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Insight into phagocytosis of mature sexual (gametocyte) stages of *Plasmodium falciparum* using a human monocyte cell line

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Abstract

During natural infection malaria parasites are injected into the bloodstream of a human host by the bite of an infected female *Anopheles* mosquito. Both asexual and mature sexual stages of *Plasmodium* circulate in the blood. Asexual forms are responsible for clinical malaria while sexual stages are responsible for continued transmission via the mosquitoes. Immune responses generated against various life cycle stages of the parasite have important roles in resistance to malaria and in reducing malaria transmission. Phagocytosis of free merozoites and erythrocytic asexual stages has been well studied, but very little is known about similar phagocytic clearance of mature sexual stages, which are critical for transmission. We evaluated phagocytic uptake of mature sexual (gametocyte) stage parasites by a human monocyte cell line in the absence of immune sera. We found that intact mature stages do not undergo phagocytosis, unless they are either killed or freed from erythrocytes. In view of this observation, we propose that the inability of mature gametocytes to be phagocytized may actually result in malaria transmission advantage. On the other hand, mature gametocytes that are not transmitted to mosquitoes during infection will eventually die and undergo phagocytosis, initiating immune responses that may have transmission blocking potential. A better understanding of early phagocytic clearance and immune responses to gametocytes may identify additional targets for transmission blocking strategies.

Graphical abstract

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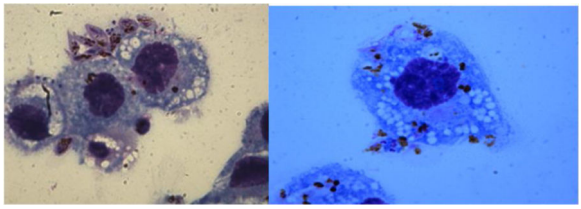
Authorship

GB and NK jointly contributed to the study's conception, design, performance and independent assessment of slides. GB performed the phagocytosis assays and NK prepared the purified parasite preparations. CW worked on establishing conditions for the phagocytosis assays and the initial standardization.

Conflict of Interest Disclosure

The authors declare no conflict of interest.



We found mature live gametocytes stage of the malaria parasite do not undergo phagocytosis (left) whereas killed or damaged gametocytes do (right). These findings have implication for malaria transmission dynamics.

Keywords

nonopsonic uptake; mature gametocytes; malaria transmission

1. Introduction

Malaria infection in a vertebrate host is initiated by sporozoites inoculated by an infected mosquito, and asexually replicating erythrocytic stages are responsible for clinical malaria. Continuation of malaria transmission depends upon differentiation and development of erythrocytic sexual stages (male and female gametocytes) which upon ingestion by a female anopheline mosquito undergo fertilization and sexual reproduction prior to transmission to a new host as sporozoites. Gametocytes are non-dividing sexual stages and their development involves maturation through morphologically and antigenically distinct stages defined as stages I through V (Carter and Miller, 1979). Studies have well established that antigens in the asexual stages are targets of innate and adaptive immune responses. Similarly, phagocytosis of erythrocytic asexual stage (Ayi et al., 2005; Kumaratilake and Ferrante, 2000) and early sexual stage I and II (Smith et al., 2003) are well described, however, there is little to no information on phagocytosis of mature stage V gametocytes that are in circulation. Previous published studies have established that the sexual stage antigens, some being pursued as candidate antigens for transmission blocking vaccines, are targets of antibody responses during natural infection (Bousema and Drakeley, 2011).

While sexual stages I to IV remain sequestered in the bone marrow, only the mature stages are ever found in the peripheral circulation (Aguilar et al., 2014; Joice et al., 2014). Since development of gametocytes occurs during blood stage infection, it is reasonable to expect that they will be targets of phagocytosis early on to initiate the adaptive immune response. Phagocytic clearance of gametocytes as yet another natural immune mechanism has not been well studied and further understanding of this mechanism will assist in identifying new targets of immunity which can potentially lower the burden of gametocytes in the human host and decrease transmission viability. It can be expected that not all of the circulating mature gametocytes are ingested in a blood meal by feeding mosquitoes, and remaining gametocytes are subject to phagocytosis. An initial role for phagocytes such as macrophages and dendritic cells that engulf pathogens or antigenic components will trigger immune responses which then will help in shaping patterns of adaptive immunity targeting malaria transmission.

2. Materials and Methods

2.1 *Plasmodium falciparum* cultures and purification of mature gametocytes

NF54 isolate of *P. falciparum* was used in these experiments. Parasites were cultured using O +ve RBCs in RPMI medium supplemented with 10% O +ve normal human serum and 0.37 mM hypoxanthine using a candle jar method (Trager and Jensen, 1976). Gametocytes were cultured according to published methods (Ifediba and Vanderberg, 1981). Purified mature gametocyte preparations were obtained from infected RBC cultures at 14–18 days. Infected RBC cultures were layered over 60% Percoll and centrifuged at 11,000×g for 30 min at room temperature. The interphase containing purified gametocytes was carefully removed and washed 3X in serum-free culture medium before use in phagocytosis assays (Rener et al., 1983). To free gametocytes from erythrocytes, purified erythrocytic gametocytes were treated with 0.1% saponin (5 min at room temperature) (Kreier, 1977). Heat killed gametocytes were prepared by incubating purified preparations of gametocytes at 65°C for 5 minutes, and bringing it back to room temperature prior to incubation with phagocytes. Asexual parasites stages (rings, trophozoites, and schizonts) were synchronized using 5% sorbitol and enriched on Percoll gradient (Lambros and Vanderberg, 1979).

2.2 Phagocytosis assay

THP-1 (American Type Culture Collection, Manassas, VA) cells were maintained in culture using RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, penicillin and streptomycin. For phagocytosis studies, cultured THP-1 cells were plated in sterile 8-well chamber slides (Lab-Tek) at a cell density of 80,000–100,000 cells/well, cultured for and activated with 30 ng/ml PMA (phorbol 12-myristate 13-acetate) for 48 hours. PMA activated monocyte/macrophage cells were incubated with purified asexual and sexual stages of parasites at 37°C/5% CO_2 for 1–4 hours (10–100 infected erythrocytes per monocyte). After incubation, wells were washed 5–6 times with serum free RPMI to remove non-adherent and non-phagocytized infected erythrocytes. In order to establish specificity of internalization of parasites, adherent but non-phagocytized erythrocytes were lysed with a brief treatment with RBC lysis solution (15 mM NH_4Cl, 100 mM NaHCO3, 1 mM EDTA). We did not observe any difference between using and not using RBC lysis method (data not shown). This brief ammonium chloride treatment effectively lyses erythrocyte without compromising monocytes. Internalized/ phagocytized infected erythrocytes were detected by Giemsa staining followed by microscopic examination to detect internalization of parasites by monocytes/macrophages. Slides were fixed in 100% methanol for a minute, and air dried. Staining was done by covering the slides with 5% Giemsa solution for 30 minutes and washed with distilled water. Slides were dried and viewed at 1000× magnification under oil immersion using Olympus BX41 microscope, and photographed using Olympus QColor 3 camera and QCapture Pro 7 Software (QImaging).

Phagocytosed parasites were quantitated by enumerating Giemsa stained THP-1 cells and parasites (gametocyte infected RBCs). Only cells with recognizable parasite structure were counted disregarding parasite debris and/or hemozoin pigment. In case of saponin released parasites, it was difficult to observe parasite structures after phagocytosis under our conditions and therefore we were unable to enumerate the phagocytosed parasites. In

addition they were too numerous to count inside the cell. We counted cells and parasites in different fields from at least 3 independent observations. Thus we counted the number of THP-1 cells, the number of parasites inside the cells and the number outside the cells in each field for each input parasite preparation (live, heat killed and saponin lysed). We did not see non-specific sticking of parasites to the slides and any parasite structure we considered as being outside were close to the edge of the THP-1 cells. Similar enumerations were done for asexual stages.

3. Results and Discussion

3.1. Activation of THP-1 cells

PMA (phorbol 12-myristate 13-acetate)-activated THP-1 cells were used to investigate phagocytosis of sexual stages of *P. falciparum*. THP-1 cells were chosen because they provided a consistent source of human monocyte cell line without bringing in heterogeneity of the phagocytic cell source, and have been used extensively in similar studies looking at phagocytosis of erythrocytic asexual stages of *P. falciparum* (Tippett et al., 2007). We first established the phagocytic assay conditions using PMA at different concentrations to activate monocytes, and fluorescent latex beads (Sigma) to determine phagocytic potential of the THP-1 cells. Based on the results of these experiments (data not shown) we selected a cell density of 80,000–100,000 cells per chamber of an 8 well chamber slide (Lab-Tek) activated with 30 ng/mL final concentration of PMA in a total culture volume of 200uL. In all subsequent experiments, THP-1 cells (Figure 1A) were incubated for 48 hours with 30 ng/mL PMA and the medium removed before addition of *P. falciparum* gametocyte preparations.

3.2. Purified live mature gametocytes

Figure 1B shows purified preparation of mature stages gametocytes. For our experiments we primarily focused on phagocytic uptake of mature stage gametocytes (stage V). In some experiments we also used stage III and IV preparations to compare any stage specific differences. After Percoll gradient centrifugation, the individual purified preparations contained about 85% gametocytes and some gametes and about 15% uninfected RBCs.

3.3. Phagocytosis of live mature gametocytes

We were interested in investigating phagocytosis of live mature gametocytes that circulate in the blood before being taken up in a blood meal by a feeding mosquito. When activated THP-1 cells were incubated with purified mature gametocytes immediately after purification, we found that purified intact gametocytes make intimate contact with phagocytic cells (Figure 2). The parasites were seen to cluster along the outside of the THP-1 cell and make firm contact. We have looked at the reproducibility of this phenomenon in fifteen independent observations and surmise from our observations that freshly purified live gametocytes are not engulfed by the THP-1 cells in the culture conditions in our assay.

This phenomenon was repeatedly observed regardless of whether the phagocytosis was allowed to occur for 30 min to an hour or for 4 hours. In rare instances we did observe that a

few gametocytes were found being internalized and we believe that some gametocytes might have died during gradient centrifugation step and thus marked for phagocytosis. In major portions of the viewed stained slides, mature stage gametocytes were found at the outside limit of the macrophages and exhibit morphological resistance to be phagocytized. Table 1A shows the quantification of the live gametocytes which are significantly found outside as described above. Thus, for a total of 428 THP-1 cells counted we found 2 gametocyte like structures inside the cytoplasm and 474 intact gametocytes outside.

3.4. Phagocytosis of heat killed or saponin-freed gametocytes

It is not clear if refractoriness of intraerythrocytic live mature gametocytes to be phagocytized is an inherent property of intact gametocytes or in some yet unknown ways, influencing the THP-1 cell from exerting its phagocytic function. In view of the observations that live intact intraerythrocytic mature gametocytes were not internalized by phagocytes, we conducted similar studies using parasites that were killed by incubation at 65°C for 5 minutes (Fig. 1C) prior to incubation with THP-1 cells. We also tested free gametocytes obtained by lysing infected RBCs with saponin (Kreier, 1977) (Fig. 1D). The rationale for these studies was that not all mature gametocytes will be ingested by the mosquito vector during transmission, and any remaining stages will eventually die and might become target of phagocytic clearance by macrophages. Also, we suggest that uptake of un-transmitted dying/dead gametocytes might be the primary source for initiating immune response against antigens specific to the gametocyte stages as we know that such antibodies are found after natural infection (Bousema and Drakeley, 2011). In sharp contrast to live intact gametocytes (Fig. 2), we observed that heat killed gametocytes (Fig. 3A and B) or saponin-liberated gametocytes and free extracellular gametes (stages that normally develop in the mosquito) were readily taken up by phagocytes (Fig. 3C and D), when incubated with THP-1 cells. Table 1A shows the quantification of the heat-killed gametocytes which are significantly found inside the cytoplasm as described above. Thus, for a total of 589 THP-1 cells counted we found 1169 gametocyte like structures inside the cytoplasm and 106 outside as described above. We believe that the gametocytes outside have not been sufficiently killed and thus not phagocytosed.

In case of the saponin released gametocytes, we found that it was extremely difficult to identify parasite structures and it mostly appeared like degradation products and were too numerous to count accurately. These structures were found intracellularly and none was seen outside the phagocytes.

3.5. Phagocytosis of asexual stages

We also performed similar phagocytosis assays using live intraerythrocytic asexual stage parasites (rings, trophozoites and schizonts) to investigate if there was any difference in the uptake of live asexual stages. Fig. 4 shows that the asexual blood stage parasites were phagocytized (panels A–C). Table 1B shows the quantification of the asexual stage parasites which were also found inside the cytoplasm. Thus, for a total of 201 THP-1 cells counted we found 44 asexual parasites inside the cytoplasm and 1 early stage gametocyte outside. This made our observations with the live mature gametocytes even more intriguing, and raising

questions on investigating possible mechanisms in future studies by which such resistance to being phagocytized occurs.

3.6. Discussion

In this study, we first addressed the primary question – do mature gametocytes undergo phagocytosis by macrophages? As reported here our studies showing refractoriness of mature gametocytes to undergo phagocytosis raises important questions about malaria transmission biology and immune interference. The inability of mature gametocytes to undergo phagocytosis may be advantageous to the parasite to optimize their transmission success. Several studies have been published on the shape, size and rigidity of the gametocytes which relate to their ability to survive splenic filtration and maintain their presence in the blood and continue the transmission cycle (Aingaran et al., 2012; McQueen et al., 2013; Nacher, 2004). This is an interesting and important phenomenon that warrants investigation to understand the mechanisms by which the gametocytes are kept out by the macrophages or mechanisms by which gametocytes avoid being phagocytized. Any immune response against these stages will act to interfere with the transmission process. Recently Banerjee et al reported on the presence of CD47 on RBCs which inhibits the phagocytosis of murine malaria asexual parasites but the studies were not targeted at gametocytes (Banerjee et al., 2015).

Delineating these mechanisms which are not entirely clear, will be the focus of further studies in our lab. Our findings provide the basis for investigating several relevant questions in future, such as-(1) are there gametocyte stage-specific differences in phagocytosis?, (2) will there be differences between monocyte cell lines and primary human monocytes?, 3) are there antibodies in immune sera from people exposed to malaria infections that opsonize and promote phagocytosis?, and (4) are there unique gametocyte surface antigens recognized by opsonizing antibodies? Characterization of any such neo-antigens may provide additional novel targets for transmission blocking immune intervention approaches.

Identification of phagocytic receptors and targets of opsonizing antibodies will enhance understanding of the role of phagocytosis in the clearance of circulating mature gametocytes, and the link to humoral arm of adaptive immunity. A better understanding of immune responses stimulated by natural infection and vaccines will help to develop effective strategies to prevent and/or treat infection by diverse pathogens. Equally important will be studies in the presence of sera from naturally infected people with demonstrated presence of antibodies against gametocyte antigens. Especially relevant is how initial innate responses orchestrate effective adaptive immune responses which remain at the center of vaccine development considerations. Phagocytosis and opsonization mediated immune activation together with presentation of gametocyte specific antigens will contribute to natural transmission blocking immunity. We do not yet know if such surface antigens could potentially be targets of opsonizing antibodies. Previous studies have reported on the presence of antibodies recognizing gametocyte surface antigens in sera from malaria exposed people (Sutherland, 2009). Specific strategies may also be developed to induce similar antibodies by vaccines aimed at overall transmission reduction.

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Highlights

- We investigated phagocytosis of the mature sexual stage of the malaria parasite
- Live gametocytes resist phagocytosis
- Killed or damaged gametocytes are readily phagocytized
- These findings have implication for malaria transmission dynamics

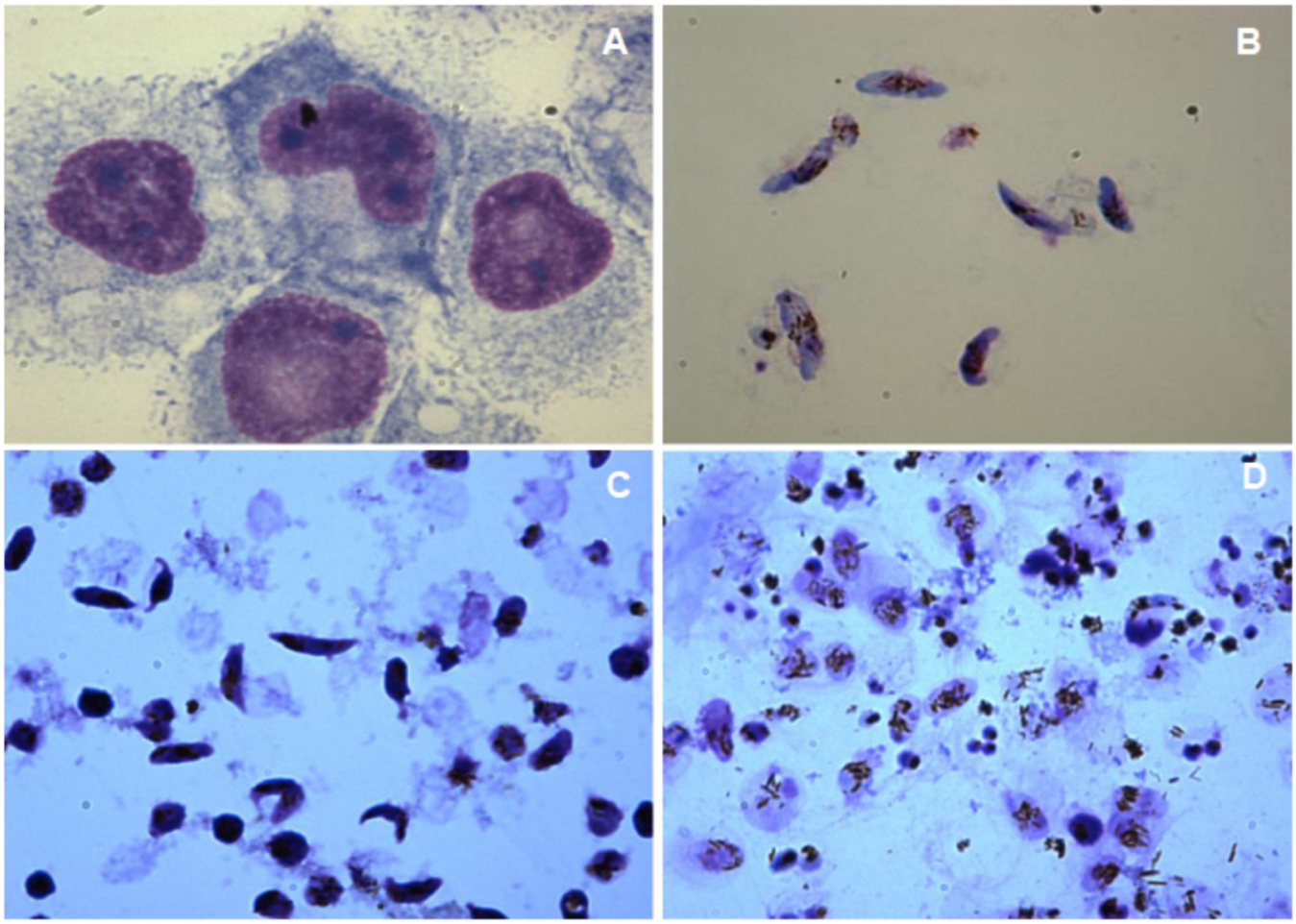

Figure 1. THP-1 cells and purified preparations of gametocytes

A – Uninfected THP-1 cells; B – live purified gametocytes; C – heat killed gametocytes; D – saponin lysed gametocytes.

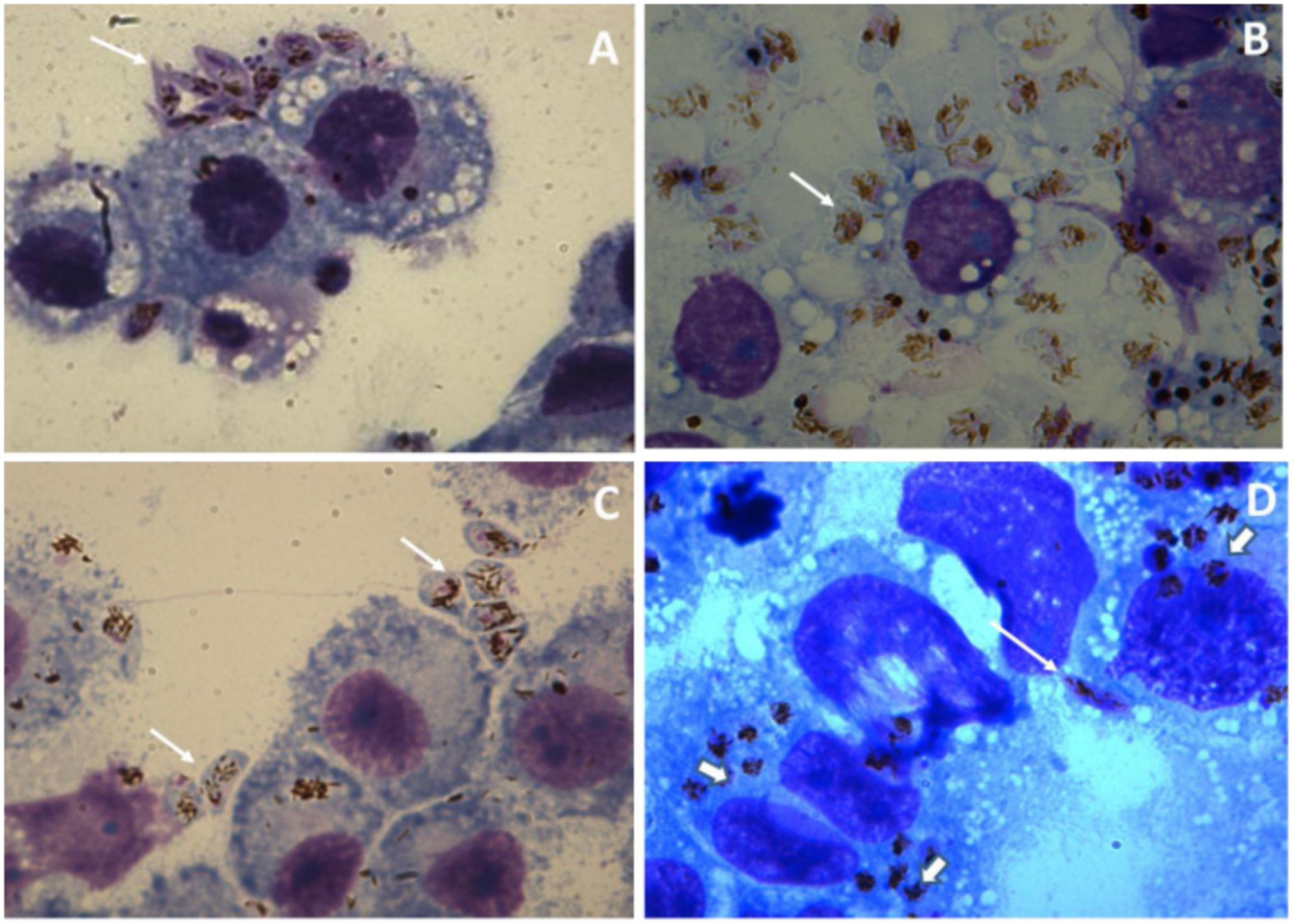

Figure 2. Nonopsonic phagocytosis of *P. falciparum* gametocytes and gametes

P. falciparum gametocytes and gametes were incubated with adherent THP-1 cells in RPMI containing 10% heat-inactivated fetal bovine serum for 4 hours as described in Materials and Methods. Panels A–C show representative results obtained from 3 independent experiments showing that live gametocytes are not taken up by the THP-1 cells and seem to line up on the outside edge of the cells (white thin arrows). Panel D shows gametes on the other hand are internalized as shown by the short white arrow.

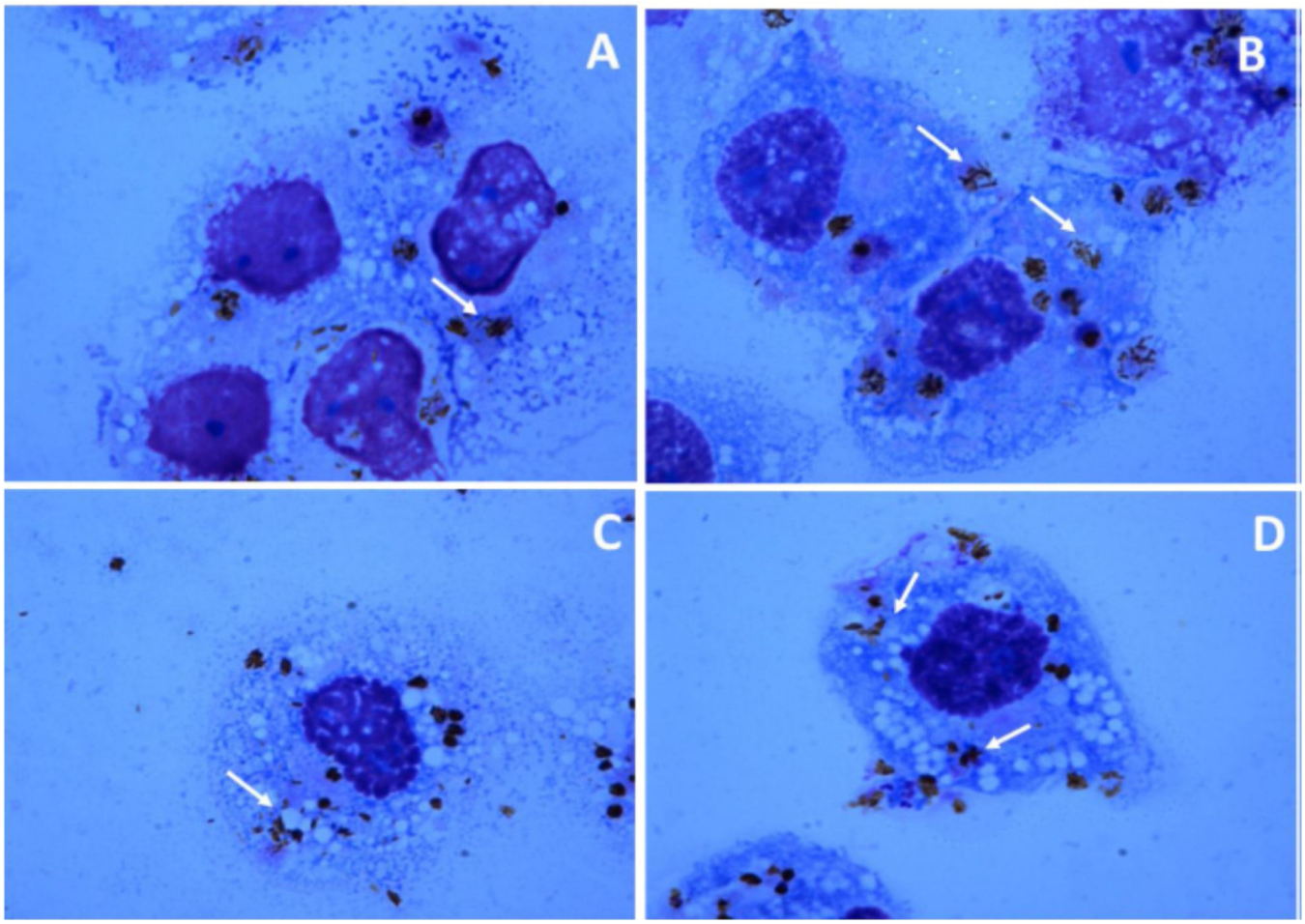

Figure 3. Phagocytosis of heat killed and saponin released gametocytes

Nonopsonic phagocytic uptake of heat killed gametocytes (A and B) and free gametocytes after saponin lysis of RBC (C and D) (arrows).

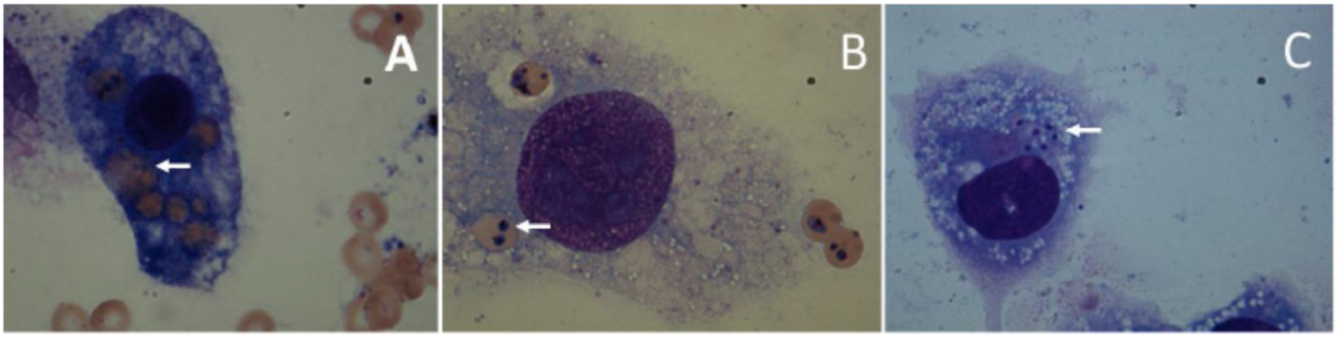

Figure 4. Phagocytosis of asexual parasites

Asexual stages undergo internalization of ring stage (A), trophozoite (B) and schizont (C).

Table 1

Quantitative assessment of phagocytosis of gametocytes (A) and asexual parasites (B).

A.					
# INDEPENDENT OBSERVATION	GAMETOCYTES	# FIELDS	THP-1 CELL	PARASITES OUTSIDE	PARASITES INSIDE
	LIVE				
I		9	175	115	0
II		6	135	119	2
III		7	118	190	0
Total		22	428	424	2
	HEAT KILLED				
I		14	262	90	627
II		10	158	16	175
III		4	169	0	367
Total		28	589	106	1169
B.					
# INDEPENDENT OBSERVATION	ASEXUAL STAGES	# FIELDS	THP-1 CELL	PARASITES OUTSIDE	PARASITES INSIDE
I		5	167	1 (gametocyte)	21
II		4	34		23
Total		9	201	1	44

Phagocytosed parasites were quantitated by enumerating Giemsa stained THP-1 cells and parasites (gametocyte infected RBCs). Only cells with recognizable parasite structure. Data is represented as THP-1 cells, the number of parasites inside the cells and the number outside the cells. In case of the saponin released gametocytes, it was extremely difficult to identify parasite structures inside THP-1 cells. Phagocytosed parasites were visible mostly as degradation products and were too numerous to count accurately. These degradation products were found intracellularly and none was seen outside the phagocytes.