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Plasmodium falciparum sexual parasites develop in human erythroblasts and affect erythropoiesis

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Abstract:
Plasmodium falciparum gametocytes, the sexual stages responsible for malaria parasites transmission from humans to mosquitoes, are key targets for malaria elimination. Immature gametocytes develop in the human bone marrow parenchyma, where they accumulate around erythroblastic islands. Notably though, the interactions between gametocytes and this hematopoietic niche have not been investigated. Here we identify late erythroblasts as a new host cell for *P.falciparum* sexual stages and show that gametocytes can fully develop inside these nucleated cells *in vitro* and *in vivo*, leading to infectious mature gametocytes within reticulocytes. Strikingly, we found that infection of erythroblasts by gametocytes and parasite-derived extracellular vesicles delay the erythroid differentiation, thereby allowing gametocyte maturation to coincide with the release of their host cell from the bone marrow. Taken together, our findings highlight new mechanisms that are pivotal for the maintenance of immature gametocytes in the bone marrow, and provide further insights on how *Plasmodium* parasites interfere with erythropoiesis and contribute to anemia in malaria patients.

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Plasmodium falciparum sexual parasites develop in human erythroblasts and affect erythropoiesis

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# Equal contribution

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Key points

- *Plasmodium falciparum* sexual parasites can fully develop within human erythroblasts
- Gametocytes and parasite-derived extracellular vesicles delay erythropoiesis to allow complete gametocyte development in nucleated cells

Scientific category: Red Cells, Iron and Erythropoiesis

ABSTRACT

Plasmodium falciparum gametocytes, the sexual stages responsible for malaria parasites transmission from humans to mosquitoes, are key targets for malaria elimination. Immature gametocytes develop in the human bone marrow parenchyma, where they accumulate around erythroblastic islands. Notably though, the interactions between gametocytes and this hematopoietic niche have not been investigated. Here we identify late erythroblasts as a new host cell for *P.falciparum* sexual stages and show that gametocytes can fully develop inside these nucleated cells *in vitro* and *in vivo*, leading to infectious mature gametocytes within reticulocytes. Strikingly, we found that infection of erythroblasts by gametocytes and parasite-derived extracellular vesicles delay the erythroid differentiation, thereby allowing gametocyte maturation to coincide with the release of their host cell from the bone marrow. Taken together, our findings highlight new mechanisms that are pivotal for the maintenance of immature gametocytes in the bone marrow, and provide further insights on how *Plasmodium* parasites interfere with erythropoiesis and contribute to anemia in malaria patients.

INTRODUCTION

Malaria remains a major public health threat with half a million deaths annually[1]. *Plasmodium falciparum* is the human parasite causing the most severe form of the disease. Sexual parasites, called gametocytes, are the only stage responsible for transmission from humans to *Anopheles* mosquitoes that spread the parasite in populations. Thus, understanding the biology of gametocyte development is crucial for successful malaria elimination. *P.falciparum* gametocytes development takes about 10 days and their maturation is divided into five stages[2,3]. Only mature stage V gametocytes circulate in the bloodstream, available for uptake by mosquitoes. In contrast, immature gametocytes from stages I to IV are sequestered in deep tissues, presumably to avoid clearance by the spleen. Recent examination of autopsies and *ex vivo* samples from malaria-infected patients revealed that immature gametocytes are enriched in the bone marrow[4-6]. The emerging role of the erythropoietic environment in hosting gametocytes suggests the presence of mechanisms that regulate homing and maintenance of sexual parasites in this niche. Unlike asexual parasites that sequester by cytoadhesion of infected erythrocytes through PfEMP1 interaction with endothelial receptors, gametocytes do not express PfEMP1 and immature gametocyte-infected erythrocytes (GIE) do not significantly adhere to endothelial cells from different organs, including bone marrow endothelial cells[7,8]. These features are consistent with the observation that gametocytes preferentially accumulate in the bone marrow extravascular space[6]. Several hypotheses have been put forward to explain the mechanism of gametocyte sequestration in the bone marrow parenchyma. For instance, immature GIE maintenance in this microenvironment may be dependent on adhesion to non-endothelial bone marrow cells, since erythrocytes infected by asexual and immature gametocytes adhere to bone marrow mesenchymal cells via trypsin-sensitive parasite ligands exposed on the erythrocyte surface[9,10]. Moreover, the increased rigidity of immature GIE may also contribute to their sequestration locally by mechanical retention[11]. Although both assumptions have yet to be validated *in vivo*, they do not fully reconcile with histological analyses of the bone marrow

parenchyma reporting that gametocytes are predominantly localized in close proximity to erythroblastic islands[6]. These specialized niches, where the terminal erythroid differentiation occurs, consist of a macrophage surrounded by differentiating erythroblasts[12]. So far, the nature of the interactions between gametocytes and these islands remains elusive. As immature GIE fail to adhere to primary human erythroblasts[13], the contiguity observed between immature gametocytes and erythroblastic islands may be the result of erythroblast infection by parasites followed by the maturation of gametocytes within these nucleated cells. Such a mechanism would allow gametocytes to benefit from direct adhesion of erythroblasts to the nursing macrophages in erythroblastic islands. In support to this hypothesis, previous studies have demonstrated that nucleated erythroid cells support *P.falciparum* invasion[14-17]. Moreover, the development of *P.falciparum* asexual parasites can take place in *ex vivo* culture of primary human erythroblasts, and immature gametocytes of stages I and II have been observed in nucleated cells *in vitro*[6,18]. These interactions may contribute to the maintenance of immature gametocytes from stage I to IV in the bone marrow parenchyma until the release of mature gametocytes-containing reticulocytes in the circulation. The consequences of these infections on human erythropoiesis are unknown but may be linked to erythroid disorders observed in malaria patients[19].

Here, we set up a protocol to produce and quantify *P.falciparum* gametocytes in a synchronized culture of primary human erythroblasts. We combined this approach with *in vivo* analyses to investigate for the first time *P.falciparum* sexual maturation processes in erythroid precursors, as well as their effects on erythropoiesis.

METHODS

Parasite culture and transfection

The *P.falciparum* NF54 clone B10[20] and the VarO line[21] were cultivated *in vitro* as described[22]. The NF54-pfs47-Hsp70-GFP line (called Hsp70-GFP) expressing GFP under the control of the constitutive promoter *hsp70* and the NF54-pfs47-Pfs16-GFP line (called Pfs16-GFP) expressing GFP under the control of the gametocyte-specific promoter *Pfs16* were obtained by transfection into the clone B10 as described in Supplemental methods.

Malaria patient bone marrow

Three bone marrow smears were obtained from a 20-year-old female patient admitted at Ispat General Hospital in Rourkela, Odisha, India, with *P.falciparum* infection (positive by microscopy and RDT), anemia (Hb: 5.2g/dl) and pancytopenia. The patient's admission parasitemia (second day of fever) was 1,320 parasites/µL before anti-malarial treatment was initiated, and no sexual forms of the parasite were detected on either thick or thin blood smears. Bone marrow biopsy was carried out as part of the standard clinical care of malaria-related anemia, and secondary analyses of the tissue were performed through an ongoing study approved by the Institutional Review Boards from the New York University School of Medicine (i12-03016), Ispat General Hospital (3137/E/05) and the London School of Hygiene and Tropical Medicine. Smears were immediately fixed by immersion in -20°C methanol for 5 minutes and left to dry prior to the immunofluorescence analyses.

Human primary erythroblasts

The human primary erythroblasts culture method is fully described in Supplemental methods. CD34+ progenitor cells were collected from bone marrow aspirate or from G-CSF mobilized peripheral blood after cytapheresis from human healthy donors who gave informed consent in accordance with the Declaration of Helsinki Principles. The study has been approved by the INSERM Institutional Review

Board IRB 00003888. Erythroid differentiation was analyzed by May-Grünwald-Giemsa staining of cytospun samples and by flow cytometry. Reticulocytes production was monitored using the DNA-labelling Draq5 probe (1/5000).

Erythroblast infection

Tightly synchronized schizonts of 40 hours post-infection (hpi) were purified by magnetic isolation and were added to human primary erythroblasts at the multiplicity of infection 2 (MOI 2). Infected cells were cultivated at 37°C, 5% CO2, 5% O2 in IMDM containing 15% BIT 9500, 1% glutamine, 1% penicillin-streptomycin, EPO (4U/ml), hypoxanthine (10mM) and heat-inactivated human serum (10%). 24 hours after infection, non-infected erythrocytes were removed by adding erythrocyte lysis buffer (Qiagen) according to manufacturer's instructions. Infected erythroblasts cultures were daily diluted to 0.8 millions cells/ml. Gametocytemia and total parasitemia were monitored by flow cytometry analysis of the Pfs16-GFP and the Hsp70-GFP lines, respectively, using a BD Accuri C6 cytometer. DNA was labelled with the Draq5 probe (1/5000) (Thermofisher) and levels of DNA content were used to discriminate erythroblasts and *Plasmodium*-infected reticulocytes.

Electron microscopy

Erythroblasts infected at 6 dpi were fixed with 2% paraformaldehyde (PFA), 2% glutaraldehyde, 1mM calcium chloride in 0.1M phosphate buffer, pH 7.2 for 10 minutes and processed for TEM as described in Supplemental methods. Samples were observed using a Hitachi Transmission Electron Microscopy HT7700 microscope. EVs were incubated on Formvar® coated grids for 2 minutes and then with uranyl acetate 1% for 2 minutes. EVs were directly observed with a JEOL 1011 transmission electron microscope. Acquisitions were performed with a GATAN Orius 1000 camera.

Fluorescent microscopy

Infected erythroblasts were fixed in 1X PBS/4% PFA and stained with Hoechst 33342 (1/20000) and PKH26. For immunofluorescence, cells or bone marrow smears were stained with anti-Glycophorin A (GPA) and anti-Pf11-1[23] antibodies. Details are provided in Supplemental methods. All samples were observed at X100 magnification using a Leica DMi8 microscope.

Gamete egress assay

At 9-10 dpi within erythroblasts or erythrocytes, Pfs16-GFP gametocytes were stained for 15 minutes at 37°C with 5µg/ml Wheat Germ Agglutinin (WGA)-Alexa Fluor 647, pelleted and then mixed in 5µl human serum supplemented with 100µM xanthurenic acid at room temperature for 30 minutes. Samples were observed at X100 magnification using a Leica DMi8 microscope. 100 cells were counted for each condition.

Purification of extracellular vesicles (EVs)

Protocol for EVs purification was adapted from[24]. Purified EVs and conditioned medium were labeled with PC7-conjugated anti-GPA (1/10) (Beckman Coulter) or with the isotypes-PC7 conjugated monoclonal antibody (1/10) (Beckman Coulter) and analyzed by flow cytometry. Total EVs were determined and counted by flow cytometry and Nanoparticle tracking analysis. Details are provided in Supplemental methods.

Determination of the oxidative stress

Two days after incubation with EVs, iECM or iECM - EV, erythroblasts were incubated with dihydroethidium (DHE) (2mM) (Sigma) during 30 minutes at 37°C, 5% CO2. Percentage of oxidized cells was determined by flow cytometry using a BD Accuri C6 cytometer.

Quantification and statistical analysis

Statistical significance was determined by Mann-Whitney test, paired *t*-test or one-way ANOVA test using GraphPad Prism 7.

RESULTS

Immature gametocytes properly develop within human primary erythroblasts

To address whether *P.falciparum* gametocytes can develop within erythroid precursors, we set up an infection protocol where purified synchronized schizonts were added to human primary erythroblasts derived from G-CSF mobilized peripheral blood or bone marrow aspirate (Figure 1A). At the time of infection, erythroblasts were mainly at the polychromatic stage (Figure 1B). As previously reported[6,18], we observed young asexual parasites within erythroblasts 24 hours after the infection (Figure 1C). We then observed trophozoites and schizonts at 2 days post-infection (dpi) and immature gametocytes at 6 dpi (Figures 1C-D). Immature gametocytes developing within erythroblasts presented typical features of sexual parasites such as a microtubular network and an inner membrane complex[25,26] (Figure 1E). Moreover, the gametocyte-specific protein Pf11-1 (PF3D7_1038400.1) was detected both in the parasite and in the infected erythroblast[27,28] (Figures 1F and S1).

To evaluate infection efficiency by flow cytometry, human primary erythroblasts were infected with two transgenic lines expressing fluorescent reporters: the Hsp70-GFP line (Figure S2) expressing GFP throughout the entire parasite life cycle under the control of the constitutive promoter *hsp70* to monitor total parasitemia, and the Pfs16-GFP line (Figure S3) expressing GFP under the control of the gametocyte-specific promoter *Pfs16* to monitor gametocytemia (Figure 1G). The infection yield was lower in erythroblasts than in erythrocytes under the same conditions of infection, however total parasitemia in nucleated cells two days after infection was 11.6%, indicating that the infection protocol is efficient (Figure 1H). Moreover, the gametocytemia in nucleated cells at 2 dpi was 3.7% (Figure 1I). These results suggest that immature gametocytes are adapted to develop in nucleated primary erythroblasts.

Infection of erythroblasts allows complete gametocytogenesis

To determine whether gametocytes undergo full maturation within erythroid precursors, erythroblast infection was followed for 10 days. We observed that nucleated cells supported immature gametocyte development from stage I to IV for 8 days, leading to the production of mature gametocytes within reticulocytes at 10 dpi (Figure 2A). The gametocytemia in nucleated cells dropped about 2-fold between 2 dpi and 4 dpi and then remained stable for several days (Figure 2B), indicating that sexual parasites can persist during several days in erythroblast culture. As the ability of mature gametocytes to undergo gametogenesis is a marker of functionality, we quantified round and egressed gametes upon a temperature drop and an increase in serum concentration supplemented with xanthurenic acid (Figure 2C). Mature gametocytes were able to undergo a release of round or motile flagellated gametes from their host cell with a similar efficiency in reticulocytes and in erythrocytes (Figures 2D-F and S4). These results indicate that gametocytes could achieve their maturation from stage I to IV in nucleated cells and suggest that resulting mature gametocytes within reticulocytes are potentially functional and may be transmissible to mosquitoes.

To address whether these *in vitro* observations can be translated *in vivo*, we analyzed three bone marrow smears from a *Plasmodium falciparum*-infected Indian patient by immunofluorescence microscopy. Gametocytes stained with antibodies against the gametocyte-specific protein Pf11-1 (Figure S1) were detected within cells that were positive for the erythroid lineage-specific GPA staining (Figure 3A). About half of the observed gametocytes were inside nucleated cells (Figure 3B). These findings confirm that gametocytes can develop in bone marrow erythroblasts *in vivo*.

Intra-erythroblastic gametocyte development occurs from the polychromatic stage

Terminal erythroid differentiation *in vitro* takes usually 8 to 10 days from pro-erythroblasts to reticulocytes[29]. Thus, erythroblast infection should theoretically take place at the early stages of terminal erythropoiesis to allow a complete development of immature gametocytes in nucleated cells. However, previous study reports that orthochromatic cells are the earliest stages that may be invaded by

P.falciparum[18]. To determine if parasites could infect erythroblasts earlier in the erythropoiesis, we tightly synchronized human primary erythroblasts and we infected them at different stages of maturation. Erythroid differentiation was monitored from pro-erythroblasts to reticulocytes (at 0 day of differentiation (dod), 2 dod, 4 dod, 6 dod and 8 dod) by May-Grünwald Giemsa staining to determine cell size, cytoplasm basophily and chromatin condensation (Figure 4A), and by flow cytometry to quantify reticulocyte production and expression of the surface markers Band 3, α-4-integrin, CD71 and GPA (Figure 4B-C). Synchronized erythroid precursors were infected at pro-erythroblast (0 dod), basophilic (2 dod), polychromatic (4 dod) and orthochromatic stages (6 dod) with the Hsp70-GFP and the Pfs16-GFP lines, then parasitemia and gametocytemia were monitored at 2 dpi, 4 dpi and 6 dpi (Figure 4D). First, we observed that parasite invasion could occur from basophilic stages, however gametocytes do not develop further in these early stages, maybe due to the low hemoglobin content (Figure 4E). Therefore, only polychromatic and orthochromatic erythroblasts sustained parasite and gametocyte development until 6 dpi (Figures 4E-F). These results indicate that the maturation length of gametocytes in erythroblasts (about 10 days, consistently with maturation time in erythrocytes[3,30]) is longer than the time usually required by uninfected erythroblasts to develop from orthochromatic stages to reticulocytes *in vitro* (about 3-4 days[31]). Therefore, our data suggest that parasites must prevent or delay the differentiation of erythroblasts into reticulocytes in order to complete their development in nucleated cells.

P.falciparum **infection induces a delay in erythroid maturation and oxidative stress in erythroblasts**

To address whether *P.falciparum* induces a delay in erythroid maturation, we monitored by flow cytometry the impact of infection on the conversion of orthochromatic erythroblasts into reticulocytes (Figure S5A). At 2 dpi, the reticulocyte rate of infected erythroblast culture was 40% vs 48.3% in the uninfected culture, and at 8 dpi it was 49.5% in the infected culture vs 66.7% in the uninfected culture (Figures 5A and S5B). This observation suggests that the differentiation of erythroblasts into

reticulocytes is delayed by *Plasmodium* parasites. More precisely, the reticulocyte rate at 8 dpi in the population of infected cells was 37% vs 58% in the population of uninfected cells within the same infected culture, indicating that gametocytes impede the maturation of their host cell (Figure 5B). Among many factors affecting erythropoiesis, oxidative stress in erythroblasts has been shown to negatively impact enucleation and reticulocyte rate[32]. To examine if the gametocyte-induced delay in erythropoiesis was related to an increase of oxidative stress in erythroblasts, we monitored the oxidation of erythroblasts by using dihydroethidium (DHE) 2 days after infection with the Pfs16-GFP line. Within the same infected culture, infected erythroblasts were five-fold more oxidized than uninfected erythroblasts at 2 dpi (Figure 5C). These results show that parasite infection induces oxidation of erythroid precursors *in vitro*, which could be linked to the delay in erythroblast maturation.

Parasite-conditioned medium induces a delay in erythroid maturation

P.falciparum has already been shown to affect host cells by modifying the culture medium[33,34]. As reticulocyte rate from uninfected erythroblasts was lower in infected culture compared to that in uninfected culture (Figure S5B), we hypothesized that parasites not only hamper the maturation of their host cells but also induce a bystander effect on the other uninfected cells in the culture. Thus, the delay in erythroblast maturation may also be due to parasite-derived factors secreted in the medium. To address this hypothesis, uninfected orthochromatic erythroblasts were exposed to a medium conditioned by a culture of *Plasmodium*-infected erythroblasts (iEbCM). After 8 days, the reticulocyte rate from erythroblasts cultivated in the iEbCM was significantly lower than that of erythroblasts cultivated with regular medium (control) or with medium conditioned by uninfected erythroblasts (uEbCM). These results indicate a parasite-specific effect on erythropoiesis (Figures 5D and S6A). A significant decrease in reticulocyte production was also induced by addition of medium conditioned by *Plasmodium*-infected erythrocytes (iECM) (Figures 5E and S6B). Due to the scarcity of human primary erythroblasts, iECM was then used for further experiments. The delay effect of iECM was observed from

4 days of culture and was dependent on the parasitemia in the culture providing the conditioned medium (Figures 5F and S7A). We then addressed the effect of media conditioned either by immature gametocyte-infected erythrocytes (gECM) (Figure S7B) or by erythrocytes infected with asexual parasites from a line which has lost the ability to produce gametocytes (Figure S7C)[21]. Both conditions triggered similar delays in reticulocyte production, indicating that the observed effect was not dependent on the parasite stage.

To decipher whether this delay was due to an enrichment in specific parasite-secreted factors or to a depletion in nutrients essential for erythroblasts development, iECM was supplemented with several components of the erythroblast culture medium: serum substitute, erythropoietin, L-glutamine, a pool of amino acids, human serum or a pool of all of these components. However, none of these conditions was able to reverse the delay phenotype (Figure S8). Although we have not exhaustively tested all the medium components, these data support the hypothesis that erythroid cellular development is affected by parasite-secreted factors rather than the consumption of essential nutrients by the parasite.

Parasite-derived extracellular vesicles delay erythropoiesis and induce oxidative stress

Upon infection with *P.falciparum*, erythrocytes produce extracellular vesicles (EVs)[35,36]. As infected erythrocytes-derived EVs have been shown to directly affect host cells by altering vascular functions of endothelial cells[37] or by activating immune cells[35,38], we addressed whether EVs could affect erythroid precursors. First, flow cytometry quantification of GPA-labelled EVs (Figure S9) showed that parasite infection increases the production of EVs by erythroblasts as similar level than by erythrocytes (Figure 6A). Electron microscopy analysis and Nanoparticle Tracking Analysis (NTA) of EVs purified from a culture of infected erythrocytes confirmed the presence of vesicular structures with characteristic EV size distribution mainly ranging from 70 to 200 nm, with a main peak near 130 nm (Figures 6B-C and S10). Importantly, adding purified EVs to uninfected erythroblasts for 8 days significantly decreased the reticulocyte rate, suggesting that EVs negatively affect erythropoiesis (Figures 6D and S11A). To confirm

this result, erythroid precursors were cultivated for 8 days with iECM or with iECM depleted in EVs (iECM - EV). After validating that iECM - EV contained 500-fold less EVs than the complete iECM, we observed that EVs depletion attenuated the iECM-induced delay in erythropoiesis (Figures 6E and S11B). These experiments provide evidence that parasite-secreted EVs contribute to the erythropoiesis delay induced by *P.falciparum*. Next, we examined if the EV-induced delay in erythropoiesis was related to an increase of oxidative stress in erythroblasts. Importantly, erythroblasts cultivated with 10 µg/mL of purified EVs or with iECM were five-fold more oxidized than erythroblasts cultivated with control medium or with iECM - EV (Figure 6F). These results show that EVs induce the same level of oxidation of erythroid precursors than parasite infection. Both mechanisms may contribute to the delay in erythroblast maturation previously observed.

DISCUSSION

Unravelling the retention mechanisms of immature GIE in the bone marrow parenchyma is necessary to decipher *P.falciparum* transmission biology. Several hypotheses have been suggested to explain gametocytes localization in the bone marrow. Sequestration of gametocytes in this niche may be mediated by cytoadhesion of immature GIE to bone marrow mesenchymal cells[10], or may depend on mechanical retention, which could result from the high rigidity of immature GIE[11,25,39]. In this study, we addressed the new hypothesis that gametocytes could mature within erythroblasts bound to the nursing macrophages of erythroblastic islands. We demonstrate that *Plasmodium* infection impedes enucleation of primary erythroblasts, thereby allowing immature gametocytes to complete their full development within these nucleated cells. Once maturation is complete inside reticulocytes, stage V gametocytes can then reach the blood circulation, where they are available for uptake and further transmission by mosquitoes. The ability to undergo a temperature-dependent release of round gametes from their reticulocyte host substantiates the functional maturity of gametocytes and suggests that they are

transmissible to mosquitoes[40]. Importantly, these observations are supported by *in vivo* analyses of bone marrow smears from a malaria-infected patient.

This discovery of a new host cell for *P.falciparum* sheds light on a poorly understood part of the parasite life cycle, and raises new questions about the cross-talk between gametocytes and these nucleated cells. In infected erythrocytes, it is well known that *P.falciparum* gametocytes drastically modify the structural and mechanical properties of the host cell membrane. This remodeling is induced by the export of several parasite-expressed proteins and plays a key role for gametocyte survival, chemosensitivity and recognition by the immune system[41]. For instance, gametocyte-induced remodeling of the erythrocyte membrane regulates deformability and permeability of the infected erythrocyte, two features involved in the ability of gametocytes to persist in circulation and their susceptibility to antimalarial drugs, respectively[11,42]. In addition, several parasite proteins exposed at the gametocyte-infected erythrocyte surface have been recently proposed as target antigens for transmission-blocking vaccines[9]. Our findings indicate that gametocytogenesis progresses in a similar manner in both erythroblasts and their mature counterparts. Further studies should address whether gametocytes export their proteins to the membrane of the infected erythroblast, and whether these proteins influence the mechanical, adhesive and immunogenic properties of the nucleated host cells. Such remodeling has crucial implications on how to target gametocytes in drug- or vaccine-based interventions, which must now be re-evaluated in the light of gametocyte development within erythroblasts.

The most striking impact of gametocyte development within erythroblasts is the inhibition of their conversion into reticulocytes. This host cell subversion is redolent of previous findings showing that *Plasmodium vivax* parasites accelerate aging of infected reticulocytes[43]. Taken together, these observations highlight how *Plasmodium* hijacks the maturation of its host cell in order to complete its own development. This is in line with the characteristic manipulation of mammalian cell functions by protozoan parasites[44]. Impaired erythropoiesis observed in *P.falciparum*-infected erythroblasts results

from both a direct effect of gametocytes on their host cell, as well as parasite factors secreted in the extracellular environment. Gametocytes might delay erythroid maturation by exporting proteins to the erythroblast nucleus. In erythrocytes, gametocytes express a wide range of proteins, and more than 10% are exported to the host cell[45]. Although most of these proteins lack any functional annotation, the presence of a nuclear localization signal in the sequence of some of them suggests that these proteins might interfere with the regulation of genes in erythroblasts. Such mechanisms could conceivably affect erythroid differentiation. We also demonstrate that parasite-derived EVs play a key role in impairing the erythroid maturation process. Although EVs produced by protozoan parasites have already been shown to contribute to the manipulation of their host cells[37,46], our results demonstrate for the first time an effect of *Plasmodium*-secreted vesicles on erythropoiesis. However, the molecular identity of the EVs-embedded factor(s) triggering this delay remains to be identified. Such factors could be nucleic acids or proteins, since EVs contain miRNAs affecting host cells[37], and recent protein-dependent effect of parasite-conditioned medium has been shown to impair erythroblast differentiation[47]. Among the multiple outcomes that parasite infection and EVs may induce in erythroid precursors, our results show that they cause oxidative stress. This is consistent with the results of a transcriptomic assay, where co-culture and infection of erythroid precursors with *P.falciparum* asexual stages increased the expression of several genes involved in the NFR2-mediated oxidative stress response[48]. As oxidative stress can lead to inhibition of erythroid maturation and dyserythropoiesis, such an increase in oxidation may be responsible for the reported delay in erythroblast enucleation[32]. Importantly, dyserythropoiesis is a major contributor to malarial anemia[19] and has been associated with a higher prevalence of gametocytes in bone marrow, suggesting a relationship between hematological disturbances and gametocyte development in this tissue[4]. Erythroblast infection by gametocytes and parasite-derived EVs secreted in the bone marrow parenchyma may therefore be involved in erythroid disorders in patients infected with

P.falciparum. Thus, our results imply that therapeutic and vaccine interventions targeting gametocytes would not only prevent transmission but also decrease anemia in malaria patients.

Collectively, our findings shed light on a new mechanism that may contribute to the maintenance of immature gametocytes in the bone marrow extravascular space, and provide new insights into the processes by which *Plasmodium* parasites subvert their host cell and may interfere with erythropoiesis in malaria patients. Future therapies aimed at disrupting these mechanisms may not only help decreasing transmission in endemic areas, but also treating malarial anemia.

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AUTHOR CONTRIBUTIONS

G.N., F.V. and C.L. conceived the project. G.N., C.R., F.V., F.D., P.A.S., B.M.-Z., P.V., M.A., A.L. and C.L. performed the experiments. P.B., N.A., R.M.M., J-J.L-R., A.M.M., D.M., S.S., F.G., A.A.A.S. and S.C.W. contributed resources or data. G.N. and C.L. wrote the article, with major input from F.V. and S.C.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURES LEGENDS

Figure 1. Immature gametocytes properly develop within human primary erythroblasts.

(**A**) Diagram illustrating the erythroblast infection protocol. Burst Forming Unit-Erythroid cells (BFU-E) expressing the cluster of differentiation 34 (CD34) were cultivated 7 days to generate Colony Forming Unit-Erythroid cells (CFU-E) expressing the cluster of differentiation 36 (CD36), then CFU-E were allowed to differentiate into late erythroblasts during 5 to 7 days. Synchronized GFP-expressing schizonts at 40 hours post invasion (hpi) were added to erythroblasts at a multiplicity of infection (MOI) of 2. After several days of culture, infected erythroblasts were analyzed by fluorescent microscopy and flow cytometry. (**B**) Distribution of the different erythroblast stages at the time of infection. Morphological analysis of erythroblasts was performed by May-Grünwald Giemsa staining of cytospin from three independent experiments. PRO-E: pro-erythroblast stage, BASO: basophilic stage, POLY: polychromatic stage, ORTHO: orthochromatic stage and RETIC: reticulocyte stage. (**C**) Erythroblast infection was observed by May-Grünwald Giemsa (MGG) staining at 1 dpi (ring stage, upper left panel), 2 dpi (trophozoite stage, upper right panel and schizont stage, lower left panel), and 6 dpi (stage III gametocyte, lower right panel). Bars represent 5 µm. (**D**) Gametocytes within erythroblasts at 6 dpi were observed by differential interference contrast microscopy. Left: stage III gametocyte, right: stage IV gametocyte. Bars represent 5 µm. (**E**) Transmission electron microscopy shows that immature gametocytes within infected erythroblasts present typical sexual structures as the microtubular network (MT) and the inner membrane complex (IMC). (EN: erythroblast nucleus, EC: erythroblast cytosol, GC: gametocyte cytosol, GN: gametocyte nucleus, ES: exo-membrane system). Bars: 1 µm (left panels) and 200 nm (right panels). (**F**) Immunofluorescence analysis (IFA) of paraformaldehyde-fixed gametocyte-infected erythroblast at 6 dpi stained with anti-Pf11-1 antibodies (green) and with anti-glycophorin A (GPA) labelling the erythroblast membrane (red). (**G**) Infection with the Hsp70-GFP line (upper panels) and with the Pfs16-GFP line (lower panels) were observed by fluorescent microscopy at 2 dpi (upper

panels and lower left panel) and 6 dpi (lower right panel). Erythroblast membrane is stained with PKH-26 (red). (**F-G**) DNA is stained with Hoechst 33342 (blue). Bars represent 5 µm. (**H**) Infection of erythrocytes or erythroblasts with the Hsp70-GFP line is evaluated by flow cytometry at 2 dpi. (**I**) Infection of erythroblasts with the Hsp70-GFP and Pfs16-GFP lines is evaluated by flow cytometry at 2 dpi. (**H**-**I**) Circles indicate the number of independent experiments (n=6) that were performed on erythroblasts derived from cytaphereses (n=5) or bone marrow aspirates (n=1) from 4 independent donors. Error bars show the standard error of the mean (SEM). ** $p < 0.01$.

Figure 2. Infection of erythroblasts allows complete gametocytogenesis

(**A**) Gametocyte maturation after erythroblasts infection with the Pfs16-GFP line was observed by light microscopy after MGG staining (upper panel) and by fluorescent microscopy (lower panel). Erythroblast membrane was stained with PKH-26 (red). DNA was stained with Hoechst 33342 (blue). Bars represent 5 µm. **(B)** Infection of erythroblasts with the Pfs16-GFP line is evaluated by flow cytometry at 2, 4, 6 and 8 dpi. Circles indicate the number of independent experiments (n=6) that were performed on erythroblasts derived from cytaphereses (n=5) or bone marrow aspirates (n=1) from 4 independent donors. (**C**) Diagram illustrating the gamete activation protocol. After infection of erythroblasts or erythrocytes with Pfs16-GFP schizonts, gametocytes were allowed to differentiate for 9 to 10 days within erythroid precursors or erythrocytes. Mature gametocytes were stained with WGA-Alexa Fluor 647 (red) for 15 minutes at 37°C and then activated in human serum and xanthurenic acid at room temperature (RT) for 30 minutes, leading to gamete activation. (**D**) GFP-positive cells were scored as either round or crescent-shaped, and plotted as percentage rounded-up. (**E**) GFP-positive cells were scored as either positive or negative for WGA-Alexa Fluor 647 staining, and plotted as percentage gamete emergence. (**D-E**) 100 cells were scored per condition. Circles indicate the number of independent experiments and error bars show the standard error of the mean (SEM). (**F**) Fluorescence microscopy of activated gametes from a culture

of infected erythroblasts (left panel) or infected erythrocytes (right panel) with the Pfs16-GFP line. Erythroid cells membrane is stained with WGA-Alexa Fluor 647 (red). Bars represent 5 µm.

Figure 3. In vivo imaging of gametocytes within erythroblasts.

(**A**) IFA of gametocyte-infected erythroblasts on methanol-fixed bone marrow smears from a *P.falciparum*-infected patient. Gametocytes were stained with anti-Pf11-1 antibody (green) and erythroblasts with anti-glycophorin A (GPA) labelling the erythroid membrane (red). DNA is stained with Hoechst 33342 (blue). i, ii: stage I-II gametocyte inside erythroblast ; iii, iv: stage III-IV gametocyte inside erythroblast ; v: stage I-II gametocyte inside reticulocyte or erythrocyte; vi: stage III-IV gametocyte inside reticulocyte or erythrocyte ; vii: stage V gametocyte inside reticulocyte or erythrocyte. Gametocyte stages were evaluated according to the size and the shape of P11-1-positive cells. Bars represent 5 µm. (**B**) Quantification of P11-1-positive cells on three bone marrow smears from the same patient. The absolute numbers of observed cells are indicated and the percentages are in parentheses.

Figure 4. Intra-erythroblastic gametocyte development occurs from the polychromatic stage

A) Morphological analysis of developing erythroblasts was performed by MGG staining from 0 to 8 days of differentiation (dod); a representative experiment is shown. Erythroblasts were mainly at pro-erythroblast stage (PRO-E) at 0 dod, basophilic stage (BASO) at 2 dod, polychromatic stage (POLY) at 4 dod, orthochromatic stage (ORTHO) at 6 dod and reticulocyte stage (RETIC) at 8 dod. Upper panel: large field, lower panel: zoom. Bars represent 5 µm. (**B**) Representative experiment of erythroblasts differentiation monitored by flow cytometry from 0 to 8 days of differentiation (dod) by cell surface expression of Band 3 and α-4-integrin. (**C**) Evolution of cell surface markers CD71, Band 3, GPA and α-4-integrin and of reticulocyte rate measured by flow cytometry during terminal erythropoiesis. (**D**) Diagram illustrating the infection protocol of synchronized erythroblasts. Synchronized schizonts at 40 hpi were

added to pro-erythroblasts, basophilic, polychromatic and orthochromatic erythroblasts at a MOI of 2. Infected erythroblasts were analyzed by flow cytometry at 2, 4 and 6 dpi. (**E-F**) Total parasitemia (E) and gametocytemia (F) in erythroblasts is evaluated by flow cytometry at 2 dpi, 4 dpi and 6 dpi after infection of erythroblasts from each stage with the Hsp70-GFP and Pfs16-GFP lines, respectively. Circles indicate the number of independent experiments and error bars show the SEM.

Figure 5. P.falciparum infection induces a delay in erythroid maturation and oxidative stress in erythroblasts

(**A**) Percentage of reticulocytes in uninfected (uEb) or infected (iEb) erythroblast culture is evaluated by flow cytometry at 8 dpi. (**B)** Percentage of reticulocytes in the population of non-infected (population 1) or Pfs16-GFP-infected (population 2) cells within an infected erythroblast culture at 8 dpi. (**C**) Percentage of oxidized erythroblasts is evaluated by flow cytometry with DHE staining in the population of uninfected (population 1) or Pfs16-GFP-infected (population 2) cells in an infected erythroblast culture at 2 dpi. (**D** and **E**) Percentage of reticulocytes in uninfected erythroblast culture at 8 days after addition of conditioned medium obtained with a culture of infected erythroblasts (iEbCM, D), uninfected erythroblasts (uEbCM, D), infected erythrocytes (iECM, E), uninfected erythrocytes (uECM, E), or control medium (Control). (**F**) Percentage of reticulocytes in erythroblast culture at 8 days after addition of iECM obtained with parasite cultures at 0.5 %, 2 % or 20 % parasitemia, or with control medium. Circles indicate the number of independent experiments and error bars show the SEM. **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, ns: non-significant difference.

Figure 6. Parasite-derived extracellular vesicles delay the erythroid maturation and induce oxidative stress

(**A**) EVs in conditioned medium obtained with a culture of uninfected erythroblasts (uEbCM), infected erythroblasts (iEbCM), uninfected erythrocytes (uECM) or infected erythrocytes (iECM) were observed and quantified by flow cytometry with a GPA-PC7 labelling. EV counts in iEbCM and iECM are normalized to uEbCM and uECM, respectively. Gating strategy is in Figure S7. (**B**) The size distribution of EVs purified from a culture of infected erythrocytes was determined by Nanoparticle Tracking Analysis (NTA). **(C)** EVs purified from a culture of infected erythrocytes were observed by electron microscopy with a negative staining. Bar represent 100 nm. (**D**) Percentage of reticulocytes in erythroblast culture at 8 days after addition of 1, 5, 10 or 20 µg/mL EVs or control medium (Control). (**E**) EVs in iECM or in iECM - EV were observed and quantified by flow cytometry with a GPA-PC7 labelling. Lower left: Percentage of reticulocytes in erythroblast at 8 days after addition of iECM, iECM - EV or control medium (Control). (**F**) Percentage of oxidized erythroblasts is evaluated by flow cytometry with DHE staining 2 days after addition of 10 µg/mL EVs, iECM, iECM - EV or control medium (Control). Circles indicate the number of independent experiments and error bars show the SEM. ***p<0.001, **p<0.01, * p < 0.05, ns: non-significant difference.

Figure 1

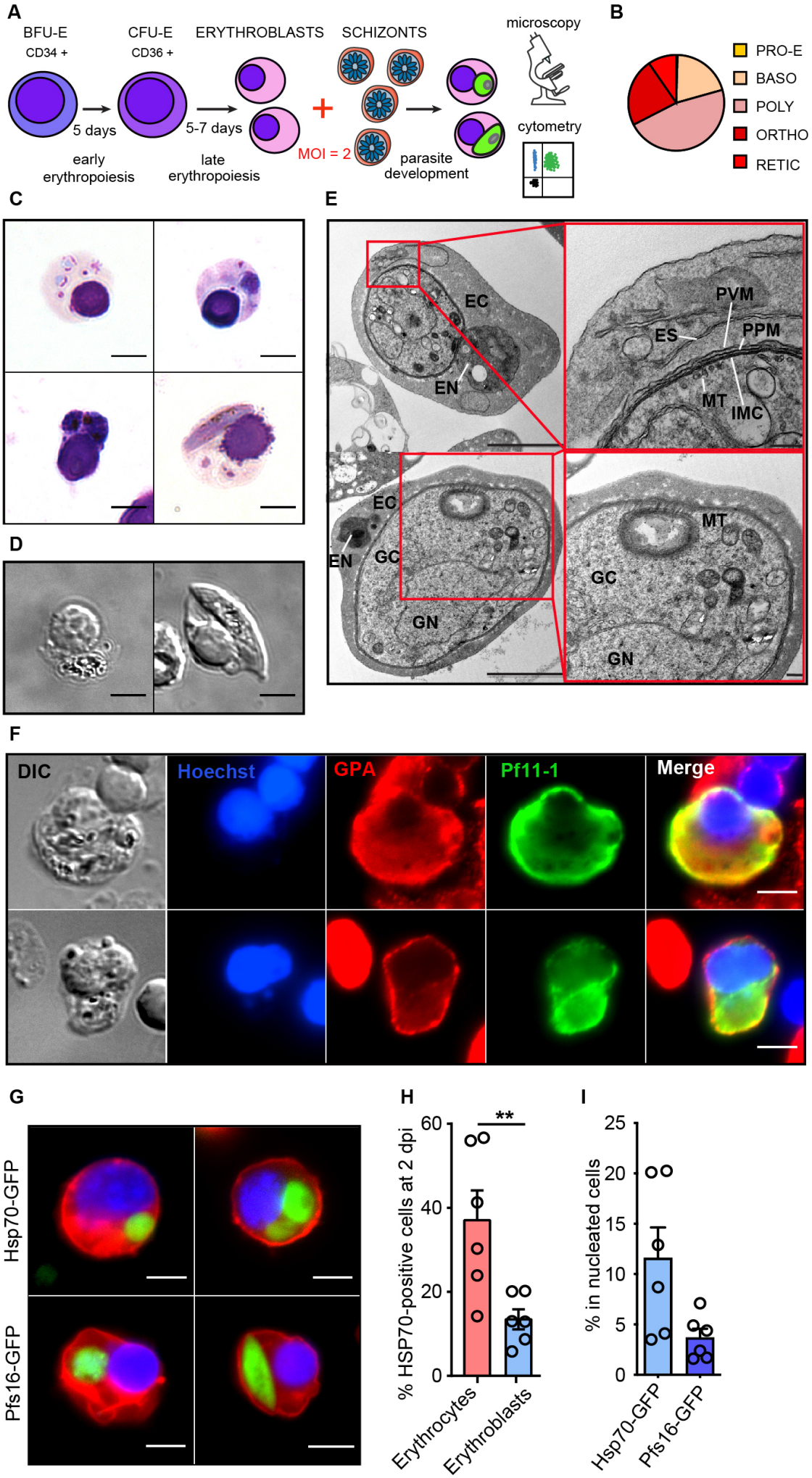
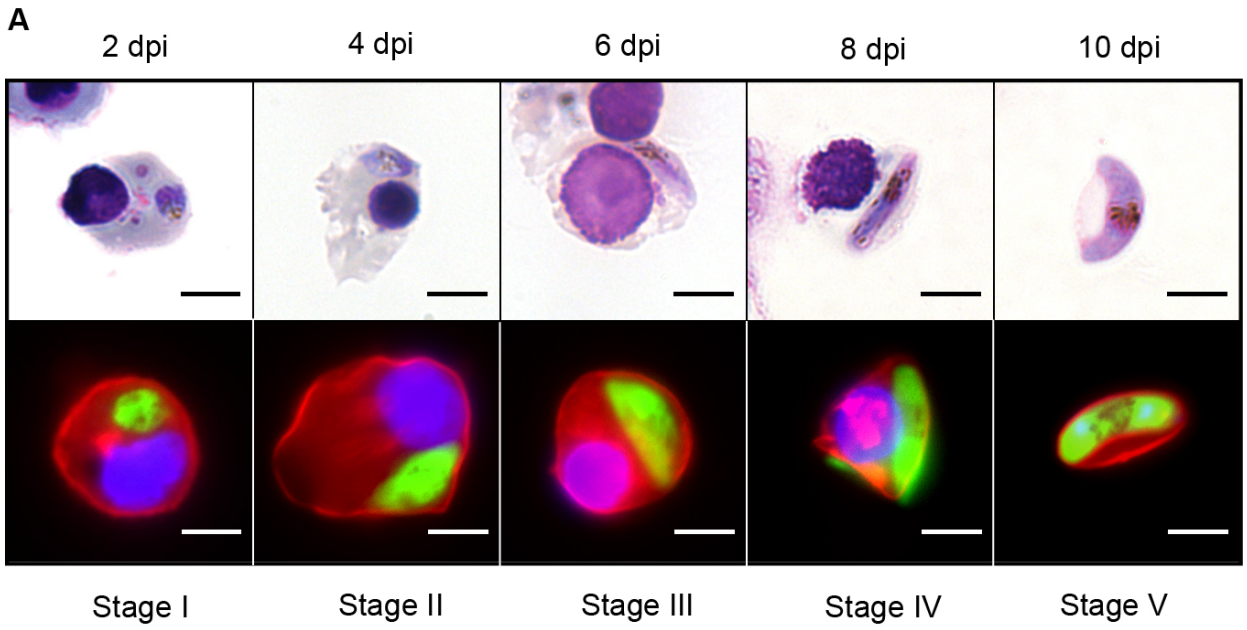
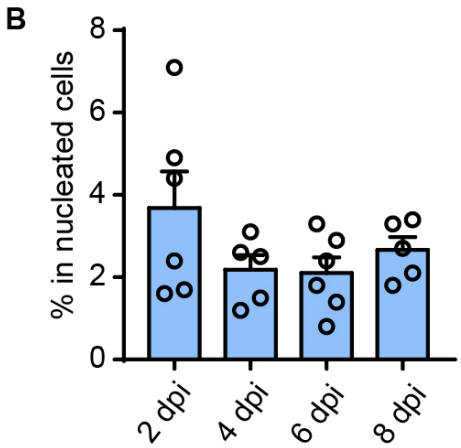
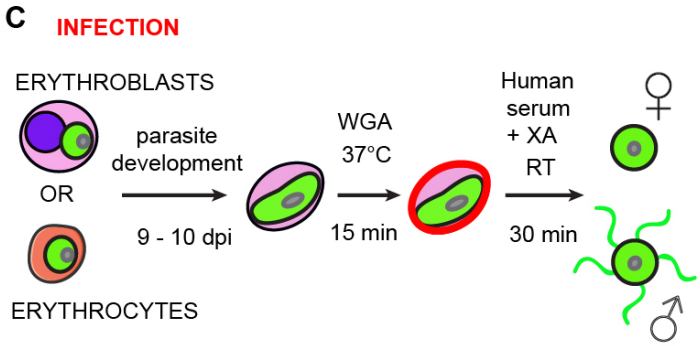
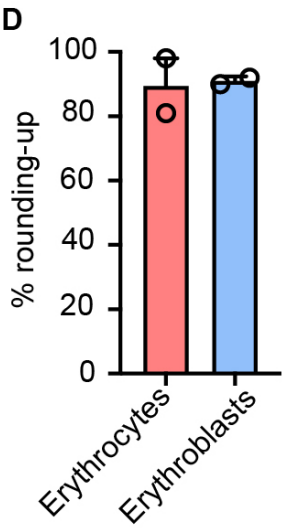


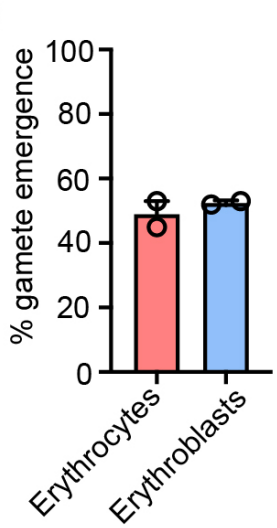
Figure 2











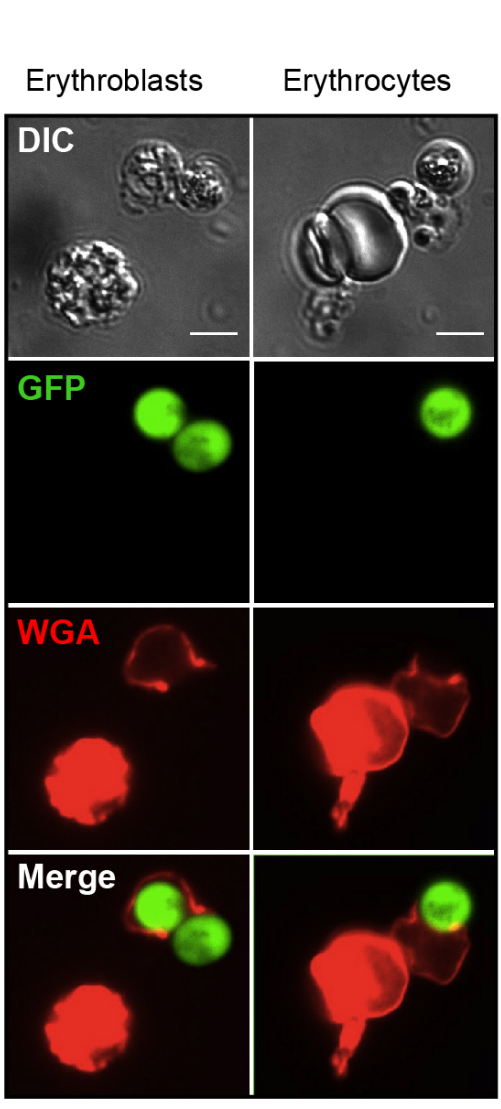
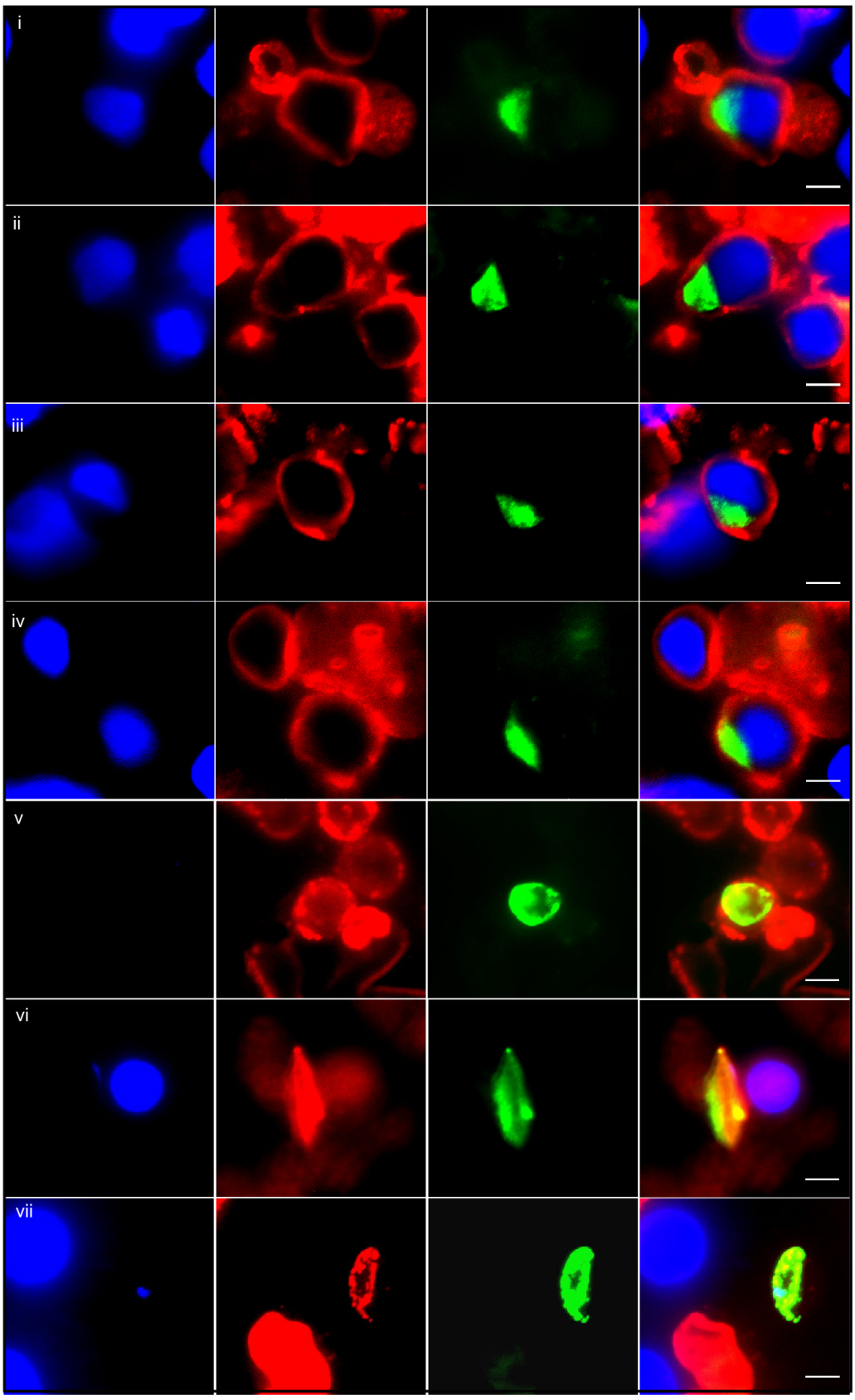


Figure 3

A



B

	Stage I-II	Stage III-IV	Stage V	Undetermined	Total
in nucleated cell	7	12	0	2	21 (44.7)
in enucleated cell	8	3	4	7	22 (46.8)
Unknown	2	2	0	0	4 (8.5)
Total	17 (36.2)	17 (36.2)	4 (8.5)	9 (19.1)	47

Figure 4

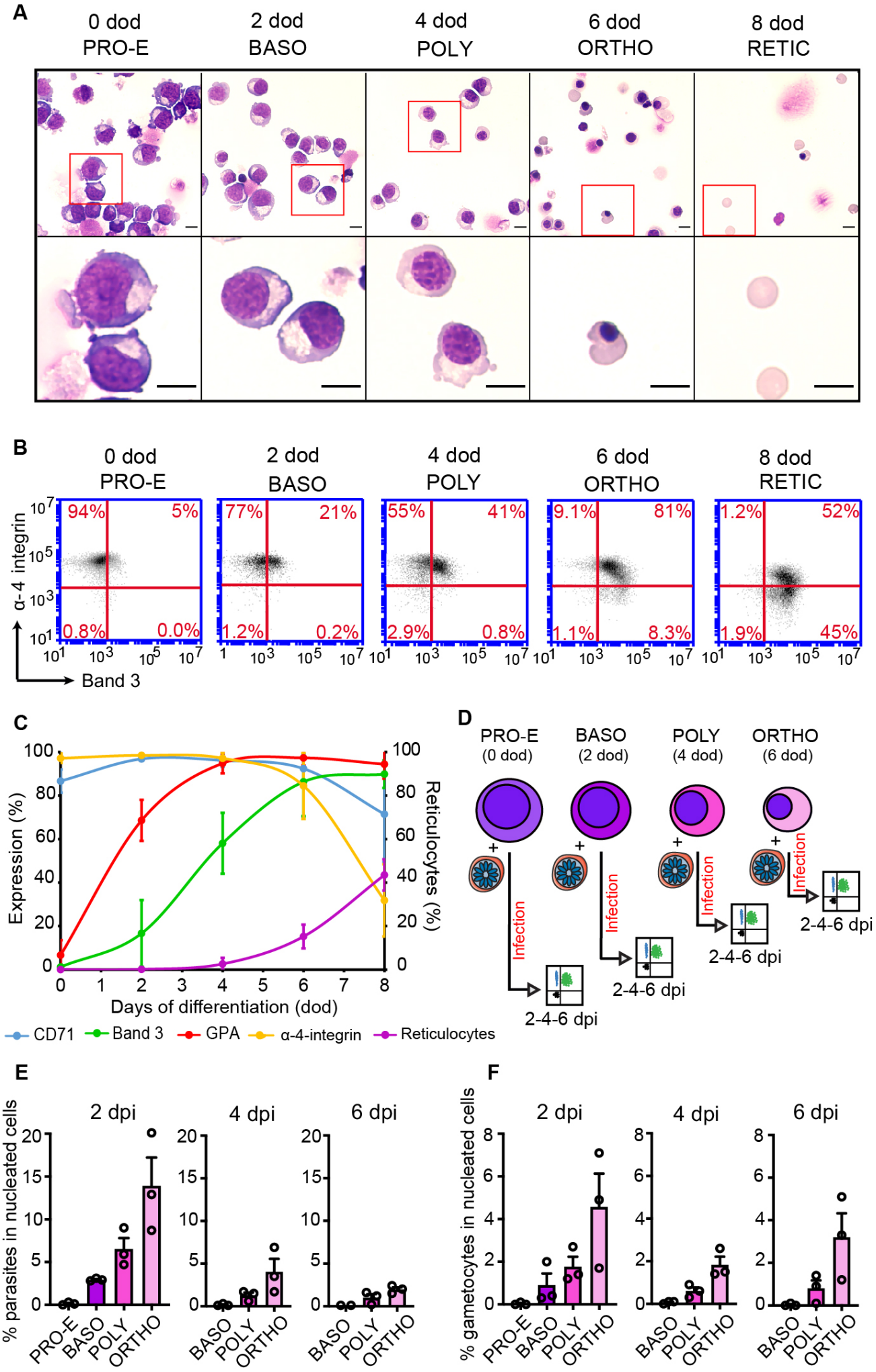


Figure 5

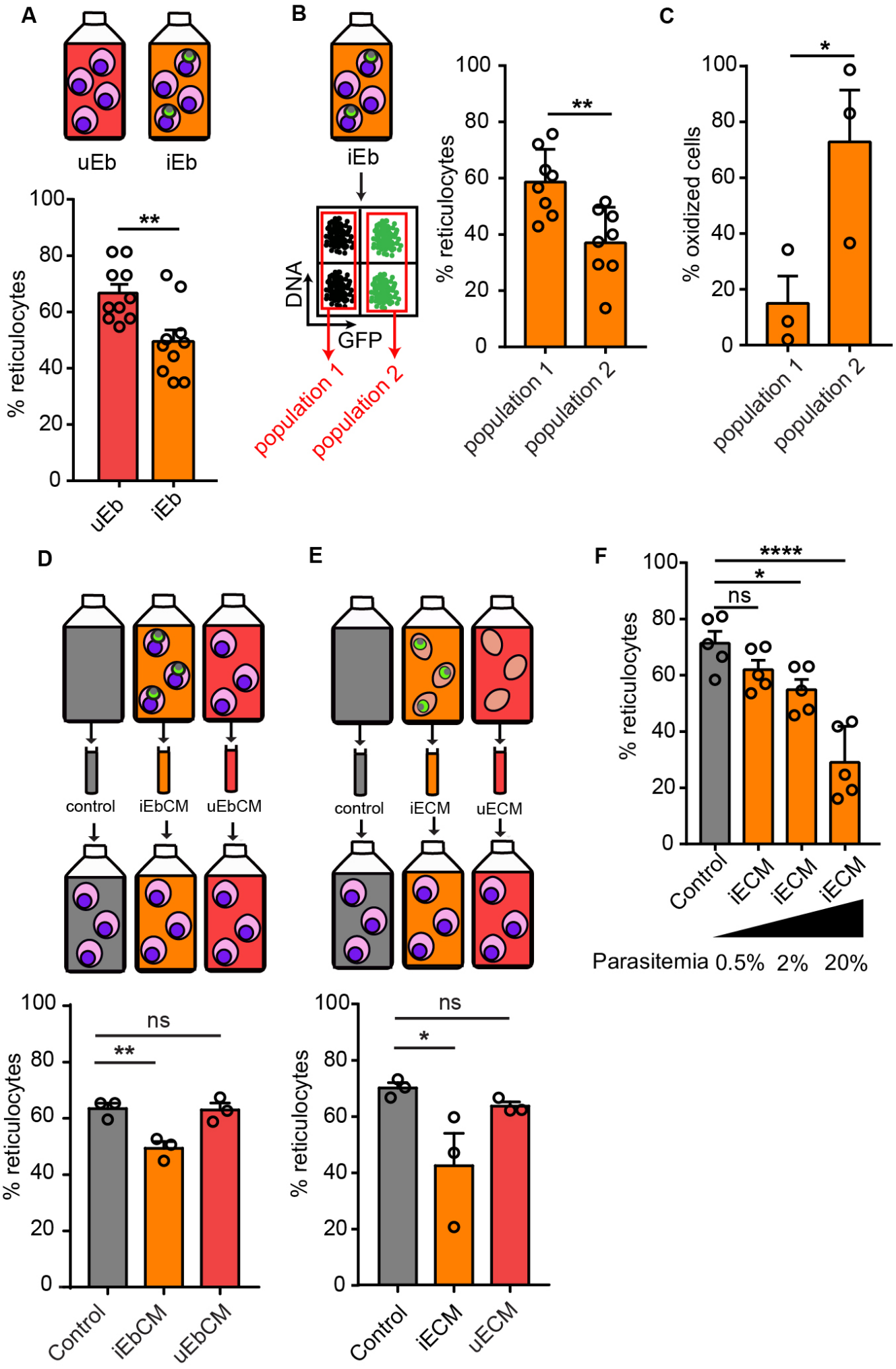


Figure 6