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Full Length Research Paper

Maternal and neonatal immune responses to *Plasmodium falciparum* infection

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Plasmodium falciparum **infection during pregnancy has severe consequences for both mother and fetus and can lead to the trans-placental passage of malarial antigens that are capable of inducing neonatal immune responses. Between July 2003-December 2004, 228 women from Omdurman Maternity Hospital, Sudan were followed monthly during antenatal period till delivery time. We examined neonatal and maternal cytokine and antibody responses to merozoite surface protein-1(MSP1-$_{19}$) in infant-mother pairs. ELISA sero-positivity rate in the maternal plasma samples was 48.9 % and 31.1% for the cord blood samples. Anti-MSP-1$_{19}$ IgG at time of delivery in neonates whose mothers had malarial infections during antenatal period was higher (p=0.000) than those who were parasite negative. In response to *P. falciparum* antigen peripheral blood mononuclear cells (PBMCs) produced significantly higher level of IFN-γ *(p* = 0.0001) than that of the paired cord blood cells (CBMCs). PBMCs from mothers infected during the antenatal period secreted significantly more IFN -γ (*p*=0.0001) and decreased IL-10 production (*p*=0.005) than non infected women. A mixed Th$_1$/Th$_2$ immune response was seen most commonly in women who had confirmed positive blood films *P. falciparum* infections; on the other hand neonates born of malaria-positive mothers mounted predominantly Th2 type immune responses as detected in their cord blood.**

Keywords: Malaria, pregnancy, cord blood, immune response, Sudan.

INTRODUCTION

Maternal malaria is a common complication in regions where malaria is endemic and pregnant women exhibited elevated susceptibility to *Plasmodium falciparum* infection, particularly among primigravidae and young pregnant women (Enato *et al*., 2009; Omer *et al*., 2011). Maternal and fetal tissues within the placenta are intimately associated, and accumulation of infected red blood cells (iRBC) at this interface between the maternal and fetal circulation may result in congenital malaria and exposure of the fetal immune system to malaria parasites and/or soluble malaria antigens (Achidi *et al*., 2005; king *et al*., 2002; Malhotra *et al*., 2005). Several studies pointed out that the production of Th-2 type cytokine (IL-4) and a Th-1 type pro-inflamatory cytokine gamma interferon (IFN)-γ by stimulated lymphocytes *in vitro* looking for a shift towards Th-1 (Raghupathy, 2001). Lymphocytes, however, are only one component of the immune system and circulating monocytes are primed to produce more Th-1 cytokines during normal pregnancy (Sacks *et al*., 2003). Moreover, changes in the levels of cytokines were reported in women with malaria at delivery (Suguitan *et al*., 2003). Malaria congenital infection and prenatal immune sensitization of the unborn child occurs not solely in women who acquire infection during gestation, but also in children born to semi-immune or sub-clinically infected mothers (D'Avanzo *et al*., 2000). At birth, fetal cord blood mononuclear cells (CBMCs) are generally less responsive than those of adults, but they can proliferate and produce cytokines

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and Abs in an Ag-specific manner *in vitro* (Splawski and Lipsky, 1991).

In utero sensitization to transplacentally transferred soluble *P. falciparum* antigens may constitute the basis for increased susceptibility to malaria episodes in early life. Importantly, it has been demonstrated that CBMCs of neonates born to mothers with pregnancy associated *Plasmodium falciparum* malaria (PAM) specifically respond to plasmodial asexual stage antigens, and that cord blood B cells produce anti-malaria specific IgM and IgE antibodies (Mutabingwa *et al*., 2005; Fievet *et al.,* 2009), providing irrefutable evidence of *in utero* sensitization. Malaria specific T cell proliferation and cytokine responses were found in CBMC of new born in Kenya, Gabon, Togo and Cameroon (King *et al.,* 2002; Brustoski *et al* 2005*;* Krish *et al*., 2004). Thus, there is substantial evidence for in utero priming of T cells to malaria, and antigens can cross the placenta and stimulate malaria specific B and T cell responses by producing cytokines and specific antibodies, i.e. parasite specific memory responses (Metenou *et al*., 2007).

The immunoglobulin G (IgG) antibodies to blood stage *P. falciparum* are acquired from the maternal circulation during gestation, although IgM and IgG synthesis can start as early the 10 week of gestation and increases with gestational age (Holt and Jones, 2000). It is generally known that only IgG crosses the placenta and higher IgG levels have been reported in maternal compared with cord sera in Africans (Deloron and Cot, 1990; Desowitz *et al*., 1993). The major merozoite surface protein of *P. falciparum* (MSP1), as a candidate for a malaria vaccine, is the precursor of several surface proteins of the merozoite (Holder, 1994; Kadekoppala and Holder, 2010). The MSP1 C-terminal 19-kDa polypeptide ($MSP1_{19}$) remains on the surface during invasion and is the target of monoclonal invasion-inhibiting antibodies (Blackman *et al*., 1990); antibodies against this fragment may therefore play an important role in protection and blocking *in vitro* growth of *P. falciparum* (Uthapibull *et al* ., 2001).

In this study we investigated the effect of maternal *Plasmodium falciparum* infection during antenatal period on the level of antibody and cytokine immune responses in the maternal peripheral blood mononuclear cells and cord blood cells in Sudanese pregnant women.

MATERIALS AND METHODS

Study design

Ethical approval was obtained from the ethical and scientific committees of the Institute of Endemic Diseases, University of Khartoum and the Directorate of Research, Federal Ministry of Health, Khartoum, Sudan.

Following informed consent of women and spouse, a total of 836 pregnant women attending the routine antenatal clinics at Omdurman Maternity Hospital, the main obstetrical referring hospital in the Sudan, were enrolled in the study during the period from July 2003 to December 2004. Study participants were those who presented at the weekly routine antenatal clinic during the study period. Consenting women provided information relative to their pregnancies including age, number of previous pregnancies and their other demographic data. The medical records of malaria episodes during mothers' pregnancy and treatment taken were recorded.

Recruitment and samples collection

Samples collected on recruitment and during the follow up were examined for malaria parasite using microscopy and PCR. We have shown previously the prevalence of *P falciparum* infection during the study period was 26.2% by microscopy and 56.5 % by PCR (Omer *et al*., 2011). Exclusion criteria were failure to give consent, twin pregnancy, significant antepartum haemorrhage or miscarriage (delivery before 24 weeks' gestation or stillbirth weighing less than 500 g). For logistic reasons, we were unable to recruit more than five women during a 24-h period. A total of 228 paired cord and maternal blood samples were collected immediately after delivery in heparinized tubes. Peripheral blood was collected by venipuncture and collection of cord blood involved direct aspiration via puncture of the ethanol sterilized umbilical vein at a site distal to the placenta to reduce to a minimal the possibility of cross-contamination by maternal antibodies and lymphocytes within 1 h of delivery. Samples were processed within 4 h of collection. The medical records of uninfected mothers at delivery time were reported to verify those who had been appropriately diagnosed and treated for *P. falciparum* malaria episodes during their pregnancy

Isolation of PBMC and CBMC

Peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) were isolated by density gradient centrifugation of heparinized blood on Ficoll- Hypaque layer (Sigma diagnostics Lot.114 H6106, USA). Cell viability was confirmed by trypan blue staining. Viability was > 99% in all tested samples.

Cytokine cultures

To compare cytokine production between maternal and fetal cells, PBMC and CBMC were cultured in triplicate at

a concentration of 1.5 X10^6 cells/ml in sterile 24 or 6–well round-bottomed cell culture plates. Cultures were activated with phytohemagglutinin (PHA Sigma # L 9132, USA) at a concentration of 40µg/ml; MSP-1 $_{19}$ antigen at 10/µg ml or cell culture medium with mononuclear cells was cultured as control. The culture plates were incubated at 37^{0}C supplemented with 5% CO_2. Cultures were harvested after 24 hours for phytohaemagglutinin (PHA) and after 48 h for MSP-$1_{19.}$

Interferon – gamma (IFN-γ) and interleukin 10 (IL-I0) levels were measured from culture supernatants of isolated PBMC and CBMC using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Europe).

ELISA for antimalarial antibodies

To measure antibodies (Abs) response responses to MSP-1 $_{19}$ in the plasma of the mothers and their neonates using ELISA (king *et al.*, 2002), flat bottom microtiter plates (Nunc-Immunoplate Maxisorp) were each coated overnight at 4°C with 100 µl/well of antigen at 0.5µg/ml of MSP-1_{19} antigen in coating buffer (15 mM NaCO3, 35 mM NaHCO3; pH 9.3). Plates were washed three times in phosphate buffered saline (pH 7.2) with 0.5% Tween 20 (PBST). After washing and blocking, plasma diluted 1/100 in 1% BSA was added to duplicate wells and incubation continued over night at 4°C, then washed three times with PBST (paired maternal cord samples were assayed in the same plate). The plates were washed, and then incubated for 3 hours at room temperature with 100 µl per well of conjugate Anti-human IgG. One hundred microlitere of the substrate was added after a final wash. The reaction was stopped by addition of 50 µl of 20% H_2SO_4 and the absorbance was read at 492 nm using ELISA reader. Plasma obtained from 18 European donors with no exposure to malaria parasites were used to calculate the cut-off values for seropositive. Plasma samples that showed an optical density (OD) greater than the cut-off values (mean+ 2 SDs) of these control sera were scored as positive for antimalarial Ab.

Statistical analysis

Pearson's X^2 or Fisher's exact test were used for qualitative variables and analysis of variance for quantitative variables using Epi-Info2002 software. Mc Nemar test was used to calculate possible associations between mother and cord antibodies seropositivity rates. Differences between parasitized subjects and non-parasitized subjects were tested by the Student's T-test or the Mann-Whitney U test. Relationships at the individual level were tested by the Spearman ranked correlation test. All tests were two-tailed and *p* values of less than 0.05 were considered significant.

RESULTS

Description of the study population

At delivery time, 228 women followed during pregnancy were further recruited for immunological studies. Paired cord and maternal samples were collected in delivery room and tested for total anti-$MSP1_{19.}$ Among the women, 124 were primigravidae and 104 were multigravidae. Around fifty per cent (112/228; 49.6%) of these women had malarial infection during antenatal period detected either by microscopy or PCR. However, 11.4% and 19.3% of the women were malaria-positive at time of delivery by microscopy and PCR respectively. Moreover none of the infected cords were detected by microscopy, but 7% were positive for malaria by PCR. Five out of the 44 women positive for malaria at delivery time, 4 primigravidae and 1 multigravidae, had malaria infection during their antenatal period. On the whole, plasmodial infection rates were significantly higher in primgraivade (p=0.005) when compared with their multigravidae counterpart.

Plasmodium falciparum infection in the mother and IgG Abs to MSP-1_{19} in paired cord and maternal blood samples

At delivery, nearly fifty percent (111/228; 48.9 %) of the mothers were seropositive for IgG antibodies to MSP-1_{19}, while only a third (77/228; 33.8%) of the cord blood contained IgG Abs to MSP-1_{19} as determined by an optical density greater than the cutoff value (0.115) derived from negative control sera (Figure.1). As expected, the anti-MSP-1_{19} IgG in mothers at time of delivery was strongly correlated with the IgG levels in cord blood ($p< 0.0001$). This correlation was not affected by microscopy or PCR parasite detection of the mother ($p>0 .05$).

Maternal peripheral infection did not considerably influence IgG seropositivity rates in cord and maternal samples. Among seropositive mothers 27.9% (31/111) and 57.5% (64/111) had microscopically and PCR positive results respectively. Only a small percentages of seronegative women were microscopically or PCR positive 3.4% (4/117) and 12% (14117) respectively (Table 1).

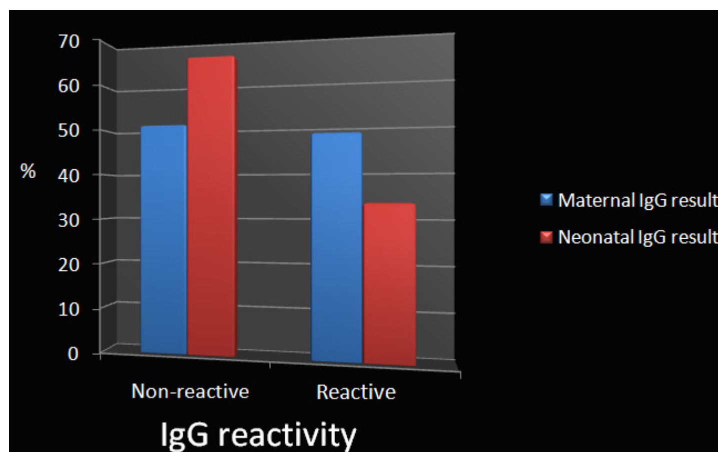

Figure 1: ELISA seropositivity rates of immunoglobulin IgG to *P. falciparum* merozoite surfaces protein 1(MSP-1_{19}) antigen in paired maternal-cord plasma. The cutoff for positivity (mean+2SD for negative control sera) was the OD of 0.115 (n=228 pair samples).

Table 1: Malarial infection during antenatal period determined by microscopy and PCR in relation to IgG seriopositivity of mothers and neonates at time of delivery.

		IGg seriopositivity at time of parturum (n=228)			
		Mothers		Neonates	
Malaria infection during antenatal period		Yes (111)	No (117)	Yes (77)	No (151)
Microscopy [a]	% (n)	% (n)	% (n)	% (n)	% (n)
Positive	15.4 **(**35)	88.6 (31)	11.4 (4)	77.1 (27)	22.9 (8)
Negative	84.6 (193)	41.5 (80)	58.5 (113)	25.9 (50)	74.1(143)
PCR [b]					
Positive	34.2 (78)	82.1 (64)	17.9 **(**14)	70.5 **(** 55)	29.5 **(**23)
Negative	150 (65.8)	44.8 (47)	68.7 (103)	14.6 (22)	85.3 (128)

[a:] asexual *Plasmodium falciparum* parasites detected by thick blood film method
[b:] detection of malaria by PCR using genus- and species-specific primer sets

Cytokines production in PBMC and CBMC of mitogen and MSP-1_{19} antigen

Mitogenic stimulation was used as a positive control to assess the overall responsiveness and cytokine production potential of the cells. The cytokine profile in supernatants of paired maternal PBMCs and CBMCs with PHA was determined. Both PBMCs and CBMCs responded to PHA showing that cells were viable. Cell culture from CBMCs produced lower (p=0.003) level of IFN –γ cell culture supernatants to PHA compared to PBMCs of mothers. On the other hand, supernatants from CBMC cultures contained higher amounts of IL-10 than maternal PBMC in response to PHA (p<0.001). Further following stimulation with *P.falciparum* antigen (MSP-1_{19}**)** cord blood mononuclear cells produce significantly low IFN –γ than that produced by PBMCs (mean level 61.5±15.3 pg/ml, p=0.001). However, no significant difference (p=0.07) was observed in the production of IL-10 between maternal PBMC and CBMC cytokine in response to *P. falciparum* MSP-1_{19} antigen (Table 2).

IFN- γ production was used as a measure of Th1 responsiveness and IL-10 production was used as a measure of Th2 responsiveness. Stimulation of PBMCs and CBMCs with MSP-1_{19} produced two groups of responders: those with Th1 and others with Th2 responses. Nonetheless, neonates born of malaria-positive mothers mounted predominantly (p=0.03) Th2 type immune responses after stimulation with MSP-1_{19}

Table 2: Cellular production of interferon (IFN -γ) and IL-10 in response to the mitogen phytohaemagglutinine (PHA) and *P. falciparum* MSP-1_{19} in mothers and their neonates.

Mothers				Neonates	
Cytokines (Pg/ml)		n	Mean ±SD	Mean± SD	Significance level
PHA	IFN –γ	29	807.7 ±87.5	105.0 ±23.4	t= 3.82 p=0.0003
	IL-10		311.7 3±6.5	441.6± 9.8	t= 3.15 p=0.0100
MSP-1_{19}	IFN –γ	21	416.5 ±47.9	61.51±5.3	t= 2.94 p=0.001
	IL-10		147.1 ±22.4	109.2±28.6	t=1.04 p=0.070

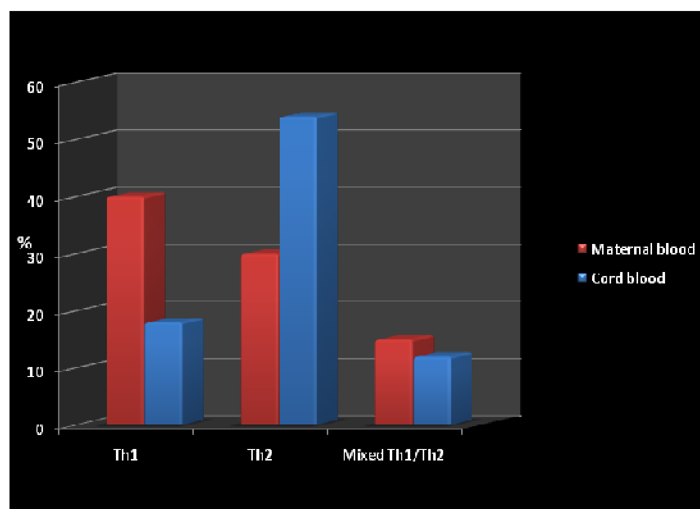

Figure 2: Th-cell cytokine pattern of malaria antigen (MSP-$1_{19.)}$ driven CBL and maternal PBMC response categorized as Th1 type (IFN- γ,), Th2 type (IL-10) or mixed Th1/Th2 type (combinations of the two groups)

when compared with their malaria parasite negative counterparts.

Comparing proportions of maternal PBMC and CBMC IFN–γ and IL-10 production in response to MSP-1_{19}

Overall 42% of PBMC and 18% for CBMC had produced IFN–γ in response to MSP-$1_{19.}$ On the other hand, the proportion of IL-10 was 30 % for PBMC and 56 % for CBMC. While 14% of mothers PBMC and 9 % newborns CBMC had a combination of Th-1 and Th-2 cytokines (Figure 2).

Maternal malaria infection during pregnancy and MSP-1_{19}-driven production of maternal and neonatal cytokines

P.falciparum infections cleared during pregnancy was correlated with the ability of the mother's PBMC to respond to MSP-$_{19}$. Maternal microscopic positivity and PCR negativity was strongly associated (p=0.001). with significant production of Th-1 cytokine IFN–γ compared to those who were PCR positive or microscopy negative On the other hand, Th-2 type cytokine, IL-10 production was higher (p= 0.005*)* in the supernatants of cultures of PBMC of pregnant women with negative results (microscopy or PCR) compared to those with microscopic or PCR positivity. No relationship (p=0.03) was observed between maternal malaria during pregnancy and infants IFN–γ production to MSP-1_{19} antigen although fewer neonates responded when mothers were microscopically positive (2/40). Neonatal production for IL-10 was higher (p=0.003) for those whose mothers had *P.falciprum* detected by PCR only compared to those whose mothers were diagnosed microscopically negative (Table 3).

DISCUSSION

Pregnant women, especially primigraviae, are more

Table 3: Maternal PMBCs and neonatal CBMCs IFN-γ and IL-10 *in-vitro* production in response to *P. falciparum* MSP-1_{19} antigen in relation to microscopy and PCR positivity.

	Maternal blood		**Neonatal cord blood**	
	IFN– γ (n=50)	*IL-10* (n=50)	*IFN–γ* (n= 49)	*IL 10* (n= 49)
	Mean levels pictogram/ml ±SD			
***P.falciparum* infection** [a]				
Microscopy				
Positive (n=16)	**560** ±107	**92** ±11 [a]	**28** ± 6.0	**168** ±23.4 [b]
Negative (n=34)	**139** ± 69	**312**±60	**10** ±0.8	**49** ±27.0
PCR				
Positive (n=36)	**113** ±31	**85** ±27 [a]	**36** ±7	**78** ±19 [a]
Negative (n=14)	**433** ±42	**236** ±77	**55**±12	**69** ±26

[ab+] *Within a row and a variable, values with different superscript alphabets differ significantly ($p < 0.05$)*

susceptible to *P. falciparum* infection as opposed to the same women before pregnancy or to their non-pregnant counterparts (Enato *et al*., 2009. Omer *et al*., 2011). In this study, we have investigated immune responses of mothers and neonates at time of delivery as a result of malaria infection during antenatal period. At delivery none of the mothers presented with an acute malaria attack, as those women who had got the infection during pregnancy generated memory immune responses and some degree of acquired immunity which will control parasite densities and prevent severe clinical malaria. In consistent with earlier findings (Delron and Cot, 1990), the cord sera anti-MSP- 1_{19} IgG antibody levels of all parity groups were lower than the maternal sera rates. This is probably due to the fact that not all IgG subclasses (IgG2 & IgG3) are transported across the placenta (Desowitz *et al*., 1993; Chiizzollni *et al*., 1991). An interesting finding of our study showed that some women at term who were IgG reactive for anti–MSP1_{19} were malaria positive for microscopy and/or PCR. This increase in the antibody levels most probably indicates current/recent malaria infection. Some studies correlated the level of passively acquired maternal IgG Abs at birth and the time to first malaria infection (Malhotra *et al*., 2005; Deloron and Cot, 1990; Hogh *et al.,*1995). Although early gestational exposure of an immature foetal immune system to exogenous antigens can induce immunological tolerance, which could increase future susceptibility to infection (Klein, 1990), late gestational exposure can prime immune responses prior to birth and improve responses to future vaccination (Gill *et al.,* 1983).

It has been thought that children exposed to parasite antigens and immune sensitized in *utero* resulted in immunological tolerance and potentially modify future susceptibility to infection (Nossal, 1989; Riley *et al.,* 2000). Also exposure to small doses of microbial antigens can prime fetal lymphocytes and improve defenses against future infections (King *et al*., 2002; Krich *et al.,* 2004). Many studies demonstrated that malaria specific T cell priming occur in utero to asexual stage antigens (King *et al*., 2002; Malhotra *et al.,*2005; Brustoski *et al*., 2005), and reduced Th-1 responsiveness in cord blood from neonates whose mothers had active placental malaria at delivery compared with neonates born to uninfected mothers (Ismaili *et al.,* 2003). Results of this study showed that CBMCs and PBMCs produced IFN–γ and IL-10 when stimulated with PHA, and lower amount to MSP-1_{19}. These results support pervious findings of low IFN –γ and high IL-10 responses of CB cells to malaria in infants living in Africa (Krich *et al*., 2004). Only one study examined the immune response in neonates born to mothers who had malaria infection during pregnancy and reported a higher median level of IFN–γ in CBMC from neonates whose mothers had been drug treated for infection during pregnancy and did not have placental malaria at delivery (Brustoski *et al.,* 2005).

The present study demonstrates the predominance of Th_1 immune response with increased IFN–γ and reduced IL-10 secretion was more evident in women who were malaria microscopy positive. Women who were negative by microscopy/PCR showed predominantly Th_2 immune response. Also interestingly women who were PCR negative showed a mixed Th_1/Th_2 immune response. A mixed Th_1/Th_2 immune response was also more evident in women who were IgG anti–MSP1_{19} reactive, while IgG non-reactive women showed a slight increase in IFN–γ and IL-10. IL-10 is a key cytokine both in protection and immunopathology during malaria. High levels of IL-10 observed during malarial episodes may be beneficial by reducing the inflammatory response, but may be detrimental by decreasing antiparasitic cellular immune responses (Edward *et al*., 2008). This is clearly shown by our data with significantly increased IFN–γ levels in

women with positive malaria microscopy. IL-10 inhibits the release of proinflammatory cytokines, including TNF-∝, by human monocytes and neutrophils, and inhibits IFN–γ secretion by Th1 lymphocytes. IL-10 concentrations were also decreased in placental serum samples from malaria-exposed women and did not differ with the placental infection status (Fried *et al*., 1998). The findings here demonstrate that foetuses can mount humoral immune responses against malarial antigens and that *P. falciparum* antibodies are transferred from mothers to newborns and contribute to the relative protection observed in young infants. These results also demonstrate that the immune system of some neonates born in the study area are primed to malaria antigens and produce predominantly IL-10 responses upon antigenic stimulation *in vitro*. Furthermore, it has been observed that maternal malaria parasitaemia directed the foetal T cells to respond further on malaria stimulation by producing predominantly Th2 type responses. Thus neonates born from malaria infected mothers may be more susceptible to malaria during their first year of life since it is known that during acute malaria attacks, proinflammatory responses are essential in bringing down the parasitemia.

CONCLUSIONS

Maternal malaria reduces IL-10 concentrations and increases IFN- γ shifting the balance toward type 1 cytokines. The immune system of some neonates born in the study area are primed to malaria antigens and produce predominantly IL-10 responses upon antigenic stimulation *in vitro*.

Maternal peripheral infection during pregnancy appears to direct foetal T cells to respond further on malaria stimulation by producing predominantly Th2 type responses.

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