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*J Immunol* 2010; 185:7115-7122; Prepublished online 27 October 2010; doi: 10.4049/jimmunol.1002293

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Elevated Levels of Soluble TNF Receptors 1 and 2 Correlate with Plasmodium falciparum Parasitemia in Pregnant Women: Potential Markers for Malaria-Associated Inflammation

Audrey Davidson Thévenon,*† James A. Zhou,‡ Rosette Megnekou,§ Simon Ako,§ Rose G. F. Leke,§ and Diane Wallace Taylor*†

Plasmodium falciparum-infected erythrocytes (IEs) sequester in the intervillous space (IVS) of the placenta causing placental malaria (PM), a condition that increases a woman’s chances of having a low-birth-weight baby. Because IEs sequester, they frequently are not observed in peripheral blood smears, resulting in women with PM being misdiagnosed and thus not treated. Because sequestered IEs induce inflammation in the IVS, detection of inflammatory mediators in the peripheral blood may provide an approach for diagnosing PM. Two counterregulatory molecules, TNF-αR (TNFR) 1 and TNFR2, modulate the pathological effects of TNF-α. Levels of these soluble TNFRs (sTNFRs) are reported to be elevated in children with severe malaria, but it is unclear if they are increased in the peripheral blood of PM-positive women with asymptomatic infections. In this study, sTNFR levels were measured throughout the course of pregnancy, as well as at delivery, in women with asymptomatic infections and those who remained uninfected. Results showed that both sTNFRs were significantly increased in the peripheral blood of women with asymptomatic malaria (p < 0.0001) and were positively correlated with parasitemia (p < 0.0001 for sTNFR1 and p = 0.0046 for sTNFR2). Importantly, levels of sTNFR2 were elevated in the peripheral blood of women who were PM-positive but peripheral blood-smear negative (p = 0.0017). Additionally, sTNFR2 levels were elevated in the blood of malaria-positive women who delivered low-birth-weight babies. In vitro studies demonstrated that syncytiotrophoblasts were not a major source of sTNFR. These data suggest that sTNFR2 may be a valuable biomarker for detection of malaria-associated inflammation. The Journal of Immunology, 2010, 185: 7115–7122.

In countries where Plasmodium falciparum is present, malaria infections in pregnant women are an important cause of maternal morbidity and neonatal mortality (1). Although women may have clinical cases of malaria during pregnancy, the disease usually remains asymptomatic. Infected erythrocytes (IEs) sequester in the intervillous space (IVS) of the placenta, creating a condition known as placental malaria (PM), which is associated with maternal anemia and poor pregnancy outcomes (reviewed in Ref. 2). Because IEs sequester, they may not be present in routine peripheral blood smears (3), making diagnosis of malaria difficult. A major consequence of PM is low-birth-weight (LBW) babies who have a reduced chance of surviving the first year of life (4).

Thus, accurate diagnosis and rapid treatment of malaria in pregnant women are of great importance. Sequestration of IE in the IVS can lead to a change in cytokine balance, including an increase in TNF-α (5, 6). Elevated TNF-α enhances the cytoadherence of IE to trophoblasts (7) and supports the recruitment of monocytes into the IVS (8), an event that correlates with LBW babies (6, 9). TNF-α is an important cytokine in PM, and its levels must be carefully regulated. Low levels are necessary for enhancing phagocytic activity and controlling parasite densities (10), but high levels of TNF-α can trigger inhibition of endocrine function and initiate extracellular matrix degradation, resulting in poor pregnancy outcomes (11, 12). The biological activity of TNF-α can be modulated, in part, by its soluble receptors, sTNFR1 and sTNFR2 (13), that are shed from the surface of a number of different cell types by proteolysis. The soluble form of the receptors can stabilize TNF-α when the cytokine is present in low concentrations in plasma or neutralize TNF-α by competing for occupation of the receptors on the cell surface when the cytokine is in excess in the local environments (14). TNFR1 is constitutively expressed in most tissues. However, TNFR2 is strongly regulated during an inflammatory response. Elevated levels of both soluble receptors in plasma have been associated with various pathological conditions (15). During pregnancy, both TNF-α (16) and its receptors are normally expressed and shed by villous trophoblasts (17, 18). The accumulation of IEs in the IVS can alter the normal tight immunologic balance at the maternal–fetal interface (19), leading to inflammation, pathological changes in the placenta, and LBW babies. Not all women with PM, however, suffer complications at delivery, and this raises the

*Department of Biology, Georgetown University, Washington, D.C. 20057; ¶Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96813; ‡AZ DataClinic, Inc., Rockville, MD 20850; and §Faculty of Medicine and Biomedical Research, The Biotechnology Center, University of Yaoundé 1, Yaoundé, Cameroon

Received for publication July 8, 2010. Accepted for publication September 28, 2010.

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health Grants U01AI-35839, SU01-A043888 (field studies and sample collection), and RO1-A071160 (laboratory studies).

Address correspondence and reprint requests to Dr. Diane Wallace Taylor, Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A. Burns School of Medicine, University of Hawaii, 651 Ilalo Street, Honolulu, HI 96813. E-mail address: dwtaylor@hawaii.edu

Abbreviations used in this paper: HBMVEC, human brain microvascular endothelial cell; IE, infected erythrocyte; IVS, intervillous space; LBW, low-birth-weight; N/BW, normal-birth-weight; ρ/μl, parasites/μl; Per Neg/Pla Pos, peripheral blood-smear negative; PM-positive; Per Pos/Pla Pos, peripheral and placental positive; PM, placental malaria; sTNFR, soluble TNF-αR; TNFR, TNF-αR.

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question of whether inhibitory or regulatory systems, such as sTNFR1 and -2, modulate immunological events within the IVS. Significant elevation of sTNFR1 and sTNFR2 has been reported in plasma of patients with severe malaria (20–25), malaria-positive pregnant women (26), and rhesus monkeys infected with \textit{P. coatneyi} (27). However, the amount of receptors, TNF-\(\alpha\), and their relationship to protection during malaria infections remains unclear (28). An association between the peripheral blood parasitemia and \(\text{TNF}\) levels has been found in some studies, suggesting that higher parasite burden induces higher levels of inflammation that, in turn, increase the shedding of these receptors. It remains unknown if the extent of inflammation is high enough in asymptomatic \textit{P. falciparum} infections for sTNFR receptors to be shed and thus detected in peripheral blood. In this study, we sought to determine if sTNFR1 and sTNFR2 were elevated in the peripheral blood of asymptomatic pregnant women who had PM. If so, detection of these receptors might be used as a biomarker for diagnosing PM. An in vitro model using trophoblasts, endothelial cells, and monocytes was also developed to identify the source of sTNFR1 and -2 in the peripheral blood of women with PM. Finally, we sought to determine if elevated levels of these receptors were predictive of malaria-associated LBW babies.

Materials and Methods

Study population and sample selection

Between 2001 and 2004, a prospective cohort study was conducted in pregnant women in Yaounde, Cameroon, where the transmission of \textit{P. falciparum} is \(\sim\)13 infectious bites/person/year (29). The study was approved by the Institutional Review Board, Georgetown University, Washington D.C., and the National Ethics Committee of Cameroon. The women were recruited at \(<14\) wk of pregnancy and followed monthly through delivery and postpartum. Nonpregnant women of child-bearing age were recruited as controls and followed for 12 mo. Before entering the study, informed consent was obtained. At enrollment, information on the women’s health, gravidity, age, and estimated length of pregnancy were recorded: monthly information on use of antimalarial drugs was recorded, and birth outcome and baby weight were obtained. Blood samples were collected during each monthly visit, the hematocrit (packed cell volume) was determined, and blood smears were prepared and examined for \textit{P. falciparum} IE as described below. Women who were blood-smear-positive for malaria were prescribed antimalarial drug treatment according to the policy of the Ministry of Health (25). Samples were selected from the above women. To determine if sTNFR were present in malaria-positive women (i.e., women who were blood-smear positive), 44 plasma samples from 19 pregnant women (2.3 ± 0.8 samples/women) and 37 samples from 15 nonpregnant women (2.5 ± 0.6 samples/women) were evaluated. To follow changes in sTNFR levels during pregnancy, 125 plasma samples from 28 pregnant women (4.5 ± 0.7 samples/women) were analyzed. All women included in this study had asymptomatic infections when they were blood-smear positive. In addition, 282 additional paired peripheral-placental IVS plasma samples collected at delivery from a prior study (30) were selected to compare the association of peripheral and IVS plasma levels of sTNFR with delivery outcome. Among the 282 samples, 102 samples were selected from women who had LBW babies (i.e., \(\leq 2500\) g) and 180 women who had normal-birth-weight (NBW) babies. To assure adequate sample sizes, an approximately equal number of PM-positive (PM\(^+\)) and PM-negative (PM\(^-\)) women were included in the LBW and NBW groups, and women in the PM\(^+\) and PM\(^-\) groups were further matched for parity, age, and parasitemia. Among the 282 samples selected, 35 were from women who were peripheral blood-smear negative but PM\(^+\) (i.e., women routinely misdiagnosed by peripheral blood smears). Characteristics of the 282 women included in the four groups are shown in Table 1. Immediately postdelivery, maternal venous blood was drawn in the presence of EDTA, and blood from the IVS was collected using the biopsy-pool method (31).

Determination of parasitemia

Thick and thin smears were made using blood collected during pregnancy and at delivery. Slides were stained with Diff-Quik (Baxter Scientific Products, Miami, FL), and thick smears were examined for parasites. If parasites were not detected after examining 100 high-power fields, the woman was considered to be malaria-negative. If parasites were detected, the number of parasites per 200 WBCs was determined, and the number of parasites per microliter was calculated using the women’s WBC count. To diagnose PM, a portion of the placental biopsy was used to prepare impression smears that were stained with Diff-Quik and examined for parasites. If present, percent of parasitemias were determined by counting the number of IEs per 2000 RBCs. The remainder of the tissue was fixed in buffered formalin, embedded in paraffin, sectioned, stained with H&E and Giemsa, and examined for parasites. PM was defined as detection of parasites in either impression smears or histological sections. In addition, PCR was used to detect the presence of \textit{P. falciparum} parasites in blood samples from 20 women who were followed throughout the course of pregnancy (32). Women who were blood-smear negative but PCR-positive were classified as having submicroscopic infections.

In vitro culture of \textit{P. falciparum} and preparation of parasite extracts

\textit{P. falciparum} parasites (D-10 and 3D7 strains) were maintained in continuous in vitro cultures using a modification of the method of Trager and Jensen (33). Parasites were cultured using human erythrocytes at a 5% hematocrit in RPMI 1640 supplemented with 4.5 g/l\(-\)glucose, 2.383 g/l HEPES, 0.02 mg/ml hypoxanthine, 1.5 g/l sodium bicarbonate, 0.11 g/l sodium pyruvate, 5% heat-inactivated human serum (PAA Laboratories, Dartmouth, MA), and 0.25% Albumax II (Invitrogen, Carlsbad, CA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO\(_2\), 5% O\(_2\), and 90% N\(_2\). Parasites were grown to 10–12% parasitemia with or without prior sorbitol synchronization (34). Cells were layered on 80–80% Percoll gradients, centrifuged at 1350 × \(g\) at room temperature, and fractions enriched for late-stage IE were pooled, washed with PBS, and frozen at \(-80^\circ\)C until used. Normal RBCs were also cultured in vitro, washed, and frozen. IEs and RBCs were freeze/thawed rapidly three times, sonicated, and centrifuged at 7000 × \(g\) for 10 min. The resulting supernatant-containing soluble parasite Ags, pellet-containing membranes, hemozoin pigments, and RBC membranes were used in the study.

In vitro studies using BeWo, THP-1, and human brain microvascular endothelial cells

Immortalized cell lines were tested in vitro for their ability to shed sTNFR in response to diverse inflammatory signals. The cell lines used are either found in infected placenta or known to be in contact with IEs during malaria infections. Specifically, forskolin-induced BeWo (choriocarcinoma cells), which represent a model for syncytiotrophoblasts lining the IVS, and PMA-activated THP-1, as a model of activated macrophages known to accumulate in the IVS during PM (35), are used. Human brain microvascular endothelial cells (HBMECs) were also used as a positive control for receptor shedding. BeWo, choriocarcinoma cells (American Type Culture Collection catalog number CCL-98, Manassas, VA) were grown in HAM’s F-12 at 37°C and then passed 12 times. HBMECs, a generous gift from V. Nerurkar (University of Hawaii, Honolulu, HI), were maintained in Eagle’s complete medium, grown in flasks coated with attachment factor (Cell Systems, Kirkland, WA), and used between passages 8 and 12. Finally, THP-1 cells (American Type Culture Collection catalog number TIB-202), a human monocyte cell line, were grown in suspension in RPMI 1640 medium. All media were supplemented with 10% FCS (VWR International, West Chester, PA), 2 mM L-glutamine (Invitrogen), 100 IU/ml penicillin, and 100 \(\mu\)g/ml streptomycin (Invitrogen). Cultures were maintained under standard conditions in a humidified incubator with 5% CO\(_2\) and 95% air.

To determine if these cells released sTNFR in response to the parasite extracts, optimal assay conditions were determined for each cell line prior to the study. BeWo cells were seeded at \(5 \times 10^5\) cells/ml, incubated for 24 h, and then treated with 10 mM forskolin for 24 h to induce the cells to form a syncytiun. Cells were then cultured for an additional 24 h in incomplete medium prior to the addition of parasite extracts. HBMECs were seeded at \(5 \times 10^5\) cells/ml. As the experiment proceeded, they were treated either directly or first activated with PMA. Briefly, THP-1 were seeded at \(1 \times 10^6\) cells/ml, treated for 18 h with 50 nM PMA that induced the adhesion of the cells, and then cultured for 48 h with fresh medium before the addition of parasite extracts. All cell lines were cultured in triplicate for 24 h with the following: PMA 50 and 200 nM; TNF-\(\alpha\) 0.1 and 10 ng/ml; LPS 0.1 and 10 \(\mu\)g/ml; RBCs at \(10^7\) and \(10^8\) cells/well; and parasite extracts equivalent to \(10^7\) and \(10^8\) IEs/well. After 24 h, supernatants from individual wells were collected, centrifuged, and stored at \(-80^\circ\)C until used. Cell viability was assessed using the MTT assay (36). Briefly, at the end of the experiment, 100 \(\mu\)l MTT stock solution (final concentration
0.5 mg/ml was added to each well and cells were incubated for an additional 4 h at 37°C. Thereafter, the MTT solution was aspirated from the wells, 200 μl 10% SDS was added to solubilize the cells. The OD of the released product was measured at 595 nm using a spectrophotometer (SpectraMax 34, Molecular Devices, Sunnyvale, CA).

Quantifications of TNFR1 and -2 in plasma and cell culture supernatants

The levels of sTNFR1 and TNFR2 in plasma were quantified in a multiplex assay using Luminex suspension array technology with Ab-coated beads obtained commercially with reagents and secondary Abs (Invitrogen, catalog number LHC3021 and LHC3031). Plasma samples were diluted 1:8 with the equimolar diluent provided in the kits (Invitrogen). To determine sTNFR1 and -2 concentrations (pg/ml), standard curves were prepared for each plate using reconstituted standards provided in the kits and linear regression analysis. The sensitivity of the assay was 2 pg/ml. To determine if the multiplex assay was detecting both free and TNF-bound receptors in plasma, 5, 0.5, and 0.05 ng/ml TNF-α was added to plasma samples with known amounts of sTNFR, preincubated for 1 h, assayed, and the amount of sTNFR in paired samples with and without added TNF-α was compared. Levels of sTNFR in cell culture supernatants were measured using commercial ELISA kits from HyCult Biotechnology (Uden, The Netherlands).

Statistical analysis

Results are reported as median or mean (± SD) levels of sTNFR (pg/ml). Spearman correlation coefficient was used to assess correlations with malaria parasitemia and sTNFR in the peripheral and IVS plasma. The between-group differences in the percentage of PM infections, LBW, and other categorical variables were compared using the χ² test. The between-group differences in sTNFR levels at delivery were tested using the Mann-Whitney or Wilcoxon rank-sum test as appropriate. Linear regression analysis was used to evaluate changes in sTNFR levels during the course of pregnancy. The between-group comparisons in repeated measures of sTNFR levels during pregnancy were made using a likelihood ratio test based on a mixed model with an unstructured covariance structure among repeated measures. All the statistical analyses were conducted using SAS software (version 9.1, Cary, NC).

Results

Levels of sTNFR1 and sTNFR2 in the peripheral blood of nonpregnant and pregnant women

sTNFR1 and sTNFR2 levels were measured in peripheral blood of 37 nonpregnant (n = 24 malaria-negative, n = 13 malaria-positive) and 44 pregnant (n = 20 malaria-negative and 24 malaria-positive) women (Fig. 1). Parasitemias were similar between nonpregnant and pregnant women (mean 3999 ± 9006 and 2941 ± 5322 parasites/μl, respectively; p = 0.49). sTNFR1 levels were similar in nonpregnant women who were slide-positive and slide-negative for malaria; however, sTNFR1 levels were significantly higher in pregnant women who were malaria-positive compared with women who were malaria-negative (p < 0.01) (Fig. 1A). In contrast, sTNFR2 levels were significantly increased in both nonpregnant and pregnant women who were slide-positive for malaria (p < 0.01 and p < 0.001, respectively) (Fig. 1B). These results document that increased sTNFR1 levels in malaria-positive women are pregnancy dependent, but that sTNFR2 is elevated in both pregnant and nonpregnant women who were slide-positive for malaria.

Levels of sTNFR throughout pregnancy in malaria-negative women

During the course of pregnancy, serum levels of sTNFR1 have been reported to increase (37). To evaluate the extent of increase, sTNFR1 and sTNFR2 levels were measured in the blood of pregnant women who remained slide-negative for malaria during the course of pregnancy. Linear regression analysis showed a small, but significant, increase in sTNFR1 levels during the course of pregnancy (p = 0.026), with levels of 774 ± 230 pg/ml at <30 wk increasing to 831 ± 135 pg/ml at 36–38 wk. In contrast, levels of sTNFR2 remained constant throughout pregnancy in malaria-negative women.

Levels of sTNFR and malaria infections

The effect of malarial infections on receptor levels during pregnancy was assessed using 125 samples from 28 women (Fig. 2). Levels of both peripheral sTNFR1 and sTNFR2 were significantly elevated in pregnant women with asymptomatic infections and the increase correlated with peripheral parasitemia. sTNFR1 levels were significantly elevated in all women who were slide-positive (p < 0.01), but not in women with submicroscopic infections (Fig. 2A). In contrast, whereas sTNFR2 levels were also significantly higher in all women who were infected with P. falciparum compared with uninfected pregnant women (p < 0.0001) (Fig. 2B), a marginal, but significant, increase of sTNFR2 was observed in women who had submicroscopic infections (p = 0.05). Importantly, the amount of sTNFR1 and sTNFR2 in plasma was directly correlated with increasing parasitemias (sTNFR1: r = 0.64, p < 0.0001; sTNFR2: r = 0.59, p = 0.0046).

Analysis of samples from women followed longitudinally showed that sTNFR2 levels increased in all women when they became blood smear-positive compared with the previous blood smear-negative visit (Fig. 3). In addition, sTNFR2 levels returned to preinfection levels at the next blood smear-negative visit. Overall, levels of sTNFR1 were not as sensitive as sTNFR2, and not all...
women had a significant increase of sTNFR1 when they were slide-positive compared with a previous malaria-negative visit (data not shown). The average fold increase for sTNFR1 was only $1.6 \pm 0.7$ compared with a 2.5 $\pm$ 1.2 fold increase in sTNFR2 levels ($p = 0.008$).

**FIGURE 2.** Levels of sTNFR1 and sTNFR2 in the peripheral blood of pregnant women with increasing parasitemias. $A$ and $B$ show sTNFR1 and TNFR2 levels, respectively. Results are based on 125 peripheral blood samples from 28 pregnant women who were followed throughout pregnancy. Negative ($n = 64$ samples from women who were slide- and PCR-negative for malaria), PCR$^+$ ($n = 15$ samples from women who were PCR$^+$ but slide-negative), and 46 peripheral blood-smear positive samples, including $n = 13$ samples from women with $\leq 350$ p/µl, $n = 22$ with 350–2000 p/µl, and $n = 11$ with $\geq 2000$ p/µl. Results are shown in box-and-whisker plot, where boxes represent the 25th and 75th percentiles, the horizontal line is the median, and bars indicate the lowest 5th and highest 95th percentiles. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

**FIGURE 3.** sTNFR2 levels of individual women at consecutive prenatal visits. Blood samples were collected at monthly prenatal visits. An increase in sTNFR2 was seen in all pregnant women ($n = 10$) when they became malaria-positive, and levels decreased at the next visit ($n = 11$ women) when they were malaria-negative.

Levels of sTNFR in peripheral plasma samples collected at delivery from women with or without PM were determined ($n = 144$ malaria-negative women, $n = 127$ women peripheral blood smear-positive and PM$^+$, and $n = 35$ women peripheral blood smear-negative but PM$^+$) (Fig. 4). Overall, levels of sTNFR were higher and more variable in samples collected following labor than during pregnancy, most likely because parturition itself is an inflammatory process (compare Figs. 2 and 4). At delivery, peripheral blood levels of sTNFR were significantly higher in women with peripheral and placental malaria than malaria-negative women (sTNFR1, $p < 0.001$; sTNFR2, $p < 0.001$) (Fig. 4). These observations were especially evident for primigravid women (data not shown). Importantly, sTNFR2 levels were significantly elevated in the peripheral blood of women who were peripheral blood smear-negative but had PM ($p = 0.0017$) (Fig. 4B, Per Neg/Pla Pos). These results suggest that one might be able to diagnose PM in blood-smear negative women by detecting sTNFR2 in their peripheral blood.

**FIGURE 4.** Peripheral sTNFR2 levels at delivery. $A$ and $B$ show results for sTNFR1 and sTNFR2, respectively, for 144 malaria-negative women, 127 women who were peripheral and placental positive (Per Pos/Pla Pos), and 35 women who were peripheral blood-smear negative, but PM-positive by impression smear and/or histology (Per Neg/Pla Pos). Results are shown in box-and-whisker plot, where boxes represent the 25th and 75th percentiles, the horizontal line is the median, and bars indicate the lowest 5th and highest 95th percentiles. **$p < 0.01$; ***$p < 0.001$. **Levels of sTNFR in the peripheral blood at delivery**
Comparison of sTNFR levels in peripheral and IVS (placenta) plasma of PM+ and PM- women

Plasma levels of sTNFR in maternal peripheral and IVS blood were compared using paired samples from 25 PM+ and 40 PM- women. sTNFR1 levels were significantly higher in the IVS compared with peripheral blood (mean 1497 pg/ml ± 1436 pg/ml and 3278 ± 2462 pg/ml, respectively; p < 0.0001), which is consistent with syncytiotrophoblasts being a major source of sTNFR1 in pregnant women. The amount of sTNFR1, however, was similar in the IVS of PM+ and PM- women (mean 2871 ± 1795 pg/ml and 3930 ± 3196 pg/ml, respectively), suggesting that IEs sequestered in the IVS did not increase shedding of sTNFR1 by the syncytiotrophoblasts. In contrast, sTNFR2 levels were higher in the peripheral blood of women with PM compared with those without (mean 7907 ± 5606 pg/ml and 4322 ± 2364 pg/ml, respectively; p = 0.005), but no difference in sTNFR2 levels in the IVS of PM+ and PM- women was found (p = 0.19). These results confirm that sTNFR1 and sTNFR2 shedding is induced by different immunological stimuli and suggests that cells in the placenta may not be the major source of sTNFR2 observed in women with PM.

sTNFR levels in peripheral and IVS plasma of women who deliver NBW and LBW babies

Previous studies have shown that inflammation within the placenta is one of the major causes of premature deliveries of LBW babies (38). Therefore, samples of IVS plasma from 282 women with and without PM who had LBW and NBW babies were tested (Table I). Results showed that sTNFR1 levels and sTNFR2 were significantly elevated in IVS plasma of mothers delivering LBW babies compared with NBW (p = 0.02 and p = 0.003, respectively) (Fig. 5A: all women). The increase in sTNFR1 was not associated with PM; in fact, high levels of sTNFR1 were found in PM- women (p = 0.03). In contrast, sTNFR2 levels in the IVS were significantly increased in women with PM who delivered LBW babies compared with NBW (p < 0.05) (Fig. 5A).

To determine if differences in sTNFR levels the IVS could be detected in the peripheral blood, paired peripheral blood samples from the mothers were tested (Fig. 5B). Among PM-negative mothers, peripheral plasma levels of neither sTNFR1 nor sTNFR2 were significantly different between women who delivered LBW and NBW babies. However, among PM+ mothers, peripheral levels of sTNFR2, but not sTNFR1, were significantly higher in women who had LBW compared with NBW babies (p < 0.05) (Fig. 5B). These results underline a physiological difference in the shedding of sTNFR1 and sTNFR2 in LBW outcomes in infected and uninfected women. They also show that levels in the peripheral blood do not always reflect those occurring within the IVS.

Production of sTNFRs in vitro by different cell types

Although a variety of cell types are known to shed sTNFR, it is unclear if cellular interaction with P. falciparum Ags can stimulate shedding for sTNFR. Accordingly, two extracts containing P. falciparum proteins (i.e., soluble proteins and the remaining particulate pellet) were incubated with BeWo trophoblasts, HBMVECs, and activated THP-1 monocytes, and the resulting supernatants were screened for sTNFR. Results showed that neither extract induced shedding of sTNFR from any of the cell lines; that is, levels were not significantly higher than those found in corresponding control cultures stimulated with normal RBCs (data not shown). In contrast, all three cell types released sTNFR in vitro when treated with 50 nM PMA (p < 0.001, for both sTNFR1 and sTNFR2 for all three cell lines), demonstrating that the cells were capable of releasing these receptors. Likewise, LPS induced the shedding of sTNFR1 from HBMVECs (p = 0.01) as well as sTNFR1 and sTNFR2 from HBMVECs (p < 0.01, p < 0.001, respectively) and sTNFR2 from activated THP-1 cells (p < 0.001). These results confirmed that shedding of these receptors can be induced from placental, endothelial, and immune cells by inflammatory signals; however, shedding was not detected in response to direct contact with soluble parasite or particulate Ags.

Discussion

sTNFRs are important counterregulatory mediators of TNF-α. Their presence in the peripheral blood signals that an inflammatory response is occurring somewhere in the body. When P. falciparum IEs sequester in the placental IVS, a cascade of inflammatory events is initiated, including the production of β-chemokines that attract activated macrophages that produce TNF-α. This study hypothesized that sTNFRs would be induced as part of the inflammatory response and that their release into the peripheral blood might provide a new approach for diagnosing PM.

Prior to this study, sTNFRs have been detected in the peripheral blood of children with severe P. falciparum infections, including those with cerebral malaria (22, 23), demonstrating that malaria can induce the extensive shedding of sTNFR. It remained unclear, however, if asymptomatic infections would be sufficient to stimulate detectable levels of sTNFR (i.e., in cases in which parasitemias and inflammatory signals were low or when parasites were sequestered within the IVS environment). In the current study, sTNFR1 and sTNFR2 were both detected in the peripheral blood of pregnant women with asymptomatic infections (Fig. 1). Upon infection, a small increase in sTNFR1 was observed only in pregnant women, whereas a significant increase in sTNFR2 was detected in both pregnant and nonpregnant women. Because sTNFR2 was elevated in asymptomatic infections, detection of sTNFR2 in the peripheral blood may prove to be a new approach for diagnosing malaria in pregnant women who are infected with P. falciparum.

Today, there is a great need for better diagnosis of women with PM. An estimated 20% to >50% of women with PM are peripheral blood smear-negative (3, 39) and thus remain untreated, putting them risk of anemia, perinatal complications, and delivering LBW babies (1). sTNFR2 has the characteristics of a good biomarker for diagnosis of malaria in pregnant women; that is, it was detected in all pregnant women when they were slide-positive, absent (or at very low levels) at the next slide-negative visit (Fig. 3), significantly elevated when peripheral parasitemias were low (<350 parasites/μl [p/μl]) (Fig. 2), detectable in women

Table I. Characteristics of the women selected for use in the study

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Women</th>
<th>Age (y)</th>
<th>Birth Weight (g)</th>
<th>Primigravidae (%)</th>
<th>Length of Gestation (wk)</th>
<th>Packed Cell Volume (%)</th>
<th>Placental Malaria (%)</th>
<th>Placental Parasitemia (%)</th>
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</thead>
<tbody>
<tr>
<td>LBW</td>
<td>102</td>
<td>22.9 ± 5.1</td>
<td>2038 ± 383*</td>
<td>52.9</td>
<td>35.4 ± 3.8*</td>
<td>30.7 ± 6.6*</td>
<td>48</td>
<td>6.3 (± 11.9)</td>
</tr>
<tr>
<td>NBW</td>
<td>180</td>
<td>22.8 ± 4.6</td>
<td>3165 ± 423*</td>
<td>54.0</td>
<td>38.6 ± 2.9*</td>
<td>33.9 ± 5.7*</td>
<td>49</td>
<td>6.4 (± 14.7)</td>
</tr>
</tbody>
</table>

Data are expressed as mean (± SD) unless otherwise indicated.

*p < 0.001.
with submicroscopic infections \( p < 0.05 \) (Fig. 2), positively correlated with parasitemia, and present in women with PM who were slide-negative \( p = 0.0017 \) (Fig. 4). In Cameroon, as well as elsewhere throughout Africa, peripheral parasitemias in primigravidae and multigravidae average \( 5000 \pm 1000 \) p/\( \mu l \) and \( 1200 \pm 500 \) p/\( \mu l \), respectively (30). Thus, detecting sTNFR2 in blood samples should be sensitive enough to detect most malaria cases. Because sTNFR2 is a marker of inflammation, it is possible that an elevation can result from pathological conditions other than malaria. In many parts of Africa, however, malaria is the most common infectious disease contracted during pregnancy. In fact, malaria is one of the few diseases women become more susceptible to during pregnancy. Detection of sTNFR2 alone is a good biomarker of inflammation but may not be sufficient to diagnose malaria. However, a combination of currently available Ag-detection rapid diagnostic tests for malaria in conjunction with detection of sTNFR2 may improve diagnosis of malaria, especially in women in whom the level of inflammation is sufficient to result in placental pathology. Our data show that women with asymptomatic infections have ongoing inflammation and produce large amounts of sTNFR2 and thus should be treated with antimalarial drugs.

The initial hypothesis was that inflammatory cells in the IVS would be the primary source of sTNFR2 in malaria-infected pregnant women; however, this does not appear to be the case. Additional cell types contribute to sTNFR2 levels found in pregnant women with PM. IL-10, which is elevated in PM (5), has been shown to induce the release of sTNFR2 from monocytes (40). Endothelial cells lining blood vessels are also a source of inducible of TNFR2, as demonstrated by in vitro studies using human endothelial cells (41) and a murine model of experimental cerebral malaria (42). The binding of late-stage IEs to endothelial cells in the deep vasculature can induce transient inflammation and contribute to sTNFR2 levels in the peripheral blood. In addition, CD4+CD25+ regulatory T cells shed sTNFR2 (43). During pregnancy, the placenta maintains immunotolerance of the fetus (reviewed in Ref. 44), and high levels of CD4+CD25+ regulatory T cells, up to 30% of all CD4+ cells in the uterus of mice, have been detected (45). Regulatory T cells have also been found in the peripheral blood of pregnant women (46). In addition, the severity of malaria-associated symptoms has been correlated with number of CD4+CD25+ regulatory T cells (47). Thus, sTNFR2 detected in the plasma of pregnant women could have been produced by multiple cell types.

Both hemozoin pigment complexed with parasite DNA and proteins and parasite GPI are known to directly stimulate monocytes to release TNF-\( \alpha \) (48, 49). Because sTNFRs help regulate TNF-\( \alpha \) levels, it seemed logical these factors might also stimulate shedding of sTNFR; however, this does not seem to be the case. Neither receptor was shed when BeWo trophoblasts, HBMVECs, and THP-1 monocytes were cultured with soluble late-stage proteins or the residual pellet-containing membrane proteins, parasite-derived hemozoin, and GPI. Because both sTNFR1 and sTNFR2 were detected in culture supernatants following culture with in-

![Figure 5. Placental and peripheral levels of sTNFR1 and sTNFR2 in women with LBW and NBW deliveries. The two figures on the left (A) show sTNFR levels in the IVS and the two on the right (B) show levels in maternal peripheral blood. Each box represents 12–180 women. Results are shown in box-and-whisker plot, where boxes represent the 25th and 75th percentiles, the horizontal line is the median, and bars indicate the lowest 5th and highest 95th percentiles. * \( p < 0.05 \); ** \( p < 0.01 \).](http://www.jimmunol.org/DownloadedFrom)
flammary stimuli (i.e., PMA and LPS), it appears that sTNFR shedding is part of the overall inflammatory process and does not occur following direct contact with parasite-derived factors known to stimulate TNF-α.

Multiple factors contribute to LBW of newborns. In the current study, low infant birth weights were due primarily to prematurity. A major cause of premature deliveries is infections that stimulate inflammation. Because syncytiotrophoblasts are a major source of sTNFR in pregnant women, it was not surprising to find elevated levels of sTNFR1 in the IVS of women who had LBW babies (p < 0.05) (Fig. 5A). Interestingly, however, was the observation that high levels were found in malaria-negative women (p = 0.05), whereas sTNFR1 levels were not elevated in the IVS of mothers with PM. Previous studies have shown that when circulating TNF-α binds to sTNFR1 expressed on syncytiotrophoblasts, the receptor complex is not shed, but rather the receptor is internalized by trophoblasts, and a signaling pathway is initiated leading to cell death (50). If this happened during PM, a cascade of events would result leading to early parturition and premature deliveries (reviewed in Ref. 51). In addition, if PM triggered internalization of sTNFR1, a decrease in neutralization of macrophage-produced TNF-α would occur, resulting in higher levels of TNF-α in the IVS, thereby increasing the risk of placental pathology associated with LBW babies. A decrease in shedding of sTNFR1 would have a significant impact on neutralization of TNF-α because sTNFR1 has a higher affinity for TNF-α than the membrane-bound receptor (52). In contrast, sTNFR2 levels were elevated in the IVS of women who had LBW babies, especially those with PM (p < 0.05) (Fig. 5). The importance of sTNFR2 in regulating IVS levels of TNF-α is unknown.

In summary, better diagnoses of malaria in pregnant women would greatly reduce maternal morbidity and neonatal mortality in regions where malaria is endemic. sTNFR1 were detected in pregnant women in response to Plasmodium falciparum infections, but differences in the amounts of this receptor in the peripheral blood of infected and noninfected women is too small for sTNFR1 to serve as a biomarker. However, elevated levels of sTNFR2 were detected in the peripheral blood of pregnant women as well as non-pregnant women with asymptomatic malaria infections. Further studies are warranted to explore sTNFR2 as a specific host biomarker of inflammation in women with PM and as a predictor for LBW babies.

Acknowledgments
We thank all of the Cameroonian women who participated in this study. We are indebted to the immense help of the entire staff of the Biotechnology Center of the University of Yaoundé I for the collection and preparation of the samples and the reading of blood smears for parasitemia data.

Disclosures
The authors have no financial conflicts of interest.

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