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Supplementary Materials for

Up-regulation of LFA-1 allows liver-resident memory T cells to patrol and remain in the hepatic sinusoids

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at immunology.sciencemag.org/cgi/content/full/2/9/eaaj1996/DC1)

Movie S1 (.mov format). Anti–ICAM-1 inhibits effector T cell migration in the hepatic sinusoids.
Movie S2 (.mov format). LFA-1–deficient cells display impaired motility in the liver.
Movie S3 (.mov format). Migration of OT-I cells in the livers of mice 1 week after sporozoite immunization.
Movie S4 (.mov format). Migration of OT-I cells in the livers of mice 4 weeks after sporozoite immunization.

Materials and Methods

Preparation of bone marrow chimeras

For bone marrow chimeras, *Rag1*$^{-/-}$ mice were irradiated with 5 Gy and were injected with 2 x 10^6 bone marrow cells (50:50 mixture of wild-type B6.SJL CD45.1$^+$ bone marrow and either mutant or wild-type CD57BL/6 (CD45.2$^+$) bone marrow. They were allowed to reconstitute for 8–13 weeks before analysis.

In vitro activation of T cells

OT-I cells were activated in vitro as follows. Splenocytes were incubated with 1µg/ml SIINFEKL peptide (Biomatik) at 37ºC for 30 minutes, washed, and cultured in complete RPMI 1640 (10% FCS, 2mM L-glutamine, 1mM Na-Pyruvate, 100U/ml Penicillin/Streptomicin, 5mM HEPES, 20µg/ml Gentimicin and 50µM β-mercaptoethanol) at 37ºC, 5% CO_2. Two days later cells were passaged and cultured in fresh media supplemented with (12.5U/ml) recombinant IL-2 (PeproTech). Cells were passaged again after 24 hours, and given fresh media with IL-2. 4 days after activation cells were ready for collection and use.

In vitro cytotoxicity assay

EL4 target cells were labeled with CellTrace™ violet (ThermoFisher) and were either pulsed with varying concentrations of SIINFEKL peptide, or left unpulsed. The cells were then incubated at 37ºC with effector OT-I CD8$^+$ T cells at a ratio of 2 effectors:1 target. After 6 hours incubation the number of remaining target cells was counted by flow cytometry. Acquisition was normalized between samples by the addition of 5µl of counting beads (Invitrogen). Lysis was

determined by measuring the number of remaining peptide pulsed target cells compared to the number of cells remaining that were not pulsed with peptide.

Adoptive transfer of $CD8^+$ T cells

In vitro activated cells were collected from tissue culture flasks, washed, layered over Histopaque® 1083 (Sigma) and centrifuged at 400rcf, for 30 minutes at 21ºC to remove dead cells. Live lymphocytes were collected from the interface between the Histopaque® and cell suspension media. 7×10^6 cells were transferred i.v to mice prior to imaging or while 2×10^6 cells were transferred prior to challenge. Naïve OT-I $CD8^+$ T cells were quantified from splenocytes via flow cytometry, and 2×10^4 $CD8^+$ OT-I cells transferred i.v to mice 1-2 days prior to sporozoite immunization.

Lymphocyte Harvesting and Flow Cytometry

Peripheral blood was collected from the tail veins of mice prior to euthanasia. Mice were subsequently anesthetised and perfused with PBS containing 2% FCS via the left cardiac ventricle, after which they were euthanized via cervical dislocation and organs were harvested. Single cell preparations of lymph nodes and spleen were made by grinding the organs through 70 micron mesh filters and washing 2 times prior to staining. For preparations of lymphocytes from the blood, red blood cells were lysed using ACK lysis buffer and the cells washed 3 times prior to staining.

Lymphocytes were isolated from the liver as follows: livers were collected and cut into small pieces with scissors. Liver pieces were mashed through metal sieves, washed, and resuspended in

a Percoll™ gradient (RPMI 1640, 35% Percoll™ (GE Healthcare), 4% PBS 10X, 0.1U/ml Heparin). Gradient was centrifuged at 500 rcf for 30 minutes at 21ºC, supernatant discarded, and lymphocytes washed.

Lymphocytes were isolated from the lung as follows: lungs were collected and stored in digestion medium (RPMI 1640, 2% FCS, 3mg/ml Collagenase P (Roche), 5µg/ml DNase (Roche), cut into small pieces with scissors, and incubated for 60 minutes at 37ºC, vortexing intermittently. Digested lungs were mashed through a cell strainer, washed, resuspended in a 20 % Percoll™ medium (RPMI 1640, 20% Percoll™, 10% FCS, 0.1U/ml Heparin) and then layered upon a 35% Percoll™ gradient (PBS 1X, 35% Percoll™, 0.1U/ml Heparin). Gradient was centrifuged at 500 rcf for 30 minutes at 21ºC, and live lymphocytes were isolated from interface between gradients.

Lymphocytes were isolated from the skin as follows: anaesthetized mice were shaved, and depilatory cream was applied to shaved skin for 5 minutes. Depilatory cream was washed off with any remaining hair and after euthanasia skin was removed and stored in digestion medium (RPMI 1640, 2% FCS, 3mg/ml Collagenase, 5µg/ml DNase). Skin was cut into small pieces with scissors and incubated at 37ºC for 90mins, vortexing intermittently. Digested skin was mashed through a metal sieve, washed, mashed through a cell strainer and washed finally.

Single cell preparations were stained for flow cytometry following incubation with TruStain Fc block (Biolegend). Antibodies used for flow cytometry analysis were: anti-CD3 (clone 17A2; Biolegend), anti-CD4 (clone GK15; eBioscience), anti-CD8a (clone 5H10-1; Biolegend);(Clone

53-6.7; eBioscience), anti-CD11a (clone M17/4; Biolegend); (Clone 2D7; Biolegend), anti-CD45.1 (clone A20; Biolegend), anti-CD183 (clone CXCR3-173; Biolegend); anti-Va2 (clone B20.1; eBioscience), anti-mouse/human KLRG1 (clone 2F1/KLRG1; Biolegend), anti-CD103 (clone 2E7; Biolegend), anti-CD69 (clone H1.2F3; Biolegend).

In most cases samples were further fixed with 4% paraformaldehyde fixation buffer (Biolegend) and stained with fixable live-dead aqua (ThermoFisher) to exclude dead cells. Flow cytometric data was collected on an LSRII flow cytometer (Becton Dickinson) and analysed using FlowJo software (FlowJo).

Assessment of Parasite Burdens

Parasite burden was measured by qRT-PCR using primers that recognize *P. berghei* specific sequences within the 18S rRNA and SYBR Green (Applied Biosystems) as outlined previously *(19)*. Parasite burdens were normalized with GAPDH expression.

Multiphoton microscopy

Mice were prepared for multi-photon microscopy essentially as described *(41)*. Briefly, mice were anaesthetized with a mix of Ketamine (100 mg/kg) and Xylazine (10 mg/kg). Throughout the surgery and imaging procedure the mouse temperature was maintained at 37ºC using a heating mat attached to feedback probe inserted in the mouse rectum. A lateral incision was made over the left lobe of the liver and any vessels cauterized by applying light pressure to the vessel until clotting occurred naturally. The mouse was then placed in a custom made holder. The liver was then exposed and directly adhered to a coverslip that was secured in the holder.

Once stable the preparation was transferred to a Fluoview FVMPE-RS multiphoton microscope system (Olympus) equipped with a XLPLN25XWMP2 objective (25x; NA1.05; water immersion; 2mm working distance). For analysis of the motility of in-vitro activated cells a 50 μm Z-stack (2 μm/ slice) was typically acquired using a standard galvano-scanner at a frame rate of 2 frames per minute. For the analysis of in vivo activated cells a single slice was acquired using the resonant scanner with 6x averaging at a rate of 3 frames/second. Images were acquired using FV30 software (Olympus) and exported to Imaris (Bitplane) for downstream processing.

Imaging analysis

Raw imaging data was analyzed with Imaris software (Bitplane). Tracking of individual cells in a Z-stack was performed using the 'Spots' function in the Surpass mode, and in a single slice by the 'Surfaces' function in Surpass mode. Detection of individual cells relied upon their relative fluorescent intensity, size (diameter ≥9μm) and used the remaining default function settings for the calculation of the motion tracks using an autoregressive motion algorithm. The polarity of in vivo activated cells was derived from the ratio of the length of the longest ellipsoid cell axis by the shortest ellipsoid cell axis giving a quantifiable measure of cell elongation

Supplementary Figures

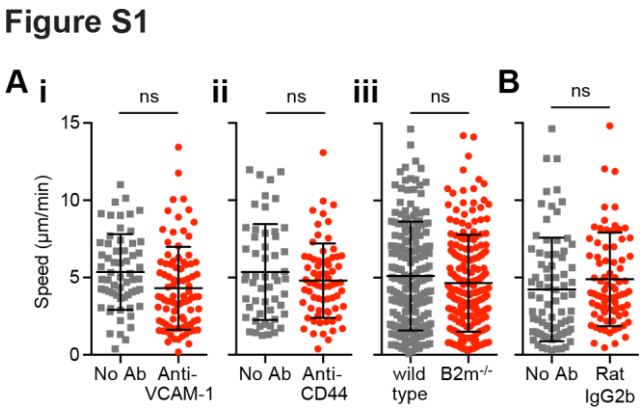

Fig. S1. CD44, VCAM-1, and β$_2$-microglobulin are not required for CD8$^+$ T cell motility in the liver. 2 hours prior to the transfer of 7 x 10^6 in vitro activated OT-I T cells mice (WT or mT/mG) were treated with blocking Abs to CD44, or VCAM-1. Cells were also transferred to mice lacking MHC Class I (β2-microglobulin$^{-/-}$) or control mice without Ab treatment. 4 hours after cell transfer mice were prepared for intravital imaging and imaged by 2-photon microscopy using a standard galvanometer-scanner to acquire a 50-micron deep Z-stack approximately every 30 seconds. (A) Speeds of cells under the different treatment conditions (B) Speeds of cells receiving Rat IgG2b antibodies (isotype control for anti ICAM-1). Data pooled from 2 experiments per condition and analyzed using linear mixed models with experiment and mouse as random effects and speed, meandering index or arrest as the fixed effects. Means and standard deviations are shown.

Figure S2

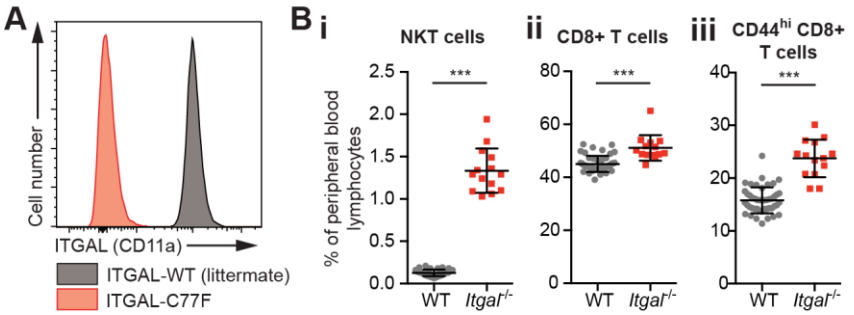

Fig. S2. Phenotype of ITGAL-C77F mice. (**A**) Expression of ITGAL (CD11a) on the surface of $CD8^+$ T cells in OT-I mice crossed to the ITGAL-C77F background (red histogram) or littermate OT-I mice expressing the WT *Itgal* allele (grey histogram). (**B**) Percentage of lymphocytes that are (i) NK T cells, (ii) Total CD8+ T cells and (iii) $CD44^{hi}$ $CD8^+$ T cells in the peripheral blood of ITGAL-C77F mice and WT littermates. Data analyzed by two-tailed Student's t test.

Figure S3

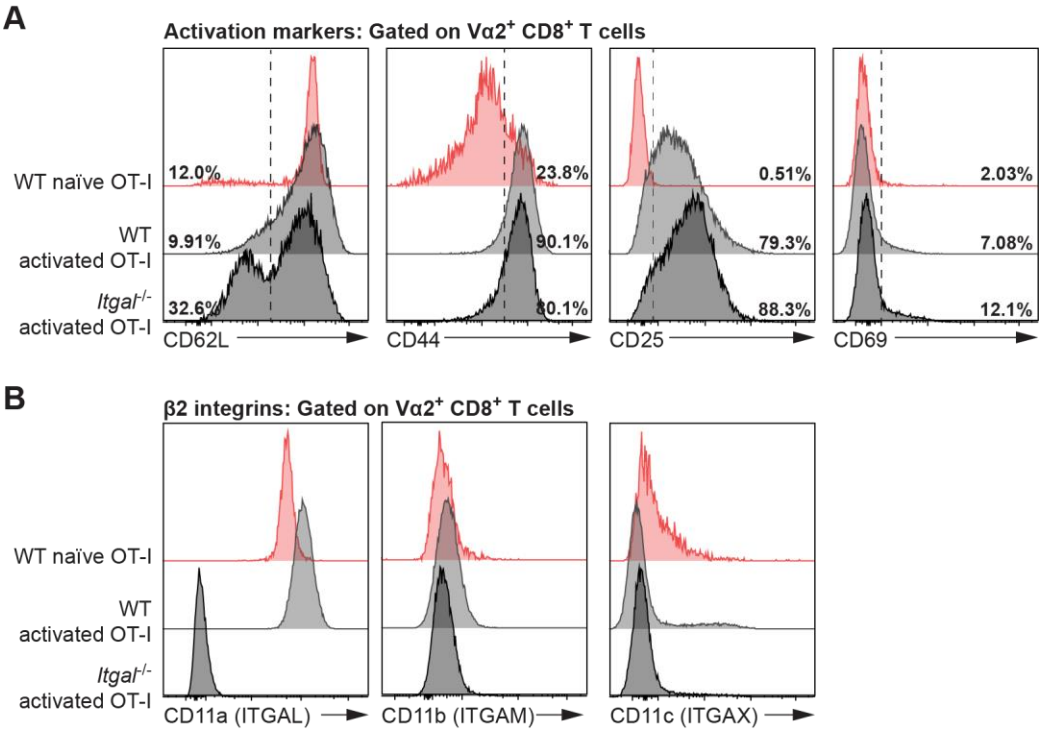

Fig. S3. Similar phenotypes of WT and *Itgal*$^{-/-}$ cells after in vitro priming. WT and *Itgal*$^{-/-}$ OT-I splenocytes were incubated with peptide pulsed splenocytes and cultured in IL-2. 4 days later the expression of (**A**) activation markers and (**B**) β2 integrins was assessed by flow cytometry. Light grey histograms show the expression of molecules on WT OT-I cells, dark grey shows their expression on *Itgal*$^{-/-}$ OT-I cells. The red histograms represent marker expression on unstimulated OT-I cells.

Figure S4

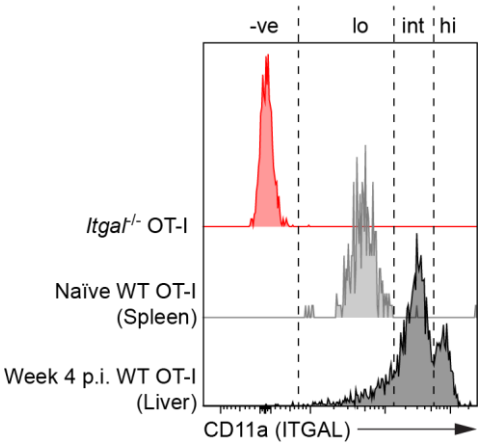

Fig. S4. Expression of CD11a on different populations of OT-I T cells. The expression of CD11a on *Itgal*-/- OT-I cells, OT-I cells in the spleens of naïve mice following adoptive transfer and the livers of mice 4 weeks after adoptive transfer and immunization are shown. Definitions of negative, low, intermediate and high expression are given above.

Figure S5

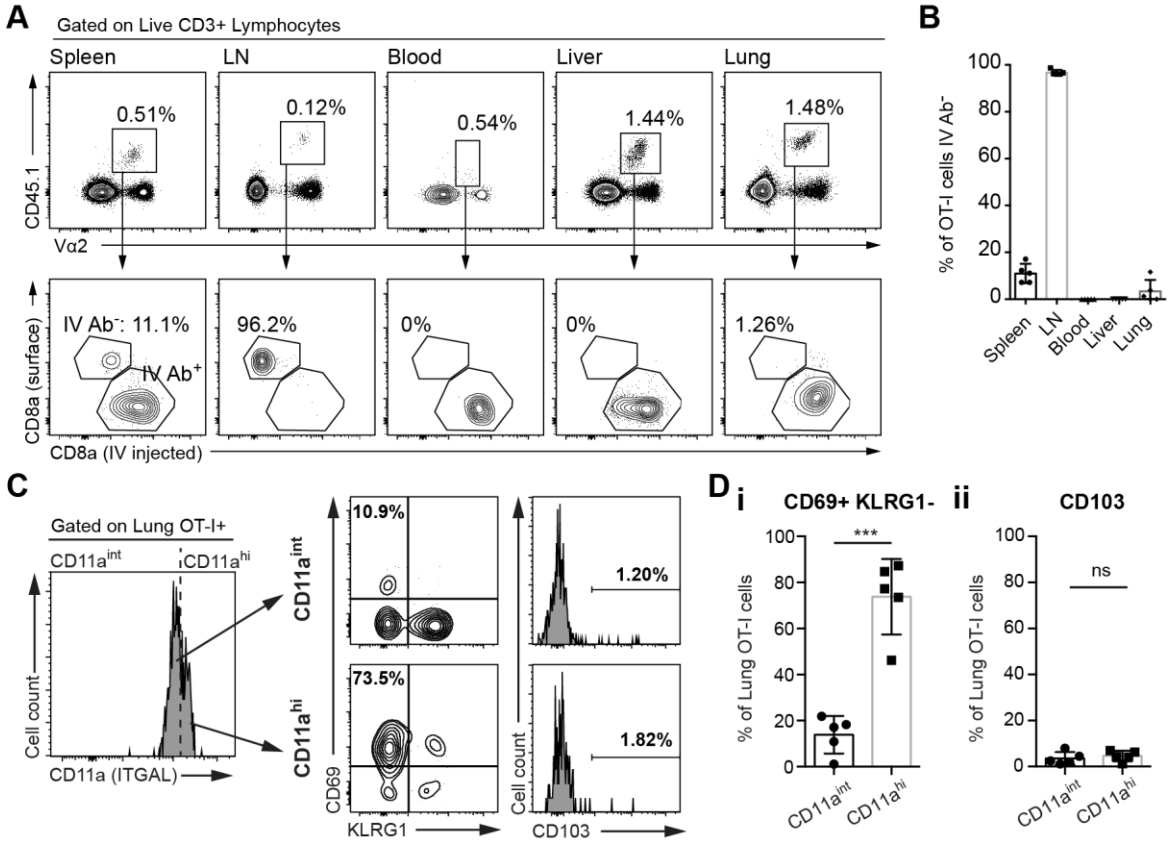

Fig. S5. CD11a^{hi} cells in the liver and lung are CD69^{+}, KLRG1$^{–}$, CD103$^{–}$, and IVAb^{+} after sporozoite immunization. 2 x 10^{4} CD45.1^{+} naïve OT-I cells were transferred to C57BL/6 mice prior to immunization with 5 x 10^{4} *P.berghei* CS^{5M} sporozoites under chloroquine cover. Four weeks post immunization and 5 minutes prior to sacrifice, mice received anti-CD8 FITC to label cell populations exposed to the circulation; following harvesting cells were counter-stained with anti-CD8-BUV395 to CD8^{+} T cells sequestered from the circulation **A**. Representative flow cytometry plots showing the proportion of OT-I T cells that were sequestered from the circulation in each organ **B.** Summary graphs showing data from one of 2 independent experiments with similar results; bars show means and standard deviations **C.** Representative flow cytometry plots of the expression of CD69, KLRG1, CD103 and CXCR3 by CD45.1^{+} CD8^{+} OT-I T cells expressing intermediate and high levels of ITGAL (CD11a^{int} and CD11a^{hi}) in the lung 4 weeks post-immunization. **D.** Summary graphs showing the proportion of cells expressing the indicated phenotypes 4 weeks post-immunization in the lung. Data are from a representative experiment from one of 4 independent experiments (for KLRG1 and CD69 expression) and 2 independent experiments (for CD103 expression). Data were analyzed by paired two-tailed T test; bars show means and standard deviations.

Figure S6

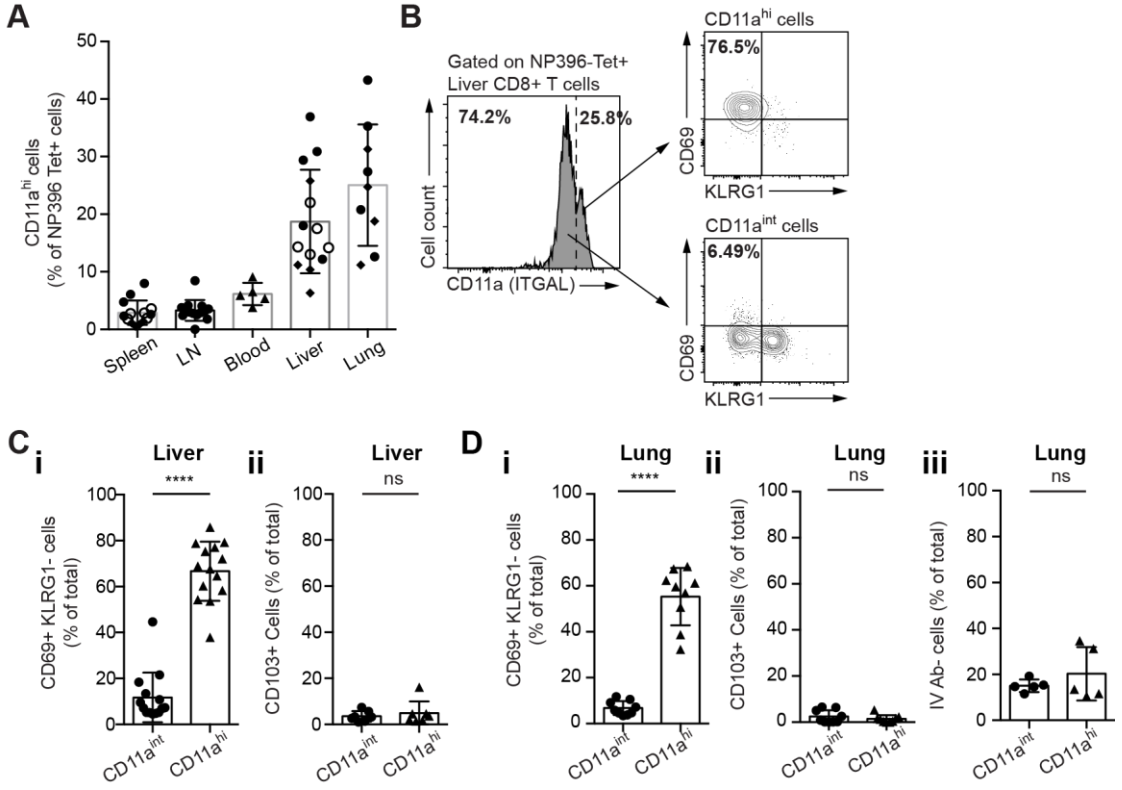

Fig. S6. LCMV infection induces populations of CD11^{hi} cells in the liver and lung that are phenotypically similar to those seen after sporozoite immunization. Mice were infected i.p. with 2 x 10^5 pfu LCMV-Armstrong 4-6 weeks later organs were harvested and the phenotype of antigen specific NP396-Tet$^+$ cells determined by flow cytometry. (**A**) Proportion of NP396-Tet$^+$ cells that are CD11a^{hi} in different organs; data pooled from 3 independent experiments and analyzed using linear mixed models with experiment and mouse as random effects and organ as the fixed effect. (**B**) Representative flow cytometry plots showing the phenotypes of CD11a^{hi} and CD11a^{int} cells with respect to CD69 and KLRG1 expression. (**C**) Summary statistics of the proportion of CD11a^{hi} and in CD11a^{int} cells in the liver that are (i) CD69$^+$ KLRG1$^-$ (data from 3 independent experiments) and (ii) CD103$^+$ (data from 2 independent experiments); analyzed using linear mixed models with mouse and experiment as a random effects and CD11a expression as the fixed effect. (**D**) Summary statistics of the proportion of CD11a^{hi} and in CD11a^{int} cells in the lung that are (i) CD69$^+$ KLRG1$^-$ (data from 2 independent experiments), (ii) CD103$^+$ (data from 2 independent experiments) and (iii) IV Ab$^-$ (data from a single experiment); analyzed using linear mixed models with mouse and experiment as a random effects and CD11a expression as the fixed effect.

Figure S7

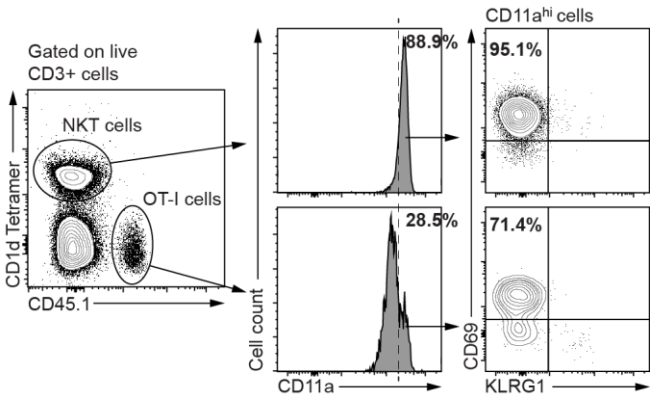

Fig. S7. NKT cells have a similar CD11a^{hi} CD69^{+} KLRG1^{-} phenotype to liver T$_{RM}$ cells. 2 x 10^4 CD45.1^{+} naïve OT-I cells were transferred to C57BL/6 mice prior to immunization with 5 x 10^4 *P.berghei* CS^{5M} sporozoites under chloroquine cover. Shown are representative flow cytometry plots of the expression of CD11a on NKT cells and OT-I T cells in the liver 4 weeks post immunization and the expression of CD69 and KLRG1 on the respective CD11a^{hi} subsets.

Figure S8

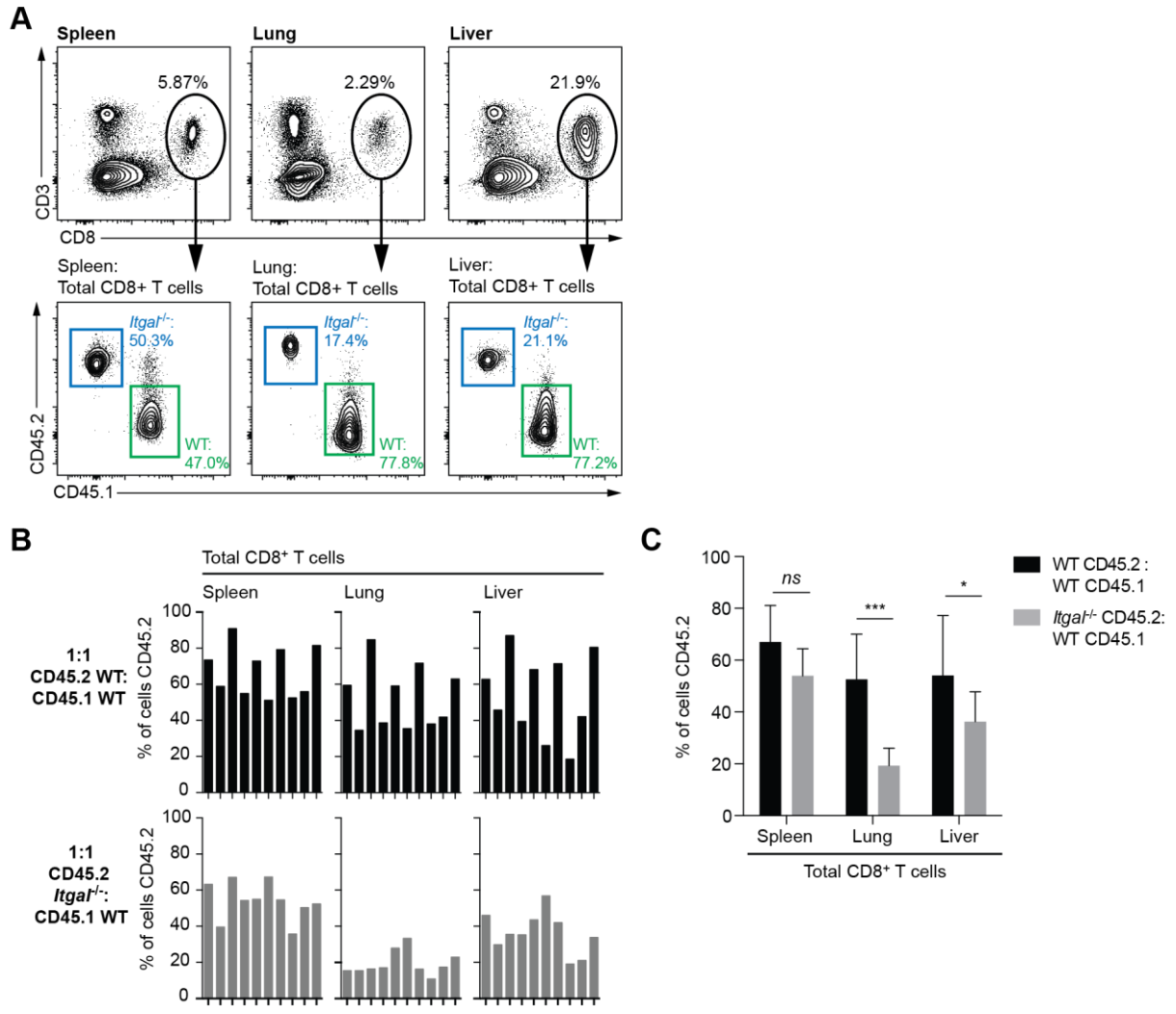

Fig S8. LFA-1 is required for the retention of cells in the liver and lung in the steady state. *Rag1*$^{-/-}$ mice were irradiated with 10 Gy and were injected with 2 x 10^6 bone marrow cells (50:50 mixture of WT B6.SJL CD45.1$^+$ bone marrow and either *Itgal*$^{-/-}$ or WT C57BL/6 CD45.2$^+$ bone marrow). They were allowed to reconstitute for 8–13 weeks before analysis. (**A**) Gating strategy for the identification of CD3$^+$ CD8$^+$ T cell populations in different organs – plots from a single representative CD45.2 *Itgal*$^{-/-}$: CD45.1 WT chimera shown. (**B**) Individual data from 20 mice (10/group) for the total CD8$^+$ T cell population across different organs and (**C**) summary data and statistics for all mice. Data were analyzed using 2-way ANOVA to analyse the effect of genotype and organ, significance values shown in D are from Sidak's multiple comparisons test. Analysis using linear mixed models gave identical results.

Figure S9

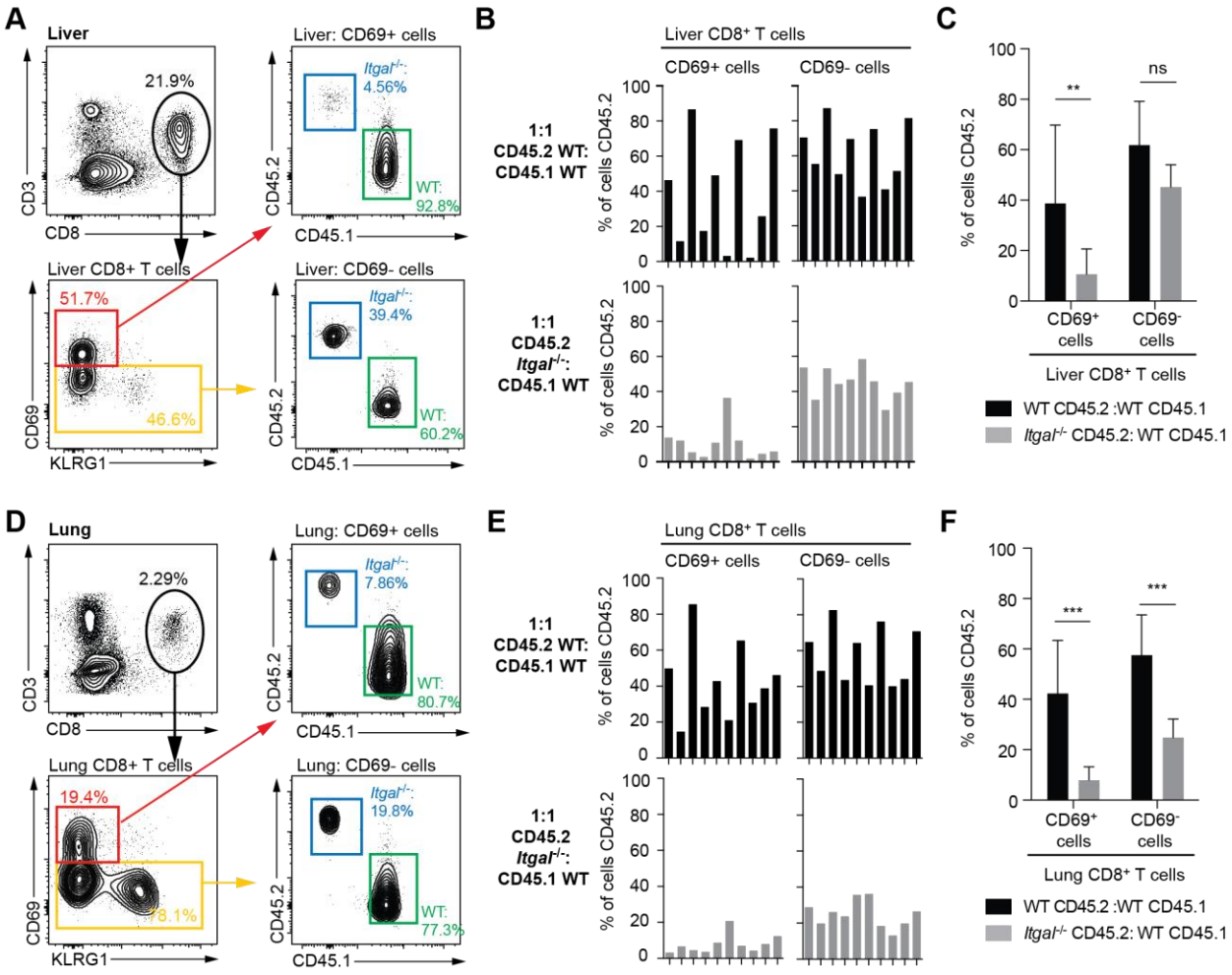

Fig. S9. LFA-1 is required for the formation of TRM cells in the liver in the steady state. Bone marrow chimeras were prepared as in Figure S8 (**A**) CD69^{+} and CD69^{-} populations within the liver – plots from a single representative CD45.2 *Itgal*$^{-/-}$: CD45.1 WT chimera shown. (**B**) Individual data from the livers of 20 mice (10/group) for the total CD8^{+} T cell population across different organs and (**C**) summary data and statistics for the livers of all mice. (**D**) CD69^{+} and CD69^{-} populations within the lung – plots from a single representative CD45.2 *Itgal*$^{-/-}$: CD45.1 WT chimera shown. (**E**) Individual data from the lungs of 20 mice (10/group) for the total CD8^{+} T cell population across different organs and (**F**) summary data and statistics for the lungs of all mice. Data were analyzed using 2-way ANOVA to analyse the effect of genotype and organ, significance values shown in C and F are from Sidak's multiple comparisons test. Analysis using linear mixed models gave identical results.

Supplementary Movie Legends

Movie S1. Anti–ICAM-1 inhibits effector T cell migration in the hepatic sinusoids. 7 x 10^6 activated GFP expressing OT-I cells were transferred to mT/mG host mice that had been treated 2 hours previously with either PBS or anti-ICAM-1. 4 hours later livers were imaged by intra-vital microscopy. A single z-slice is shown. The sinusoids of the liver can be seen as the tdTomato expressed in these mice is membrane bound and thus effectively labels the sinusoidal endothelium. White lines show the paths of the cells. The major tick interval is 30 μm.

Movie S2. LFA-1–deficient cells display impaired motility in the liver. 7 x 10^6 activated GFP expressing wild type OT-I cells were co-transferred to host mice with 7 x 10^6 activated *Itgal*$^{-/-}$ $CD8^+$ T cells labelled with CellTrace^{TM} Violet. 4 hours later the liver was imaged by intra-vital microscopy. A 50 μm deep z-stack is shown. Hepatocytes can be observed via the auto-fluorescence of the liver. White tracks show the paths of WT (GFP) cells and yellow tracks show the paths of *Itgal*$^{-/-}$ (CTV) cells. The major tick interval is 50 μm.

Movie S3. Migration of OT-I cells in the livers of mice 1 week after sporozoite immunization. C57BL/6 mice received 2 x10^4 GFP expressing OT-I cells prior to immunization with 5 x10^4 *P. berghei* CS^{5M} sporozoites under choloroquine cover. 1 week later the livers were imaged by intra-vital microscopy. A single slice acquired using a resonant (high speed) scanner is shown. White lines show the paths of cells. The major tick interval is 50 μm. The different types of T cell motility are highlighted by the annotation.

Movie S4. Migration of OT-I cells in the livers of mice 4 weeks after sporozoite immunization. C57BL/6 mice received 2 x10^4 GFP expressing OT-I cells prior to immunization with 5 x10^4 *P. berghei* CS^{5M} sporozoites under choloroquine cover. 4 weeks later the livers were imaged by intra-vital microscopy. A single slice acquired using a resonant (high speed) scanner is shown. White lines show the paths of cells. The major tick interval is 50 μm.