**In vivo CD8+ T Cell Dynamics in the Liver of Plasmodium yoelii Immunized and Infected Mice**

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### Abstract

*Plasmodium falciparum* malaria remains one of the most serious health problems globally and a protective malaria vaccine is desperately needed. Vaccination with attenuated parasites elicits multiple cellular effector mechanisms that lead to *Plasmodium* liver stage elimination. While granule-mediated cytotoxicity requires contact between CD8+ effector T cells and infected hepatocytes, cytokine secretion should allow parasite killing over longer distances. To better understand the mechanism of parasite elimination in vivo, we monitored the dynamics of CD8+ T cells in the livers of naive, immunized and sporozoite-infected mice by intravital microscopy. We found that immunization of BALB/c mice with attenuated *P. yoelii* 17XNL sporozoites significantly increases the velocity of CD8+ T cells patrolling the hepatic microvasculature from 2.69±0.34 μm/min in naive mice to 5.74±0.66 μm/min, 9.26±0.92 μm/min, and 7.11±0.73 μm/min in mice immunized with irradiated, early genetically attenuated (Pyuis4-deficient), and late genetically attenuated (Pyyabb/f-deficient) parasites, respectively. Sporozoite infection of immunized mice revealed a 97% and 63% reduction in liver stage density and volume, respectively, compared to naive controls. To examine cellular mechanisms of immunity in situ, naive mice were passively immunized with hepatic or splenic CD8+ T cells. Unexpectedly, adoptive transfer rendered the motile CD8+ T cells from immunized mice immotile in the liver of *P. yoelii* infected mice. Similarly, when mice were simultaneously inoculated with viable sporozoites and CD8+ T cells, velocities 18 h later were also significantly reduced to 0.68±0.10 μm/min, 1.53±0.22 μm/min, and 1.06±0.26 μm/min for CD8+ T cells from mice immunized with irradiated wild type sporozoites, Pyyabb/f-deficient parasites, and *P. yoelii* CS280–288 peptide, respectively. Because immobilized CD8+ T cells are unable to make contact with infected hepatocytes, soluble mediators could potentially play a key role in parasite elimination under these experimental conditions.

### Citation

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### Competing Interests

The authors have declared that no competing interests exist.

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### Introduction

Despite considerable accomplishments in the fight against malaria over the past years, *Plasmodium falciparum*, the deadliest of all human malaria parasites, still remains responsible for more than half a million annual deaths worldwide, predominantly in young children in Africa [1]. In the face of the inevitable development of parasite drug resistance and potential vector resistance to insecticides, a malaria vaccine that can protect the 40% of the world’s population at risk of malaria infection is therefore urgently needed. Sera and immune cells of protected individuals have identified the circumsporozoite protein (CSP) as a therefore urgently needed. Sera and immune cells of protected individuals have identified the circumsporozoite protein (CSP) as a

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Shown to elicit high levels of sterile immunity in humans and experimental animals following exposure to bites of irradiated *Plasmodium*-infected mosquitoes [6,7]. Immunization with radiation-attenuated sporozoites (RAS) remains the “gold standard” for *P. falciparum* malaria vaccine development [7–14]. Protection can also be achieved in mice by immunization with *P. berghei* or *P. yoelii* RAS [6,15,16], genetically attenuated parasites (GAP) such as the *P. yoelii* GAP Pyuis3(−), Pyuis4(−), Pyyabb/f(−), and PyP52/P36 double knockout in the BALB/c mouse model [17–21] or the *P. berghei* GAP Phuis3(−), Phuis4(−), PbbP52(−), and PbbP36p(−) in the C57Bl/6 mouse model [22–25]. Similarly, chemically attenuated *P. berghei* sporozoites [26], viable *P. yoelii* sporozoites under drug cover [27], and a prime boost regimen consisting of CSP peptide-coated dendritic cells and recombinant *Listeria monocytogenes* can result in immunity [15,28]. Vaccination with late liver stage-feeding GAP without progression to blood stage development appears to generate the most efficient antimalarial immunity in mice, presumably because memory T cells targeting a broader spectrum of LS antigens are induced [18,29].
The intricate life cycle of the *Plasmodium* parasite in the mammalian host provides a unique challenge for malaria vaccine development. After transmission into the skin by the bite of an infected mosquito [30,31], *Plasmodium* sporozoites travel to the liver, glide along the sinusoidal endothelium, leave the bloodstream by traversing Kupffer cells [32–34], the resident hepatic macrophages, migrate through several hepatocytes, and develop to large intracellular liver stage (LS) parasites, which eventually differentiate to tens of thousands of erythrocyte-infective merozoites. During this extensive migration phase [35,36], CSP and other parasite antigens are continuously released from the sporozoite surface [37–39] and translocated into the cytosol of mammalian cells [33,40,41], suggesting that essentially all parenchymal and non-parenchymal liver cells are exposed these antigens. Because most non-parenchymal liver cells, in addition to dendritic cells (DC), can function as antigen presenting cells (APC) [42,43], sporozoite antigens released into the hepatic microenvironment are likely internalized and processed by local hepatic APC, in particular Kupffer cells, and presented to immune cells patrolling the liver sinusoids. Although not formally proven, this notion is supported by the finding that vaccination with attenuated sporozoites stimulates Kupffer cells to upregulate MHC class I and produce IL-12 [44]. In agreement with the tolerogenic properties of the liver [42,45–48], however, infectious sporozoites down-modulate the production of proinflammatory cytokines and block the respiratory burst in Kupffer cells thus crippling their properties of the liver [42,45–48], however, infectious sporozoites and produce IL-12 [44]. In agreement with the tolerogenic effector memory T cells, lost in the absence of cytokines [49], significantly increases the background [64]. LS development was initiated by intravenous injection of at 1–2×10^6 freshly dissected sporozoites in mice. Sporozoites were isolated from salivary glands of infected mosquitoes by gently crushing the glands with a micropette in a microtube (Kimble Chase, 749520-0000) with minimal RPMI medium (Invitrogen, 11835-055) with 2% fetal bovine serum (HyClone) [32,65]. After sequential centrifugation at 700 rpm and 500 rpm, sporozoites were recovered from the supernatant fractions and resuspended to a final volume of 200 µl with RPMI.

**Immunization and challenge**

To produce radiation-attenuated sporozoites (Py-RAS), freshly isolated Py17XNL sporozoites were irradiated within a gamma irradiator (MDS Nordion Gammacell 1000 Elite) to a central dose of ≥12,049 cGy and a minimum dose of ≥10,266 cGy. Recipient BALB/c mice were injected intravenously with 50,000 Py-RAS, with 2 subsequent booster injections of 20,000 Py-RAS each, at 15 days intervals. A second and third group of BALB/c mice were immunized with freshly isolated Pyuis4(+) and Pyfabb/f(−) GAP sporozoites using the same dosing scheme. To elicit large numbers of Py-CS280–288 epitope-specific CD8+ T cells, a four group of BALB/c mice were primed with 1×10^6 splenic dendritic cells coated with P. yoelii CS280–288, and boosted 7 d later with 1×10^7 – 1×10^8 CFU recombinant *Listeria monocytogenes* expressing CS280–288, as described previously [66,67]. For ex vivo analysis of LS, immunized mice were challenged with 300,000 viable PyGFP-XNL sporozoites. As positive controls, naive mice were challenged at the same time by inoculation of the same number of sporozoites from the same pool of dissected sporozoites.

**Ethics statement**

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, NYU School of Medicine (Protocol number 120213-01). All surgery was performed under ketamine-xylazine-acepromazine anesthesia, and all efforts were made to minimize suffering.

**Parasites**

Wild type *P. yoelii* strain 17XNL (PyXNL), P. yoelii 17XNL expressing GFP (PyXNL-GFP) [62], and P. yoelii 17XNL GAP with a deletion at PyUIS1 [Pyuis1(−)] [17] or Pyfabb/f/PyFabb/f [Pyfabb/f(−)] [18] were propagated in female Swiss Webster mice (Taconic Farms) and cycled in female *Anopheles stephensi* mosquitoes.
Splenic and hepatic lymphocyte isolation and adoptive transfer

For adoptive transfer, the entire population of hepatic or splenic CD8+ cells was harvested from immunized mice 2 weeks after the second booster, and suspended in 300 μl RPMI. Single cell suspensions of splenic lymphocytes were obtained by mechanically disrupting spleens and filtering through a 100 μm cell strainer (Fisherbrand, Fisher Scientific, 22-363-549). Erythrocytes were lysed with ACK Lysis Buffer (Lonzza, 10-548E) for 10 min at RT, washed and resuspended in PBS pH 7.0. Total numbers of splenic and CD8+ cells (eBiosciences, 12-0081) were determined using a BD Accuri C6 flow cytometer (Becton Dickinson). For isolation of intrahepatic lymphocytes (IHL), livers from Py-RAS immunized mice were perfused using a modified perfusion method by Crispe et al. [68]. Briefly, livers were perfused with 95% O2/5% CO2 balanced buffers to remove contaminating erythrocytes from the sinusoidal space, homogenized, digested with collagenase, and enriched via density gradient centrifugation with Optiprep Density Gradient Medium (Sigma-Aldrich). CS280–288 specific CD8+ T cells were either surface- or intracellularly labeled by intravenous injection of 2 μg conjugated anti-mouse CD8a (Clone 53–6.7, BD Pharmingen) antibodies. CD8+ T cells were visualized intravascularly using fluorochrome-conjugated anti-mouse CD8a antibody after transfer, labeling conditions that do not interfere with the ability of cytotoxic T cells to crawl or to kill antigen-presenting target cells in vivo or in vitro (Cabrera, Movila, Nacer, and Frevert, unpublished data) [70–72].

Anesthesia, surgery, and intravitral microscopy

For intravital microscopy, mice were anesthetized with a cocktail of 50 mg/kg Ketamine HCl (Ketaset, Fort Dodge Animal Health, 0856-4403-01), 10 mg/kg Xylazine (Lloyd Laboratories, 4821), and 1.7 mg/kg Acepromazine Maleate (Butler Animal Health Supply, 003845) (KXA mix) as described [35,64,65]. Anesthetized mice were intravenously or intraperitoneally inoculated with 648 nmol of the nuclear stain Hoechst 33342 (Invitrogen). To visualize hepatocytes, mice received intravenous inoculation of fluorochrome-conjugated anti-mouse CD8a (Clone 53–6.7) antibodies on days 0, 2, 4, 6, 8, 10, 14, 17, 19, and 21 post-infection. To evaluate hepatic cell death, immunized mice were intravenously injected with 40 nmol of the fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a antibody; for Additional comments, please see Additional file 1. 4821), and 1.7 mg/kg Acepromazine Maleate (Butler Animal Health Supply, 003845) (KXA mix) as described [35,64,65]. The mouse peritoneal cavity was opened along the rib cage and the liver exposed for IVM as described [35,64,65] using an inverted Leica DMIRE2 microscope equipped with a temperature controlled Ludin chamber, and analyzed with a Leica TCS SP2 AOBS confocal system (40x HCX PL APO 1.25–0.75 oil lens; 488 nm Argon/ Krypton line; and HeNe laser lines at 543 nm, 594 nm and 633 nm. Periodic reinjection of KXA mix at 60–90 minute intervals allowed imaging for at least 4 h.

Cellular in vivo markers

Prior to imaging, mice were intravenously or intraperitoneally inoculated with 648 nmol of the nuclear stain Hoechst 33342 (λex = 405 nm; Invitrogen). To visualize hepatocytes, mice received 18.4 nmol MitoTracker Deep Red (λex = 633 nm; Invitrogen) or 14.9 nmol MitoTracker Green FM (λex = 633 nm; Invitrogen) as described previously [64]. CD8+ T cells were either surface-labeled by intravenous injection of 2 μg phycoerythrin (PE)- conjugated anti-mouse CD8a (Clone 53–6.7, λex = 488 nm; eBiosciences) or loaded after purification with CellTracker Red CMTPX (Invitrogen) or Cell Trace Calcein Red-Orange AM (λex = 594 nm) or Cell Trace Calcein Violet AM (λex = 405 nm) and then intravenously inoculated as described previously [64].

Ex vivo analysis of LS number and size

Anesthetized mice were intravenously injected with fluorescent tracers and antibodies and sacrificed 20 min later. The liver was dissected out and immersed in ice-cold PBS pH 7.0 for preparation of 200 μm thick tissue sections (speed 0.60 mm/s and amplitude 1.0 mm) using a Leica VT 1200 S vibratome. Liver sections were kept hydrated in PBS pH 7.0, lined up on microscope slides, covered with a 22×50 coverslip (Fisherbrand No 1.5, Fisher Scientific; 12-544-D), and sealed with nail polish. The sections of fresh unfixed liver tissue were immediately systematically scanned visually. Each 40x field of view was defined as a dimension of 573×373×~50 μm observed immediately using a Leica DMIRE2 microscope and subsequently analyzed with a Leica TCS SP2 AOBS confocal system (40x HCX PL 1.25–0.75 oil lens). A field of view with no LS parasite was still counted as part of the total number of fields of view. XYZ stacks were collected for LS parasite volume determination.

Image processing

After acquisition with Leica LCS software, 2D, 3D, or 4D datasets were reconstructed and processed in Imaris 7.4.2 (Bitplane). LS volume was measured using isosurface reconstructions and tracks were measured by tracking ROIs as spots or isosurfaces. Volume and track statistics obtained from Imaris software were exported to Microsoft Excel and SigmaPlot 12.0 (Systat Software Inc.). The arrest coefficient is defined as the proportion of time individual CD8+ T cells exhibit a velocity of less than 2 μm/min. Composite figures were generated in Adobe Photoshop (Adobe Systems Inc.).

Statistic analyses

Velocity and arrest coefficient statistics were calculated using One-way ANOVA on Ranks. LS number and size statistics were calculated using unpaired t-tests. Errors are standard error of the mean ± SEM.

Results

The goal of this study was to better understand the cellular dynamics associated with the elimination of the liver of Plasmodium LS by CD8+ effector T cells. In a first set of experiments, we generated CD8+ effector memory T cells specific for P. yoelii 17XNL LS by immunizing BALB/c mice with Py-RAS or GAP sporozoites using standard intravenous immunization schemes [69]. Using our recently developed novel imaging techniques for the hepatic microvasculature [64], we monitored the behavior of these CD8+ T cells in the sinusoidal microvasculature of immunized mice and, for comparison, naive mice by IVM. CD8+ T cells were visualized in vivo either by loading with CellTracker or CellTrace dyes prior to adoptive transfer or by intravenous inoculation of fluorochrome-conjugated anti-mouse CD8a antibody after transfer, labeling conditions that do not interfere with the ability of cytotoxic T cells to crawl or to kill antigen-presenting target cells in vivo or in vitro (Cabrera, Movila, Nacer, and Frevert, unpublished data) [70–72].

CD8+ T cell effector function

Pyfabb/l(−) sporozoite immunized BALB/c mice have been shown to be develop CD8+ T cell mediated protective immunity [18]. To assess the effector function of the CD8+ T cells within the immunized mice, naive or Pyfabb/l(−) immunized mice were challenged with 3×107 viable PyXNL-GFP sporozoites and sacrificed 18 h or 42 h later. Ex vivo measurement at 18 h and 42 h after challenge of LS number to measure parasite density in the liver tissue and LS size to detect changes in growth rate revealed a reduction of 81% and 97%, respectively, in PyXNL-GFP LS in Pyfabb/l(−) immunized mice relative to naive control mice (Fig. 1A). In addition, the volume of the few remaining LS was significantly reduced by 63% (p<0.05) at 42 h post infection, from 14,177±1556 μm3 in the control mice to 5306±2517 μm3 in the immunized mice (Fig. 1B). In combination, the 97% and 63% reduction in LS density and volume, respectively, translates into a near-complete prevention of LS development in the immunized mice.
**CD8+ T cell motility in naive and immunized mice**

To visualize effector CD8+ T cells *in situ*, we used intravital microscopy combined with intravenous injection of a PE-conjugated anti-CD8a antibody to visualize the cells within the liver microenvironment. CD8+ T cells in naive mice had a rounded shape and either remained stationary in the sinusoids or moved at bloodstream velocity in the livers of naive mice ([Fig. 2A, Video S1](#)). In contrast, CD8+ T cells from immunized mice imaged 2 weeks after the second booster immunization exhibited the characteristic amoeboid shape and behavior of activated effector T cells with a leading edge and a trailing uropod and actively controlled the liver sinusoids of BALB/c mice immunized with Pyfabb/f(−) sporozoites ([Fig. 2B, Video S2](#)). Similar observations were made in mice immunized with Py-RAS ([Fig. 2C, Video S3](#)) or Pyuis4(−) ([Fig. 2D, Video S4](#)) sporozoites, which also develop high levels of CD8+ T cell mediated immunity [73–75].

To quantify these IVM observations, we measured velocities of individual CD8+ T cells in the livers of naive and immunized mice ([Fig. 3A](#)). CD8+ T cells from naive mice crawled at the slow speed of 2.69±0.34 μm/min, which has been defined as locally confined movement [76]. In contrast, velocities of CD8+ T cells from the sporozoite immunized mice measured 2 weeks after the second booster immunization were significantly higher (Py-RAS = 5.74±0.66 μm/min, Pyuis4(−) = 9.26±0.92 μm/min, and Pyfabb/f(−) = 7.11±0.73 μm/min; p<0.05). The CD8+ T cell velocities amongst the groups of mice immunized with Py-RAS, Pyuis4(−), and Pyfabb/f(−) were not significantly different (p>0.05). Calculation of the corresponding arrest coefficients ([Fig. 3B](#)) revealed that the majority of the CD8+ T cells (66%) in naive mice moved at a speed of less than 2 μm/min. In contrast, the arrest coefficients of the CD8+ T cell populations in mice immunized with Py-RAS, Pyuis4(−), or Pyfabb/f(−) sporozoites were significantly lower (p<0.05), with only 39%, 33%, and 39%, respectively, of the cells moving at less than 2 μm/min. Thus, the velocity of CD8+ T cells monitoring the liver sinusoids in immunized mice was significantly increased compared to naive mice. Considering that effector memory T cells represent the majority of liver resident CD8+ T cells one week after challenge [25], the increased velocity likely reflects an antigen-specific effect.

**Behavior of CD8+ T cells after adoptive transfer into Plasmodium infected mice**

Accumulating evidence suggests that immunization with attenuated sporozoites generates a reservoir of hepatic CD8+ T cells, which is maintained by Kupffer cell and DC derived IL-15 in the presence of a depot of parasite LS antigens, and that resident CD44hiCD45RBhiCD122hiCD62Llo/hi CD8+ central memory T cells are required for the proliferation of IFN-γ producing CD44hiCD45RBhiCD122hiCD62Llo effector memory T cells capable of conferring protection against reinfection [55,77–79]. Based on these findings, we performed all preliminary experiments with purified IHL [68]. To do this, naive BALB/c mice were intravenously inoculated with 1–2×106 PyXNL-GFP sporozoites. The mice were surgically prepared for IVM and a well-immobilized area of the exposed liver lobe was selected for long-term observation of LS [35,65]. The mice were then inoculated with 106 fluorescently labeled CD8+ T cells, which had been isolated from the livers of Py-RAS or Pyuis4(−) sporozoite immunized mice. Initially, multiple sets of experiments were conducted in which the timing of T cell purification relative to sporozoite inoculation, and IVM timing relative to infection and adoptive transfer were varied in an effort to optimize the conditions that favor interactions between CD8+ T cells and infected hepatocytes in the recipient mice. When the CD8+ T cells remained essentially immobile independently of the experimental conditions tested, we settled for a prime-boost regimen that mirrors an immunization scheme that protects mice against challenge with 10,000 PyXNL sporozoites [20]. CD8+ T cells were harvested from donor mice 2 weeks after the second booster and transferred into recipient mice 18 h 42 h post-infection, followed immediately by IVM examination.

To exclude the possibility that liver perfusion or other procedures required for purification of intrahepatic lymphocytes (IHL) [68] abrogated motility, we repeated the experiments with...
splenic T cells. Surprisingly, the velocity of immune splenic CD8+ T cells was not significantly different (p>0.05) from that of immune IHL at 18 h or 40 h after adoptive transfer into PyXNL-GFP infected mice (Fig. S1). Both hepatic and splenic CD8+ T cells were immotile when imaged immediately after adoptive transfer into uninfected naive control mice (data not shown). Because no differences could be observed for the overall behavior of CD8+ T cells purified from liver versus spleen under the different infection, immunization, and IVM conditions tested, including transfer into uninfected control mice, all subsequent experiments were done with splenic CD8+ T cells.

At 16–18 h post-infection, young LS did not yet occupy the entire cytoplasm of the infected hepatocyte and frequently had not yet retracted their sporozoite ends (Fig. 4A, Video S5). Only beyond 20 h after infection did the LS appear completely rounded (data not shown). When harvested from donor mice at least 2 weeks after the second booster immunization with Py-RAS and transferred (1 million) into mice harboring late stage LS, splenic CD8+ T cells moved at 3.24±0.32 μm/min (Fig. 5A), which is significantly slower than the CD8+ T cells observed in the Py-RAS immunized mice (5.74±0.66 μm/min; p<0.05). By 42 h post-infection, P. yoelii LS are almost mature and exceed the size of normal uninfected hepatocytes (Fig. 4B, Video S6). Splenic CD8+ T cells harvested from Py-RAS immunized mice 2 weeks after the second booster immunization and adoptively transferred (1 million) into mice harboring late stage LS also moved at the significantly slower velocity of 2.44±0.43 μm/min. In the PyXNL-GFP infected BALB/c mice, the average arrest coefficients for CD8+ T cells adoptively transferred from Py-RAS immunized mice revealed significantly higher numbers of CD8+ T cells with track velocities of less than 2 μm/min (p<0.05) at 18 h (64%) and 42 h (76%) post infection when compared to the CD8+ T cells in the livers of Pyuis4(−) sporozoite immunized mice (9.18±0.94 μm/min), the mean velocities of splenic CD8+ T cells with track velocities of less than 2 μm/min (p<0.05) at 18 h (64%) and 42 h (76%) post infection when compared to the CD8+ T cell arrest coefficient in immunized mice (Fig. 5B). Similarly, when compared to mean velocities of CD8+ T cells in the livers of Pyuis4(−) sporozoite immunized mice (9.18±0.94 μm/min), the mean velocities of splenic CD8+ T cells adoptively transferred into PyXNL-GFP infected mice were significantly lower when adoptive transfer was done at 18 h (1.45±0.57 μm/min) and 42 h (1.51±0.14 μm/min) post infection (Fig. 6A). Again, the corresponding arrest coefficients followed the pattern observed for Py-RAS immunization: compared to the CD8+ cells in Pyuis4(−) sporozoite immunized mice (39%), 82% and 85% of the CD8+ T...
cells at 18 h and 40 h, respectively, had a velocity of less than 2 μm/min after adoptive transfer (p < 0.05) (Fig. 6B). Significantly, none of the adoptively transferred CD8+ T cells observed (N = 197) made direct contact with LS at any time least during the 4 h IVM monitoring of a total of 25 infected mice. If in rare instances CD8+ T cells were arrested in the vicinity of infected hepatocytes, they showed no evidence for a change in shape, which would precede formation of an immunological synapse indicating recognition of cognate peptide on infected hepatocytes [72].

Simultaneous sporozoite infection and adoptive CD8+ T cell transfer

Since RAS parasite arrest occurs at early stages of development, we addressed the possibility that CD8+ T cells recognize infected hepatocytes only at early stages of LS development. BALB/c mice were inoculated with 1–2×10^6 PyXNL-GFP sporozoites immediately followed by adoptive transfer 10^6 splenic CD8+ T cells harvested from Py-RAS or Pyfabb/(−) immunized mice 2 weeks after the second booster immunization and monitored by IVM 18 h later. Compared to the CD8+ T cell velocity in the Py-RAS immunized donor mice before transfer (3.74 ± 0.66 μm/min; Fig. 3A), the average velocity of the Py-RAS CD8+ T cells after transfer into the PyXNL-infected mice was 0.68 ± 0.10 μm/min (Fig. 7A, Video S7) and thus significantly lower (p < 0.05). The corresponding arrest coefficient for CD8+ T cells after transfer was 96% (Fig. 7B) compared to 39% for the immunized donor mice before transfer (Fig. 3B). Similarly, the average velocity of CD8+ T cells from Pyfabb/(−) immunized mice was 1.53 ± 0.22 μm/min after transfer into PyXNL-infected mice (Fig. 7A), which is significantly lower (p < 0.05) than the value of 7.11 ± 0.73 μm/min in the Pyfabb/(−) immunized donor mice before transfer (see Fig. 3A). Again, the arrest coefficient for CD8+ T cells in the PyXNL-infected mice after transfer was 77% (Fig. 7B) compared to 33% in the Pyfabb/(−) immunized donor mice before transfer (Fig. 3B). Thus, although slightly more motile than CD8+ T cells from Py-RAS immunized mice, CD8+ T cells from Py-fabb/(−) mice also remained locally confined [76]. IVM as well as ex vivo examination of the entire liver surface provided no evidence for LS development 18 h post infection in the recipient mice. Others used PCR to show that 20–40 million cloned CD8+ T cells strongly inhibited the development of 100 P. yoelii sporozoites in the liver, even when adoptively transferred as late as 20 h after infection [80,81]. In another study, inoculation of 6 million activated P. yoelii CSP tetramer-positive CD8+ T cells resulted in a near-complete

![Figure 3. Tracking CD8+ cells in naïve and immunized mouse livers.](A) CD8+ T cells were identified by anti-CD8a-PE labeling in the livers of naive mice (circles) or mice immunized with Py-RAS (triangles), Pyfabb/(−) (diamonds), or Pyfabb/(−) (squares) and monitored via IVM. Velocities (A) and arrest coefficients (B) were calculated from the tracks of individual CD8+ T cell from at least 2 infected mice per group. One-way ANOVA on Ranks shows that the velocities of the CD8+ T cells from all immunized mice are significantly higher and the arrest coefficients significantly lower than those of the CD8+ T cells from naive mice (p < 0.05). The same statistical analysis does not reveal any significant difference between the CD8+ T cell velocities between the groups of immunized mice (p > 0.05). * = p < 0.005, ns = not significant. CD8+ T cells of immunized mice were monitored at least 2 weeks after the second booster.

![Figure 4. Behavior of adoptively transferred CD8+ T cells in the liver of infected mice.](A) At least 2 weeks after the second booster, CD8+ T cells were purified from the spleens of Py-RAS immunized mice and loaded with CellTracker Red. One million CD8+ T cells were transferred into recipient mice 18 h (A) or 42 h (B) post infection with PyXNL-GFP, and immediately imaged by IVM. CD8+ T cells (red) remained immobile and failed to migrate to or make contact with hepatocytes infected with PyXNL-GFP LS (green). Hepatocyte autofluorescence is shown in green in (A) and red in (B). Nuclei were stained with Hoechst (blue) in (A). Scale bars 20 μm.

![Figure 3A](http://journal.pone.0070842.g003)

![Figure 3B](http://journal.pone.0070842.g004)
inhibition of the development to LS of 50,000 simultaneously inoculated sporozoites [82]. It should be noted that in our efforts to observe interactions between CD8+ T cells and LS, we inoculated sporozoite numbers that exceed those typically used to confirm protection after challenge by several logs of magnitude. Thus, while we expect the adoptively transferred CD8+ T cells to

Figure 5. Tracking adoptively transferred Py-RAS activated CD8+ T cells in the livers of infected mice. LS were identified in a well-immobilized area of the liver and labeled CD8+ T cells in the vicinity of the LS were monitored to calculate velocities (A) and arrest coefficients (B). Py-RAS CD8+ T cells in the liver of immunized mice (circles) were used as controls. CD8+ T cells were purified 2 weeks after the second booster from the spleens of Py-RAS immunized mouse and 1 million was adoptively transferred into recipient mice 18 h (triangles) or 42 h (squares) after infection with PyXNL-GFP. Based on ANOVA on Ranks, the velocity and arrest coefficients of all adoptively transferred cells differed significantly from Py-RAS in the liver of immunized mice (p<0.05). The same test showed only a significant difference in the values between the two 18 h time points. At least nine infected mice were used per experimental condition. * = p<0.05, ns = not significant.

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Figure 6. Tracking adoptively transferred Pyuis4(−) activated CD8+ T cells in the livers of infected mice. Mice were infected with 1–2 million PyXNL-GFP sporozoites. Activated CMTPX labeled CD8+ T cells were then purified 2 weeks after the second booster from the spleens of Py-RAS immunized mice and 1 million was adoptively transferred into the infected mice at 18 h (triangles) or 42 h (squares) after infection with PyXNL-GFP. Anti-mouse CD8a labeled T cells in Pyuis4(−) immunized mice (circles) were used as controls. ANOVA on Ranks show that the mean velocities (A) and arrest coefficients (B) of all adoptively transferred CD8+ T cells differ significantly from those of the CD8+ T cells in Pyuis4(−) immunized mice. At least three infected mice were used per experimental condition. * = p<0.05, ns = not significant.

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have reduced the parasite biomass in the liver, some LS likely survived under these experimental conditions.

Sterile protection of BALB/c mice by Py-RAS immunization requires the induction of a large percentage of parasite-specific CD8+ T cells [15,59]. To increase the likelihood of detecting Py-specific CD8+ T cells within the overall pool of adoptively transferred immune cells in our IVM studies, we utilized a heterologous prime/boost immunization protocol that has been shown to generate high levels of CD8+ memory T cells specific for the Py CTL epitope CS 280–288 [66,67]. In this protocol, BALB/c mice were primed with 1×10^6 splenic dendritic cells coated with P. yoelii CS 280–288 and boosted 7 d later with 1×10^7 CFU recombinant Listeria monocytogenes expressing CS 280–288, as described previously [66,67]. Another group of BALB/c mice was then infected with PyXNL-GFP, as above, followed by immediate inoculation of 10^6 purified splenic CD8+ T cells from the Py-CS 280–288 immunized mice. Py-CS 280–288 specific TCR transgenic mice were primed with 1×10^6 splenic dendritic cells coated with P. yoelii CS 280–288 and boosted 7 d later with 1×10^7 CFU recombinant Listeria monocytogenes expressing CS 280–288, as described previously [66,67].

Discussion

The major finding of this study is that adoptive transfer renders CD8+ T cells immobile in the sinusoidal microvasculature for a period of at least 3 days. While motile and actively patrolling the liver of immunized donor mice, few of the adoptively transferred CD8+ T cells exhibited significant motility, neither in infected nor in uninfected mice. This loss of motility was observed for CD8+ T cells from donor mice immunized with Py-RAS, Pyuis4(−), or Pyfabb/f(−) sporozoites, as well as for Py-CS 280–288 epitope-specific CD8+ T cells. In this study, adoptive transfer was done under conditions known to confer protection against challenge with a small number of viable sporozoites [20]. To allow monitoring of multiple LS per IVM session, mice were infected with 2–3 logs higher sporozoite numbers than what has been typically used for challenge. Although the resulting higher LS density should have facilitated parasite recognition, surprisingly none of the adoptively transferred CD8+ T cells made contact with infected hepatocytes under any of the experimental conditions used. While motile CD8+ T cells should be able to use the entire arsenal of contact-dependent and -independent cytotoxic mechanisms to eliminate Plasmodium LS from the liver, we speculate that the initially immobile adoptively transferred CD8+ T cells have only soluble mediators at their disposal and use cytokines, in particular IFN-γ, for parasite killing. A central role of IFN-γ in protection against P. yoelii was demonstrated using a DC prime/L. monocytogenes boost regimen, a model in which CD8+ memory T cells represent the only Plasmodium-specific immune cells [66]. We further speculate that adoptive transfer of CD8+ T cells from granzyme or perforin deficient mice immunized with this prime boost regimen would prevent liver infection to a similar degree as transfer of CD8+ T cells from immunized wild type mice.

CD8+ T cells use redundant effector mechanisms for Plasmodium LS elimination

It has been known for several decades that immunization with radiation-attenuated sporozoites stimulates a strong protective response [6,7,83]. However, the cellular interactions involved in the elimination of Plasmodium LS from the liver in vivo are still unknown. CD8+ T cells are instrumental in protection and it is...
currenlty assumed that effector T cells use redundant mechanisms of cytotoxicity that operate in parallel [53,59,66]. While CD8+ T cells are clearly capable of parasite killing via immunological synapse formation in hepatocyte monocultures in vitro [36,59], the situation in the natural hepatic microenvironment, i.e. in the presence of non-parenchymal cells, blood flow, and a normal liver architecture, is more complex [51,59]. Studies using a variety of different in vivo models, including knockout mice, antibody-mediated ablation, synthetic cytokines and adoptive T cell transfer, suggest that direct CD8+ T cell-mediated cytotoxicity, involving perforin, granzyme B, Fas, Fasl, or TRAIL, represents the predominant mechanism of protection [51,58,66,84,85] and that immunity can be achieved in the absence of IFN-γ or IFN-γ signaling [51,66,73,80,86–89]. On the other hand, a role of IFN-γ and TNF-α in parasite killing is clearly documented, both in vitro and in vivo [25,51,66,81,85,90–96]. Although the IFN-γ mediated iNOS upregulation in infected hepatocytes is considered crucial for protection [92,97,98], the exact mode of operation of this cytokine in vivo is not established (reviewed in [51,53,66]).

Despite the utility of adoptive transfer experiments for the study of CD8+ T cell function in response to vaccination against malaria, this approach remains sparsely used because inordinate numbers of CTLs are required to effectively eliminate liver stage malaria parasites. Our finding that adoptive transfer renders CD8+ T cells immobile and thus unable to form an immunological synapse with infected hepatocytes for a period of at least 3 days may explain this and also shed light on some of the apparent contradictions in the literature. In agreement with the notion that CD8+ T cells are initially immobile and thus unable to use contact-dependent mechanisms of cytotoxicity to attack and eliminate Plasmodium LS from the liver, antibody-mediated blockage of IFN-γ abrogated protection within 2 days [81,93,99]. Protection was not abolished, however, when IFN-γ was neutralized in actively immunized mice [51] or when mice were challenged 8 days after transfer of CD8+ T cells from IFN-γ deficient mice [73]. Together, these and our data suggest that CD8+ T cell mobility and a complete repertoire of cytotoxic mechanisms are regained within a week after adoptive transfer. This notion is supported by the finding that CD8+ T cells from IFN-γ KO mice protect recipient mice 8 days after adoptive transfer [73] and that CD8+ T cells are able to protect IFN-γ KO mice against infection with P. yoelii, presumably by using a contact-dependent cytotoxic mechanism [66]. Based on these reports and our IVM observations, we propose a model in which CD8+ T cells actively monitor the liver sinusoids of immunized mice and eliminate LS via a combination of redundant mechanisms that include classical granule-mediated cytotoxicity [51,66] as well as the cytokines IFN-γ and TNF-α [66,94,98,100], with additional IFN-γ provided by hepatic NK cells through a positive feedback loop that involves Kupffer cell or DC-derived IL-12 [101–103]. Thus, adoptive transfer induced T cell immobilization could be used as a model to elucidate the mode of action of cytokines against infected hepatocytes in the absence granule-mediated T cell cytotoxicity. Further analysis of the response of resident intrahepatic CD8+ T cells of immunized mice to challenge with viable sporozoites and of adoptively transferred CD8+ T cells to immunization with attenuated parasites should reveal how accurately the adoptive transfer model represents the events that occur during a natural challenge.

Lymphatic fluid transport in the liver

The liver is widely recognized as a lymphatic organ with unique immunological properties [43,45,46,104–106], but its role in lymph formation and the resulting implications for liver immunology have been largely discounted to date. According to the current model, plasma continuously enters the space of Disse through the sinusoidal sieve plates, and flows as lymph retrogradely around the sinusoids towards the perportal space of Mall [107–110]. This blood/lymph counterflow concept has important implications for hepatic immunity (reviewed in [61]). We speculate that the perisinusoidal lymphatic counterflow would transport secreted cytokines along the space of Disse towards the portal field thus directly exposing infected hepatocytes to these cytotoxic mediators, if Plasmodium-specific effector T cells were to eliminate LS via cytokines such as IFN-γ from within the sinusoidal lumen. The potential use of soluble mediators for CD8+ T cell mediated Plasmodium LS killing is reminiscent of data from a murine Leishmania model, in which CD8+ T cells were shown to exert their protective activity by generating a gradient of IFN-γ that reaches more than 80 μm beyond the site of antigen presentation thus engaging a minority of infected cells and promoting pathogen clearance in the absence of immunological synapse formation [60]. Unlike in the skin, however, where interstitial fluid diffuses slowly through the intercellular spaces, the liver parenchyma is highly vascularized, both in terms of blood and lymph vessels, and contains, except for the portal fields, very little connective tissue. Cytokine dissemination in the liver must therefore be expected to occur considerably faster than in the skin. Considering the high sinusoid-to-lymph filtration rate [110–112], the enhanced pressure gradients created by leukocytes moving through the sinusoidal lumen [113], and the highly anastomozed nature of the sinusoidal microvasculature, we speculate that immobilized CD8+ T cells could conceivably exploit both the anterograde blood flow in the sinusoids and the retrograde lymph flow in the perisinusoidal spaces of Disse to take control of a substantial portion of the liver lobule (see [61] for a recent review).

Implications for cross-protection

As adoptively transferred CD8+ effector T cells confer protection under the experimental conditions used here, but cannot detect antigen presented on infected hepatocytes, they could potentially kill LS in a species-independent manner. Accordingly, immunization with P. falciparum sporozoites or attenuated P. berghei or P. yoelii sporozoites resulted in cross-protection against heterologous challenge [18,23,26,114–118]. Although cross-protection appears to depend on a blood factor [117], several experimental systems have shown that antibodies are not involved [91,114,119] (reviewed in [115]). Comparison of the various attenuation and immunization strategies used over the past years suggests that the superior cross-protective immunity elicited by genetically modified late-stage arresting parasites may be due to improved IFN-γ production [18]. Together with the motility data presented here, we speculate that CD8+ T cells are able to recognize parasite antigens on non-parenchymal cells, in particular Kupffer cells [47,120], and kills LS, including those located at somewhat larger distances, in a species-independent fashion by secretion of large amounts of IFN-γ into the sinusoidal microvasculature. Interestingly, proinflammatory cytokine mediated cross-protective mechanisms have also been reported to operate against malaria blood stages as well as other intracellular blood-borne pathogens (reviewed in [121]).

LS development in immunized mice

Under natural exposure conditions, effector T cells are unlikely to encounter mature Plasmodium LS. First, most sporozoites are immobilized by antibodies after transmission by mosquito bite and thus unable to exit the skin [30]. The few parasites that do reach the liver are likely opsonized and phagocytosed by Kupffer cells...
Should individual sporozoites succeed in infecting hepatocytes, Plasmodium-specific liver resident CD8+ effector memory T cells would likely kill the growing LS before they reach maturity [79,124]. In agreement with this notion, sporozoite infection of immunized mice resulted in a drastic reduction in LS number compared to naïve mice (Figure 1). Surprisingly, we also found that the few surviving LS were of a significantly smaller size. Neither Hoechst nor Mitotracker staining provided any evidence for chromatin condensation or mitochondrial damage suggesting that parasite growth retardation was not due to host cell death.

Further, DNA staining revealed that the small LS formed merozoites at very late stages of development, which would not have been the case if host cell or parasite death had been the reason for the small parasite size. Examination of the blood of these mice by Giemsa staining and wet mounts starting 2 days after challenge revealed a few fluorescent merozoites, but no detectable increase in parasitemia over the course of 2 weeks. For these reasons, we speculate that other factors, for example cytotoxic effects exerted by proinflammatory cytokines or parasite-specific antibodies some of which are reportedly able to inhibit the intracellular LS development [125,126], were responsible for the observed reduction in growth rate. While inflammatory cytokines such as IFN-γ are generally thought to eliminate LS via the NO pathway, an intriguing alternative may be that severely growth-inhibited, and thus barely detectable, miniscule parasites persist in the liver of immune mice and contribute to the formation of the depot of LS antigen that is required for maintenance of protracted protective immunity [78].

Possible causes for CD8+T cell immobilization

There are several scenarios we considered that could explain the observed reduced CD8+ T cell motility after adoptive transfer. First, it could be argued that the hepatic environment of naïve mice differs substantially from that of immunized mice. A general change in the immunized animal could potentially explain why CD8+ T cells appear to regain the ability to kill via classical contact-dependent cytotoxicity within 8 days after transfer. This hypothesis could be tested by measuring the velocity of CD8+ T cells transferred from one into another immunized mouse. Second, only a small number of antigen specific T cells were transferred and thus utilized under our experimental conditions. However, the lack of motility of the Py-CS280–288 specific CD8+ T cells in the time frame considered and the fact that CD8+ T cells of both hepatic or splenic origin equally showed reduced motility, despite the liver harboring considerably more parasite-specific CD8+ effector memory T cells than the spleen [22,25,127], would argue against this possibility. Third, the transfer protocol could be the culprit, and we consider this the most likely explanation. Adoptive transfer rendered the entire population of splenic CD8+ T cells immobile in the liver, suggesting that T cell immobility after transfer was antigen-independent, and therefore likely not recoverable by another immunization. We favor a model in which CD8+ T cells undergo a general phenotypic change during the adoptive transfer procedure. For example, the activation status of the memory T cells could have been affected after removal from the donor mice. Alternatively, de novo expression of adhesion molecules on the surface of CD8+ T cells could have been stimulated. In agreement with the higher expression levels of the integrins ICAM-1 and VCAM-1 in the liver compared to other tissues [128], activated CD8+ T cells were shown to be trapped in the liver in two ways, by an active antigen-dependent mechanism based on ICAM-1/LFA-1 and a passive antigen-independent mechanism based on VCAM-1/VLA-4 [129]. According to the current model, activated CD8+ T cells are initially sequestered in the liver by passive adhesion. Antigen recognition on the surface of hepatocytes or endothelia then increases the affinity of the interaction, mainly with ICAM-1, thus extending T cell residence time in the liver [129]. It is therefore conceivable that the transferred CD8+ T cells upregulated LFA-1 in response to the purification and adoptive transfer procedures and arrested in the liver by binding to ICAM-1. Of note, ICAM-1 is constitutively expressed on both sinusoidal endothelia and Kupffer cells [128]. Alternatively, high levels of the adhesion molecule CD44 is considered the most reliable surface marker of CD8+ T cell activation and has been implicated in effector memory CD8+ T cell cytotoxicity against Plasmodium LS [74,124,130–132]. CD44 is involved in the sequestration of neutrophils in the liver [133] suggesting that this ligand of hyaluronic acid could also contribute to the observed CD8+ T cell immobilization in the liver. Insight into these molecular events, in combination with direct intravitreal observations, will provide critical information on the value of adoptive transfer techniques for analysis of the mechanisms leading to CD8+ T cell mediated elimination of Plasmodium LS, and perhaps other intracellular pathogens from the liver.

Conclusions

Based on our findings and published evidence, we propose a model in which CD8+ T cells can recognize antigen presented by non-parenchymal hepatic APC, in particular Kupffer cells, and take advantage of the unique fluid transport pathways of the liver, namely the anterograde bloodstream and the retrograde lymphatic flow, for efficient dissemination of pro-inflammatory mediators towards infected hepatocytes. The ensuing NO formation then allows destruction of the intracellular parasites. Cytokine-enhanced cell-mediated cytotoxicity could potentially remove the necessity for effector T cells to screen every single hepatocyte to eliminate a minute number of Plasmodium LS from a huge organ such as the liver. In combination with classical granule-mediated cytotoxicity, this contact-independent mechanism would not only dramatically increase the efficiency of finding the infamous needle in the haystack, but should also provide cross-protection against other Plasmodium species. A better understanding of these effector functions will guide the rational design of future malaria vaccines and adjuvant formulations that stimulate immune responses comparable to or better than those observed during parasite infection to interrupt the clinically silent liver phase of the Plasmodium life cycle.

Supporting Information

Figure S1 Velocities of IHL and splenic CD8+ T cells in PyXNL-GFP infected mice. IHL (Liver) or splenic (Spleen) CD8+ T cells were purified from immunized mice 2 weeks after the second booster with Py-RAS and adoptively transferred into PyXNL-GFP infected recipient mice. Velocities were measured at 18 h or 40 h post infection. At least four infected mice were used per experimental condition, NS = not significant (p>0.05). (TIF)

Figure S2 Frequency of CS280–288 specific TCR-transgenic CD8+T cells. Naïve BALB/c mice (Thy1.2) were seeded with 2000 naïve Thy1.1 CS280–288 specific TCR-transgenic CD8+ T cells and adjuvant formulations that stimulate immune responses comparable to or better than those observed during parasite infection to interrupt the clinically silent liver phase of the Plasmodium life cycle.
Thy1.1 (bottom) to determine the frequency of CS280–288-specific TCR-transgenic cells. 

**Video S1** Behavior of CD8+ T cells in a naive Tie2-GFP liver. Recording of multiple Z-stacks over time (XYZT) showing a naive mouse with GFP+ endothelia (green), anti-CD8a-PE labeled CD8+ T cells (green), MitoTracker labeled hepatocyte mitochondria (red), and Hoechst stained nuclei (blue). See Figure 2A for representative still image. Scale bars 20 μm. 

**Video S2** Behavior of CD8+ T cells in a Pyfabf(−) immunized mouse liver. Recording of multiple Z-stacks over time (XYZT) showing a Pyfabf/b- immunized mouse liver with anti-CD8a-PE labeled CD8+ T cells (green), MitoTracker labeled hepatocyte mitochondria (red), and Hoechst stained nuclei (blue). See Figure 2B for representative still image. Scale bars 20 μm. 

**Video S3** Behavior of CD8+ T cells in a Py-RAS immunized mouse liver. Recording of multiple Z-stacks over time (XYZT) showing a Py-RAS immunized mouse liver with anti-CD8a-PE labeled CD8+ T cells (green), MitoTracker labeled hepatocyte mitochondria (red), and Hoechst stained nuclei (blue). See Figure 2C for representative still image. Scale bars 20 μm. 

**Video S4** Behavior of CD8+ T cells in a Pyuis4(−) immunized mouse liver. Time sequence (XYZT) showing a Pyuis4(−) immunized mouse liver with anti-CD8a-PE labeled CD8+ T cells (red) and MitoTracker labeled hepatocyte mitochondria (green). See Figure 2D for representative still image. Scale bars 20 μm. 

**Video S5** Behavior of Py-RAS activated CD8+ T cells in a PyXNL-GFP infected mouse liver. Recording of multiple Z-stacks over time (XYZT) showing an 18 h PyXNL-GFP LS (bright green) with a nearby Calcein Red Orange labeled CD8+ T cell (red). Hoechst labeled nuclei are blue, the tissue was visualized by collecting autofluorescence (green). See Figure 4A for representative still image. Scale bars 20 μm. 

**Video S6** Behavior of Py-RAS activated CD8+ T cells in a PyXNL-GFP infected mouse liver. Recording of multiple Z-stacks over time (XYZT) showing a PyXNL-GFP LS (green) and several CD8+ T cells labeled with CellTrace Calcein Violet (blue) in the sinusoidal space at 42 h post infection. Hepatocyte mitochondria were visualized with MitoTracker Deep Red (red). See Figure 4B for representative still image. Scale bars 20 μm. 

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**Author Contributions**

Conceived and designed the experiments: MC UF. Performed the experiments: MC LLP. Analyzed the data: MC UF. Contributed reagents/materials/analysis tools: UF JTH. Wrote the paper: MC UF.

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