

CD8⁺ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes

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The success of immunization with irradiated sporozoites is unparalleled among the current vaccination approaches against malaria, but its mechanistic underpinnings have yet to be fully elucidated. Using a model mimicking natural infection by *Plasmodium yoelii*, we delineated early events governing the development of protective CD8⁺ T-cell responses to the circumsporozoite protein. We demonstrate that dendritic cells in cutaneous lymph nodes prime the first cohort of CD8⁺ T cells after an infectious mosquito bite. Ablation of these lymphoid sites greatly impairs subsequent development of protective immunity. Activated CD8⁺ T cells then travel to systemic sites, including the liver, in a sphingosine-1-phosphate (S1P)-dependent fashion. These effector cells, however, no longer require bone marrow-derived antigen-presenting cells for protection; instead, they recognize antigen on parenchymal cells—presumably parasitized hepatocytes. Therefore, we report an unexpected dichotomy in the tissue restriction of host responses during the development and execution of protective immunity to *Plasmodium*.

After an animal has been immunized with a model antigen, lymph nodes draining the site of inoculation are the venue for a tightly regulated series of events that profoundly influences the subsequent fate of cellular immunity¹. During microbial infection, identifying such a site is usually more complex because of the intricate life cycles and different tissue specificities of microorganisms. Sporozoites of the malaria parasite *Plasmodium* are deposited in the skin of vertebrate hosts through the bites of infected mosquitoes and subsequently journey to the liver to infect hepatocytes^{2–4}. The sporozoites induce robust protective immunity in humans and other animals^{5,6}, but the precise lymphoid and nonlymphoid tissues involved in the induction and effector phases of this immune response have not been defined.

In murine models of malaria infection, the acquired resistance to liver-stage parasites is contingent upon the presence of CD8⁺ T cells^{7–10}, and recently the circumsporozoite protein expressed in sporozoites and in liver-stage parasites was found to be an immunodominant target of T-cell responses¹¹. T-cell receptor transgenic CD8⁺ T cells specific for the circumsporozoite protein epitope SYVPSAEQI (CS-TCR Tg CD8 cells) have been used to extensively characterize the mechanisms by which CD8⁺ cells are activated and by which they inhibit parasite development^{12–14}. After intravenous immunization with irradiated sporozoites, CD8⁺ T cells are primed by dendritic cells (DCs)¹⁵ and then rapidly accumulate in the spleen and liver¹². However, the precise site where DCs acquire and present parasite antigen following sporozoite infection through the skin has not been identified. Because sporozoites invade and differentiate exclusively

within hepatocytes, it is thought that DCs acquire antigen from apoptotic hepatocytes and then prime the T cells in lymph nodes draining the liver^{16–18}.

Recent studies have used intravital imaging of sporozoites injected by infected mosquitoes to show deposits of parasites in the skin. Skin-injected sporozoites can travel to the regional lymph nodes⁴ or remain in the skin for an extended period while some trickle into the bloodstream to reach the liver^{19,20}. The potential for multiple sporozoite-host interaction sites led us to analyze each site's contribution to the induction of protective anti-sporozoite immunity.

Although parasites may also prime cellular immune responses elsewhere, intrahepatic effector CD8⁺ T cells are probably the most important factor in protection against malaria. Parasites remain in the liver for only a short duration and there may not be sufficient time to recruit lymphocytes from other organs²¹. Consistent with this idea, circumsporozoite protein-specific effector CD8⁺ T cells must be located within the liver to elicit an effective immune response against live malaria, as extrahepatic effector cells are not sufficient for protection against live parasites²². However, it is still unclear whether CD8⁺ T cells directly recognize parasite antigen on the infected hepatocytes or whether they require formal antigen presentation by 'professional' antigen-presenting cells (APCs) for effector function.

Here we describe studies aimed at identifying the anatomical origin of CD8⁺ T-cell priming against liver-stage malaria parasites in a mouse infection model that mimics natural exposure to parasite-infected

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mosquitoes. We also use radiation bone marrow chimeras to define the cellular requirements for the optimal anti-parasite activity of these cells.

RESULTS

CD8⁺ T cells are primed in skin-draining lymph nodes

Our first goal was to identify the anatomical sites where CD8⁺ T-cell priming occurs after injection of *P. yoelii* sporozoites into the skin. To enhance the sensitivity of our detection methods, we increased the frequency of naive T-cell precursors by adoptively transferring CS-TCR Tg CD8 cells into BALB/c mice. Mice were then immunized by the bites of irradiated *Anopheles stephensi* mosquitoes infected with *P. yoelii* sporozoites. An interferon (IFN)- γ secretion assay was used to determine the number of primed epitope-specific T cells in auricular lymph nodes draining the ear where sporozoites were injected (dALN), lymph nodes draining the contralateral ear (nALN), spleen, liver parenchyma, and celiac lymph nodes (CLN) draining the liver.

In three independent experiments, epitope-specific IFN- γ -secreting CD8⁺ T cells were consistently found 2 d after immunization only in the dALN (Fig. 1a). On day 3, a small number of primed cells were also detected in the liver, and occasionally in the spleen, as well as in nALN and CLN (Fig. 1a). When mice were immunized with *P. yoelii* sporozoites microinjected through a syringe, identical results were obtained (Fig. 1b).

We monitored the temporal development of anti-circumsporozoite protein CD8⁺ T-cell responses after immunizing the mice with sporozoites, either by mosquito bite or by microinjection. Activated T-cell counts peaked after 4–5 d in all tissues examined (Fig. 1c,d); this peak was followed by a contraction phase. At all time points, the absolute number of epitope-specific CD8⁺ T cells in the dALN was larger than that in the nALN, CLN or liver (Supplementary Table 1 online). Between 4 d and 10 d, the total number of activated IFN- γ -producing CS-TCR Tg CD8 cells found in the spleen exceeded the number recovered from the dALN, reflecting either the migration

of activated T cells from the dALN or additional T-cell priming within the spleen after the parasites reached the bloodstream. The spleen has been shown to support naive CD8⁺ T-cell priming against sporozoites injected intravenously¹². Because data obtained with mosquito bites and sporozoite microinjection were similar, we microinjected parasites for all subsequent experiments to precisely control parasite numbers for accurate quantitative comparisons. Immunization in the ear induced a strong protective immunity, an effect that was reversed if immunized mice were treated with antibodies to mouse CD8 (Supplementary Fig. 1 online).

To determine whether sporozoite antigen was being presented at the draining cutaneous LN, we purified DCs from the dALN of BALB/c mice immunized with sporozoites and incubated the DCs *in vitro* with carboxyfluorescein succinimidyl ester (CFSE)-labeled naive CS-TCR Tg CD8 cells. Dendritic cells from the dALN 60 h after immunization induced a robust proliferation of naive CD8⁺ T cells *in vitro* (Fig. 2), as did DCs obtained 36 or 48 h after immunization (data not shown). When mice were immunized at the base of the tail, identical results were obtained (Supplementary Fig. 2 online). In contrast, DCs obtained from nonimmunized mice (data not shown), from the spleen or from the CLN, did not stimulate appreciable CS-TCR Tg CD8–cell proliferation (Fig. 2 and Supplementary Fig. 2). Together, these data reveal an unexpected early induction of CD8⁺ T-cell responses to the circumsporozoite protein epitope of malaria sporozoites by DCs in skin-draining lymph nodes.

CD8⁺ T-cell priming requires cross-presentation of antigen

Because sporozoites can productively infect only hepatocytes, the early CD8⁺ T-cell response in skin-draining lymph nodes raises questions regarding the mechanism by which DCs acquire malaria parasite antigen in the skin. In recent reports, treatment of mice with Toll-like receptor ligands has been shown to induce the maturation of DCs and a concomitant decrease in their endocytic or phagocytic activity and ability to cross-present antigen to CD8⁺ T cells^{23,24}, whereas direct

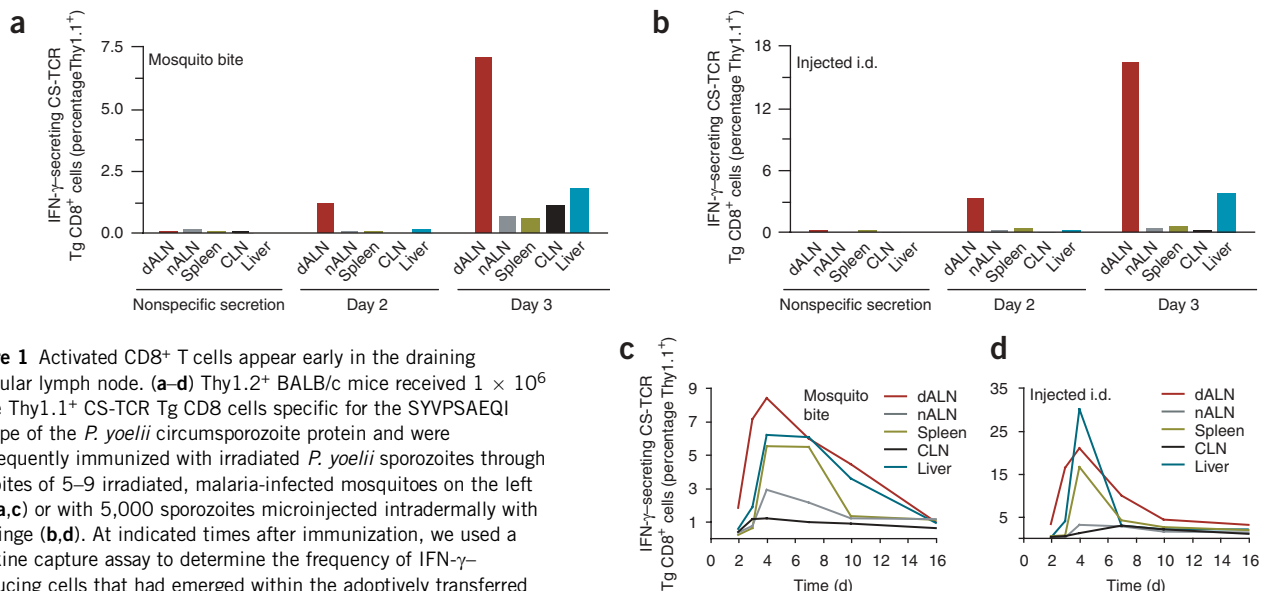


Figure 1 Activated CD8⁺ T cells appear early in the draining auricular lymph node. (**a–d**) Thy1.2⁺ BALB/c mice received 1×10^6 naive Thy1.1⁺ CS-TCR Tg CD8 cells specific for the SYVPSAEQI epitope of the *P. yoelii* circumsporozoite protein and were subsequently immunized with irradiated *P. yoelii* sporozoites through the bites of 5–9 irradiated, malaria-infected mosquitoes on the left ear (**a,c**) or with 5,000 sporozoites microinjected intradermally with a syringe (**b,d**). At indicated times after immunization, we used a cytokine capture assay to determine the frequency of IFN- γ -producing cells that had emerged within the adoptively transferred Thy1.1⁺ CD8⁺ T lymphocyte population. Nonspecific secretion was determined by restimulation without peptide (shown from day 2). Flow cytometry was used to evaluate the frequency of IFN- γ -producing, epitope-specific CD8⁺ T cells (gated on Thy1.1⁺ T lymphocytes) in different tissues, including the dALN, nALN, spleen, CLN and liver 2–3 d after immunization (**a,b**). Nonspecific IFN- γ secretion without peptide restimulation was used as a control. The analysis was extended to 4, 7, 10 and 16 d after immunization by mosquito bite or intradermal (i.d.) injection (**c,d**). Data shown are representative of three independent experiments. Representative data shown from one of the three independent experiments.

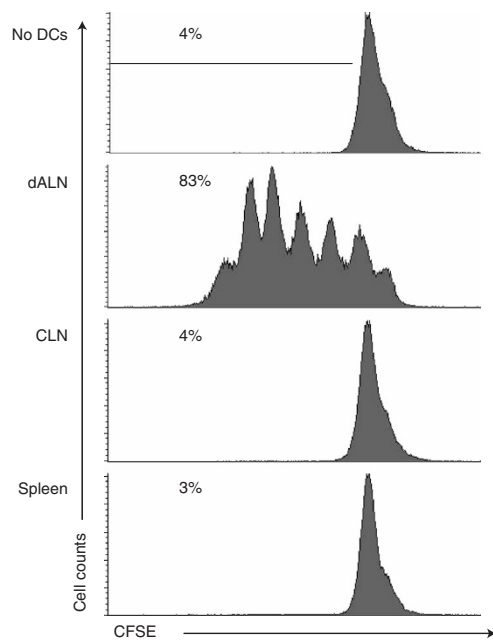


Figure 2 Dendritic cells from the dALN support naive T-cell proliferation *in vitro*. Thy1.2⁺ BALB/c mice were immunized with 50,000 sporozoites injected intradermally in each ear, and 60 h later, DCs from various organs were isolated as indicated. 5×10^4 purified DCs were incubated with an equal number of CFSE-labeled naive SYVPSAEQI-specific Thy1.1 CS-TCR Tg CD8 T cells. T-cell proliferation was measured after 60 h by analyzing CFSE dilution profiles (gated on Thy1.1 and CD8) using FACS. T cells incubated without DCs were used as a control. Numbers in the upper left corner indicate the percentage of CS-TCR Tg CD8⁺ T cells that had proliferated (denoted by horizontal bar in top panel) (CFSE^{low}).

priming is not affected. When we treated mice with CpG (the ligand for Toll-like receptor-9) before immunization with sporozoites, CD8⁺ T-cell activation was markedly reduced (Fig. 3a). Similarly, DCs from the dALN of CpG-treated sporozoite-immunized mice induced 50% less proliferation of naive CS-TCR Tg CD8 cells *in vitro* compared to that induced by DCs from untreated sporozoite-immunized controls (Fig. 3b). Notably, CpG treatment did not affect the overall number of DCs recovered from the dALN. These data suggest that optimal induction of CD8⁺ T cells is at least partially dependent on cross-presentation of exogenous antigen by immature DCs that have intact antigen uptake capabilities. In addition, induction of this T-cell response also required metabolically active parasites, as heat-inactivated sporozoites induced a minimal CD8⁺ T-cell response (Fig. 3c).

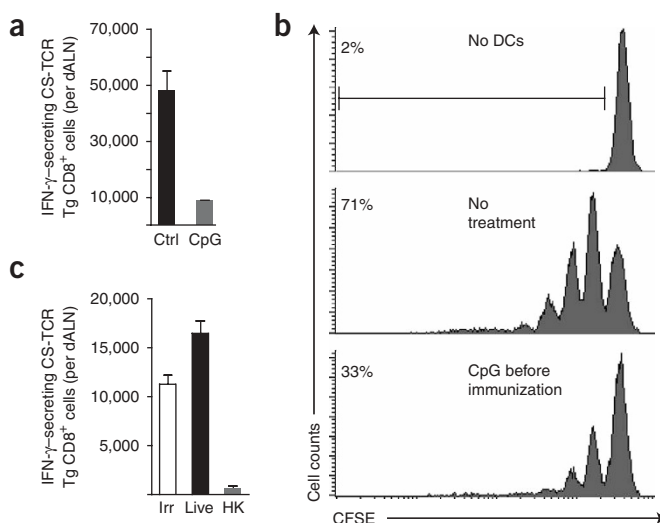
Activated CD8⁺ T cells migrate from dALN to other tissues

The accumulation of IFN- γ -secreting CS-TCR Tg CD8 cells in the liver and lymphoid tissues other than the dALN that we observed 4–5 d after immunization (Fig. 1c,d) might have resulted either from the migration of CS-TCR Tg CD8 cells primed in the dALN or from priming of CD8⁺ T cells *in situ*. To determine the contribution of cells

primed in the dALN to the systemic distribution of CD8⁺ T cells, we prevented lymphocyte migration with the drug FTY720, which downregulates sphingosine-1-phosphate receptor-1 (S1P1), reducing lymphocyte egress from lymph nodes^{25,26}. CS-TCR Tg CD8 cells were transferred into BALB/c mice. The mice were then treated with FTY720 before immunization with sporozoites in the ear, and the presence of activated CD8⁺ T cells was evaluated in different tissues. In mice treated with FTY720, the total number of IFN- γ -secreting, antigen-specific CD8⁺ T cells in the dALN increased about 2.6-fold (Fig. 4) as a result of an overall greater proportion of transgenic cells (1.2% compared with 0.5% of all lymphocytes, Supplementary Fig. 3 online) and a greater fraction of these transgenic cells that secreted IFN- γ (53% compared with 24% of all lymphocytes, Supplementary Figure 3). The dALN is the only site where the absolute number of activated CD8⁺ cells increased with drug treatment; in contrast, in the nALN, spleen, CLN and liver, the number of activated cells was reduced by 70%, 85%, 35% and 83%, respectively (Fig. 4). FTY720 did not affect sporozoite infectivity when given before parasite injection (data not shown).

The importance of this initial priming was further substantiated by the ablation of lymph nodes draining the site of parasite injection in BALB/c mice before the transfer of CS-TCR Tg CD8 cells and subsequent immunization with sporozoites in the ear. Mice subjected to lymphadenectomy had 62% fewer activated CS-TCR Tg CD8 cells in the liver compared to sham-operated mice, whereas the amount in the spleen was not affected (Fig. 5a). When both the dALN and the spleen were removed before immunization, the number of primed CD8⁺ T cells in the liver was further reduced by 88% (Fig. 5b), whereas the amount was not affected in the liver-draining CLN. On the basis of these observations, we conclude that extrahepatic lymphoid tissues, the skin-draining lymph node and perhaps also the spleen are important in establishing effector cells in the liver.

Figure 3 Antigenic epitopes from viable parasites are cross-presented by dendritic cells. (a) SYVPSAEQI-specific CS-TCR Tg CD8 cells were adoptively transferred into BALB/c mice and 10 nmol CpG DNA was injected intravenously 24 h before immunization with 10,000 sporozoites in the left ear. The frequency of primed IFN- γ -secreting cells was evaluated in the draining ALN 6 d later and compared against control (Ctrl) untreated groups. Histograms represent absolute numbers of IFN- γ -secreting CS-TCR Tg CD8 cells per dALN \pm s.e.m., averaged over three or four mice per group. (b) BALB/c mice were treated with CpG DNA as in a and immunized with 50,000 sporozoites in each ear. After 60 h, DCs from the dALN of untreated or CpG-treated mice were isolated and their ability to stimulate naive CS-TCR Tg CD8⁺ T-cell proliferation was assayed as in Fig. 2. T cells incubated without DCs served as a control. Horizontal bar in top panel indicates cells that have proliferated (CFSE^{low}). Representative data shown from one of three independent experiments. (c) Priming of CS-TCR-Tg CD8 cells in the draining ALN was measured 6 d after immunization with irradiated (Irr), live or heat-killed (HK) sporozoites. Histograms denote absolute numbers of IFN- γ -secreting CS-TCR Tg CD8 cells in the dALN \pm s.e.m., from four or five mice per group.



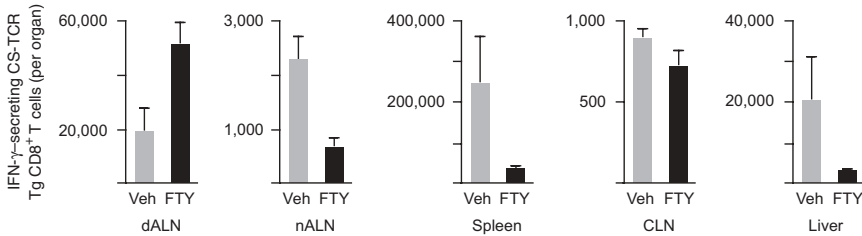


Figure 4 FTY720 prevents T-cell activation in nALN, CLN, liver and spleen. Tg CD8 cells (1×10^6) were adoptively transferred into naive BALB/c mice, which were then immunized with 5,000 irradiated *P. yoelii* sporozoites injected in the ear. Mice were given daily injections of 1 mg/kg FTY720 (black bars) or vehicle (gray bars) intraperitoneally, beginning the day before immunization. After 7 d, the presence of primed Tg CD8 cells in the dALN, nALN, spleen, CLN and liver was determined using the IFN- γ secretion assay after peptide restimulation *ex vivo*. Histograms denote absolute numbers of IFN- γ -secreting Tg cells recovered from each organ after vehicle or drug treatment, averaged over three or four mice per group, with error bars representing the s.e.m.

In view of the importance of the dALN and the spleen in T-cell homing to the liver, we next assessed their relevance in inducing anti-parasite activity in CD8⁺ T cells. BALB/c mice subjected to lymphadenectomy, splenectomy or both received CS-TCR Tg CD8 T cells, were immunized with sporozoites in the ear and 10 d later were challenged with viable parasites. A strong inhibition of parasite development was observed in sham-operated immunized mice compared to nonimmunized groups (Fig. 5c). In contrast, when the dALN and spleen were ablated before immunization, parasite load in the liver was similar to that in nonimmunized mice. Splenectomy alone did not affect inhibition of parasite development, supporting the conclusion that skin-draining lymph nodes are the primary source of protective CD8⁺ T cells. The effect of lymphadenectomy alone could not be evaluated because the healing process after surgery causes efferent and afferent lymphatic vessels to coalesce and drain into the thoracic duct, allowing DCs to circulate to other lymphoid organs^{27,28}.

Effector CD8⁺ T cells recognize antigen on parenchymal cells

On the basis of data reported here and in previous studies that have highlighted the importance of CD11c⁺ DCs in priming CD8⁺ T-cell responses¹⁵, we explored a possible role for these cells in triggering or supporting anti-parasite effector mechanisms. Transgenic BALB/c mice expressing the diphtheria toxin receptor (DTR) under control of the promoter for *ITGAX* (the gene encoding CD11c) received previously activated CS-TCR Tg CD8 cells and were then treated with diphtheria toxin to eliminate the CD11c⁺ DCs. Next, the mice were challenged with live sporozoites. Mice that had received effector CD8⁺ T cells strongly inhibited parasite development regardless of whether their DCs had been eliminated or not (Fig. 6a). Thus, CD11c⁺ DCs

seem to have no appreciable role in the anti-parasite activity of effector CD8⁺ T cells.

We then evaluated a possible contribution from CD11c⁻ APCs of the hematopoietic lineage during the effector phase (Fig. 6b,c). To do this, we created radiation bone marrow chimeras in which the expression of the H-2K^d class I MHC molecule, recognized by the CS-TCR Tg CD8 cells, was restricted to the hematopoietic lineage by transferring donor BALB/c (H-2K^d) bone marrow to irradiated C57BL/6 (H-2K^b) recipients (BALB/c \rightarrow B6). In reverse chimeras, bone marrow precursors from C57BL/6 were transferred into irradiated BALB/c mice (B6 \rightarrow BALB/c) to restrict H-2K^d expression to nonhematopoietic parenchymal cells.

The chimeric mice received effector CS-TCR Tg CD8 cells (previously primed in normal BALB/c mice) and were then challenged with live parasites. Unexpectedly, the effector CS-TCR Tg CD8 cells that were transferred into B6 \rightarrow BALB/c chimeric mice strongly inhibited parasite development in the liver, whereas these effector T cells were completely ineffective in reducing liver parasite loads in BALB/c \rightarrow B6 chimeric mice (Fig. 6c). These results were in stark contrast to the requirement for hematopoiesis in naive T-cell priming, as shown by the BALB/c \rightarrow B6 chimeras. Unlike the B6 \rightarrow BALB/c chimeras,

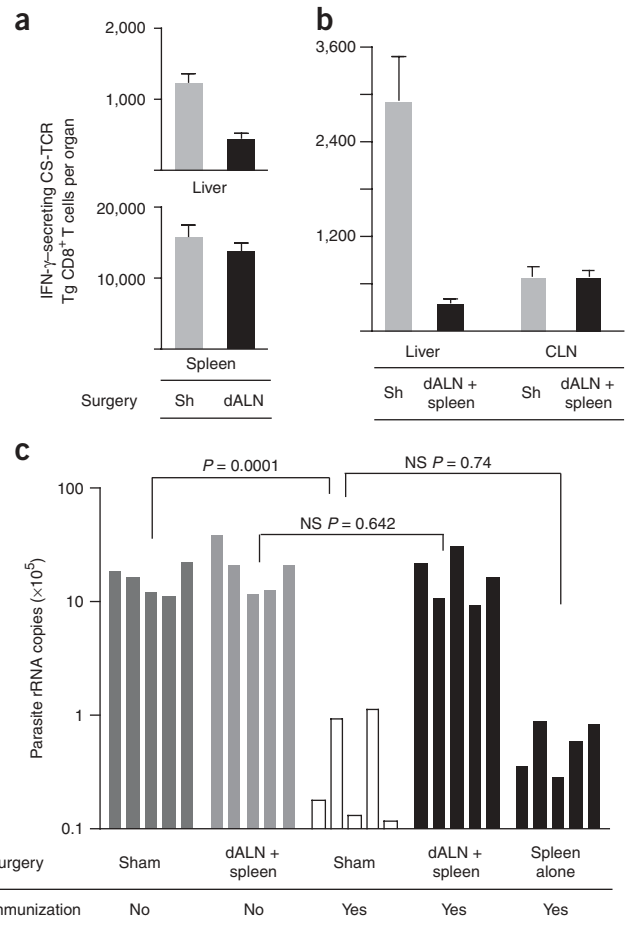


Figure 5 The dALN and spleen support T-cell homing to the liver and protective immunity. (a,b) BALB/c mice underwent surgical ablation of the draining ALN alone (a) or of dALN and spleen (b) 4 weeks before adoptive transfer of Tg CD8 cells. The mice were immunized with 10,000 *P. yoelii* sporozoites in the left ear. Sham (Sh) surgery on separate groups of mice served as controls. The amounts of IFN- γ -secreting CS-TCR Tg CD8⁺ T cells in the liver, spleen and CLN were determined 10 d after immunization and plotted as absolute numbers \pm s.e.m. of IFN- γ -secreting CS-TCR Tg CD8⁺ T cells recovered from each organ; $n = 5$ mice. (c) BALB/c mice underwent lymphadenectomy (dALN), splenectomy or both, or sham surgery, before immunization with 7,000 irradiated *P. yoelii* sporozoites in the ear. After 10 d, mice were challenged with 20,000 live sporozoites intravenously, and parasite load in the liver was determined by quantitative real-time PCR. Each bar represents a single mouse. Student's *t*-test was used to assess the statistical significance of differences between groups. NS, not significant.

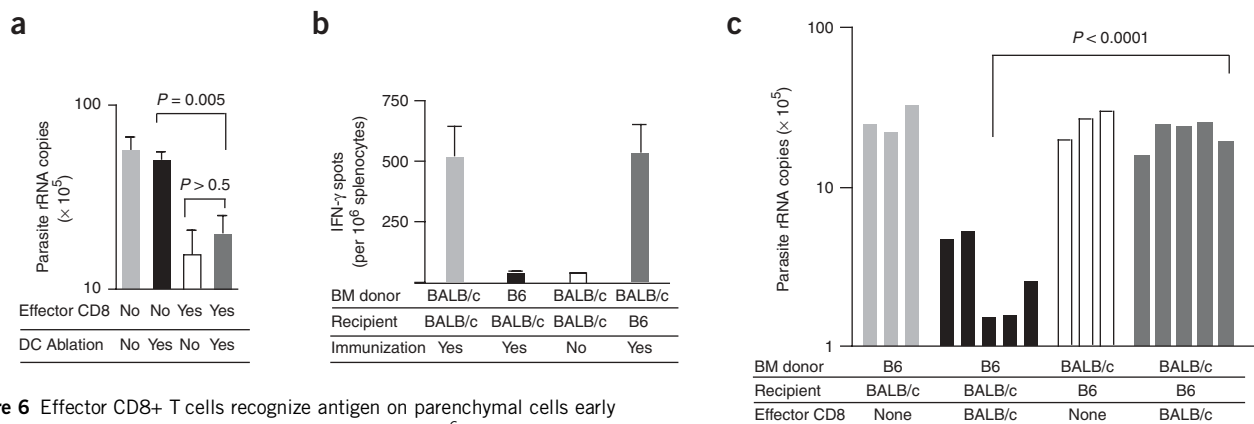
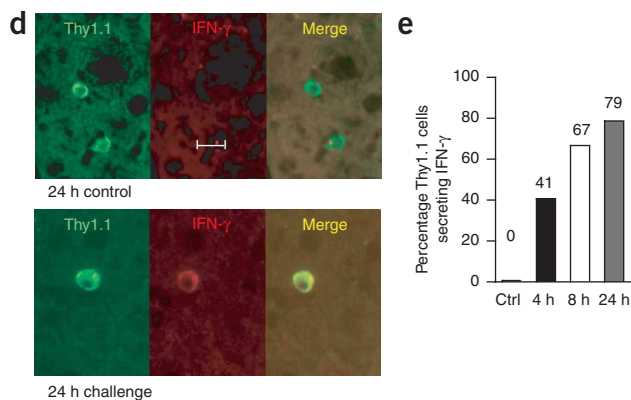


Figure 6 Effector CD8⁺ T cells recognize antigen on parenchymal cells early after infection. **(a)** Normal BALB/c mice received 5×10^6 CS-TCR Tg CD8⁺ T cells and were immunized 2 d later with a recombinant vaccinia virus expressing the *P. yoelii* CS protein. After 14 more days, 2×10^6 purified effector Tg CD8 cells from immunized mice were transferred into CD11c-DTR transgenic mice that carry the diphtheria toxin receptor on their DCs. Then, 1 d later, recipient mice were given vehicle or diphtheria toxin intraperitoneally to ablate DCs and were challenged 20 h after this with 35,000 live sporozoites. After 40 h, parasite ribosomal RNA in the liver was quantified by real-time PCR. Error bars represent means \pm s.e.m. ($n = 3$). **(b)** B6 \rightarrow BALB/c and BALB/c \rightarrow B6 chimeric mice received naive CS-TCR Tg CD8⁺ T cells 12 weeks after bone marrow reconstitution and were immunized 1 d later with 50,000 irradiated sporozoites given intravenously. The IFN- γ ELISPOT assay was used to evaluate the proportion of CS-specific, IFN- γ -secreting CD8 T cells in splenocytes isolated from immunized or nonimmunized mice. Error bars represent means \pm s.e.m. ($n = 4$). **(c)** Effector CS-TCR Tg CD8 T cells (2×10^6) purified from vaccinia-immunized mice described in **a** were adoptively transferred into chimeric mice. After 2 d, recipient mice were challenged with 35,000 live sporozoites. The anti-parasite activity of effector CS-TCR Tg CD8⁺ T cells was evaluated by quantifying parasite burden in the liver 40 h later by real-time PCR. BALB/c mice that received no effector CD8⁺ T cells served as controls. Each bar represents an individual mouse. Liver sections from two mice per group were examined (> 100 fields per group). **(d)** Thy1.1⁺ BALB/c mice received transgenic Thy1.1⁺ CD8⁺ T cells and were immunized with a recombinant vaccinia virus as in **a**. 14 d later, immunized mice received 500,000 sporozoites intravenously and the number of IFN- γ -producing CS-TCR Tg CD8⁺ T cells was evaluated histologically in liver sections. Sample sections of the liver show IFN- γ -producing, CS-TCR CD8⁺ T cells in the livers of control and sporozoite-challenged mice. Cells were stained for Thy1.1 (green) and IFN- γ (red) 24 h after challenge. Scale bar represents 10 μ m. **(e)** Percentage of Thy 1.1⁺ cells secreting IFN- γ at various times after sporozoite challenge. Statistical differences between groups were determined using Student's two-tailed *t*-test.



BALB/c \rightarrow B6 mice developed strong epitope-specific responses in response to immunization (Fig. 6b). Therefore, antigen presentation by cells of the hematopoietic lineage is not necessary for effector function of CD8⁺ T cells. Instead, antigen presentation by parenchymal liver cells, probably parasitized hepatocytes, is crucial, suggesting that successful elimination of liver-stage parasites might require a direct interaction between CD8⁺ T cells and hepatocytes.

We further investigated effector CD8⁺ T-cell reactivation in the liver after live sporozoite challenge, using a histological approach. BALB/c mice received CS-TCR Tg CD8 cells and were immunized with a recombinant vaccinia virus expressing the SYVSAEQI epitope of the circumsporozoite protein. After sporozoite challenge, their livers were processed for immunohistochemistry. Responsive effector CD8⁺ Thy1.1⁺ T cells were tracked with an antibody specific for IFN- γ (Fig. 6d). This cytokine has a controversial role in protective immunity^{29,30}, which is not addressed in these experiments—it is used here only as an activation marker. Circumsporozoite-specific effector CD8⁺ Thy1.1⁺ T cells in the liver produced IFN- γ unexpectedly early (Fig. 6e); it was detectable 4 h after sporozoite challenge, increasing to 80% by 24 h. In unchallenged mice, in contrast, effector T cells were quiescent with regard to IFN- γ production (Fig. 6d,e). These data highlight the very swift response of circumsporozoite-specific effector CD8⁺ T cells to live sporozoites.

DISCUSSION

The bite of infected mosquitoes delivers *Plasmodium* sporozoites into the skin of a vertebrate host^{2–4}, from whence they enter the bloodstream and eventually reach the liver—the only organ supporting productive sporozoite infection. In this report, we show that a protective anti-sporozoite CD8⁺ T-cell response, however, originates early in lymphoid tissues linked not to the liver but to the cutaneous infection site. These data contrast with the prevalent idea that, because the parasite develops exclusively in hepatocytes, CD8⁺ T-cell responses against liver stages of *Plasmodium* originate in hepatic tissues^{16–18}. In fact, we did not find appreciable evidence for CD8⁺ T-cell priming in the CLN, although the target antigen in this model (circumsporozoite protein) is expressed by sporozoites in early phases of liver infection³¹. Because only a few of the skin-deposited sporozoites reach the liver, the amount of circumsporozoite protein available to the celiac nodes may be limiting. Alternatively, the peculiarities of the liver micro-environment, which have been thought to provide tolerance-inducing signals, may hamper T-cell priming^{32,33}. Protective T-cell responses against liver-stage antigens³⁴ originating in the CLN are a possibility yet to be verified.

The early activation of circumsporozoite protein-specific CD8⁺ T cells is driven by a high frequency of DCs bearing sporozoite antigen in the draining cutaneous nodes (relative to the frequency in any other

lymphoid site). Such a localized enrichment may be facilitated by the recently reported extended residence of the parasite at the initial injection site in the skin^{4,20}, which would create a readily accessible antigen depot for DCs. Alternatively, sporozoites migrating to the draining lymph node may directly load antigen onto resident DCs. As previously shown in other studies^{4,20}, we found that *P. yoelii* sporozoites reach the skin-draining lymph nodes, where they retain full infectivity and are detectable 0.5–8 h after skin injection (**Supplementary Fig. 4** online). Our observation that antigen presentation is substantially diminished after treatment of mice with TLR ligands supports a requirement for the internalization of parasite antigen by immature DCs and suggests that sporozoite antigens may be cross-presented similarly to antigens from viruses that do not directly infect macrophage-like cells. The forms of antigen being internalized may include whole sporozoites, sporozoites associated with phagocytic cells, or vesicular aggregates of circumsporozoite protein that are known to be secreted by active parasites³⁵. In addition, DCs could acquire circumsporozoite protein antigen by nonphagocytic routes as a result of the sporozoites' unique property of cell traversal. Because sporozoites localize within CD11c⁺ DCs⁴, their passage through these cells might lead to intracellular trails of circumsporozoite protein³⁶ being deposited in the DC cytosol. Most of the antigen-loading processes suggested above require live parasites, and this might explain why CD8⁺ T-cell responses are drastically reduced in response to heat-killed sporozoites, as shown in this and previous studies³⁷.

In contrast to the obligatory need for DC-dependent antigen presentation in naive CD8⁺ T-cell priming, our experiments with radiation bone marrow chimeras reveal that effector CD8⁺ T cells must recognize antigen on host parenchymal cells to eliminate parasites from the liver. This excludes any role for bone marrow-derived hematopoietic liver cell types such as Kupffer cells, cholangiocytes and stellate cells^{38,39}, or for blood-derived DCs or macrophages, in antigen presentation to effector CD8⁺ T cells, although the sporozoites interact with many of these cell types^{36,40,41}. The parenchymal cells that elicit effector cell function are therefore most likely to be infected hepatocytes⁴². The recognition of antigen by liver-resident CD8⁺ T cells occurs rather quickly, as they make IFN- γ just a few hours after sporozoite challenge.

Apart from delineating the cellular events underlying protective immunity to sporozoites, our data also suggest criteria that may be relevant to the choice of candidate vaccine antigens used to elicit immune responses early in *Plasmodium* infection. Because ablation of the cutaneous lymph node and spleen greatly diminishes protective immunity acquired after sporozoite immunization, perhaps antigens that are expressed before the liver infection will be most effectively presented by DCs to induce and boost a strong CD8⁺ response. However, as effector CD8⁺ T cells must recognize the epitopes on parenchymal cells, expression of this antigen must also continue into the liver stage. Although the circumsporozoite protein antigen fulfills these requirements in spite of its reduced expression after hepatocyte invasion, additional molecules with robust expression during the liver stage may also prove to be useful candidates for an effective vaccine.

METHODS

Mice. We purchased 5- to 8-week-old female BALB/c mice from Taconic. Transgenic mice expressing a TCR specific for the SYVPSAEQI epitope of the *P. yoelii* circumsporozoite protein (CS-TCR Tg CD8 cells) were derived as previously described¹². CD11c-DTR mice were maintained as heterozygotes¹⁵. The Institutional Animal Care and Use Committee of The Johns Hopkins University approved all experimental procedures involving mice.

Drugs and immunizations. We dissolved FTY720 (Toronto Research Chemicals) in saline (0.2 mg/ml) and injected mice intraperitoneally at a dose of 1 mg/kg every 48 h beginning the day of immunization. *Plasmodium yoelii* (strain 17X NL) sporozoite isolation⁴³ and recombinant vaccinia virus expressing the SYVPSAEQI epitope^{10,44} have been described previously.

Surgical excision of dALN and splenectomy. We anesthetized mice by intraperitoneally administering a 2.5% solution of Avertin (Sigma-Aldrich) at 0.01 ml per gram of body weight. We then gently extracted the lymph node or spleen, sutured the underlying muscles and closed the skin incisions with wound clips.

Bone marrow reconstitution. We administered two injections of an antibody to asialo GM1 to deplete natural killer cells in the recipient mice and irradiated the animals by exposure to a single dose of 8.5 Gy (BALB/c) or 9.5 Gy (C57BL/6). We isolated bone marrow cells from the tibia and femur of donor mice, depleted T cells using antibody against Thy1.1 (clone J1J.10) and complement, and transferred 5–10 $\times 10^6$ cells into recipient mice. Reconstitution was allowed to proceed for 12 weeks, after which the MHC haplotype of CD45⁺ leukocytes was determined. The protocol resulted in 85%–90% reconstitution efficiencies.

Cell isolation and depletion. We isolated naive Tg CD8 cells from SYVPSAEQI-specific CD8⁺ TCR-transgenic mice and calculated the proportion of tetramer⁺ CD8⁺ T cells by flow cytometry. We generated effector cells by adoptive transfer of 2 $\times 10^6$ naive transgenic cells into Thy1.2-congenic mice and subsequent immunization with 5 $\times 10^6$ PFU recombinant vaccinia virus carrying the SYVPSAEQI epitope, then isolated lymphocytes from the spleens and lymph nodes 14 d later. When necessary, purified populations of CD8⁺ T cells were obtained using the mouse CD8⁺ T-cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Intrahepatic lymphocytes were isolated as described⁴⁵. Splenic myeloid dendritic cells were prepared as described⁴⁶. Dendritic cells were depleted from CD11c-DTR transgenic mice by intraperitoneal injection of diphtheria toxin at 4 ng per gram of body weight (Sigma).

IFN- γ secretion assay and enzyme-linked immunosorbent spots. We used the mouse IFN- γ secretion assay detection kit (Miltenyi Biotec) to measure IFN- γ secretion from different lymphocyte populations according to the manufacturer's instructions. Briefly, we restimulated 1 $\times 10^7$ cells *in vitro* using SYVPSAEQI peptide-loaded A20 cells⁴⁷ for 1.5–3 h and then coated cells with an IFN- γ capture antibody. The timing of stimulation was optimized to prevent nonsecreting cells from contributing a false positive signal. A 45-min period of cytokine secretion followed, and cells were stained with a PE-labeled IFN- γ -detecting antibody (Miltenyi Biotec), FITC-labeled antibody to Thy1.1 (clone His51; BD Biosciences) and APC-labeled antibody to CD8 (clone 53-6.7; eBioscience) before fluorescence-activated cell sorting (FACS) analysis. Enzyme-linked immunosorbent spots were analyzed as described⁴⁷ to quantify SYVPSAEQI-specific, IFN- γ -secreting cells.

Ex vivo antigen-presentation assay. We labeled purified naive transgenic CD8⁺ T cells with 0.6 μ M CFSE using the Vybrant Cell Tracker kit (Invitrogen) and mixed 5 $\times 10^4$ DCs purified as described above with an equal number of CFSE-labeled CD8 transgenic cells in a single V-bottom well of a 96-well plate. We harvested the cells 60–65 h later and assayed the CFSE dilution within CD8⁺ Thy1.1⁺ transgenic cells as an index of antigen presentation.

Parasite quantification in the liver and lymph nodes. In challenge experiments, we inoculated live sporozoites intravenously to ensure uniform trafficking of parasites to the liver and quantified them as previously described⁴⁸. We harvested lymph nodes at various times after inoculation and isolated RNA from individual lymph nodes using TRIzol reagent (Invitrogen). Real-time PCR was conducted using SYBR Green (Applied Biosystems) as described⁴⁸.

Immunofluorescence. Liver tissue was processed for immunofluorescence assays. We cut frozen sections (8 μ m) using a Leica cryostat and labeled them using a mouse monoclonal antibody to rat CD90.1 (BD Pharmingen) and rat IFN- γ (XMG1.2). Thy 1.1⁺ (also called CD90) cells were incubated with a biotinylated antibody to mouse IgG, then with fluorescein-avidin, and

then visualized. IFN- γ producing cells were detected with rat IgG-specific antibody conjugated to Alexa 594. Single-fluorochrome images were captured using a SPOT RT camera (Diagnostic Instruments) and merged to produce multicolor images.

Data acquisition and analysis. We acquired FACS data on a FACSCalibur machine and analyzed the data using CellQuest software (BD Biosciences). We read enzyme-linked immunosorbent spot plates with an Immunospot plate reader and analyzed the data using Immunospot software (Cellular Technology). Graphs were prepared and analyzed using GraphPad software. Student's *t*-test was used for pairwise comparisons to determine statistical significance.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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