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Stage-Dependent Release of *Plasmodium Falciparum* Homolog of T-Cell Immunomodulatory Protein and Association of Plasma Levels with Suppression of Macrophage-Inflammatory Protein-1 Chemokine Production in Infected Individuals

Donkeu Christiane[1, 2], Efange Noella[1, 3], Atchombat Derrick[1, 2], Emoh Cynthia[1, 4], Fogang Balotin[1], Biabi Marie Florence[1], Mbianda Nana Chris Marco[1, 2], Essangui Estelle[1], Maloba Franklin, Cheteug Glwadys[1], Kemleu Sylvie[1], Nsango Sandrine[1, 5], Eboumbou Carole[1,5], Perraut Ronald[1], Regis Grailhe[6], Douglas H Cornwall[7], Njiokou Flobert[2], Tracey J Lamb[7] and Ayong Lawrence[1,*]

[1]Malaria Research Unit, Centre Pasteur du Cameroun, BP1274 Yaounde, Cameroon
[2]Department of Animal Biology and Physiology, University of Yaounde 1, BP 812 Yaounde, Cameroon
[3]Department of Biochemistry, University of Buea, BP 63 Buea, Cameroon
[4]Department of Biochemistry, University of Yaounde 1, BP 812 Yaounde, Cameroon
[5]Department of biological sciences, Faculty of Medicine and Pharmaceutical sciences, University of Douala, BP 2701 Douala, Cameroon
[6]Center for Core Technologies, Institut Pasteur Korea, South Korea
[7]Department of pathology, University of Utah, 15 N Medical Drive, Salt Lake City 84112,United States of America

[*]**Corresponding Author:** Ayong Lawrence, Malaria Research Unit, Centre Pasteur du Cameroun, BP1274 Yaounde, Cameroon, Tel.: +237222232015, E-mail: layong05@yahoo.co.uk

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Abstract

The *Plasmodium falciparum* T-cell immunomodulatory protein homolog, *Pf*TIP, is a novel invasion-related protein that is thought to play additional roles in modulating host immune responses to the infection. We examined the cellular localization and secretion patterns of *Pf*TIP in laboratory cultured *Plasmodium falciparum* parasites and in naturally infected individuals, and investigated the association between plasma levels of the protein and host immune responses. We show by immunofluorescence microscopy and enzyme linked immunosorbent assays that *Pf*TIP is expressed in all major asexual forms of the parasite and exported in a stage-dependent manner to the infected host erythrocyte surface, where it is released predominantly during schizont development. The protein was detected in the plasma of infected individuals at levels indepen-

dent of peripheral blood parasitaemia. High plasma levels of naturally induced anti-*Pf*TIP antibodies strongly associated with the submicroscopic carriage or absence of parasites in peripheral blood. Interestingly, rabbit antibodies to an immunodominant peptide of the *Pf*TIP protein significantly reduced parasite growth *in vitro*. Compared with the *P. falciparum*-specific protein, *Pf*HRP-2, that correlated positively with MIP-1, we observed a strong negative correlation between plasma levels of *Pf*TIP and MIP-1 indicating a potential modulation of vascular activation. TNF-α and IL-10 levels were generally low and a balanced TNF-α/IL-10 ratio was observed regardless of individual parasitaemia or *Pf*TIP plasma levels. Taken together, the data suggest a role for *Pf*TIP in host cell invasion by *P. falciparum* and in suppressing host inflammatory responses in favor of parasite survival.

Keywords: Plasmodium falciparum; T-cell immunomodulatory protein homologue; host cell invasion; anti-inflammatory activity

Introduction

Malaria is a debilitating disease caused by protozoan parasites of the genus *Plasmodium,* which are transmitted to humans through the bite of various mosquito vectors [1]. Of the five *Plasmodium* species known to cause malaria in humans, *P. falciparum* kills the most, followed by *P. vivax*, with *P. malariae*, *P. ovale* and *P. knowlesi* causing milder disease [2]. To ensure survival within the host, *Plasmodium* parasites have evolved a range of immune evasion strategies that include the expression of adhesion molecules on the surface of infected erythrocytes critical for parasite sequestration and escape of vulnerable parasite stages from splenic clearance, antigenic variation involving multiple virulence factors, and secretion of immune modulators aimed primarily at inhibiting deleterious pro-inflammatory host responses [3, 4].

Indeed, many parasite proteins can interact with immune cell surface receptors to prevent cell activation. For example, hepatic stages of the parasite release circumsporozoite surface proteins (CSP) that suppress NF-*k*B signaling, thereby modulating host inflammatory responses to the infection [5]. CSP also interferes with LRP-1 (low-density lipoprotein receptor-related protein) and proteoglycans on the surface of Kupffer cells, increasing intracellular cAMP/EPAC levels and preventing the formation of reactive oxygen species (ROS) capable of killing the parasite [6, 7]. Similarly, *Plasmodium* gametocytes express on their surface a 6-cysteine protein family member, Pfs47, capable of suppressing nitration responses in the mosquito midgut, critical for activation of the complement-like system, thus allowing its efficient transmission by the vector [8, 9].

Within the bloodstream, asexual forms of malaria parasites express a family of multigene proteins, including *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1), repetitive interspersed repeats (RIFIN), and subtelomeric variant open reading frame (STEVOR) capable of binding to inhibitory receptors on immune cells and suppressing their activation [3, 10]. *Pf*EMP-1 suppresses NF-*k*B activity in monocytes and macrophages, leading to suppression of cytokine and chemokine production [11]. In addition, *Pf*EMP-1 binds to dendritic cells via CD36 and CD51, significantly reducing their ability to present antigens while also suppressing cytokine release by innate immune cells such as NK and γδ T cells [12]. On its part, RIFINS are known to bind human inhibitory receptors such as LILRB1, LILRB2 and LAIR1 expressed on NK cells, monocytes, B and T cells that are capable of recognizing MHC class 1 molecules as ligands to suppress immune responses to host cells [13, 14]. Indeed, immune evasion by malaria parasites is complex, and studies have yet to fully unravel the underlying mechanisms.

Recent studies with the mouse malaria model, *P. berghei*, identified the T cell immunomodulatory protein homolog, *Pb*TIP, as a novel immune inhibitory factor of malaria parasites, demonstrating its release and binding to macrophages to suppress inflammatory cytokine responses [15, 16]. The human malaria parasite, *P. falciparum* expresses a TIP homolog that shares 85% amino acid sequence similarity with *Pb*TIP and 29.6% identity with human TIP [17]. Earlier studies on *Pf*TIP revealed a role in

host erythrocyte invasion [18]. However, nothing is known about the expression and localization patterns of the protein in human malaria parasites or its interaction with host immune system. In this study, we sought to investigate the potential dual role of *Pf*TIP in invasion and immune suppression.

Given the aforementioned knowledge gap, we integrated immunological profiling of plasma samples from a malaria endemic population in Cameroon and *in vitro* studies of laboratory cultured *P. falciparum* malaria parasites. We show by immunohistochemistry and experimental analyses of human samples from Cameroon that *Pf*TIP release in *P. falciparum* is associated with suppression of pro-inflammatory MIP-1, a chemokine largely produced from endothelial cells that attracts monocytes and macrophages to the inflamed endothelium. We data further suggest that anti-*Pf*TIP antibodies may protect against parasite invasion of host cells, indicating a dual role for *Pf*TIP.

Materials and Methods

Parasite Cultures: *P. falciparum* (strain 3D7) parasites were obtained from the Biodefense and Emerging Infections (BEI) Research Resources (Manassas, VA) and maintained in O+ human red blood cells (RBCs) in RPMI 1640 medium as previously described [19, 20]. When needed, parasites were synchronized at the ring stage by sorbitol (5%) treatment [21] and further cultivated through one complete cycle prior to use.

Anti-*PfTIP* Peptide Antibodies: Antisera were raised in rabbits against the tetradecapeptide NGVKSSRIGVQKSQ, derived from the membrane proximal segment (residues 589-602) of *Pf*TIP, and affinity purified (GenScript Inc, USA) prior to use in this study. The reactivity and specificity of the obtained anti-*Pf*TIP antibodies (concentration of 2.631 mg/ml) against a *P. falciparum* (strain 3D7) whole cell extracts or the free *Pf*TIP peptide was confirmed by Western immunoblot and indirect ELISA analyses, respectively.

Plasma Samples: A total of 121 plasma samples from residents of a malaria endemic area (96 *P. falciparum*-infected and 25 uninfected) were tested. In addition, 20 plasma samples from unexposed Europeans were purchased (EFS, France) and used as non-endemic controls in the immunoassays. All malaria endemic samples were collected from five villages in the Esse health district in Cameroon during a previous study in 2018 [22]. *P. falciparum* infection was detected by thick smear microscopy and multiplex PCR, and the participants sub-divided into symptomatic and asymptomatic groups based on the presence or absence of fever (axial temperature ≥37.5°C) in the 48 hours preceding sample collection, or microscopic and submicroscopic infection groups based on sample positivity by thick blood smear microscopy and/or multiplex PCR targeting the 18S rRNA gene of *Plasmodium* parasites [23].

Indirect Immunofluorescence Assay: *P. falciparum* parasitized cultures were fixed in 4% formaldehyde and then permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS), pH 7.2 prior to mounting on poly-L-lysine-coated coverslips. To test for surface exposure of the *Pf*TIP protein on *P. falciparum*-infected erythrocytes, aliquots of the paraformaldehyde--fixed cells were mounted unpermeabilized on a second coverslip. Coverslips were blocked with 3% bovine serum albumin in PBS for 30 minutes and incubated with affinity purified anti-*Pf*TIP antibodies (1:100 dilution in 3% BSA/PBS) for 2 hours at room temperature. Upon washing three times with PBS, the coverslips were incubated with goat anti-rabbit Alexa Fluor 594 antibody conjugates (Molecular Probes) diluted 1:1000 in 3%BSA/PBS for 1 hour at room temperature. The coverslips were then washed four times in PBS and mounted with a drop of mounting solution (Prolong Diamond Antifade Mountant with DAPI, Invitrogen). The coverslips were then examined under a 100x oil objective using a fluorescence microscope (Leica DM 1000). Captured images were further processed using the open-source software Fiji-Image J.0.

Antigen Release Assay: Doubly synchronized *P. falciparum* parasites at the early ring stage (6-8 hours post-invasion) were washed twice with complete medium and re-introduced into culture at ~5% parasitaemia and 3% hematocrit. The culture was

maintained for 12 hours and the spent media corresponding to the 6-18 hours post-invasion (hpi) time point was recovered by centrifugation (Universal 320R, Hettich) at 2 000 rpm and 4°C for 5 minutes. The resulting cell pellet was washed twice and reintroduced to culture for a further 12 hours prior to collection of spent media corresponding to the 18-30 hpi time point. This process was repeated for two additional steps to recover spent media corresponding to the 30-42 hpi and 42-6 hpi time--points, respectively. Recovered samples were stored at -80°C until needed for antigen capture ELISAs. Briefly, levels of parasite antigens (*Pf*TIP, *Pf*MSP-5, *Pf*BIP, or *Pf*HRP-2) in the recovered spent media (1:2 diluted in PBS/1%BSA) were estimated by sandwich ELISA using purified rabbit anti-*Pf*TIP (this study), rabbit anti-*Pf*MSP-5 (MRA-320, BEI Resources), and rat anti-*Pf*BIP (MRA-1247, BEI Resources) each diluted 1:500 in PBS, or mouse anti-*Pf*HRP-2 monoclonal antibody (MA5-18245, Invitrogen, USA) at 0.1µg per well as capture antibodies. Antigen capture was achieved following incubation for two hours at room temperature. A pool of highly immune plasma from malaria endemic residents, diluted 1:50 in PBS/1%BSA, was used as detection antibodies for the captured *Pf*TIP, *Pf*MSP-5 and *Pf*BIP antigens, and the resulting complexes revealed using horse radish peroxidase (HRP)-conjugated goat anti-human IgG secondary antibodies at1:10 000 in PBS/1%BSA. Meanwhile, a distinct monoclonal anti-*Pf*HRP-2 antibody (MA5-18243, Invitrogen, USA) was biotinylated using a one-step antibody biotinylation kit (130-093-385, Miltenyi Biotec, Germany) and used at 1: 1000 dilution as detection antibodies (one hour incubation at room temperature) followed by detection with a streptavidin horse radish peroxidase conjugate (A8592, Invitrogen, USA) diluted 1: 1000 in PBS/1%BSA for 1 hour. In both set-ups, 3,3,5,5–tetramethylbenzidine (Thermo Scientific, USA) was used as the chromogenic substrate, and the reaction in each well terminated with 50 µl of 0.1N sulphuric acid. Absorbances were read at 450 nm using a MULKTISKAN FC spectrophotometric plate reader (Thermo Scientific, USA), and the level of each antigen in the collected culture supernatant normalized against its intracellular level in a ring-stage derived protein extract.

Plasma Antigen Levels: Plasma levels of *Pf*TIP and *Pf*HRP-2 antigens were estimated by sandwich ELISA. Microtiter plates were coated with either rabbit anti-*Pf*TIP antibodies (0.5µg/well) or monoclonal anti-*Pf*HRP-2 (MA5-18245, Invitrogen, USA) antibodies at 0.1µg per well overnight at 4°C, and blocked with PBS/1%BSA buffer prior to addition of 100µl of diluted plasma (1:2 in PBS/1%BSA). Plates were incubated at room temperature for two hours, washed three times with PBS-T buffer, and 100 µl of immune plasma pool at 1:50 dilution (anti-*Pf*TIP coated plates) or biotinylated anti-*Pf*HRP-2 monoclonal antibodies (MA5-18243, Invitrogen, USA) at 1: 1000 dilution (anti-*Pf*HRP-2 coated plates) was added for a further 1 hour at room temperature. The plates were again washed and incubated with 100 µl of secondary antibodies (goat anti-human IgG-HRP conjugate at 1: 10 000 dilution or streptavidin horse radish peroxidase conjugate (A8592, Invitrogen, USA) diluted 1: 1000 in PBS/1%BSA) for 1 hour at room temperature. After washing as above, plates were incubated with the HRP enzyme substrate, 3,3,5,5 –tetramethylbenzidine (Thermo Scientific, USA) for 10 min at room temperature. The reaction was terminated with 50 µl of 0.1N sulphuric acid and absorbances read at 450nm using a MULTISKAN FC microtiter plate reader (Thermoscientific, USA).

Invasion Inhibition Assay: Doubly synchronized *P. falciparum* parasites were grown until the early schizont stage, and 300 µl distributed in each well of a 24-well culture plate after adjusting parasitaemia and hematocrit to 5% and 1.5%, respectively. Purified anti-*Pf*TIP antibodies were added at a final 1:10 dilution and incubated for 24 hours prior to parasitaemia determination by Sybr Green I fluorescence method as previously described (Efange et al., 2022). Briefly, 80 µl of the re-suspended cultures were transferred into a dark microtiter plate and 40 µl of Sybr Green I staining solution (3X) were added and further incubated in the dark for 30 minutes at 37°C. The fluorescence emission in each well was read using a Fluoroskan Ascent plate reader. The fold change in fluorescence unit in each well relative to mean fluorescence units obtained prior to antibody treatment was calculated and used as a measure of increase in culture parasitaemia. The experimental controls comprised culture wells treated with either 5 mM EGTA (positive control) or PBS (negative control).

Plasma anti-*PfTIP* Antibody Levels: Microtiter plates were coated with *Pf*TIP peptide (0.2µg/well) overnight at 4°C. Plates were rinsed and blocked with PBS/1%BSA for 1 hour at room temperature and then incubated at room temperature for 1h30 with plasma (100µl of 1:200 diluted sample). Upon four washes, plates were incubated with HRP-conjugated goat anti-human

IgG (100 µl of 1:10 000 diluted samples) for 1 hour at room temperature. The plates were again washed and incubated with 100 µl of the HRP enzyme substrate, 3,3,5,5 –tetramethylbenzidine (Thermo Scientific, USA), for 10 min at room temperature. The reaction was stopped with 50 µl/well of sulphuric acid and optical densities were read at 450nm using a MULTISKAN FC microtiter plate reader (Thermoscientific, USA). Plasma from unexposed European blood donors was used as a negative control, while a pool of immune plasma from adults living in Cameroon was used as a positive control. Results were expressed as optical density units after background subtraction.

Plasma Cytokine and Chemokine Levels: Plasma concentrations of 38 immune mediators including interleukin (IL)-1α, IL-1β, IL-1RA, IL-2 IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, Interferon (IFN)-α2, IFN-γ, tumor necrosis factor (TNF)-α, TNF-β, IFN-γ induced protein (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein (MIP)-1α, MIP-1β, Eotaxin, Fractalkine, human growth-regulated oncogene (GRO), Macrophage-derived chemokine (MDC), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), sCD40L, FMS-like tyrosine kinase 3 ligand (Flt-3L), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), transforming growth factor alpha (TGF-α) and vascular endothelial growth factor (VEGF) were determined using a Milliplex® MAP Human cytokine/chemokine magnetic bead-based panel (MILLIPLEX MAP kit, Cat. No. HCYTMAG-60K-PX38, EMD Millipore Corporation, Germany) according to the manufacturer's instructions. Briefly, 25 µL each of premixed magnetic capture beads were incubated with equal volumes of plasma or diluted standard in a 96-well microplate and placed on a horizontal plate shaker for 2 hours. After washing using a magnetic plate, plates were further incubated with 25 µL/well of biotinylated detection antibodies for 1 hour at room temperature. The resulting antibody-cytokine complexes were revealed by adding 25 µL of streptavidin-PE to each well and incubating for 30 minutes at room temperature. Median fluorescence intensities were read on a Luminex® MAGPIX® Analyser (XMAP Technology, Luminex USA) equipped with xPONENT software version 4.2. The relative concentration of each cytokine (pg/ml) in each sample was determined based on the automatically generated standard curve for each analyte. Analyte amounts less than the limit of detection for each cytokine were attributed this lowest value.

Statistical Analyses: Except for cytokine analyses that were performed once, all other biological assays involved at least three experimental replicates and the resulting means or medians used for data comparison. Plots were produced using GraphPad Prism version 6.0 (San Diego, CA, USA) or Microsoft Excel software. Normality was determined using the Shapiro-Wilk test, and for $P>0.05$, the data were considered normally distributed. Comparison between two unpaired groups were assessed using Student's t-test for normally distributed data or Mann-Whitney rank sum for non-normally distributed data. Association between continuous variables were established using Spearman rank correlation analyses, and P-values <0.05 were considered statistically significant. For cytokine analysis models were run in R studio (2022.12.0+353) running R version 4.2.2 [24]. We employed a mixed linear model approach using the LME4 package [25]. Cytokine level was used as the response with Plasma PfHRP-2 or Plasma PfTIP as the fixed-effect of the model. Sex and age were used as random effects in the model and P values were corrected for multiple comparisons using the Sattherthwaite correction. To obtain p-value estimates from the t-values we used the lmerTest package [26].

Results

Stage-Dependent Export and Secretion of *Pf*tip

*Pf*TIP is a 719 amino acid polypeptide with a predicted signal peptide at its N-terminus (aa 1-28) and a single transmembrane domain at its C-terminus (aa 678-697). To functionally characterize the protein, monospecific antibodies were raised in rabbits against a predicted immunodominant segment (aa 589-602) in the extracellular domain of *Pf*TIP and used for expression analyses by immunoblot and immunofluorescence microscopy analyses. As shown in Figure 1A, the protein was detected in all three

major intra-erythrocytic forms (rings, trophozoites and schizonts) of *P. falciparum*, associated mainly with intracellular membrane-like spots, or surrounding developing merozoites and the infected host erythrocyte in schizont-stage parasites. A single protein band of size approximately 82 kD, corresponding to the predicted molecular mass of *Pf*TIP, was observed by Western blotting using total protein extracts of all three major parasite stages (Supplementary Figure S1). In non-permeabilized cells (Figure 1B), the protein was detected only in trophozoite- and schizont- infected erythrocytes and associated with the host erythrocyte surface, indicating exposure of the N-terminal membrane proxima domain of the protein to the host cell surface. Together, these findings suggest a stage-dependent export of *Pf*TIP in *P. falciparum*, and insertion on the host erythrocyte membrane with its adhesin domains exposed to the extracellular matrix.

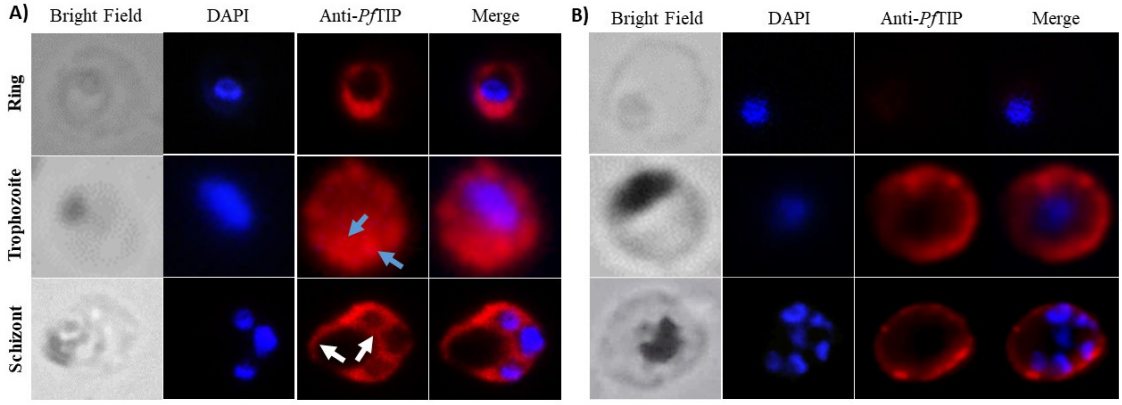

Figure 1: Expression and developmental stage-dependent export of *PfTIP* in *P. falciparum*. A) Immunofluorescence assay (IFA) with Triton-X100- permeabilized cells, showing expression of *Pf*TIP in the three major asexual stages (rings, trophozoites, schizonts) of *P. falciparum*, and its association with intracellular spots (blue arrows) and peripheral membrane structures (white arrows)**, B)** IFA with non-permeabilized cells, showing the association of *Pf*TIP with host erythrocyte membrane surface in trophozoite and schizont infected erythrocytes, but not in ring-stage parasites.

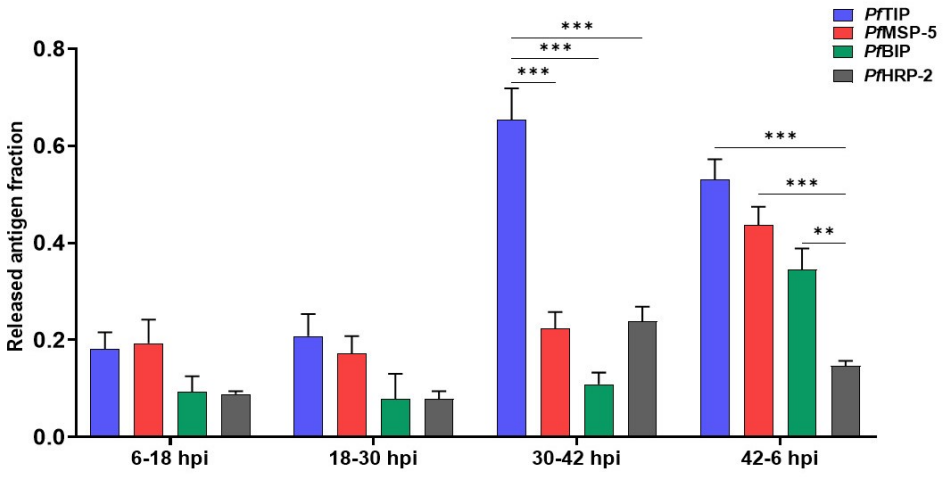

Figure 2: Stage-dependent release of *PfTIP* in *P. falciparum*. Bar diagram showing mean fractions of each antigen (*Pf*TIP, *Pf*MSP-5; *Pf*BIP or *Pf*HRP-2) in culture supernatants from different post-invasion time intervals (6-18, 18-30, 30-42 and 42-6h-pi) relative to their intracellular levels in ring-stage parasites. Error bars represent standard deviations obtained from three replicate wells. Data indicate significant release of *Pf*TIP antigen in schizonts (30-42hpi) compared to rings (6-18hpi) and trophozoites (18-30hpi).

To further determine whether or not exported *Pf*TIP was released, *P. falciparum* culture supernatants were analyzed at various post-invasion time intervals for presence of the protein. As shown in Figure 2, measured extracellular fractions of the *Pf*TIP protein in developing rings (6-18 hpi) and trophozoites (18-30 hpi) relative to its intracellular levels at the ring stage were similar to those of the control proteins *Pf*BIP (endoplasmic reticulum lumen protein), *Pf*HRP-2 (host erythrocyte compartment associated exported protein) and *Pf*MSP-5 (merozoite surface protein). Interestingly, the extracellular antigen fraction of *Pf*TIP was significantly greater than for *Pf*BIP, *Pf*HRP-2 or *Pf*MSP-5 in developing schizonts (30-42 hpi), indicating a selective secretion of the protein in schizont-infected erythrocytes. The extracellular fractions of *Pf*TIP, *Pf*MSP-5 and *Pf*BIP were similar at the egress/invasion stages (42-6hpi), but significantly higher than the extracellular fractions of *Pf*HRP-2, consistent with the release of most parasite proteins during egress and the decreased expression of *Pf*HRP-2 in schizont-stage parasites.

*Pf*TIP levels were also investigated in the plasma of naturally infected individuals by ELISA method. As shown in Figure 3A, the protein was detected in human plasma at significantly higher levels in infected participants (microscopic and submicroscopic carriers) than in non-endemic controls and uninfected endemic residents. No significant difference was observed in plasma levels of the protein when comparing individuals with microscopic and submicroscopic parasitaemia (Figure 3A). Furthermore, consistent with secretion of the protein by parasite stages not often present in peripheral blood, plasma levels of the protein did not correlate with peripheral parasitaemia (Figure 3B), nor with plasma levels of the *P. falciparum* diagnostic marker *Pf*HRP-2 (Figure 3C). In contrast, a strong positive correlation was obtained between *Pf*HRP-2 and peripheral parasitaemia (Figure 3D), consistent with the intracellular nature of *Pf*HRP-2, whose presence in plasma may result from schizont rupture during merozoite egress from infected erythrocytes. Collectively, the data suggest the active release of *Pf*TIP in infected individuals and a stage-specific role for the protein, presumably involving interactions with host ligands and the survival of vulnerable parasite stages.

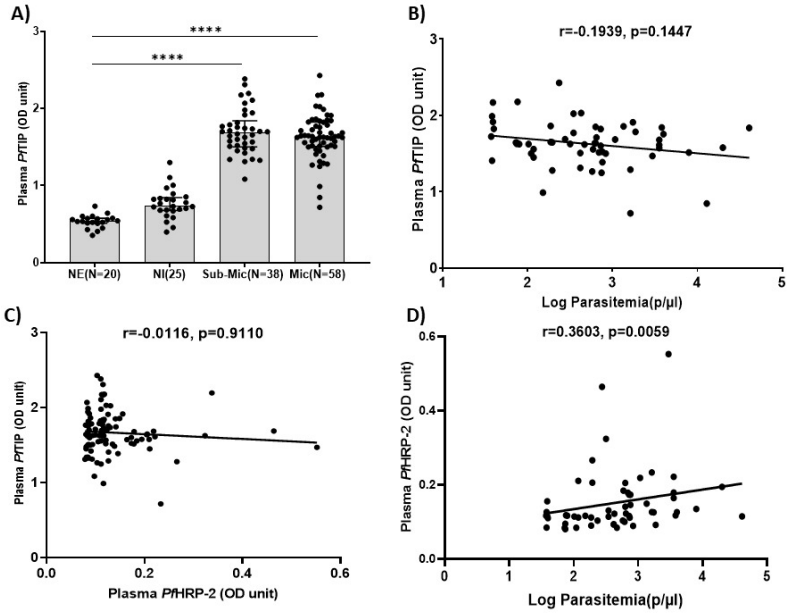

Figure 3: Infection-related presence of *PfTIP* in blood plasma. Plasma levels of *PfTIP* were determined by sandwich ELISA using rabbit anti-*Pf*TIP IgGs as capture antibodies and a pool of human immune sera as probing antibodies. **(A)** Bar plots showing the median optical density values in each population group (NE, NI, Sub-Mic, Mic) and the interquartile ranges (25th and 75th percentile). *NE*: non-exposed controls, *NI*: non-infected endemic residents, *Sub-Mic*: Submicroscopic infections, *Mic*: microscopy-positive samples. Spearman correlation plots show the independence of *Pf*TIP plasma levels from **B)** peripheral parasitaemia in microscopy-positive individuals and **C)** actual parasite biomass represented by *Pf*HRP-2 antigenemia in the study population. **D)** A positive correlation was observed between *Pf*HRP-2 plasma levels and peripheral parasitaemia. ****P<0.0001.

Potential Role of *Pf*TIP in Invasion

*Pf*TIP localizes to membrane structures surrounding developing merozoites in schizont infected erythrocytes (Figure 1A). To determine whether or not *Pf*TIP is essential for merozoite egress and host cell invasion, synchronized parasites at the schizont stages were cultured in the presence of anti-*Pf*TIP antibodies and the effect on culture parasitaemia was investigated. As shown in Figure 4A, exposure of parasites to anti-*Pf*TIP antibodies significantly reduced culture parasitaemia ~3-fold compared to parasitaemia in untreated cultures. Contrarily to the untreated cultures, which predominantly comprised parasites at the late ring stages, the treated cultures comprised mainly of early rings (Figure 4B). Schizont stage parasites were absent in all wells following the treatment, thus indicating the lack of egress inhibition. Consistent with *Pf*TIP's role in invasion, significantly lower levels of anti-*Pf*TIP antibodies were detected in microscopically infected participants compared to submicroscopically infected or uninfected individuals (Figure 4C). Among the microscopically positive individuals, a negative albeit non-significant correlation (r=-0.1080, p=0.4460) was observed between plasma anti-*Pf*TIP antibody levels and peripheral blood parasitaemia (median parasitaemia: 521.47 parasites/µl, range: 37.38-40,905.66 parasites/µl). Taken together, the data suggest a role for *Pf*TIP in host erythrocyte invasion and the potential role of anti-*Pf*TIP antibodies in immune protection.

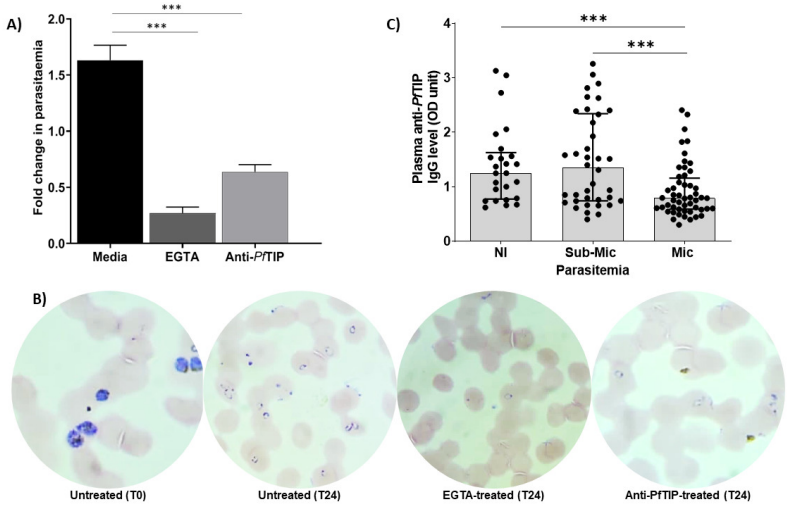

Figure 4: Invasion inhibitory activity of anti-*PfTIP* antibodies. A) *P. falciparum* (Strain 3D7) parasites at the early schizont stage were incubated with culture media alone (untreated control) or treated with 5 mM EGTA (treatment control) or with purified rabbit anti-*Pf*TIP IgGs for 24 hours under normal culture conditions. Bar diagrams show significant reduction in parasitaemia in the EGTA and anti-*Pf*TIP treated cultures compared with the untreated control. **B)** Representative micrographs of Giemsa-stained thin smears showing the dominant parasite stage in each culture at the start (T0) and end (T24) of the incubation period. **C)** Combined dot and bar plots showing the association of circulating anti-*Pf*TIP antibody levels with *P. falciparum* infection status (non-infected, submicroscopic or microscopic) among the endemic residents. Bar plots represent median optical densities and the interquartile ranges (25^{th} and 75^{th} percentile), whereas dots represent each participant's absorbance readings. *NI*: non-infected endemic residents, *Sub-Mic*: Submicroscopic infections, *Mic*: microscopy-positive individuals. *** P<0.001

Potential Role of *Pf*TIP in Immune Modulation

Interactions between *Pf*TIP and host immune cells were assessed by determining the association of its plasma levels with the levels of 38 different host cytokines and chemokines. For comparison, associations between plasma levels of the *P. falciparum*-specific protein *Pf*HRP-2 and the cytokines measured were also determined. A fixed effects model with Sattherthwaites correction did not yield any positive associations other than TGF-, transforming growth factor involved in cell proliferation, differentiation and development for *Pf*TIP. However modelling cytokines individually indicated a negative correlation between plasma

levels of *Pf*TIP and MIP-1 (Figure 5A) whereas a positive correlation was observed between plasma levels of *Pf*HRP-2 and MIP-1 (Figure 5B). No other associations were observed between *Pf*TIP and all other cytokines studied, indicating the high specificity of the association between *Pf*TIP and MIP-1. These above findings suggest a possible role of *Pf*TIP in suppression of vasculrear activation and/or recruitment of CCR5-expressing cells to sites of *Pf*TIP release. MIP-1, also called CCL4, is a chemokine produced by endothelial cells as well as a number of different immune cells during inflammation, with roles in the attraction of immune cells to sites of infection. Thus, *PfTIP* may be involved in the maintenance of a less inflammatory immune environment in the study population, comprising mainly of weakly infected individuals (median parasitaemia: 521.47 parasites/µl, range: 37.38-40,905.66 parasites/µl). Indeed, TNF-α levels increased with increasing levels of the regulatory cytokine, IL-10 (Figure 6A), and the ratio of TNF-α to IL-10 remained stable irrespective of *Pf*TIP levels (Figure 6B) or parasitaemiaure (Figure 6C&D).

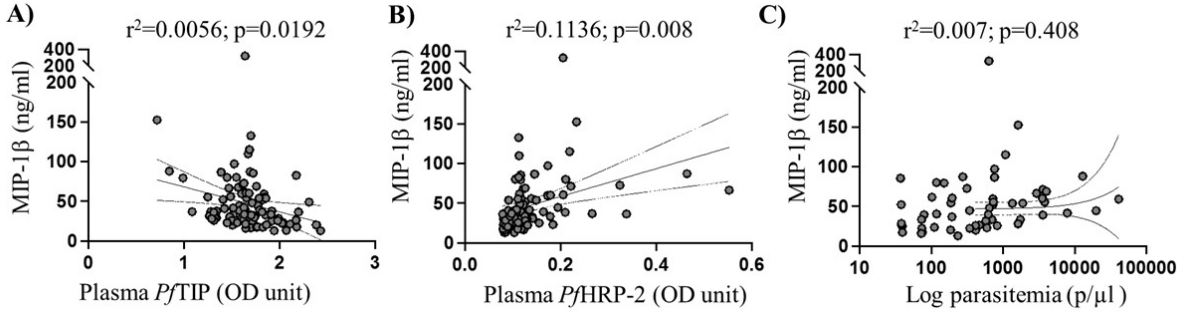

Figure 5: Association of *Pf*TIP plasma levels with suppression of MIP-1 chemokine production. Linear mixed model plots showing negative correction between *Pf*TIP and MIP-1 (A), a positive correlation between *Pf*HRP-2 and MIP-1 (B), and absence of significant association between peripheral parasite density and MIP-1 (C). Cytokine level was used as the response with Plasma *Pf*HRP-2 or Plasma *Pf*TIP as the fixed-effect of the model. Sex and age were used as random effects in the model and P values were corrected for multiple comparisons using the Sattherthwaite correction. The lmerTest package was used to obtain p-value estimates from the t-values.

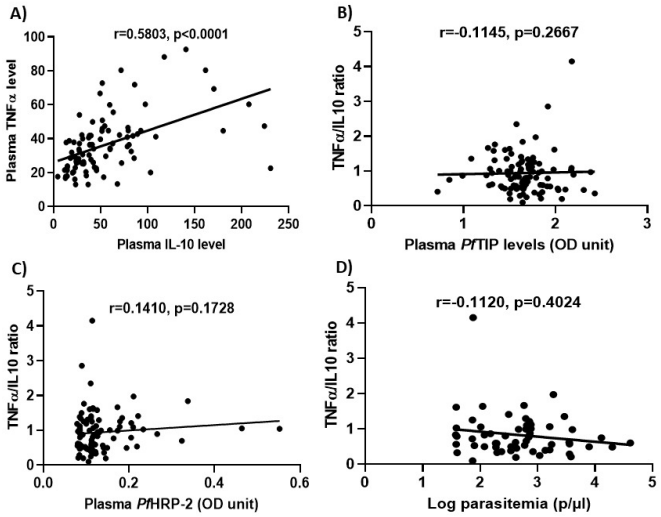

Figure 6: Concurrent increase in TNF-α and IL-10 levels with parasitaemia and maintenance of a balanced inflammatory response in the study participant. Spearman correlation plots showing strong positive association between TNF-α and IL-10 plasma levels (A), and a balanced TNF-α/IL-10 ration irrespective of *Pf*TIP plasma levels (B), *Pf*HRP-2 plasma levels (C), or peripheral parasitaemia (D).

Discussion

T-cell immunomodulatory proteins (TIP) are a family of integrin-α domain-containing adhesins that are widely expressed in eukaryotes, including in apicomplexans and parasitic helminths [15-17, 27, 28]. In mammals, TIPs exhibit mainly immunoregulatory functions, suppressing excessive inflammatory responses [29]. The role of TIP homologs in parasitic organisms remains a subject of discussion, with recent data suggesting a role for the *P. falciparum* TIP homolog in host cell invasion and a role of *P. berghei* TIP in immune regulation [16, 18]. We sought to investigate the potential dual role of *Pf*TIP in host cell invasion and immune regulation. Our data support a role for *Pf*TIP in invasion and a potential role in suppression of pro-inflammatory responses to *P. falciparum* infection.

*Pf*TIP is abundantly expressed in all major intra-erythrocytic stages of *P. falciparum* and, like its counterpart in *P. berghei, C.-parvum* and *Echinococcus multilocularis* [16, 27, 28], is secreted into the extracellular space. In a life-cycle stage-dependent manner, the protein decorated the surface of infected erythrocytes with its adhesin domains exposed to the exterior. Export of *Pf*TIP into the host cell compartment likely involved a non-PEXEL-dependent mechanism, as this *Plasmodium* export element is absent within the *Pf*TIP sequence. Consistent with a role in intercellular interactions, *Pf*TIP was also associated with membrane structures surrounding developing merozoites in schizont-infected cells. Blocking antibodies directed against a membrane proxima segment located within the extracellular portion of the protein delayed host erythrocyte invasion, resulting in significant reductions in culture parasitaemia. Collectively, these findings indicate a role for *Pf*TIP in host cell invasion and a potential role of anti-*Pf*TIP antibodies in protection. Indeed, plasma levels of anti-*Pf*TIP IgG negatively associated with parasitaemia in infected individuals, indicating a protective role for natural anti-*Pf*TIP antibodies. Like most exported adhesins, including members of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) family [20-33], *Pf*TIP is likely to play additional roles in cytoadherence of *Plasmodium*-infected erythrocytes.

*Pf*TIP was detected in culture supernatants with up to 60% of its intracellular levels at the ring stage secreted during schizont development. The reasons for the stage dependence of the *Pf*TIP secretion process are unknown, but may depend on the availability of its export machinery or release factors, notably parasite proteases. Importantly, plasma levels of the protein correlated negatively with plasma levels of the pro-inflammatory chemokine MIP-1. The association of *Pf*TIP with MIP-1. is consistent with observations in *P. berghei* and *E. multilocularis* as well as in some human inflammatory diseases (16, 27, 29]. Our study is the first to associate a TIP family member with lower MIP-1 levels. MIP-1 is a chemokine that is produced by endothelial cells in vascular inflammation as well as innate and adaptive immune cells. Reduced levels of MIP-1 when plasma *Pf*TIP levels are higher is highly suggestive of reduced vascular inflammation and is likely to reduce trafficking of CCR5-expressing immune cells such as T cells to areas of parasite sequestration. In fact, although a non-significant correlation was observed with most other cytokines known to contribute to inflammation during malaria parasite infection [34], their plasma levels in the study population were generally low. A balanced TNF-α/IL-10 ratio, reminiscent of early events in inflammatory responses to infection with *Plasmodium* parasites [35, 36], was observed irrespective of individual parasitaemia or *Pf*TIP plasma level, suggesting a concurrent modulation of both pro- and anti-inflammatory responses in the study population. Such modulation may be essential for sustaining low-level parasitaemia and the asymptomatic carriage of the parasite in infected subjects.

Overall, this study confirms the multifunctional role of TIP family members, with the *P. falciparum* TIP homolog, *Pf*TIP, exhibiting a role in host cell invasion and immune modulation. The detected growth inhibitory characteristics of anti-*Pf*TIP antibodies against the parasite suggest a potential role for this antigen as a vaccine candidate. Furthermore, the presence of schizont-released *Pf*TIP in blood plasma also suggests the potential use of this protein as a diagnostic marker.

Supplementary Figures

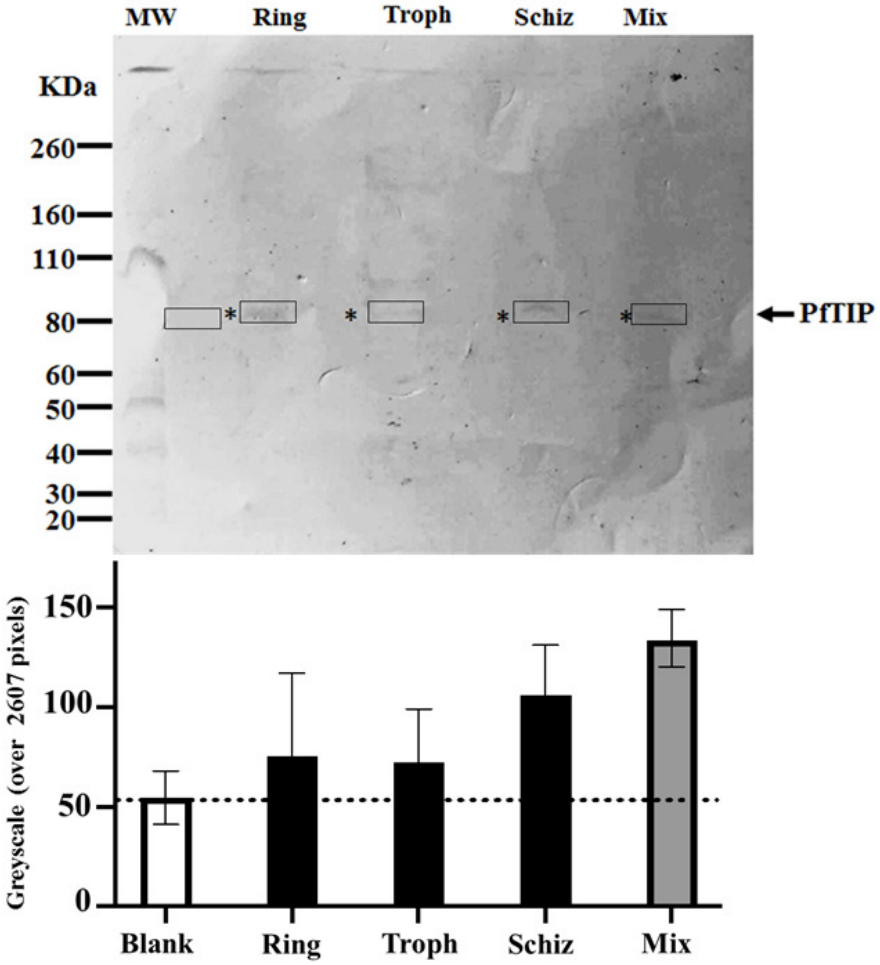

Figure S1: Immunoblot showing expression of endogenous *Pf*TIP in all three major intra-erythrocytic forms (rings, trophozoites and schizonts) of *P. falciparum*. Immunoblots were developed using a chromogenic substrate and imaged using a hand-held camera (A). The positions of the 82 kDa *Pf*TIP band in each test lane (Ring, Troph, Schiz or Mix) are highlighted by asterisks (*). Ring, Troph, Schiz and Mix represent lanes loaded with 10µg of total proteins extracted from saponin-isolated rings, trophozoites, schizonts or mixed stage cultures, respectively. B) The bar graph represents the mean grayscale of the rectangle with the error bars representing the maximum and minimum pixel grayscale of the rectangle. Grayscale was quantified within a 2607-pixel rectangle

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