SAPIENZA
UNIVERSITÀ DI ROMA

Role of the human GTPase Rac1 in *Plasmodium falciparum* infection of erythrocytes

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1. Introduction

1.1. Malaria.

Malaria is an infectious disease that still represents one of the world's deadliest infectious diseases, together with tuberculosis and AIDS. It is caused by protist parasites belonging to the *Plasmodium* genus, transmitted to humans by the bite of female mosquitoes of the *Anopheles* genus. There are about 200 different *Plasmodium* species, but only five of them are able to infect humans: *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*, that causes the disease in monkeys of the *Macaca fascicularis* species and that have recently been identified also in humans in some regions of South-East Asia (1).

According to the World Health Organization (WHO), malaria is the first parasitosis for number of casualities worldwide, with 219 million cases and 435 thousand deaths in 2017, mainly in children under five years of age (Tab. 1).

Number of Malaria cases (000)					
Africa	America	Middle-West	Sout-East Asia	West Pacific	World
194 000	875	4 300	14 600	1 600	216 000

Tab 1. Extimated number of Malaria cases in 2017. Numbers are expressed in thousands[1].

Around 92% of malaria cases and 91% of lethal outcomes occur in sub-Saharan Africa, where *P. falciparum*, the most lethal among *Plasmodia* is predominant. Other high-risk regions are South-East Asia, Middle-West and Central and South America (Figure 1).

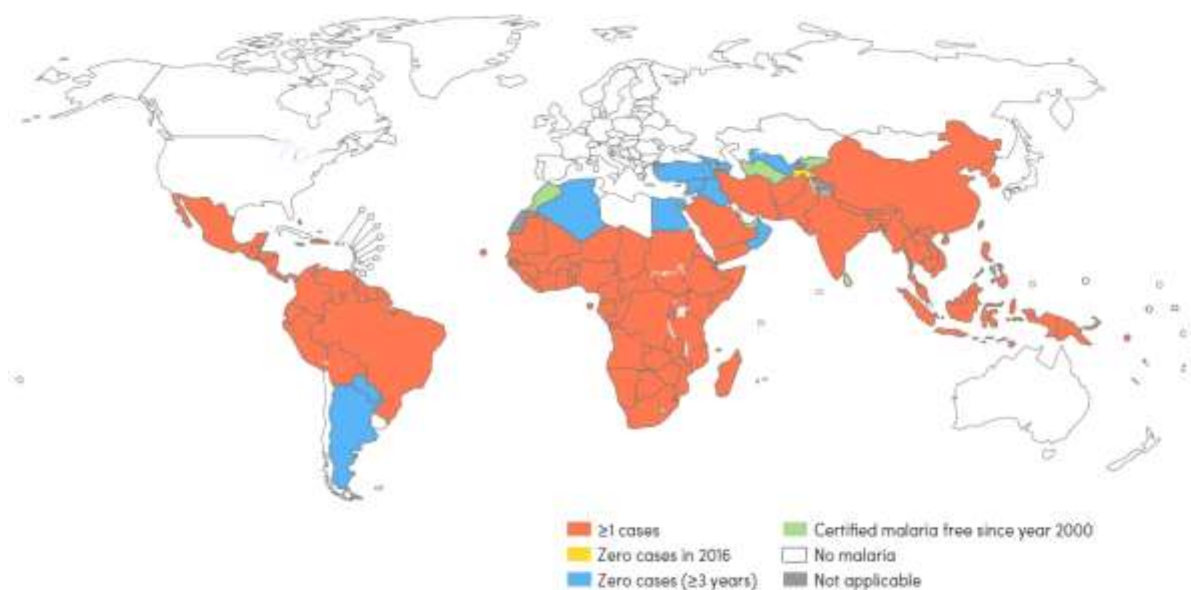

Figure 1. Autochthonous Malaria cases in 2016 [1].

Poor communities, less able to afford preventive measures and medical treatment, are the most affected in endemic countries. In regions with high malaria transmission, the most exposed groups are newborns, children under five years of age, pregnant women and immunosuppressed patients (HIV/AIDS), with an increase also in the number of lethal outcomes. Migrants and travellers are often a source of malaria cases in regions that have been declared malaria-free from a long time (2).

1.2. Malaria control.

In the last 15 years a huge economic effort was made to control the disease in endemic regions and funding have constantly increased since the year 2000 (Figure 2). 74% of fundings have been destined to Africa and only in 2016, 576 million dollars have been invested for research (2), making research on malaria the second most funded in the infectious disease field worldwide, after HIV/AIDS.

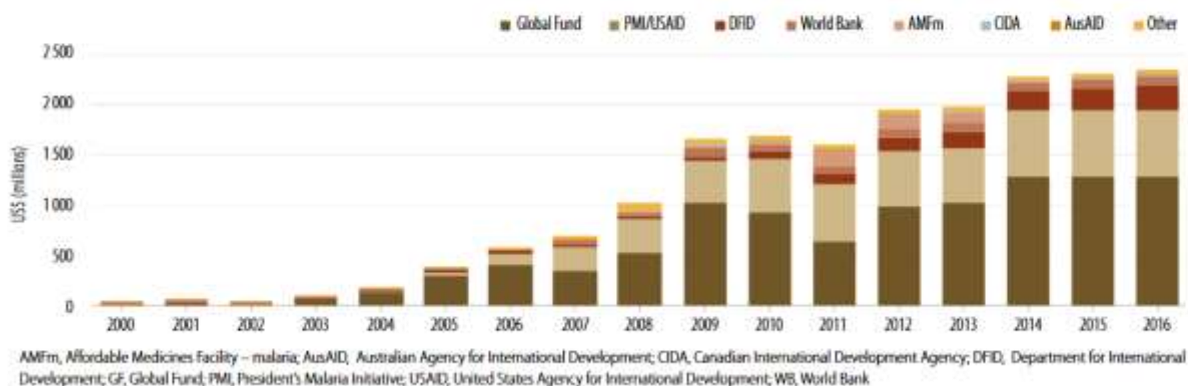

Figure 2. Malaria research fundings from 2000 to 2016, expressed in millions of american dollars [1].

Thanks to economic investments, to the development of new drugs and to the promotion of malaria prevention, mainly with the use of insecticide-treated bed nets and indoor residual spraying, the number of malaria cases and casualties in endemic regions constantly decreased between 2010 to 2016 (Tab. 2). However, In 2016, World Health Organization reported an increase of 5 million cases over 2015. Since then, no significant progress in reducing global malaria cases was made. Malaria is concentrated in countries with the least resourced health systems. Democratic Republic of the Congo and Nigeria together account for more than 35% of the total estimated malaria deaths. The slowest declines were reported in the high-burden countries, where an adequate malaria control and monitoring is difficult. Of these, Nigeria, Madagascar and the Democratic Republic of the Congo had the highest estimated increases in the last three years, each greater than half a million cases (2).

	Number of cases (000)						
	2010	2011	2012	2013	2014	2015	2016
Total	237 000	225 000	217 000	210 000	210 000	211 000	216 000
	Number of deaths (000)						
	2010	2011	2012	2013	2014	2015	2016
Global	591 000	529 000	487 000	465 000	459 000	446 000	445 000

Tab 2. Global number of malaria cases and of deaths from 2010 to 2016, expressed in thousands [1].

The fight against malaria is difficult because of different factors: i) insecticide resistance (3) ii) antimalarial drug resistance (4) iii) inadequate monitoring of asymptomatic cases especially in regions with low transmission (5) iv) The only vaccine approved for use against malaria (RTS,S, trade name “Mosquirix”), shows low efficacy and its effect wanes over time (6).

1.3. Anti-malarial drugs resistance

One of the causes of malaria increase in the last years could be *Plasmodium* ability to acquire resistance to available anti-malarial drugs. In the last century, several molecules active against *P. falciparum* have been used in the fight against malaria, resistance to currently available antimalarial drugs periodically emerge. Since 2014, drug-resistant parasite strains have been reported for all the existing classes of antimalarial compounds. Already 400 years ago, it was observed that acutely ill patients were cured by period fever after treatment with infusions of the bark from “chinchona” plants from Peruvian Amazon. Such an activity was later attributed to the alkaloid quinine, isolated in 1820 (7). It was thank to quinine-based prophylaxis and to other prevention measures, that malaria was officially eradicated from Italy in 1970 (8). Quinin had however heavy side-effects and was subsequently replaced by its derivative chloroquine, that has been for a long time the standard drug for malaria prophylaxis and treatment. At the beginning of the 50’s, the first

P. falciparum strains resistant to chloroquine emerged in Thailand and then in Central-America, spreading then in all the other endemic countries in the 70's (9). Chloroquine interferes with hemozoin crystal formation (10). Chloroquine resistance is conferred by mutations in the *P. falciparum* gene encoding the chloroquine resistance transporter (PfCRT), expressed on the membrane of the parasite digestive vacuole. The *pfcrt* gene shows an extraordinary sequence diversity among geographically distinct isolates and codes for a 45 kDa protein with ten transmembrane domains (11, 12). Another mutation that confers resistance to chloroquine is in the *pfmdr1* gene, that encodes a 162-kDa protein residing in the digestive vacuole membrane of the parasite (12). Both PfMDR1 and PfCRT1 mutant parasites show resistance to other antimalarial drugs, including quinine, lumefantrin, halofantrine and artemisinin (13). *Plasmodium* resistant strains have emerged also for other drugs, like pyrimethamine (14), mefloquine (15) and, more recently, piperaquine (16).

Nowadays, the most effective malaria therapy consist in the combined use of arthemisinin derivatives compounds with a second drug (Artemisinin-based Combination Therapies, ACT). Artemisinin is activated by ferrous heme and causes the production of reactive radicals that interfere with haemoglobin digestion and kill most of the parasites (17). The second drug eliminates, in a longer time, the remaining ones with a different mechanism of action. In this way the risk of survival of resistant parasite is definitely reduced (18).

However, in 2014 the first cases of artemisinin-resistant parasites have been reported in South-East Asia (Vietnam, Cambodia and Thailand), were the other resistant strains have already emerged in the past. Today, *P. falciparum* resistant strains have emerged for all the classes of available anti malarial drugs (19, 20). Moreover, together with anti-drug resistance in parasites, also *Anopheles* mosquitos resistant to pesticides are emerging, making also the fight against malaria transmission more difficult (21). Of 73 countries with ongoing malaria transmission monitoring data, 60 countries reported resistance to at least one class of insecticides, while 50 reported resistance to two or more insecticide classes (3).

1.4. Novel anti-malarial strategies: the host-targeted therapies

The threat of emerging malaria strains resistant to currently available drugs has made the search for novel drug targets compelling. An emerging strategy to limit the insurgence of drug-resistance is to target host molecules that pathogens exploit in order to enter or develop inside the host cell. This approach is less likely to generate drug resistance than conventional antimicrobial therapies, since the pathogen needs to redirect its entire infection strategy to compensate for a missing interaction with a host molecule and could possibly be effective against different *P. falciparum* strains. In other words, a drug can become ineffective by a single mutation in the parasite genome, but to compensate for the absence of a host factor, the parasite should change in a more important way its infection strategy (22, 23). In fact, the majority of mutations that confer resistance to known antimalarial drugs are point mutations in genes encoding for molecular pumps and channels of the parasite plasma membrane or for proteins involved in pathways affected by anti-malarial drugs (4).

Host-targeted molecules have already been proven effective against different intracellular pathogen like *Mycobacterium tuberculosis* (24), *Helicobacter Pylori* (25), *Hepatitis C* (26), *Dengue virus* (27). Different strategies have been proposed for host- targeted interventions that aim to eliminate parasite or to modulate the host inflammatory response (28). The treatment of human erythrocytes with Conoidin A, an irreversible inhibitor of the host peroxidase Prx2, impairs DNA synthesis and hemozoin production, disrupt nuclear integrity and prevents growth of *P. falciparum*, making it more sensitive to chloroquine (29). Some host-targeted compounds have already been tested in clinical trials. For instance, in *Hepatitis C* treatment, *alisporivir* inhibits viral replication by binding host cyclophilin A, a protein that regulates HCV replication inside the host cell (30), and *celgosivir* in combination with other drugs blocks viral envelope formation and viral release, acting on alpha-glucosidase I, an enzyme that processes the N-linked oligosaccharides of viral envelope glycoproteins. (31). For HIV/AIDS, *maraviroc*, a compound that binds the host chemokine receptor CCR5 used by the virus to enter inside host cells, is already in clinical use and has a synergistic effect with other anti-retroviral drugs (32).

1.5. *Plasmodium falciparum* life cycle

Plasmodia are protozoan belonging to the Apicomplexa phylum, which only includes endocellular parasites. *Plasmodium* has a complex life cycle that takes place in two different organism: a mosquito of the *Anopheles* genus as a definitive host, and a vertebrate as intermediate host. Each *Plasmodium* species can only infect a specific intermediate host.

Plasmodium falciparum infects humans by the blood feeding of an infected mosquito. Together with the mosquito saliva a great number of haploid *P. falciparum* sporozoites enters the blood stream. In an hour, sporozoites reach the liver and enter inside the hepatocytes, where they will develop for nearly a week. In this time, they become trophozoites and then hepatic schizonts, replicating their genome by schizogony (multiple nuclear division by mitosis followed by subsequent cellular division). When the schizont is mature, it breaks the hepatocyte releasing around 40.000 merozoites in the bloodstream and beginning the hematic cycle of the parasite. Merozoites invade human erythrocytes and inside them, the parasite undergoes a cycle of asexual replication that takes about 48 hours. This cycle ensures the maintenance of the parasite in the blood, but is not useful for parasite transmission. In face of the host immune response, a sub-population of parasites, instead of replicating, differentiates into sexual forms, called gametocytes. This process, called gametocytogenesis, leads to the formation of male and female sexual stages (micro- and macro-gametocytes respectively), characterized by a sickle-like shape that gives the name to this species (from the latin, falx). Gametocytogenesis lasts about 14 days and immature gametocytes are sequestered mainly in the bone marrow and in the spleen. Mature gametocytes are then released into the bloodstream, ready to be ingested by a mosquito during its blood meal. In the stomach of the mosquito, mature gametocytes differentiate in gametes. Each microgametocyte replicates three times, forming eight male gametes, while a macrogametocyte forms a single female gamete. In the mosquito midgut, female and male gametes mate, forming a zygote and then a motile ookynete, the only diploid form of all the parasite life cycle. The ookinete exits from the mosquito gut by penetrating through the gut epithelim and forms an oocyst on the basal lamina. With a single meiosis and several mitosis, the oocyts forms thousands of sporozoites that spread through the mosquito hemolymph,

reaching the salivary glands. The following blood meal of the mosquito will take the parasite to the next human host (Figure 3) (33).

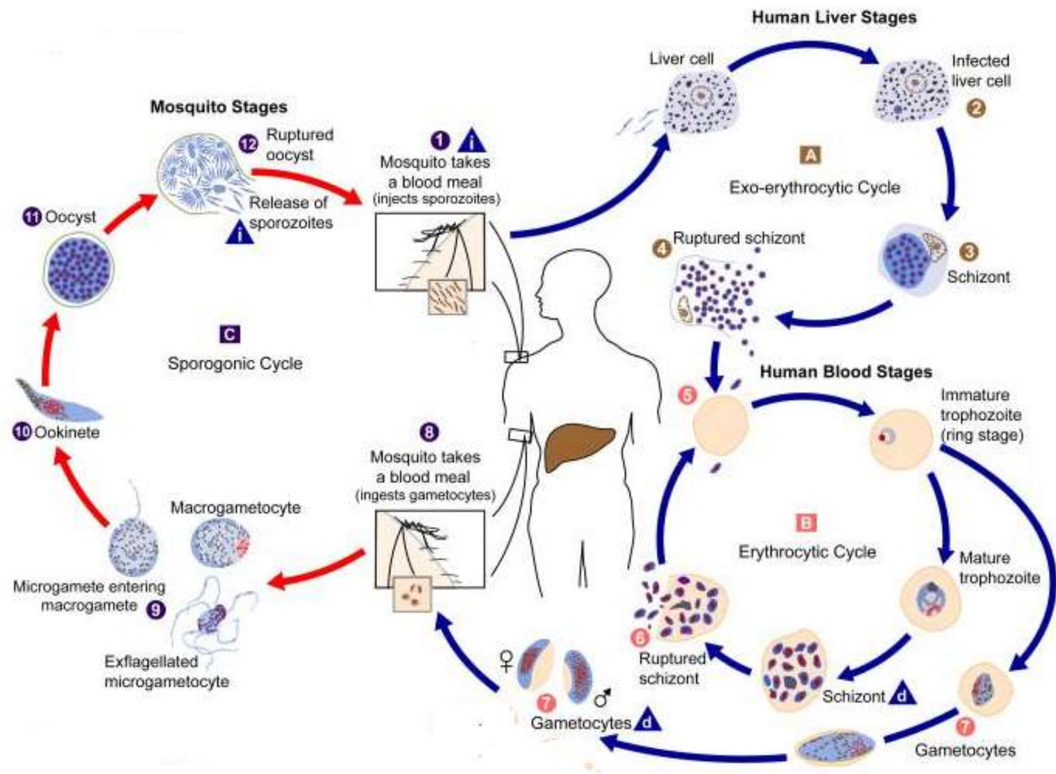

Figure 3. ***Plasmodium falciparum*** **life cycle**. 1. 1. The bite of an infectious mosquito releases the sporozoites in the blood. 2. Sporozoites enter inside hepatocytes and feed on its cytoplasm. 3. Nuclear replication begins, forming the hepatic schizont. 4. Mature schizonts break, releasing merozoites in the bloodstream. 5. Merozoites invade the erythrocyte, where they grow, feeding on its cytoplasm. 6. At the end of the replication, the schizont breaks, releasing merozoites in the bloodstream and the blood cycle starts again. 7. Some parasites undergo gametocytogenesis, differentiating in microgametocytes and macrogametocytes. 8. An *Anopheles* mosquito ingests the gametocytes, stimulating them to produce gametes. 9. A male and a female gamete mate. 10. The ookinete migrates to the mosquito gut and here undergoes meiosis. 11. The oocyts produce thousands of sporozoites by several cycles of mitosis. 12. The oocyst breaks, releasing the sporozoites in the coelomatic cavity of the insect; sporozoites then reach the salivary glands, ready to be released again at the next bite (Modified Image from (34)).

1.5.1. Pathology

Malaria pathology is directly linked to the biology and life cycle of the parasite. The first symptoms show when the parasites infect the erythrocytes. The pathologic phase begins with a series of febrile episodes every second day. The frequency of the febrile episodes is due to the synchronous release of the parasites in the bloodstream: each 48 hours, when blood schizonts break, together with merozoites they also release hemozoine (a pyrogenic molecule resulting from haemoglobin digestion). Other symptoms include spleen hypertrophy, as a result of its filtering activity to remove infected erythrocytes and anaemia due to erythrocyte lysis caused by the parasites. Anaemia, if associated with precarious health conditions and/or malnutrition, can lead to death.

As previously mentioned, most severe malaria cases are caused by *P. falciparum*. This parasite has a fast life cycle and a high replicative capacity and, as a consequence, can reach high parasitemia levels compared to other *Plasmodia*. Moreover, *P. falciparum* has another characteristic that is fundamental from a clinical point of view: mature parasites express adhesive proteins that are exposed on the surface of the erythrocyte, forming the so called "knobs". These are sticky membrane protrusions that bind to endothelial receptors, causing the adhesion of the parasitized erythrocyte to the capillary endothelium and to other erythrocytes. In this way, the parasite can circumvent the removal by the spleen. This mechanism leads to a secondary effect. These aggregates of erythrocytes can block the blood vessels, leading to ischemia and damaging the organs. In particular, cerebral malaria can lead to irreversible neural damage, coma and death.

1.5.2. Hematic stages

P. falciparum shows different morphologies for each different stage of its life cycle. The different blood stages can be easily recognized by analysis at the optical microscope of GIEMSA-stained blood smears. Merozoites, the parasite invasive forms, are the only blood stages free in the bloodstream and thus exposed to the host immune response. They are the smallest form of the parasite inside the blood, most of their volume is occupied by the

nucleus and by the organelles of the apical complex (the structure that leads the invasion process and that characterizes the whole apicomplexan phylum). The merozoite actively penetrates inside the erythrocyte by forming an invagination of the host membrane that will form the parasitophorous vacuole membrane, a membrane that will surround the parasite during its whole intraerythrocytic life.

From invasion until the first nuclear replication, the parasite will mainly feed and grow, preparing for the subsequent replication. This form, the trophozoite, will last for 32 hours and shows two different morphologies. The first one has a flat peripheral nucleus and is called "ring stage". The second one is recognizable by the appearance of hemozoin. With the first nuclear replication, the schizont phase begins. It will last around 16 hours, forming a maximum of 32 merozoites by schizogony (Figure 4).

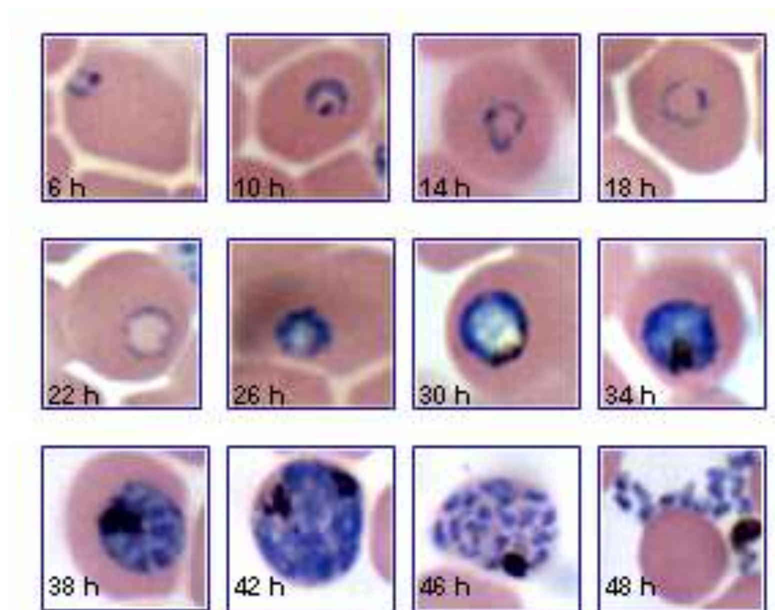

Figure 4. Blood smears stained with Giemsa. Number indicates hours from host cell invasion by the merozoite. From 6 to 22h: ring-stage trophozoite. From 26 to 30h: mature trophozoite. From 34 to 46 hours: schizonts with 2, 16 and 32 nuclei. 48 hours: merozoites release from a ruptured schizont (35).

In the peripheral blood of a malaria patient, only ring stages and gametocytes can be detected, because trophozoites and schizonts adhere to the capillary endothelium (33).

1.5.3. Erythrocyte invasion process

Merozoites are about 1 µm long and are characterized by a complex apparatus at the apical pole of the cell, the *apical complex*, typical of all of the organisms belonging to the Apicomplexa phylum. This complex is necessary to the parasite to penetrate inside the host cell. The complex includes different types of secretory organelles called micronemes, rhoptries and dense granules (Figure 5).

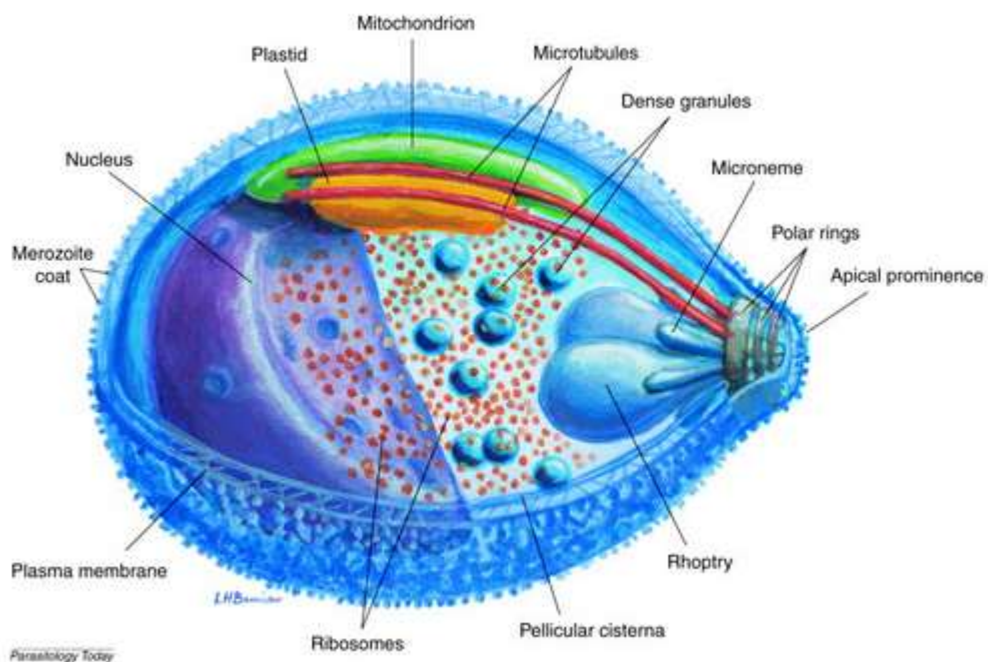

Figure 5. Representation of the structure of a *P. falciparum* merozoite (modified from (36)).

The first step in erythrocyte infection is the recognition and initial attachment to the host cell, followed by the release of intracellular Ca^{++} in the parasite that stimulates the release of the secretory organelles content. The factors released contribute to create a series of host/parasite interactions. The invasion process can be divided in four phases. 1) Initial

contact between merozoite and erythrocyte 2) Re-orientation of the merozoite in order to expose its apical complex to the erythrocyte membrane. 3) Formation of a tight junction, called *moving junction*. 4) The merozoite slides through the moving junction, entering into the erythrocyte and remaining enclosed in the parasitophorous vacuole membrane (PVM), which reseals at the caudal pole of the parasite. The PVM includes both host proteins and parasite proteins (Figure 6).

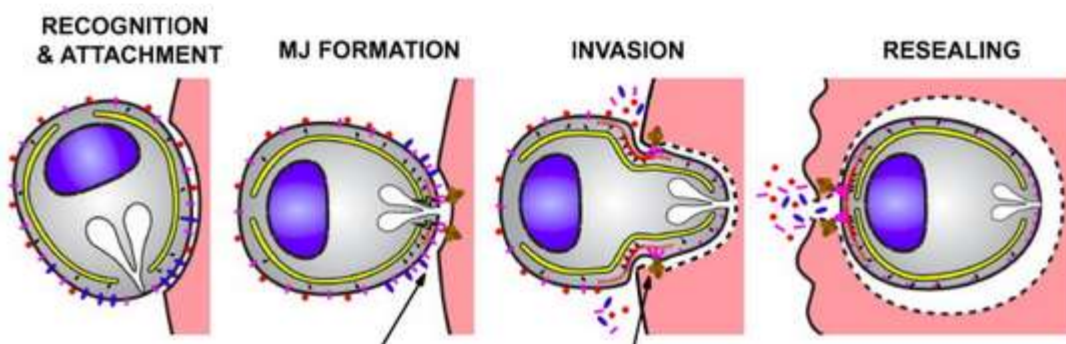

Figure 6. Mechanism of erythrocyte invasion by P. falciparum merozoite (Modified from (37)).

In the first contact between parasite and erythrocyte, a fundamental role is played by the Merozoite Surface Proteins (MSPs), a family of proteins exposed on the merozoite surface that forms low-affinity bonds with proteins of the erythrocyte membrane like Band3 or Glicophorin A (GPA). In the following phase, adhesive proteins are secreted from micronemes and rhoptries. These proteins bind with a stronger affinity to host cell membrane receptors, creating a “binding affinity gradient” that allows the correct re-orientation of the merozoite, with its apical pole in tight contact with the cellular membrane (38). In the third phase, Rhoptry Neck-Derived Proteins (RON2, RON4, RON5) are released from rhoptries, forming the RON complex, that translocates to the host cell membrane and acts as a receptor for the protein Apical Membrane Antigen 1 (AMA1), secreted from the micronemes, forming a third point of anchoring, the moving junction (37). In the fourth and last phase, the merozoite slides through the moving junction, forming the parasitophorous vacuole membrane (PVM) from the erythrocyte membrane, where also proteins secreted by

the parasite, like RAP (Rhoptry associated protein) and EXP1 (Exported protein 1) (39, 40) will localize. When the parasite has fully entered the host cell, the moving junction closes behind the merozoite. Now the parasite can start the intraerythrocytic growth inside the PVM that has a protective function and allows the parasite to import nutrients from the cytoplasm.

An important consideration in terms of drug-development is that erythrocytes invasion requires a series of co-ordinated and often irreversible events to occur in sequence, with even small perturbations of this complex process likely to limit parasite survival *in vivo*. In fact, several parasite factors involved in this process are under study for the development of novel malaria vaccines. The importance of key parasite-RBC protein interactions on the merozoite surface highlights the possibility of blocking them directly by using chemical compounds. Importantly, a drug blocking merozoite ability to invade would immediately and permanently stop the parasite's lifecycle, including the subsequent transmission to mosquitos (41).

1.6. GTPases

Several biological processes, such as cellular growth and differentiation, signal transduction, protein synthesis and vesicular transport, are regulated by proteins that bind to the nucleotide guanine. These proteins are defined as “molecular switches”, since they are able to modify their conformation between an active and an inactive state, depending on the phosphorylation state of the bound nucleotide. These proteins are also called “G proteins”, because they are able to hydrolyze the nucleotide Guanine-Tri-Phosphate (GTP) in Guanine-Di-Phosphate (GDP), which imply consequently a conformational change. G proteins are divided in two classes: Heterotrimeric G proteins, composed by three subuinits, and small monomeric G proteins. Despite these proteins are heterogeneous in structure and in the processes they control, they have common characteristics, like the “switch” mechanism inside the so called *G domain*.

The *G domain* is formed by a *P loop*, a motif with a specific aminoacidic sequence able to bind GTP through the alpha and beta phosphates and by two motifs, known as *switch I* and *switch II*, that coordinate the terminal gamma phosphate of the bound triphosphate nucleotide (Figure 7).

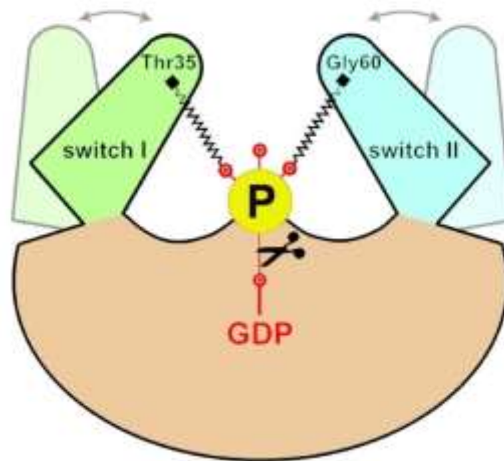

Figure 7. Representation of the switch mechanism. Switch I and II domains are bound to the terminal phosphate through the –NH groups in the principal chain of the invariant residues Thr and Gly. The phosphate release after GTP hydrolysis allows the switch region to relax in a different conformation (modified from(42)).

The protein is active when is GTP-bound, with the gamma phosphate interacting via hydrogen bond with the –NH groups of two invariant residues of Thr and Gly, present respectively in the switch I and switch II regions, coordinated with a Mg^{++} ion. In this conformation, the G domain is in a state that can be defined as "spring loaded". GTP to GDP hydrolysis causes the release of the gamma phosphate and the subsequent relaxation of the switch regions to a conformation that is specific for GDP, the inactive conformation. This conformational change is limited to the switch regions. The interactions that other effector proteins have with the active and the inactive forms of the protein determine which cellular mechanism is controlled by a particular G protein (39).

Once the GTP is hydrolized to GDP and the protein is in its inactive form, the GDP will dissociate from the GTPase in order to allow a new GTP molecule to bind.

Since the dissociation is very slow, G-proteins need other proteins, the GEFs (*guanine nucleotide exchange factors*) to discharge GDP. These proteins bind to G proteins and promote the GDP release, inducing conformational changes that open the binding site, thus activating the signal. Each G-protein is able to interact with different GEFs, thus integrating signal that come from different sources.

In the cytosol, GTP concentration is ten-fold higher than the GDP one. As a consequence, once the GDP is released, the G protein will soon bind a new GTP molecule and turn back to its active state.

Since the catalytic activity of the G proteins is not very efficient, the rate of GTP hydrolysis required to turn back to the inactive state would be very low. So, a second class of proteins, the GAPs (*GTPases activating proteins*) interact with G proteins in their active conformation, improving the catalytic activity and allowing the protein to turn back to the inactive state, thus shortening the duration of the signal (Figure 8). *Guanine-nucleotide-dissociation inhibitors* (GDIs), which maintain the GTPase in an off-state, preventing GDP dissociation, are a third class of GTPases regulators, with inhibitory function. (43).

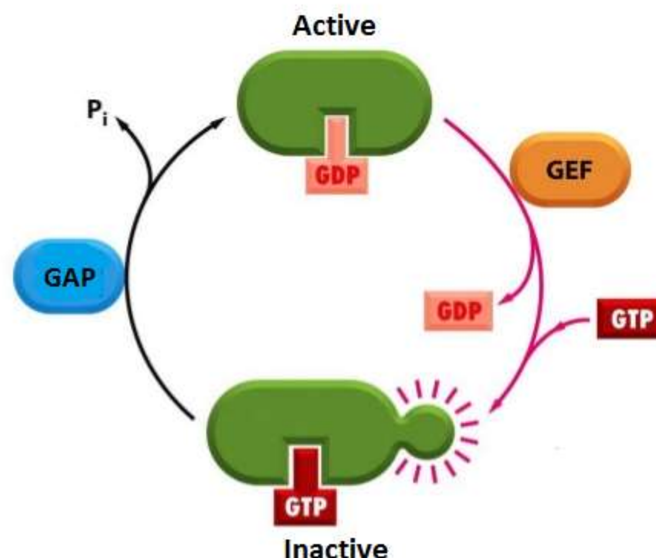

Figure 8. Activation/inactivation cycle of a monomeric G protein (modified from : http://www.molecularlab.it/public/data/GFPina/200611319058_Fig.%2015-54.JPG)

1.6.1. Rho GTPases and Rac subfamily

Part of the Ras family, the Rho GTPases are small G proteins of about 21-30 kDa. They are ubiquitous proteins, found in all the eukaryotes, including yeast and some plants. These proteins control the signal transduction pathways regulating the response to membrane surface receptors, modulating a lot of intracellular processes such as actin cytoskeleton regulation, cell polarity, gene transcription, microtubule dynamics, vesicular trafficking (44). The Rho GTPase family includes 20 proteins, divided in 8 subfamilies, based on their primary sequence, domain structure and functions, divided in two groups: the classic Rho GTPases, able to cycle between the active GTP-bound form and the inactive GDP-bound form, and the atypical Rho GTPases, constitutively bound to GTP (45, 46) (Figure 9).

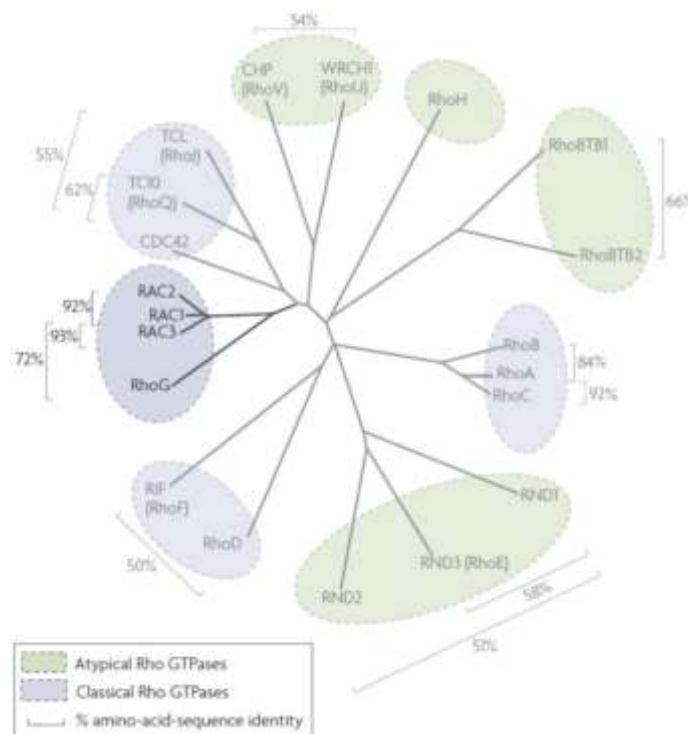

Figure 9. Phylogenetic tree of the Rho GTPase family of proteins, based on structure similarity. In bold the Rac subfamily, with the three proteins Rac1, Rac2, Rac3, showing an aminoacidic sequence identity higher than 90% and Rho G with a lower sequence identity (72%) (Modified from (45)).

The Rac subfamily includes the four most studied Rho GTPases. Rac1, Rac2, Rac3 (sharing 88% of sequence identity) and RhoG, the latter being more divergent from the others (only 72% sequence identity) and consequently less prone to share redundant functions with the other three proteins or to be recognized by antibodies and inhibitors specific for the other Rac GTPases (47). Even though the first three show distinct functions in the large part of tissues, they may sometimes show an overlap in their roles (48). Rac3 is known to be specifically expressed in the central nervous system (49) and animals knockout for this GTPase show no obvious erythropoietic phenotypes (50) Rac1 and Rac2 instead, were shown to be expressed in the erythropoietic line (51), but according to published data, only Rac1 was identified by proteomic analysis in mature human erythrocytes (http://rbcc.hegelab.org).

1.6.2. Rac1 (Ras-related C3 Botulinum Toxin Substrate 1)

Ras-related C3 Botulinum toxin substrate 1 or Rac1 (Figure 10) is the most studied protein of the Rac subfamily. It is a small GTPase, with a size of about 21 kDa, transcribed from the *rac1* gene, located on chromosome 7. Rac1 is ubiquitously expressed in tissues (52).

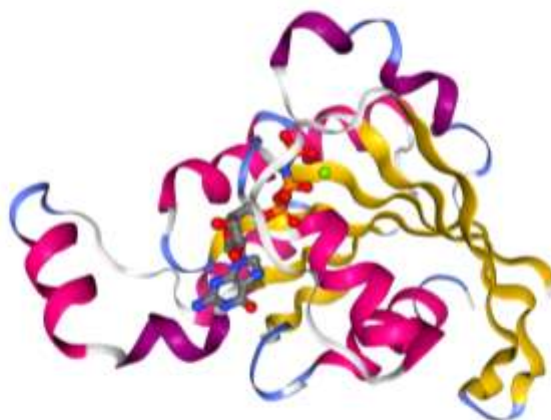

Figure 10. Cartoon of the crystalline structure of Rac1 bound to GTP. In green, the Mg^{++} ion. (From: http://www.rcsb.org/3d-view/3TH5)

Rac1 activity is involved in a variety of biological processes and is finely tuned by various post-translational modifications that regulate its localization and activation and by interacting proteins like GEFs and GAPs. Rac1 can localize to different subcellular compartments. Rac1 can translocate to the nucleus thanks to a nuclear localization signal in its C-terminal polybasic region (53). Here it can either interact directly with nuclear proteins like the transcriptional factor STAT3 (Signal Transducer and Activator of Transcription 3), regulating Rac1 activity by the promotion of its phosphorylation. Rac1 can also localize to mitochondria. In the cytoplasm Rac1 can be alone or associated with other proteins. Rac1 can be translocated to the plasma membrane by interactions with membrane-associated GEFs, adhesion molecules or tyrosin-kinase receptors (TRK) (54) or by post-transcriptional modifications like palmitoylation at its C-terminal region followed by geranylgeranylation that address Rac1 to cholesterol-rich membrane domains called *lipid rafts* (55). When localized at the membrane, Rac1 rules several processes like phagocytosis, lamellipodia formation, membrane ruffling and cell to cell adhesion mediated by E-cadherin (54). More than 30 different GEFs are able to activate Rac1 and several GAPs are able to inactivate it, regulating its switch both in a spatial and in a temporal way. GEFs that activate Rac1 are divided in two families, called Dbl and DOCK. The DBL GEFs have a Dbl homology domain (DH), while the DOCKs lack this domain, whose function is performed by an high-conserved region called DOCK-Homology Region 2 (DHR2) (46).

Together with Rac2, Rac1 plays an important role in actin cytoskeleton dynamics and in cell survival and proliferation (55). In particular, in the erythropoietic line, Rac1 is involved in proliferation and differentiation of erythroid precursors to mature erythrocytes, following the stimulation by cytokines like SCF (steam cell factor), with subsequent phosphorylation of the ERK kinase (Extracellular Signal-regulated Kinase). This activates a pathway in which Rac1 plays a pivotal role. Mice KO for *Rac1* and *Rac2* show erythrocytes with a disorganized membrane cytoskeleton. These RBCs show increased level of adducin phosphorylation on Ser726. In the erythrocyte, actin protofilaments are stabilized at their rapidly exchanging ends by adducin. Adducin phosphorylation on Ser726 causes its dissociation from spectrin, altering the meshwork that adducin, spectrin and actin form together beneath the plasma

membrane (56). Rac1 is also involved in the enucleation of erythroid cells, a fundamental step on the way to the mature erythrocytes. Rac1 inhibition blocks the formation of the contractile actine ring on the plasma membrane of enucleating erythroblasts, totally impairing the enucleation process (57).

Because of its role in cell motility, Rac1 is essential in the development of many cancers. In fact, a common feature of metastasis is a deregulation of Rac1, with consequent deregulation of cell motility (58). Because of its importance in cancers, Rac1 has been widely studied in the last years as a possible therapeutic target to fight cancer (59). As a consequence, several cell permeable inhibitors of Rac1 have already been developed and are commercially available (60-63). Moreover, the crystal structures of Rac1, Rac1-GTP and Rac1 in association with the GEF Tiam1 are available (64-66), possibly facilitating structure assisted drug design.

1.6.3. Rac1 role in intracellular infections.

Interestingly, Rac1 was shown to be involved in the invasion of the host cell by many intracellular pathogens (67). Several bacteria, such as *Campylobacter jejuni* (68), *Neisseria gonorrhoeae* (69)and *Staphylococcus aureus* (70) activate Rac1 during the invasion of the host cell, leading to membrane ruffling and to the formation of membrane protrusions that allow pathogen entry. Others, such as bacteria belonging to the *Clostridium* genus, modulate Rac1 activation to disrupt epithelial junctions and endothelial integrity (71). Bacteria apply two different strategies to modulate Rac1 activity: i) by secreting soluble virulence factors, such as CNF1 from *Escherichia coli*, that bind to the host cell surface and are then internalized by endocytosis. These factors can act both on proximal and on distal host cells and can be delivered to target cells by membrane vescicles produced by the bacteria (72, 73); ii) by injecting virulence effectors directly into the host cell through needle-like appendages, i.e. SopE and SptP from *Salmonella typhimurium* (74). Rac1 activation state is modulated by pathogen factors in three ways: i) by secreting factors that mimic Rac1 protein modulators, like GEfs, GAPs and GDIs (75), ii) by generating post translational modifications that act on Rac1 activation state or on its membrane localization (76), or iii) by regulating

Rac1 degradation by the ubiquitin-proteasome system (77). The outcome of Rac1 modulation is in most cases a localized disruption of the actin cytoskeleton that allows pathogen entry into the host cell. It was shown that treatment with Rac1 inhibitors reduces invasion rates of several intracellular bacteria (78-82) and also of the HIV-1 virus, maybe interfering with its bond to the host cell (82).

Rac1 was shown to play a role also in infection by intracellular parasites. The transfection of a host cell infected by *Trypanosoma cruzi* with a dominant-negative form of Rac1 reduces the rate of parasite internalization, while the transfection of a dominant-positive form of the protein increases the rate (83). Moreover, macrophages transfected with an inactive form of Rac1 show a significant reduction in infection rate of *Leishmania donovani* (84). Interestingly Rac1 plays a role also in the infection of *Toxoplasma gondii,* belonging to the same phylum as the malaria parasite *P. falciparum*. In *T. gondii* the host GTPase is recruited to the parasitophorous vacuole in its active form. The amount of active Rac1 in infected cells is higher than in the control. Both transfection of a dominant-negative form of Rac1 in the host cell or its down-regulation by RNA interference lead to a significant reduction in parasite invasion rates, demonstrating that Rac1 is necessary for the invasion of the host cell by *T. gondii* (85) **.**

Interestingly, Rac1 also plays a role in the egress of *P. falciparum* schizonts from infected human erythrocytes. Comparing the proteomes of uninfected RBCs and infected RBCs a few hours before parasite egress, it was shown that Rac1 disappears from the host cell membrane at around 35 hpi (hours post infection), together with others proteins, including adducin. The loss of Rac1 and adducin should help the parasite to dismantle the host plasma membrane in order to egress from it (86).

2. Aim of the work.

The continuous emergence of *P. falciparum* strains resistant to anti-malarial drugs is contributing to the malaria case increase reported in the last years. Efforts to reduce the spread of malaria are impaired also by the development of resistances to pesticides by *Anopheles* mosquitoes. It is thus evident that, despite the huge economic investments and the continuous search of new drugs against the parasite and its vector insect, these organisms manage to mutate and acquire resistances, thus nullifying all of these efforts. The insurgence of artemisinin-resistant *P. falciparum* strains in South-East Asia makes fundamental and urgent the identification of new pharmacological targets less prone to induce resistances.

A strategy to limit the development of new resistances could be to look for pharmacological targets on the host organism rather than on the parasite. Such an approach has already been successfully used with other intracellular pathogens as *Hepatits C* and *HIV* (30-32, 87, 88). Preliminary studies seem promising also for parasites and *P. falciparum* in particular. Conoidin A, an inhibitor of the host peroxidase Prx2, prevents *P. falciparum* growth and makes the parasite more susceptible to chloroquine (29).

Aim of this work is to assess whether Rac1, a host erythrocyte protein known to be involved in infection of a large number of intracellular pathogens, plays a role also in *P. falciparum* infection of the host erythrocytes. Because of its role in cancers, Rac1 has been extensively studied. Several cell permeable Rac1 inhibitors have already been developed and are commercially available (60-63). Moreover, the crystal structures of Rac1, Rac1-GTP and Rac1 in association with the GEF Tiam1 are available (64-66), making it easier to design new inhibitory compounds. Thus, Rac1 could be a promising candidate for the development of new anti-malarial drugs directed to the host.

We will here describe Rac1 localization during *P. falciparum* infection of human erythrocytes. Then, we will investigate Rac1 role during the invasion process and the subsequent intraerythrocytic growth, in order to assess its contribution during the different phases of parasite infection.

3. Materials and methods

3.1. Western blot

Whole blood collected from 7 donors was washed in RPMI 4 times in order to remove plasma, platelets and leucocytes. The number of erythrocytes was determined by using flow cytometry analysis with cell counting beads (Thermo Fisher). Purified erythrocyte membranes were obtained by lysis of red blood cells in hypotonic lysis buffer (5 mM Na-phosphate, 0.5 mM EDTA, pH 8), followed by 4 washings to eliminate the soluble fraction.

For western blot analysis, erythrocyte membranes were lysed in 2X loading buffer (100 mM tris-HCl pH 6.8, 4% SDS, 0,2% bromophenol blu, 20% glycerol, DTT 200 mM) and boiled for 5 minutes . Purified GST-tagged Rac1 protein (Cytoskeleton) was used to show the specificity of the antibody against Rac2. Purified His-tagged Rac2 protein (Sino Biological) was used as a positive control. All samples were then subjected to SDS-PAGE under reducing conditions, followed by transfer to nitrocellulose membrane (Sartorius). Antibodies used: anti-Rac1 mouse monoclonal 1:1000 (Cytoskeleton) anti-Pan Rac mouse monoclonal, (Santa Cruz Biotech) 1:1000, anti Rac-2 rabbit polyclonal (ThermoFisher) 1:1000. The filters were then incubated with anti-mouse or anti-rabbit HRP-conjugated antibodies (Pierce) and the immunocomplexes were visualized using chemioluminescence ECL detection system (Luminata Forte Western HRP Substrate, Millipore).

3.2. Immuno-fluorescence assays

Blood smears were fixed in 4% paraformaldehyde/0,015% glutaraldehyde for 30 minutes at room temperature. Cells were then permeabilized with 0,1% Triton X100 in PBS 1X for 10 minutes and incubated for 1 hour with the following primary antibodies: Rac1 mouse monoclonal antibody (ProteinTech) 1:200, Active Rac1 mouse monoclonal antibody (NewEast Bioscience) 1:200, Rac1 rabbit polyclonal antibody (ProteinTech) 1:20, Exp1 antibody (40) 1:50, Ron 4 antibody (89) 1:100, Band3 antibody 1:100. Samples were then washed in PBS and incubated with the secondary antibodies: anti mouse fluorescein

(Invitrogen) 1:200, anti-rabbit rhodamine (ThermoFisher) 1:200 and with the nuclear marker DAPI (Life Technologies) 500 ng/ml. Samples were washed again in PBS and smears were mounted in Vectashield™ (Vector Laboratories).

Smears were then analysed with a Leitz DMRB microscope, equipped with BP 340-380, BP 470-490 and BP 515-560 filters to visualize respectively DAPI, fluorescein and rhodamine fluorescence. The same fields were then observed in bright light and acquired with a Leica DFC340-FX camera. Images were analyzed with LAS (v 3.8, Leica Microsystems) software and then elaborated using the program ImageJ, using the plug-in Coloc2 for co-localization and the "grey scale media" to measure fluorescence.

3.3. Parasite cultures of 3D7, D10 and W2 strains

CQ-sensitive D10 (90), CQ-resistant W2 (91) strain and 3D7 (92) strain of *P. falciparum* were used in this work. Cultures were maintained according to Trager and Jensen with slight modifications (93). Briefly, all the strains were cultured at 5 % hematocrit (human type A-positive red blood cells for D10 and W2; 0-positive red blood cells for 3D7) in complete medium at 37°C in a standard gas mixture consisting of 1% O_2, 5% CO_2, 94% N_2. Complete medium is made up of RPMI 1640 (EuroClone, Celbio, Gibco) with the addition of 0.01% hypoxantine (Sigma-Aldrich), 20mM Hepes (Euroclone), 2mM glutamine (Euroclone). All the parasites were cultured in the presence of 1% AlbuMaxII (lipid-rich bovine serum albumin, Life Technologies), except for the 3D7 strain, which was cultured in the presence of 10% naturally-clotted heat-inactivated 0+ human serum (Interstate Blood Bank, Inc.), which ensures constant and high gametocyte production.

3.4. Antiplasmodial activity against D10 and W2 strains

Compounds were dissolved in DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1%, which is nontoxic to the parasite). Drugs were placed in 96 well flat-bottom microplates and serial dilutions made. Asynchronous

cultures with parasitemia of 1–1.5% and 1% final haematocrit were aliquoted into the plates and incubated for 72 h at 37 °C. Parasite growth was determined spectrophotometrically (OD650) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method in control and drug-treated cultures (94). Antiplasmodial activity is expressed as the 50% inhibitory concentrations (IC50). Each IC50 value is the mean ± standard deviation of at least three separate experiments performed in duplicate.

3.5. Invasion assays

Parasites were pre-synchronized 44 hours before the assay by 60% Percoll gradient [52]. Just before the invasion assay, cultures were double purified by 60% Percoll gradient and MACS column magnetic separation system (Miltenyi Biotec) (95). Purified schizonts were allowed to reinvade human RBCs for 6 hours in a 96-well plate (1% hematocrit, at a parasitemia ranging from 2% to 8%). The T0 sample was fixed just after schizont seeding. Human RBCs were fixed in 4% paraformaldehyde/0,075% glutaraldehyde for 30 minutes at room temperature, then washed in PBS and stained with 2 uM Hoechst 33342 (Thermo Fisher) for 30 minutes at 37°C in the dark, in order to distinguish parasitized RBCs from uninfected RBCs. Cells were then washed in PBS and counted by using a GALLIOS cytometer (Beckman Coulter), acquiring 200,000 events per sample. Initial gating was carried out with unstained, uninfected erythrocytes to exclude erythrocyte autofluorescence. Invasion rates were calculated by subtracting the number of schizonts at the end of the assay from the number of schizonts in the T0 sample, thus obtaining the number of merozoite-releasing schizonts. Then, the number of ring stage parasites was normalized on the number of merozoite-releasing schizonts, to obtain the number of invading parasites per egressed schizont in each sample. This value was finally normalized on the control sample, thus obtaining the invasion rate value.

3.6. Growth assays

Parasites were pre-synchronized 44 hours before the assay by 60% Percoll gradient [52] and put back into culture in complete medium with fresh human blood, in order to obtain synchronous mature schizonts for the assay. Just before the beginning of the assay, synchronous schizonts were purified by 60% Percoll gradient and allowed to invade fresh RBCs for two hours in a 24-well plate in complete medium. The culture was then treated with 5% sorbitol to kill all stages except young ring-stage parasites resulting from the recent invasion. Cells were then put back in complete medium in the presence or absence of Rac1 inhibitory compounds. Blood smears were taken immediately after sorbitol treatment (T0) and at 20, 27 and 39 hours, corresponding to mature ring stage, trophozoite stage and schizont stage. Blood smears were then counted by optical microscopy in order to assess the parasitemia.

3.7. Merozoite enrichment

Parasites were pre-synchronized 44 hours before the assay by 60 % Percoll gradient [52] and put back into culture in complete medium with fresh human blood. Just before the assay, parasites were purified by 60% Percoll gradient and put back in a small volume (5 ml) of complete medium with the protease inhibitor E64 at 10 µM concentration. E64 is known to block schizont egress in a reversible way, without affecting merozoite viability and invasiveness (96). After 6 hours, when schizonts are fully mature, cells were washed and resuspended in complete medium at room temperature. At this temperature, merozoite half-life is considerably increased (97). When E64 is removed, merozoite release is complete in about 10 minutes. Human erythrocytes were added to the culture in order to allow merozoite to invade them. Blood smears were taken 5 minutes after the beginning of merozoite release. In these conditions, around 5 invasion events were visible in each field at a 100x microscope magnification.

4. Results

4.1. Rac1 expression in mature erythrocytes.

Three of the Rac family members Rac1, Rac2, Rac3 show a high sequence homology (see paragraph 1.6.1.) and may be recognised by the same antibodies or be inhibited by the same chemical compounds. We thus decided to investigate which Rac GTPases are expressed in mature erythrocytes. Rac3 is known to be absent in human RBCs (56) while Rac2 is expressed in hematopoietic cells (55-57), but only Rac1 was identified by previous proteomic analyses of mature human erythrocytes (http://rbcc.hegelab.org).

In order to confirm the presence of Rac1 in mature erythrocytes and the absence of Rac2, we performed a western blot analysis of mature human erythrocytes with an antibody specific for Rac1 and an antibody specific for Rac2. First, we validated antibody specificity. To do so, we loaded on a gel purified GST-tagged Rac1 protein and purified histidine-tagged Rac2. Proteins were then transferred to a nitrocellulose membrane and hybridized with anti-Rac1 and anti Rac2 antibodies. Anti-Rac1 antibody did not recognize 100 ng of purified Rac2-His protein and anti-Rac2 antibody did not recognize 233 ng of purified anti-Rac1-GST protein, corresponding to 100 ng of Rac1, thus confirming their specificity (Figure 11).

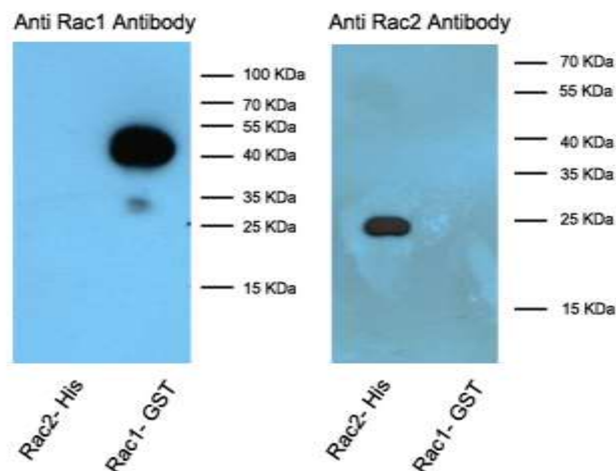

Figure 11. Antibody validation. We loaded 233 ng of purified Rac1-GST protein and 100 ng of purified Rac2-His protein. Signals were detected by using an anti-Rac1 specific antibody (left panel) and an anti-Rac2 specific antibody (right panel).

Once demonstrated the selectivity of our antibodies, we sought to investigate the expression of Rac1 and Rac2 in mature human erythrocytes. A sample of $5x10^7$ erythrocyte membranes was loaded on a gel, then transferred to a nitrocellulose membrane and hybridized with either an anti-Rac1 antibody or with an anti-Rac2 antibody (Fig. 12).

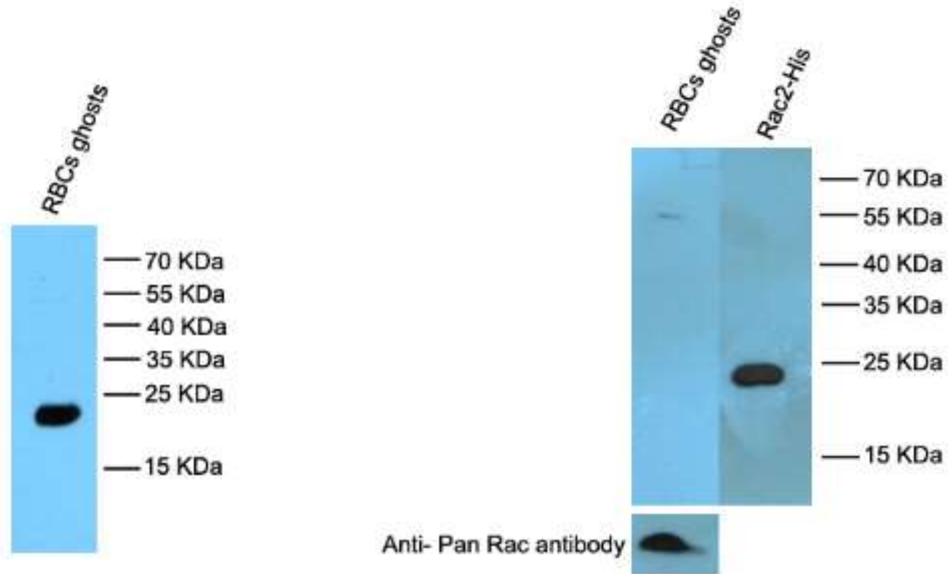

Figure 12. Western blot analysis of human erythrocyte membranes. Left panel: A sample of $5x10^7$ erythrocyte membrane was hybridized by using an antibody specific for Rac1. Right panel: $5x10^7$ erythrocyte membranes and 100 ng of purified Rac2-His protein were detected using an anti-Rac2 specific antibody. Anti-Pan Rac antibody was used as a loading control.

While we see a significant signal of Rac1 in $5x10^7$ erythrocyte membranes, no signal was detected with the anti-Rac2 antibody in the same membrane amount. This result confirms that Rac1 is the only detectable Rac GTPase in mature human erythrocytes, as previously reported in proteomic analysis.

We then sought to approximately assess the amount of the GTPase present in erythrocyte membranes. To do this, we compared the signal of a sample of $5x10^7$ erythrocyte membranes to the signal resulting from different amounts of purified Rac1-GST protein. The signal of Rac1 in the membrane sample was comparable to the signal resulting from 100 ng of Rac1-GST protein, equivalent to about 44 ng of Rac1 (Fig. 13). The amount of Rac1 in 10^7 erythrocyte membranes is thus approximately 10 ng.

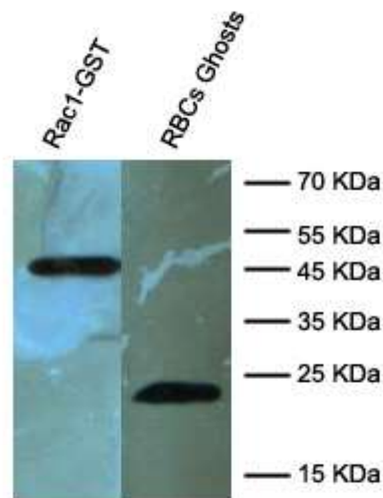

Figure 13. Quantification of Rac1 signal. A sample of $5x10^7$ human erythrocytes membranes and 100 ng of purified Rac1-GST protein were hybridized with an anti-Rac1 antibody.

4.2. Rac1 localization on *P. falciparum.*

4.2.1. Rac1 localizes in proximity to the PVM.

Once demonstrated that Rac1 is expressed in mature human erythrocytes, we sought to assess its cellular localization in non-infected erythrocytes and in erythrocytes infected by *P. falciparum*. We thus performed an immunofluorescence assays of fresh RBCs. Band3 is an erythrocyte membrane marker (Fig. 14). This experiment showed that Rac1 localizes on the erythrocyte membrane of uninfected RBCs, with a dotty pattern.

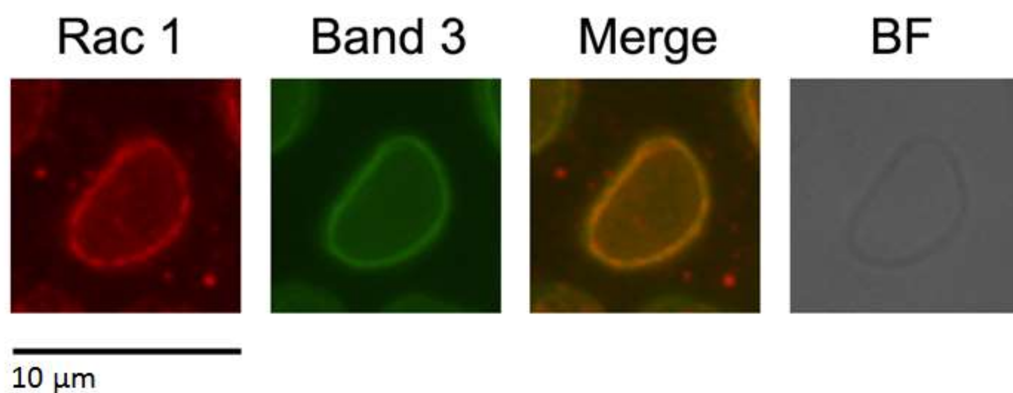

Figure 14. Rac1 subcellular localization in uninfected human erythrocyte. Immunofluorescence assay of human erythrocytes. Primary antibody against Band3 was used to mark the erythrocyte membrane. BF: Bright field.

Then, in order to assess Rac1 localization during *P. falciparum* infection, we performed a time course experiment.

Parasites from the 3D7 strain were synchronized by Percoll gradient and hallowed to reinvade human RBCs for 1 hour. After removing schizonts by 5% sorbitol treatment, smears were taken at 1, 6, 25, 31 hours post invasion (hpi). The time points were chosen in order to be able to study all the stages of *P. falciparum* blood cycle (Fig. 15).

Parasite nuclei are stained with DAPI (since erythrocytes lack a nucleus, the only genetic material belongs to the parasite). EXP1 is a parasite protein that marks the parasitophorous vacuole membrane (PVM) (98). Rac1 is marked with a monoclonal antibody (Protein Tech).

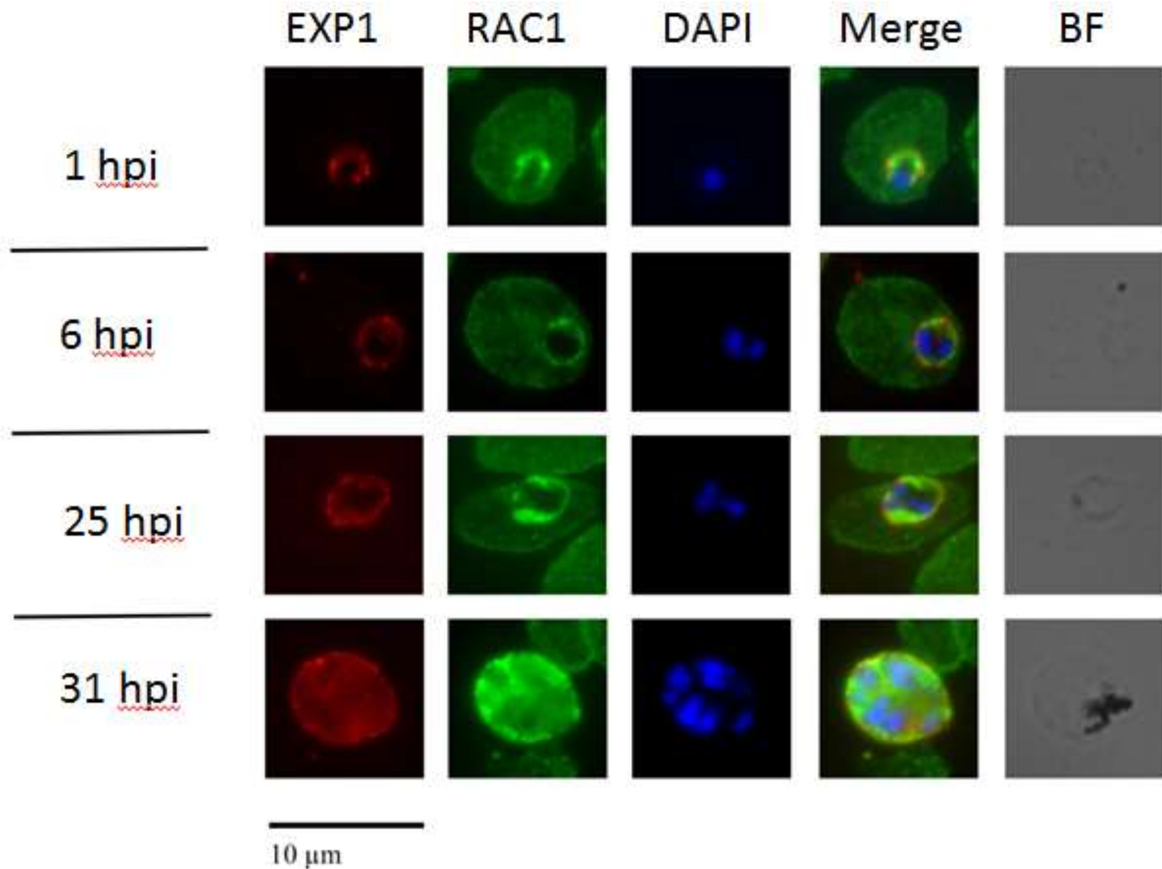

Figure 15. Rac1 subcellular localization in infected erythrocytes. Immunofluorescence assay of human erythrocytes, infected with synchronous parasites from the *P. falciparum* 3D7 strain, fixed at 1, 6, 25, 31 hours post invasion (hpi). Primary antibody against EXP1 was used to mark the parasitophorous vacuole membrane. Parasite nuclei were stained with DAPI. BF: Bright field.

It is clear from immunofluorescence images that Rac1 localizes in proximity to the PVM, in all the parasite stages, but does not completely colocalize with it. In order to assess the possible co-localization between Rac1 and the PVM marker EXP1, we calculated by using ImageJ, the Pearson's correlation coefficient between the signals of the two proteins. The value of the

coefficient is 71,75 ±9,4%, too low to exclude a casual overlap between the two signals. So, Rac1 doesn't colocalize perfectly with the PVM marker and we can only conclude that it is internalized and localized in proximity to the PVM.

4.2.2. Temporal diffusion of Rac1 around the PVM.

Rac1 initially shows a half circle localization (1 hpi, Figure 15), redistributing later on around the PVM (6 hpi, Figure 15). To confirm this, we counted the different forms on the blood smears produced in the *time course* experiment, to evaluate the phenotypic differences at different time points. We counted the number of parasite showing a Rac1 signal with an open ring shape (identified ad *semicircles*) and those showing a Rac1 signal with a closed ring shape (identified as *rings*) both in the samples at 1 and 6 hpi (Fig. 16 A). The results are shown in the pie chart in Fig. 16 B.

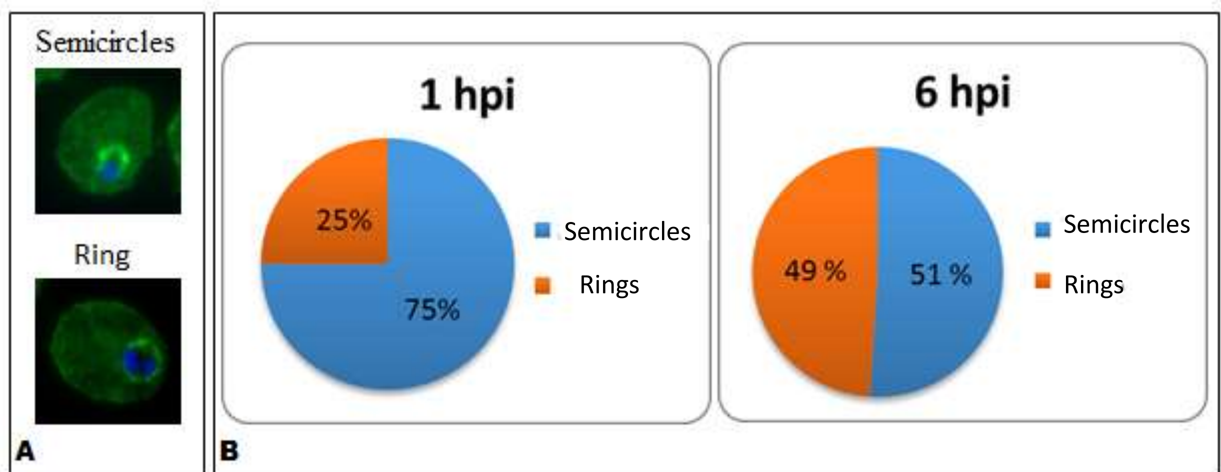

Figure 16. Rac1 distribution over time on *P. falciparum* PVM. A) Immunofluorescence with anti-Rac1 antibody of human erythrocytes infected with synchronous P. falciparum parasites, fixed 1 hour and 6 hours post invasion (hpi) **B)** Percentage of semicircles and ring phenotypes in the sample at 1 hpi and 6 hpi.

The results show that at 1 hpi, just after invasion of the host cell, most of the parasites show the Rac1 signal with a shape of an open circle, while at 6 hpi, the percentage of the two forms is similar, meaning that Rac1 diffuse around the parasite. At 20 hpi, all the parasite show Rac1 on all the PVM. These observations mean that Rac1 diffuse around the parasite during its intraerythrocytic life cycle.

4.3. Rac1 localization during *P. falciparum* invasion of human erythrocytes.

In order to study the subcellular localization of Rac1 during the invasion, we performed immunofluorescence assays on a sample enriched in merozoites. To obtain this enrichment, we treated synchronous schizonts with the protease inhibitor E64, that reversibly inhibits merozoite release. The sample was then washed and smears were taken 5 minutes later, in order to have several invasion events per field at 100X microscope magnification. The immunofluorescence was performed using a polyclonal antibody against Rac1, an antibody against Ron4 (Rhoptry Neck Protein 4), a protein involved in the formation of the *moving junction* during erythrocyte invasion by merozoite and an antibody against Rac1-GTP, specifically recognizing only the active form of the GTPase (Fig. 17).

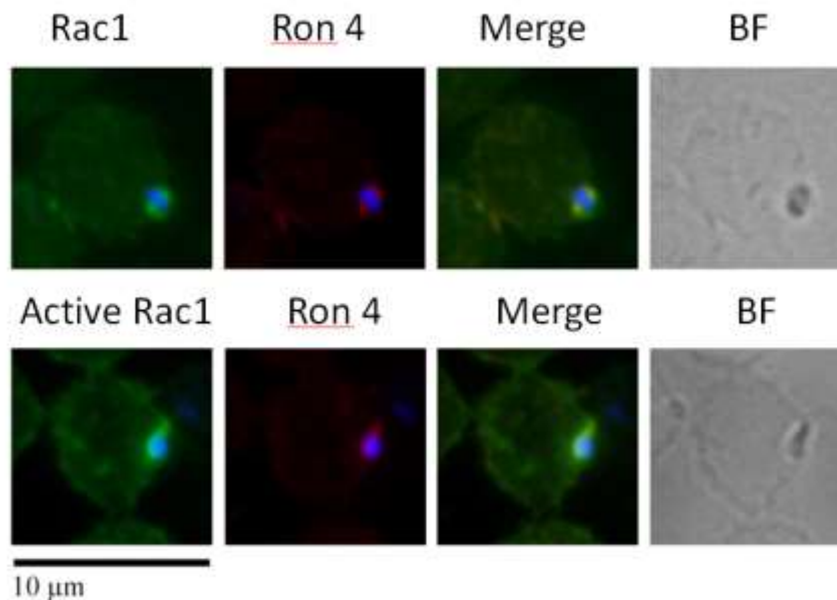

Figure 17. Subcellular localization of Rac1 during the invasion process. Immunofluorescence of a merozoite-enriched culture. Ron4 is used as a moving junction marker. Nuclei are stained with DAPI. Upper panel: During invasion, Rac1 co-localizes with the moving junction marker Ron4. Lower panel: Rac1 is activated by the parasite at the site of parasite entrance. BF: Bright field

As shown in Fig. 17, during invasion the *moving junction* marker Ron4 localizes with Rac1, which is recruited to the invasion site. Our results also show that Rac1 is here in its active form, GTP-bound (Fig. 16, lower panel).

4.4. Rac1 localization during the intraerythrocytic development of *P. falciparum.*

4.4.1. Rac1 depletion from erythrocyte membrane and recruitment on the trophozoite.

During parasite intraerythrocytic growth, Rac1 is further recruited from the parasite and depleted from the erythrocyte membrane, consistently with what found in (86). In this study, the authors compared by subtractive proteomic analysis membranes from uninfected erythrocytes and from erythrocytes infected by parasites at 35 hpi and identified Rac1 as one of the proteins depleted by the parasite from the host membrane.

At 6 hpi Rac1 signal intensity on erythrocyte membranes is similar in infected and non-infected cells (Fig. 18, top panel), while at 25 hpi Rac1 signal intensity is significantly lower on membranes of infected erythrocyte compared to non-infected ones.

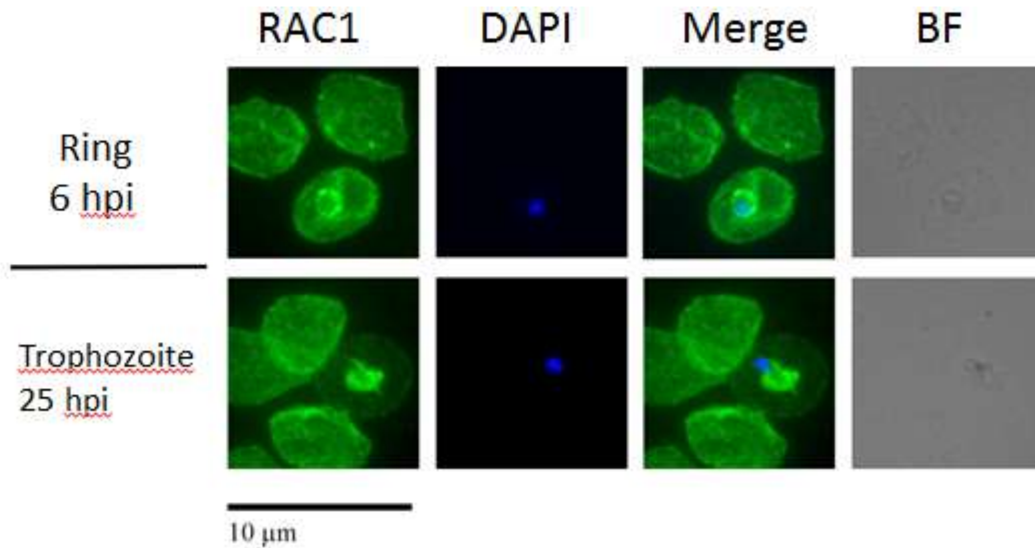

Figure 18. Depletion of Rac1 from infected erythrocyte membranes. At the trophozoite stage, the fluorescence intensity of Rac1 antibody decreases on the membrane of infected erythrocytes, compared to uninfected erythrocytes. BF= bright field

This observation suggests that Rac1 is actively recruited by the parasite, being gradually depleted from the host erythrocyte, during its development inside the host cell.

4.4.2. Rac1 activation on *P. falciparum.*

In order to assess if the parasite activates Rac1 during its intraerythrocytic development, a sample of synchronous ring stage parasites was analysed by immunofluorescence, using an antibody against Rac1 and an antibody specific against Rac1-GTP, the active form of the protein. In the lower panel, it is clear that the signal of the active form of Rac1 is significantly higher on the parasitophorous vacuole, compared to the erythrocyte membrane, while on the upper panel the signal from total Rac1 is comparable on the parasitophorous vacuole and on the erythrocyte membrane (Fig. 19).

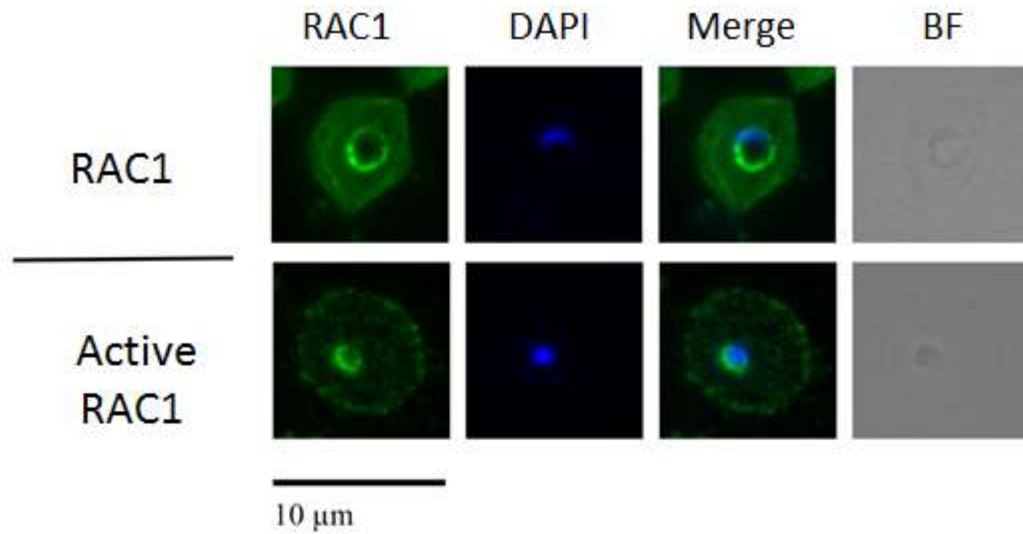

Figure 19. Subcellular localization of active Rac1. Erythrocytes infected with ring-stage parasites were stained with an antibody against Rac1 (upper panel) and an antibody that specifically recognize the active form of the GTPase (lower panel). Comparing the two images it is clear that Rac1 in its active form is found preferentially on the parasitophorous vacuole, while the total Rac1 is both on the parasitophorous vacuole and on the erythrocyte.

In order to confirm these results, the intensity of Rac1 and active Rac1 signals were quantified with the ImageJ software, measuring the intensity of the fluorescence on the PVM and on the erythrocytes membranes.

Once obtained the data on the signal intensities, the ratio between the signal on the PVM and the signal on the erythrocyte membrane was assessed, both for Rac1 and for its active form. Results are showed in the graphic (Fig. 20).

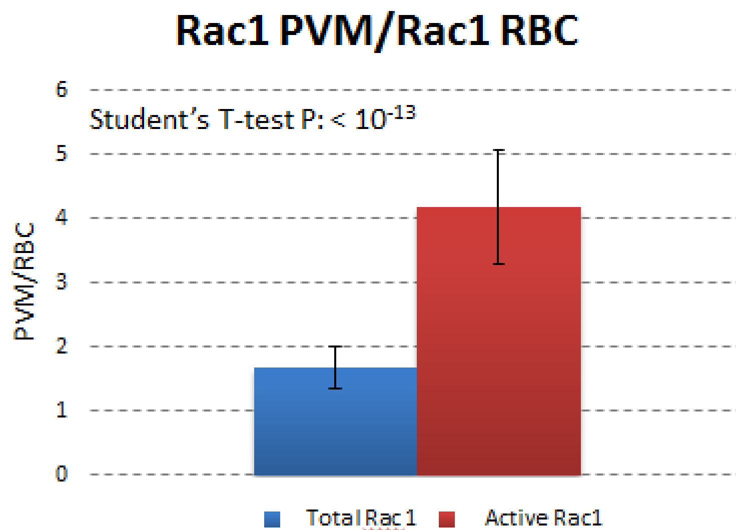

Figure 20. Amount of fluorescence signal from Rac1 and active Rac1. The graphic shows the ration between the fluorescence intensities on the PVM and on the erythrocyte membrane (see fig. 19) from total Rac1 and from its active form. The ratio between the PVM and the erythrocyte membrane is significantly higher for the active form of the protein, compared to the total Rac1, thus demonstrating that Rac1 is activated by the parasite on the PVM.

From the analysis it is clear that the ratio between Rac1 signal between the PVM and the erythrocyte is significantly higher from the antibody that recognizes the active form of the protein, compared to the signal from the antibody that recognizes all of the protein. This demonstrates that on the parasitophorous vacuole Rac1 is preferentially present in its active form, GTP-bound and that the protein is activated by the parasite, thus confirming the hypotesis that the parasite exploits the host Rac1 protein.

4.5. Functional analysis of Rac1 during *P. falciparum* infection of human erythrocytes.

4.5.1. Antiplasmodial activity of Rac1 chemical inhibitors.

In order to investigate Rac1 role in *P. falciparum* infection, we purchased two Rac1 chemical inhibitors and tested their effect on asyncrhonous *P. falciparum* cultures from two different strains – the chloroquine sensitive D10 and the chloroquine resistant W2 - in collaboration with Sarah D'Alessandro and Silvia Parapini, from "Statale" University of Milan. These inhibitors block Rac1 function in two different and independent ways. **EHT-1864** binds to Rac1 with high affinity and specificity on the site that catalizes the hydrolisis of GTP to GDP, thus inhibiting the GTPase activity and blocking, as a consequence, its signalling (61). The inhibitor **1A116** instead, inhibits Rac1 by binding to the surface containing Trp65, a residue critical for interaction with GEFs (63), thus inhibiting Rac1 activation by these proteins. Both inhibitors, tested for their specificity against Rac1, did not affect the activation state of the closely related GTPase CDC42 (61, 63).

Parasites were cultured for 72 hours in presence of Rac1 inhibitors at different concentrations. The viability of the parasites was then assessed by measuring the parasite lactate dehydrogenase (pLDH) activity (Tab. 3) (94).

Compounds	D10 IC_{50} (nM)	W2 IC_{50} (nM)
EHT1864	3876,9 ± 738,0	7748,5 ± 1821,0
1A116	3.209 ± 1.173	6.888 ± 1.407
CQ	16,3 ± 3,4	418.1 ± 75,3

Tab. 3 Antimalarial activity of EHT-1864 and 1A116 inhibitors on *P. falciparum* parasites. The inhibitors were tested on the chloroquine (CQ)-sensitive D10 line and the CQ-resistant W2 lines. CQ was used as positive control. The values are the average ± standard deviation of three biological replicates, each performed in duplicate.

Both Rac1 chemical inhibitors show antimalarial activity in both cell lines, revealing a contribution of Rac1 to *P. falciparum* survival during erythrocyte infection and thus making thus these molecules adequate to study Rac1 functional contribution to *P. falciparum* infection of human erythrocytes.

4.5.2. Rac1 contribution to *P. falciparum* invasion of human erythrocytes.

Once we assessed Rac1 role in *P. falciparum* viability, we then sought to specifically assess wether the GTPase has a functional role in *P. falciparum* invasion of human erythrocytes. To do so, we performed invasion assays in presence of the Rac1 inhibitors EHT-1864 and 1A116 at concentrations ranging from 10 to 100 uM. Synchronous schizonts were double purified by 60% Percoll gradient followed by MACS column purification system and allowed to reinvade fresh erythrocytes in the presence of Rac1 inhibitors for about 6 hours. The number of newly infected erythrocytes was estimated by normalizing the number of the rings on the number of schizonts that released merozoites during the assay, according to this formula:

Invasion rate: Rings/ [Schizonts T0h - Schizonts T6h]

Where the number of schizonts releasing merozoites was calculated as the difference between the number of schizonts at the end of the assay (schizonts T6h) and their the number at the beginning of the assay (schizonts T0h). Invasion rates from the samples treated with Rac1 inhibitors were then normalized on the invasion rate of the control sample, obtaining the % of invasion rate reduction. The assay was performed in three biological replicates, each composed by three technical replicates (Fig. 21). Both the compounds resulted to significantly inhibit parasite invasion of the host cell in a dose-dependent manner.

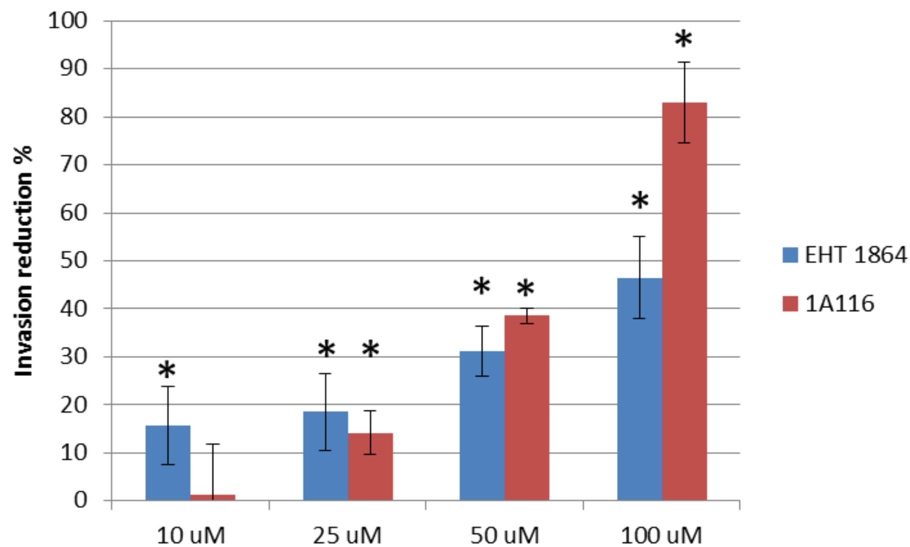

Figure 21. Dose-dependent reduction of *P. falciparum* invasion rates in the presence of the Rac1 inhibitors EHT-1864 and 1A116. The number of newly infected erythrocytes at the end of the assay was normalized on the control. The data shown here are the result of three independent experiments, each performed in three technical replicates. T-test compared to control: *= P <0,05

It is clear from the results that there is a significative dose-dependent correlation between the reduction of *P. falciparum* invasion rates and the concentration of the inhibitors. These results confirm that Rac1 plays a role in the invasion process of human erythrocytes by the parasite.

4.5.3. Rac1 contribution to *P. falciparum* intraerythrocytic growth.

Once we assessed Rac1 contribution to *P. falciparum* invasion of human erythrocytes, we sought to assess if the GTPase plays a role also in the subsequent intraerythrocytic growth of the parasite. To do so, we prepared a synchronous *P. falciparum* culture and took blood smears at different time points during the parasite growth inside the host cell. Syncrhonous schizonts were purified by 60% Percoll gradient and then seeded on fresh human RBCs and

allowed to invade the cells for two hours. Schizonts were then killed by 5% sorbitol treatment, leaving in culture only young ring-stage parasite. Parasites were then resuspended in complete culture medium with the two Rac1 chemical inhibitors EHT 1864 and 1A11A at concentrations corresponding to ten-fold the IC_{50} concentration assessed in asynchronous culture (39 µM and 32 µM respectively). Blood smears were taken at 20, 27 and 39 hours post invasion, corresponding to mature ring, trophozoite and schizont stages. Parasitemia was assessed by counting the parasites on Giemsa stained smears. Parasitemia decreased significantly in 1A116-treated sample compared to control, and the reduction increases over time. No evident reduction of the parasitemia was revealed in the sample treated with EHT-1864 (Fig. 22).

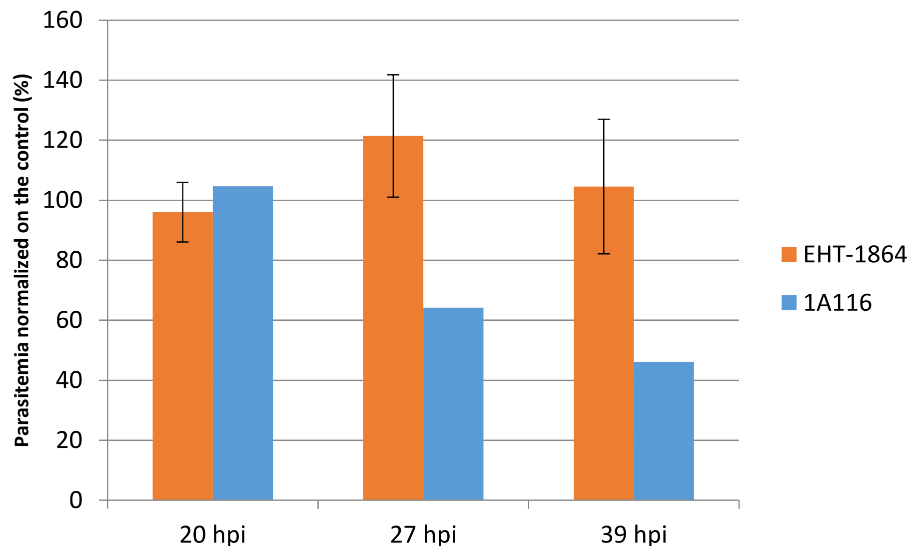

Figure 22. Effects of Rac1 chemical inhibitors on *P. falciparum* intraerythrocytic growth. Blood smears were taken at 20, 27 and 39 hours post invasion, corresponding to mature ring, trophozoite and schizont stages. Parasitemia was normalized on the control. The data shown for EHT-1864 are the result of three indipendent biological replicates, while for 1A116 the data are the preliminar result of a single experiment.

Although no significant reduction in parasitemia was observed in the sample treated with EHT-1864, it is clear that parasites treated with this Rac1 inhibitor show a delay in their growth, compared to untreated control. At 39 hpi, parasites treated with EHT-1864 are smaller than the control and show a lower number of nuclei (Fig. 23). In 1A116-treated sample, the surviving parasites are likewise smaller at 27h and 39h, compared to the control and show an aberrant morphology (Fig.23).

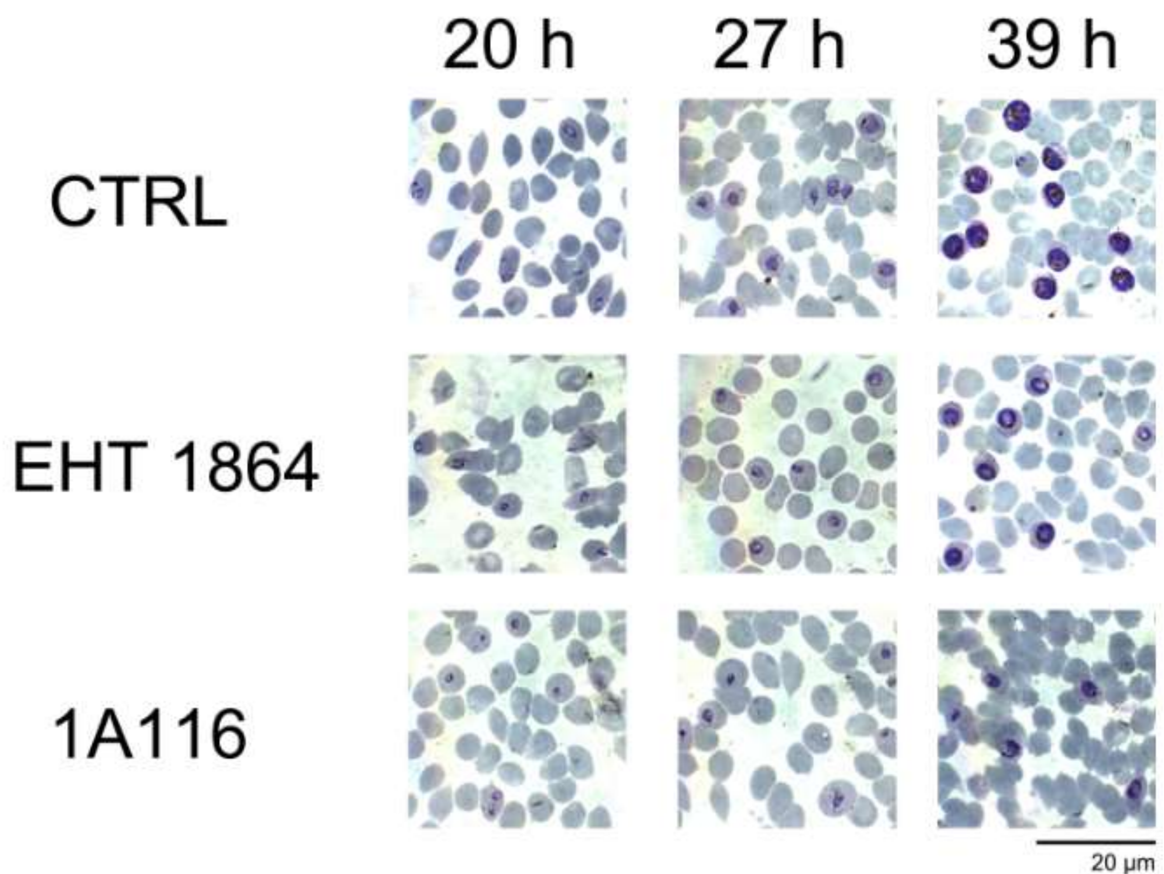

Figure 23. Morphology of *P. falciparum* parasites treated with Rac1 chemical inhibitors. Images from a Giemsa-stained smear of human erythrocytes infected with synchronous *P. falciparum* parasites, taken at 20, 27 and 39 hours post invasion, corresponding to mature ring, trophozoite and schizont stages of the parasite. The microscopy magnification is 100X.

These experiments show that two different Rac1 chemical inhibitors affect *P. falciparum* intraerythrocytic growth, revealing a role for the GTPase also in this phase of *P. falciparum* asexual cycle.

4.6. Effect of Rac1 chemical inhibitors on *P. falciparum* survival.

In order to further confirm Rac1 role in *P. falciparum* infection and in the search for more effective inhibitory molecules, in collaboration with Sarah D'Alessandro and Silvia Parapini, from "Statale" University of Milan, other eight commercially available Rac1 chemical inhibitors were tested on asynchronous cultures. All of them showed antimalarial activity with IC_{50} below 40 µM both on the chloroquine-sensitive D10 strain and on the chloroquine-resistant W2 strain. Among them, two (EHop-016 and Aza-1) showed nanomolar IC_{50} (Tab. 4).

Compounds	D10 IC_{50} (nM)	W2 IC_{50} (nM)
EHT1864	3876,9 ± 738,0	7748,5 ± 1821,0
1A116	3.209 ± 1.173	6.888 ± 1.407
NSC23766	5022,7 ± 1467,9	4048,9 ± 499,5
553511	11890,4 ± 7368,4	37473,8 ± 8669,1
EHop-016	138,8 ± 16,0	321,5 ± 28,5
ML141	10703,8 ± 289,0	17262,6 ± 1840,3
Aza-1	252,7 ± 33,6	758,0 ± 271,6
MLS532223	6853,8 ± 735,3	9512,5 ± 1661,2
MLS573151	31873,0 ± 6542,3	39693,3 ± 10884
ZINC 69391	17499,3 ± 4640,0	12559,3 ± 5254,4
CQ	16,3 ± 3,4	418.1 ± 75,3

Tab. 4 Antimalarial activity of different Rac1 inhibitors on *P. falciparum* parasites. The inhibitors were tested on the chloroquine (CQ)-sensitive D10 line and the CQ-resistant W2 lines. CQ was used as positive control. The values are the average ± standard deviation of three biological replicates, each performed in duplicate.

These results strongly confirm the role of the GTPase Rac1 in *P. falciparum* infection of human erythrocytes and suggest that Rac1 could be a promising target for the development of novel anti-malarial drug.

5. Discussion

Here we analyzed the localization and function of the human GTPase Rac1 in infection of human erythrocytes by *P. falciparum*, both during the invasion process and the subsequent intraerythrocytic parasite growth.

We first demonstrated that Rac1 is recruited and activated during invasion of RBCs to the parasite entry site. This observation suggests that parasites exploit the host signalling machinery in order to enter the host cell. We know from the literature that this strategy is used by several intracellular pathogens, that stimulate host receptors in order to exploit its signalling pathways (99, 100). In most cases, the outcome of highjacking Rac1 signalling pathway is the manipulation of the host actin cytoskeleton. It is indeed well known that intracellular pathogens entry into the host cell and subsequent intracellular development are strictly associated with their ability to manipulate the host cytoskeleton. For instance, *Listeria monocytogenes* promotes its internalization in human host cells by activating Rac1 signalling. This leads to the formation of actin filaments that will produce membrane protrusions around the bacterium adhesion site, facilitating its internalization (101). Moreover, during invasion of the host cell, *C. jejuni* activates the GEFs DOCK 180 and Tiam1 in order to activate Rac1, causing local rearrangement of the actin cytoskeleton at the site of entry (68). *S.aureus* requires the formation of actin-rich membrane protrusions that aid the bacterium engulfment. Inactivation of *rac1* gene decreases the invasion rate, while the transfection of a dominant-positive form of the protein increases the invasion rate (70) Also the intracellular apicomplexan parasite *Cryptosporidium parvum* requires host cell actin polymerization in order to penetrate inside the host cell (102). Interestingly, it was also shown that both *T. gondii* tachyzoites and *P. berghei* sporozoites require host actin polymerization in order to enter inside the host cell (103). However a recent work showed that treatment with reversible actin polymerization inhibitors does not prevent *P. falciparum* entry into the host cell and stated that the participation of host cell actin to *P. falciparum* invasion appears unlikely (104). Our data contradict this conclusion, supporting the hypothesis of a role of the host actin in *P. falciparum* invasion of the host cell, as already reported for other apicomplexan parasites and for *P. berghei* sporozoites (102, 103).

We here showed that erythrocyte treatment with two Rac1 inhibitors significantly reduced parasite invasion rates. Being Rac1 the only Rac protein identified by western blot in mature erythrocytes, these inhibitors were most probably acting specifically on Rac1. The two inhibitors block Rac1 activation with different mechanisms, one blocking the catalytic site of the GTPase and thus its enzymatic activity, the other inhibiting Rac1 interaction with its activating partner proteins, the GEFs. Moreover, invasion rate reduction was dose-dependent in both cases. It is thus very unlikely that the observed phenotype might be due to off target molecules.

Since treatment with Rac1 inhibitors did not induce a complete inhibition of parasite invasion, we cannot claim that Rac1 plays an essential role in *P. falciparum* invasion of host erythrocytes. However, this could be due to different reasons. First, the inhibitors could not have been effective in inhibiting the whole Rac1 molecules present in the cell, still consenting the parasite to carry out invasion with lower efficiency. Another explanation could be that parasites exploit also alternative pathways for invasion, beyond the one involving Rac1. Finally, Rac1 could also have a secondary role in the invasion process, like for instance Semaphorin-A (105) and Glicophorin-A (106).

After invasion, Rac1 localizes in proximity to the PVM and diffuses on it over time during the parasite intraerythrocytic growth. We also demonstrated that during parasite intraerythrocytic growth Rac1 is depleted from the erythrocyte membrane, being accumulated and activated in proximity to the PVM. This result suggests that the parasite recruits and activates Rac1 in order to exploit its signalling also in this phase of the life cycle.

It was previously suggested that *P. falciparum* may remove Rac1 from the erythrocyte membrane in order to destabilize the host cytoskeleton to allow the egress of daughter merozoites. By comparing the proteomes of uninfected RBCs and RBCs infected by parasites ready to egress (35 hpi), it was shown that Rac1 disappears from the host cell membrane together with other proteins, including adducin. The authors state that the removal of Rac1 and adducin may contribute to the parasite dismantling of the host plasma membrane (86). These results are consistent with our IFA results that showed that Rac1 is depleted from the erythrocyte membrane and recruited to the PVM.

We also demonstrated a functional role for Rac1 in intraerythrocytic growth of the parasite, by using two Rac1 chemical inhibitors in growth assays. Both the inhibitors affect either parasite survival or its growth rate. Based on available information, it is difficult to assess the reasons why parasites activate Rac1 in this phase of the life cycle, but we hypothesize it may be related to actin cytoskeleton regulation. Rac1 signalling could be involved in modulating the actin cytoskeleton, to facilitate parasite volume increase. It was already reported that host actin plays a role in *P. berghei* intracellular growth inside hepatocytes, possibly involving PVM remodelling. It was reported that hepatocyte actin accumulates around the PVM and important actin reorganization events take place between 10 and 16 hpi (107). A recent work demonstrated that this is the case also in erythrocytes infected by *P. falciparum* (108). Our results are consistent with these reports.

Another possible role for Rac1 could be in intracellular vescicle trafficking. In fact, actin is known to be involved in intracellular trafficking of *Maurer's clefts*, parasite-induced membranous compartments that *P. falciparum* forms inside the host erythrocyte in order to export factors involved in cytoadherence on the surface of the host cell (109). This could imply that Rac1 chemical inhibitors could be even more effective in vivo, by acting on *Maurer's clefts* trafficking, thus impairing RBCs cytoadherence, that is the main cause of severe clinical symptoms in *P. falciparum* infections.

Rac1 has several advantages as a pharmacological target, compared to other molecules. First, due to its role in several pathologies and cellular mechanisms, Rac1 is a widely studied molecule: more than 7500 articles were published about this GTPase. Rac1 crystal structure, as well as several of its interacting proteins are known and numerous Rac1 inhibitors are already available. Among these inhibitors, some have already been tested *in vivo*, like EHop-016 and others, like Azathioprine, are already in clinical use. We here tested different Rac1 inhibitors for their anti-malarial activity. Ten different inhibitors were able to kill the parasite and two of them (EHop-016 and Aza-1) showed antimalarial activity at nanomolar IC_{50}. All these facts could facilitate the production of a drug able to selectively modulate the activity of this well-known GTPase.

Moreover, the development of an antimalarial drug acting on the host rather than on the parasite, could be less prone to induce resistance in the parasite. In fact, while a mutation on the parasite genome could easily reduce the efficacy of a drug that acts on the parasite, it would be less simple for the parasite to change the entire infection strategy in order to circumvent the absence of an host factor (22). This approach, defined "host directed therapies (HDT)" was effective in clinical use against *Hepatitis C* (30, 31) and HIV/AIDS (32). Moreover, host-targeted molecules have already been proven effective against different intracellular pathogen (24-27) . Moreover, Conoidin A, a Prx2 host peroxidase inhibitor was shown to prevent parasite growth and make it more sensitive to cloroquine (29). A host-directed drug could also act on different parasite strains and maybe also against different *Plasmodium* species. Because of the role of Rac1 in several tissues, host-targeted therapies directed against this protein could have toxic effects. A possible strategy to overcome this issue could be to specifically address inhibitory molecules against human erythrocytes by tissue-specific drug delivery strategies.

In conclusion, we here propose the human GTPase Rac1 as a promising target for the development of novel antimalarial drugs addressed against the host. Other studies should be improved in order to better understand the mechanisms that parasites use in order to exploit Rac1 signalling during the infection of human erythrocytes. An interesting approach could be to knock out *rac1* gene in erythrocyte precursors and then induce them to differentiate in mature erythrocytes that will be infected with *P. falciparum*. Co-immunoprecipitation experiments would be useful in order to define which molecules interact with Rac1 in erythrocytes and during *P. falciparum* infection. Finally, use *of targeted molecular design* based on the knowledge of Rac1 crystal structure, could allow the synthesis of inhibitory molecules directed against Rac1 or its interactors, effective and well tolerated for a human pharmacological therapy by improving their effectiveness and specificity.

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