RESEARCH ARTICLE

Antigen Export during Liver Infection of the Malaria Parasite Augments Protective Immunity

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ABSTRACT **Protective immunity against preerythrocytic malaria parasite infection is difficult to achieve. Intracellular *Plasmodium* parasites likely minimize antigen presentation by surface-expressed major histocompatibility complex class I (MHC-I) molecules on infected cells, yet they actively remodel their host cells by export of parasite factors. Whether exported liver-stage proteins constitute better candidates for MHC-I antigen presentation to CD8$^+$ T lymphocytes remains unknown. Here, we systematically characterized the contribution of protein export to the magnitude of antigen-specific T-cell responses against *Plasmodium berghei* liver-stage parasites in C57BL/6 mice. We generated transgenic sporozoites that secrete a truncated ovalbumin (OVA) surrogate antigen only in the presence of an amino-terminal protein export element. Immunization with live attenuated transgenic sporozoites revealed that antigen export was not critical for CD8$^+$ T-cell priming but enhanced CD8$^+$ T-cell proliferation in the liver. Upon transfer of antigen-specific CD8$^+$ T cells, liver-stage parasites secreting the target protein were eliminated more efficiently. We conclude that *Plasmodium* parasites strictly control protein export during liver infection to minimize immune recognition. Strategies that enhance the discharge of parasite proteins into infected hepatocytes could improve the efficacy of candidate preerythrocytic malaria vaccines.**

IMPORTANCE **Vaccine development against *Plasmodium* parasites remains a priority in malaria research. The most advanced malaria subunit vaccine candidates contain *Plasmodium* surface proteins with important roles for parasite vital functions. A fundamental question is whether recognition by effector CD8$^+$ T cells is restricted to sporozoite surface antigens or extends to parasite proteins that are synthesized during the extensive parasite expansion phase in the liver. Using a surrogate model antigen, we found that a cytoplasmic antigen is able to induce robust protective CD8$^+$ T-cell responses, but protein export further enhances immunogenicity and protection. Our results show that a cytoplasmic localization does not exclude a protein's candidacy for malaria subunit vaccines and that protein secretion can enhance protective immunity.**

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Multiple immunizations with live, attenuated, metabolically active *Plasmodium* sporozoites remain the benchmark for malaria vaccine development (1, 2). Recent clinical trials confirmed that repeated exposure to *Plasmodium falciparum*-infected mosquitoes combined with treatment to suppress blood-stage parasites confers sterile and lasting protection against malaria in a human challenge model (3, 4). Likewise, intravenous administration of high doses of irradiation-attenuated, aseptic, purified, and cryopreserved *P. falciparum* sporozoites can confer substantial, even sterile, antimalarial immunity in humans (5). Experimental vaccinations with irradiated sporozoites in murine models provided compelling evidence that sterilizing immunity is principally mediated by CD8$^+$ T cells directed against liver-stage parasites (6–8).

In one murine infection model, H-2^d-restricted (BALB/c) mice, protective immunity correlates with the magnitude of CD8$^+$ T cells that recognize the *Plasmodium* circumsporozoite protein (CSP) (9–11), but whether these responses contribute to naturally acquired antimalarial immunity remains unresolved (12). CSP is surface expressed on *Plasmodium* sporozoites, shed during parasite transmigration of cellular barriers, and remains detectable after hepatocyte invasion (13–15). Opsonization of sporozoites inhibits CSP presentation by dendritic cells (DCs) (16), possibly because the parasites are immobilized (17) and this process interferes with T-cell priming. Immobilized heat-killed parasites fail to induce a protective CD8$^+$ T-cell response (6, 18), strongly suggesting that invasion of live parasites is central for T-cell activation and protection. Mice with a tolerance for CSP still develop protective immunity after immunization with irradiated sporozoites, indicating that additional antigens contribute to protection (19). Moreover, it has been shown that the sterile protection induced by immunization with irradiated sporozoites or sporozoites under chloroquine prophylaxis is independent of CSP (20, 21).

In the robust C57BL/6 (H-2^b)/*Plasmodium berghei* vaccine and infection model, CSP is not recognized by CD8$^+$ T cells, and the

major sporozoite adhesin, thrombospondin-related anonymous protein (TRAP), was identified as an immunodominant and protective antigen (22). Additional, hitherto unrecognized protective antigens likely include preerythrocytic surface parasite proteins, which are presented by DCs in the priming phase and by infected hepatocytes to $CD8^+$ effector T cells, which in turn eliminate liver-stage parasites (8, 23, 24).

A recent study showed that presentation of CSP that contained the very potent H-$2K^d$ ovalbumin (OVA) epitope to $CD8^+$ T cells occurs by the two classical cellular pathways (16); during the priming phase, DCs display the antigen by cross-presentation via the endosomal pathway, whereas epitope presentation on infected hepatocytes during the effector phase involves antigen secretion to the host cell cytoplasm. Accordingly, DC priming in draining lymph nodes and/or the spleen via phagocytosis is expected to stimulate extensive T-cell responses to diverse secreted and nonsecreted parasite antigens, and antigen presentation to effector $CD8^+$ T cells on major histocompatibility complex class I (MHC-I) molecules of infected hepatocytes is much more restricted. Sporozoite antigen presentation by DCs and hepatocytes is TAP dependent, i.e., parasite molecules must reach the cytoplasm to be presented (16). Nevertheless, the mechanism of translocation of liver-stage-parasite protein into the hepatocyte cytoplasm and the entry into the MHC-I presentation pathway are incompletely understood. Protein export is an important parasite-mediated process to remodel an infected cell and typically involves a short, degenerate amino-terminal *Plasmodium* export element (PEXEL) (25–27). While this motif was initially reported to be important for CSP translocation into the hepatocyte cytoplasm (28), it was recently shown that it does not affect the capacity of $CD8^+$ T cells to recognize and eliminate infected hepatocytes (16).

Despite the recent progress in gaining a better understanding of the molecular mechanisms of liver-stage immunity, it is unclear whether recognition by effector $CD8^+$ T cells is restricted to sporozoite surface antigens or extends to parasite proteins that are synthesized during parasite development and exported to the host hepatocyte. In support of the former notion, it has been proposed that hepatocytes only present antigens that are secreted into the cytoplasm after invasion or during cell traversal (29, 30). Recent genome-wide immune profiling approaches in the two murine models provided evidence for each of these propositions (22, 31). Support for a striking immunodominance of sporozoite antigens was reported for *P. berghei*/H-2^b (C57BL/6) mice, which returned two sporozoite-specific epitopes (22), while work in the *Plasmodium yoelii*/H-2^d (BALB/c) model identified a previously unrecognized intracellular parasite protein that is highly unlikely to be exported to the infected liver cell (31).

Here, we systematically analyzed the contribution of *Plasmodium* preerythrocytic cytoplasmic and exported antigens to protection induced by vaccination with live attenuated sporozoites. For this purpose, we generated two transgenic parasite lines that express the OVA surrogate antigen with and without an export targeting sequence. This approach allowed us to address the potential role(s) of protein export in antigen presentation and priming of protective immune responses. Our findings support the notion that exported antigens are presented by MHC-I molecules more efficiently, resulting in superior T-cell activation and protection.

RESULTS

Generation of *P. berghei* OVA-expressing parasites. We first determined to what extent the localization of *P. berghei* antigens in infected cells affects MHC-I presentation. To this end, we generated a *P. berghei* parasite line that expresses a truncated version of the OVA surrogate antigen, encompassing amino acids 142 to 389 and including the well-characterized $CD8^+$ and $CD4^+$ T-cell epitopes. We placed OVA under the control of a preerythrocytic-stage-specific promoter, *UIS4* (15), and targeted a dispensable *P. berghei* locus with an insertion vector to generate transgenic OVA parasites (Fig. 1A; see also Fig. S1 in the supplemental material). In addition, we generated the *expOVA* parasite line, which expresses OVA fused to the CSP export sequence together with an N-terminal PEXEL motif (22, 32), using the same strategy (Fig. 1A and Fig. S1).

We confirmed that both transgenic OVA-expressing *P. berghei* parasite lines were able to infect mosquitoes and mice similarly to the parental parasite strain (see Fig. S2 in the supplemental material). Since we observed no differences in either gliding motility (data not shown) or *in vivo* infectivity, we conclude that the additional copy of the surrogate antigen expression cassette did not affect the parasite's life cycle progression or infection of mice.

Validation of surrogate antigen localization. To verify whether the surrogate antigen was efficiently exported, we infected Hepa 1-6 cells and examined them for the presence of OVA using a specific antibody. As predicted, OVA was confined to the parasite interior, while expOVA largely displayed a distinct pattern on structures outside the parasite (Fig. 1B). The exported OVA molecules were always surrounded by parasite membrane material, as shown by positive staining for UIS4, a specific marker of the parasitophorous vacuole membrane (PVM). We did not detect the OVA signal free in the host cell cytoplasm but inside vesicles, which are often detached from the PVM (32, 33). In contrast, the staining patterns of *OVA* parasites revealed the OVA signal inside the parasite at all time points, and no OVA staining was observed in the membranous structures extending into infected cells (Fig. 1B). We also determined the expression levels of OVA protein in both transgenic parasite cell lines (Fig. 1C). We quantified the fluorescence levels by immunofluorescence assay (IFA) in infected hepatoma cells and did not observe any differences between the parasite lines. Together, these data demonstrate that the surrogate expOVA antigen is effectively exported into the parasite's liver-stage tubulovesicular network (LSTVN).

At late-liver-stage development, the OVA signal was very weak (Fig. 1D), as expected for proteins expressed under the control of the *UIS4* promoter (15, 34). We therefore excluded the possibility that OVA is present during blood infection. The OVA signal was present in free sporozoites, in good agreement with the promoter activity (Fig. 1E). Together, these findings show that the two transgenic lines differ in the spatial distribution but not the temporal expression of the surrogate antigen OVA.

$CD8^+$ T-cell cytotoxicity *in vivo* does not depend on OVA secretion. To interrogate whether OVA export is critical for antigen presentation and T-cell activation *in vivo*, we determined the cytotoxic activity of antigen-specific T cells isolated from mice that were immunized with the two transgenic OVA parasite lines (Fig. 2). Mice received 3×10^5 nonactivated, OVA-specific $CD8^+$ T cells (OT-1 cells) by intravenous (i.v.) injection. Next, mice were i.v. immunized with 10,000 irradiation-attenuated normal or

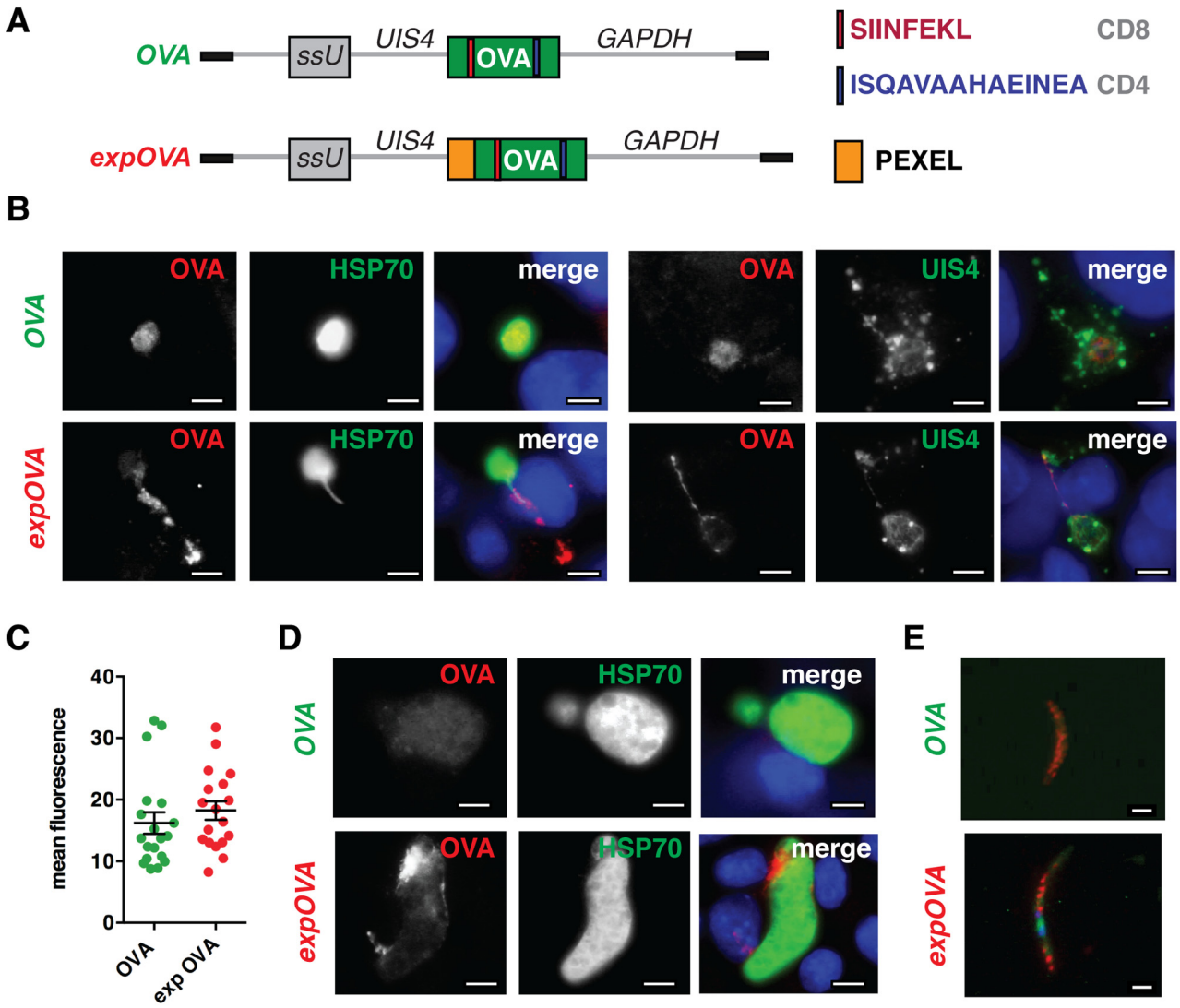

FIG 1 Transgenic *OVA* and *expOVA Plasmodium* sporozoites and liver-stage parasites express and export the surrogate antigen ovalbumin (OVA). (A) Schematic of transgenic *P. berghei* parasite lines that express a truncated version of the surrogate antigen OVA. The H-2^{b}-restricted CD8^{+} T-cell epitope (SIINFEKL) and CD4^{+} epitope (ISQAVAAHAEINEA) are highlighted in red and blue, respectively, in the schematic. Antigen expression is controlled by the preerythrocytic-stage-specific UIS4 promoter. The *expOVA* parasites express OVA fused to an N-terminal protein export element (PEXEL), whereas *OVA* parasites produce a cytoplasmic version of the antigen. (B) Indirect immunofluorescence microscopy of early liver-stage parasites (18 h after infection). (C) Quantification of fluorescence signal revealed by IFAs of infected Hepa 1-6 cells at 24 h postinjection. Bars and whiskers show means ± standard deviations. Results are representative of two independent experiments ($n \geq 8$). (D, E) Late liver-stage parasites (48 h after infection) (D) and *P. berghei* sporozoites (E). Parasites were stained with a polyclonal anti-OVA antibody (red) and the nuclear dye Hoechst 33342 (blue). In addition, liver-stage parasites were stained with an anti-*P. berghei* Hsp70 antibody (green). (B, D) To visualize the liver-stage tubulovesicular network (LSTVN), early liver-stage parasites were also stained with an anti-*Pb*UIS4 antiserum. Scale bars: 5 μm (B, D); 2 μm (E).

transgenic sporozoites. Six days later, the animals received 1×10^7 lymphocytes that were loaded with the SIINFEKL peptide and labeled with a high concentration of carboxyfluorescein succinimidyl ester (CFSE) and 1×10^7 unloaded lymphocytes labeled with a low concentration of CFSE (Fig. 2A). After 18 h, the spleens were removed and the proportions of the two CFSE-labeled target cell populations were determined by fluorescence-activated cell sorting (FACS) analysis (Fig. 2B). To control for endogenous T-cell activation, mice were immunized in the absence of OT-1 cell transfer (see Fig. S3 in the supplemental material). Control mice and animals immunized with attenuated normal sporozoites displayed a minor reduction of the target cell population (Fig. 2C and Fig. S3). In contrast, attenuated transgenic OVA sporozoites induced specific CD8^{+} T-cell cytotoxicity, indicating that the surrogate antigen was adequately processed and presented by MHC-I molecules *in vivo* (Fig. 2C). Interestingly, both parasite populations induced a similar degree of T-cell-specific lysis.

Antigen export enhances CD8^{+} T-cell proliferation in the liver. Next, we evaluated CD8^{+} T-cell proliferation in animals infected with the two OVA-expressing parasite populations. To this end, we transferred either 2×10^6 or 8×10^6 CFSE-labeled OT-1 CD45.2 cells intravenously into CD45.1 C57BL/6 recipient mice (Fig. 3A). One day, later, mice were immunized with 10,000 irradiation-attenuated normal and transgenic sporozoites.

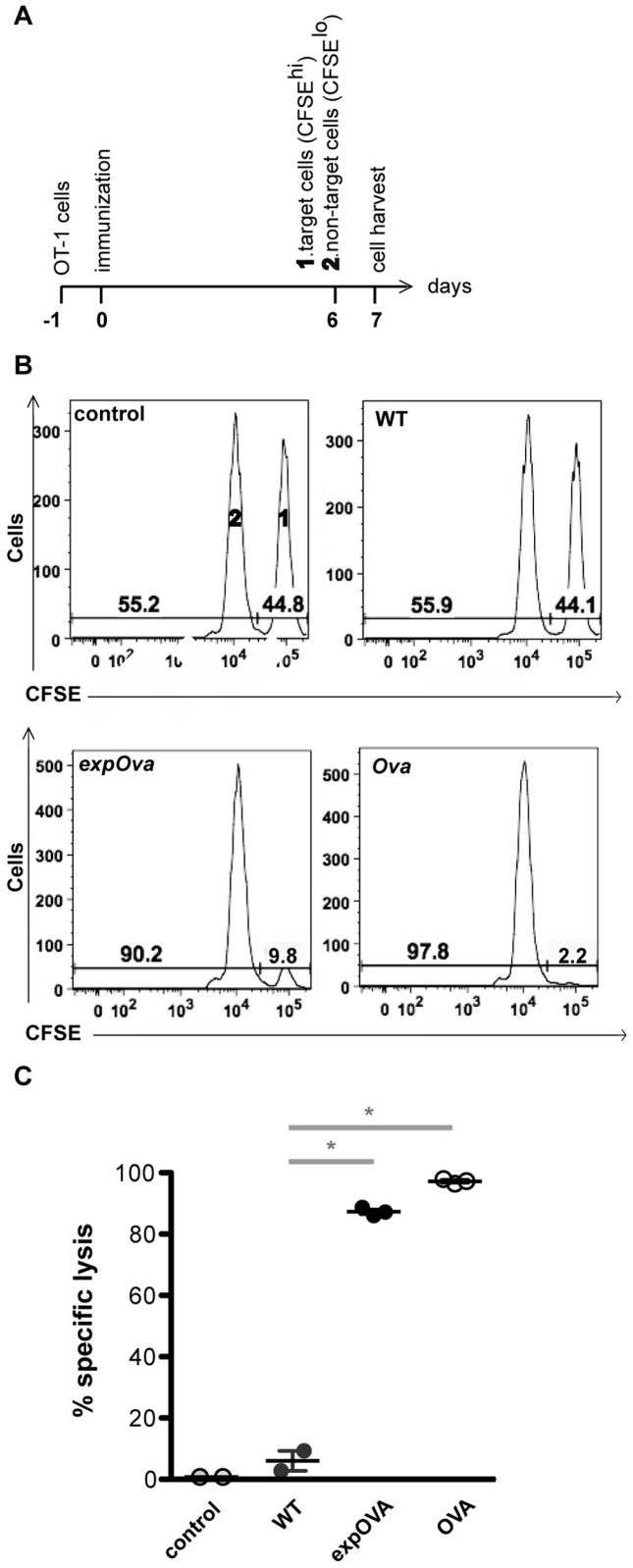

FIG 2 CD8$^+$ T-cell responses induced by intraparasitic and exported antigen are cytolytic *in vivo*. (A) Schematic diagram of methodology. Mice received 3 $\times$ 10^5 OT-1 cells and were either left untreated or immunized by i.v. injection of 10,000 irradiated wild-type (WT), *expOVA*, or *OVA* sporozoites. Six days later, target cells were prepared by pulsing syngeneic splenocytes with the

(Continued)

Three days later, livers and spleens were harvested to recover CD45.2$^+$ CD8$^+$ T cells (Fig. 3A) and quantify their CFSE intensity (Fig. 3B). T-cell proliferation was measured by quantification of CFSE^{low} CD45.2$^+$ CD8$^+$ cells in these organs (Fig. 3C). In both organs, CD8$^+$ T cells isolated from mice immunized with *expOVA* parasites showed the highest proliferation. A substantial proportion (~10 to 25%) of CD45.2$^+$ CD8$^+$ T cells recruited to the liver proliferated upon infection with *OVA* sporozoites. This proportion was significantly enhanced and reached up to ~40% in mice infected with *expOVA* sporozoites. This difference was less pronounced in the splenic CD45.2$^+$ CD8$^+$ T-cell population (Fig. 3C). We did not detect any proliferation of OT-II (CD4) T cells specific for ovalbumin by employing a similar strategy (data not shown).

A cytoplasmic surrogate antigen stimulates IFN-γ secretion. Since sterile protection induced by immunization with live irradiation-attenuated sporozoites is associated with gamma interferon (IFN-γ) secretion by proliferating CD8$^+$ T cells, we evaluated the ability of CD8$^+$ T cells to secrete IFN-γ after *in vitro* restimulation with the SIINFEKL peptide. We transferred 3 $\times$ 10^5 nonactivated OT-1 cells into mice, and 24 h later, the recipient animals were immunized with 10,000 irradiated normal or transgenic OVA sporozoites, followed by a second immunization 1 week later. CD8$^+$ T-cell responses were measured in both spleen and liver 7 days after the last immunization (Fig. 4A) (22).

We observed that the IFN-γ levels in splenic and hepatic lymphocytes isolated from mice immunized with *expOVA* parasites were higher than the levels in mice immunized with *OVA* parasites, although this difference was not significant, indicating that antigen localization is not a major contributing factor to IFN-γ secretion (Fig. 4A and B). As a control for T-cell restimulation, we included the TRAP$_{130-138}$ peptide (amino acids 130 to 138 of TRAP), which is an H-2^b-restricted *Plasmodium*-specific immunoprotective epitope present in the thrombospondin-related anonymous protein (TRAP) (22). The surface protein TRAP is released by sporozoites during gliding motility, and it is also present in liver-stage parasites. Lymphocytes isolated from the spleens of mice immunized with OVA and expOVA-expressing parasites showed high proportions of IFN-γ after restimulation with TRAP peptide (Fig. 4B). Intriguingly, we observed a significant reduction of CD8$^+$ CD44$^+$ CD62L$^-$ cells secreting TRAP-specific IFN-γ in mice immunized with sporozoites expressing the surrogate antigen compared to the levels of these cells in mice immunized with wild-type (WT) sporozoites; however, this reduction was only significant for hepatic lymphocytes isolated from mice immunized with *OVA* parasites (Fig. 4B). Finally, we note that the magnitudes of CD8$^+$ CD44$^+$ CD62L$^-$ cells secreting IFN-γ specific for endogenous (TRAP) and surrogate (OVA) differed in the two organs (Fig. 4B). The TRAP responses were superior and in-

Figure Legend Continued

SIINFEKL or no peptide prior to labeling with CFSE and transfer to mice (1 $\times$ 10^7 pulsed cells/mouse each). After 18 h, spleens of recipient mice were harvested and analyzed by CFSE fluorescence. (B) Representative histogram plots showing the fate of target cells in naive mice (top left), mice immunized with irradiated WT sporozoites (top right), and mice immunized with *expOVA* (bottom left) or *OVA* (bottom right) sporozoites. (C) Quantification of *in vivo* cytolytic activity. Bars and whiskers show means $\pm$ standard deviations. *, $P <$ 0.05 (Kruskal-Wallis test). Results are representative of one of two experiments with three mice per group per experiment.

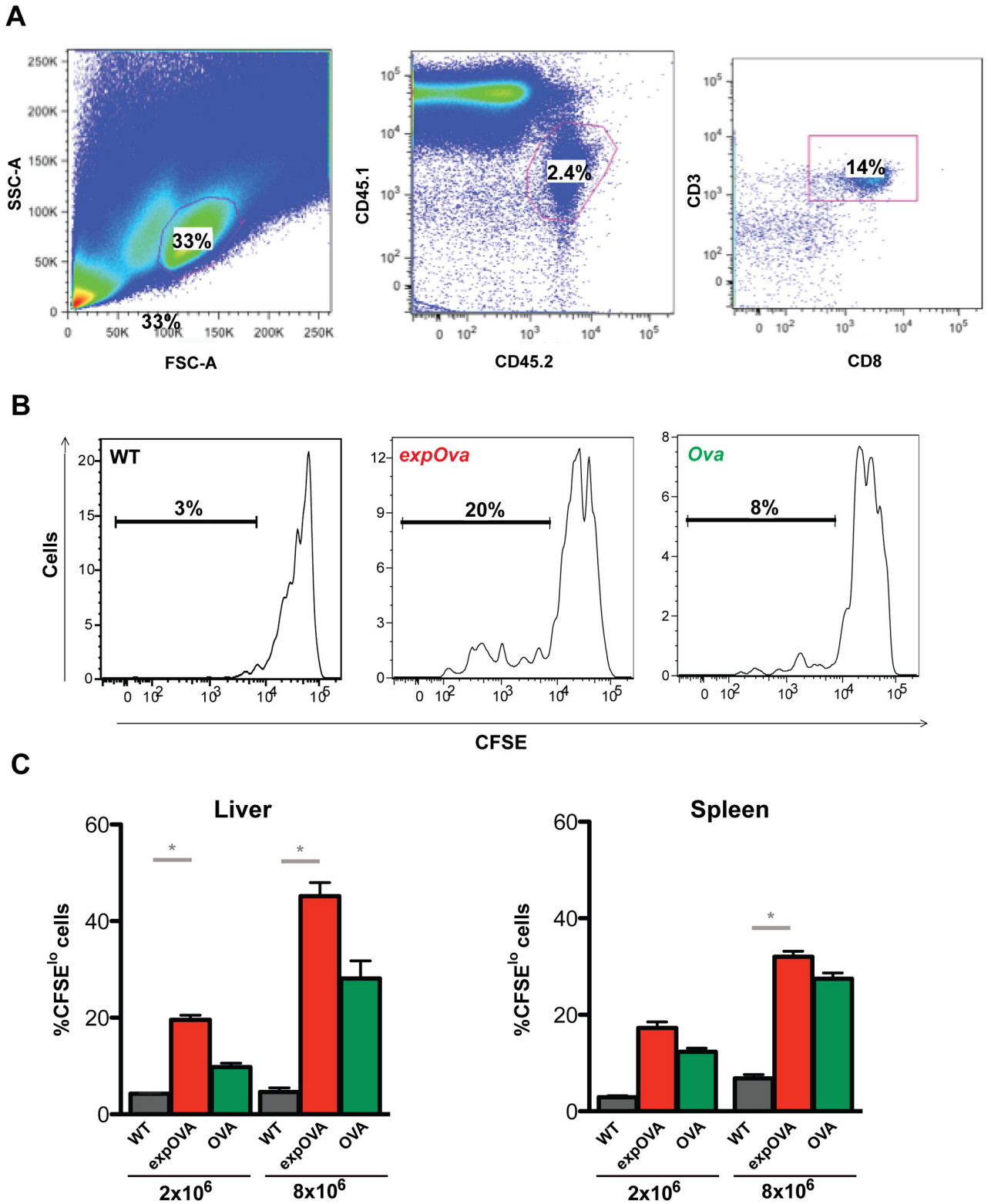

FIG 3 Antigen export enhances CD8$^+$ T-cell proliferation *in vivo*. C57BL/6 CD45.1 mice received OT-1 cells as indicated in panel C; the cells were obtained from CD45.2 donor mice and labeled with CFSE prior to transfer. After 18 h, mice received 10,000 irradiated WT, *expOVA*, or *OVA* sporozoites. Three days later, spleens and livers were harvested from recipient mice and cells stained with CD8, CD3, CD45.1, and CD45.2 antibodies. (A) Representative histogram plots showing the gating strategy for CD45.2$^+$ CD8$^+$ T cells. SSC, side scatter; FSC, forward scatter. (B) Representative histogram plots showing proliferation of hepatic CD45.2$^+$ CD8$^+$ T cells from mice immunized with WT (left), *expOVA* (center), or *OVA* (right) sporozoites. (C) T-cell proliferation from livers and spleens of sporozoite-immunized mice ($n = 3$ each). Shown is the percentage of the CFSE population of OT-1 CD8$^+$ CD45.2 T cells originating from the liver (left) or spleen (right). The amount of cells transferred is indicated. *, $P < 0.05$ (Kruskal-Wallis test).

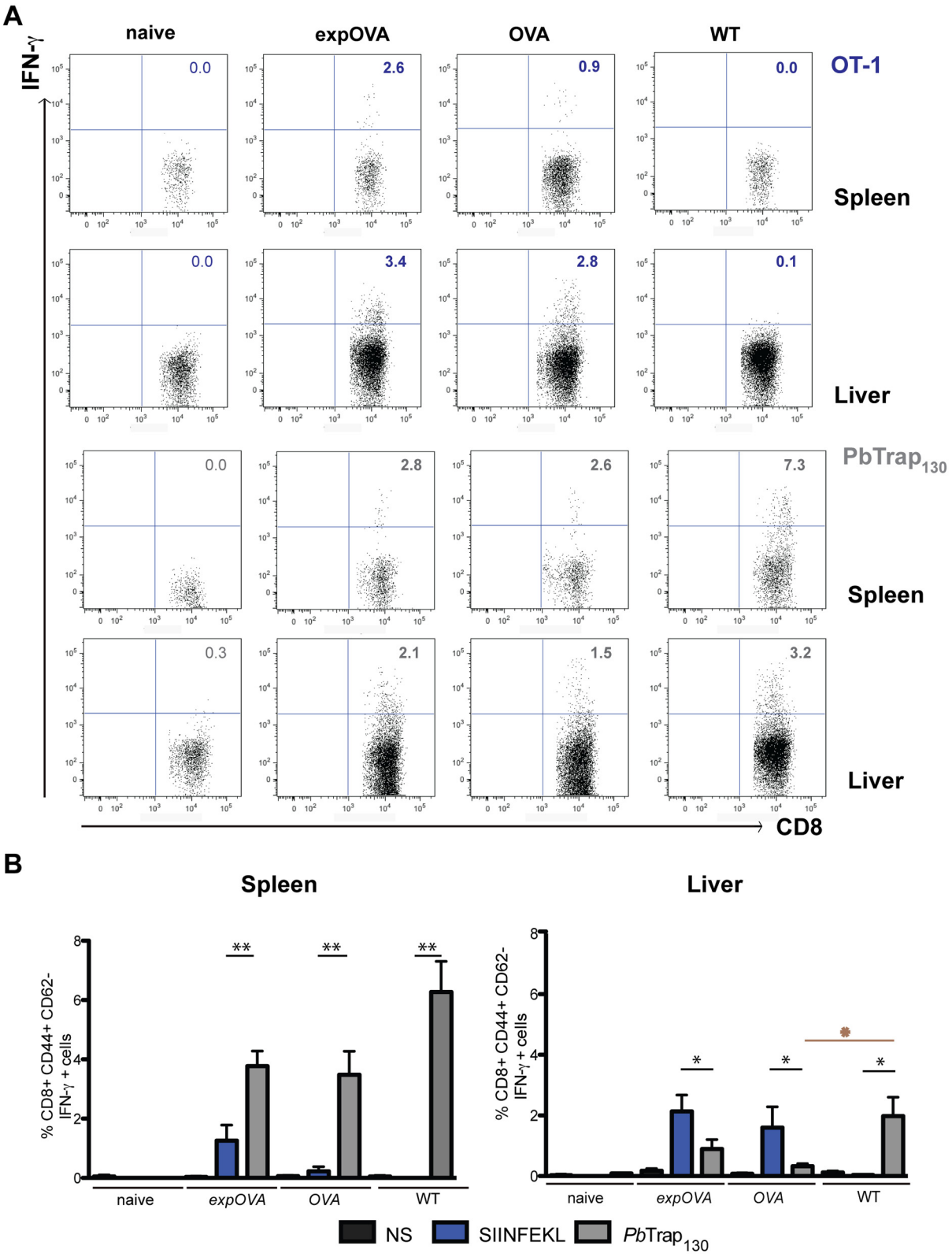

FIG 4 IFN-γ production in CD8$^+$ T cells recognizing OVA and *Plasmodium berghei* sporozoite-derived antigens. (A) Mice were immunized weekly with two doses of 10,000 irradiated *P. berghei* sporozoites. Seven days after the last immunization, cells from livers and spleens were tested for their capacity to produce IFN-γ after restimulation *in vitro* with SIINFEKL peptide. As controls, splenic and hepatic cells were left unstimulated or stimulated with a sporozoite-specific peptide, $TRAP_{130-138}$ ($PbTrap_{130}$). Representative flow cytometry plots show IFN-γ production by CD8$^+$ T cells in spleens and livers of mice. (B) Percentages of IFN-γ$^+$ CD8$^+$ T cells are shown as means ± standard deviations. Results are representative of two independent experiments ($n = 3$ or 4 mice). *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

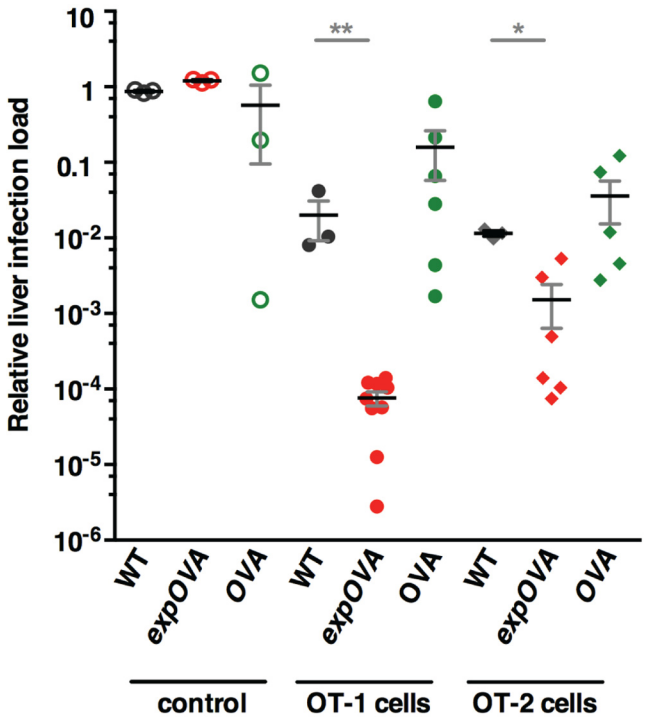

FIG 5 Immunization with *expOVA* sporozoites enhances protection against reinfection. (A) Quantification of parasite liver loads in immunized mice that received OT-1 or OT-2 cells. C57BL/6 mice received 2×10^5 OT-I (CD8) T cells (OT-1) or OT-II (CD4) T cells (OT-2). Next, mice were immunized once with 10,000 irradiated WT (black), *expOVA* (red), or *OVA* (green) sporozoites. Control mice were immunized once without prior T-cell transfer. Twelve days after the last immunization, animals were challenged by i.v. injection of 10,000 sporozoites of the corresponding genotype. After 42 h, livers were removed and parasite loads were quantified by real-time PCR. Bars and whiskers show means ± standard deviations. *, $P < 0.05$; **, $P < 0.01$ (Mann-Whitney test).

ferior to OVA responses in the spleen and liver, respectively. Taken together, these results suggest that cytoplasmic and surface antigens can differ in their capacity to be presented in both organs. Further experiments will be necessary to explain these observations.

Antigen export improves vaccine-induced protection. To address the functional relevance of antigen-specific $CD8^+$ and $CD4^+$ T cells to protection in this immunization and infection model, we tested the contribution of OVA-specific T cells to inhibition of *Plasmodium* liver-stage development *in vivo*. We transferred 2×10^5 nonactivated OT-I (CD8) and OT-II (CD4) T cells each into mice, and 24 h later, immunized the recipient animals with 10,000 irradiated normal or transgenic OVA sporozoites (Fig. 5). Ten days later, the mice were challenged with the respective sporozoite lines used for immunization. Control mice did not receive OVA-specific T cells before immunization.

In mice that received OT-1 T cells, we observed a very profound reduction in parasite liver loads in those that were immunized and challenged with *expOVA* sporozoites. In marked contrast, mice that received irradiated *OVA* or WT sporozoites displayed similar degrees of reduction, which can be attributed to endogenous protective antigens.

OT-II-cell-recipient mice treated with *expOVA* sporozoites showed only modest reductions in liver loads compared to the reductions in mice immunized and challenged with WT sporozoites. Reductions were also apparent in comparison to the liver loads in the mice immunized with *OVA* sporozoites, but the difference did not reach significance. To test whether $CD4^+$ T cells play a helper role in $CD8^+$ T-cell-mediated protection in this model, we next cotransferred 2×10^5 nonactivated OT-1 and OT-II T cells each into mice and, 24 h later, immunized the recipient animals with 10,000 normal or transgenic OVA sporozoites (see Fig. S4 in the supplemental material). Ten days later, the mice were challenged with the respective sporozoite lines used for immunization. A second cohort was treated similarly, but instead of only one immunization, they received two immunizations with irradiation-attenuated sporozoites (Fig. S4). As expected, mice treated with irradiated WT sporozoites mounted a less effective immune response, since the WT sporozoites cannot activate OT-1 and OT-2 cells. The reductions of parasite loads in livers of *expOVA* sporozoite-immunized and -challenged mice were significantly smaller than those in mice receiving *OVA* or normal sporozoites and were more pronounced after the second immunization.

Taken together, these results indicate that antigen export is an important factor for MHC-I antigen presentation. Furthermore, OVA export does not improve antigen presentation by MHC-II, suggesting that the $CD4^+$ T-cell contribution to protection is negligible in this model.

DISCUSSION

We generated reporter malaria parasites expressing a surrogate antigen that either localizes to the parasite cytoplasm or is exported, allowing us to discriminate between cross-presentation of phagocytized antigens and parasite-mediated antigen export. The finding that live attenuated *OVA* and *expOVA* sporozoites were both capable of efficiently inducing high levels of $CD8^+$ cytotoxic T cells indicates that $CD8^+$ T-cell priming does not depend on the antigen fate in the parasite. It is plausible that all cytoplasmic antigens can be acquired via phagocytosis by antigen-presenting cells (APCs), such as DCs or macrophages, and cross-presented to specific T cells or presented directly to hepatocytes (30).

Intriguingly, our study revealed a marked difference in the proliferation of $CD8^+$ T cells in the livers and spleens of mice that received *expOVA* sporozoites. Previous work showed that presentation of the endogenous sporozoite antigen CSP does not require the protein export motif (16). Throughout intrahepatic development, CSP is typically engulfed inside the PVM (14, 15, 35) but can also be found in punctate structures in the host hepatocytes during the early stages of infection (35, 36). We designed the expression of the surrogate antigen such that the protein abundance increases after PVM formation, allowing us to distinguish between a cytoplasmic and an exported antigen. Therefore, our model antigen differs fundamentally from the endogenous CSP protein in that it is not released during transmigration. Moreover, OVA is not part of the inner membrane complex (IMC) and, hence, is not released into the host hepatocytes in early liver stages after IMC dismantling. Therefore, this surrogate antigen model depends on the presence of the engineered secretory and PEXEL elements to be exported to the LSTVN (27). Our data suggest greatly improved MHC-I presentation on infected hepatocytes upon antigen export, leading to very efficient elimination of infected cells by antigen-specific $CD8^+$ T cells. Therefore, we conclude that the antigen localization influences both the T-cell priming induced by irradiated sporozoites and the elimination of liver-

stage parasites. It is tempting to speculate that during intrahepatic development, the parasite tightly restricts the export of immunogenic proteins in order to minimize visibility by the immune surveillance.

Candidate *Plasmodium* antigens for subunit vaccine development are typically parasite surface proteins which mediate parasite invasion of hepatocytes or red blood cells. High levels of circulating antibodies can reduce parasite motility and host cell invasion. Thus, antibodies against sporozoite surface proteins can inhibit sporozoite entry to the liver, contributing at least partially to protection (37). Sporozoite surface proteins are also released during cell transit, upon either breaching of cellular barriers or transmigration of hepatocytes (38) and, hence, constitute valid candidates for presentation to effector $CD8^+$ T cells. It follows that this process, as part of the parasite life cycle progression, could also serve the purpose of distracting the immune system, for instance, by directing antigen-specific T cells to traversed instead of infected hepatocytes, but this hypothesis remains largely untested thus far. One study that used *spect*$^-$ sporozoites, which are defective in cellular transmigration, reported that the activation of CSP-specific $CD8^+$ T cells was markedly (~5-fold) reduced compared to their activation by normal sporozoites (29). However, the contribution of traversed cells to $CD8^+$ T-cell activation and parasite killing was reported to be negligible (29). Thus far, prioritizing antigens that can elicit potent T-cell-mediated immunity based on parasite biology has been demanding.

Here, we addressed whether distinct targeting of an otherwise identical surrogate antigen affects the magnitude of antigen-specific T-cell responses. For this purpose, we expressed a nonsurface antigen under the control of a preerythrocytic-stage promoter as a surrogate for a protective antigen. An attractive hypothesis is that migrating *expOVA* sporozoites secrete the antigen, which can then be acquired by nonparenchymal APCs and cross-presented to T cells. This assumption would explain the higher levels of $CD8^+$ T-cell proliferation in the livers and spleens of mice immunized with attenuated *expOVA* parasites. However, our attempts to detect OVA in trails deposited during parasite gliding or inside cells after transmigration were unsuccessful (G. N. Montagna, unpublished data).

Plasmodium sporozoites, unlike those of other protozoan pathogens, such as *Toxoplasma* or *Leishmania*, do not infect professional APCs and only invade and develop inside hepatocytes. Apoptotic hepatocytes can be phagocytosed by DCs, but according to *in vitro* studies, the number of infected apoptotic cells is not sufficient for DC uptake and T-cell priming (39). It was postulated that DCs could acquire parasite antigens from live infected hepatocytes through phagocytosis or trogocytosis (40). Alternatively, it has been suggested that infected hepatocytes can also directly prime T cells in the liver (30). Unlike DCs, hepatocytes are expected to be more selective, since they can only present antigens that have access to the cytoplasm. According to this model, OT-1 $CD45.2^+$ T-cell proliferation is expected to be elevated in mice immunized with *expOVA* sporozoites due to more efficient MHC-I presentation. This is in good agreement with our data. A number of recent *in vitro* and *in vivo* studies have provided evidence for direct T-cell stimulation by infected hepatocytes (30, 41, 42), and the role of hepatocytes in immune surveillance in the liver clearly deserves further investigation (43).

Another important finding with potential implications for malaria vaccine development is superior protection when the target antigen contains a signal sequence and a PEXEL element. Two recent imaging studies showed that *Plasmodium*-infected hepatocytes associate with $CD8^+$ T-cell clusters *in vivo* and *in vitro*, likely correlating with parasite elimination (44, 45). Specific killing was achieved by $CD8^+$ T cells that were specific for either the potent H-$2K^d$-restricted CSP epitope (44) or the SIINFEKL peptide in a cytoplasmic OVA molecule (45). In support of these studies, our results suggest that an abundant antigen can contribute to immune protection. Yet, we provide evidence that an exported antigen improves the elimination of infected cells by antigen-specific $CD8^+$ T cells.

Early studies showed that the protozoan *Trypanosoma cruzi*, which has direct access to the host cell cytoplasm, releases proteins that constitute the major source of MHC-I peptides for the generation of parasite-specific CTLs (46). In the case of apicomplexan parasites, the PVM acts as a molecular sieve, which likely restricts the transport of *Plasmodium* antigens to the host cell cytoplasm (33, 47). In *Toxoplasma*-infected cells, antigens gain access to the host cell cytoplasm, thus facilitating subsequent MHC-I presentation via fusion of the PVM with the host cell endoplasmic reticulum (ER) (48). In marked contrast, the developing *Plasmodium* liver-stage parasite does not appear to associate with the host ER but develops an extensive and highly dynamic LSTVN, which associates with the late endosome/lysosome compartment (32). This contact zone might serve as the entry point of *Plasmodium* antigens, such as expOVA, to ultimately get access to MHC-I presentation via the lysosome and *trans*-Golgi network. This scenario would differ markedly from the classical presentation pathway reported for the sporozoite antigen CSP (16).

In conclusion, our findings suggest that abundant *Plasmodium* liver-stage proteins that contain the PEXEL signature for protein export and gain access to the PVM represent excellent candidates for protective antigens. Our findings in *Plasmodium* are very consistent with reports on the influence of antigen localization on T-cell activation for other intracellular pathogens, including bacteria and protozoa (46, 49, 50). Further studies are warranted to gain a better understanding of the cellular events that lead to the entry of *Plasmodium* liver-stage antigens into the MHC class I presentation machinery. These insights are important to understand the escape mechanisms employed by the parasite to silence potential immune responses during the crucial intrahepatic population expansion phase and, ultimately, for rational design of an efficacious vaccine against malaria.

MATERIALS AND METHODS

Experimental animals. All animal work was conducted in accordance with German Animal Protection Law (Tierschutzgesetz). The protocol was approved by the ethics committees of the Max Planck Institute for Infection Biology and the Regional Office for Health and Social Affairs, Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin: LAGeSo Reg# G0469/09).

***Plasmodium* life cycle.** For all experiments, *P. berghei* clone 507 (strain ANKA), which expresses green fluorescent protein (GFP) under the constitutive EF1alpha promoter (51), was used. Parasites were maintained in NMRI mice. For mosquito transmission, mice were monitored for high proportions of differentiated gametocytes and microgametocytes capable of exflagellation. *Anopheles stephensi* mosquitoes were allowed to blood feed for 15 min on anesthetized mice and maintained under a 14-h light/10-h dark cycle in 75% humidity at 20°C. Mosquitoes were dissected at days 10, 14, and 17 to determine infectivity, midgut sporozoite numbers, and salivary gland sporozoite numbers, respectively. To detect liver-

stage parasites in hepatocytes, ~50,000 hepatoma (Hepa 1-6) cells were seeded in 8-well chamber slides and grown to semiconfluence. *P. berghei* sporozoites were added, incubated for 90 min at 37°C, and washed off. After 18 or 48 h, infected cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 1% Triton X-100, and parasites visualized by using a primary antibody against *P. berghei* heat shock protein 70 (Hsp70) (52). To detect OVA, a commercial polyclonal antiserum was used (C6534; Sigma-Aldrich). To visualize the parasite's parasitophorous vacuolar membrane (PVM), an antiserum against UIS4 of *P. berghei* (*Pb*UIS4) was used. Cells were fixed with 4% PFA and permeabilized with 1% Triton X-100. The IFA results were recorded with a Zeiss Axio Observer.Z1 microscope (Zeiss) with a charge-coupled device camera (AxioCam, Zeiss) using AxioVision software (Zeiss). The images were processed with Photoshop, and fluorescence was quantified using FIJI (ImageJ). To determine the prepatent period, which is defined as the time to detection of the first parasites in the peripheral blood, 10,000 sporozoites were injected intravenously into C57BL/6 mice and parasitemia assayed by daily examination of Giemsa-stained blood films. For natural transmission experiments, C57BL/6 mice were infected by 5 to 8 mosquito bites, and parasitemia was examined daily.

Recombinant protein expression and antiserum production. A carboxy-terminal fragment of *Pb*UIS4 encompassing amino acid residues 77 to 220 was fused to the amino terminus of the glutathione *S*-transferase (GST) protein in the pGEX 1-lambda-T expression plasmid as described previously (53). For the production of specific antiserum, 50 μg of GST-*Pb*UIS4 suspended in phosphate-buffered saline (PBS) was injected intraperitoneally into male mice (NMRI). Two boosters of 10 μg each were given at days 15 and 25 postinjection, and 2 weeks after the second boost, blood was collected and serum isolated. The specificity of the anti-*Pb*UIS4 antiserum was evaluated by reactivity toward the His_6-tagged version of the protein.

Generation of OVA parasites. To generate *P. berghei* parasites expressing OVA, a 747-bp fragment of chicken OVA corresponding to amino acids 142 to 389 was amplified with primers OVA_sin and OVA_rev, using an OVA-containing plasmid as the template (kindly provided by Alexandra Lorenz, Deutsches Rheuma-Forschungszentrum, Berlin, Germany). The *UIS4* promoter was amplified from *P. berghei* genomic DNA using primers UIS4_5'UTR_fd and UIS4_5'UTR_UIS4_PEXEL_reverse. To amplify a 1.1-kb fragment of the *P. berghei* small subunit (*ssU*) rRNA locus, primers *PbSSU*_fd and ssU_rv were used, with *P. berghei* genomic DNA as the template. The resulting fragments were cloned into the *P. berghei* transfection vector $B3D^+$, leading to plasmid pOVA-$BD3^+$. For export of OVA, a 221-bp fragment from the PEXEL motif of CSP was amplified using the primers PEXEL_fd and PEXEL_rv. The corresponding OVA fragment was amplified using the primers OVA_fv2 and OVA_rev. The resulting fragments were cloned into $B3D^+$, generating plasmid pexpOVA-$BD3^+$. Parasites were transfected with Apa1-digested pOVA-$BD3^+$ and pexpOVA-$BD3^+$ plasmids by using the Nucleofector device (Amaxa GmbH) and injected into naive mice. After transfection, recombinant parasite populations were selected using pyrimethamine (51). Clonal parasite lines were obtained by limited dilution into 10 recipient NMRI mice. Genotyping of recombinant parasite populations was performed by PCR with the following primer combinations: Tg_pro and OSssU_test_rev (test 1), OSssU_test_for and OVA_rev (test 2), OVA_fv2 and OVA_rev (test 3), and OSssU_test_for and ssU_rv (*ssU* rRNA). All primers are listed in Table S1 in the supplemental material.

Cytolytic-T-cell assays. Spleen cells from transgenic OT-1 mice containing 3×10^5 $CD8^+$ T cells isolated with CD8α (Lys2) microbeads (Miltenyi Biotec) were transferred intravenously to C57BL/6 mice. After 24 h, mice were immunized with 10,000 irradiated sporozoites (*OVA*, *expOVA*, and WT). At day 6 postimmunization, the *in vivo* cytotoxicity assay was performed. To this end, splenocytes from female wild-type C57BL/6 mice were incubated at 1×10^7 cells/ml in complete RPMI medium alone (unpulsed cells) or 1×10^7 cells/ml with SIINFEKL peptide (10 μg/ml; pulsed cells) for 2 h. The cells were washed and incubated at 5 $\times 10^7$ cells/ml in the dark with CFSE (Molecular Probes Europe, Leiden, Netherlands), either at 1.5 μM (unpulsed cells; $CFSE^{low}$) or 15 μM (peptide-pulsed cells; $CFSE^{high}$), for 15 min at 37°C, washed again, and resuspended in PBS at 1×10^8 cells/ml. Twenty million cells of a 1:1 mixture of the $CFSE^{low}$/$CFSE^{high}$ target cells were adoptively transferred into test mice, and 18 h later, the splenocytes of test mice were analyzed by flow cytometry. The percentage of target cell killing was calculated using the following formula: 100 − (((% peptide-pulsed cells in immunized mice/% unpulsed cells in immunized mice)/(% peptide-pulsed cells in control mice/% unpulsed cells in control mice)) × 100).

T-cell proliferation assay. Spleen cells from transgenic OT-1 CD45.2 mice were isolated and labeled with CFSE according to the manufacturer's instructions (Molecular Probes). Amounts of 2×10^6 or 8×10^6 OT-1 CD45.2 cells were transferred intravenously to C57BL/6 CD45.1 mice. After 24 h, the animals were immunized with 10,000 irradiated normal or transgenic sporozoites. After 72 h, lymphocytes were isolated from spleens and livers by using a Percoll gradient. The cells were resuspended in PBS–2% bovine serum albumin buffer and stained with antibodies to mouse CD8 (clone 53-6.7, peridinin chlorophyll protein [PerCP]; BD Pharmingen), CD3 (clone 145-2C11, Pacific Blue; e-Bioscience), CD45.2 (clone 104, APC-780; e-Bioscience), and CD45.1 (clone A20, APC; e-Bioscience). FACS analysis was performed in a BD LSRII flow cytometer.

***In vitro* stimulation of immune cells and intracellular IFN-γ staining.** For intracellular cytokine staining, single-cell suspensions at a density of 2×10^6 cells per well were cultured in 96-well flat bottom plates in the presence of $TRAP_{130-138}$ peptide (10 μg/ml) or SIINFEKL peptide (10 μg/ml) and brefeldin A (1:1,000, GolgiPlug; BD Biosciences). After 5 h, surface staining was performed using antibodies to CD3e (145-2C11, phycoerythrin [PE]-Cy7), CD62L (MEL-14, PE), CD8a (53-6.7, PerCP-Cy5.5), and CD44 (IM7, Pacific blue) for 1 h on ice, washed twice in FACS buffer, and fixed with 4% paraformaldehyde. Cells were permeabilized using BD Perm/Wash buffer, which was also used throughout the staining procedure. To detect intracellular IFN-γ, cells were stained with antibody to IFN-γ (XMG1.2, APC) for 45 min. Cell acquisition was performed on a BD LSRII flow cytometer, and data were analyzed using FlowJo software.

Quantification of parasite liver loads by real-time PCR. For quantification of parasite loads in the liver by real-time quantitative reverse transcription (qRT)-PCR, C57BL/6 mice were challenged by intravenous injection of 10,000 normal or transgenic sporozoites. Mice were sacrificed 42 h later, and livers removed and homogenized. Total RNA was isolated with the RNeasy kit (Qiagen), and cDNA synthesized with the RETROScript kit (Ambion). Real-time PCR was performed with the ABI 7500 sequence detection system and Power SYBR green PCR MasterMix (Applied Biosystems), using gene-specific primers for *P. berghei* 18S rRNA (gi:160641) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (gi:281199965) genes as described previously (54).

Statistical analysis. Statistical significance was assessed using the two-tailed Student's *t* test, Mann-Whitney test, and Kruskal-Wallis test. All statistical tests were computed with GraphPad Prism 5 (GraphPad Software).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01321-14/-/DCSupplemental.

Figure S1, TIF file, 1.4 MB.
Figure S2, TIF file, 0.3 MB.
Figure S3, TIF file, 0.2 MB.
Figure S4, TIF file, 0.2 MB.
Table S1, DOCX file, 0.1 MB.

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