Supporting Information

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SI Materials and Methods

Reagents. All chemicals were reagent-grade from Sigma. Antibodies against CD4, CD8α, CD8β, B220, Thy1.1 (OX-7 clone), CD11a, KLRG1, CD127, CD44, CD62L, and MHC H2-K^d were obtained from BD or eBioscience. Tetramers against the circumsporozoite protein (CSP) and L3 epitopes were provided by the National Institutes of Health Class I Tetramer Facility (Emory University, Atlanta, GA). Blocking antibodies against CD4 (GK1.5) and CD8 (2.43) were from the University of California, San Francisco Antibody Facility (San Francisco, CA). Recombinant *Listeria monocytogenes* (Lm) expressing the L3 epitope was constructed by using PCR primers to insert the L3 protein sequence encoding the TAFMGYKSGMSHIVREV peptide into a shuttle vector containing a large segment of the ovalbumin protein-coding sequence, which was then stably integrated into *L. monocytogenes* as described (1).

***In Silico* Library Protein Selection and Minigene Library Synthesis.** Standard bioinformatics tools were used to assemble a library of 687 proteins including 623 expressed in the *Plasmodium yoelii* liver stage (2) and 67 containing a possible export domain predicted by ExportPred (3) by using a cutoff of 5 (Dataset S1). Only four proteins were in common between these strategies. Peptides from these proteins that could potentially bind to BALB/c mouse MHC class I H-2^d molecules were identified by using NetMHC 3.0 (4). Each peptide-coding sequence was extended four codons upstream and downstream from its core nonamer coding sequence. The final library comprising 1,987 peptides with the strongest predicted H-2^d binding properties plus the immunodominant H2-K^d–restricted CSP peptide SYVPSAEQI were sorted *in silico* into pools containing 10 sequences each and inserted into a standard minigene design (5). The antisense library was commercially synthesized on a microarray and provided as a single oligonucleotide mixture. From the complete oligonucleotide library, pools of 10 minigenes (each pool sharing a unique, pool-specific primer) were amplified by PCR to segregate the library into small, defined, nonredundant pools in 96-well plates. A 3′ untranslated region and a polyA tail were added. PCR products were purified and in vitro transcribed with a cap analog to produce 96-well arrays of fully translatable mRNA pools, with each pool encoding a defined set of candidate peptides (5). A high-fidelity oligonucleotide encoding GYKSGMSHI made by column synthesis was also obtained as a control minigene (IDT).

IFNγ ELISPOT. Erythrocyte-depleted cell suspensions were made from spleens or livers of euthanized animals on the days indicated in the text. Antigens were added to 96-well enzyme-linked immunosorbent spot (ELISPOT) plates by using either individual peptides (1×10^{-10} to 1×10^{-6} M; Genemed Synthesis) or P815 mastocytoma cells transfected (AMAXA Nucleofector; Lonza) with pools of the aforementioned minigenes. Mouse splenocytes (1×10^6 per well) were added as effector cells, and IFNγ ELISPOT assays (eBiosciences) were performed over 24 h (peptides only) or 40 h (high-throughput screens; HTSs). In some studies, antibodies against CD4, CD8, or H2-K^d were used to block specific responses. For screening, the spot count was determined for each well. To adjust for background differences of different immunization regimens in minigene screening studies, spot counts were compared against mock-transfected control wells from the same ELISPOT plate for the same immunization type and deemed positive if the spot count for two separate screening wells for a given pool was >95% confidence interval (CI) for the corresponding mock-transfected controls in the same plate. For smaller-scale nonminigene screening ELISPOTs, a value corresponding to the upper limit of the 95% CI for triplicate mock-transfected wells in a single 96-well ELISPOT plate were subtracted to generate a normalized spot forming unit (nSFU) count.

Heat-Treated Parasite Immunizations. In some studies, *P. yoelii* 17XNL sporozoites (2×10^4) or PBS-washed *P. yoelii* 17XNL or *Plasmodium berghei* ANKA-infected RBCs (1.5×10^7) were incubated at 55 °C for 30 min and then injected into the tail vein of mice.

Tetramer-Specific Magnetic-Activated Cell Sorting Purification and Cytotoxic T Lymphocyte Phenotyping. Tetramer pulldown assays were performed as described (6) by simultaneously staining for PE-labeled CSP$_{280–288}$/K^d (SYVPSAEQI) and antigen-presenting cell (APC)-labeled L3$_{48–56}$/K^d (GYKSGMSHI) tetramers by using anti-PE and anti-APC beads and columns from Miltenyi. Cells were subsequently phenotyped by flow cytometry using antibodies against CD8, CD11a, KLRG1, CD127, CD44, and CD62L using an LSRII instrument (BD).

Immunization with Peptide-Pulsed DCs or L3-Expressing *Listeria monocytogenes*. Bone marrow-derived DCs were cultured for 8 d in RPMI medium 1640 without glutamine (Invitrogen) supplemented with Glutamax-I (Invitrogen), sodium pyruvate (1 mM; Invitrogen), nonessential amino acids (Invitrogen), penicillin/streptomycin (100 U/mL each; Invitrogen), murine GM-CSF and IL4 (both 20 ng/mL; PeproTech), and 1.5% BALB/c mouse sera (Innovative Research). Media was changed on days 3, 5, and 7. On day 7, media was supplemented with lipopolysaccharide (0.1 mg/mL; Sigma) and peptides (1×10^{-6} M), as appropriate. The next day, cells were washed in serum-free RPMI medium, counted, and injected i.v. into mice.

Protection and Liver-Stage RT-PCR Studies. Livers of mice immunized with GAP or challenged with WT sporozoites were harvested 40–44 h after injection. Nucleic acids were extracted from fresh whole perfused livers by using TRIzol (Invitrogen). Samples were DNase-treated (Turbo DNA-free kit; Invitrogen) and quantitative RT-PCR was performed by using the SuperScript III One-Step SYBR Green RT-PCR kit (Invitrogen). Forward/reverse primer pairs (IDT) were as follows: mouse GAPDH (CCTCAACTACATGGTCTACAT/GCTCCTGGAAGATGGTGATG); *P. yoelii* 18S rRNA (GGGGATTGGTTTTGACGTTTTTGCG/AAGCATTAAATAAAGCGAATACATCCTTA). The ΔC_T was calculated as C_T of the mouse glyceraldehyde-3-phosphate mRNA minus the C_T of *P. yoelii* 18S rRNA for an individual animal. A dilution series was used to determine reaction linearity to calculate the percentage reduction in liver-stage parasites. Liver-stage expression of additional transcripts were also tested by using the following forward/reverse primer pairs: PY00204 (GCAAGTGCTAACGCAATGG/AGAATAAAAGAAACGGAAAGATAGCAG), PY02405 (TCCGGACGTGAAATACTTTCC/AAAAGAGAAATGAAGAGGTCAAACG), PY02619 (GTAACAGCAAGAAATGAGGGC/AATCGTTAGAAAGAGTTAATTTTGACAAG), PY03995 (AAGAAAGGTTTTTTCCATCATTATTTCC/AGGGAATATTTATAATTGGGAATGACTT), PY05060 (CACCATCTTCACCATCTTTATCAC/CTAATGAGGATACTGAAGCTAGCG), PY05756 (CAATCTCCTGGTCTTGGTTCA/ATTTCTTATGGGCATTTAAACTAAAACC), PY05881 (AGGTCATGGAACAAAAGGTGT/GCCATGCACCAATACAAGCA), PY07509 (GGTTCGTTTGGTTCGTTTGG/AAAGGCCCAATTTAGTAAACCATC). All reactions consisted

of 3 min at 50 °C, 5 min at 95 °C and finally 40 cycles of 15 s at 95 °C and 30 s at 55 °C. Transcripts were confidently detected after one immunization if real-time melting point analysis gave the expected melting temperature for the amplicon.

1. Zehn D, Lee SY, Bevan MJ (2009) Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458(7235):211–214.
2. Sacci JB, Jr., Aguiar JC, Lau AO, Hoffman SL (2002) Laser capture microdissection and molecular analysis of Plasmodium yoelii liver-stage parasites. *Mol Biochem Parasitol* 119(2):285–289.
3. Sargeant TJ, et al. (2006) Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. *Genome Biol* 7(2):R12.
4. Hoof I, et al. (2009) NetMHCpan, a method for MHC class I binding prediction beyond humans. *Immunogenetics* 61(1):1–13.
5. Hondowicz BD, et al. (2012) Discovery of T cell antigens by high-throughput screening of synthetic minigene libraries. *PLoS ONE* 7(1):e29949.
6. Moon JJ, et al. (2009) Tracking epitope-specific T cells. *Nat Protoc* 4(4):565–581.

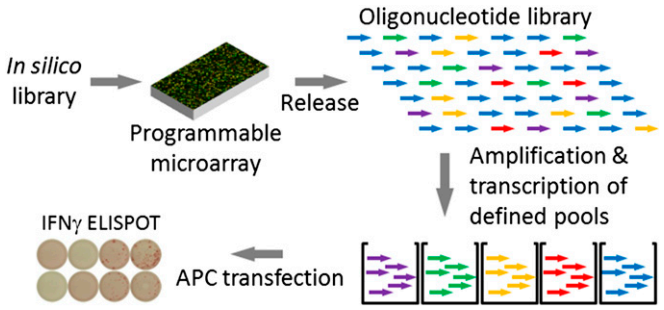

Fig. S1. A minigene approach to high-throughput T-cell target screening. Computationally selected protein-coding sequences are designed *in silico* as antigen-encoding minigenes flanked by terminal primer sequences. Antisense sequences are synthesized on and cleaved from a microarray, resulting in a complex oligonucleotide pool. Distinct pools are segregated by using pool-specific PCR, and in vitro transcription of pooled oligonucleotides results in mRNA pools that can be transfected into APCs for use in ELISPOT assays.

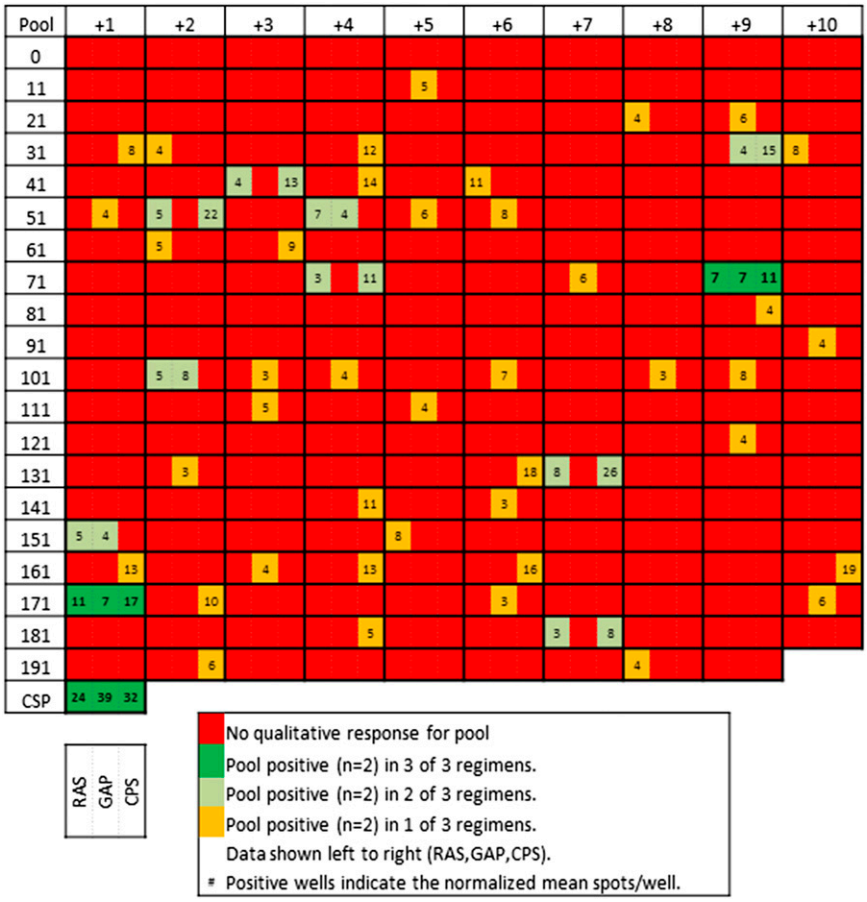

Fig. S2. Complete data from minigene HTS pool screening of mice immunized once with radiation-attenuated sporozoite (RAS), genetically attenuated parasite (GAP), or sporozoites with chloroquine prophylaxis (CPS) regimens. Data are from two separate screens for each immunization approach with each pool tested in singlet during each individual screen. Screening data were normalized as in *Materials and Methods* and deemed positive for a given sporozoite regimen if both individual screens showed a response >95% CI of the corresponding mock-transfected controls. In such cases, the nSFU was determined, and the average calculated and shown here. Pool 1 is in the upper left corner and Pool 199 is in the lowest right hand corner; rows contain 10 pools each as indicated by the markings on the top row and left column. A CSP control well is shown at the last row. Within each cell, data are grouped from left to right as RAS, GAP and then CPS data. Colors in the key denote whether a pool was positive in none (red), one (orange), two (light green) or all three (green) immunizations.

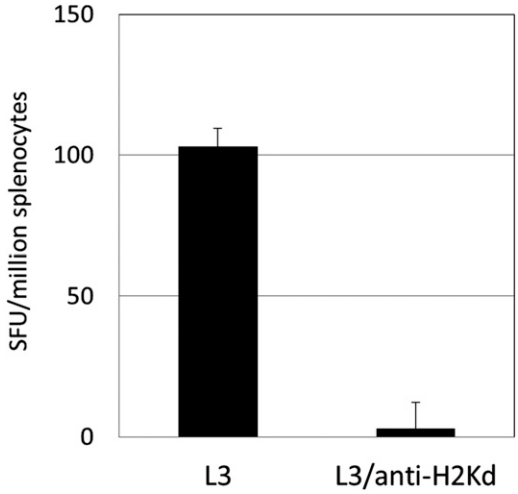

Fig. S3. Antibodies against H2-K^d block CPS sporozoite-induced L3-specific T-cell responses in an IFNγ ELISPOT assay. Mice were immunized with CPS, and splenocytes were evaluated by ELISPOT by using 100 nM of the corresponding peptides 6 d after immunization. Two mice per experimental group; error bars show the mean $\pm$ SD.

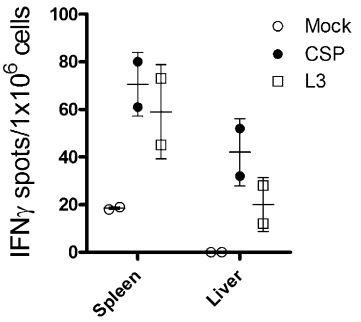

Fig. S4. L3-specific T cells are detected in the liver of sporozoite-immunized mice. Single-cell suspensions of lymphocytes from the spleen or liver of CPS-treated mice were tested by ELISPOT using 100 nM CSP or L3 peptides 6 d after immunization. Two mice per experimental group; error bars show the mean ± SD.

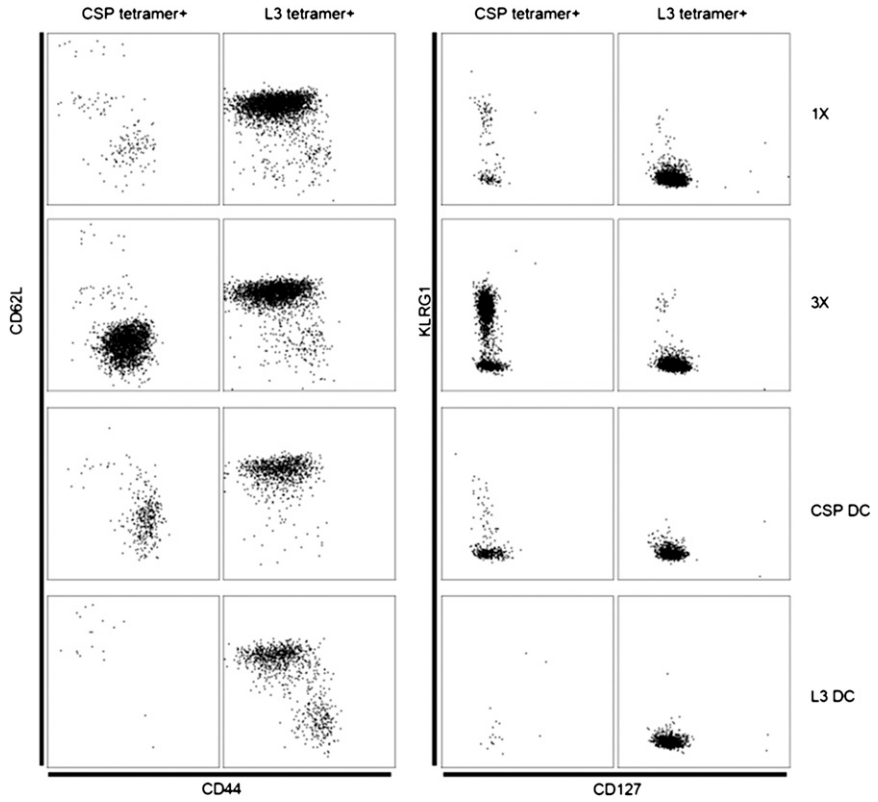

Fig. S5. Despite initial activation by single sporozoite immunization or L3-pulsed DCs, L3-specific cells do not expand or remain strongly activated after repeated sporozoite immunizations. Flow cytometry staining of L3 (blue) or CSP (red) tetramer-positive CD11a^{HI} cells for CD44/CD62L and CD127/KLRG1 in mice immunized once (1×) or three times (3×) with GAP or once with CSP DC or L3 DC as indicated. Three mice per experimental group; two experiments total; representative mice shown.

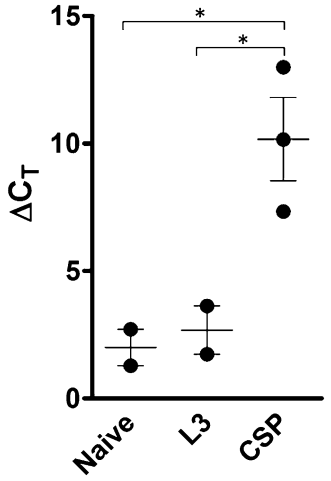

Fig. S6. L3-specific cytotoxic T lymphocytes do not protect against malaria sporozoite challenge. Mice were immunized with mock, CSP-, or L3-peptide pulsed DCs, challenged 6 d later with 2×10^4 wild-type sporozoites and tested by liver-stage RT-PCR as in Fig. 5 at 44 h after challenge. Whereas CSP-treated DCs could induce protection, L3-treated DCs did not. $^*P < 0.05$ (unpaired *t* test; 2–3 mice per group; representative of two experiments).

Table S1. Detection of *P. yoelii* mRNA transcripts in total liver RNA 44 h after GAP immunizations

Sporozoite doses	PY02619	PY05756	PY02405	PY03995	PY00204	PY07509	PY05060	PY05881
1	3/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3
2	0/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
0 (naïve)	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Other Supporting Information Files

Dataset S1 (PDF)