Plasmodium vivax Strains Use Alternative Pathways for Invasion

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Plasmodium vivax has 2 invasion ligand/host receptor pathways (*P. vivax* Duffy-binding protein/Duffy antigen receptor for chemokines [DARC] and *P. vivax* reticulocyte binding protein 2b/transferrin receptor [TfR1]) that are promising targets for therapeutic intervention. We optimized invasion assays with isogenic cultured reticulocytes. Using a receptor blockade approach with multiple *P. vivax* isolates, we found that all strains utilized both DARC and TfR1, but with significant variation in receptor usage. This suggests that *P. vivax*, like *Plasmodium falciparum*, uses alternative invasion pathways, with implications for pathogenesis and vaccine development.

Keywords. *Plasmodium vivax*; DARC; TfR1; parasite invasion; receptor blockade.

Invasion of red blood cells by *Plasmodium* species is a rapid, multistep process that relies on interactions between numerous parasite-produced invasion ligand proteins and host receptors on the surface of the host cell [1], termed invasion pathways. Blocking these interactions is a promising avenue for vaccine development [2]. Variation in invasion pathway usage has been observed in the often lethal *Plasmodium falciparum* parasite, and is a potential factor in immune evasion [3] and pathogenesis [4]. Little is known about the invasion pathways of *Plasmodium vivax*, the second most prevalent malaria parasite, which, in contrast to *P. falciparum*, is restricted to invading reticulocytes, the youngest red blood cells (RBCs). Here we investigated the variation in usage of the 2 known sets of invasion

ligand/host receptor pairs for *P. vivax* invasion: (1) *P. vivax* Duffy-binding protein (PvDBP), a high-priority vaccine candidate [5, 6], which binds to the Duffy antigen receptor for chemokines (DARC/Duffy) [7]; and (2) *P. vivax* reticulocyte binding protein 2b (PvRBP2b), which binds to the transferrin receptor (TfR1/CD71)/transferrin (Tf) complex [8].

MATERIALS AND METHODS

CD34⁺ Cell Culture

Bone marrow-derived CD34⁺ hematopoietic stem cells (HSCs) (Lonza) were cultured as described previously [9] with the addition of 2-PCPA/tranlylcypromine during the expansion phase of the culture as detailed in the [Supplementary Methods](#).

Sample Collection

Clinical *P. vivax* isolates were collected with informed consent and cryopreserved in liquid nitrogen as described previously [10] at Goa Medical College and Hospital in Bambolim, Goa, India, as part of the Malaria Evolution in South Asia International Centers for Excellence in Malaria Research project at the University of Washington.

P. vivax Short-Term Culture and Invasion Assays

Plasmodium vivax short-term ex vivo isolates were thawed and cultured as described elsewhere [10] in Iscove's Modified Dulbecco's Medium (IMDM) + Glutamax (Gibco) with 10% (v/v) heat-inactivated AB⁺ serum and 0.5% (v/v) gentamycin in modular incubator chambers (Billups Rothenberg) gassed with 10% carbon dioxide, 5% oxygen, and 85% nitrogen and maintained at 37°C.

Between 40–46 hours postthaw, cultures were pelleted and loaded over a MACS LS column (Miltenyi Biotech) with a 23G needle to restrict flow. The sample was washed once with 5 mL IMDM + 0.5% (w/v) bovine serum albumin (BSA) and then eluted with 5 mL IMDM + 0.5% (w/v) BSA by removing it from the magnet. Cells were pelleted at 500g for 5 minutes and then transferred to sterile 1.5-mL Eppendorf tubes, pelleted at 500g for 2 minutes, and resuspended to 1 mL. Cell concentration was determined via hemocytometer reads. A total of 50 µL of the sample was used to make a cytospin and the sample was stained with Giemsa. The total number of late-stage parasites (stage IV/V) was determined and invasion assays were set up aiming for 1%–3% schizontemia. Each assay well contained 5 × 10⁵ cells in a total of 30 µL of culture medium in a half-area 96-well plate (BD Falcon). Carryover of uninfected RBCs was on average <5% (average, 4.33% [range, 1.02%–7.67%]). OKT-9 (BioXCell), mouse IgG1 isotype control MOPC-21 (BioXCell), or melanoma growth-stimulating activity (MGSA; Peprotech) was added from azide-free stocks to the appropriate final

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concentrations. Cells were returned to the incubator for 16–20 hours, at which point cytospin slides were made and stained with Giemsa (Sigma-Aldrich).

Slide Counting

Assay slides were blinded and counted under immersion oil using a $\times 100$ objective on a Zeiss Primo Star microscope in conjunction with a 1:9 eyepiece reticle. A whole-field counting method was employed whereby all cells in the large reticle box were counted across multiple fields (range, 1000–2500 cells). Parasitized cells were counted anywhere in the field. Parasitemia was estimated as follows: number of parasitized cells / [number of total cells in large reticle $\times 5.506$ (scale factor for whole field)] $\times 100\%$.

A minimum of 25 ring-stage parasitized cells was required in control slides for a sample to be counted for an assay (this corresponds to a minimum of $\sim 0.2\%$ parasitemia). All data analysis was performed in GraphPad Prism version 8.4 software.

RESULTS

To enable quantitative invasion pathway comparisons between *P. vivax* strains, we improved upon previous assays that used variably enriched cord-blood reticulocytes [5, 6, 11] through the use of isogenic reticulocytes (cultured RBCs [cRBCs]), obtained through in vitro differentiated bone marrow–derived CD34⁺ HSCs [12] from a single Duffy-positive donor. However, as primary cells have limited ability to expand in culture, we adapted a previously used JK-1 erythroleukemia cell protocol [13]. Growth of CD34⁺ HSCs in the presence of 2-PCPA/tranylcypromine enabled both greater expansion (~ 100 -fold) of undifferentiated cells and reproducible synchronous differentiation (Supplementary Figure 1A–I), resulting in $>70\%$ reticulocytes and a smaller fraction of orthochromatic cells (nucleated reticulocytes) (Figure 1A), providing an isogenic and relatively homogenous source of target cells.

We used cryopreserved Indian *P. vivax* clinical isolates from Goa that were short-term cultured in vitro [10] to obtain purified schizonts for inclusion in small-format invasion assays with cRBCs (Supplementary Figure 2A). We readily observed ring-stage parasites in both enucleated and nucleated reticulocytes (Figure 1B), and as neither cell type was preferentially invaded (Supplementary Figure 2B and 2C), we pooled all ring-stage counts. We corrected for the number of unburst schizonts remaining at the end of the assay to calculate the parasitized erythrocyte multiplication rate (Supplementary Figure 2D–F).

To determine the dependence on the PvDBP/DARC and PvRBP2b/TfR1 ligand-receptor interactions, we developed a host-directed receptor blockade approach that avoids complications of strain-specific inhibition due to invasion ligand (sequence and expression level) polymorphism, as previously observed in studies using anti-PvDBP antibodies [5, 6, 11]. We used the cytokine MGSA and a monoclonal anti-TfR1 antibody

(OKT-9) to inhibit PvDBP/DARC [14] and PvRBP2b/TfR1 [8] interactions, respectively (Figure 1C). A dose-dependent inhibition of parasite invasion across a dilution series of both OKT-9 and MGSA was observed (Figure 1D). Half maximal inhibitory concentration (IC_{50}) values from 11 different *P. vivax* isolates were measured (Supplementary Figure 3A and 3B). No systematic effects on IC_{50} values were observed with different cRBC batches (Supplementary Figure 4A and 4B), nor was there strong evidence for inoculum effects relating to parasite number, with the range of IC_{50} values from both dilution experiments and biological replicates falling within a narrow 0.5- to 1.5-fold range of the average IC_{50} (Supplementary Figure 4C–I), supporting the robustness of the assay.

All Goan *P. vivax* strains tested were inhibited by receptor blockade of DARC. Between the 11 isolates we observed a 4.6-fold range of MGSA IC_{50} values (Figure 2A). Similarly, we observed that all Goan *P. vivax* isolates could be inhibited by blocking the newly identified TfR1 receptor, suggesting that, like DARC, it is universally employed by *P. vivax* isolates. However, inhibition was associated with a considerably greater range (12.1-fold) in OKT-9 IC_{50} values, with several distinct outliers (Pv2122, Pv2140, and Pv2083) (Figure 2B). When comparing IC_{50} values for both DARC and TfR1 receptor blockade, we found multiple patterns (Figure 2C) including isolates with IC_{50} values outside of the range of the 95% confidence intervals (CIs) ($\pm 2.23 \times$ standard error of the mean) of the average IC_{50} value for either MGSA or OKT-9 alone, as well as isolates outside of the combined range of 95% CIs of both MGSA and OKT-9 together.

The wide difference in usage of PvDBP/DARC and PvRBP2b/TfR1 pathways may also lead to reduced effectiveness of vaccine strategies targeting a single invasion ligand alone. To address this, we tested for evidence of synergy when blocking both TfR1 and DARC together by measuring invasion with different ratios of OKT-9 and MGSA with isolate Pv2083 (Figure 2D). We observed a significantly increased invasion inhibition in the presence of both inhibitors, compared to each one alone, suggesting that a combinatorial approach may be an attractive vaccine strategy.

DISCUSSION

Understanding the extent and magnitude of variation in usage of *P. vivax* invasion pathways is an essential but understudied factor in prioritizing blood-stage vaccine candidates. We developed a *P. vivax* invasion assay that has multiple advantages, including (1) use of isogenic reticulocytes from Duffy-positive CD34⁺ HSCs from a single individual allowing high reproducibility compared to assays using variably enriched cord blood reticulocytes from different donors [5, 6, 11]; (2) use of cryopreserved Goan clinical isolates, stored in multiple vials allowing for assays in biological replicates; (3) clearly defined parasite invasion metrics including measurement of assay reproducibility; and (4) use of a host-targeted approach (MGSA

Figure 1. A, Differentiation of cultured red blood cells from multiple batches reproducibly resulted in >70% reticulocytes. Insets show representative images for different stages. B, Giemsa-stained microscopy images of ring-stage *Plasmodium vivax* parasites (black arrowheads) in reticulocytes (top row) or orthochromatic cells (bottom row). C, Schematic showing inhibition of *P. vivax* Duffy-binding protein (PvDBP) binding to Duffy antigen receptor for chemokines (DARC) with melanoma growth-stimulating activity (MGSA) and *P. vivax* reticulocyte binding protein 2b (PvRBP2b) binding to transferrin receptor (TfR1)/transferrin (Tf) with OKT-9. D, Dose-response invasion inhibition curves for OKT-9 and MGSA for 2 biological replicates of Pv2167. Average and standard deviation are shown from 2 technical replicates. Nonlinear regression r^2 values are shown for each fit.

to inhibit PvDBP/DARC and OKT-9 to inhibit PvRBP2b/TfR1) that avoids strain-specific inhibition observed in anti-PvDBP antibody studies [5, 6, 11].

While the use of a host-targeted approach circumvents the issue of invasion ligand sequence variation, one possible limitation is agglutination of cells at high concentrations of OKT-9 antibody leading to nonspecific invasion inhibition; however, this was not observed visually, microscopically, or by flow cytometry. Another observation was that OKT-9 invasion inhibition required much higher concentrations of antibody (µg/mL range) compared to MGSA (ng/mL range). We believe that this may be in part due to the much greater abundance of TfR1

(up to 1×10^6 copies/cell on in vitro-cultured reticulocytes [15]) compared to DARC (5×10^3 copies/cell on RBCs [16]), as well as differences in affinity/avidity of the reagents and ability to block receptor binding through steric hindrance. Another potential confounding factor is that the health of *P. vivax* parasites may be driving the differences in invasion inhibition that we observe. While we cannot exclude this possibility, we do not believe it is a major factor as we did not observe any association between ring-stage parasitemia and IC_{50} values for either MGSA or OKT-9 (Supplementary Figure 4G and 4H), suggesting that a parasite's ability to invade (as a proxy for parasite health) does not affect inhibition.

Figure 2. Variation in Half maximal inhibitory concentration (IC_{50}) values for 11 *Plasmodium vivax* (Pv) isolates (average and standard deviations) for OKT-9 (A) and melano-ma growth-stimulating activity (MGSA; B). Variation was significant for OKT-9 as assessed by analysis of variance (***) and Tukey post hoc tests (Supplementary Figure 5). C, OKT-9 vs MGSA IC_{50} values showed no significant association (Spearman $r = 0.345$; $P = .299$). D, Significant differences (** $P < .01$, 2-tailed paired t test) were observed between the expected and observed inhibition when OKT-9 and MGSA were combined at different relative ratios (bottom panel). Expected inhibition was calculated as the sum of the relative inhibition ratio of each inhibitor alone.

Large variations in usage of the PvDBP/DARC invasion pathway have been reported to be linked with duplication of PvDBP in Cambodian *P. vivax* isolates [6]. However, such duplications have not been observed in Goan *P. vivax* isolates [17] and indeed the variation we observed was similar to the IC_{50} inhibition range for single-copy PvDBP isolates (4.5-fold, 5 isolates) [6]. While variation in usage of the PvRBP2b/TfR1 invasion pathway has not previously been reported, our data suggest that all Goan *P. vivax* isolates utilize this pathway but to different extents, and more variably than the PvDBP/DARC invasion pathway. The range of different patterns of usage of PvDBP/DARC and PvRBP2b/TfR1 between isolates is strongly suggestive of alternative invasion pathways, similar to those well characterized for *P. falciparum* [3]. Reliance on different invasion pathways between isolates could be an important determinant of immune evasion and cellular age tropism, as well as *P. vivax* pathogenesis.

Our observation of inhibition synergy when both MGSA and OKT-9 are used in combination merits further investigation with a greater number of *P. vivax* isolates as it suggests that targeting distinct invasion pathways may lead to generation of a more potent blood-stage malaria vaccine. While we did not observe complete inhibition of parasite invasion when the 2 inhibitors were combined, we believe that this is most likely due to the relatively low concentration of each inhibitor used, and we likely would have observed greater inhibition at higher concentrations. However, we cannot exclude the possibility that alternative invasion pathway(s) may also be operating, and indeed this is a critical future research objective. From a vaccine

development perspective, we would be interested in the future to test whether we are able to observe invasion inhibition synergy with antibodies against the cognate invasion ligands PvDBP and PvRBP2b for the receptors that we have blocked in our study. Such antibodies have been extensively characterized for PvDPB [5, 11, 18], but identifying strain-transcendent antibodies remains challenging. In addition, future studies could seek to understand the basis of PvDPB and PvRBP2b pathway variation such as differences in expression or sequence variation of invasion ligands. More globally, studies are needed to understand the variation in usage of all *P. vivax* invasion ligand/host receptor interactions, beyond PvDBP/DARC and PvRBP2b/TfR1, to prioritize combinatorial vaccine candidates.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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