

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

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ABSTRACT

Babesia divergens is an intra-erythrocytic parasite that causes malaria-like symptoms in infected people. As the erythrocyte provides the parasite with the infra-structure to grow and multiply, any perturbation to the cell should impact parasite viability. Support for this comes from the multitude of studies that have shown that the sickle trait has in fact been selected because of the protection it provides against a related Apicomplexan parasite, *Plasmodium*, that causes malaria. In this paper, we examine the impact of both the sickle cell anemia and sickle trait red blood cell (RBC) environment on different aspects of the *B. divergens* life-cycle, and reveal that multiple aspects of parasite biological processes are altered in the mutant sickle anemia RBC. Such processes include parasite population progression, caused potentially by defective merozoite infectivity and/or defective egress from the sickle cell, resulting in severely lowered parasitemia in these cells with sickle cell anemia. In contrast, the sickle trait RBC provide a supportive environment permitting *in vitro* infection rates comparable to those of wild-type RBC. The elucidation of these naturally occurring RBC resistance mechanisms is needed to shed light on host-parasite interaction, lend evolutionary insights into these related blood-borne parasites, and to provide new insights into the development of therapies against this disease.

Introduction

The human erythrocyte serves as the common host cell for two major Apicomplexan parasites, *Plasmodium* and *Babesia*. Both species invade, develop and egress from the red blood cell (RBC) following specific developmental programs that contribute to the majority of clinical symptoms associated with these infections.¹⁻³ Shielded within the host RBC, these intra-erythrocytic parasites differentiate and multiply while concealing their presence from the immune system. After filling the available intra-cellular space, the resultant parasite progeny egress as merozoites, and invade new RBC, carrying on the cycle of growth and proliferation. This cycle depends on intricate interactions between host RBC and parasite molecules. Thus, any perturbations to either the composition or arrangement of proteins on or within the host RBC can impact the parasite's development and survival, and thus increase host resistance to parasite infection.

Much effort has been spent in determining the different means by which the human host can suppress this active parasite replication to limit the damage caused by the continuous destruction of the host RBC. One of the most commonly encountered mechanisms discovered is the genetic disorders that are found in the RBC.⁴ As the *Plasmodium* parasites have long co-existed with the human host, they have exerted extraordinary adaptive pressure on the human species.⁵ Consequently, in humans, multiple genetic polymorphisms have been selected for several hemoglobin disorders that provide intrinsic protection against severe malaria complications and are convincingly supported by clinical data.^{6,7} Hemoglobin (Hb) is the oxy-

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gen-carrying component and major protein of the RBC and is normally formed as a tetramer of two α -globins and two β -globins which constitute adult hemoglobin A (HbA). The major hemoglobinopathies result from mutations that either decrease the production of α - or β -globins (in α - and β -thalassemia) or sickling of the erythrocyte (in sickle HbS, HbC, and HbE diseases).^{8,9} Remarkably, small genetic variations confer dramatic levels of protection from malaria.^{10,11} HbS is the result of a single point mutation (Glu→Val) on the sixth codon of the β -globin gene. Homozygotes for hemoglobin S (HbSS) with two affected β chains develop sickle cell disease (SCD), in which polymerized Hb causes RBC to sickle and occlude blood vessels, and results in high morbidity and mortality.¹² Heterozygotes for sickle hemoglobin (HbAS) have sickle cell trait and are generally asymptomatic. Despite the obvious deleterious nature of HbSS, it is now widely accepted that the persistence of the sickle mutation in human populations is due to the protection from malaria afforded to heterozygous individuals.^{13,14} Multiple divergent mechanisms have been put forward to explain this resistance to malaria, including enhanced macrophage uptake, impaired growth and maturation of parasite, and decreased deposition of parasitized RBC in deep post capillary beds, but no single convincing explanation has yet been given.^{1,15,16}

Babesiosis has long been recognized as a veterinary problem of great significance, but only in the last 50 years has it been recognized as an important pathogen in man.² The four identified *Babesia* species that have so far been definitively confirmed to infect humans are *B. microti*,¹⁷ *B. divergens*,¹⁸ *B. dumcani*,^{19,20} and *B. venatorum*.²¹⁻²⁴ As sampling has become expansive and techniques have become more sensitive, there is evidence that more *B. microti*-like and *B. divergens*-like *spp.* are able to cause human infection (as reported in detail by Yabsley and Shock).²⁵ However, the general life cycle within humans remains the same. *Babesia* parasites are intracellular obligates that target RBC, and the parasite's ability to first recognize and then invade host RBC is central to the disease pathology. Besides its natural route of transmission *via* the infected tick, the parasite is also transmitted by transfusion of infected blood as its RBC host provides an optimum vehicle to facilitate its transmission. In fact, as the frequency of clinical cases has risen, there has been an associated increase in transfusion-transmitted *Babesia* (TTB), mainly reported for *B. microti*,²⁶⁻²⁸ making babesiosis the most frequent transfusion-transmitted infection in the US. Patients with sickle cell anemia, especially those on chronic transfusion therapy, are at high risk for severe TTB.^{29,30} Whether the sickle red cells themselves are responsible for the increased susceptibility of these patients to TTB or whether this is due to other related factors, such as a compromised immune system, has not been investigated. In this paper, we focus on the ability of the *Babesia* parasite to invade, grow in and egress from sickle trait and sickle cell anemia erythrocytes. Use of *in vitro* invasion and development assays were developed in our laboratory,³¹ as our primary outcome provided a rare opportunity to systematically examine the cellular determinants of parasite development in the sickle cell anemia setting. These enabled a comparison between various components of the parasite life-cycle in RBC obtained from various hemoglobin genotypes, HbAA, HbAS and HbSS, and revealed altered parasite population progres-

sion, parasite maturation and egress phenotypes in the HbSS cells.

Methods

Ethics statement

Human blood from healthy volunteer donors was used to culture *B. divergens* (Bd) *in vitro*. SCD patients' RBC were obtained from residual anticoagulated blood samples from same day collections from patients with sickle cell anemia (hemoglobin genotype SS) who had not been transfused for at least three months prior to sample draw. Patients provided consent for use of de-identified blood for research purposes on a Montefiore Medical Center Institutional Review Board (IRB) approved protocol. HbAA RBC and sickle trait RBC were identified from New York Blood Center (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 RBC as previously described.^{32,33} A⁺ RBC were collected in 10% CPD and washed 3X with RPMI 1640 medium for the complete plasma and white cell removal.

Free merozoites isolation

High concentration of viable free merozoites was isolated from unsynchronized cultures at high parasitemia (40%), as described previously.^{31,34}

Assessment of invasion, development and egress in various red blood cells

Fresh cultures were seeded with purified merozoite suspension at 20% (v/v) of culture volume. To define time points to accurately estimate invasion in the different RBC (HbAA, HbAS; HbSS), invasion was assayed in the first set of samples at 5 minutes (min), 1 hour (h) or 6 h post invasion. 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 three replicate cultures for each RBC sample.

Light microscopy

Blood smears were fixed with methanol and stained with 20% Giemsa (Sigma-Aldrich, St. Louis, MO, USA) 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 RBC over 72 h, as previously described³¹ with modifications.

Statistical analysis

Parasitemia was defined as the total number of infected RBC (iRBC) in every 100 RBC, not taking into consideration the number of parasites seen in a given cell when measured by flow

cytometry. 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 describe.³⁴ The percentage of inhibition mediated by the different Hb genotypes was determined by assuming that the parasitemia from control HbAA cells was equivalent to 0% inhibition or enhancement.

Results

Response of *B. divergens* to the different host cell hemoglobin environments

To assess the hosting ability of the sickle cell anemia cells, purified merozoite preparations were used to infect HbSS and HbAS RBC from sickle and sickle trait patients, respectively, in parallel with HbAA RBC as wild-type control. Multiple independent experiments were performed with each cell type and processes such as invasion, development and egress were chronologically observed during the asexual erythrocytic cycle of *B. divergens*.

The first indication that there were differences in parasite development in the RBC of different Hb genotypes was observed while defining the ideal time points to evaluate various aspects of the parasite life-cycle (Table 1). A single sample each of HbAA, HbAS and HbSS RBC was used in an invasion assay using purified merozoite preparations. One-hour post-mixing was found to be the ideal time point to evaluate invasion and monitor the initial parasitemia. At the 5 min time point, the parasitemia was too low to assess differences between the three cell types, although high synchronicity of parasites was achieved. At the 6 h time point, the initial synchronicity was lost by events of late merozoite invasion and the cultures were affected by the long exposure to the ruptured RBC remnants that were introduced by the inoculation (*data not shown*). Because of these factors, 1 h post- mixing of merozoite preparations and RBC was used as the time-point to assess invasion in the experiments that followed.

B. divergens was able to efficiently invade RBC of all three genotypes: HbAA, HbAS and HbSS. However, parasite culture expansion was drastically compromised in the sickle cell (SS) environment which can be seen by the comparison of parasitemia progression in the HbSS culture as compared to both HbAS and HbAA cultures (Table 1) (culture growth inhibition of 36%, 59% and 70% respectively at 24 h, 48 h and 72 h post invasion of the HbSS culture when compared to HbAA) (Figure1A). In contrast to the HbSS cultures, HbAS cells were found to provide a favorable environment for parasite progression, with only modest differences in parasitemia observed when compared with the wild-type HbAA (culture growth inhibition of 4.3%, 0% and 1% respectively at 24 h, 48 h and 72 h post invasion of HbAS culture compared to HbAA) (Table 1 and Figure 1A).

HbSS red blood cells from diverse donors exhibit comparable degrees of impairment of parasitemia progression while HbAS red blood cells from diverse donors permit normal parasite progression

To assess the effect of the individual variability in sickle cell anemia and sickle trait RBC from different individuals on the parasite response to these RBC environments, RBC from 11 different sickle cell (Table 2) and five sickle trait (Table 3) patients were infected with purified merozoite preparations. Each experiment was run together with several wild-type RBC controls (HbAA) originating from different individuals. The cultures were monitored from invasion to 48-72 h post invasion.

On monitoring the parasite population sizes over time, it was clear that, although there was individual variation among sickle cells from different sickle cell anemia patients, the HbSS cells in every tested culture did not support normal culture progression, as the parasitemia was drastically reduced in all 11 sickle cell cultures (Table 2). This inhibition of HbSS-culture expansion was statistically significant at 48 h ($P<0.05$), when HbAA RBC controls exhibited a robust approximately 3-4-fold increase in parasitemia, compared to the HbSS cells which remained static, exhibiting parasitemia close to the initial starting values. Eight HbSS cultures continued to be monitored at 72 h post invasion, to confirm if significant inhibition ($P<0.05$) was maintained. Inhibition was found to be sustained and, once again, all eight cultures showed a plateau in parasitemia, not appreciably changed from their 48 h parasitemia, unlike the HbAA cultures which approach 40-50% parasitemia at 72 h (Table 2). From the perspective of parasitemia progression, there was thus an increased inhibition over time; approximately 73-92% inhibition of growth in HbSS cultures when compared to HbAA controls at 72 h (Figure 1B). In contrast, all HbAS cultures were able to reach a similar parasite population size as the control HbAA cultures at 72 h, averaging 45% parasitemia, with no significant variations in parasitemia in individual HbAS donor cells at different time points ($P>0.05$) (Table 3). Overall, all parasite cultures established in the HbAS-cells exhibited a similar increase in parasitemia when compared to the HbAA control (Tables 1 and 3), indicating the parasites capability for population progression in HbAS RBC, unlike the impaired culture growth phenotype observed in HbSS RBC. Table 4 shows the aggregate values of inhibition of parasite population progression in the HbSS cells and HbAS cells with reference to the parasitemias in HbAA cells at the three time points tested. Despite the HbSS cells and HbAS originating from diverse donors, the inhibition seen in the HbSS population is significantly higher than that seen in the HbAS population which has close to parasitemia values in the HbAA population, at the 48 h (68% inhibition in HbSS vs. 15% in HbAS) and 72 h (84% inhibition in HbSS vs. 4% in HbAS) time points in culture ($P<0.05$) (Table 4).

Table 1. Parasitemia assessed from cultures grown in single samples of each hemoglobin (Hb) genotype-HbAA, HbSS, HbAS cultures (5 min-6 hours post inoculation of cultures with free merozoites) and at 24-hour intervals after inoculation (24-72 hours).

Sample	5 min	1 h	6 h	24 h	48 h	72 h
HbAA	0.6 [0.03]	2.1 [0.03]	3.0 [0.06]	4.7 [0.00]	14.4 [0.25]	43.2 [0.15]
HbSS	0.9 [0.01]	2.7 [0.02]	3.1 [0.06]	3.0 [0.00]	5.9 [0.06]	12.8 [0.10]
HbAS	0.4 [0.04]	2.8 [0.02]	3.3 [0.00]	4.5 [0.00]	15.1 [0.12]	42.9 [0.32]

Parasitemia values represent average percentage. [StDv]: Standard Deviation among triplicates; h: hours; min: minutes.

Similar invasion efficiencies were obtained in HbAA, HbAS and HbSS red blood cells

To understand the specific defect induced in the parasite life-cycle by the sickle environment, the population structure of cultures was analyzed based on the parasite load within the infected RBC (number of parasite genomes by FACS analysis). By monitoring the distribution of sub-populations of infected RBC, the life cycle processes of invasion, parasite development and egress were evaluated in sickle cell anemia compared with sickle trait and control cells.

The invasion of merozoites in all cell types was measured by the percent parasitemia in the different cultures at the 1 h time point, established earlier to be the optimum point to assess invasion efficiency. The sickle RBC were able to support efficient merozoite invasion (HbSS-1 h parasitemia ranged from 0.8% to 2.3%) (Tables 1 and 2). When the invasion efficiency of HbSS RBC was compared

to that of control HbAA RBC, no significant difference in parasite invasion was observed among cultures ($P>0.05$). In fact, the subtle increase in initial infection rates of HbSS *versus* HbAA RBC was found to be an artefactual consequence of the presence of Howell-Jolly bodies (H-JB) in HbSS cells. These bodies, known as micronuclei, contain small erythrocyte DNA inclusions (approx. 1 μm) that are the consequence of cytogenetic damage.^{35,36} To confirm that the higher DNA content in infected HbSS cells was indeed from H-J bodies, their quantification was first performed by their detailed counts on Giemsa stained smears of uninfected HbSS cells, followed by quantification of DNA content in uninfected HbSS cells (FACS analysis with Vybrant®DyeCycleTMGreen) (Figure 2). Our estimates of these bodies in all HbSS samples were in the range of 0.1-0.3% (shown for 3 independent HbSS samples in *Online Supplementary Table S1*), which when subtracted from the parasitemias found for the same infected

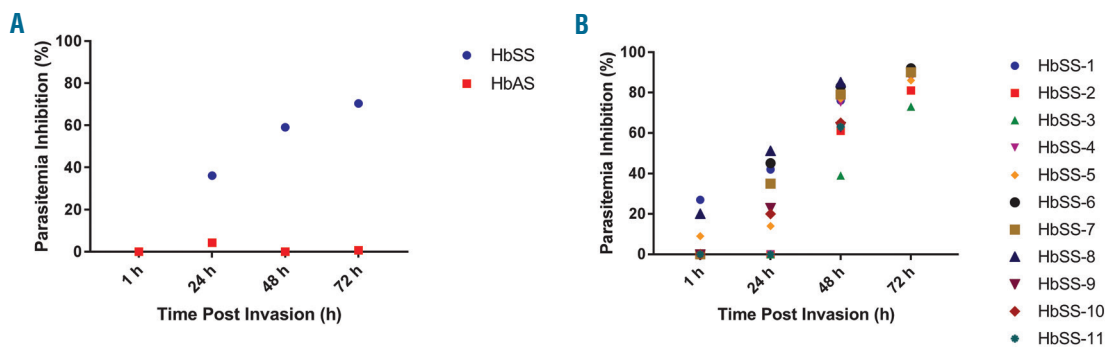


Figure 1. The expansion of *B. divergens* population was inhibited in HbSS cultures. Comparison of the growth inhibition rate between parasite cultures grown in HbSS cells and HbAS cells relative to growth in wild-type HbAA as measured by parasitemia. (A) Between cultures originating from a single HbSS and single HbAS donor. Values calculated based on data from Table 1. (B) Among HbSS cells from 11 different sickle cell disease patients, showing that, despite variation in parasitemia in individual cultures, all exhibit high degrees of inhibition of culture progression. Values calculated based on data from Table 2.

Table 2. Parasitemia of *B. divergens* cultures measured 1-72 hours post-invasion in sickle (HbSS) and wild-type (HbAA) red blood cells from different donors.

Sample*	1 h	24 h	48 h†	72 h†
HbAA-I	1.1 [0.01]	4.0 [0.40]	14.5 [0.35]	37.3 [1.35]
HbSS-1	0.8 [0.03]	2.3 [0.21]	3.4 [0.12]	3.9 [0.23]
HbSS-2	1.5 [0.01]	4.5 [1.18]	5.6 [0.12]	7.2 [0.21]
HbSS-3	1.9 [0.02]	5.1 [0.84]	8.9 [0.38]	9.9 [0.10]
HbAA-II	1.1 [0.03]	4.1 [1.06]	20 [0.92]	43.4 [0.21]
HbSS-4	2.0 [0.02]	4.6 [1.56]	5.1 [0.07]	5.5 [0.28]
HbSS-5	1.0 [0.02]	3.5 [1.13]	4.8 [0.07]	5.9 [0.00]
HbAA-III	1.9 [0.02]	5.5 [0.26]	21 [0.15]	49.3 [0.75]
HbSS-6	2.3 [0.01]	3.0 [0.00]	3.5 [0.14]	4.1 [0.07]
HbSS-7	2.0 [0.03]	3.6 [0.06]	4.5 [0.20]	4.9 [0.12]
HbAA-IV	1.0 [0.03]	2.9 [0.06]	12 [0.25]	ND
HbSS-8	0.8 [0.03]	1.4 [0.10]	1.7 [0.12]	ND
HbSS-9	1.5 [0.02]	2.2 [0.07]	4.3 [0.03]	ND
HbSS-10	1.5 [0.04]	2.3 [0.02]	4.0 [0.10]	ND
HbSS-11	1.5 [0.01]	3.0 [0.35]	4.2 [0.15]	ND

*Four independent experiments. (HbSS 1-11) sickle samples (HbAA I-IV) wild-type control samples. Parasitemia values represent average percentage. [StDv]: Standard Deviation among triplicates. †Significant difference between wild-type controls HbAA and tested HbSS samples. $P<0.05$. ND: not determined; h: hours.

HbSS cells, results in equivalent invasion efficiencies for HbSS and HbAA cells. Additionally, all the parasite cultures were followed a few hours later to ensure that parasite development could be recorded to yield comparable parasitemia. Images of invaded parasites can be very easily discriminated from that of the H-J bodies, with the parasite seen as a distinct ring *versus* the solid staining pattern of the body (Figure 2). Overall, all cells were found to support parasite invasion equally well.

Effect of sickle Hb (HbSS) and sickle trait Hb (HbAS) on the intra-erythrocytic parasite development

The relative differences in parasitemia in the cultures (Tables 1-3) and in the distribution of their infected RBC sub-populations (Figure 3) described not only parasite competence for population expansion in that cell type, but also their developmental and differentiation status when monitoring the chronological emergence of each sub-parasite population within the infected RBC. The supplementary histogram peaks detected at 24 h post invasion (Figure 3) indicating parasite proliferation showed that the HbSS sickle cells, permitted intra-erythrocytic proliferation and differentiation of the parasites; however, they

may not be providing an optimum environment for culture propagation as there was no appreciable increase in parasitemia after 24 h.

As mentioned before, at 1 h post invasion (1 h histograms) (Figure 3), all cultures exhibited similar FACS profiles, with the 1N population seen as the dominant sub-population as expected, as shown in the representative samples. The distribution over time of these sub-populations of iRBC (frequency of events acquired in the histogram peaks corresponding to 1N-; 2N-; 4N-; and >4N-iRBC sub-populations), when translated into the line charts as presented in *Online Supplementary Figure S1*, showed that the conventional pattern of population formation was adopted by *B. divergens* in all RBC, irrespective of genotype, during the first 24 h. During this first intra-erythrocytic cycle, with the intense intra-cellular proliferation (characterized by the emergence of infected RBC with higher intra-cellular parasite load) and the low ratio of invasion/egress (as evidenced by the relatively stable culture sizes over 24 h as expected, even for the control cultures) *Babesia* similarly built its populations in all three cell types, exhibiting the common gain of heterogeneity by the presence of different sub-populations of iRBC.

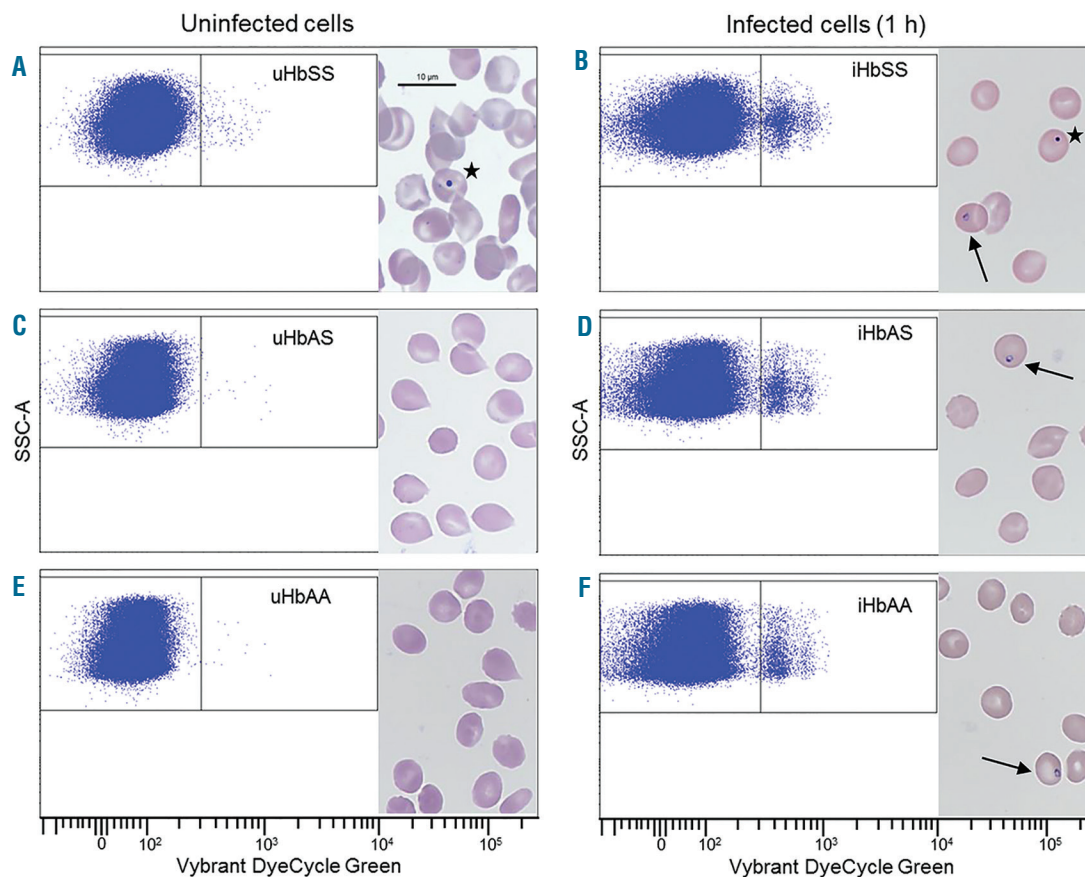


Figure 2. Invasion efficiency is similar in HbAA, HbAS and HbSS cells. FACS analysis of DNA content in representative (A,C,E) uninfected red blood cells (RBC) and (B,D,F) infected RBC, along with Giemsa stained smears. (A) HbSS cells reveal DNA positive population (0.1-0.3%) from Howell Jolly (H-J) Bodies in cells, Giemsa image shows a dense H-J body, (marked by asterisk). (C) HbAS cells and (E) HbAA cells do not have DNA positive population and Giemsa smear shows absence of bodies. (B) Parasite cultures in the same HbSS cells show higher Vybrant positive population than uninfected, being composed of both cells containing H-J bodies and parasites. The difference between panels (B) and (A) yields invasion parasitemias (see Table 4 for data). Giemsa smear shows distinct ring formed 1 hour (h) post invasion, presenting a clear difference from H-J body. (D and F) 1 h post-invasion cultures of (D) HbAS cells and (F) HbAA cells showing Vybrant positive population, and 1 h post-invasion Giemsa image showing newly invaded parasites (marked by arrows).

Table 3. Parasitemia of *B. divergens* cultures measured 1-72 hours post invasion in sickle trait (HbAS) and wild-type (HbAA) red blood cells from different donors.

Sample	1 h	24 h	48 h	72 h
HbAA-V	4.5 [0.50]	8.7 [0.15]	26.7 [0.82]	43.1 [2.35]
HbAA-VI	3.8 [0.22]	7.7 [0.15]	26.7 [0.38]	47.2 [1.25]
HbAA-VII	3.3 [0.12]	7.6 [0.21]	26.0 [0.26]	48.2 [0.70]
HbAS-1	2.8 [0.04]	6.7 [0.23]	23.8 [1.01]	41.8 [3.35]
HbAS-2	3.1 [0.01]	6.1 [0.06]	16.9 [0.32]	41.8 [2.95]
HbAS-3	3.6 [0.03]	7.5 [0.17]	22.2 [0.06]	44.7 [1.04]
HbAS-4	4.9 [0.03]	8.4 [0.00]	23.6 [0.10]	47.4 [2.35]
HbAS-5	3.9 [0.11]	7.4 [0.00]	21.2 [0.12]	45.8 [1.50]

Parasitemia values in percentage average. [StDv]: Standard Deviation among triplicates; h: hours.

During the second proliferative cycle (between 24-48 h post invasion) the emergence of >4N- and the increase of 4N-iRBC revealed the continued cellular proliferation over time. However, unlike the population seen in the control HbAA cells after 24 h, the parasite population in the HbSS RBC turned structurally stable, with the frequency of sub-populations remaining constant up to the last time point assayed at 72 h. The set of data from four representative HbSS cultures presented in *Online Supplementary Figure S1* illustrates that, despite the individual biological features of RBC from different individuals with SCD, a general common pattern of population structure was adopted by the parasite in sickle cells (HbSS). Once the heterogeneity of stages and sub-parasite populations was achieved at the 24 h time point in the HbSS cells, the proportion of sub-populations were kept relatively stable throughout the monitored 72 h period. Comparing these frequencies with those from control HbAA samples showed that the maintenance of relative proportions of sub-populations was not random. Although the parasitemia along with the sub-population structure varied among the sickle cell anemia samples, the specific proportions of 1N-, 2N-, 4N- and >4N-iRBC were built in the 11 samples that stayed constant from 24-72 h [frequency averages in HbSS iRBC: 1N-HbSS-iRBC dropped from approx. 68% at 1 h to 47% at 24 h; remained constant throughout 48 h as 44% and at 72 h as 51%; 2N-HbSS-iRBC from the initial 26% at 1 h slightly changed to 28% up to 72 h; 4N reached 20% at 24 h and then stayed relatively unchanged up to 48 h (21%) and 16% (72 h); >4N reached 7% at 24 h, 10% at 48 h and 5 % at 72 h] (*data not shown*). Unlike the HbSS cultures, control HbAA cultures exhibited the typical fluctuating dynamics of parasite culture.

Unlike the parasites in the HbSS cell cultures, parasites grown in wild-type cells (HbAA-RBC) efficiently performed sequential cycles of invasion and egress, as evidenced by the increase in 1N-iRBC (invasion clearly identified by the arrow between 48-72 h) (*Online Supplementary Figure S1A*) and the decrease in 4N- and >4N-iRBC (egress identified by the decrease in quantified frequency 4N- and >4N-iRBC; *data not shown*) with a progressive increase in total parasite population size (levels of parasitemia over time HbAA-III; Table 2). This fluctuation among sub-populations of parasites in HbAA cultures proved that conditions in HbAA cells were conducive to parasite life-cycle processes that resulted in population growth. Similarly, parasite cultures in HbAS cells exhibited the variation in infected RBC-sub populations expected

Table 4. Inhibition of parasite culture expansion compared to wild-type (HbAA) measured 24-72 hours post invasion in sickle cell anemia (HbSS) and sickle trait (HbAS) from different donors.

Sample	24 h	48 h	72 h
HbSS (n=12)	22 [19]	69 [12]	84 [8]
HbAS (n=6)	10 [8]	15 [11]	4 [4]

Values expressed as average percentage of inhibition compared to control HbAA [StDv]: Standard Deviation among triplicates; h: hours.

over time, based on the sequential process of invasion, development and egress (*data not shown*). This normal equilibrium among the infected RBC hosting different intra-parasitic loads observed in HbAA and HbAS contrasted with the static proportions of the sub-populations among the HbSS cultures (*Online Supplementary Figure S1B-E*) observed after 24 h, and suggested a potential inhibitory environment in the HbSS cell may account for the low parasitemia found in HbSS cultures.

Effect of HbSS cell environment on the morphological differentiation of the parasite

As shown above, HbSS RBC did not interfere with the intra-erythrocytic parasite proliferation and differentiation since iRBC were seen carrying high intracellular parasite loads (4N and >4N) right from the first 24 h life cycle (Figure 3A). In addition, the analysis of the sequential appearance of the different morphological stages (light microscopy of Giemsa smears) in the HbAA, HbAS and HbSS cultures confirmed this successful intracellular proliferation, and showed that parasite differentiation into the different morphological stages occurred successfully in all three types of cultures (Figure 4). However, closer examination of parasite morphology in HbSS cells revealed some abnormalities. The heterogeneity of the sub-populations of iRBC reported from the FACS analysis of these cultures (Figure 3) was also observed by microscopic analysis and can be described as a change in frequency of infected RBC hosting different numbers of attached or detached parasites (Figure 4). The predominant parasite stages seen at later time points in sickle cells were of the detached variety and consisted of rings (describes any unattached intracellular parasite) present in single, double, quadruple and multiple forms. Many of the parasites were seen unattached to each other and assumed either circular, pear or deformed shapes (Figure 4). However, there were parasitized RBC that also assumed conventional morphological stages, seen as “paired-fig-

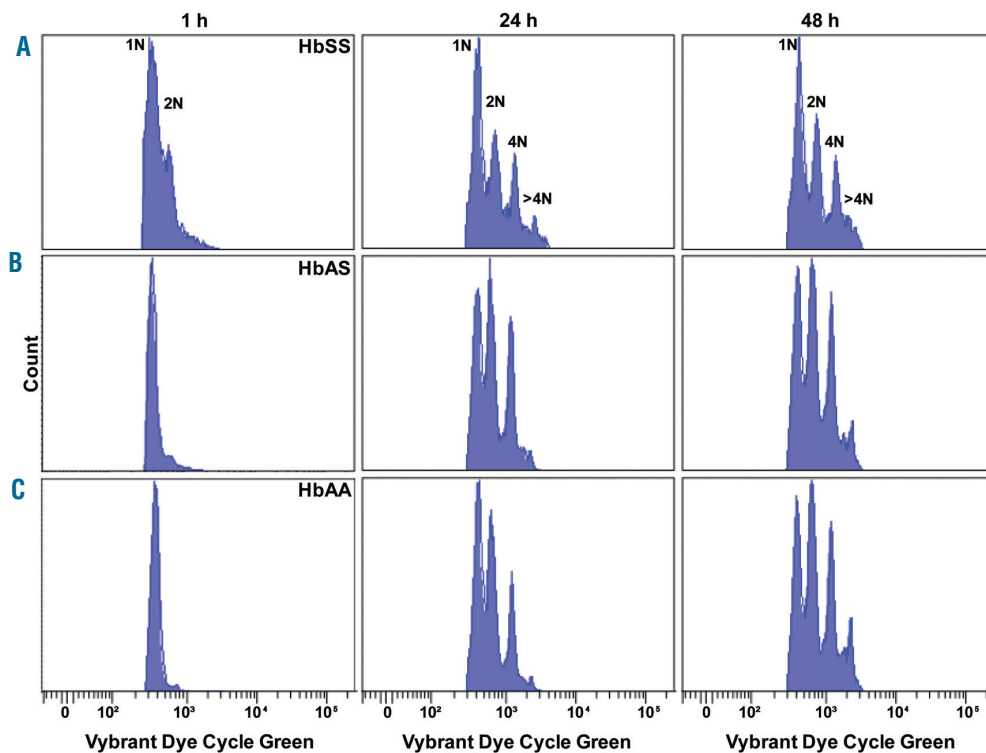


Figure 3. Distribution of parasite sub-populations cultured in three types of red blood cells (RBC), based on genome content reveals all sub-populations are represented in the cultures. (A) HbSS. (B) HbAS. (C) HbAA. Samples were collected at 1 hour (h), 24 h and 48 h and the percentage of each infected RBC sub-population was determined by FACS using VybrantRDyeCycleTMGreen dye to quantify the amount of parasitic DNA within infected cells, where (N) is the number of parasite genomes. (Top left) HbSS cells show higher numbers of 2N cells at 1 h time point compared to (left middle) HbAS and (left bottom) HbAA cells confirming a higher frequency of multiple invasion events in HbSS. (Middle and right panels) Parasite population structure at 24 and 48 h showing all sub-populations represented in all three cell genotypes.

ures" (2 attached parasites) and/or Maltese Cross (4 attached parasites) and/or double paired-figures (also as 4 parasites attached 2 by 2) and were seen co-existing with the detached ring parasites, showing that cellular division and differentiation of *B. divergens* followed the sequential transformation of stages from attached into unattached forms after completing cytokinesis before their egress. In addition, some of the infected RBC in HbSS cells hosted multiple parasites (≥ 4 -8 or more) which were detected with variable frequency among HbSS cultures (Figure 4).

Thus, the morphological analysis supported the FACS analysis of population structures at the various sampled time points of the different cultures, HbAA, AS and SS. Importantly, the parasites in the HbSS RBC exhibited features that signaled they were ready for egress, i.e. the presence of both-infected RBC hosting multiple parasites as well as the presence of detached parasites in these cells. However, these parasites were not successful at starting new intra-erythrocytic cycles, as evidenced by the lack of increase in parasitemia.

Parasite infectivity is impaired in HbSS cells as the addition of fresh red blood cells does not rescue parasitemia in HbSS cultures

Success of the parasite culture propagation as measured by an increase in parasitemia requires successful invasion, successful production and maturation of merozoites which then need to successfully egress. This in turn depends on several factors involving host cells and para-

sites. Sickle cell anemia RBC are notorious for exhibiting increased fragility³⁷ as compared to HbAA RBC and this could be a factor in the inability of the cultures to support parasitemia after 24 h.

To test whether the lack of increase in parasitemia was a consequence of defective egress, sickle cell fragility or defective merozoite maturation, we examined progression of cultures in which the introduction of fresh RBC (either HbAA and HbSS) into 24 h HbSS parasite cultures was performed. After 1 h, all cells supported *Babesia* invasion equally well, as shown, following the mixing of merozoite inoculum and RBC (*Online Supplementary Table S2*). The cultures were then allowed to progress for 24 h at which point, the parasitemia in both the HbAA and HbSS cultures had risen slightly (HbAA-A: 3.2% vs. HbSS-A: 3.4%) (*Online Supplementary Table S2*), in line with the results reported for the 12 HbSS cultures above (Tables 1 and 2). The cultures were then split, either staying the same with only medium change (flasks A) or receiving fresh SS cells (flasks B) or AA cells (flasks C), maintaining hematocrits of 5%. At 48 h, the parasitemia in the original HbAA-A culture had doubled (from 3.2% to approx. 6.5%) while that of the original HbSS-A culture reported only a small increase in parasitemia (from 3.4% to approx. 4%), following the pattern reported for the original 12 HbSS cultures (Tables 1 and 2). The HbAA-B culture which was diluted with fresh HbAA RBC to receive half of the inoculum exhibited half the parasitemia of the undiluted HbAA-A culture, once again as expected (3.2%)

(*Online Supplementary Table S2*). The two HbSS cultures that received fresh RBC, either HbAA (HbSS-C) or HbSS (HbSS-B), had lower parasitemias (2.1% and 2.5%, respectively; 24 h time point) than that of HbAA-B culture, proving that the nature or condition of the host cells did not play a role in the inhibition of new cycles of parasitemia seen in the HbSS cultures (HbSS-C and HbSS-B). At later time points (48 h after addition of fresh cells), this result of non-rescue was further strengthened when cultures in HbAA-B exhibited a robust increase in parasitemia (16%), contrasting with HbSS-B and HbSS-C and the stable parasitemia of (3.6% and 2.3%, respectively). The original half culture which had only medium change (HbAA-A and HbSS-A) followed the previously reported inhibition patterns of in culture progression showing approximately 18% inhibition in HbSS-A culture growth, compared to the HbAA-A culture. Neither the addition of fresh HbSS RBC or HbAA RBC could significantly rescue the HbSS cultures, with the original HbSS-A parent culture at 4.7% and the culture receiving fresh HbSS cells at 3.6% parasitemia. Surprisingly, the culture that received

fresh HbAA cells was even lower demonstrating an infection rate of 2.3%, indicating that parasite development within the HbSS RBC was potentially impaired, resulting in the formation of merozoites unable to invade fresh RBC at frequencies typically seen *in vitro* parasite cultures. The formation of mature merozoites that egress from the RBC are critical to start new intra-erythrocytic cycles by invading new host cells, and these merozoites are required to be in an optimum invasive state to support new cycles.

Parasite egress from sickle red blood cells may also be compromised

One of the factors that may impact the progress in parasitemia in HbSS cells is the ability of the parasite to egress from the host sickle cell and invade new host cells. The FACS profile of the parasite population in the HbSS cells was carefully examined to look for this inhibition of egress. The typical pattern of parasite-holding within the RBC that we have reported for *B. divergens*,³¹ when there is an egress defect, where the *B. divergens* parasite population builds its 4N and >4N populations to high levels that

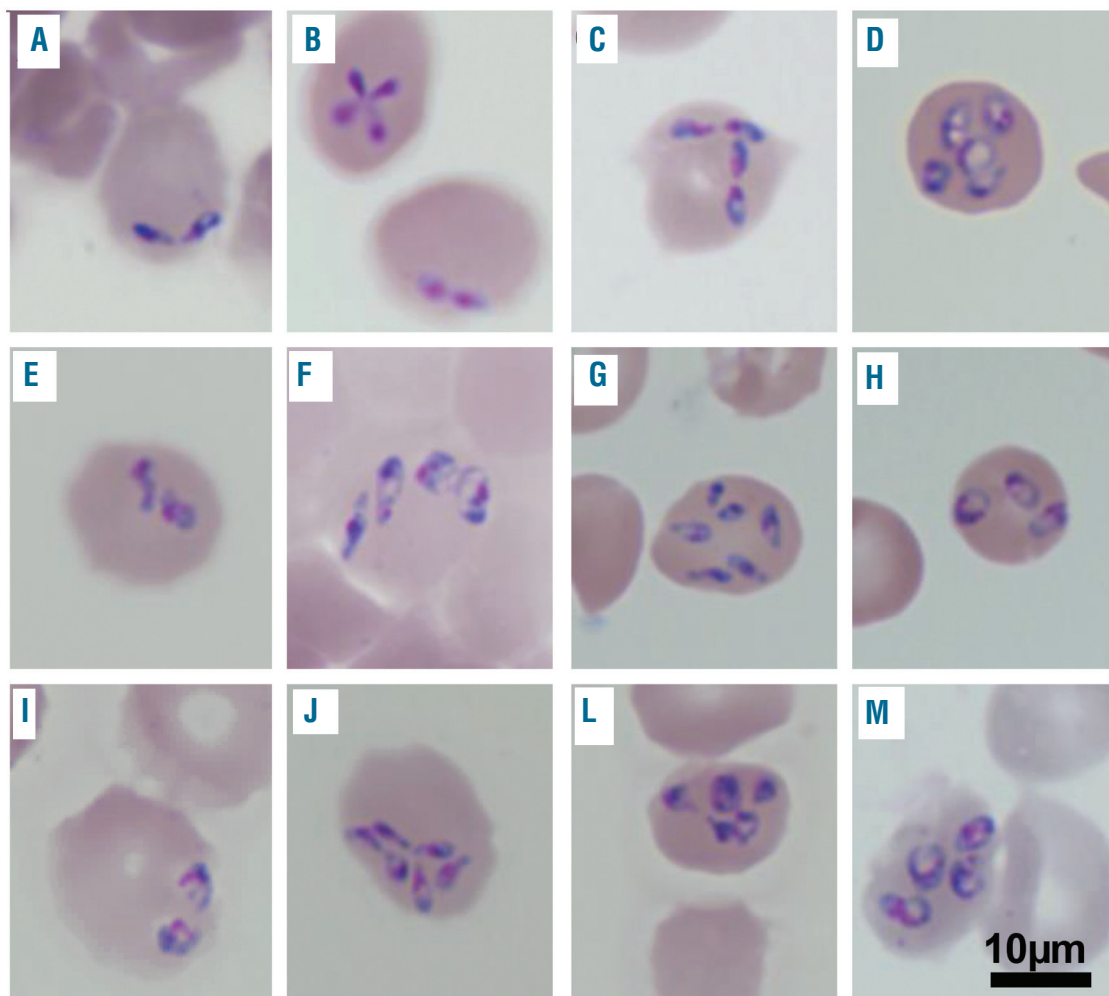


Figure 4. Parasites grown in HbSS cells exhibit atypical morphology although normal parasite forms are also seen in the same cultures. (A-C) Normal morphology of parasite seen as paired figures or Maltese Crosses or double paired figures (D-M) Unusually high numbers of detached rings seen at high frequency in all HbSS parasite cultures.

can be visualized by both FACS analysis and Giemsa stained smears, was not seen in these sickle cell cultures (Figures 3 and 4). In fact, the presence of free merozoites was noted in most culture supernatants (*data not shown*), although it is difficult to quantitatively estimate if these numbers are normal as compared to wild-type RBC. However, unlike the fluctuating proportions of the parasite sub-populations seen in both HbAA and HbAS cultures, relatively stable proportions of parasite sub-populations in the sickle RBC cultures, after the 24 h time point was a characteristic noted in all 12 HbSS cultures (*Online Supplementary Figure S1*). This novel holding pattern could potentially signify an inability of the parasites to egress, which in turn could cause the relatively static parasitemia observed in these cultures. Thus, inefficient parasite egress from the host HbSS cells infected in the first round of invasion may not permit new cycles of invasion to take place.

Discussion

Variant RBC are produced from some of the most common human genetic polymorphisms, and their high incidence has been ascribed to the evolutionary selection by life-threatening falciparum malaria.^{38,39} The sickle hemoglobin genotype (HbAS/HbSS) is the best-characterized human genetic polymorphism associated with malaria. In this paper, we have explored the effect of the sickle hemoglobin mutation on a related intra-erythrocytic apicomplexan parasite, *B. divergens*. There are several stages in the parasite's erythrocytic cycle at which RBC mutations can affect parasite infection. The first would be erythrocyte invasion by *Babesia* merozoites; the next stage susceptible to inhibition is the intra-erythrocytic parasite development. This category would include impairments in the parasite's ability to meet its nutritional requirements or changes in the host cell milieu that would be fatal to the parasite. Finally, impairment of red cell rupture and release of infective merozoites at parasite maturity could inhibit increase in infection. Use of *in vitro* growth assays as our primary outcome, along with a robust sample size of SCD RBC, provided a rare opportunity to systematically examine the cellular determinants of parasite growth in the sickle setting. We present here evidence for atypical population progression, a potential loss of merozoite infectivity, and defective egress of the parasite in these hemoglobinopathic cells (Tables 1-4 and *Online Supplementary Table S2*). Interestingly, using the Townes mouse model of SCD, and *B. microti*, we have shown that a similar inhibition results in dramatically low infection rates in HbSS mice as compared to HbAS and HBAA mice.⁴⁰

Invasion of the human RBC is the central pathogenic step in the life-cycle of *Babesia*. When *Babesia* spp. sporozoites are first injected into the human host with a tick bite, they target the host RBC immediately, unlike *Plasmodium* spp. which are required to undergo an exo-erythrocytic phase in hepatic cells. It is the parasite's ability to first recognize and then invade host RBC that is central to symptomatic human babesiosis, and the parasites invade RBC using multiple complex interactions between parasite proteins and the host cell surface, which have not been fully elucidated.^{2,41,42} Like *Plasmodium*, *B. divergens* has been shown to use GPA and GPB as invasion receptors.³³ *In vitro* studies with *P. falciparum* have suggested a

decreased invasion and growth of the parasite in sickle cells;⁴³⁻⁴⁵ however, some of the older studies have not been able to differentiate between the various phases of the parasite lifecycle. Hence, a deficiency in egress or invasion would both be visualized as an overall decrease in parasitemia. The use of our platform combining *in vitro* invasion and growth assays with synchronized cultures monitored by both FACS analysis and Giemsa smears provided an excellent system to systematically dissect the phase of the erythrocytic cycle impacted by the HbSS environment. The increased DNA content of HbSS cells that contributed to the subtle higher invasion percentages was found to be due to the presence of the Howell-Jolly bodies which artefactually increased the DNA load of the cell. When the contribution of the bodies was subtracted, a similar rate of invasion was obtained in all Hb genotypes, indicating a potential difference between malaria and *Babesia* mechanisms of invasion in sickle cells.

The development of the parasite in HbSS cells in the first 24 h was normal and exhibited all conventional forms reported *in vitro* cultures by us earlier.³⁴ Thus, rings, paired figure and Maltese Cross forms were all documented in culture. However, a larger than expected proportion of cells hosted detached rings (Figure 4). The multiple unattached parasites feature assumed by parasites in HbSS cultures suggests that the parasite is able to complete cytokinesis shortly after the nuclear duplication, allowing the conclusion of cell division which may not allow the accumulation of attached morphological stages such as Maltese-Cross or paired-figures, as seen in HbAA cultures. After 24 h, the progression of cultures is stalled in HbSS cells as seen by FACS analysis (Figure 3A and *Online Supplementary Figure S1B-E*) where the sub-populations remain in static proportions unlike the dynamic movements seen among sub-populations in HbAA (Figure 3C and *Online Supplementary Figure S1A*) and AS cells (Figure 3B). As this profile is representative of the parasite population and not individual parasites, it is apparent that, overall, there is no growth in the population despite small increases seen in parasitemia, reflecting a minority of parasites successfully initiating new cycles of infection. Microscopic analysis of the parasites in HbSS cells reveals normal morphology in terms of size, shape and staining patterns. Ultra-structural analysis may shed more light on potential defects in these parasites, if present.

Egress is a phase of the cycle that, if impacted, can lead to disastrous outcomes for the parasite population progression. *In vitro* studies with *P. falciparum* suggest a link between the hydration status of the host RBC and parasite invasion and egress.^{46,47} The high water-permeability of the RBC ensures their continued osmotic equilibrium in plasma so that they can shrink or swell by the loss or gain of a fluid isosmotic with surrounding plasma. This homeostatic balance is disrupted in HbSS cells, resulting in altered ion fluxes, ion content regulation, and hydration states in the circulation.⁴⁸ Malaria parasites have to breach both the parasitophorous vacuolar membrane (PVM) and erythrocyte membrane in order to egress. The altered exit of malaria merozoites from the dehydrated RBC was linked to the reduction of osmotic pressure within the parasitophorous vacuole that was needed to lyse the compartment prior to lysis of the RBC membrane. However, intra-erythrocytic *Babesia* parasites are free in the cytoplasm of the RBC without being enclosed in a vacuole as the PVM is a transient structure found fleetingly after

invasion of the *Babesia* merozoite. Thus, the lack of osmotic pressure in the infected sickle RBC may not impact *Babesia* parasite egress. Additionally, FACS analysis of the cultures at later time points do not support the typical loss of egress phenotype, which presents as a build-up of 4N and >4N populations in a single cell, as seen in our previous work with egress inhibitors.³¹ Although there were cells that hosted multiple parasites (>4N), these appeared to be a result of the normal proliferative cycle to build the population structure, but they were not the majority of infected cells. However, the novel holding pattern reported here (Online Supplementary Figure S1), where the sub-populations are maintained after 24 h in the same ratios, indicate that egress may be compromised in these cells. Thus, it is possible that HbSS cells were not as efficient in supporting egress as HbAA cells, but other factors also contribute to the low infection rates seen in these cultures. Production of viable, infective merozoites within HbSS cell appeared to be another limitation of these cultures. Although free merozoites were seen in most HbSS culture supernatants, they did not appear to be able to successfully initiate new rounds of infection. Rescue experiments with both fresh HbSS and HbAA cells indicate that the defect may not lie in the unavailability of optimal host cells but rather with the merozoite, despite them presenting normal morphology (Online Supplementary Table S2). Detailed morphological examination of these zoites by electron microscopy may provide clues to their lack of infectivity.

Despite years of research effort, the mechanism of protection of the sickle trait in malaria remains unclear. A number of cellular, biochemical and immune-mediated mechanisms have been proposed, and it is likely that multiple complex mechanisms are responsible for the observed protection. Invasion and growth of *P. falciparum* *in vitro* within HbAS RBC is reduced in low oxygen tension growth conditions (<5% O₂).^{43,49} Some have proposed that the increased sickling of infected HbAS RBC, due to polymerization of sickle Hb, may be a mechanism for impaired growth under low O₂ conditions.^{50,51} However, in our *in vitro* assays, which utilize micro-aerophilic conditions with 5% O₂, no inhibition of *Babesia* growth was observed in the HbAS cells, in which all five samples yielded comparable support of parasite growth as that seen in control HbAA RBC. Unlike *Plasmodium*, which digests Hb to meet its nutritional needs, *Babesia* does not digest any of the Hb, which may account for the lack of

inhibition of intra-erythrocytic proliferation seen in the HbAS cells (no significant difference between HbAA and HbAS cells; $P>0.05$) (Table 3). As the assays reported here are purely *in vitro* assays, the effect of *in vivo* parameters like increased cytoadhesion,⁵² splenic retention,⁵³ and altered immune system response, as seen in enhanced phagocytosis and induction of inflammatory cytokines following endothelial activation, are not factored in, which may lead to an overall protection against the parasite *in vivo*, as seen in malaria. Future studies documenting the incidence of babesiosis in the sickle cell trait population compared to that in the HbAA individuals will confirm these findings.

In this paper, we present evidence for altered parasite population progression caused potentially by defective merozoite maturation and/or defective egress from the sickle cell anemia RBC. The latter two phenomena when present alone or together may explain the inability of HbSS cells to support high infection rates *in vitro*. It is not clear whether the parasite would behave similarly *in vivo*. We have recently shown in a mouse SCD model similar inhibition of parasite population progression, and the results presented in this study confirm the inability of the *Babesia* parasite to thrive in the sickle cell anemia setting.⁴⁰ In a significant contrast to malaria, sickle trait cells seemed to sustain parasite infection rates comparable to those of wild-type RBC, and, once again, these *in vitro* studies need to be supplemented by comparable *in vivo* analyses under conditions of differing oxygen tension, which has been shown to play a role in suppressing malaria parasitemia. Patients with SCD are on chronic transfusion therapy and may develop severe transfusion-associated *Babesia* infection.²⁹ Studies dissecting the cellular and molecular mechanisms of parasite growth within sickle cell patients that may impact the pathogenesis of babesiosis in sickle cell and other hemoglobinopathies are needed to define potential novel therapies against this disease.

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