Expression of human CD81 differently affects host cell susceptibility to malaria sporozoites depending on the *Plasmodium* species

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Summary

*Plasmodium* sporozoites can enter host cells by two distinct pathways, either through disruption of the plasma membrane followed by parasite transmigration through cells, or by formation of a parasitophorous vacuole (PV) where the parasite further differentiates into a replicative exo-erythrocytic form (EEF). We now provide evidence that following invasion without PV formation, transmigrating *Plasmodium falciparum* and *Plasmodium yoelii* sporozoites can partially develop into EEFs inside hepatocarcinoma cell nuclei. We also found that rodent *P. yoelii* sporozoites can infect both mouse and human hepatocytes, while human *P. falciparum* sporozoites infect human but not mouse hepatocytes. We have previously reported that the host tetraspanin CD81 is required for PV formation by *P. falciparum* and *P. yoelii* sporozoites. Here we show that expression of human CD81 in CD81-knockout mouse hepatocytes is sufficient to confer susceptibility to *P. yoelii* but not *P. falciparum* sporozoite infection, showing that the narrow *P. falciparum* host tropism does not rely on CD81 only. Also, expression of CD81 in a human hepatocarcinoma cell line is sufficient to promote the formation of a PV by *P. yoelii* but not *P. falciparum* sporozoites. These results highlight critical differences between *P. yoelii* and *P. falciparum* sporozoite infection, and suggest that in addition to CD81, other molecules are specifically required for PV formation during infection by the human malaria parasite.

Introduction

Malaria remains the most important parasitic human disease, responsible for millions of deaths each year. *Plasmodium* infection is initiated by the inoculation of sporozoites in the host by a female *Anopheles* mosquito. Following transmission by the mosquito, sporozoites enter the bloodstream and rapidly reach the liver and infect hepatocytes, where they further differentiate into a replicative exo-erythrocytic form (EEF) that will ultimately give rise to thousands of merozoites that initiate the pathogenic erythrocytic cycle. Infection of hepatocytes by *Plasmodium* sporozoites involves the formation of a membrane-bound compartment called the parasitophorous vacuole (PV), where the parasite resides for further development. Sporozoites can also enter cells directly without PV formation, by disrupting the cell plasma membrane (Mota *et al.*, 2001). After gliding through the cell cytoplasm, the parasites eventually egress by rupturing the plasma membrane again.

One characteristic feature of the cell-traversal mode of entry is the absence of cell type selectivity, because
sporozoites from several *Plasmodium* species have been shown to migrate through various types of cells from various host species *in vitro* (Mota *et al*., 2001). This mode of cell entry reflects the need for the parasite to migrate through different tissues of the host, first in the skin then in the liver sinusoids, before it reaches hepatocytes (Baldacci and Menard, 2004; Frevert, 2004). Several parasite proteins have been recently identified which are involved in this process, including the sporozoite microneme proteins essential for cell-traversal (SPECT)-1 and SPECT-2 (Ishino *et al*., 2004; 2005; Kaiser *et al*., 2004), and a protein with a phospholipase activity (Matuschewski *et al*., 2002; Bhanot *et al*., 2005).

In contrast with transmigration, infection of host target cells is a much more restricted process. The PV formation presumably depends on the antero–posterior translocation of a tight junction formed by sporozoite adhesins bound to their cognate receptors on the host cell surface (Baldacci and Menard, 2004; Kappe *et al*., 2004). The nature of the parasite and host components forming the junction still remains unknown. It is likely that the specificity of molecular interactions between the parasite and the host cell accounts for the selectivity of host cell types supporting the development of EEFs. Sporozoites from the deadly human parasite *Plasmodium falciparum* only infect hepatocytes from human and a few non-human primate species both *in vivo* (Druijhe *et al*., 1982; Meis *et al*., 1990; Zapata *et al*., 2002) and *in vitro* (Mazier *et al*., 1985; Millet *et al*., 1991). There is currently no cell line supporting *P. falciparum* sporozoite infection and EEF development, in contrast with *Plasmodium vivax* and rodent plasmodial species, which are able to develop not only in primary hepatocytes (Mazier *et al*., 1982; Mazier *et al*., 1984) but also in hepatoma cell lines (Hollingdale *et al*., 1983; 1985). In particular, sporozoites from the rodent parasite *Plasmodium yoelii* can infect mouse hepatoma cell lines (Mota and Rodriguez, 2000), while another rodent parasite, *Plasmodium berghei*, can infect not only mouse but also human hepatoma cells, such as the human hepatocarcinoma cell line HepG2 (Hollingdale *et al*., 1983; Mota and Rodriguez, 2000).

The narrow host cell tropism of *P. falciparum* sporozoites could be determined by several factors, including surface receptors and host molecules required for the intracellular growth of the parasite. We have recently reported that the tetraspanin CD81, a receptor for the hepatitis C virus (HCV) (Pileri *et al*., 1998; Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005), is required on hepatocytes for infection by *P. falciparum* and *P. yoelii* sporozoites (Silvie *et al*., 2003). In the absence of evidence of a direct interaction between CD81 and the parasite, it seems that CD81 does not act directly as a receptor for the sporozoites but is rather involved indirectly. Here, we investigated whether CD81 expression contributes to the host cell tropism during infection by *P. falciparum* and *P. yoelii* sporozoites.

**Results**

**Host specificity of *P. falciparum* and *P. yoelii* sporozoites in vitro**

To gain further insight into host–parasite interactions during sporozoite infection, we first analysed the host species specificity during infection of hepatocytes by *P. falciparum* and *P. yoelii* sporozoites. Primary hepatocytes isolated from human and mouse liver fragments were inoculated with *P. falciparum* or *P. yoelii* sporozoites, and infection was monitored by counting EEFs after immunolabelling. As expected, we observed *P. falciparum* EEFs in human hepatocytes and *P. yoelii* EEFs in mouse hepatocytes (Fig. 1A and B). Interestingly, while *P. falciparum* sporozoites failed to infect mouse hepatocytes (Fig. 1A), *P. yoelii* sporozoites efficiently infected human hepatocytes *in vitro*.

![Fig. 1. *P. falciparum* and *P. yoelii* sporozoite host species tropism.](image-url)
(Fig. 1B), raising EEFs with a similar morphology as seen in mouse hepatocytes. Notably, some parasites displayed typical features of maturing EEFs 48 h post infection, as evidence by staining with anti-HSP70 antibodies and DAPI (Fig. 1C).

Expression of human CD81 in transgenic mice confers susceptibility to \(P.\) yoelii but not to \(P.\) falciparum sporozoite infection

We have previously reported that expression of the tetraspanin CD81 on hepatocytes is required for infection by \(P.\) falciparum and \(P.\) yoelii sporozoites (Silvie et al., 2003). To investigate whether CD81 accounts for the host specificity during infection by \(P.\) falciparum sporozoites, we tested whether expression of human CD81 (hCD81) in mouse hepatocytes could confer susceptibility to infection by \(P.\) falciparum and \(P.\) yoelii sporozoites. Primary hepatocytes isolated from hCD81 transgenic mice deficient for endogenous mouse CD81 (Masciopinto et al., 2002) were inoculated with \(P.\) falciparum and \(P.\) yoelii sporozoites. While CD81-deficient mouse hepatocytes are completely refractory to infection by \(P.\) yoelii sporozoites (Silvie et al., 2003), expression of human CD81 in transgenic mice completely restored \(P.\) yoelii sporozoite infectivity \textit{in vitro} (Fig. 2A). As expected, an anti-human CD81 mAb could block infection of hCD81 transgenic but not wild-type mouse hepatocytes, while reciprocally an anti-mouse CD81 mAb was effective only in wild-type cells (Fig. 2A and B). Human CD81 transgenic mice were also fully susceptible to \(P.\) yoelii sporozoite infection \textit{in vivo}, because all mice (\(n = 5\)) injected intravenously with \(4 \times 10^3\) \(P.\) yoelii sporozoites developed a patent blood-stage infection 3 days post injection, similarly to wild-type control mice. These results clearly demonstrate that human CD81 expressed in mouse hepatocytes is functional as regards to \(P.\) yoelii sporozoite infection.

We also attempted to infect hCD81 transgenic mouse hepatocytes with \(P.\) falciparum sporozoites, but we constantly failed in obtaining any \(P.\) falciparum EEF in these cells (data not shown), demonstrating that expression of human CD81 is not sufficient to promote \(P.\) falciparum sporozoite infectivity in mouse hepatocytes. Based on the fact that \(P.\) falciparum sporozoites infect human but not mouse hepatocytes, and together with the absence of evidence of a direct interaction between CD81 and the parasite (Silvie et al., 2003), these results suggest that additional human molecules may be required during host cell infection by \(P.\) falciparum sporozoites.

\textit{Plasmodium yoelii} and \(P.\) falciparum EEFs develop intranuclearly in hepatocarcinoma cells

Human hepatocarcinoma HepG2 cells have been widely used as target host cells for \(P.\) berghei EEF cultures (Hollingdale et al., 1983). HepG2 cells do not express CD81 (Berditchevski et al., 1996; Charrin et al., 2001), and it has been reported that they do not support EEF development of \(P.\) falciparum (Hollingdale et al., 1984; Mazier et al., 1985) and \(P.\) yoelii (Calvo-Calle et al., 1994; Mota and Rodriguez, 2000). Unexpectedly, when we examined HepG2 cells infected with \(P.\) yoelii sporozoites, we constantly found small numbers of EEFs in these cells. However, while in hepatocytes EEFs are found in the cytoplasm next to the host cell nucleus, parasites observed in HepG2 cells (\(n = 195\)) were all abnormally located in the infected cell nucleus. Intranuclear localization was confirmed by confocal microscopy analysis (Fig. 3). These intranuclear forms displayed a limited degree of maturation, although most of the parasites had undergone several rounds of nuclear divisions, as shown by DAPI staining.
Limited numbers of *P. falciparum* EEFs were also observed in HepG2 cells, and as seen with *P. yoelii* these parasites (*n* = 88) were all located in the infected cell nucleus (Fig. 3).

The mouse hepatoma cell line Hepa1–6 supports infection and complete development of *P. yoelii* sporozoites (Mota and Rodriguez, 2000). Hepa1–6 cells express CD81 (Fig. 4A) and infection by *P. yoelii* sporozoites depends on CD81, as described with primary hepatocytes (Silvie *et al.*, 2003). Indeed, the knock-down of mouse CD81 using small interfering RNA (siRNA) (Fig. 4A) reduced the number of infected Hepa1–6 cells (Fig. 4B), while the knock-down of the tetraspanin CD9 (Fig. 4A) had no effect on infection (Fig. 4B), consistent with our previous observation that CD9 is not required during *P. yoelii* infection (Silvie *et al.*, 2003). Also, anti-mouse CD81 mAbs could efficiently block infection of Hepa1–6 cells by *P. yoelii* sporozoites (Fig. 4C). Interestingly, *P. yoelii* intranuclear EEFs were also observed in Hepa1–6 cells (Fig. 5A). Intranuclear localization of *P. yoelii* EEFs had not been reported in Hepa1–6 cells before, although in our hands intranuclear EEFs represented 10–25% of EEFs in these cells (Fig. 5B and C). Interestingly, anti-CD81 antibodies and siRNA targeting CD81 reduced the number of cytoplasmic *P. yoelii* EEFs but had no effect on intranuclear EEFs in Hepa1–6 cells (Fig. 5B and C).

Fig. 3. *P. yoelii* and *P. falciparum* EEFs develop intranuclearly in HepG2 cells. HepG2 cells were infected with *P. yoelii* (5 × 10⁴) or *P. falciparum* (1 × 10⁵) sporozoites and cultured for 36 h or 72 h, respectively, before immunofluorescence labelling with anti-HSP70 antibodies (green) and DAPI (blue) and examination by confocal fluorescence microscopy. EEFs (arrowheads) are clearly located in the infected cell nucleus. Bar, 10 μm.

Fig. 4. Infection of mouse hepatoma Hepa1–6 cells by *P. yoelii* sporozoites depends on CD81.
A. Hepa1–6 cells transfected with siRNA oligonucleotides targeting CD81 (siCD81), CD9 (siCD9) or with a control siRNA (cont) were stained with anti-CD81 mAb Eat1 (left panels) (Maecker *et al.*, 2000) or anti-CD9 mAb 4.1F12 (right panels) (Le Naour *et al.*, 2000) and analysed by flow cytometry.
B. *P. yoelii* EEF number in Hepa1–6 cells transfected with siRNA oligonucleotides targeting CD81 (siCD81) or CD9 (siCD9), or with a control siRNA.
C. Effects of anti-mouse CD81 mAbs Eat1 and Eat2 (Maecker *et al.*, 2000) on *P. yoelii* infection of Hepa1–6 cells.
HepG2 cells genetically engineered to express human CD81 support the complete development of P. yoelii but not P. falciparum EEFs

Because HepG2 cells do not express CD81, we wondered whether the exclusively intranuclear localization of P. falciparum and P. yoelii EEFs was due to the lack of CD81. Therefore, we investigated the effects of CD81 expression in HepG2 cells on infection by the two Plasmodium species. We tested two different HepG2 cell lines retrovirally transduced to stably express human CD81. These two HepG2/CD81 cell lines differ in the level of CD81 surface expression as shown by FACS analysis (Fig. 6A), and thus are here referred to as HepG2/CD81<sup>+</sup> and HepG2/CD81<sup>++</sup>. Confirming previous observations, CD81 was not detected on the surface of parental HepG2 cells, whereas HepG2/CD81<sup>++</sup> cells express ~4 times more CD81 than HepG2/CD81<sup>+</sup>. P. yoelii sporozoites readily infected HepG2/CD81 cells, rising high numbers of EEFs (Fig. 6B). Furthermore, a vast majority of these EEFs were located in the infected cell cytoplasm, close to the host cell nucleus, which corresponds to the normal localization of EEFs (Fig. 6C). These EEFs were bigger than the intranuclear forms seen in HepG2 cells, and displayed a degree of maturation that was comparable to that seen in mouse and human hepatocytes in vitro. Interestingly, the level of infection correlated well with the level of expression of CD81, as indicated by the higher number of EEFs in HepG2/CD81<sup>++</sup> as compared with HepG2/CD81<sup>+</sup> (Fig. 6B). To test whether infectious merozoites could form in these EEFs, naive BALB/c mice were injected with P. yoelii-infected HepG2/CD81<sup>++</sup> cells. Four out of five mice injected with HepG2/CD81 cultures 48 h post infection developed blood-stage infections, 4 ± 1 days after injection. This confirms that P. yoelii sporozoites can completely develop in HepG2/CD81 cells, in a way similar to the growth of P. berghei in HepG2 cells (Hollingdale et al., 1983).

We also analysed the fate of P. falciparum sporozoites in HepG2/CD81<sup>+</sup> and HepG2/CD81<sup>++</sup> cells. In sharp contrast with the very efficient infection of these cells by P. yoelii sporozoites, only limited numbers of EEFs could be observed (< 25 per well), even in cells expressing the higher level of CD81, and as seen with the parental CD81-negative HepG2 cells, these EEFs (n = 93) were all located in the infected cell nuclei (data not shown). This shows that CD81 expression in HepG2 cells is not sufficient to permit the development of cytoplasmic P. falciparum EEFs.

Subcellular localization of Plasmodium EEFs reflects the mode of cell entry of sporozoites

Although most of P. yoelii EEFs were found in the cytoplasm of infected HepG2/CD81 cells, intranuclear EEFs were also observed in these cells, in relatively small numbers, and thus representing only a minor fraction of the EEFs cells (2–10%, Fig. 6B). Importantly, the number of intranuclear EEFs was similar in HepG2/CD81<sup>+</sup> and HepG2/CD81<sup>++</sup> cells (Fig. 6B). Furthermore, siRNA targeting CD81 and anti-CD81 antibodies reduced the number of P. yoelii cytoplasmic EEFs but had no effect on the number of intranuclear EEFs in HepG2/CD81<sup>+</sup> cells. Data not shown, as seen with Hepa1–6 (Fig. 5B and C), confirming that intranuclear localization of P. yoelii EEFs occurs independently of CD81. Intranuclear EEFs were still observed 72 h post infection in HepG2 cells, although their number tended to decrease (Fig. 6D). The number of EEFs in HepG2/CD81 cells also decreased over time, probably due to the complete maturation of the parasites (Fig. 6E).

Interestingly, when we assessed infection at early time points (1–3 h) after inoculation of HepG2/CD81 cells with P. yoelii sporozoites, we could observe not only cytoplasmic sporozoites but also intranuclear sporozoites, sug-
suggesting that intranuclear development of EEFs results from the intranuclear localization of sporozoites at the time of or early after invasion. Sporozoites can enter cells through two distinct pathways, either by disruption of the host cell plasma membrane, followed by migration of the parasite through the cell, or by formation of a PV, which is essential for the EEF development in hepatocytes (Mota et al., 2001). We have previously reported that CD81 is required for invasion through the vacuole pathway, but is dispensable for sporozoite migration through cells (Silvie et al., 2003). Because intranuclear localization of \textit{P. yoelii} sporozoites did not depend on CD81, we hypothesized that these forms may result from the development of sporozoites arrested in the host cell nucleus while they were transmigrating. To directly test this hypothesis, we performed a cell wounding and repair assay using the membrane-impermeant macromolecular tracer dextran (Mota et al., 2001). Disruption and resealing of the cell membrane, which occurs when sporozoites migrate through cells, allows the entry and trapping of dextran in traversed cells. After infection by \textit{P. yoelii} sporozoites in the presence of rhodamine-labelled dextran, all examined HepG2 \( (n = 82) \) (Fig. 7A) and HepG2/CD81 cells \( (n = 54) \) (Fig. 7B) harbouring a parasite in their nucleus were dextran-positive, both at early and late stages of infection, clearly demonstrating that indeed intranuclear localization of \textit{P. yoelii} parasites results from invasion of the host cell by traversing sporozoites. Parasites in dextran-negative cells, resulting from invasion with PV formation, where only found in the cytoplasm of HepG2/CD81 cells (Fig. 7B, middle and lower panels), but not in HepG2 cells.

As mentioned above, sporozoites could be observed in the cytoplasm of both HepG2 and HepG2/CD81 cells early after infection. In HepG2/CD81 cells, a majority of cytoplasmic sporozoites were found in dextran-negative...
cells 1 h (55 ± 4%, n = 30) and 3 h (64 ± 1%, n = 63) post infection, reflecting sporozoite entry with PV formation. In sharp contrast, essentially all the HepG2 cells harbouring a sporozoite in their cytoplasm were dextran-positive, both at 1 h (92 ± 9%, n = 26) and 3 h (95 ± 8%, n = 25) post infection, showing that \textit{P. yoelii} sporozoites fail to enter HepG2 cells by forming a vacuole. To further determine whether sporozoites which localized to the host cell nucleus formed a vacuole inside the nucleus, we performed immunofluorescence assays using the mAb NYLS3, which recognizes \textit{P. yoelii} HEP17 protein, a marker for the PV membrane (Charoenvit \textit{et al.}, 1995). In infected HepG2/CD81 cells, NYLS3 labelled the periphery of cytoplasmic EEFs, consistent with PyHEP17 localization to the PV membrane (Charoenvit \textit{et al.}, 1995) (Fig. 8A). In contrast, PyHEP17 distribution in infected HepG2 cells was not restricted to the periphery of the intranuclear parasites, as would be expected if these EEFs were contained in a PV, and was more diffuse. Indeed, while intranuclear EEFs were entirely contained inside the host cell nucleus as determined with the labelling with anti-HSP70 antibodies (as in Figs 3 and 7A),
PyHEP17 was found outside of the nucleus, in the cytoplasm of infected cells (Fig. 8B). This pattern indicates that intranuclear *P. yoelii* EEFs are not enclosed in a PV inside the infected cell nucleus.

Finally, to determine whether the absence of *P. falciparum* EEF development in HepG2/CD81 cells was due to a lack of invasion or to a defect in the subsequent steps of parasite maturation, we analysed the mode of entry used by *P. falciparum* sporozoites to enter HepG2 versus HepG2/CD81 cells. In both cell types, at early time points after infection, we could observe not only cytoplasmic sporozoites but also intranuclear sporozoites (Fig. 9). As seen with *P. yoelii*, all the cells harbouring a *P. falciparum* sporozoite in their nucleus were dextran-positive, confirming that intranuclear *P. falciparum* EEFs result from the differentiation of sporozoites arrested and/or trapped in the host cell nucleus during cell-traversal activity. In contrast with *P. yoelii*, essentially all the HepG2/CD81 cells (96.6 ± 3.1%, n = 83) and the HepG2 cells (95.4 ± 1.1%, n = 80) containing a *P. falciparum* sporozoite in their cytoplasm were also dextran-positive. These observations indicate that *P. falciparum* sporozoites invade HepG2 and HepG2/CD81 cells using cell-traversal mode of entry, but fail to form a PV in these cells.
zoites into EEFs can take place independently of sporozoite–host cell interactions linked to PV formation. These results extend the previous description of *P. berghei* sporozoite transformation in the absence of host cells, simply induced upon temperature switch to 37°C in the presence of serum factors (Kaiser et al., 2003).

Previous studies have reported the intranuclear localization and differentiation of *P. berghei* sporozoites, although the pathway used for cell entry had not been characterized (Meis et al., 1984; Suhrbier et al., 1986). Intranuclear *P. berghei* were found not only in vitro in HepG2 cells but also in vivo in rat Kupffer cells, still they were not observed in hepatocytes (Meis et al., 1984). Interestingly, as shown by electron microscopy, there was no evidence of a PV membrane surrounding intranuclear *P. berghei* sporozoites (Meis et al., 1984). This result is consistent with our data, which demonstrate that intranuclear localization of *P. yoelii* and *P. falciparum* sporozoites is linked to invasion without PV formation, as shown by dextran assays and NYLS3 labelling. It is noteworthy that early after infection, sporozoites could be found both in the cytoplasm and in the nucleus of dextran-positive cells, as a result of invasion without PV formation. Nevertheless, only intranuclear sporozoites further developed into EEFs, while cytoplasmic parasites rapidly disappeared from HepG2 cell cultures, maybe because they were degraded or because their presence induced the host cell death. In this regard, the circumsporozoite protein (CSP), which covers the sporozoite surface, can bind to ribosomes and inhibit protein synthesis (Frevert et al., 1998). Based on this observation, one can speculate that the presence of sporozoites lying directly in the cytoplasm of infected cells also has detrimental effects on the cell protein synthesis and viability. In contrast, localization in the nucleus may provide a particular niche suitable for both parasite differentiation and maintenance of the infected cell viability. If one essential role of the PV is to physically isolate the parasite from the infected cell cytoplasm, our results indicate that the nuclear envelop can replace a conventional PV to fulfil such a function.

If *P. yoelii* intranuclear EEFs may also form in vivo, it is unlikely that these intranuclear parasites can cause a blood-stage infection. Indeed, we failed in obtaining infection in mice injected with *P. yoelii*-infected HepG2 cells (data not shown), where only intranuclear EEFs can develop. Although we cannot exclude that the absence of infection in injected mice was due to the very low numbers of parasites in HepG2 cultures, another likely explanation is the absence of complete maturation of intranuclear EEFs. In HepG2/CD81 cells, many cytoplasmic *P. yoelii* parasites had the typical aspect of mature EEFs 48 h post infection, with high numbers of small nuclei (as in Fig. 1C), consistent with the differentiation of individual merozoites. This aspect was never observed with intranu-

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**Discussion**

Infection of hepatocytes by *Plasmodium* sporozoites is a prerequisite for initiation of malaria, and therefore represents an attractive target for antimalarial strategy. *Plasmodium* sporozoites can enter host cells by two distinct pathways, either through disruption of the plasma membrane followed by parasite migration through cells, or by formation of a vacuole where the parasite further differentiates into an EEF (Mota et al., 2001). Here we provide evidence that host cell invasion by migrating sporozoites, without PV formation, can result in the development of intranuclear EEFs for both *P. yoelii* and *P. falciparum*. Intranuclear EEFs were observed in hepatocytic cell lines but not in primary hepatocyte cultures. Unlike hepatocytes, which do not proliferate in vitro, hepatocytic cell lines undergo continuous cell divisions in culture, so we hypothesize that sporozoites may readily access the host cell nucleus during mitosis, upon nuclear envelop breakdown. Our data demonstrate that transformation of sporozoites into EEFs can take place independently of sporozoite–host cell interactions linked to PV formation. These results extend the previous description of *P. berghei* sporozoite transformation in the absence of host cells, simply induced upon temperature switch to 37°C in the presence of serum factors (Kaiser et al., 2003).

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**Fig. 9.** *P. falciparum* sporozoites only use cell-traversal activity to invade HepG2 cells. HepG2 cells were infected with *P. falciparum* sporozoites in the presence of rhodamine-dextran (red), and incubated for 5 h before labelling with an anti-PfCSP antibody (green) and DAPI (blue), and analysis by fluorescence microscopy. Note that cells infected with intracytoplasmic (right panels) or intranuclear (left panels) sporozoites are both dextran-positive. Bar, 10 µm.
clear EEFs inside HepG2 cells, where the parasite nuclear material was typically limited to a few indistinct DAPI-stained aggregates. It should be noted that the growth of intranuclear EEFs caused a major shrinkage of the infected host cell nucleus, which was reduced to a thin layer surrounding the parasite (see Fig. 7A and 8B). It is likely that such a compression of the host nucleus has detrimental effects on cell functions and viability, which in turn may prevent the full maturation of the parasite. Nevertheless, the partial development of intranuclear parasites in vivo may participate in the development of immune responses against *Plasmodium* pre-erythrocytic stages. In this regard, the partial EEF development resulting from infection by radiation-attenuated (Suhrbier et al., 1990; Silvie et al., 2002) or genetically attenuated (van Dijk et al., 2005; Mueller et al., 2005a,b) sporozoites is associated with the induction of protective immune responses against subsequent sporozoite challenge (Nussenzweig et al., 1967; Hoffman et al., 2002; van Dijk et al., 2005; Mueller et al., 2005a,b).

In sharp contrast with the cell-traversal mode of entry, productive infection of hepatocytes requires the formation of a PV, which is essential for differentiation of EEFs and infectious merozoites. Although this has not been definitively established with *Plasmodium* sporozoites, PV formation presumably results from an invagination of the host cell plasma membrane due to the antero–posterior translocation of a moving junction formed by sporozoite ligands bound to their cognate receptors on the hepatocyte (Baldracci and Menard, 2004; Kappe et al., 2004). The range of host cells supporting the formation of the PV and the subsequent EEF development likely depends on the specificity of these ligand–receptor interactions. *P. falciparum* sporozoites can infect human and chimpanzee hepatocytes (Mazier et al., 1985; Meis et al., 1990), but not mouse liver cells in vitro. We have shown previously that the host tetraspan CD81 is required for *P. falciparum* sporozoite invasion (Silvie et al., 2003). Because human and chimpanzee CD81 are identical on the amino acid level (Flint et al., 1999), but differ from the mouse sequence, we wondered whether CD81 could account for *P. falciparum* host selectivity during infection of hepatocytes. We found that expression of human CD81 is not sufficient to promote infection of transgenic mouse hepatocytes by *P. falciparum* sporozoites, showing that the narrow host tropism of *P. falciparum* sporozoites is not linked to CD81 alone. In contrast, we found that *P. yoelii* sporozoites readily infect and form EEFs not only in mouse but also in human primary hepatocytes, clearly demonstrating the absence of host species barrier for this parasite during infection of hepatocytes. It should be noted, however, that *P. yoelii* sporozoites are not responsible for malaria in man, due to host species barriers outside the liver. Notably, infection of human erythrocytes by merozoites from rodent malaria parasites has never been reported.

It has long been known that human hepatocarcinoma HepG2 cells are readily infected by *P. vivax* and *P. berghei* sporozoites, but do not support *P. falciparum* (Hollingdale et al., 1984; Mazier et al., 1985) and *P. yoelii* EEF development (Calvo-Calle et al., 1994; Mota and Rodriguez, 2000). The molecular mechanisms underlying this refractoriness were unknown. CD81, which is required for *P. falciparum* and *P. yoelii* but not *P. berghei* sporozoite invasion (Silvie et al., 2003), is not expressed on the surface of HepG2 cells (Berditchevski et al., 1996; Charrin et al., 2001). We now report that expression of human CD81 in HepG2 cells is sufficient to render them fully permissive to infection by *P. yoelii* sporozoites, confirming that the refractoriness of HepG2 cells to *P. yoelii* is due to the absence of CD81 expression. In sharp contrast, expression of human CD81 in HepG2 cells is not sufficient to render them permissive to infection by *P. falciparum*. The only *P. falciparum* EEFs that we observed in these cells were all intranuclear, deriving from transmigrating sporozoites that had entered the cell without PV formation. Based on this result, it is likely that in addition to CD81 *P. falciparum* requires other molecules, to establish a junction and invade host cells by forming a PV. Such molecules are expressed and functional in human hepatocytes but not in HepG2 cells, even when CD81 is present.

The availability of a cell line susceptible to *P. falciparum* sporozoite infection would constitute a major breakthrough for the development and testing of malaria pre-erythrocytic vaccines. The demonstration that HepG2 cells can be engineered to support infection by *P. yoelii* paves the way for the design, through additional genetic manipulations, of a cell line supporting *P. falciparum* infection. To this aim, it will be essential to better characterize the mechanisms underlying sporozoite invasion and EEF development, and notably to identify the host factors specifically required for infection by *P. falciparum* sporozoites.

**Experimental procedures**

**Parasites and cells**

*Plasmodium yoelii* (265BY strain) and *P. falciparum* (NF54 strain) sporozoites were obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands. Mouse hepatoma cells Hepa1–6 (ATCC CRL-1830) were cultured in DMEM (Invitrogen, Cergy Pontoise, France) supplemented with 10% FCS (Biowest, Nuaillé, France), 2 mM glutamine, 50 μg ml⁻¹ penicillin, 50 μg ml⁻¹ streptomycin and 100 μg ml⁻¹ neomycin (Invitrogen). Human hepatocarcinoma cells HepG2 cells (ATCC HB-8065) and HepG2 stably expressing CD81 (HepG2/CD81⁺) (Bartsch et al., 2003) were cultured in DMEM supplemented as above, in culture dishes coated with rat tail collagen I (Becton Dickinson, Le Pont de Claix, France). Primary mouse hepatocytes were isolated from wild-type and hCD81 transgenic mCD81-deficient mice.
Weil 200 pmol of siRNA by electroporation (300 V, 500 microfarads) for 48 h before flow cytometry analysis or sporozoite infection. 

P. falciparum triplicate wells by immunofluorescence, as described (Silvie et al., 2003). For antibody-mediated inhibition assays, anti-human CD81 mAb TS81 (Charrin et al., 2000) and MT81 (Silvie et al., 2000) were added to the cultures at the time of sporozoite inoculation and removed 3 h later. Inhibition results were analysed for statistical significance using the one-way ANOVA followed by the Tukey multiple comparison test.

In vivo experiments

Wild-type C57BL/6 mice (n = 5) and CD81-knockout human CD81 transgenic mice (n = 5) were injected intravenously with 4 \times 10^5 P. yoelli sporozoites. BALB/c mice (n = 5) were injected intraperitoneally with 1 \times 10^6 P. yoelli-infected HepG2 or HepG2/CD81 cells. Mice were then examined daily for the presence of malaria parasites in their peripheral blood.

Construction of the CD81 lentiviral vector and transduction of HepG2 cells

The TRIP3-EG1α-CD81 plasmid was constructed by inserting the human CD81 cDNA sequence in the TRIP3-EG1α vector (Sirven et al., 2001). Vector particles were produced by transient calcium phosphate cotransfection of 293T cells by the TRIP3-EG1α-CD81 plasmid, an encapsidation plasmid and a vesicular stomatitis virus envelope expression plasmid, as described (Sirven et al., 2001). HepG2/CD81 cells were obtained by transducing HepG2 cells with concentrated lentiviral particles.

Sporozoite invasion assay

Hepa1–6 or HepG2 cells (15 \times 10^4 per well) or primary mouse (9 \times 10^4 per well) or human (18 \times 10^4 per well) hepatocytes were seeded in 8-chamber plastic Laboratory-Tek slides (Nalge Nunc International, Cergy Pontoise, France), 24–48 h (except for human hepatocytes, 3–7 days) prior to inoculation with P. yoelli (3–5 \times 10^4 sporozoites per well) or P. falciparum sporozoites (1 \times 10^6 sporozoites per well). After 3 h at 37°C, cultures were washed and further incubated in fresh medium for 36 h (P. yoelli) or 72 h (P. falciparum) before quantification of infected cells in triplicate wells by immunofluorescence, as described (Silvie et al., 2003). For antibody-mediated inhibition assays, anti-human CD81 mAb TS81 (Charrin et al., 2001) or anti-mouse CD81 mAbs Eat1, Eat2 (Maecker et al., 2000) and MT81 (Silvie et al., submitted) at 25 µg ml⁻¹ were added to the cultures at the time of sporozoite inoculation and removed 3 h later. Inhibition results were analysed for statistical significance using the one-way ANOVA followed by the Tukey multiple comparison test.

Small interfering RNA

We used small double stranded RNA oligonucleotides targeting mouse CD81 (5’-CGT GTC ACC TTC AAC TGT A-3’), mouse CD9 (5’-GAG CAT CTT CGA GCA AGA GAA-3’), or human CD81 (5’-GCA CCA AGT GCA TCA AGT A-3’). A siRNA oligonucleotide targeting human CD53 (5’-CAA CTT CGG AGT GCT CTT C-3’) was used as a control siRNA throughout the study. Cells (5–10 \times 10^4 cells in 400 µl of RPMI) were transfected with 200 pmol of siRNA by electroporation (300 V, 500 microfarads) (Weil et al., 2002) using the Gene Pulser apparatus (Bio-Rad, Ivry, France). Following siRNA transfection, cells were cultured for 48 h before flow cytometry analysis or sporozoite infection.

Immunofluorescence assays

After fixation of the cultures with cold methanol, EEFs were stained using a mouse polyclonal serum raised against Plasmodium heat shock protein 70 (HSP70) (Renia et al., 1990) or with the anti-PyHep17 mAbs NYS1 (Charoenwit et al., 1995), followed by goat anti-mouse FITC or TRITC conjugate (Sigma) and DNA stain diamidino-phenylindole (DAPI, Sigma), before examination by fluorescence microscopy. The HSP70 labelling was used to count EEFs. P. falciparum and P. yoelli sporozoites were stained using the anti-PICSP mAb E9 (Stuber et al., 1990) and the anti-PyCSP mAb NYS1 (Charoenwit et al., 1987), respectively, using a double labelling procedure as described (Silvie et al., 2002). For flow cytometric analysis, cells were detached using a non-enzymatic solution (Intrivogen), washed and stained with saturating concentrations of primary antibody as indicated. After washes in culture medium, cells were incubated with 10 µg ml⁻¹ FITC-labelled secondary antibody (Becton-Dickinson, San Jose, CA, USA), washed and stained with 1% formaldehyde in PBS. All incubations were performed for 30 min at 4°C. Analysis of cell-surface staining was performed using a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Cell wounding and membrane repair assay

HepG2 and HepG2/CD81 cells were incubated for 3 h with P. yoelli or P. falciparum sporozoites in the presence of 1 mg ml⁻¹ rhodamine-dextran, lysine fixable, 10 000 MW (Molecular Probes, Leiden, the Netherlands), as described (Mota et al., 2001). Cultures were then washed to remove extracellular dextran and further incubated as indicated in the figure legends before fixation with 4% paraformaldehyde and analysis by fluorescence microscopy.

Acknowledgements

We thank M. Tefit, K. Farhadi and T. Houpert for technical assistance. The HepG2/CD81 cells were provided by F.L. Cosset (Ecole Normale Supérieure, Lyon, France); the anti-PICSP mAb by G. Pluschke (Swiss Tropical Institute, Basel, Switzerland) and the anti-PyCSP mAb NYS1 and the anti-PyHep17 mAb NYS1 by Y. Charoenwit (Naval Medical Research Center, Silver Spring, Maryland). O.S. was supported by a fellowship from Inserm. This work was supported in part by grants from Association Nouvelle Recherche Biomédicale-Vaincre le Cancer, Association pour la Recherche sur le Cancer, and by US National Institutes of Health Grant AI45900 and European Union FP5 contract number QLK2-CT-2002-00774.

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