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Supporting Online Material for

Manipulation of Host Hepatocytes by the Malaria Parasite for Delivery into Liver Sinusoids

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Published 3 August 2006 on *Science* Express
DOI: 10.1126/science.1129720

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Other Supporting Online Material for this manuscript includes the following (available at www.sciencemag.org/cgi/content/full/1129720/DC1):

Material and Methods

Cells and parasites. HepG2 and Hepa1-6 cells were obtained from the European cell culture collection and were maintained in EMEM supplemented with 10% FCS and 2 mM glutamine. *P. berghei* (Anka) sporozoites were prepared from infected *Anopheles stephensi* mosquito salivary glands and were co-cultured with HepG2 cells for 2 h in supplemented EMEM. Free sporozoites were removed by washing with complete medium and the infected HepG2 cells were incubated in a CO_2 incubator at 37° C for indicated times.

TEM and SEM. For Transmission electron microscopy, floating cells were fixed by addition of 3% glutaraldehyde/0,1 M phosphate buffer followed by 0,1% osmium tetroxide. Cells were dehydrated in 80% ethanol and embedded in EPON resin mixture. Ultrathin sections of grey interference colour were observed with a Zeiss electron microscope.

Scanning electron microscopy. *P. berghei* infected cells were washed twice in PBS, fixed in 2% glutaraldehyde in sodium cacodylate buffer, and postfixed with 1% osmium. Samples were dehydrated at increasing ethanol concentrations (30 to 100%). After critical-point drying, samples were treated with gold and analyzed with a Philips SEM500 scanning electron microscope.

Drug treatment. Floating infected cells were treated with 1 µg/ml ionomycin for 1 h before the cells were analysed for Ca^{2+} release and phosphatidyl serine exposure using the Annexin-V-Alexa 568 kit from Roche. Infected HepG2 cells were treated with 100 µM zVAD-fmk (Promega) or 10µg/ml L-trans-epoxy-succinyl-leucylamido-(4-guanidino)butane E64 (Sigma) at 55 and 61hpi and analysed for the appearance of floating cells.

In vivo infection. C57/Bl6 mice were kept and treated according to national animal care guidelines. Mice were infected intravenously with $2x10^5$ to $4x10^5$ *P. berghei* sporozoites. Livers were removed and fixed with 4% Paraformaldehyde (PFA). Livers were dissected out and post-

fixed overnight in 4% PFA. After embedding in paraffin, livers were sectioned and stained with either haematoxylin/eosin or with chicken anti *P. berghei* hsp90 antiserum.

To test the infectivity of liver-derived merozoites, floating cells were separated at 63 hpi and culture supernatant was collected and incubated for another hour at 37°C to make sure that free merozoites which were present in the medium are not viable anymore. For each experiment 2 groups of each 4 mice were infected with either floating cells or with culture supernatant of 63h infected HepG2 cells without floating cells to control the viability of free merozoites. Parasitemia was followed up for 6 weeks or until the animals died. To test the viability of merozoites in Annexin-V positive cells, floating cells (75 hpi) were first labelled with Annexin-V-Alexa 568, transferred into 6 well plates and diluted in PBS. Single cells were separated using extra long micro pipet tips (Biorad) while viewing them under an IF microscope and were collected in a 24 well plate. Cell pools were again analysed for positive Annexin-V staining before 4 mice were infected with each 20 Annexin-V-Alexa 568 positive cells. As control, 4 mice were injected with Annexin-V-Alexa 568-negative floating cells isolated 63 hpi (20 cells/mouse), which were stained, analysed and separated as described above with the exception that Annexin-V-negative cells were collected.

Intravital imaging. For infection we injected 0.5-1.5x10^6 *P. berghei* sporozoites that express the green fluorescent protein into the tail vein of C57/Bl6 or nude mice. From 42 to 51 hpi the infected rodent was anaesthetised i.p. with a mixture of ketamine (125 mg/kg, Imalgene, Merial) and xylazine (12.5 mg/kg, Rompum, Bayer) and a small left-ventral abdominal incision was made to expose the left liver lobe. The exposed lobe was immobilised on a coverslip with cyanocrilate glue. The anaesthetised animal was placed on the motorised stage of an inverted Axiovert 200 microscope (Zeiss) and covered with a homothermic blanket system at 37 °C (Harvard Apparatus). Images were acquired using an UltraView RS Nipkow-disk confocal system (Perkin-Elmer) with an ORCA II ER CCD camera (Hamamatsu). Continuous single movies were recorded for up to 30 minutes covering a volume of 20-50 μm in depth (individual z-stacks of 1-2

μm) with a Plan Neofluar objective 25x/0.8 Imm. Korr (Zeiss) plus optovar (1.6x). To label the blood circulation far-red fluorescent albumin (50 μg; Invitrogen) was injected prior to recording. Image files were processed using ImageJ (http://rsb.info.nih.gov/ij/).

Ca^{2+} detection in parasitized cells. Suspensions of infected floating cells in serum free EMEM were loaded with Fluo-4/AM (3 μM, Molecular Probes) for 30 min at 37° C. For simultaneous staining of infected cells with Fluo-4/AM and Annexin-V-Alexa^{TM} 568, the cell suspension was first loaded with Fluo-4/AM, transferred in Annexin-V binding buffer and stained with Annexin-V-Alexa 568 according to the manufacturers protocol (Roche). After washing in PBS, cells were analysed with a Zeiss Axioskop 2 plus Microscope and the Improvision software (Openlab 4.01).

Detection of mitochondrial membrane potential. HepG2 cells were infected with GFP expressing parasites and cultivated for 50-65 hours. Cells were treated with 25 nM TMRE for 15 min at 37 °C in the dark and subsequently analysed with a Zeiss Axiovert Immunofluorescence microscope and the Improvision software (Openlab 4.03).

TUNEL. For the TUNEL test, DNA ends were labelled using an *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany). Adherent infected HepG2 cells were fixed in 2 % PFA and then stained according to the manufactures protocol. Floating cells were first immobilised on poly-L lysine-coated coverslips and then fixed and stained.

Immunofluorescence analysis. Cells were washed with PBS before fixation with 2 % PFA. Floating cells were then immobilised on poly-L lysine coated coverslips. After permeabilisation with ice-cold 100 % methanol, fixed cells were incubated with primary antibodies overnight at 4°C in PBS supplemented with 10 % FCS. The primary antibodies used were chicken anti-*P. berghei* Exp1, rabbit anti-cytochrome c (Transduction laboratories), anti-merozoite mouse serum, mouse anti-*P. berghei* hsp90. Corresponding secondary antibodies used were anti-mouse antibodies labelled with Alexa Fluor 594 (Molecular Probes), anti-rabbit antibodies labelled with

Cy3 and anti-chicken antibodies conjugated with Cy 2 (Dianova, Germany). Cells were washed in PBS and stained with Hoechst 33258 (Molecular Probes, 1:2000 in PBS) for 1 min.

Image acquisition and manipulation

Images were obtained with a Zeiss Axioskop 2 plus immunofluorescence microscope and a Hamamatsu video camera. The imaging medium was Dako® fluorescent mounting medium for all fixed probes and PBS for living cells. Acquisition software was from Improvision (Openlab release 4.01). Nearest neighbour acquisition or contrast enhancement was applied before import into Adobe Photoshop. Images were cropped to size and saved as TIF files without further manipulation.

SUPPLEMENTARY FIGURES:

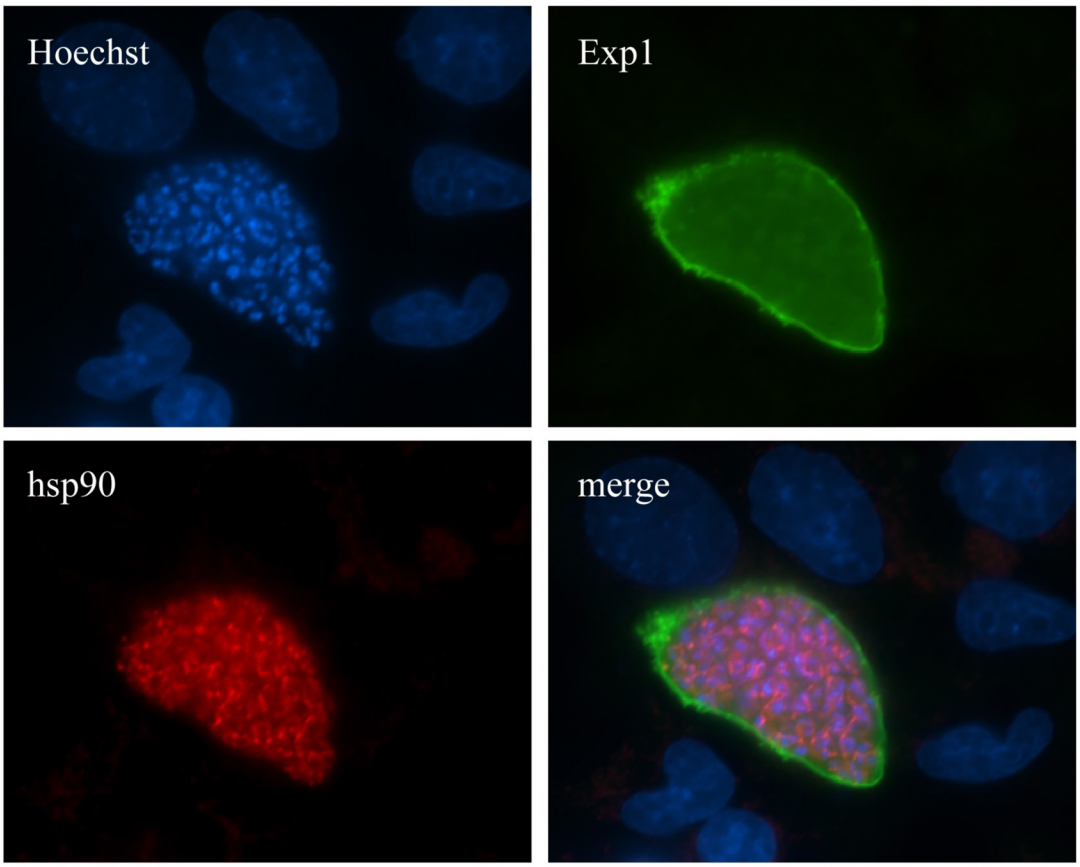

Figure S1. IF analysis of fully developed *P. berghei* liver schizonts in HepG2 cells at 48 hpi. Infected cells were fixed and subsequently stained with chicken polyclonal antibodies against PbExp1 and mouse polyclonal antiserum against Pbhsp90. Secondary antibodies (anti-chicken conjugated with Cy2 and anti-mouse conjugated with Alexa 594 (Molecular Probes) were used to visualize the detected antigens. DNA is stained with Hoechst 33258 (blue).

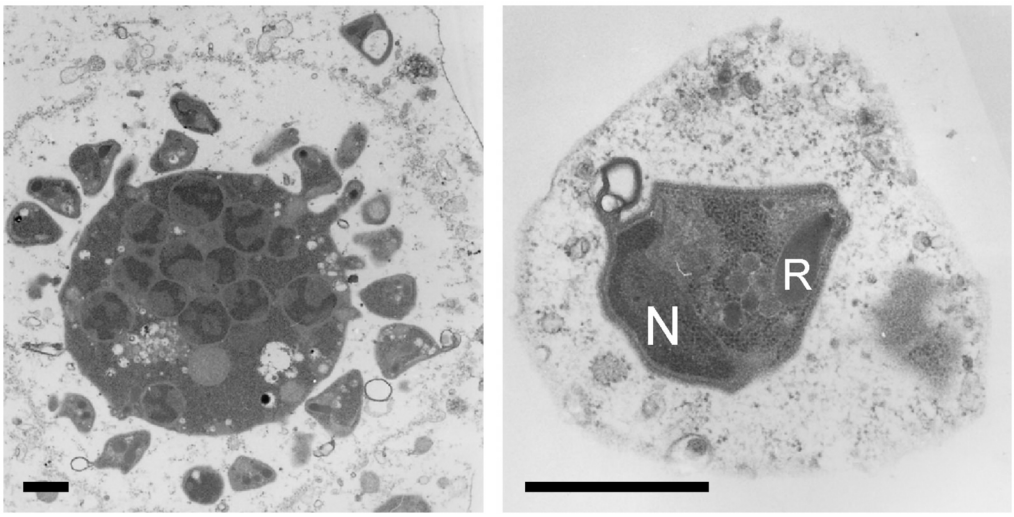

Figure S2. TEM of a meroblast and a single in vitro developed exoerythrocytic merozoite. Note that merozoites bud of the meroblast within the host cell. N, merozoite nucleus; R, rhoptry; Scale bars: 1 µM

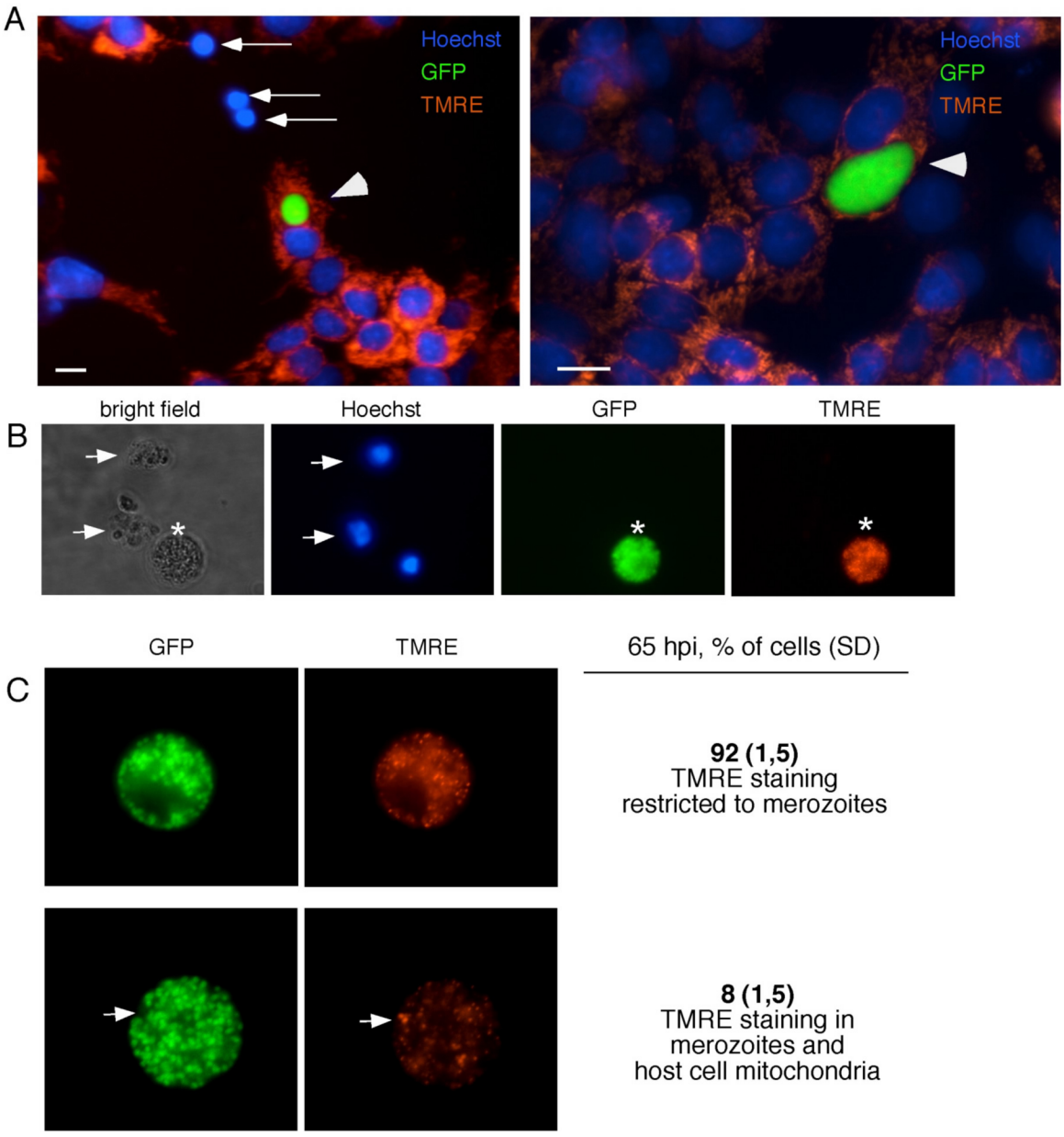

Figure S3. Mitochondria in detached cells lose membrane potential. HepG2 cells were infected with 2 x 10^4 GFP expressing *P. berghei* sporozoites and cultured for 55 - 65 h. (A) adherent cells (50 hpi) were analysed as a positive control for TMRE staining. As expected the status of mitochondria in these infected adherent cells does not differ from non-infected cells. 99 % (SD=1) of more than 300 cells counted in three independent experiments exhibited the depicted phenotype. Note TMRE-negative apoptotic HepG2 cells (arrows). Infected cells are marked with an arrowhead. (B and C) in detached cells (65 hpi), the majority of host cell mitochondria lost membrane potential. Instead, merozoites are labelled indicating that the parasite is viable, whereas host cell mitochondria are not anymore functional. (B) note the TMRE-negative apoptotic cells (arrows), the detached infected cell is labelled by an asterisk. (C) more examples of TMRE-stained detached cells and quantitative assessment of cells with no, or residual TMREpositive host cell mitochondria. Arrow labels a TMRE-positive body, presumably a host cell mitochondrion (GFP-negative).

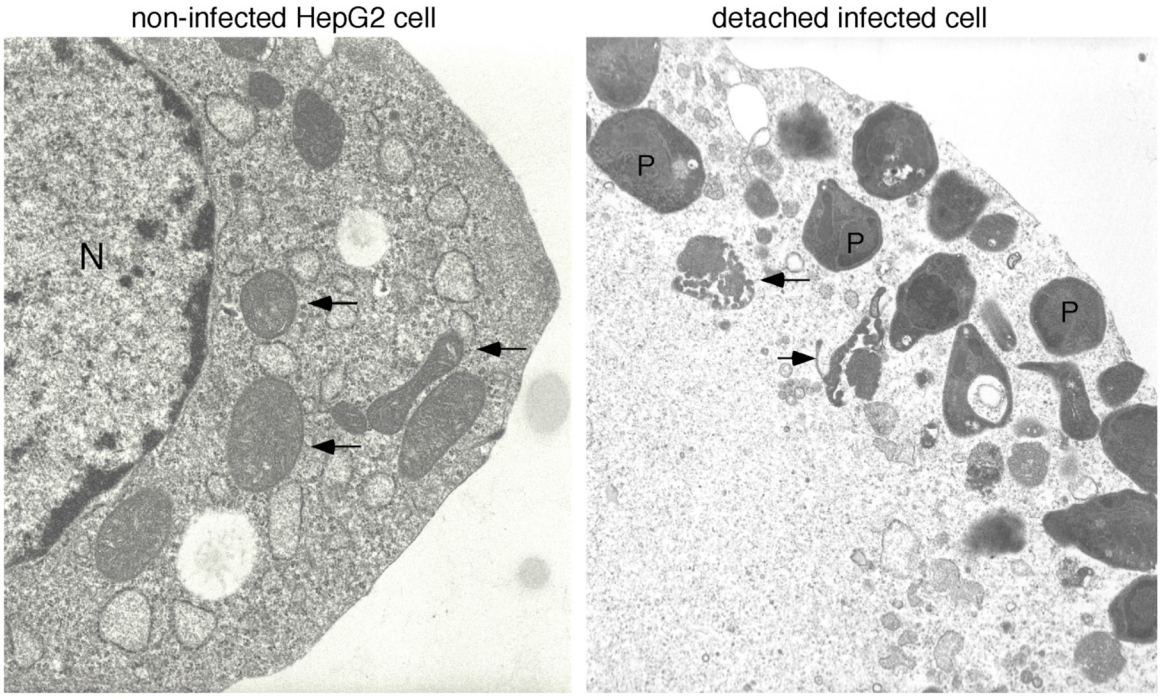

Figure S4. TEM of a non-infected HepG2 cell and a detached cell containing merozoites. HepG2 cells were trypsinised and thereafter treated as detached cells (left image, arrows indicate mitochondria). Non-infected cells served as a positive control to demonstrate intact mitochondria structures. Detached cells (right image) contain few organelles, which might correspond to mitochondria. Arrows label presumed mitochondria in detached cells.

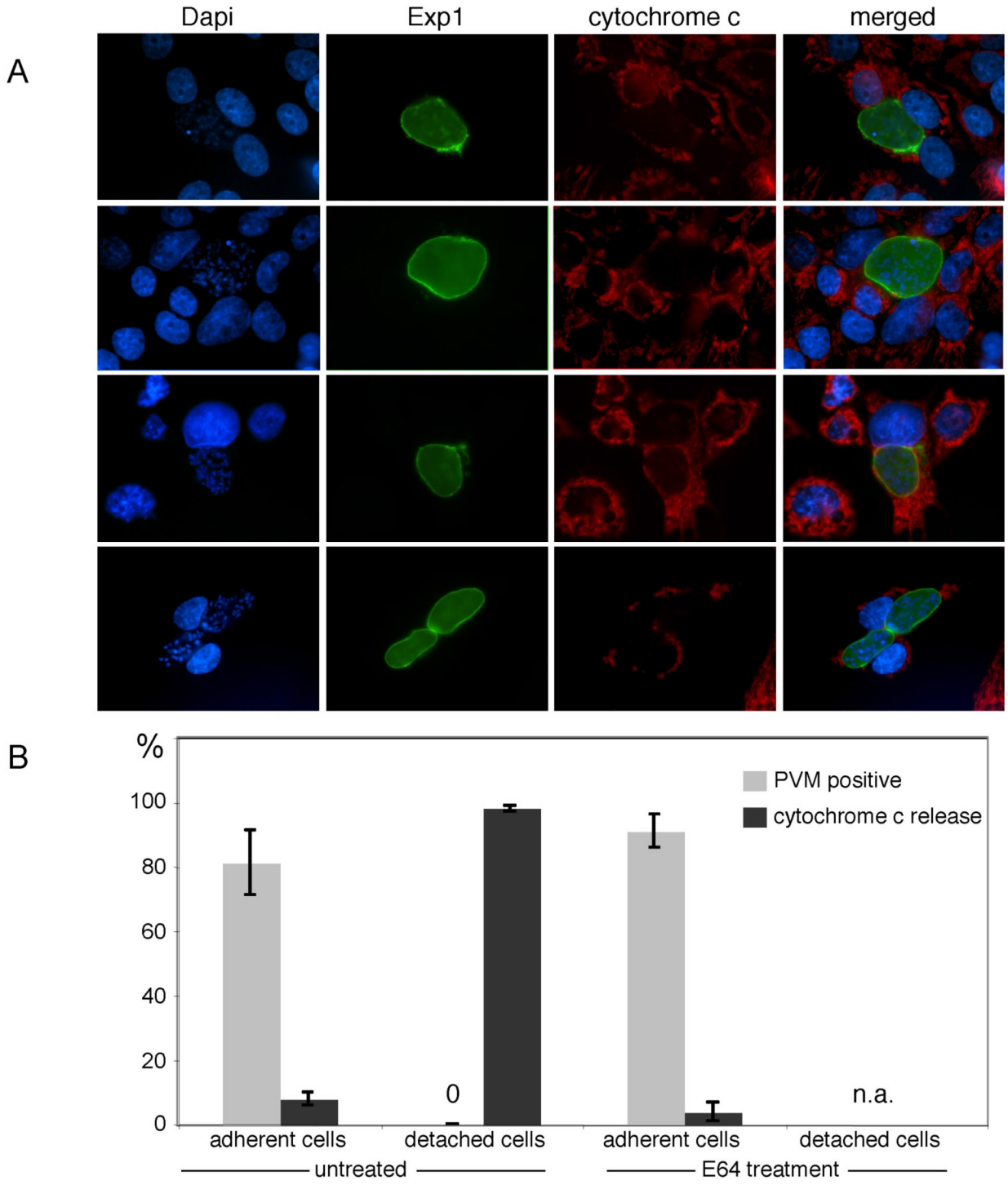

Figure S5. (A) E64 treatment inhibits cell detachment and maintains integrity of mitochondria. HepG2 cells were infected with 2 x 10^4 *P. berghei* sporozoites and cultured for 55 h. Infected cultures were then treated with 10 µg/ml E64 and cultivated for additional 8 h before they were PFA-fixed and stained with an anti-*P. berghei* Exp1 chicken antiserum and an anti-cytochrome c rabbit antiserum followed by anti-chicken Cy2 antibody (green) and anti-rabbit Cy3 antiserum (red). DNA was stained with Hoechst 33258. (B) quantitative evaluation. HepG2 cells were infected as described above and cultured for 63 hours. E64 was added for the last 8 h, control cultures were left untreated. 63 hpi, detached cells were collected from the culture supernatant and transferred to poly L-lysine slides, fixed and stained with the anti-Exp1 and anti-cytochrome c antiserum. Adherent cells were fixed at 63 hpi and stained accordingly. Both, adherent cells and detached cells were additionally stained with Hoechst 33258. Note that E64 treatment inhibits cell detachment and therefore quantitative evaluation of detached, treated cells was not applicable (n.a.). For the other groups, 100 infected cells were counted and the status of the PVM and cytochrome c release was investigated. Percentage (±SD) of positive cells was calculated from 3 independent experiments.

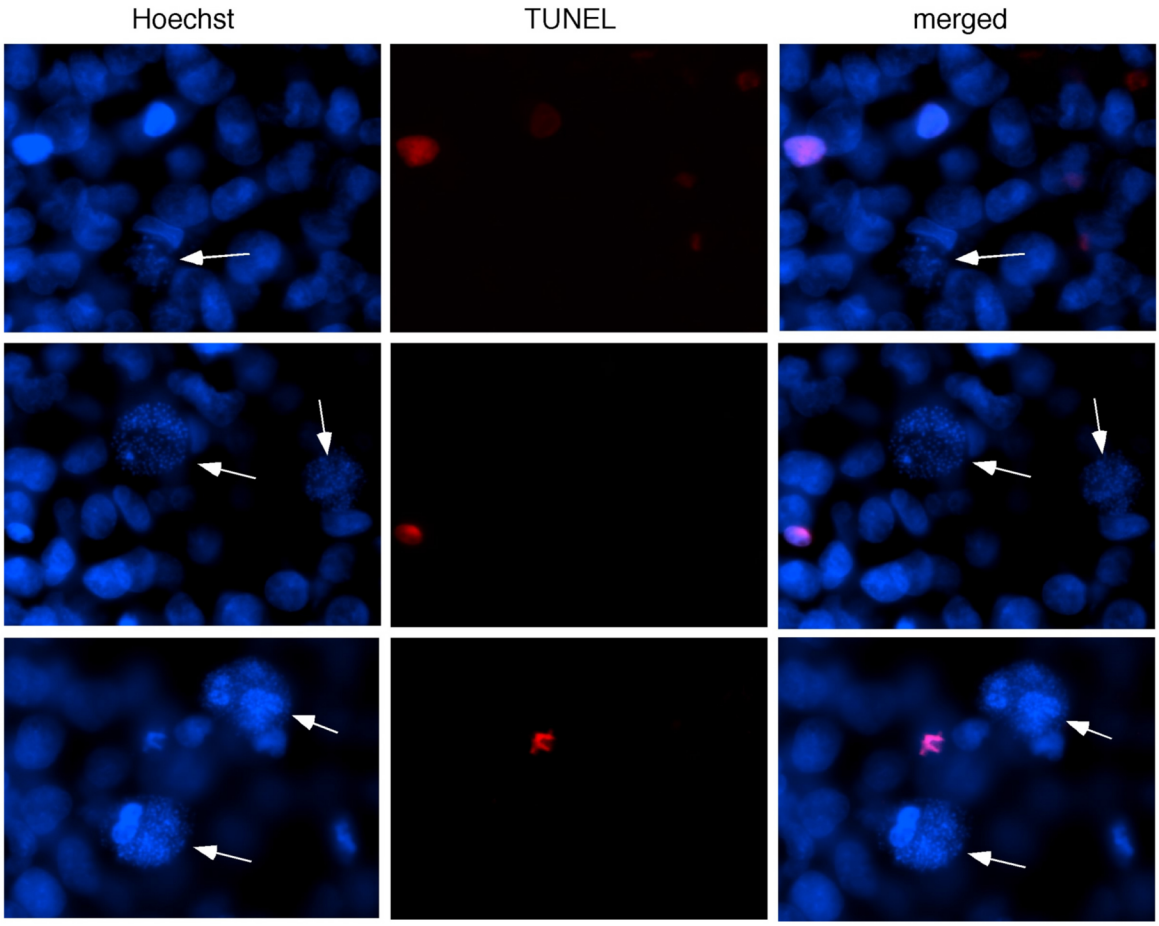

Figure S6. TUNEL staining of late infected cells. Infected cells were cultured for 63 hours and then fixed with 2 % PFA and subsequently stained for fragmented DNA using the TUNEL assay. Different phases of cell detachment are shown. In order to present a positive control for TUNEL staining, images including apoptotic non-infected cells (red stained nuclei) are depicted. Infected cells are labelled with arrows. Nuclei are stained with the Hoechst 33258 dye.

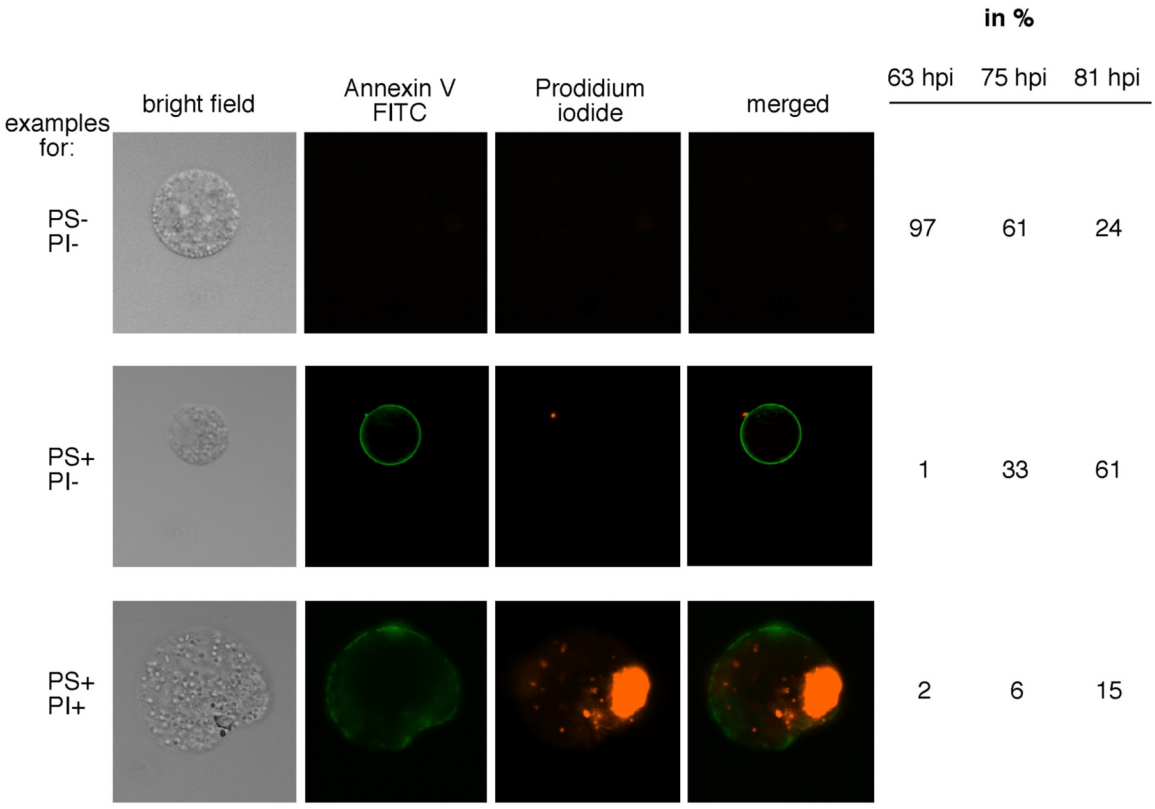

Figure S7. Detached cells initially maintain cell membrane integrity. HepG2 cells were infected with 2 x 10^4 *P. berghei* sporozoites and cultured for 63 - 81 h. Detached cells were collected and co-stained with propidium iodide and Annexin-V-FITC. Note the single PI-positive free merozoite in the middle panel attached to the detached cell. Detached cells, however, do not become PI-positive, although they lose membrane asymmetry (become PS positive) indicating that the cell undergoes ordered cell death and not necrotic cell death. The few PI-positive cells detected 63 - 75 hpi were most likely physically damaged by pipetting. After 81 hpi, PI-positive cells are more frequent presumably because of complete membrane breakdown in the final stages. For every time point 100-200 cells were analysed and the percentage of PI and PS positive cells calculated. The experiment was repeated three times with similar results. The complete statistical evaluation of the experiment (incl. SD) is presented in Figure 4a.

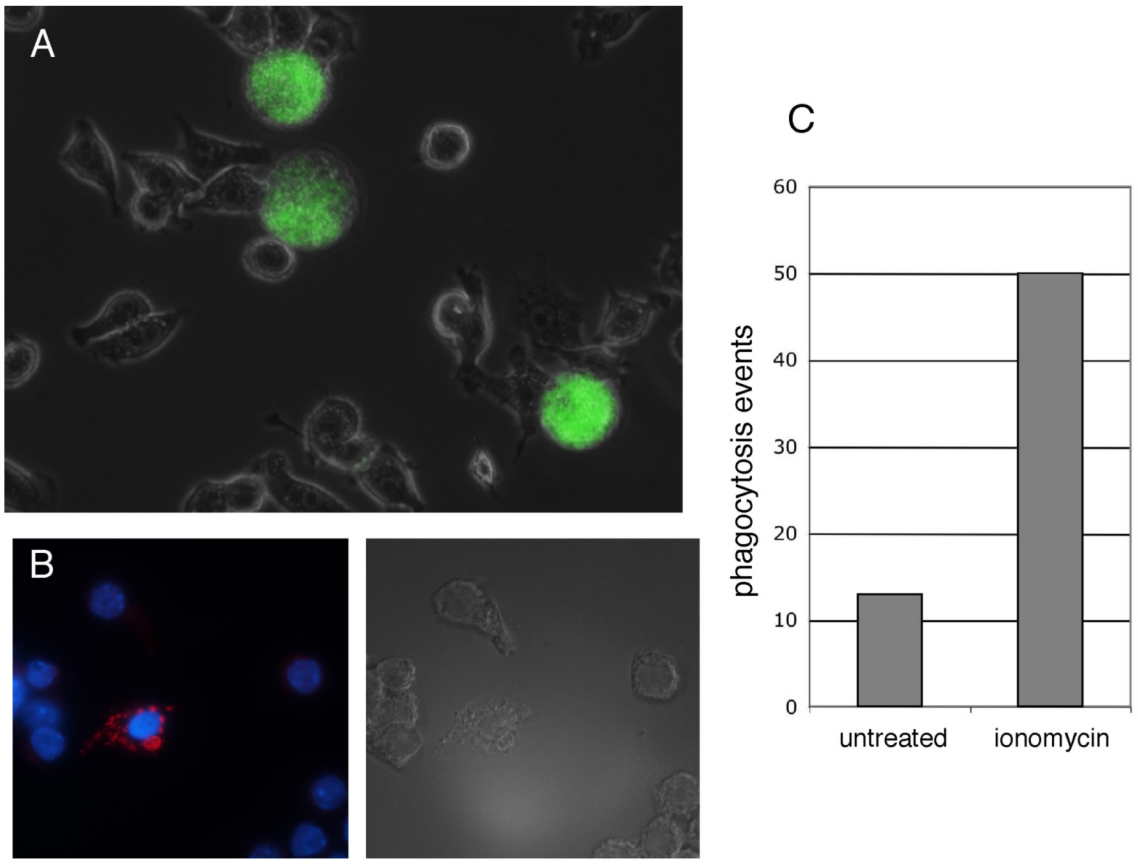

Figure S8. Detached cells are not phagocytosed by RAW macrophages. About 200 green fluorescent infected detached cells were treated with ionomycin (B, positive control), or left untreated (A) and were then added to cultures of RAW macrophages and co-cultivated for 2 hours. Phagocytic events were counted either by scoring GFP-positive macrophages, or by fixation and subsequent staining with parasite specific anti-sera (B). (C) quantitative assessment of phagocytic events observed after fixation and staining. The experiment was repeated twice with similar results.

SUPPLEMENTARY FIGURE VIDEOS:

Supplementary Videos 1 to 4

Time-lapse intravital microscopy of a *P. berghei*-infected hepatocyte and budding vesicles (merosomes). Mice were infected with GFP-expressing *P. berghei* sporozoites and a liver lobe was exposed for microscopy at 51 hpi. Developing merosomes were followed up for 150 minutes. Video S1 shows parasite material (green) transported to a liver sinusoid labelled with red fluorescent BSA (red). S2 shows both, retraction of this parasite material to the mother cell and formation of a new merosome at a different place of the same cell. Video S3 and S4 show how this merosome increases with time by continous transfer of parasite material from the mother cell to the budding merosome. Time is individually indicated in minutes or seconds.