Inflammatory responses due to *Plasmodium falciparum* infection during pregnancy and childhood

Stéphanie Boström

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SUMMARY

In areas where malaria is endemic, pregnant women and children are the groups most at risk of developing severe and life-threatening disease. Malaria during pregnancy may have detrimental consequences both for the mothers and their offspring as well as affecting infants during their first years of life.

Cytokines and chemokines are major mediators during an immune response and are crucial in the defense against pathogens. They regulate immune-cell development, direct cells to sites of infection and in general modulate the immune system. The balance between pro and anti-inflammatory cytokines is crucial in determining the outcome of an infection. Therefore, the aim of these studies was to evaluate inflammatory responses in pregnant women and children infected with *Plasmodium falciparum*. To do this, two separate cohort studies were employed: 1) A longitudinal, prospective study conducted in Tanzania where pregnant women were enrolled and followed up during pregnancy, 2) A case-control study carried out in two sympatric ethnic groups, the Dogon and the Fulani, in Mali. The Fulani have been shown to be less susceptible to malaria as compared to other sympatric ethnic groups.

A panel of selected cytokines, chemokines and antibodies was analyzed in plasma from the participating pregnant women and children to evaluate the impact of *Plasmodium* infection. Our results show that upon infection with *P. falciparum*, the cytokine and chemokine levels in the pregnant women are changed. The anti-inflammatory factor IL-10 and the inflammatory factor IP-10 were found to be increased at all time points when the pregnant women had an infection and might therefore be important biomarkers for inflammation during pregnancy.

Fulani children exhibited higher inflammatory cytokine levels and humoral immune responses towards the parasites as compared to Dogon children. When the children were further subdivided into infected and uninfected individuals, the infected Dogon children showed increased levels of MCP-1, MIG and IP-10 but decreased levels of RANTES compared to their uninfected counterparts. In contrast, there were no differences in the levels between infected and uninfected Fulani. This suggests that the Fulani have a higher inflammatory response which potentially could protect them against *P. falciparum* infections already at an early age. Taken together, our results demonstrate that infections with *P. falciparum* induce both inflammatory and anti-inflammatory factors and suggest that the balance between these factors is important for the outcome with respect to disease severity.
LIST OF PAPERS

This thesis is based on the original papers listed below, which will be referred to by their roman numerals in text:


* These authors contributed equally to this work.
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ABBREVIATIONS

ADCC  antibody-dependent cellular cytotoxicity
APC  antigen-presenting cell
CSA  chondroitin sulphate A
DAMP  danger-associated molecular pattern
DC  dendritic cell
FoxP3  transcription factor forkhead box P3
ICAM  inter-cellular adhesion molecules
IDO  indoleamine 2,3-dioxygenase
iE  infected erythrocyte
IFN  interferon
Ig  immunoglobulin
IL  interleukin
IP  inducible protein
IVS  intervillous space
LAP  latency-associated protein
LBW  low birth weight
LPS  lipopolysacharide
MCP  monocyte chemotactic protein
MHC  major histocompatibility complex
MIG  monokine induced by IFN-γ
MIP  macrophage inflammatory protein
MS  multiple sclerosis
NF-kβ  nuclear factor-kβ
NK  natural killer
NO  nitric oxide
PAMP  pathogen-associated molecular pattern
P. falciparum  Plasmodium falciparum
PfEMP1  P. falciparum erythrocyte membrane protein 1
PM  placental malaria
PRR  pattern recognition receptor
RA  rheumatoid arthritis
RANTES  regulated upon activation normal T cell expressed and secreted
ROS  reactive oxygen species
SOCS  suppressor of cytokine signaling
STAT  signal transducer and activator of transcription
Tc  T cytotoxic cell
TGF  transforming growth factor
Th  T helper
TLR  toll-like receptor
TNF  tumor necrosis factor
Treg  regulatory T cell
uNK  uterine natural killer
uPAR  urokinase receptor
VEGF/Flt1  vascular endothelial receptor
THE IMMUNE SYSTEM

The immune system is crucial for our defense against invading microorganisms and relies on a complex network of organs, cells and molecules responsible for maintaining the body’s homeostasis and hindering pathogens from infecting us. It is capable of recognizing self from non-self and distinguishing what is dangerous and what is not, thus keeping us safe in a surrounding constantly exposed to environmental microbes. We are equipped with many effective means for blocking potential pathogens from entering our body. The skin and the mucus membranes act as physical barriers while lytic enzymes and low pH in the stomach work as chemical barriers to reduce pathogenic invasion. Mucosal surfaces further extend the protection against invading pathogens by secretion of antimicrobial peptides. Besides these physical and chemical barriers, the immune system is equipped with immune cells that conduct surveillance for pathogens that do manage to break through. These immune cells are transported in the body, via the blood and lymphatic vessels, into specialized immune compartments such as the spleen, thymus, and lymph nodes where they expand and multiply for an efficient immune response. There are two branches of the immune system, the innate and the adaptive arms, which together act to disarm pathogens but that have major differences in terms of their specificity, their speed of action and the generation of memory.

INNATE IMMUNITY

The innate immune system ensures rapid limitation of microbial invasion through phagocytosis, release of inflammatory mediators, activation of the complement system as well as synthesis of acute phase proteins, cytokines and chemokines. These processes are activated by specific stimuli, represented by structures that are unique to microorganisms, such as lipopolysacharides (LPS), mannose and teichoic acids that constitute pathogen-associated molecular patterns (PAMPs) which are conserved among microorganisms and are commonly found on the surface of pathogens. The innate immune system recognizes these structures through pattern recognition receptors (PRRs) among which the toll-like receptor family (TLRs) are the most studied. Up until today, 10 different TLRs have been identified in humans. Somewhere located in the cell membrane and others inside the cell, which enables an effective way of detecting both intra and extra-cellular pathogens. TLR1, TLR2, TLR4, TLR5 and TLR6 are found on the cell surface and recognize structures that are representative for
both gram positive and gram negative bacteria such as peptidoglycan, LPS and flagellin. TLR3, TLR7, TLR8 and TLR9 are located on the endolysosome and recognize viral DNA and RNA in the cytoplasm. Other PRRs are the C-type lectin receptors, nucleotide oligomerization domain-like receptors and retinoic acid inducible gene-like receptors. Activation of the PRRs by their ligands initiates signaling cascades in the cell that ultimately lead to activation of nuclear factor-kB (NF-kB) pathways and consequently to the induction of pro-inflammatory cytokines and increased antimicrobial activities. It has also been proposed that activation of innate immune system is not only based on the recognition of PAMPs but also relies on the presence of danger signals or danger-associated molecular patterns (DAMPs) released by injured cells, for example the release of uric acid.

The main effector cells in the innate immune system are macrophages, neutrophils, dendritic cells (DCs) and natural killer (NK) cells. Neutrophils are professional phagocytes that usually are the first responders to migrate towards the site of inflammation or bacterial stimuli. Once at the inflammatory site, they start to phagocytose the pathogen and also enhance their production of reactive oxygen species (ROS) that efficiently kills the pathogen. NK cells are important in our nonspecific defense and have cytotoxic activity against virus-infected cells or tumor cells. They recruit neutrophils and macrophages and activate DCs, T and B cells by secreting a variety of potent cytokines. Monocytes circulate in the blood before they enter tissues where they differentiate into macrophages and DCs. Two major monocyte subpopulations have been described, the classical (CD14+CD16-) and the inflammatory (CD14+CD16+) subset that differ in terms of effector functions and trafficking potential. Monocytes and macrophages are efficient phagocytes that engulf microbes and cellular debris. They are also potent cytokine producers upon bacterial stimuli.

During inflammation, antigen-presenting cells (APCs) that are specialized in the uptake, processing and presentation of antigens to cells of the adaptive immune system. DCs are the most specialized APC, capturing and presenting antigens to naïve lymphocytes, and they are therefore considered to be a bridge between the innate and the adaptive immune system. DCs are found in peripheral tissue such as the skin, liver and intestine where they capture antigens, become activated and subsequently migrate to the lymph nodes. Inside the lymph node, they present antigens to T cells thereby initiating an immune response. The innate immune system is clearly important in the fight against pathogenic organisms but it has its limitations. It works rapidly and is immediately initiated whenever sensing a threat, but it is on the expense of being non-specific and results in no memory. For this reason the innate immune system
may be sufficient to clear or control an infection, until the adaptive arm of the immune system takes over to ensure complete elimination of the threat.

**ADAPTIVE IMMUNITY**

Unlike the innate immune system, the adaptive immune system confers a high degree of specificity and diversity and is able to remember captured pathogens by the production of antigen-specific memory cells. The innate arm is said to activate the adaptive immune system and therefore the initial quality of the innate immune response may direct the proceeding activation of the adaptive immune responses. Traditionally, adaptive immunity is divided into two different branches; 1) the cell-mediated response, in which the effector cells are antigen-specific T cells, and 2) the humoral response, which is made up by antibodies produced by antigen-specific B cells. Antigen recognition occurs through their surface receptors, which display a high degree of diversity and specificity due to germ line gene rearrangement. T cells recognize processed antigens when they are presented in association with the major histocompatibility complex (MHC) I or II on the surface of APCs. When a specific T cell recognizes the antigen presented by an APC, such as the B cell, the T cell provides additional help to the B cell, whereafter both the T and the B cells expand and generate highly specific effector cells and long lasting memory cells.

T cells are essential in the development of the cell-mediated immunity. They originate from the bone marrow but fully mature in the thymus into two major groups; the T helper (Th) CD4+ and the T cytotoxic (Tc) CD8+ cells. In general, Tc are important for the clearance of virus-infected and tumor cells, while the Th cells mainly regulate other cells of the immune system by secreting cytokines. The Th cells can be further differentiated into Th1 and Th2 cells depending on their cytokine expression profiles. Th1 cells produce pro-inflammatory cytokines such as tumor necrosis factor (TNF), interferon (IFN)-γ, interleukin (IL)-1 and IL-2. These cytokines activate Tc cells and macrophages to stimulate cell-mediated immunity and inflammation and are characterized by the transcription factor T-bet and STAT4. On the other hand, Th2 cells mainly produce IL-4, IL-5 and IL-13 which are important in regulating B-cell maturation and subsequent production of antibodies. Th2 cells have a major function in clearing helminthic infections and they are characterized by transcription factor GATA-3 and STAT6. A positive feedback loop exists in which the cytokines produced by one subgroup of the Th cells are inhibiting the differentiation and cytokine secretion of the other subgroup and
vice versa. However, recently the Th1/Th2 paradigm had to be revised with the discovery of a third subset of effector T cells, named Th17 cells which selectively produce IL-17. The Th17 cells are characterized by the transcription factor ROR-α. This population has shown to play an important role in clearing extracellular pathogens, induce tissue inflammation and has also been implicated in the pathogenesis of certain diseases, such as rheumatoid arthritis (RA), psoriasis, multiple sclerosis (MS), Crohn’s disease and allergic asthma. Therefore the Th1/Th2 concept is a bit simplistic and has nowadays been expanded into a broader Th1/Th2/Th17 cell paradigm.

All the functions of the effector T cells are regulated by the CD4+ regulatory T (Treg) cells. The Treg cells are an important group of T cells, which are involved in the induction and maintenance of tolerance towards self by suppressing self reactive T cells. They reside in the thymus as naïve T cells but when they go to the periphery and become activated they acquire a memory phenotype. There are several types of Treg cells distinguished by their different expression of cell surface molecules and cytokine production. The natural occurring Treg cells reside in the thymus and are CD4+CD25+ and express high levels of the transcription forkhead box P3 (FoxP3) and are potent producers of IL-10 and transforming growth factor (TGF-β). Treg cells can also be induced from peripheral T cells. The induced Tr1 and Th3 cells are characterized by secretion of IL-10 and TGF-β, respectively.

B cells are key cells in the humoral immune response. They are produced in the bone marrow as immature B cells that become fully matured in the spleen and one of their important tasks in the immune system is to generate immunoglobulins (Ig). When a naïve B cell recognizes a pathogen through the B-cell receptor it engulfs it and internalizes it and processes it to finally present it in association with the MHC II molecule on its surface. When this occurs, a T cell can recognize the antigen and bind to the B cell and together with additional co-stimulatory molecules the B cell becomes activated. Upon activation of a naïve B cell, clonal expansion occurs which results in the production of antibody-secreting plasma cells and memory-B cells. The B cell receptors may undergo somatic hypermutations (point mutations) in the germinal centers, which increases its affinity and specificity for an antigen, thereby making it more and more prone to capture the antigen it previously has encountered. Importantly, each B cell can only produce one specific antibody.

The Ig molecules can be membrane bound or secreted and the two forms have different functions. As a membrane form, the Ig function as a receptor, while the secreted form mainly
works as a neutralizing agent that blocks foreign pathogens or activates other mediators. After activation, the B cells may also undergo class switching in which the Ig production is shifted from one antibody class to another one that further directs the immune response. There are five different isotypes: IgM, IgD, IgG, IgA and IgE. The antibodies are effective in clearing pathogens and their effector functions are mainly to promote opsonisation (by binding to the surface antigens on the pathogen) to activate the complement system (which is part of the innate immune system and consists of a complex pathway of proteins and molecules that interact to mediate direct lysis of cells) or to mediate antibody-dependent cellular cytotoxicity (ADCC).

IgM is the first antibody that encounters an antigen and is a potent activator of the complement system. After binding to the pathogen, IgM with or without complement promotes clearance of the pathogen by activating phagocytic cells, by limiting pathogen dissemination throughout the host as well as by enhancing the presentation of pathogen-derived antigens to the adaptive immune system.17

IgG is one of the key antibody isotypes involved in the humoral immune response. It represents almost 70% of all isotypes in the blood and is like IgM, a potent activator of the complement system. IgG is the only antibody that can pass extensively across the placental barrier to provide passive immunity from a mother to her fetus.18 In humans, IgG can be divided into 4 different subclasses IgG1-IgG4. IgG1 has the highest prevalence in blood followed by IgG2, IgG3 and IgG4.19 IgG1 and IgG3 are generally considered to be cytophilic, while IgG2 and IgG4 are not.20 In terms of activating complement, IgG3 and IgG1 are the most effective once, while IgG2 is a weak activator whereas IgG4 does not seem to be able to activate it at all. In humans, a Th1 response induces IgG1 and IgG3 subclasses, whereas a Th2 type of response mediates IgE and IgG4 production.

IgD is co-expressed together with IgM on most B cells and is in general found in very low levels in blood (less than 1%) and has a very weak capacity to activate complement.21

IgA is a key antibody involved in mucosal immunity.22 This antibody class works together with nonspecific protective factors, such as the mucus to block microbial adhesion to epithelial cells without causing a tissue-damaging inflammatory reaction.

IgE is the subtype mostly known for its involvement in allergic reactions and protection against helmints.23
THE INFLAMMATORY RESPONSE

Inflammation is a crucial component of the body’s immune system. It is part of the first line of defense that takes place when an infectious agent comes into our body or when we have a tissue injury. It consists of vascular responses, migration and activation of leucocytes, secretion of soluble mediators and systemic reactions. Inflammation is manifested by redness, heat, swelling, pain and loss of function. Tissue resident cells such as macrophages, DCs and mast cells secrete a variety of soluble mediators such as histamine, prostaglandins and cytokines that enhance cell function at the inflamed site. Local production of the chemokines IL-8 and monocyte chemotactic protein (MCP)-1 induces chemotaxis and results in recruitment of neutrophils to the inflamed site and later also monocytes which can differentiate into macrophages and DCs at the inflamed site. Furthermore, adhesion molecules of the selectin family, the inter-cellular adhesion molecules (ICAM) and vascular cell adhesion molecule (VCAM)-1 on endothelial cells, becomes highly expressed and aid in the recruitment of cells. The whole purpose of the process is to remove the inducing stimulus and to start local tissue recovery. There is an increased vascular diameter and vascular permeability of the blood vessels, which enables leukocytes to migrate into the inflamed tissue supported by the highly expressed adhesion molecules on endothelial cells and guided by induced chemotaxis. The neutrophils and macrophages are specialized phagocytes, but they also have an important role in mediating the acute phase response through the production of IL-1, IL-6 and TNF. Once the pathogen is cleared or when the tissue has been repaired, the signal that triggered the inflammation needs to be inhibited in order to sustain normal homeostasis. A failure to regulate this suppression may have detrimental consequences for the host, leading to chronic inflammation and in the end severe tissue damage. One major signaling pathway involved in this task is represented by the suppressor of cytokine signaling (SOCS) proteins that mediate a negative feedback loop to cytokine signaling. For the final resolution of inflammation the immune cells recruited to the site have to be removed, which may happen by inducing apoptosis followed by clearance by macrophages.
CYTOKINES

Cytokines are low-molecular-weight regulatory proteins that are secreted by many cells of the immune system in response to a number of stimuli. They are involved in virtually all physiological responses in the body and are key players in coordinating immune responses between cells, by binding to a variety of receptors and to induce cell-specific immune responses. A cytokine can work in three different ways; by binding to receptors on the membrane of the same cell that secretes it (autocrine action), by binding to receptors on a nearby cell (paracrine action) or by binding to receptors on targets cells in distant parts of the body (endocrine action) but the last mentioned happens very rarely. There are different families of cytokines depending on their function. However, any given cytokine can have different actions depending on which cell it influences or if it works in antagonistic or synergistic ways with other cytokines. In addition, most cytokines have more than one biological activity, probably depending on the local context in which the cytokine is produced. The IFNs are key molecules in the early defense against infections and are in many cases the first line of resistance to many viral infections. The ILs, which form a large group of cytokines, are mainly produced by/and deliver signals to leukocytes. They are involved in regulation of cell division and differentiation. Chemokines are another group of important chemoattractant cytokines that regulate cell trafficking of various types of leukocyte populations through seven-transmembrane G-protein-coupled receptors. They are produced in response to exogenous stimuli, such as viruses and bacterial LPS as well as endogenous stimuli such as IL-1, TNF and IFNs. Chemokines mainly act on neutrophils, monocytes, lymphocytes and eosinophils and influence their ability to migrate, especially to inflamed sites by inducing chemotaxis. Thus, chemokines have an important role in host defense mechanisms. Chemokines are divided into several families based on the position of their cysteine residues. The two major families that have been classified are the CXC or α chemokines (e.g. IL-8, IFN-γ inducible protein (IP)-10, and platelet factor 4), and the CC or β-chemokines (e.g. MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES). Interest in chemokines and their function has grown since it became clear that besides having chemotactic properties they also play a role in angiogenesis, hematopoiesis and development. A brief description of the cytokines and chemokines of relevance in the papers will follow.
PRO-INFLAMMATORY CYTOKINES

IL-1β and TNF

IL-1β and TNF are two acute phase proteins that are rapidly induced upon tissue injury or when the immune system senses microbes. They are produced by many cells of the immune system but mostly by activated monocytes, macrophages and DCs. TNF can also be produced by activated NK cells, T cells and B cells. Both of these factors are potent pro-inflammatory cytokines and must therefore be kept tightly regulated. As a consequence, under normal conditions they are not found in high concentrations in the circulation which otherwise could be dangerous for the host. They are both involved in systemic and local responses to infection and inflammation by generating fever, activating lymphocytes and inducing other systemic acute phase proteins, such as C-reactive proteins and fibrinogen by the liver. IL-1β is regulated by the NALP3 inflammasome and is kept in an inactive form as a pro-peptide. Upon recognition of microbes or danger signals by the inflammasome, the caspase-1 enzyme becomes activated and cleaves the precursor form of IL-1β into its active form, which then can be secreted. IL-1 signals through the IL-1 receptor and ligation to the receptor activates signal transduction pathways involving, for example, NF-kβ, AP-1 and p38 MAPK. Likewise, TNF signals through the TNFR1 and TNFR2 receptors and after binding to the receptor mediates apoptosis and differentiation or proliferation, by activating signaling pathways similar to IL-1β. As mentioned above, these cytokines are major mediators during infection and inflammation and elevated levels of these cytokines have been found in systemic shock, autoimmune diseases and chronic inflammatory diseases. For this reason, new therapies aiming to block IL-1β and TNF are under investigation. Currently, antibodies specific for TNF have been approved for the treatment of Crohn’s disease and anti-TNF therapy using soluble TNFR2 have also been approved for treatment of RA.

IL-6

IL-6 is one of the key cytokines mediating fever and acute phase responses. In many cases it mediates its effects in combination with IL-1β and TNF. IL-6 plays an important role in the resolution of inflammation and is believed to be a regulatory switch directing the early innate defense towards acquired immunity. Many cells can produce and secrete IL-6 in response to IL-1β/TNF stimulation or after triggering by PRRs. Depending on the immune response, IL-6
can act both as a pro-inflammatory and as an anti-inflammatory cytokine. During the initiation of inflammation, IL-6 encourages production of acute phase proteins and activation of macrophages as well as recruiting immune cells such as lymphocytes to the site of infection and/or tissue injury. In the late stage of infection, IL-6 takes a protective role and counteracts the manifestations of certain inflammatory responses by stimulating the development of adaptive immune responses and inducing apoptosis in neutrophils, thereby down-modulating production of reactive oxygen species (ROS) and other toxic compounds and aiding in the resolution of inflammation. IL-6 signaling is mediated by two pathways, either by binding to the membrane-bound IL-6 receptor (IL-6r) or by binding to its soluble form of the IL-6r. Both pathways activate the signal transducing glycoprotein gp130 followed by activation of gene transcription. Because of the many functions of IL-6 it has been implemented in various inflammatory diseases such as RA, MS, inflammatory bowels disease and Castleman's disease.\textsuperscript{34}

**IL-12p70**

The IL-12 family of cytokines has a fundamental role in the induction of Th1-associated immunity by inducing differentiation of Th1 cells. IL-12 is produced primarily by monocytes, macrophages, DCs and B cells. The major functions of this cytokine are to induce IFN-γ production in NK cells and T cells, to enhance NK and Tc-cell cytotoxicity and to induce differentiation of naïve T cells into Th1-effector cells, thus having a central role in cell-mediated immunity. IL-12 is composed of two covalently linked subunits (IL-12p35 and IL-12p40) and together they comprise the biologically active form IL-12p70. Two new members of IL-12 have also been described:\textsuperscript{35} IL-23 and IL-27, which share the same features as IL-12 but are composed of different subunits. IL-23 contains p40 and p19 subunits, while IL-27 comprises the p28 and EB13 subunits. The main signaling pathway induced by the IL-12 family is the JAK/STAT pathway in which IL-12 mainly activates STAT4, whereas IL-23 and IL-27 mainly activate STAT3 and STAT1, respectively. The importance of the IL-12 family is evident from the fact that a deficiency in either IL-12 or its receptors has been shown to lead to impaired cell-mediated immunity and enhanced susceptibility to diseases.\textsuperscript{36}
**Interferons**

The IFNs are a multigene family of inducible cytokines that exhibit a diversity of biological functions in the immune system. Their most important functions are their anti-viral and anti-tumoral activity but they also exert immunomodulatory effects on other immune cells. They are broadly grouped into type I IFN and type II IFN. Type I IFN include IFN-α and IFN-β and are induced, for example, by viral infections, while type II IFN include IFN-γ induced by mitogenic or antigenic stimuli. Most virus-infected cells can produce IFN-α/IFN-β when they are grown in cell culture. In contrast, only certain specific cells produce IFN-γ, including NK cells and activated T cells. The IFNs binds to different receptors, such as the IFNAR-1/IFNAR-2 that are ubiquitously expressed for type I IFNs and the IFNγR-1/IFNγR-2, that are expressed only on activated T cells, macrophages and NK cells, for the type II IFN, but they all share downstream signaling molecules and regulate the same genes. Binding of the IFNs to their receptors leads to a) increased expression of intrinsic proteins, b) induced apoptosis by virus-infected cells, c) cellular resistance to virus infection and, d) activation of NK cells and DCs.

**ANTI-INFLAMMATORY CYTOKINES**

**IL-10**

IL-10 is a unique cytokine with regulatory properties and is one of the most important anti-inflammatory cytokines known today. It works as a negative feedback molecule that effectively blocks the biological functions of cells such as T cells, monocytes and macrophages, usually by blocking the NF-kβ transcriptional pathway. The pivotal role of this cytokine is evident by the fact that the same cells that initiate inflammation also induce the production of IL-10, thereby limiting the extent of the inflammation to ensure a balanced immune response. For this reason, many cell types can produce IL-10 including monocytes, B cells and the majority of all T-cell subpopulations as well as DCs and NK cells. Thus, the principle function of IL-10 is to limit and ultimately terminate the inflammatory response. In addition, it also regulates growth and/or differentiation of B cells, NK cells, cytotoxic and Th cells, DCs, mast cells, granulocytes and endothelial cells. IL-10 signals through the IL-10 receptor, which is composed of at least two subunits of the interferon receptor family which mostly signal via the JAK/STAT pathway. IL-10 modulates the expression of cytokines,
soluble mediators and cell surface molecules with important consequences for the cells’ ability to activate and sustain immune- and inflammatory responses. For example, it potently suppresses key functions in monocytes/macrophages such as the production of several cytokines (IL-1β, IL-6, IL-12, IL-18 and TNF), chemokines (MCP-1, RANTES, IL-8 and IP-10), the expression of MHC class II molecules and the co-stimulatory molecules CD80 and CD86. Further, IL-10 inhibits the production of IL-12 and the expression of co-stimulatory molecules on various DC subsets, and also exerts its anti-inflammatory properties by inducing the differentiation of Treg cells. The capacity of IL-10 as a suppressive agent is also evident by the fact that the sequence of IL-10 has close homology to several virus genomes, including Epstein Barr virus.

TGF-β

TGF-β belongs to a family of regulatory cytokines that have pleiotropic functions in a broad range of cell types. It is mostly recognized for its involvement in maintaining tolerance through the regulation of lymphocyte proliferation, differentiation and survival. In addition, TGF-β plays an essential role in the suppression of inflammation but has lately also shown to have a positive role in these responses. For example, TGF-β induces Treg cells in the presence of IL-2, while in the presence of IL-6 it induces pathogenic IL-17 producing Th17 cells. TGF-β regulates multiple responses in the immune system, including suppression of effector Th cell differentiation, inhibition of T and B cell proliferation and effector cytokine production, such as IL-2, IFN-γ and IL-4 and suppression of NK cells, DCs and macrophages. In mammals, three isoforms of TGF-β (TGF-β1, TGF-β2, TGF-β3) have been identified, among which TGF-β1 is the predominant form expressed in the immune system. TGF-β is synthesized in an inactive form composed of the TGF-β dimer in association with the latency-associated protein (LAP). This latent TGF-β molecule can either be secreted or it can bind to latent-TGF-β-binding protein that deposits the protein complex in the extracellular matrix. For TGF-β to become activated it has to bind to the TGF-β activator that triggers LAP degradation. Binding of the active form of TGF-β to one of its two receptors (TGF-βRI and TGF-βRII) initiates signaling pathways that primarily activate the Smad transcription factors. The importance of TGF-β as a master regulator becomes evident by the fact that knock-out mice lacking TGF-β suffer from extreme inflammatory dysregulation and die prematurely and.
CHEMOKINES

IL-8

IL-8 (CXCL8) is a chemokine highly involved in inflammation. It is produced by a variety of cells of the immune system i.e. monocytes, granulocytes, lymphocytes, fibroblasts and endothelial cells. The primary function of IL-8 is to induce chemotaxis of responsive cells mostly neutrophils, which is its primary target cell. Neutrophils respond to IL-8 by chemotaxis to inflammatory sites and subsequent up-regulation of surface adhesion molecules, increased phagocytosis, production of ROS and exocytosis, which are key functions during inflammation. While neutrophils are the primary target cell, other cell types such as endothelial cells, macrophages, mast cells and keratinocytes also respond to IL-8, which underscores its importance as a chemoattractant factor during inflammation. Like many other chemokines, IL-8 signals through two G-protein coupled receptors (CXCR1 and CXCR2) which are expressed mostly on neutrophils. CXCR1 is specific for IL-8 whereas CXCR2 can bind to other chemokines as well.47

RANTES (CCL5)

RANTES (regulated upon activation normal T cell expressed and secreted) was first considered a T-cell-specific chemokine but is now known to be produced by a number of other cell types, such as epithelial cells, fibroblasts, platelets, macrophages as well as endothelial cells. RANTES induces the migration of leucocytes by binding to specific receptors in the seven-transmembrane G-protein coupled receptors family, namely CCR1, CCR3, CCR4 and CCR5. RANTES is a potent chemoattractant for T cells and monocytes, but also brings other cell types such as DCs, esinophils, NK cells and mast cells to the site of infection.48 Increased RANTES expression has been found in various inflammatory disorders and pathologies for example in atherosclerosis, RA and inflammatory airway disorders.49 In addition, RANTES is important in the defense against viral infections, and has been found to be degranulated from virus-specific CD8+ T cells.50 Further, the importance of RANTES in antiviral immunity can be appreciated by the fact that certain viruses have evolved evasion mechanisms from RANTES. One example is the human cytomegalovirus, which expresses a chemokine homolog that sequesters RANTES.51 One of the receptors for RANTES (CCR5)
has also been discovered to be a co-receptor for HIV. For this reason, therapeutic strategies targeting RANTES and CCR5 are being tested for treatment against HIV infection.\textsuperscript{52}

**MIG (CXCL9) and IP-10 (CXCL10)**

MIG (Monokine induced by IFN-γ) and IP-10 (IFN-γ-inducible protein 10) belong to the CXC or α subfamily of chemokines and are produced by a variety of leucocytes such as monocytes, endothelial cells and fibroblasts in response to IFN-γ and TNF. They both have potent chemotactic activities and direct the trafficking to the site of infection of primarily activated T cells, NK cells and NKT cells. Expression of IP-10 is often seen in many Th1-type inflammatory diseases.\textsuperscript{53} IP-10 and MIG are structurally closely related and share the same high-affinity receptor; CXCR3.

**Monocyte chemotactic protein (MCP)-1 (CCL2)**

MCP-1 is produced by a variety of cell types, either constitutively or after induction by oxidative stress, cytokines or growth factors. Many cells can produce and secrete MCP-1 upon stimulation such as fibroblasts, epithelial, endothelial and smooth muscle cells but the two most important sources are monocytes and macrophages. MCP-1 signals through the CCR2 receptor which activates MAPK and ultimately the NF-κβ transcription factor. It is a potent chemoattractant for monocytes but it is also important for memory T cells and NK cells. Along with IL-8, MCP-1 is very often found in tissues during inflammation, where it triggers adhesion of monocytes to vascular endothelium, thus having an important role in extravasation. MCP-1 expression has been noted in many inflammatory diseases during progression including athrosclerosis, RA and cancers.\textsuperscript{54, 55} In these cases, the massive influx of macrophages to the site of infection is thought to be partly responsible for the tissue damage and the exacerbation of the disease.
PREGNANCY AND THE IMMUNE SYSTEM

IMMUNE TOLERANCE

A fundamental role of the immune system is to protect the host from pathogens. Maternal immunity during pregnancy is therefore somewhat complex since it both needs to accommodate and accept fetal antigens and at the same time maintain protection against microbes and other pathogens. In other words, the maternal immune system requires substantial regulation for fetal survival but not to such an extent that it compromises protection of the mother. The mechanisms involved in tolerance of the fetus by the maternal immune system remain somewhat elusive. Studies by Owen, who showed that dizygotic cattle twins retain cells from the other twin post-natally led Billingham, Brent and Medawar to formulate the idea of immunological tolerance. However, Sir P. Medawar was the first to recognize the idea of fetal-maternal tolerance and proposed the concept of the fetal allograft to explain the special immune relationship between the mother and the fetus. The idea he proposed was that the semi-allogeneic fetus was able to survive because the immunological interaction between the mother and the fetus was suppressed, possibly due to lack of presentation of fetal antigens to maternal cells due to a physical separation of maternal and fetal tissue by the placenta. Although his explanations were not fully correct, their hypothesis had a profound influence on this research area during the following years. These original ideas were later extended and revised by Wegmann et al., who postulated that pregnancy is purely a Th2 phenomenon meaning that the Th1 cells that mediate cytotoxic responses are inhibited, while the number of Th2 cells are increased to support the production of antibodies and sustained pregnancy. This Th2-biased hypothesis has been the belief for many years and received support from clinical evidence of women with RA who experience a temporary remission of their symptoms during pregnancy, while systemic lupus erythematosus, in which the principal pathology is mediated by excessive autoantibody production (humoral-mediated), tends to flare up during pregnancy. The Th2 hypothesis is currently under debate. However, it has been clearly shown that the Th1/Th2 ratio is changed in the decidua during pregnancy, which is probably supported by increases in local progesterone levels (a potent Th2 inducer) and secretion of Th2 cytokines, such as IL-4, IL-5 and IL-10. However, a strict Th2 dominance throughout pregnancy does not seem to be essential and recent data indicate that a successful early pregnancy requires help of cytokines which traditionally are associated with Th1 responses. For example, IFN-γ has previously been
considered as a potentially harmful cytokine during pregnancy, since it could lead to pregnancy complications through embryotoxic activity or damage of the placental tissue, but in murine models it has shown to be needed for proper vascularisation during placental development.\textsuperscript{66} Th1 cytokines are thought to be detrimental for a sustained pregnancy since administration of IL-2, IFN-\(\gamma\) and TNF-\(\alpha\) to pregnant mice has been shown to lead to fetal resorption,\textsuperscript{67} and high levels of Th1 cytokines have been found in women who develop spontaneous abortions.\textsuperscript{68-70} For this reason, anti-inflammatory factors such as IL-10 and TGF-\(\beta\), are needed to sustain pregnancy. In addition, others have suggested that the Th1 cytokines might be irrelevant to pregnancy and that the appearance of Th1 cytokines may be a reflection of cellular immunity that becomes activated as a response to pregnancy complications, such as pre-eclampsia and spontaneous fetal loss.\textsuperscript{71} Therefore the original prediction that pregnancy is a Th2 phenomenon might have been too simplified. A recent review by Mor et al.,\textsuperscript{72} suggests that pregnancy is both a pro- and anti-inflammatory condition, depending upon the stage of gestation. Furthermore, one important immune evasion strategy by the fetus is the absence of classical MHC class I (HLA-A and HLA-B) and class II molecules on the trophoblast. The expression of non-classical HLA-G does not appear to stimulate Tc activity\textsuperscript{73} and actively inhibit NK cells\textsuperscript{74} and is therefore believed to be involved in implantation through modulation of maternal decidual leucocytes.\textsuperscript{75} Other factors suggested to help in sustaining maternal tolerance of the fetus include expression of Fas ligand on fetal cells to eliminate activated T cells\textsuperscript{76} and local production of the T-cell suppressive substance indoleamine 2,3-dioxygenase (IDO) expressed by fetal syncytiotrophoblasts and decidual macrophages.\textsuperscript{77} Also, placental exosomes, which display proteins that down regulate the cytotoxicity of T and NK cells, have been found in maternal circulation.\textsuperscript{78, 79}

**IMMUNE ACTIVATION DURING PREGNANCY**

Increasing knowledge has confirmed that both the innate and the adaptive immune system are essential for a successful pregnancy. The maternal immune system changes both locally in the placental environment and in the peripheral circulation. Maternal adaptive immune responses are generally suppressed, while the innate components of the immune system are activated systemically.\textsuperscript{80} During normal pregnancy, the human decidua, or implantation site, is populated by large numbers of immune-competent cells such as macrophages, NK cells and Tregs,\textsuperscript{81-84} but there are no B cells present. These cells (macrophages, NK and Tregs) have
shown to be essential during the first trimester since depletion of them results in termination of the pregnancy,\textsuperscript{85} which suggests that they have effects on placental development, implantation or decidual formation. A special NK-cell subset exists, named the uterine NK (uNK) cell, which is the major immune cell type in the decidua representing almost 70\% of all decidual leucocytes. uNK cells are phenotypically and functionally different from peripheral NK cells\textsuperscript{86} and have been shown to be important for the vascularisation early during pregnancy\textsuperscript{87}, and decreased numbers of uNK cells have been associated with intrauterine growth restriction.\textsuperscript{88} Macrophages represent about 20-25\% of the decidual leucocytes but their numbers may vary depending on hormones affecting the influx of leucocytes and monocytes into the decidua. Also, cytotrophoblasts may stimulate migration of macrophages by local production of MIP-1.\textsuperscript{89} Since there are very few immune defense systems within the placental tissue it is thought that the macrophages residing in the decidua have an important role in non-specific host defense within the placenta. They could facilitate clearance of apoptotic cells and debris as well as secreting cytokines and immunosuppressive prostaglandins, which could hamper the function of Tc cells and uNK cells.

\textbf{THE PLACENTA}

The placenta is a highly specialized organ, primarily of fetal origin, that develops after the successful implantation of the blastocyte into the endometrium (Fig 1). The outer layer of the blastocyst becomes the trophoblasts that form the outer layer of the placenta. This can be further divided into two layers the underlying cytotrophoblasts and the overlaying syncytiotrophoblasts. Soon after the implantation, fetal blood vessels are established called villi, which are surrounded by the syncytiotrophoblasts. Maternal blood will perfuse the decidua, via the spiral arteries, and into the intervillous space (IVS) at around 10 weeks of gestation.\textsuperscript{90} Failure in the remodeling process of the vessels might lead to pregnancy complications such as early pregnancy loss, intrauterine growth restriction and pre-eclampsia.\textsuperscript{91,92} As a result of this remodeling, fetal villi are bathed in maternal blood which creates the platform for maternal-fetal exchange of nutrients, respiratory gases and waste products. The placenta further stimulates production of hormones, growth factors and cytokines that aid in this uptake and delivery system. Thus, the primary function of the placenta is to supply the fetus with oxygen and nutrients throughout delivery, while discarding waste products. The placenta also serves as an interface between the mother and
the fetus and separates the fetus blood supply from the mothers. However, this barrier is not complete and small molecules can passively diffuse through the placenta. One exception is antibodies that are actively transported across the placental barrier to give passive protection to the fetus from the mother that usually persists for up to 6 months after birth. The main antibody class involved is IgG, but small amounts of IgA and IgM antibodies are also thought to be able to cross the placenta.\textsuperscript{93} The placenta also produces high levels of several mediators that are thought to drive Th2 immunity, while dampening Th1 responses, such as IL-4, IL-10, prostaglandins and progesterone\textsuperscript{94-96} to support fetal tolerance.

\textbf{Figure 1.} The structure of the human placenta. Adapted from:

MALARIA

MALARIA IS A MAJOR HEALTH PROBLEM

Malaria remains one of the most common infectious diseases in the world. In 2009, an estimation of 225 million cases of malaria were reported by the World’s Health Organization and despite endless efforts to prevent its spread almost one million deaths still occurs annually. Malaria is caused by a protozoan parasite of the genus *Plasmodium* and currently five species (*P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi*) are known to cause human malaria. *P. falciparum* is the deadliest of the five known species, causing the most severe form of the disease and is responsible for most of the deaths. The parasites are widely distributed in tropical and subtropical regions making African countries especially affected. In 2009, malaria was reported to be endemic in 35 African countries. In malaria-endemic areas, it is mostly children under the age of five and pregnant women who experience the most severe complications associated with the disease. Complications such as severe anemia and cerebral malaria are thought to be the major cause of morbidity and mortality in children, but the host’s immunological response could also be contributing to the pathology. Following repeated infections, immunity increases with age, however, sterile immunity is almost never achieved and parasites can still be found in the blood circulation without the infected person displaying any clinical symptoms.

THE LIFE CYCLE OF THE MALARIA PARASITE

The life cycle of *P. falciparum* is complex and involves both human and mosquitoes as hosts to be able to replicate and survive. The lifecycle starts when an infected *Anopheles* mosquito takes a blood meal and injects sporozoites, via the saliva, into the human host (Fig. 2). Within 2-30 minutes the sporozoites migrate to the liver, where they infect hepatocytes and the asexual stage of the life cycle is started. After approximately one week, the infected hepatocytes burst and fully developed merozoites are released which starts to invade erythrocytes in the blood circulation, thereby initiating the intra-erythrocytic stage of the life cycle. This stage is responsible for all the clinical manifestations often seen during a malaria episode. Inside the erythrocytes, young parasites undergo a series of maturation steps resulting in the formation of trophozoites and ultimately into schizonts. When the schizonts rupture, newly made merozoites are released ready to infect new erythrocytes. This cycle of
rupture and reinfection of erythrocytes has a periodicity of 48-78 hours, depending on the *Plasmodium* species, and gives rise to the typical fever episodes characteristic for malaria. Some parasites differentiate into female and male gametocytes that can be picked up by a new feeding mosquito. These gametocytes fuse inside the mosquito’s midgut and after a series of developmental steps new sporozoites are formed and migrate to the salivary glands, thereby completing the parasite’s life cycle.

**Figure 2.** The life cycle of *P. falciparum*. Adapted from Jones MK *et al.* Reprinted with permission from NPG.

**CLINICAL MANIFESTATIONS OF MALARIA**

The majority of *P. falciparum* infections cause flu-like symptoms such as fever, headache, malaise and nausea that may resolve by itself without the patient taking any drug-treatment. In some cases however, the infection can lead to severe and life threatening disease such as cerebral malaria, severe malarial anemia and acute respiratory distress, which mostly occur in young children and in the pregnant women. The outcome of an infection is never the same for
two individuals and disease severity may vary depending on the host genetics, age, previously acquired immunity as well as transmission rate in the area and parasite strain. For that reason, the disease can vary from being mild asymptomatic, to symptomatic and to severe disease. In high malaria-endemic areas, infants and young children are more susceptible to develop the life threatening diseases, while in low-malaria transmission areas, even adults may develop severe symptoms.

**INNATE AND ADAPTIVE IMMUNE RESPONSES IN MALARIA**

Innate and adaptive immune responses can together limit the peak of parasitemia and reduce the load of circulating parasites. How this may be achieved is presented in Figure 3. An immune response to the malaria parasite begins when the mosquito injects sporozoites into the skin and/or blood stream. Antibodies against antigens on the sporozoites surface, such as the circumsporozoite protein, are able to limit infection by inhibiting invasion of hepatocytes. In addition, early interactions between circulating parasites and cells of the innate immune system including DCs, monocytes, macrophages, NK cells, NKT cells and γδ T cells are important in the initial limitation and control of parasite loads. These cells can directly destroy infected hepatocytes through perforins and granzymes or by cross linking death receptors, thereby inducing apoptosis. The major role of the innate immune system during infection with *P. falciparum* appears to be the production of cytokines such as IL-12 and IFN-γ, which are critical for the development and regulation of type 1 immune responses involving CD4+ T cells, B cells and other effector cells. The APC, especially the DCs, become activated by parasite ligands that are recognized by TLR leading to subsequent up-regulation of their co-stimulatory molecules and adhesion molecules and their capacity to secrete cytokines, in particular IL-12. CD4+ T cells are of major importance for the induction of blood-stage immunity, while the CD8+ subset has been shown to be cytolytic against liver stages of the parasite. CD4+ T cells produce cytokines which are involved in further activation of the innate immune system but also in the support of anti-malaria-specific antibody production. They also produce IL-2 that further activates NK cells to produce IFN-γ, which activates macrophages to efficiently kill parasites by secretion of NO and ROS. In addition, IFN-γ-producing CD4+ T cells also assist in the induction and regulation of CD8+ T cells and of NK and NKT cells. IFN-γ is a key cytokine during *P. falciparum* infection and is associated with reduced susceptibility to malaria.
Role of cytokines

Cytokines are major mediators during *P. falciparum* infection but many contradictory results have been obtained regarding their involvement in protection and/or pathology as a result of using different animal models and different *Plasmodium* strains. However, available data are consistent with a requirement for an early production of pro-inflammatory Th1 cytokines, including TNF, IL-12 and IFN-γ that may limit the progression from uncomplicated malaria to severe and life-threatening complications. High levels of IFN-γ as part of a Th1-driven immune response have been associated with a more favorable outcome in most animal models of malaria, an effect that has been attributed to the monocyte-macrophage activating capacity of IFN-γ, that rapidly kills malaria blood stage parasites by NO and ROS production. Likewise, in humans, high IFN-γ levels have been associated with resistance to reinfection in children and to be increased in uncomplicated compared to severe malaria cases, further supporting a critical role for IFN-γ in limiting progression of the disease. Furthermore, an important role for IL-12 in early responses against *Plasmodium*
has been proposed. IL-12 appears to be critically linked to IFN-γ production, thereby allowing an early and sustained Th1 response.\textsuperscript{107} Injection of IL-12 into mice before infection with \textit{Plasmodium} provides full protection through an IFN-γ dependent mechanism.\textsuperscript{108} In young African children who presented with mild or severe \textit{P. falciparum} infection, the plasma levels of IFN-α and IL-12 were found to be lower in children with severe rather than mild malaria, and IL-12 correlated inversely with parasitaemia.\textsuperscript{109} A similar picture as for IFN-γ emerges for TNF-α in early responses against \textit{Plasmodium}. Treatment with anti-TNF-α antibodies results in a tendency towards longer times for parasite clearance. Furthermore, increased plasma levels of TNF-α together with IFN-γ, IL-1β and IL-6 and reduced production of IL-4 and TGF-β have been reported in patients with cerebral malaria.\textsuperscript{110} Elevated levels of IL-1 have also been associated with increased severity of the disease.\textsuperscript{111, 112} Paradoxically, the same cytokines that are implicated in protection against malaria have also been found to be involved in the pathogenesis of the disease. For example, TNF seems to be important in the early responses to malaria and in late pathological manifestations. Excess production of TNF is likely to be involved in the appearance of symptoms such as fever and headache associated with severe disease.\textsuperscript{113} In addition, low levels of IL-10 or a low IL-10/TNF ratio are associated with malarial anaemia in African children,\textsuperscript{114-116} which suggests that the clinical course of severe malaria is influenced by a relative imbalance between TNF and IL-10 levels. It is widely accepted that Th2 cytokines down-regulate Th1-derived cytokines. Elevated levels of IL-10 have been reported in severe malaria.\textsuperscript{117} \textit{In vitro} studies and studies in mice have shown that IL-10 inhibits TNF-α production and also down regulates the MHC class II expression on monocytes/macrophages.\textsuperscript{118} Thus, IL-10 appears to have important roles in counteracting the potentially harmful host pro-inflammatory response to malarial antigens. Overall, the outcome of \textit{P. falciparum} infection may depend on the fine balance between pro and anti-inflammatory cytokines.

The role for chemokines during infection is to direct the innate and adaptive cells to sites were they are needed.\textsuperscript{119} A range of chemokines are elevated during malaria infection, especially some pro-inflammatory chemokines such as MIP-1α, MIP-1β and IL-8.\textsuperscript{120-123} MIP-1α and MIP-1β are critical chemoattractants for monocytes and after recruitment they activate monocytes/macrophages to produce TNF-α, IL-1 and IL-6.\textsuperscript{124}
Role of antibodies

The most direct evidence that antibodies are important mediators in protective immunity to malaria comes from old classical passive transfer experiments, in which antibodies from malaria-immune adults were given to children with severe malaria. The treatment was found to successfully reduce the parasite loads to nearly undetectable levels in these children. Additional studies in mice deficient in their Fcγ receptors further supported a role for antibodies in protection. Several mechanisms may explain how these antibodies are thought to neutralize the parasite. Antibodies reactive to malaria antigens may inhibit hepatocyte and erythrocyte invasion by binding to the surface of free sporozoites and merozoites. They can also bind to surface antigens on already infected erythrocytes, thereby inhibiting the intraerythrocytic development of the parasite. They may also facilitate phagocytosis of whole infected erythrocytes or merozoites by promoting opzonisation and increase the phagocytic capacity of monocytes and macrophages. In addition, antibodies may, beside their direct role in inhibiting parasite invasion and/or growth, also induce ADCC via the FcR on the effector cell and the induction of toxic mediators such as TNF or NO. Further, antibodies may also block parasites from binding to scavenger receptors, such as CD36 or ICAM-1 on endothelial cells, which the parasites commonly use as binding sites to avoid clearance by the spleen and establish an infection.

Several epidemiological studies have underlined the importance of having high circulating levels of malaria-specific antibodies, especially levels of IgG, in relation to protection against severe malaria. This protection seems to be largely dependent on the balance between the cytophilic i.e IgG1 and IgG3 and the non-cytophilic i.e IgG2 and IgG4 antibodies. Of note, the cytophilic IgG1 and IgG3 subclasses have been associated with protection against *P. falciparum* malaria in various endemic areas and some have even suggested that the non-cytophilic antibodies could possibly compete with the cytophilic types, thereby blocking their protective effects. Interestingly, in some areas IgG2 antibodies in combination with certain genetic mutations in the FcγRIIa have found to be protective and especially in combination with low IgG4 levels. In contrast, IgG4 does not seem to have any protective role.
PROJECT BACKGROUND

MALARIA DURING PREGNANCY

25 million pregnant women are currently at risk to develop malaria and malaria during pregnancy accounts for almost 10,000 maternal and 200,000 neonatal deaths every year. However, these figures might be underestimated when considering the significant impact of malaria on perinatal morbidity and mortality as well as exaggeration by co-infections, such as HIV.

The malaria parasites seem to have evolved ways to evade the immune system and especially the complex placental environment. That the pregnant women down-regulates her cell-mediated immunity is known, but the only evidence for increased susceptibility to infections during pregnancy is for intracellular parasites, such as *Plasmodium*. This suppressive state of Th1 immunity and up-regulation of Th2 responses has been used as an explanation for the women’s increased susceptibility. However, this explanation can not by itself explain some of the features associated with malaria during pregnancy. For example, why the prevalence is higher in primigravidae compared to secundigravidae or multigravidae or why the severity of the disease including symptoms and other complications tends to decrease over successful pregnancies is not known. The understanding of why pregnant women become more susceptible to develop malaria is still incomplete, but one possible reason is that *P. falciparum* infected parasites are able to bind to special proteins in the intervillous spaces of the placenta, creating the condition known as placental malaria (PM). This condition is probably one of the most complex interactions between a host and a pathogen, taking place in the placenta known today.

One reason for *P. falciparum’s* ability to invade and infect the host so efficiently is the remarkable ability of these parasites to adhere and sequester. During the erythrocytic stage, young ring-stage parasites circulate in the blood and mature into trophozoites, which start to express adhesion molecules that are displayed on the erythrocyte membrane. These adhesive ligands make it possible for the parasites to adhere to endothelial tissue. One of the major family of proteins that have been identified as one of these adhesive ligands on the erythrocyte is the *P. falciparum* erythrocyte membrane protein 1 (*PfEMP1*), encoded by the *var* multigene family. Adults residing in endemic areas acquire a wide range of antibodies towards these proteins after multiple exposures and are therefore said to be semi-immune.
However, in the pregnant women, the development of a new organ, the placenta, represents a new niche to which infected erythrocytes (iE) can and will adhere. These parasites express a unique repertoire of *Pf*EMP1 proteins that the primigravidae women have not encountered before. A particular variant of *Pf*EMP1 has been named var2CSA which has the capacity to bind to chondroitin sulphate A (CSA) expressed on the surface of trophoblasts in the IVS. Binding of iE in the placental tissue leads to physiological changes, such as thickening of the trophoblast membrane, intervillous infiltrates of immune cells, and deposition of malarial pigment, called hemozoin, in phagocytic cells. As a consequence of placental malaria, babies are usually born with a low birth weight (LBW) and the babies have been shown to have a reduced chance of surviving their first 2 years of life. LBW can be caused by intrauterine growth retardation or pre-term delivery as a result of placental insufficiency and impaired blood flow, but the exact mechanism is still not known. What really occurs inside the IVS once the parasites adhere is not completely clear. The binding of iE to CSA in the placental is thought to stimulate syncytiotrophoblasts and cells in the placenta to release various chemokines. Elevated levels of chemokines have been described, especially levels of MCP-1, MIP-α and β as well as IP-10 in placentas from infected women. MIP-1α and β recruits additional macrophages and MCP-1 plays an important role in recruiting monocytes to the infected placenta. Overall these factors are responsible for the massive infiltration of inflammatory cells into the placenta. The inflammatory milieu with increased plasma cytokine levels causes a shift of the placenta cytokine balance which is necessary for elimination of parasites. On the other hand, excessive levels may also contribute to pathology, especially high TNF levels have been shown to be a risk factor for LBW. Thus, the balance between pro and anti-inflammatory factors seems to be important.

**MALARIA AND ETHNIC GROUPS IN WEST AFRICA**

The genetic and cultural background of the two sympatric ethnic groups Fulani and Dogon are by now very well established. In brief, the Fulani are nomadic pastoralists that have been settled in the study area in Mali (where samples included in paper II were taken from) for the last 200 years, but they have also settled in other countries of West Africa. The Dogon are on the other hand, traditional farmers who migrated to this particular study area more than 50 years ago. There is no intermarriage between the two ethnic groups. The Fulani of West
Africa is a particular interesting ethnic group because of their lower incidence of malaria compared to other sympatric ethnic groups living in Mali\textsuperscript{152} and in Burkina Faso.\textsuperscript{153} These tribes live under similar social, cultural and geographical conditions but the Fulani have been shown to be less susceptible to developing malaria. They suffer less clinical symptoms, have fewer parasites in their blood\textsuperscript{152, 153} and have been found to have a stronger humoral immune response as compared to other ethnic groups\textsuperscript{132-134, 154}. Furthermore, they also have increased spleen rates\textsuperscript{155} and increased numbers of malaria-specific IL-4 and IFN-γ producing cells\textsuperscript{154} but lower frequencies of Tregs\textsuperscript{156} compared to their neighboring ethnic groups. In addition, APC subsets from Fulani and Dogon children has also been shown to respond differently to different TLR stimulations,\textsuperscript{157} suggesting that there is a difference in the immune regulation between these two groups. However, the fundamental reason for the Fulani’s relative resistance to \textit{P. falciparum} is as yet unknown.
PRESENT STUDY

OBJECTIVES

The overall aim of this work is to examine the impact of *P. falciparum* infection on inflammatory responses in pregnant women and children residing in African countries.

More specifically we aimed to:

- Carry out comprehensive analysis of inflammatory markers in pregnant women throughout their pregnancy and at delivery to find possible biomarkers for pregnancy-associated malaria (Paper I).

- Examine cytokine, chemokine and antibody levels in children from two African ethnic groups, which differ in their susceptibility to *P. falciparum* infection to investigate their involvement in protection and/or pathology of malaria (Paper II).
SUBJECTS AND METHODS

Subject demographics, information of the study areas and methods used are found in detail in the corresponding papers. In this section, there will only be a brief description of the study subjects and experimental procedures.

The first study was a longitudinal cohort study of pregnant women and their babies, conducted in Tanzania and Benin from 2008 until 2010. At both study sites, 1000 pregnant women were enrolled and followed up during pregnancy with a series of scheduled antenatal visits up until delivery. Here we present data from the cohort in Tanzania on a subgroup of 121 pregnant women (42 infected and 79 uninfected). The infected women were selected based on having a positive RDT and came to the hospital for all three antenatal visits and at delivery. Infected women were individually matched to 2 separate uninfected controls of similar age (±4), gestational age (±2 weeks) and the same gravidity.

The second study was conducted from October to November 2008 in a rural area of the Dogon valley in Mali where people from the Dogon and Fulani ethnic groups live together in sympatry. In total, 77 children (age 2-10) were randomly included in the study and were divided according to their ethnicity and slide reading result. Out of these children, 40 children belonged to the Dogon ethnicity of whom 20 were slide positive for *P. falciparum* and 20 were negative. Consequently, 37 children belonged to the Fulani ethnic group of whom 14 were slide positive and 23 were negative.

From both study sites, venous blood from the participating pregnant women and children presented with or without *P. falciparum* infection was obtained. For the pregnant women, venous blood was obtained from each time point the women came to the hospital. Plasma was collected and the samples were shipped back to Sweden and analyzed for inflammatory markers (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF, IFN-α, IFN-γ, RANTES, MIG, MCP-1, IP-10, vascular endothelial growth factor (VEGF/Flt1), urokinase receptor (uPAR), angiopoietin (Ang)-1 and Ang-2) and antibody titers (malaria-specific total IgG, IgM and IgG subclasses (IgG1-IgG4)) using ELISAs or CBA.
RESULTS AND DISCUSSION

Paper I

Pregnancy is a unique state in life in which the women carries, nourishes and supports the growing fetus until it is time for delivery. The mother’s immune system is strongly modulated to perform this task considering that it has to be able to accept fetal antigens in her womb without attacking it. The placenta has a key function during this process as a source for immunoregulatory molecules. It sustains fetal tolerance and at the same time supports the fetus with nutrients, while working as a barrier for invading pathogens. The \textit{P. falciparum} parasite has evolved ways of evading the immune system during pregnancy and uses the placenta as a hiding place to avoid clearance by the spleen by binding to proteins in the placental tissue.\textsuperscript{139,140} For this reason, and due to the many difficulties in developing a vaccine in combination with the wide spread drug resistance, it leaves the pregnant women and her fetus vulnerable. Therefore there is a major need for malaria prevention and rapid diagnosis followed by accurate treatment of pregnant women. Over the past century important studies have accumulated and have laid the groundwork for the knowledge we possess regarding placental malaria. Many have characterized the effect of infection with \textit{P. falciparum} and/or placental malaria on the mother and her fetus at enrollment or specifically at delivery using cross-sectional designs.\textsuperscript{158} In this study we aimed to assess aspects of the timing of infection during pregnancy by using a longitudinal design.

We started out to investigate selected markers in peripheral blood plasma from pregnant women without malaria since such data are currently lacking in an African population. The factors we analyzed were IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF, IFN-α, IFN-γ, RANTES, MIG, MCP-1, IP-10, VEGF/Flt1, uPAR, Ang-1 and Ang-2. We found that most of the factors were stable during the course of pregnancy, from inclusion all the way up until delivery, suggesting that many of the factors are tightly regulated. Especially factors previously associated with poor pregnancy outcomes such as IFN-γ and IFN-α were very stable. Interestingly, some factors that we measured, especially levels of IL-6, IL-8, IP-10, uPAR and VEGF/Flt1 increased at delivery compared to the other time points. These findings are in agreement with those of others\textsuperscript{159} who have reported increased levels of some of these factors at the end of a normal pregnancy, probably as a result of initiating labor.
To be able to investigate the influence of an infection on the selected markers we analyzed the factors in pregnant women with or without a *P. falciparum* infection. It is widely accepted that infection with *P. falciparum* during pregnancy can alter the cytokine and chemokine balance both in the placenta as well as in the periphery\textsuperscript{121, 148, 150, 151, 160, 161} and that this might lead to poor pregnancy outcomes.\textsuperscript{141, 143, 151, 160, 161} As a result of infection, we observed increased levels of MIG, MCP-1 and IP-10 in infected women compared to uninfected controls. One fundamental feature often seen during placental malaria is the massive inflammatory infiltrates of monocytes and macrophages into the placenta, probably as a cause of parasite adherence.\textsuperscript{141-143} The increased concentration of the measured chemokines might aid the recruitment of these cells into the placenta as a means for the phagocytic cells to move to the infected site and efficiently kill the parasites. Increased levels of several chemokines have previously been reported during placental malaria\textsuperscript{121, 122, 148} and especially IP-10 have been shown to be produced by intervillous blood mononuclear cells isolated from infected placentas\textsuperscript{149} further supporting this. Of all the molecules we quantified, the level of RANTES was the only one that decreased in association with infection. RANTES appears to be important in various inflammatory diseases, however the role for RANTES during malaria is not completely known. Low levels of RANTES have previously been reported in severe malaria cases\textsuperscript{123, 162} and of note in association with cerebral malaria.\textsuperscript{163} Studies in mice deficient in RANTES production have shown a dysfunction of virus-specific CD8\textsuperscript{+} T cells with imbalanced cytokine production and viral control.\textsuperscript{164} RANTES is considered to be a chemoattractant for T cells but also act on monocytes and DCs. These studies suggest that RANTES has an important role in regulating and/or sustaining immune responses during viral infections.

We also confirmed increased levels of IL-10 in the infected women compared to controls, which has been reported earlier.\textsuperscript{165} However, in this study by using a longitudinal design, we also showed that IL-10 together with IP-10 levels increases in the infected women irrespective of their gestational age. IL-10 is a cytokine that has been involved in both protection and in the pathogenesis of malaria. High levels might be beneficial for the host by reducing exaggerated harmful inflammation, but may also be detrimental by suppressing the anti-parasitic specific responses that are needed for parasite clearance. On the other hand, IP-10 is pro-inflammatory and might be induced when schizont ruptures and malaria antigens are released. Thus, this factor has previously been associated with mortality in *P. falciparum* mediated cerebral malaria.\textsuperscript{166, 167} In addition, IP-10 has also been shown to be produced in high
amounts of uNK cells.\textsuperscript{168} Both IP-10 and IL-10 might be important markers for inflammation during placental malaria.

From a clinical point of view, it is known that primigravidae women are more susceptible to malaria infection with more severe symptoms and clinical complications when infected as compared to multigravidae women.\textsuperscript{169, 170} In line with this, we show that babies born from infected primigravidae women have a lower birth weight compared to secundigravidae and multigravidae women. Likewise, we could also show that primigravidae women have lower heamoglobulin levels and white blood cell count when infected as compared to secundigravidae and multigravidae women. This may suggest that the infection is more severe during the first pregnancy and that this could affect the health of both the mother and of her fetus.

Overall, to the best of our knowledge there are no comprehensive prospective, longitudinal studies that describe cytokine and chemokine profiles during pregnancy and at delivery. In this study, we evaluated a panel of selected markers in plasma from infected and uninfected pregnant women and found that levels of IL-10 and IP-10 might be valuable for diagnostic purposes when investigating inflammation during pregnancy malaria.
Studies in African countries comparing different aspects of the immune responses elicited by the malaria parasite have been conducted in the Fulani and their sympatric ethnic groups. In these studies it has been shown that the Fulani are less susceptible to develop malaria compared to their neighboring groups, although they live under the same parasite pressure. However, most of the studies performed on these ethnic groups have been done on asymptomatic adults and very little is known about these responses in children from these ethnic groups. The focus of our study has therefore been to investigate the inflammatory and antibody responses in infected and uninfected children from the Fulani and Dogon ethnic groups living in Mali. More specifically our study aimed to investigate whether these differences that have already been established in adults could also be evident during childhood.

Cytokines and chemokines are important mediators during *P. falciparum* infection and the balance between pro and anti-inflammatory cytokines may be important in terms of the clinical outcome of the disease. In this study the inflammatory cytokines; IL-1beta, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-α and IFN-γ and the chemokines; RANTES, MIG, MCP-1 and IP-10 were analysed in plasma samples from children belonging to the two ethnic groups. For IL-1β, IL-10 and TNF no conclusive results could be found due to the fact that many samples had levels of these cytokines below the detection limit. When comparing the Fulani and the Dogon children, regardless of infectious status, we found higher levels of all tested cytokines in the Fulani children compared to Dogon. The same pattern of increased cytokine levels were also found in infected Fulani children when compared to infected Dogon children. This indicates that Fulani children have higher baseline levels of these tested cytokines compared to Dogon children.

Interestingly, when comparing infected children between the ethnic groups, only IFN-γ was significantly higher in infected Fulani. This is in agreement with what has been reported by McCall and colleagues who reported that mononuclear cells from Fulani are able to produce much more IFN-γ after stimulation with parasite antigens compared to cells from the Dogon. High levels of IFN-γ are crucial early during an infection to limit the first wave of parasitemia. The increased levels seen in infected Fulani compared to infected Dogon might be one reason for Fulani’s relative better protection against malaria.
Chemokines are essential mediators in directing cells in the immune system. However, chemokines and their distribution in plasma have never been investigated in Dogon and Fulani ethnic groups. Our results showed that the Fulani children have higher basal chemokine levels compared to the Dogon. Further, upon infection the levels increased in infected Dogon compared to uninfected counterparts, while this marked up-regulation was not evident in infected Fulani compared to their uninfected peers. The stable levels of MIG, MCP-1 and IP-10, all of which are potent chemoattractants for immune cells, such as monocytes, macrophages and T cells further suggests that Fulani children already are more prepared to combat infections compared to their neighboring ethnic group.

We also measured the anti-malaria specific antibodies; IgG, IgM and IgG subclasses (IgG1-IgG4) in plasma from these children. We confirmed the higher titers of anti-malaria-specific antibodies in Fulani compared to Dogon children, as previously reported by others in adults. In fact, our study showed significantly higher levels of anti-malaria specific total IgG, IgM as well as higher levels of the IgG1, IgG2 and IgG3 but not IgG4 subclasses in Fulani compared to Dogon children. Interestingly, when children were divided into infected and uninfected individuals, we observed decreased levels of IgG3 antibodies in infected Fulani as compared to their uninfected counterparts. This result may suggest that the IgG3 antibodies have bound parasite-derived antigens and formed immune complexes and hence are therefore found in lower levels in circulation.

Taken together, our results clearly show that the Fulani children have a stronger inflammatory response compared to the Dogon. This was evident from the fact that Fulani had higher baseline levels of several cytokine and chemokine levels in their circulation. We also confirmed the higher titers of malaria-specific antibodies in the Fulani children compared to Dogon children. Furthermore, upon infection with \textit{P. falciparum} the infected Dogon children had increased levels of several chemokines tested compared to their uninfected counterpart, and these differences were not evident between infected and uninfected Fulani children. These findings suggest that Fulani are more prone to combat pathogens than Dogon children and that these differences that we observed already are established at an early age.
GENERAL CONCLUSIONS

In this work, we investigated the inflammatory response due to infection with *P. falciparum* in pregnant women and children in two African cohorts. We show that an infection during pregnancy causes a change in the cytokine profile of several of the factors we investigated. When the women’s cytokine profile was assessed longitudinally we found transient increases in the levels of IL-10 and IP-10, regardless of the gestational age of the women. These results suggest that IL-10 and IP-10 might be important biomarkers for inflammation during placental malaria. We also confirmed that primigravid women are more affected by the disease when infected, as reflected by lower haemoglobin levels and white blood cell count as well as more often giving birth to low birth weight babies, compared to infected secundigravidae and multigravidae.

We also show that the inflammatory response differs in children from two ethnic groups with known different susceptibility to contract malaria. We show that Fulani children, like their adult population, have increased cytokine and malaria-specific antibody titers as compared to their sympatric ethnic group Dogon. We also show, for the first time, that Fulani children have stable chemokine levels in their circulation upon an infection, while this was not the case in Dogon children. These findings suggests that there are marked differences in the cytokine and chemokine levels as well as antibody titers between Fulani and Dogon that are established already at an early age.
FUTURE PERSPECTIVES

Our results from both paper I and II showed that the cytokine and chemokine levels changed upon *P. falciparum* infection. However, the malaria transmission in Tanzania (where the first study was conducted) is very low. Therefore, we will continue to investigate these responses in pregnant women living in areas where they are more frequently exposed to malaria parasites (such as the site in Benin) to see if we can repeat the findings we observed in Tanzania in women with multiple infections during pregnancy. To further evaluate this, cells from infected and uninfected pregnant women will be phenotypically analyzed for cell surface markers and co-stimulatory molecules and their association with maternal plasma levels and occurrence of *P. falciparum* infection will be evaluated. These analyses will be performed on inclusion and delivery samples and prospective analyses will be conducted.

Furthermore, from the study site in Tanzania, we have biochemical and haematological data from each participating women from all time points when they came to the hospital. A follow up analysis with the same women as in paper I will be performed to investigate the pathophysiology of *P. falciparum* infection during pregnancy. Different parameters such as haemoglobin, white blood cell counts, platelets, alanine transaminase and creatinine etc. will be analyzed in these women. We will use internal control values from the women’s own antenatal visit at which they were uninfected to create a second control group and determine which factors that might be associated with *P. falciparum* infection. We will also evaluate if primigravidae differ compared to secundigravidae or multigravidae of these parameters.

In addition, the results from paper I showed an increase in the levels of IL-10 and IP-10 when the pregnant women had an infection. It would be interesting to set up *in vitro* assays in which cell populations know to be important in placental malaria (for example monocytes) are stimulated with these two factors. The aim would be to investigate how the inflammatory milieu (caused by the parasites) influences these cells migration, co-stimulatory capacity and secretion of cytokines to broaden the picture on how the parasites would influence these cells. Buffy coats will be used to purify monocytes (and their subsets) that will be put in co-cultures together with IP-10 or IL-10 (to mimic the inflammatory milieu in the pregnant women) and later challenge these cells with parasite antigens to see how this would affect these cells ability to migrate but also to investigate their cell surface receptors, co-receptors and what type of cytokines they produce at protein (from supernatants) or mRNA level.
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Massor med kramar till er!
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