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Akanni Adédédji Samad IBITOKOU

Paludisme Associé à la Grossesse : Conséquences immunologiques chez la femme enceinte et le nouveau-né

Thèse dirigée par Dr. Nadine Fievet

Soutenue le 18 Juin 2013

Jury

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“There are known knowns. These are things we know that we know. There are known unknowns. That is to say, there are things that we know we don't know. But there are also unknown unknowns. There are things we don't know we don't know”.

Donald Rumsfeld

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Résumé

L'objectif de ce travail était d'étudier les conséquences immunologiques du paludisme gestationnel chez la femme enceinte et le nouveau-né. Ce travail de recherche fait partie du projet STOPPAM, un suivi de cohorte de femmes enceinte et de leurs enfants, réalisés dans deux pays d'Afrique : le Bénin et la Tanzanie. Les objectifs de STOPPAM étaient d'élucider les mécanismes et le déroulement de la pathologie du paludisme associé à la grossesse (PAG) et de quantifier les conséquences du PAG sur la santé de la mère et du jeune enfant.

Nous avons, dans un premier temps, caractérisé les cellules immunocompétentes chez des femmes infectées et non-infectées au cours de la grossesse. Deux études ont été ainsi réalisées sur deux sous-groupes de femmes prélevées au début de la grossesse et à l'accouchement. Sur une vingtaine de données appariées entre le début de la grossesse et l'accouchement, nous avons mis en évidence une diminution de fréquence des lymphocytes T indépendamment de l'infection palustre.

Au cours du PAG, nous avons déterminé sur deux sous-populations de femmes enceintes, au début de la grossesse et à l'accouchement : i) les fréquences et le niveau d'activation des cellules médiatrices de l'immunité et ii) les concentrations de cytokines et de chimiokines plasmatiques. Nos résultats ont permis de montrer, d'une part que le paludisme gestationnel induit des changements qualitatifs et quantitatifs sur le profil cellulaire et cytokinique, en fonction de l'âge gestationnel et en fonction de la date de l'infection. L'originalité de ces études réside dans l'identification au début de la grossesse, de marqueurs cellulaires et cytokiniques, tels que les lymphocytes B et les Treg d'une part, l'IL-10, l'IP-10 et le MIG d'autre part, qui pourraient être un facteur de risque d'infection placentaire à l'accouchement. Ces observations suggèrent la nécessité de la prise en charge des infections au début de la grossesse dans le contrôle du paludisme gestationnel.

La deuxième partie de notre travail s'est intéressée aux conséquences du PAG sur les réponses innées du jeune enfant. Dans une première étude, nous avons caractérisé les cellules présentatrices d'antigènes dans le sang du cordon en fonction du paludisme placentaire ou de l'inflammation du placenta. Nos résultats montrent une altération partielle des cellules présentatrices d'antigènes du nouveau-né, induite par la présence de pigment malarique dans le placenta. Nos résultats soulignent l'importance de l'âge de la mère au moment de la grossesse et de la parité, sur les cellules présentatrices d'antigène du nouveau-né. Ces résultats suggèrent une modulation des réponses cellulaires néonatales par le PAG.

La dernière partie de ce travail a considéré les réponses cytokiniques dans le sang de cordon et le sang périphérique du jeune enfant, à des ligands de TLR sur une cohorte de 134 enfants. Les observations issues de cette étude démontrent que l'activation du système immunitaire inné du jeune enfant peut être modifiée par une l'infection survenue à l'accouchement chez la mère. Nos résultats montrent que la susceptibilité au paludisme au cours de la première année de vie dépend de la période de stimulation des récepteurs TLR du système immunitaire néonatal.

L'ensemble des travaux réalisés au cours de cette thèse permet de comprendre des mécanismes cellulaires et inflammatoires impliqués dans le paludisme gestationnel chez la femme enceinte et chez l'enfant. Nos résultats ont permis d'identifier des bio-marqueurs liés au PAG.

Table des matières

Liste des figures et tableaux

Liste des abréviations

°C: degré Celsius
Ang-1&2: Angiopoietin-1&2
AIRD: Agence Institut de Recherche pour le Développement
BDCA: Blood dendritic cells antigens
CBA: Cytometry Beads array
CD: Cluster of differentiation (Complexe Membranaire)
CERPAGE: Centre d'étude et de recherche sur le paludisme associé à la grossesse et à l'enfant
CIDR: Cystein InterDomain Rich region
CMH: Complexe Majeur d'Histocompatibilité
CPA: Cellule(s) Présentatrice(s) d'Antigène(s)
CPDA: Citrate Phosphate Dextrose Anticoagulant
CpG-ODN: CpG oligodeoxynucleotide (ODN)
CR1: Complement receptor (récepteur de complement)
CSA: Chondroïtine Sulfate A
CTA: Combinaison Thérapeutiques à base d'Artémisine
DBL: Duffy Binding Like
DC: Cellules dendritiques (Dendritic Cells)
ELISA: Enzyme-linked immunosorbent assay
FACS: Fluorescent Activated Cell Sorting
FcR: Fc receptor (récepteur Fc)
GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor
hCG: Hormone gonadotrophique chorionique
HLA: Human Leukocytes Antigens
HOMEL : Hôpital de la Mère et de l'Enfant Lagune
HP : Hématie parasitée
Hz : Hémozoïne
ICAM-1 : Intercellular adhesion molecule-1
IFN-γ : Interféron gamma
Ig : Immunoglobuline
IL: Interleukine
IP-10: IFN-γ-inducible protein-10
ISBA: Institut des Sciences Biomédicales Appliquées
kDa : kilo Dalton
KIR: Killer Immunoglobulin like Receptor
LPS: Lipopolysaccharide
MCP-1: Monocytes chemotactic protein-1
mDC: myeloïde Dendritic Cell (cellule dendritique myéloïde)
MIG: Monokine induced by IFN-γ
MILD: Moustiquaire Imprégnée à Longue Durée d'action
MSP : Merozoïte surface protein
NK : Natural Killer
OMS: Organisation Mondiale de la Santé
PAG: Paludisme Associé à la Grossesse
PAMP: Pathogen-associated molecular patterns
PBMC: Peripheral Blood Mononuclear Cells (Cellules mononucléées du sang périphérique)
pDC: plasmacytoïde Dendritic Cell (cellule dendritique plasmacytoïde)
PfEMP1 : *P. falciparum* Erythrocyte Membrane Protéine 1
pLDH: *Plasmodium* lactate déshydrogénase
PRR: Pattern Recognition Receptors
RANTES: Regulated on activation normal T-cell expressed and secreted
STOPPAM: Strategies To Prevent Pregnancy Associated Malaria

TDR: Test de Diagnostic Rapide
Teff: Lymphocyte T effecteur
TH1/TH2: Lymphocytes T auxiliaire(s) de type 1&2 (T helper type 1&2)
TLR: Toll like receptor
TNF: Tumor necrosis factor
TPI-SP: Traitement Préventif Intermittent-Sulfadoxine Pyrimétamine
Treg: Lymphocyte T régulateur
UMR : Unité mixte de recherche
uPAR: Urokinase plasminogen activator receptor
VEGF/Flt1: vascular endothelial growth factor
VSA: Variant Surface Antigens (Antigènes variants de surface)

INTRODUCTION

Le paludisme est responsable de plusieurs millions de décès dans le monde dont 80% environ en Afrique sub-saharienne. Les personnes immunodéprimées, les femmes enceintes et les jeunes enfants restent les cibles les plus vulnérables. Le paludisme est une affection causée par un protozoaire appartenant au genre *Plasmodium*. Cinq espèces sont responsables de la maladie chez l'homme *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* et *P. knowlesi* [1-3]. Parmi ces dernières, *P. falciparum* est l'espèce qui provoque les formes cliniques les plus graves, notamment le paludisme cérébral. L'infection est transmise à l'homme par la piqûre d'un *Culicidea* femelle du genre *Anopheles*.

Les nouvelles stratégies de lutte et de contrôle du paludisme présentés en 2011 par le partenariat « Roll Back Malaria » ont entraîné une diminution de la transmission et une réduction du nombre de cas [4]. En effet, les traitements curatifs par des Combinaisons Thérapeutiques à base d'Artémisinine (CTA), la lutte anti-vectorielle par la distribution des moustiquaires imprégnées à longue durée d'action (MILD), la sensibilisation et la prise en charge rapide et gratuite des cas de paludisme dans certains pays ont entraîné la baisse de la prévalence du paludisme dans le monde. Cependant, ces mesures restent fragiles et nécessitent d'être consolidées en raison des résistances et de la politique de gestion par les états. En dépit du contrôle, la recherche sur le paludisme permettra de faire des choix spécifiques sur de nouvelles combinaisons thérapeutiques, et de proposer des candidats vaccins.

La grossesse est décrite comme un état physiologique au cours duquel l'immunité maternelle est modulée, pour favoriser le développement de l'allogreffe fœtale [5, 6]. Cette immuno-modulation se traduit par l'inhibition des réponses pro-inflammatoires de type TH1 en faveur de réponses anti-inflammatoires de type TH2. Cependant, cet état favorise la susceptibilité des femmes enceintes aux infections comme le paludisme. Par ailleurs, la séquestration et l'adhésion des parasites de *P. falciparum* au tissu trophoblastique induit des réactions inflammatoires importantes dans le placenta [7]. Ces réactions inflammatoires se traduisent par le recrutement des cellules immunocompétentes et la production de cytokines et chimiokines inflammatoires dans le placenta. En outre, l'inflammation locale peut perturber les échanges entre la mère et le fœtus [8]. La prévention du paludisme chez la femme enceinte repose actuellement sur l'administration d'un traitement préventif intermittent au cours de la grossesse (TPIGross) par la sulfadoxine-pyriméthamine (SP). Chez les enfants, la prise en charge des accès palustres simples est faite avec les CTA. Cependant, ces stratégies nécessitent des mesures d'accompagnement, telles que les tests de diagnostic rapide (TDR) et de nouvelles molécules thérapeutiques.

Cette thèse vise à apporter des informations sur les mécanismes de régulation des réponses immunitaires liées au paludisme gestationnel et à l'exposition *in utero* du nouveau-né. Dans le cadre de la préparation d'un essai vaccinal contre le paludisme gestationnel, notre but est d'étudier les conséquences du paludisme gestationnel sur les réponses cellulaires de la femme enceinte et au cours de la première année de vie du nouveau-né.

PREMIERE PARTIE : REVUE BIBLIOGRAPHIQUE

Chapitre 1: Paludisme chez la femme enceinte et le nouveau-né

1.1. Généralités sur le paludisme

Le *Plasmodium* est un parasite intracellulaire ayant un cycle biologique complexe, caractérisé par une multiplication asexuée dans l'organisme humain, et la reproduction sexuée chez l'anophèle femelle (**Figure 1**) [9].

- Cycle chez l'anophèle

L'anophèle femelle ingère des gamétocytes lors d'un repas sanguin sur un individu infecté. Ceux-ci migrent vers l'estomac du moustique et se transforment en gamètes. Par un processus d'exflagellation du gamète mâle, les gamètes femelles sont fécondés et il en résulte un zygote appelé ookinète. Celui-ci s'implante sous la paroi stomacale en formant l'oocyste. Après une division méiotique suivie par plusieurs mitoses, les sporozoïtes sont générés. Ils se libèrent après éclatement de l'oocyste pour se concentrer au niveau des glandes salivaires en attendant la prochaine piqûre infectante. Ce cycle se déroule en 10 à 40 jours, suivant la température extérieure et les espèces en cause [10].

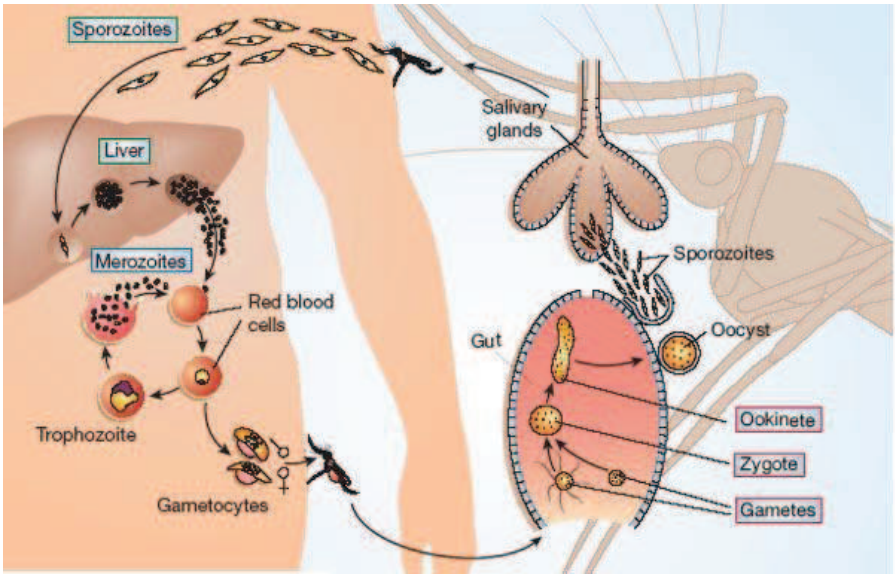
- Cycle chez l'homme

Au cours de son repas sanguin, l'anophèle infecté injecte avec sa salive des centaines de sporozoïtes. Ils restent environ une demi-heure dans la circulation sanguine avant de gagner le foie, où se développe la phase hépatique ou pré-érythrocytaire.

Au cours de la phase pré-érythrocytaire, les sporozoïtes libérés par l'anophèle envahissent les hépatocytes et prennent le nom de cryptozoïtes. Après environ une semaine de maturation et de division, ils se transforment en schizonte, forme mature du parasite d'environ 40 à 100 micromètres et contenant quelques milliers de noyaux, appelés corps bleus. L'éclatement du schizonte libère de nombreux mérozoïtes qui passent dans la circulation sanguine pour entamer le cycle érythrocytaire [11]. Le cycle pré-érythrocytaire dure en moyenne 6 jours pour *P. falciparum*, 8 jours pour *P. vivax*, 8-9 jours pour *P. knowlesi*, 9 jours pour *P.ovale* et 13 jours pour *P.malariae*.

Au cours de la phase érythrocytaire, les mérozoïtes envahissent les érythrocytes et se transforment en trophozoïtes. Chaque cycle érythrocytaire dure 48 heures pour *P. vivax, P. ovale ou P. falciparum*, 72 heures pour *P. malariae*, et 24 heures pour *P. knowlesi*, rythmant ainsi les accès fébriles tels que la fièvre tierce ou quarte pour certaines espèces. Lors de l'éclatement des rosaces, l'hémozoïne, pigment résultant de la destruction de l'hématie par le parasite, est libérée puis

phagocytée par des polynucléaires neutrophiles ou des monocytes qui deviennent métallifères. Après plusieurs cycles schizogoniques, certains trophozoïtes vont être détournés du cycle érythrocytaire pour former les gamétocytes, première étape d'une phase sexuée chez l'hématozoaire. Dans le sang s'amorce enfin le cycle sexué ou sporogonique. Les gamétocytes continueront leur développement s'ils sont absorbés par un anophèle femelle lors de son repas sanguin pour continuer le cycle [10, 11].



Source : Menard et al, Nature 2005 **[9]**

Figure 1 : Cycle de développement de *Plasmodium falciparum* chez l'homme et l'anophèle.

1.1. Physiopathologie

La virulence du paludisme dépend de la capacité des parasites à adhérer aux récepteurs endothéliaux. La protéine parasitaire impliquée dans la cyto-adhérence est appelée « protéine membranaire de l'érythrocyte » ou *PfEMP1* (*P. falciparum*-erythrocyte membrane protein-1) [12-14]. Le *PfEMP1* appartient à la famille des gènes *Var* représentée par environ 60 copies par génome haploïde [15] et il est formé de trois parties ; i) une partie extracellulaire composée d'un segment N-terminal variable, ii) une partie transmembranaire (TM) et iii) un segment conservé C-terminal intracellulaire. La diversité antigénique du paludisme réside dans l'expression d'un seul variant de *PfEMP1* à la surface des hématies parasitées (HP), favorisant la diversité de séquestration des souches parasitaires. Le *PfEMP1* a la capacité d'adhérer à plusieurs récepteurs cellulaires via ses multiples domaines. Il est composé de plusieurs domaines DBL (Duffy Binding like) et de régions inter-domaines riches en cystéines (CIDR) [16, 17]. Les DBL sont classés en 5 groupes (α-ε) en fonction de leur séquence.

Différentes études ont associé les *PfEMP1* à la pathogénèse du paludisme. Le neuropaludisme implique l'adhésion de *PfEMP1* au récepteur ICAM-1 (intercellular adhesion molecule-1) dans les capillaires cérébraux [14]. Au cours de l'accès grave, le *PfEMP1* se lie aux récepteurs endothéliaux tels que le CD36, dans les capillaires viscéraux et cérébraux et provoque ainsi une anoxie tissulaire à l'origine du coma. L'adhésion des *PfEMP1* à la chondroïtine sulfate A (CSA) caractérise le paludisme placentaire [18-20] (**Figure 2**). Cette partie sera plus détaillée dans les chapitres suivants.

Parmi toutes les espèces plasmodiales infectant l'homme, *P. vivax* est la deuxième espèce adhérant à la CSA après *P. falciparum* [21]. Au cours de l'accès simple, la fièvre intervient après libération des pigments malariques, dans la circulation après hémolyse.

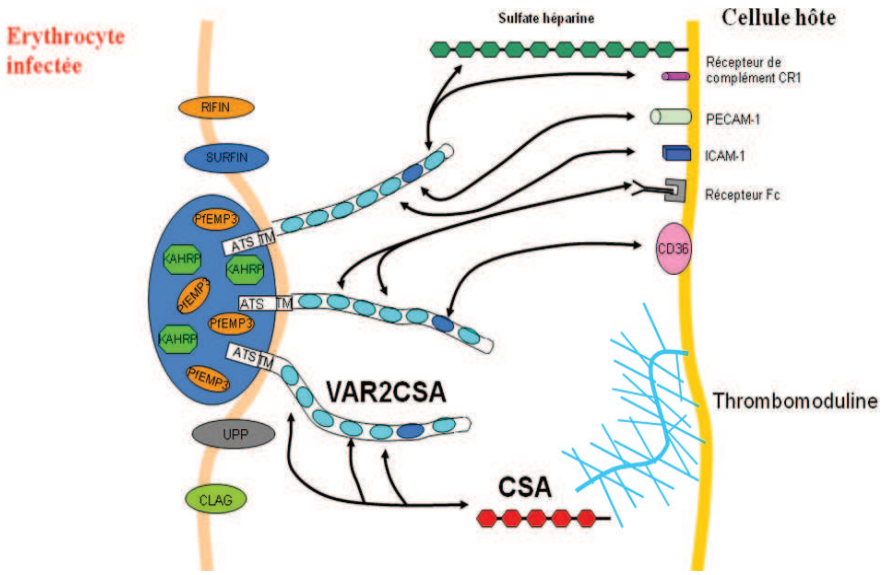


Figure 2 : Interactions moléculaires du *Pf*EMP1 avec la cellule hôte [22]

1.2. Diagnostic biologique

Les outils de diagnostic du paludisme reposent sur la mise en évidence directe ou indirecte de la présence de *Plasmodium* dans le sang.

Le diagnostic direct repose sur les techniques de microscopie : frottis mince et goutte épaisse. Après séchage, fixation au méthanol pour le frottis et coloration au Giemsa, une goutte de sang est observée au microscope optique. Le frottis mince, où les globules rouges sont intacts, permet de faire le diagnostic différentiel entre les espèces plasmodiales. La goutte épaisse représente l'examen de référence de l'OMS. Elle se réalise par éclatement des hématies d'une goutte de sang, sur une lame. Sa sensibilité est 20 à 30 fois plus élevée que celle du frottis mince.

La densité parasitaire (DP) est estimée sur goutte épaisse, par microlite de sang selon la formule suivante :

$$DP = \frac{\text{Nombre de parasites}}{\text{Nombre de leucocytes comptés}} \text{ X Nombre total de leucocytes}$$

Le diagnostic indirect consiste en la mise en évidence de la présence du parasite par des techniques qui ne reposent pas sur l'observation directe du parasite. Ces techniques permettent indirectement d'identifier une infection palustre passée. Les différents outils utilisés sont :

- Tests de Diagnostic Rapides (TDR) qui révèlent en 5 à 15 mn la présence ou l'absence d'un antigène de *Plasmodium* dans le sang du patient. Les antigènes les plus fréquemment recherchés par cette technique sont : HRP-II (histidine-rich protein-II), spécifique de *P. falciparum* ; pLDH (*Plasmodium* lactate déshydrogénase) commune aux 4 espèces de *Plasmodium* ou spécifique de l'une des espèces.
- La PCR (réaction de polymérisation en chaîne) qui permet la détection d'infections occultes avec des parasitémies très faibles, la surveillance des gènes de résistance et le génotypage des espèces. Cette technique plus performante, nécessite des équipements plus lourds et très coûteux.
- Recherche d'immunoglobulines spécifiques de *Plasmodium* par sérologie ou ELISA.

1.3. Stratégies de lutte contre le paludisme

Les stratégies de lutte contre le paludisme existant aujourd'hui, sont essentiellement axées sur la prévention par l'utilisation des moustiquaires imprégnées de longue durée d'action (MILD), l'assainissement de l'environnement domestique et les traitements avec les combinaisons thérapeutiques à base de dérivés d'artémisinine (CTA). La lutte anti-vectorielle est basée sur l'utilisation d'insecticides domestiques capables d'éliminer les larves et les anophèles adultes. Malheureusement, cette méthode accélère l'apparition d'anophèles résistants aux insecticides. De plus, il est nécessaire de compléter l'utilisation d'insecticides par la lutte contre l'insalubrité, l'assainissement des points d'eaux stagnantes, et l'utilisation de moustiquaires. Des travaux de recherche sont en cours en vue d'un vaccin antipaludique sûr et efficace.

Le traitement préventif intermittent repose sur l'utilisation de médicaments antipaludiques à des doses curatives administrées systématiquement en l'absence de signes morbides. Cette méthode préventive est désormais recommandée chez les femmes enceintes, avec l'utilisation de

sulfadoxine-pyriméthamine (TPI-SP). Le TPI-SP comporte deux prises supervisées à un mois d'intervalle à compter de la 18ème semaine d'aménorrhée. Son efficacité a été démontrée par la baisse de prévalence d'infection placentaire ou de l'anémie maternelle [23].

Plusieurs essais vaccinaux ont été réalisés. Les vaccins candidats sont basés sur divers antigènes issus des différents stades du cycle évolutif parasitaire. Les nouveaux types de candidats vaccins existants de nos jours sont ceux basés sur l'ADN parasitaire. Trois catégories de vaccins sont en phase d'essai contre le paludisme :

- Les vaccins anti-stade exo-érythrocytaire ou pré-érythrocytaire

Le vaccin idéal dans ce domaine serait celui capable d'induire de fortes concentrations d'anticorps anti-sporozoïtes pour prévenir l'invasion des cellules hépatiques par les sporozoïtes et les lymphocytes T cytotoxiques capables d'éliminer les hépatocytes infectés.

Plusieurs essais cliniques ont eu lieu chez l'homme sans succès, avec divers vaccins de ce type. Dans ce domaine, le vaccin RTS, S/AS01 des laboratoires GlaxoSmithKline (GSK) a montré une protection de 34,8 % contre la forme sévère du paludisme chez les jeunes enfants tous âges confondus après la phase III de l'essai [24]. Mais des résultats complémentaires sur la durée de cette protection et l'évaluation des réponses immunitaires induites par ce vaccin sont nécessaires.

- Les vaccins anti-stade érythrocytaire

Il existe deux classes de vaccins dans ce groupe ; les vaccins anti-invasion des hématies et le vaccin anti-complication de la maladie. Ces types de vaccins contrôleraient les manifestations cliniques du paludisme. Plusieurs candidats vaccins de ce type ont fait l'objet d'essais cliniques chez l'homme. A cet effet, les vaccins en phase d'essai ciblent la protéine de surface des mérozoïtes (MSP : Merozoite Surface Protein) ou certaines parties conservées de la protéine *PfEMP1* à cause de la variabilité antigénique de cette protéine.

- Les vaccins bloquant la transmission

C'est une catégorie de vaccin qui induit la production d'anticorps empêchant la maturation vers les stades sexués du parasite chez l'homme et la fécondation chez le moustique.

- Une quatrième catégorie pourrait être discernée, avec des anticorps bloquant la cytoadhérence des hématies parasitées. Chez la femme enceinte, des travaux récents ont identifié les domaines de la protéine VAR2CSA impliqués dans l'adhésion des parasites à la CSA dans le

placenta. Il a, en outre, été montré qu'au cours des grossesses successives, les mères produisaient des anticorps protecteurs contre VAR2CSA, réduisant les conséquences du paludisme gestationnel [25, 26]. Par ailleurs, la capacité de plusieurs domaines du VAR2CSA à induire la production d'anticorps anti-adhésion a été démontrée. Entre autre domaine, l'Id1-DBL2x récemment identifié par sa capacité à induire une production d'anticorps anti-adhésion des HP aux CSA donne un espoir dans le choix des molécules vaccinales chez les femmes enceintes [27, 28].

1.4. Paludisme associé à la grossesse

1.4.1. Mécanismes favorisant l'infection palustre chez les femmes enceintes

Les femmes enceintes sont plus susceptibles aux infections, notamment au risque d'infection palustre. Plusieurs hypothèses expliquent cette susceptibilité. Il a été rapporté que les femmes enceintes seraient plus attractives aux anophèles et, par conséquent, pourraient subir plus de piqûres de moustiques que les femmes non-enceintes [29]. Une seconde hypothèse concerne la cytoadhérence des parasites dans le placenta qui leur permettrait d'échapper aux réponses inflammatoires [30]. La troisième hypothèse met en exergue l'immuno-modulation, favorisée par l'augmentation de facteurs plasmatiques comme le cortisol et la prolactine, qui inhiberaient les réponses inflammatoires nécessaires au contrôle des parasites [31]. La combinaison de toutes ces hypothèses participerait à la susceptibilité au paludisme de la femme enceinte.

1.4.2. Aspects épidémiologique et parasitologique du PAG

L'OMS estime que 50 millions de femmes enceintes seraient exposées chaque année au paludisme (Rapport OMS 2012). Les conséquences et les caractéristiques cliniques vont dépendre des niveaux de stabilité de transmission de paludisme.

En zone d'endémie stable comme au Bénin, le risque d'infection palustre est élevé chez les femmes primigestes comparées aux multigestes en raison d'une immunité protectrice acquise par ces dernières au cours des grossesses successives impaludées [26, 32, 33]. Les infections sont le plus souvent pauci-symptomatiques et les accès graves sont rares. Les principales conséquences du PAG sont l'anémie maternelle et le faible poids de naissance [33-35]. Le faible poids de naissance serait une conséquence du retard de croissance intra-utérin.

Dans les zones de faible transmission, les formes cliniques du PAG sont souvent symptomatiques associées à des accès graves, une anémie sévère et un risque plus élevé de faible poids de naissance. Les infections palustres entraîneraient des fausses couches spontanées et des mort-nés [36, 37]. Ici le faible poids de naissance serait associé à la prématurité.

L'anémie maternelle peut être associée à plusieurs facteurs tels que : la carence en fer ou en acide folique, les co-infections aux helminthiases ou au VIH [36-38]. Les densités parasitaires dans le placenta sont beaucoup plus importantes que dans le sang périphérique, et l'architecture placentaire est souvent altérée [39-41]. La principale et la plus fréquente caractéristique du paludisme placentaire est une inflammation intervilleuse, parfois massive, observée à l'examen histologique [42, 43].

Lors de l'invasion érythrocytaire, *P. falciparum* exprime un facteur de virulence, le *PfEMP1*, qui est exporté à la surface de la cellule hôte [44]. Comme cela avait été décrit dans le chapitre précédent, la protéine *PfEMP1* est une molécule capable d'adhérer à des récepteurs de différents tissus [45]. Les souches de *P. falciparum* impliquées dans le PAG expriment des *PfEMP1* spécifiques de la CSA, sucre présent sur des protéoglycanes localisés dans le placenta (tel que la chondroïtine sulfate proteoglycan (CSPG) et la thrombomoduline). Le gène surexprimé et impliqué spécifiquement dans l'adhérence de *PfEMP1* à la CSA chez les femmes enceintes, reste le *var2csa* contrairement au *var1csa* [46]. Sur les 60 variants comprenant la famille des *PfEMP1*, *var2csa* est l'un des plus conservé dans des isolats parasitaires [47]. La spécificité de *var2csa* pour le CSA est due à un sous-groupe de domaines DBL. Plusieurs domaines DBL sont impliqués dans la cyto-adhésion des HP au tissu trophoblastique du placenta [48-53]. Plusieurs équipes s'intéressent au mécanisme de cyto-adhérence des HP dans le placenta en vue d'identifier des domaines qui pourraient intervenir dans la conception d'un vaccin contre le PAG. Ainsi la capacité inhibitrice des anticorps anti domaine Id1-DBL2x de VAR2CSA a été récemment décrite. Ce domaine aurait des propriétés immunogènes importantes dans la stimulation des réponses immunitaires spécifiques contre le PAG [28]. Les anticorps dirigés contre ce domaine inhiberaient l'adhésion des HP à la CSA.

1.5. Paludisme chez le nouveau-né

Le paludisme représente une des premières causes de morbidité et de mortalité infantile en Afrique sub-saharienne. Malgré les résultats encourageants observés dans les stratégies de prise en charge des cas de paludisme, les nouveau-nés et les jeunes enfants restent les cibles les plus touchées. Plusieurs hypothèses existent pour justifier la susceptibilité et la sensibilité à l'infection palustre chez les enfants. En effet, il a été démontré que les enfants vivant en zone d'endémie palustre stable, acquièrent progressivement en fonction de l'exposition, l'immunité protectrice contre le paludisme tandis que ceux exposés *in utero* aux antigènes palustres sont plus susceptibles à l'infection au cours de leur première année de vie [54]. D'autres études ont montré que des facteurs environnementaux comme l'exposition aux anophèles et les conditions climatiques,

peuvent influencer la survenue de la première infection chez l'enfant [55, 56]. L'anémie grave (Hb<5g/dl) est l'une des conséquences du paludisme chez l'enfant. La forme sévère de la maladie chez l'enfant est le neuro-paludisme, induite par la séquestration des HP dans le cerveau. Cette forme du paludisme se manifeste par un coma, des convulsions, des hallucinations et peut entraîner des complications mortelles.

Les nouvelles stratégies de prise en charge des accès palustres simples utilisent les traitements par des CTA à cause des cas de résistance confirmée avec la SP [57]. Malgré les stratégies de prévention mises en place pour la prévention, la morbidité et la mortalité liées au paludisme chez les enfants restent très élevées. Le problème majeur à résoudre dans le cas du paludisme chez les enfants reste la prise en charge et la surveillance.

Chapitre 2 : Immunité, grossesse et paludisme

La grossesse a longtemps constitué une énigme immunologique pour comprendre comment l'embryon exprimant des protéines pour 50% d'origine paternelle n'était pas rejeté par le système immunitaire de la mère.

Des avancées récentes ont permis de confirmer ou de faire évoluer les 4 hypothèses de Medawar sur l'acceptation de l'allogreffe fœto-placentaire [58]:

- Le placenta constituerait une barrière physique qui isolerait le fœtus du système immunitaire de la mère. Le fœtus se développerait dans un lieu immunologiquement privilégié.
- Les antigènes du fœtus et de ses annexes seraient immatures et formeraient une entité immunologiquement neutre non reconnue par le système immunitaire de la mère.
- Le système immunitaire de la mère serait immunodéprimé et ne réagit pas à l'expression par le fœtus des molécules allogéniques.
- Des mécanismes actifs de régulation et de reconnaissance entre le fœtus et le système immunitaire de la mère se mettent en place pour aboutir à une tolérance materno-fœtale.

Plus tard, la grossesse a été décrite comme un état physiologique caractérisé par un déséquilibre des réponses TH1/TH2, la première qui favorise la réponse à médiation cellulaire étant réprimée au profit de la seconde qui stimule la réponse humorale. Cette immuno-modulation transitoire augmente la susceptibilité des femmes enceintes à certaines infections (paludisme, syphilis, tuberculose) qui peuvent avoir des conséquences pour la mère, le fœtus et le nouveau-né [59]. Cette hypothèse a été confirmée par la prépondérance des réponses anti-inflammatoires observées au cours de la grossesse [60, 61].

La grossesse n'est plus considérée comme un seul événement immunologique via la balance TH1/TH2 mais comme un processus d'immuno-modulation qui évolue en trois périodes pendant la grossesse [62]. Un nouveau paradigme « TH1/TH2/TH17, lymphocytes T régulateurs et cellules dendritiques (DC)» est proposé pour expliquer l'immuno-modulation particulière d'origine placentaire nécessaire à l'acceptation et au maintien de l'intégrité fœto-placentaire [63, 64]. Les cellules trophoblastiques fœtales et les cellules immunes maternelles interagissent pour leur bénéfice réciproque pendant les 3 phases immunologiques distinctes de la grossesse.

Lors de la première phase (1er trimestre), l'implantation de l'embryon dans l'espace utérin et la placentation requièrent une réponse inflammatoire forte ; pendant la deuxième phase, qui correspond à la croissance et au développement fœtal des 2ème et 3ème trimestres pro parte, la mère, le placenta et le fœtus sont en symbiose avec des réponses anti-inflammatoires TH2 prédominantes ; enfin pendant la troisième phase, la préparation à l'accouchement, les réponses pro-inflammatoires sont nécessaires aux contractions de l'utérus et à l'expulsion du nouveau-né et du placenta [65]. Cette immuno-modulation fait intervenir de nombreux effecteurs cellulaires et moléculaires présents à l'interface materno-fœtale.

Il est décrit deux interfaces immunologiques qui se succèdent pendant la grossesse. Premièrement, l'interface cytotrophoblaste extravilleux/sang maternel/cellules déciduales qui disparaît à la fin du premier trimestre et qui correspond à la mise en place des artères spiralées dans la décidue. Et deuxièmement, l'interface sang maternel/syncytiotrophoblaste qui se met en place à partir de 9 semaines de gestation pour devenir l'interface dominante. Les mécanismes immunologiques de tolérance à l'interface materno-fœtale sont nombreux avec un dialogue materno-fœtal de mouvements cellulaires et cytokiniques qui évoluent tout au long de la grossesse.

2.1. Les cellules médiatrices de l'immunité

Les cellules médiatrices de l'immunité (CMI) jouent un rôle primordial dans le lien entre les réponses immunitaires innée et adaptative. Elles assurent leur fonction à travers la production de plusieurs facteurs solubles. Quelques populations cellulaires intervenant dans cette médiation sont décrites dans les lignes suivantes.

2.1.1. Les cellules dendritiques

Les cellules dendritiques (DC) constituent une population hétérogène de cellules d'origine hématopoïétique, importantes dans l'initiation et la régulation des réponses immunitaires innée et adaptative. Les DC sont des cellules présentatrices d'antigènes « professionnelles », c'est-à-dire

capable d'initier une réponse immune primaire à travers la présentation d'antigènes aux lymphocytes T naïfs. Elles sont également capables de s'attaquer directement aux pathogènes et d'interagir avec d'autres cellules effectrices de l'immunité innée et adaptative, les lymphocytes T, B et les cellules NK [66, 67]. Elles sont regroupées en deux grandes familles, les myéloïdes (mDC) et les plasmacytoïdes (pDC) qui se distinguent sur des bases phénotypiques mais complémentaires [66]. Les DC sont générées dans la moelle osseuse. Cependant, les monocytes seraient des précurseurs des mDC qui migrent vers les organes lymphoïdes pour parachever leur maturation [68, 69]. Les pDC quant à elles peuvent être d'origine lymphoïde et myéloïde [70]. Par ailleurs, il a été montré que les mDC induisent la production de cytokines de type TH1 telles que l'IFN-γ et le TNF-α tandis que les pDC pouvent induire la production de cytokines de type TH2 comme l'IL-4 et l'IL-5 [71, 72].

La caractérisation des DC est souvent difficile puisqu'elles représentent une population moins représentée dans la circulation sanguine. Les mDC sont caractérisées chez l'homme par l'expression de l'antigène BDCA-1 (Blood Dendritic Cell-Antigen) ou le $CD1c^+$ alors que les pDC sont caractérisées par les BDCA-2 (CD303). Une sous-population de mDC caractérisée par BDCA-3 a été décrite mais n'exprime pas le BDCA-1. Les DC du sang périphérique sont phénotypiquement différentes des DC tissulaires car elles expriment plus faiblement que ces dernières les marqueurs de maturation tels que le CD83 ou le CD86. Elles seraient donc immatures et en transit dans le sang périphérique avant d'atteindre leur maturation dans les tissus [73]. Les DC se différencient également par la production de cytokines induite après activation des récepteurs PRR (TLR) présents à leur surface ou par contact intercellulaire. En effet, les lymphocytes B contrôlent la maturation et l'activation des DC, participant ainsi à la régulation du système immunitaire [74].

2.1.2. Les monocytes et les macrophages

Les monocytes et les macrophages représentent un groupe de cellules hétérogènes très important dans la défense immunitaire innée. Dans la circulation sanguine, les monocytes sont considérés comme des précurseurs pour les macrophages et les DC, car peuvent générer *in vitro* ou *in vivo*, ces dernières après stimulation par certains facteurs solubles comme l'IL-4 ou le M-CSF (Macrophage-Colony Stimulating Factor) [75-77].

Les monocytes sont engendrés au niveau de la moelle osseuse par les myéloblastes et libérés dans la circulation sanguine. Ils représentent environ 10% de la population leucocytaire sanguine et participent à l'homéostasie tissulaire. Il existe trois classifications de monocytes selon l'expression

des marqueurs CD14 (récepteur pour le LPS) et CD16 (Fcγ récepteur III) [78]. Les monocytes classiques caractérisés par le phénotype $CD14^{++}CD16^{-}$, les intermédiaires $CD14^{++}CD16^{+}$ et les non-classiques $CD14^{+}CD16^{++}$ [73]. Les monocytes classiques constituent environ 90% de la population totale de monocytes circulants et expriment à leur surface, la molécule CMH-II moins que les monocytes $CD16^{+}$. Les monocytes expriment à leur surface plusieurs récepteurs de chimiokines leur facilitant l'adhésion et la migration vers les organes lymphoïdes. Ils peuvent induire la production de plusieurs cytokines et chimiokines nécessaire à l'élimination des pathogènes. Les monocytes non classiques secrètent une forte concentration de TNF-α et faible d'IL-10 après stimulation à travers les TLR [79, 80].

Les macrophages sont des cellules importantes dans le processus d'élimination des agents pathogènes de l'organisme. Ils exercent leur action par le mécanisme de phagocytose induite à travers les récepteurs d'opsonine (FcR) et de complément (CR). Les macrophages sont identifiés par les marqueurs CD14, CMH-II et le CD68, décrits comme marqueurs de phagocytes [81]. Deux différentes classifications ont été proposées pour les macrophages en fonction des types de cytokines produites. Deux sous-populations de macrophages ont été décrites : les macrophages M1 et M2. Les M1 sont activés par les cytokines pro-inflammatoires telles que l'IFN-γ, alors que les cytokines de type TH2 (IL-4 et IL-13) peuvent induire l'activation des M2. Ces deux sous-populations cellulaires interviennent dans le contrôle des pathogènes.

2.1.3. Les cellules NK T lymphocytes (NKT)

Les cellules NK T sont des cellules du système immunitaire inné caractérisées par des marqueurs communs aux lymphocytes T et NK [82]. Leur rôle dans la régulation des réponses immunitaires innées et adaptatives a été décrit [83]. Cependant, les NK T influent sur un large éventail de réponses immunologiques y compris l'auto-immunité [84], l'immunité tumorale [85-87], l'induction de la tolérance [88-90] et les maladies infectieuses [91-93]. Cette diversité fonctionnelle est due à la capacité des NK T à produire des cytokines de type TH1 ou TH2. Il existe deux catégories de cellules NK T ; les NK T classiques, encore connues sous le nom de type I ou iNKT et les NK T non-classiques de type II.

Les NK T classiques reconnaissent et se lient aux glycoprotéines comme l'α-galactosylceramide (α-GalCer), présentée par le CD1d à travers le CMH-I [94-96]. Les NK T classiques sont caractérisées par l'expression de marqueurs CD161 ou par $CD3^{+}CD56^{+}$ souvent décrits dans la littérature [97, 98]. Cette catégorie de NK T est peu représentée chez l'Homme.

Les NK T non classiques présentent des différences fonctionnelles par rapport aux NK T classiques. Elles présentent un large répertoire de récepteurs T (TCR) leur permettant de reconnaître les antigènes [99, 100]. Elles ne reconnaissent pas l'α-GalCer et ont un rôle suppresseur sur les NK T classiques [101]. Le rôle des NK T au cours des maladies infectieuses et spécifiquement le paludisme reste encore à déterminer. Elles représentent l'une des sources de production précoce d'IFN-γ pendant la pathologie liée au paludisme.

2.2. Les étapes précoces de la grossesse et la réponse immunitaire de la mère

Juste après la fécondation, un afflux massif de macrophages et de lymphocytes T au niveau de l'utérus en réponse à la présence du fluide séminal et des spermatozoïdes va permettre la première phase de l'implantation. Cette phase qui correspond à l'adhésion de l'embryon et à l'invasion trophoblastique est conditionnée par une importante inflammation via des cytokines (IL-1β, IL-6, TNF-α), des facteurs de croissance (GM-CSF, CSF-1) et des enzymes, qui s'attenue pour permettre l'afflux d'un autre type cellulaire, les cellules NK.

Les cellules NK sont essentielles à la croissance fœto-placentaire et à la décidualisation qui correspond à l'invasion de l'endomètre maternel par les cellules trophoblastiques fœtales pour aboutir à la vascularisation de la décidue [102-110]. L'accumulation des NK dans l'espace utérin (NKu) est maximale au début de la grossesse avec 70% des cellules de la décidue puis diminue à partir de la vingtième semaine pour disparaître au terme. Les NKu produisent principalement de l'IFN-γ, de l'IL-18, du VEGF (Vascular Endothelial Growh Factor), de l'angiopoïétine, du TGF-β (Tumor Growth Factor) et du PLGF (Placental Growth Factor) favorisant la vascularisation de la décidue et la croissance placentaire [111-114]. Ils produisent aussi du LIF (Leukemia Inhibitory Factor) cytokine indispensable à l'implantation de l'œuf. Comme les NK circulants, la fonction des cellules NKu est assurée par les récepteurs KIR (Killer Immunoglobulin like Receptor) et ILT (Immunoglobulin-Like Transcript) capables de moduler leur activation ou leur inhibition.

2.3. Mécanismes d'échappement du fœtus à la réponse immunitaire de la mère

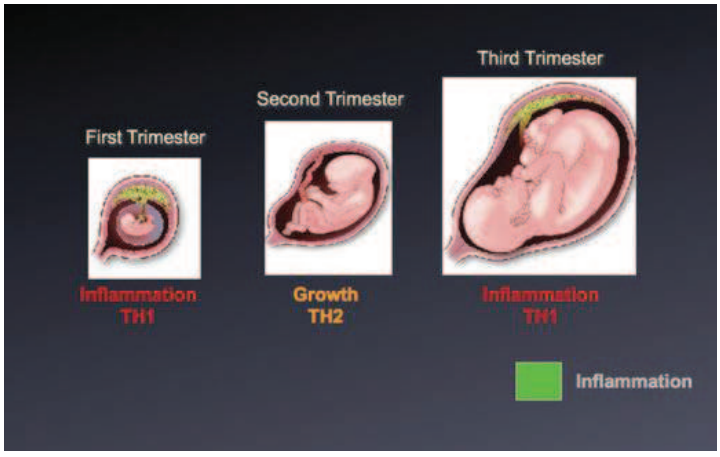
La progestérone, hormone immunosuppressive produite par le corps jaune et le placenta, participerait à la régulation des réponses immunitaires maternelles contre l'allogreffe fœtale [115].

L'absence d'expression de molécules HLA ou CMH de type I (HLA-A et B) ou II par le trophoblaste, empêche sa reconnaissance par les lymphocytes NK, T $CD8^+$ ou B d'origine maternelle [116, 117]. Les trophoblastes expriment au contraire des formes particulières de molécules HLA non classiques (HLA-C, HLA-E, HLA-G), contrôlées en partie par la production

d'IL-10 participant ainsi à la tolérance du fœtus. Les signaux d'inhibition sont induits par les récepteurs KIR des NKu, se liant aux déterminants allotypiques du groupe d'allèles CMH ou par les récepteurs de type C-lectine (CD94/NKG2A) spécifiques des groupes non spécifiques de CMH (HLA-E) [118]. Les récepteurs participant à l'activation sont le FcγRIII (Fc-gamma receptor III) [119-121]. L'action lytique des NKu sera inhibée par la liaison des KIR à l'antigène HLA-G exprimé à la surface des cytotrophoblastes extravilleux participant ainsi à la mise en place des artères spiralées.

Au second trimestre de gestation, les Treg ont un rôle majeur dans le maintien de la tolérance fœtale. Leur activité anti-inflammatoire se développe via différents mécanismes [102, 122-124]. Les Treg produisent de l'IL-10 et le TGF-β pour inhiber des lymphocytes T activés. La production d'Indoléamine 2, 3-dioxygénase (IDO) par les cellules dendritiques ou les macrophages maternels, active les Treg [125-129]. L'hormone gonadotrophique chorionique (hCG) produite par les trophoblastes serait impliquée dans la migration des Treg. Par interaction avec d'autres cellules endothéliales, les Treg inhibent l'activation des cellules maternelles via les molécules membranaires PD-1 ou CTLA-4 fortement exprimées à leur surface et induisent l'apoptose des lymphocytes T maternels activés par l'induction de galectine-1 [130]. L'antigénicité de l'allogreff-fœtale est donc modifiée et contrôlée.

Le 3ème trimestre nécessite des réponses inflammatoires de type TH1 prépondérante pour assurer ses fonctions d'expulsion (**Figure 3**).



Source : Mor et al, 2011 [65]

Figure 3 : Immunologie des différentes phases de la grossesse.

2.4. Aspects immunologiques du PAG

Le contrôle des réponses pro-inflammatoires au second trimestre de la grossesse contribuerait à l'augmentation de la prévalence du paludisme pendant cette période [33, 65]. Le PAG induit des réponses immunitaires locales au niveau placentaire et périphérique pouvant conduire à des conséquences physiopathologiques délétères pour le fœtus et à la mère.

2.4.1. Aspects immunologiques au niveau placentaire

La séquestration des HP favorise l'infiltration de cellules immunocompétentes dans les espaces intervilleux du placenta, la détérioration du tissu placentaire [37, 131, 132] et l'accumulation des pigments malariques dans les cellules phagocytaires [133]. Les monocytes et les macrophages induisent des réactions inflammatoires importantes via des concentrations élevées d'IL-1β, IL-8, TNF-α et IFN-γ [33, 134-137]. De plus, des concentrations élevées de facteurs chimioattractants tels que le MCP-1, MIP-1α & β, I-309 et l'IP-10 sont associées au recrutement des monocytes et des macrophages dans les placentas infectés [7, 33, 135, 136, 138]. L'IL-10 a été également observée en concentration élevée dans le placenta et serait impliquée dans la régulation des réponses inflammatoires [136, 139-141].

La présence d'HP dans le placenta active les monocytes via l'expression de CD54 et le CMH-II, alors que les lymphocytes placentaires présentaient une incapacité fonctionnelle à répondre aux monocytes [142]. Ces résultats suggèrent, d'une part l'incapacité des lymphocytes T à induire des réponses immunitaires efficaces contre le parasite et d'autre part, le recrutement des monocytes et des macrophages vers le placenta.

L'inflammation placentaire favorise des lésions placentaires et ainsi déséquilibre les échanges fœto-maternels tel que le transport d'aminoacides nécessaire au maintien de la croissance intra-utérine [133, 143]. La perturbation de l'angiogénèse à travers des concentrations élevées du VEGF/Flt1 au second trimestre de la grossesse, a été aussi rapportée [144]. Les conséquences de cette inflammation locale sont l'anémie maternelle, le retard de croissance *in utero* et le passage trans-placentaire d'antigènes parasitaires solubles vers le fœtus [143, 145].

2.4.2. Aspects immunologiques au niveau périphérique

Au cours du PAG, l'infection périphérique est caractérisée par une densité parasitaire faible et l'absence des formes trophozoïtes et schizontes du parasite sauf dans les cas rares d'accès graves. Une réponse inflammatoire importante traduite par des concentrations plasmatiques élevées d'IL-

10, de TNF-α et de G-CSF est également présente [146-148]. Des corrélations entre la densité parasitaire et des médiateurs solubles de l'inflammation tels que les récepteurs solubles de TNF (TNFR), l'IL-10 et le G-CSF, ont été montrées lors des infections asymptomatiques chez les femmes enceintes. Ces résultats suggèrent l'utilisation de ces facteurs dans le choix de bio-marqueurs de l'inflammation liée au PAG [146-148]. L'identification de bio-marqueurs devra tenir compte des facteurs cellulaires. Une altération des cellules dendritiques (DC) et des monocytes du sang périphérique, caractérisée par la diminution de leur fréquence et l'inhibition des marqueurs d'activation CD86 et CD80 a été observée chez les femmes impaludées à l'accouchement. Ces observations permettent d'émettre l'hypothèse d'une migration des DC vers les organes lymphoïdes [139]. Le pigment malarique induirait l'activation et la maturation des DC via l'expression de CD80, CD86 et des récepteurs de chimiokines CXCR4, favorisant leur migration vers les organes lymphoïdes [149]. Toutes les études sur-citées ont été réalisées à l'accouchement. Des études longitudinales permettraient de mieux comprendre et d'identifier des facteurs cellulaires et solubles pouvant être utilisés comme bio-marqueurs de l'inflammation liée au PAG.

2.4.3. Immunité spécifique du PAG

La prémunition contre le paludisme, s'acquiert de façon lente et progressive après une exposition répétée. Chez les femmes enceintes, il se développe une immunité mémoire spécifique contre le VAR2CSA en fonction du nombre de grossesses. Ceci explique la susceptibilité des femmes primipares à l'infection. Cette mémoire immunologique est composée, d'une part de réponses cellulaires caractérisées par une production élevée d'IL-2 et d'IL-4 spécifique des souches parasitaires adhérant à la CSA [150] et d'autre part, des réponses humorales spécifiques caractérisées par des concentrations élevées d'anticorps inhibant l'adhérence des HP dans le placenta [151]. Les niveaux d'anticorps élevés contre les domaines du VAR2CSA varient en fonction du niveau de transmission et sont associés à une absence d'infection placentaire chez la femme enceinte [152]. L'immunité protectrice contre le PAG nécessite une régulation des réponses cellulaires effectrices par la production d'anticorps, de cytokines et de chimiokines. Peu d'études existent sur les réponses cellulaires spécifiques du PAG chez la femme enceinte. Les études génomiques réalisées dans le placenta montrent que l'infection chronique est associée à l'expression des facteurs de transcription T-bet dans les lymphocytes B par conséquent une production élevée d'anticorps [153]. Il a été montré que le domaine CIDR1α du *PfEMP1* induit une activation des cellules B [154] et une adhésion non spécifique des IgM au domaine CIDR du *PfEMP1* favorisant leur échappement au système immunitaire [153, 155]. Les recherches sont en cours pour déterminer des domaines similaires au CIDR dans le VAR2CSA afin de comprendre les mécanismes impliqués

dans cette adhésion non-spécifique. Le tableau 2 présente un résumé du rôle des cytokines et chimiokines étudiées au cours du PAG [7].

Tableau 1: Rôle des cytokines et chimiokines au cours du PAG

Cytokines et chimiokines	***Fonctions au cours du PAG***	***Références***
IL-10	Suppression d'activités TH1 ou régulation	[135, 136, 139, 140, 146, 148]
IL-12p70	Favorise les réponses TH1, favorise production IFN-γ	[137, 156, 157]
TNF-α	Induction de la phagocytose chez les macrophages	[136, 140, 145, 147]
IFN-γ	Induction de la phagocytose chez les macrophages	[137, 140, 158]
IL-8, MCP-1	Recrutement des macrophages dans le placenta, adhésion des monocytes aux cellules endothéliales	[136, 138]
MIG ; IP-10	Recrutement des macrophages dans le placenta	[157]

Chapitre 3 : Immunité du nouveau-né et paludisme

3.1. Développement de l'immunité in utero

Les mécanismes de développement de l'immunité débutent *in utero* mais n'achèvent leur maturation qu'après la naissance. Ce développement se fait à travers plusieurs processus: la mise en place de réponses immunitaires innées et adaptatives, les échanges cellulaires entre la mère et le fœtus, le transfert d'immunoglobulines maternelles au fœtus et le système de régulation des réponses immunitaires néonatales.

3.1.1. Mise en place du système immunitaire in utero

Le système immunitaire inné et adaptatif du nouveau-né, se met en place progressivement à travers les mécanismes d'hématopoïèse intra-utérins. Les précurseurs de cellules présentatrices d'antigènes constituent les premières cellules en développement dans le sac vitellin à partir de 4 semaines de gestation. Leur capacité à induire la production de cytokines pro-inflammatoires (IL-6, TNF-α et IL-8) et à présenter l'antigène aux lymphocytes T reste faible par rapport à celle de l'adulte. L'expression des molécules CMH-II est diminuée sur ces cellules. Les cellules NK et les précurseurs de neutrophiles sont générés au niveau du foie fœtal à 6 semaines de gestation tandis que le système du complément se met en place à partir de la 8ème semaine. L'activité cytotoxique des NK et du système du complément devient complète après la naissance [159, 160].

Le développement des cellules du système immunitaire adaptatif se fait en deux phases.

1. Aux environs de 7 semaines de gestation, les lymphocytes pré-T migrent du foie fœtal vers le thymus. Ils acquièrent dans le thymus la capacité d'expression de marqueurs tels que le

TCR et le CD3. Les lymphocytes T naïfs sont présents dans le sang de cordon à partir de 13 semaines de gestation [159]. Ces cellules restent naïves puisqu'elles n'ont jamais rencontré d'antigène [161].

2. Des précurseurs de lymphocytes B, exprimant le $CD19^{+}$ induisent le développement des lymphocytes B dans le foie fœtal à partir de 8 semaines de gestation. Dès la $12^{ème}$ semaine, les lymphocytes B naïfs, exprimant les récepteurs BCR sont détectables dans le sang de cordon.

Au cours du développement fœtal, les cellules du système immunitaire sont qualitativement matures mais restent naïves en raison de l'absence d'infection [162]. Seules les cellules de type TH2 et les Treg sont activées au cours du développement fœtal en absence d'infection utérine [162]. Le mécanisme de mise en place du système immunitaire néonatal peut être perturbé par des infections maternelles, des pathologies associées à la grossesse, telles que l'éclampsie, le retard de croissance *in utero* ou par un dérèglement dans les échanges materno-fœtaux [163, 164].

3.1.2. Echanges materno-fœtaux

Plusieurs échanges immunologiques se font entre la mère et le fœtus, à travers le syncytiotrophoblaste et les vaisseaux endothéliaux. Ces échanges peuvent se faire par transfert passif ou actif [165]. Les cellules trophoblastiques peuvent être identifiées dans la circulation sanguine de la mère alors qu'il existe des contradictions sur le passage trans-placentaire des cellules maternelles [166, 167]. La mise en évidence du passage de cellules du fœtus à la mère est utilisée pour le diagnostic de pathologies anténatales.

3.1.3. Transfert d'immunoglobulines de la mère vers le fœtus

Le transfert de certaines substances de faible poids moléculaire (<500 Da), telles que le fer et les acides aminés est coordonné par le transfert unidirectionnel alors que le transfert des immunoglobulines G (poids moléculaire 160 kDa) se fait à travers les récepteurs Fc endothéliaux à partir du premier trimestre de la grossesse [168]. Les sous-classes d'immunoglobulines G (IgG1, IgG2, IgG3 et IgG4) représentent les seules immunoglobulines pouvant traverser la barrière placentaire [165, 169]. Des études sur des grossesses pathologiques ont montré que les IgA et IgM pouvaient traverser la barrière placentaire mais les mécanismes liés à ce transfert restent à déterminer, en raison de leur poids moléculaire élevé [170]. La présence d'IgA, IgE et IgM dans le sang de cordon signe une production de novo par le nouveau-né. Le transfert d'IgG vers le fœtus participe à sa protection jusqu'à neuf mois environ, en attente de la mise en place du système immunitaire du nouveau-né [171]. Malgré une exposition limitée *in utero* aux antigènes et une

protection temporaire induite par les anticorps maternels, le système immunitaire du nouveau-né reste naïf et quiescent [172, 173].

Les cytokines et chimiokines ne passent pas la barrière placentaire. Le passage d'IL-6 a été rapporté [174] mais cette information a été contredite par une autre équipe démontrant l'absence de transfert de cytokines pro-inflammatoires [175].

3.1.4. La régulation du système immunitaire néonatal

Au moment d'une infection intra-utérine, les réponses immunitaires du fœtus sont régulées par un mécanisme de contrôle programmé. En effet, les cellules hématopoïétiques fœtales seraient programmées pour induire la différenciation des lymphocytes T en Treg. Cette différenciation est nécessaire à la tolérance du fœtus [176]. Ce contrôle de la réponse immunitaire par des fréquences élevées de Treg chez le fœtus sera prépondérant jusqu'à la naissance.

Les travaux de Holt et coll (2009), ont montré qu'une exposition *in utero* aux facteurs environnementaux, induirait une programmation des cellules dendritiques maternelles à la sensibilisation de la réponse immunitaire fœtale. Cette sensibilisation va dans le sens du contrôle des allergies au cours du développement fœtal. Ces résultats suggèrent un mécanisme par lequel l'exposition de la mère aux bactéries environnementales pourrait protéger le nouveau-né contre les allergies [177]. Des études *in vitro* et *in vivo* ont montré que l'exposition à *Trypanosoma cruzi*, peut être associée à une production de cytokines pro- (IFN-γ, TNF-α) et anti-inflammatoires (IL-10) chez des nouveau-nés non-infectés [178, 179]. Ces résultats suggèrent le développement de réponses inflammatoires nécessaires à la protection contre une transmission verticale chez ces enfants. Les nouveau-nés issus de femmes infectées par *P. falciparum* à l'accouchement développent très tôt une infection palustre, un épisode fébrile ou une gastro-entérite [54, 180-184]. L'ensemble des travaux sur-cités démontrent que l'exposition *in utero* du fœtus aux pathogènes, peut orienter ses réponses immunitaires vers la susceptibilité à la maladie ou vers une régulation. Le mécanisme de régulation des réponses immunitaires du nouveau-né lors d'une exposition *in utero* est mal connu.

3.2. L'immunité du nouveau-né

Le changement soudain d'environnement par le nouveau-né après l'accouchement l'expose aux microorganismes. L'activité cytolytique faible des NK, l'immaturité des DC et les différences fonctionnelles des lymphocytes T et des monocytes par rapport à l'adulte, favoriseraient la susceptibilité du nouveau-né aux infections. La production d'IL-10, et d'IL-17 et l'abondance des lymphocytes Treg suggèrent une tolérance caractéristique du système immunitaire néonatal [173].

Le nouveau-né a donc besoin d'assurer sa propre défense vis-à-vis des pathogènes. Les contacts répétés avec des pathogènes induiraient une maturation progressive de son système immunitaire. Le système immunitaire du nouveau né est naïf mais dispose de toutes ses capacités fonctionnelles pour évoluer progressivement vers une immunité protectrice. En effet, les cellules NK du nouveau-né sont capables de produire de l'IFN-γ après une stimulation avec de l'IL-12 et de l'IL-15 [185]. La maturation du système immunitaire néonatal est caractérisée par la reconnaissance par les cellules de l'immunité innée (CPA, NK) des motifs moléculaires antigéniques connus sous le nom de PAMP (Pathogen-associated molecular patterns). Cette liaison favorise la production de cytokines et de chimiokines impliquées dans les réactions inflammatoires. La reconnaissance des PAMP peut se faire de façon directe ou indirecte. La liaison directe se traduit par l'adhésion aux récepteurs de surface les reconnaissant appelés "PRR" (Pattern Recognition Receptors) alors que la liaison indirecte implique la reconnaissance de molécules sériques (opsonines) à la surface du pathogène accompagnée du processus d'opsonisation. La mise en place de l'immunité à médiation cellulaire se fait progressivement chez le nouveau-né après une primo-infection ou par certains vaccins [186, 187].

Trois familles de PRR ont été décrites : les récepteurs Toll (TLR : Toll Like-Receptors), les récepteurs de type lectine (CLR : C-type lectin receptors) et les récepteurs intra-cytoplasmiques de type NLR (nucleotide oligomerization domain (NOD)-like domain). Les récepteurs TLR sont les plus étudiés et, dans le cadre de cette thèse, nous nous sommes intéressés à ce groupe de récepteurs. Il existe à ce jour, 10 récepteurs TLR décrits chez l'homme [188] et distribués sur différents types de cellules immunitaires (Tableau 1) [189, 190]. Les récepteurs TLR induisent la production des cytokines ou chemokines nécessaires à l'élimination du pathogène à travers l'activation cellulaire. Les TLR sont constitué d'un domaine intra-cytoplasmique qu'ils partagent avec la famille des récepteurs de l'IL-1 (IL-1R) et d'un domaine extracellulaire riche en leucine appelé TIR (Toll/IL-1R/R). Les TLR1, TLR2, TLR4, TLR5 et TLR6 exprimés à la surface des cellules reconnaissent des motifs microbiens protéiques ou lipidiques (exogènes) alors que les TLR3, TLR7, TLR8 et TLR9 sont intracellulaires (localisés dans les endosomes/lysosomes de la cellule hôte) et reconnaissent les acides nucléiques [191]. Du fait de leur implication dans l'initiation des réponses immunes effectrices, les TLR font l'objet de plusieurs études dans le cadre de la recherche des cibles thérapeutiques car ils constituent des cibles de choix en matière d'immuno-modulation. Cependant, les réponses immunitaires innées induites par l'activation des TLR chez le nouveau-né diffèrent de celles induites chez l'adulte [192].

Tableau 2: Distribution cellulaire des récepteurs TLR [189, 190]

Récepteurs TLR	***Type cellulaire***	***Ligands et origines***
TLR1	myéloïdes (m) et plasmacytoïdes (p) DC, Monocytes et Macrophages	Triacyl Lipoproteïne (bacterie)
TLR2	Macrophages, mDC et Monocytes	Zymozan, Peptidiglycan (champignons et bacteries)
TLR3	mDC, lymphocyte T	ARN double brin (virus), Poly I.C
TLR4	Macrophages, Monocytes	Lipopolysaccharide (LPS), proteine (bacterie, virus)
TLR5	mDC, Monocytes	Flagelline (bacterie)
TLR6	pDC, Monocytes	Diacyl Lipoproteïne (champignons et bacteries)
TLR7	pDC,	ARN double brin (virus)
TLR8	mDC, Monocytes	ARN double brin (virus)
TLR9	pDC, Monocytes	CpG-ADN, (virus, protozoaire)
TLR10	mDC, pDC	-

3.3. Aspects immunologiques du nouveau-né liés au paludisme

La compréhension de l'immunité néonatale par rapport à l'infection palustre permet de comprendre la susceptibilité à la maladie pendant la croissance. Les nouveau-nés issus de mères infectées ont un risque élevé de faire leur premier accès palustre au cours de la première année de vie [54]. Les mécanismes immunologiques liés à cette susceptibilité restent encore à déterminer. Des études réalisées dans le sang de cordon permettent de comprendre les réponses immunitaires spécifiques du paludisme chez les nouveau-nés. L'immunité du nouveau-né de mère infectée est caractérisée par une suppression de réponses inflammatoires par des fréquences élevées des cellules Treg [193-197]. L'immaturité des cellules dendritiques (faible expression de CMH-II) favoriserait leur incapacité à initier des réponses spécifiques T chez les nouveau-nés exposés *in utero* [193]. Ces résultats montrent la régulation des réponses cellulaires chez les nouveau-nés exposés *in utero* [194, 196-199]. Cependant, une stimulation avec des ligands de TLR démontre la capacité des cellules du sang de cordon de nouveau-né exposés, à produire de l'IFN-γ [200]. La capacité des nouveau-nés exposés à produire des cytokines inflammatoires dépend de la parité et de la période d'exposition. Dans une étude prospective, les enfants exposés *in utero* ont été suivis jusqu'à l'âge de trois ans [197]. Les résultats de cette étude montrent que les enfants ayant été exposés présentent une tolérance immunitaire se manifestant par des concentrations élevées d'IL-10 après stimulation avec des antigènes parasitaires. Ces résultats suggèrent l'acquisition *in utero* d'une tolérance immunitaire vis-à-vis du paludisme qui induirait une modulation des réponses immunitaires néonatales par rapport aux enfants non exposés. Les mécanismes liés à ce retard de réponse, restent encore à déterminer. Nous proposons dans cette thèse d'étudier un des mécanismes à travers la stimulation avec des ligands de TLR.

Chez les jeunes enfants infectés par *P. falciparum*, des fréquences élevées de DC ont été observées ce qui suggère une réponse spécifique importante mais la présence d'antigènes parasitaires inhiberait l'activation de ces cellules [201-204].

Les travaux d'Asito et coll (2011), ont montré que les jeunes enfants vivant en zone d'endémie palustre présentent une altération de réponse des cellules B mémoires [205]. En effet, des fréquences élevées de lymphocytes B et une expression de facteurs du complément dans le sang périphérique, suivie par celle de cytokines inflammatoires IL-10, IL-13, IL-31, IL-33 ont été associées aux accès graves [206, 207]. Dans d'autres études, des fréquences élevées de DC immatures ont été observées chez des enfants infectés par *P. falciparum* [202, 204]. Ces données suggèrent qu'une réponse inflammatoire importante chez l'enfant au cours de l'infection palustre pourrait conduire à des accès graves. Par conséquent, la prise en charge des jeunes enfants est indispensable dans le contrôle du paludisme.

DEUXIEME PARTIE : PROBLEMATIQUE ET OBJECTIFS DE LA THESE

L'infection à *Plasmodium falciparum* au cours de la grossesse a des conséquences importantes sur les réponses immunologiques de la mère et du nouveau-né. L'objectif de cette thèse est d'apporter des éléments nouveaux sur ces modifications immunologiques qui seront utiles à la compréhension de la pathologie et au développement d'un vaccin chez la femme enceinte mais aussi chez l'enfant. Le paludisme associé à la grossesse (PAG) est caractérisé par la cyto-adhérence d'un antigène parasitaire particulier VAR2CSA. Ce phénomène s'accompagne de réponses inflammatoires locales impliquant l'ensemble des cellules médiatrices de l'immunité [7, 34, 153]. Le PAG a pour conséquences : l'anémie maternelle, l'avortement, la prématurité et le retard de croissance utérin [33, 42]. Ces conséquences peuvent trouver leur origine dans les infections survenues très tôt pendant la grossesse avant que la prévention ne soit possible [208, 209]. Il a été montré que les femmes enceintes acquièrent progressivement en fonction de la parité, une immunité protectrice contre l'adhésion des parasites dans le placenta [26]. Par ailleurs, les parasites des infections précoces (<13 semaines de gestation) exprimeraient déjà un génotype et phénotype placentaires démontrant ainsi l'importance d'un contrôle de la séquestration des HP par l'immunité quand un traitement n'est pas encore possible [210].

L'étude des réponses immunitaires associées au paludisme gestationnel a souvent été réalisée à l'accouchement. D'une part, l'existence d'activités inflammatoires locales dans le placenta et le contrôle de ces activités par les réponses TH2 et les lymphocytes T régulateurs ont été observés au cours du paludisme placentaire [137, 146, 153]. D'autre part, une seule étude a montré de faibles fréquences de DC immatures dans le sang périphérique et le sang intervilleux du placenta chez les femmes infectées par *P. falciparum* [139]. Cette étude suggère une migration des DC vers les sites d'infection. Par ailleurs, plusieurs molécules ont été proposées comme bio-marqueurs de l'inflammation liée au PAG [146, 147, 211] mais les données existantes nécessitent une validation sur des études de cohorte. Pour mieux comprendre les mécanismes immunologiques liés au PAG, nous proposons de documenter les changements immunologiques qui pourraient intervenir chez la femme enceinte, dans un contexte de temps et de pathologie. Un des aspects de cette thèse est l'étude des réponses cellulaires mises en place au début de la grossesse et à l'accouchement. Nos objectifs spécifiques sont :

- Caractériser phénotypiquement les cellules immunitaires dans le sang périphérique de la femme enceinte
- Quantifier les cytokines et les chimiokines plasmatiques impliquées dans l'inflammation au cours du PAG.

Les enfants nés de mères impaludées développent leur première parasitémie vers l'âge de 3 mois alors que les enfants nés avec un placenta non parasité développent leur première parasitémie vers 6 mois [54]. Cette observation a été confirmée par d'autres cohortes [180-182, 184] et la présence d'immunoglobulines G (IgG) anti-parasites placentaires d'origine maternelle dans le sang de cordon de nouveau-nés issus de mères impaludées [212]. Chez les nouveau-nés de mères infectées, des concentrations importantes d'IgM et IgE ont été retrouvés dans le sang de cordon. Ces résultats démontrent que l'acquisition *in utero* d'immunité anti-plasmodiale pourrait influencer les réponses immunitaires futures de l'enfant [213-216]. L'infection placentaire pourrait induire chez le nouveau-né des fréquences élevées de lymphocytes T régulateurs dans le sang de cordon et, par conséquent, des concentrations élevées d'IL-10 [33, 194, 197, 200]. Ceci induirait la suppression de réponses de type TH1 dans le sang de cordon et favoriserait la susceptibilité à l'infection [196, 199]. L'étude réalisée sur une cohorte d'enfants confirme ces résultats et suggère une tolérance induite par l'exposition *in utero* au paludisme [197]. Cependant, les mécanismes immunologiques impliqués dans l'orientation de cette tolérance restent encore à déterminer. C'est dans ce contexte que se situe notre travail de thèse afin de comprendre les mécanismes d'orientation des réponses immunitaires innées du nouveau-né et du jeune enfant.

Nous étudierons les conséquences d'une exposition *in utero* sur les fréquences et l'activation des cellules présentatrices d'antigènes (CPA) dans le sang de cordon pour, ensuite, étudier les mécanismes de stimulation ou d'inhibition des réponses immunitaires innées à travers les récepteurs TLR.

Ce projet de thèse fait partie d'un vaste projet financé par l'Union Européenne, STOPPAM (Strategies TO Prevent Pregnancy Associated Malaria), regroupant 4 équipes européennes (France, Suède, Danemark et Pays-Bas) et 2 équipes africaines (Tanzanie et Bénin). Il a pour objectif principal, d'élucider les mécanismes et le déroulement de la pathologie du paludisme associé à la grossesse et de quantifier les conséquences du paludisme associé à la grossesse sur la santé de la mère et du fœtus. Les résultats issus de ce projet permettront d'optimiser les stratégies de prévention contre le paludisme et de proposer des candidats vaccins anti-parasites placentaires. Sur le plan immunologique, il permettra de comprendre des mécanismes immunologiques liés aux conséquences du PAG chez la femme enceinte, le nouveau-né et le jeune enfant.

La finalité de cette étude de thèse est l'identification de marqueurs cellulaires et solubles liés à la pathologie associée au PAG.

TROISIEME PARTIE : CADRE ET METHODES D'ETUDE

Organisation de la thèse en alternance

La plus grande partie de ce travail a été réalisée grâce à une bourse de thèse de trois ans, du département AIRD-DPF de l'Institut de Recherche pour le Développement (IRD). Ce financement m'a permis d'effectuer pendant les trois années des alternances entre les équipes du CERPAGE (Centre d'étude et de recherche sur le paludisme associé à la grossesse et à l'enfant) à Cotonou (Bénin), de l'UMR216 IRD à Paris (France) et de l'Institut Wenner-Gren de l'Université de Stockholm (Suède).

Chapitre 1 : Etude préliminaire mère-cordon réalisée à l'HOMEL

Dans une première étude préliminaire, nous nous sommes intéressés à l'impact du PAG sur les réponses cellulaires du nouveau-né. Cette étude a été réalisée en 2007-2008 avec la collaboration de l'Hôpital de la Mère et de l'Enfant de la Lagune (HOMEL) de Cotonou. Nous avons enrôlé 59 femmes enceintes à l'accouchement. Des tests de diagnostic rapide (TDR) ont été réalisés quelques heures avant l'accouchement (au cours du travail) chez ces femmes, pour le diagnostic palustre. Le sang de cordon a été prélevé sur anticoagulant et acheminé vers le laboratoire du CERPAGE. Des biopsies et des appositions placentaires ont été également réalisées. Pour chaque femme infectée, un ajustement sur une femme non-infectée a été fait selon l'âge et la parité.

Chapitre 2 : Site d'études et déroulement de l'étude STOPPAM

Au Bénin, le projet STOPPAM a été mis en place et coordonné par le CERPAGE implanté au sein de la Faculté des Sciences de la Santé à Cotonou, Bénin. Il est associé à l'Université d'Abomey-Calavi à travers l'Institut de Sciences Biomédicales Appliquées (ISBA). En Tanzanie, l'étude STOPPAM a été mise en place et coordonnée par l'Université Radboud de Nijmegen (Pays-Bas), l'Université de Copenhague (Danemark) et l'Institut National de Recherche Médicale de Tanzanie. Les différents partenaires de ce projet sont représentés sur la figure 4.

Figure 4 : Les différents partenaires impliqués dans le projet STOPPAM

2.1. Description de l'étude STOPPAM

Au Bénin, l'étude STOPPAM s'est déroulée de février 2008 à avril 2011 à Comé (70 Km de Cotonou, capitale économique). En Tanzanie, elle s'est déroulée entre septembre 2008 et octobre 2010 à Korogwe (100 Km au Nord-Est de Tanga). Il s'agissait d'un suivi longitudinal d'une cohorte de 1000 femmes enceintes sur les deux sites et d'une cohorte de 210 enfants sur le site du Bénin. La figure 5 représente le site d'étude au Bénin.

Figure 5 : Site d'étude STOPPAM au Bénin

Les critères d'inclusion étaient : l'âge gestationnel inférieur à 24 semaines, la résidence depuis plus de 6 mois à moins de 10 Km du dispensaire et l'intention d'accoucher au dispensaire. Un

consentement écrit et éclairé a été signé par les femmes qui acceptaient de participer à l'étude. L'étude STOPPAM a reçu l'aval des comités d'éthique dans chaque pays du consortium, Faculté des Sciences de la Santé de l'Université d'Abomey-Calavi pour la partie Béninoise et du « Medical Research Coordinating Committee » en Tanzanie.

Pendant la grossesse les données cliniques et parasitologiques ainsi que les échantillons biologiques étaient recueillis à chaque consultation prénatale (CPN) de l'inclusion à l'accouchement et aussi pendant les consultations d'urgence. Les enfants étaient vus deux fois par mois avec en alternance, une visite à domicile et une visite au dispensaire avec aussi des consultations en urgence (**Figure 6**). Lorsqu'une infection était détectée au cours du suivi et des visites, les femmes étaient systématiquement traitées selon les recommandations nationales du ministère de la santé béninois et tanzanien, avec une dose de quinine (8mg/kg/jour en trois fois pendant sept jours) et les enfants au Coartem© (20 mg d'artéméther et 120 mg de luméfantrine).

Les échantillons biologiques étaient convoyés quotidiennement au laboratoire pour des analyses hématologiques, biochimiques, parasitologiques et l'étude de l'immunité à médiation cellulaire sur chaque site. La cohorte de Tanzanie a servi de complément de données et de confirmation pour les résultats obtenus dans cette thèse.

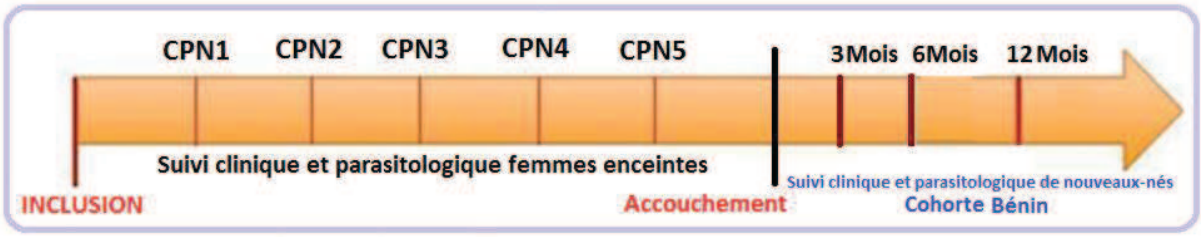


Figure 6 : Suivi des femmes enceintes et des enfants au cours du projet STOPPAM

2.2. Transmission palustre sur les deux sites de STOPPAM

La transmission palustre au Bénin est hyper-endémique avec deux pics correspondant aux deux saisons de pluies (avril-juillet, septembre-novembre). Le taux d'inoculation entomologique est de 20 à 35 piqûres infectantes par personne et par an [181, 217]. Les deux vecteurs de transmission palustre au Bénin sont *Anopheles gambiae* suivi d'*Anopheles funestus*. L'espèce plasmodiale prépondérante est *Plasmodium falciparum* suivi de quelques cas de *Plasmodium malariae*. La transmission placentaire au Bénin dans l'étude STOPPAM était de 11,3 %.

La transmission palustre en Tanzanie devient faible d'après les données entomologiques existantes et *Plasmodium falciparum* est l'espèce prépondérante [218]. Les données

entomologiques sur le taux d'inoculation manquent et il était estimé à moins de 30 piqûres infectantes par personne et par an dans les zones rurales en 2003 [219].

Chapitre 3 : Méthodes d'étude

L'originalité de l'étude STOPPAM était de pouvoir disposer de l'échographie obstétricale pour estimer l'âge exact de la grossesse. L'étude STOPPAM a enrôlé 1037 femmes enceintes au Bénin et 1000 en Tanzanie. Environ, 6000 prélèvements veineux ont été réalisés au cours de cette étude sur chaque site.

3.1. Echantillonnage

Nous avons choisi, dans cette thèse, de travailler à deux moments de la grossesse (début de grossesse et accouchement) sur des sous-populations issues de la cohorte STOPPAM pour des raisons techniques et financières.

Chez les femmes enceintes : 131 femmes ont été incluses lors de leur première visite prénatale, et suivies pendant toute leur grossesse, et 111 femmes différentes (en partie) ont été incluses à l'accouchement après leur suivi. Le groupe de femmes incluses à la première visite est subdivisé en deux sous-groupes de femmes infectées et non infectées par le critère TDR (Parascreen, IDA Foundation, Netherlands) plus la goutte épaisse (GE), réalisés sur du sang capillaire, celui des femmes à l'accouchement est subdivisé en femmes infectées (TDR et GE positifs à l'accouchement), femmes exposées (infection palustre au moins une fois durant la grossesse) et femmes non infectées (TDR ou GE négatifs au cours de la grossesse). Des prélèvements de sang veineux ont été effectués.

Chez les nouveau-nés: 217 enfants ont été répartis en 3 groupes, 99 nés de mères non-infectées, 71 nés de mère ayant été au moins une fois infectée au cours de la grossesse mais non-infectée à l'accouchement, enfin 47 nés de mère infectées à l'accouchement. Des prélèvements veineux à 3, 6 et 12 mois ont été effectués. Les enfants avec un statut VIH positif ou inconnu ainsi que ceux pour lesquels nous n'avons pas pu prélever 2 ml de sang veineux (80) ont été exclus de l'étude. Ce qui réduit à 137 le nombre d'enfants suivis.

3.2. Immunophénotypage des cellules présentatrices d'antigènes (CPA) et des lymphocytes T par cytométrie en flux

3.2.1. Séparation des cellules mononucléées par Ficoll

Les échantillons de sang ont été acheminés au laboratoire dans les 4 heures suivant le prélèvement. Ils sont centrifugés à 1500 tours/minute pendant 10 minutes, ensuite les plasmas sont conservés à -80°C, en prévision du dosage de cytokines et chimiokines. Le ficoll-hypaque (Pharmacia Uppsala, Sweden) a été effectué sur le sang total afin de récupérer les cellules mononucléées (CMN). Ces cellules sont récupérées et lavées avec de l'eau physiologique, puis suspendues à une concentration de $2x10^6$ cellules/ml dans du milieu RPMI 1640 (Roswell Park Memorial Institute, USA) contenant de la L-glutamine (Gibco Eragny, France), du sérum de veau fœtal (Gibco Eragny, France) à 10% et de la gentamycine à 50µg/ml. La viabilité des cellules a été déterminée grâce à la solution de bleu Trypan. Pour tous nos échantillons, la viabilité était supérieure à 99%.

3.2.2. Phénotypage *ex vivo* des CPA et lymphocytes

Chez les femmes enceintes : Les fréquences des sous-populations cellulaires ont été déterminées après marquage extracellulaire par des anticorps monoclonaux (Miltenyi, Biotech, Bergisch-Gladbach, Germany). Les mDC ont été caractérisées par le $CD1c^+$ et les pDC par le $CD303^+$. Les monocytes sont définis par le $CD14^+$, les lymphocytes B par $CD19^+$, les Treg par le phénotype $CD4^+CD25^+CD127^-$ les NK T par le $CD3^+CD56^+$ et les NK par $CD56^+$. Les niveaux d'intensité de fluorescence des marqueurs d'activation cellulaire tels que le CMH-II et le CD86 ont été mesurés sur les DC, monocytes et lymphocytes B, puis le $Foxp3^+$ sur les Treg (**Figure 7**). Les stratégies d'analyse sont représentées sur les **Figure 8 & 9**.

Chez les enfants : L'immunophénotypage des cellules mononucléées du sang de cordon est fait selon la procédure utilisée chez les femmes enceintes. En plus des populations cellulaires décrites chez les femmes enceintes, les mDC ($BDCA3^+$, $CD141^+$) ont été recherchées dans le sang de cordon. Les populations de Treg, de NK et de NK T n'ont pas été étudiées dans cette thèse dans le sang de cordon.

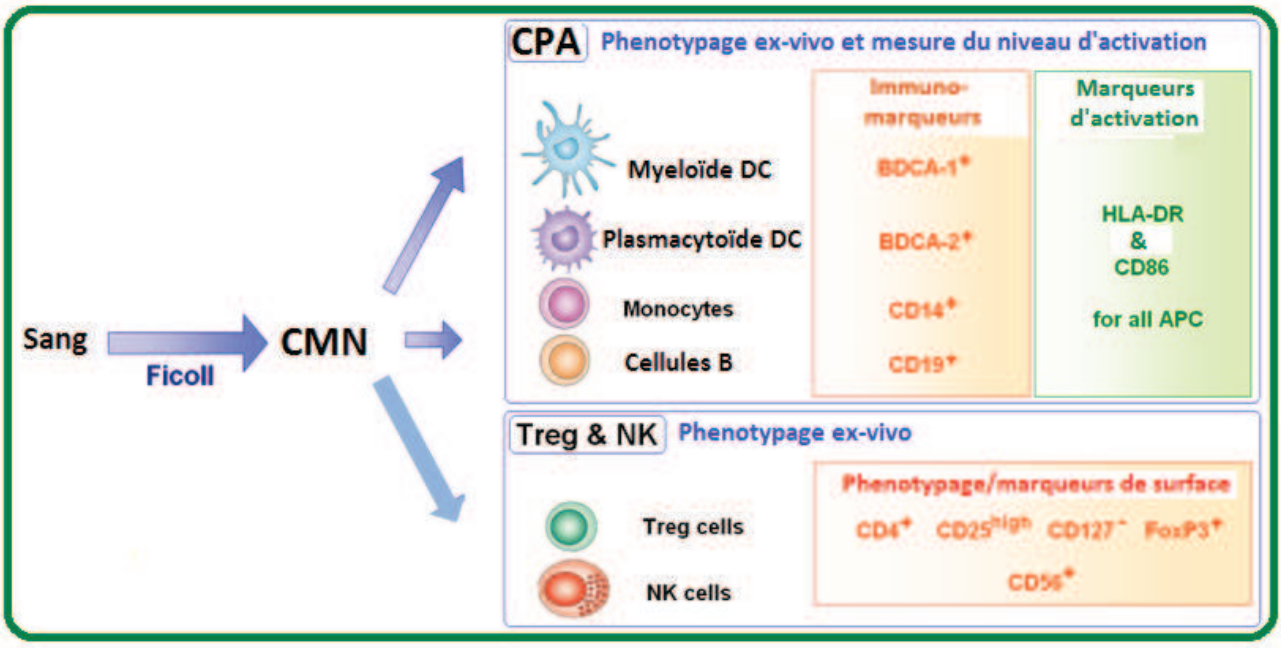


Figure 7 : Immunophénotypage des cellules médiatrices de l'immunité par cytométrie en flux

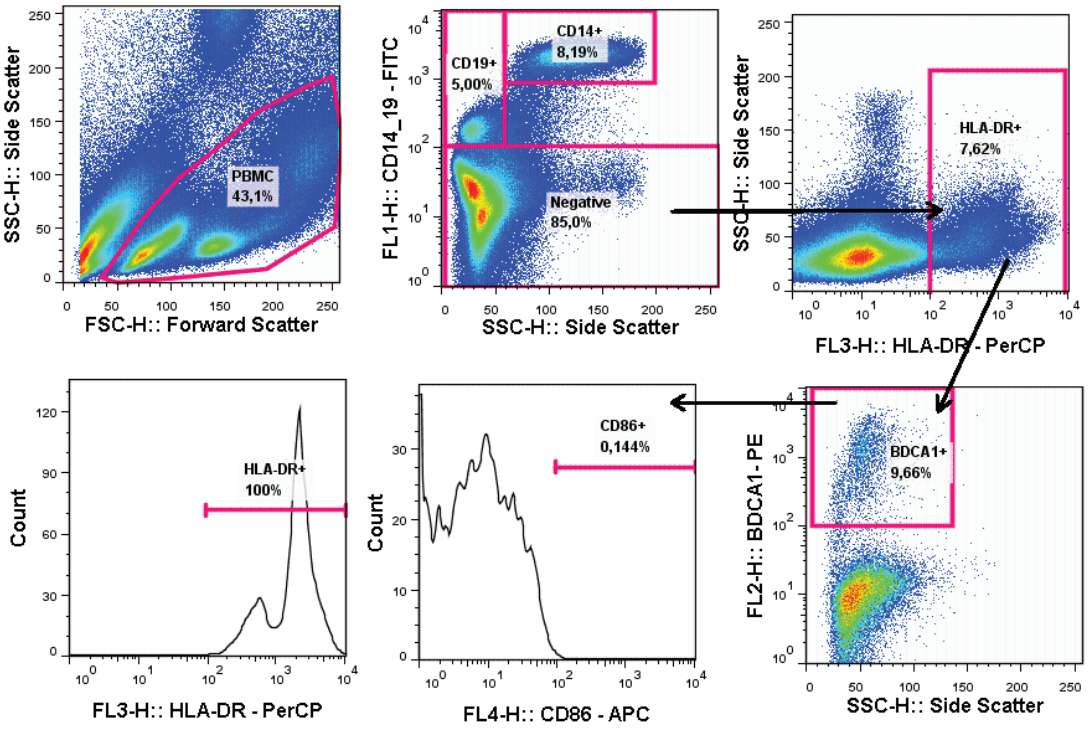


Figure 8 : Stratégie d'analyse phénotypique des CPA

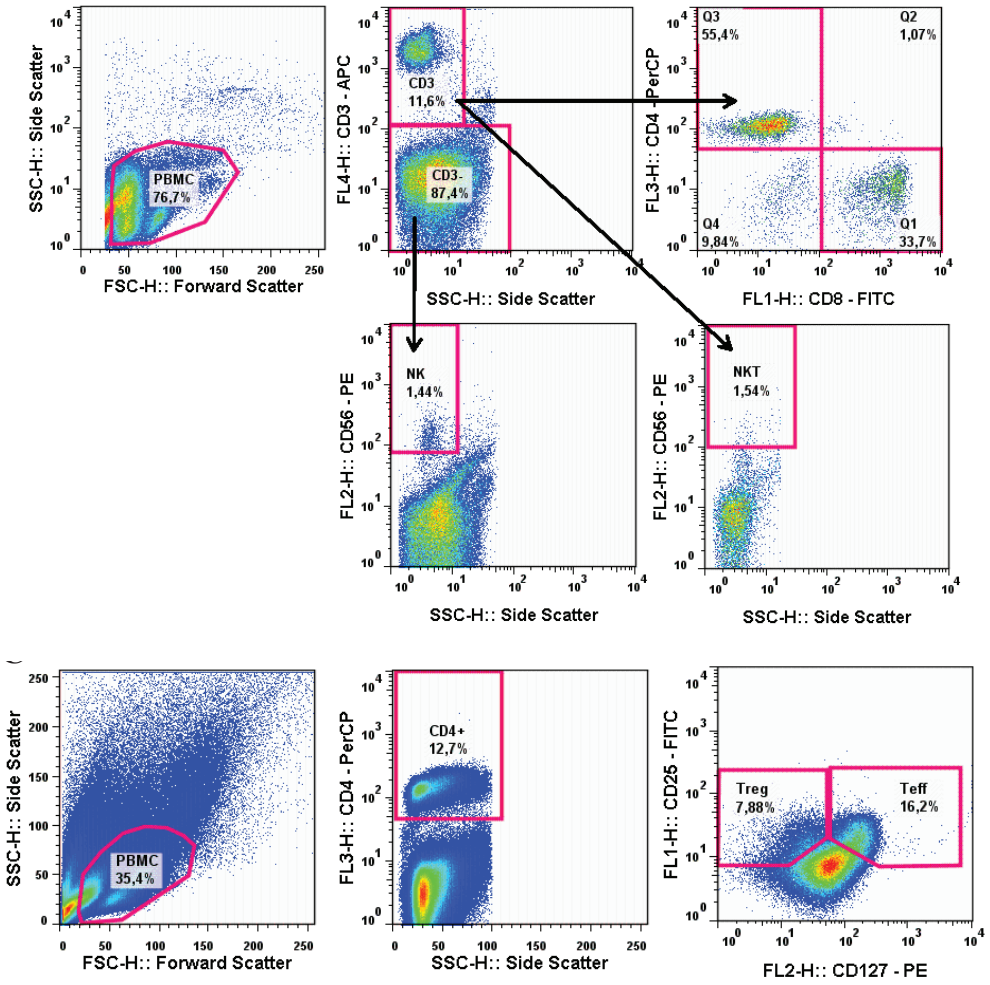


Figure 9 : Stratégie d'analyse des cellules NK, NK T et Treg

3.3. Quantification des cytokines pro- et anti-inflammatoires dans les plasmas et les surnageants de culture

Chez les femmes enceintes : Les concentrations plasmatiques des cytokines: IL-1β, IL-6, IL-10, TNF-α, IL-12p70, des chimiokines: CCL5/RANTES, MCP-1/CCL2, IP-10 et MIG/CXCL9 ont été mesurées par CBA (Cytometric Beads Array). Les concentrations d'IFN-α, IFN-γ et des facteurs de vascularisation: Angiopoiétineen-1&2, uPAR et le VEGF-R1/Flt1 soluble, sont mesurées par la technique ELISA. Les deux techniques ont été utilisées selon les recommandations réglementaires.

Chez les enfants : Des stimulations spécifiques ont été faites sur des cellules mononucléées du sang de cordon, avec des ligands de TLR suivants : LPS (TLR4), le Poly IC (TLR3), le résiquimod (TLR7-8) et le CPG ODN (TLR9) pendant 20 heures. Les surnageants de culture sont conservés à -80°C. La quantification des cytokines IL-10, IL-6, IFN-γ et TNF-α est faite par la technique CBA (**Figure 10**).

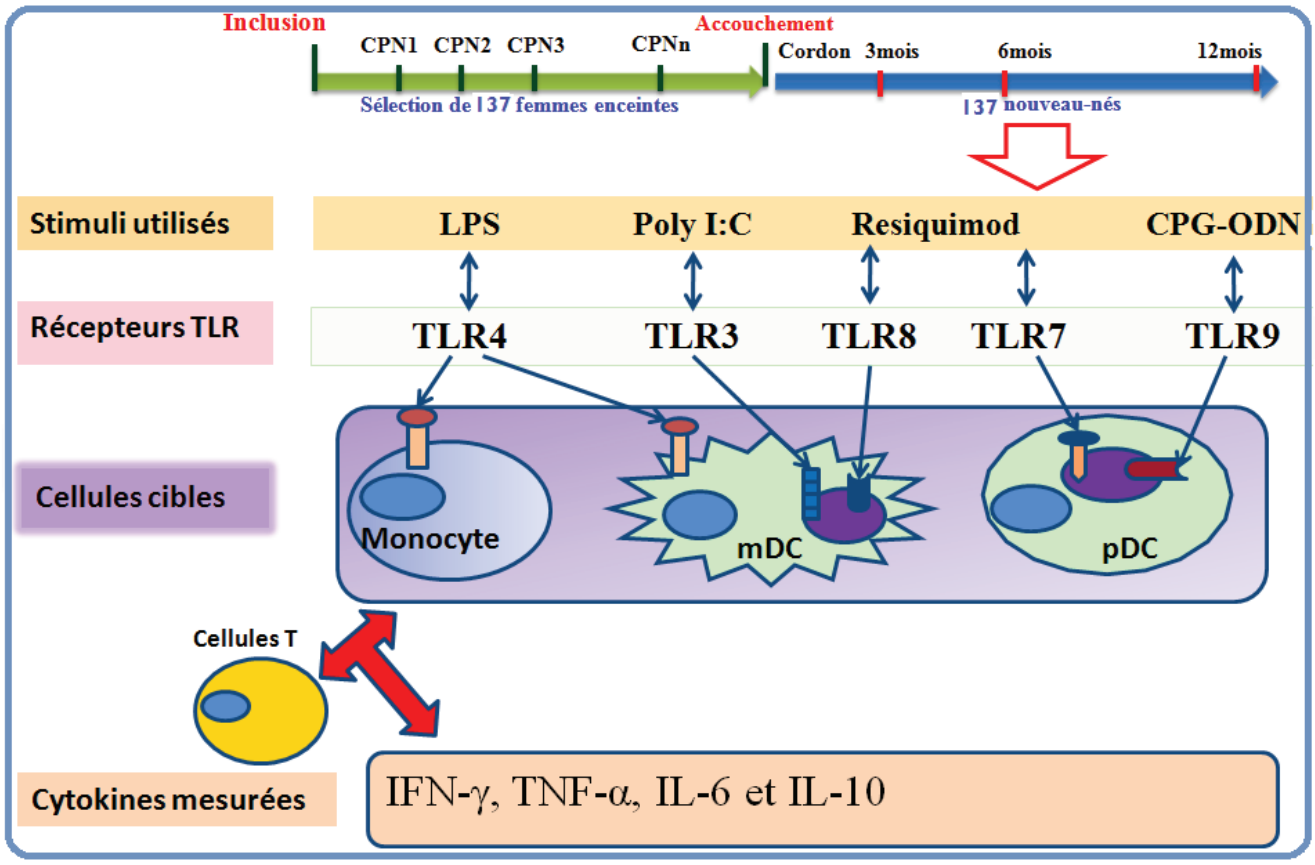


Figure 10 : Protocole expérimental de mesure de cytokines dans les surnageants de cultures chez les nouveau-nés inclus dans notre étude.

3.4. Analyse statistique

Les données de l'immunophénotypage ont été acquises par le cytomètre BD FACS Calibur (BD Biosciences, France). L'analyse des données est faite grâce au logiciel FlowJo et CellQuest Pro. Les concentrations de cytokines et chimiokines ont été obtenues après analyse avec le logiciel FCAP array.

Les analyses statistiques ont été réalisées par les outils STATA 12 (StataCorp, College Station, TX USA) et Prism 5.0 (Graph pad Inc, CA, USA). Les tests paramétriques et non paramétriques ont été utilisés selon les données.

QUATRIEME PARTIE: RESULTATS

ARTICLE 1: Peripheral blood cell signatures of Plasmodium falciparum infection during pregnancy.

Ibitokou S, Oesterholt M, Brutus L, Borgella S, Agbowaï C, Ezinmègnon S, Lusingu J, Massougbodji A, Deloron P, Troye-Blomberg M, Varani S, Luty AJF & Fievet N.

PLoS ONE (2012), 7(12): e49621.

Contexte : La séquestration des hématies parasitées dans le placenta caractérise le paludisme associé à la grossesse (PAG). Ceci a pour conséquences principales une anémie maternelle et un faible poids de naissance (avec un retard de croissance et une prématurité). Il a été démontré que l'infection placentaire altère les cellules présentatrices d'antigènes du sang périphérique ou du sang intervilleux placentaire chez la femme enceinte [139]. Par ailleurs, les conséquences du paludisme gestationnel chez la femme enceinte dépendent de la période de l'infection [208, 209]. Cependant, les données longitudinales sur le plan immunologique sont difficilement accessibles à cause de l'indisponibilité du placenta. Afin de mieux documenter les profils cellulaires au cours du PAG et la physiopathologie du PAG chez la femme enceinte, nous nous sommes intéressés aux phénomènes immunologiques au début de la grossesse et à l'accouchement.

Objectifs de l'étude : Cette étude vise en premier lieu, à évaluer l'impact du PAG sur les fréquences et l'activation des cellules mononucléées (CMN) du sang périphérique au début de la grossesse et à l'accouchement, ensuite à identifier les profils cellulaires qui pourraient être impliqués dans la physiopathologie du paludisme gestationnel.

Méthode : Dans le cadre de l'étude STOPPAM, 131 femmes enceintes ont été sélectionnées au début de la grossesse et 111 à l'accouchement au Bénin, 38 femmes à l'inclusion et 27 à l'accouchement en Tanzanie. L'infection palustre a été identifiée par le TDR et par une goutte épaisse, positives. Les femmes infectées ont été ajustées selon l'âge gestationnel et la parité avec des femmes non-infectées. Deux sous-groupes : infecté et non infecté ont été constitués au début de la grossesse alors qu'à l'accouchement, nous avons tenu compte des infections palustres observées lors du suivi pour constituer trois groupes (infectées, exposées et non-infectées).

Résultats :

1ère partie : impact de l'infection palustre sur les profils cellulaires au début de la grossesse et à l'accouchement.

Au début de la grossesse (qui correspond au second trimestre dans cette étude) et à l'accouchement, l'infection à *P. falciparum* est associée à l'augmentation des fréquences de lymphocytes B exprimant le marqueur d'activation CD86 à leur surface.

Au second trimestre, l'infection palustre est aussi associée à une anémie, à une diminution de fréquence de monocytes exprimant le CMH-II (immature) et à une diminution de fréquence de lymphocytes T régulateurs. A l'accouchement, l'infection palustre est associée à une diminution de fréquence de pDC et à une augmentation de cellules T effectrices et de mDC exprimant faiblement le CMH-II.

2ème partie : analyse prospective sur l'association des profils cellulaires mesurés au début de la grossesse, sur la physiopathologie à l'accouchement.

Afin de mesurer l'impact des changements de profils cellulaires au cours du PAG sur la physiopathologie de la maladie, nous avons recherché dans un modèle prédictif, le rôle des changements de profils cellulaires sur l'anémie maternelle. Le risque d'anémie maternelle à l'accouchement était associé à une fréquence élevée de monocytes exprimant fortement le marqueur CD86 au second trimestre.

Discussion: Nos résultats montrent qu'au second trimestre de gestation, le PAG est associé à des fréquences élevées de monocytes immatures et de cellules B activées. Les monocytes et les cellules B sont impliqués dans l'inflammation du placenta au cours du paludisme placentaire [133, 153, 220]. Une diminution de fréquence de pDC a été déjà décrite au cours du PAG [139]. L'ensemble de ces cellules expriment des récepteurs de chimiokines chimiottractives comme le CXCL3, CXCL9 et CCL2. Notre hypothèse rejoint celle proposée par Diallo et coll. (2008), sur la migration des cellules immunocompétentes vers les sites d'infection. Les facteurs solubles liés à cette migration seront étudiés dans les études suivantes.

Conclusion : Les observations issues de cette étude démontrent qu'à l'inclusion, le PAG peut induire des changements quantitatifs et qualitatifs sur les cellules immunocompétentes. Les cellules B activées à l'inclusion seraient en transit via le sang périphérique pour rejoindre les sites d'infection ou le placenta alors que les Treg seraient recrutés dans le placenta pour assurer le maintien de l'immuno-modulation. Notre étude constitue une première, démontrant l'activation des cellules B et la diminution des fréquences de T régulateurs au cours du PAG.

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PLOS ONE

Peripheral Blood Cell Signatures of *Plasmodium falciparum* Infection during Pregnancy

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Abstract

Sequestration of *Plasmodium falciparum*-infected erythrocytes in placental intervillous spaces causes inflammation and pathology. Knowledge of the profiles of immune cells associated with the physiopathology of pregnancy-associated malaria (PAM) is scarce. We conducted a longitudinal, prospective study, both in Benin and Tanzania, including ~1000 pregnant women in each site with systematic follow-up at scheduled antenatal visits until delivery. We used *ex vivo* flow cytometry to identify peripheral blood mononuclear cell (PBMC) profiles that are associated with PAM and anaemia, determining the phenotypic composition and activation status of PBMC in selected sub-groups with and without PAM both at inclusion and at delivery in a total of 302 women. Both at inclusion and at delivery PAM was associated with significantly increased frequencies both of B cells overall and of activated B cells. Infection-related profiles were otherwise quite distinct at the two different time-points. At inclusion, PAM was associated with anaemia, with an increased frequency of immature monocytes and with a decreased frequency of regulatory T cells (Treg). At delivery, infected women presented with significantly fewer plasmacytoid dendritic cells (DC), more myeloid DC expressing low levels of HLA-DR, and more effector T cells (Teff) compared to uninfected women. Independent associations with an increased risk of anaemia were found for altered antigen-presenting cell frequencies at inclusion, but for an increased frequency of Teff at delivery. Our findings emphasize the prominent role played by B cells during PAM whenever it arises during pregnancy, whilst also revealing signature changes in other circulating cell types that, we conclude, primarily reflect the relative duration of the infections. Thus, the acute, recently-acquired infections present at delivery were marked by changes in DC and Teff frequencies, contrasting with infections at inclusion, considered chronic in nature, that were characterized by an abundance of immature monocytes and a paucity of Treg in PBMC.

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Introduction

Pregnancy is characterized by still generally poorly defined changes in the immunological equilibrium needed to protect the mother and the fetus from invading pathogens whilst at the same time tolerating the highly immunogenic paternal alloantigens in order to sustain fetal integrity. Through their capacity to modulate immunological responses, maternally-derived regulatory T cells (Treg) are now thought to play a pivotal role in the tolerance of the fetus by the mother's immune system, a role reflected by their reportedly dramatic increase in numbers during pregnancy [1–4]. Dendritic cells (DC), particularly those DC located in the decidual tissues, are central controllers of the materno-foetal tolerance process through their overall influence, governed by the presence of Treg, on immune responses in general [3]. A further level of maternal-foetal tolerance extends to the expression by fetal trophoblasts of non-classical human leucocyte antigens (HLA) class I molecules, such as HLA-G. Such molecules do not trigger the natural killer (NK) cell-mediated cytotoxic response elicited by abnormal expression of HLA molecules that commonly occurs on cells that are stressed or infected [3]. For obvious reasons, the knowledge we have of such aspects is derived from examination of placental tissues at delivery and/or of peripheral blood, with the latter providing the only accessible 'window' through which one can view changes in cell numbers and phenotypes as a function of gestational age. Indeed, data from recently conducted longitudinal studies have revealed increasing evidence of significant changes in both the quantity and the quality of Treg, DC and other cell types during normal pregnancies in high-income countries [5–8].

Infections during pregnancy can represent profound disturbances to the delicate materno-foetal equilibrium, especially infections that are localised to the placenta itself. In the public health context of low-income countries, one of the most prominent and important examples of such an infection is, without doubt, *Plasmodium falciparum*, the protozoan parasite that causes malaria. Its prominence as such reflects the burden of maternal and neonatal disease and death for which it is directly responsible worldwide [9]. The clinical and pathological outcomes include maternal anaemia, premature birth and low birthweight, which are a direct consequence of infection of the placenta by *P. falciparum* [10]. That tissue specificity arises from the adhesive interactions between at least one parasite-derived protein, referred to as VAR2CSA, that is inserted into the membrane of *P. falciparum*-infected erythrocytes (*Pf*iE), and a host receptor, chondroitin sulphate A (CSA), that is expressed on syncytiotrophoblasts [11]. Those interactions lead to accumulations of *Pf*iE in the intervillous spaces of the placenta that are accompanied by an inflammatory response, predominantly involving monocytes and to some extent also B and NK cells and neutrophils [12,13]. Numerous published studies have documented the nature of the immune response elicited by placental infection with *P. falciparum*, using peripheral venous and/or placental blood collected at delivery. Such studies have quantified levels of cytokines and chemokines in plasma, and have also identified the phenotypes of the cells as well as non-specific and parasite-specific response profiles, following stimulation *in vitro*, of mononuclear cell populations [14–16]. A single study has documented specific infection-related alterations in DC populations at delivery [17]. There are, however, no published reports of similar data collected longitudinally during pregnancy in women with and without *P. falciparum* infection. The study presented here is therefore a first step in the attempts to fill this large gap in our knowledge. Within the overall framework of the STOPPAM project, the study's primary objective was thus to evaluate the impact of pregnancy-associated malaria (PAM) on the phenotypic composition and activation status of peripheral blood mononuclear cells (PBMC), and to attempt to identify PBMC profiles that are associated with particular outcomes e.g. maternal anaemia, in order to better understand the pathogenesis of PAM. As such, we designed the study to provide two 'windows' through which to observe cellular profiles in women with or without infection by *P. falciparum*, the first at inclusion into the study (during the second trimester for the majority of participants) and the second at delivery. Identical procedures were used, based on standardized flow cytometric staining methods with PBMC *ex vivo*, in two geographically separated study sites in sub-Saharan Africa, one in Benin and the other in Tanzania, that differ distinctly with respect to the patterns of transmission of malaria. In both sites, detailed clinical and parasitological data were collected from each participant at inclusion into the study and thereafter throughout pregnancy up to and including delivery. The resulting databases thus allow for in-depth assessments of outcomes related to the range of immunological variables evaluated.

Materials and Methods

Ethics statement

The STOPPAM study received ethical clearance from the ethics committees of the Health Science Faculty of the University of Abomey-Calavi, Benin, and of the National Institute for Medical Research of Tanzania.

Study population

The study populations comprise sub-groups drawn from the cohorts of pregnant women that participated in a longitudinal study known as "Strategies TO Prevent Pregnancy Associated Malaria" (STOPPAM) that was conducted in parallel in the two study sites i.e. Benin and Tanzania. After giving written informed consent, ~1000 pregnant women at ≤24 weeks' gestational age were included both in Comé, located in the Mono province 70 km west of Cotonou, the economic capital of Benin, and in Korogwe, located in the Tanga Region of north-eastern Tanzania. Transmission of malaria in the area of the Beninese study site is considered as moderate-high (the entomological inoculation rate (EIR) was 20.5 in neighbouring Tori Bossito) [18], whilst in the Korogwe area transmission was, historically, high (EIR = 90) [19] but has recently declined sharply [20]. Perennial transmission with seasonal peaks characterizes both sites. The STOPPAM study design has been described in detail elsewhere [21]. Briefly, ultrasound examinations were used to determine gestational age, and women were followed from inclusion up to and including delivery through a series of scheduled ante-natal visits (ANV) during which ultrasonographic, clinical and parasitological assessments were conducted. Women were also encouraged to attend the clinic ('emergency' visits) in the event of any perceived illness or other pregnancy-related problem. At all visits (ANV or emergency), infection with *P. falciparum* was identified through the use of rapid diagnostic tests (RDT), and those with a positive RDT were given appropriate anti-malarial treatment. Retrospective parasitological confirmation of infections comprised microscopical examination of routinely prepared, giemsa-stained thick and thin blood smears. All women received two standard curative treatment doses, spaced at least 1 month apart, of sulphadoxine-pyrimethamine according to the national policies for intermittent preventive treatment in pregnancy (IPTp). The sub-groups selected for cellular immunological studies both at inclusion and at delivery described here were identified on the basis either of their current or their past infection status, with a case-control design. At inclusion, on the basis of an informed estimate of a prevalence of 10%, we expected 100 women to present with infection with *P. falciparum*. For immunological assessments at inclusion we therefore planned to include a total of 200 women, comprising 100 'cases' of women infected with *P. falciparum* and 100 'controls' of uninfected woman matched to 'cases' by age, gravidity and gestational age. Cases were recruited sequentially according to their inclusion into the overall study, with the matched uninfected controls selected and recruited as soon as practically possible after each case. At delivery infected women ('cases' who may or may no have been infected earlier) were again included into the immunological sub-study chronologically, but 2 different uninfected 'control' sub-groups, matched by age and gravidity to cases, were defined using the detailed clinical and parasitological histories available as a result of the follow-up: (i) an uninfected group comprising those with no evidence of infection with *P. falciparum* at inclusion or subsequently throughout follow-up or at delivery, (ii) an exposed group comprising those uninfected at delivery but who had at least one infection episode at inclusion or during follow-up. In each site, a majority of those selected at inclusion and delivery were different individuals although samples from a minority were included in both. The low prevalence of *P. falciparum* infection in Tanzanian women resulted in markedly lower-than-expected numbers for inclusion in sub-groups at both time-points. Data from women in either study site with proven seropositivity for HIV or with unknown HIV serostatus were not included in the analyses.

P. falciparum parasites detection in blood smears

Plasmodial parasite detection in freshly-drawn blood was performed using a rapid diagnostic test (RDT) (Parascreen*, Zephyr Biomedical Systems, Goa, India), and retrospectively through standard high-power microscopical examination of thin and thick blood smears prepared from peripheral blood and of placental impression smears.

Blood collection and cell preparation

Peripheral venous blood samples were collected at inclusion and at delivery in vacutainers containing citrate phosphate dextrose adenine (CPDA) anticoagulant. For the detailed immunological studies described here, samples were taken from 131 and 111 Beninese women at inclusion and at delivery, and from 38 and 27 women in the Tanzanian cohort, respectively. The blood samples were transported to the research laboratories in the respective study sites and were processed for immunological assessments within 4 hours. Peripheral blood mononuclear cells (PBMC) were isolated using Leucosep tubes (Greiner-Bio) according to the manufacturer's description and were subsequently used for the immunophenotyping described below.

Dendritic cell, B cell and monocyte immunophenotyping

PBMC were washed in staining buffer (PBSx1, EDTA 5 mM, 2% FBS) for 10 min at 150 g and resuspended at a concentration of 10 million cells/ml. Cells were then incubated with 10 µl of FcR Blocking reagent (Miltenyi Biotec, Gladbach, Germany) for 10 minutes in the dark at 4°C to prevent non-specific labelling. Specific surface labelling was then performed by adding anti-BDCA-1-Phycoerythrin (PE) for myeloid dendritic cells (mDC) and anti-BDCA-2-PE for plasmacytoid DC (pDC) detection (all Miltenyi Biotec), anti-CD14-Fluorescein isothiocyanate (FITC) for monocytes, anti-CD-19-FITC for B cells, and the combination of anti-HLA-DR-Peridinin chlorophyll protein (PerCP) and anti-CD86-Allophycocyanin-cyanin (APC) (all BD Pharmingen, San Diego, CA) antibodies for cells' activation status. After incubating for 30 minutes at 4°C in the dark, cells were then washed and fixed with FACS lysing solution (BD Pharmingen). The cells were finally resuspended in 300 µl of staining buffer, acquired using BD FACSCalibur and analyzed using CellQuest Pro or FlowJo 7.6 software. Gating strategies are shown in Figure 1A.

T cell, NK cell and T regulatory cell immunophenotyping

Cells were resuspended and incubated with 10 µl of FcR Blocking reagent (Miltenyi Biotec) for 10 minutes in the dark at 4°C. Cells were then incubated for 30 minutes in the dark, with specific antibodies. Anti-CD3-APC, anti-CD8-FITC, anti-CD4-PerCP and anti-CD56-PE (BD Pharmingen) were used for T lymphocyte and NK cell labelling, whilst anti-CD25-FITC and anti-CD127-PE (BD Pharmingen) were used for regulatory T cell (Treg) labelling. Anti FoxP3-APC (BD Pharmingen) was added for Treg labelling according to the manufacturer's recommendations after permeabilisation and fixation with PermFix (BD Pharmingen). Cells were acquired in 300 µl of PBS 3% FBS using BD FACSCalibur and analyzed by CellQuest Pro or FlowJo 7.6 software. Gating strategies are shown in Figure 1B & C.

Data analysis

When using matching for inclusion of controls our original intention was to enhance statistical power through the use of paired tests. In practice, resource restrictions precluded collection of samples from the intended number of women. In addition, the implementation of various exclusion criteria (HIV infection, missing information and/or data) resulted in an overall reduction in sample sizes and a consequently reduced number of 'pairs'. In order to maximize the use of available data, we therefore chose to apply unpaired tests throughout, and retained gravidity, gestational age and age - the specific criteria used for matching - as variables in all analyses. The decline in transmission of malaria in the Tanzanian site resulted in a markedly lower prevalence of infection compared with Benin, and hence smaller sub-group sizes. Although it therefore lacked the same level of statistical power we nevertheless opted to maximize the use of the hard-won Tanzanian dataset to validate, where possible, the findings from Benin.

Data analysis was performed using STATA/MP 11.2 (StataCorp, College Station, TX USA) and Prism 5.0 (Graph pad Inc). For each continuous biological variable, the median of the distribution for uninfected women was used as the threshold value for the transformation of the variable into categorical variable (i.e. below or above the threshold). Categorical variables were compared with chi2 ($\div^2$) or Fisher's exact tests and continuous variables compared with non-parametric tests (Mann Whitney). Multiple logistic, ordered logistic and linear regressions were performed using respectively "logit", "ologit" and "regress" commands in STATA in order to identify biological variables associated with the risk for or the level of *P. falciparum* infections among pregnant women both at inclusion and at delivery in the Beninese cohort. The threshold value of statistical significance in univariate analyses for inclusion of variables into multivariate analyses was set at $p<0,2$. Results of analyses of data from Benin were confirmed in a multiple logistic regression by using data from the Tanzanian cohort as an external set of validation. In order to assess whether any of the biological variables measured were independently associated with the risk of anaemia at inclusion and/or at delivery, similar procedures to those described above were used. A prospective evaluation of the association between biological variables at inclusion and anaemia at delivery was also conducted using similar procedures for selection.

Results

Demographic and other characteristics of the study populations

In order to compare data between *P. falciparum*-infected and uninfected pregnant women, sub-groups were selected from within the whole STOPPAM cohort in each site. In Benin the sub-groups selected comprised 131 women at inclusion (62 infected and 69 uninfected) and 111 at delivery (37 with infection at delivery, 27 'exposed' women who had been infected at least once during pregnancy but were uninfected at delivery, 47 uninfected throughout pregnancy, Tables 1 & 2). In Tanzania, the sub-group at inclusion comprised 38 women (20 infected and 18 uninfected), whilst at delivery it comprised 27 women (9 infected at delivery, 7 'exposed' and 11 uninfected throughout pregnancy, Table 2). To assess whether the women included in the sub-groups for immunological analysis were representative, relevant parameters were compared between them and the whole study cohort. As expected because of the bias inherent to the criteria used for their selection at inclusion (*P. falciparum* infections are more common in primigravidae, who are by definition younger), the sub-group of 131 Beninese women was of significantly lower gravidity and was significantly younger than the whole cohort (Table 1), but at delivery no such differences were apparent (Table 1). The smaller size of the Tanzanian cohort meant that similar comparisons of the sub-groups were not meaningful.

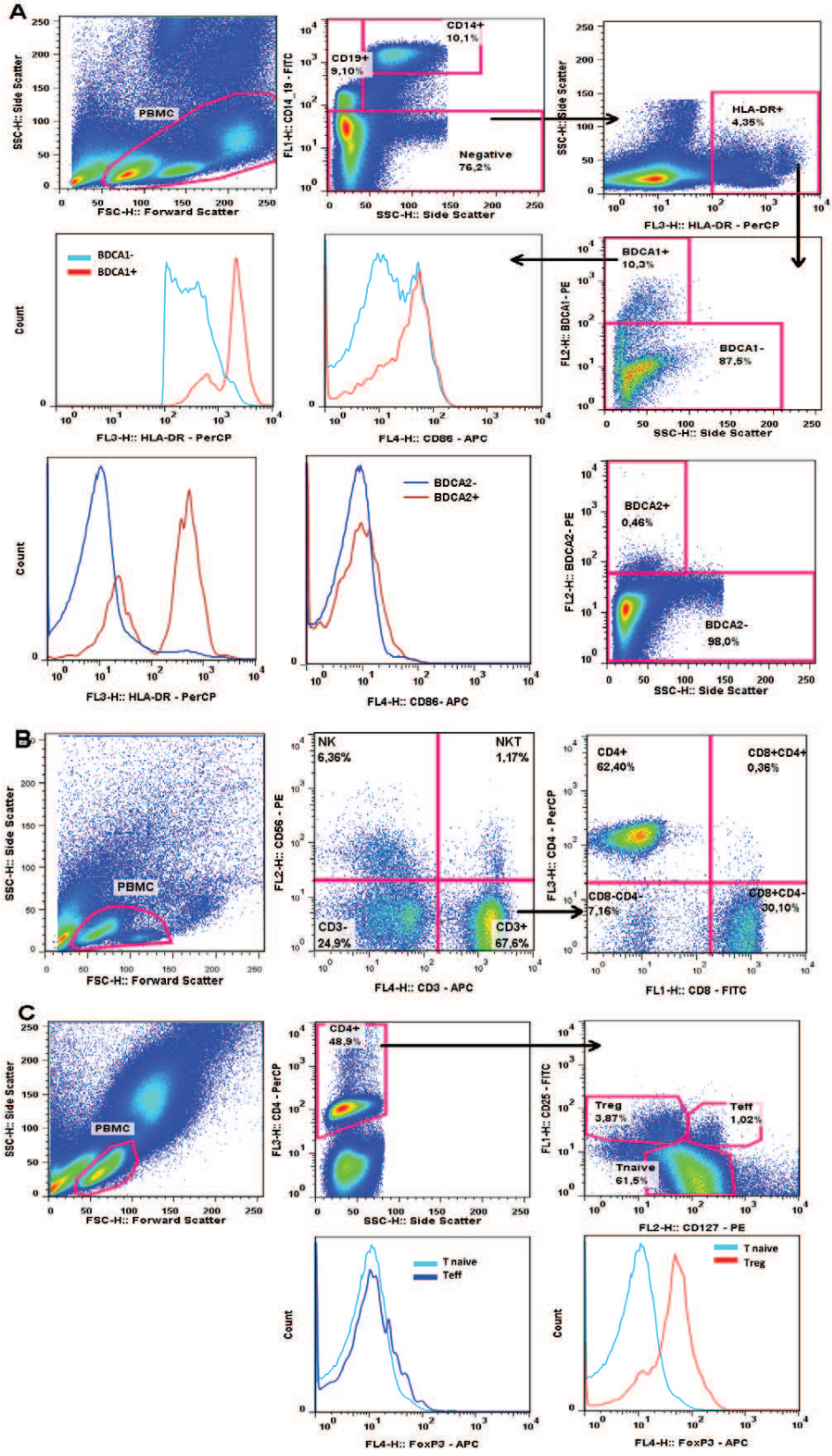
A
PBMC
SSC-H:: Side Scatter
FSC-H:: Forward Scatter
CD14+
10,1%
CD19+
9,10%
Negative
76,2%
FL1-H:: CD14_19 - FITC
HLA-DR+
4,35%
FL3-H:: HLA-DR - PerCP
BDCA1-
BDCA1+
Count
BDCA1+
10,3%
BDCA1-
87,5%
FL2-H:: BDCA1 - PE
FL4-H:: CD86 - APC
BDCA2-
BDCA2+
BDCA2+
0,46%
BDCA2-
98,0%
FL2-H:: BDCA2- PE
FL4-H:: CD86- APC
B
PBMC
NK
6,36%
NKT
1,17%
CD3-
24,9%
CD3+
67,6%
FL2-H:: CD56 - PE
FL4-H:: CD3 - APC
CD4+
62,40%
CD8+CD4+
0,36%
CD8-CD4-
7,16%
CD8+CD4-
30,10%
FL3-H:: CD4 - PerCP
FL1-H:: CD8 - FITC
C
PBMC
CD4+
48,9%
Treg
3,87%
Teff
1,02%
Tnaive
61,5%
FL3-H:: CD4 - PerCP
FL1-H:: CD25 - FITC
FL2-H:: CD127 - PE
T naive
Teff
T naive
Treg
FL4-H:: FoxP3 - APC

Figure 1. Cytometry-based gating for definition of phenotypes. (A) Monocytes ($CD14^+$), B cells ($CD19^+$) were gated from PBMC. pDC were directly gated from the $CD14^-CD19^-$ population whilst mDC were gated from the $CD14^-CD19^-HLA\text{-}DR^+$ population. CD86 and HLA-DR expression were determined by MFI (Mean Fluorescence Intensity). (B) NK, NK T and $CD3^+$ cells were gated from PBMC. $CD4^+$ and $CD8^+$ T cells were gated from $CD3^+$. The gating strategies for Treg ($CD4^+CD25^+CD127^-$) and Teff ($CD4^+CD25^+CD127^+$) are presented in (C). Cell frequencies were determined as a percentage of PBMC, and relative FoxP3 expression level determined as a function of FoxP3 expresssion by naïve $CD4^+$ T cells ($CD4^+CD25^-$).
doi:10.1371/journal.pone.0049621.g001

We wished to assess the influence of *P. falciparum* infection on multiple parameters, and therefore used univariate analysis in a first step to make comparisons within the sub-groups. In Benin, women infected with *P. falciparum* at inclusion showed a non-significant trend to be younger, whilst a significantly higher proportion were anaemic and significantly fewer declared possessing a bednet compared with the uninfected group (Table 2). The same set of parameters did not differ significantly between the sub-groups of Tanzanian women at inclusion, although the proportion of those infected who were also anaemic was almost double that in the uninfected group (Table 2). At delivery, we wished to make as detailed a comparison as possible and therefore used the information generated by the longitudinal surveillance of women through pregnancy to identify 3 different groups of women based on their *P. falciparum* infection histories since inclusion into the study, namely (i) those never found infected from inclusion through to delivery (including at emergency clinic visits outside scheduled ANV), (ii) an 'exposed' group comprising those infected at least once during pregnancy but who were uninfected at delivery and (iii) those infected at delivery with or without recorded infection during pregnancy. We found that a higher proportion of infected mothers in Benin were younger and anaemia was more common amongst them compared with the uninfected group (Table 2). A separate logistical regression analysis, using dichotomised data for the sub-group at delivery to compare the infected group with all women found to be uninfected at delivery (i.e. both exposed and uninfected groups combined together), revealed a higher proportion of primigravidae in the infected group (data not shown). No such differences were seen at delivery in the Tanzanian mothers but the small sample sizes are restrictive in this case (Table 2).

P. falciparum infection-related changes in the peripheral blood mononuclear cell composition at inclusion

The principal focus of this study concerned the effects of infection with *P. falciparum* during pregnancy on PBMC profiles. At inclusion into such a study, when access to placental blood is not practicable, it cannot be determined with absolute certainty that an infection detected in peripheral blood extends to the placenta. Current knowledge indicates that the state of placental vascularization first allows access to intervillous spaces by maternal blood, and hence by *P. falciparum*-infected erythrocytes (*Pf*iE), between the 10^{th}–12^{th} weeks of gestation. Our own findings concerning VAR2CSA expression, nevertheless, suggest that *Pf*iE do indeed have access to cells expressing CSA (i.e. of placental origin) before the start of the 2^{nd} trimester in >90% of women (Tuikue Ndam N., unpublished observations). Within the selected sub-group of women in Benin there were eight (8) infected women with a gestational age (GA) <12 weeks at inclusion. Data from these women were not excluded from the analysis presented here.

Univariate analysis of the Beninese dataset included immunological variables as well as age, gestational age, *P. falciparum* infection status, and bednet possession. Figure 2 illustrates the flow cytometry-based characterization by phenotypic markers of the different populations of cells within PBMC that we identified for comparisons. A number of variables were found to display associations with *P. falciparum* infection detectable at inclusion (Table 2). These included, as outlined above, age, anaemia and bednet possession. Amongst the cellular phenotypes of antigen-presenting cells, the frequencies of mDC/pDC and monocytes did not differ according to infection status but the frequency of B cells was significantly higher in PBMC of infected versus uninfected women (Figure 3A–D). Within T cell subsets, the only difference found concerned the significantly lower frequency of Treg in PBMC of infected versus uninfected women (Figure 3E–H). With

Table 1. Characteristics of sub-groups compared with the whole cohort in Benin at inclusion and at delivery.

	Inclusion					Delivery				
Variables	**Whole cohort**		**Sub-group**			**Whole cohort**		**Sub-group**		
	n		**n**		**p***	**n**		**n**		**p***
Gestational age in weeks	982	18.1 (5.0)	131	17.7 (4.1)	0.31	623	39.5 (2.3)	111	39.5 (1.5)	0.75
Gravidity	1037	3.4 (2.0)	131	3.0 (1.9)	0.02	630	3.5 (2.1)	111	3.3 (1.9)	0.25
Age in years	1023	26.4 (6.2)	131	25.2 (6.1)	0.04	618	26.7 (6.3)	110	26.2 (5.9)	0.50
% possessing a bednet	1037	32.1	131	28.2	0.37	630	29.5	111	34.2	0.32
% with haemoglobin <11 g/dl	1029	60.7	130	66.9	0.17	578	46.4	102	40.2	0.25
Parasites/μL	176	1538 (299)	59[†]	2399 (699)	0.19	70	15266 (1142)	33[‡]	13627 (1250)	0.83

Values are means (standard deviation) except for parasitaemia which are medians (interquartile ranges).
*Student t test or χ^2 for proportions.
[†]3 subjects were positive by RDT but negative by microscopy.
[‡]4 subjects were positive by RDT but negative by microscopy.
doi:10.1371/journal.pone.0049621.t001

Table 2. Univariate analysis of sub-groups segregated according to the presence or absence of *P. falciparum* infection at inclusion and at delivery.

	Inclusion						Delivery							
	Benin			Tanzania			Benin				Tanzania			
Variables	Infected	Uninfected	p	Infected	Uninfected	p	Infected	Exposed	Uninfected	p	Infected	Exposed	Uninfected	p
	(n = 62)	(n = 69)		(n = 20)	(n = 18)		(n = 37)	(n = 27)	(n = 47)		(n = 9)	(n = 7)	(n = 11)	
Gestational age in weeks	17.4 (4.0)	17.9 (4.2)	0.44*	18.0 (2.7)	18.4 (3.6)	0.76*	39.3 (1.9)	39.8 (1.3)	39.6 (1.3)	0.35‡	39.4 (1.1)	39.5 (1.0)	38.9 (3.0)	0.80‡
Gravidity	2.9 (1.9)	2.9 (2.0)	0.97*	2.2 (1.8)	2.2 (0.8)	0.26*	3.3 (2.2)	2.6 (1.1)	3.7 (2.0)	0.10‡	3.2 (1.3)	1.7 (0.7)	2.8 (1.5)	0.07‡
Age in years	24.2 (5.9)	26.1 (6.2)	0.08*	23.3 (4.6)	24.0 (5.3)	0.32*	25.6 (6.5)	24.0 (4.2)	28.1 (5.7)	0.01‡	28.3 (7.3)	23.3 (4.1)	25.4 (6.3)	0.28‡
% possessing a bednet	19.3	36.2	0.03**	70.0	66.7	1.0**	29.7	25.9	42.5	0.20†	55.6	57.1	81.8	0.21†
% with Hb <11 g/dl	80.3	55.1	<0.01**	60.0	33.3	0.12**	53.1	44.0	28.9	0.03†	50.0	71.4	45.4	0.78†
Parasites/μL	2399 (345)	-	-	1107 (71)	-	-	12492 (1035)	-	-	-	4008 (1460)[a]	-	-	-

Values are means (standard deviation) except for parasitemia which are medians (interquartile ranges).
*Student t test,
**χ^2 for proportions,
†Tendency test,
‡Anova test.
[a]data from 2 subjects with parasitaemia.
doi:10.1371/journal.pone.0049621.t002

respect to activation status, HLA-DR expression on both mDC and monocytes was significantly lower whilst CD86 expression on both pDC and B cells was significantly higher in PBMC of infected versus uninfected women (Figure 4A–H).

All the variables displaying associations with infection in univariate analysis were subsequently included in multiple logistic regression analysis. The latter confirmed the independent association between maternal anaemia and infection (Table 3), but the association with bednet possession was lost. Variables that remained significantly different in PBMC of infected compared with uninfected women after multivariate analysis included the lower frequency of Treg, the higher frequency of B cells overall and the higher frequency of B cells expressing high levels of CD86 (Table 3). We next conducted a sub-analysis using ordered logistic regression that categorised women according to their parasite density as uninfected or with a parasitaemia above or below the infected group's median value. This revealed significant associations for the same set of variables as for infection alone, namely that higher parasitaemia loads were associated with younger age (OR 5.3, 95% CI 2.0–14.0; $p<0.05$), more frequent anaemia (OR 2.8, 95% CI 1.0–7.5; $p<0.01$), significantly fewer Treg (OR 3.8, 95% CI 1.6–9.2; $p<0.01$) and a significantly higher proportion of B cells expressing high levels of CD86 (OR 3.4, 95% CI 1.4–8.2; $p<0.01$). Linear regression analysis showed that higher parasitaemia loads were also associated with significantly reduced levels ($p\leq0.001$ in both cases) of expression of both HLA-DR and CD86 on monocytes (data not shown).

Our overall analysis strategy comprised validation of the findings generated by the analysis of the Beninese dataset through identical analysis of the Tanzanian dataset. Using the latter, we therefore next conducted multiple logistic regression analysis in the same way as with the Beninese dataset, incorporating the same variables for assessment as a function of the presence or absence of *P. falciparum* infection. This revealed a significantly higher proportion of B cells with a high level of expression of CD86 (OR 13.8; IC 95% 1.6–120.6; $p<0.05$), and a significantly lower proportion of Treg (Treg/Teff ratio) (OR 8.4; IC 95% 1.2–57.5; $p<0.05$) in infected versus uninfected Tanzanian women at inclusion, confirming the results of analysis of the Beninese data.

Of note, we found no evidence of differences in the frequencies of T ($CD4^+/CD8^+$, Figure 3G, H), NK or NKT cells (data not shown) as a function of the presence or absence of *P. falciparum* infection at inclusion.

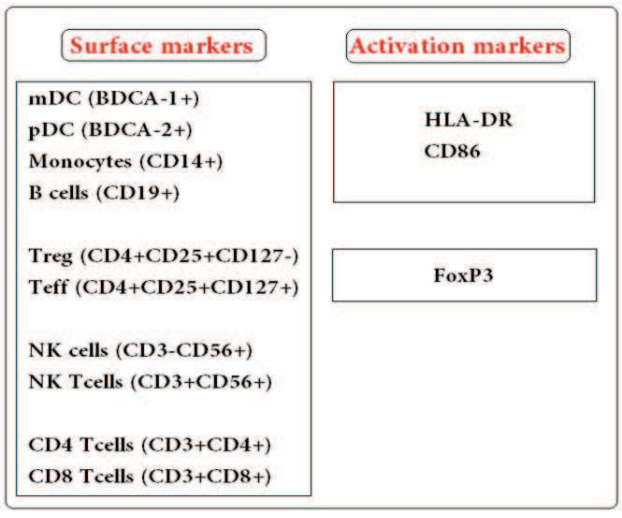

Figure 2. Basic flow cytometry-based phenotypic characterization of different cell populations investigated. The left panel lists the cell types identified with different surface markers and the right panel lists the separate markers used to characterize antigen-presenting cells activation status (HLA-DR & CD86) and to distinguish $CD4^+$ regulatory T cells (Treg, expressing the transcription factor FoxP3) from effector T cells (Teff).
doi:10.1371/journal.pone.0049621.g002

P. falciparum infection-related changes in the peripheral blood mononuclear cell composition at delivery

Univariate analysis of the Benin dataset at delivery included the same set of variables as at inclusion. The analysis revealed differences with respect to a comparatively restricted number of variables, specifically concerning significantly higher frequencies of B cells and effector T cells (Teff) but lower frequencies of pDC in PBMC of infected versus uninfected women (Figures 3B,D,F & 4H). In multiple logistic regression analysis PBMC from infected women were shown to contain significantly more B cells that expressed higher levels of CD86, significantly fewer pDC, significantly more mDC expressing a low level of HLA-DR, and significantly more Teff cells compared to uninfected women (Table 4).

We then performed a separate analysis combining the uninfected and 'exposed' groups into a single 'non-infected' group for comparison with the infected group that was itself dichotomised into those with either 'low' or 'high' parasite loads. Ordered logistic regression analysis in this case showed increasing parasite load to be associated with a significantly lower frequency of pDC (OR 4.8, 95% CI 1.4–16.9, $p=0.01$), a non-significant trend for reduced expression of HLA-DR on mDC (OR 3.4, 95% CI 0.8–13.7, $p=0.09$), and a significant increase in the frequency of Teff cells (OR 4.0, 95% CI 1.0–16.4, $p=0.05$). Sample sizes here were too small to allow analysis by linear regression, and the small number of women included in the Tanzanian dataset at delivery (Table 2) precluded validation of the findings from the Beninese data.

As was the case for the analysis of data at inclusion, we found no evidence for differences in the frequencies of monocytes (Figure 3C), $CD4^+/CD8^+$ T cells (Figure 3G,H), NK or NKT cells (data not shown) as a function of the presence or absence of *P. falciparum* infection at delivery.

Predictors of maternal anaemia

Of those mothers with relevant data available, 87/131 and 41/102 Beninese were anaemic (Hb<11 g/dL) at inclusion and delivery, respectively. As was the case for evaluation of association with infection, univariate analysis here included immunological variables as well as age, gestational age, *P. falciparum* infection status and bednet possession. Analysis at inclusion revealed the strongest ($p<0.01$) associations for anaemia and infection, age and reduced HLA-DR expression on mDC. Subsequent multivariate analysis showed independent associations between anaemia and infection, a gestational age at inclusion more than 17 weeks, and 3 separate PBMC-specific variables, namely (i) reduced HLA-DR expression on pDC, (ii) lower frequency of mDC, and (iii) higher expression of CD86 on monocytes (Table 5). Univariate analysis of data at delivery revealed only a single variable (increased frequency of Teff) associated with anaemia with a level of significance below the 5% level. In multivariate analysis, the increased frequency of Teff remained the only variable to display a significant association with anaemia at delivery (Table 6).

In a prospective analysis, we examined the predictive value for anaemia at delivery of variables measured at inclusion. Apart from age, infection status, bednet possession and gravidity, the

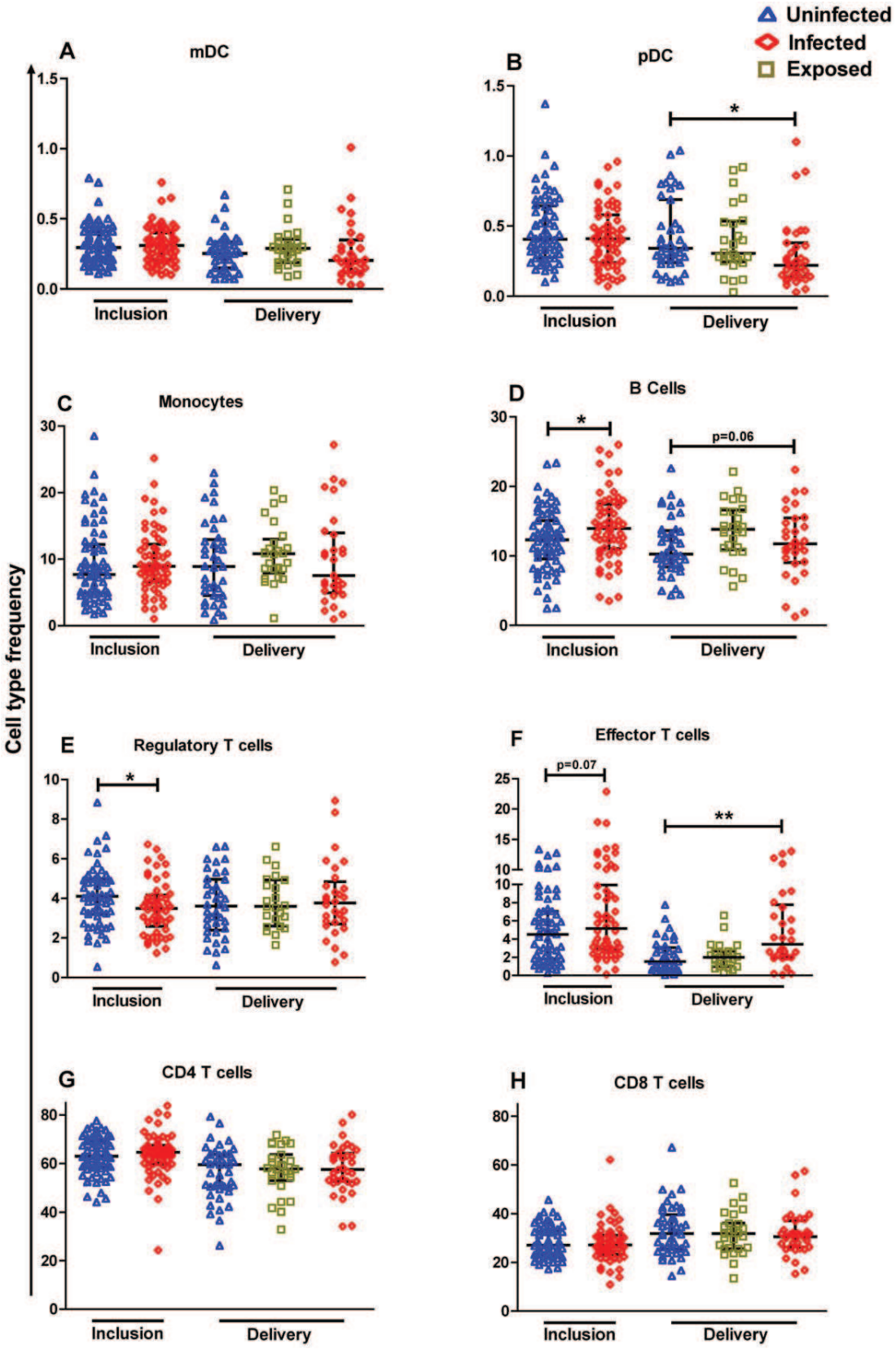
Uninfected
Infected
Exposed
Cell type frequency
A
mDC
B
pDC
C
Monocytes
D
B Cells
E
Regulatory T cells
F
Effector T cells
G
CD4 T cells
H
CD8 T cells
Inclusion
Delivery
*
**
p=0.06
p=0.07

Figure 3. *P. falciparum* infection-related changes in peripheral blood mononuclear cell frequencies of pregnant Beninese at inclusion and at delivery. Scatter plots include bars depicting medians with interquartile ranges of antigen-presenting cells (A, B, C and D) and T cell subset frequencies (E, F, G and H) from 69 uninfected compared to 62 infected women at inclusion, and from 47 uninfected, 27 exposed and 37 infected women at delivery. The statistical significance of differences between profiles segregated according to the presence or absence of infection were determined using the non-parametric Mann Whitney U test for data at inclusion combined with the non-parametric Kruskall Wallis test for data at delivery. *$p<0.05$, **$p<0.01$.
doi:10.1371/journal.pone.0049621.g003

immunological variables retained for multivariate analyses as a function of univariate analyses were monocyte frequency, monocyte CD86 expression, B cell HLA-DR expression, Treg and Teff freqeuncy. The multivariate model showed that the risk of anaemia at delivery was associated with a higher frequency of monocytes overall and of monocytes expressing high levels of CD86 amongst PBMC at inclusion (Table 7).

Discussion

The immunological study we present here constituted a part of our larger STOPPAM study. STOPPAM inevitably had a major impact on various parasitological and immunological outcomes in the mothers participating in it. This is due to both the active (scheduled ante-natal clinic visits) and the passive (unscheduled 'emergency' visits) modes of detection of infection integral to it, allied to the fact that curative anti-malarial treatment was given whenever *P. falciparum* infection was detected. In addition, the participants received intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp SP) on two occasions during pregnancy as per national policy in the two countries concerned. Any interpretation of our findings must therefore necessarily take into account these facts.

In the discussion that follows, we discuss the profiles of the different cell types we observed sequentially, focusing on those that our analysis revealed to vary significantly as a function of the presence of infection with *P. falciparum*, whilst also comparing and contrasting the profiles observed at inclusion versus delivery. It is of particular note that maternal anaemia was independently associated with *P. falciparum* infection present at inclusion but not at delivery. In this context, the women's infection histories, when allied to molecular genotypic characterisation of parasite isolates from them (Tuikue Ndam N., unpublished data), revealed that 90% of infections at delivery (29/32 women in the selected immunology sub-group for whom relevant data was available) were acquired within the preceding 4 weeks. This finding, coupled with the complete absence of malarial haemozoin pigment in placental biopsy samples from those with infections at delivery (Fievet N., unpublished data), leads us to conclude that the overwhelming majority of infections detected at delivery were acute i.e. recently acquired in nature. In contrast, we can make no similarly conclusive statement concerning the duration of *P. falciparum* infections detected at inclusion. The median gestational age at inclusion was 18 weeks, which allows a theoretical time-frame for acquisition of infections prior to inclusion of 2–3 weeks or, potentially, 1–4 months. Despite this possible range, for reasons outlined below, our conclusions are based on the assumption that a majority of the *P. falciparum* infections we detected at inclusion were in fact chronic rather than acute in nature i.e. the immunological assessments we made directly reflect the differing character of the infections at inclusion and delivery. In this context, it is also notable that we found gestational age-related changes to PBMC profiles that were independent of womens' *P. falciparum* infection status (Ibitokou S et al, manuscript submitted)

Compared to PBMC of uninfected women we found a significantly increased frequency of immature monocytes i.e. cells expressing reduced levels of both HLA-DR and the co-stimulatory molecule CD86 [22], in the PBMC of infected women at inclusion but not in those of infected women at delivery. The latter finding is consistent with that of an earlier study in which we showed that the expression of markers of activation on monocytes present in PBMC at delivery was similar, regardless of the presence or absence of infection [16]. Circulating immature monocytes are found during malaria attacks in children [23], and such cells are indicative of an on-going inflammatory response [22]. There is general consensus that monocytes form the principal component of the inflammatory response to *Pf*iE in the placenta [13,24,25]. Earlier studies, including our own, have shown that the monocytes present in infected placentas are activated, as reflected by upregulated expression of HLA-DR, CD86 and CCR5 [16,26]. We therefore speculate that chronic on-going *P. falciparum* infections, as seen at inclusion of women into the current study, lead to a localized and persistent turnover of monocytes in the placenta requiring replacement from the bone marrow pool. By corollary, recently acquired (acute) infections, as found here at delivery, have not yet depleted the locally-available population sufficiently to require recruitment of immature cells from the bone marrow. In work to be reported elsewhere we show that the concentrations of the chemokines interferon-gamma-inducible protein (IP)-10, in particular, and monocyte chemotactic protein (MCP)-1 were elevated in the peripheral plasma of infected mothers, clearly indicating on-going monocyte chemoattraction (Boström S & Ibitokou S, unpublished data). Enhanced activity of various chemokines, including both IP-10 and MCP-1, is reported to be associated with placental infection by *P. falciparum* [13,27–32]. Moreover, it is well-established that chemokines show specific binding affinities for glycosaminoglycans (GAG) [33–35], thereby establishing concentration gradients via which cells are recruited to sites of inflammation. The CSA expressed by syncytiotrophoblasts that acts as the receptor for *Pf*iE in the placenta is itself a GAG, and its potential interactions with chemokines thus clearly merit further research.

We found no significant infection-associated alterations to DC frequencies in PBMC at inclusion, although reduced expression of HLA-DR on mDC, possibly indicating functional impairment of this subset, was seen. Infection at delivery, on the other hand, was associated with loss of circulating pDC. These observations are entirely consistent with those reported in studies of DC from Senegalese mothers with *P. falciparum* infections at delivery [17]. They also accord well with DC profiles seen during malaria attacks in Kenyan children and, in the case of loss of pDC, in Thai adults [23,36,37]. It is known that pDC express CXCR3 [33], a receptor with specificity for the chemokines monokine-induced by IFN-γ (MIG) and IP-10, as well as CCR2, the ligand for which is MCP-1. MIG, IP-10 and MCP-1 were all present at significantly higher levels in the peripheral plasma of infected women (Boström S & Ibitokou S, unpublished data), potentially providing a stimulus for migration of DC from the peripheral circulation to lymphoid tissues at the site of infection. A prominent, potentially pivotal role of pDC in the evolution of immune responses to malarial parasites, as suggested recently [38], is not evident from our data, but more detailed investigation would be necessary to clarify this point.

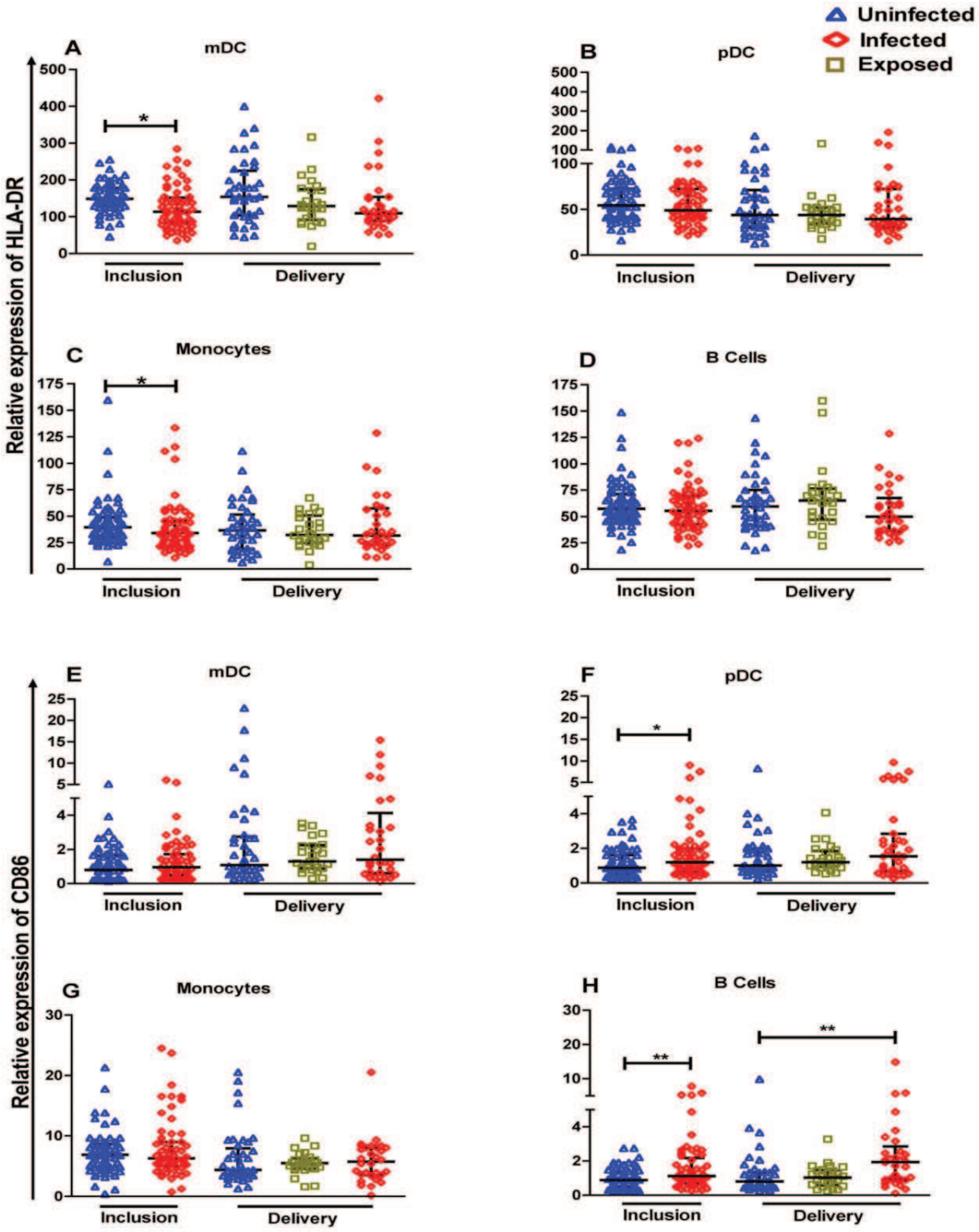

Figure 4. *P. falciparum* infection-related changes in activation status of antigen-presenting cells at inclusion and delivery in pregnant Beninese. Scatter plots include bars depicting medians with interquartile ranges of HLA-DR expression (A–D) and CD86 expression (E–H) on APC of 69 uninfected compared to 62 infected women at inclusion and of 47 uninfected, 27 exposed and 37 infected women at delivery. The statistical significance of differences in levels of expression were determined using the non-parametric Mann Whitney U test for data at inclusion combined with the Kruskall Wallis test for data at delivery. *$p<0.05$, **$p<0.01$, ***$p<0.001$.
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Table 3. Multiple logistic regression analysis for independent associations of selected parameters with the presence of *P. falciparum* infection in the Benin sub-group at inclusion.

Variables	Odds Ratio	95% CI	p
% with hemoglobin <11 g/dl	4.2	1.5—11.7	<0.01
% aged ≤25 years	2.7	1.0—6.9	<0.05
% B cells (≥median)[a]	2.5	1.0—6.1	<0.05
% B cells expressing $CD86^{hi}$ (≥median)[a]	3.3	1.3—8.1	= 0.01
% Treg (<median)[a]	3.1	1.3—7.7	= 0.01

[a]median value of uninfected group used for dichotomisation.
χ^2 Hosmer-Lemeshow test (15ddl) = 13.9, p = 0.53.
doi:10.1371/journal.pone.0049621.t003

The increased frequency in PBMC of B cells in association with *P. falciparum* infections - including higher proportions expressing elevated levels of the activation marker CD86 - was a consistent finding at both time-points in the present study. This is indicative of the strong involvement of B cells in the placental inflammation associated with *P. falciparum* infections during pregnancy [13]. Overall B cell frequencies during acute *P. falciparum* infections in children are reported to be either unchanged [39] or increased, as seen here [40]. Since B cells are known to express toll-like receptor (TLR) 9, upregulation of CD86 on B cells may result from the same parasite antigen-mediated pathway reported for pDC [36]. As is also the case for pDC mentioned above, B cells are known to express the chemokine receptor CXCR3, and they could thus be assumed to be responding in the same way to the elevated concentrations of the MIG and IP-10 chemokines present in the plasma of infected women. The CXCL13-CXCR5 axis of B cell recruitment has also been reported to play a prominent role in this context [13]. A plausible conclusion from our own and others data would therefore be that B cell responses, and by inference antibody production and secretion locally in the placenta, are primary components of the maternal immune response to infection with *P. falciparum* during pregnancy. Such B cell responses are thought to be activated in a T cell-independent manner [13]. A potent non-specific T cell-independent activator of B cells that is able to bind multiple molecules, including IgM, has been identified in *P. falciparum* [41,42], and VAR2CSA, the parasite-derived ligand for CSA, displays a similar capacity for non-specific binding of IgM [43]. Given the candidacy of VAR2CSA as a vaccine to prevent malaria in pregnancy, priority should be given to fully characterizing its domain(s) potentially mediating this particular property and, equally, to determine whether it extends to non-specific activation of B cells. Activated B cells, furthermore, are purported to play a prominent role in the regulation of human DC responses, particularly on the monocyte-mDC maturation axis [44]. The importance of elucidating whether such interactions occur in the context of PAM and/or a candidate vaccine thus seems clear enough.

Table 4. Multiple logistic regression analysis for independent associations of selected parameters with the presence of *P. falciparum* infection in the Benin sub-group at delivery.

Variables	Odds Ratio	95% CI	p
% pDC (<median)[a]	2.3	1.0—5.7	= 0.06
% mDC expressing $HLA\text{-}DR^{low}$ (<median)[a]	3.4	1.3—8.6	= 0.01
% B cells expressing $CD86^{hi}$ (≥median)[a]	2.8	1.1—7.2	<0.05
% Teff (≥median)[a]	3.3	1.3—8.4	= 0.01

[a]median value of uninfected group used for dichotomisation.
doi:10.1371/journal.pone.0049621.t004

Table 5. Multiple logistic regression analysis for independent associations of selected parameters with the presence of anaemia in the Benin sub-group at inclusion.

Variables	Odds Ratio	95% CI	p
P. falciparum infection[a]	4.9	1.9—12.9	<0.01
% pDC expressing $HLA\text{-}DR^{low}$ (<median)[a]	3.1	1.2—7.5	<0.05
% mDC (<median)[a]	2.9	1.2—7.1	<0.05
% Monocytes expressing $CD86^{hi}$ (≥median)[a]	2.8	1.1—7.2	<0.05
Gestational age in weeks (>17 weeks)[a]	3.3	1.4—7.9	<0.01

[a]median value of non-anaemic group used for dichotomisation.
χ^2 Hosmer-Lemeshow test (17ddl) = 7.91; p = 0.97.
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The changes in Treg and Teff profiles that were associated with *P. falciparum* infections differed at inclusion and delivery. These changes are of particular interest given the potentially pivotal role Treg may play in determining the outcome of infection [45,46]. At inclusion, Treg were found at a significantly lower frequency in PBMC of infected women compared to uninfected women, whilst at delivery the frequency of Teff relative to Treg was significantly higher. Reduced frequencies of Treg in PBMC have not been reported during *P. falciparum* infections, in fact they have been reported to be elevated during infections of non-pregnant Africans [46]. Our observation is nevertheless consistent with reports in the literature of 'sequestration' of Treg during non-plasmodial infections that display tissue- or organ-specificity. During pregnancy, *P. falciparum* displays a clear tissue-specificity, localising to the placenta, that is in marked contrast to the normally systemic distribution of *Pf*iE in non-pregnant hosts. The apparent 'loss' of Treg from PBMC of infected women that we observed at inclusion thus probably reflects their migration to the infected placenta, mediated, as for monocytes and other cell types, by the increased concentrations of chemokines such as IP-10. The anti-inflammatory functions of Treg involve production of, for example,

Table 6. Multiple logistic regression analysis for independent associations of selected parameters with the presence of anaemia in the Benin sub-group at delivery.

Variables	Odds Ratio	95% CI	p
% mDC expressing $HLA\text{-}DR^{low}$ (<median)[a]	2.5	0.9—6.8	= 0.07
% Teff (≥median)[a]	3.0	1.1—8.1	<0.05

[a]median value of non-anaemic group used for dichotomisation.
χ^2 Hosmer-Lemeshow test (2ddl) = 0; p = 1.0.
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Table 7. Prospective analysis of association between variables at inclusion and anaemia at delivery.

Variables	Odds Ratio	IC 95%	p
% Monocytes (>median)[a]	3.1	1.04—9.06	<0.05
% Monocytes expressing CD86^{hi} (≥median)[a]	3.2	1.04—9.73	<0.05
% B cells expressing HLA-DR^{hi} (≥median)[a]	2.6	0.88—7.46	= 0.08

[a]median value of non-anaemic group used for dichotomisation.
χ^2 Hosmer-Lemeshow test (4ddl) = 0.91; p = 0.92.
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cytokines such as IL-10, which could at least partially explain the increased concentrations of IL-10 we observed in peripheral plasma in parallel with the different chemokines mentioned earlier (Boström S & Ibitokou S, unpublished data). The impact that any of the infection-mediated changes in circulating Treg observed here may have on the reported gestational age-related fluctuations in the frequency of Treg in PBMC during normal, healthy pregnancies remains to be determined [4,47,48]. Whether such fluctuations occur during 'normal' healthy pregnancies in African populations in any case requires confirmation. The increased frequency of Teff associated with infections at delivery but not with those at inclusion may be a further reflection of the acute nature of the infections present at delivery, whilst the generalized lymphopenia that is a classical feature of symptomatic *P. falciparum* was not seen at either time-point, a finding that may reflect both the asymptomatic character and the comparatively low-level parasitaemias of the infections.

In the specific context of anaemia during pregnancy, the associations with PBMC profiles we observed at inclusion and delivery involved DC/monocytes and effector T cells, respectively. The aetiology of anaemia during pregnancy is acknowledged to be complex, but one plausible explanation for those differences might be, as suggested by others [49], the relationship between the time-course of inflammatory responses - exemplified here in particular by the increased frequency of activated (CD86^{high}) monocytes associated with anaemia at inclusion - and *P. falciparum* infection, in the sense that inflammation and hence anaemia will tend to 'lag behind' infection. In the same context, we interpret the increased frequency of Teff in PBMC at delivery, associated with both infection and anaemia, as an indication of the presence of T cell subsets (e.g. effector memory cells) that are able to respond rapidly to infection. The contrasting association between anaemia and reduced HLA-DR expression on DC, on the other hand, might plausibly reflect the known suppressive effects of IL-10 - present at significantly higher levels in the plasma of infected women - in this context [50].

In summary, we have identified a number of features of PBMC associated with *P. falciparum* infection and with anaemia during pregnancy. The striking association between infection and increased B cell frequencies in PBMC, irrespective of gestational age, emphasizes the role such cells appear to have in the context of infection-induced placental inflammation. Our data also serve to clearly highlight the prominent role played by monocytes in that inflammatory response. They further suggest that Treg provide a balancing anti-inflammatory response through, we assert, their migration to the placenta as the primary site of *P. falciparum* infection during pregnancy. That Treg may respond and be reallocated in such a way is entirely consistent with current knowledge of the pathology-limiting controlling role such cells are thought to play during tissue-specific infections.

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Author Contributions

Conceived and designed the experiments: SI MO JL AM PD MTB SV AJFL NF. Performed the experiments: SI MO CA SE NF CS SB. Analyzed the data: SI MO LB SB AJFL NF CS. Wrote the paper: SI MTB SV AJFL NF.

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ARTICLE 2: Gestational age-related changes in the peripheral blood cell composition of sub-Saharan African women.

Ibitokou S, Brutus L, Vianou B, Oesterholt M, Massougbodji A, Deloron P, Troye-Blomberg M, Fievet N & Luty AJF.

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Contexte : Le concept de l'immuno-modulation au cours de la grossesse a été décrit et des réponses de type TH1 et TH2 permettent le maintien de l'équilibre fœto-maternel. Le 1er et le 3ème trimestre de la grossesse sont caractérisés par l'implantation de l'œuf et la placentation d'une part, et d'autre part l'accouchement et l'expulsion du nouveau-né [65]. Ces phénomènes requièrent des réponses pro-inflammatoires générées par les cellules de l'immunité pour maintenir l'équilibre physiologique de la grossesse. Les études longitudinales récentes ont montré des changements qualitatifs et quantitatifs importants dans les populations cellulaires en fonction de l'âge gestationnel au cours des grossesses normales [122, 124, 221-223]. A partir des données de l'article précédent, nous avons souhaité rechercher, la composition des cellules immunocompétentes chez les femmes incluses au Bénin et en Tanzanie au cours du projet STOPPAM.

Méthode : L'originalité de cette étude réside dans l'utilisation des données appariées à l'inclusion et à l'accouchement pour évaluer les différences en fonction de l'âge gestationnel. Parmi les 131 inclusions et 111 accouchements enrôlés au Benin, les données paires inclusion-accouchement de 24 femmes ont permis de valider nos analyses. Dans un premier temps, nous avons comparé dans la cohorte du Bénin, les fréquences cellulaires des femmes non-infectées à l'inclusion (69) par rapport aux femmes non-infectées à l'accouchement (74), ensuite celles des femmes infectées à l'inclusion (62) comparées aux femmes infectées à l'accouchement (37). Dans un second temps, les populations cellulaires qui présentaient des différences significatives dans les deux groupes ont été sélectionnées et introduites dans un modèle d'analyse appariée sur les 24 paires de femmes sélectionnées. Les données de l'étude en Tanzanie ont été utilisées pour compléter et pour la confirmation des données du Benin.

Résultats : Indépendamment de l'infection palustre, les fréquences de lymphocytes T CD4$^+$, T effecteurs et de monocytes exprimant le CD86 diminuaient entre l'inclusion et l'accouchement alors que celles de lymphocytes T CD8$^+$, mDC exprimant le CD86 sont augmentées dans une analyse non appariée. Lorsqu'on inclut ces paramètres dans une analyse appariée sur 24 paires de femmes, les fréquences de T CD4$^+$ et de T effecteurs sont diminuées à l'accouchement comparées à l'inclusion.

Discussion et conclusion : Nos résultats montrent des variations dans les populations lymphocytaires au cours de la grossesse indépendamment de l'infection palustre. Ces résultats confirment ceux déjà décrits chez les populations non africaines sur la diminution de fréquence de T $CD3^+$ entre le second et le troisième trimestre de grossesse et apportent des informations complémentaires sur la diminution des fréquences de lymphocytes T effecteurs au cours de la grossesse chez les populations africaines. Cette étude est une première décrivant les changements de populations cellulaires au cours de la grossesse chez les femmes africaines.

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Gestational age-related changes in the peripheral blood cell composition of sub-Saharan African women

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ABSTRACT

Gestational age-related changes in the cellular composition of peripheral blood have not been described in sub-Saharan African settings. We conducted longitudinal cohort studies in Beninese and Tanzanian mothers with quantification of peripheral blood mononuclear cell-types ex vivo using flow cytometry. Between the second trimester and delivery the frequency of $CD4^+$ T cells declined significantly, contrasting with a non-significant increase in $CD8^+$ T cells, but no changes in T-regulatory, NK or NKT cell frequencies. Antigen-presenting cell profiles were also unaltered, although non-significant trends were evident. These changes resemble in some respects those reported during pregnancies in developed countries, but differ in others.

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1. Introduction

The concept of pregnancy as an immunosuppressive state permitting the foetal allograft to implant and grow has been well described (Munoz-Suano et al., 2011). Cell-mediated immunity plays a pivotal role in the tolerance of the foetus by the mother's immune system, but discrepancies exist in the results of several studies that have assessed changes in the distribution and activation levels of different circulating cell populations during pregnancy. Thus, studies conducted in developed countries have variously reported an increased frequency of peripheral blood $CD8^+$ T cells in women in their third trimester compared with non-pregnant women (Luppi et al., 2002a), no changes in the frequencies of $CD4^+$ or $CD8^+$ T cells at any stage of pregnancy (Kuhnert et al., 1998; Luppi et al., 2002b), and a decreased frequency of 'helper' ($CD4^+$) T cells in late pregnancy (Watanabe et al., 1997). A progressive gestational age (GA)-related increase in circulating monocytes' activation status has also been reported (Luppi et al., 2002b). Data from recent longitudinal studies in high-income countries have revealed evidence of significant

Abbreviations: APC, antigen-presenting cells; ANV, antenatal visit; BD, Becton–Dickinson; CPDA, citrate phosphate dextrose adenine; DC, dendritic cell; EDTA, ethylenediamine tetraacetic acid; FACS, fluorescent activated cell sorter; GA, gestational age; HIV, human immunodeficiency virus; NK, natural killer cells; PBMC, peripheral blood mononuclear cell; *P. falciparum*, *Plasmodium falciparum*; RDT, rapid diagnostic test; Teff, effector T cells; Treg, regulatory T cells.

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GA-related changes in both the quantity and the quality of regulatory T cells (Treg), dendritic cells (DC) and other cell types in peripheral blood (Bachy et al., 2008; Della Bella et al., 2011; Kolte et al., 2011; Leber et al., 2010; Somerset et al., 2004). Whether similar changes occur during pregnancies in sub-Saharan African settings is currently unknown.

2. Materials and methods

2.1. Study design, location and population

The study described here comprised cellular immunological assessments conducted in sub-groups of pregnant women who were participating in a longitudinal study entitled "Strategies to Prevent Pregnancy Associated Malaria" (STOPPAM). The STOPPAM study was conducted in parallel in two study sites in Benin and Tanzania and its overall design has been described in detail elsewhere (Huynh et al., 2011; Minja et al., 2012). Malaria, primarily due to infection with *Plasmodium falciparum*, is endemic in the areas of both sites, displaying seasonal peaks, but transmission is higher in the Benin site. After giving written informed consent, ~1000 pregnant women at ≤24 weeks' GA were included both in the area of Comé, located in the Mono province 70 km west of Cotonou, Benin, and in Korogwe, located ~100 km inland from the coast in the Tanga Region of north-eastern Tanzania. Clinical and parasitological data were systematically collected at inclusion and at subsequent scheduled ante-natal visits (ANV), when ultrasound examinations were also performed allowing accurate determination of GA. Data were also collected at non-scheduled ('emergency') visits whenever women presented at the clinics because of illness. The sub-groups we studied here comprised, firstly, women selected sequentially at inclusion who presented with *P. falciparum* infection as determined by the combination of rapid diagnostic tests (RDT) and microscopic examination of peripheral blood smears. Using a case–control approach, we subsequently selected, as closely in time as possible, an uninfected 'control' woman matched for age, gravidity and gestational age to the corresponding previously selected 'case'. A second sub-group was selected at delivery comprising women with *P. falciparum* infections identified by RDT and examination of placental impression smears, and uninfected women in whom both RDT and smear were negative at delivery. At all times, women in whom infection with *P. falciparum* was identified by RDT were given anti-malarial treatment the same day according to national guidelines. Women who were seropositive for HIV or with no HIV diagnosis were not included in the present analyses. Ethics approval for the STOPPAM study was obtained from the ethics committees of the Health Science Faculty of the University of Abomey-Calavi, Benin, and the National Institute of Medical Research of Tanzania.

2.2. Blood collection and cell preparation

Peripheral venous blood samples were collected from selected women at inclusion and at delivery in vacutainers (BD Biosciences, France) containing citrate phosphate dextrose adenine (CPDA) anticoagulant. For the detailed immunological studies described here, samples were taken on two occasions: (i) at inclusion and (ii) a maximum of 8 h prior to delivery, from a total, respectively, of 131 and 111 Beninese women and from 38 and 27 Tanzanian women, all of whom had uncomplicated vaginal deliveries. The blood samples were transported to the study sites' research laboratories and processed for immunological assessments within four hours of collection. Haemograms were obtained using an automated Sysmex KX-21N analyser according to the manufacturer's instructions. Peripheral blood mononuclear cells (PBMC) were isolated using Leucosep tubes (Greiner-Bio, France) according to the manufacturer's procedure and were subsequently used for immunophenotyping by flow cytometry. The procedures, including gating strategies, used for flow cytometry-based quantification of cells ex vivo have been described in detail elsewhere (Ibitokou et al., 2012). Briefly, PBMC were stained with appropriate combinations of standard cell-surface- and cytoplasmic marker-specific monoclonal antibody reagents to allow identification of (i) T (subsets, anti-CD3-APC/anti-CD4-PerCP/anti-CD8-FITC/anti-CD25-FITC/anti-CD127-PE/anti-FoxP3-APC) and B lymphocytes (anti-CD19-FITC), (ii) NK & NK T (anti-CD3-APC/anti-CD56-PE) cells, (iii) monocytes (anti-CD14-FITC) and (iv) dendritic cells (DC, subsets, anti-BDCA1-phycoerythrin (PE)/anti-BDCA2-PE), as well as estimating the activation status of antigen-presenting cells (APC: monocytes, DC, B cells) through measurement of HLA-DR (anti-HLA-DR-PerCP) and CD86 (anti-CD86-APC) expression. Of note, we used a definition of Treg ($CD4^+CD25^+CD127^-$) here that clearly defines cell populations with the highest expression level of FoxP3, thereby distinguishing it from the population we defined as effector T cells (Teff, $CD4^+CD25^+CD127^+$, Fig. 1). A FACSCalibur four-colour flow cytometer (BD Biosciences, France) running CellQuest Pro software was used throughout.

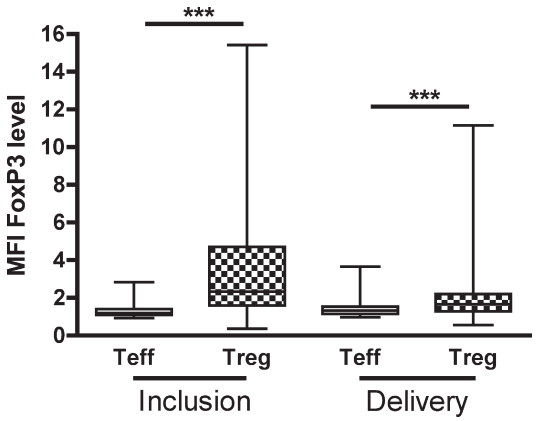

Fig. 1. Comparison of FoxP3 expression levels in Treg ($CD4^+CD25^+CD127^-$) and Teff ($CD4^+CD25^+CD127^+$) in 107 pregnant Beninese at inclusion and in 87 at delivery. Box-plots represent medians with 75th and 25th percentiles and whiskers for 90th and 10th percentiles. *p*-values were determined by the non-parametric Mann–Whitney *U* test. Significant differences are depicted by *** for $p < 0.001$.

2.3. Statistical analyses

Data analyses were performed using STATA/MP 11.2 (StataCorp, College Station, TX, USA) and Prism 5.0 (Graph pad Inc, CA, USA).

In order to assess GA-related changes in the PBMC composition, we compared the median values of PBMC frequencies or of antigen-presenting cells' expression of activation markers (HLA-DR/CD86) between a set of pregnant women of ≤24 weeks' GA and a second set of women at delivery using the non-parametric Mann–Whitney *U* test. First, we compared parameters of interest separately in groups of *P. falciparum*-infected and uninfected women. We selected those parameters that showed significant GA-related changes in both infected and uninfected women for use in a second round of analysis for which we grouped data from all women together (infected with uninfected) to compare the selected parameters between unpaired samples at inclusion and delivery. In order to avoid the problems of multiple testing, we used Bonferroni correction by dividing the nominal alpha level by the number of comparisons and rejecting the null hypothesis if the *p* value was less than or equal to the corrected significance level. The corrected *p* value for comparison of these unpaired data was therefore 0.002 (0.05/23).

In order to validate the findings from analyses of unpaired samples, we sought to circumvent the issue of individual bias inherent to the latter. For this purpose we compared PBMC (for the six parameters that had shown significant GA-related changes in unpaired samples) in paired samples collected at inclusion and delivery from a sub-group of 24 Beninese women. Since paired samples are, by their very nature, not independent, we used the non-parametric Wilcoxon signed-rank test to determine the significance of differences, and again used Bonferroni correction (0.05/6, $p \leq 0.008$) in order to avoid bias due to multiple testing.

3. Results

The sub-groups of women included in the analyses here were drawn from the larger cohorts of the STOPPAM study. In order to exclude the possibility of selection bias, we therefore first compared selected parameters – essentially demographic in nature, but including the prevalence of anaemia – in the sub-groups of Beninese women with the same parameters in the whole cohort at the two time-points. Table 1 illustrates the results of those comparisons, revealing that median gestational ages were significantly longer in the delivery sub-group compared with the whole cohort, but that all other variables tested were similar. In order to exclude other potential sources of bias, we also compared the basic clinical and haematological characteristics of the sub-groups of Beninese women segregated according to the presence or absence of infection with *P. falciparum* (Table 2). At inclusion, as previously reported (Ibitokou et al., 2012), a higher proportion of those with *P. falciparum* infections were anaemic, whilst the median axillary temperature was also significantly higher in the infected group, even though only one of the infected women presented with a fever (38.0 °C). At delivery none of the parameters measured differed according to the women's infection status.

Our own recent data have shown that *P. falciparum* alters the composition of PBMC in infected versus uninfected women (Ibitokou et al., 2012). We therefore first compared the frequencies of cell types at inclusion and at delivery within groups of Beninese and Tanzanian women segregated according to the presence or absence of infection. In the Beninese women there was a significant decline in the frequency of $CD4^+$ T cells with a parallel increase in the frequency of $CD8^+$ T cells from inclusion (mean GA: 18 weeks) to delivery, regardless of their infection status (Fig. 2A and B). The decline in $CD4^+$ T cells' frequency was paralleled by a decline in the frequency of effector T cells (Teff, $CD4^+CD25^+CD127^+$) and a consequent increase in the regulatory T cell (Treg, $CD4^+CD25^+CD127^-$)/Teff ratio over time concurrent with stable Treg frequencies (Fig. 2C–F). The relative level of expression of FoxP3 (R Treg), however, as a marker of the activation status of Treg, declined significantly (Fig. 2E and F). The frequency of natural killer (NK, $CD56^+$) cells was unchanged, whilst that of NK T ($CD3^+CD56^+$) cells increased significantly, but in the uninfected group only (data not shown). Amongst

Table 1
Characteristics of sub-groups compared with the whole cohort in Benin women at inclusion (A) and at delivery (B).

Variables	Whole cohort		Sub-group		p^a
	n		*n*		
(A) Inclusion					
Gestational age in weeks	975	17 (6)	102	17.8 (4.7)	0.06
Gravidity	1029	3.0 (3.0)	107	3.0 (4.0)	0.11
Age in years	1015	25.0 (9.0)	107	25.0 (10.0)	0.17
% possessing a bednet	1029	32.1	107	28.0	0.39
% with haemoglobin < 11 g/dl	1022	60.6	106	67.9	0.14
(B) Delivery					
Gestational age in weeks	629	39.7 (1.6)	87	40.0 (1.8)	0.03
Gravidity	635	3.0 (3.0)	87	3.0 (3.0)	0.61
Age in years	623	26.0 (9.0)	86	26.5 (7.0)	0.48
% possessing a bednet	635	30.2	87	35.6	0.30
% with haemoglobin < 11 g/dl	582	46.4	82	40.2	0.29

Values are medians (interquartile ranges).

[a] Non-parametric Mann–Whitney *U*-test or Chi-squared test for proportions.

Table 2
Clinical and haematological characteristics of sub-groups of Beninese women at inclusion and delivery.

Variables	Inclusion ($n = 131$)			Delivery ($n = 111$)		
	Uninfected ($n = 69$)	Infected ($n = 62$)	p^a	Uninfected ($n = 74$)	Infected ($n = 37$)	p^a
Systolic pressure (cm Hg)	10.0 (2.0)	10.0 (1.0)	0.88	11.0 (1.0)	11.0 (1.0)	0.25
Diastolic pressure (cm Hg)	6.0 (1.0)	6.0 (1.0)	0.71	7.0 (2.0)	8.0 (2.0)	0.13
Axillary temperature (°C)	36.8 (0.5)	37.0 (0.6)	< 0.01	36.8 (0.5)	36.8 (0.5)	0.63
Haemogram						
% Lymphocytes	32.0 (11.0)	32.0 (11.0)	0.55	32.0 (12.0)	33.5 (15.0)	0.85
% Basophils	0.0 (0)	0.0 (0)	0.34	0.0 (0)	0.0 (0)	0.36
% Eosinophils	0.0 (1.0)	0.0 (2.0)	0.08	0.0 (1.0)	0.0 (0.0)	0.73
% Neutrophils	60.0 (11.0)	60.0 (12.0)	0.29	60.0 (13.0)	61.0 (12.5)	0.88
% Monocytes	6.0 (2.0)	6.0 (3.0)	0.07	7.0 (1.0)	6.0 (2.0)	0.06
% with vomiting	5.8	3.7	0.60	2.7	2.9	0.96
% with headache	21.7	17.0	0.51	2.7	8.6	0.17
% with anaemia	55.1	80.3	< 0.01	32.4	45.9	0.16

Values are medians (interquartile ranges)
[a] Comparisons made using the non-parametric Mann–Whitney *U* test or Chi-squared test for proportions.

antigen-presenting cell (APC) populations, GA-related declines in the frequencies of both myeloid and plasmacytoid dendritic cells (mDC, pDC) as well as of B cells were also evident, but these changes were not consistently significant across the uninfected and infected groups, whilst monocyte frequencies were unchanged from inclusion to delivery irrespective of infection status (Fig. 3A–D). In the Tanzanian dataset not all changes were consistent across the uninfected and infected groups, but similar GA-related changes as seen in Beninese women in the frequencies of $CD4^+$ and $CD8^+$ T cells were evident, as well as in those of mDC and B cells, whilst the frequency of monocytes increased (data not shown). Separate comparisons of the activation status of different cell types revealed significantly increased levels of expression of CD86 on mDC over time in both uninfected and infected Beninese women (Fig. 4A and B), but a contrasting decrease in CD86 expression on monocytes (Fig. 4A and B) that was also evident in Tanzanians (data not shown). Lastly, there were non-significant trends for GA-related decreases in the expression of HLA-DR on pDC in both uninfected and infected Beninese women (Fig. 4C and D).

We sought to confirm the findings described above firstly through analyses of the eight parameters (%$CD4^+$ T cells, %$CD8^+$ T cells, %Teff, R Treg, %mDC, CD86 expression on mDC and on monocytes, HLA-DR expression on pDC) that displayed changes related to GA, by comparing data from 107 Beninese women at inclusion with 87 at delivery regardless of their infection status. Table 3 shows that, in all cases, the differences between immunological parameters at inclusion versus delivery were strongly significant and 6/8 were below the level ($p \leq 0.002$) set by correction for multiple testing.

In a final step, we analysed a separate set of data from another 24 Beninese women for whom relevant, i.e. paired samples were available at inclusion and delivery, in order to validate the GA-related changes indicated by the analyses of unpaired data (Table 3). These analyses confirmed the following changes in PBMC profiles between inclusion and delivery: (i) a decline in the frequencies of $CD4^+$ T cells and of Teff; (ii) an increase in the frequency of $CD8^+$ T cells; (iii) reduced expression of the activation marker CD86 on monocytes (Table 3). After adjustment for multiple tests only the reductions in the frequencies of $CD4^+$ T cells and of Teff remained significant (Table 3).

4. Discussion

Our data reveal significant gestational age-related alterations between the second trimester and delivery in the PBMC composition of African women with uncomplicated pregnancies. The most striking of these alterations

Table 3
Comparison of selected peripheral blood mononuclear cell (PBMC) profiles between inclusion and delivery in unpaired and paired groups of Beninese women.

Variables	Unpaired			Paired		
	Inclusion ($n = 107$)	Delivery ($n = 87$)	p	Inclusion ($n = 24$)	Delivery ($n = 24$)	p
% CD4	63.0 (10.4)	57.9 (13.3)	<0.0001	63.8 (9.1)	58.9 (9.4)	**0.005**
% CD8	27.1 (9.0)	31.3 (12.3)	0.0011	28.4 (9.4)	31.8 (11.1)	0.042
% Teff	4.7 (5.4)	1.8 (1.8)	<0.0001	5.6 (8.3)	2.4 (3.0)	**0.008**
R Treg (FoxP3)	2.2 (3.0)	1.6 (0.7)	<0.0001	2.0 (2.7)	1.7 (0.7)	0.159
% mDC	0.3 (0.2)	0.3 (0.2)	0.025	–	–	–
% mDC–$CD86^{hi}$	0.9 (1.3)	1.3 (2.0)	**0.0013**	0.5 (1.5)	1.2 (2.1)	0.133
pDC HLA-DR (MFI)	54.3 (34.2)	46.9 (29.8)	0.0121	–	–	–
Monocyte CD86 (MFI)	6.9 (3.8)	4.9 (3.6)	**0.0033**	6.7 (6.0)	5.3 (3.2)	0.022

Inclusion and delivery groups include women with and without *P. falciparum* infections; values are medians (interquartile ranges) compared using the Mann–Whitney *U* test/Wilcoxon signed-rank test for unpaired/paired data respectively; after Bonferroni correction, p values ≤ 0.002 (unpaired comparisons included 23 variables) or ≤ 0.008 (paired comparisons, six variables) are considered significant (p values in bold face); MFI: mean fluorescence intensity; R Treg: ratio of FoxP3 expression in Treg vs. naïve T cells ($CD4^+CD25^-CD127^+$).

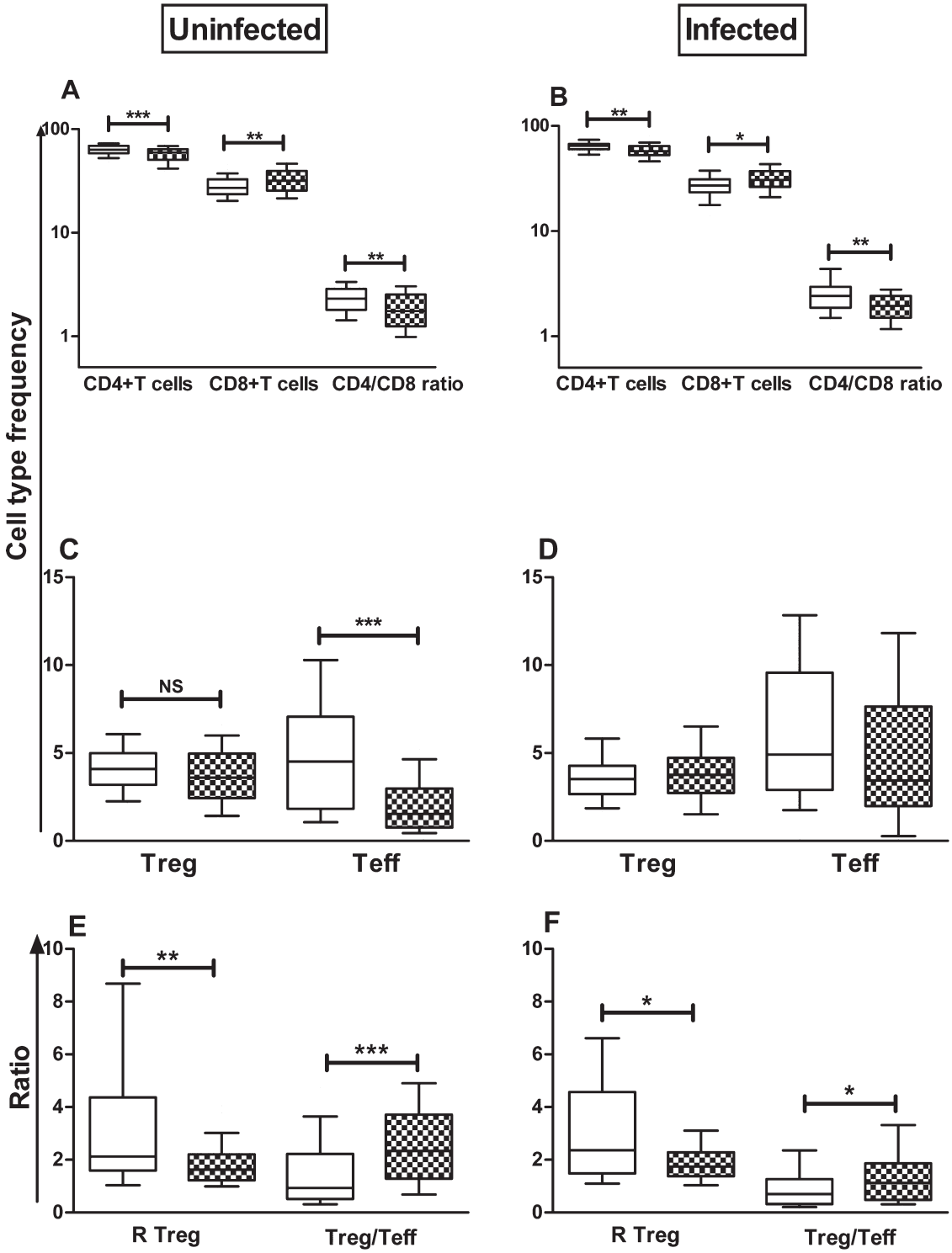

Fig. 2. Peripheral blood composition changes according to gestational age. Frequencies *ex vivo* of different PBMC lymphocyte populations compared at inclusion and delivery respectively in 69 and 47 uninfected Beninese women (left panels) and in 62 and 37 *P. falciparum*-infected Beninese women (right panels). Box-plots represent medians with 75th and 25th percentiles and whiskers for 90th and 10th percentiles. R Treg: ratio of FoxP3 expression in Tregvs naïve T cells ($CD4^+CD25^-CD127^+$). *p*-values were determined by the non-parametric Mann–Whitney *U* test. Significant differences are depicted by * if $p < 0.05$, ** if $p < 0.01$ and *** if $p < 0.001$.

concerned reciprocal decreases and increases in, respectively, the frequencies of $CD4^+$ and $CD8^+$ T lymphocytes, although the latter did not reach statistical significance in a comparison of a limited number of paired samples. Coincidentally, we have previously reported that the frequencies of these same cell types did not vary at all as a function of infection with *P. falciparum* at either inclusion or delivery (Ibitokou et al., 2012), possibly reflecting the fact that almost all such infections in these women were asymptomatic at the time of detection. Lymphopaenia, i.e. a decreased frequency of $CD3^+$ cells as a feature of late pregnancy in Japanese mothers, has been reported (Minagawa

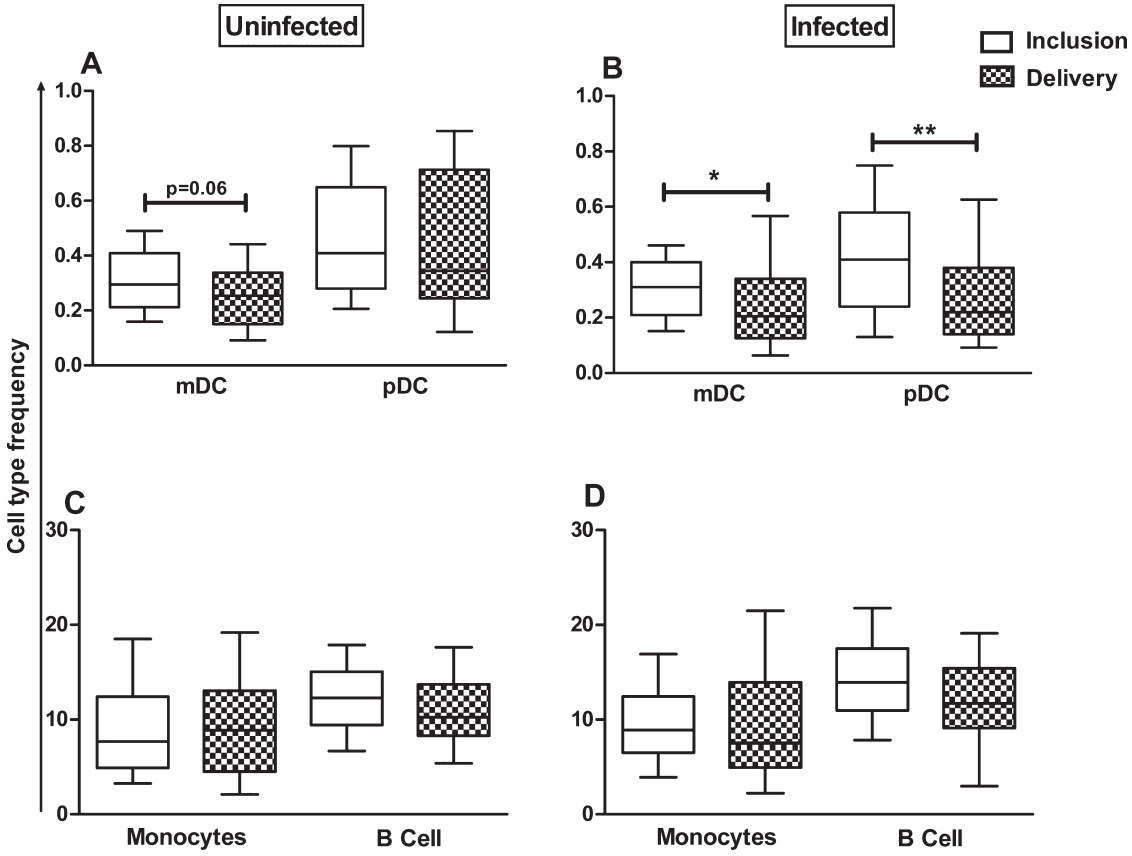

Fig. 3. Peripheral blood composition changes according to gestational age. Antigen presenting cell frequencies in PBMC *ex vivo* compared at inclusion and delivery respectively in 69 and 47 uninfected Beninese women (left panels) and in 62 and 37 *P. falciparum*-infected Beninese women (right panels). Box-plots represent medians with 75th and 25th percentiles and whiskers for 90th and 10th percentiles. *p*-values were determined by the non-parametric Mann–Whitney *U* test. Significant differences are depicted by * if $p < 0.05$, and ** if $p < 0.01$.

et al., 1999), but another recent study indicated no pregnancy-related change in total peripheral $CD4^+$ T cell counts in healthy Danish pregnancies (Kolte et al., 2011). The data we present here concerning the significant gestational age-related reduction of the frequencies both of $CD4^+$ T cells and of the Teff sub-population they contain are thus unequivocal, although any functional relevance of these changes in the context of pregnancy and/or maternal cell-mediated immunity remains to be defined.

Stable frequencies of circulating Treg during the second and third trimesters of pregnancy, as we saw here, have been reported in non-African pregnant populations (Ernerudh et al., 2011; Richardson and Weinberg, 2011; Somerset et al., 2004), although higher frequencies of Treg in the second versus the third trimester of healthy pregnancies have also been reported (Kolte et al., 2011). A caveat here is that the definition of 'Treg' by phenotypic profile is not consistent, potentially confounding comparison of results across different studies. Notably, Kolte et al. (2011) did define Treg using a set of phenotypic markers ($CD4^+CD25^+CD127^-[FoxP3^+]$) similar to ours, suggesting that Treg dynamics may indeed differ between uncomplicated pregnancies in African and non-African populations. Sub-Saharan African populations, including women of childbearing age, are exposed to infection by a comparatively broader array of pathogens than Europeans, for example, including helminths that induce strong Treg responses. We did not assess helminth infections here, but we speculate that the lack of any gestational age-related change in Treg frequency in our study may reflect such factors. Of note, we found that women infected with *P. falciparum* at inclusion into the STOPPAM study had significantly fewer circulating Treg compared with uninfected women, but this difference was not evident in samples taken at delivery (Ibitokou et al., 2012).

Increased frequencies of circulating NK and NK T cells in late pregnancy of healthy Japanese women, but no such changes in pregnancies of healthy British women have been reported (Minagawa et al., 1999; Southcombe et al., 2010). Our results are thus consistent with those of the latter study. An increased frequency of mDC with increasing gestational age has been reported for non-Africans (Darmochwal-Kolarz et al., 2003), but that was not the case in our data and, furthermore, we did not find gestational age-related increases in the activation status of either mDC or monocytes that have also been reported in non-African populations (Bachy et al., 2008; Della Bella et al., 2011; Luppi et al., 2002a,b). Although statistically non-significant, the trend in our data was actually the opposite, suggesting a reduced activation status of both monocytes and pDC with increasing gestational age (Table 3).

We conclude that uncomplicated pregnancies in sub-Saharan African settings do indeed display some, but not all, of the pregnancy-related changes in PBMC profiles reported to date in non-African populations. The consistent and marked changes we did observe concerned the

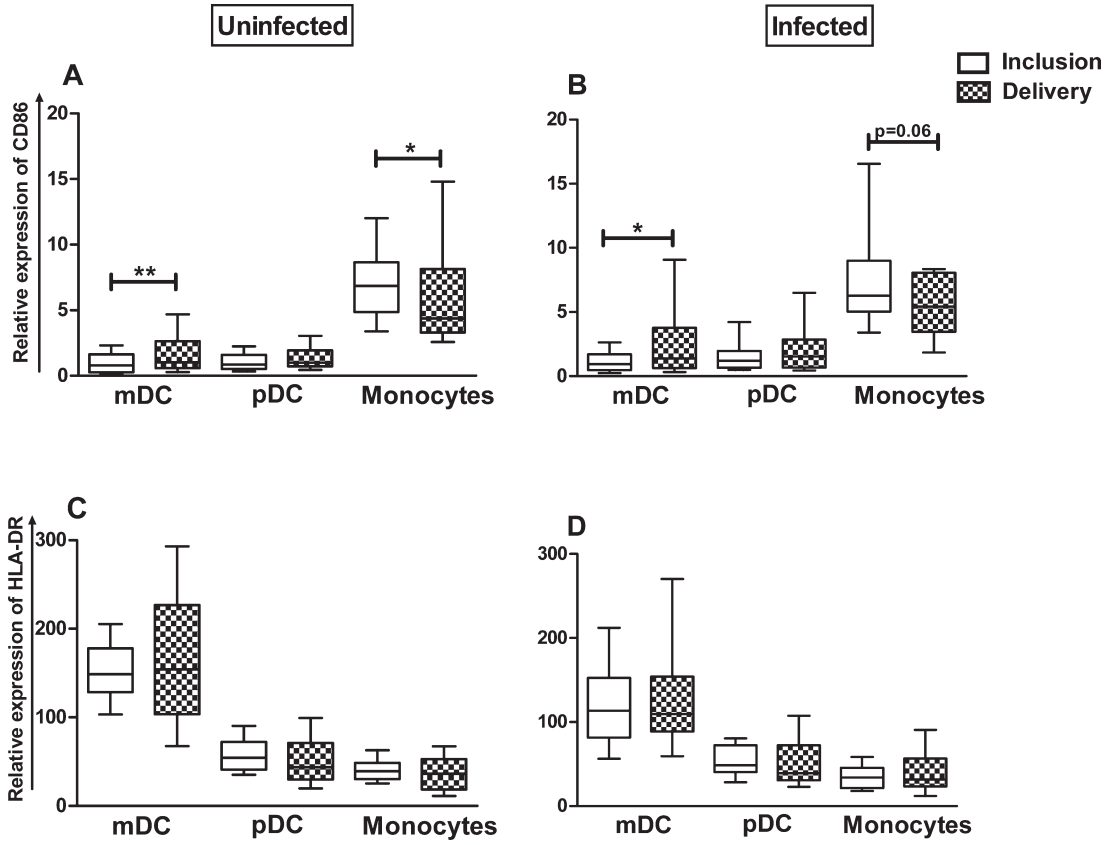

Fig. 4. Antigen presenting cells' activation status *ex vivo* related to gestational age. The level of expression of CD86 and of HLA-DR on mDC, pDC and monocytes at inclusion and delivery in uninfected (A and C) and infected (B and D) Beninese women. Box-plots represent medians with 75th and 25th percentiles and whiskers for 90th and 10th percentiles. *p*-values were determined by the non-parametric Mann–Whitney *U* test. Significant differences are depicted by * if $p < 0.05$, and ** if $p < 0.01$.

decline in the frequencies of $CD4^+$ T and Teff cells between the second trimester and delivery, declines that occurred independently of the presence or absence of infection with *P. falciparum*. It is of note that, in separate studies, we and others have shown placental infection with *P. falciparum* to be associated with pre-eclampsia in African women (Adam et al., 2011; Ndao et al., 2009). In non-African populations, the frequency of peripheral Treg is significantly lower in women with pre-eclampsia than in those with normal pregnancies (Prins et al., 2009; Steinborn et al., 2008; Toldi et al., 2012). Elucidating any specific gestational age-related changes in a particular PBMC profile that may be relevant to pre-eclampsia in African women, be it that of Treg or of other cells, will require larger, focused and more in-depth studies.

Conflict of interest statement

All authors declare that they have no conflict of interest. The funding sources had no involvement in the design, collection, analyses, and interpretation of data, writing of the manuscript or decision to publish.

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Authors' contributions

SI, NF, PD, MTB and AJFL, conceived, designed and coordinated the study. SI, BV and MO participated in the sample collection and processing. SI, NF and AJFL designed and supervised the immunoassays. SI and LB performed statistical analyses. SI, BV and MO carried out the immunoassays. SI drafted the first version of the manuscript. All authors read and approved the final manuscript.

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ARTICLE 3: Inflammatory factors and Cell-Mediated Immune responses during Pregnancy-Associated Malaria.

Ibitokou S, Boström S, Brutus L, Ndam NT. Massougbodji A, Deloron P, Troye-Blomberg M, Fievet N & Luty AJF.

Manuscrit en préparation.

Contexte : Le paludisme placentaire favoriserait l'infiltration des cellules immunocompétentes dans le placenta [7]. La migration des cellules vers les organes lymphoides est assurée par les chimiokines. Les travaux de Conroy et coll (2011), ont montré que les marqueurs périphériques pouvaient prédire l'infection placentaire à l'accouchement indépendamment de la présence de parasites circulant [224]. Par ailleurs, plusieurs marqueurs de l'inflammation liée au PAG ont été identifiés [136, 146, 148]. Des études longitudinales sur l'identification des indicateurs de l'infection très tôt au cours de la grossesse sont nécessaires pour valider ces marqueurs.

Méthode : Nous avons utilisé dans cette étude les mêmes sous-groupes de femmes que ceux décrits dans les articles 1&2. Une des particularités de cette étude est l'utilisation de la PCR pour l'identification des infections sub-microscopiques. Les cytokines et chimiokines suivantes ont été mesurées par les techniques CBA et ELISA : IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ, RANTES (Regulated on Activation Normal T-cell Expressed and Secreted), MIG (monokine induced by IFN-γ), MCP-1 (monocytes chemotactic protein-1), IP-10 (IFN-γ-inducible protein-10), VEGF/Flt1 (Vascular Endothelial Growth Factor), uPAR (urokinase receptor), Ang-1 et Ang-2 (angiopoietin-1&2) selon les recommandations réglementaires.

Résultats : Dans un premier temps, nous avons étudié l'impact des infections sub-microscopiques sur le profil des cellules immunocompétentes et ensuite les concentrations de cytokines et chimiokines plasmatiques à l'inclusion et à l'accouchement. Dans un second temps, nous avons recherché des bio-marqueurs cellulaires et solubles qui pourraient être prédictifs d'une infection placentaire ou impliqués dans des conséquences liées au PAG chez la femme enceinte et le nouveau-né.

Infection à *P. falciparum*, profils cellulaires et concentrations plasmatiques des bio-marqueurs. Au début de la grossesse et à l'accouchement, nous avons observé des concentrations élevées des cytokines et chimiokines IL-10, MIG et IP-10 chez les femmes infectées comparées aux femmes non-infectées. L'infection à *P. falciparum* est associée également à des fréquences élevées de lymphocytes T effecteurs et de B exprimant le CD86 à l'inclusion et à l'accouchement.

Etude prospective des bio-marqueurs sur les conséquences du PAG. L'identification des bio-marqueurs associés au risque d'infection palustre au second trimestre de la grossesse, nous permet, grâce à un modèle logistique, la recherche de bio-marqueurs prédictifs d'une infection placentaire, d'une anémie maternelle et d'un faible poids de naissance à l'accouchement. Des concentrations élevées d'IL-10 et une diminution d'IL-1β, associées à une fréquence élevée de monocytes au second trimestre de la grossesse, sont des facteurs de risque d'anémie maternelle à l'accouchement. Des concentrations diminuées de RANTES, une diminution d'IL-6, de mDC exprimant le CD86 sont facteurs de risque du faible poids chez le nouveau-né.

Nous n'avons pas observé de corrélation entre les infections sub-microscopiques et les concentrations de cytokines ou les fréquences cellulaires dans cette étude.

Discussion et conclusion : Les résultats sur les profils cellulaires, issus de cette étude confirment ceux de l'article 1 et apportent des informations complémentaires sur le fait que les changements de profils observés au cours du PAG sont associés à la présence de parasites et non aux antigènes parasitaires. Nos observations sur les concentrations de cytokines et chimiokines, démontrent l'importance de l'IL-10 et d'IP-10 dans le PAG. Nous avons aussi identifié le MIG comme une chimiokine associée à l'infection palustre chez la femme enceinte. Les concentrations élevées d'IL-10 au cours du PAG interviendraient dans le maintien de la régulation inflammatoire au cours de la grossesse et sont facteur d'anémie maternelle dans notre étude. Les chimiokines IP-10 et MIG seraient associées au recrutement des cellules immunocompétentes vers les sites d'infection ou dans le placenta.

Title: Inflammatory factors and Cell-Mediated Immune responses during Pregnancy-Associated Malaria

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Abstract

Background: Pregnancy associated malaria (PAM) is public health problem. Recent studies have proposed such cytokines and chemokines as makers for PAM. We also showed peripheral blood cells profile changes during PAM. In this study we investigated the impact of *P. falciparum* infection during pregnancy on inflammatory factors.

Methods: In longitudinal, prospective study carried out in Benin, immunophenotyping and activation levels of cell-mediated immune responses as well as plasma levels of several inflammatory mediators have been analyzed on subgroup of 131 pregnant women at inclusion and 111 at delivery using flow cytometry, standard cytometric bead arrays and ELISA. Infected pregnant women indentified as thick/thin smear and qPCR positive were matched to uninfected controls based on *P. falciparum* status and gravidity.

We compared levels of cytokines, chemokines and peripheral mononuclear cells in *P. falciparum*-infected and uninfected pregnant women at inclusion and at delivery, and used multiple logistic regressions at inclusion to develop predictive PAM outcomes at delivery.

Results: Increased plasma levels of IL-10, MIG, IP-10 and higher frequency of B cells and activating B cells were observed in infected *P. falciparum* women at inclusion and delivery. Increased levels of IL-10, IL-1β, higher monocytes were predicted for anemia at delivery. Increased IL-6, RANTES and higher mDC expressing CD86 were associated to risk of low birth weight.

Conclusions: Our results suggest that PAM is associated to cytokines, chémokines and peripheral blood cells changed in pregnant women. IL-10, IP-10 and activated B cells could be used as biomarkers for PAM.

Introduction

The concept that pregnancy is associated with immune suppression for fetal allograft tolerance has been well described [1-3]. In healthy pregnancies, components of the cellular arm of the immune response have been shown to populate the human decidua during the first trimester, and are necessary for placental development [4]. In areas where *Plasmodium falciparum* is endemic, pregnancy, and especially the first, is associated with an increase in susceptibility to infection with the parasite. Sequestration of *P. falciparum*-infected erythrocytes (*Pf*iE) in the placenta, via their adherence to the chondroitin sulphate A (CSA) expressed by syncytiotrophoblasts, is the major pathological characteristic of pregnancy-associated malaria (PAM), with potentially multiple detrimental outcomes [5]. *Pf*iE induce inflammation, characterized particularly by monocytic infiltration of the infected placenta [6-8]. One other well-described consequence of PAM is an altered cytokine and chemokine balance in the peripheral and in the placental blood [9-14].

We recently showed that *P. falciparum* infection during pregnancy is associated with modified peripheral blood mononuclear cell (PBMC) profiles, consistently characterised by enhanced B cell activity [15], a finding consistent with the prominent B cell component of the inflammatory response to *Pf*iE in the placenta reported by others (Muehlenbachs, Fried et al. 2007). B cells are recognized as having multiple functions not only as antigen presenting cells (APC) and antibody producers but also, for example, as regulators of dendritic cell (DC) function and maturation through their production of the cytokine interleukin(IL)-10 [16]. The polyclonal activation of B cells is a recognized feature of infection with *P. falciparum* that, in the case of PAM, may be linked to the demonstration of non-specific binding of IgM antibody to the parasite-derived ligand for CSA expressed on *Pf*iE [17].

The concentrations of several molecules, including the cytokines IL-10, TNF-α, IFN-γ and the chemokines IP-10, MIG, have been shown in several studies to be increased in placental plasma at delivery in women with PAM [11, 12, 18]. Here we used samples collected both during pregnancy and at delivery to examine in more detail the association between plasma cytokine/chemokine concentrations and PAM, including samples from women with sub-microscopical infections. We also wished to determine whether the PBMC profiles determined in the same samples retained their predictive value when analyzed in parallel, following the hypothesis that both cytokines/chemokines and cells are implicated in susceptibility to PAM.

Materials and Methods

Ethics statement

The study described here received ethical clearance from the ethics committees of the Health Science Faculty of the University of Abomey-Calavi, Benin.

Study population

The study populations comprised sub-groups drawn from the cohort of pregnant Beninese women that participated in a longitudinal study known as “Strategies TO Prevent Pregnancy Associated Malaria” (STOPPAM).STOPPAM was conducted in parallel in two study sites in Benin and Tanzania. After giving written informed consent, ~1000 pregnant women at ≤24

weeks' gestational age were included in the district of Comé, located in the Mono province 70 km west of Cotonou, the economic capital of Benin. The STOPPAM study design in Benin has been described in detail elsewhere [19], as has the procedure used to select women for the immunological sub-study described here [15]. Briefly, we selected firstly a sub-group of women at inclusion into the study who were identified as being infected with *P. falciparum* (for methods, see below) and a control, uninfected group, appropriately matched for age, gravidity and gestational age. Secondly, at delivery, a sub-group of women with varying infection histories during pregnancy – identified from the results of our own active and passive surveillance – was identified. The latter included women with no evidence of infection from inclusion through to delivery ('uninfected'), women with no infection at delivery but with a history of infection earlier in pregnancy ('exposed'), and women with infections at delivery who may or may not have been infected earlier ('infected').

Sample collection

Venous blood samples, collected in vacutainers with citrate phosphate dextrose adenine (CPDA) anticoagulant, were transported to the research laboratories within 4hours. Plasma samples were separated and stored at -80°C. Peripheral blood mononuclear cells (PBMC) were isolated using Leucosep tubes (Greiner-Bio) according to the manufacturer's description and were subsequently used for immunophenotyping as described [15].

Detection of P. falciparum infection

Infection status was determined using a rapid diagnostic test (RDT) (Parascreen, Zephyr Biomedical Systems). Thin and thick smears of peripheral and placental intervillous blood, to identify plasmodial parasites, were prepared using standard methods and were double-read retrospectively by routine microscopical examination performed by two experienced technicians. Quantitative (q) polymerase chain reaction (PCR) was performed after DNA extraction from filter paper blood spots using the chelex method as described elsewhere [20]. Women identified as infected had positive PCR and thin/thick smear test results whilst uninfected women had negative results with both PCR and thin/thick smears. Women with a positive PCR result but a negative blood smear result constituted a separate group identified as having sub-microscopic infections.

Cytometric Bead-Arrays for quantification of cytokines and chemokines

Plasma levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α cytokines were analyzed using human inflammatory kit (CBA, BD Biosciences, San Diego, CA, USA) and regulated on activation normal T cell expressed and secreted (RANTES), monokine-induced by IFN-γ (MIG), monocytes chemotactic protein (MCP)-1 and IFN-gamma-inducible protein (IP)-10 with human chemokines kit (CBA, BD Biosciences, San Diego, CA, USA). The procedure used adhered to the manufacturer's recommendations and has been described in detail elsewhere [21].

ELISA

Plasma concentrations of alpha (α) and gamma (γ)- Interferon (IFN) were determined using commercial ELISA kits (Mabtech, Stockholm, Sweden), as were those of soluble vascular endothelial growth factor receptor (VEGF-R1/Flt1), soluble urokinase plasminogen activator receptor (suPAR), and the angiopoietins (Ang-1 and Ang-2) (R&D, Minneapolis, MN). The methods used conformed to the manufacturer's recommendations and have been described in detail elsewhere [21].

Peripheral blood mononuclear cells immunophenotyping

PBMC were washed and re-suspended at a concentration of 10 million cells/ml. Cells were then labeled using a panel of surface and intracellular marker-specific monoclonal antibodies as described elsewhere [15]. Events were acquired using a BD FACSCalibur and data analyzed using CellQuest Pro or FlowJo 7.6 (Oregon, USA) software.

Data analysis

Data analysis was performed using STATA/MP 12.0 (StataCorp, College Station, TX USA) and Prism 5.0 (Graph pad Inc). Data were expressed as median and interquartile range. Comparison between groups was made with non-parametric Kruskal-Wallis and Mann Whitney tests. Categorical variables were compared with chi2 (χ^2) tests. Multiple logistic regressions were performed using "logit" commands in STATA in order to identify factors associated with the risk for or the intensity of *P. falciparum* infections in pregnant women both at inclusion and at delivery. In order to assess whether any of the cytokines, chemokines or other factors measured were independently associated with the risk of anaemia or with particular PBMC profiles during pregnancy, multiple logistic regression were used. A stepwise procedure was performed to select a model including factors associated with infection. Prospective analysis of the association between factors measured at inclusion and either placental malaria, maternal anemia or low birth weight identified at delivery was also conducted using multiple logistic regression using data collected at inclusion. Two-tailed *P*-values <0.05 were considered significant.

Results

Detection of P. falciparum infections

The study described here included sub-groups of Beninese women participating in the 'STOPPAM' study. These sub-groups have previously been shown not to differ in demographic characteristics from the whole cohort [15]. Here we wished to determine specifically whether and how circulating plasma levels of cytokines and chemokines during pregnancy might be associated with peripheral blood cell profiles in the same women and/or with the presence or absence of infection with *P. falciparum*. Since such infections may remain below the level of detection of the routinely-used microscopical method of diagnosis, here we performed additional analyses using a sensitive species-specific PCR-based method to identify so-called 'occult' infections. The latter were detected in a total of 38/242 women: in a subgroup of 131 assessed at inclusion – the majority of whom were in the second trimester of their pregnancy – 52 were uninfected whilst 61 had infections identified by microscopy and 18 had sub-microscopic infections identified by PCR; in 111 women assessed

at delivery 54 were uninfected, 37 had infections detected by microscopy and 20 sub-microscopic (**Table 1**). In the sub-group assessed at inclusion, those with infections detectable by microscopy were significantly younger, significantly less likely to possess a bednet and a significantly higher proportion was anaemic compared to those who were uninfected (**Table 1**). Of note, the same variables in those harboring sub-microscopic infections at inclusion did not differ significantly with respect to either of the other two groups, whilst in the sub-group assessed at delivery there were no differences in any variable between any of the groups.

Circulating levels of cytokines and chemokines associated with P. falciparum infection at inclusion and at delivery

There are few published studies concerning the effects of sub-microscopic infections during pregnancy, with only one, to our knowledge, identifying an association between such infections and a poor pregnancy outcome [low birth weight] [22]. We therefore first addressed the changes in the levels of circulating cytokines and chemokines as a function of women's infection status. At inclusion there was a significantly higher level of IL-10 in those with sub-microscopic infections compared to uninfected women, but otherwise no differences (**supplementary Table1**). This evidence of a modified immune status in women with sub-microscopic infections led us to merge them as a group, in all subsequent analyses, with the group of women harboring microscopically-detectable infections.

Univariate analyses revealed significantly increased levels of IL-6, IL-10, MIG and IP-10 at inclusion in the infected compared with the uninfected women, whilst at delivery significantly higher levels of IL-10, MIG, MCP-1, IP-10 and lower levels of Ang-1 were observed in infected versus uninfected women (**Table 2 & Figure 1**).

P. falciparum infection is associated altered cellular profiles during pregnancy

As we have previously shown, *P. falciparum* infection is associated with altered peripheral blood mononuclear cell (PBMC) profiles both at inclusion and at delivery in our study population [15]. Here, using PCR-based methods that identified women previously classified as uninfected but who were revealed in fact to be harboring sub-microscopic *P. falciparum* infections, we wished to determine the effects of such infections, if any, on PBMC profiles at inclusion and at delivery, as for the cytokines/chemokines described above. For the full range of variables analyzed, we found no significant differences at either time-point between those with sub-microscopic infections and either of the other two groups (**supplementary Table 2**). For subsequent analyses we therefore merged the two infected groups of women at each time-point.

In univariate analyses at inclusion, the frequency of mDC expressing HLA-DR in infected versus uninfected women was significantly lower whilst there were non-significant trends for (i) more $CD86^{hi}$ B cells, (ii) more Teff (and consequently a lower Treg/Teff ratio), and (iii) fewer NK cells in infected women (**Table 3**). Similar analyses at delivery revealed significantly higher frequencies of both Teff and of $CD86^{hi}$ B cells were observed in infected women, but significantly lower frequencies of pDC and of NKT cells compared to uninfected women (**Table 3**).

Multiple regression analyses of associations between cytokines, chemokines and peripheral blood cell profiles with P. falciparum infections during pregnancy

In order to determine the independent associations between PAM and the different cytokines, chemokines and peripheral blood cell populations identified by univariate analyses, we next included all the variables in multiple logistic regression analyses. The models tested also included anaemia, bednet possession, gravidity and maternal age at inclusion and at delivery. The model including cytokines and chemokines at inclusion showed an increased risk of PAM to be associated with elevated levels of IL-6, IL-10 and IP-10. The same model with variables at delivery showed that the risk of PAM increased as a function of higher IL-10 and IP-10 levels, with primigravidity and with younger age (data not shown). In the same model, in which cytokines were replaced with PBMC, anaemia, primigravidity, maternal age below the median and a higher frequency of $CD86^{hi}$ B cells were associated with PAM at inclusion. For PAM at delivery, the model showed again that the risk was increased with a higher frequency of $CD86^{hi}$ B cells, but also with fewer pDC and NK cells and with a lower Treg/Teff ratio (data not shown).

The final model that combined cytokines, chemokines and PBMC showed that, at inclusion, the risk of infection increased as a function of with higher levels of IL-6, IL-10, IP-10 (**Table 4**), and that, at delivery, it was associated with higher levels of IL-10 and IP-10 and with a lower Treg/Teff frequency (**Table 5**).

Factors predicting placental malaria, anaemia and low birth weight at delivery

Various cytokines and chemokines have been proposed to have predictive value for the identification of infection with *P. falciparum* during pregnancy. We were therefore interested to know the extent to which the different variables we measured at inclusion, including the PBMC profiles, might be predictive of maternal anemia or other PAM outcomes identified at delivery. For this purpose we included a range of factors measured at inclusion in multiple logistic regression analyses. Table 6 shows that reduced frequencies of Treg as well as increased concentrations of suPAR at inclusion were both predictive of placental infection at delivery, whilst young age was of borderline significance in this regard. Tables 7 and 8 illustrate factors measured at inclusion that were found to be predictive of, respectively, maternal anemia and low birth weight determined at delivery. Higher IL-10 but lower IL-1β, as well as increased frequencies of monocytes and of activated Treg ($FoxP3^{hi}$) were all associated with an increased risk of maternal anemia at delivery. An increased risk of low birth weight was associated with higher RANTES, lower of IL-6 and fewer mDC expressing CD86 at inclusion (Table 7&8).

Discussion

We have previously shown that most of inflammatory factors studied here are stable during pregnancy regardless malaria infection [21]. In this cohort, it was shown that the majority of infection detected at inclusion were chronic whilst acute at delivery [15]. Both at inclusion and at delivery, malaria infection during pregnancy can induce increased levels of cytokines and chemokines such as TNF-α, IFN-γ, IL-10, MCP-1 and IP-10 in peripheral blood or in placenta blood [9, 11, 13, 14, 18, 21, 23]. This phenomenon induces immune cells infiltration

in the placenta and increasing malaria outcomes [6-8, 24, 25]. Therefore, studies that developed inflammatory factors associated cells profile aspects in pregnant women are lack. The study presented here is the first which combining inflammatory factors and cells profiles in longitudinal cohort.

We confirmed in this study that anaemia and bed-net possession were associated to *P. falciparum* infection at inclusion as described previously [15]. In this context, more efforts will be necessary from national or international organization working on malaria eradication, to protect pregnant women early in this area of study.

In the context of sub-microscopic *P. falciparum* infection, we shown increased level of IL-10 in sub-microscopic infected compared to uninfected group at inclusion. Some studies have demonstrated that higher level of IL-10 during normal pregnancy is associated to infant's outcomes [26] and often associated to malaria outcomes in malaria endemic area [22, 27]. Sub-microscopic malaria infection is more important in region where malaria prevalence is lower [28]. Our observation is consistent with those reported from Ghana showing higher level of IL-10 in pregnant women with asymptomatic malaria [29]. Of note, we found no symptoms-associated malaria in the sub-microscopic infected group in this study.

Infection during pregnancy alters peripheral or placental blood cells [15, 30-33] and therefore enhanced cytokines and chemokines level in plasma [18, 21]. We found elevated level of IL-6, IL-10, MIG and IP-10 in infected pregnant women at inclusion and at delivery. IL-10 has been demonstrated as an important cytokines in the maintenance of pregnancy and inhibitor of antigen presenting cells by inhibiting HLA-DR expression and effectors function of Tcells [34, 35]. In this context, it is notable that immature mDC was found in our study. Higher level of IL-10 during malaria induces regulatory responses controlled by Treg and consequently increasing susceptibility of infection. Indeed lower frequency of Treg was observed in our study. Peripheral blood cells like B cells could induce IL-10 production by naïve T cells in response to antigens presentation. Moreover our data suggest that IL-10 observed in infected pregnant women can be produce by others cells than Treg. We showed higher activated B cells in infected pregnant women. Elevated level of IL-6 have previously been shown associated to pre-eclampia in pregnant women [36] and correlated to severity of malaria in adults [37]. MIG and IP-10 are α-chemokines induced by IFN-g and attracting activated cells to the placenta. Dendritic cells (mDC and pDC) have been found in lower frequency expressing lower HLA-DR in this study. MIG and IP-10 have been reported associated to low birth weight [23] and MIG as sensitive measure of IFN-γ production [38]. A lower level of IFN-γ has been found in this study necessary for fetal allograft protection. However more data will need on those chemokines in the context of PAM in association with cells profile.

We also found increased levels of MCP-1 and decreased of Angiopoietin-1 in infected pregnant women compared to uninfected group. MCP-1 is monocyte chemotractant factors. We previously showed higher frequency of monocytes in placenta and lower in peripheral blood [15, 31]. This result could be associated to acute status of infection found at delivery facilitating adhesion of monocytes to placental endothelial cells [9, 14].

P. falciparum infection has been reported to associate with a decrease of Ang-1 in maternal plasma [39] as we observed here. Angiogenesis factors decreased are associated to pregnancy

outcomes [39]. A decreased Ang-1 in our study associated to cells migration in the placenta trough chémokines responses could be risk for pregnancy outcomes in our study.

The specific focused of this study was to identify of a combination of parameters included peripheral cells associated to cytokines and chemokines levels in infected pregnant women. We found that parameters associated to *P. falciparum* infection at inclusion were different to those associated to PAM at delivery. Of note IL-10 and IP-10 were found associated to malaria infection during pregnancy suggesting their levels changes at time of infection during pregnancy [21].

Prospective analysis showed younger pregnant women, higher level of uPAR and lower Treg frequency at inclusion can predict placental malaria at delivery. Such studies have shown that placental malaria is age-dependant.

Overall, our study suggests that PAM can induce changes in cytokines, chémokines and cells profiles during pregnancy.

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Table 1. Characteristic of study subjects

	Inclusion				Delivery			
Variables (median, ir)	Uninfected (n=52)	Sub-infected (n=18)	Infected (n=61)	P*	Uninfected (n=54)	Sub-infected (n=20)	Infected (n=37)	P*
Age (year)	25.5 (8.0)[§]	23.5 (7.0)	22.0 (8.0)[§]	**0.047**	27 (9)	25 (5.5)	25 (10)	0.41
% Primigravidea	30.7	27.8	26.2	0.86	12.9	10.0	24.32	0.24
Gestational age (weeks)	18.5 (4.6)	16.9 (4.8)	17.4 (5.1)	0.12	39.6 (1.3)	40.1 (1.4)	39.4 (2.3)	0.36
% Possessing a bednet	36.5[¶]	33.3	19.6[¶]	0.12	38.9	30.0	29.7	0.60
% Hb <11g/dl	50.0[++]	66.6	81.6[++]	**0.002**	31.5	35.0	45.9	0.36
Parasites density by qPCR; mean [33]/ul	0	52.0 (210.7)	714.6 (2981.4)	-	0	5.3 (105.8)	4119.8 (24088.9)	-

**Kwallis and chi2 tests. [§]Mann Whitney test shows p=0.015; [¶] chi2 test shows p =0.045; [++] chi2 test shows p<0.001*

Table 2: Cytokines and chemokines levels at inclusion and at delivery

	Inclusion			Delivery		
Variables (med, ir)	Uninfected (n=52)	Infected (n=79)	p*	Uninfected (n=54)	Infected (n=57)	p*
Il-1β	1.0 (0.0)	1.0 (0.0)	0.91	1.0 (0.0)	1.0 (0.5)	0.32
IL-6	1.0 (0.0)	1.0 (1.5)	**0.002**	12.1 (15.0)	13.8 (31.5)	0.15
IL-8	9.6 (12.2)	11.8 (17.0)	0.24	114.0 (423.2)	99.73 (40.7)	0.81
IL-10	1.0 (0.0)	12.3 (19.9)	**<0.0001**	1.8 (2.6)	6.0 (26.3)	**<0.0001**
IL-12p70	1.0 (0.0)	1.0 (0.0)	0.59	1.0 (0.0)	1.0 (0.0)	0.11
TNF-α	1.0 (0.0)	1.0 (0.0)	0.71	1.0 (0.0)	1.0 (0.0)	0.27
RANTES	58383.3 (61130.3)	63393.4 (67572.9)	0.84	64620.4 (63759.7)	51956.9 (55946.6)	0.19
MIG	2272.3 (1701.4)	3494.0 (2617.9)	**0.0001**	1242.2 (746.0)	2648.7 (4812.7)	**0.0001**
MCP-1	1 (2.3)	1 (3.8)	0.38	10.4 (10.0)	13.1 (24.3)	**0.029**
IP-10	1806.1 (968.7)	2634.1 (1802.6)	**<0.0001**	1789.8 (1165.0)	2870.5 (3422.6)	**0.0010**
Ang-1	18844.0 (4458.6)	19190.3 (6182.6)	0.77	20004.8 (21454.1)	14852.7 (25660.9)	**0.050**
uPAR	2046.5 (753.8)	2149.6 (936.6)	0.36	2014.3 (1214.6)	2562.0 (1829.4)	0.17
IFN-α	3.5 (0.0)	3.5 (0.0)	0.50	3.5 (5.5)	3.5 (5.8)	0.69
IFN-γ	1.0 (0.0)	1.0 (0.0)	0.51	1.0 (6.5)	1.0 (0.0)	0.18
VEGF/Flt1	747.7 (1243.6)	746.9 (1347.0)	0.80	4547.1 (4759.1)	4221.6 (5851.2)	0.91

Data were presented in median and interquartile range. P-values were calculated by non-parametric Mann Whitney test.

435 **Table 3**: Cells profiles at inclusion and at delivery
436

	Inclusion			Delivery		
Variables (med, ir)	Uninfected (n=52)	Infected (n=79)	p*	Uninfected (n=54)	Infected (n=57)	p*
Monocytes	7.6 (7.7)	8.9 (5.5)	0.47	9.4 (7.4)	9.8 (7.4)	0.70
B cells	12.5 (6.5)	13.2 (6.3)	0.13	11.8 (4.9)	12.0 (7.0)	0.53
myelDC	0.3 (0.2)	0.3 (0.2)	0.88	0.28 (0.1)	0.2 (0.2)	0.26
plasmDC	0.41 (0.3)	0.4 (0.3)	0.49	0.3 (0.3)	0.2 (0.3)	**0.040**
MonoCD86	7.0 (3.8)	6.1 (4.1)	0.58	5.05 (3.2)	5.4 (4.4)	0.89
BcellCD86	0.9 (0.91)	1.1 (1.0)	**0.072**	0.93 (0.8)	1.3 (1.5)	**0.025**
myelDCCD86	0.8 (1.3)	0.8 (1.4)	0.69	1.3 (2.2)	1.2 (2.2)	0.58
plasmDCCD86	0.8 (1.3)	1.1 (1.1)	0.27	1.1 (1.2)	1.3 (1.6)	0.45
MonoHLADR	39.2 (20.3)	34.0 (20.7)	0.20	32. 8 (24.1)	35.2 (32.5)	0.42
BcellHLADR	56.2 (20.3)	56.2 (26.1)	0.82	62.6 (30.7)	54.2 (39.8)	0.42
myelDCHLADR	148.6 (49.1)	125.2 (67.4)	**0.0044**	139.2 (74.3)	121.8 (94.5)	0.40
plasmDCHLADR	54.2 (31.7)	52.3 (31.6)	0.75	40.6 (31.5)	48.6 (30.9)	0.23
Treg	3.8 (2.05)	3.6 (2.1)	0.30	3.6 (2.4)	3.6 (2.1)	0.92
Teff	4.0 (5.2)	5.1 (6.5)	**0.07**	1.6 (2.0)	2.6 (4.02)	**0.0019**
RatioTregTeff	0.9 (1.8)	0.7 (1.1)	**0.06**	2.3 (2.3)	1.4 (1.4)	**0.0012**
RTreg	2.3 (2.7)	2.1 (3.0)	0.54	1.6 (0.6)	1.7 (0.8)	0.12
RTeff	1.2 (0.2)	1.2 (0.41)	0.57	1.3 (0.23)	1.3 (0.4)	0.78
NK	4.8 (4.3)	3.9 (3.4)	**0.052**	5.3 (5.3)	4.8 (5.4)	0.14
CD3	67.9 (14.6)	68.1 (12.1)	0.38	65.0 (11.7)	62.2 (17.4)	0.55
NKT	1.4 (1.6)	1.6 (1.6)	0.77	2.4 (1.5)	1.88 (2.0)	**0.034**
CD4	63.6 (10.4)	63.6 (8.8)	0.42	56.7 (12.7)	58.9 (12.1)	0.42
CD8	27.4 (9.1)	27.1 (8.3)	0.88	31.8 (13.5)	31.0 (9.2)	0.40
RatioCD4CD8	2.3 (1.2)	2.3 (1.0)	0.69	1.8 (1.2)	1.9 (1.1)	0.38

437 Data were presented in median and interquartile range. P-values were calculated by non-
438 parametric Mann Whitney test.
439

Table 4: multiple logistic regression analysis for independent associations of selected parameters with the presence of malaria infection at inclusion

Factors at INC	Odds ratio	IC 95%	p*
IL-6 (>median)[a]	3.72	1.09 - 12.64	0.035
IL-10 (>median) [a]	17.29	6.23 - 47.96	<0.0001
IP-10 (>median) [a]	3.03	1.01 - 9.12	0.048
Anemia	2.92	0.98 - 8.73	0.054

[a]median value of uninfected group used for dichotomisation. Chi2 Hosmer-Lemeshow test (4ddl)=0.61; p=0.96

Table 5: Multiple logistic regression analysis for independent associations of selected parameters with the presence of malaria infection at delivery

Factors at Delivery	Odds ratio	IC 95%	p*
IL-10 (>median) [a]	7.82	2.38 – 25.67	0.001
IP-10 (>median) [a]	3.37	1.11 – 10.22	0.032
Ratio Treg/Teff (<median) [a]	2.60	0.87 - 7.80	0.086

[a]median value of uninfected group used for dichotomisation. Chi2 Hosmer-Lemeshow test (6ddl)=9.75; p=0.13

Table 6: Factors associated to placental malaria predicting at delivery

Variables	Odds Ratio	IC 95%	p
%Treg (>median)	0.15	0.03—0.90	0.038
uPAR (>median)	10.6	1.19—94.7	0.034
Age (≤median)	5.8	0.99—34.2	0.052

Chi^2 de Hosmer-Lemeshow test (5ddl)=0.41 ; p=0.99

Table 7: Factors associated to maternal anemia predicting at delivery

Variables	Odds Ratio	IC 95%	p
IL-10 (>median)	5.3	1.49—19.3	0.010
IL-1β (>median)	0.05	0.004—0.59	0.017
B cell HLADR (>median)	3.0	0.85—10.6	0.088
RTreg (>median)	4.5	1.19—16.7	0.026
% Monocytes (>median)	7.0	1.86—26.0	0.004

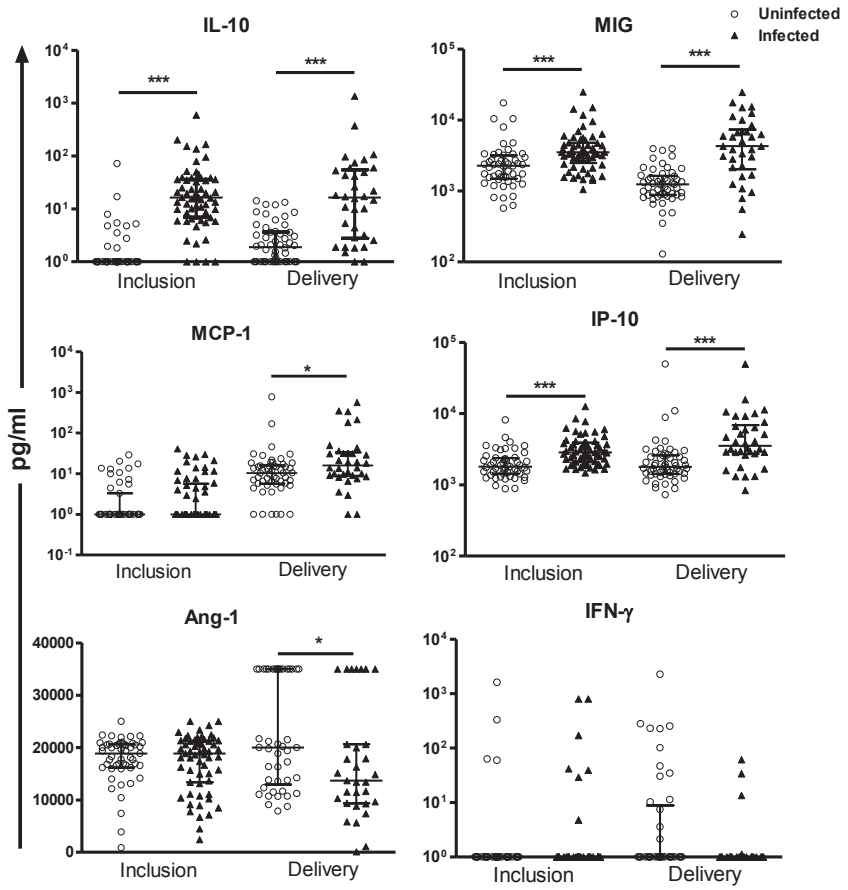
Chi^2 de Hosmer-Lemeshow test (7ddl)=4.35 ; p=0.74

462 **Table 8**: Factors associated to low birth weight predicting at delivery
463

Variables	Odds Ratio	IC 95%	p
Rantes (>median)	5.09	1.40—18.51	0.009
IL-6 (>median)	0.18	0.04—0.94	0.040
mDC expressing CD86 (>median)	0.11	0.02—0.46	0.008

464 Chi^2 de Hosmer-Lemeshow test (4ddl) =1.58; p=0.81
465
466
467 **Figure 1**: Circulating levels of cytokines and chemokines associated with P. falciparum
468 infection at inclusion and at delivery

469



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Supplementary Table 1

	Inclusion			Delivery		
Variables (med, ir)	Uninfected (n=52)	Sub-infected (n=18)	p	Uninfected (n=54)	Sub-infected (n=20)	p
Il-1β	1.0 (0)	1.0 (0)	0.70	1.0 (0)	1.0 (0.2)	0.73
IL-6	1.0 (0)	1.0 (0)	0.93	12.1 (15.0)	10.3 (16.1)	0.88
IL-8	9.6 (12.3)	9.6 (7.1)	0.83	114.0 (423.2)	122.2 (243.6)	0.89
IL-10	1.0 (0)	3.1 (9.6)	**0.0002**	1.9 (2.6)	2.3 (2.8)	0.14
IL-12p70	1.0 (0)	1.0 (0)	0.29	1.0 (0)	1.0 (0)	0.42
TNF-α	1.0 (0)	1.0 (0)	0.72	1.0 (0)	1.0 (0)	0.24
RANTES	58383.3 (61130.3)	68798.7 (67151.2)	0.30	64620.4 (63759.6)	58421.5 (79230.2)	0.40
MIG	2272.3 (1701.4)	2210.3 (3909.2)	0.21	1242.2 (746.0)	1142.9 (1307.5)	0.84
MCP-1	1.0 (2.3)	1.0 (0)	0.73	10.4 (10.0)	8.9 (25.2)	0.53
IP-10	1806.1 (968.7)	1925.6 (1289.7)	0.62	1789.8 (1165.0)	1953.6 (779.4)	0.81
Ang-1	18844.0 (4458.6)	19500.0 (2676.1)	0.45	20004.8 (21454.0)	26499.4 (25674.4)	0.63
uPAR	2046.6 (753.8)	2173.0 (596.0)	0.51	2014.3 (1214.6)	2087.9 (1542.2)	0.97
IFN-α	3.5 (0)	3.5 (0)	0.54	3.5 (5.5)	4.8 (37.8)	0.23
IFN-γ	1.0 (0)	1.0 (0)	0.65	1.0 (6.5)	1.0 (6.6)	0.91
VEGF/Flt1	747.7 (1243.6)	757.6 (625.1)	0.76	4547.2 (4759.1)	5850.9 (5787.7)	0.68

478
479 **Supplementary Table 2**

	Inclusion			Delivery		
Variables (med, ir)	Uninfected (n=52)	Sub-infected (n=18)	p*	Uninfected (n=54)	Sub-infected (n=20)	p*
Monocytes	7.6 (7.7)	8.3 (7.0)	0.74	9.5 (7.4)	9.8 (5.0)	0.7
B cells	12.5 (6.5)	11.3 (5.0)	0.77	11.8 (4.9)	12.5 (7.4)	0.4
myelDC	0.3 (0.2)	0.3 (0.1)	0.93	0.3 (0.1)	0.2 (0.2)	0.7
plasmDC	0.4 (0.3)	0.4 (0.4)	0.87	0.3 (0.3)	0.3 (0.4)	0.9
MonoCD86	7.0 (3.7)	5.6 (3.2)	0.22	5.0 (3.2)	5.2 (1.9)	0.9
BcellCD86	0.9 (0.9)	0.7 (0.6)	0.61	0.9 (0.8)	0.9 (1.2)	0.9
myelDCCD86	0.8 (1.3)	0.5 (1.0)	0.28	1.3 (2.2)	1.2 (1.4)	0.9
plasmDCCD86	0.8 (1.3)	0.8 (0.6)	0.32	1.1 (1.2)	1.0 (0.8)	0.9
MonoHLADR	39.2 (20.3)	37.2 (23.4)	0.50	32.7 (24.1)	40.7 (36.2)	0.3
BcellHLADR	56.5 (20.3)	61.5 (34.8)	0.37	62.6 (30.7)	58.3 (44.5)	0.5
myelDCHLADR	148.6 (49.2)	148.5 (53.7)	0.85	139.2 (74.3)	143.3 (142.1)	0.6
plasmDCHLADR	54.2 (31.7)	57.2 (35.3)	0.37	40.6 (31.5)	50.5 (27.1)	0.06
Treg	3.8 (2.0)	4.6 (1.7)	0.49	3.6 (2.4)	3.4 (2.3)	0.7
Teff	4.0 (5.2)	4.7 (6.5)	0.74	1.6 (2.0)	2.1 (1.6)	0.2
RatioTregTeff	0.9 (1.8)	0.9 (1.5)	0.94	2.3 (2.3)	1.5 (1.5)	0.1
RTreg	2.3 (2.7)	1.9 (1.3)	0.26	1.6 (0.6)	1.8 (0.7)	0.2
RTeff	1.2 (0.2)	1.3 (0.3)	0.54	1.3 (0.2)	1.4 (0.5)	0.07
NK	4.8 (4.3)	3.6 (2.8)	0.21	5.3 (5.3)	3.7 (6.9)	0.08
CD3	67.9 (14.6)	66.2 (14.2)	0.30	65.0 (11.7)	62.4 (19.9)	0.4
NKT	1.5 (1.6)	1.7 (0.9)	0.96	2.4 (1.5)	1.8 (2.6)	0.1
CD4	63.6 (10.4)	59.2 (14.7)	0.06	56.7 (12.7)	59.7 (13.5)	0.5
CD8	27.4 (9.1)	25.6 (10.4)	0.80	31.7 (13.4)	32.2 (10.4)	0.4
RatioCD4CD8	2.3 (1.2)	2.3 (1.0)	0.38	1.8 (1.2)	1.7 (1.3)	0.4

ARTICLE 4: Biomarkers of Plasmodium falciparum infection during pregnancy in women living in northeastern Tanzania.

Boström S, **Ibitokou S**, Oesterholt M, Schmiegelow C, Persson J-O, Minja D, Lusingu J, Lemnge M, Fievet N, Deloron P, Luty AJF & Troye-Blomberg M.

PLoS ONE (2012), 7(11): e48763.

Contexte : Dans l'optique de rechercher des bio-marqueurs inflammatoires liées au PAG, cette étude a été réalisée sur un suivi longitudinal des femmes incluses de la cohorte STOPPAM en Tanzanie

Méthode : Nous avons sélectionné rétrospectivement parmi les 1000 femmes enrôlées, 121 femmes enceintes selon leur statut infectieux au cours de la grossesse (42 infectées et 79 non infectées). Les critères de sélection pour le groupe infecté était : femme enceinte ayant été infectée une fois au cours de la grossesse (TDR et goutte épaisse positive), ayant suivi les 3 consultations prénatales et ayant un statut VIH négatif. Chaque femme infectée sélectionnée était appariée selon l'âge et l'âge gestationnel, avec deux femmes non-infectées pendant le cours de leur grossesse.

Les marqueurs suivants de l'inflammation, de la croissance fœtale et de la vascularisation ont été mesurés par les techniques CBA et ELISA : IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ, RANTES (regulated on activation normal T-cell expressed and secreted), MIG (monokineinduced by IFN-γ), MCP-1 (monocytes chemotactic protein-1), IP-10 (IFN-γ-inducible protein-10), VEGF/Flt1 (vascular endothelial growth factor), uPAR (urokinase receptor), Ang-1 et Ang-2 (angiopoietin-1&2).

Résultats :

Profils des marqueurs inflammatoires au cours de la grossesse indépendamment de l'infection palustre. Nous nous sommes intéressés dans un premier temps à la cinétique des facteurs mesurée au cours de la grossesse. Nous avons observé que la plupart des facteurs plasmatiques mesurés restent stables au cours de la grossesse, excepté quelques-uns dont les concentrations élevées sont associées à l'accouchement.

Impact de l'infection palustre sur les concentrations de cytokines et chimiokines au cours de la grossesse. Au cours du PAG, nous avons comparé les concentrations de cytokines et chimiokines des femmes infectées par rapport à celles du groupe non-infecté. Nos résultats montrent que l'infection palustre était associée à la concentration élevée d'IL-6, IL-10, MIG, MCP-1 et IP-10

alors que celle de RANTES est diminuée. Nous avons observé des pics d'IL-10 et d'IP-10 significativement différents à chaque fois que la femme enceinte est infectée au cours de la grossesse.

Prédiction de l'infection palustre. Grace à une régression logistique, nous avons identifié certains bio-marqueurs associés au risque d'infection palustre chez la femme enceinte. Nous avons ensuite déterminé si ces bio-marqueurs sont prédictifs d'infection chez la femme enceinte. En effet, l'association entre les concentrations élevées d'IL-10, d'IP-10 et le RANTES diminué étaient prédictifs de l'infection palustre chez la femme enceinte.

Discussion et conclusion : Des études précédentes ont montré des concentrations élevées d'IL-10 au cours du PAG [146]. Une faible concentration de RANTES avait été associée à des accès pernicieux chez les jeunes enfants [225, 226]. Le rôle de l'IP-10 au cours du PAG n'est pas totalement décrit. Nos résultats confirment que l'IL-10 et l'IP-10 pourraient être des bio-marqueurs associés au PAG.

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Biomarkers of *Plasmodium falciparum* Infection during Pregnancy in Women Living in Northeastern Tanzania

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Abstract

In pregnant women, *Plasmodium falciparum* infections are an important cause of maternal morbidity as well as fetal and neonatal mortality. Erythrocytes infected by these malaria-causing parasites accumulate through adhesive interactions in placental intervillous spaces, thus evading detection in peripheral blood smears. Sequestered infected erythrocytes induce inflammation, offering the possibility of detecting inflammatory mediators in peripheral blood that could act as biomarkers of placental infection. In a longitudinal, prospective study in Tanzania, we quantified a range of different cytokines, chemokines and angiogenic factors in peripheral plasma samples, taken on multiple sequential occasions during pregnancy up to and including delivery, from *P. falciparum*-infected women and matched uninfected controls. The results show that during healthy, uninfected pregnancies the levels of most of the panel of molecules we measured were largely unchanged except at delivery. In women with *P. falciparum*, however, both comparative and longitudinal assessments consistently showed that the levels of IL-10 and IP-10 increased significantly whilst that of RANTES decreased significantly, regardless of gestational age at the time the infection was detected. ROC curve analysis indicated that a combination of increased IL-10 and IP-10 levels and decreased RANTES levels might be predictive of *P. falciparum* infections. In conclusion, our data suggest that host biomarkers in peripheral blood may represent useful diagnostic markers of *P. falciparum* infection during pregnancy, but placental histology results would need to be included to verify these findings.

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Introduction

Plasmodium falciparum infections during pregnancy cause substantial maternal and neonatal morbidity and mortality [1]. Malaria during pregnancy, often referred to as placental malaria (PM), results from infected erythrocytes (iE) binding to chondroitin-sulphate A (CSA) in the placenta [2]. A major consequence of PM is low birth weight (LBW) [3]. The reason for this is not completely understood but may be due to impaired uteroplacental blood flow [4], metabolic/growth hormone disturbances [5], alterations of the syncytiotrophoblast layer [6], or impaired trophoblast invasion, leading to intrauterine growth retardation. This makes prompt and accurate diagnosis of PM extremely important, but the combination of the asymptomatic character of infections and the frequent paucity of iE in peripheral blood smears makes diagnosis difficult. In a recent study from Malawi [7], the authors investigated whether a different suite of biomarkers could predict placental infection at delivery in the absence of circulating parasites. The results from that study suggested that host biomarkers in peripheral blood may indeed improve the detection of PM when parasites are undetected in circulation.

PM is usually more frequent and more severe in primigravidae as they lack antibodies that inhibit iE binding to CSA. Sequestration of iE in intervillous spaces leads to monocytic inflammatory infiltration in the placenta [8,9]. This inflammation may affect cellular functions by altering the cytokine and chemokine balance both in the periphery and in the placental blood [10–15]. Fetal and maternal cells secrete inflammatory and immunoregulatory molecules in response to sequestered iE [11]. In this context it is notable that PM and pre-eclampsia share many features including an altered cytokine balance and some studies have demonstrated an increased risk of preeclampsia among pregnant women with malaria [16].

Pregnancy represents a state of immunological tolerance in which maternal pro-inflammatory T helper (Th) lymphocyte type 1 cytokines are down-regulated to protect the fetus from allograft rejection [17]. Th1-type responses in the placenta are detrimental for the fetus: high levels of pro-inflammatory cytokines have been shown to be incompatible with successful pregnancy in mice [18]. In humans, PM results in elevated levels of both tumor necrosis factor (TNF)-α and interferon (IFN)-γ in placental plasma, affecting the delicate cytokine balance [12,13]. These cytokines may help eliminate parasites by enhancing the phagocytic activity of monocytes/macrophages, but uncontrolled inflammatory responses in the placenta could be pathological, interfering with normal maternal-fetal exchange. Chemokines mediate the initial inflammatory responses to pathogens via chemotactic interactions with their corresponding receptors expressed on multiple leucocyte cell-types. Binding of iE in the placenta leads to chemokine secretion that stimulates leucocyte infiltration and initiates an inflammatory cascade. Several chemokines are increased during PM [11], some associated closely with monocytic infiltrates [10].

A recent study from Cameroon [19] reported an association between plasma soluble TNF receptor-2 levels and LBW in women infected by *P. falciparum*, suggesting that biomarkers in peripheral blood might discriminate women with poor pregnancy outcomes as a function of malarial infection status. Since PM induces a local host response in the placenta, and soluble components from the placental compartment may circulate in the peripheral blood, investigating host proteins as possible candidate biomarkers might be a good way to detect PM. In the study described here peripheral venous plasma concentrations of several pro- and anti-inflammatory molecules and angiogenic factors were measured on multiple occasions during pregnancy and at delivery in a cohort of Tanzanian women, and their association with infection by *P. falciparum* was evaluated.

Methods

Ethics Statement

Written informed consent was obtained from all mothers before inclusion, and ethical clearance was obtained from the Tanzanian Medical Research Coordinating Committee (NIMR7HQ/R.8a/Vol.IX/688).

Study Area

This study was carried out between September 2008 and October 2010 in the Korogwe district, located about 100 kilometers inland from the coastal city of Tanga, northeastern Tanzania. Historically, malaria transmission in the area was reported to be intense and perennial but with seasonal peaks during and following the rainy seasons from March to July and from October to December [20]. However, malaria transmission has markedly declined in recent years [21]. *P. falciparum* is the predominant malaria species in the area [21].

Study Design

STOPPAM (''Strategies To Prevent Pregnancy Associated Malaria''), a longitudinal cohort study of pregnant women, was conducted in parallel in two separate sites in Tanzania and Benin. In both study sites, 1000 pregnant women with a gestational age $\leq$24 weeks based on ultrasound evaluation were included and followed during pregnancy with a series of scheduled antenatal visits (ANV) until delivery. Delivery samples were collected within 24–48 hours of delivery. Details of the study design and procedures used have been published elsewhere [22]. Here we present data from a part of the study focused on immunological aspects conducted in the Tanzanian study site.

Parasitological Diagnoses and Treatment

For diagnosis of plasmodial infection at each ANV and at delivery, Parascreen^{TM} (Zephyr Biomedical Systems) rapid diagnostic tests (RDT) were used except during May-July 2009 and July-September 2009 when ParacheckPf® (Orchid Biomedical Systems) and ParaHIT®f (Span diagnostics Ltd) were used, respectively. Thick and thin blood smears were also systematically made at each visit, as well as placental impression smears at delivery. Smears were routinely stained with Giemsa and read by two expert microscopists. PCR-based detection was not used. All women presenting with infection diagnosed by RDT at any ANV received anti-malarial treatment according to the national guidelines.

Study Population

For the sub-study described here a group of 121 pregnant women (42 infected and 79 uninfected) was retrospectively identified for the assessment of potential bio-markers in peripheral venous plasma. The infected women were selected based on the following criteria: (i) *P. falciparum* infection - defined by the combination of a positive RDT and the presence of parasites in blood/placental impression smears - once during pregnancy, (ii) attendance at all three scheduled ANV at gestational ages 26 (ANV2), 30 (ANV3), and 36 (ANV4) and at delivery, with corresponding plasma samples available and (iii) being HIV seronegative and not pre-eclamptic. Each infected woman was matched to two separate uninfected controls of similar age ($\pm$4 years), gestational age ($\pm$2 weeks) at the time the infection was detected, and gravidity. Of note, of the 1000 pregnant women enrolled 78 were identified at some point during pregnancy to be infected with *Plasmodium*. Of these 78 women, 42 met the above mentioned criteria to be included in this sub-group for analysis of biomarkers. The characteristics of these pregnant women are summarized in Table 1.

Sample Collection

Venous blood samples from the women were collected at all visits in vacutainers (Greiner bio-one, Denmark) with citrate phosphate dextrose adenine anticoagulant. After centrifugation, undiluted plasma was collected, aliquoted and stored at -80°C until use in assays.

Cytometric Bead Arrays

Levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF, regulated on activation normal T cell expressed and secreted (RANTES), monokine-induced by IFN-γ (MIG), monocytes chemotactic protein (MCP)-1 and IFN-gamma-inducible protein (IP)-10 were measured in plasma using cytometric bead arrays (CBA, BD Biosciences, San Diego, CA, USA) according to the manufacturer's recommendations. The samples and standards were acquired on a flow cytometer (FACSCalibur, Becton Dickinson, France) and analyzed using FCAP Array software v1.0.1 (BD/Softflow, Hungary). Calibration was performed on the flow cytometer before acquisition using BD FACSComp^{TM} and BD CaliBRITE^{TM} beads. The lower detection limits were 3.6, 7.2, 2.5, 3.3, 3.7, 1.9, 1.0, 2.5, 2.7 and 2.8 pg/ml for IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70, RANTES, MIG, MCP-1 and IP-10, respectively. All samples from a given woman were assayed simultaneously and the positive women were always assayed together with the corresponding control women's plasma samples.

Table 1. Description of the pregnant women included in the present study.

Characteristics	Uninfected	Infected	*p*
Number of subjects (n)	79	42	
Age of mother, (mean ± SD), years	24±5.3	24±5.3	
Mean parasitemia, (min-max ± SD), (parasites/μl)	-	27969.2 (39.5–390749±17132.4)	
Primigravidae	22	12	
Secundigravidae	33	17	
Multigravidae ≥3	25	13	
Neonatal birth weight (median ± IQR), g	3200±600	3000±780	0.067[b]

[b]Mann-Whitney non-parametric U test.
doi:10.1371/journal.pone.0048763.t001

ELISA

Commercially available ELISA-based kits for IFN-α, IFN-γ (Mabtech, Stockholm, Sweden), vascular endothelial growth factor receptor 1 (VEGF R1/Flt1), urokinase receptor (uPAR), Angiopoietin (Ang)-1 and Ang-2 (R&D system, Minneapolis, MN) were used according to the manufacturer's recommendations. The enzyme-substrate reaction was developed using p-nitrophenyl phosphatase (Sigma, St Louis, MO, USA) for IFN-α and IFN-γ and tetramethylbenzidine substrate (R&D systems) for the others, measuring optical densities in a multiscan ELISA reader at 405 and 450 nm, respectively. The concentrations were calculated from standard curves established with corresponding purified recombinant human proteins. The lower detection limits were 7, 2, 78, 16, 27, 78 pg/ml for IFN-α, IFN-γ, VEGF R1/Flt1, uPAR, Ang-1 and Ang-2, respectively.

Statistical Analysis

Statistical differences in plasma protein concentrations between *P. falciparum* infected women and controls were evaluated by the Mann-Whitney U non-parametric test. Statistical significance was declared when $p<0.05$. To detect significant changes in plasma protein concentrations during healthy pregnancies, Friedman's test was performed with observations from all five time points for the non-infected women. Multiple logistic regression was used to assess the association between different molecules and *P. falciparum* infection. A stepwise procedure was performed to select a model including the factors with the strongest association with infection. The predictive power of such a model was summarized using receiver operating characteristics (ROC) curves, and area under ROC curve (AUC). The observations used for these analyses were from the time point for infection together with the corresponding matched observations. The statistical software packages used were StatView 5.0.1 and Stata 12.

Results

Cytokines, Chemokines and Other Factors During Pregnancy in Plasma Obtained from *P. falciparum* Negative Pregnant Women

Little is known about cytokine and chemokine levels during normal pregnancies and to our knowledge baseline levels at different gestational ages of African populations have not been reported. We therefore first determined the levels of a panel of markers (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF, RANTES, MIG, MCP-1, IP-10, IFN-α, IFN-γ, Ang-1, Ang-2, uPAR and VEGF R1/Flt1) in the plasmas of 79 women who remained infection-free from inclusion through to delivery (Fig. 1). The levels of the majority of the cytokines (Fig. 1A), chemokines (Fig. 1B) and angiogenic factors other factors (Fig. 1C) were not statistically significantly different over time with the exception, at delivery, of IL-6, IL-8, IP-10, uPAR and VEGF R1/Flt1 that increased notably ($p<0.0001$ for all). TNF and IL-1β were in most cases undetectable (Fig. 1A).

Inflammatory Factors and *P. falciparum* Infection

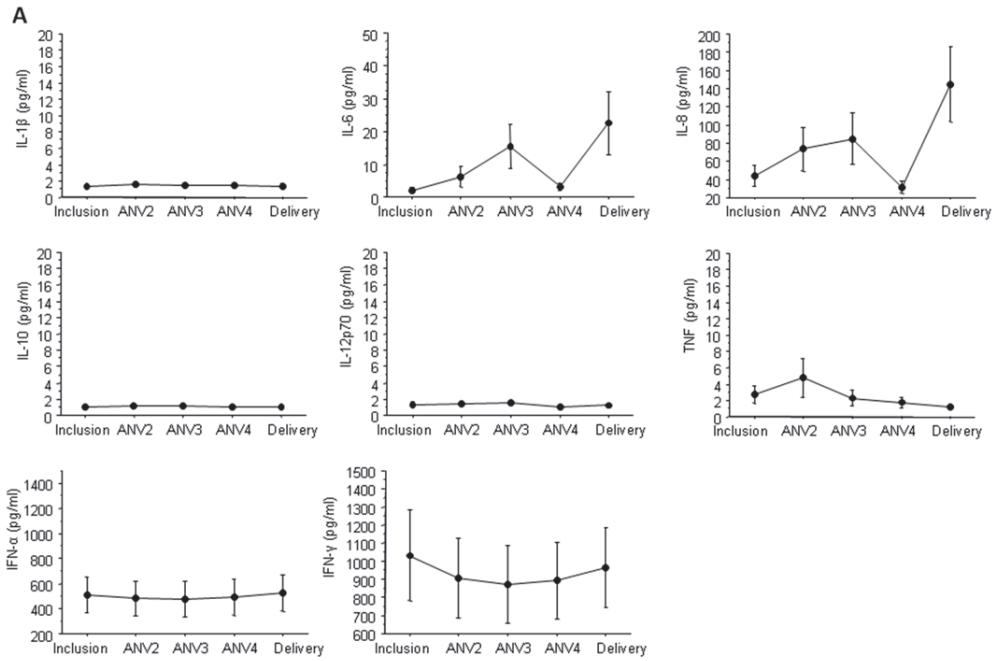
To evaluate the effect of *P. falciparum* infection we compared the concentrations of the different molecules in the plasmas of infected and uninfected pregnant women (Fig. 2). The level of RANTES was significantly lower whilst the levels of IL-6, IL-10, MIG, MCP-1 and IP-10 were all significantly higher in the infected women. Levels of IL-8 and uPAR were also altered as a result of infection, but in neither case did the difference reach statistical significance. The levels of Ang-1, Ang-2, IFN-α, IFN-γ and VEGF R1/Flt1 were unaffected by infection (Fig. 2). Of note, there were no differences at any time in the levels of either TNF or IL-1β between infected and uninfected individuals (data not shown).

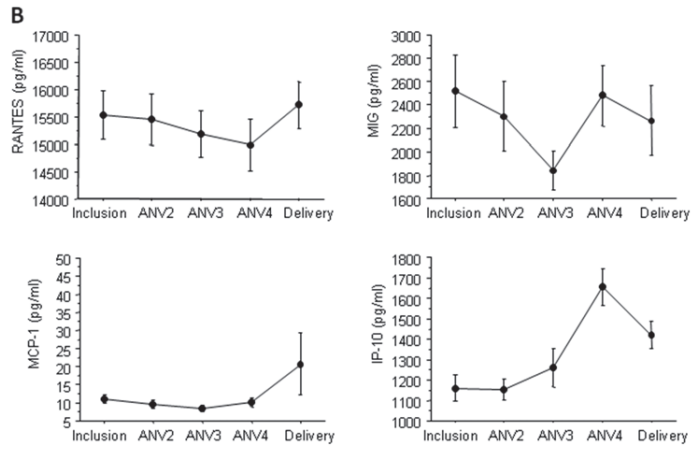
Longitudinal Assessment of IL-10 and IP-10 during Pregnancy

We next determined whether the levels of the different molecules changed as a function of women's gestational age at the time of infection with *P. falciparum*. For this purpose, the infected and matched uninfected women were grouped according to the gestational age at the time infection was identified (Fig. 3). The results showed that the levels of both IL-10 and IP-10 increased significantly when women were infected, irrespective of gestational age. Following anti-malarial treatment the levels decreased to background levels as reflected consistently by the assessments of samples taken at the subsequent ANV. Of all the molecules evaluated in this way, only IL-10 and IP-10 showed this consistent infection/treatment-related change in profile (Fig. 3 and data not shown).

Levels of Markers Differ Based on Gravidity and Infection Status

Primigravidae are at greatest risk of infection with *P. falciparum* and are more likely to suffer severe complications and to have poorer pregnancy outcomes compared to multigravidae. We therefore assessed the levels of different factors according to gravidity (Fig. 4). Infected primigravidae and secundigravidae had significantly higher levels of MCP-1 (Fig. 4A) but lower levels of RANTES (Fig. 4B) compared to their uninfected counterparts. These differences were not seen in the multigravidae, although RANTES displayed the same trend towards lower concentrations


A
IL-1β (pg/ml)
IL-6 (pg/ml)
IL-8 (pg/ml)
IL-10 (pg/ml)
IL-12p70 (pg/ml)
TNF (pg/ml)
IFN-α (pg/ml)
IFN-γ (pg/ml)
Inclusion ANV2 ANV3 ANV4 Delivery


B
RANTES (pg/ml)
MIG (pg/ml)
MCP-1 (pg/ml)
IP-10 (pg/ml)
Inclusion ANV2 ANV3 ANV4 Delivery

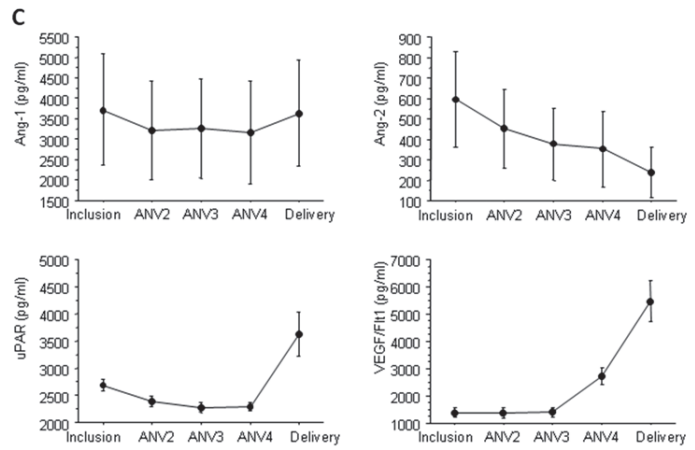

C
Ang-1 (pg/ml)
Ang-2 (pg/ml)
uPAR (pg/ml)
VEGF/Flt1 (pg/ml)
Inclusion ANV2 ANV3 ANV4 Delivery

Figure 1. Baseline plasma levels of several inflammatory factors and others throughout pregnancies not complicated by malaria. Plasma levels of (A) cytokines, (B) chemokines, and (C) angiogenic factors in plasma samples from 79 uninfected pregnant women measured throughout their pregnancy. Samples were analyzed at inclusion, at antenatal visit (ANV) 2, 3 and 4 and at delivery by ELISA or CBA. The dots represents the mean value and the bars the standard deviation.
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in infected women. Levels of IP-10 were significantly higher in all infected women, irrespective of gravidity, compared to uninfected women (Fig. 4C). The levels of IL-10 were significantly increased in infected primigravidae and multigravidae but not in secundigravidae (Fig. 4D).

Predicting *P. falciparum* Infection during Pregnancy Associated Malaria

We included a number of biomarkers in our analysis that have previously been shown to be altered in the peripheral blood of women with placental malaria. To investigate their predictive value for infection, selected factors were analyzed using multiple logistic regression (Table 2). The results show that the likelihood of having been infected with *P. falciparum* increases by a factor of 2.85, 2.82 and 0.32 with the doubling of concentration of IP-10, IL-10 and RANTES, respectively. We further analyzed the diagnostic accuracy of these three putative biomarkers using ROC curve analysis (Fig. 5). Individually all markers displayed moderate predictive ability with areas under the curve (AUC) between 0.61–0.77 (data not shown). However, when combining the factors identified by the multiple logistic regression analyses, we found the combination of elevated IL-10 and IP-10 levels with decreased RANTES levels to be predictive of infection, with the highest AUC of 0.83 (Fig. 5).

Discussion

An essential component of the sub-study described here comprised the detailed longitudinal clinical and parasitological surveillance of women during pregnancy that was an integral aspect of the overall STOPPAM study. This enabled us to make assessments of sequential samples from a sub-group of women in whom a single defined asymptomatic infection with *P. falciparum* was identified, along with equivalent samples from appropriately matched women who remained infection-free up to and including delivery. The study design thus sets it apart from other published studies. Amongst the latter, those that focus on individuals with asymptomatic *P. falciparum* are scarce. Children with asymptomatic *P. falciparum* infections have elevated plasma IFN-γ, TNF and IL-4 levels [23], whilst a cross-sectional study of third trimester pregnancies reported increased G-CSF and IL-10 in women with asymptomatic infections [24]. To our knowledge, few studies have used a longitudinal design to assess aspects of the timing of infection during pregnancy and the relationship with potential biomarkers in plasma. The advantage of using such a prospective study design is that it gives a full picture of changes in the levels of

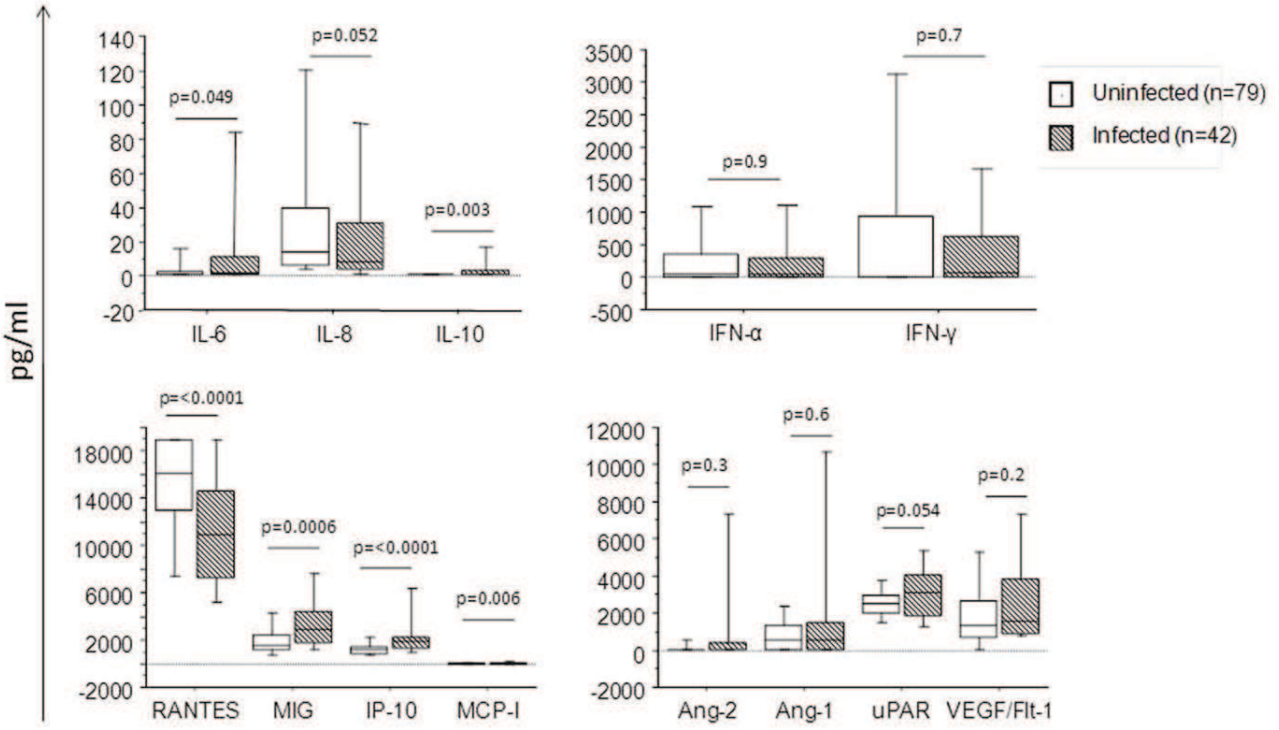

Figure 2. Plasma levels of several inflammatory and other factors in pregnant women with or without *P. falciparum* infection. Plasma levels of selected factors were measured in 42 *P. falciparum* infected women, who were infected once at single time points during pregnancy, and matched to 79 uninfected controls. The factors were measured using ELISA or CBA. The boxes represent the values between 25% and 75% quartile and the line indicates the median. The whiskers indicate the 10% and 90% percentiles. The *p*-values were determined by non-parametric Mann-Whitney U test.
doi:10.1371/journal.pone.0048763.g002

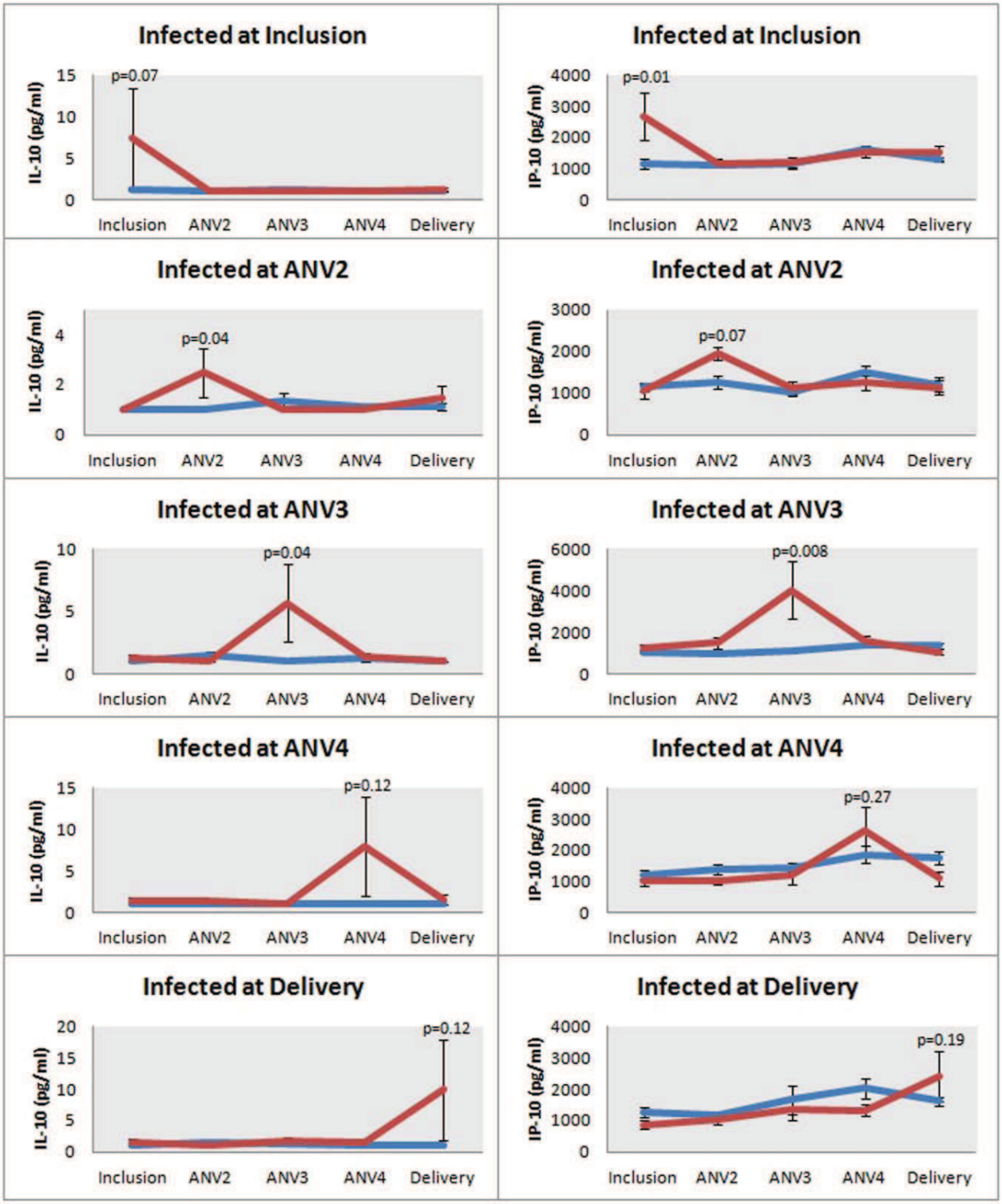

Figure 3. Longitudinal assessment of IL-10 and IP-10 during pregnancy. All women that were infected during their pregnancy were grouped according to the time point when the women were infected. For each group of infected women, samples from the negative control women at the same occasion were grouped together in the same graph. The three antenatal visits (ANV) were at gestational ages 26 (ANV2), 30 (ANV3), and 36 (ANV4). At inclusion: uninfected (n = 24), infected (n = 13); at ANV2: uninfected (n = 13), infected (n = 7); at ANV3: uninfected (n = 15), infected (n = 8); at ANV4: uninfected (n = 13), infected (n = 7); at Delivery: uninfected (n = 14), infected (n = 7). The red lines illustrate mean values for the infected women and the blue line illustrates the mean values for the uninfected control women. The statistical significance of differences in the concentrations between infected and uninfected women at the different time-points is illustrated.
doi:10.1371/journal.pone.0048763.g003

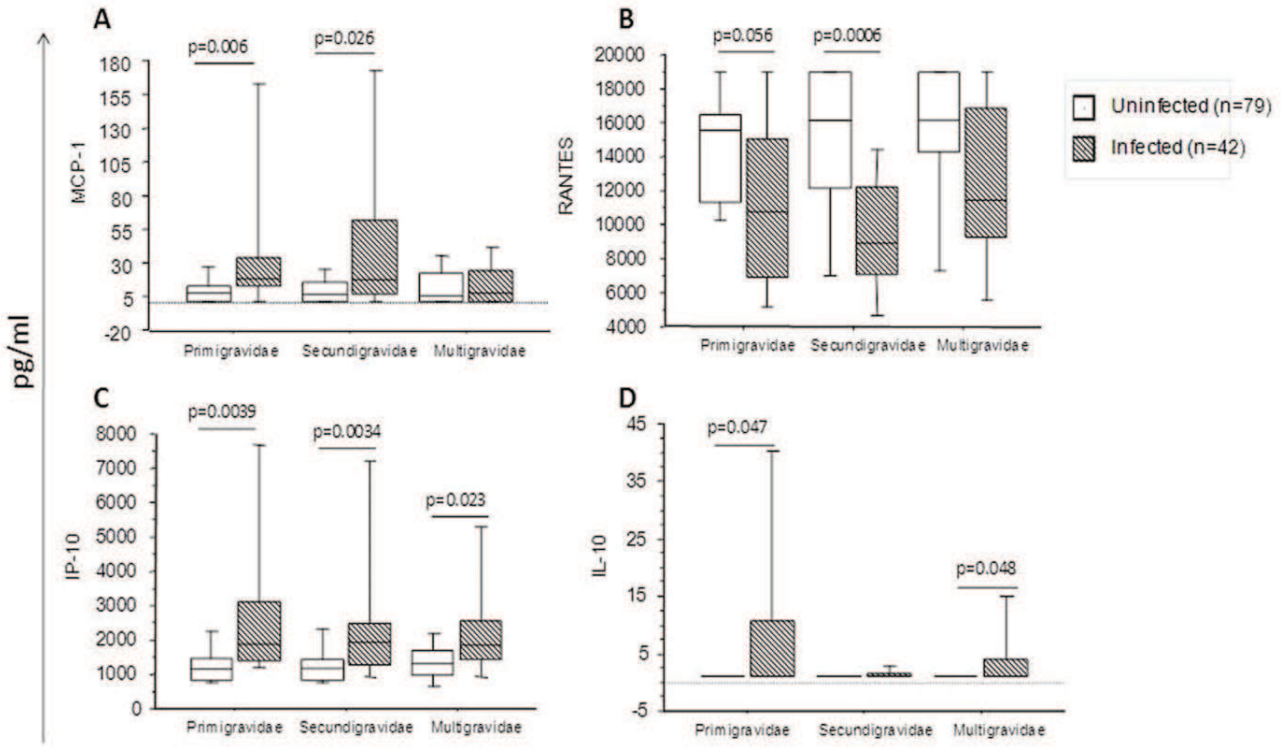

Figure 4. Comparison of inflammatory factors in peripheral plasma from *P. falciparum* infected and uninfected women stratified by gravidity status. Plasma levels of (A) MCP-1 (B) RANTES, (C) IP-10 and (D) IL-10 according to gravidity of the women. Infected primigravidae (n = 12), uninfected primigravidae (n = 22), infected secundigravidae (n = 17), uninfected secundigravidae (n = 33), infected multigravidae (n = 13) and uninfected multigravidae (n = 25). The boxes represent the values between 25% and 75% quartile and the line indicates the median. The whiskers indicate the 10% and 90% percentiles. *P*-values were determined by non-parametric Mann-Whitney U test.
doi:10.1371/journal.pone.0048763.g004

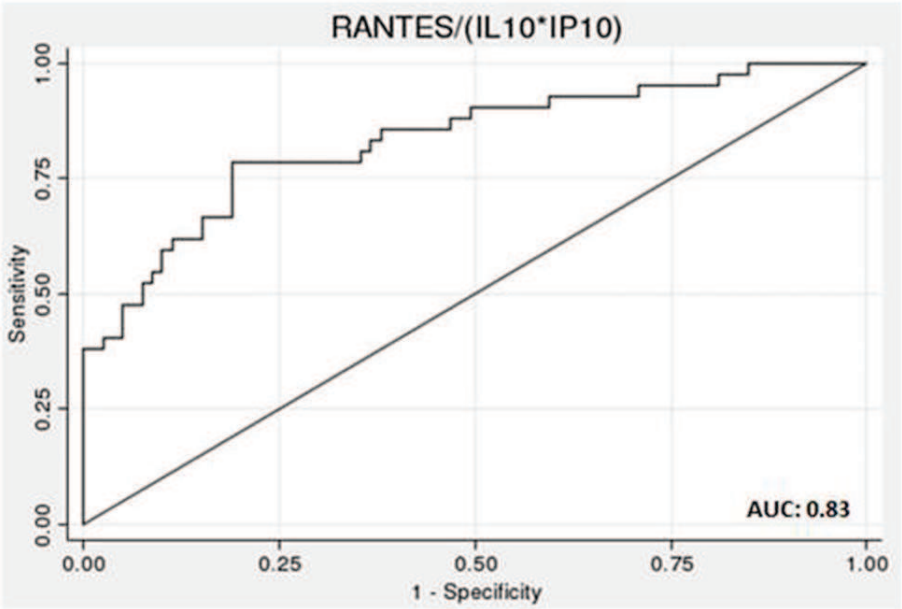

Figure 5. Assessment of biomarkers in predicting *P. falciparum* infection during pregnancy. Receiver operating curve (ROC) for the multiple logistic regression model, with IL-10, IP-10 and RANTES as predictors. The area under the ROC curve (AUC) with 95% confidence interval is 0.83 (0.75, 0.91).
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Table 2. Estimated odds ratio with 95% confidence interval for the multiple logistic regression model.

Factor	Odds ratio	95% CI	*p*
IP-10	2.85	1.39–5.84	0.004
IL-10	2.82	1.21–6.58	0.016
RANTES	0.32	0.15–0.68	0.004

Concentration values of factor 2 were log2-transformed which means that odds ratio estimate applies for a doubling of concentration.
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such biomarkers as a function both of gestational age and of a defined infection event rather than the 'snapshot' image that a cross-sectional design gives. For example, a longitudinal study involving a relatively small number of women in Cameroon identified changes in plasma angiopoietin levels during pregnancy as a function of infection with *P. falciparum* [25,26]. Such infections were shown by Thévenon and colleagues – using samples from the same study in Cameroon – to be associated with altered TNF receptor profiles [19]. Importantly, we have shown that, over the course of a normal pregnancy, the concentrations of the majority of the factors measured remained stable with few fluctuations except at delivery. Notably, however, at times when the women were infected with *P. falciparum* the levels of several molecules changed, in particular those of IL-6, IL-10, MIG, MCP-1 and IP-10 that increased, while the level of RANTES decreased. Integral to our study design was the fact that the comparisons made were with the levels of the same factors in two uninfected women, who were matched for several potentially confounding parameters. Consistently increased concentrations in relation to infection, regardless of gestational age, were only seen for IL-10 and IP-10. The latter two factors were also found to be present at significantly higher concentrations in infected primigravidae, the group known to be most susceptible to malaria during pregnancy. ROC curve analyses revealed the combination of IL-10, IP-10 and RANTES to be strongly predictive of *P. falciparum* infection during pregnancy.

The fact that the levels of most factors studied were stable, from inclusion right through to delivery, indicates a strict degree of regulation during pregnancy. In particular, the levels of IFN-α and IFN-γ, factors known to be associated with spontaneous abortions and preterm delivery [18] were very stable. Notable also was the absence of detectable levels of TNF in the peripheral plasma of both uninfected and infected women. Numerous studies have shown TNF to be elevated in placental plasma during *P. falciparum* infection [12,13,15]. This again suggests tight regulation restricting potentially harmful molecules to isolated areas such as the placenta. Emphasizing the latter, one of those studies did quantify TNF levels in a small number of peripheral plasma samples at delivery and found that the relatively low concentrations detected were weakly associated with *P. falciparum* infection [15]. Of note, other factors measured in our study, especially IL-6, IL-8, IP-10, uPAR and VEGF R1/Flt1, increased markedly at delivery. These findings are consistent with those reported by others [27], showing increased IL-6 levels at the end of normal pregnancies, possibly contributing to the process of initiation of labor. Increased levels of IL-6 at delivery have been reported in pre-eclampsia [28], indicating that IL-6 can be harmful, possibly playing a role in the inflammation and endothelial dysfunction associated with pre-eclampsia.

That infection with *P. falciparum* during pregnancy alters the cytokine balance in both placenta and periphery is widely accepted. We consequently found elevated levels of MIG, MCP-1 and IP-10 in infected women compared to uninfected controls. MIG, MCP-1 and IP-10 are all chemokines that attract different immune cell populations to the sites of infection. MIG and IP-10 are α (CXC) chemokines that are produced by a variety of leucocytes in response to IFN-γ and TNF and are chemoattractants for activated T and NK cells and macrophages. MCP-1 is a β (CC) chemokine produced primarily by monocytes, macrophages and endothelial cells and is a potent monocyte chemoattractant [29]. Monocytes and macrophages predominate in the inflammatory infiltrate of infected placentas [8,9]. Our findings point to MIG, MCP-1 and IP-10 as pivotal in recruiting such cells into the placenta. Little is known concerning IP-10 and MIG in the context of placental malaria, although IP-10 has been shown to be produced by cultured intervillous blood mononuclear cells isolated from the placentas of women infected with *P. falciparum* [30]. High levels of IP-10 are found in pre-eclampsia [31], and have recently been shown to be involved in the pathogenesis of cerebral malaria, both in mice [32] and in humans [33]. In the latter study IP-10 was identified as a biomarker associated with mortality in *P. falciparum*-mediated cerebral malaria. IP-10 has both pro- and anti-inflammatory properties, and has been proposed to be a potential link between inflammation and anti-angiogenesis in preeclampsia [34]. We found that IP-10 levels were increased irrespective of gravidity, emphasizing its association with infection, and lending support for this chemokine as a potential biomarker.

We also found increased levels of uPAR in the infected women compared to uninfected controls, although this did not reach statistical significance. Elevated levels of uPAR have previously been shown to predict LBW in maternal malaria [35], and to be associated with parasitaemia in children with acute *P. falciparum* infections [36]. Various immune cells express uPAR which can be shed from the cell surface [37]. Given the fact that activated monocytes have increased expression and release uPAR, it could be that monocytes within the placenta contribute to the high blood levels of this factor during malaria infection. In addition, high levels of pro-inflammatory cytokines, or presence of adherent and circulating parasitized erythrocytes could also contribute to enhanced uPAR release from vascular endothelial cells, but the exact source for this molecule is still not known.

Of all the molecules quantified, only the concentration of RANTES decreased upon infection. Low circulating levels of RANTES have previously been shown to be associated with severe malaria [38], and especially with mortality in children with cerebral malaria [39]. Thrombocytopenia is frequent in severe malaria cases and is associated with increased mortality in children [40]. Since platelets are a major reservoir of RANTES in the circulation [41], it has been suggested that lower levels of RANTES in patients with severe malaria may be due to parasite-induced thrombocytopenia [42]. Of relevance to our study is the fact that pregnant women with acute uncomplicated malaria become more thrombocytopenic than non-pregnant women [43]. The possible pathological relevance of the decreased amounts of RANTES in women with *P. falciparum* during pregnancy – all of whom, it should be stressed, were asymptomatic at the time of diagnosis – therefore remains to be clarified.

Increased levels of IL-10 at delivery in the infected women have been reported by Kabyemela and colleagues [44]. Here, our longitudinal study has extended that finding, revealing that IL-10, in tandem with IP-10 levels, increase in infected women irrespective of their gestational age. IL-10 is a key cytokine both

in the protection and in the pathogenesis of malaria. High levels of IL-10 may be beneficial to the host by reducing inflammatory responses, but on the other hand may be detrimental by suppressing protective anti-parasitic Th1-type responses. Low levels of IL-10 or a low IL-10/TNF ratio are associated with malarial anemia in African children [45]. Elevated levels of IL-10 in infected pregnant women may, thus, plausibly play a role in the down-regulation of pathological parasite-induced Th1-type responses in order to maintain a healthy pregnancy. The anti-inflammatory properties of IL-10 are mediated through blockade of monocytes/macrophage functions including the production of pro-inflammatory cytokines such as IL-6, TNF and IL-1 [46]. The latter are primary mediators of acute phase responses that regulate the induction of acquired immune responses. The asymptomatic nature of the infections in our pregnant women could, thus, reflect the suppressive effects of increased amounts of IL-10. The increased IL-10 levels seen in asymptomatic pregnant Ghanaian women infected with *P. falciparum* in their third trimester further confirm of our observations [24]. The important role IL-10 plays in suppressing Th1 responses during pregnancy is reflected by the increased levels of IL-10 seen during normal healthy pregnancies compared to non-pregnant controls [47].

The combination of biomarkers may be a better way to provide better diagnostic or prognostic accuracy than single markers. In an attempt to identify the best biomarker(s) of *P. falciparum* infection during pregnancy from amongst the panel of molecules quantified, we first used a logistic regression model that revealed IL-10, IP-10, MIG and RANTES as potentially useful in this regard. We then used ROC curve analysis as the most appropriate means of determining predictive values. The conclusion from those analyses is that the combination of increased IL-10 and IP-10 with decreased RANTES levels was most predictive of infection. While the result of this study is promising, the specificity of this combination requires further detailed investigation in a larger sample that should optimally include women with symptomatic as well as asymptomatic *P. falciparum* infections and also needs to be validated in other populations with differing levels of malaria endemicity. In this context, it should be noted that, in the Benin cohort of the STOPPAM study, PCR-based detection of 'occult' infections with *P. falciparum* at inclusion – undetected by either RDT or microscopy - has revealed significant associations with elevated plasma IL-10 levels (N Tuikue Ndam, unpublished data).

One major limitation of this study is the relatively small sample size, but this lack of power is at least partly offset by our use of samples from two closely matched controls per case. Placental histological evidence of infections that could have shed further light on the issue was not available to us due to technical problems during placental biopsy preparations and storage. We did not assess all potential biomarkers, including some more recently identified, but the plasma samples are still available and, resources permitting, could easily be screened to identify other candidates.

Conclusion

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 an African cohort. Our study shows that IL-10, IP-10 and RANTES are increased upon infection with *P. falciparum* and therefore might be valuable for diagnostic purposes during pregnancy-associated malaria. The biomarkers that we have identified will need to be validated together with other biomarkers that have recently been associated with placental infection alongside malaria rapid diagnostic tests and PCR to compare accuracy and whether they could be combined to improve PM diagnosis. Our results contribute to the overall picture of *P. falciparum*-induced changes in cytokine and chemokine levels during pregnancy but more detailed studies are needed to further clarify the mechanisms underlying the patho-physiology of pregnancy-associated malaria.

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Author Contributions

Conceived and designed the experiments: SB SI MO CS DM JL ML NF PD AJFL MTB. Performed the experiments: SB SI. Analyzed the data: SB SI JP MTB AJFL. Contributed reagents/materials/analysis tools: MTB AJFL JP JL ML NF PD. Wrote the paper: SB SI MTB AJFL JP.

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ARTICLE 5: Plasmodium falciparum exposure in utero, maternal age and parity influence the innate activation of foetal antigen presenting cells.

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Contexte : Les jeunes enfants vivant en zone d'endémie palustre constituent une des populations à risque qu'il faudrait protéger en priorité contre le paludisme. Une meilleure compréhension des mécanismes d'acquisition et de construction naturelle de l'immunité anti-palustre pendant les premières années de la vie, permettrait de proposer une meilleure prise en charge des cas. L'objectif de ce travail est d'étudier les répercussions du paludisme gestationnel sur les réponses immunitaires du nouveau-né. Cette étude constituait, un préliminaire des conséquences du paludisme gestationnel sur les réponses immunitaires du nouveau-né.

Méthode : Cette cohorte a été réalisée en collaboration avec l'Hôpital de la Mère et de l'Enfant Lagune (HOMEL) de Cotonou, Bénin entre Juillet 2006 et Janvier 2007. Le sang de cordon a été prélevé systématiquement après accouchement sur anticoagulant et convoyé vers le laboratoire dans les 4 heures. Nous avons inclus au total 59 femmes enceintes dont 30 infectées et 29 non-infectées.

Nous avons premièrement, caractérisé les cellules présentatrices d'antigènes (CPA) à travers leurs fréquences et leur niveau d'activation dans le sang de cordon. Ensuite, nous avons stimulé spécifiquement des TLR sur les CPA pendant 8 heures pour mesurer leur capacité à produire des cytokines inflammatoires. Le Lipopolysaccharide (LPS), l'acide polyinosinic-polycytidylic (Poly I : C), CPG-ODN et l'hémozoïne synthétique (Hz) ont été utilisé comme stimuli.

Résultats : Nos résultats montrent que la présence de pigments malariques dans les monocytes placentaires est associée à l'activation partielle des cellules dendritiques du sang de cordon. Cette activation s'explique par la forte expression des molécules CMH-II à la surface des DC mais pas de CD86. La présence de pigments malariques dans le placenta influence aussi la production d'IL-10 et de TNF-α dans le sang de cordon après stimulation des CPA par le CPG-ODN (TLR9). Une forte concentration d'IL-10 et de TNF-α est ainsi obtenue chez les nouveau-nés de mères présentant du pigment malarique dans le placenta. Enfin, nous avons montré que l'âge maternel et la parité sont deux paramètres qui influencent les fréquences et l'activation des CPA dans le sang de cordon et devraient être pris en compte dans l'étude des réponses immunitaires du nouveau-né.

Discussion et conclusion : Les résultats de cette étude suggèrent que les réponses immunitaires du nouveau-né peuvent être modulées par l'exposition *in utero* aux antigènes palustres. L'altération des CPA dans le sang de cordon induirait une mauvaise présentation d'antigène aux lymphocytes T naïfs et par conséquent une mauvaise orientation des réponses immunitaires efficientes. Les concentrations élevées d'IL-10 et de TNF-α, en présence du pigment malarique démontrent une activation partielle des cellules présentatrices d'antigènes du sang de cordon, traduite par leur exposition aux antigènes parasitaires.

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Plasmodium falciparum exposure *in utero*, maternal age and parity influence the innate activation of foetal antigen presenting cells

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Abstract

Background: Malaria in pregnancy is associated with immunological abnormalities in the newborns, such as hampered T-helper 1 responses and increased T-regulatory responses, while the effect of maternal *Plasmodium falciparum* infection on foetal innate immunity is still controversial.

Materials and methods: The immunophenotype and cytokine release by dendritic cells (DC) and monocytes were evaluated in cord blood from 59 Beninese women with or without malaria infection by using flow cytometry.

Results: Accumulation of malaria pigment in placenta was associated with a partial maturation of cord blood myeloid and plasmacytoid DC, as reflected by an up-regulated expression of the major histocompatibility complex class II molecules, but not CD86 molecules. Cells of newborns of mothers with malaria pigment in their placenta also exhibited significantly increased cytokine responses upon TLR9 stimulation. In addition, maternal age and parity influenced the absolute numbers and activation status of cord blood antigen-presenting cells. Lastly, maternal age, but not parity, influenced TLR3, 4 and 9 responses in cord blood cells.

Discussion: Our findings support the view that placental parasitization, as indicated by the presence of malaria pigment in placental leukocytes, is significantly associated with partial maturation of different DC subsets and also to slightly increased responses to TLR9 ligand in cord blood. Additionally, other factors, such as maternal age and parity should be taken into consideration when analysing foetal/neonatal innate immune responses.

Conclusion: These data advocate a possible mechanism by which PAM may modulate foetal/neonatal innate immunity.

Background

Pregnancy-associated *Plasmodium falciparum* malaria (PAM) results, sometimes, in massive intervillous inflammation that contributes to placental insufficiency, impaired intra-uterine growth and consequently to low birth weight in the newborns and a higher risk of dying early in life [1-4].

Infants born to women with PAM are more predisposed to *P. falciparum* infection in their first year of life [5-7]. Immunological mechanisms are generally considered to play an important role in causing this susceptibility. *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 cord blood mononuclear cells (CBMC) of neonates born to mother with PAM specifically respond to plasmodial asexual stage antigens, and that cord blood B cells produce anti-malaria specific IgM and IgE antibodies [5,8-10], providing irrefutable evidence of *in utero* sensitization.

In this context, active infection in the placenta by *P. falciparum* was associated with hampered T-helper 1 (Th1) responses, as reflected by reduced IFN-γ production upon T-cell stimulation [9]. In addition, the anti-inflammatory IL-10 cytokine is more frequently produced by CBMC of those born to mothers with PAM compared with non-infected mothers [11]. $CD4^+CD25^{high}$ regulatory T-cells (Treg) are a principal source of IL-10 in such cases [12]. Treg are found at higher frequency in cord blood (CB) of neonates born to mothers with PAM at delivery as compared to unexposed newborns [12].

Because of their key function in the initiation and regulation of adaptive immune responses, it is reasonable to assume that antigen presenting cells (APC), such as monocytes and dendritic cells (DC), contribute to the modulation of foetal immune responses upon exposure to *P. falciparum in utero*. Indeed, DC seem to play an important role in both protective and dysfunctional immune responses against malaria in murine models [13,14]. DC comprise a heterogeneous population of cells; myeloid DC (MDC) that orchestrate T-cell responses through a fine modulation of IL-12 secretion, while plasmacytoid DC (PDC) are an essential component of innate and adaptive immunity through secretion of type I interferons (IFN) in response to pathogens [15]. A minor blood MDC population, blood DC antigen (BDCA)-3^+ cells, has been described sharing the same ontogeny as the more frequent BDCA-1^+ MDC subset [16,17].

The foetal/neonatal immune system exhibits quantitative and functional differences from the adult one and neonatal DC have reduced ability in delivering co-stimulatory signals to T-cells as a consequence of their incomplete maturation [18]. They also exhibit a markedly decreased capacity in secreting IL-12 and IFN-α [19,20]. This probably contributes to the development and relative predominance of Treg in CB [21], although seemingly less marked in Africans vs. Europeans [22].

Whether and how *P. falciparum* infection in the mother may affect foetal innate immunity is poorly understood. One study conducted in The Gambia reported lower lipopolysaccharide (LPS)-induced IFN-γ and IL-12 activity in CBMC of newborns of mothers with PAM as compared to uninfected mothers [9]. A more recent study revealed that CBMC of neonates born of Gabonese mothers with *P. falciparum* infection exhibit significantly increased IFN-γ responses upon stimulation with toll-like receptor (TLR)3 and TLR4 ligands [22].

Contrasting findings have also been reported on the characterization of DC subsets in CBMC of neonates born to *P. falciparum*-infected mothers. One study reported a significantly higher frequency of MDC [23], while another reported profoundly reduced numbers of PDC [24] as compared to unexposed newborns.

The mechanistic hypothesis behind the present study is that malaria infection in the mother may cause a dysfunctional activation of foetal APC by parasite-derived products that cross the placenta. An altered activation of foetal APC could be responsible for the impaired T-cell response that is observed in infants born to mothers with PAM.

Using flow cytometry, subpopulations of DC and monocytes were evaluated in CB of neonates from Beninese women with or without malaria infection. In addition, the impact of *P. falciparum* exposure *in utero*, on the innate activation of foetal APC was examined by stimulating CBMC with specific TLR ligands; LPS was employed to activate TLR4 on monocytes and BDCA-1^+MDC, polyinosine-polycytidilic acid (PolyI:C) to selectively stimulate TLR3 expressed in MDC, and CpG-A ODN to specifically activate TLR9 expressed in PDC [16,17,25].

Methods

Study population

Pregnant women were enrolled after informed consent from July 2006 to January 2007; in the Hospital "Mother and Child Lagune", the main obstetrical referring hospital in Cotonou. This study was approved by the Science and Health Faculty Ethics Committee. To identify women with malaria infection, a rapid immuno-chromatographic test (Cypress®, Langdorp, Belgium) was performed on finger-pricked capillary blood before delivery. Thirty *P. falciparum*-infected women and twenty-nine uninfected women matched for parity and age were enrolled in the study. Twenty-five ml heparinized CB were collected immediately after delivery. According to national policy,

pregnant women receive intermittent preventive treatment with sulphadoxine-pyrimethamine (SP). Despite this usage of SP was declared by only 47% of the women, while the remaining mothers declared having taken chloroquine (CQ) as chemoprophylaxis.

Determination of P. falciparum *status of the mothers at delivery*

Thin and thick smears were prepared from maternal peripheral, placental intervillous and cord blood, stained with Giemsa and examined for the presence and density of parasites. Malaria infection in the mothers at delivery was defined by the presence of parasites in the placental and/or maternal peripheral blood. The presence of malaria pigment (MP) was also evaluated in leukocytes of placental intervillous blood (Table 1).

CBMC cultures

Mononuclear cells were isolated from CB by centrifugation over Ficoll-Hypaque (Pharmacia Uppsala, Sweden). Cells were washed twice and resuspended in RPMI 1640 medium with L-glutamine (Gibco Eragny, France) supplemented with 10% foetal bovine serum (FBS, Gibco) and 50 μg/ml gentamycin to a final concentration of 2×10^6 CBMC/ml. Viability was > 99% in all tested samples as determined by Trypan blue staining.

To assess production of IL-12, CBMC were stimulated for 8 hours with LPS (100 ng/ml; Sigma Aldrich, St. Louis MO), PolyI:C (20 μg/ml; Sigma Aldrich), or synthetic haemozoin (Hz, 5 μg/ml) in the presence of Brefeldin-A (BD Pharmingen, San Diego, CA) during the last five hours of incubation. Hz was prepared from haemin chloride as described [26]. Endotoxin levels in the Hz preparation were found to be below the threshold (<0.125 units/ml) by the Limulus-amoebocyte lysate assay (Biowhittaker, Cambrex). To assess cytokine production by CBMC upon contact with TLR9 ligands, CpG-A ODN 2216 (3 μg/ml; Metabion GmbH, Martinsried, Germany) was employed.

Immunophenotype of APC

CBMC were resuspended in staining buffer (PBS 2% FBS, 5 mM EDTA). Cells were first incubated with FcR blocking reagent (Miltenyi Biotech, Bergisch-Gladbach, Germany) and then with anti-CD14-FITC, anti-CD19-FITC, anti-BDCA-1-PE, anti-BDCA-2-PE, anti-BDCA-3-PE (Miltenyi Biotech), anti-HLA-DR-PerCP and anti-CD86-APC (BD Pharmingen), or alternatively mouse isotype controls (BD Pharmingen). Cell acquisition was performed with a FACSCalibur flow cytometer (BD Pharmingen) and analysis was performed by CellQuest software as described in Figure 1A.

Intracellular cytokine staining for IL-12

After stimulation, cells were incubated with FcR blocking reagent and stained with anti-HLA-DR-PerCP, anti-CD14-FITC, anti-CD19-FITC, anti-BDCA-1-PE, anti-BDCA-3-PE or isotype controls for 10 min at 4°C. Cells were then fixed with FACS lysing solution, washed and incubated in a permeabilization buffer (staining buffer with 0.25% saponin and 5% AB human serum) for 15 min at 4°C. After centrifugation, cells were stained with anti-IL-12-APC (BD Pharmingen) or alternatively APC-conjugated

Table 1: Summary of the study population.

Characteristics	Study group	
	P. falciparum-positive	*P. falciparum*-negative
Number of subjects n = 59	30	29
Age of mother, mean ± SD, years	25.4 ± 6.1	26.3 ± 5.2
Pregnancies, no., mean ± SD	2.2 ± 1.5	2.1 ± 1.1
1-2 pregnancies (n = 41)	21	20
≥ 3 pregnancies (n = 18)	9	9
Ratio of malaria prevention (CQ/SP[a]) (30/27)	19/10	11/17
Declaration of malaria infection during pregnancy (%)	33.3	3.7
Reported use of bednet (%)	83.3	82.8
Neonate birth weight, mean ± SD, g	3052.8 ± 443.3	3059.6 ± 412.5
Neonate gender, female/male (21/34)	10/19	11/15
P. falciparum density at delivery:		
peripheral blood, mean ± SD, iRBC[b]/μl	19,037 ± 55,257	0
intervillous blood, mean ± SD, iRBC/μl	245,764 ± 475,906	0
cord blood, mean ± SD, iRBC/μl	0	0
intervillous blood leukocytes with MP[c], n = 59	19	0

[a] CQ/SP; Chloroquine/sulfadoxine-pyrimethamine
[b] iRBC; infected red blood cells
[c] MP; malaria pigment

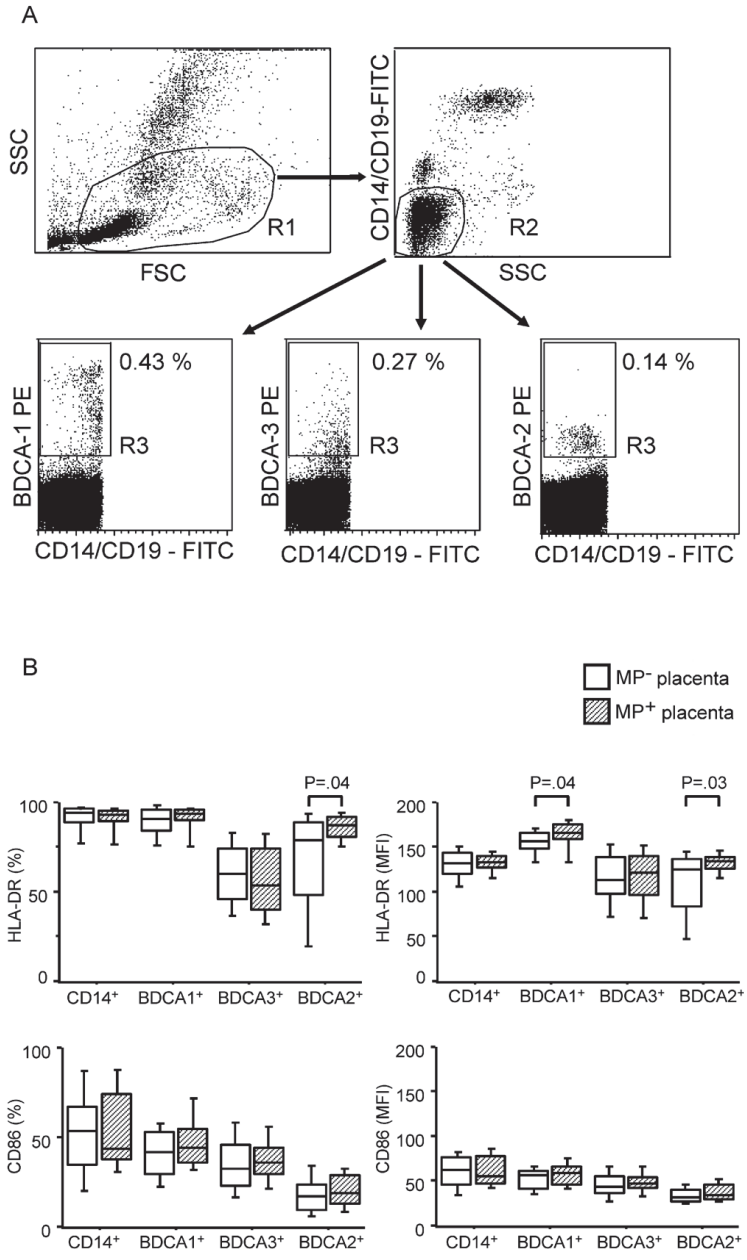

Figure 1

(A) Flow cytometric identification of MDC (BDCA-1$^+$ or BDCA-3$^+$) and PDC (BDCA-2$^+$) in CBMC from one subject. R1 gate was set to include only viable mononuclear cells, as determined by forward scatter (FSC) and side-scatter (SSC) characteristics. Furthermore, cells were gated to be both CD14 and CD19 negative (R2) and either BDCA-1, BDCA-3, or BDCA-2 positive (R3). One million events were analysed for DC, 300,000 events for monocytes and 100,000 events for isotype control enumeration. The percentages represent relative levels of the different DC populations from the selected subject. (B) Partial activation of cord blood BDCA-1$^+$ and BDCA-2$^+$ cells is related to the presence of MP in placenta. The expression levels of HLA-DR and CD86 were measured by flow cytometry on foetal APC subsets from 16 MP-positive (diagonal striped bars) and 39 MP-negative mothers (white bars). Boxplots illustrate the medians and the 25th and 75th percentiles. Y-axes report percentage of positive cells and mean fluorescence intensity (MFI) for the activation markers CD86 and HLA-DR in different APC subsets. P-values were calculated by Mann-Whitney U test. The significance limit was $P < 0.05$.

isotype control for 30 min at 4°C, and then analysed by flow cytometry. BDCA-1$^+$ and BDCA-3$^+$ cells that did not express CD19 and CD14 were gated together as MDC.

Determination of cytokine levels in plasma samples and supernatants

IFN-α levels were measured with an ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). The assay sensitivity was 12.5 pg/ml. A panel of pro-inflammatory and anti-inflammatory cytokines including IL-6, IL-10, IL-12, (MIP)-1α/CCL3; TNF-α and IFN-γ were quantified by the Human Cytokine Cytometric Bead Array Kit (BD Pharmingen) using flow cytometry. The assay sensitivity was 1.6 pg/ml; 0.13 pg/ml; 0.6 pg/ml; 0.2 pg/ml; 1.2 pg/ml; and 1.8 pg/ml for IL-6, IL-10, IL-12, MIP-1-α/CCL3, TNF-α and IFN-γ respectively. Results were formatted using the BD CBA Analysis Software.

Statistical analysis

Background values on cytokines in supernatants obtained from unstimulated cells were subtracted from data acquired from cultures in the presence of stimuli. Normally distributed variables were analysed by unpaired *t* test. Data that were not normally distributed even after log-transformation were analysed by the non-parametric Mann-Whitney test. To test if the age of the mother was related to parity, Spearman rank correlation was employed. Linear regression analysis on log-transformed data was used to identify dependent variables for a multivariate analysis. The significance limit was $P < 0.05$.

Results

Partial activation of foetal DC is related to the presence of MP in placenta

The absolute numbers of APC subpopulations and expression of activation markers are described in Table 2. CBMC contained MDC at a higher frequency than PDC, resulting in a mean BDCA-1$^+$/BDCA-2$^+$ cell ratio of 4.4 ± 5.2.

Segregation on the basis of maternal malaria infection (i.e. presence of parasites in placental and/or maternal peripheral blood) did not show differences either in the absolute number of foetal APC or in the expression levels of MHC class II and CD86 molecules in different APC subsets studied (additional file 1). However, foetal APC status segregated on the basis of presence or absence of MP in the placenta, revealed a significant up-regulation of the MHC-class II expression, but not of CD86, on BDCA-1$^+$ and BDCA-2$^+$ DC in CB obtained from MP-positive mothers as compared to MP-negative mothers (Figure 1B). Thus, a partial activation of foetal DC is related to the presence of MP in the placenta, and not to maternal infection at delivery.

Impact of age and parity of the mother on the frequency and activation status of foetal APC

The absolute numbers of monocytes and BDCA-1$^+$ MDC in CB were negatively associated with maternal age and parity (Figure 2A and 2B). In addition, maternal age showed a positive correlation with higher expression levels of CD86 on monocytes (Figure 2A). We also observed significantly increased MHC-class II expression on BDCA-2$^+$ DC in CB from multigravidae as compared to mothers undergoing first or second pregnancy (Figure 2B).

As expected, maternal age and multiparity were related (Spearman coefficient = 0.47; P = 0.0002). The multivariate analysis showed an effect of both age and parity on CB monocytes and BDCA-1$^+$ cells absolute numbers (age: β = +0,55 (0,10-1,00) with P = 0,02 and parity: β = +0,42 (-0,06-0,90) with P = 0,08).

Malaria status of the mother does not affect immunophenotype of foetal APC upon TLR and Hz stimulation

By examining MHC class II expression, CB monocytes and MDC were activated by LPS and PolyI:C stimulation

Table 2: APC absolute numbers and immunophenotype in cord blood samples.

	APC subset			
Parameter: median (± interquartile)	**Monocytes**	**MDC**		**PDC**
	CD14$^+$	**BDCA-1$^+$**	**BDCA-3$^+$**	**BDCA-2$^+$**
% of total CBMC	13.00 ± (7.10)	0.62 ± (0.42)	0.19 ± (0.18)	0.25 ± (0.18)
absolute no./ml	624,787 ± (571,630)	23,850 ± (30,430)	8,160 ± (12,225)	9,680 ± (9,032)
% HLA-DR$^+$ cells	93.87 ± (7.09)	93.53 ± (11.01)	59.95 ± (28.55)	82.64 ± (31.69)
HLA-DR, MFI[a]	132.71 ± (17.69)	162.50 ± (18.81)	118.26 ± (40.81)	129.9 ± (39.59)
% CD86$^+$ cells	50.28 ± (34.37)	44.67 ± (22.15)	35.95 ± (20.98)	18.47 ± (13.41)
CD86, MFI	62.36 ± (30.17)	57.51 ± (21.12)	45.54 ± (17.80)	33.29 ± (13.77)

Immunophenotyping was performed on 55 cord blood samples; 27 from malaria infected women and 28 from uninfected women. APC were quantified as a percentage of the total CBMC. Absolute numbers of MDC, PDC and monocytes were calculated from CBMC counts. HLA-DR and CD86 expression levels were then examined in different APC subsets.

[a]; MFI, mean fluorescence intensity

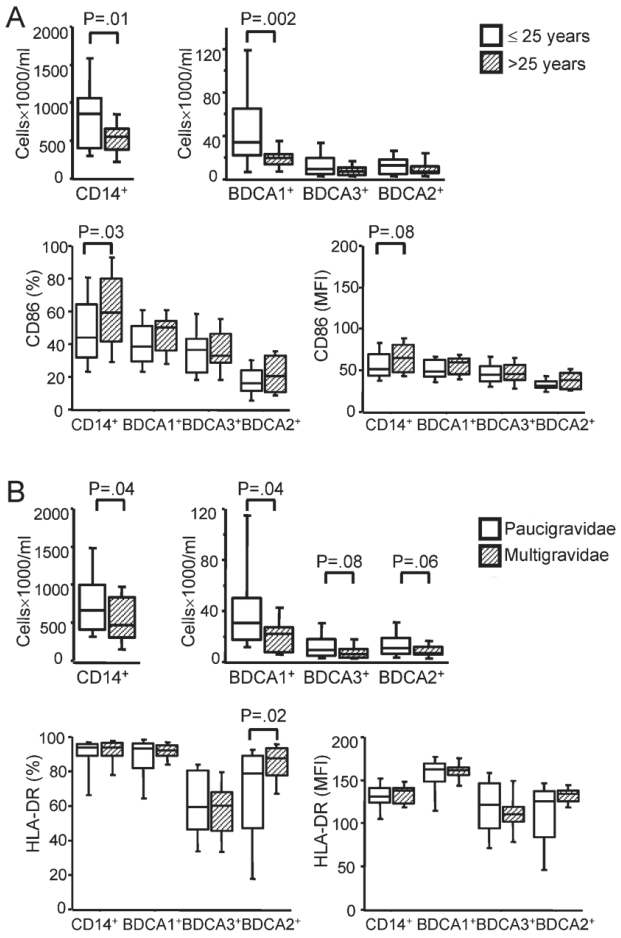

Figure 2
Association between APC numbers and activation and maternal age or parity. (A) Data were segregated into 2 groups according to the median value for maternal age. Absolute numbers and CD86 expression levels (as percentage of positive cells and mean fluorescence intensity, MFI) on different foetal APC subsets were analysed as a function of maternal age in 31 women ≤ 25 years of age and 24 women > 25 years of age Boxplots illustrate the medians and the 25th and 75th percentiles. (B) Women were divided for parity as paucigravidae (1st and 2nd pregnancy) and multigravidae (≥ 3 pregnancies) as we previously observed that women at first and second pregnancy exhibited the same risk of malaria infection (Fievet, unpublished data). Absolute numbers and HLA-DR expression levels (as percentage of positive cells and mean fluorescence intensity, MFI) on different foetal APC subsets were analysed as a function of parity in 37 women undergoing first or second pregnancy and 18 multigravidae. Boxplots illustrate the medians and percentiles. P-values were calculated by Mann-Whitney U test. The significance limit was $P < 0.05$.

(Table 3). In addition, when we added synthetic Hz as stimulus, we found monocytic activation, as shown by significantly increased expression levels of MHC class II molecules, while the MHC class II levels on MDC were not affected (Table 3). However, there was no difference in MHC class II expression following stimulation with TLR3 and TLR4 ligands or synthetic Hz on CB monocytes and MDC when segregated according to the presence of MP in the placentas (Table 3). Thus, MP positivity did not affect further phenotypic *ex- vivo* maturation of foetal APC by diverse TLR ligands or Hz.

Plasmodium falciparum *infection in the mother induces amplification of TLR9 response in foetal leukocytes*

Cytokine secretion in APC is triggered by the recognition of microbial pathogens through TLR [15]. The ability of foetal APC to secrete cytokines by stimulating CBMC with different TLR ligands was investigated. IFN-α was evaluated in supernatants of unstimulated, CpG-A- and Hz-stimulated CBMC cultures. As previously reported [19,27], unstimulated and synthetic Hz-stimulated CBMC did not secrete any IFN-α, while CpG-A-stimulated cells, most likely PDC, produced low levels of this cytokine (0.12 ± 13.79 pg/ml; median ± interquartile). No difference was observed in IFN-α production upon CpG-A stimulation when CBMC were segregated according to accumulation of MP in placenta: (0.01 ± 12.04 pg/ml vs 1.90 ± 18.21 pg/ml; MP-negative vs MP-positive women; p = 0.45).

The levels of IL-6, IL-10, IL-12, MIP-1α/CCL3α IFN-γ and TNF-α were also quantified in supernatants of CBMC that were either unstimulated or stimulated with synthetic Hz or different TLR ligands. TLR3, TLR4 and, to a lesser extent, TLR9 stimulation induced release of both pro- and anti-inflammatory cytokines (Figure 3). The presence of MP in the placenta influenced TLR-mediated CBMC cytokine responses such that the production of IL-10 and TNF-α was significantly higher upon TLR9 stimulation and there was a similar trend for increased production of IFN-γ after TLR-3 stimulation in CBMC of neonates of mothers with MP-positive placenta as compared to MP-negative mothers (Figure 3).

To further analyse the production of IL-12 by different APC subsets upon TLR3 and TLR4 stimulation, we performed intracellular cytokine staining. Only a minor population of foetal APC produced IL-12. In fact, 2.7% ± 3.4% and 2.6% ± 3.8% of IL-12-producing monocytes (median ± interquartile) were observed in response to LPS and PolyI:C, respectively, and 2.7% ± 3.5% and 2.0% ± 3.6% of IL-12-producing MDC (median ± interquartile) were found in responses to LPS and PolyI:C, respectively. However, no differences were observed when comparing foetal APC of mothers with or without MP accumulation in placenta (table 4).

Fifty-seven plasma samples from CB of mothers were analysed for cytokine levels. In all samples we found detecta-

Table 3: Immunophenotype of fetal APC upon TLR and Hz stimulation.

		Monocytes			MDC		
Parameter median (± interquartile)	**stimulus**	**Total CB**	**MP-Negative**	**MP-Positive**	**Total CB**	**MP -Negative**	**MP-Positive**
% HLA-DR$^+$ cells	unstimulated	89.34 ± (13.90)	89.33 ± (16.57)	89.79 ± (7.27)	88.78 ± (19.83)	87.33.40 ± (19.83)	92.62 ± (20.13)
	Hz	91.03 ± (10.10)	90.46 ± (11.97)	92.27 ± (8.31)	90.99 ± (12.22)	89.40 ± (13.55)	91.37 ± (7.88)
	LPS	96.73 ± (6.22)	96.73 ± (7.61)	96.45 ± (3.14)	93.24 ± (11.08)	93.24 ± (11.66)	93.38 ± (15.23)
	Poly I:C	96.79 ± (4.68)	96.79 ± (7.99)	96.70 ± (2.27)	93.99 ± (12.57)	93.08 ± (9.54)	92.09 ± (16.42)
HLA-DR, MFI[a]	unstimulated	120.81 ± (20.14)	121.15 ± (24.36)	119.54 ± (16.48)	143.85 ± (23.19)	143.57 ± (21.28)	149.29 ± (22.77)
	Hz	122.08 ± (20.42) *	125.36 ± (23.00)	121.47 ± (15.15)	146.96 ± (14.93)	151.30 ± (14.69)	156.06 ± (20.47)
	LPS	135.72 ± (16.78)*	137.56 ± (18.02)	133.23 ± (13.82)	152.40 ± (16.28)*	145.49 ± (19.31)	150.67 ± (13.84)
	Poly I:C	136.53 ± (15.66)*	137.27 ± (19.29)	136.41 ± (13.51)	150.16 ± (14.93)*	150.61 ± (16.72)	149.60 ± (16.35)

Immunophenotyping on stimulated cells was performed on 43 cord blood samples (total CB); 12 from women with MP accumulation in placenta (MP-Positive) and 31 without MP in placenta (MP-negative). All comparison between MP-Negative and MP-Positive were not significant.
[a]; MFI, mean fluorescence intensity
*; $P < 0.05$

ble levels of IFN-α, IL-6, IL-10, IL-12, MIP-1-α/CCL3 and TFN-γ but no IFN-γ. The CB plasma levels of the different cytokines were not influenced by the presence of malaria infection in the mother (Figure 4A) or by MP accumulation in placenta (Figure 4B).

Thus, cytokine responses to TLR3, 4 and 9 ligands and to Hz were observed, with amplification of TLR9-mediated responses in CBMC from MP-positive mothers.

Maternal age influences TLR3, 4 and 9 responses of foetal leukocytes and cytokine levels in foetal plasma

Segregation of samples into two groups according to the median value for maternal age revealed significant differences in cytokine production after TLR3, 4 and 9 stimulation of CBMC. The levels of TNF-α, IFN-γ, MIP-1α/CCL3 and IL-10 produced in response to LPS by CBMC of newborns of mothers ≤ 25 years were significantly higher than those produced by CBMC of neonates born to mothers > 25 years. In addition, the amount of TNF-γ and IL-10 produced by CBMC in response to TLR3 and TLR9 ligands were negatively associated with maternal age (Figure 5). Parity had no influence on cytokine secretion by CBMC upon TLR stimulation (Figure 6).

Significant differences were also observed in CB cytokine levels according to maternal age. Foetal plasma from mothers > 25 years of age exhibited significant lower levels of IL-10 as compared to younger mothers (Figure 4C). Thus, productions of cytokines by CBMC and plasma levels for IL-10 were higher in children born to the younger mothers.

When an effect of both MP-positivity in placenta and maternal age on cytokines in supernatants was observed, the multivariate analysis showed that maternal age was predominant and that effect of MP-positivity disappeared for IL-10 in response to TLR9 ligands (β = 1.23 (0.56-1.89) P = 0.001). However, both MP-positivity and maternal age had an effect on TNF-α secretion upon TLR9 stimulation (MP: β = 0.88 (0.024-1.74), P = 0.04; Age: β = 1.04 (0.23-1,74), P = 0.04).

Discussion

The consensus view is that *in utero* sensitization to *P. falciparum* antigens is a common phenomenon during PAM. Parasites do not usually cross the placental barrier and such sensitization is most probably caused by the transplacental passage of soluble *P. falciparum* antigens [8,28]. Accordingly, no parasite-positive smears were detected in CB samples of malaria-infected women in this study population.

Pregnant women declared to receive either SP or CQ as malaria prevention during pregnancy. In contrast to a previous study performed in the same area [29], no differences were observed for *P. falciparum* infection rate according to the type of prophylaxis used by the mothers. However, the number of subject included in this study was low and the project was not designed to examine this matter.

In this study, the foetal BDCA-1$^+$ and BDCA-2$^+$ DC subsets expressed significantly higher levels of MHC class II molecules upon PAM, as indicated by the presence of *P. falciparum* MP in placenta, which is in agreement with

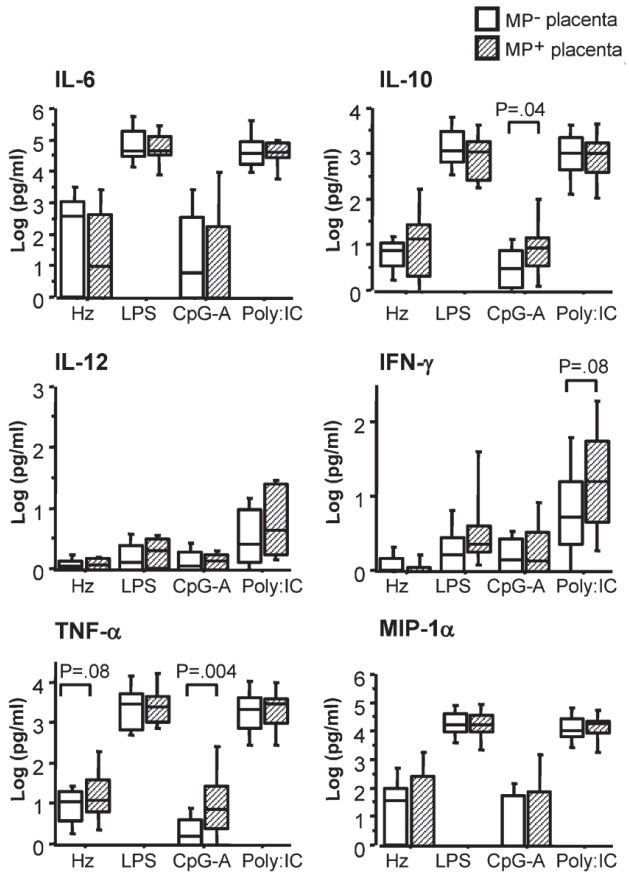

Figure 3
TLR-induced cytokine responses in CBMC obtained from women with or without MP accumulation in placenta. CBMC (2 million/ml) were stimulated or not with synthetic Hz (5 μg/ml), LPS (100 ng/ml), CpG-A (3 μg/ml) or PolyI:C (20 μg/ml). After 18 hours, supernatants were collected and analysed for IL-6, IL-10, IL-12, IFN-α, TNF-α and MIP-1α/CCL3 levels. Data represent median values and percentiles for 59 individuals; 16 MP-positive (diagonal striped bars) and 43 MP-negative mothers (white bars). Cytokine levels in unstimulated cells were subtracted from the values shown. P-values were calculated by Mann-Whitney U test or t test (see Subjects, Materials and Methods). The significance limit was $P < 0.05$.

previous data [23]. The observation that CD86 expression on foetal DC was unaffected by PAM suggests that *P. falciparum* stimulation *in utero* induces only partial activation of these cells. Failure to provide DC with a sufficiently strong costimulatory signals can impair the ability to form stable interactions with T-cells, as recently shown in a murine model of malaria [30]. Partial DC maturation can lead to altered T-cell activation and induction of tolerance [31-34], possibly contributing to impaired immune responses that have been observed in the offspring of mothers with PAM [9,11].

The findings presented in this study diverge from those of studies on peripheral blood DC from children with acute malaria, where expression levels of MHC class II on the BDCA-1⁺ DC are reduced compared to healthy controls [35,36]. Also, no increase in foetal BDCA-3⁺ DC was detected upon maternal malaria infection in this study like others have shown in children with severe malaria [35]. Circulating APC are continuously exposed to *P. falciparum*-infected erythrocytes during malaria episodes in children, which may exert a contact-mediated inhibitory effect on DC functionality, as demonstrated by *in vitro* studies [4,37]. Conversely, infected erythrocytes are rarely detected in CB of those born to mothers with PAM [23,38]. Thus, foetal APC would rarely if ever encounter parasitized red-blood cells, but would be primarily exposed to and influenced by parasite-derived soluble compounds.

Interestingly, TLR9 stimulation led to increased pro- and anti-inflammatory responses of CBMC of neonates whose mothers had MP accumulating in placentas, and there was a tendency towards increased IFN-γ response upon TLR3 stimulation in the same group. Responses *via* other TLR ligands, such as LPS were amplified in CBMC but did not change appreciably as a function of maternal malaria infection. Thus, foetal different TLR responses are independently modulated by *in utero* exposure to *P. falciparum*, consistent with a recent study [22].

In humans only PDC and B cells express TLR9 [39]. In this study, CpG-A, a TLR9 ligand that specifically stimulates

Table 4: Accumulation of MP in placenta does not affect IL-12 production by APC upon TLR3 and TLR4 stimulation.

		Monocytes		MDC	
Parameter median (± interquartile)	**stimulus**	**MP-Negative**	**MP-Positive**	**MP-Negative**	**MP-Positive**
IL-12 (% positive cells)	LPS	3.55 ± (4.13)	2.70 ± (3.15)	3.13 ± (3.72)	2.13 ± (1.72)
	Poly:IC	2.33 ± (3.37)	3.99 ± (4.49)	1.95 ± (4.01)	2.38 ± (3.09)

Intracytoplasmic IL-12 production by APC upon TLR3 and TLR4 stimulation was analysed on 43 cord blood samples; 31 from MP-negative women and 12 MP-positive women. All comparisons between MP-negative and MP-positive women were not significant.

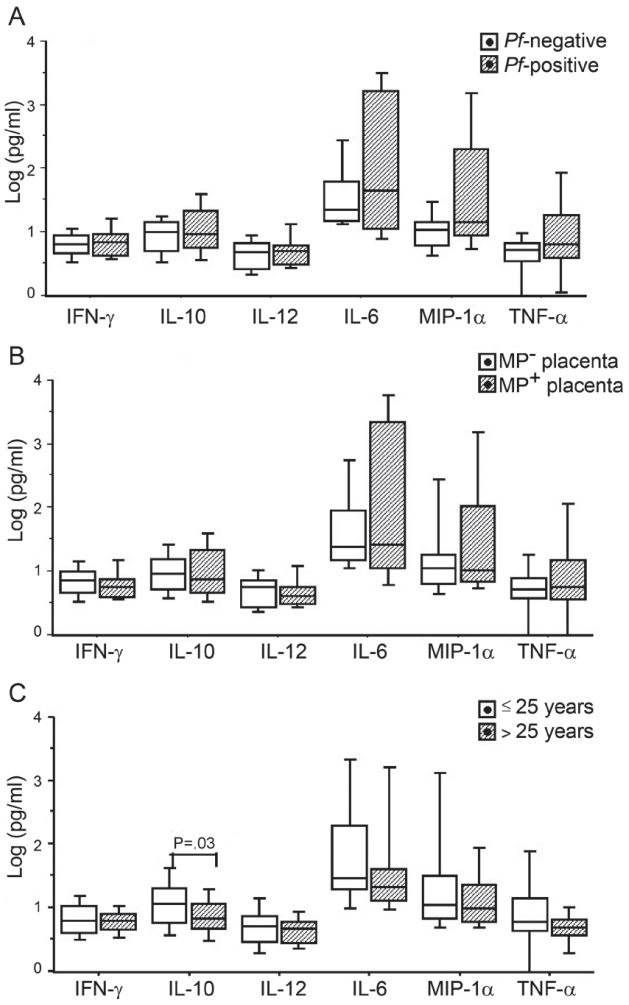

Figure 4
Cord blood cytokine levels are affected by maternal age, but not by maternal P. falciparum infection or MP accumulation in placenta. Foetal plasma samples were collected at delivery and subsequently analysed for IL-6, IL-10, IL-12, IFN-γ, TNF-α and MIP-1α/CCL3 levels. (A) Data represent median values and percentiles for 57 individuals divided in 27 *P. falciparum* negative mothers (Pf-negative, white bars) and 30 *P. falciparum* positive mothers (Pf-positive, diagonal striped bars). (B) Women were divided in 39 MP-negative mothers (white bars) and 18 MP-positive (diagonal striped bars). (C) Women were divided in 33 mothers ≤ 25 years of age (white bars) and 24 women > 25 years (diagonal striped bars) Values were calculated by Mann-Whitney U test or t test (see Subjects, Materials and Methods). The significance limit was $P < 0.05$.

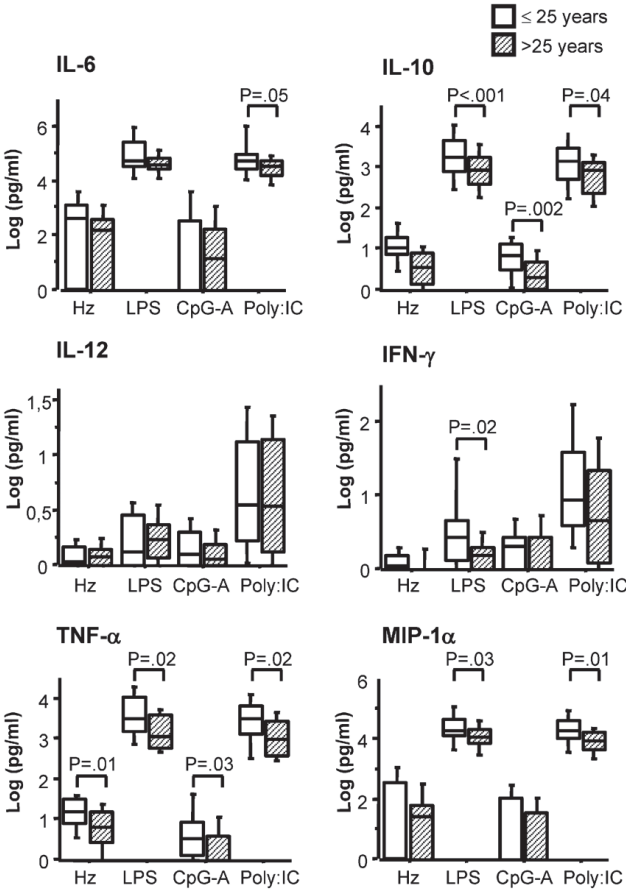

Figure 5
TLR-induced cytokine responses in foetal leukocytes are influenced by maternal age. CBMC (2 million/ml) were stimulated with synthetic Hz (5 μg/ml), LPS (100 ng/ml), CpG-A (3 μg/ml) or Polyl:C (20 μg/ml). After 18 hours, supernatants were collected and analysed for IL-6, IL-10, IL-12, IFN-α, TNF-α and MIP-1α/CCL3 levels. Cytokine levels in unstimulated cells were subtracted from the values shown. P-values were calculated by Mann-Whitney U test or t test (see Subjects, Materials and Methods). The significance limit was $P < 0.05$. Data represent median values and percentiles for 57 individuals; 33 mothers ≤ 25 years of age (white bars) and 24 women > 25 years (diagonal striped bars) (Data on maternal age were missing for 2 subjects, that were therefore not included in this analysis).

PDC [25,40], was employed. In concordance with the findings presented in this study, MP or alternatively plasmodial DNA bound to MP activate the TLR9 pathway in human and murine PDC [27,41,42]. This would suggest a role for MP derived from maternal parasitic infection in inducing foetal BDCA-2+ DC partial maturation and increased sensitization to TLR9 ligands. Nevertheless, only low levels of IL-10 and TNF-α were detected in CBMC cultures upon TLR9 stimulation. This was not unexpected given the low frequency of cells able to specifically respond to such stimulus. The biological significance of a slightly increased release of IL-10 and TNF-α by CBMC upon TLR9 stimulation after *in utero* exposure to *P. falciparum* is uncertain.

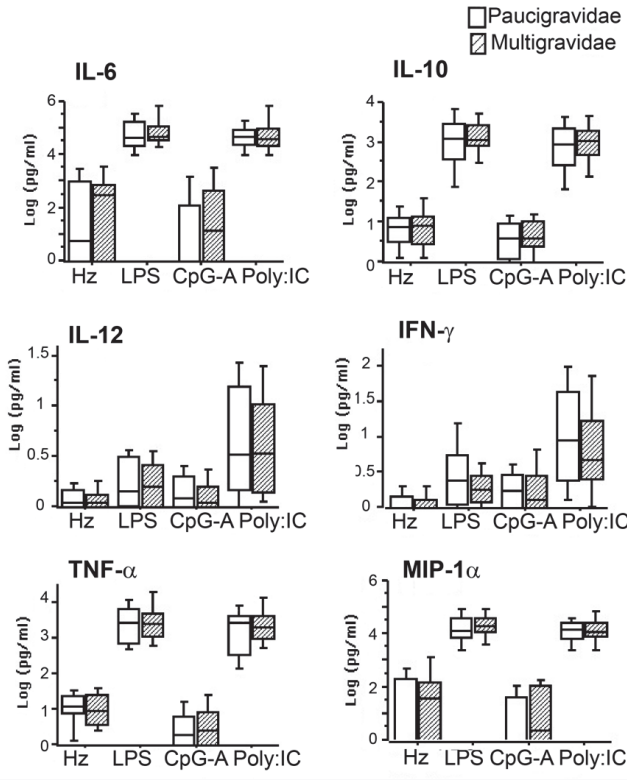

Figure 6
TLR-induced cytokine responses in foetal leukocytes are not influenced by parity. CBMC (2 million/ml) were stimulated with synthetic Hz (5 μg/ml), LPS (100 ng/ml), CpG-A (3 μg/ml) or PolyI:C (20 μg/ml). After 18 hours, supernatants were collected and analysed for IL-6, IL-10, IL-12, IFN-α, TNF-α and MIP-1α/CCL3 levels Cytokine level in unstimulated cells was subtracted from the values shown. P-values were calculated by Mann-Whitney U test or t test (see Subjects, Materials and Methods). The significance limit was $P < 0.05$. Data represent median values and percentiles for Women were segregated on the basis of parity as 41 paucigravidae (1st and 2nd pregnancy; white bars) and 18 multigravidae (≥ 3 pregnancies; striped bars).

Notably, MP was the only indicator of maternal malaria infection that was significantly associated with partial activation of foetal DC and to amplified innate response to TLR9 ligation, while other markers of maternal parasitization at delivery such as the presence of parasites in peripheral and/or placental blood, were unrelated to DC activation in the exposed newborns. It has been recently postulated that accumulation of MP in leukocytes is a good indicator of total parasite burden, including parasite sequestration [43], and therefore we can consider accumulation of MP in placenta as a marker of high intensity of maternal malaria infection and/or of prolonged parasite exposure. In addition, accumulation of MP in placental leukocytes has been associated with increased monocyte activation and inflammation [44]. As a hypothesis, accumulation of MP may represent a specific activation stimulus and inflammation at the placental level and this may cause partial and inadequate activation of APC in the foetal compartment.

Additionally, maternal age and parity should be taken into consideration when analysing foetal/neonatal innate immunity. Women of higher parity and increased age delivered babies in whom significantly fewer blood APC were found, but these cells exhibited an enhanced activation status. Maternal age but not parity also influenced the APC cytokine responses upon TLR stimulation, such that CBMC of offspring of younger mothers exhibited an increased ability to respond to TLR3, 4 and 9 ligands. These data are in agreement with published data on African [23] and Caucasian [45] women and suggest that maternal age and obstetric history may influence foetal/neonatal immune parameters.

Consequences of increased maternal age and/or multiple parities in terms of neonatal responses to pathogens are poorly understood. Two recent studies indicate that the frequency of malaria episodes is higher among infants of malaria-infected multigravidae as compared to primigravidae [6,7]. The intrinsic effect of multiple pregnancies on malaria susceptibility in the offspring may be at least partially explained by our finding of a significantly reduced number of myeloid APC in foetal blood from multigravidae. How maternal age or alternatively parity can affect the number, activation status and cytokine secretion capacity of cord blood APC is presently unknown.

In conclusion, placental parasitization, as indicated by the presence of MP in placental leukocytes, is significantly associated with partial maturation of different DC subsets and to slightly increased responses to a TLR9 ligand in cord blood. As semi-maturation of DC leads to tolerance [46], such partial foetal APC activation may contribute to the altered T-cell responses often observed in newborns of mothers with PAM [5-7].

These observations advocate a possible mechanism by which PAM may modulate foetal/neonatal innate immunity. Further evaluation of APC activation and downstream T-cell responses is ongoing in a large cohort of newborns and infants from mothers with PAM to assess the impact of altered DC activation on the neonatal cell-mediated immunity.

As it is known that neonatal immune responses are largely dependent on the innate branch of immunity and can be improved through selective TLR stimulation [47,48], our results should be considered in the development of effec-

tive vaccine strategies for infants living in areas where malaria is endemic.

Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

NF: conceived the study, participated in its design and coordination, acquired the data and contributed to data analysis and interpretation and drafted the manuscript. SV: participated in the design and coordination of the study, contributed in data analysis and interpretation and drafted the manuscript. IS: acquired the data and contributed to their analysis and interpretation. VB: contributed in interpretation and statistical analysis of data and in critically revising the manuscript. SL: contributed in performing the immunoassays, and contributed in their interpretation, participated in critically revising the manuscript. RP: participated in the design of the study, in the analysis of the data and in critically revising the manuscript. AM: participated in the design and coordination of the study and in critically revising the manuscript. AH: participated in the design of the study, in the analysis of the data and in critically revising the manuscript. MT: participated in the design of the study, in the analysis and interpretation of data and in critically revising the manuscript. PD: participated in the design and coordination of the study and contributed to draft the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Presence of* P. falciparum *parasite in maternal and/or placental blood at delivery does not influence the activation status of cord blood DC and monocytes. *The expression levels of HLA-DR and CD86 were measured by flow cytometry on foetal APC from 27* P. falciparum*-positive (diagonal striped bars) and 28* P. falciparum*-negative mothers (white bars).*

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Ethical issues

Informed consent was obtained from all donors. The "Faculté des Sciences de la Santé" Committee of Beninese University approved this study.

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ARTICLE 6: Malaria modifies neonatal and early-life Toll-like receptor cytokine responses.

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Infection and Immunity, (2013) sous presse.

Contexte : Dans l'article précédent, nous avons démontré que l'exposition *in utero* des nouveau-nés au parasite du *P. falciparum* pouvait induire une altération des cellules présentatrices d'antigènes dans le sang de cordon. Nous avons montré aussi que cette altération serait induite à travers la stimulation des TLR. Il est cependant nécessaire de connaître l'orientation des stimulations induites par le parasite *P. falciparum* et la durée de celles-ci chez le jeune enfant. L'objectif de cette étude est d'étudier l'impact de l'exposition *in utero* au PAG sur les réponses immunitaires innées du nouveau-né et du jeune enfant.

Méthode : Dans une étude prospective de 137 paires de femmes enceintes et nouveau-nés, nous avons mesuré la production de cytokines inflammatoires (IL-6 , IFN-γ, TNF-α et l'IL-10) dans le sang de cordon et le sang périphérique des nouveau-nés à 3, 6 et 12 mois, à travers la stimulation de récepteurs TLR spécifiques.

Résultats:

- Indépendamment de l'infection palustre, la production de cytokines inflammatoires après stimulation des TLR, augmente en fonction de l'âge excepté pour l'IL-6 et l'IL-10.
- L'exposition à *P. falciparum* à l'accouchement induit chez le jeune enfant, des concentrations élevées d'IL-6, d'IL-10 et de TNF-α produites par la stimulation des TLR3, TLR4 et TLR9.
- Les analyses prospectives montrent que la production d'IL-10 dans le sang de cordon après stimulation par les TLR3 et TLR7/8 est associée au risque élevé d'infection palustre au cours de leur première année de vie.

Discussion : L'augmentation de la concentration de cytokine en fonction de l'âge suggère une maturation progressive des cellules immunocompétentes pendant les premiers mois de vie de l'enfant. Nos résultats concordent avec ceux de Burl et coll (2011), montrant l'ontogénie de l'immunité innée au cours de la première année de vie [227]. Des concentrations élevées d'IL-6, d'IL-10 et de TNF-α chez les nouveau-nés exposés suggèrent un contrôle des réponses de type TH1 médié par l'IL-10 chez le nouveau-né exposé [173].

Conclusion : Nos résultats montrent que la susceptibilité à l'infection palustre chez l'enfant au cours de sa première année de vie est contrôlée par le niveau de stimulation des TLR. L'IL-10 pourrait jouer un rôle important dans la survenue du risque de l'infection.

Malaria modifies neonatal and early-life Toll-like receptor cytokine responses

Running title : Malaria modifies early-life TLR cytokine responses

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Abstract

Protection from infections in early-life relies extensively on innate immunity, but whether and how maternal infections modulate infants' innate immune responses, thereby altering susceptibility to infections, is not known. *Plasmodium falciparum* causes pregnancy-associated malaria (PAM) and epidemiological studies have shown that PAM enhances infants' susceptibility to infection with *P. falciparum*. We investigated how PAM-mediated exposures *in utero* affect innate immune responses and their relationship with infection in infancy.

In a prospective study of mothers and their babies in Benin we investigated changes in toll-like receptor (TLR)-mediated cytokine responses related to *P. falciparum* infections. Whole-blood samples from 134 infants at birth, 3, 6 & 12 months of age were stimulated with agonists specific for TLR3, TLR4, TLR7/8 & TLR9. TLR-mediated IL-6 & IL-10 production was robust at birth then stabilized, whereas TNF-α and IFN-γ responses were weak at birth then increased. In multivariate analyses, maternal *P. falciparum* infections at delivery were associated with significantly higher TLR3-mediated IL-6 and IL-10 responses in the first 3 months of life ($p<0.05$), and with significantly higher TLR3-/TLR7/8-/TLR9-mediated TNF-α responses between 6-12 months (all $p<0.05$). Prospective analyses showed that higher TLR3-/TLR7/8-mediated IL-10 responses at birth were associated with a significantly higher risk of *P. falciparum* infection in infancy (all $p<0.05$).

Neonatal and infant intracellular TLR-mediated cytokine responses are conditioned by *in utero* exposure through PAM late in pregnancy. Enhanced TLR-mediated IL-10 responses at birth are associated with an increased risk of *P. falciparum* infection, suggesting a compromised ability to combat infection in early-life.

Introduction

Chronic infections during pregnancy, including those of parasitic origin, are a frequent occurrence in low-income areas of the world in general, and in sub-Saharan African countries in particular, and they affect foetal immunity such that infants' responses to vaccination are diminished and their susceptibility to infection is increased (1, 2). Maternal infection with *Trypanosoma cruzi*, for example, without vertical transmission, stimulates foetal innate and adaptive immune responses such that exposed but uninfected neonates produce higher concentrations of pro-inflammatory and anti-inflammatory cytokines than their unexposed counterparts (3, 4). Maternal infection with *Schistosoma* spp. has also been shown to be associated with foetal inflammation, characterised by increased levels of IL-1β and TNF receptor II in cord blood of those born to infected mothers (5). With an annual estimate of 50 million or more mothers at risk, pregnancy-associated malaria (PAM) due to *Plasmodium falciparum* is a well-recognized and well-described example of such an infection, representing a major public health burden measurable by the adverse pregnancy outcomes it causes (Reviewed in: (6-10). Quite apart from the association between PAM and low birthweight and the poor prognosis of survival that goes with it, a number of studies have documented the increased susceptibility to malaria of infants born to mothers with *P. falciparum* infection detected at delivery (11-15). PAM has also recently been shown to be associated with an increased risk of fever episodes of non-malarial causes in infancy (16). Taken together, these findings suggest that exposure to *P. falciparum in utero* alters foetal and/or neonatal immune development resulting in enhanced susceptibility to malaria in infancy, and, furthermore, that such alterations may also affect susceptibility to infections other than malaria.

Evidence of altered foetal/neonatal cellular immunological activity comes from reports of cord blood cell frequencies, their activation status and their antigen-specific proliferative and/or cytokine responses resulting from placental infection with *P. falciparum* and consequent exposure of the foetal immune system to parasite-derived antigens *in utero* (Reviewed in: (17). At the level of induction and acquisition of specific T cell activity, the conclusion from several of these studies is that regulatory T cells (Treg) producing the immunosuppressive cytokine interleukin (IL)-10 play a pivotal role in down-modulating cord blood Th1-type responses to *P. falciparum* antigens (18-23). The latter studies, however, only examined the 'downstream' nature of the altered immunological responses, giving little, if any, information on the character of the 'upstream' innate immune interactions, for example, that may have led to the observed changes in T cell activity.

Innate immunity involves the family of so-called 'professional' antigen-presenting cells (APC), a family that includes myeloid and plasmacytoid dendritic cells (m and pDC) as well as monocytes and B cells. These cells play a pivotal role in recognition of pathogens and orchestrating the subsequent adaptive immune

response (24). A major component of pathogen recognition by APC comprises a family of pattern recognition receptors referred to as Toll-like receptors (TLR) that interact with a range of highly conserved microbial components, leading, conventionally, to a burst of pro-inflammatory cytokine activity. In early life, the components of the adaptive immune system, although present, are insufficiently mature and an infant's response to infection therefore relies extensively on innate immunity (Reviewed in (25)). At all times, the balance between production of pro- and anti-inflammatory mediators is tightly regulated to allow efficient, protective immune responses to develop whilst preventing the pathological consequences of excessive inflammation (26). In the particular context of control of inflammatory activity in early life, a pivotal role for neonatal B cells producing IL-10 as a result of TLR9 activation has been reported (27), a role that is consistent with reports of robust TLR ligand-mediated IL-10 responses present at birth both in non-African and in African populations (28-30).

In the context of pregnancy, we and others have shown that placental infection with *P. falciparum* at delivery is associated with altered foetal innate immune responses, with modified frequency of cord blood DC (31), partial activation of cord blood APC (32) and modulated cord blood cytokine responses to TLR ligation (33). Two independent studies in non-pregnant individuals have reported the capacity of *P. falciparum* infection to cause pro-inflammatory priming of responses to subsequent TLR ligation, a capacity that distinguishes the parasite from most other microbial pathogens (34, 35). Thus, although the detailed mechanisms remain to be characterised, with controversy remaining over the precise nature of the parasite-derived TLR ligands, it is clear that *P. falciparum* generates an array of responses through interaction with different TLR that include at least TLR2, 4 & 9 (36). The above-mentioned cross-sectional studies present a limitation, i.e. the role of PAM in influencing early life cellular immunological responses has been investigated only at delivery, leaving the potential impact of *in utero* exposure to *P. falciparum* on the normal profile of maturation of the innate immune system in infancy as yet unknown.

Here, we evaluated the development of TLR-mediated cytokine responses both at birth and during the first 12 months of life in a large cohort of children born to mothers with different malaria histories, following the hypothesis that PAM and, potentially, infection and/or malaria episodes in early life would affect the development of infants' innate immune responses. Since the timing of occurrence of PAM during pregnancy is associated with foetal and infant malaria outcomes (37, 38), parasitological data were collected from all mothers starting with inclusion, during the second trimester, and thereafter throughout pregnancy up to and including delivery. These data were then combined with measures of innate immune activity at birth and during infancy. Through prospective evaluation during the first year of life we also evaluated the independent influence of being born premature and of infection and/or malaria episodes during infancy on the same TLR-mediated responses.

Materials and Methods

Ethics statement

The STOPPAM study was approved by the ethics committee of the Health Science Faculty of the University of Abomey-Calavi, Benin, and by the ethics committees of the Research Institute for Development (IRD) in France. Written informed consent was obtained from all mothers, who additionally provided consent for their babies, prior to inclusion in the study. All procedures adhered to Declaration of Helsinki principles.

Study design

The STOPPAM (Strategies TO Prevent Pregnancy Associated Malaria) project was conducted in parallel in Benin and in Tanzania from 2008 to 2011. In Benin, the study took place in the district of Come, Mono province, located 70 km west of the economical capital, Cotonou. Malaria transmission in this area can be characterised as hyperendemic, with two peaks during the rainy seasons (April-July, September-November), and an entomological inoculation rate estimated at 35-60 infective bites per person per year (13). The STOPPAM study design has been described in detail elsewhere (37). In total, 1037 women with a gestational age below 24 weeks were enrolled in 3 clinics (Come, Akodeha and Ouedeme Pedah) in the Come district. Clinical and parasitological data were subsequently collected at monthly follow-up visits and at delivery. According to the existing national policy for malaria, women received intermittent preventive treatment during pregnancy (IPTp) with the anti-malarial drug combination sulphadoxine-pyrimethamine (SP) on two separate occasions during scheduled antenatal visits (ANV) spaced at least 1 month apart in the second/third trimesters. Women diagnosed with malaria (positive malaria rapid diagnostic test, RDT, see below) received a standard treatment regimen of quinine unless the diagnosis coincided with a scheduled IPTp dose in which case SP was given instead of quinine. Women were encouraged to present at the maternity clinic to receive care whenever necessary between ANV. Such visits are referred to as 'emergency' visits.

For an immunological sub-study conducted as part of the STOPPAM project in Benin, a sub-group of 217 pregnant women from the enrolled cohort was selected at delivery on the basis of their recorded history of infection with *P. falciparum* (uninfected during pregnancy, n = 99; infected during pregnancy but uninfected at delivery, n = 71; infected at delivery, n =47). The cohort of infants of these selected women was actively followed-up at home from birth to 12 months of age, with clinical assessments every two weeks and parasitological assessments (see below) every month. Cellular immunological studies were performed with cord and infants' peripheral blood that was collected at 3, 6 and 12 months of age. Infants from whom fewer than 3 blood samples were collected during the follow-up period were excluded from the study as

were infants either of mothers who subsequently tested seropositive for HIV or for whom the HIV serostatus was unknown**.**

P. falciparum infection status

To assess the impact of *P. falciparum* infection on neonatal immunity, clinical and parasitological data were collected from mothers at each ANV and emergency visit, and from infants as described above. RDT (Parascreen™, Zephyr Biomedical Systems©) were used for mothers at all routine ANV and emergency visits and for infants whenever fever was detected during active surveillance. Retrospective confirmation of infection comprised parasitological diagnosis via standard microscopical examination of thick blood smears (TBS) that were prepared at monthly intervals from both mothers and infants. Briefly, smears were stained with Giemsa and examined by two experienced laboratory technicians for the presence and density of parasites. Smears were considered negative if no asexual stage *Plasmodium* parasite was detected by counting high-power fields containing the equivalent of 500 leucocytes. Parasites were counted against 200 leukocytes and parasite density was calculated based on an estimate of 8000 leukocytes/µl of blood. At delivery, TBS was performed on maternal peripheral and on cord blood samples, and an impression smear of placental blood was also examined. Infection of mothers at delivery was thus defined by the presence of parasites in placental and/or in peripheral blood, whilst infections earlier in pregnancy and during infancy were defined either by a positive RDT or by a positive TBS.

Blood collection and cell stimulation with TLR agonists

Cord (10 ml) and peripheral (2 ml) venous blood samples were collected in tubes containing citrate phosphate dextrose adenine (CPDA) as anticoagulant. All samples were transported within 4 hours to the laboratory of the Research Center for Malaria during Pregnancy and Infancy (CERPAGE) in Cotonou, where stimulation assays were performed the same day. Whole blood samples were diluted 1:1 with RPMI, and separate aliquots (200 µl) of diluted whole blood distributed in Facs tubes were either left unstimulated or stimulated either with polyinosinic:polycytidylic acid (poly I:C, TLR3 ligand; 20 µg/ml; Sigma Aldrich, Schnelldorf, Germany), with ultrapure lipopolysaccharide from *E. coli* (LPS, TLR4 ligand; 100 ng/ml; Sigma Aldrich, Schnelldorf, Germany), with resiquimod (R848, TLR7/8 ligand; 1 mg/ml; Sigma Aldrich,), or with CpG oligonucleotide type A (CpG ODN2216, TLR9 ligand; 3µg/ml; Metabion, Martinsried, Germany). After 24 hours of incubation at 37°C in 5%CO_2, culture supernatants were collected by centrifugation and stored at -80°C for cytokine determination.

The different TLR ligands used in these assays exert their effects on distinct immune cell subsets: poly I:C interacts with TLR3 and RIG-like receptors expressed within mDC (39), natural killer cells and lymphocytes (40, 41); LPS activates TLR4 expressed on monocytes and mDC (42); R848 activates TLR7 and TLR8, with TLR7 being mainly expressed within pDC and B cells (40, 43) whereas TLR8 is found within monocytes

and mDC (44, 45); the type A CpG 2216 interacts with TLR9 expressed within pDC but not B cells (46). The choice of stimulants reflects a desire, based on our own knowledge and experience (32), to focus as far as possible on DC-mediated activity.

Cytokine measurement

We quantified the cytokines IL-6, IL-10, IFN-γ and TNF-α in 50 µl of sampled supernatants through the use of the commercially-available Cytometric Bead Array (CBA soluble protein Flex set assay, BD Biosciences, Grenoble, France), conducted according to the manufacturer's instructions on a FACsCalibur 4-colour cytometer. The assay sensitivity was 1.6 pg/ml for IL-6, 0.13 pg/ml for IL-10, 0.8 pg/ml for IFN-γ and 1.2 pg/ml for TNF-α. When the cytokine concentration in a sample was below the detection level of the test, an arbitrary value was assigned that corresponded to half of the sensitivity value for the specific cytokine concerned. Results were formatted using the BD CBA Analysis Software. Spontaneous cytokine production by unstimulated samples was assessed independently of that of stimulated samples, whilst the latter were analysed after subtracting the corresponding unstimulated sample values. Pilot studies of cytokine kinetics following stimulation with TLR agonists showed the 24hr incubation time to be optimal for the selected cytokines but sub-optimal for pDC-specific IFN-α (maximal at 8hr), which was therefore not included in the panel.

Statistical analysis

The association between TLR-mediated cytokine responses and infant age was analysed using the non-parametric Kruskal-Wallis test. To determine changes in TLR-mediated cytokine production as a function of malaria during pregnancy and/or infancy, the analysis proceeded as follows: for each cytokine response we built a multivariate linear mixed model (LMM), which allows to take into account the correlation between the repeated measurements as well as the potentials confounders in the relation between malaria and the TLR level. The analysis was performed in two steps; first a univariate model which aimed to select potential confounders, followed by a multivariate model. In the univariate step, we investigated the association between baseline characteristics and cytokine levels upon TLR stimulation. The baseline characteristics included in the analysis were gravidity, maternal anaemia (Hb < 11 g/dl), prematurity (<37 weeks), low birth weight (< 2500 g) and infant gender.

All variables related to *P. falciparum* infection were included in the final multivariate model, whilst infant age and baseline characteristics were used to adjust for confounding of the association between TLR-mediated cytokine responses and either *P. falciparum* infection during pregnancy (segregated into three periods according to the time of occurrence, see below), *P. falciparum* infection during infancy (also segregated into three time periods, see below), or other selected variables. To graphically illustrate the predicted effect of maternal infection on the TLR responses of infants, we then computed the mean predicted

TLR-mediated cytokine levels of infants born from uninfected mothers at each time-point, as well as those of the same infants if they were born to an infected mother.

Maternal infections were segregated into the following intervals: (i) infection before the third trimester of pregnancy, (ii) infection during the third trimester of pregnancy but more than 10 days before delivery, (iii) infection from 10 days prior to delivery up to and including delivery. Designation of the latter group was based on the premise that infections detected during an emergency visit occurring 10 days or less before delivery, and therefore treated, were too close in time to delivery to be separable from it, and because most of those concerned were also found to be infected at delivery.

During the first year of life, infection/malaria episodes were also segregated into 3 intervals (i) those occurring before three months of age, (ii) those occurring between 3 and 6 months of age, (iii) those occurring between 6 and 12 months of age, and were separately assessed for associations both with spontaneous (unstimulated) and with TLR stimulation-mediated cytokine production.

In a separate analysis we determined whether TLR-mediated cytokine responses at birth were associated with the occurrence of malaria during the first 12 months of age. For this purpose we employed a logistic mixed model; risk factors were first analyzed in a univariate model and then, after adjusting for potential confounders, in a multivariate model. TLR responses in cord blood were considered as a baseline for prediction of the development of *P. falciparum* infection during infancy. Malaria episodes recorded each month during the follow-up of infants were considered as the dependent variables and coded as "infected" or "not infected". The model allowed estimation of the predictive values of the TLR-mediated cytokine levels (for any set of covariates) of all infants at each time-point.

Statistical significance in all multivariate analyses was considered if p-values < 0.05. All analyses were performed using the R statistical package (R Development Core Team; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) and graphs made with graphPad (Prism 5.0).

Results

Characteristics of the study population

Between November 2008 and April 2011, 217 mother/infant pairs were enrolled in the study, but of these 83 were excluded due to insufficient numbers of blood samples (80) and HIV sero-status (3 either HIV+ or unknown). Maternal and infant characteristics of the 134 remaining pairs are presented in **Table 1**. Mothers' mean age (± standard deviation, SD) was 26.3 (± 0.2) years, 18.7% were primigravid and 13.4% had anaemia at delivery. Babies' mean birth weight was 3020 ± 410 g (mean ± SD). Fifteen newborns (11.2%) had a low birth weight (≤2500 g), 9 (6.7%) were born premature.

There were 41 (30.6%) women in whom infections before the third trimester of pregnancy were identified, whilst 24 (17.9%) were infected during the third trimester of pregnancy (but not within 10 days of delivery), and 29 (21.6%) were infected in the 10 days prior to or at delivery. Twenty-one women (15.7%) had an

infected placenta as determined by placental impression smear. Seven infants (5.2%) were infected during the first 3 months of life, 13 (9.7%) were infected between 3 and 6 months of age and 34 (25.4%) between 6 and 12 months of age.

Age-dependent maturation of TLR-mediated cytokine responses

Whole blood samples from 134 infants were stimulated with TLR agonists at birth (cord blood) and at 3, 6 and 12 months of age (peripheral venous blood). At all time-points the production of IL-6, IL-10 and TNF-α by agonist-stimulated cells was higher than the amounts spontaneously secreted by unstimulated control samples (**Table 2, Figure 1**). Conversely, only very limited TLR-mediated IFN-γ production above unstimulated levels was observed regardless of time-point, with the highest values seen in response to the TLR7/8 agonist R848 (**Table 2, Figure 1**). Overall, the latter agonist induced the highest levels of all cytokines, whilst the TLR9 agonist, CpG Type A, induced comparatively the weakest cytokine responses at all time-points.

The concentrations of cytokines spontaneously released by unstimulated cells increased with age: univariate analyses showed that the amounts of IL-6 and TNF-α increased significantly from birth to 6 months of age (all $p<0.001$) and of IL-10 from birth to 3 months of age ($p<0.001$) (**Table 2, Figure 1)**. Consequently, analyses of the age-dependent maturation of TLR agonist-mediated cytokine responses were conducted after subtraction of cytokine concentrations in supernatants of unstimulated cells from those in supernatants of stimulated cells.

The cytokine responses to the TLR3, TLR4 and TLR7/8 agonists exhibited similar age-related patterns, characterised by robust secretion of IL-6 and IL-10 at birth that either remained stable over time (TLR7/8) or diminished significantly with increasing age (TLR3 & 4, all $p<0.04$ by Kruskal-Wallis, **Figure 1**). In contrast, TNF-α and IFN-γ production in response to these three agonists was weak at birth then increased significantly to reach plateau levels 3-6 months of age (all $p<0.001$ by Kruskal-Wallis, **Figure 1**). In all cases, TLR9 agonist-mediated cytokine responses in cord blood were low but subsequently increased with age, with significantly enhanced production of IL-6, IL-10 and TNF-α (all $p<0.001$ by Kruskal-Wallis, **Figure 1)**.

In multivariate analyses that adjusted for potential confounders (maternal anaemia, premature birth, maternal/infant infection), the various profiles of age-dependency of TLR agonist-mediated cytokine responses described above were confirmed (**Table 3**).

Maternal P. falciparum infection at delivery affects the profile of TLR agonist-mediated cytokine responses in infants

We next examined whether *P. falciparum* infection during pregnancy influenced TLR-mediated cytokine responses in the offspring using segregation into 3 gestational age-related intervals [(i) infection before the

third trimester of pregnancy, (ii) infection during the third trimester of pregnancy but more than 10 days before delivery, (iii) infection from 10 days before delivery up to delivery].

Univariate analyses revealed no differences in spontaneous cytokine release by cells from infants born to mothers with different infection histories (**Table 2**), but did show that TLR ligand-mediated cytokine release by infants' cells was modulated by maternal infection occurring either during the 3rd trimester or close to/at delivery. The changes related to infection close to/at delivery concerned significantly increased production of (i) IL-6, IL-10 and TNF-α upon TLR3 stimulation, (ii) IL-6 upon TLR4 stimulation and (iii) of IL-10 upon TLR9 stimulation, in comparison with cells of children born to mothers who were uninfected at delivery (**Table 2**). There were also associations of borderline significance between infections late in pregnancy and increased TNF-α production upon TLR4 and TLR7/8 stimulation (**Table 2**), as well as between infections earlier during the 3rd trimester and decreased IL-10 production following TLR4, TLR7/8 and TLR9 stimulation (data not shown). The concentration of IFN-γ in supernatants of cells stimulated with TLR3, TLR4 and TLR9 agonists were too low to identify differences in secretion of this cytokine whilst no differences were observed in infants' TLR7/8–induced production of IFN-γ when segregated according to maternal infection history (data not shown).

The multivariate analyses of infants' TLR-mediated cytokine secretion as a function of maternal infection history were designed to evaluate whether maternal infection affected either spontaneous or TLR-mediated cytokine responses in infancy whilst adjusting for potential confounding co-variables identified in the univariate analyses, i.e. maternal anaemia, premature birth, age of the infant. Using the profiles obtained with those born to uninfected mothers as reference values, the associations that remained significant following these multivariate analyses concerned only infections occurring close to/at delivery and not those earlier in pregnancy. In terms of spontaneous release of cytokines, the production of both IL-10 and TNF-α was enhanced in cord blood whilst that of IL-6 was reduced at 6 months of age (**Table 3**). In the context of TLR3 stimulation, the association between maternal infection at delivery and significantly enhanced production of IL-6, IL-10 and TNF-α found in univariate analyses remained, with higher production (i) of IL-6 at birth and at 3 months (ii) of IL-10 at three months and (iii) of TNF-α at 6 months of age, compared to those born to mothers who were uninfected at delivery (**Table 3**). Similarly, for TLR7/8 stimulation, the association between maternal infection at delivery and significantly enhanced production of TNF-α (at 6 months of age) remained, as did the association for increased TLR9 agonist-mediated IL-10 production - at 12 months of age - in parallel with increased TNF-α (**Table 3**), although production of the latter cytokine was significantly lower in infants aged 6 months. In marked contrast to the altered responses to TLR3/7-8/9 stimulation, multivariate analyses revealed no significant differences in TLR4-mediated cytokine responses of infants when segregated according to maternal infection history.

A graphical depiction of the multivariate model-derived predicted i.e. coefficient-derived TLR-mediated cytokine responses of infants as a function maternal infection close to/at delivery is shown in **Figure 2.** The

overall picture this gives is one of maternal infection-related enhancement of TLR3-mediated production of both IL-6 and IL-10 at birth and at 3 months of age, and enhancement of TLR3-, TLR7/8- & TLR9-mediated TNF-α and IL-10 production at 6 or 12 months of age.

TLR-mediated cytokine production in cord blood as a predictor of P. falciparum infection in infants

We next wished to ascertain whether the TLR-mediated cord blood cell cytokine responses shown to be altered or not as a result of maternal infection were predictive of *P. falciparum* infections during infancy. Univariate analyses showed that high IL-10 production, following stimulation of TLR3, TLR4 or TLR7/8 in cord blood, as well as high TNF-α production following TLR7/8 stimulation, was associated with an increased risk of malaria during the first year of life (**Table 4**). Multivariate analyses confirmed the independent associations for an increased risk of *P. falciparum* infection in infancy with high TLR3- and with high TLR7/8-mediated cord blood cell IL-10 production (**Table 4**).

P. falciparum infection in infants influences TLR agonist-mediated cytokine response profiles

Infants' innate immune responses, whilst having been 'conditioned' *in utero* as a result of maternal *P. falciparum* infection, will also be potentially altered by *P. falciparum* when the infant itself is infected. Our next step was therefore to incorporate the prospectively-collected data on *P. falciparum* infections in the first year of life into the multivariate model of TLR-mediated cytokine responses. In order to control for the possible influence of passively acquired (maternal antibody-mediated) anti-plasmodial immunity, infection and/or malaria episodes in infants were segregated into three different time-periods for the assessments of independent associations with either spontaneous or TLR agonist-mediated cytokine responses. Regardless of age, infection arising during infancy had no observable effect on spontaneous cytokine release (**Table 2**), but univariate analyses did show significantly increased TLR7/8-mediated IL-10 as a function of infection after 6 months of age, and significantly decreased TLR9-mediated IL-10 production related to infection between birth and 3 months of age (**Table 2**). Multivariate analyses confirmed both those associations, whilst also revealing independent associations with infection between birth and 3 months of age for increased TLR7/8-mediated IL-6, as well as for decreased TLR9-mediated TNF-α production **(**data not shown).

Influence of maternal anaemia, gravidity, baby gender & prematurity on the profile of TLR-mediated cytokine responses over time

In univariate analyses, maternal anaemia at delivery was associated with increased spontaneous release of IL-6 and with decreased spontaneous release of TNF-α (both $p<0.05$), but not with any change in TLR-mediated cytokine production (data not shown). In multivariate analyses, neither of the associations with

anaemia remained. In univariate analyses, no cytokine production of any type was affected either by gravidity status (primi- versus multi-gravid) or by the baby's gender (data not shown).

Univariate analyses of data from those born premature (delivery <37 weeks) revealed significantly elevated levels of IL-6, IL-10 and in response to TLR9 ligation (data not shown). Multivariate analyses confirmed the independent influence of being born premature with increased TLR9-mediated TNF-α production as well as with increased TLR4-mediated IL-10 and increased TLR7/8-mediated IFN-γ compared to full-term babies (all $p<0.05$, data not shown).

Discussion

The published evidence of *in utero* exposure to *P. falciparum* resulting in long-term changes to the immunological responses of infants is limited, to the best of our knowledge, to a single prospective birth cohort study that documented altered parasite antigen-specific responses that persisted through childhood (23). In parallel, epidemiological studies including our own, have clearly documented the altered susceptibility of infants to *P. falciparum* infection or disease (11-16). Whether and how specific components of the innate immune system of such 'tolerant' infants may contribute to their apparent inability to control the parasite has not been documented. The study reported here is thus the first prospective birth cohort study to address this issue by investigating in detail the effect of both the presence and timing of maternal *P. falciparum* infection on an essential component of innate immunity in early life, namely TLR-mediated cytokine responses.

The overall age-related patterns of cytokine responses to TLR ligands we observed here in infancy are consistent with those reported in the only study of its kind in sub-Saharan African (Gambian) infants published to date that used whole blood and a short-term (18-24hr) culture period i.e. conditions almost identical to those we used (28). The principal similarities include the presence of robust IL-6 and IL-10 responses at birth and of pro-inflammatory TNF-α and IFN-γ responses that increased markedly from birth through the first 3 months of life. In contrast to the Gambian study, we did not detect appreciable TLR7/8-mediated production of IFN-γ at birth. This difference most likely relates to the fact that we used just a single dual TLR7/8 agonist (R848) compared to the panel of 3 different agonists used in the Gambian study that allowed the identification of fine differences in TLR7- versus TLR8-mediated activity, differences our study could not distinguish. Also, in the same context of TLR7/8-mediated activity, we did not find the same dramatic age-related increase in the amounts of IFN-γ produced, again a difference that may be explained by the choice of agonist. Nevertheless, and as seen both in Caucasian (47, 48) and Gambian infants (28), we did find that R848 was the agonist that consistently induced the strongest pro-and anti-inflammatory cytokine responses in Beninese infants, whilst responses induced by the TLR9 agonist were relatively weak. Cytokine

production in response to the TLR9 agonist nevertheless increased from birth up to 6 months of age, a pattern not seen in the comparable studies of European or African infants (28, 49). These disparate findings may be explained (i) by the comparatively broad target populations of R848 that can stimulate pDC, mDC, monocytes and B cells (50), and (ii) the TLR9 ligand we used (ODN CpG 2216) that exerts selective activity only on pDC (46) as opposed to both pDC and B cells (28, 49). Our data also confirm published observations of a decrease in the production of IL-10 in response to TLR4 agonists post-natally (29).

In the context of the principal focus of our study i.e. maternal infection-related changes to neonatal/infant TLR-mediated responses, the most notable finding is that exposure to *P. falciparum in utero* just prior to or at delivery significantly modulated infants' subsequent cytokine responses arising specifically from ligation of the endosomally-expressed TLR3, 7/8 & 9, whereas responses arising from ligation of the surface-expressed TLR4 were unaffected. All of the observed changes involved increased production, either early or late in the first year of life, of both pro-(IL-6, TNF-α) and anti-(IL-10) inflammatory cytokines. The same exposure *in utero* also led to significantly increased spontaneous secretion of both TNF-α and IL-10 by cord blood cells, but reduced IL-6 secretion by 6 month old infants' cells. As has been noted by others (51), the relative predominance of the Th17 cell-promoting cytokine IL-6 along with IL-10 in infants' TLR-mediated cytokine repertoire almost certainly contributes to the well-documented deficiency in Th1 cell-type (IFN-γ-led) responses (25). The latter are responses that are commonly considered to be an essential component of anti-malarial immunity (52). Thus, maternal infection and consequent foetal exposures that reinforce TLR-mediated IL-6 & IL-10 responses in early life, rather than inducing a switch to a 'mature' Th1-type of cytokine response, could quite plausibly be implicated in enhancing infants' susceptibility to *P. falciparum* infection. That enhanced IL-6 activity may indeed have such effects is supported by the fact that (i) *P. falciparum*-induced IL-6 is known to be associated with susceptibility to malaria in children (53), and (ii) several studies have reported the circulating concentration of IL-6 to be significantly higher in children with severe malaria (54). The results of the prospective assessment of cord blood cells' TLR-mediated cytokine profiles we conducted here provide support for a similarly detrimental effect of IL-10 (Table 4).

Three recent studies have substantially expanded the knowledge of the interactions between *P. falciparum* and TLR. All revealed that, somewhat unexpectedly, exposure to the parasite *in vivo* or *in vitro* leads to a form of pro-inflammatory priming of TLR-mediated responses, although, notably in the context of the findings we report here, no significant change in parasite-induced TLR3-mediated cytokine responses was found (34, 35, 55). Paradoxically, in this context, our data clearly identify enhanced TLR3-mediated cytokine responses in early life as one of the major modified outcomes of exposure to *P. falciparum in utero*. Current knowledge nevertheless gives no indication of a parasite-derived TLR3-specific ligand that may be at the origin of this effect (56, 57). These findings are particularly striking because the significantly

increased TLR3-mediated IL-6 and IL-10 production by exposed infants' cells at birth and/or at 3 months of age runs exactly counter to the significant age-related decline in production of these same two cytokines occurring over precisely the same time-period (Figure 1). We also observed significantly increased TLR3-mediated TNF-α production by 'exposed' 6 months old infants' cells, but this, in contrast to IL-6 and IL-10, is on a background of an increasing age-related production profile. Pertinent perhaps here is the fact that TLR3, amongst the panel of TLR that we investigated, is the only one expressed within T lymphocyte populations as well as in a prominent APC population (mDC), possibly implicating maternal infection-induced up-regulation of TLR3 expression by foetal T cells. Further, we employed a TLR3 ligand – polyI:C – that is also known to stimulate non-TLR pattern recognition receptors in mDC (39). We did not detect the pattern of TLR3-/4-mediated reduced TNF-α and enhanced IFN-γ production by cord blood cells associated with placental infection at delivery reported in a Gabonese study by Adegnika and colleagues (33). Although the agonists used were the same in both studies, the substantially different culture conditions used (purified cord mononuclear cells cultured for 3 days versus whole blood for 24hr) possibly explain the different outcomes.

Our findings relating to infants' TLR7/8-mediated cytokine responses, although comparatively less pronounced in scope, echo those for TLR3 in the sense that, here again, none of the relevant published studies have thus far identified a *P. falciparum*-derived ligand for either TLR7 or 8. Despite this, maternal infection at delivery was associated with enhanced TLR7/8-mediated TNF-α responses in 6-month old infants, as was also the case for TLR3. In the case of TLR9, for which there is a recognized parasite-derived ligand (57), the enhancing effects of maternal infection on cytokine responses in early life were even more prolonged, with significantly stronger IL-10 and TNF-α production apparent in 12-month old infants.

The fact that only maternal *P. falciparum* infections occurring close to or at delivery affected the innate immune responses we measured, whilst infections during the second or third trimester of pregnancy appeared to have no such influence, is intriguing for two main reasons. Firstly, in cord blood innate immune (γδ) T lymphocyte subsets we have previously documented distinct 'activated' phenotypes *ex vivo* associated with maternal infections that were successfully treated earlier in pregnancy but not with infections detected at delivery (58). These findings suggest that different components of the foetal innate immune response may be affected differently according to the timing, type or duration of exposure to parasite-derived molecules. Secondly, our data imply that plasmodial infections acquired by mothers very late in pregnancy can have sustained effects on infants' innate immune responses, raising the question of the possible implication of such changes in the increased frequency of non-plasmodial febrile illnesses with which such infants present (16).

Two other aspects of our results also deserve mention. Firstly, we identified the impacts of *P. falciparum* infections occurring early (0-3 months of age) or late (6-12 months of age) during infancy that included, respectively, enhanced IL-6 or enhanced IL-10 responses after TLR7/8 ligation, but, for infections occurring early in infancy, reduced IL-10 and TNF-α responses after TLR9 ligation. Inappropriately elevated IL-10 activity may contribute to sustaining infants' susceptibility to infection by suppressing Th1-type responses, and, similarly, impaired responses to TLR9 activation may lead to increased susceptibility to pathogens that bind TLR9, such as DNA viruses (59-62). Secondly, we observed for the first time that, upon TLR4 stimulation, premature newborns produced more IL-10, and also, upon TLR7/8 or TLR9 stimulation, more IFN-γ or TNF-α, respectively. These data suggest up-regulation of the MyD88-dependent pathway in pre-term infants, which contrasts with previous findings in a non-African population (63).

In conclusion, we report the first prospective study to examine the influence of pregnancy-associated malaria on the maturation of TLR responses in infants. Notably, our results show the profound effects of maternal infection close to or at delivery on an infant's innate immune responses, and indicate that some of the immunological consequences of PAM we measured here are long-lasting. Further studies are needed to better define the consequences of the increased production of the 'pro-inflammatory' cytokines TNF-α and IL-6 on adaptive T cell-mediated immunity and of the 'suppressive' cytokine IL-10 on the development of regulatory T cells during the first year of life. In this same STOPPAM study we have found that the frequency of circulating Treg during infancy of those born to mothers with placental infection at delivery is consistently higher than in infants born to uninfected mothers (Fievet N et al, unpublished observations), suggesting but not proving causality vis-à-vis enhanced susceptibility to *P. falciparum* infection. How specific cells and the cytokines they produce in early life may also separately influence infants' responses to routine infant and/or candidate malaria vaccines is an area that needs to be urgently addressed.

Abbreviations

APC: (antigen presenting cell); ANV: (antenatal visit); BD: (Becton Dickinson); CBA: (cytometric bead array); CBMC: (cord blood mononuclear cell); CERPAGE: research center for pregnancy associated malaria and children; CPDA: (citrate phosphate dextrose adenine); CpG ODN: cytosine phosphodiester guanine oligodeoxynucleotide); DC: (dendritic cell); ERK: (extracellular signal-regulated kinases); HIV: (human immunodeficiency virus); IFN-γ: (interferon gamma); IL-6: (interleukin-6); IL-10: (interleukin-10); IRD: (Research Institute for Development); LMM: (linear mixed models); LPS: (lipopolysaccharide); mDC: (myeloid dendritic cell); MyD88: (myeloid differentiation 88); pDC: plasmacytoid dendritic cell); PAM: (pregnancy associated malaria); *P. falciparum*: (*Plasmodium falciparum*); Poly I:C: (Polyinosinic:polycytidylic acid); R848: (resiquimod); RDT: (rapid diagnostic test); STOPPAM: (Strategies

TO Prevent Pregnancy Associated Malaria); TBS: (thick blood smear); TLR: (Toll-like receptor); TNF-α: (tumor necrosis factor alpha).

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Authors' contributions

KG, SV, SI, AM, PD, MTB, NF, and AJFL conceived, designed and coordinated the study. KG, SI, SE, ON, participated in the sample collection and processing. KG, SI, SV, NF and AJFL designed and supervised the immunoassays. PH and GC performed statistical analysis. KG, SI, ON, SE, and AA carried out the immunoassays. KG, SI, NF and SV drafted the first version of the manuscript. All authors read and approved the final manuscript.

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776 Table 1. Characteristics of the mother-infant pair study population (n=134)
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Mother		**n (%)**
Age (years)	≤ 20	29 (21.6)
	21-25	43 (32.2)
	26-30	29 (21.6)
	≥ 31	33 (24.6)
Gravidity status	Primigravid	25 (18.7)
	Multigravid	109 (81.3)
Anemia at delivery (Hb<11g/dl)		18 (13.4)
Infected before 3rd trimester of pregnancy		41 (30.6)
Infected during 3rd trimester of pregnancy up until 11 days before delivery		24 (17.9)
Infected 10 days or less prior to or at delivery		29 (21.6)
Infected placenta		21 (15.7)
No sign of infection		40 (29.9)
Infant		
Female		64 (47.8)
Premature (gestational age ≤ 37 weeks)		9 (6.7)
Residence	Rural	35 (26.1)
	Semi-rural	99 (73.9)
Low birth weight (< 2500 g)		15 (11.2)
Infected before 3 months of age		7 (5.2)
Infected between 3 and 6 months of age		13 (9.7)
Infected between 3 and 6 months of age		34 (25.4)
No sign of infection		80 (59.7)

778

Table 2. Univariate analysis of spontaneous and TLR agonist-mediated cytokine responses in cord/infant whole blood as a function of *P. falciparum* infection detected either in the mother at delivery or during infancy

			TLR3		TLR4		TLR7/8		TLR9		Unstimulated	
			Coef (SD) [A]	p	Coef (SD)	p	Coef (SD)	p	Coef (SD)	p	Coef (SD)	
IL-6	***P. falciparum* (mother)**	at delivery [B]	39697 (18685)	**0.02**	23266 (17497)	**0.04**	15335 (22503)	ns	-8615 (9930)	ns	-0,2 (0,4)	ns
	***P. falciparum* (infant)[C]**	M0-M3	-7969 (35165)	ns	-18425 (32309)	ns	64449 (40690)	***0.06***	-16961 (17797)	ns	-0,7 (0,8)	ns
		M4-M6	-13092 (26431)	ns	-31224 (24277)	***0.07***	-34977 (30934)	ns	-9364 (13902)	ns	-0,3 (0,6)	ns
		M7-M12	-15297 (17943)	ns	-1602 (16736)	ns	-8256 (21346)	ns	-11586 (9569)	ns	-0,2 (0,4)	ns
IL-10	***P. falciparum* (mother)**	at delivery	623 (283)	**<0.01**	325 (274)	ns	-191 (520)	ns	98 (278)	**0.02**	0,1 (0,4)	ns
	***P. falciparum* (infant)**	M0-M3	136 (534)	ns	-438 (499)	ns	64 (948)	***0.06***	-759 (497)	**0.05**	-0,1 (0,8)	ns
		M4-M6	191 (402)	ns	31 (379)	ns	596 (715)	ns	-301 (389)	ns	0,5 (0,6)	ns
		M7-M12	300 (272)	ns	297 (261)	ns	1133 (483)	**0.03**	159 (267)	ns	-0,4 (0,4)	ns
TNF-α	***P. falciparum* (mother)**	at delivery	1772 (941)	**<0.01**	2005 (1207)	***0.06***	4023 (2807)	***0.08***	-334(921)	ns	0,1 (0,4)	ns
	***P. falciparum* (infant)**	M0-M3	1747(1741)	ns	-1347 (2202)	ns	992 (5080)	ns	-1016(1652)	ns	-0,3 (0,7)	ns
		M4-M6	-298 (1312)	ns	-1194 (1671)	ns	-3363 (3854)	ns	-1777(1288)	ns	0,5 (0,5)	ns
		M7-M12	535 (891)	ns	1268 (1151)	***0.09***	1739 (2666)	ns	1029(885)	***0.08***	0,2 (0,4)	ns

[A] positive/negative coefficients indicate cytokine concentrations above/ below control (uninfected) levels; SD: standard deviation; [B] denotes the influence of infection at delivery or in the time-period ≤ 10 days prior to delivery on neonatal/infant cytokine responses in the first 12 months of life; [C] denotes the influence of infection during different periods of early life on spontaneous or TLR-stimulation mediated cytokine responses over the whole 12 month period ; M0: cord blood, M3, M6, M12: blood drawn at 3, 6 & 12 months of age

Table 3. Multivariate (LMM) analyses of alterations in spontaneous and TLR-mediated cytokine responses in whole blood as a function of infants' age and of maternal *P. falciparum* infection at delivery

			TLR3		TLR4		TLR7/8		TLR9		Unstimulated	
			Regression coef. (SD) [C]	p	Regression coef. (SD)	p	Regression coef. (SD)	p	Regression coef. (SD)	p	Regression coef. (SD)	p
IL-6												
Infant age[A]	3 months		-31335 (15138)	**<0.05**	-1.32 (0.76)	**??**	14919 (22093)	ns	1.79 (0.69)	**<0.01**	2.00 (0.54)	**<0.01**
	6 months		-38365 (15232)	**<0.05**	-2.33 (0.77)	**<0.01**	31892 (21825)	ns	2.84 (0.70)	**<0.01**	3.16 (0.54)	**<0.01**
	12 months		-7910 (14822)	ns	-1.60 (0.75)	**<0.05**	34536 (21579)	ns	1.38 (0.68)	**<0.05**	2.00 (0.53)	**<0.01**
P. falciparum	at delivery[B]	& M0	75243 (27539)	**<0.05**	1.25 (0.99)	ns	-6932 (36838)	ns	1.03 (1.03)	ns	0.11 (0.81)	ns
maternal		& M3	58659 (28714)	**<0.05**	-0.75 (1.15)	ns	9411 (38250)	ns	0.10 (1.07)	ns	-0.66 (0.85)	ns
infection		& M6	33481 (28406)	ns	0.98 (1.19)	ns	40462 (37592)	ns	-1.83 (1.07)	ns	-1.19 (0.83)	**0.05**
		& M12	8772 (27539)	ns	0.64 (1.19)	ns	-16047 (36470)	ns	0.63 (1.02)	ns	0.82 (0.84)	ns
IL-10												
Infant age[A]	3 months		-547 (268)	**<0.05**	-2.05 (0.67)	**<0.01**	-1428 (518)	**<0.05**	2.04 (0.67)	**<0.01**	0.62 (0.40)	ns
	6 months		-1064 (269)	**<0.01**	-1.48 (0.67)	**<0.05**	-664 (512)	ns	3.44 (0.67)	**<0.01**	1.81 (0.40)	**<0.01**
	12 months		-1217 (262)	**<0.01**	-2.34 (0.66)	**<0.01**	-1197 (506)	**<0.05**	0.94 (0.66)	ns	1.04 (0.40)	**<0.05**
P. falciparum	at delivery[B]	& M0	652 (454)	ns	-0.84 (1.01)	ns	-772 (858)	ns	0.85 (1.00)	ns	1.01 (0.69)	**0.05**
maternal		& M3	1300 (476)	**<0.05**	1.84 (1.05)	ns	462 (891)	ns	0.24 (1.03)	ns	-0.10 (0.72)	ns
infection		& M6	328 (470)	ns	0.22 (1.05)	ns	56 (876)	ns	-0.29 (1.03)	ns	-0.65 (0.70)	ns
		& M12	411 (454)	ns	0.89 (1.01)	ns	-337 (850)	ns	2.11 (0.98)	**<0.05**	0.69 (0.71)	ns
TNF-α												
Infant age[A]	3 months		4529 (1129)	**<0.01**	2.15 (0.76)	**<0.05**	21850 (3398)	**<0.01**	3.62 (0.64)	**<0.01**	1.99 (0.46)	**<0.01**
	6 months		2254 (1135)	**<0.05**	2.70 (0.76)	**<0.01**	21817 (3361)	**<0.01**	5.07 (0.64)	**<0.01**	2.83 (0.46)	**<0.01**
	12 months		5171(1107)	**<0.01**	2.32 (0.74)	**<0.01**	23242 (3327)	**<0.01**	2.42 (0.62)	**<0.01**	1.81 (0.45)	**<0.01**
P. falciparum	at delivery[B]	& M0	7.4 (1827.3)	ns	0.38 (1.14)	ns	92 (5147)	ns	-0.79 (0.95)	ns	1.12 (0.74)	**0.05**
maternal		& M3	1004 (1918)	ns	0.99 (1.18)	ns	-1908 (5360)	ns	-0.56 (0.98)	ns	-0.71 (0.78)	ns
infection		& M6	5238 (1894)	**<0.05**	0.45 (1.18)	ns	15856 (5262)	**<0.01**	-2.10 (0.98)	**<0.05**	-0.36 (0.76)	ns
		& M12	491 (1827)	ns	1.36 (1.14)	ns	-679 (5092)	ns	2.85 (0.93)	**<0.05**	0.77 (0.77)	ns

[A] the reference values used for comparison are those recorded in cord blood (M0); [B] denotes the influence of infection at delivery or in the time-period ≤ 10 days prior to delivery on neonatal/infant responses measured at designated time-points; M0: cord blood, M3, M6, M12: blood drawn at 3, 6 & 12 months of age; [C] Positive/negative coefficients indicate cytokine concentrations above/below control (uninfected) levels; SD: standard deviation.

Table 4. Prospective assessment of the predictive value of TLR agonist-mediated cytokine production in cord blood for infection with *P. falciparum* in the first year of life

		Univariate			Multivariate[A]		
		OR	CI 95%	p-value	Adjusted OR	CI 95%	p-value
TLR3	IL-6	1.17	[0.97; 1.42]	0.09	1.07	[0.88 ; 1.30]	0.50
	IL-10	1.24	[1.03; 1.49]	0.02	1.22	[1.00; 1.50]	**0.05**
	TNF-α	1.04	[0.94; 1.16]	0.44	0.98	[0.88; 1.09]	0.75
TLR4	IL-6	1.23	[0.96; 1.59]	0.09	1.12	[0.84 ; 1.48]	0.43
	IL-10	1.24	[1.01; 1.54]	0.04	1.19	[0.93; 1.54]	0.16
	TNF-α	1.00	[0.90; 1.12]	0.93	0.95	[0.85; 1.06]	0.37
TLR7/8	IL-6	1.13	[0.95; 1.35]	0.15	0.94	[0.79 ; 1.12]	0.46
	IL-10	1.40	[1.06; 1.86]	0.01	1.38	[1.00; 1.90]	**0.04**
	TNF-α	1.18	[1.03; 1.35]	0.01	1.11	[0.97; 1.27]	0.11
TLR9	IL-6	1.00	[0.89; 1.12]	0.95	0.94	[0.80; 1.11]	0.48
	IL-10	1.02	[0.90; 1.14]	0.80	0.96	[0.82; 1.14]	0.66
	TNF-α	1.10	[0.97; 1.24]	0.12	1.17	[0.99; 1.38]	0.06

[A] Adjusted on *P. falciparum* infection history of mother, gravidity, infant age, low birth weight

Note: the concentration of IFN-γ in supernatants of cells stimulated with TLR agonists was too low to allow appropriate analyses

Figure 1 Spontaneous and TLR agonist-mediated cytokine production in neonatal/infant whole blood. In each case, agonist-mediated responses presented have had the corresponding cytokine concentrations in supernatants of unstimulated cells subtracted. Box-plots illustrate medians with 25th & 75th percentiles and whiskers for 10th & 90th percentiles. M0: cord blood; M3, M6, M12: peripheral venous blood at 3, 6, 12 months of age. Unsti: unstimulated samples. The statistical significance of differences in cytokine activity detected at different ages was determined using the non-parametric Kruskall-Wallis test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

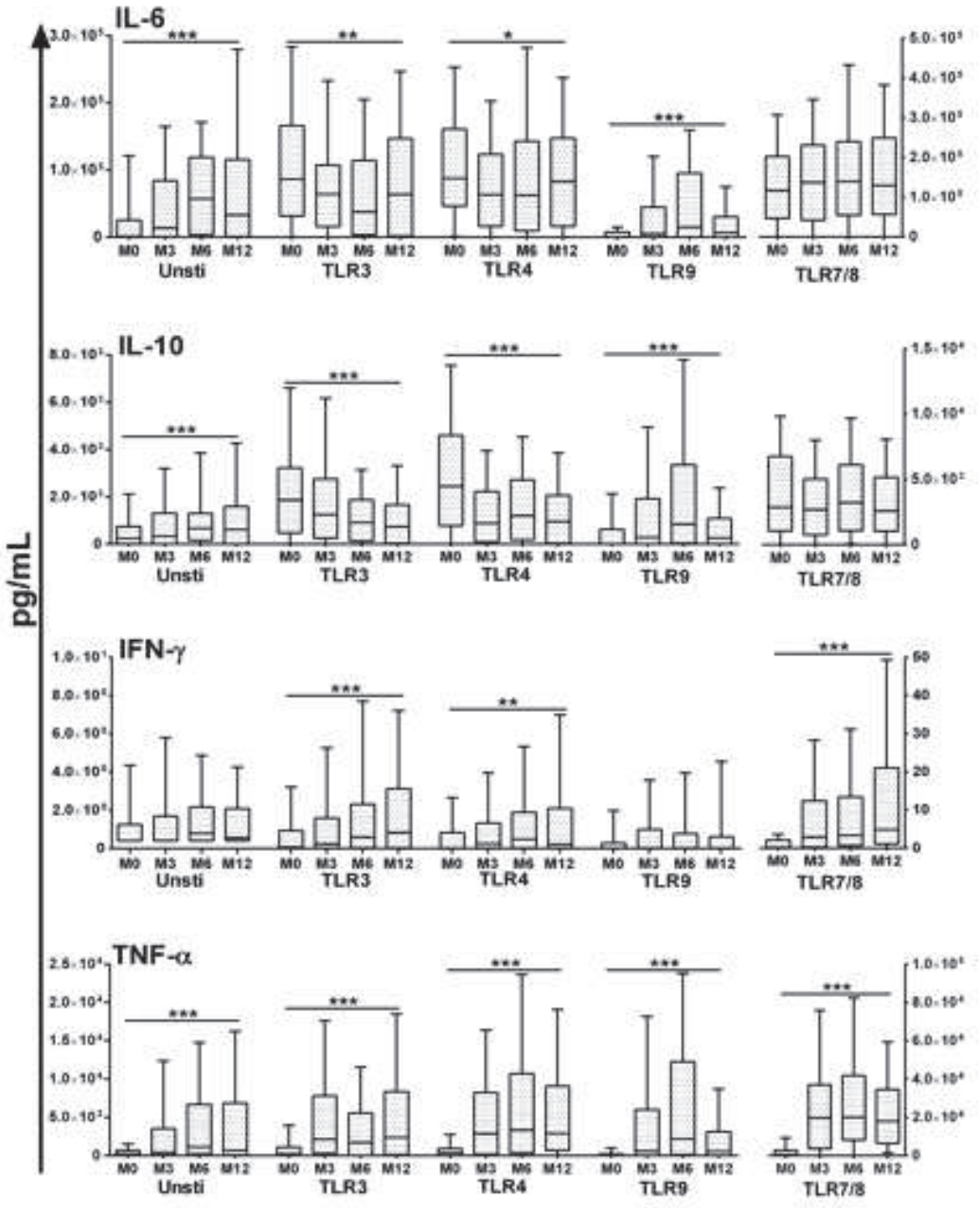
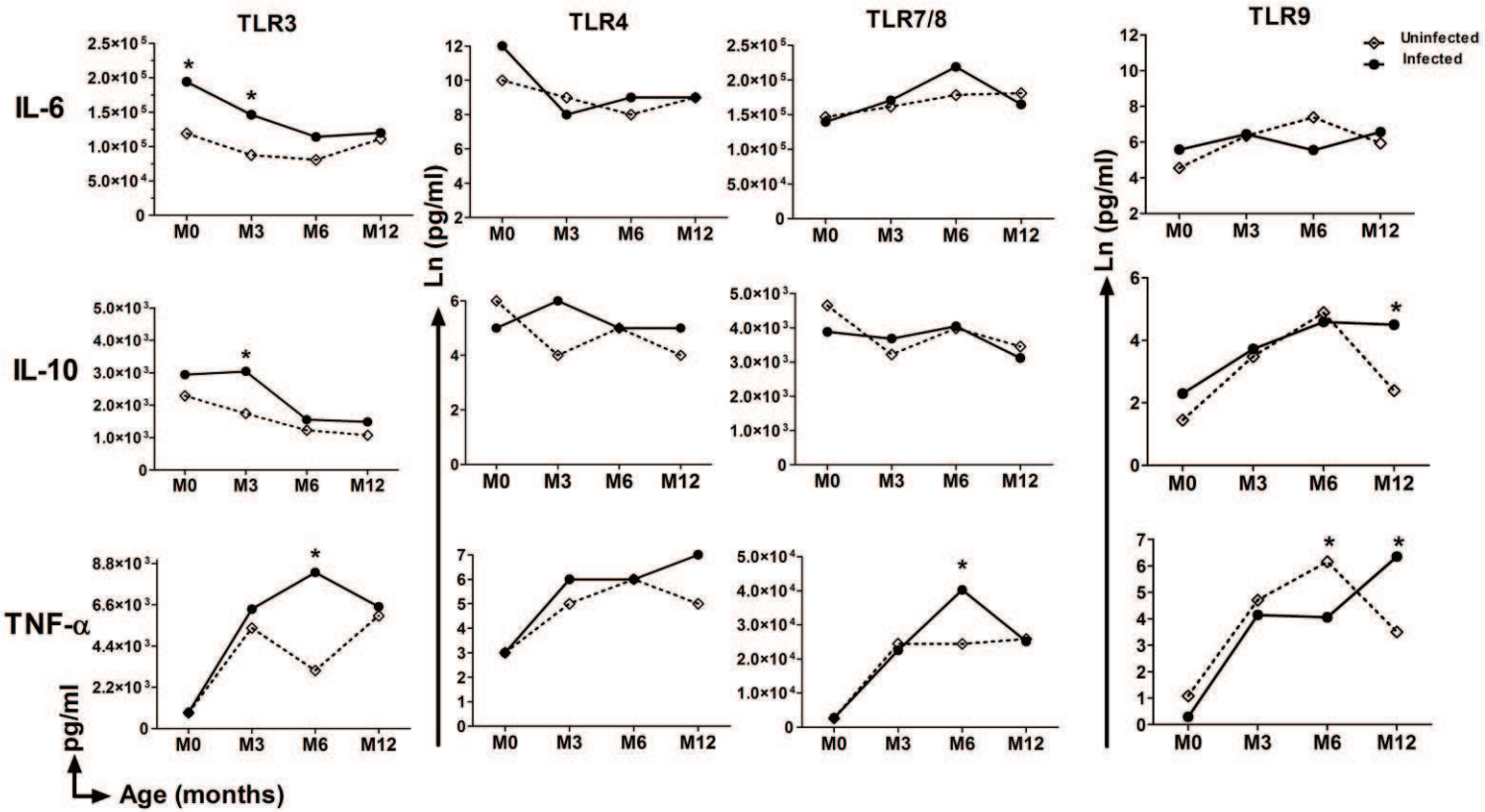


Figure 2 GLMM-based predictive profiles of TLR agonist-mediated cytokine production by neonatal/infant whole blood as a function of the presence or absence of PAM at delivery. M0: cord blood; M3, M6, M12: peripheral venous blood at 3, 6, 12 months of age. The statistical significance of differences was determined using the GLMM model. * $p < 0.05$



DISCUSSION GENERALE ET CONCLUSION

La plupart des études antérieures portant sur les conséquences immunologiques du paludisme associé à la grossesse (PAG) ont été réalisées à l'accouchement, soit sur le sang périphérique ou intervilleux de la mère, soit sur le sang de cordon. L'intérêt majeur de la cohorte STOPPAM a été de permettre d'étudier l'impact du PAG sur les réponses cellulaires à différentes périodes de la grossesse et d'évaluer les conséquences d'une exposition *in utero* au paludisme sur les réponses innées du nouveau-né. Cette thèse apporte des informations complémentaires sur le mécanisme de mise en place des réponses immunitaires chez la femme enceinte impaludée et chez le jeune enfant issu d'une mère exposée à l'infection, à partir d'une cohorte longitudinale de femmes et de leurs enfants.

Nos hypothèses dans cette thèse, sont que i) les infections palustres acquises au cours de la grossesse, induiraient une modulation des réponses cellulaires de la femme enceinte, et, par conséquent, augmenteraient sa susceptibilité aux infections ou l'aggravation d'une pathologie associée à la grossesse, ii) chez les nouveau-nés, une exposition *in utero* à des antigènes palustres pourrait altérer la mise en place des réponses immunitaires néonatales. La compréhension du mécanisme d'orientation des réponses immunes au cours du PAG, permettra d'apporter de nouvelles informations à prendre en compte dans la recherche vaccinale sur le PAG.

Au cours du projet STOPPAM, 1037 femmes enceintes ont été incluses au Benin et 1000 en Tanzanie et ont fait l'objet d'un suivi clinique et parasitologique rigoureux et régulier du début de la grossesse jusqu'à l'accouchement avec en moyenne cinq consultations prénatales et consultations d'urgence. L'originalité de cette cohorte est la réalisation de 4 échographies pendant la grossesse qui a permis de déterminer le terme exact et de suivre la croissance fœtale. L'étude des réponses immunitaires sur l'ensemble de la cohorte STOPPAM était impossible en raison du coût prohibitif des réactifs. Par conséquent, nous avons choisi de sélectionner des sous-échantillons de femmes à l'inclusion et à l'accouchement et des enfants suivis. A l'inclusion et à l'accouchement nous avons ajusté le groupe de femmes infectées à un groupe témoin de femmes non-infectées, en tenant compte de l'âge et de la gestité. Ces groupes sont restés représentatifs de la cohorte générale sur les deux sites, ce qui est important pour la généralisation de nos conclusions. Par ailleurs, nous avons souhaité réaliser les différents phénotypages cellulaires sur cellules fraîches afin de préserver les populations cellulaires très faiblement représentées dans le sang périphérique, telles que les DC et les Treg. Nos comparaisons concernent donc des groupes de femmes différentes au deuxième trimestre et à l'accouchement. Cependant, dans un sous-échantillon de 24 femmes, nous avons pu comparer avec des tests appariés, les réponses cellulaires au second trimestre et à l'accouchement, deux périodes immunologiquement différentes [65].

Les femmes enceintes n'ont pas déclaré leur grossesse dans un centre de santé avant la fin du premier trimestre. L'un de nos critères d'inclusion était l'âge gestationnel inférieur à 24 semaines. L'âge gestationnel médian dans notre population d'étude était de 17 semaines, ce qui veut dire que notre « début » de la grossesse est équivalent au début du second trimestre [209]. Les infections à l'inclusion, sont considérées comme des infections chroniques, alors que les infections à l'accouchement sont considérées comme récentes. Ceci s'explique par le fait que les femmes enrôlées à l'inclusion ne prenaient pas de traitements alors que des traitements ont été administrés systématiquement aux femmes en cas d'infection au cours du suivi STOPPAM. Malgré nos efforts pour inviter les femmes à consulter plus tôt, les informations sur l'état immunologique au début ou au cours du premier trimestre de la grossesse n'ont pas été accessibles.

La grossesse constitue un état physiologique caractérisé par une immuno-modulation du système immunitaire de la femme enceinte, qui favorise la tolérance de l'allogreffe fœtale. Différents mécanismes immunologiques et hormonaux caractérisent les trois phases de la gestation. Dans le groupe de femmes appariées, nous avons montré une diminution significative des fréquences de cellules T $CD4^+$, et plus particulièrement des lymphocytes T effecteurs, entre le second trimestre et l'accouchement, indépendamment de la survenue d'une infection palustre. Nos résultats vont dans le même sens que ceux rapportés chez les femmes caucasiennes montrant une lymphopénie au cours de la grossesse [228]. Par contre, des données récentes sur les femmes danoises ne montrent pas de différence dans les populations lymphocytaires [223]. Ces résultats suggèrent qu'au cours de la grossesse non pathologique, la diminution de lymphocytes T $CD4^+$ et Teff serait liée au mécanisme de tolérance qui caractérise la grossesse. Cette observation soulève cependant la question de la capacité fonctionnelle de ces cellules au cours de la gestation. Par ailleurs, nous n'avons pas observé de fréquence élevée de Treg au second trimestre comparé à l'accouchement, tel que cela avait été montré dans l'étude de Kolte et coll. (2011) [223]. La principale différence entre notre étude et celle de Kolte peut se traduire par la situation géographique des deux populations d'étude. Nous avons travaillé au sein des populations Africaines vivant en zone endémique de plusieurs pathologies telles que les helminthiases et le paludisme, qui pourraient induire des fréquences importantes de lymphocytes Treg alors que l'étude de Kolte a été réalisée chez les femmes danoises. Des fréquences élevées de mDC exprimant le CD86 ont été observées par Bachy et coll (2008), chez les femmes enceintes de plus de 37 semaines de gestation [221]. Cette observation est manquante dans notre étude. Les différences avec nos résultats pourraient s'expliquer par le type d'analyse effectuée. Dans notre étude nous avons fait des analyses appariées sur 24 femmes enceintes alors que l'étude de Bachy a utilisé les analyses non paramétriques sur 17 femmes enceintes sélectionnées au troisième trimestre de gestation. Des études réalisées au premier, second et troisième trimestre de grossesse, ont montré des fréquences

élevées de pDC, exprimant le CD86 [222] et, d'autre part, une diminution de pDC au second trimestre comparée au troisième trimestre [229]. Nos résultats sont en désaccord avec ces observations et les effectifs et le type d'analyse effectuée pourraient en être la raison. Nos observations concernent également des différences dans les fréquences de monocytes en fonction de l'âge gestationnel, tel que cela a été rapporté [230]. Nous pouvons retenir que les grossesses non pathologiques chez les femmes enceintes sont caractérisées par une diminution de fréquences de lymphocytes T CD4 et Teff dans notre population d'étude.

Les conséquences immunologiques liées au calendrier de l'infection palustres sont encore mal connues. Nous avons montré que l'infection palustre au second trimestre, était associée à une augmentation de la fréquence de monocytes immatures, c'est-à-dire exprimant faiblement le CMH-II et le CD86 [80]. Des résultats similaires ont été rapportés chez des enfants avec des signes cliniques du paludisme [202], ce qui serait un indicateur de la mise en place des réponses inflammatoires dans les organes profonds [80]. De plus, le consensus sur la présence des concentrations élevées de monocytes et de macrophages dans les espaces inter-villeux du placenta au cours du PAG existe [133, 153, 220], ce qui renforce l'hypothèse de leur recrutement vers le placenta [7]. Chez les mêmes femmes, nous avons observé des concentrations élevées de chimiokines IP-10 à l'inclusion et MCP-1 à l'accouchement. Ces dernières ont des propriétés chémoattractantes importantes dans l'accumulation des monocytes vers les sites d'infection, notamment le placenta. Des concentrations élevées de MCP-1 et d'IP-10 ont été observées dans le placenta infecté par *P. falciparum* [136, 138, 157]. Ces chimiokines ont une affinité spécifique pour les glycosaminoglycanes (GAG) [231-233]. La chondoïtrine sulfate A (CSA) est une GAG présente dans le placenta dont le rôle dans l'adhésion aux chimiokines reste encore à déterminer. Nos résultats suggèrent que les infections chroniques présentes au second trimestre de la grossesse seraient associées à une immaturité de monocytes dans le sang périphérique et par conséquent à leur migration vers le placenta alors qu'à l'accouchement, caractérisé par les infections aigües, le processus de recrutement de monocytes ne serait pas encore établi.

Nous avons montré une diminution des fréquences de mDC exprimant le CMH-II, chez les femmes infectées au second trimestre. Ce résultat pourrait indiquer une modulation de cette population cellulaire au cours du PAG. Nous avons aussi observé, que l'infection palustre à l'accouchement était significativement associée à une diminution de fréquence des pDC. Ce résultat confirme ceux de Diallo et coll. (2008), qui montrent une altération des pDC à l'accouchement chez les femmes sénégalaises impaludées [139]. Nos observations concordent également avec les études réalisées chez les enfants kényans et chez les adultes thaïlandais infectés [202, 204, 234]. Le récepteur de chimiokines CXCR3, ligand pour le MIG et l'IP-10 est exprimé par les pDC [231].

Des concentrations élevées de MIG et d'IP-10 observées dans notre étude chez les femmes impaludées pourraient traduire le signal de migration des DC du sang périphérique vers les sites d'infection.

Une activation des cellules B (exprimant le CD86) a été observée au cours du PAG au second trimestre et à l'accouchement. Chez les enfants impaludés, des fréquences élevées de cellules B ont été rapportées [235] et restent inchangées dans d'autres études [236]. L'activation de lymphocytes B impliquerait une production d'anticorps ou leur migration vers les sites d'infection. Les lymphocytes B expriment comme les pDC, le TLR9 et le CXCR3. L'activation des cellules B pourrait se faire par les parasites du sang périphérique à travers le TLR9, tel que cela avait été observé chez les pDC [234]. L'expression de CXCR3 par les cellules B pourrait également induire un signal au MIG et IP-10, pour leur migration vers les sites d'infection. Nos résultats suggèrent, qu'au cours du PAG, les parasites de *P. falciparum* induisent l'activation des cellules B à produire des anticorps localement dans le placenta. Il a été montré que, le domaine CIDR1α du *Pf*EMP1 adhère aux IgM et pourrait induire l'activation non-spécifique des cellules B [154, 237]. Dans le cas du PAG, l'adhésion des IgM au VAR2CSA permet aux parasites d'échapper à la phagocytose [155]. Ces observations marquent d'un accent particulier la nécessité de caractériser les domaines du VAR2CSA potentiellement impliqués dans l'activation des cellules B au cours du PAG. Compte ténu du rôle important des cellules B dans la maturation des cellules dendritiques [74], il paraît important d'étudier les interactions entre les cellules B et les DC avant la mise en place d'un candidat vaccin contre le PAG.

Une diminution des fréquences de lymphocytes Treg au second trimestre de gestation, et une augmentation des T effecteurs à l'accouchement, ont été observées chez des femmes impaludées de notre étude. La caractérisation des Treg au cours du PAG est encore peu étudiée. Cependant des fréquences élevées de Treg ont été observées chez des adultes infectés par *P. falciparum* [238] et au cours des grossesses normales au niveau de l'interface materno-fœtale [239]. L'évidence d'une spécificité tissulaire des Treg a été démontrée au cours de la grossesse [240]. En effet les Treg périphériques seraient recrutés par le placenta pour participer à une régulation locale. De la même manière, une diminution des fréquences de Treg a été observée au cours des grossesses pré-éclamptiques et dans les cas d'avortements [163, 164, 241, 242]. Nous n'avons pas enregistré des cas d'avortement et de pré-éclampsie dans notre étude. Nos résultats vont dans le sens d'une migration des Treg vers le placenta pour assurer la régulation. Les concentrations élevées d'IL-10, observées dans notre étude pourraient participer à cette régulation locale. Les fréquences élevées de Teff à l'accouchement seraient liées à l'infection aigüe qui caractérise cette période de la grossesse dans notre cohorte.

Nous avons montré des concentrations élevées d'IL-10, MCP-1, MIG et IP-10 chez les femmes impaludées, indépendamment de l'âge gestationnel, sur les deux sites d'étude. Ces résultats confirment ceux précédemment publiés sur les concentrations plasmatiques d'IL-10 au cours du PAG [136, 146, 148]. Les chimiokines telles que le MCP-1, le MIG et l'IP-10 sont impliquées dans la migration des cellules immunocompétentes vers les sites d'infection et induisent la production d'IFN-γ. Des concentrations élevées de ces chimiokines participeraient au recrutement des cellules immunocompétentes telles que les monocytes, les macrophages les cellules B et les DC vers les sites d'infection au cours du paludisme placentaire [136, 243]. Le rôle de l'IP-10 et du MIG au cours du paludisme et spécifiquement du PAG est encore mal connu. L'IL-10 intervient dans la régulation des réponses immunitaires à travers la suppression et l'inhibition de la production de cytokines telles que l'IL-1β, le TNF-α et l'IL-6 par les macrophages [244]. De plus l'IL-10 inhibe l'activité cytotoxique des macrophages contre les pathogènes [245]. Des concentrations élevées d'IL-10 au cours du PAG favoriserait des parasitémies élevées et par conséquent une susceptibilité à l'infection. La cohorte de Tanzanie a permis de mettre en évidence, que l'augmentation de l'IL-10 et d'IP-10 dépend de l'infection au cours de la grossesse. Ces résultats renforcent le choix de l'IL-10 et d'IP-10 comme bio-marqueurs pour étudier les conséquences du PAG sur les pathologies de la grossesse.

Nous avons recherché par une analyse prospective si les paramètres immunologiques mesurés au deuxième trimestre de la grossesse pouvaient être un facteur de risque de pathologies liées au PAG. Des fréquences élevées de monocytes exprimant le CD86, de cellules B exprimant le CMH-II, associées à des concentrations élevées d'IL-10 et d'IL-1β favoriseraient le risque d'anémie maternelle à l'accouchement. L'IL-10 inhiberait l'érythropoïèse à travers la suppression des facteurs de croissance cellulaire, favorisant ainsi l'anémie [33, 246]. Les monocytes et les cellules B sont impliqués dans les réponses inflammatoires et leur activation induit la production de cytokines telles que l'IL-10, l'IL-12, l'IFN-γ, le TNF-α, l'IL-1β et l'IL-6 au cours du PAG [7, 137]. Une des conséquences de ces activités inflammatoires serait l'anémie maternelle ou le retard de croissance utérin [7, 132].

Au final, nous avons identifié des changements phénotypiques et cytokiniques associés à l'infection à *P. falciparum* et à l'anémie pendant la grossesse. L'association entre l'infection et l'augmentation des fréquences des cellules B, indépendamment de l'âge gestationnel, met l'accent sur le rôle de ces cellules dans l'inflammation placentaire. Nos résultats montrent clairement le rôle des monocytes dans cette réponse inflammatoire et suggèrent que les Treg migrent vers le placenta, site primaire de l'infection à *P. falciparum* pendant la grossesse.

Au second trimestre, nous avons exploré les modifications phénotypiques et cytokiniques liées au PAG lorsque le placenta n'est pas encore accessible en spéculant qu'elles pourraient expliquer celles du sang intervilleux placentaire. Par ailleurs dans la cohorte STOPPAM, les parasites circulant à partir du premier trimestre (<13 semaines de gestation) chez la femme enceinte, présentent déjà le phénotype VAR2CSA [210], suggérant un tropisme placentaire des hématies parasitées très tôt au cours de la grossesse. Cette observation suppose que les résultats obtenus au second trimestre pourraient être semblables à partir du premier trimestre de la grossesse. Par ailleurs, si les modifications phénotypiques et cytokiniques associées à l'infection à *P. falciparum* au niveau placentaire peuvent expliquer ou participer à l'aggravation de pathologies de la grossesse nous ne savons pas si les réponses immunitaires spécifiques vis-à-vis des antigènes parasitaires pourraient être affectées. Le tableau 3 résume l'ensemble des résultats obtenus chez les femmes enceintes.

Un point important dans cette étude, était de savoir si les parasitémies sub-microscopiques suffisent à induire les phénomènes observés chez les femmes enceintes porteuses d'une infection microscopique. Les stratégies de lutte contre le paludisme ont réduit considérablement les niveaux des parasitémies en cas d'infection palustre. Pour étudier ceci, les infections sub-microscopiques ont été recherchées par PCR. Nous avons recherché la part des infections sub-microscopiques dans les sous-groupes de femmes au second trimestre et à l'accouchement. Nous n'avons pas observé d'association entre les infections sub-microscopiques et les changements de fréquences cellulaires ou de concentration de cytokines dans notre population d'étude. Ces observations suggèrent que les changements de fréquences cellulaires et de concentrations de cytokines observés au cours du PAG sont associés à la présence de parasites et non à de l'ADN parasitaire circulant. Ce qui nourrit l'hypothèse d'un seuil de parasitémie minimal nécessaire pour induire des changements de profils cellulaires et cytokiniques au cours du PAG.

Les mécanismes immunologiques liés aux conséquences du PAG sur l'immunité du nouveau-né et du jeune enfant sont peu connus. Dans une étude préliminaire, nous avons montré que la présence de pigment malarique dans les macrophages du sang intervilleux, induit une activation partielle des DC dans le sang de cordon. Cette activation est traduite par une forte expression de CMH-II sur les mDC (BDCA-1^+ DC) et les pDC (BDCA-2^+ DC) alors que l'expression de CD86 reste inchangée. L'expression élevée de CMH-II sur les DC de nouveau-nés exposés au PAG a été également observée dans une étude réalisée au Gabon [193]. Une conséquence de cette maturation partielle des DC serait, un défaut de présentation d'antigène ou d'induction de stimulation suffisante aux lymphocytes T, ceci favoriserait une tolérance vis-à-vis des antigènes parasitaires [247, 248]. Contrairement à nos résultats, chez les jeunes enfants kényans

et les dogons au Mali, infectés par *P. falciparum*, une diminution des fréquences de BDCA-1^+ DC et BDCA-2^+ DC exprimant le CMH-II a été observée dans le sang périphérique. De plus des concentrations élevées de BDCA-3^+ DC ont été rapportées chez ces jeunes enfants contrairement à notre étude [202, 204, 249]. Il a été montré que le contact avec les hématies parasitées (HP) inhiberait la maturation des DC, via les TLR [201, 203]. L'activation partielle des DC du sang de cordon chez les nouveau-nés exposés dans notre étude, serait induite par le passage trans-placentaire d'antigènes solubles vers le fœtus. Nous avons observé des concentrations élevées de TNF-α et d'IL-10, après stimulation du TLR9, dans le sang de cordon de nouveau-nés dont le placenta contenait des macrophages avec du pigment malarique. Seules les pDC et les cellules B expriment le TLR9 chez l'homme [250]. Nous avons utilisé dans notre étude, le CPG-A qui est un ligand spécifique des pDC. L'hémozoïne ou l'ADN parasitaire associé au pigment, active le TLR9 [251]. Nos résultats suggèrent donc que la présence de pigments dans le placenta induit une activation partielle des pDC via le TLR9, traduite par la production d'IL-10 et de TNF-α. Des études plus approfondies sont nécessaires pour étayer cette observation.

Nos résultats ont également montré que les enfants issus de mères âgées de plus de 25 ans ou multipares ont des fréquences de DC et monocytes faibles dans le sang de cordon. Ce résultat met un accent particulier sur l'importance de l'âge maternel, la parité et les fréquences de CPA du sang de cordon et suggère la prise en compte de ces paramètres dans l'étude des réponses immunitaires du nouveau-né au cours du PAG.

Dans la cohorte d'enfants STOPPAM, nous nous sommes intéressés à l'impact d'une exposition au PAG sur la maturation des réponses immunitaires néonatales au cours de la première année de vie. Nos résultats ont montré des concentrations élevées de cytokines anti- et pro-inflammatoires en fonction de l'âge indépendamment de l'infection palustre. Ces résultats confirment ceux de Burl et coll. (2011) sur l'ontogénie des réponses immunitaires néonatales après une stimulation des récepteurs TLR [227]. Les principales similarités entre les deux études concernent les concentrations élevées d'IL-6 et d'IL-10 dans le sang de cordon et l'augmentation de TNF-α et d'IFN-γ à partir de la naissance jusqu'à 3 mois, indépendamment des TLR. Par contre, nous n'avons pas observé de production d'IFN-γ via la stimulation du TLR7/8 tel que cela a été montré chez les jeunes enfants Gambiens. Cette différence serait liée au type de ligand du TLR7/8, utilisé dans notre étude. Nous avons utilisé le R848 ayant une double action alors que dans l'étude de Burl, trois panels de ligands ont été utilisés pour le TLR7 et le TLR8 afin de déceler les différences dans les réponses de ces récepteurs. Comme dans les études réalisées chez les enfants Gambiens [227] et caucasiens [252, 253], nous avons observé que la stimulation du TLR7/8 induit

des concentrations plus importantes de cytokines alors que la stimulation du TLR9 induit des concentrations de cytokines plus faibles.

Pour ce qui concerne l'objectif de notre étude, la principale observation est une modulation des réponses spécifiques aux TLR3, TLR7/8 et TLR9 alors que les réponses spécifiques au TLR9 ne sont pas affectées, chez les enfants issus de mères infectées 10 jours avant ou à l'accouchement. Les modifications observées impliquent la production de cytokines pro-inflammatoires (TNF-α et d'IL-6) et anti-inflammatoire (IL-10) via les TLR3, TLR7/8 et TLR9 chez les nouveau-nés pendant la première année de vie. La production d'IL-10 et d'IL-6 a été associée à un défaut de production d'IFN-γ [173]. De plus une production importante d'IL-6 a été observée chez des enfants avec des accès palustres graves et cette cytokine serait associée à la susceptibilité à l'infection [254, 255]. Chez des jeunes enfants, un défaut de contrôle de la parasitémie a été observé en présence de concentration élevée d'IL-10 [256]. Les mêmes enfants inclus dans notre étude présentent des fréquences élevées de Treg à 3 mois chez les enfants issus de mères infectées 10 jours avant ou à l'accouchement (données non publiées). Nos résultats suggèrent une modulation de réponses pro-inflammatoires chez les jeunes enfants issus de femmes infectées à l'accouchement.

Dans une étude de cohorte réalisée au Kenya chez des enfants exposés *in utero*, il a été observé qu'une stimulation spécifique de cellules T avec des antigènes palustres (MSP-1), induisait des concentrations élevées d'IL-10 et par conséquent participerait à la tolérance des jeunes enfants à l'infection [197]. L'intérêt de notre étude était d'étudier les réponses spécifiques des cellules présentatrices d'antigènes (CPA) aux ligands de TLR. Nos observations suggèrent une altération des réponses spécifiques des CPA à induire la production de cytokines pro-inflammatoires nécessaires au contrôle de l'infection chez les jeunes enfants exposés.

En raison des traitements curatifs administrés aux femmes impaludées pendant le suivi, nous n'avons pas mis en évidence la présence de pigments malariques comme cela avait été le cas dans l'article 5 (Fievet 2009).

Dans une analyse prospective nous avons montré que la production d'IL-10, à travers la stimulation de TLR3 et TLR7/8, était un facteur de risque d'infection palustre chez les enfants exposés à l'accouchement. Ces résultats suggèrent qu'une altération des réponses immunitaires à la naissance prédispose à l'infection au cours de la première année de vie. Les cellules cibles dans cette stimulation sont celles impliquées dans la présentation d'antigènes : monocytes, mDC et pDC. L'altération des récepteurs TLR, tels que le TLR9, augmente la susceptibilité aux infections virales et bactériennes (herpès, ou cytomégalovirus) [257, 258] et pourrait affecter les réponses spécifiques nécessaire à la vaccination [259].

En résumé, le paludisme associé à la grossesse induit des changements qualitatifs et quantitatifs dans le processus de mise en place des réponses cellulaires immunes de la mère et du nouveau-né. La sensibilisation *in utero* à des antigènes et les réponses inflammatoires du placenta à une infection chez la mère pourraient affecter le développement normal du système immunitaire fœtal et conduire à des conséquences physiopathologiques néfastes lors des infections futures du nouveau-né ou plus tard dans la vie. Suite à une infection, les réponses pro-inflammatoires du placenta ont des effets bidirectionnels sur le système immunitaire de la mère et du fœtus en créant un environnement inflammatoire délétère pour la mère et le fœtus. Cela est illustré par des réponses altérées à la vaccination et le développement d'allergies [259]. A l'heure où le développement de nouveaux vaccins applicables dès la naissance apparaît comme une priorité, il est essentiel de mieux comprendre les conséquences du paludisme gestationnel et de préciser les bases moléculaires et cellulaires des réponses immunologiques du nouveau-né.

L'identification des facteurs, tels que l'IL-10 et l'IP-10, au début de la grossesse comme facteur de risque d'une infection à l'accouchement suggère de nouveaux outils pour la surveillance des pathologies associées à la grossesse. Ces facteurs pourraient servir de bio-marqueurs au cours du PAG. La contribution de ce travail à la compréhension du mécanisme de mise en place des réponses immunitaires au cours du PAG bénéficie d'une approche originale qui porte sur l'utilisation des cellules fraîches du sang humain recueilli à deux périodes de la grossesse. Ceci permet d'être plus proche des conditions physiologiques *in vivo*. La confirmation par des approches plus spécifiques sur l'activation des cellules B et des Treg au cours du PAG pourrait conforter nos observations. L'identification au moyen de techniques moléculaires de la régulation des fonctions des TLR sera un apport pour des cibles thérapeutiques contre le paludisme. Par ailleurs la protection des femmes enceintes au second trimestre de la grossesse réduirait le risque d'infection placentaire et l'absence d'une infection placentaire serait bénéfique pour la mise en place des réponses innées du jeune enfant. Comme approche plus intéressante, nous envisageons dans le futur étudier les réponses spécifiques au VAR2CSA, des cellules mononucléées du sang périphérique de la femme enceinte. Ceci permettra de mieux comprendre les mécanismes immunologiques liés au PAG.

	Second trimestre	Accouchement
Grossesse normale *(24 paires de femmes)*	%T CD4$^+$ ↗ %Teff ↗	T CD4 ↘ Teff ↘
Grossesse impaludée *(Second trimestre, 69 non infectées vs 62 infectées)*	% Lympho B CD86$^+$ ↗ % Lympho B ↗ % Monocytes HLA-DR$^+$ ↘ % mDC HLA-DR$^+$ ↘ % Treg ↘ IL-6 ↗ IL-10 ↗ IP-10 ↗ MIG ↗ Ang-1 ↘	% Lympho B CD86$^+$ ↗ % pDC CD86$^+$ ↗ % pDC ↘ %Teff ↗ IL-10 ↗ IP-10 ↗ MCP-1 ↗
Etude prospective		
Facteurs de risque	%Monocytes ↗ IL-10 ↗ IL-1β ↘ uPAR ↗ %Treg ↘ IL-10 ↗ IP-10 ↗ IL-6 ↘ RANTES ↘ %mDC CD86$^+$ ↘	Anémia à l'accouchement Infection placentaire Faible poids de naissance

↗ Augmentation

↘ Diminution

Tableau 3 : Résumé des résultats

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Summary

The objective of this thesis was to investigate the immunological consequences of pregnancy associated malaria (PAM) in pregnant women and their newborns. The study populations comprise sub-groups drawn from a longitudinal study known as "Strategies TO Prevent Pregnancy Associated Malaria" (STOPPAM) that was conducted in parallel in the two study sites i.e. Benin and Tanzania. The STOPPAM objectives were to elucidate the mechanisms and course of PAM and quantify the effects of PAM on mothers and their childhood's health.

First, we characterized cell mediated-immunity from *P. falciparum*-infected or non-infected pregnant women. Two studies were carried out in two sub-groups of pregnant women at early pregnancy and at delivery. In the first study, we observed a decrease frequency of T cells independently of malaria infection in the twenty-four pregnant women matched at early pregnancy and at delivery.

In the second study, we characterized: i) the frequency and the level of activation of cell mediated-immunity and ii) quantify the plasma level of cytokine and chemokine in sub-groups at early pregnancy and at delivery. We observed that malaria infected pregnant women enhanced qualitative and quantitative changes in cells frequencies early during pregnancy and at delivery. The main finding of this study was the B cells activation at any time of infection and the decreased frequency of Treg at early pregnancy. PAM enhanced also higher levels of IL-10, IP-10 and MIG in our sub-groups. These bio-markers were identified as risk factors of placental infection at delivery. These observations suggest the management of malaria control in pregnant women in early pregnancy.

The second part of this thesis was focused on the impact of PAM on neonatal innate immune responses. In a first study, we characterized antigen-presenting cells (APC) in cord blood as a function of placental malaria infection or inflammation of the placenta. Our results showed that the presence of malaria pigment in the placenta was associated to partial activation of cord blood-dendritic cells. Our results highlight the importance of the age of the mother at the time of pregnancy and parity on APC activation in cord blood. These results suggest that PAM can induce neonatal innate responses alteration.

Then we considered the cytokine responses in cord blood and in peripheral blood of newborn, to TLR ligands in a cohort of 134 children. The observations from this study demonstrate that infection occurred close to/at delivery in the mother can induce modulation of young-children innate immune system, through the TLR. Our results suggest that susceptibility to malaria infection during the first year of life could depend on the period of stimulation of TLRs neonatal immune system.

All the work done in this thesis provides an understanding of the cellular mechanisms involved in inflammatory and gestational malaria in pregnant women and in children. Our results have identified biomarkers associated with PAG.