SUPPLEMENTARY APPENDIX

Altered parasite life-cycle processes characterize *Babesia divergens* infection in human sickle cell anemia

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Supplemental Methods

Ethics Statement: Human blood from healthy volunteer donors was used to culture *B. divergens* (Bd) *in vitro*. Sickle Cell Disease (SCD) patient RBCs were obtained from residual anticoagulated blood samples from same day collections from patients with sickle cell disease (hemoglobin genotype SS) who had not been transfused for at least 3 months prior to sample draw. Patients provided consent for use of de-identified blood for research purposes on a Montefiore Medical Center IRB approved protocol. HbAA RBCs and sickle trait RBCS were identified from NYBC blood donors and confirmed through genotypic analysis. All blood was used within a few hours of drawing. The blood was de-identified and approved for use by the NYBC IRB. All blood donors gave informed written consent for use of their blood for research purposes.

B. divergens in vitro culture: *B. divergens* (Bd Rouen 1986 strain) were maintained in human RBCs at 5% hematocrit in complete medium (RPMI 1640; supplemented with 50 μg/mL hypoxanthine; 0.24% (v/v) sodium bicarbonate; and 10% human serum) under low oxygen atmosphere (5% O_2, 90%CO_2, 5% N_2) at 37^{o}C, as previously described (32, 33). A+ RBCs were collected in 10% CPD and washed 3X with RPMI 1640 medium for the complete plasma and white cells removal.

Free merozoites isolation*:* High concentration of viable free merozoites was isolated from unsynchronized cultures at high parasitemia (40%) as described previously (31, 34). Briefly, ~12x10^{9} infected RBC were resuspended in 6 mL of fresh complete medium and filtered, at room temperature, once with 5μm and twice with 2 μm filters (Versapor® membranes) using 1 mL syringe. Centrifugation (670 g; 2 min) was applied only after the last filtration. The supernatant (suspension of free merozoites) was used as inoculum of new cultures for parasite synchronization. All inoculum preparations were checked for potential contamination with carry-over RBCs by Light Microscopy and Flow Cytometry.

Assessment of Invasion, Development and Egress in Various RBC: Fresh cultures were seeded with purified merozoite suspension at 20% (v/v) of culture volume. To define timepoints to estimate invasion accurately in the different RBCs, (HbAA, HbAS; HbSS) invasion was assayed in the first set of samples at 5 min, 1 h or 6 h post invasion, when cultures were centrifuged (670 g; 2 min; room temperature) and

medium replaced by fresh complete medium to remove the lysed RBC components introduced into the culture during the inoculation. At additional time points (24-72 h), samples were collected to assess the culture progression and sub-population dynamics from the perspective of parasite development and egress. Analysis was carried out at specific intervals of 24 h, 48 h and 72 h for the majority of cultures (6 cultures were monitored for 48 h). The culture size (parasitemia) and the parasite proliferation analysis were carried out by FACS (described below). Characterization of parasite morphology and development was performed by light microscopy; Cells were obtained from 3 replicate cultures for each RBC sample.

Assessment of Impact of Fragility of HbSS RBC on Parasite Population Progression:

Parasite cultures were initiated using the same merozoite preparation in HbAA and HbSS RBCs. Hematocrits of 5% were maintained throughout the experiments. Invasion efficiencies were calculated at 1 h and parasitemia progression at 24 h, after which the cultures were divided into two. Half of each culture was allowed to progress with only medium change, while the other half was split into 2, with one portion receiving either fresh HbSS or HbAA RBCs. Parasitemia in all cultures were then assessed at 48h.

***Light Microscopy*:** Blood smears were fixed with methanol and stained with 20% Giemsa (Sigma-Aldrich, St. Louis, MO) for the morphological analysis of parasites. A minimum of 2000 cells was scanned for assessment of changes in morphology using a Nikon Eclipse E 600 microscope.

Flow Cytometry: The dual-color staining protocol was used to monitor the parasite cycle within the RBCs over 72hs as previously described (31) with modifications. Briefly, human erythrocytes were identified by BV421 Mouse Anti-Human CD235a (BD Biosciences, San Jose, CA) that specifically binds to Glycophorin A (GPA). Parasites were identified by DNA staining with Vybrant®DyeCycle^{TM}Green dye at 10 μM (Life Technologies, Corporation, Carlsbad, CA) at 37^{o}C for 30 min and protected from light. The presence of Howell-Jolly bodies was evaluated by staining the different types of uninfected RBC with Vybrant®DyeCycle^{TM}Green prior to invasion–Samples were analyzed in 1 mL of cell suspension at a concentration of 1x10^{7} infected cells/mL in fresh complete medium without previous fixation or washing procedures. Samples were analyzed on an LSR Fortessa SORP analyzer (BD Biosciences, San Jose, CA), equipped with a 488nm blue laser for Vybrant®DyeCycle^{TM}Green detection

(530/30 nm BP) and a 405 nm violet laser for BV421-GPA detection (450/50 nm BP) 10,000 target events (iRBCs) (or ~4.10^5 total events when iRBC at low frequency) were collected at low flow rate to achieve the maximum resolution among sub populations of iRBC and further analyzed with BD FACSDiva™ Software, version 6.2 (BD Biosciences, San Jose, CA). All parameters were processed using log scaling.

***Statistics*:** Parasitemia was defined as the total number of infected RBCs (iRBCs) in every 100 RBCs not taking into consideration the number of parasites seen in a given cell when measured by flow cytometry. As RBCs do not contain a nucleus, iRBCs and their Sub-populations were identified as a function of the presence and their number of intra erythrocyte parasites / genome (intra erythrocyte parasite load) where "1 N" refers to one genome copy based on the method previously described (34). Inhibition of culture growth was calculated as a percentage of parasitemia of control cultures. The percentage of inhibition mediated by the sickle hemoglobin genotype was determined by assuming that the parasitemia from control HbAA cells was equivalent to 0% inhibition or enhancement. Data reproducibility was ensured by running independent experiments, each in triplicate. The statistics (StDv, Standard Deviation; T-Test) were calculated by Microsoft Excel (2007) or SAS Software, version 9.4 2012 (SAS Institute Inc., Cary, NC, USA). Significance level was set at $P< 0.05$.

SUPPLEMENTAL REFERENCES

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Table S1. DNA Content in uninfected HbSS, HbAS and HbAA RBC as measured by the size of Vybrant Green positive population. Parasitemia obtained using the same HbSS, HbAS, HbAA cells in cultures 1 h post inoculation of cultures with free merozoites.

Sample (*)	**uRBC**	**iRBC (1 h)**
HbAA-I	0.0 [0.00]	1.0 [0.50]
HbSS-1	0.1 [0.01]	1.2 [0.67]
HbAS-1	0.0 [0.00]	1.2 [0.98]
HbAA-II	0.0 [0.00]	1.0 [1.22]
HbSS-2	0.3 [0.02]	1.4 [1.54]
HbAS-2	0.0 [0.00]	1.4 [0.84]
HbAA-III	0.0 [0.00]	2.3 [1.17]
HbSS-3	0.2 [0.02]	2.7 [1.42]
HbAS-3	0.0 [0.00]	2.4 [1.23]

Parasitemia values in percentage average

[StDv]: Standard Deviation among triplicates.

Table S2. Parasitemia of *B. divergens* cultures measured 1 h -72 h post-invasion in Sickle and Wild Type RBCs along with parasitemia in HbSS cultures supplemented by the addition of fresh Sickle or Wild-Type RBCs at 24 h and followed for another 48h

Sample	**1 h**	**24 h**	**48 h**	**72 h**
HbAA-A	2.1 [0.01]	3.2 [0.06]	6.4 [0.01]	25 [0.08]
HbSS-A	2.8 [0.07]	3.4 [0.06]	4.0 [0.11]	4.7 [0.06]
		0 h	**24 h**	**48 h**
HbAA-B*	-	1.6 [0.01]	3.2 [0.04]	16 [0.41]
HbSS-B[#]	-	1.7 [0.03]	2.5 [0.24]	3.6 [0.34]
HbSS-C*	-	1.7 [0.09]	2.1 [0.07]	2.3 [0.01]

Parasitemia values represent average percentage

[StDv]: Standard Deviation among triplicates

* Fresh HbAA RBCs added at 24h (corresponding to 0 h of new cultures)

Fresh HbSS RBCs added at 24h (corresponding to 0 h of new cultures)

FIGURE S1

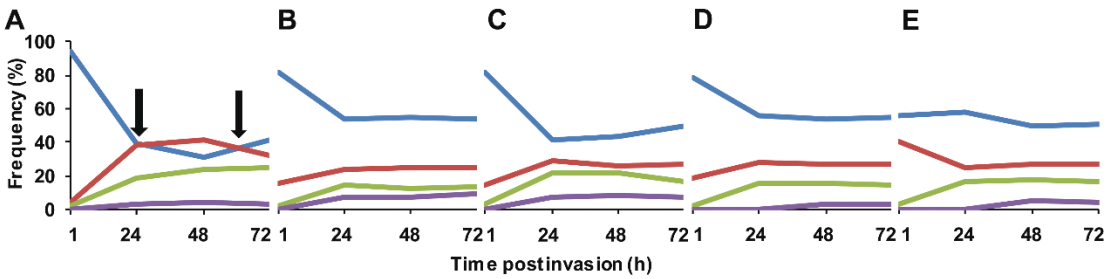

Figure S1: Sickle cell (HbSS) environment halts fluctuating dynamics of the parasite population structure. Parasitic population structure of 5 representative *B. divergens* cultures in different Hb genotypes: (A) HbAA RBC control and (B-E) 4 different HbSS-RBC cultures. Each curve represents the frequency of sub-populations of infected RBCs bearing different number of parasites: Blue: 1N-iRBC Red: 2N-iRBC Green: 4N-iRBC and Purple: >4N-iRBC populations. The 1-24 h time frame represents the first life cycle of the parasites where the sequential proliferative cycles results in the emergence of other iRBC sub populations. **A:** *B. divergens* in the control HbAA cells maintain a dynamic balance among sub-populations using sequential events of egress (identified by the decrease in frequencies of 4N and >4N- iRBC sub populations) and of invasion (identified by the increase in the frequency of 1N-iRBC sub population). Arrows indicate the expected overlap between 1N- and 2N-iRBC curves in control HbAA which demarcate the end of 2 normal life cycles, one at ~24 h and the second at ~48 h post invasion. **B-E**: In HbSS RBCs, once different sub-populations were established, the proportion of the different sub-populations was kept relatively stable up to 72h.