Title: HIV-1 infection and antibodies to *Plasmodium falciparum* in adults

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Word count for Abstract: 201

Word count for text: 3496

ABSTRACT

Background: Co-infection with human immunodeficiency virus (HIV) may increase susceptibility to malaria by compromising naturally acquired immunity.

Methods: In 339 adults (64% HIV infected), we measured antibodies to *Plasmodium falciparum* variant surface antigens (VSA) and antibodies that opsonise infected erythrocytes using parasite lines FCR3, E8B and R29, and antibodies to merozoite antigens, AMA-1 and MSP2. We determined the relationship between malaria antibodies, HIV infection, markers of immune compromise, and risk of incident parasitemia.

Results: HIV-infected adults had significantly lower mean levels of opsonising antibody to all parasite lines ($P<0.0001$), and lower levels of antibody to AMA-1 ($P=0.01$) and MSP2 ($P<0.0001$). Levels of IgG to VSA were not affected by HIV status. Opsonising antibody titres against some isolates were positively correlated with CD4 count. There were negative associations between HIV-1 viral load and opsonising antibodies to FCR3 ($P=0.04$), and levels of IgG to AMA-1 ($P\leq0.03$) and MSP2-3D7 ($P=0.05$). Lower opsonising antibody levels on enrolment were seen in those who became parasitemic during follow up, independent of HIV infection ($P\leq0.04$ for each line).

Conclusion: HIV-1 infection decreases opsonising antibodies to VSA, and antibody to merozoite antigens. Opsonising antibodies were associated with lack of parasitemia during follow up, suggesting a role in protection.

Keywords: *Plasmodium falciparum*, HIV, malaria, antibody, IgG, phagocytosis, merozoite antigens, variant surface antigens

Introduction:

Co-infection with *Plasmodium falciparum* malaria and human immunodeficiency virus (HIV) is common in sub-Saharan Africa. Together these infections cause approximately 4 million deaths each year [1], and interactions between them are well recognised [2, 3]. By compromising host immunity, HIV infection increases susceptibility to malaria and reduces the efficacy of antimalarial drugs [4-7]. In pregnant women, HIV infection is associated with greater risk of severe anemia, decreased antimalarial antibody and increased density of placental parasitemia [8-11], and HIV-malaria co-infection leads to poor pregnancy outcomes such as low birthweight, preterm birth and post-neonatal mortality [2, 12-14].

In non-pregnant adults, HIV infection is associated with increased risks of parasitemia and uncomplicated malaria, of severe malaria, and of treatment failure[3]; these risks increase with advancing immunosuppression. The effect on malaria immunity, and how this effect translates into increased susceptibility, is less well understood. In one study, HIV infection was associated with lower levels of antibody to apical membrane antigen-1 (AMA-1), but not to variant surface antigens (VSA) on infected erythrocytes (IE) [5, 7].

Anti-malarial antibody has an important role in malaria immunity. AMA-1 and merozoite surface proteins (MSPs) have roles in erythrocyte invasion and these antigens have been considered as vaccine candidates [15]. Variant surface antigens (VSAs) on the infected

erythrocyte (IE) surface are involved in sequestration [16-18], and are believed to be major targets for immunity to blood stage infection.

In a longitudinal cohort study conducted before widespread availability of anti-retroviral drugs, malaria was shown to increase HIV viral load, and HIV infection predisposed to malaria parasitemia [19, 20]. Using samples from that study, we examined the humoral immune response to malaria in adults with and without HIV-1 infection. We hypothesised that HIV might have differential effects on different types of antibody to malaria blood stage antigens, and that adults lacking antibodies to one or more such targets were at increased risk of malaria infection during follow up. We measured antibody levels to VSA on IE, levels of functional antibodies that opsonised IE for phagocytosis, and antibody levels to merozoite antigens, in adults followed prospectively for 6 months, and determined whether antibody levels correlated with measures of HIV-1-mediated immune compromise including viral load and CD4 T cell counts. We examined whether antibody levels predicted risk of parasitemia during follow up.

Methods

Ethics statement

Ethical clearance for this study was obtained from the University of Malawi College of Medicine Research Ethics Committee and the Melbourne Health Human Research Ethics Committee. Written informed consent for HIV testing and enrolment in this study for all the participants was obtained in the initial study [20].

Study Design

The serum samples used in this study came from a cohort that has previously been described [19, 20]. Briefly, the study was conducted in Thyolo district, Malawi. Participants were enrolled before the malaria season and were followed until the end of the malaria season (From 1 October 2000 to 30 June 2001). Serum was available for 339 of 349 adults aged 18-70 years. Participants were recruited following testing for HIV-1 infection using rapid tests, namely Determine (Abbott), Uni-gold (Trinity Biotech) and Hemostrip HIV (Saliva Diagnostics). Serostatus was based on two agreeing rapid tests and out of serum available, 216 were HIV infected. Adults with AIDS-defining features, recent history of tuberculosis, or who had symptomatic malaria parasitemia at baseline were excluded. Participants were followed every 8 weeks in a dedicated clinic, where free treatment was available for malaria or other intercurrent illness. Following treatment of malaria parasitemia, additional visits were performed 3, 7, 14 and 28 days after administration of malaria chemotherapy. Parasitemias were treated with the then current national policy treatment for uncomplicated malaria, sulfadoxine/pyrimethamine (SP, Fansidar; Roche Isando). At enrolment, demographic data were collected and venous blood was taken to detect malaria parasites by microscopy of Giemsa stained thick blood films; to measure CD4 cell count by f;low cytometry (FACScount; Becton Dickinson) and HIV-1 RNA concentration was quantified by polymerase chain reaction (Amplicor HIV monitor 1.5 assay; Roche Molecular Systems); and to measure hemoglobin concentration. Enrollment serum was separated, stored at -70^{o}C and assayed in Malawi, or transported on dry ice to Melbourne.

Cell and Parasite Culture

P. falciparum parasite lines used in this assay were FCR3 which binds to CD36; E8B, which binds to ICAM-1 and CD36; and R29 which forms rosettes and binds to complement receptor 1 (CR1) on erythrocytes [21]. Parasite lines were cultured in human group O^+ erythrocytes (Australian Red Cross Blood Service) and maintained at 5-10% parasitemia in RPMI 1640-HEPES medium supplemented with 0.25% AlbumaxII (Gibco), 5% heat-inactivated non-immune human sera (Australian Red Cross Blood Service) and 25 mM $NaHCO_3$. Cultures were selected for knob expression by flotation in 0.75% gelatin in RPMI-1640 fortnightly and adhesion to receptors was regularly checked to ensure high levels of binding. R29 was regularly selected to increase rosetting. In the assay using R29 IE, 100 U/mL Heparin (Sigma) were added in the wash buffer and medium in order to prevent rosetting.

Undifferentiated THP-1 (uTHP-1) cells used in the phagocytosis assay were maintained at density below $5x10^5$ cells/mL in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 2 mM glutamine and 25 mM of HEPES (all from Gibco).

Phagocytosis Assay

Phagocytosis was measured as described by Ataide and colleagues with minor modifications [22]. Trophozoite-stage IE were purified by density gradient centrifugation by layering on a gradient of 80%, 60% and 40% Percoll (Amersham) in RPMI 1640-HEPES. Purified IE on the 60% layer were collected, were washed three times with RPMI-1640-HEPES and stained with 10 μg/mL of ethidium bromide (EtBr, Bio-Rad Laboratories) for 30 minutes. Stained IE were washed five times with RPMI-HEPES before being resuspended at 3.3 x 10^7cells/mL. 3.3 μL of

test serum, patient pooled serum (positive control for E8B-ICAM) or rabbit antiserum to human red blood cells (positive control for FCR3 and R29 with working concentration of 90 μg/mL, MP CAPPEL) that had been previously plated in 96-well U-bottom plate were incubated with 30 μl of IE suspension (1:10 dilution) for 1 hour. A no-serum control was used as negative control to establish appropriate gates for the flow cytometry. IE were incubated with 5 x 10^4 uTHP-1 cells/well at 1:10 ratio for 40 minutes at 37^{o}C in humidified 5% CO_2. Phagocytosis was stopped by centrifugation at 4^{o}C at 350 g for 5 minutes). Unphagocytosed IE were lysed by adding FACS Lysing solution (prepared in 1:10 dilution in distilled water; BD Biosciences) for 10 minutes. Lysis was stopped by adding cold FACS Buffer solution (phosphate-buffered saline (PBS (-$Ca^{2+,}$ -Mg^{2+}), 2 % FBS and 0.02% NaNO3). cells were washed three times with FACS Buffer before the cells were fixed with cold 2% paraformaldehyde in PBS.

Cells were acquired in a CYAN hypercyt flow cytometer. THP-1 cells were gated based on forward (FSC) and side scatter (SSC) properties. No-serum controls were used to set up the gating of non-Fc-receptor-mediated phagocytosis, which was set to be less than 5%. The phagocytosed iRBC were recognized by EtBr fluorescence (FL2 channel). Sample duplicates were acquired for 55 seconds per well; 8000-15,000 THP-1 cells were acquired per well.

IntelliCyt HyperView® (IntelliCyt Corp) was used to analyze the data and the opsonizing antibodies were measured by calculating the percentage of THP-1 cells that phagocytosed the IE relative to the percentage of the positive control.

IgG to Variant Surface Antigen assay

Levels of IgG to VSA were measured as described with minor modifications [11]. Trophozoite-stage IE were harvested at 5-8% parasitemia and washed three times with PBS/1% newborn calf serum (NCS, Gibco). IE were prepared at 0.2% hematocrit in PBS/1% NCS and incubated with patient serum or control at 1 in 20 dilution (final volume 50 µl) in 96-well U-bottom plates at room temperature for 30 minutes, in duplicate. Cells were washed as before and incubated for 30 minutes at room temperature with rabbit anti-human IgG (Dako; 1:100 in PBS/1% NCS,). After washing thrice as before, Alexafluor 488-conjugated donkey anti-rabbit IgG (1:500 in PBS/1% NCS, Invitrogen) and 10 µg/mL EtBr were added and samples were incubated in the dark for 30 minutes at room temperature. Cells were washed thrice, resuspended and fixed with 2% Paraformaldehyde in PBS.

Cells were acquired in a CYAN hypercyt flow cytometer. RBC were gated on FSC and SSC properties and IE were gated according to EtBr fluorescence (FL2 channel). The geometric mean fluorescence intensity (MFI) of Alexafluor 488 (FL1 channel) generated by the gated IE population was used as a relative measure of human IgG binding to iRBC for each serum sample. The positive control was a pool of serum with known high antibody recognition to the specific parasite lines, and negative controls were from 9 unexposed Australian donors.

The data were analyzed in Intellicyt Hyperview Analysis and samples MFI was calculated relative to the MFI of positive and negative controls.

ELISAs to measure IgG to merozoite antigens

Microtitre plates were coated with recombinant proteins AMA1 and MSP2 (3D7 and FC27 types) at 2 μg/mL in PBS (kindly provided by Prof. R. Anders) with a final volume of 100 μl per well and were left overnight at 4^{o}C. After washing three times with PBS containing 0.05% of Tween (PBS/Tween), plates were blocked with 5% skim milk powder in PBS (Blotto) for one hour at room temperature. Plates were washed five times with PBS/Tween and samples were diluted 1/1000 in Blotto before being added to plates (final volume of 50 μl, in duplicate) and left to incubate for 2 hours at room temperature. Plates were again washed three times in PBS/Tween, and horseradish peroxidase-conjugated sheep anti-human IgG (1:2000, 50 μl per well; Silenus) was added. Plates were incubated for 2 hours at room temperature and washed three times with PBS/Tween and twice with deionized water. Peroxidase substrate was added and color was developed. Plates were read at 415 nm on a Biorad plate reader, and optical density (OD) results were obtained.

Statistical Analysis.

Results were analysed using Stata version 11.0 (Stata Corporation, College Station, Texas, USA). Non-normally distributed variables such as level of phagocytosis and level of IgG to VSA and to merozoite antigens were analyzed using Mann-Whitney rank sum tests. P-values <0.05 were considered to be significant.

Results

Characteristics of study population:

Of 339 adults enrolled in this study 216 (64%) were HIV-1 infected (Table 1). Compared to uninfected adults, HIV-1 infected adults had a lower mean hemoglobin concentration ($P<0.0001$); no other significant differences were observed between groups.

Effect of HIV-1 serostatus on antibody level and function

HIV-1 infected adults had significantly lower mean levels of opsonising antibody against each of the three parasite lines tested, FCR3 ($P<0.0001$), E8B-ICAM ($P<0.0001$) and R29 ($P<0.0001$) (figure 1 A).

Antibody levels to VSA were not different between HIV-infected adults and non-infected adults for parasite line FCR3 ($P=0.25$), E8B-ICAM ($P=0.23$) or R29 ($P=0.49$) (figure 1 A).

HIV-1 infected adults had significantly lower mean levels of IgG to merozoite antigens than non-infected adults. These differences were observed for responses to AMA-1 and to both 3D7 and FC27 MSP2 types (AMA-1: $P=0.01$; MSP2-FC27: $P<0.0001$; MSP2-3D7: $P<0.0001$) (figure 1).

Effect of HIV-1 severity on antibody level and function

The relationship between HIV-related immune suppression (as measured by HIV-1 RNA concentration and CD4 T cell count at enrolment) and antimalarial antibody levels was examined. CD4 counts were categorized as high (≥400 cells/mL), intermediate (200-399 cells/mL) and low (<200 cells/mL) [20]. There were positive associations between CD4 cell counts and amounts of opsonising antibody to FCR3 and E8B-ICAM. Significantly - decreased levels of IgG to VSA with -decreasing CD4 count were seen in E8B-ICAM and R29 but not FCR3. A similar decreasing trend with lower CD4 cell count was observed in levels of IgG to AMA-1, MSP2-3D7 and MSP2-FC27, but this was not significant (table 2).

HIV-1 viral load was categorized as low (<10,000 copies/mL), moderate (10,000 to 99,999 copies/mL) and high (≥100,000 copies/mL) [20]. There was a negative association between the concentration of HIV-1 and functional antibodies to FCR3 but not E8B and R29. In contrast, IgG to VSA showed a borderline significant positive correlation for FCR3 but not E8B and R29. With increasing HIV-1 concentration there was a significant decrease in mean levels of IgG to AMA-1 and MSP2-3D7 but not MSP2-FC27 (table 2).

Relationship between antibodies at enrolment and malaria infection during follow up

Among adults without malaria on enrolment, those who had parasitemia on follow up had lower mean opsonising antibody levels on enrolment than those who did not develop parasitemia, for all parasite lines (FCR3: P=0.01; E8B-ICAM: P=0.01; R29: P=0.04). After adjusting for HIV status, levels of opsonising antibody to FCR3 (P= 0.03) and E8B (P=0.03) but not R29 (P=0.21) remained significantly associated with lack of parasitemia. By contrast, levels of IgG to VSA and to merozoite antigens were not associated with parasitemia on follow up after adjusting for HIV status (Figure 2).

Discussion

HIV-infected adults are more vulnerable to malaria parasitemia, and to symptomatic and severe malaria, and each of these risks increases with the severity of HIV-related immunocompromise [5, 20, 23]. However, little is known regarding the effect of HIV infection on malaria immunity in non-pregnant adults. To understand how HIV affects immunity to malaria we measured parameters of humoral immunity in both HIV-infected and uninfected Malawian adults. These

parameters included levels of antibodies that opsonised IE, levels of IgG to VSA of three parasite lines, and levels of IgG to three merozoite antigens.

The cohort we studied is a unique resource of adults of known HIV status followed prospectively for malaria infection. Predating the widespread availability of antiretroviral treatment (ART) in Malawi, the cohort allows us to observe the relationships between HIV, malaria and malaria immunity in a large number of ART treatment-naïve adults, with appropriate HIV negative controls. Antiretroviral drugs reconstitute the individual's immune system, although this may not eliminate malaria risk [24], and protease inhibitors and cotrimoxazole prophylaxis both have direct antimalarial effects [25, 26], meaning that studies such as the present one cannot now be performed.

HIV-infected adults had significantly lower levels of opsonising antibodies to the three parasite lines tested. This finding is consistent with previous studies showing an association between HIV and decreased opsonising antibodies to pregnancy-associated IE in pregnant women [9, 10, 22, 27]. We have previously demonstrated that in vitro HIV infection of macrophages substantially decreases their ability to phagocytose opsonised IE [28]. Reduced levels of opsonising antibodies and of phagocytosis in HIV-infected adults suggest incompetency in clearing parasites, and this could explain the association between HIV-1 positivity and increased risk of parasitemia seen in this cohort [20].

Immunity against *P. falciparum* malaria requires, in part, acquisition of a broad repertoire of antibodies to *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), a family of

variant proteins expressed at the IE surface [29, 30]. We compared the level of antibodies to three parasite lines, one that forms rosettes and that is associated with severe malaria [31 , 32], and two that are representative of those that might cause uncomplicated malaria in older children and adults [33, 34 , 35]. Fewer HIV-infected than HIV-negative adults had high levels of opsonising antibodies to these lines (Figure 2), which suggests a reduced capability to produce a repertoire of functional antibodies against different variants of proteins expressed on IE, potentially contributing to reduced parasite clearance.

In this cohort, levels of antibody to VSA, measured as antibody binding to the IE surface, were not affected by HIV infection. We made similar observations in another study of non-pregnant adults [7]. Unlike opsonising antibodies, these antibody responses appear to be unchanged by HIV infection, perhaps because HIV infection has limited effects on established antibody responses, such as those associated with life-long exposure to malaria. By contrast, HIV-infected pregnant women of all gravidities have considerably impaired production of IgG to pregnancy-specific VSA[11]. Expression of this unique member of the PfEMP1 family is restricted to pregnant women, in whom it mediates sequestration of IE in the placenta [36]; thus women in their first pregnancy are encountering this antigen for the first time. HIV infection may have a greater impact on development of antibody responses to newly-encountered antigens than on maintenance of IgG antibody responses to VSA in individuals with previously-established antibody responses.

Our findings showed that levels of IgG antibody to merozoite antigens were on average significantly lower in HIV-infected than uninfected adults. Reduced antibody levels to AMA-1

in HIV-infected adults have also been observed in adults, children and pregnant women, however responses to MSP2 were minimally affected [7, 11, 37]. Reasons why HIV-infected adults had lower levels of IgG to merozoite antigens but not to IE are not known, but the persistence of antibody in the absence of boosting might provide some insights. In African immigrants with no or limited ongoing malaria exposure, antibody responses to merozoite antigens and to IE show a broadly similar decrease over time [38], whereas in pregnant Thai women the half life of antibody to merozoite antigens was shorter than that for antibody to the VSA, VAR2CSA [39]. If responses to the merozoite antigens tested have shorter half-lives in our Malawian population, the effect of HIV infection on these responses would be manifest sooner.

The differential effect of HIV on opsonising antibody levels, compared to total IgG to VSA, is unlikely to be due to assay characteristics. Opsonising assays are performed at or above saturating concentrations [22], whereas for antibody to VSA a 50% reduction in antibody titre is associated with a similar decrease in measured antibody levels (W Hasang, unpublished). Thus opsonising antibodies would be less, not more, susceptible to minor decreases in antibody level. The difference is likely to be biologically relevant, as opsonising antibody has been more strongly associated with protection from complications of malaria in several studies [40-42]. Cytophilic IgG3 antibodies that opsonise IE and promote phagocytosis via Fc receptors [43, 44] predominated in healthy Gabonese adults [45], and IgG1 and IgG3 antibodies were dominant in children with malaria infection [46]. HIV decreases these IgG1 and IgG3 responses to pregnancy associated IE [27], and IgG3 has the shortest half life of antibody subclasses, and so may be most affected by decreases in antibody production. Whether HIV differentially affects IgG subclass responses to VSA or other malaria antigens is unknown, but levels of IgG1 or IgG3 antibodies

specific to H1N1 influenza virus do not appear to be affected by HIV infection [47, 48]. There could be more subtle differences in functional activity of antibodies to IE produced by HIV infected adults, and further research that includes antibody subclass is required to confirm this finding.

In HIV positive adults, opsonising antibodies and VSA antibodies were generally decreased with lower CD4 count, and opsonising antibodies were also negatively correlated with HIV-1 viral load, suggesting that functional antibody to malaria antigens is particularly affected by the degree of immunosuppression. Weaker associations were seen between antibodies to merozoite antigens and indices of immune suppression. In the cohort, there were moderate but inconsistent associations between CD4 count or viral load and risk of parasitemia, and a stronger relationship between low CD4 count and symptomatic malaria [20]. Taken together these findings suggest that decreases in opsonising antibodies, which showed the strongest correlation with increased immune suppression, could be responsible, at least in part, for the increased predisposition to parasitemia and clinical malaria seen in the most immunocompromised participants. Although we did not see a direct relationship between HIV infection and increased episodes of malaria, in the complete study cohort HIV-infected adults had a higher incidence of parasitemia than controls [20]. This suggests that, in this Malawian community, poor antibody responses in HIV-infected adults may be contributing to their increased risk of parasitemia.

Levels of opsonising antibody (and not antibodies to VSA or merozoite antigens) were associated with lower risk of infection with malaria during follow up and this was independent of HIV status. This finding suggests that opsonising antibodies directed against IE that are

commonly recognised in adults have a role in protection against parasitemia. Clinical malaria was uncommon in this cohort, and it would be of interest to determine whether opsonising antibodies are particularly protective against symptomatic disease in appropriate cohorts, to add to published observations of associations with protection against anemia, low birth weight and treatment failure [9, 10, 40] Taken together, these studies suggest that high levels of opsonising antibodies could be a marker for protection against parasite infection. Opsonising antibodies could contribute to protection by enhancing clearance of IE by blood monocytes or tissue macrophages. It has also been shown that antibodies of the cytophilic subclasses IgG1 and IgG3 were dominant among healthy adults, children and pregnant women [44-46].

HIV-infected adults who are constantly exposed to malaria have altered humoral immunity to *P. falciparum*, with lower levels of opsonising antibodies to IE. Low levels of opsonising antibodies potentially explain the increase of parasitemia seen in HIV infected adults, and were associated with decreased risk of malaria infection during follow up in this study. Measuring opsonising antibodies to IE expressing locally-prevalent surface antigens may be a useful correlate of the risk of malaria re-infection.

Footnote Page

(1) Acknowledgements: We thank all the participants who took part in the study. Prof. R. Anders is thanked for providing recombinant AMA1 and MSP2 (3D7 and FC27) proteins.

(2) Financial support: The laboratory work was supported by the National Health and Medical Research Council of Australia.

(3) Potential conflicts of interest: None reported

(4) This information was presented in part at the Molecular Approaches to Malaria 2012 conference, 19-23rd February 2012, Lorne, VIC, Australia

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Figure legends

Table 1. Characteristic of study population based on HIV-1 status

NOTE: P value based on Students' t-test, Chi square or Wilcoxon Rank-Sum as required

Figure 1. Anti-malarial antibody levels and functions based on HIV-1 serostatus.

A) Levels of opsonising antibodies to parasite lines FCR3, E8B-ICAM and R29. Adults infected with HIV had significantly lower opsonising antibodies to the three different parasite lines; B) Levels of IgG to VSAs of FCR3, E8B-ICAM and R29 were not different between HIV-infected adults and non-infected adults; C) Levels of IgG to merozoite antigens AMA-1 and MSP2 (FC27 and 3D7) were significantly lower in HIV infected individuals. P values by Mann-Whitney's rank sum test. Boxes represent median (center line) and interquartile ranges. Whiskers represent 95% Cl. Dots represent outliers.

Table 2. Anti-malarial antibody based on the indicators of HIV severity within HIV infected adults.

P value based on Wilcoxon Rank-Sum test

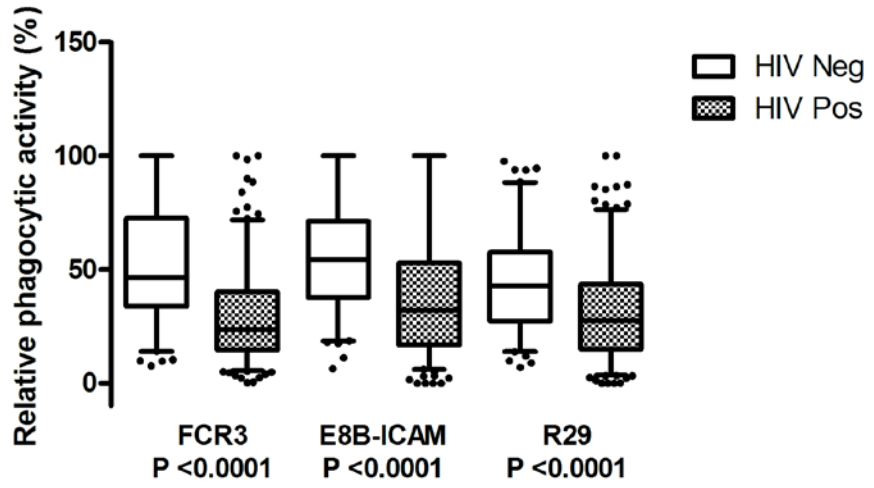
Figure 2. Relationship of anti-malaria antibody levels at enrolment and parasitemia during follow up. Serum was collected at enrolment, participants were followed prospectively for 6 months, and incidence rate of parasitemia was recorded. A) Levels of opsonising antibodies to parasite lines FCR3, E8B-ICAM and R29 were significantly lower in adults who became parasitemic on follow up. After adjusting for HIV status, levels of opsonising antibody to FCR3 (P= 0.03) and E8B (P=0.03) but not R29 (P=0.21) remained significantly associated with protection; Levels of B) IgG to variant surface antigens of FCR3, E8B-ICAM and R29 and C) IgG to AMA-1 and MSP2 (FC27 and 3D7) did not differ between individuals who did and did not develop parasitemia. P value by Mann-Whitney's rank sum test. Boxes represent median (center line) and interquartile ranges. Whiskers represent 95% Cl. Dots represent outliers.

Table 1. Characteristics of study population based on HIV-1 status

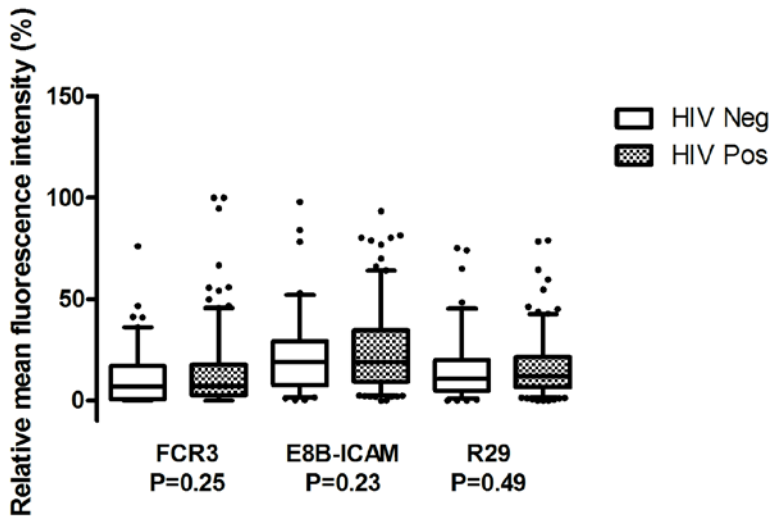
Characteristic	Non HIV-1 infected adults (n=123)	HIV-1 infected adults (n=216)	P Value
Number of women, N (%)	56 (45.5)	88 (40.7)	0.39
Mean weight, kg (SD)	55.5 (9.7)	54 (7.4)	0.11
Mean age, years (SD)	31.9 (10.2)	32.7 (8.8)	0.29
Mean hemoglobin, g/dl (SD)	13.9 (1.7)	12.6 (1.9)	**<0.0001**
Parasitemia episodes during follow up, N (%)	48 (39)	100 (46.5)	0.18
Mean log viral load HIV-1 RNA copies/µl (SD)	N/A	4.8 (0.7)	
Median CD4 cell count at baseline, cells/mL(IQR)	N/A	347 (233-498.5)	

NOTE: P value based on Students' t-test, Chi square or Wilcoxon Rank-Sum as appropriate

A


Relative phagocytic activity (%)
150
100
50
0
HIV Neg
HIV Pos
FCR3
P <0.0001
E8B-ICAM
P <0.0001
R29
P <0.0001

B


Relative mean fluorescence intensity (%)
150
100
50
0
HIV Neg
HIV Pos
FCR3
P=0.25
E8B-ICAM
P=0.23
R29
P=0.49

C

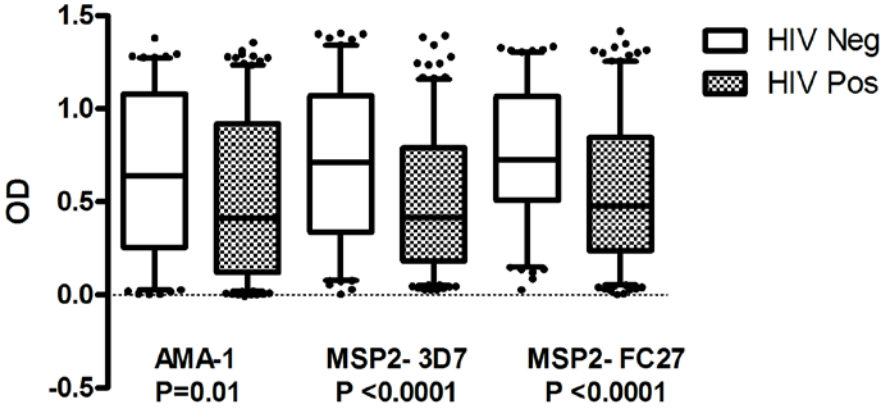

OD
1.5
1.0
0.5
0.0
-0.5
HIV Neg
HIV Pos
AMA-1
P=0.01
MSP2- 3D7
P <0.0001
MSP2- FC27
P <0.0001

Figure 1. Anti-malarial antibody levels and functions based on HIV-1 serostatus. A) Levels of opsonising antibodies to parasite lines FCR3, E8B-ICAM and R29. Adults infected with HIV had significantly lower opsonising antibodies to the three different parasite lines; B) Levels of IgG to VSAs of FCR3, E8B-ICAM and R29 were not different between HIV-infected adults and non-infected adults; C) Levels of IgG to merozoite antigens AMA-1 and MSP2 (FC27 and 3D7) were significantly lower in HIV infected individuals. P values by Mann-Whitney's rank sum test. Boxes represent median (center line) and interquartile ranges. Whiskers represent 95% Cl. Dots represent outliers.

Table 2. Anti-malarial antibody based on markers of HIV severity within HIV infected adults.

Variables	n (%)	Opsonic antibodies			IgG levels to VSA			IgG levels to merozoite antigens		
		FCR3 P value (Z)	E8B P value (Z)	R29 P value (Z)	FCR3 P value (Z)	E8B P value (Z)	R29 P value (Z)	FCR3 P value (Z)	E8B P value (Z)	R29 P value (Z)
CD4 count (c/mL)										
>400	74 (40.7)	---	---	---	---	---	---	---	---	--
200-399	76 (41.8)	**0.02** **(**-2.4)	0.3 (-1.1)	0.8 (0.20)	0.24 (-1.2)	0.6 (-0.6)	0.15 (-1.5)	0.47 (-0.7)	0.06 (-1.9)	0.66 (-0.4)
<200	32 (17.6)	0.09 (-1.7)	**0.001** **(**-3.2)	0.96 (0.05)	0.9 (-0.09)	**0.03** **(**-2.2)	**0.02** **(**-2.4)	0.21 (-1.2)	0.19 (-1.3)	0.09 (-1.7)
HIV-1 RNA concentration (copies/mL)										
<10,000	78 (41.9)	---	---	---	---	---	---	---	---	--
10,000-99,999	82 (44.1)	0.08 (-1.8)	0.39 (-0.9)	0.87 (1.6)	0.09 (1.7)	0.21 (1.3)	0.76 (-0.3)	**0.03** **(**-2.2)	**0.05** **(**-1.9)	0.13 (-1.5)
≥100,000	26 (14.0)	**0.04** **(**-2.0)	0.64 (-0.5)	0.2 (1.3)	**0.05** **(**2.0)	0.79 (-0.2)	0.72 (-0.4)	**0.02** **(**-2.4)	0.08 (-1.8)	0.1 (-1.6)

NOTE: P value base on Wilcoxon Rank-Sum test. Dashed lines represent the reference groups.

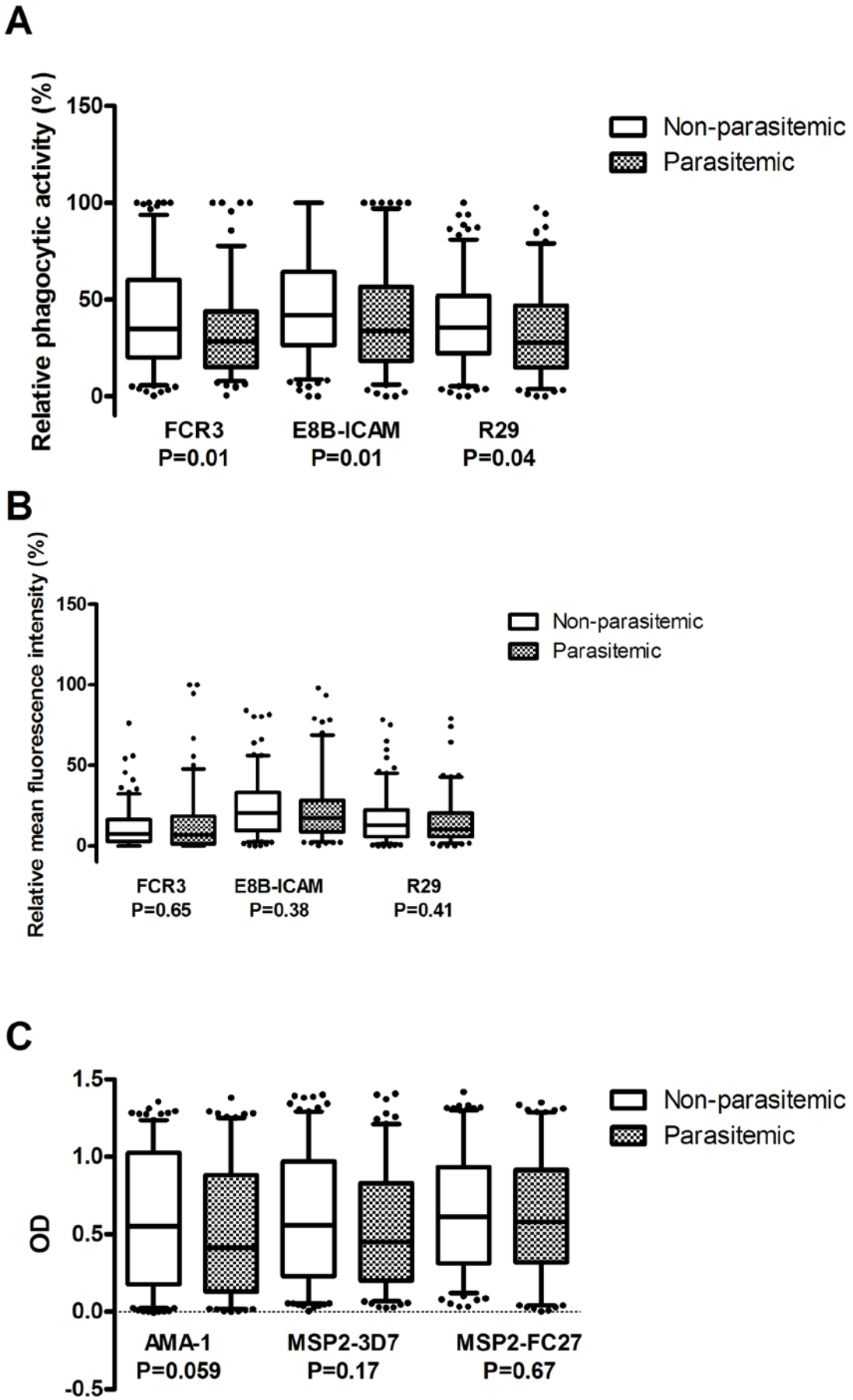

A
Relative phagocytic activity (%)
150
100
50
0
Non-parasitemic
Parasitemic
FCR3
P=0.01
E8B-ICAM
P=0.01
R29
P=0.04
B
Relative mean fluorescence intensity (%)
150
100
50
0
Non-parasitemic
Parasitemic
FCR3
P=0.65
E8B-ICAM
P=0.38
R29
P=0.41
C
OD
1.5
1.0
0.5
0.0
-0.5
Non-parasitemic
Parasitemic
AMA-1
P=0.059
MSP2-3D7
P=0.17
MSP2-FC27
P=0.67

Figure 2. Relationship of anti-malaria antibody levels at enrolment and parasitemia during follow up. Serum was collected at enrolment, participants were followed prospectively for 6 months, and incidence rate of parasitemia was recorded. A) Levels of opsonising antibodies to parasite lines FCR3, E8B-ICAM and R29 were significantly lower in adults who became parasitemic on follow up. After adjusting for HIV status, levels of opsonising antibody to FCR3 (P= 0.03) and E8B (P=0.03) but not R29 (P=0.21) remained significantly associated with protection; Levels of B) IgG to variant surface antigens of FCR3, E8B-ICAM and R29 and C) IgG to AMA-1 and MSP2 (FC27 and 3D7) did not differ between individuals who did and did not develop parasitemia. P value by Mann-Whitney's rank sum test. Boxes represent median (center line) and interquartile ranges. Whiskers represent 95% Cl. Dots represent outliers.

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Title:
HIV-1 Infection and Antibodies to Plasmodium falciparum in Adults

Date:
2014-11-01

Citation:
Hasang, W., Dembo, E. G., Wijesinghe, R., Molyneux, M. E., Kublin, J. G. & Rogerson, S. (2014). HIV-1 Infection and Antibodies to Plasmodium falciparum in Adults. JOURNAL OF INFECTIOUS DISEASES, 210 (9), pp.1407-1414. https://doi.org/10.1093/infdis/jiu262.

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