Dendritic cells and *Plasmodium falciparum*: studies *in vitro* and in the human host.

Pablo Giusti

Stockholm 2009
“Seamos realistas y hagamos lo impossible”

Ernesto Guevara de la Serna
Summary

Malaria is one of the world’s most threatening diseases. About half the world’s population is at risk of infection and the infection claims a million lives each year. A vast majority of the deaths occur in children below the age of 5 in sub-Saharan Africa. Survivors typically acquire immunity only after long time of repeated exposure and immunity is rapidly lost. Immunity is created by the activation of naive T cells and their differentiation into effector cells. The most potent activators of naive T cells are dendritic cells (DCs). The life cycle of DCs is adapted to find and process microbes in order to be able to present their antigens to T cells and thereby activate them. Antigen presentation typically takes place in the lymph nodes and that is why migration to these areas is an essential part of the DC life cycle. Various studies have shown that DC function may be hampered by the malaria parasite or its components.

We have investigated activation and migratory capacities of DCs upon in vitro exposure of the malarial pigment hemozoin and Plasmodium falciparum infected red blood cells. Furthermore, we have assessed the activation status of blood DCs in the Fulani, a traditionally nomadic population that respond better to malaria infection and exhibit less clinical symptoms than other ethnicities living under similar conditions, and a neighbouring ethnic group, the Dogon, in Mali.

Our results indicate that DCs are semi-activated upon malaria exposure in vitro, including enhanced migratory capacity, partial up-regulation of co-stimulatory markers and no IL-12, which may lead to inappropriate T-cell priming. We also observed that DCs from the Fulani have a higher degree of activation than DCs from the Dogon upon malaria exposure in vivo. We hypothesize that this increased DC activation may be the reason for the relatively increased protection against malaria.

Taken together, our findings suggest that improper DC activation may contribute to poor immunity in Malaria.
List of Papers

This thesis is based on the following manuscripts which will be referred to by their roman numerals:


II. Arama Charles *, Giusti Pablo *, Boström Stéphanie, Dara Victor, Traore Boubacar, Dolo Amagana, Doumbo Ogobara, Varani Stefania and Troye-Blomberg Marita. *Low frequency of circulating dendritic cells is associated with higher cell activation, raised specific antibody levels and increased pro-inflammatory responses in Fulani, an ethnic group with low susceptibility to malaria.* Manuscript in preparation.

* These authors contributed equally to this work.
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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood dendritic cell antigen</td>
</tr>
<tr>
<td>CC</td>
<td>Chemotactic cytokines</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
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<td>Dendritic cell</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol anchors</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>Hz</td>
<td>Hemozoin</td>
</tr>
<tr>
<td>HLA</td>
<td>Human-leukocyte antigen</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor-associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>IFN regulatory factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysacharide</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocytes-chemoattractant protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage-inflammation protein</td>
</tr>
<tr>
<td>MoDC</td>
<td>Monocyte-derived dendritic cells</td>
</tr>
<tr>
<td>MØ</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural-killer cells</td>
</tr>
<tr>
<td>NF-kb</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pathogen-recognition receptors</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation Normal T Expressed and Secreted Protein</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic-T cell</td>
</tr>
<tr>
<td>Th</td>
<td>Helper-T cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory-T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis factor</td>
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</table>
Introduction

The immune system

The mammalian immune system is an evolutionary product of the co-existence of a wide variety of microbes with multi-cellular organisms. Consequently, the human immune system has evolved to become a very complex network, involving everything from peptides to signalling molecules to specialized cells dispersed all throughout the body. The immune system is commonly divided into two different branches that are called the innate and the adaptive immunity.

On the one hand, one branch has evolved to detect danger signals and indiscriminately attack microbes. This branch is called the innate immune system. Innate immunity is very similar in all humans and does not, from a short time perspective, seem to adapt to changes in the surrounding environment. The innate protection is achieved by creating obstacles at many different levels to impair the pathogens from colonizing the human body. The first barriers are physical, such as the skin and mucosal surfaces of the body. In this context it is important to remember that everything from mouth, to stomach, to the end of the intestine can be regarded as a tunnel through the body and is actually to be regarded as the body’s exterior surfaces. These surfaces are colonized by enormous amounts of microorganisms that, for the most part, are not pathogenic. To this end the immune system in the gut is somewhat specialized to induce tolerance against harmless microorganisms. There are also physiological barriers like the acidic environment in the stomach. Then there are the antibacterial peptides and complement proteins in circulation that recognize pathogens and initiate a cascade of proteolytic activity ultimately leading to lysis of the bacteria. In addition, there are the cells of the innate immune system equipped with different kinds of receptors to recognize infectious stimuli.
However, since living conditions in different social, cultural and geographical environments can be very variable, there is a need for adaptation to different circumstances. The other branch of the immune system has evolved to specifically recognize and remember the pathogens that it has been exposed to. For that purpose some lymphocytes have evolved to recognize pathogens specifically and to remember them. That is what is known as the adaptive immune system and is, in contrast to the innate, unique in every human.

In summary, the innate immune system is unspecific, fast acting and lacks memory while the adaptive is specific, slower to act and has a memory component. The outcome is an immune system that can act swiftly against all pathogens and learn to respond more efficiently against any pathogen common to a given individuals specific environment. These branches are, however, not two entirely separate systems but the adaptive is dependent on the innate components to function and the innate immune responses are much more efficient when aided by adaptive components.

The cells of the immune system are found throughout the body, particularly in the circulation and in the lymphatic tissues. They all originate from the bone marrow and, at certain stages of development, in the liver. These cells are collectively called leukocytes and are of either lymphoid or myeloid origin. The lymphoid are B-, T-, natural killer (NK) - cells and possibly some subsets of dendritic cells (DCs). The granulocytes, monocytes and most of the DCs stem from a myeloid progenitor. A vast majority of the circulating immune cells are granulocytes, in round numbers, granulocytes comprise about 60% of leukocytes in blood, about 30% are lymphocytes and the final 10% are monocytes (Figure 1).
Figure 1. Cells of the Immune system

The cells of the innate immune system are monocytes, macrophages (MØ), granulocytes, DCs and NK cells. All of them, except for the NK cells, have endocytic properties enabling them to constantly survey their surroundings.

The cells of both the adaptive and innate immunity communicate through the secretion of cytokines. There is an important subgroup of cytokines that governs cell motility and they are called chemotactic cytokines (CC). These substances are shared between all the cells of the immune system and will therefore be discussed in a separate chapter after a closer look at the two separate branches.
Innate Immunity

Recognition strategies
Perhaps the most essential task for the immune system is to be able to distinguish what is to be regarded as friendly and what is to be regarded as dangerous. To solve this task, an array of receptors has evolved to recognize components that are essential and unique to microbes. An example is the cell-wall component lipopolysaccharide (LPS), in gram negative bacteria, that potently activates the innate immunity.

The mechanisms that have evolved to know what is dangerous and what is not have, perhaps in an oversimplified manner, been referred to as self/non-self recognition. It is now known, however, that these mechanisms recognize not only foreign antigens but also self derived “danger signals” and even healthy self (1). Among the microbes there are recurring pathogen associated molecular patterns (PAMPs) particular or essential to their function. The components of the innate immunity have evolved to take advantage of that and initiate adaptive immune responses (2). The cells of the innate immune system express receptors that recognize these PAMPs that are called pattern-recognition receptors (PRR) (3). There is also a family of receptors called C-type-lectin receptors. The C-type-lectin receptors recognize carbohydrate structures on glycosylated surface proteins of pathogens. Some examples are the mannose receptor, DC-SIGN and Dectin-1. Another important mechanism for recognizing pathogens is Fc receptors which binds the constant region of antibodies. Because of this they are dependent on adaptive immunity and should not be counted as a part of innate immunity.

Toll like receptors
The most investigated family belonging to the PRRs in humans are the toll-like receptors (TLRs). The TLRs were first discovered in drosophila and it became clear that they were related to the IL-1 receptor suggesting a role in immunity (4). This has been proven as toll mutants were shown to be more susceptible to fungi infections (5). One after another, the roles and ligands for the different receptors were discovered (6-9). Ten different TLRs have
so far been characterized in humans. They all have specific ligands and localizations (Table 2) and are expressed on different subtypes of DCs which will be further discussed in the chapter of DCs. TLR1, TLR2, TLR4, TLR5 and TLR6 recognize recurring PAMPs in bacteria, fungi, parasites and some viruses and are found in the outer cell membrane. However, the TLRs that are specialized in recognition of intracellular bacteria and viruses, such as TLR3, TLR7, TLR8, and TLR9 are located intracellularly. The motifs that they recognize are not necessarily unique to viruses. In fact, nucleic acids are also present in human cells, but since TLR are localized in endolysosomes there should not be any nucleic acids from the host present and reactions against self-derived nucleic acids can therefore be avoided.

**Signalling**

TLRs are membrane-spanning proteins with one amino terminal extracellular domain of leucine-rich repeats, that is responsible for binding the ligand, and an intracellular signalling domain. The TLRs can form either homodimers or heterodimers. For example, it has been shown that TLR2 forms a homodimer or heterodimers with either TLR1 or TLR6 (10). In this manner different dimer formations can recognize different ligands. The signalling domain of the TLRs is homologous to the signalling domain of the IL-1R and is called Toll/IL-1R domain (TIR). In general this domain signals through TIR-TIR interactions with an adaptor protein, thus recruiting an IL-1 receptor-associated kinase (IRAK) family protein and the TNF receptor associated factor 6 (TRAF6). This complex activates Map kinases, that eventually leads to the activation of transcription factors (11,12).

All TLRs except TLR3 signal through the adaptor protein myeloid differentiation primary response gene 88 (MyD88). TLR3 signals through the TIR-domain-containing adapter-inducing interferon-β (TRIF) leading to IFN regulatory factor (IRF) 3 and IFN-α secretion (13). TLR4 however, can use both of these pathways. TLR 1, 2, 4 and 6 can also signal through toll-interleukin-1 receptor domain containing adaptor protein (TIRAP).
However, signalling through the same adaptor protein can have different outcomes. Both TLR7 and TLR9 signal through MyD88 and engage IRF7 which leads to secretion of IFN-α (14). When the TLR4 is activated through the MyD88-dependent pathway it leads to IL-12p70 secretion (15) but when the alternative pathway via TRIF is activated this results in IRF3 activation and secretion of type 1 IFNs (13,16). The impacts of different TLR activation on adaptive immune responses have been summarised in a recent review (Figure 2) (15).

Briefly, IL-12p70 is strongly induced by TLR4, TLR5 and TLR8 and only weakly induced by TLR2 in its different dimer complexes that instead lead to IL-10 production. TLR4 ligation can lead to either IL-12p70 or IFN-α release whereas viral recognition by TLR3, TLR7 and TLR9 induces IFN-α secretion. When the different TLR complexes involving TLR2 are activated that would result in T-helper (Th) 2 or Treg skewing of the immune responses.

Figure 2. Reprinted from Manicassamy S, Pulendran B. Modulation of adaptive immunity with Toll-like receptors. Semin Immunol (2009), doi:10.1016/j.smim.2009.05.005 with permission from Elsevier
Conversely, activation of TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 would lead to a Th1-type of response. Thus, depending on which TLR is engaged, the outcome will be different and thereby a “custom made” default response mechanism exists to deal with different types of antigens.

**Innate immune cells**

*Monocytes*

Monocytes are characterized as mononuclear leukocytes expressing the LPS co-receptor CD14. The circulating monocytes constitute a reservoir for tissue specific macrophages (MØ) and DCs. They may also have a role in maintaining tissue homeostasis by cleaning up debris. About 20 years ago a subpopulation of monocytes expressing CD16 (Fcγ Receptor III) was described (17). The classical CD14++CD16- cell expresses higher levels of CD14 and no CD16 (Fcγ Receptor III), while the inflammatory CD14+CD16+ cell type expresses CD16 and lower levels of CD14. A more recent review has better defined the classical and inflammatory monocytes based on studies of cytokine secretion and T-cell stimulation (18). It seems like the inflammatory monocytes are more prone to trans-endothelial cell migration and differentiation to DCs (19).

*Macrophages*

Macrophage (MØ) means big eater and tissue specific MØ subsets have been described in various organs such as lung, liver and nervous tissues. Intracellular macrosialin or CD68 has been widely used for identification of macrophages (20), although it has been proposed that it is not a marker of macrophages (21) but rather of phagocytosis. In analogy with the Th1/Th2 activation of T cells a M1/M2 characterisation of MØ has been suggested. Classical activation of MØs has been defined as the response to LPS, with IFN-γ secretion and induction of cytotoxic-Th1 immune response; these are the ones called M1. The alternative activation induced by IL-4, IL-10 and IL-13 of monocytes consequently leads to a M2 polarized MØ thus promoting a Th2 type of response (22,23). However, there have been objections to the
oversimplification of this concept (24) where a more complex picture of MØ activation is presented. Nevertheless, a subcategorizing of M2 polarized MØs has been suggested (25) where the M2 MØs now include all MØs that are not M1. This leads to a concept where the M1 is well defined but the M2 is still a heterogeneous group of MØs. Recently, a model that suggests dividing MØs in host defence, wound healing and immune regulation has been proposed (26) that would better account for the role of MØs in homeostasis.

**Dendritic Cells**

DCs are a very heterogeneous group of cells both when it comes to their origins and their distribution as specialized tissue specific cells in different organs (27). Unlike MØs, some DC subsets may have a lymphoid origin. DCs are the most prominent professional APCs because their life cycle is adapted to find, process and present antigens to naive T cells in lymphoid tissues. The immature DCs (iDCs) travel the peripheral tissues expressing high amounts of PRRs, particularly TLRs, and a repertoire of chemokine receptors (CCR) that are specialized to detect inflammatory mediators. Upon encountering a pathogen, DCs become activated and alter the expression of their surface molecules (28). The surface antigen CD83 is one of the most important maturation markers on DCs and it has been shown that CD83 knockout mice were blocked in the generation of CD4⁺ T cells (29). The DC-maturation process also involves down regulation of PRRs and inflammatory CCRs, such as CCR1, CCR2 and CCR5. Simultaneously, DCs up regulate co-stimulatory molecules and CCRs with ligands expressed in lymphoid tissues such as CCR7 and CXCR4 (30). This CCR switch enables DCs to migrate to T-cell rich areas in secondary lymphoid organs and encounter massive amounts of naive T cells (31). When the proper combination of T-cell receptor (TCR) on a CD4⁺ T cell and human-leukocyte antigen (HLA) class II complex expressed on a DC is found, co-stimulation and cytokine secretion increases. This triggers an activation programme in the T cell that then starts to grow and divide so that thousands of clones will be active in a matter of days.
There are many subpopulations of DCs in circulation and different models for the development of DCs have been suggested (32). Most commonly blood DCs are described as two distinct populations; the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs) with complementary functions (33). However, it seems that there is certain plasticity even in these two subtypes of DCs (34). A more careful characterisation has resulted in specific surface marker for different DC subsets in circulation (Table 1) (35).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>CD1c</th>
<th>CD11c</th>
<th>CD123</th>
<th>CD2</th>
<th>CD16</th>
<th>CD32</th>
<th>CD64</th>
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<tr>
<td>BDCA1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>BDCA2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BDCA3</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD16⁺DC</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Nd</td>
<td>Nd</td>
</tr>
</tbody>
</table>

Table 1. Expression of surface molecules on distinct blood DC subsets. Abbreviation: Nd- no data. References: (35-37).

The major mDC subpopulation in circulation is defined as lineage negative, CD1c⁺ CD11c⁺ CD123⁻ cells. They are characterised by their expression of the blood dendritic cell antigen (BDCA)-1 also called CD1c. Conversely, BDCA-2 (CD303) is strictly expressed on pDCs. These cells are defined as lineage negative, CD123⁺ and CD11c⁻ cells. In addition, BDCA-3 (CD141) is expressed on a subpopulation of mDCs that are also defined as lineage negative, CD1c⁺ CD11c⁺, CD123⁻ cells. Unlike the BDCA-1⁺ DCs, these cells lack the expression of BDCA-1, CD2, CD32 and CD64. The monocytes and the different DC subsets differ in the expression of TLRs. Different subtypes of DCs are directed towards different kinds of pathogens and therefore express different TLRs (Table 2). The pDCs express TLR1, TLR6, TLR7, TLR9 and TLR10, while mDCs express TLR1, TLR2, TLR3, TLR5, TLR6, TLR8 and TLR10 (38).
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand and origin</th>
<th>location</th>
<th>mDC</th>
<th>pDC</th>
<th>MoDC</th>
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</thead>
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<tr>
<td>TLR1</td>
<td>Triacyl LpP (bacteria)</td>
<td>surface</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TLR2</td>
<td>Zymozan, PGN (fungi, G+ bacteria)</td>
<td>surface</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA (viruses)</td>
<td>surface</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, proteins (G- bacteria, viruses)</td>
<td>surface</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin (bacteria)</td>
<td>surface</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl LpP, Zymosan (bacteria, fungi)</td>
<td>surface</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA (virus)</td>
<td>internal</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA (virus)</td>
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<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>CpG-rich DNA, Hemozoin (virus, protozoa)</td>
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<td>+</td>
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<td>TLR10</td>
<td>Nd</td>
<td>surface</td>
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</table>

Table 2. TLR receptors localization and ligands. Abbreviations: mDC-myeloid DC, pDC-plasmacytoid DC, MoDC-monocyte derived DCs, LpP-Lipoprotein, PGN-Peptidoglycan, ds-double stranded, ss-single stranded, Nd-No data. References: (38,39).

The pDCs primarily recognize and react to viral infections and respond by secreting high amounts of IFN-α (40). On the other hand mDCs primarily respond to LPS and other bacterial, viral and parasitic stimuli by secreting high levels of TNF-α, IL-6, IL-8, IL-12 and up regulate the maturation markers HLA-DR and CD80 when exposed to LPS (41). This has also been subject of more recent reviews and the different roles in pathogen recognition and subsequent responses of pDCs and mDCs have been well characterized (42,43).

The BDCA-1+ mDC subset expresses higher levels of CD86 and HLA class II molecules than the BDCA-3+ mDC subpopulation (35). The function of the rare BDCA-3+ mDC subpopulation is still not clear, but a possible immunomodulatory role has been suggested for this cell subset upon parasitic infection in humans (44).

Taken together, the DC specialisation in pathogen recognition, their transition from immature to mature state and their high capacity to activate naive T cells, make them a crucial link between the innate and adaptive immune responses. It is well established that the secretion of pro-inflammatory Th1 cytokines like IL-12 and type 1 IFNs is essential in directing adaptive responses. It has also been shown that DC-derived IL-12 induces differentiation of naive B-cells (45) and induces class switching to IgG and IgA, thus regulating humoral responses (46). Moreover, the typical pDC type 1 IFN secretion discussed earlier can also induce plasma-cell differentiation and Ig production (47,48).
Several protocols have been established to obtain purified mDCs from precursors in vitro (49-51). DCs derived from CD34+ cord blood cells differentiate along two independent pathways in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α. After 5-6 days, two subsets (one CD1a-CD14- and one CD1a+CD14+) can be observed; after 12 days in culture, all cells are CD1a-CD14-. The CD1a+ precursors differentiate into Langerhans cells that contain Birbeck granules, whereas the CD14+ precursors lead to CD1a- mDC that do not produce Birbeck granules and that possess the characteristics of interstitial DCs (52). It is also believed that monocytes represent a pool of circulatory precursor cells that are capable of differentiating into mDCs that resemble the features of interstitial DCs (28). Monocyte-derived DCs (MoDCs) with the typical phenotype of CD1a-CD14- cells can be obtained in vitro by stimulation with IL-4 and GM-CSF for 6 days (51) (256). In vitro-generated monocyte-derived DCs are considered to have inflammatory properties (53). These cells can further mature upon incubation with various stimuli, such as bacterial LPS, inflammatory cytokines (TNF-α and IL-1β) and T-cell signals (e.g. CD40 ligand, CD40L). In the first study of this thesis we have employed monocyte-derived DCs to resemble inflammatory DCs that develop upon infection in vivo.

**Granulocytes**

Granulocytes are by far the most prevalent leukocytes in the circulation. Their morphology is somewhat different from other leukocytes in that they have a segmented nucleus and their cytoplasm contains granules. The granules contain highly reactive oxygen or nitrogen-derived compounds, highly acidic contents or enzymes that digest bacterial components. The granulocytes can be subdivided in three categories based on their staining characteristics. The vast majority are neutrophils that quickly move to the site of infection and once there, they release their granules and phagocytose as much bacteria as they can until they die. The pus that is formed in wounds upon infection is remnants of neutrophils and their phagocytosed bacteria. The other two subcategories of granulocytes, the eosinophils and the basophils, are...
believed to be more adapted to clear parasite infections as they express IgE receptors (54,55).

Another class of densely granulated cells are the mast cells that contain histamine granules and have been implicated in allergic reactions (56).

**Natural Killer Cells**

NK cells were named after their ability to find and kill tumour or virus infected cells without prior activation (57). They express a variety of receptors that can bind to HLA class I molecules. When NK cells meet a cell with no expression or an aberrant expression of HLA class I, they release granules containing perforin and granzyme that will lyse the host cell. This is known as the missing self hypothesis (58). NK cells express immunoglobulin-like receptors that have either inhibitory or activating motif, as signalling sequences, on their intraplastic domain (59). The sum of the signals that the NK cell receives upon an encounter with another cell will determine whether or not that cell should be killed (60). NK cells are characterized by the expression of CD56 but not TCRs or CD3. It is also possible to subdivide them according to the levels of CD56 and CD16 they express (61). The CD56\textsuperscript{high} NK cells are more prone to secrete high levels of cytokines while the CD56\textsuperscript{dim} NK cells have a higher cytotoxic activity.

**Effector molecules**

The complement system consists of about 30 serum proteins that attach directly to the surface of pathogens or antibodies bound to the surface of pathogens (62). When activated they recruit not only other complement proteins but also acts as chemotactic stimuli to leukocytes. The final outcome, of the complement activating each other, is lysis of the pathogen they are bound to and recruitment of cells to the site (63). In addition, there are some peptides in circulation with bactericidal properties they were first found in plants but now various antimicrobial peptides have been found in human as well.
Adaptive Immunity

The adaptive immune system has to be selective, in that it has to discriminate self from non-self, specific in order to recognize every particular pathogen and respond accordingly, flexible in order to confront changes in the pathogen and have a memory to recognize previous infectious agents. The destiny and developmental pathways of the lymphocytes make them well suited to solve these tasks.

Recognition strategies

During maturation, B and T lymphocytes randomly rearrange the joining (J), diversity (D) and variable (V) segments of their genome. These segments code for antigen-binding molecules and are responsible for specifically recognising a particular antigen. In the case of T cells, gene rearrangement occurs in the genes coding for the α, β, γ and δ chains of the T-cell receptors (TCRs) while in B cells gene rearrangement occurs in the immunoglobulin (Ig) genes coding for the heavy and light chains of the B-cell receptor (BCR).

Naive T cells gather in T-cell areas of the secondary lymphoid organs where they await an activated APC that will present the right combination of peptide loaded HLA complex and thereby activate them. A similar antigen from the same microbe may also bind a BCR thus preparing the B cell for the encounter of an activated T cell. In this encounter the B cell will become potently activated, expand clonally and produce vast amounts of antibody.

Most T cells recognize antigens only when presented to the TCR in the context of an HLA molecule. The TCR cannot be expressed alone on the T-cell surface but co expression of TCR and CD3 is mutually required for the successful expression of either (64). The TCR can be composed of one α and one β chain and in that case it lacks an intracellular signalling domain. It can also be composed of one γ and one δ chain this gives rise to the γδ-T cells. They recognize antigens in a different manner than the αβ-T cells and are discussed further later in the text. The intracellular signalling upon binding of the TCR goes through CD3 and triggers
the activation of various intracellular pathways leading to elevated Ca$^{2+}$, actin remodelling and activation of NF-$\kappa$B (65).

**Adaptive immune cells**

*T Cells*

T lymphocytes are a heterogenous group of cells that have undergone gene rearrangement and express a TCR. The heterogeneity consists mainly in their manner of activation and consequently their effector functions. They range from the most pro-inflammatory Th1 to the most anti-inflammatory regulatory T cells (Tregs).

*Development and Selection*

The random rearrangement of the TCR genes creates a large enough variety of antigen-binding molecules so that for almost any given antigen, there will be a TCR to recognize it. To avoid the release of potentially harmful T cells from the thymus to the periphery there is a selection process in the thymus. During early thymic development, before the TCR genes are rearranged, the cells express neither CD4 nor CD8 and are therefore termed double negative (66). Since the gene recombination is random the specificity of the respective TCR is also random. When the cells have rearranged their TCR, they express it on the surface along with both co-receptors CD4 and CD8. At this stage they are termed double positive and a selection process takes place where less than 5% of the cells survive (67). The T cells are presented to self antigens in association with human-leukocyte antigen (HLA) molecules. During positive selection the T cells that can bind self HLA molecules survive. If a T cell undergoing the positive selection process is rescued by a cell expressing HLA class I it will maintain the expression of CD8 but loose CD4 and vice versa. During the negative selection those who bind too firmly to the HLA are potentially self reactive and therefore eliminated. The objective is that the mature T cells released from the thymus all recognize self but should not be self reactive. The T cells leaving the thymus can be subdivided into sub categories the T-helper (Th), T-cytotoxic (Tc) and T-regulatory cells (Treg).
**T helper cells**
The classical Th cells express CD4 and can be further subdivided in Th1 and Th2 cells according to cytokine secretion and their functional properties (68,69). When the naive Th cell is activated by an APC the co stimulation and the cytokines secreted are what will decide what type of helper cells they become. The classical Th cells are the Th1 / Th2 cells. Th1 cells are induced by high IL-12 that in turn leads to IFN-γ secretion by the Th1 cells. IFN-γ secretion creates a positive feedback loop and causes up regulation of transcription factor T-bet that enhances Th1 response (70). Similarly IL-4 induces the transcription factor GATA3 that also maintains the expression of IL-4 and thus, a Th2 phenotype.

**T-cytotoxic cells**
The T-cytotoxic cells (Tc) express CD8 but not CD4 and therefore only recognizes antigens in association with HLA class I. Consequently, the cells bind to all nucleated cells in the body and when an aberrant expression of HLA-antigen complex is encountered, as it would be in a cancer or virus infected cell, the Tc are activated. Upon activation the Tc cells kill the other cell by induction of apoptosis or the release of granules with perforin and granulosin that lyse the membrane of the infected cell (71).

**Regulatory T cells**
An additional CD4+ subtype expressing high levels of CD25 was found more than 10 years ago exhibiting immunosuppressive properties (72). These T cells are released from the thymus expressing CD4+CD25++ and the transcription factor FoxP3 and are called natural regulatory T cells (nTreg). In addition there are Tregs that are induced in the periphery that secrete high amounts of IL-10 and TGF-β these are referred to as inducible Tregs (iTregs). The nTregs are believed to act in a contact dependent manner while the iTregs are cytokine dependent (73).

**Gamma delta T cells**
About 1-10% of the T cells have TCR composed of one γ and one δ subunit instead of the αβ subunits. It was suggested that a γδ clone could recognize a viral protein independently of
antigen presentation (74) and that recognition of non-peptidic antigens required cell contact between T cells (75). The distribution of γδ-T cells differs from that of the αβ-T cells in that they are mainly found in blood and peripheral tissues but rarely in the lymphoid organs. This fact agrees well with the theory that these cells do not require APC to recognize antigens but recognize them either alone or in a different context (76). The γδ-T cells are now considered to work as a link between innate and adaptive immunity (77).

**T helper 17**
Recently, another type of Th cell has emerged because of its cytokine production it has been called the Th17 cell. These cells secrete high amounts of IL-17, but not IL-4 or IL-12 (78). Although it is not yet clear what drives Th17 differentiation it seems to be dependent on the transcription factor Ror-γ that is induced by TGF-β and the transcription factor STAT-3 that is regulated by IL-6, IL-21 and IL-23 (79). In humans it is not yet clear which cytokines induce the Th17 while in mice this cell subset is induced by IL-6 and TGF-β (78).

**Natural killer T cells**
This subset of cells expresses the αβ-TCR and are therefore defined as T cells. They do however display properties of NK cells and seem to be important for recognizing antigen displayed on non-classical HLA molecules.

**B cells**
Much like the random rearrangement of the TCR in T cells, B cells in the bone marrow rearrange their genome in the variable region coding for Ig. The Igs are expressed either as a membrane bound form, i.e. the BCR, or secreted as antibodies and their production is exclusive to B lymphocytes. The naive B cell expresses the BCR on the plasma membrane and secretes low amount of IgM antibodies. When the BCR binds its specific antigen it is internalised, processed and displayed on an HLA II molecule on the surface. At this stage, an activated T cell whose TCR can bind the HLA-antigen complex on the B cell to form the immunological synapse, can thereby potently activate the B cell. This leads to clonal
expansion of the B cell; the clones undergo somatic hypermutation in the epitope binding region and as a result increase the affinity to the antigen. In this way the antibodies will have higher affinity to the antigen the longer the infection persists. B cells can also be activated without the aid of Th cells through for example TLR ligation. Upon activation the B cells become either plasma cells that produce high amounts of antibodies or memory cells. After activation, the B cells also undergo class switching where the Ig production is shifted from mainly IgM to IgG or IgE subtype.

**Effector molecules**
The effector molecules of adaptive immunity are the antibodies. An antibody is composed of two heavy and two light chains each consisting of one constant and one variable region. The two heavy chains are linked to each other and to one light chain each by disulfide bridges. After the rearrangement of the heavy and the light chain the B cells are released into circulation. Each B cell expresses its BCR bound in the membrane as IgM or IgD isotype and undergoes activation when BCR binds its specific antigen. An activated B cell undergoes a recombination of the heavy-chain genes coding for the constant region but not the variable region allowing it to keep its specificity but switching isotype (80). There are five different isotypes or classes of antibodies; IgA, IgD, IgE, IgG and IgM, and the isotype is determined by the constant region of the Ig heavy chain. It is this constant region that interacts with complement proteins and FcRs thereby regulating the immune response (81). In addition, in humans there are four subclasses of IgG antibodies the IgG1, IgG2, IgG3 and IgG4 and two subclasses of IgA the IgA1 and IgA2.
Coordinated Immune responses

As already mentioned above the innate and adaptive branches of the immune system are not two separate systems. Therefore it is impossible to generally write about immune responses under two separate headings. The connecting factor between innate and adaptive immunity is antigen presentation. Finally, molecules governing cell motility and signalling are shared between the two branches of immunity and are therefore discussed here with regards to coordinated immune response.

Leukocyte movement

In order to efficiently mount a proper immune response the cells need to travel to the site of infection. Cell movement is guided by a class of small proteins called chemotactic cytokines (CC) or more commonly, chemokines. They were named because of their ability to induce chemotaxis i.e. cell movement towards a gradient of a certain substance. The chemokines are divided according to structural properties and the C symbolises the location of highly conserved cystein residues in the peptide chain of chemokines. The CC are divided into two major subgroups; the CC and the CXC (82). and there has been an addition of two smaller subgroups; i.e. the C and the CX₃C subgroups to this major families (83). The chemokine receptors (CCRs) have a 7 transmembrane domain and are coupled to a G protein for intracellular signalling (84).

Inflammatory CCRs

CCR1, CCR2 and CCR5 have various ligands and share many of them (Table 3) (85). They typically bind inflammatory stimuli such as the macrophage inflammatory protein (MIP)-1α and MIP-1β, the Monocytes-Chemoattractant Protein (MCP)1-4 and the Regulated on Activation Normal T Expressed and Secreted Protein (RANTES) (85). In this manner cells that express these CCRs will sense inflammation and start to move towards the site of inflammation.
**Lymphoid CCRs**

Contrary to the inflammatory CCRs, CXCR4 and CCR7 do not have many known ligands.

CXCR4 is expressed on T and B lymphocytes and dramatically affects the development of B cells and cerebral tissues (86). CCR7 is expressed on naive T cells and it has been shown that lack of this receptor severely impairs primary immune responses (87). The CXCR4 ligand stromal-derived factor (SDF)1/CXCL12 and the two ligands for CCR7, CCL19 and CCL21 are expressed mainly in lymphoid tissues (Table 3).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Location</th>
<th>iDC</th>
<th>mDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>CCL3/MIP-1α, CCL5/RANTES</td>
<td>Inflammation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CCR2</td>
<td>MCP1-4</td>
<td>Inflammation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CCR5</td>
<td>MIP-1α, CCL4/MIP-1β, CCL5/RANTES</td>
<td>Inflammation</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CCR7</td>
<td>CCL19/ELC/MIP-3β, CCL21/SLC</td>
<td>Lymphatic tissue</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXCL12/SDF-1</td>
<td>Lymphatic tissue</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3. A few examples of CCRs expressed on immature and mature DCs and some of their ligands. Abbreviations: CCL-Chemokine ligand, iDC- immature DC, mDC-mature DC, MCP-monocytes-chemoattractant protein, MIP-Macrophage inflammatory protein. References: (83,85)

**Antigen presentation**

It is well established that two signals are required in order to activate T cells (88) and it was suggested that a third signal also is needed (89) which has now been widely accepted.

The three signals required to properly activate a CD4⁺ T-cell are:

1. Binding of the T-cell receptor to a HLA class II antigen bound complex
2. Binding of co-stimulatory molecules
3. Cytokine signalling

**Human Leukocyte Antigen**

The HLA molecules are what define an individual’s immunological “self”. They are the reason for organ rejection upon transplant. If the transplant has a different HLA expression it will not be considered self but instead be treated as foreign and attacked. T cells can only bind an antigen when presented to them as a complex with HLA molecules. The classical HLA
proteins and are subdivided into class I and II. The HLA class I molecules are expressed on all nucleated cells of the body and bind to the distal part of the CD8 molecule present on T cells. The expression of HLA class II molecules is restricted only to professional APCs they bind the distal part of the CD4 receptor present on T cells.

**Antigen uptake**

APCs are the cells that activate the adaptive branch of immunity. For this purpose it is necessary for APCs to internalize the pathogen, and this is done by a mechanism called endocytosis. Endocytosis is a process by which the cell takes up extracellular contents by budding of inwards forming an endosome of the plasma membrane and internalizing it to the cytoplasm. There are different forms of endocytosis. There is pinocytosis or cell drinking and phagocytosis or cell eating. Pinocytosis is formation of vesicles seemingly at random that serves to sample the environment for soluble antigen in a non-specific manner. Phagocytosis is receptor-mediated uptake of an antigen bound to receptors on the surface such as the C-type lectin family of receptors or the Fc receptors (90). This leads to reorganisation of the cytoskeleton, thus, surrounding the complex and internalising the antigen. Depending on the nature of the antigen and the receptor there may be different inflammatory responses (91).

**Antigen processing**

All nucleated cells present endogenous peptide fragments on HLA class I molecules. This is done through a mechanism called the cytosolic pathway. The professional antigen-presenting cells (APCs) can take up particles from the extracellular environment, process them and present peptide fragments on HLA class II molecules. That is called the endocytic pathway.

**The endocytic pathway**

Typically, the endosome is fused with lysosomes containing digestive enzymes in an acidic environment to form an endolysosome where the pathogen is degraded. The peptide fragments are loaded on HLA molecules intracellularly and transported to the surface of the cell.
The cytosolic pathway
Instead of loading foreign peptide fragments on the HLA class II molecules the HLA class I molecules are loaded with endogenous fragments of cytosolic proteins. The cytosolic proteins are degraded by the proteasome and transported to the endoplasmatic reticulum. In the endoplasmatic reticulum they are loaded on HLA class I molecules and put on the surface.

Cross-priming
The protein production in a tumor or infected cell is different from a healthy cell. The display of these products will be recognized as aberrant and killed by NK cells (92). In order to be able to activate CD8\(^+\) T cells antigens must be presented on HLA class I molecules. Professional APCs have an alternative pathway that enables them to present exogenous antigens on HLA class I molecules, thus inducing antigen-specific CD8\(^+\) T cells (93). This phenomenon is called cross priming and is restricted to DCs and to lesser extent macrophages. Cross priming is induced by viral recognition receptors in particular TLR9 (94,95).

Co-stimulation
As a fundamental part in the antigen-presentation process, APCs express a wide variety of co-stimulatory molecules. These will aid in the binding between the cells and create what is called the immunological synapse. Two very important co-stimulatory molecules are the CD80/B7.1 and the CD86/B7.2 that bind to CD28 and CD152 expressed on T cells. Ligation of CD28 will lead to T-cell activation while ligation of CD152 will suppress activating signalling. Co-stimulatory molecules can also skew T-cell responses in different directions. The ligands specific for the TNFR family members CD134/OX40 and CD137/4-IBB are two other molecules that are known to have an impact on T-cell responses. For example, if an APC express high amounts of the co-stimulatory factor OX40L that binds OX40 on the T cells they will be more prone to differentiate into CD4\(^+\) cells (96), while if 4-IBBL, expressed on the APC, binds CD137 or 4-1BB that are present on the T cells, the T cells will be more
prone to become CD8\(^+\) cells (97). Recently it has been proven that both the ligands OX40 and 4-1BB can support proliferation of naturally occurring regulatory T cells (Tregs) while OX40 can also antagonise the induction of peripherally induced Tregs (98). Finally, evidence shows that T-cell priming in the absence of proper co-stimulation (99,100) or of activating cytokines will instead lead to anergic T cells or to differentiation of Tregs (101).

Inflammation

At the site of inflammation complement proteins and other inflammatory mediators act as signal substances that call the attention of inflammatory cells. As soon as the cells reach the site of inflammation cytokines such as IL-1, IL-6 and TNF-\(\alpha\) as well as inflammatory chemokines such as IL-8 are secreted. This will lead to an increased permeability of the vessels increasing the blood flow at the inflammation site. Simultaneously, the vessel walls express adhesion molecules like selectins which contribute to adhesion of circulating cells to the vessel wall, followed by transendothelial migration. The granulocytes are early at the site of inflammation and kill the pathogen by release of granules containing toxic compounds and by phagocytosis. Monocytes will also phagocytose the pathogen and secrete pro inflammatory factors until the infection is resolved. Finally tissue specific APCs will also be activated, internalize the pathogen and prepare for encountering naive T cells. If the pathogen is removed the inflammation is resolved and tissue balance is restored. If the pathogen persists there is increased risk for a permanent inflammation leading to tissue damage.

Intercellular communication

For coordinating an attack against a pathogen there are many types of signalling molecules that the cells use for intercellular communication. Cytokines have traditionally been given names according to whatever function they were discovered for, but as they are sequenced they become renamed to an interleukin (IL).
Cytokines are a means of communication between leukocytes and other cells. They are typically small proteins induced by activated immune cells to act on other immune cells. There is not a very clear distinction between hormones and cytokines. For example, it has been suggested that IL-6 acts in a hormone-like manner as it is secreted by muscle cells during exercise and has major metabolic effects (102).

**Interferons**
These cytokines were discovered over 50 years ago and named interferons (IFNs) because they interfered with viral replication (103). Nowadays, it is known that there are many subgroups of IFNs and that they are found in all vertebrates where they have been investigated (104). In this thesis I will only discuss the type 1 and the type 2 IFNs.

**Type I IFN**
The classical antiviral type 1 IFNs are the IFN-α and IFN-β. The NK cells and the pDCs are very potent producers of IFN-α in response to activation by viral antigens. IFN-α acts on other immune cells to elicit an anti viral response and also functions as an analgesic.

**Type II IFN**
IFN-γ is the hallmark cytokine of Th1 responses and is secreted by activated T cells and NK cells as a response to IL-12 (105). IFN-γ acts in response to virus or intracellular bacteria and tumor cells. IL-12 is secreted by mDCs, MØs and B cells in response to inflammatory stimuli (106). It acts on NK and T cells to induce the production of IFNs and induces differentiation of naive T cells to activated effector-T cells.

**IL-1β**
IL-1β does not seem to play a role in homeostasis but is a potent pro-inflammatory cytokine that induces fever. IL-1β is produced by monocytes, MØs and DCs in response to inflammation. It is implicated in inflammation through induction of cyclo-oxygenase 2 and inducible nitrous oxide synthase. It is an inflammatory cytokine that helps to increase
expression of adhesion molecules on the endothelium at a site of inflammation and acts as a bone marrow stimulant increasing the number of neutrophils in circulation (107).

**IL-4**

IL-4 was originally known as the B-cell differentiation factor and is the hallmark cytokine for Th2 responses (108). It is secreted by various subsets of T cells and granulocytes (109) and acts on two different receptors. The type 1 receptor binds only IL-4 while the type 2 receptor can also bind IL-13 although the response is not as strong as compared to IL-4 (110).

**IL-6**

IL-6 was initially named B-cell differentiation factor upon its discovery (111) but was later renamed to IL-6. IL-6 is induced by inflammatory cytokines such as TNF-α and IL-1β but is inhibited by IL-4 and IL-13. Nevertheless, this cytokine has been shown to skew CD4+ T cells to a Th2 phenotype (112). More recently it has been suggested that IL-6 inhibits differentiation of Tregs but promotes Th17 by increasing expression of RORγt while inhibiting Foxp3 (113).

**IL-8**

IL-8 is secreted by almost all cells of the innate immune system during an inflammatory response upon activation through TLRs. It is a chemokine and its major role is to recruit T cells to the site of inflammation (114).

**IL-10**

IL-10 was first described as the substance secreted by Th2 cells inhibiting Th1 actions (115) under the name of cytokine-synthesis inhibitory factor. Later, this cytokine was also shown to inhibit the expression of co-stimulatory molecules as well as MHC class II molecules (116,117). IL-10 is now widely recognized as an anti inflammatory cytokine secreted by monocytes, MØs, DCs and some T cells to counteract inflammatory stimuli. It inhibits the
production of a great number of cytokines, even its own production, but the inhibition of IL-1 and TNF-α is crucial to its anti-inflammatory effects (118).

**IL-12 family**

IL-12 was the first member of the IL-12 family of interleukins initially discovered as NK-cell stimulatory factor (119). More recently, IL-23 (120) and IL-27 (121) were added to the IL-12 family. IL-12 is the classical Th1-inducing cytokine secreted by neutrophils, monocytes, MØs and DCs and induces IFN-γ release in NK cells and T cells. Although similar in many ways, the members of the IL-12 family have varying structures and their actions and are differentially regulated depending on the nature of the microbial stimuli. This is evidenced by the fact that LPS induces IL-12 and IL-23 in DCs whereas peptidoglycan induces IL-23 but not IL-12 (122).

**Tumour Necrosis Factor-α**

TNF-α is a member of the TNF super family of proteins and is a pro-inflammatory cytokine initially discovered for killing of tumour cells. In infectious diseases, TNF promotes inflammation and helps to combat the pathogen. However, too high levels of this cytokine are responsible for tissue damage and pathogenicity (123). TNF-α can trigger apoptosis via the activation of pro-caspases of the TNF receptor and thus induce cell death.

**Malaria**

**Malaria burden**

There are as many as 3.3 billion people that live at risk of infection and 247 million cases where reported in 2006. The vast majority of the victims are children that live in Sub-Saharan Africa. About 98% of the cases in Africa are caused by *Plasmodium falciparum* and 85% of the deaths are children below the age of 5 (124). Another group at risk are pregnant women that are susceptible to pregnancy associated malaria (125). Malaria is not only one of the most
devastating infectious diseases in the world it is also tightly connected to poverty. Not only is the most severe impact of malaria on the poorest populations in the poorest countries but these populations are also kept in poverty as an effect of infection (126,127).

Parasite life cycle
The word malaria is Italian and means “bad air” because the disease was initially thought to be airborne. The infection is caused by a protozoan parasite of the genus *Plasmodium* and is probably one of the oldest diseases of mankind (128). The definitive host is the mosquito of the genus *Anopheles* and the parasite is transmitted from mosquitoes to intermediate hosts such as reptiles, birds and mammals. There are five species infecting humans and the four “classical” are *Plasmodium falciparum, vivax, malariae, ovale*. Recently, several cases of human *Plasmodium knowlesi* that was formally believed to only infect macaques have been reported from Southeast Asia (129-135).

*P. falciparum* remains, however, by far the most lethal species of the genus *Plasmodium*. The sexual stage of the parasite lifecycle takes place in the mosquito gut and thousands of sporozoites are released from there to travel to the salivary glands (Figure 3). The sporozoites are then transmitted to humans by the bite of an infected mosquito. The human stage of infection is asexual. The sporozoites enter the blood stream and immediately infect the liver where the parasites develop into merozoites. The length of the liver stage depends on the species of *Plasmodium*. When the merozoites are released from the liver they infect red blood cells (RBCs) in circulation and undergo replication. This stage is known as the blood stage and RBCs go from ring stage infected RBCs to trophozoites and finally schizonts. At the rupture of the schizont, thousands of new merozoites are released into the blood stream. A small portion of the released parasites are gametocytes, which are responsible for sexual stages in the mosquito gut. It has been shown that, when the parasitemia is reduced during anti-malarial treatment, the amount of gametocytes increases (136).
Hemozoin

When the parasites degrade hemoglobin as a source for amino acids, the free heme group that is left is toxic to the parasites. It is therefore transformed into innocuous crystals called malarial pigment or hemozoin (Hz) as described in (137). Hz is released into the circulation and taken up by APCs. The detection of circulating leukocytes containing Hz has been suggested as a good method of diagnosing malaria because it can be correlated to total parasite burden. In fact, one study has shown that this better predicts the prognosis then the peripheral parasite count (138).

Methods have been developed to form hemozoin crystals synthetically from hemin (137) and the product is usually referred to as synthetic (s)Hz or β-hematin. Except for the effect in humans that will be discussed in the next section, several studies in vivo in mice have assessed the immunogenicity of Hz. It was shown that sHz induces potent pro inflammatory responses and recruitment of leukocytes, mainly monocytes and neutrophil populations (139-141).
Project background

Immune response to malaria

Innate responses

Different parts of the parasites have been shown to bind the TLRs and elicit innate immune responses and it has been suggested that MyD88 plays an important role in malarial pathogenesis (142). Glycosylphosphatidylinositol anchors (GPIs) of protozoan parasites have been implicated as ligands for TLR2 (143). TLR4 has been shown to induce pro inflammatory responses to GPI from another apicomplexan parasite Trypanosoma cruzi (144). Even one of the intracellular nucleic acid recognising TLRs, TLR9, has been suggested to be the receptor for Hz and binding of Hz to TLR9 would induce cell activation (145). However, a recent study suggests that malarial DNA attached to Hz is responsible for TLR9 activation and Hz would only function as a carrier of malarial DNA to the endolysosomes (146). Recent studies indicate that children with a polymorphism in the TLR4 gene are predisposed to severe malaria (147) and that TLR4 and TLR9 polymorphisms in pregnant women infected with malaria are associated with increased risk of low birth weight in the offspring (148). These studies suggest a role for innate immunity and in particular TLRs in the responses against malaria. In addition, it has been shown that whole blood stimulation with malaria antigens increase the secretion of pro- and anti- inflammatory cytokines upon subsequent specific TLR stimulation (149). This clearly indicates that P. falciparum modulates TLR activity.

Antigen-presenting cells in malaria

As crucial cells of innate immunity and by expressing several TLRs, APCs may play a fundamental role in the host response against the parasites. It has been suggested that T cells are hampered in immune responses to malaria and that their suppressed function may be
mediated by DCs (150,151). A lower expression of HLA-DR on peripheral blood DCs in children with acute malaria as compared to healthy children has been observed in vivo (152). In vitro observations have shown increased pro as well as anti-inflammatory mediators when exposing APCs to Hz as reviewed in (153). Hz mediates activation of pDCs via TLR9 although it is unclear whether the ligand is Hz itself, parasite DNA or both in a complex (145,146,154). Monocytes loaded with Hz secrete high TNF-α and IL-1β (155) but lower levels of IL-12p70 by an IL-10 dependent mechanism (156). They also exhibit impaired up-regulation of MHC class II (157) and inhibited differentiation to DCs (158). As for human MoDCs, it has been shown that maturation in response to LPS was impaired when the immature DCs were exposed to P.falciparum infected red blood cells (iRBCs) prior to the LPS challenge (159). On the other hand, enhanced maturation was observed when immature DCs were exposed to parasite derived Hz (160). The heterogeneity of DCs, the different parasite strains, the different type of Hz employed and the time of exposure are all factors that could possibly explain these discrepancies (161,162). Taken together, these studies indicate that P.falciparum can influence the function of various APC subsets and suggest that immune responses to malaria may be modulated by hampering APC function, in particular DCs.

Antibodies and protective immunity

Immunity to malaria is both stage and species specific. It is acquired gradually and is lost if the individual leaves the endemic area for a prolonged time period. T cells are essential to acquire and regulate immunity to malaria (163). In mice, immunity to Plasmodium chaubaudi is mainly cell mediated while immunity to Plasmodium yoeeli is mainly antibody mediated thus proving that both T and B-cell mediated immunity are important for protection (164). It has been known since the early sixties that Ig play an important role in malarial immunity. Already over 40 years ago it was shown that transferring γ-globulins from adults living in endemic areas to children, both from the Gambia (165) and from east Africa (166) exerted a
therapeutic function. It was also shown that monocytes could inhibit parasite growth when incubated with sera from immune individuals (167). More recently it has been suggested that the more cytophilic subclasses such as IgG1 and IgG3 (168) are more important for protection against the infection (169).

Cytokines and clinical implications in malaria

The clinical stage of malaria is the blood stage of infection and the characteristic fever returning at each rupture of the schizonts is a hallmark for malaria. Pro-inflammatory cytokines seem to play an important role in the development of severe malaria. In fact, high levels of TNF-α and low levels of IL-10 have been associated with disease severity and IL-12 was found to be lower in the severe cases as compared to the mild cases in Gabonese children (170). More recently, high plasma levels of IL-1β have been associated with the development of cerebral malaria (171) and high levels of both IL-6 and IL-10 were associated with severe malaria as compared to uncomplicated malaria controls (172).

Fulani and Dogon in northern Mali

The Fulani are traditionally a nomadic pastoral people that have settled in various African countries. The inhabitants of the Fulani and the Dogon village in this area do not intermarry. The Fulani still have cattle but are now sedentary in northern Mali and have lived in the study area for at least 200 years. The Dogon are farmers and migrated to this particular area about 50 years ago. Many studies have been performed showing that the Fulani are less susceptible to malaria than their sympatric counterparts i.e. other ethnicities living under similar geographical economic and social conditions. It has been shown that the Fulani respond more potently to the infection than other populations under similar conditions. The Fulani exhibit higher malaria-specific antibody titres, increased spleen rate, and have higher proportions of
malaria specific IL-4 and IFN-γ producing cells as compared to their sympatric neighbours (173-176). In addition, it has been shown recently that a population of Fulani in Burkina Faso have lower Treg activity than a neighbouring sympatric group (177). The underlying reasons for the different susceptibility to malaria infection of the Fulani have not yet been completely clarified.
Present Study

Aims

First Study
The goal of the first study was to characterize the behavior of MoDCs in malaria by challenging these cells with iRBCs or Hz and analyze their phenotype, migratory behaviour and cytokine secretion. We hypothesize that an impaired activation of DCs by the parasite or by its derived products may contribute to the poor development of immunity to malaria.

Second study
It is well established that the Fulani are less susceptible and mount a relatively stronger immune response to malaria than their sympatric neighbouring populations. The underlying mechanisms for that are still not well understood. We hypothesize that the prevalence or activation status of different DC subsets in circulation of the Fulani children could be related to the relative protection against malaria seen in the Fulani.

Methods
Here follows only a brief description of the methods used in the different projects. A more detailed description of the methods is included in each manuscript.

First Study
Monocytes were purified from peripheral blood mononuclear cells (PBMCs) from healthy blood donors using Ficoll separation. The monocytes were then cultured in the presence of GM-CSF and IL-4 to produce MoDCs. The MoDCs were then challenged with sHz, crude malaria antigen or iRBCs. The phenotype of the cells was analyzed using FACS, their
migratory properties were assessed using a Boyden chamber and their culture supernatant was analysed for cytokine secretion.

Second study
Study area
The study area is in northern Mali in the area of Sahel just south of the Sahara desert. Malaria transmission is seasonal and the rainy season starts in July and lasts until October. The rate of infected mosquitoes is similar between the Fulani and Dogon villages but the Dogon have had more clinical episodes of malaria infection (175).

Methods
A total of 40 Dogon children were enrolled of whom 20 were slide positive for *P. falciparum* and 20 were negative. In the Fulani there were a total of 37 children enrolled, 14 were infected and 23 were uninfected. Venous blood from infected and uninfected children of the Fulani and Dogon ethnicities was collected. The plasma was frozen in order to analyze levels of cytokines and antibodies in circulation. PBMCs were isolated and stained for DC subtypes and activation markers and analysed by FACS.

Results and Discussion

First Study
Various studies have investigated the effect of Hz or iRBCs on monocytes and MoDCs as recently reviewed in (178,179). It is now known that MoDCs that have been exposed to high doses of iRBCs do not mature properly in response to LPS (159). It has also been shown that Hz has a similar effect on MoDCs (158). In this study, we further analyzed the effect of sHz and iRBCs on MoDC activation, CCR expression and migratory capacity. As a positive control we used a combination of TNF-α and prostaglandin E2 (hereafter referred to as TNF-
α/PGE2). We choose this combination because of the enhanced migratory capacity of MoDCs exposed to these stimuli (180).

Our data show that upon incubation with sHz, MoDCs significantly increased the expression of CD80 and CXCR4. We also observed a slight up regulation of CD83, HLA-DR and CCR7. Nevertheless, upregulation of these maturation markers upon sHz stimulation was significantly lower than the one observed upon TNF-α/PGE2 stimulation. This may indicate that MoDCs exposed to sHz are poor inducers of adaptive immune responses. A similar pattern of activation was found when MoDCs were incubated with iRBCs. These results are in line with other findings that reported only partial maturation of DCs upon incubation with malaria derived products (160,181).

The increased expression of the lymphoid CCRs on DCs in response to malaria stimuli led us to test the migratory ability of these cells in vitro towards the ligands for CCR7 and CXCR4, CCL19/MIP-3β and CXCL12/SDF-1α, respectively. The results revealed that immature MoDCs exposed to 20 µg sHz increased their migration towards both ligands as compared to the cells that where incubated with medium alone. The increased migration towards lymphoid chemotactic ligands homing to lymph nodes that we observed in immature MoDCs exposed in vitro to malaria derived products may indicate that immature, circulating DCs are induced to travel to the lymph nodes during a natural *P. falciparum* infection.

Conversely, when MoDCs were exposed to sHz before TNF-α/PGE2 was added to the culture, they failed to properly up regulate CCR7. Other than just inducing chemotaxis towards lymphoid organs, this receptor has been reported to enhance maturation, survival and migratory speed of DCs (182). Thus, we hypothesize that malaria derived products could negatively affect DC responses in the presence of other maturation stimuli.

The kinetics of sHz effect on MoDCs was also evaluated and cells were phenotypically analyzed at 12, 36 and 60 hours upon incubation with sHz. The results indicated that sHz
induced a short lasting up regulation of CD83 on the surface of MoDCs as compared to TNF-α/PGE2, thus further indicating that MoDCs are only partially activated during malaria infection.

To better characterize the response of MoDCs to malaria derived products, we analyzed the cytokine release by DCs in response to sHz and iRBCs. An increased release of IL-6, IL-10 and TNF-α was seen when MoDCs were incubated with 20 µg sHz. Similarly, incubation of DCs with iRBCs increased the secretion levels of the same cytokines with the addition of IL-1β.

It has been shown that IL-10 secreted by monocytes-MØs upon contact with Hz decreases the levels of IL-2, IL-12 and IFN-γ in PBMC cultures (183), thus down-regulating Th type 1 responses. In addition, increased levels of TNF-α, IL-1β and IL-6 were reported to be implicated in severe malaria. Recently, the serum levels of IL-1β, IL-6, IL-10 and TNF-α have been associated to severe complications during malaria infection in various studies (171,172). Furthermore, it has been shown that rapid induction of IL-1β may help to control malaria infection, but persistence of this cytokine can lead to anemia (184).

Secretion of pro-inflammatory cytokines is generally important in the initial phases of malaria infection but maintained high levels are associated with inflammatory induced tissue injury. We suggest that mDCs can be crucial cells in inducing the large inflammatory response that is often observed during acute P. falciparum infection.

Taken together, our results show partially increased expression of maturation markers, increased migration towards lymphoid chemokines and increased secretion of IL-10 but not IL-12 by MoDCs upon contact with malaria derived products. These in vitro observations suggest that DC responses in malaria are potent enough for DCs to make contact with naive T cells in the lymph nodes. However, proper induction of adaptive immunity requires high expression of co-stimulatory molecules and IL-12 release by DCs. It is therefore possible that
the actions of Hz on mDCs, with induction of partial DC maturation and increased IL-10 secretion, may lead to insufficient activation of T cells or even induction of T cells with regulatory properties upon infection in the natural host. The poor activation of T cells may in turn lead to impaired adaptive immune responses and therefore insufficient clearance of the parasites. We therefore hypothesize that an impaired activation of DCs by the parasite or by its derived products may contribute to the poor development of immunity to Malaria.

Second study
Many studies have been conducted in different African countries comparing different aspects of the immune responses to malaria of the Fulani and their sympatric ethnic groups (175,185). It has been confirmed that the Fulani show less clinical symptoms of infection than sympatric ethnic groups although they are exposed to the same parasite pressure (173). The Fulani exhibit higher antibody titres, enlarged spleen rates and higher levels of cytokines in circulation. Our study was conducted to investigate some aspects of the innate immune responses in children belonging to the Fulani and Dogon ethnic groups in Mali. In particular, the study aim was to investigate whether the properties of circulating DCs of Fulani and Dogon children could account for their different response to malaria infection.

We confirmed the higher levels in circulation of Malaria specific antibodies in Fulani as compared to Dogon. In fact, our findings showed significantly higher levels of malaria specific IgG, IgM and higher levels of IgG1, IgG2 and IgG3 but not IgG4 subclasses in the Fulani children as compared to Dogon children. For the IgG3 subclass of antibodies, decreased levels were found in the Fulani children undergoing *P. falciparum* infection as compared to uninfected Fulani. This result may suggest that the IgG3 antibodies have bound the parasite-derived antigen, formed immune complexes and thus, could not act in an antibody-cell-mediated way (168), although the immune complexes could act through Fc-receptors and thereby induce inflammatory cytokines (186).
The level of IFN-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-12(p70) and TNF-α were measured in serum of children belonging to the two ethnic groups. For IL-1β, IL-10 and TNF-α no conclusive results could be found due to the fact that many samples exhibited values that were below the detection limit of our assay.

When comparing the Fulani and the Dogon, regardless of infectious status, the levels of IL-6, IL-8, IL-12, IFN-α and IFN-γ were higher in the Fulani children. Thus, similarly to what was observed for the antibodies, the Fulani had higher levels of several inflammatory cytokines in plasma as compared to the Dogon children.

The infected Dogon children had significantly higher levels of IL-6 and IL-12 while increased levels of IL-6 and IL-12 were not observed when comparing uninfected and infected Fulani. This may suggest a different skewing of immune responses of the Dogon as compared to the Fulani. When comparing only infected children from the two ethnicities, differences were only found for IFN-γ but not the other cytokines, and higher IFN-γ levels were observed in the Fulani ethnic group as compared to the Dogon.

Taken together, cytokine analysis of plasma samples in the two ethnic groups revealed that upon *P. falciparum* infection an IFN-γ mediated response seems to be increased in the Fulani, while a different pro-inflammatory pattern lacking IFN-γ up-regulation would be activated in the Dogon.

We also analyzed three subtypes of blood DCs as well as their activation status in the children involved in the study. We observed increased frequency of circulating pDCs and BDCA-3+ mDCs in the infected Dogon as compared to the uninfected Dogon. Conversely, the infected Fulani exhibited lower frequency of the same subsets than the infected counterpart.

The different levels of DCs in the blood of the two ethnic groups undergoing malaria might be explained by the different activation status of these cells. In fact, with regard to the expression of HLA class II molecules, the DC activation status was consistently higher in pDCs of the
infected Fulani as compared to the uninfected children belonging to the same ethnic group, while HLA class II expression on BDCA-1⁺ and BDCA-3⁺ mDCs and on pDCs was significantly lower in infected Dogon than in the uninfected peers. The increased activation status of circulating pDCs in infected Fulani children may indicate that these cells have migrated to lymphoid organs to trigger T and B-cell responses and are therefore no longer found in circulation, thus possibly explaining the reduced frequency of these cells subsets in the blood of infected Fulani children. Conversely, our findings suggest that malaria infection impairs activation of circulating DCs in the Dogon ethnic group, thus possibly affecting downstream cellular and humoral immune responses.

Our findings suggest that there is a difference in the prevalence and activation status of blood DCs, cytokine secretion and Ig levels between the Fulani and the Dogon in response to malaria infection. Such differences are likely to be important for the relatively stronger protection against malaria seen in the Fulani as compared to the Dogon.
General Conclusions

We have shown that malaria derived products can activate MoDCs in vitro leading to migration of DCs towards lymphoid ligands and to secretion of both pro and anti-inflammatory cytokines. However, such activation is partial, and may lead to an impaired DC functionality. Additionally, we have shown that blood DCs in the Fulani express higher levels of activation markers during malaria infection, while blood DCs in the infected Dogon exhibit significant lower levels of activation markers than the uninfected counterpart. Moreover, the Fulani had higher levels of IFN-γ in plasma as compared to the Dogon children, indicating a more efficient response to this parasitic infection (187). We also know that any malaria naive adult that becomes infected typically becomes severely ill and unable to efficiently combat the infection.

Impaired DC activation upon malaria infection, as indicated in the Dogon population in our study, may result in a deficient and delayed adaptive immune response as commonly seen in malaria. This may not be the case in the Fulani, where we observed increased DC activation and a more Th-1 skewed response upon Malaria infection. We know that DCs play a pivotal role in initiating immune responses and directing adaptive immunity. The early IFN-γ skewed response seen in the Fulani is the most beneficial response for the host and we suggest that such a response may well depend on the manner of DC activation upon infection.
Future work

The immunogenic effects of Hz and in particular its impact on APCs are still not clarified. In relation to the first study an interesting aspect to study is whether the increased migration towards lymphoid ligands can be reproduced upon stimulation of DCs with iRBCs instead of Hz. Another interesting follow up would be to test the capacity of Hz-induced “partially matured” DCs to activate T cells.

In relation to the second study a follow up during the dry season would yield very important information about the innate immune system of the children included in the study when not exposed to the malaria parasite. In addition, to test the response of APCs obtained from these children in vitro to Hz and iRBCs, similar to the experimental setup used in the first study presented here, might yield important information of the function of individual cell types in the different ethnicities upon exposure to malaria. This may even lead to proof that the initial response to P. falciparum infection is indeed decisive in the outcome of the infection and thus may in the future lead to focus efforts of preventive strategies to the initial, innate response to the parasite.
Acknowledgements

I would like to thank everybody at the department of immunology as well as other departments, institutes and universities that have in any way contributed to my education and development within the fields of malaria and immunology.

First and foremost my gratitude goes to my supervisor Marita Troye-Blomberg for giving me the opportunity to work with you and always taking your time to help me with answers to all my questions and suggest solutions to my problems.

I am also very grateful to my co-supervisor Stefania Varani for support and endless comments, sometimes difficult but I really am grateful and I know that in the end it is all for the best, that has greatly improved the quality of this work and hopefully my own abilities as an author. Grazie mille!

Another great support in writing this thesis was Carmen Fernandez muchísimas gracias por toda la ayuda no solo con esta tesis pero también con todo lo demás.

I am also grateful to Klavz Bersins, Eva Sverremark-Ekström and Eva Severinson for aiding in all kinds of worries from cross-words to C-course problems.

For all things practical Gelana, Maggan, Ann och Anna-lena tack, tack och tack för kaffet, korsorden medmera men först och främst att ni orkar med all röra och kaos jag sprider runtomkring mig.

All the other students that I have, or have had, the pleasure to work with, “Allergy girls”, “TB boys”, “Hispanic crew” and prominent Malaria student investigators. To those that are now roaming about in the great big world outside these walls, in particular Lisa (Vemskajanuskyllapå) Isralesson. And of course those whose company I still have the pleasure to enjoy.
For the time spent in and outside the lab, country and continent, it has been great fun and I am sure it will stay that way for years to come. I especially want to mention Charles “Malian genius” Arama, Ebba “duvetvem” Sohlberg and Stéphanie “TanzanianElisaOracle” Boström. I am very glad to have crossed paths with you and for being able to share your experiences be they good, bad or even everyday ones.

To all the former students at the department not mentioned here, the corridor in front or at all the different departments where important issues are being resolved, be it by Chen girls or by Portuguese mafia.

Last but not least, all my family past, present or future, by blood or picked up along the way I will keep you forever with me.

Para toda mi familia, pasada, presente y porvenir, de sangre o recuparada por los caminos los llevaré por siempre conmigo. Mamma, Pappa, Erre och Marre vad hade jag varit utan er?

Hasta la Victoria siempre!
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Synthetic hemozoin induces partial maturation of human dendritic cells and increases their migration towards lymphoid chemokines.

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Abstract

Acute and chronic *Plasmodium falciparum* infections alter the immune competence of the host possibly through changes in dendritic cell (DC) functionality. DCs are the most potent activators of T cells and migration is integral to their function. Mature DCs express lymphoid chemokine receptors (CCRs) which enables them to migrate to the lymph nodes where they encounter naïve T cells. The present study aimed to investigate the impact of the malaria pigment hemozoin (Hz) or infected erythrocytes (iRBCs) on the expression of CCR. Monocyte derived DCs partially matured upon incubation with synthetic Hz or iRBCs as indicated by a moderately increased expression of CD80, CD83 and HLA-DR. Both stimuli also provoked the release of pro-inflammatory and anti-inflammatory cytokines such as IL-6, IL-10 and TNF-α and induced up-regulation of the lymphoid chemokine receptor CXCR4, which was coupled to an increased migration to lymphoid ligands. Taken together, these results suggest that the partial maturation of DCs upon stimulation with malaria-derived products and the increased IL-10 secretion may lead to insufficient activation of T cells or even induction of T cells with regulatory properties. The poor activation of T cells may in turn lead to impaired adaptive immune responses and therefore insufficient clearance of the parasites.
Introduction

*Plasmodium falciparum* (Pf)-malaria is one of the most severe infectious diseases in the world where as many as 3.3 billion people live at risk of infection and 247 million cases where reported in 2006. The vast majority of victims are children below 5 years of age (1). Immunity to malaria is developed only after repeated exposure and is not long lasting (2). Several studies have demonstrated impairment of immune responses in acute and chronic *Pf* malaria (3).

Previous studies indicate that an early pro-inflammatory cytokine-mediated mechanism is crucial to control parasitemia and for the clearance of parasites. Excessive pro-inflammatory responses, however, can cause severe disease (4). The rapid production of cytokines implies release either from pre-existing memory T cells or from cells of the innate immune system, such as antigen presenting cells (APCs) or natural killer (NK) cells. Among APCs, dendritic cells (DCs) are the most potent in initiating, controlling and regulating functionally distinct T cell responses (5,6). Three signals are required from a DC to potently activate naive T cells and they consist of high expression of peptide-loaded human leukocyte antigen (HLA) class II molecules, co-stimulatory molecules and cytokine secretion. For proper stimulation of T cells, upon encountering a pathogen in the periphery DCs need to migrate to secondary lymphoid organs in order to encounter them (7). The migratory capacities of DCs are guided by the expression of chemokine receptors (CCRs), which in turn is regulated by DC maturation. In the periphery, the expression of CCR1 and CCR5 enable DCs to detect inflammation and find the causative agent. Upon maturation, those inflammatory CCR are down regulated and lymphoid CCRs, like CXCR4 and CCR7, whose ligands are expressed in lymphatic tissues are instead up regulated (5).

The *Pf* parasite has been shown to affect DC responses through the action of the malaria pigment hemozoin (Hz) or through adhesion of *Pf* infected red blood cells (iRBCs) to these
cells. However, the data obtained so far are somewhat contradictory. Coban et al. reported activation of monocyte-derived DCs (MoDCs) upon Hz stimulation, as indicated by elevated expression of maturation markers and IL-12 secretion (8). On the other hand, Hz-loaded monocytes do not differentiate properly into MoDCs, and Hz-loaded MoDCs exhibit an impaired maturation in response to lipopolysaccharide (LPS), suggesting that Hz could have an inhibitory role in the development of inflammatory DCs (9). It has been shown that high-doses of iRBCs inhibit LPS-induced maturation of MoDCs and alter their immunostimulatory abilities (10). It was later suggested that this effect could have been induced by apoptosis of DCs upon contact with high doses of iRBCs (11). Yet another study has recently demonstrated that low doses of iRBCs induce semi-maturation of MoDCs and that these cells release TNF-α, IL-6 and IL-10 but not IL-12 (12).

In vivo, the proportion of circulating activated DCs is reduced in children with Pf malaria (13). Recent evidence shows that a minor blood myeloid DC population, the blood dendritic cell antigen (BDCA)-3+ DCs, is expanded in children with severe malaria as compared to healthy controls, which was associated with augmented IL-10 plasma levels and impaired DC allostimulatory ability, indicating an immunomodulatory function for this APC subset (14). Therefore, despite contradictory in vitro results, analysis of DCs during natural malaria infection suggests that these cells are activated during infection, and importantly exhibit an immunomodulatory phenotype as compared to classical type-1 DC activation which leads to a Th1 skewed T cell response.

In the present study, the phenotype and function of MoDCs were evaluated upon exposure to synthetic Hz (sHz), parasite lysates and iRBCs. As a measure of function the expression of CCRs and their capacity to migrate towards chemoattractants were investigated.
Materials and Methods

Dendritic cell cultures

Peripheral blood mononuclear cells (PBMCs) from anonymous blood donors (Karolinska Hospital, Stockholm, Sweden) were isolated on a density gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and used for further cell purification. MoDCs were generated by modification of a described method (15). Briefly, monocytes were isolated by negative selection using MACS microbeads according to the manufacturer's instructions (Monocyte Isolation Kit II, Miltenyi Biotech, Bergisch Gladbach, Germany). Purified monocytes were resuspended in complete RPMI 1640 supplemented with 10% fetal calf serum (FCS), 35 ng/ml IL-4, and 50 ng/ml GM-CSF (R&D Systems, Minneapolis, MN, USA). Cell differentiation was monitored by light microscopy and flow cytometry. At day 5, cells were harvested and 89 % ± 1 % of the cells exhibited a typical MoDC phenotype (CD14-CD1a+). Parasite lysate, sHz or iRBCs were then added to the differentiated MoDC cultures. In order to induce maturation of the MoDC, 1 µM of prostaglandin E2 (PGE2) (R&D Systems) and 50 ng/ml of TNF-α (R&D Systems) were added to the culture medium at day 5 if nothing else is stated.

Preparation of synthetic hemozoin

Synthetic Hz, also called β-hematin, is structurally similar to Hz formed naturally by parasites (16,17) and was prepared from hemin chloride as previously described (18) with some modifications (19). Briefly, 75 mg of porcine hemin (Sigma-Aldrich, Steinheim, Netherlands) were dissolved in 14.5 ml NaOH 0.1 M and incubated at 60°C. Within 10 min, 1.45 ml HC1 (1M) and 8.825 ml sodium acetate (12,8 M) were added and the solution was incubated for two hours at 60°C constantly stirring at a rate of ~150 rpm. The solution was then centrifuged at 15000 g for 13 min, and the pellet was resuspended in Tris-HCl buffer (100 mM, pH=8) containing 2.5% SDS (KEBO Lab, Stockholm, Sweden) and incubated for 30 min 37°C to
dissolve heme aggregates. The solution was then centrifuged at 15000 g for 13 min and the pellet resuspended in pre-warmed NaHCO$_3$ (100 mM, pH=9.2). After washing twice, Hz was dried at 37°C. The resulting pellet was weighed and re-suspended to 10 mg/ml in sterile water. In agreement with previous findings (20), the spectrum of Hz exhibited the presence of intense bands at 1710, 1660, 1299, 1279, 1209 cm$^{-1}$ (Figure 1). Two intense bands at 1660 and 1209 cm$^{-1}$ were present in our preparation indicating a high grade of purity of the sample (18). The endotoxin level in the preparation was below 0.05 endotoxin units (EU) EU/ml (Bacteriology Lab, Sahlgrenska Hospital, Göteborg, Sweden) in our highest working concentration.

Parasite cultures

*Pf* parasite strains were cultured using human O$^+$ RBCs (Karolinska Hospital, Stockholm, Sweden) in RPMI 1640 medium containing 25mM Hepes, 2mM L-glutamine, 50 μg/ml gentamycin, and 0.2% sodium bicarbonate and supplemented with 0.5% Albumax (Gibco™, Paisley, UK). The Tanzanian laboratory strain F32 was maintained in continuous cultures in candle jars as previously described (21). Parasite cultures were regularly tested for *Mycoplasma* contamination by the mycoplasma detection kit Mycoalert® (Lonza, Rockland ME, USA). The parasite cultures were kept synchronous by treatment with 5% D-sorbitol in distilled water as previously described (22). In order to produce parasite lysates, late stage parasites of the F32 strain were purified on 60% Percoll gradient centrifugation, sonicated as previously described (23) and stored at -80°C until used.

Intact iRBCs harbouring late parasite stages (schizonts and late trophozoites) were harvested when the parasitemia reached approximately 10% by using magnetic separation as previously described (24).

Briefly, parasite cultures were pelleted and re-suspended in phosphate-buffered saline (PBS) supplemented with 2% bovine serum albumin (BSA) and 2 mM EDTA. Parasites were added
to CS columns (Miltenyi Biotech) and incubated 10 min before washing the column. The column was then removed from the magnet and the parasites were rinsed out with PBS. Percentage of late stage iRBCs was determined by staining with acridine orange and counting under UV-microscope. Uninfected RBCs run in parallel under the same conditions were used as negative controls. iRBCs and uninfected RBCs were derived from the same donor in each experiment.

**MoDCs-parasites co-cultures**

In order to prevent burst of the infected erythrocytes during co-culture, iRBCs were incubated in aphidicolin which arrests the parasite-cell cycle in the trophozoit stage (25). Briefly, synchronous parasite cultures at ring stage (8-10% parasitemia) were cultured in 15 µg/ml of aphidicolin (Sigma-Aldrich, St. Louis, USA) for maximum 24 hours. When the parasites had reached trophozoit stage the cultures were purified using magnetic separation as previously described (24). Immature MoDCs were harvested and resuspended at 10⁶ cells/ml in 24-well plates. Purified trophozoites (90% ± 1% purity) were added at the ratios of iRBCs/DCs 10:1, and 100:1 or uninfected RBCs at the high ratio. MoDCs and uninfected or iRBCs were co-cultured for 10 hours and TNF-α/PGE₂ was used as a positive maturation control.

**Immunophenotype**

All antibodies used were mouse monoclonal antibodies either unconjugated or conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Conjugated antibodies used for cell-surface staining included those recognizing CD1a, CD14, CD80, CD83, CD86, HLA-DR, (Pharmingen, San Diego, CA, USA). Unconjugated antibodies were CCR7, CXCR4 (R&D Systems) that were made detectable using a secondary polyclonal goat anti-mouse PE conjugated antibody (Dako Cytomation, DAKO A/S, Denmark). Data acquisition and analysis were performed on a FACSCalibur flow cytometer (Beckton Dickinson, Mountain View, CA) using BD CellQuest™Pro version 5.2.1 software. FACS data are presented here as mean
fluorescence intensity (MFI) indicating the level of expression of a certain marker on an individual cell.

Assessment of migratory properties of MoDCs

The human recombinant chemokines used were the chemokine (C-X-C motif) ligand, (CXCL)-12/stromal cell-derived factor (SDF)-1α and the CCL19/MIP-3β (PeproTech Inc., Rocky Hill, NJ, USA). Both chemokines were used at a final concentration of 100 ng/ml in RPMI 1640 medium containing 1% FCS. Cell migration was evaluated using a 48-well Boyden chamber (Neuroprobe, Pleasanton, CA, USA) with 5-µm-pore-size polycarbonate filters. Medium without FCS was used as control of background migration. Cells were allowed to migrate for 1.5 hours after which MoDCs on the membrane were stained and counted. All samples were assayed in duplicates, and the number of cells that migrated in five visual fields (original magnification, x100) was determined for each well, as previously described (26). The net number of cells that migrated was calculated by subtracting the number of cells that migrated in response to medium alone from the number of cells that migrated in response to chemokines.

Cytokine measurement in the supernatant of cell cultures

Supernatants from MoDC cultures were used to analyse cytokine production in the presence or absence of malaria-derived material. Supernatants were collected when the cells where harvested and stored at -70°C. Supernatants were analyzed for the levels of interferon (IFN)-γ, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α and TNF-β using a Human Th1/Th2 11plex bead assay (Bender MedSystems, Vienna, Austria) according to manufacturer’s instructions. Data acquisition and analysis were performed by FACSCalibur and the FCAP Array software (Soft Flow, Hungary Ltd. Hungary).
Statistical analysis

Data was analyzed using the Wilcoxon’s rank-sum test. Comparisons were considered statistically significant at p < 0.05.
Results

sHz induces partial maturation of MoDCs and increases lymphoid CCR expression

In order to evaluate the effect of sHz on the phenotype of MoDCs, cells were stimulated with sHz or TNF-α/PGE$_2$ overnight. MoDCs cultured in medium alone were used as a negative control. As expected, cells that were stimulated with TNF-α/PGE$_2$ showed a statistically significant increased expression of CD80 and CD83 (Figure 2). After stimulation with sHz, an upregulation of CD80 was observed (p=0.046 and p=0.018, for 10 or 20 µg/ml, respectively). Non-stimulated cells expressed low levels of CD83 and there was a statistically significant upregulation of this maturation marker when the cells were stimulated with 10 µg/ml sHz (p=0.043), while the highest concentration of sHz (20 µg/ml) did not induce a significant increase of CD83 on MoDCs. TNF-α/PGE$_2$ induced a strongly increased expression of HLA-DR and CCR7 molecules, while the expression levels of these markers were only slightly, not significantly, increased upon sHz stimulation (Fig 2c and d). Conversely, similarly to the effect caused on MoDCs by TNF-α/PGE$_2$, sHz induced significant increased expression of CXCR4 as compared with unstimulated MoDCs when added at the highest concentration (20 µg/ml) (p=0.028) and a similar increase was observed using 10 µg/ml although this was not significant (p=0.0797). Thus, sHz induces partial maturation of MoDCs and increased lymphoid CXCR4 expression on the cell surface.

To evaluate the kinetics of MoDC activation upon sHz or TNF-α/PGE$_2$ stimulation, a phenotypic analysis at different time points after incubation with these stimuli was performed. Given the results obtained in the previous section the kinetics of activation markers was analyzed only after stimulating cells with 20 µg/ml of sHz. At first, data was evaluated without taking into consideration the time points to confirm the partial activation. The spontaneous MFI expression of CD80 was 56 +/- 3 (mean−value +/- standard error) and increased to 85 +/- 4 when stimulated with 20 µg/ml sHz and 96 +/- 4 with TNF-α/PGE$_2$.
stimuli. For CD83 the background expression was 21 +/- 4 and increased to 35 +/- 6 upon sHz stimuli and to 68 +/- 6 when stimulated with TNF-α/PGE₂. HLA-DR expression increased from 75 +/- 3 to 91 +/- 2 upon sHz stimulation and to 100 +/- 1 using TNF-α/PGE₂.

The data were then evaluated by considering different time intervals, and increased expression of CD80 and HLA-DR was maintained after 60 hours of stimulation regardless of the type of stimulus (Figure 3a and c). For CD83, there was a tendency of continuously decreased expression when stimulated with sHz. The statistical analysis showed that the decrease between the 12 and 60 hour time points was significant for unstimulated cells (p=0.021) or cells stimulated with sHz (p=0.043) (Figure 3b). When the cells were stimulated with TNFα/PGE₂ however, no statistically significant differences were seen (p=0.19). Thus, the partial activation that was observed in MoDCs stimulated with sHz was not fully maintained at 60 hours of incubation.

**sHz impairs the up regulation of CCR7 induced by maturation stimuli on MoDCs**

In addition to the stimulatory effect of sHz seen on unstimulated MoDCs we investigated whether sHz could interfere with the maturation induced by TNFα/PGE₂. As shown in Figure 4, addition of sHz to the cultures prior to TNF-α/PGE₂ stimulation significantly impaired the up-regulation of CCR7 (p= 0.042 and p=0.017, for 10 µg and 20 µg of sHz, respectively). No differences were observed for the expression levels of CD80, CD83, HLA-DR and CXCR4 in MoDCs matured with TNF-α/PGE₂ in the presence or absence of sHz (data not shown). Thus, sHz selectively blocks the CCR7 up-regulation induced by TNF-α/PGE₂ on MoDCs.
Contact with iRBCs induces partial maturation of MoDCs

Next, we examined whether parasite lysates or intact iRBCs could induce activation of MoDCs. MoDCs from four donors were incubated for 18 hours with 15 or 75 µg/ml of parasite lysate. The cells were subsequently harvested and stained for surface expression of CD80, CD83, HLA-DR, CXCR4 and CCR7 and analyzed by FACS. No significant differences were observed in the investigated markers upon contact with parasite lysates (data not shown).

To evaluate the effect of iRBCs on MoDCs, cells were incubated with aphidicolin-treated iRBCs, (F32 laboratory strain) or uninfected RBCs, for 12 hours and then stained for CD83, HLA-DR, CXCR4 and CCR7 before FACS acquisition. The expression levels of the different markers did not differ significantly between MoDCs cultured in medium alone or with uninfected RBCs. Similarly, low doses of iRBCs (10:1, iRBCs:MoDCs) did not significantly alter the expression of the investigated markers (data not shown). Conversely, the high iRBC to MoDCs ratio revealed a strong tendency, similar to that seen for TNF-α/PGE₂, for an up regulation of MFI values from 82 +/- 4 (mean−value +/- standard error), 26 +/- 6 and 58 +/- 14 in the unstimulated cells to 90 +/- 11, 39 +/- 1 and 89 +/- 12 for HLA-DR, CCR7, and CXCR4, respectively. The levels of HLA-DR, CCR7 and CXCR4 were 99 +/- 8, 51 +/- 9 and 96 +/- 12, respectively, in cells cultured with TNF-α/PGE2 (Figure 5). In addition, TNF-α/PGE2 stimuli lead to an increased CD83 expression from 20 +/- 15 to 44 +/- 16. No difference was seen in cells stimulated with high ratio of iRBCs as compared to uninfected RBCs (data not shown). Thus, high doses of iRBCs seem to induce an incomplete maturation
of MoDCs. Importantly, intact parasitized erythrocytes are required to induce this effect, since parasite lysates could not induce any maturation/activation of DCs.

**sHz-induced maturation of MoDCs is coupled to increased migratory capacity in response to lymphoid chemokines.**

Upon maturation, MoDCs acquire the ability to migrate in response to lymphoid chemokines by up regulating the CXCR4 and CCR7 molecules (27). Since we found that sHz induced a partial maturation of MoDCs with an up regulation of CXCR4, the capacity of sHz-treated MoDCs to migrate in response to inflammatory and lymphoid ligands was assessed. MoDCs treated with TNF-α/PGE₂ exhibited a significantly increased migratory capacity, as compared to untreated cells, in response to the CXCR4 (n=6) and CCR7 (n=5) ligands, CXCL12/SDF1-α (p=0.03) and CCL19/MIP3-β (p=0.04), respectively (Figure 6). An increased migration towards CXCL12/SDF1-α (p=0.03) as well as for CCL19/MIP3-β (p=0.04) was also observed in sHz-treated MoDCs as compared to untreated cells.

**MoDCs exhibit increased secretion of pro- and anti- inflammatory cytokines after stimulation with sHz or high concentrations of iRBCs.**

To investigate whether malaria-derived stimuli could induce the production of soluble mediators in MoDCs, the levels of selected cytokines were measured in the cell-culture supernatant obtained from sHz-, iRBC- and un- treated MoDCs. Baseline production of all inflammatory cytokines in unstimulated MoDCs was negligible except for IL-8, as shown in
Figure 7a and b. In agreement with published data (28), the levels of all examined cytokines were unaffected upon stimulation with TNF-α/PGE2 (Figure 7a and b). MoDCs stimulated with 20 µg of sHz released significantly higher levels of IL-6 (p=0.009) and IL-10 (p=0.021) as compared to unstimulated cells (Figure 7a). The secretion of IL-6 was also increased when stimulated with 10 µg of sHz (p=0.05). There was also a slight increase in TNF-α secretion in sHz-stimulated cultures as compared to unstimulated cultures, but this did not reach statistical significance. No differences were found in IL-1β, IL-8 or IL-12p70 secretion.

Next, we analyzed cytokines release by MoDCs after stimulation with iRBCs. At the low (10:1) iRBCs:MoDCs ratio no significant increment in the release of IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α or TNF-β was observed (data not shown). However, using a high (100:1) iRBCs:MoDCs ratio there was a significantly increased secretion of IL-1β (p=0.021), IL-6 (p=0.021), IL-10 (p=0.021) and TNF-α (p=0.043) by MoDCs. Of note, the secretion of IL-12p70 was very low upon iRBCs stimulation in all donors examined.

When comparing the levels of cytokines secreted by DCs upon stimulation with sHz and iRBCs the same cytokines were elevated with both stimuli except for IL-1β that was not increased upon sHz stimulation. The levels of IL-6, IL-10 and TNF-α were higher when MoDCs were stimulated with iRBCs as compared to sHz stimulation. Thus, these results indicate that both sHz and iRBCs induce secretion of a number of pro-inflammatory cytokines in MoDCs.
Discussion

Previous studies evaluating the effect of malaria-derived products have found that immune cells exposed to malaria-derived antigens are impaired in their activation and subsequent function. In this study a partial activation of MoDCs upon exposure to both iRBCs and sHz was observed, as demonstrated by an increased, albeit moderate, expression of maturation markers, as well as lymphoid CCRs on the surface of DCs as compared to the TNF-α/PGE2 induced maturation. The up-regulation of CXCR4 and the slight up regulation of CCR7 on the surface of MoDCs was coupled to a significantly increased migration towards their respective ligands CXCL12 /SDF1-α and CCL19/MIP3-β. A kinetic study revealed that MoDCs, unstimulated or activated by sHz could not maintain their expression of CD83 for a 60 hour period. When the cells where stimulated with TNF-α/PGE2, however, they did maintain elevated levels of CD83 expression. These data strengthen the results indicating that malaria derived products activate MoDCs, but not as potently as TNF-α/PGE2.

It is known that the quality of the interaction between T cells and DCs affects the outcome of the immune response (5,6) and that co-stimulation and cytokine release are crucial factors in antigen presentation and the modulation of T-cell responses (5,6). In our experiments, iRBC- and sHz- exposed MoDCs were not fully matured with regard to proper up-regulation of maturation markers nor did these cells release IL-12, which is crucial to activate a type 1 response including IFN-γ secretion (29). In addition, our iRBC- and sHz- exposed MoDCs released IL-10.

We therefore propose that the partial activation of myeloid DCs in combination with the release of IL-10 might lead to impaired T-cell activation, in line with what has been observed in vitro (10) and during natural infections (14). Alternatively, it is possible that this DC phenotype may lead to the induction of a regulatory-T cell (Treg) phenotype. Tregs have been reported to contribute to immune evasion during malaria in mice and humans, indicating
that this is one mechanism whereby the malaria parasites could subvert the host immune system (30). In support of this, evidence shows expansion of Treg population in a controlled model of experimental malaria in humans (31), which was related to a more rapid parasite growth (32). In addition, a field study in Kenya reported that expanded CD4+CD25\textsuperscript{high} cell population, potentially Tregs, was associated with a higher risk of clinical malaria (33).

We also observed that sHz selectively impaired the up-regulation of CCR7 induced by TNF-\(\alpha\)/PGE2 stimulation. Recent evidence indicates that, apart from chemotaxis, CCR7 controls the cytoarchitecture, the migratory speed, and the maturation rate of DCs (34). Therefore, blocking the CCR7 up-regulation on maturing MoDCs may be an effective tactic for the malaria parasite to block DC functionality, thus impairing the initiation of the T-cell responses, as seen in other parasitic infections (35). In support of this, it has been observed that Hz-containing DCs reside in the T-cell areas of the spleen, but fail to induce a proper T-cell effector function in a murine model of malaria infection (3).

Increased secretion of a number of pro-inflammatory cytokines such as IL1-\(\beta\), IL-6, and TNF-\(\alpha\) by MoDCs upon contact with \(Pf\)-derived stimuli was observed in our study. Malaria creates a large inflammatory response during acute infection and a correlation has been found between high levels of serum TNF-\(\alpha\) and IL-10, severe malaria and high parasitemia in African children (36). Recently, the serum levels of IL1-\(\beta\), IL-6, IL-10 and TNF-\(\alpha\) have been associated with severe complications during malaria infection in various studies (37,38).

Our results indicate that myeloid DCs may be crucial cells in inducing the large inflammatory response that is often observed during acute \(Pf\) infection in the natural host. In conclusion, contact of MoDCs with sHz or iRBCs induced partial maturation of these cells, provoked the release of some inflammatory cytokines and up-regulated lymphoid CCR on the cell surface. It was also observed that the up regulated expression of CXCR4 was coupled to
increased migration to the related ligand. This might suggest that *Pf* induces MoDCs to migrate to lymphoid organs. On the other hand, it appears that MoDCs activated by malaria stimuli might not be fully equipped to potently activate T cells or that they may skew the T-cell differentiation towards a regulatory phenotype. This phenomenon may contribute to altered *Pf* specific immunity that is often observed in malaria-infected patients.

**Acknowledgements**

This work was supported by grants from the Swedish Agency for Research and Development with Developing Countries (Sida/SAREC), as well as a grant within the BioMalPar European Network of Excellence (LSHP-CT-2004-503578)
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Figure legends

**Figure 1. Characteristics of sHz preparation.**

The spectrogram exhibits characteristic peaks of hemozoin. The scale represents the sHz as prepared according to the Tripathi’s modification of the Egan’s protocol. The spectrum exhibited the presence of intense bands at 1710, 1660, 1299, 1279, 1209 cm$^{-1}$ characteristic of hemozoin.

**Figure 2. sHz induces partial maturation of MoDCs and causes upregulation of lymphoid CCR expression.**

MoDCs were incubated with or without sHz (10 or 20 µg/ml), TNF-α and PGE2 were added or not as maturation stimuli in designated wells. After 18 hours of incubation, the cells were harvested, stained for surface markers and analyzed by FACS. Y-axis reports mean fluorescence intensity (MFI) for CD80, CD83, HLA-DR, CXCR4 and CCR7. The boxplots illustrate the medians and the 25th and 75th quartile and the whiskers represent the 10% and 90% percentiles for 5 donors. Data were analyzed using Wilcoxon’s rank sum test * = P<0.05. ** = P<0.01.

**Figure 3 Kinetics of expression levels for different activation markers on MoDCs upon sHz stimulation.**

MoDCs (1x10$^6$ cells/ml) were left unstimulated or were stimulated with sHz (20 µg/ml) or the maturation stimuli (TNF-α and PGE2) and harvested after 12, 36 or 60 hours of incubation.
Cells were stained for surface markers and analyzed by FACS. The graphs illustrate the result of one representative donor out of four independent experiments.

**Figure 4. sHz impairs the upregulation of CCR7 upon DC maturation.**

MoDCs were incubated with or without sHz (10 (n=5) or 20 (n=8) µg/ml) for 4 hours, when the maturation stimuli (TNF-α and PGE2) were added. Cells were incubated for additional 18 hours, harvested, stained for surface markers and analyzed by FACS. Y-axis indicates mean fluorescence intensity (MFI) for CCR7. The boxplots illustrate the medians and the 25th and 75th quartiles and the whiskers represent the 10% and 90% percentiles. Data were analyzed using Wilcoxon’s rank sum test *; P<0.05.

**Figure 5. Incubation with iRBCs induces partial maturation of MoDCs including upregulation of lymphoid CCR expression.**

MoDCs were co-cultured with uninfected RBCs (ctrl RBC) or iRBCs at the ratio of 100:1 (RBCs/DCs). Maturation stimuli (TNF-α and PGE2) were added in separate wells as a positive control for maturation. Cells were incubated for 12 hours, harvested, stained for surface markers and analysed by FACS. The mean fluorescence intensity (MFI) of HLA-DR, CD83, CXCR4 and CCR7 is illustrated in the diagram as mean values and standard error of the mean. Data were analyzed using Wilcoxon’s rank-sum test.

**Figure 6. Partial maturation of MoDCs induced by sHz is coupled to increased migration in response to lymphoid chemokines.**

MoDCs were stimulated with sHz (20µg/ml) or the maturation stimuli (TNF-α/PGE2) and incubated for 18 hours. Cells were then harvested and seeded in the upper compartments of a Boyden chamber while 100 ng/ml of CXCL12/SDF1-α or CCL19/MIP3-β were added to the
lower compartments. Medium without FCS was used as control of background migration. Cells were incubated for 1.5 hours to allow migration after which the cells that had migrated to the lower compartment were counted in 5 representative fields for each well. The net number of cells that migrated was calculated by subtracting the number of cells that migrated in response to medium alone from the number of cells that migrated in response to chemokines. The diagram represents mean values ± standard error of the mean of 6 donors for CXCL12/SDF1-α and 5 donors for CCL19/MIP3-β. Data were analyzed using Wilcoxon’s rank sum test *; P<0.05.

**Figure 7. Increased secretion of pro and anti-inflammatory cytokines by MoDCs upon stimulation with high concentrations of sHz and iRBCs.**

(a) MoDC were incubated with 10 and 20 µg/ml of sHz and cell culture supernatants were subsequently analyzed for cytokines. Each graph illustrates the mean values and SEM of 4 donors and the concentration in pg/ml of each cytokine. (b) MoDCs were incubated with a high and a low concentration of iRBCs and cell culture supernatants were subsequently analyzed for cytokines. Each graph illustrates the mean values and standard error of the mean of 4 donors and the concentration in pg/ml of each cytokine.
Figure 2
Figure 3

(a) CD80
(b) CD83
(c) HLA-DR

- TNF-α/PGE₂
- 20 µg/ml shZ
- Unstimulated MoDCs
Figure 4

CCR7

MFI

- + - +10μg/ml sHz
- - + +20μg/ml sHz
+ + + +TNF-α/PGE₂

Figure 4
Figure 5
Figure 6

![Graph showing net migrated cells for Mip3-β and SDF1-α](image)

- Unstimulated MoDCs
- +20µg sHz
- +TNF-α/PGE₂

* Indicates statistical significance.
Figure 7a
Figure 7b
Low frequency of circulating dendritic cells is associated with higher cell activation, raised plasma levels of anti-malarial antibodies and increased pro-inflammatory responses during *Plasmodium falciparum* infection in Fulani, an ethnic group with low susceptibility to malaria.

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Abstract

The Fulani ethnic group from West Africa is relatively better protected against malaria infection as compared to other sympatric ethnic groups, but little is known about the mechanisms behind this lower susceptibility to *Plasmodium falciparum* (*Pf*) infection. Dendritic cells (DCs) are the most potent activators of naive T cells, and thereby play an important role in regulating the adaptive immune responses. It has been shown that the DC subset expressing the blood-dendritic-cell antigen (BDCA)-3 is more prevalent in the circulation of children with severe malaria. In this regard, we aimed to investigate whether two ethnic groups exposed to similar malaria pressure but differing in their response to malaria exhibit differences in terms of number and activation status of circulating DCs, plasma levels of cytokines and anti-malaria specific antibodies.

The present study has been conducted in children during the malaria season of 2008. All children belonged to either the Fulani or the Dogon ethnic groups, and they were divided into two groups i.e. one with active *Pf* infection and one with no active infection.

In agreement with previous studies, the Fulani exhibited higher plasma levels of anti-malarial antibodies as compared to the Dogon. In addition, plasma levels of IFN-γ, IFN-α and a number of pro-inflammatory cytokines were higher in the Fulani children as compared to the Dogon, regardless of infection. During *Pf* infection, serum levels of IFN-γ were increased in the Fulani, while a different pro-inflammatory pattern that lacked IFN-γ was seen in the Dogon. Interestingly, when analysing the expression of activation markers on different DC subsets, our results showed that malaria infection induced activation of pDCs in Fulani but impaired activation in the Dogon. Taken together, this suggests a marked difference in innate responses that could clearly affect adaptive immune responses already early in life. In conclusion, the findings presented here suggest are likely to be important for the relative protection against malaria seen in the Fulani as compared to the Dogon.
Introduction

Malaria is one of the most important infectious diseases in the world and has a severe impact on child mortality. Mali in West Africa has seasonal malaria, with infections peaking during the late rain period from September to November. Previous studies performed in a rural area in Mali, conducted during the malaria transmission season, have shown different susceptibility to *Plasmodium falciparum* (*Pf*) infection between two different ethnic groups; the Fulani and the Dogon. These populations live under similar social, cultural and geographic conditions (1) and are exposed to identical malaria pressure. However, the Fulani show less clinical symptoms of malaria, and parasites are less frequently detected in their blood (2). In addition they exhibit higher titres of *Pf*-specific IgG subclasses (3), and IgM antibodies (4,5).

It has been shown that individuals undergoing malaria infection respond less to other infections as well, indicating that at least some functions of the immune system are somehow impaired by the parasite (6). Immunity to malaria is achieved only after multiple infections and is not long lasting. This may indicate a failure in the adaptive branch of the immune system to achieve proper memory responses. The initiators of adaptive immune responses are the antigen-presenting cells (APCs) and the most potent APCs are dendritic cells (DCs). Two main populations of DCs have been described in peripheral blood; the plasmacytoid DCs (pDCs) and the myeloid DCs (mDCs), and the latter can be further divided into three subtypes; the blood dendritic cell antigen-1 (BDCA-1⁺) DCs, the BDCA3⁺ DCs and the CD16⁺ DCs. It has been shown that mDC differentiation and function are impaired *in vitro* by the malaria parasite (7) or by products derived from infection (8). In addition, HLA-DR levels are decreased on circulating DCs of *Pf*-infected children (9) as compared to healthy controls and higher numbers of circulating BDCA-3 DCs have been detected in blood of severely infected children combined with reduced stimulatory ability, indicating that malaria
may impair DC functionality and disclosing a modulatory role for the BDCA-3⁺ DC subset in immunity (10).

During viral infections pDCs are essential components of the innate immunity (11,12). In addition, pDCs have been shown to play a crucial role in B-cell activation and in the induction of antigen-specific and polyclonal IgG production in response to viruses (12,13). The role of this DC subset during malaria infection is still under investigation. It is known that the function of pDCs is affected by Pf and that this cell subset is activated by asexual blood stage parasites in vitro with increased interferon (IFN)-α secretion and enhanced ability to stimulate γδ T cells (14). In addition, higher IFN-α levels in plasma have been observed in patients suffering of mild as compared to severe malaria (15,16), suggesting a possible role of pDCs in protection against severe malaria in humans.

Using flow cytometry, we evaluated the prevalence of different DC subpopulations in peripheral blood mononuclear cells (PBMCs) obtained from Fulani and Dogon children with or without malaria infection in a rural area of Mali. In addition, the plasma samples collected from the corresponding individuals were analysed for the levels of anti-malarial IgM, IgG subclasses as well as a number of pro-inflammatory and anti-inflammatory cytokines.
Materials and Methods

Study population

The study was conducted in a rural area of the Dogon valley of Mali where malaria is mesoendemic with intense transmission during the rainy season. The study area and the study population have been described in detail elsewhere (1). Children between 2 and 10 years of age belonging to either the Fulani or the Dogon ethnic groups were included in the study. Forty children from the Dogon population and thirty seven from Fulani were recruited. The study included healthy children and patients that were classified as uncomplicated malaria. A thick blood smear was made from each volunteer. The slides were stained in 3% Giemsa and examined for the presence of Pf parasites. Presence of malaria infection was defined as a positive thick smear with or without any malaria symptom. Axillary temperature was measured in all patients and symptomatic malaria was defined as fever (≥37.5°C), or history of fever, plus the presence of any density of parasites in the blood. Among the Dogon, 20 children were undergoing malaria infection and 20 children were healthy, while among Fulani 14 children were suffering of malaria infection and 23 were healthy. Ethical approval was obtained from the institutional review boards of the University of Bamako Mali (N°08_64/FMPOS). For each volunteer, both community and an individual consent form from the parents were obtained, before the inclusion of the children in the study.

Blood collection and cell preparation

Venous whole blood samples were collected from healthy and infected children of both ethnicities. From each volunteer, 6 ml of peripheral blood were collected in heparin tubes (BD Vacutainer® Plasma Tube, 143 USP Units of Sodium Heparin freeze dried) and were transported to the laboratory of Bandiagara for further experiments.
PBMCs were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) and used for APC immunophenotyping and expression of activation markers. Plasma was separated by centrifugation; plasma samples were stored at -80°C and used for cytokine and antibody studies.

**Determination of circulating levels of anti-malaria antibodies**

Determination of malaria-specific antibodies was done using a standard sandwich enzyme linked immunosorbent assay (ELISA) using a crude malaria antigen preparation (17) and performed as previously described (3)(18). Briefly, 96-well ELISA plates (Costar, Corning, NY, USA) were coated with crude malaria antigen (10 µg/ml) over night at 4°C. Biotin-conjugated mouse anti-human monoclonal antibodies and alkaline phosphatase conjugated streptavidine (Strep-ALP) (Mabtech AB, Nacka, Sweden) were used for each subclass. For detection of malaria-specific IgG and IgM, goat-anti-human IgG-ALP (Mabtech AB) and goat-anti-human IgM-ALP (Jackson ImmunoResearch Laboratories, PA, USA) were used. The assays were developed with p-nitrophenyl phosphate disodium salt (pNPP) (Sigma-Aldrich, St Louis, MO, USA) as substrate, and the optical densities were measured at 405nm in an ELISA plate reader (VmaxTM Kinetic Microplate Reader, Menlo Park, CA, USA). The concentrations of anti-malarial antibodies were calculated from standard curves obtained from sandwich ELISA with 6 dilutions of myeloma proteins of the IgG1-4 isotypes (Biogenesis, Poole, England) or for total anti-malarial IgG and IgM antibodies, with highly purified IgG or IgM antibodies, respectively (Jackson ImmunoResearch Laboratories).

**Determination of cytokine levels in serum using ELISA**

The levels of IFN-α and IFN-γ were measured using commercial ELISA kits (Mabtech). All samples were assayed in duplicates according to manufacturer’s recommendation. The
cytokine concentrations were calculated from a standard curve obtained in a sandwich ELISA with eight dilutions of lyophilized native human IFN-α standard (range 10,000-3 pg/ml) and recombinant human IFN-γ standard (range 3,000-1.0 pg/ml). The assays sensitivity was 2 pg/ml for IFN-α and 7 pg/ml for IFN-γ, respectively.

**Determination of cytokine levels in serum using cytometric bead array**

Plasma levels of IL-1β, IL-6, IL-8, IL-10, IL-12(p70) and TNF-α were determined by using cytometric bead array (CBA) technology (BD Biosciences, San Diego, CA) according to manufacturers’ recommendation. Briefly, 24 µl of bead populations with distinct fluorescent intensities, coated with cytokine-specific capture antibodies were added to 24 µl of serum sample, and 24 µl of phycoerythrin (PE)-conjugated anti-human inflammatory cytokine antibodies. Simultaneously, standards for each cytokine (range 0-10000 pg/ml) were also mixed with cytokine capture beads and PE-conjugated reagent. The lower limit of detection for the various cytokines were as followed; IL-1β (7.2), IL-6 (2.5), IL-8 (3.6), IL-10 (3.3), IL-12(p70) (1.9) and TNF-α (3.7) pg/ml. All data were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and CellQuest software. The software used for the analysis was the FCAP Array software (Soft Flow, Hungary Ltd. Hungary).

**Immunophenotype**

Purified PBMCs were fixed and frozen immediately after purification as previously described (19,20). Briefly, cells were resuspended in PBS to a concentration of 2 million cells/ml and treated with DNAse (Sigma-Aldrich) for 5 minutes at 37°C at a concentration of 666 units/ml. Cells were then washed and resuspended in 3 ml of prewarmed (37°C) 4% paraformaldehyde (PFA) (Sigma-Aldrich), incubated for 5 minutes at RT and then washed in cold PBS +1%
foetal bovine serum (FBS, Gibco, Paisley, UK). Finally, cells were resuspended in 1 ml PBS +10% DMSO and frozen at -70°C. For staining, the cells were thawed and resuspended in PBS 5 mM EDTA 2% FBS. FcR blocking reagent and mouse monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or PE, peridinin chlorophyll protein (PerCP) or allophycocyanin were added. Antibodies used for cell-surface staining included those recognizing BDCA-1, BDCA-2, BDCA-3, CD3, CD14, CD19, and mouse isotype controls IgG1 and IgG2a (Miltenyi Biotec, GmbH, Germany) and CD16, CD56, CD86, HLA-DR (Pharmingen, San Diego, CA, USA). The cells were finally resuspended in PBS 1% PFA, acquired and analysed using a FACSCalibur (Beckton Dickinson) and the BD CellQuest™Pro version 5.2.1 software.

**Statistical analysis**

Statistical significance was determined using non-parametric tests. A Kruskal-Wallis test was employed to compare when more than two groups were involved, if the test exposed a significant difference the Mann-Whitney U-test for significance between two unpaired groups was employed. For all tests $p \leq 0.05$ was considered significant and no correction was made for using multiple tests. The data were analyzed using the StatView software.
Results

Specific antibody responses to *Pf* are higher in Fulani children than in the Dogon children

Plasma levels of total antimalarial IgG, IgM as well as IgG1, IgG2, IgG3 and IgG4 were measured in plasma of both uninfected and infected Fulani and Dogon. When considering both uninfected and infected subjects, higher titres of anti-malarial IgG and IgM were found in the Fulani population as compared to the Dogon (p<0.001, Figure 1A and B). No differences were observed between the infected and uninfected children within the two ethnic groups except for IgM that was higher in the infected Dogon compared to the uninfected Dogon (p=0.05, Figure 1B), while this was not the case in the Fulani (p=0.17). However, the IgM levels in the infected Dogon were significantly lower as compared to the infected Fulani (p<0.01). Similarly, when analyzing the plasma levels of antimalarial IgG subclasses in the Fulani as compared to the Dogon, regardless of infection, we observed that IgG1 (p<0.0001), IgG2 (p<0.0001), IgG3 (p<0.0001) but not IgG4 (p=0.1957) were higher among the Fulani than the Dogon. The same differences were observed when comparing only the uninfected children from the two groups. When comparing the infected children of both ethnicities, the Fulani had higher levels of IgG1 (p<0.030) and IgG3 (p<0.001) than the Dogon. However, no intraethnic differences were observed for IgG1, IgG2 and IgG4 between the infected and uninfected children. Nevertheless, the Fulani children undergoing *Pf* infection had significantly lower levels of IgG3 as compared to their uninfected peers (p=0.045), while the Dogon children undergoing *Pf* infection exhibited higher levels of IgG3 in plasma than the uninfected Dogon children (p=0.034; Figure 2C).
Intraethnic and interethnic difference in cytokine plasma levels

The plasma of the children participating in the study were analysed for IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α. When comparing the Fulani to the Dogon, regardless of infectious status, we found higher levels of IL-6 (p = 0.0003; Figure 3A), IL-8 (p < 0.0001; Figure 3B), IL-12p70 (p = 0.0102; Figure 3C), IFN-α (p = 0.0027; Figure 3D) and IFN-γ (p = 0.0044; Figure 3E) in the Fulani population as compared to the Dogon. When comparing only the uninfected children, plasma levels of IL-6 (p < 0.0001), IL-8 (p < 0.0002), IL-12 (p = 0.0010) and IFN-α (p < 0.0025) were significantly higher in the Fulani than in the Dogon children. Differences were only found for IFN-γ (p = 0.0175), but not the other cytokines, when comparing infected children from the two ethnicities, and higher IFN-γ levels were observed in the Fulani as compared to the Dogon.

Finally, differences were also observed within the same ethnic group depending on the malaria status. The Dogon children undergoing infection had significantly higher levels of IL-6 (p < 0.0001) and IL-12 (p < 0.0001) in the plasma than the uninfected Dogon subjects. In the Fulani, the only significant difference observed, was that the infected children had lower levels of IL-8 (p = 0.0485) in plasma than the uninfected children belonging to the same ethnic group. The plasma levels of IL-1β were below detection limit for all samples (not listed). Similarly, IL-10 detectable plasma levels were found only in four samples out of 76 totals (not listed).

Interethnic and intraethnic differences in circulating dendritic cell frequency and activation

The percentage or absolute numbers of circulating BDCA-1 + DCs did not show any interethnic or intraethnic difference (Figure 4A and B). However, the infected Dogon
exhibited significantly lower levels of HLA-DR expression on BDCA-1+ cells as compared to uninfected Dogon (p<0.001), and infected Fulani (p=0.002, Figure 5A). When analyzing BDCA-2+ pDCs and BDCA-3+ mDCs distribution, no interethnic differences were observed between the uninfected Fulani and Dogon. However, differences were observed within the same ethnic groups upon malaria infection. In fact, the infected Dogon exhibited significantly higher frequency of circulating pDCs+ and BDCA-3+ mDCs than uninfected children of the same ethnic group (p=0.034 and p=0.016, respectively), while the infected Fulani had a reduced frequency of pDCs and BDCA-3+ mDCs in the circulation as compared to uninfected Fulani (p=0.060 and p=0.038, respectively, Figure 4A). No difference in absolute numbers of circulating BDCA-3+ mDCs was seen in any of the examined groups (Figure 4B) while there was a significant increase of blood pDCs in infected Dogon children as compared to the uninfected Dogon (p=0.020, Figure 4B). Additionally, pDCs of infected Fulani exhibited higher expression levels of HLA-DR as compared to the uninfected Fulani (p=0.009), while the expression of HLA-DR on pDCs was lower in the infected Dogon as compared to their uninfected peers (p=0.035; Figure 5A). Expression levels of the activation marker CD86 was also slightly increased on pDCs in infected Fulani as compared to uninfected children, while the expression of CD86 was significantly lower in the infected Dogon as compared to uninfected Dogon (p<0.001; Figure 5B). When analyzing BDCA-3+ mDCs, the infected Fulani had slightly higher levels of HLA-DR on this cell subset than the uninfected subjects belonging to the same ethnic group (p=0.057), while the BDCA3+ mDCs of infected Dogon had significantly lower levels of HLA-DR than the uninfected Dogon (p=0.027; Figure 5A).

Finally, when analyzing CD86 expression on pDCs (p < 0.0129) and BDCA-1+ (p < 0.0061) and BDCA-3+ (p = 0.0002) mDCs of the Fulani as compared to the Dogon regardless of infection, the levels were significantly higher in Fulani than in Dogon for all DC subsets. In addition, expression of HLA-DR was higher on the BDCA-3+ mDCs in the Fulani
ethnic group as compared to the Dogon (p=0.05) but not on the BDCA1 (p = 0.1578) or the pDCs (p = 0.1224).
Discussion

In this study, we investigated some immunological aspects of \textit{Pf} malaria in terms of prevalence of different subsets of DCs as well as certain activation markers, plasma levels of cytokines, and specific anti-malaria antibodies in children from two different ethnic groups living in a malaria endemic area in Mali. These populations, the Fulani and the Dogon, have differences in the susceptibility to \textit{Pf} infection.

Previous studies have shown higher levels of antimalarial antibodies in asymptomatic adults belonging to the Fulani as compared to other sympatric ethnic groups (3,5,21,22). In line with these findings our data show that Fulani children also have higher plasma levels of anti-malarial IgG subclasses except IgG4 as compared to the Dogon children. Although this has been established previously in adults, we here show that the higher humoral specific immune response of the Fulani is evident already early in life.

During \textit{Pf} infections, the presence of anti-malarial IgG3 is associated with protection against malaria in humans, possibly by an antibody-dependent mechanism inhibiting the growth of parasites (23,24). Interestingly, we observed that the plasma levels of anti-malarial IgG3 were higher in the uninfected Fulani than in the uninfected Dogon, suggesting a protective role of these antibodies in the Fulani. However, the levels of antimalarial IgG3 were found to be lower in the infected Fulani children than in the uninfected subjects of the same ethnic group. The lower IgG3 levels seen in the infected Fulani could be due to the rapid formation of \textit{Pf} specific IgG3 antibody-antigen complexes, thereby making the antigen-binding part of the antibody incapable of interacting with infected erythrocytes. Thus, antibodies in the form of immune complexes cannot function in antibody-dependent cellular inhibition assays (ADCI) an important suggested killing mechanism of anti-malaria IgG3 antibodies (25). However, immune complexes can act through Fc-receptors and thus play an important role in enhancing inflammatory responses as seen in the Fulani (2).
Cytokines play a central role in the pathogenesis of malaria. The balance between pro- and anti-inflammatory cytokines may be important in the clinical outcome of malaria (26-28).

When analyzing uninfected subjects, the levels of IFN-α, IL-6, IL-8 and IL-12p70 in plasma were higher in the Fulani than in the Dogon children. This may indicate that the Fulani children exhibit higher levels of basal activation of their innate immune response as compared to the Dogon.

We found significantly lower level of IL-8 in the infected Fulani children as compared to the uninfected subjects belonging to the same ethnic group. These low levels were similar to those seen in both infected and uninfected Dogon. The clinical significance of reduced IL-8 levels in infected Fulani as compared to uninfected peers is presently unknown.

The levels of IL-6 and IL-12 were higher in infected Dogon than uninfected children of the same ethnic group. Earlier studies have shown that acute-phase levels of IL-12p70 in plasma were inversely correlated with parasitemia (15). Thus, the high level of this cytokine observed in infected Dogon could be the consequence of the ongoing acute Pf infection which is exacerbated in Dogon children as compared to the Fulani.

Conversely, we found higher plasma levels of IFN-γ in the infected Fulani children as compared to the infected Dogon. This is in line with recent findings indicating that mononuclear leukocytes from the Fulani produce a markedly stronger IFN-γ response to in vitro stimulation with Pf than leukocytes from Dogon (McCall, Unpublished manuscript). Studies in both humans and mice indicate that the magnitude of early IFN-γ response is a crucial determinant of the outcome of malaria infection (29-31). Our findings suggest that the elevated IFN-γ responses in Fulani undergoing Pf infection may play an important role in the differences that we observe in the immune response of the Fulani as compared to the Dogon.
Taken together, these results may indicate that one of the underlying causes for the relative protection against malaria, of the Fulani, may be related to increased IFN-γ secretion that seems to be absent in the Dogon.

DCs initiate adaptive immune responses by providing antigenic stimulation to T and B cells. Thus, DCs provide a crucial link between the innate and adaptive immune response and are potent in the uptake, processing and presentation of antigens to T cells (32). We found lower frequency of pDCs and BDCA-3+ mDCs in the circulation of infected Fulani as compared to uninfected children belonging to the same ethnic group, while the same DC subsets were relatively increased in numbers in infected Dogon as compared to uninfected Dogon. Interestingly, analysis of HLA-DR expression revealed that circulating pDCs and BDCA-3+ mDCs in the Fulani were more activated in the infected children. DC activation usually results in up-regulation of lymphoid chemokine receptors on the cell surfaces which will result in an increased cellular migration to lymph nodes. DC migration may therefore account for the lower frequency of pDCs and BDCA-3+ mDCs that we observed in peripheral blood of infected Fulani. However, absolute numbers of circulating pDCs and mDCs were not affected by malaria infection in Fulani, and reduced frequency of these DC subsets in infected subjects may also be explained by a relative increase in some other leukocyte population. It is known that the Fulani have a higher spleen rate than other sympatric ethnic groups (33,34) that could be coupled to an increase in both T- and B-cell subsets.

In the Dogon children the malaria infection caused a strongly reduced expression of HLA-DR in circulating pDCs and BDCA-1+ and BDCA-3+ mDCs. These results are in accordance with previous findings in Kenyan children where it has been shown that children with acute malaria exhibited reduced expression levels of HLA-DR on mDCs (9). HLA class II expression on DCs is fundamental for presenting antigens to T cells and inducing their activation (32,35). It has been previously shown that PBMCs from children
with severe malaria induced less T-cell proliferation than PBMCs from healthy children (36,36). Thus, the reduced expression of HLA-DR on the surface of all DC subsets in Dogon children undergoing malaria infection may indicate impaired DC functionality, which in turn, may contribute to impaired cell-mediated responses against the parasite.

Taken together, our results show that malaria infection induces activation of pDCs in Fulani, while the same infection impairs HLA-DR expression in all examined DC subsets in Dogon. Since no differences were observed when comparing only the uninfected children from the two ethnicities. These different responses observed in the two ethnic groups may be the reason that we see a higher expression of activation markers when comparing the two groups without considering the infectious status.

Since pDCs play a crucial role in triggering humoral immunity (12,13) increased pDC responses in Fulani may support antibody production which could explain the higher Ig levels observed in Fulani as compared to other ethnic groups living in the same areas (3,5,21,33) On the other hand, impaired DC activation upon malaria infection, as reflected here in the Dogon population, may result in a deficient immune response as commonly seen in malaria (37). To further evaluate the innate response in the Fulani we are presently analysing the cytokine secretion of the PBMCs after stimulation with specific TLR ligands.

In conclusion, the findings presented here suggest that there is a difference in the prevalence and activation status of blood DCs, cytokine secretion and Ig levels between the Fulani and the Dogon in response to malaria infection already in early life. Such differences are likely to be important for the relative stronger protection against malaria seen in the Fulani as compared to the Dogon.
Acknowledgements

This work was supported by grants from the Swedish Agency for Research and Development with Developing Countries (Sida/SAREC), as well as a grant within the BioMalPar European Network of Excellence (LSHP-CT-2004-503578) and from the European & Developing Countries Clinical Trials Partnership (EDCTP).
References


Figure legends

Figure 1. Specific antibody responses to Pf are higher in Fulani children than in the Dogon children

Blood plasma samples from 77 children were analysed for antimalarial IgG (A) and IgM (B). The samples were subdivided according to ethnicity and slide positivity to malaria; i.e. uninfected Dogon (n=20), infected Dogon (n=20), uninfected Fulani (n=23) and infected Fulani (n=14). The y-axis depicts the concentration of the respective antibody/ml of plasma. The boxplots illustrate the medians and the 25th and 75th quartile and the whiskers represent the 10% and 90% percentiles. Data were analyzed using Mann-Whitney rank sum test. *; p≤0.05. **; p<0.01. ***; p<0.001.

Figure 2. Malaria specific IgG subclass responses to Pf in Fulani and Dogon children.

Blood plasma samples from 77 children were analysed for antimalarial IgG1 (A), IgG2 (B), IgG3 (C) and IgG4 (D). The samples were subdivided according to ethnicity and slide positivity to malaria; i.e. uninfected Dogon (n=20), infected Dogon (n=20), uninfected Fulani (n=23) and infected Fulani (n=14). The y-axis represent µg of the respective antibody/ml of plasma. The boxplots illustrate the medians and the 25th and 75th quartile and the whiskers represent the 10% and 90% percentiles. Data were analysed using Mann-Whitney rank sum test. *; p≤0.05. **; p<0.01. ***; p<0.001.
Figure 3. Intraethnic and interethnic difference in cytokine plasma levels.

Blood plasma samples from 77 children were analyzed for cytokines. The levels of IL-6 (A), IL-8 (B) and IL-12p70 (C) were measured using cytometric bead array. The levels of IFN-α (D) and IFN-γ (E) were analyzed using commercial ELISA kits. The samples were subdivided according to ethnicity and slide positivity to malaria; i.e. uninfected Dogon (n=20), infected Dogon (n=20), uninfected Fulani (n=23) and infected Fulani (n=14). The y-axis depicts picograms (pg) of the respective cytokine/ml of plasma. The boxplots illustrate the medians and the 25th and 75th quartile and the whiskers represent the 10% and 90% percentiles. Data were analyzed using Mann-Whitney rank sum test. *; p≤0.05.***; p≤0.001.

Figure 4. Interethnic and intraethnic differences in the frequency and absolute numbers of circulating DC.

PBMCs from 63 children were isolated using Ficoll and subsequently fixed and frozen. The cells were harvested and stained using monoclonal antibodies against known subtypes of blood DCs and analyzed using FACS Calibur. The samples were subdivided according to ethnicity and slide positivity to malaria, i.e. uninfected Dogon (n=20), infected Dogon (n=20), uninfected Fulani (n=13) and infected Fulani (n=10). The y-axis in (A) depicts the percentage of circulating DC subsets while the y-axis in (B) depicts the absolute number of circulating DCs/ml of blood. The boxplots illustrate the medians and the 25th and 75th quartile and the whiskers represent the 10% and 90% percentiles. Data were analyzed by Mann-Whitney rank sum test. *; p≤0.05.
Figure 5. Interethnic and intraethnic differences in blood DCs activation markers

PBMCs from 63 children were isolated using Ficoll and subsequently fixed and frozen. The cells were harvested and stained using monoclonal antibodies against known subtypes of blood DCs and activation markers, such as HLA-DR (A) and CD86 (B). Cells were subsequently analyzed using FACS Calibur. The samples were subdivided according to ethnicity and slide positivity to malaria, i.e. uninfected Dogon (n=20), infected Dogon (n=20), uninfected Fulani (n=13) and infected Fulani (n=10). The y-axis depicts mean fluorescence intensity (MFI) of activation markers. The boxplots illustrate the medians and the 25th and 75th quartile and the whiskers represent the 10% and 90% percentiles. Data were analyzed by Mann-Whitney rank sum test. *; p≤0.05. **; p<0.01. ***; p<0.001.
Fig 1

A

B

Uninfected Dogon
Infected Dogon
Uninfected Fulani
Infected Fulani
Fig 2

A

B

C

D

***

***

***

***

Uninfected Dogon
Infected Dogon
Uninfected Fulani
Infected Fulani

\[ \text{IgG2 (µg/mL)} \]

\[ \text{IgG4 (µg/mL)} \]
Fig 3

A

B

C

D

E

- Uninfected Dogon
- Infected Dogon
- Uninfected Fulani
- Infected Fulani
Fig 5

A

HLA-DR expression (MFI)

BDCA-1⁺  BDCA-2⁺  BDCA-3⁺

Uninfected Dogon  Infected Dogon  Uninfected Fulani  Infected Fulani

B

CD86 expression (MFI)

BDCA1⁺  BDCA2⁺  BDCA3⁺

Uninfected Dogon  Infected Dogon  Uninfected Fulani  Infected Fulani