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Malaria Enhances Expression of CC Chemokine Receptor 5 on Placental Macrophages

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Malaria and human immunodeficiency virus (HIV) coinfections are common in pregnant women in sub-Saharan Africa. The current study shows that placentas of malaria-infected women contain 3 times as much CC chemokine receptor 5 (CCR5) RNA as placentas of women without malaria. By immunohistochemistry, $CCR5^+$ maternal macrophages were seen in placentas from malaria-infected women but not in placentas from malaria-uninfected women. In addition, CCR5 also was found on fetal Hofbauer cells in placentas from both groups. Thus, malaria infections increase the potential reservoir for HIV in the placenta by increasing the number of HIV target cells.

Malaria and human immunodeficiency virus type 1 (HIV-1) are among the most prevalent infectious diseases in sub-Saharan Africa, and coinfections are common [1]. These coinfections may be of great significance in pregnant women, because it is possible that malaria may enhance mother-to-child transmission of HIV. In a recent study in The Gambia, vertical transmission rates for HIV-1 were twice as high during the rainy malaria season than during the dry season [2]. This may have been the result of an effect of malaria infection on viral replication. In vitro, exposure to malaria antigens causes increased HIV replication in peripheral blood mononuclear cells [3]. In Malawian adults, malaria infections were associated with 7-fold higher HIV virus loads [4].

HIV cell entry is complex. HIV requires the CD4 molecule plus another coreceptor for efficient entry. Several chemokine receptors, especially CXCR4 and CCR5, appear to be important coreceptors [5–9]. CCR5 appears to play an important role in mother-to-child transmission of HIV: black infants who are homozygous for a promoter polymorphism (59396T) were at a 6-fold higher risk of being infected than were heterozygotes or homozygotes [10]. However, little is known about the distribution of chemokine receptors in the placenta [11].

About 20%–25% of HIV-infected infants become infected within the uterus before delivery [12]. Infants infected in this manner might be unaffected by short-course antiretroviral therapies [11]. Because placentas of malaria-infected mothers often contain high numbers of macrophages [13, 14] and because placental macrophages can be reservoirs for HIV [15], we studied whether malaria infections might increase placental virus load and promote intrauterine transmission of HIV.

Materials and Methods

Study population. Placental biopsy specimens were obtained from participants in a prospective cohort study of primigravid women in Mangochi, Malawi, and were frozen directly in liquid nitrogen. This area of Malawi has high-level transmission of *Plasmodium falciparum.* Recruitment of subjects and methods of evaluation were done as described elsewhere [16, 17].

All available placental biopsy specimens from women with cord blood parasitemias were selected as the infected group ($n = 23$). For the control group, placentas were used from women who had no indication of malaria infection during pregnancy ($n = 21$). Hemozoin levels were undetectable in the control group placentas, and none of the women from this group had positive antenatal or delivery parasitemia. Placental hemozoin concentrations were determined as described elsewhere [18].

Ribonuclease protection assay. Total RNA was extracted from the placental biopsy specimens, as described elsewhere [16]. Two chemokine-specific riboprobe template sets, hCR-8 and hCD-1, were used (BD Pharmingen). The hCR-8 template set contained riboprobes specific for human STRL33, US28, CCR3, CCR5, CXCR4, CCR8, GPR15, GPR1, V28, CCR2b, L32, and GAPDH. The hCD-1 template set contained riboprobes specific for human TCRδ, TCRα, CD3ϵ, CD4, CD8α, CD8β, CD19, CD14, CD45, L32, and GAPDH. All riboprobe syntheses and subsequent steps of probe purification, placental RNA (15 μg) probe hybridization,

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The study protocol was approved by the Malawi Health Science Research Committee and by the University of Michigan Health Sciences Institutional Review Board.

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RNase treatment, and purification of protected RNA duplexes were done as described elsewhere [16]. Protected RNA was resolved by gel electrophoresis, using the QuickPoint Rapid Nucleic Acid Separation System (NOVEX), according to the manufacturer's instructions. Radiolabeled, protected RNA bands were visualized by using a phosphorimager (Storm series 400) and were quantified by using ImageQuant software (both from Molecular Dynamics), as described elsewhere [16, 19]. Each sample was analyzed in 2 separate experiments, and the relative phosphorimager values obtained were averaged. Duplicates were <20% of each other. Each probe was normalized against the internal housekeeping signal (L32), to control for variability in each assay, as described elsewhere [16].

Immunohistochemistry (IHC) and immunofluorescence. Frozen placental tissue sections were prepared, fixed, and stored, as described elsewhere [16]. A frozen lymph node biopsy specimen from a patient with malignant lymphoma was used as the positive control tissue. The incubations detailed below were done at room temperature.

IHC single-staining procedure. Tissue sections were rehydrated in PBS (Life Technologies Gibco BRL) containing 0.1% bovine serum albumin (BSA, ELISA-grade; Sigma), were rinsed in deionized water, and then were incubated in 1% H_2O_2 (diluted in PBS and 0.1% BSA) for 30 min, to neutralize endogenous peroxidase activity. After slides were rinsed in deionized water and then in PBS and 0.1% BSA, samples were treated with 1:10 dilution of blocking serum (normal goat serum [BioGenex] diluted in PBS and 0.1% BSA) for 30 min. The slides were then exposed (for 30 min to 1 h) to 4 μg/mL anti-human CCR5 (clone 2D7), 2 μg/mL anti-human macrophage mannose receptor (clone 19.2), or 2 μg/mL anti-human macrophage CD68 (clone KP1). All were monoclonal antibodies purchased from Pharmingen. Purified mouse IgG1 (2 μg/mL), κ isotype standard (clone MOPC-21), was used as the control antibody for anti-CD68 anti-mannose receptor staining, whereas purified mouse IgG2a (4 μg/mL), κ isotype standard (clone C1.18.4), was used as a control for the anti-CCR5–specific antibody. After being treated with antibodies, the slides were rinsed with PBS and 0.1% BSA and then were incubated for 15 min with biotin-conjugated goat anti-mouse immunoglobulin–specific polyclonal antibody (Vector Laboratories) diluted 1:200. All antibody dilutions were done in common antibody diluent (BioGenex). The samples were rinsed in PBS and 0.1% BSA and were treated for 15 min with preformed avidin and biotinylated horseradish peroxidase macromolecular complex reagent (Vectastain Elite; Vector Laboratories) diluted 1:100. Samples then were rinsed with PBS/0.1% BSA, were rinsed again with PBS only, and then were incubated with the substrate 3-amino-9-ethyl carbazole (Vector Laboratories). Color was allowed to develop for 10 min, and then the samples were rinsed in PBS to stop the reaction. The slides then were rinsed a final time in deionized water. Gill's no. 1 hematoxylin (Fisher Scientific) was used as a counterstain. Slides were mounted with aqueous mounting media (Biomeda) and were allowed to dry for 24 h before being viewed with a microscope.

Immunofluorescence double-staining procedure. Tissue sections were rehydrated with PBS containing Triton X-100 (0.01%). They were first incubated with rabbit polyclonal anti-CCR5 (10 μg/mL; Chemicon International) for 1 h, and the slides then were washed in PBS and were incubated with anti-rabbit IgG–fluorescein isothiocyanate (Jackson Immunochemicals), 1:150, for 30 min. This and all subsequent incubations were done in the dark. Slides were washed with PBS, were incubated with prediluted anti-CD68 (Zymed) for 1 h, and then were washed with PBS again. The slides then were incubated with anti-mouse IgG-Texas Red (Vector Laboratories), 1:75, for 30 min, were washed with PBS, and then were washed with water. Coverslips were mounted with aqueous mounting media (Biomeda), and the slides were stored at −20°C until viewed under a fluorescent microscope.

Statistical analyses. Analyses of means, SDs, and 2-tailed Student *t* tests for unequal variances were done by use of Microsoft Excel 97.

Results

Placental mRNA receptor expression associated with malaria infection. Placental mRNA levels for CD4, CD14, CCR3, CCR5, and CXCR4 were quantitated by ribonuclease protection assay and were normalized to the housekeeping gene L32 (table 1). There was no difference in CD4 or CD14 expression between the control and infected groups, which suggests that there was no difference in the population sizes of either $CD4^+$ T cells or macrophages and dendritic cells that are $CD14^+$. Of interest, there was also no difference in expression levels for CXCR4, a coreceptor for T-tropic HIV. However, expression of CCR5, the coreceptor for M-tropic viruses, and CCR3 were increased ~3-fold in the infected placentas ($P = .01$ and $P = .03$, respectively).

Detection of CCR5 in placental tissue sections by IHC and immunofluorescence. Increased expression of CCR5 could facilitate virus entry into macrophages. However, since CCR5 is expressed on many cell types, we needed to know whether the increase in placental CCR5 mRNA corresponds to increased CCR5 expression on HIV-receptive cell types. Through IHC, we identified 2 distinct cell populations that expressed the coreceptor CCR5. In both the control and infected groups, the $CCR5^+$ staining was most common on cells within the chorionic villous, which appeared to be Hofbauer cells (figure 1*E* and 1*F*). Hofbauer cells are fetal macrophages located in the stroma that are known to express CD68 and the anti-mannose receptor [20, 21]. The same cells that stained with anti-CCR5 appeared

Table 1. Placental chemokine receptor and surface marker mRNA levels for women with and without malaria.

Receptor	mRNA Levels, μg/mL[a]		Ratio infected: uninfected	*P*
	Infected subjects ($n = 23$)	Control subjects ($n = 21$)		
CD4	0.166 ± 0.067	0.158 ± 0.056	1.05	NS
CD14	0.422 ± 0.112	0.412 ± 0.101	1.02	NS
CCR5	0.028 ± 0.032	0.009 ± 0.004	3.11	.01
CCR3	0.026 ± 0.031	0.009 ± 0.005	2.89	.03
CXCR4	0.091 ± 0.058	0.067 ± 0.018	1.36	.07

NOTE. Data are mean ± SD, unless otherwise indicated. NS, not significant.
[a] Calculated from the ratio of volume of signal to that of L32, a housekeeping gene, in the same lane on the gel.

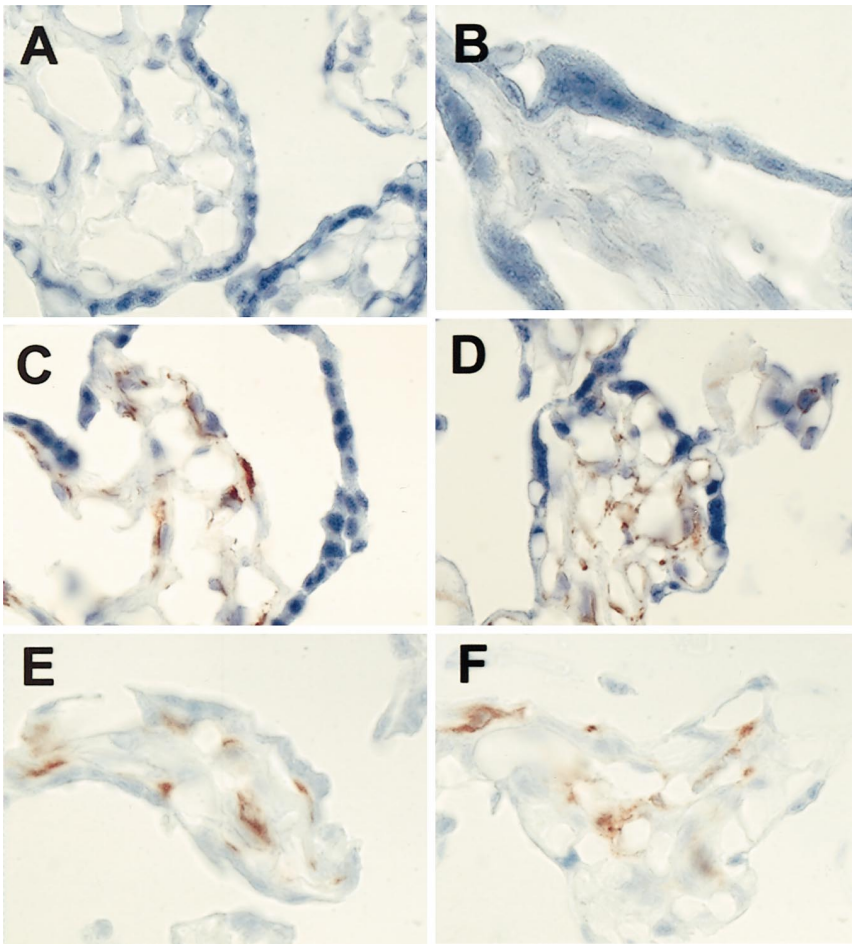

Figure 1. Immunohistochemical staining for CD68 and CC chemokine receptor 5 (CCR5) in chorionic villi in placentas from malaria-infected and nonparasitized mothers. Placental sections were stained with control IgG *(A* and *B),* anti-CD68 *(C* and *D),* and anti-CCR5 *(E* and *F). A, C,* and *E,* Samples from a malaria-infected mother; *B, D,* and *F,* samples from a control placenta. CD68 staining pattern of stromal cells, which are consistent with tissue macrophages or Hofbauer cells, is similar to CCR5 staining in both types of placentas. Original magnification, ×400.

to stain with anti-CD68 (figure 1*C* and 1*D*) and anti-mannose receptor (not shown) but not with control IgG (figure 1*A* and 1*B*). Intervillous maternal macrophages also stained positively for CCR5 (figure 2*G*–2*I*) but were found only in the placentas from malaria-infected women. CCR5 is expressed on maternal macrophages containing intracellular hemozoin and on macrophages with no hemozoin. Similar appearing clusters of intervillous macrophages also stained with anti-CD68 (figure 2*D*–2*F*), as expected [14], but not control IgG (figure 2*A*–2*C*).

To establish the identity of the $CCR5^+$ cells, we doubly stained sections by immunofluorescence with anti-CCR5 and anti-CD68. Anti-CD68 was used to identify both maternal macrophages and Hofbauer cells. All $CCR5^+$ intervillous cells were CD68 positive (figure 3*A* and 3*B*), confirming that they were indeed macrophages. All $CCR5^+$ Hofbauer cells were CD68 positive (figure 3*C* and 3*D*) in both infected (shown) and uninfected placentas (not shown). There was no staining with any of the control antibodies (not shown).

Discussion

In this study, we compared CCR5 expression in placentas from malaria-infected and uninfected women. In both types of placentas, CCR5 is expressed on fetal Hofbauer cells. In addition, maternal macrophages express CCR5 in the malaria-infected placentas. As determined on the basis of RNA levels, CCR3 and CCR5 expression is increased 3-fold by malaria infection. The increase appears to be selective, since there is no increase in CD4, CD14, or CXCR4 RNA.

This is the first study, to our knowledge, of CCR5 expression in situ in placentas. The observation of CCR5 on maternal macrophages and fetal Hofbauer cells is consistent with pre-

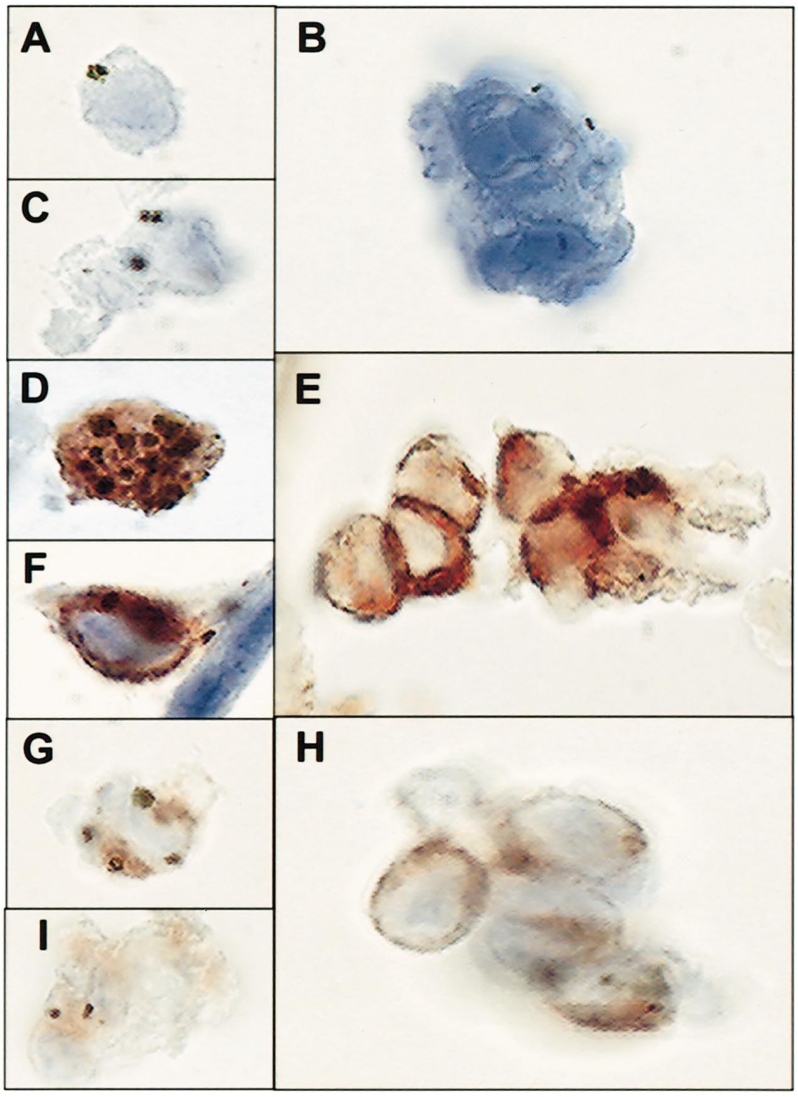

Figure 2. Immunohistochemical staining for CD68 and CC chemokine receptor 5 (CCR5) of individual and clusters of intervillous mononuclear cells in placenta from malaria-infected mother. Cells were stained with control IgG *(A–C),* anti-CD68 *(D–F),* or CCR5-specific IgG *(G–I)*. Brown spots are hemozoin pigment. Original magnification, ×1000.

vious studies of HIV-infected mothers, which demonstrated the presence of HIV in these cells by IHC and in situ hybridization [1, 22, 23]. The presence of CCR5 on both these cell types lends credence to the possibility that they are involved in intrauterine transmission of HIV.

There are several reasons to believe that CCR5 is important in mother-to-child transmission of HIV in Africa. First, almost all strains of HIV that are transmitted in this manner are CCR5-trophic (M-trophic, R5) [24, 25]. Second, offspring of HIV-infected black mothers who are homozygous for a CCR5-promoter polymorphism have a 6-fold higher risk of being infected at birth [10]. Thus, increases in placental CCR5 might indeed be of clinical relevance in mother-to-child HIV transmission.

There was no apparent difference between infected and uninfected placentas in the distribution or staining intensities of $CCR5^+$ Hofbauer cells. The major difference appeared to be that $CCR5^+$ maternal macrophages were present in the malaria-infected group but were not present in the malaria-uninfected group. Thus, it is likely that the difference in CCR5 RNA levels between the 2 groups was due to its expression by maternal macrophages. Previous studies have shown that malaria infections lead to the accumulation of maternal macrophages in the placenta [13, 14]. In aggregate, evidence suggests that malaria causes an increase in HIV-receptive maternal macrophages within the placenta.

A modest number of maternal macrophages was seen in sec-

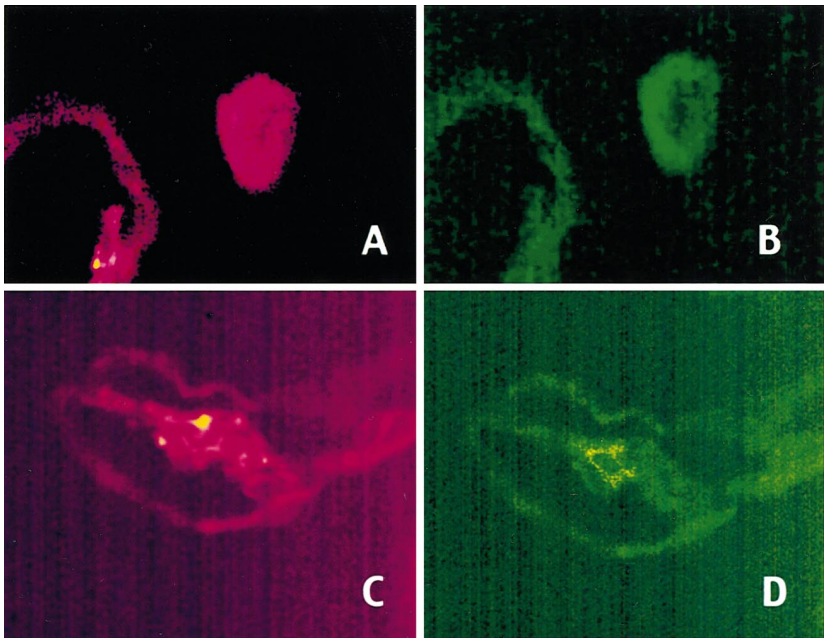

Figure 3. Immunofluorescence double staining for CD68 *(red)* and CC chemokine receptor 5 (CCR5; *green*). Shown are an intervillous macrophage stained for CD68 *(A)* and CCR5 *(B)* and a Hofbauer cell stained for CD68 *(C)* and CCR5 *(D)*. Original magnification, ×1000 (oil immersion).

tions from infected placentas, and none was seen in sections from uninfected placentas. Pronounced infiltration of the intervillous spaces by mononuclear cells was not seen. However, this usually only occurs in a small percentage of malaria-infected placentas [13, 14]. In all sections of these placentas, the fetal Hofbauer cells greatly outnumbered maternal macrophages (data not shown). Since Hofbauer cells also produce CD14 [26], it is not surprising that the levels of CD14 RNA in infected and uninfected placentas were similar.

In this study, CCR5 was found on macrophages both with and without hemozoin. The presence of hemozoin within the macrophage is evidence that the cell had previously phagocytosed either an intact malaria-infected erythrocyte or fragments of parasites. Thus, our data suggest that phagocytosis of parasite material does not directly induce CCR5. This contrasts with our previous study, in which tumor necrosis factor–α and interleukin-8 were expressed only in placental hemozoin-laden maternal macrophages [16]. This suggests that CCR5 induction may be indirectly induced by the proinflammatory cytokines elicited in the placenta by malaria infections. Alternatively, CCR5 expression may be in a dynamic state of up-regulation and down-regulation on inflammatory cells during recruitment.

Interactions between malaria and HIV during pregnancy have important public health consequences. HIV-infected pregnant women had more frequent and higher malaria parasitemias [27, 28] and responded more poorly to presumptive antimalarial therapy [29]. Offspring of HIV- and malaria-coinfected pregnant women in Malawi were 3–8 times more likely to die in their first year of life than were offspring of mothers with either HIV or malaria infections alone [30]. Mother-to-child transmission of HIV-1 and HIV-2 was studied recently in The Gambia, where malaria transmission occurs almost exclusively during the rainy season [2]. HIV-1 and HIV-2 transmission rates, which were 14.8% and 1.7%, respectively, in the dry season, rose to 29.4% and 7.1%, respectively, during the rainy season. This effect was hypothesized to be due to an effect of malaria, although malaria parasitemias were not assessed in any of the mothers. In aggregate, these reports suggest that coordinated efforts to prevent HIV and malaria in pregnant women are needed.

Many of the women in this study may have been HIV infected, and HIV could affect the immune environment of the placenta [31]. Although no HIV tests were done at the time of enrollment, reverse transcription–polymerase chain reaction analysis was done on all the biopsy specimens, and they were negative for HIV [16]. Nevertheless, some women in either group could still have been infected with HIV. However, one would not expect the incidence of HIV infection to be different in the infected and uninfected groups; no association between cord blood parasitemia and HIV infection was seen in a large cohort study of malaria- and HIV-infected mothers [28].

In conclusion, CCR5 expression is increased in placentas of malaria-infected women. Further studies are needed to determine whether this increased expression could contribute to intrauterine transmission of HIV. If such an association is found, it is possible

that interventions to prevent maternal malaria might aid in the prevention of mother-to-child HIV transmission.

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