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Phosphothioate oligodeoxynucleotides inhibit *Plasmodium* sporozoite gliding motility

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Summary

***Plasmodium* sporozoites, transmitted to the mammalian host through a mosquito bite, travel to the liver, where they invade hepatocytes, and develop into a form that is then able to infect red blood cells. In spite of the importance of innate immunity in controlling microbial infections, almost nothing is known about its role during the liver stage of a malaria infection. Here, we tested whether synthetic CpG phosphothioate (PS) oligodeoxynucleotides (ODNs), which bind to Toll-like receptor 9 (Tlr9), could have a protective effect on *Plasmodium berghei* infection in hepatocytes. Surprisingly, CpG PS-ODNs potently impair *P. berghei* infection in hepatoma cell lines independently of Tlr9 activation. Indeed, not only CpG but also non-CpG PS-ODNs, which do not activate Tlr9, decreased parasite infection. Moreover, the ability of PS-ODNs to impair infection was not due to an effect on the host but rather on the parasite itself. In fact, CpG PS-ODNs, as well as non-CpG PS-ODNs, impair parasite gliding motility. Furthermore, our analysis reveals that PS-ODNs inhibit parasite migration and invasion due to their negative charge, whereas development inside hepatocytes is undisturbed. Altogether, PS-ODNs might represent a new class of prophylactic antimalaria agents, which hamper hepatocyte entry by *Plasmodium* sporozoites.**

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Introduction

The intracellular protozoan parasite *Plasmodium* is the aetiological agent of malaria, an arthropod-borne infectious disease that still kills nearly one million people every year (WHO report 2008). *Plasmodium* utilizes a mosquito of the genus *Anopheles* as a vector to enter its mammalian host. The infectious cycle then relies on the development of different parasitic forms, in different tissues of the host. First, *Plasmodium* sporozoites, transmitted through the bite of an infected mosquito, migrate through skin cells and enter the blood or lymph vessels (Amino *et al.*, 2008). Once in the blood stream, sporozoites are rapidly and specifically retained in the liver sinusoids. This step is mediated by electrostatic interactions between liver-specific cell surface heparan sulfate proteoglycans (HSPGs) and parasite surface molecules, such as circumsporozoite protein (CSP) (Frevert *et al.*, 1993; Pinzon-Ortiz *et al.*, 2001). Sporozoites then cross the sinusoidal layer, possibly through Kupffer cells, specialized macrophages located in the liver, to reach hepatocytes (Frevert *et al.*, 2006). After traversing several hepatocytes, individual sporozoites invade final target cells with formation of a parasitophorous vacuole (Mota *et al.*, 2001). In these vacuoles, sporozoites develop and replicate into thousands of merozoites, the red blood cell-infectious parasite forms (Sturm *et al.*, 2006). It is only after merozoite release into the blood and their invasion and development inside erythrocytes that the infection will become symptomatic (Haldar *et al.*, 2007).

Even though innate immunity represents the first line of host defence and is essential for the activation and orientation of the adaptive immune response, its role during the malaria liver stage remains elusive. Toll-like receptors (TLRs) are well characterized germline-encoded pattern recognition receptors that trigger the innate immune response after ligation to highly conserved microbial components, named pathogen-associated molecular patterns (Medzhitov and Janeway, 1997). Among the TLRs, Tlr9 is activated by unmethylated CpG-rich DNA, such as

microbial DNA, or artificially, by short synthetic oligodeoxynucleotides (ODNs) containing two to three CpG motifs (Kumagai *et al*., 2008). Tlr9 activation induces a T-helper 1 type immune response leading to the production of cytokines including interferon-γ and interleukin-12 (Jakob *et al*., 1998; Hacker *et al*., 1998), which are required to combat intracellular pathogens, such as *Plasmodium* (Klinman, 2004). Indeed, a previous study in mice has shown that CpG ODNs are able to induce protection against infection by *Plasmodium yoelli* sporozoites. This CpG ODN-mediated protection seems to be dependent on interleukin-12 and interferon-γ (Gramzinski *et al*., 2001; Chen *et al*., 2009). Although Tlr9 is mainly expressed in immune cells, studies revealed that it is also expressed in hepatocytes (Liu *et al*., 2002; Sanchez-Campillo *et al*., 2004). Moreover, it was suggested that hepatocytes might be able to sense and respond to CpG ODNs (Sanchez-Campillo *et al*., 2004). We therefore addressed the question of whether direct stimulation of Tlr9 in hepatocytes by CpG ODNs could influence infection by *Plasmodium* sporozoites.

Here we show that several CpG phosphothioate (PS)-ODNs potently impair *Plasmodium berghei* infection in hepatoma cells in a dose-dependent manner. Unexpectedly, we found that the mechanism of inhibition was Tlr9-independent. More precisely, our data demonstrate that PS-ODNs *in vitro* do not have a direct effect on the host response to the infection but on the parasite's ability to glide, traverse and invade hepatocytes.

Results

CpG PS-ODNs reduce hepatocyte infection by sporozoites independently of Tlr9 activation

Single-stranded CpG ODNs are 20–30 nucleotides long and usually contain a PS backbone, in which one of the non-bridging oxygen atoms of phosphodiester (PD)-ODNs is replaced by a sulfur atom, enhancing stability to nucleases, cellular uptake and immunostimulatory properties (Fig. 1A) (Krieg, 2002). To test whether activation of Tlr9 could have any effect on infection by *P. berghei* sporozoites *in vitro*, we pre-treated human Huh7 hepatoma cells with a potent agonist of human Tlr9, CpG PS-ODN 2007 (Latz *et al*., 2007). One hour later, Huh7 cells were infected with green fluorescent protein (GFP)-expressing *P. berghei* parasites, and 24 h later infection was measured by quantifying the proportion of GFP-positive cells using fluorescence activated cell sorting (FACS) (Prudencio *et al*., 2008). Addition of CpG PS-ODN 2007 to Huh7 hepatoma cells caused a significant and dose-dependent inhibitory effect on infection (Fig. 1B), without affecting the viability of Huh7 cells (data not shown).

The ODNs in which the CpG motifs have been changed to GpC are also able to bind to Tlr9 but do not trigger Tlr9 signalling (Latz *et al*., 2007). Surprisingly, we found that the control GpC PS-ODN 2007 impaired parasite infection to a similar degree as CpG PS-ODN 2007 (Fig. 1B). Other PS-ODNs, CpG PS-ODN 2006, CpG PS-ODN 1826 and their non-CpG PS-ODN derivatives had similar effects on the infection both in Huh7 cells as well as in the mouse hepatoma cell line Hepa1–6, illustrating that the observed inhibition was not restricted to PS-ODN 2007 or to a particular cell line (Fig. S1). On the contrary, CpG PD-ODN 2007 did not cause a significant inhibition of infection (Fig. 1B). Altogether, these results demonstrate that the inhibitory activity of the CpG PS-ODNs is independent of the CpG motif but rather related to the chemical structure of the PS backbone.

Although it has been previously reported that Tlr9 is expressed in hepatoma cell lines (Liu *et al*., 2002; Sanchez-Campillo *et al*., 2004), we were not able to detect any significant Tlr9 expression or any induction of pro-inflammatory cytokines (IL-6, IL-8 and IFNs) 6 h after addition of CpG PS-ODN 2007 to Huh7 cells (data not shown). This strongly suggests that Tlr9 signalling is defective in Huh7 cells. In immune cells, CpG PS-ODNs are endocytozed into subcellular compartments where they bind and activate Tlr9 within minutes (Latz *et al*., 2004). Therefore, we next tested whether Huh7 cells internalized CpG PS-ODNs. When Huh7 cells were incubated for 1 h with fluorescently labelled CpG PS-ODNs, several DNA-positive vesicular structures were found, showing that CpG PS-ODNs were indeed taken up by the cells (Fig. 1C). To test whether this cellular uptake of PS-ODNs is required to inhibit infection by *Plasmodium*, Huh7 cells were pre-treated for 1 h with CpG PS-ODN 2007 and then washed before infection with *P. berghei* sporozoites. As shown in Fig. 1D, sporozoite infection was similar to the control situation, suggesting that once CpG PS-ODNs are internalized into Huh7 cells they are not able to impair the infection. Overall, the data show that the protective effect of CpG PS-ODNs in Huh7 cells is independent of Tlr9 activation.

PS-ODNs interfere with P. berghei *sporozoite migration and invasion into hepatocytes*

Liver infection by sporozoites consists of several consecutive steps: traversal of several host cells, hepatocyte attachment and entry, and intracellular development inside the parasitophorous vacuole. To investigate at which of these stages PS-ODNs reduce parasite infection of hepatocytes, we first explored whether PS-ODNs inhibit the traversal and invasion steps of infection. In order to quantify cell traversal and invasion Huh7 cells were infected with GFP-expressing *P. berghei* sporozoites

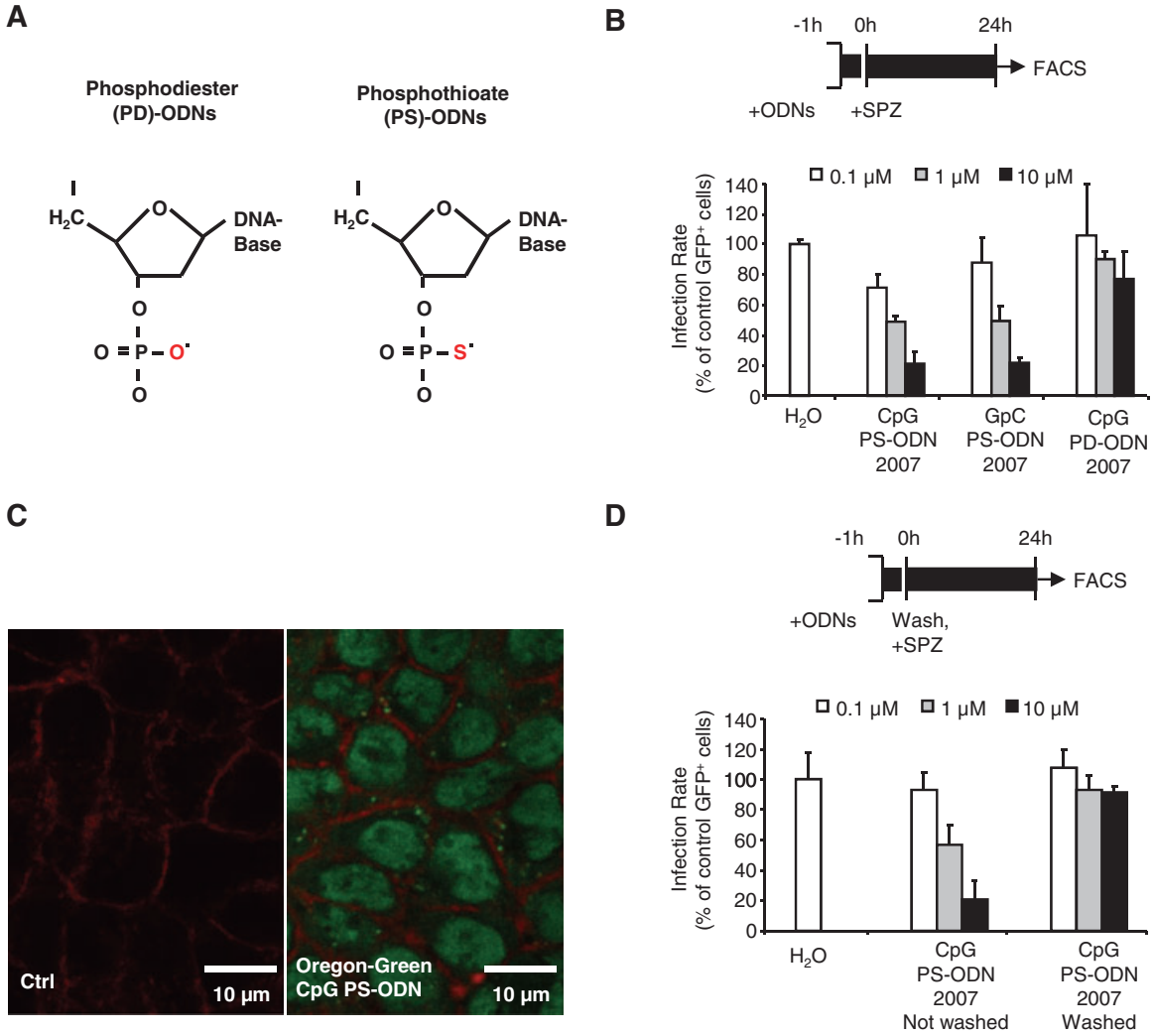

Fig. 1. CpG PS-ODNs inhibit *in vitro* sporozoite infection in a TIr9-independent manner.
A. Chemical structure of PD- and PS-ODN sugar backbones.
B. PS-ODNs inhibit sporozoite infection in Huh7 cells. Huh7 cells were incubated with increasing concentrations of CpG PS-ODN 2007, GpC PS-ODN 2007 and CpG PD-ODN 2007 1 h prior to GFP-expressing *P. berghei* sporozoite addition. Infection rates were measured 24 h post infection by FACS. This experiment was repeated three times and yielded similar results. The graphs show the average of triplicate samples with standard deviations of one representative experiment. CpG PS-ODN 2007 and GpC PS-ODN 2007-treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, hypothesis (H) rejected with *P*-value ≥ 0.05.
C. PS-ODNs are taken up by Huh7 cells. Cells were incubated with 1 µM of Oregon Green-labelled CpG PS-ODN 2007 at 37°C for 45 min.
D. PS-ODNs removal from the medium before infection does not lead to a decrease in sporozoite infection. Huh7 cells were treated with CpG PS-ODN 2007 for 1 h and washed twice with PBS, before addition of GFP-expressing *P. berghei* sporozoites. Infection was determined 24 h later by measuring the number of GFP-positive cells by FACS. The graphs show the average of triplicate samples with standard deviations of one representative experiment. CpG PS-ODN 2007-treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value ≥ 0.05.

in the presence of rhodamine-dextran (RD). Two hours after sporozoite addition, cells were collected and analysed by FACS. Cells that had been traversed by the parasite were identified as RD-positive, whereas GFP-positive/RD-negative cells were counted as invaded by sporozoites. This assay is based on the observation that sporozoites disrupt the host cell membrane during traversal, allowing RD internalization, while membranes stay intact during the final invasion with parasitophorous vacuole formation (Mota *et al.*, 2001; Prudencio *et al.*, 2008). Cell treatment with PS-ODNs led to a dose-dependent reduction in traversal and invasion, indicating that PS-ODNs interfere with the ability of parasites to traverse and to invade cells (Fig. 2A).

In order to distinguish between the cell attachment and entry processes, we performed a double staining assay that allows discriminating between intracellular and extracellular parasites (Renia *et al.*, 1988). We evaluated

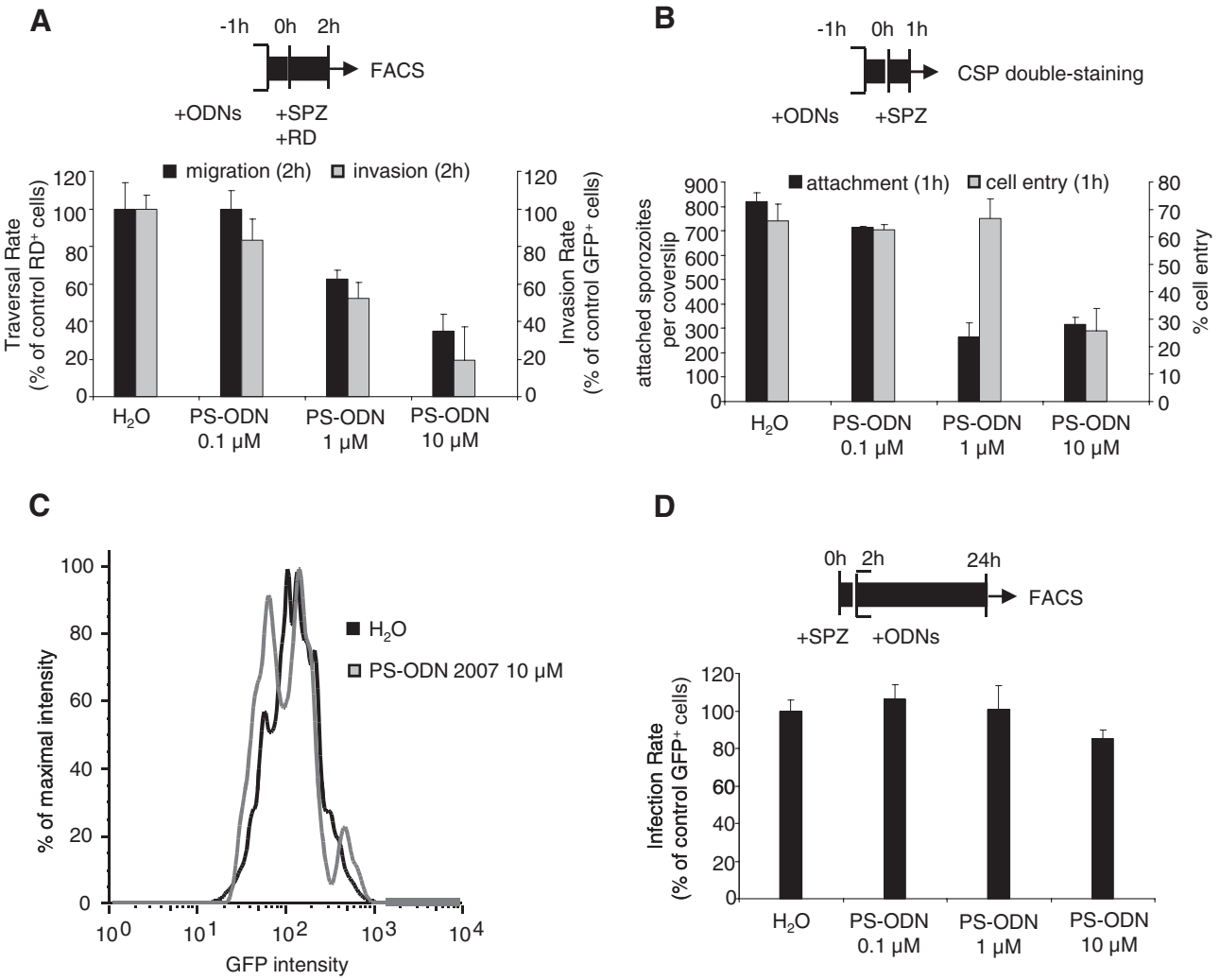

Fig. 2. PS-ODNs interfere with parasite attachment to hepatocytes and cell entry.
A. PS-ODN treatment inhibits sporozoite migration and invasion. Different doses of PS-ODNs were added to Huh7 cells 1 h before infection with GFP-expressing *P. berghei* sporozoites. At the time of infection, RD was added to the medium and 2 h post infection cells were harvested for FACS analysis. To monitor the cell traversal rate, the percentage of RD-positive/GFP-negative cells was measured and compared with control values. The invasion rate was determined as the percentage of GFP-positive cells. The graphs show the average of triplicate samples with standard deviations of one representative experiment. For migration and invasion PS-ODN $\geq$ 1 µM treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value $\geq$ 0.05.
B. PS-ODNs affect sporozoite attachment to Huh7 cells and cell entry. Huh7 cells were pre-incubated 1 h before sporozoite addition with different concentrations of PS-ODNs. After 1 h of infection, cells were washed twice with PBS to remove unbound sporozoites. The cells were treated with a double-staining procedure, as described in *Experimental procedures*, permitting the differentiation between intracellular and extracellular parasites. Attached sporozoites were counted as the number of extracellular sporozoites per coverslip. The percentage of internalized sporozoites was calculated using the following equation: [(total number of parasites – extracellular number of parasites)/total number of parasites] × 100. The graphs show the average of triplicate samples with standard deviations of one representative experiment. For attachment PS-ODN 2007 $\geq$ 1 µM and for cell entry PS-ODN 2007 $\geq$ 10 µM treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value $\geq$ 0.05.
C. PS-ODNs do not impair *P. berghei* development within Huh7 cells. Huh7 cells were incubated with 10 µM PS-ODN 2007 1 h prior to sporozoite addition. The graphs represent the GFP intensity of infected cells measured by FACS 24 h after sporozoite addition.
D. Infection is not inhibited by PS-ODNs when they are added after invasion. PS-ODNs were added to Huh7 cells 2 h after addition of GFP-expressing sporozoites and infection was measured by FACS 24 h later. The graphs show the average of triplicate samples with standard deviations of one representative experiment.

attachment as the number of extracellular sporozoites per coverslip after washing. The percentage of internalized sporozoites was calculated as a ratio of the number of sporozoites that entered cells to the number of total sporozoites, as previously described (Sinnis, 1998). We observed that sporozoites attached significantly less in the presence of 1 µM PS-ODNs as compared with its absence (Fig. 2B). In contrast, parasites that still attached

under these conditions then displayed a rate of internalization similar to that found in non-treated cells (Fig. 2B). This result demonstrates that at 1 μM PS-ODNs impair cell invasion through the inhibition of sporozoite attachment to cells. Interestingly, in cells treated with 10 μM PS-ODNs not only sporozoite attachment but also cell entry was significantly impaired. This suggests that PS-ODNs used at higher concentrations interfere with the cell entry machinery.

Finally, FACS measurement showed that similar GFP intensities, which are proportional to parasite development, were observed for PS-ODN-treated and non-treated control cells 24 h after sporozoite addition (Fig. 2C). This indicates that once the sporozoites have entered the cell their development is not affected by PS-ODNs. Moreover, when PS-ODNs were added to the cells 2 h after sporozoite infection no significant reduction in the infection rate was observed at 24 h post infection (Fig. 2D). In summary, our results demonstrate that PS-ODNs inhibit parasite traversal and invasion but not its development.

PS-ODNs impair P. berghei *sporozoite motility*

We next sought to test whether PS-ODNs could have a toxic effect on sporozoites and thereby impair hepatoma cell infection. Sporozoites were pre-incubated with CpG PS-ODN 2007 and 45 min later the sporozoite/PS-ODN mixture was added to Huh7 cells and diluted to reduce the ODN concentration. The proportion of GFP-positive cells was measured 24 h later by FACS. No significant difference in infection rates was observed between cells infected with sporozoites pre-treated with CpG PS-ODN 2007 and those infected with non-treated sporozoites (Fig. 3A). This result demonstrates that PS-ODNs are not toxic for the parasites and confirms that PS-ODNs must be present during the time of infection in order to affect it.

One important feature of *Plasmodium* sporozoites is their ability to move by gliding (Vanderberg, 1974). Gliding is a substrate-dependent form of motility that does not involve a change in cell shape. Gliding motility is easily visualized by using anti-CSP antibodies to stain the CSP trails left by *Plasmodium* on the substrate (Stewart and Vanderberg, 1988). We monitored the effect of increasing concentrations of CpG and GpC PS-ODNs on sporozoite gliding by quantifying the number of sporozoites that left trails on anti-CSP monoclonal antibody-coated microscopy slides. Concentrations of 1 μM PS-ODN and above inhibited sporozoite gliding motility (Fig. 3B and C). However, washing sporozoites after PS-ODN treatment was sufficient to restore a normal gliding motility (data not shown). CpG ODNs containing a PD backbone also did not affect gliding motility, confirming the importance of the sulfate groups in the PS-ODN backbone (Fig. 3B and C).

To further confirm the inhibition of the gliding motility by PS-ODN treatment, we next filmed sporozoite motility in the presence of PS-ODNs. Time-lapse imaging confirmed that PS-ODN treatment impaired gliding capacity, though, the effect was less pronounced than that observed with the CSP staining method (Fig. 3D). This difference could be explained by the fact that in the presence of negatively charged molecules less CSP is captured by anti-CSP antibodies. Overall, this analysis shows that PS-ODNs have an inhibitory effect on sporozoite motility. Furthermore, the fact that this effect is lost after washing suggests a low affinity, reversible interaction between PS-ODNs and the sporozoites.

The negative charge of PS-ODNs is essential to inhibit P. berghei *sporozoite invasion*

As PS-ODNs are negatively charged we hypothesized that they might interfere with the sporozoite motility because of their polyanionic nature. Indeed, we also observed an inhibition of sporozoite motility when sporozoites were treated with different nucleic acid polyanion polymers, such as polyinosine (polyI), polyinosinic : polycytidylic acid [poly(I : C)] and salmon sperm DNA (Fig. 4A). In addition, sulfated glycoconjugates (dextran sulfate, heparin), another class of polyanions, also significantly reduced sporozoite motility (Fig. 4A). It is therefore likely that the effect of PS-ODNs on sporozoite motility is a consequence of their negative charge.

To test whether the negative charge of PS-ODNs is required to impair the sporozoite–hepatocyte interaction, we treated Huh7 cells with various nucleic acid polyanion polymers. Increasing concentrations of polyI, poly(I : C) and salmon sperm DNA all dramatically inhibited parasite invasion at similar doses to PS-ODNs (Fig. 4B). In sharp contrast, addition of PS-ODNs complexed to DOTAP cationic liposomes did not inhibit parasite invasion (Fig. 4C). Altogether, these results confirm the importance of the negative charge of PS-ODNs to efficiently inhibit sporozoite motility and invasion.

Discussion

In this study we tested whether synthetic CpG PS-ODNs, ligands of Tlr9, could have a protective effect against *P. berghei* infection in hepatocytes. Our analysis reveals that sporozoite infection is efficiently impaired not only by CpG PS-ODNs, but also by GpC PS-ODNs, which do not activate Tlr9. We could show that, in our experimental system, the mechanism of the CpG PS-ODN action on infection is not dependent on Tlr9 stimulation but rather on a direct disruption of parasite motility and of its ability to migrate through and invade hepatocytes.

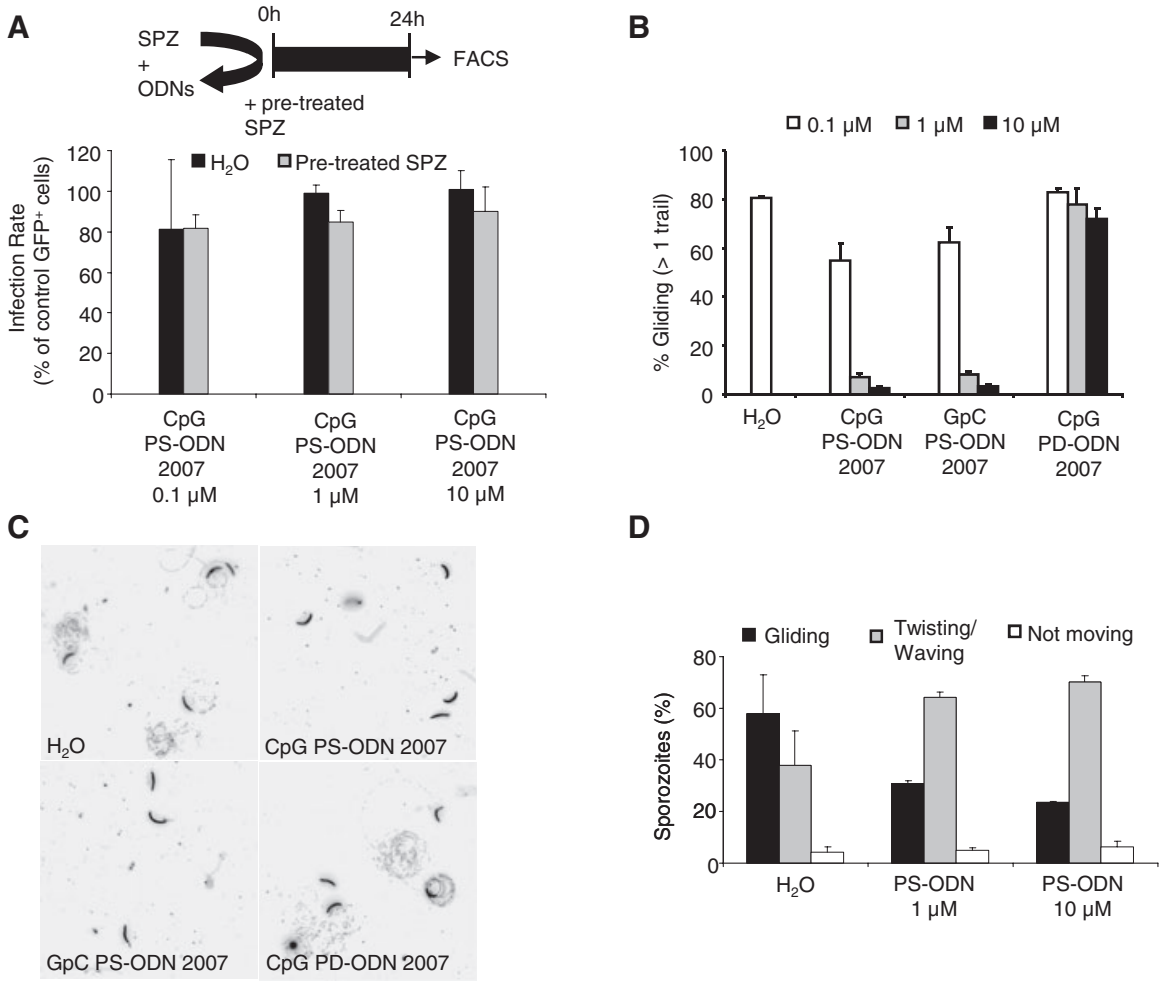

Fig. 3. PS-ODNs impair *P. berghei* sporozoite gliding motility.
A. Pre-treatment of sporozoites with PS-ODNs does not affect parasite infectivity. Sporozoites were incubated with CpG PS-ODN 2007 or solvent (control), for 45 min at room temperature, before addition to Huh7 cells. The samples were diluted to reduce the PS-ODN concentration. PS-ODNs were added to the control wells to reach the same ODN concentration as in the experimental wells. No difference in infection rate was observed 24 h post infection between cells infected with sporozoites pre-treated with CpG PS-ODN 2007 and those infected with non-treated sporozoites. The graphs show the average of triplicate samples with standard deviations of one representative experiment.
B and C. PS-ODNs decrease sporozoite gliding motility. Gliding motility was monitored by the capacity to produce the characteristic circles, in the presence or absence of CpG PS-ODN 2007, GpC PS-ODN 2007 or CpG PD-ODN 2007, by staining for the CSP as described in *Experimental procedures*. Mean values of the gliding frequencies as obtained upon examination of at least 100 sporozoites are presented. CpG PS-ODN 2007 and GpC PS-ODN 2007 $\geq$ 1 µM treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value $\geq$0.05 (B). Representative immunofluorescence staining of trails produced by non-treated and ODN-treated sporozoites are shown (C).
D. Gliding phenotypes of PS-ODN-treated sporozoites. Time-lapse micrographs of sporozoites gliding on uncoated glass slides were taken every 3 s during 2 min. Sporozoite movement in circles, in a pendulum-like (waving) or twisting-like movement was observed and recorded. PS-ODN-treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value $\geq$ 0.05.

Accumulating evidence shows the potential of CpG PS-ODNs to mediate protection against various infectious diseases (Klinman, 2004). A previous *in vivo* study in mice has shown that CpG PS-ODNs are able to induce protection against *P. yoelli* infection (Gramzinski *et al.*, 2001; Chen *et al.*, 2009). However, to the best of our knowledge, no *in vitro* study has addressed the effects of CpG PS-ODNs on hepatocyte infection by *Plasmodium*. This, along with the reports on Tlr9 expression in human hepatocyte cell lines (Liu *et al.*, 2002; Sanchez-Campillo *et al.*, 2004), prompted us to explore whether CpG PS-ODNs could have a protective effect against parasite infection by activating Tlr9 in hepatocytes. Our results clearly show that CpG PS-ODNs inhibit infection in Huh7 and Hepa 1–6 cells independently of Tlr9 stimulation. This conclusion is based on the observations that: (i) PS-ODNs missing the Tlr9-binding CpG motif also impaired infection, (ii) no significant upregulation of IL-6, IL-8 or IFNs was detected after 6 h of exposure to CpG and (iii) removal of CpG PS-ODNs before infection was sufficient

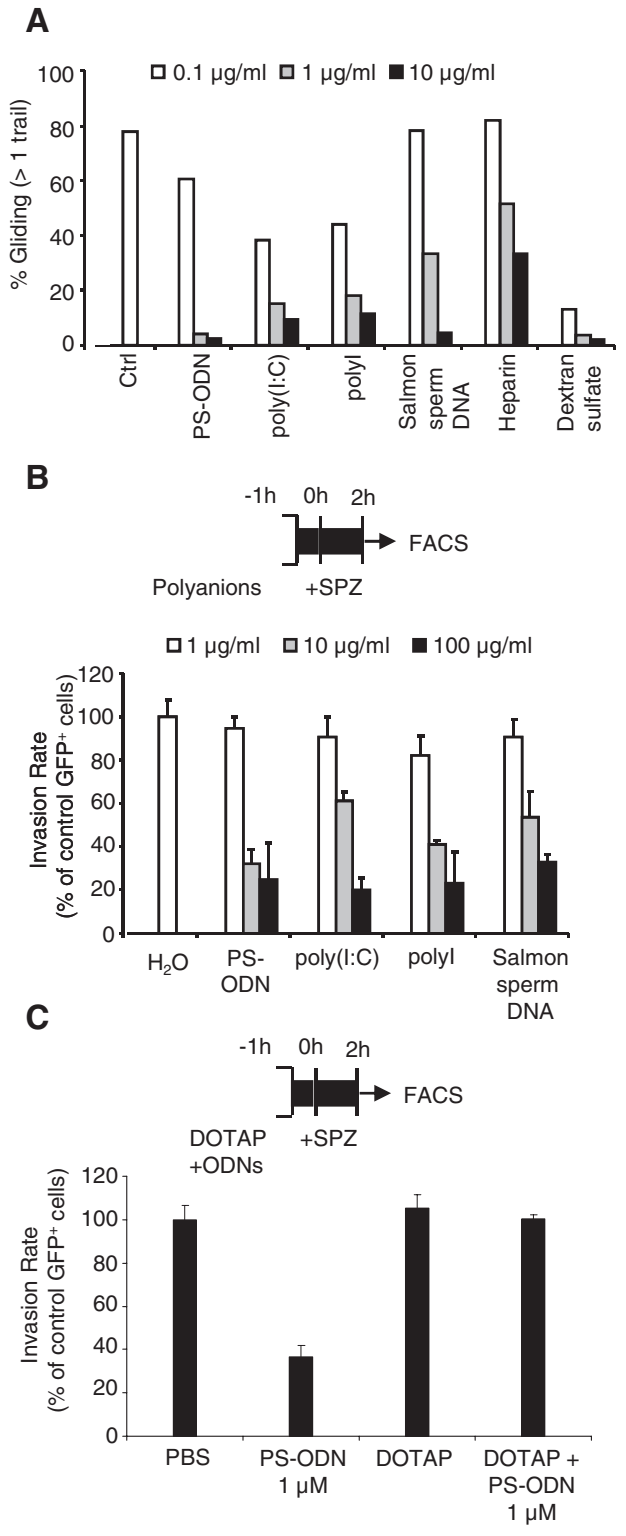

Fig. 4. The negative charge of PS-ODNs is essential to inhibit invasion.
A. Incubation of parasites with increasing concentrations of polyI, poly(I : C), salmon sperm DNA, heparin and dextran sulfate resulted in a significant inhibition of gliding motility. Mean values of the gliding frequencies as obtained upon examination of at least 100 sporozoites are presented. Polyanion ≥1 µM treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value ≥ 0.05.
B. The polyanions polyI, poly(I : C) and salmon sperm DNA are able to inhibit parasite invasion at similar concentrations as PS-ODNs. The invasion rate was measured by FACS 2 h after sporozoite addition as the percentage of GFP-positive cells. The graphs show the average of triplicate samples with standard deviations of one representative experiment. Polyanion ≥1 µM treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value ≥0.05.
C. Treatment of Huh7 cells with PS-ODNs complexed to DOTAP does not inhibit sporozoite invasion. Huh7 cells were incubated 1 h before sporozoite infection with PS-ODN/DOTAP complexes and invasion rate was measured 2 h after infection as the percentage of GFP-positive cells by FACS. The graphs show the average of triplicate samples with standard deviations of one representative experiment. PS-ODN-treated sample was tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with *P*-value ≥ 0.05.

to abolish their protective effect on pre-treated Huh7 cells. It is interesting to note that similar observations have been made with viruses. *In vitro* studies showed that CpG PS-ODNs potently inhibit HIV and cytomegalovirus infection independently of Tlr9 (Schlaepfer *et al.*, 2004; Luganini *et al.*, 2008).

In the HIV model it was proposed that CpG PS-ODNs specifically interact with the virus envelope and thus block fusion with the cell membrane (Schlaepfer *et al.*, 2004). For cytomegalovirus, the authors also suggested that PS-ODNs interfere with the cell entry process but attempts to elucidate the mechanism of inhibition have failed to reach a definitive conclusion (Luganini *et al.*, 2008). In the case of *Plasmodium* infection, the Tlr9-independent protective effect of PS-ODNs also raised the possibility that PS-ODNs could interfere directly with the parasite. Indeed, our results show that PS-ODNs impair parasite gliding motility and that a DNase-resistant PS backbone seems to be essential for this activity, as PD-ODNs were not able to reduce sporozoite gliding activity. In addition, we show that different polyanions, such as nucleic acid molecules [polyI, poly(I : C) and salmon sperm DNA] as well as sulfated polysaccharides (dextran sulfate and heparin) are able to inhibit parasite motility at similar concentrations to PS-ODNs. Previous biochemical experiments have shown that negatively charged molecules, such as heparin and dextran sulfate, bind to positively charged residues of CSP, a constitutively secreted protein that is essential for parasite adhesion to the substrate (Stewart and Vanderberg, 1991; Pancake *et al.*, 1992). This suggests that PS-ODNs could also have the ability to bind via an electrostatic interaction to CSP, which could be sufficient to disrupt parasite attachment to the substrate and thus impair its gliding motility. We further demonstrate that PS-ODNs affect traversal and invasion of hepatocytes but not parasite development

inside the vacuole. More precisely, we could distinguish between cell attachment and cell entry impairment, and show that at low concentrations PS-ODNs reduce invasion through the disruption of the parasite's ability to attach to hepatocytes. In contrast, the remaining sporozoites that could still bind to the cell were not affected in the entry process. This clearly resembles the action of heparin, which is also able to impair sporozoite attachment but not cell entry (Pinzon-Ortiz *et al*., 2001). As it has been reported that the sporozoite CSP is not only required for gliding but also for attachment to hepatocytes, PS-ODNs could disturb this process by binding to CSP, as we hypothesize in the case of the gliding motility. This electrostatic interaction would prevent CSP to bind to its main hepatocyte surface ligand HSPG, thus disturbing cell attachment (Frevert *et al*., 1993; Pinzon-Ortiz *et al*., 2001). Last, we also found that higher concentrations of PS-ODNs are able to inhibit not only cell attachment but also parasite cell entry. The thrombospondin-related anonymous protein (TRAP) is another sporozoite surface protein that is required for cell attachment and cell entry (Matuschewski *et al*., 2002). Upon cell traversal TRAP is released at high local concentrations at the apical end of the parasite (Gantt *et al*., 2000; Mota *et al*., 2002). TRAP also contains a region that binds to HSPGs as well as heparin, suggesting that PS-ODNs may also have affinity to this protein (McCormick *et al*., 1999). The low versus high concentration effects of PS-ODNs on, respectively, cell attachment and cell entry could be explained either by a higher avidity of PS-ODNs for CSP versus TRAP, or by the differences in location and thus accessibility of these proteins. Indeed, CSP is located in a more diffuse pattern at the surface of the sporozoite than TRAP, which reaches high concentration at the apical end. It is therefore possible that a certain threshold concentration of PS-ODNs is necessary to effectively access and bind to released TRAP molecules. Taken together, these results suggest that the primary mechanism of action of CpG PS-ODNs against sporozoite infection, in our experimental system, depends on the inhibition of parasite invasion. However, we cannot exclude the possibility of an additional Tlr9-mediated effect in other systems, such as primary mouse hepatocytes.

Our results reveal that PS-ODNs represent a new class of potent inhibitors of sporozoite migration and invasion into hepatocytes. This finding is interesting as PS-ODNs could be new drug candidates for the prevention of malaria infection. It will now be essential to evaluate whether PS-ODNs are also effective *in vivo* and whether they could be developed as prophylactic drugs with a sufficient therapeutic window for use in human. Interestingly, Tlr9-independent *in vivo* efficacy of PS-ODNs was recently demonstrated in a mouse model of another apicomplexan parasite *Cryptosporidium parvum* infection: prophylactic oral administration of CpG PS-ODNs and their non-CpG derivatives were equally effective in reducing the infection (Barrier *et al*., 2006). In this context, our demonstration of a Tlr9-independent CpG PS-ODNs protection against *Plasmodium* sporozoite infection is highly relevant, as it suggests that *in vivo* CpG PS-ODNs administration might have a dual protective activity by both stimulating the innate immune system in a Tlr9-dependent manner and directly inhibiting parasite invasion of hepatocytes.

Experimental procedures

Cells and parasites

The human hepatoma cell line Huh7 was cultured in RPMI medium, supplemented with 10% fetal calf serum (Gibco/Invitrogen), 1% nonessential amino acid (Gibco/Invitrogen), 1% penicillin/streptomycin (Gibco/Invitrogen), 1% glutamine (Gibco/Invitrogen) and 1% Hepes at pH 7 (Gibco/Invitrogen).

The mouse hepatoma cell line Hepa1–6 was maintained in DMEM 10% fetal calf serum, 1% penicillin/streptomycin and 1 mM glutamine. Both cell lines were maintained at 37°C with 5% CO_2.

The GFP-expressing *P. berghei* ANKA sporozoites were obtained from dissection of infected *Anopheles stephensi* mosquito salivary glands (Franke-Fayard *et al*., 2004). After grinding, the suspension was filtered through a 70 μm cell strainer (Falcon) to remove mosquito debris.

Reagents

The ODNs were commercially synthesized either with a PS or a natural PD backbone, by Thermo Scientific. The following ODNs were used in this study: CpG ODN 2007 tcgtcgttgtcgttttgtcgtt, CpG ODN 2007 containing Oregon Green at the 5′ end, GpC ODN 2007 tgctgcttgtgcttttgtcgtt, CpG ODN 2006 tcgtcgttttgtcgttttgtcgtt, GpC ODN 2006 tgctgcttttgtgcttttgtgctt, CpG ODN 1826 tccatgacgttcctgacgtt, GpC ODN 1826 tccatgagcttcctgagctt. The polyanions, heparin, dextran sulfate, salmon sperm DNA, polyI and poly(I : C) were purchased from Sigma. Dextran tetramethylrhodamine 10000 molecular weight and lysine fixable (fluoro-ruby) were purchased from Molecular Probes/Invitrogen and DOTAP from Roche Diagnostics.

Anti-CSP 3D11 antibody (directed against the repeat region of *P. berghei* CSP) was kindly provided by Victor Nuessenzweig (New York Medical School); anti-mouse ALEXA594 and anti-mouse ALEXA488 were bought from Molecular probes.

ODN complexation with DOTAP

The complexation of CpG PS-ODN 2007 with DOTAP was performed accordingly to the manufacturer's instructions. Briefly, PS-ODNs were suspended in 120 μl of PBS, and then mixed with 30 μl of DOTAP solution. This mixture was incubated for 15 min at room temperature and added to cells cultured in 850 μl of complete RPMI medium.

ODN uptake assay

The ODN uptake was measured as previously described (Roberts *et al*., 2005). Confluent Huh7 cells were incubated with Oregon Green-labelled CpG PS-ODN 2007 for 45 min in 500 ml complete RPMI medium. Cells were washed with ice-cold PBS and incubated with 12.5 mg ml^{-1} dextran sulfate for 10 min on ice to remove residual ODNs bound to the cell surface. Cells were then washed with PBS, fixed with 4% paraformaldehyde and analysed with a Zeiss LSM510 Meta laser scanning confocal microscope.

Sporozoite gliding motility assays

Glass coverslips were coated with 10 μg ml^{-1} of a *P. berghei* specific anti-CSP monoclonal antibody for 1 h. Freshly dissected sporozoites were resuspended in complete RPMI medium, spun for 5 min at 3000 r.p.m. on glass coverslips and incubated at 37°C for 1 h. Sporozoites and trails were visualized using anti-CSP antibody followed by anti-mouse-ALEXA488 conjugated antibody. Slides were washed and photographs of the 'gliding circles' were obtained using a Leica DM5000B widefield fluorescence microscope at 40× magnification. Live imaging of sporozoite gliding motility was performed with a Zeiss Axiovert 200 M widefield fluorescence microscope.

In vitro *infection assays*

For determination of sporozoite infectivity *in vitro*, 30 000 freshly dissected sporozoites were added to subconfluent monolayers of Huh7 cells. FACS analysis at 2 and 24 h after sporozoite addition was performed to determine the percentage of parasite-containing cells and parasite-GFP intensity within infected cells. For measurements of traversal and invasion, 1 mg ml^{-1} RD was added to the cells immediately prior to sporozoite infection. Cell samples for FACS analysis were processed as previously described (Prudencio *et al*., 2008).

Sporozoite attachment/cell entry assay

The attachment/cell entry assay was performed as previously described (Renia *et al*., 1988). Huh7 cells were fixed with 4% paraformaldehyde 1 h after sporozoite addition and extracellular (non-invading) parasites were stained with monoclonal anti-CSP antibodies followed by anti-mouse ALEXA594. In order to quantify intracellular parasites, the hepatocytes were permeabilized with 1% Triton X-100 in PBS for 5 min allowing the intracellular parasites to be stained using monoclonal anti-CSP antibodies in conjunction with anti-mouse-ALEXA488. Nuclei were stained with 1 μg ml^{-1} diamidino-phenylindole. All sporozoites were ALEXA488-positive, whereas only extracellular sporozoites appeared red. Analysis and counting of stained parasites were performed using a Leica DM5000B widefield fluorescence microscope. The internalization rate was calculated as the ratio of the number of intracellular parasites to the total number of parasites present per coverslip, using the following equation: [(total number of parasites – extracellular number of parasites)/total number of parasites] × 100 (Sinnis, 1998).

Statistical analysis

Data were analysed with Kruskal–Wallis test for statistical significance. The test is based on the hypothesis (H) that all samples are the same. *P*-values bigger than 0.05 indicate that the data are significantly different. The program R was used for the analysis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CpG and GpC PS-ODNs impair *P. berghei* infection in Hepa 1–6 cells. Hepa 1–6 cells were incubated 1 h prior to sporozoite addition with 10 μM of CpG PS-ODN 2006, GpC PS-ODN 2006, CpG PS-ODN 1826 and GpC PS-ODN 1826. Infection rates were measured 24 h post infection by FACS. PS-ODN-treated samples were tested against non-treated control samples by Kruskal–Wallis rank sum test, H rejected with P-value ≥ 0.05.

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