concluded that the increase in HIV-1 viral load when contracting malaria could be sustained long enough to augment the risk of virus transmission. Unfortunately, the possible molecular/cellular mechanisms by which the parasite might influence the HIV-1-related disease outcome remain unclear. Considering that both pathogens represent major public health issues and that they can be present in the same endemic regions, studies attempting to clarify the complex interplay between them are crucial for the development of therapeutic strategy in a co-infection context (WHO, 2005).

HIV-1 infection is associated with immune hyperactivation that ultimately lead to systemic immunosuppression, a condition in which the immune system fails to limit the emergence of opportunistic infections. It has been previously reported that this immune activation is in part mediated by microbial translocation occurring upon mucosal immune dysfunction in HIV-1-infected individuals (Paiardini et al., 2008). Once entered in the tissues or the bloodstream, microbial products activate innate immune cells like macrophages and dendritic cells (DCs), which in turn stimulate CD4+ T lymphocytes. This phenomenon is beneficial for HIV-1 since activated CD4+ T cells are the most permissive targets permitting viral replication. Even if cell-free viruses can directly infect DCs, viral transmission through cell-to-cell interactions was described as a more efficient way to get powerful virus infection (Cameron et al., 1992; Turville et al., 2004; Piguet and Steinman, 2007). DCs are one of the most efficient cells transferring viral particles to CD4+ T lymphocytes. In their immature form, DCs capture HIV-1 in the mucosa and transmit them to surrounding effector CD4+ T cells, which massively replicate HIV-1 (Turville et al., 2004; Lederman et al., 2006). Virus transmission by DCs has been described to occur through a two-phase transfer mechanism (Turville et al., 2004). Initially, an early transfer occurs when viruses, bound onto plasma membrane or located within endosomal compartments in immature DCs (iDCs), are concentrated and transmitted through the DC-CD4+ T cell synapse. This is followed by another round of transfer (i.e. late transfer), relying on productive virus replication in iDCs. Indeed, as elegantly shown by...
Turville et al. (2004), during the first 24 h of co-culture HIV-1 particles are rapidly transferred from DCs to CD4+ T cells without requiring de novo synthesis of newly formed virions. Because significant amounts of viruses are degraded within 24 h, early transfer rapidly diminishes with time and allows a long and more stable transfer dependent on the ability of iDCs to complete de novo viral production. Although viral transfer can take place in peripheral tissues and mucosal interfaces, most of the cell-mediated viral transfer takes place in secondary lymphoid organs. Maturation of iDCs loaded with HIV-1 triggers their migration towards draining lymph nodes where virions are potently transferred to CD4+ T cells during the formation of the virological synapse. Most studies have shown that mature DCs are less susceptible to productive HIV-1 infection than iDCs (Camerone et al., 1992; Granelli-piperno et al., 1998; Bakri et al., 2001), but the efficiency of viral dissemination to CD4+ T cells is enhanced following DC maturation (Izquierdo-useros et al., 2007).

Although malaria-associated immunosuppression is not very well understood, it has long been associated with the parasitic production of malaria pigments, better known as hemozoin (HZ) (Metzger et al., 1995; Coban et al., 2002; Doherty, 2007). Moreover, it has been demonstrated that the number of HZ-containing phagocytic cells is correlating well with disease severity (Hanscheid et al., 2007). HZ is synthesized by *P. falciparum* to detoxify the free haem in infected red blood cells and released in circulation upon reythrocyte rupture (Pisciotta and Sullivan, 2008). A minimum of 95% of the haem localized in the digestive food vacuole of the parasite is converted into HZ (Egan et al., 2002). The approximate size of the HZ crystals is thought to be about 1 μm × 0.4 μm × 0.2 μm (Egan et al., 1999), and approximately three to five crystals are produced per parasite (Noland et al., 2003). HZ is made of unit head-to-tail haem dimers where each crystal dimer consists of two haem molecules covalently linked through reciprocal iron-carboxylate coordinate bonding (Pagola et al., 2000). Previous studies indicate that HZ can affect the disease pathogenesis by various processes. For example, ingestion of HZ impairs priming of heterologous immune responses during *Plasmodium chabaudi* infection in mice by two major mechanisms. First, by preventing migration of CD4+ T cells into B-cell areas, a phenomenon leading to a less effective B-cell expansion and antibody production and, second, by inhibiting interactions between CD4+ T cells and DCs without affecting antigenic presentation (Millington et al., 2006; 2007). Discrepancies have been described depending on the *Plasmodium* species, the inoculation dose of malaria pigment and the type of DCs under investigation (Urban and Todryk, 2006). For example, ingestion of crude HZ (i.e. HZ complexed with lipid and proteins) leads to an impairment of monocyte function, DC differentiation and DCs maturation (Skorokhod et al., 2004; Skorokhod et al., 2005, Urban and Todryk, 2006), whereas purified HZ was found to upregulate surface molecules such as CD83, CD86 and CD1a, which are maturation markers for DCs (Coban et al., 2002).

Even though *P. falciparum* and HIV-1 co-infections are considered as major public health concerns, there is still a paucity of data with respect to the possible impact of malaria pigments on the establishment and dissemination of HIV-1 infection in targeted cells. Accordingly, the present study investigates whether HZ engulfment by immature monocyte-derived DCs (iMDDCs) can change their susceptibility to HIV-1 infection and their ability to transfer viral particles to CD4+ T lymphocytes.

## Results

**DC-mediated transmission of HIV-1 is affected by the malaria pigment**

As previously mentioned, HIV-1 can be transferred from DCs to CD4+ T cells via a process involving two distinct kinetic processes: an initial transfer phase (i.e. early transfer leading to *trans*-infection) followed by a second kinetic phase (i.e. late transfer leading to *cis*-infection). We first monitored the impact of the presence of sHZ in iMDDCs on both early and late transfer of HIV-1 towards CD4+ T cells. Results depicted in Fig. 1 (left panel) indicate that, at an early time point following initiation of the co-culture, sHZ-loaded iMDDCs transmit viruses in greater amounts (~twofold increase) as compared with what is seen with untreated cells. The p24 content measured in cell-free supernatants after 2 days of co-culture is a pool of newly synthesized virions coming from both iMDDCs and infected CD4+ T lymphocytes following viral transfer. At later time points, most virions present in the supernatant are produced by CD4+ T cells. To study the impact of sHZ on the early transfer phase, we focused on the first days of co-culture. In addition to discriminate which transfer type is favoured in presence of sHZ (i.e. early and/or late), the antiviral compound efavirenz (EFV) was added in some samples because this non-nucleoside reverse transcriptase inhibitor prevents the late transfer without affecting the early phase. Indeed, when cells are pretreated with EFV before being used for HIV-1 transfer studies, this treatment prevents productive infection of iDCs, including iMDDCs, allowing only the first transfer phase to take place (Gilbert et al., 2007; Barat et al., 2008; Lambert et al., 2008). As so, only viruses located on the cellular surface or recycled via the endosomal pathway will be transferred from DCs to CD4+ T cells. Furthermore, transfer can only be measured at 2 days following initiation of the co-culture as it is the minimum time required for a complete measurable virus cycle to take place in CD4+ T cells.
cells. Results indicate that treatment with EFV decreased viral transfer by 83% in co-cultured cells unexposed to sHZ, thus indicating that most of viral transmission comes from late transfer. However, virus propagation mediated by sHZ-treated iMDDCs is less affected by EFV leading to a 53% reduction. These data suggest that, in sHZ-loaded iMDDCs, about half of viruses are transmitted from early transfer and the other half results from de novo virus production by iMDDCs. A similar trend was obtained when iMDDCs are first treated with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) (data not shown), two powerful maturation agents for DCs. This observation is consistent with reported data showing an augmentation of the early transfer but a decrease of viral infection in those matured cells (Sanders et al., 2002; Fahrbach et al., 2007; Izquierdo-useros et al., 2007). Taken together, these results seem to indicate that exposure of iMDDCs to sHZ affects the process of HIV-1 propagation to autologous CD4+ T cells by possibly triggering maturation of DCs.

**sHZ phagocytosis prevents HIV-1 infection in human iMDDCs**

Next, we wanted to corroborate whether exposure of iMDDCs to sHZ influences the outcome of HIV-1 infection in this cell type. To this end, iMDDCs were initially treated with sHZ and next inoculated with HIV-1 before harvesting cell-free supernatants at several days following infection. Results illustrated in Fig. 2 (left panel) demonstrate that

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**Fig. 1.** sHZ modulates HIV-1 transfer from iMDDCs to autologous CD4+ T cells. iMDDCs were either left untreated (Ctrl) or treated with sHZ (10 µg ml⁻¹) for 48 h, incubated with NL4-3Balenv for 1 h, extensively washed to eliminate unbound and finally co-cultured with autologous CD4+ T cells. In some samples, iMDDCs were also treated with EFV before virus infection. Cell-free supernatants were harvested at the indicated time points in the left panel and at 2 days following initiation of the co-culture in the right panel. Virus production was estimated by performing a p24 test. The data shown in the left panel represent the mean ± SEM of triplicate samples from a single donor and are representative of seven separate experiments with distinct donors. Results depicted in the right panel represent the means ± SEM of triplicate samples from a total of seven independent experiments carried out with different donors. Statistical analysis was performed on the results from all experiments. Asterisks denote statistically significant data (*P < 0.05; ***P < 0.001).

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**Fig. 2.** sHZ-loaded iMDDCs are less permissive to productive HIV-1 infection. iMDDCs were left untreated (Ctrl) or treated for 48 h either with a single dose (left panel) or increasing concentrations of sHZ (right panel). Next, cells were incubated with NL4-3Balenv for 1 h. Cell-free supernatants were harvested at the indicated time points in the left panel and at 12 days following infection in the right panel. Virus production was estimated by performing a p24 test. The data shown in the left panel represent the mean ± SEM of triplicate samples from a single donor and are representative of three separate experiments with distinct donors. Results depicted in the right panel represent the means ± SEM of triplicate samples from a total of three independent experiments carried out with different donors. Statistical analysis was performed on the results from all experiments. Asterisks denote statistically significant data (*P < 0.05; **P < 0.01).
HIV-1 production in iMDDCs is significantly reduced by sHZ. Experiments conducted with increasing concentrations of sHZ indicate that virus production is diminished by 41%, 82% and 87% when cells are subjected to sHZ at 5, 10 and 25 μg ml⁻¹ respectively (right panel). As expected, replication of HIV-1 was almost completely abolished upon a treatment with LPS and IFN-γ (data not shown). Importantly, cell viability was not affected by the chosen concentrations of sHZ as monitored by the fluorescent cytotoxic MTS assay (data not shown). A comparable diminution in HIV-1 replication was seen when iMDDCs were treated with sHZ and natural HZ directly isolated from *P. falciparum* cultures (PfHZ) (Fig. 3), which provides additional significance to our findings.

**sHZ induces DC maturation**

The mechanism responsible for the lower susceptibility of sHZ-loaded iMDDCs to acute HIV-1 infection could be due to a possible modulation of expression of some cell surface receptor/coreceptor involved in the process of virus entry. Although surface expression levels of CD4 are not affected by sHZ (data not shown), a marked decrease in the percentage of CCR5-positive cells was caused by sHZ (Fig. 4A). The sHZ-mediated diminution of CCR5 expression is comparable to the situation seen when treating iMDDCs with LPS/IFN-γ (i.e. 6 ± 1.7, 2.5 ± 0.5 and 1.4 ± 0.8 for untreated, sHZ- and LPS/IFN-γ-treated iMDDCs respectively). Intracellular staining showed that the diminished CCR5 cell surface expression following sHZ treatment was paralleled by a concomitant reduced intracellular expression (Fig. 4B).

Given that a downregulation of CCR5 is a typical hallmark of the DC maturation process, our next series of investigations were aimed at defining whether sHZ can induce the establishment of a mature-like phenotype in the treated iMDDCs. *In vivo* maturation of iDCs upon reverse transmigration across the endothelial barrier has been established to take place within 48 h (Randolph *et al.*, 1998). Taking this into account, we performed comparative microscopy and flow cytometry analyses using, as positive controls, iMDDCs subjected to a treatment with LPS/IFN-γ because this combination of agents has been shown to induce a complete maturation program (Alldawi *et al.*, 2005). Upon microscopic analysis of iMDDCs cul-

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**Fig. 3.** Virus replication in iMDDCs is similarly affected by sHZ and natural HZ. iMDDCs were left untreated (Ctrl) or treated for 48 h with sHZ or natural HZ (10 μg ml⁻¹). Next, cells were incubated with NL4-3Balenv for 1 h. Cell-free supernatants were harvested at the indicated time points following infection. Virus production was estimated by performing a p24 test. The data shown in the left panel represent the mean ± SEM of triplicate samples from a single donor and are representative of three separate experiments with distinct donors.

**Fig. 4.** CCR5 expression in iMDDCs is reduced by sHZ. iMDDCs were either left untreated or treated for 48 h with sHZ (10 μg ml⁻¹) or LPS/IFN-γ (100 ng ml⁻¹ and 1000 U ml⁻¹ respectively). Next, flow cytometry analyses were carried out to assess either cell surface (A) or intracellular expression of CCR5 (B). The mean fluorescence intensities are displayed at the top of each bar (A). Results are depicted as percentages of CCR5-expressing cells compared with untreated iDCs (hypothetical value of 100%). (B) The data shown represent the means ± SEM of triplicate samples from a total of either five (A) or two (B) independent experiments carried out with different donors. Statistical analysis was performed on the results from all experiments. Asterisks denote statistically significant data (*P < 0.05; **P < 0.001).
In the presence of sHZ, we noticed the appearance of a cellular phenotype resembling the one seen in LPS/IFN-γ-treated cells (Fig. 5A). Indeed, sHZ- and LPS/IFN-γ-stimulated iMDDCs display pseudopod-like structures and adhere more firmly to the bottom of the well, two features characterizing mature DCs. To further confirm that capacity of sHZ to induce DC maturation, we performed detailed phenotypic analyses by flow cytometry. We concentrated our efforts on some specific maturation makers, such as the calcium-dependent pattern-recognition lectin DC-SIGN, which is reduced upon maturation of DCs (Lai et al., 2006), CD83, which is a member of the immunoglobulin superfamily expressed primarily by mature DCs (Kuwano et al., 2007), and HLA-DR, which has been reported to be upregulated upon DC maturation (Obermaier et al., 2003). The three studied cell surface markers were all modulated similarly by an exposure of iMDDCs to either LPS/IFN-γ combination or sHZ (Fig. 5B–D). For example, as for LPS/IFN-γ-treated cells, the presence of sHZ resulted in a marked reduction of

**Fig. 5.** sHZ induces DC maturation. iMDDCs were either left untreated or treated for 48 h with sHZ (10 μg ml⁻¹) or LPS/IFN-γ (100 ng ml⁻¹ and 1000 U ml⁻¹ respectively). Thereafter, phenotypic changes were monitored using microscopic (A) and flow cytometric analyses (B–D). The following three specific maturation markers were studied in our work: DC-SIGN (B), CD83 (C) and HLA-DR (D). The intensity of fluorescence was obtained by multiplying the percentage of positive cells by the mean fluorescence intensity (MFI). The data shown represent the mean ± SEM of triplicate samples from a single donor and are representative of three separate experiments with distinct donors. Asterisks denote statistically significant data (**P < 0.01).
DC-SIGN expression (i.e. 1603 ± 159, 622 ± 186 and 617 ± 192 for untreated, LPS/IFN-γ and sHZ-treated cells respectively) and an enhanced expression of both CD83 (72 ± 31, 692 ± 279 and 408 ± 204 for untreated, LPS/IFN-γ and sHZ-treated cells respectively) and HLA-DR (476 ± 85, 1106 ± 441 and 842 ± 428 for untreated, LPS/IFN-γ and sHZ-treated cells respectively).

It can thus be concluded that treatment of iMDDCs with subcytotoxic concentrations of sHZ is sufficient to induce maturation of this cell type, which will in turn affect the DC-dependent propagation of HIV-1.

**Discussion**

It is now well accepted that DCs and HIV-1 interact through a complex molecular networking (reviewed in Wu and KewalRamani, 2006; Piguet and Steinman, 2007). Once captured by iDCs, viral entities can either remain bound onto the cell surface, be stored in intracellular vesicles and/or lead to a productive infection. Upon exposure to a danger signal, HIV-1-loaded iDCs will mature and migrate towards lymphoid tissues enriched in CD4+ T cells, a process promoting viral dissemination. It has been demonstrated that mature DCs exhibit a reduced susceptibility to viral infection than iDCs but transmit more efficiently viral particles to target CD4+ T cells (Granelli-piperno et al., 1998; Piguet and Steinman, 2007). The reduced sensitivity of mature DCs to productive HIV-1 infection, as compared with iDCs, is thought to be due to various factors including an important degradation of internalized virions concentrated in endosomal compartments, expression of some restriction factors and low expression levels of HIV-1 receptor and co-receptors. For example, while iDCs express high levels of CCR5 on their surface, mature DCs express much lower amounts of this chemokine receptor, which is a marker for peripheral tissue homing (Sallusto et al., 1998; Sato et al., 2001). Instead, mature DCs express the chemokine receptor CCR7, which is essential for their trafficking and targeting to secondary lymphoid organs (Sallusto et al., 1998; Sato et al., 2001; Humrich et al., 2006). Hence, it is not surprising that maturation of DCs renders this cell population less susceptible to infection by R5-using isolates of HIV-1 since downregulation of CCR5 prevents efficient viral fusion and thus productive infection (Zaitseva et al., 1997; Granelli-piperno et al., 1998; Cavrois et al., 2006). It is also important to note that exposure of iDCs to maturation-inducing stimuli, like LPS, can trigger synthesis of soluble factors such as type-I IFNs that can repress HIV-1 replication. It was demonstrated that LPS inhibits virus production in macrophages again through a type-I IFN-mediated process (Equils et al., 2006; Liu et al., 2006; Simard et al., 2008). However, it is very unlikely that the sHZ-mediated diminution of virus production in iDCs cultured alone is due to the expression of those cytokines. This based on our findings that treatment of human primary macrophages with sHZ does not induce secretion of detectable levels of type-I IFNs (Diou, J.).

Here we report, for the first time, that treatment of iMDDCs with physiological concentrations of sHZ (see below for more details) induces a maturation phenotype resembling the one mediated by a treatment with LPS/IFN-γ when we assessed expression of some specific surface markers. Indeed, as described in the literature, DCs are considered mature when, for example, cellular markers, such as CD83 and HLA-DR, are increased and DC-SIGN is decreased (Granelli-piperno et al., 1998; Obermaier et al., 2003; Arrighi et al., 2004; Kuwano et al., 2007). Our findings concur with a previous work by Coban and colleagues who have reported that HZ purified from *P. falciparum*-infected erythrocytes enhances maturation of immature human myeloid DCs (Coban et al., 2002). Our results are also supported by previous observations indicating that HZ is efficiently engulfed inside DCs. For example, confocal microscopy examination and biochemical analyses have already showed a massive and persistent presence of HZ inside DCs (Skorokhod et al., 2004), confirming stability without shedding of ingested HZ as previously shown in monocyte-derived macrophages (Schwarzer et al., 1999; Schwarzer et al., 2001). HZ clumps were visualized to be homogeneously distributed inside the cells, they did not adhere to their external surface nor induce apoptosis of both immature and mature DCs (Skorokhod et al., 2004). While the objective of the current study was not to improve knowledge about phagocytosis of HZ by DCs, we recently performed live cell microscopy on sHZ-loaded monocyte-derived human primary macrophages and proposed that HZ is phagocytosed by a process relying on the formation of filopodia extension. Following phagocytosis, sHZ seems to remain in specialized phagosomes without being degraded and without killing the cell (Diou et al., 2009). Additional studies are warranted to define whether engulfment of sHZ in DCs follows the same pathway as in macrophages. Furthermore, we provide evidence that the DC-mediated transfer of HIV-1 to autologous CD4+ T cells is affected when iMDDCs are first exposed to sHZ. Indeed, we found that, in contrast to the situation prevailing with iMDDCs for which 83% of viral transmission was abrogated in the presence of EFV, viral transfer from sHZ-loaded iMDDCs was inhibited by about 53%. These results suggest that half of the transferred viruses come from newly synthesized virions and the other half originate from surface bound or internalized endosome-associated viruses. We postulated that sHZ-treated iMDDCs are less susceptible to productive virus infection, a hypothesis that was confirmed when iMDDCs
either left untreated or treated with sHZ were acutely infected with HIV-1 and cultured alone in absence of CD4+ T cells.

The mechanism(s) by which the sHZ-mediated maturation process of IMDDCs might influence the transit of HIV-1 into DCs remains unclear. It is possible that the intracellular transport of HIV-1 is affected by the ingestion of sHZ since malaria pigments can occupy as much as 30% of the total volume of the cell (Schwarzer et al., 2008). We have obtained some preliminary data suggesting that the trafficking of HIV-1 within sHZ-loaded human primary macrophage is impaired. Indeed, while entry and reverse transcription events remain untouched, integration of HIV-1 proviral DNA is significantly diminished in sHZ-treated macrophages (Diou et al., 2009). Therefore, we can speculate that the same phenomenon might possibly occur in IMDDCs. However, since sHZ phagocytosis induces cell maturation, it is also plausible that the inhibition of viral infection is associated with a downregulation of CCR5. The reduced expression of surface CCR5 is a well-established phenomenon that occurs during the maturation process. It has been shown that it can occur at the mRNA level, either by decreased transcription or increased degradation. Flow cytometry analyses revealed that sHZ is also mediating a diminution of intracellular CCR5, which supports the idea that HZ does not induce retention of CCR5 within the cell but rather promotes an increased degradation or a decreased synthesis of this molecule. It was established that LPS stimulation resulted in a complete downregulation of CCR5 mRNA levels after 40 h of treatment (Sallusto et al., 1998; Ip and Lau, 2004). Surprisingly, a previous report has shown that malaria causes a threefold increase in CCR5 expression (Tkachuk et al., 2001). However, it should be stated that this study has not scrutinized the contribution of HZ in this phenomenon and the superior CCR5 expression was detected in placental macrophages.

It should be noted that discrepant results have been described in the literature when using sHZ and other forms of HZ. For example, a previous study mentioned that P. falciparum-infected erythrocytes could adhere to IMDDCs, inhibit their maturation and subsequently reduce their capacity to stimulate both memory and naive T cell responses (Urban and Todryk, 2006). Although phagocytosis of parasitized erythrocytes was shown to inhibit important cellular functions of human macrophages and DCs (Schwarzer et al., 1992; Skorokhod et al., 2004), purified HZ was found to enhance maturation of DCs (Coban et al., 2002). With crude HZ preparations, the role of malaria pigments and those of other contaminating proteins, membranes and glycosylphosphatidylinositol are difficult to distinguish (Coban et al., 2002). Other studies describe conflicting results on the impact of HZ on the host immune system, particularly on DC functions, but only in murine models (Langhome et al., 2004; Perry et al., 2004; Coban et al., 2005; Millington et al., 2006; 2007).

In conclusion, we report here that the malaria pigment HZ induces DC maturation as evidenced by upregulation of CD83 and HLA-DR as well as downregulation of DC-SIGN. The sHZ-mediated phenotypic maturation of IMDDCs is comparable to what is seen when using the well-known maturation compounds, LPS/IFN-γ. In addition, our results indicate that treatment of IMDDCs with sHZ will affect transmission of HIV-1 by IMDDCs towards CD4+ T cells and DC susceptibility to productive virus infection. The physiological significance of the present work is high considering that during parasitic infection the concentration of HZ after erythrocytes rupture may be as high as 100 μg ml⁻¹ (Parroche et al., 2007) and can persist in macrophages of infected individuals for several months (Schwarzer et al., 1998). Additional and more comprehensive basic studies are needed to fully understand the cellular consequences of such deadly interactions. This is essential to discover promising drug combinations and promote new prevention strategies to treat worldwide co-infected individuals.

**Experimental procedures**

**Reagents**

Recombinant human interleukin-2 (rHL-2) and efavirenz (EFV) were obtained from the AIDS Repository Reagent Program (Germantown, MD, USA). LPS was purchased from Sigma (St Louis, MO, USA), IL-4 and IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA), whereas granulocyte macrophage colony-stimulating factor (GM-CSF) was a generous gift from Cangene (Winnipeg, MB, Canada). The culture medium of IMDDCs consisted of RPMI-1640 supplemented with 10% fetal bovine serum, penicillin G (100 U ml⁻¹), streptomycin (100 U ml⁻¹), primocine (Amixa Biosystems, Gaithersburg, MD, USA) and glutamine (2 mM).

**Antibodies**

The following antibodies were all purchased from eBioscience (San Diego, CA, USA): fluorescne isothiocyanate (FITC)-tagged anti-DC-SIGN monoclonal Ab (clone eB-h209), phycoerythrin (PE)-conjugated anti-human CD1a (clone HI149), FITC-tagged anti-human CD14 (clone 61D3) and PE-conjugated anti-human HLA-DR (clone LN3), while FITC-tagged anti-human CD83 (clone HB15e) and PE-tagged anti-CCR5 (clone 2D7) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-p24 (clone HB15e) and PE-tagged anti-CCR5 were obtained from the AIDS Repository Reagent Program and the American Type Culture Collection (Manassas, VA, USA) respectively. Anti-p24 antibodies were purified by using MAbTrap protein affinity columns according to the manufacturer’s instructions (Pharmacia Technology AB, Uppsala, Sweden).
Cells

Human embryonic kidney 293T cells were obtained from the ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (FCS). Peripheral blood mononuclear cells from healthy donors were isolated by Ficoll-Hypaque gradient and plated in 75 cm² flasks (1 × 10⁷ cells ml⁻¹) for 2 h in order to separate, by adherence to plastic, monocytes (CD14⁺) from the other non-adherent cells. To generate iMDDCs, purified CD14⁺ cells were cultured in complete culture medium that was supplemented every other day with a cocktail of GM-CSF (1000 U ml⁻¹) and IL-4 (200 U ml⁻¹) during 6 days. The percentage of cells expressing the surface markers CD3 and CD19 was evaluated to assess contamination with T and B cells respectively. Experiments were performed with cell preparations that contained a minimal amount of contaminants (i.e. DC: purity > 95%; CD4⁺ T cells: purity > 98%) (Lambert et al., 2008). Autologous CD4⁺ T cells were isolated using a negative selection kit according to the manufacturer’s instructions (StemCell Technologies) and the AutoMACS technology (Miltenyi Biotec, Auburn, CA, USA). These cells were activated with phytohemagglutinin-L (PHA-L; 1 µg ml⁻¹) and maintained in complete culture medium supplemented with rHL-2 (30 U ml⁻¹) at a density of 2 × 10⁵ cells ml⁻¹.

Plasmids and virus production

pNL4-3Balenv is a R5 (macrophage)-tropic strain where the NL4-3 env gene is replaced with a R5-tropic Bal strain (kindly provided by R. Pomerantz, Thomas Jefferson University, Philadelphia, PA, USA) (Dornadula et al., 1999). Viruses were produced by the calcium phosphate co-precipitation method in 293T cells as described previously (Fortin et al., 1997). The virus-supernatants were harvested at day 2 after infection, filtered through a 0.22 µm cellulose acetate syringe filter, ultracentrifugated, and normalized for virion content by using an in-house enzymatic assay specific for the major viral p24 protein. In this test, 183-H12-5C and 31-90-25 are used in combination to quantify p24 levels and the lower sensitivity of this test is 31.25 pg ml⁻¹ (Bounou et al., 2002).

HZ production

Synthetic hemozoin (sHZ) production was modified from a previous methodology (Jaramillo et al., 2003) but optimized in our laboratory. Briefly, 90 mg of haemin chloride (Sigma-Aldrich) was solubilized in DMSO (polymerization solvent) and added to a 4 M acetic acid solution at pH 5.0. The suspension was stirred with a magnet for 6 h at 65°C. After adding a volume of 10% SDS, the suspension was centrifuged at 12 500 g for 25 min. The pellet was then sonicated twice at the lowest setting in 100 mM sodium bicarbonate, pH 9.0, 0.5% SDS and centrifuged again. The pellet was then washed three times in SDS 2%, at least five times in sterile H₂O and once in endotoxin-free phosphate-buffered saline (PBS) to wash out residual SDS. The final pellet was resuspended in endotoxin-free PBS and aliquoted to obtain a solution of 652 µg ml⁻¹ sHZ. Natural HZ directly isolated from P. falciparum cultures was kindly provided by D.J. Sullivan (The Malaria Research Institute, Baltimore, MD, USA).

HZ quantification

Total haem content of HZ was determined, as described by Sullivan et al. (1996). Polymerized haem was incubated for 1 h in 2% SDS/20 mM NaOH to solubilize polymer into monomeric haem, which has a molar extinction coefficient at 400 nm of 1 × 10⁵.

HIV-1 infection

After differentiation, DCs were plated at 1 × 10⁶ cells in a final volume of 100 µl in 96-well plates. After treatment or not (Ctrl) with increasing concentration of sHZ for 24 h, cells were infected with a fixed amount of virus (i.e. 5 ng of p24 per 5 × 10⁴ cells). Every 3 days and for a period lasting 9 days, half of the medium was removed and kept frozen at 20°C until assayed. Virus production was estimated by measuring p24 levels in culture supernatants by ELISA.

HIV-1 transfer assay

iMDDCs (33 × 10⁵ cells in 100 µl) were either untreated or treated with sHZ (5 µg ml⁻¹) or LPS/IFN-γ (100 ng ml⁻¹ and 1000 U ml⁻¹ respectively) for 48 h and then exposed to HIV-1 (10 pg p24) for 60 min at 37°C. Next, the virus–cell mixture was extensively washed 3 times with PBS to remove unadsorbed virions. In some experiments, cells were also treated for 15 min with the antiviral drug EFV (50 nM) before pulsing iMDDCs with virions. Finally, iDCs were co-cultured with autologous PHA-activated CD4⁺ T cells at a 1:3 ratio in complete RPMI-1640 medium supplemented with rHL-2 (30 U ml⁻¹) in 96-well plates in a final volume of 200 µl. Virus production was estimated by measuring p24 levels in cell-free culture supernatants.

Flow cytometry analysis

Before staining, cells were incubated for 15 min at 4°C with 10% pooled human sera to block non-specific binding sites and washed once with PBS supplemented with 0.5% bovine serum albumin (BSA). To monitor cell surface expression of CD83, DC-SIGN and HLA-DR, samples from various healthy donors were incubated with specific antibodies or with an appropriate isotype-matched irrelevant control antibody (for non-specific staining) for 30 min at 4°C. Cells were then washed with PBS/0.5% BSA and fixed in 2% paraformaldehyde and analysed by FACS (Epics ELITE ESP; Coulter Electronics). Intracellular staining for CCR5 was performed using Cytofix/Cytoperm Fixation/Permeabilization Solution Kit according to the manufacturer’s instructions (BD Biosciences).

Statistical analysis

Results presented are expressed as means ± SEM of at least triplicate samples. All experiments were repeated at least three times and each figure combines the results obtained with all the different donors. Statistical significance between groups was determined by analysis of variance. Calculations were made with Prism version 3.03. P-values of < 0.05 were considered statisti-
cally significant. The statistical significance of the results was defined by performing a one-way analysis of variance with Dunnett’s post tests to compare treated and control samples or with Bonferroni’s post tests to compare all pairs of columns. For the percentages of inhibition, illustrated in Fig. 3, statistical analysis was performed by using arcsine transformation followed by a one-way analysis of variance with Bonferroni’s post tests.

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