Plasmodium berghei-Infected Primary Hepatocytes Process and Present the Circumsporozoite Protein to Specific CD8+ T Cells In Vitro

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Plasmodium berghei-Infected Primary Hepatocytes Process and Present the Circumsporozoite Protein to Specific CD8+ T Cells In Vitro

Silaïyuv E. Bongfen,* Ralph Torgler,* Jackeline F. Romero,* Laurent Renia,†‡§ and Giampietro Corradin**

A substantial and protective response against malaria liver stages is directed against the circumsporozoite protein (CSP) and involves induction of CD8+ T cells and production of IFN-γ. CSP-derived peptides have been shown to be presented on the surface of infected hepatocytes in the context of MHC class I molecules. However, little is known about how the CSP and other sporozoite Ags are processed and presented to CD8+ T cells. We investigated how primary hepatocytes from BALB/c mice process the CSP of Plasmodium berghei after live sporozoite infection and present CSP-derived peptides to specific H-2Kd-restricted CD8+ T cells in vitro. Using both wild-type and spect−/− P. berghei sporozoites, we show that both infected and traversed primary hepatocytes process and present the CSP. The processing and presentation pathway was found to involve the proteasome, Ag transport through a postendoplasmic reticulum compartment, and aspartic proteases. Thus, it can be hypothesized that infected hepatocytes can contribute in vivo to the elicitation and expansion of a T cell response. The Journal of Immunology, 2007, 178: 7054–7063.

The feasibility of vaccine development against malaria liver stages is indicated by studies (1, 2) which show that sterile immunity against the liver stage can be obtained by immunization of animals and humans with irradiated and, recently, genetically attenuated sporozoites (3). Irradiated sporozoite-induced protective immunity has been shown to involve IFN-γ, CD4+, and CD8+ T cells directed against liver stage parasites, and in vivo depletion of these effectors has led to blood infection upon a live sporozoite challenge (4–10).

Even though sporozoites pass through Kupffer cells before infecting hepatocytes (11), hepatocytes are the only cells that sustain complete development of malaria liver stages after infection with sporozoites. Maintenance of the anti-sporozoite protective CD8+ T cell response requires hepatic stages, since protection against sporozoite challenge is abrogated if these hepatic stages are eliminated (12). Furthermore, immunization with irradiated Plasmodium berghei-infected hepatocytes (but not infected liver nonparenchymal cells) conferred protection against a sporozoite challenge (13). It has been suggested that in malaria, hepatocytes express MHC class I-peptide complexes on their surface and that the recognition of these complexes by CD8+ T cells is necessary for protection (reviewed in Ref. 14). This notion is supported by studies which show that irradiated sporozoite-immunized β2m−/− mice are not protected against sporozoite challenge, despite induction of IL-12, IFN-γ, and proliferating T cells (15).

It was widely believed that only endogenous Ags could be presented on MHC class I molecules to CD8+ T cells. A large body of evidence now shows that peptides derived from exogenous Ags are also capable of binding to MHC class I molecules and stimulating CD8+ T cells, a process known as cross-presentation. Several models of cross-presentation have been described (16–21), especially for professional APCs. In addition, the use of exogenous Ags such as long synthetic peptides to immunize mice (22, 23) and humans (24) to obtain MHC class I-restricted CTLs also supports the concept of cross-presentation. Besides professional APCs, non-professional APCs have also been shown to be capable of cross-presenting long polypeptides to CD8+ T cells (25, 26).

The mechanisms involved in malaria Ag presentation to T cells remain unclear, because the low number of Plasmodium-infected hepatocytes in the liver has prevented the development of direct Ag presentation studies. The question is further complicated by the fact that sporozoites do not infect the first hepatocyte they encounter, but traverse through many hepatocytes before infecting a final one (27). As they traverse, they leave behind trails of the circumsporozoite protein (CSP)3 in the traversed cells, some of which reseal their membranes and survive and are, in principle, capable of presenting the Ag. This suggests that sporozoite Ags could either be presented by the infected cell, the traversed cell, or both. It has been suggested (28) that migration of sporozoites through hepatocytes is essential for final infection. However, a recent study (29) has identified a sporozoite microneme protein essential for cell traversal (SPECT) and has shown that sporozoites

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3 Abbreviations used in this paper: CSP, circumsporozoite; SPECT, sporozoite microneme protein essential for cell traversal; wt, wild type; BFA, brefeldin A; LLnL, N-acetyl-l-leucyl-l-leucyl-l-norleucinal; Oβ-LLL, carbobenoxyl-leucyl-leucyl- leucinal; ICS, intracellular cytokine staining; RT, room temperature; DAPI, 4’,6’-diamidino-2-phenylindole; EEF, exoerythrocytic form; GdCl, gadolinium chloride; DC, dendritic cell; HSP, heat shock protein.

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deficient in the spect gene are incapable of cell traversal, but capable of infection and normal development. spect−/− parasites are therefore an essential tool in determining the ability of the infected hepatocyte to process and present sporozoite Ags to CD8+ T cells.

Despite evidence for the importance of hepatic stages to maintain a protective immune response and suggestions that hepatocytes present MHC class I-peptide complexes on their surface, very few studies (30, 31) have attempted to address the question of direct Ag presentation by hepatocytes in an in vitro or in vivo assay.

In this study, using mouse primary hepatocytes, wild-type (wt) and spect−/− P. berghei sporozoites, we investigated the cell (traversed or infected hepatocytes or both) involved in processing and presentation of the CSP of P. berghei to specific CD8+ T cells. We also investigated the pathway of this processing and presentation by the primary hepatocytes after infection with wt sporozoites. We formally show that both infected and traversed hepatocytes are able to process the native CSP present on live parasites for presentation to specific CD8+ T cells. Our data show that this processing is predominantly dependent on the proteasome and also on aspartic proteases.

Materials and Methods

Peptides

The peptide PbCS245–253, representing the H-2Kd-restricted CTL epitope of the CSP of P. berghei and the peptide PICS227–235, an HLA-A2.1-restricted peptide from the CSP of P. falciparum, were synthesized by standard solid-phase F-moc chemistry. Peptide stock solutions (2 mg/ml) were prepared in 1× PBS and stored at −20°C.

Cells

The APCs were primary hepatocytes isolated from BALB/c mice by a two-step perfusion of liver lobules with collagenase as previously described (32), with slight modifications. Briefly, mice were sacrificed by CO2 inhalation, dissected, and a liver lobule was cut out. The lobule was perfused for 10 min with Ca2+-free HEPES buffer (pH 7.6) at 37°C and at a rate of 5 ml/min. The lobule was then perfused with type IV collagenase (Sigma-Aldrich) solution (Ca2+-free HEPES buffer containing 0.04% type IV collagenase and 0.075% CaCl2) for 5 min at 37°C. The perfused lobule was incubated for 10 min at 37°C in the collagenase solution. Using sterile pipettes, the tissue was gently teased apart to release cells. Cells were washed once with Ca2+-free HEPES buffer at 800 rpm for 30 s at 4°C. The pellet was gently resuspended in DMEM, layered on 60% Percoll, centrifuged again at 800 rpm for 30 s at 4°C. The pellet was resuspended in DMEM, layered on 60% Percoll, centrifuged again at 800 rpm for 30 s at 4°C. The pellet was resuspended in complete culture medium (DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, 1% HEPES, and 0.05 mM 2-ME), and resuspended in complete culture medium (DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, 1% HEPES, and 0.05 mM 2-ME) until a concentration of 105 cells was obtained.

In this study, mouse primary hepatocytes, wild-type (wt) and spect−/− P. falciparum sporozoites, were also used.

Inhibitors

The inhibitors used were brefeldin A (BFA), N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLNL), carbobenzoxy-L-leucyl-L-leucinal-leucinal (Cbz-LLL), lactacystin, pepstatin, leupeptin, chloroquine, and ammonium chloride (NH4Cl). All inhibitors were obtained from Sigma-Aldrich. In preliminary experiments, we showed that none of these inhibitors were toxic for the primary hepatocytes at the concentrations used (data not shown).

IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay was performed as previously described (33). Briefly, 105 primary hepatocytes were seeded per well in 1% gelatin-coated 48-well culture plates and incubated overnight at 37°C to enable them to adhere. Hepatocytes were washed with culture medium and infected for different lengths of time with P. berghei sporozoites (25,000/well). Uninfected cells were used as a negative control. All cells were washed twice with culture medium and cocultured with 5000 PbCS-C7 CD8+ T cells per well for 4 h at 37°C. T cells were then transferred to ELISPOT plates previously coated with anti-mouse IFN-γ Ab and blocked with 1% BSA. The ELISPOT plates were incubated for 20–24 h at 37°C, after which the plates were washed and developed. Spots were visualized and counted with an automated ELISPOT reader.

Intracellular cytokine staining (ICS)

Primary hepatocytes (105) were seeded per well in 1% gelatin-coated 48-well culture plates and incubated overnight at 37°C. Cells were washed and infected with 25,000 wt, spect−/−, or midgut sporozoites per well for 8 h at 37°C. As controls, heat-killed wt sporozoites or material from salivary glands of uninfected mosquitoes were incubated with hepatocytes for 8 h before coculture with specific CD8+ T cells. As a positive control, cells were pulsed with 1 μg/ml of the PbCS245-253 peptide for 2 h at 37°C. Unpulsed/uninfected cells were used as a negative control. After pulsing and infection, cells were washed and cocultured with 5000 PbCS-C7 CD8+ T cells per well for 4 h at 37°C in the presence of 10 μg/ml of the intra-cellular transport inhibitor BFA. T cells were then harvested, washed, and stained with FITC-conjugated anti-mouse CD8 Ab (BD Biosciences) for 30 min at 4°C. Cells were washed twice with PBS-3% FCS and fixed for 10 min at room temperature with prewarmed 4% paraformaldehyde. After two washes, with 0.5% saponin-1× PBS, cells were stained overnight at 4°C with PE-conjugated anti-mouse IFN-γ Ab (BD Biosciences) diluted in the saponin solution. Cells were washed with PBS-3% FCS and analyzed for CD8+ and IFN-γ+ T cells by flow cytometry.
In separate experiments, primary hepatocytes were prepared from a BALB/c mouse 24 h after i.v. injection of 10 mg/kg body weight of gadolinium chloride (GdCl) to depress Kupffer cell function (34). These hepatocytes were then plated on 1% gelatin-coated 48-well plates and ICS was conducted as described above.

**Inhibition assay**

In the inhibition assays, the inhibitors were used at the following concentrations: LLnL (50 μM), Cbz-LLL (10 μM), lactacystin (20 μM), pepstatin (36 μM), leupeptin (110 μM), BFA (35 μM), chloroquine (10 μM), and NH₄Cl (10 mM). Hepatocytes were incubated for 2 h at 37°C with the different inhibitors before pulsing with peptide or infection with sporozoites. Pulsing or infection of inhibitor-treated cells was performed either without washing off the inhibitor or after washing. Cells were then washed and cocultured with specific CD8⁺ T cells. The inhibition assays were completed as described for ICS.

**Chromium release assay**

Primary hepatocytes (10⁵) were plated per well in a 1% gelatin-coated 96-well plate and incubated overnight at 37°C. They were washed and either pulsed with 1 μg/ml of the PbCS₂₄₅–₂₅₃ peptide (using the HLA-A2.1-restricted CTL epitope from the CSP of *P. falciparum*, PbCS₃₂₇–₃₃₅, as a negative control) for 1 h at 37°C, or infected with 25,000 wt sporozoites for 16 h. Seventy-five microcuries of chromium was then added and
incubation was continued for another hour. Cells were washed and cocultured with PbCS-C7 CTLs at different E:T ratios in the presence or absence of 10 μg/ml Fas-Fc for 4 h at 37°C. The supernatants were collected and read with a gamma counter.

Detection of apoptotic hepatocytes

Primary hepatocytes (8 × 10^4) were seeded per well in 8-well LabTek chamber slides and incubated overnight at 37°C. Cells were washed and either infected with 2 × 10^4 P. berghei sporozoites for 8 h or pulsed with the PbCS245-253 peptide for 2 h at 37°C. Hepatocytes were washed off with 1× PBS and the hepatocytes were fixed for 10 min with ice-cold methanol. After washing, cells were blocked with 1% BSA-1× PBS for 1 h at room temperature (RT). Hepatocytes infected with sporozoites were stained for the intracellular parasite by incubation with anti-heat shock protein (HSP) 70 Ab (2E6) (35) for 1 h at RT, followed by washing and staining for 1 h at RT with a 1/100 dilution of anti-mouse IgG Ab conjugated to Alexa Fluor 488. Cells were washed and all cells were stained with a 1/100 dilution of 4′,6-diamidino-2-phenylindole (DAPI) to detect apoptotic nuclei. The cells were then viewed under a fluorescence microscope.

Quantification of intracellular sporozoites

Primary hepatocytes (8 × 10^4) were seeded per well in 8-well LabTek chamber slides and incubated overnight at 37°C. Cells were pretreated with different inhibitors for 2 h at 37°C. Wild-type sporozoites (2 × 10^4) were added without washing off inhibitors and incubated for 2 h at 37°C. Cells were washed with 1× PBS and either fixed with cold methanol (for detection of both extracellular and intracellular sporozoites) or with cold 1% glutaraldehyde (for detection of extracellular sporozoites only) for 10 min. All cells were washed and blocked for 1 h at RT with 1% BSA-1× PBS, followed by incubation with an anti-PbCS repeat Ab for 1 h at RT. After washing, cells were stained for 1 h at RT with a 1/100 dilution of anti-mouse IgG Ab conjugated to Alexa Fluor 488. Cells were then washed, mounted in 50% glycerol, and observed under a fluorescence microscope. Sporozoites were counted in duplicate wells. The number of extracellular sporozoites (glutaraldehyde fixation) was subtracted from the number of sporozoites in wells with methanol fixation to yield the number of intracellular sporozoites.

Quantification of exoerythrocytic forms (EEFs)

Primary hepatocytes (8 × 10^4) were seeded per well in 8-well LabTek chamber slides and incubated overnight at 37°C. Cells were infected with 2 × 10^4 wt or spect−/− P. berghei sporozoites per well for 20 h at 37°C. Cells were washed with 1× PBS and fixed for 10 min with ice-cold methanol. Cells were blocked with 1% BSA-1× PBS for 1 h at RT following washing with 1× PBS. Infected hepatocytes were then stained with anti-HSP 70 Ab for 1 h at RT. After washing, cells were stained for 1 h at RT with a 1/100 dilution of anti-mouse IgG Ab conjugated to Alexa Fluor 488. Cells were then washed, mounted in 50% glycerol, and observed under fluorescence microscope. EEFs were counted over the entire well.

Quantification of the migration capacity of sporozoites

Sporozoites migrate through several hepatocytes breaching their plasma membranes, before infecting a final one by formation of a parasitophorous vacuole (27). The wounded cells release cytosolic contents into the...
microenvironment (R. Torgler, S. E. Bongfen, J. F. Romero, A. Tardivel, M. Thome, and G. Corradin, submitted for publication). We exploited this ability of sporozoites to wound cells and release cytosolic contents to quantify their migration capacity.

HepG2 cells (10^5) were seeded per well in a 96-well plate and incubated for 48 h at 37°C to enable them to adhere. Seventy-five microliters of chromium was then added and incubated for 1 h at 37°C. Cells were washed several times to eliminate all residual chromium, then infected with 5 × 10^4 wt or spect^-/- sporozoites for 3 h at 37°C. The resulting supernatant was collected and read with a gamma counter. The amount of chromium released was proportional to the number of sporozoites added and hence to hepatocytes wounded due to the migratory action of sporozoites.

Statistical analysis

The one-way ANOVA and two-tailed Student t test were used to determine the significance of variation between test samples. All p values ≤0.05 were considered to be significant.

Results

Primary hepatocytes can process and present the P. berghei CSP, and do so optimally 8–20 h after infection with sporozoites

Our preliminary in vitro data showed that primary hepatocytes are capable of processing and presenting sporozoite Ags after infection (data not shown). However, the level of CD8^+ T cell activation varied with the length of time of hepatocyte infection. To find out the optimal time necessary for processing and presentation of the CSP after infection with sporozoites, hepatocytes were infected with sporozoites for different time periods over a total period of 24 h. Secretion of IFN-γ by the PbCS-C7 CD8^+ T cells after coculture with infected hepatocytes was determined by an ELISPOT assay. Optimal activation of the T cells occurred when hepatocytes were infected with the sporozoites for a time period between 8 and 20 h. Activation was lower when T cells were cocultured with hepatocytes infected with sporozoites for <8 h or >20 h. (Fig. 1). This suggests that the optimal time necessary for hepatocytes to process and present the CSP to CD8^+ T cells after infection with sporozoites is between 8 and 20 h.

To establish the purity of the hepatocyte preparation, primary hepatocytes were stained with different Abs (see Materials and Methods) to exclude the presence of contaminating cells, and the preparation was found to contain essentially hepatocytes (data not shown). To further prove that the Ag presentation was performed by hepatocytes and not by Kupffer cells that could contaminate the hepatocyte preparation, primary hepatocytes were prepared from a BALB/c mouse 24 h after i.v. injection of 10 mg/kg body weight of GdCl. GdCl is known to deplete Kupffer cells in vivo (34). Hepatocytes from both a wt and GdCl-treated mouse were then used in ICS. As shown in Fig. 2a, hepatocytes depleted of Kupffer cells presented the CSP of P. berghei just as well as hepatocytes from wt mice. Similarly, presentation of the PbCS_245–253 peptide by both preparations of hepatocytes was comparable (Fig. 2b).

Taking into consideration that T cells could be activated by contaminants of parasite preparations, hepatocytes were incubated with material from uninfected mosquito salivary glands as a control. Fig. 2c shows that the activation of T cells observed is specific for sporozoites, because T cells were not activated in the presence of uninfected salivary gland material. To eliminate the possibility of Ag from extracellular degradation being presented, hepatocytes were incubated with heat-killed and with midgut sporozoites (which contain the mature CSP, but neither migrate through nor infect hepatocytes). T cells were not activated in either case, implying that migration and/or infection of sporozoites are necessary for the CSP to be processed and presented by hepatocytes (Fig. 2d).

To further demonstrate that hepatocytes are indeed presenting the peptide, ICS was performed with hepatocytes from C57BL/6 mice (H-2K^b). As can be seen in Fig. 2e, T cells were only activated when they were cocultured with hepatocytes from BALB/c mice, which express the correct MHC molecules (H-2K^b).

Both infected and wounded (traversed) hepatocytes process and present the CSP

Sporozoites migrate through several hepatocytes before infecting a final one by formation of a parasitophorous vacuole (27). During migration, they leave behind a trail of the CSP. Some of the traversed cells reseal their membrane and survive, and thus can, in principle, present the CSP to T cells.

To find out which cells were presenting the CSP (traversed or infected cells or both), an ICS assay was done with both wt and spect^-/- sporozoites, whose migratory ability through hepatocytes is totally or profoundly inhibited (Fig. 3a) (29), but which infect and develop normally in hepatocytes (Fig. 3b). As shown in Fig. 3c, T cells are activated when hepatocytes are infected with either wt or spect^-/- sporozoites. The level of activation of T cells by wt sporozoites is about 5-fold that observed with spect^-/- sporozoites. Given that spect^-/- parasites are capable of infection but not migration, and that migration and/or infection are necessary for CSP processing and presentation (Fig. 2d), these results suggest that both infected and uninfected (traversed) hepatocytes present
the CSP to T cells, with most of the presentation being done by the traversed cells. To further show that infected cells present the CSP, hepatocytes were infected with either wt or \textit{spect} \textsuperscript{-/-} sporozoites and cocultured with specific CD8\textsuperscript{+} T cells at different E:T ratios for 20 h. The number of EEFs in each case was then determined. Fig. 3\textit{di} shows that EEFs from hepatocytes infected with wt and \textit{spect} \textsuperscript{-/-} sporozoites are equally eliminated, indicating that infected cells do present the CSP. The perfect correlation ($r = 0.997$) between the elimination of EEFs from wt or \textit{spect} \textsuperscript{-/-} sporozoite-infected cells as a function of E:T ratio (Fig. 3\textit{dii}) indicates a null or limited contribution of parasite killing due to activation of CD8\textsuperscript{+} T cells by traversed cells.

**Processing and presentation of the CSP by primary hepatocytes are dependent on the proteasome and on vesicular transport through the Golgi apparatus**

The presentation of proteasome-generated cytosolic peptides by MHC class I molecules requires vesicular transport from the endoplasmic reticulum through the Golgi complex to the cell surface. The dependence of the processing of the CSP on the proteasome was tested using the proteasome inhibitors LLnL, Cbz-LLL, and lactacystin. Processing and presentation of the CSP after infection of hepatocytes with sporozoites was strongly inhibited ($p < 0.05$) by the proteasome inhibitors (Fig. 4\textit{a}). In contrast, presentation of the PbCS\textsubscript{245-253} peptide was unaffected (Fig. 4\textit{b}). Likewise, sporozoite-infected hepatocytes lost their capacity to present the CSP when they were treated with the intracellular transport inhibitor BFA, whereas presentation of the PbCS\textsubscript{245-253} peptide was not affected.

These results indicate that after sporozoite infection, processing and presentation of the CSP follow a proteasome-dependent pathway.

To establish that the inhibitors added did not affect the biosynthetic machinery of the parasite, inhibitor-treated cells were washed before infection, and ICS was conducted as before. No difference in activation of T cells was observed, whether or not the inhibitors were washed before infection (data not shown), indicating that the inhibitors do not have an effect on the parasite, but inhibit a process in the hepatocyte.

**Effect of lysosomal protease inhibitors and inhibitors of lysosomal acidification on processing of the CSP**

Sporozoites are a source of exogenous Ags and, as shown in other systems (26), it is possible that processing of sporozoite proteins could depend, at least in part, on the vacuolar pathway. To test the involvement of endolysosomal proteases in CSP processing, hepatocytes were treated with leupeptin and pepstatin before infection and cocultured with the specific CD8\textsuperscript{+} T cells.
with sporozoites or pulsing with the PbCS$_{245-253}$ peptide. Processing and presentation of the CSP were not significantly affected by leupeptin ($p > 0.05$). However, activation of T cells was significantly reduced ($p < 0.05$) when hepatocytes were treated with the aspartic protease inhibitor pepstatin. To further establish the role of the endosomal compartment, the hepatocytes were treated with chloroquine and ammonium chloride, which alter the acidification of lysosomes and endolysosomes. No significant effect on processing and presentation was observed (Fig. 5a). As expected, presentation of the PbCS$_{245-253}$ peptide was not affected by any of the inhibitors (Fig. 5b).

The different inhibitors used have no effect on sporozoite infection of hepatocytes

To show that the inhibitors which had an effect on processing and presentation did not affect the rate of hepatocyte infection by sporozoites, hepatocytes were infected with sporozoites in the presence or absence of inhibitors for 2 h. Infected cells were
stained for intracellular sporozoites using a mAb against the repeats of the \textit{P. berghei} CSP. As shown in Fig. 6, the number of intracellular sporozoites did not vary significantly (\(p > 0.05\)) whether or not infection was done in the presence of inhibitors.

**Hepatocytes are lysed by CD8\(^+\) T cells after pulsing with the PbCS CTL epitope or after incubation with sporozoites**

In addition to production of cytokines such as IFN-\(\gamma\), CD8\(^+\) T cells also induce target cell death via a perforin/granzyme B- or Fas-dependent mechanism. To further prove that hepatocytes are able to process and present the CSP after infection and to show that the CD8\(^+\) T cells used were functional, chromium release assays were conducted to find out whether hepatocytes could be lysed by CD8\(^+\) T cells after pulsing with the optimal CTL epitope or after infection with sporozoites.

Hepatocytes pulsed with the specific PbCS\textsubscript{245–253} peptide were found to be efficiently lysed by specific CD8\(^+\) T cells, as opposed to hepatocytes pulsed with a CTL epitope of \textit{P. falciparum}, PFC\textsubscript{327–335}, even at high E:T ratios (Fig. 7a). The killing of peptide-pulsed hepatocytes observed here seemed to be primarily due to perforin/granzyme B, and not via Fas-Fas ligand, because addition of soluble Fas-Fc did not inhibit killing. This killing was further shown by staining the peptide-pulsed hepatocytes for apoptotic nuclei 2 h after coculture with specific CTLs. In this case, massive apoptosis was observed, as shown by the presence of fragmented nuclei (Fig. 7b, fourth panel). No apoptosis was observed when hepatocytes were cocultured with CTLs in the absence of peptide (Fig. 7b, third panel) or when peptide alone was added to hepatocytes (Fig. 7b, second panel).

Hepatocytes were also lysed by specific CTLs after infection with sporozoites, as shown by a chromium release assay (Fig. 7c). The lysis was greatly increased when the specific peptide was added to infected hepatocytes. However, by microscopically examining cocultures of CD8\(^+\) T cells and hepatocytes after sporozoite infection, we found that infected hepatocytes did not appear to undergo apoptosis, as shown by the absence of apoptotic nuclei in infected cells 2 h after coculture with specific CTLs in the presence (Fig. 7d, fourth panel) or absence (Fig. 7d, third panel) of peptide. Interestingly, the infected cells bordering infected cells were apoptotic whether or not peptide was added (Fig. 7d, third and fourth panels, arrowheads). Importantly, no apoptosis was observed when infected cells were cultured alone (Fig. 7d, first panel) or in the presence of IFN-\(\gamma\) for 2 h (Fig. 7d, second panel).

**Discussion**

The aim of this study was to provide evidence that primary hepatocytes are able to process the \textit{P. berghei} CSP after infection with sporozoites and to present the optimal \textit{P. berghei} CSP CTL epitope to specific CD8\(^+\) T cells. In this light, we show that both infected and uninfected hepatocytes (traversed by sporozoites) process and present the CSP, and that processing is largely dependent on proteasomes and, to a lesser extent, aspartic proteases.

There is a continuous discussion about the cell type involved in the processing and presentation of the CSP after sporozoite infection. Three cell types may be implicated in this process: Kupffer cells, DCs, and hepatocytes.

Although little is known about the role of Kupffer cells, there is no doubt that DCs can present sporozoite-derived Ags, and a protective status is attained in mouse experimental models in which a relatively high number of sporozoites are injected (36–38). However, in the field, where the number of sporozoites injected via mosquito bites is low, and with a high proportion of these sporozoites going directly to the hepatocyte within minutes (11), DC presentation may not be the relevant pathway for priming. Thus, the crucial, still unresolved question is: can infected hepatocytes present Ags to T cells in a primary and secondary response? Our data formally prove that at least in vitro, both infected and traversed hepatocytes can present sporozoite-derived Ags to cloned CD8\(^+\) T cells. spect\textsuperscript{-}\(\gamma\) \textit{P. berghei} sporozoites have been shown to be incapable of cell traversal, but capable of infection and normal development in hepatocytes (29). Our finding that CD8\(^+\) T cells could be activated when hepatocytes were infected with spect\textsuperscript{-}\(\gamma\) sporozoites (Fig. 3c) shows that infected hepatocytes present the \textit{P. berghei} CSP CTL epitope to T cells, because in this case we have a situation of infection only and no minimal traversal. This is further proven by the fact that in the presence of T cells, EEFs from spect\textsuperscript{-}\(\gamma\) parasites could be eliminated as well as those from wt parasites (Fig. 3d). The activation observed is unlikely to be due to uptake and processing of extracellular CSP by hepatocytes, because activation of T cells was observed when hepatocytes were incubated with heat-killed or midgut sporozoites (Fig. 2d).

The long-lasting protection observed with the irradiated sporozoite model is also consistent with continuous presentation of sporozoite-derived Ags by infected hepatocytes to primed T cells. It has been, in fact, shown that as early as 12 h after infection, no liver mononuclear cells contain parasite Ags (38), suggesting that the only cells that contain parasite Ags for a long period of time are hepatocytes. In addition, elimination of hepatic stages after irradiated sporozoite immunization abrogates protection (12). More recently, long-lasting protection has been obtained by immunization with live sporozoites of mice under chloroquine treatment (39). This protection was abrogated by eliminating hepatic forms with primaquine. Although the role of primaquine in Ag presentation in vivo has not been elucidated, these data suggest that activation of primary responses may be mediated by infected hepatocytes. It is known that priming of immune responses requires expression of costimulatory molecules by the APCs. In fact, costimulatory molecules have been shown to be expressed in the cytoplasm of hepatocytes during hepatitis C viral infection (40) and in biliary atresia patients with liver dysfunction (41). It is, therefore, possible that sporozoite infection of hepatocytes induces expression of costimulatory molecules necessary for priming T cell responses.

Although not formally proven, our in vitro microscopy data show that sporozoite-infected hepatocytes do not undergo nuclear fragmentation, suggesting that they are resistant to CTL-induced apoptosis. This is in conformity with studies which have shown that infected cells are protected from apoptosis (42, 43). In addition, the finding that Fas- and perforin-deficient mice could still be protected from \textit{P. berghei} (44) and \textit{P. yoelii} (45) infection suggests that other mechanisms are implicated in the destruction of hepatic forms. It is interesting to note that even though infected cells did not undergo apoptosis, neighboring noninfected cells (probably traversed by sporozoites) readily underwent apoptosis upon addition of specific CTLs (Fig. 7d, third panel). This suggests that the CS protein left behind by sporozoites traveling through or between hepatocytes (27, 46) is presented by traversed hepatocytes. This finding is further substantiated by the fact that hepatocytes infected with spect\textsuperscript{-}\(\gamma\) sporozoites do not activate T cells as much as those infected with wt sporozoites, suggesting that traversed hepatocytes process and present the CSP to T cells. This presentation by traversed hepatocytes could represent an escape mechanism elaborated by the parasite to evade the host immune system, by directing specific T cells to traversed hepatocytes which present malaria-derived peptides. However, further studies are needed to determine whether this mechanism is operative in vivo. Nevertheless, the fact that protection could still be observed in Fas- and perforin-deficient mice (44) suggests that infected cells do not undergo apoptosis in vivo.
We found that optimum processing and presentation occur 8–20 h after infection of hepatocytes with sporozoites (Fig. 1). This is in agreement with previous studies conducted in vivo (47). In that study, it was shown that maximum CD8\(^+\) T cell activation occurred within the first 8 h after immunization with normal or irradiated \textit{P. yoelii} sporozoites, and lasted for \(\approx 48\) h.

The dependence of processing on the proteasome is consistent with studies which showed that sporozoites deposit the CSP in the cytoplasm of cells, beginning from the time of their attachment to the cell surface and peaking 4–6 h after invasion (48). In addition, parasite Ags have also been shown to be inserted into the parasitophorous vacuole membrane and into the hepatocyte cytoplasm (49). Recently, a pentameric motif, the \textit{Plasmodium} export element (termed Pexel), was discovered and shown to be necessary for trafficking of \textit{Plasmodium falciparum} proteins in the infected erythrocyte (50, 51). This motif is present in the \textit{P. berghei} CSP and could be used to traffic the CSP from the parasitophorous vacuole to the cytoplasm in infected hepatocytes.

After infection of human hepatoma cells with sporozoites, the parasites were found not to be localized in an acidified vacuole, and the released CSP did not enter the endocytic compartment of the host cell (48). In agreement with this, we found that CD8\(^+\) T cell activation was not affected by chloroquine and NH\(_4\)Cl, suggesting that an acidic compartment may not be involved in CSP processing. It has, however, been shown that cross-presentation of Ags by DCs occurs through a phagolysosome, which is resistant to acidification (52). Whether this mechanism also occurs in hepatocytes has not been determined. Interestingly, pepstatin inhibited processing, suggesting that cellular or parasitic aspartic proteases play a role in processing the CSP. These proteases could possibly cleave the CSP in the parasitophorous vacuole before its exit into the hepatocyte cytoplasm.

On the other hand, additional studies using spectr\(\text{-}^\gamma\) parasites and inhibitors of Ag processing and presentation as shown here are needed to clearly distinguish the requirements of processing and presentation of live sporozoites from those of proteins left behind by the traveling parasite.

In summary, our results clearly show that primary hepatocytes process and present the CSP of \textit{P. berghei} live sporozoites via a pathway involving the proteasome and aspartic proteases. The biological significance of hepatocyte processing and presentation is very likely associated with the maintenance of the protective immune response induced by irradiated sporozoites and in subsequent protection against a parasite challenge, both of which have to be formally proven in vivo. On the other hand, in our opinion, the role of infected hepatocytes in the elicitation of a primary immune response still remains elusive.

The finding that infected hepatocytes also present parasite Ags has profound consequences for vaccine development. The CSP is the most widely studied pre-erythrocytic stage vaccine candidate and is an immunodominant protective Ag in irradiated sporozoites (53). However, the developing parasite also expresses other Ags inside the infected cell. If these Ags are presented on the surface of the infected hepatocyte, they could be potential candidates for a pre-erythrocytic stage vaccine.

Finally, a clear understanding of the mechanisms involved in processing and presentation of sporozoite Ags could assist in the design of effective vaccines capable of stimulating CD4\(^+\) and CD8\(^+\) T cell responses.

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Disclosures

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