Supporting Information

Benseler et al. 10.1073/pnas.1112251108

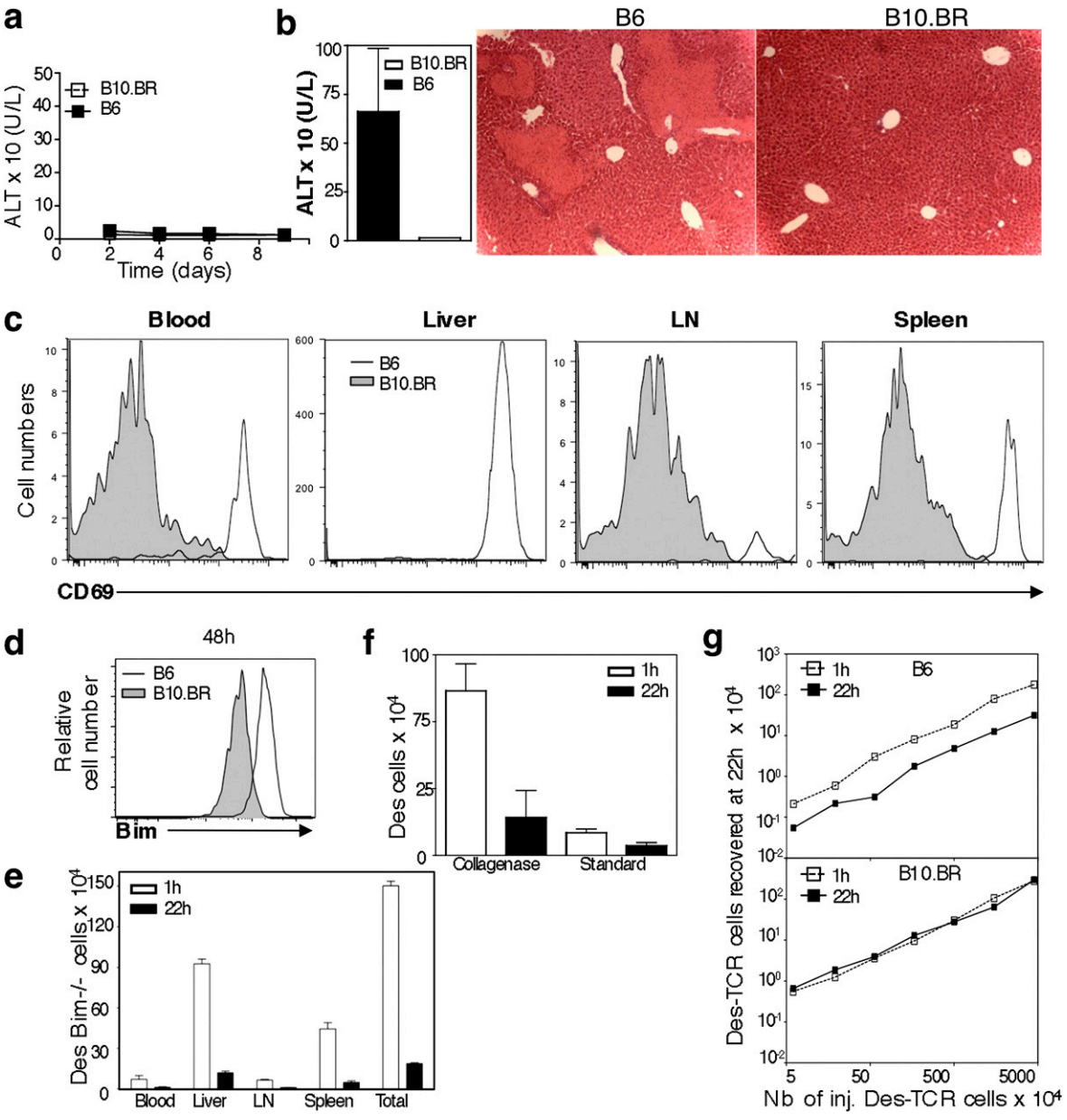

Fig. S1. Rapid CD8$^+$ Des T-cell loss after intrahepatic activation is an efficient and Bim-independent process that is not easily saturated. (*A*) ALT levels of B10.BR and B6 mice adoptively transferred with 10^8 LN cells from Des mice showing that no sign of hepatitis was detected. (*B*) Unlike naive Des T cells, effector Des T cells induced severe hepatitis in B6 recipients. Effector T cells were generated in vitro by culturing Des LN cells for 3 d with B6 splenocytes and then grown for 10–15 d in the presence of exogenous IL-2 as described (1). Effector Des T cells (5×10^6) were adoptively transferred into B6 and B10.BR recipient mice. (*B Left*) ALT levels in serum recipient mice at 1 d after transfer. (*B Right*) Hematoxylin and eosin staining of liver sections from recipient mice at 1 d after transfer (original magnification 500×). B6 but not B10.BR mice developed severe hepatitis with multiple infarcts. Error bars represent SEM of three mice per group. Data are representative of two independent experiments. (*C*) FACS profile 5 h after adoptive transfer of 1.5×10^6 CFSE-labeled naive Des LN cells demonstrates the up-regulation of the early activation marker CD69 in TCR transgenic cells purified from the blood, liver, LN and spleen of B6 mice compared with B10.BR controls. (*D*) CFSE-labeled CD8$^+$ Des RAG-1$^{-/-}$ T cells were adoptively transferred into B6 and B10.BR mice. Livers were harvested at 48 h and donor lymphocytes, identified by CD8 and CFSE, were analyzed by flow cytometry for expression of Bim. T-cell activation in the B6 liver led to the up-regulation of Bim. (*E*) Total number of CD8$^+$ Des$^+$ T cells in different organs at 1 and 22 h after transfer of CFSE-labeled Des Bim$^{-/-}$ T cells into B6 mice. Data are representative of at least two experiments. (*F*) Leukocytes from the recipient B6 liver were either purified by collagenase digestion or mechanical separation and percoll gradient (standard as described (2) at 1 and 22 h after adoptive transfer. Results demonstrate that failure to recover cells was observed regardless of the protocol used.

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Data are representative of at least three experiments. (*G*) Total cell number of CFSE$^+$ CD8$^+$ T cells isolated from the liver in B6 and B10.BR mice injected with a range (0.06–45 $\times$ 10^6) of Des T cells at 1 and 22 h after transfer. Cell loss was observed regardless of the number of donor T cells injected, suggesting that the process eliminating the T cells was nonsaturable.

1. Manjunath N, et al. (2001) Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J Clin Invest* 108:871–878.
2. Bertolino P, Trescol-Biémont MC, Rabourdin-Combe C (1998) Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur J Immunol* 28: 221–236.

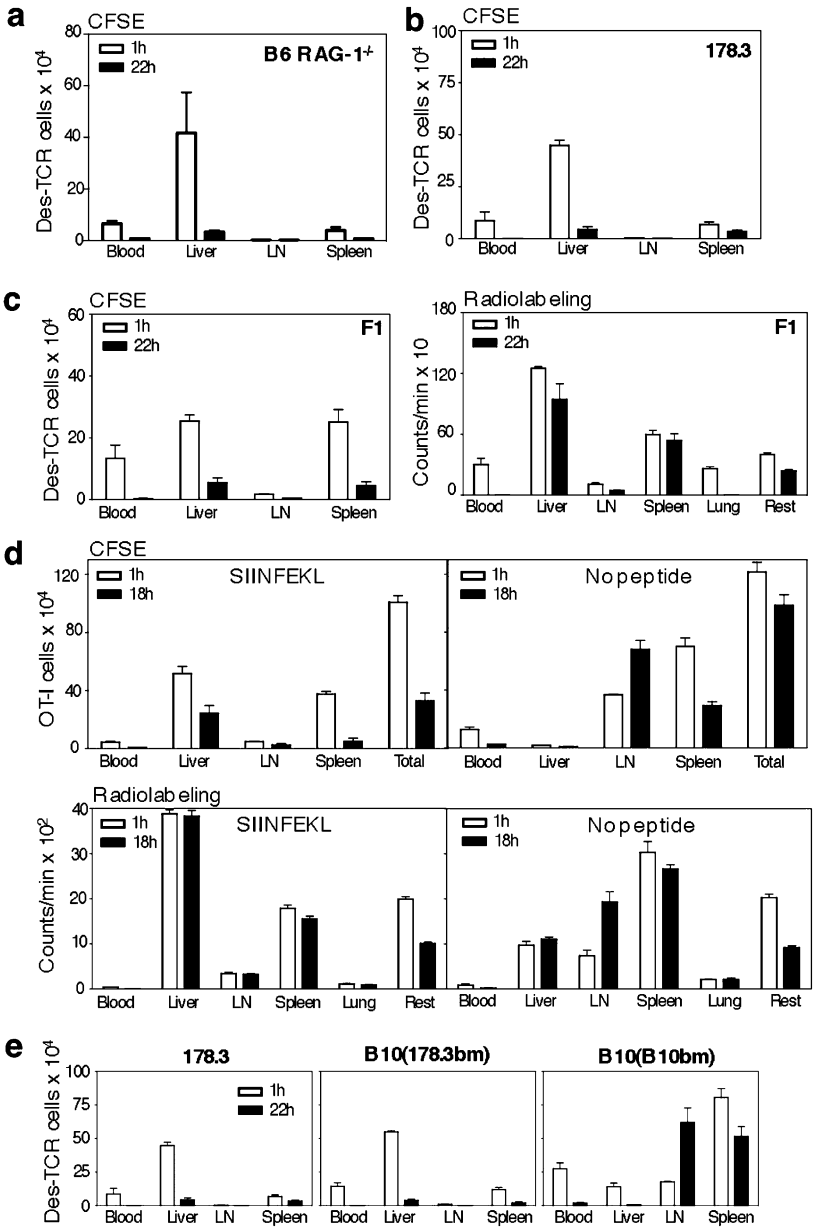


Fig. S2. Loss of CD8$^+$ Des T cells is antigen-specific and independent of host lymphocytes and NK cells. Distribution of CFSE-and/or radiolabeled transgenic CD8 T cells at 1 and 18 or 22 h after adoptive transfer of Des T cells into B6 RAG-1$^{-/-}$ (*A*), 178.3 (*B*), and F_1 (*C*) recipients and into B6 mice injected with SIINFEKL peptide and OT1 T cells (*D*), a totally syngeneic model. CFSE labeling experiments indicate that T cells were retained at 1 h, but could not be recovered from antigen-expressing recipients, suggesting that T cells were lost. Radiolabeling experiments suggest that the T-cell content was still contained in the liver despite the inability to recover viable cells. (*E*) Distribution of CFSE-labeled Des-RAG-1$^{-/-}$ T cells at 1 and 22 h after adoptive transfer into 178.3 and B10.BR reconstituted with 178.3 (B10(178.3bm)) or B10 (B10(B10bm)) bone marrow. T cells were retained at 1 h but could not be recovered from the livers of antigen-expressing recipients, suggesting that T cells disappeared in the liver of these mice. The 178.3 control is the same graph as the one shown in *E* as this control group was performed at the same time as the two chimeras groups. Data represent the mean ± SEM of three mice per group. All Data except for *A* (one experiment) and *E* (2 experiments) is representative of three independent experiments.

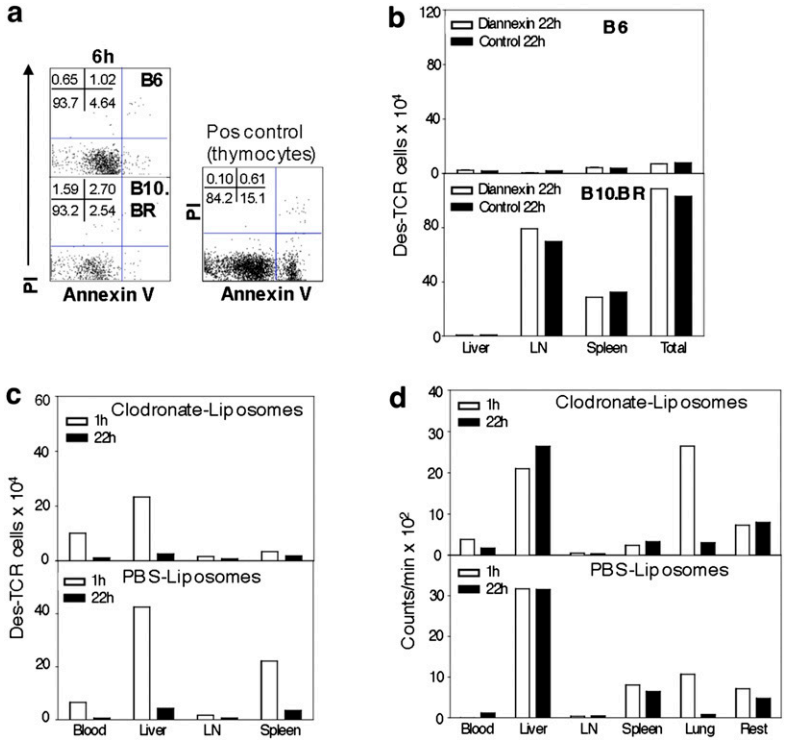


Fig. S3. T-cell loss was nonapoptotic and did not require macrophages and phosphatidylserines (PS). (*A*) Annexin V staining of donor T cells harvested at different time points. Positive controls are thymocytes cultured for 6 h at 37 °C. Donor T cells were identified by gating on CFSE$^+$ CD8$^+$ T cells. (*B*) PS-mediated uptake is not critical for the rapid clearance of T cells observed at 22 h. To test the possibility that donor T cells are cleared after expression of PS on their surface as reported for recently activated T lymphocytes (1), recipient B6 and B10.BR mice were treated with a homodimer of annexin V (Diannexin) that specifically blocks PS-mediated binding (2). However, PS blockade did not prevent donor T-cell loss seen at 22 h. Error bars for B6 mice represent SEM of three mice per group. Data are representative of at least three independent experiments. (*C* and *D*) Macrophages were not critical in the clearance of donor T cells. Recipient mice were treated with chlodronate liposomes, which efficiently depleted phagocytic F4/80$^+$ liver cells as determined by immunohistochemistry. (*C*) CD8$^+$ Des T-cell numbers at 1 and 22 h in B6 recipient mice treated with clodronate- or control PBS-liposomes 48 h before the adoptive of Des T cells. (*D*) Radioactive counts of ^{51}Cr-labeled CD8$^+$ Des T cells in mice treated as in *C*. Interestingly, clodronate-liposome treatment caused a redistribution of cells to the lung with a reduction in the liver and spleen at 1 h, but failed to prevent deletion. Data are representative of at least two independent experiments.

1. Elliott JI, et al. (2005) Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat Cell Biol* 7:808–816.
2. Kuypers FA, Larkin SK, Emeis JJ, Allison AC (2007) Interaction of an annexin V homodimer (Diannexin) with phosphatidylserine on cell surfaces and consequent antithrombotic activity. *Thromb Haemost* 97:478–486.

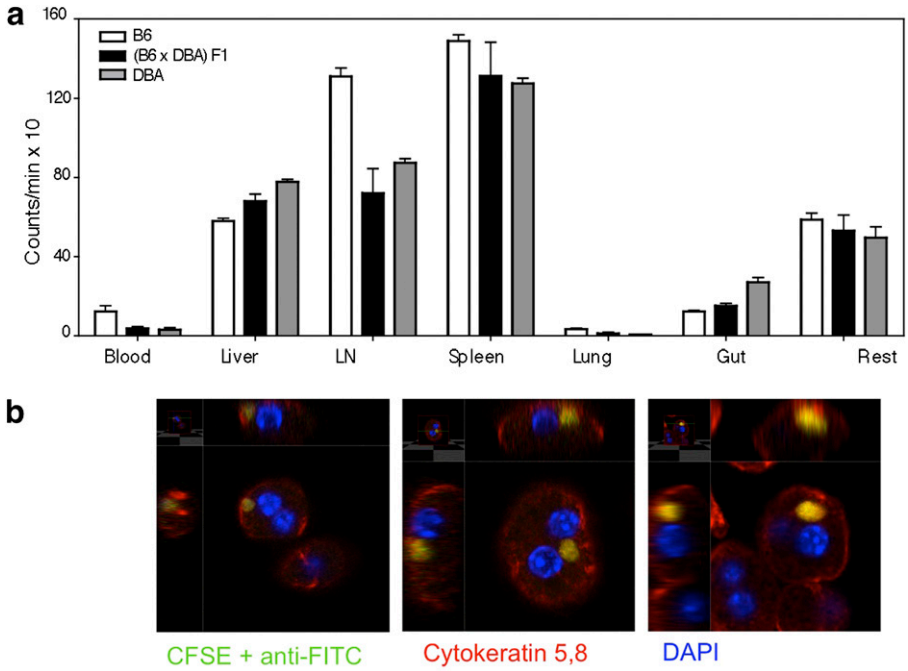


Fig. S4. "Wildtype" H-2^b CD8$^+$ T cells were retained in the livers of allogeneic recipient mice and were found contained inside hepatocytes. (*A*) Organ distribution of radioactivity after transfer of purified ^{51}Cr-labeled CD8$^+$ T cells isolated from B6 LN, 22 h after adoptive transfer into fully allogeneic (DBA and (B6 x DBA)F_1) or syngeneic B6 mice. A subset of adoptively transferred cells was retained in the liver of allogeneic mice only, resulting in lower cell numbers migrating to LN. (*B*) Purified hepatocytes from DBA mice 14 h after adoptive transfer of CFSE labeled allogeneic B6 CD8$^+$ LN cells. CFSE positive remnants of T cells (in yellow) were detected (by using anti-FITC-AlexaFluor 488 antibody) inside hepatocytes. Blue, DAPI; red, Cytokeratin 5,8.

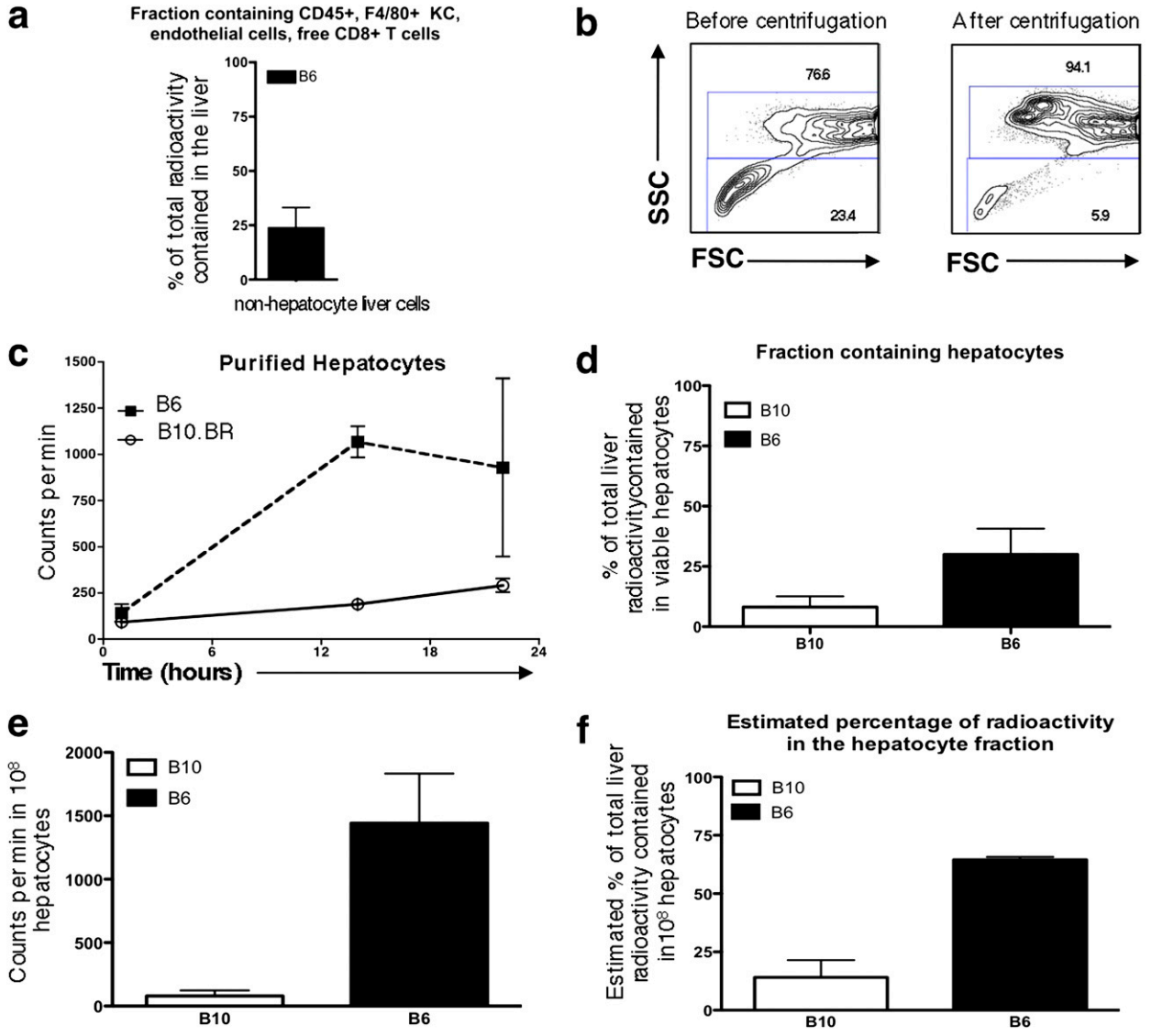


Fig. S5. Most of the radioactivity in the liver was detected in the hepatocyte fraction, suggesting that T-cell invasion of hepatocyte is a dominant mechanism by which T cells are eliminated in the liver. (*A*) Percentage of the total liver radioactivity contained in nonhepatocyte cells. B6 mice were injected with Des RAG-$1^{-/-}$ T cells and 22 h later, livers were perfused with Collagenase IV. Nonhepatocyte cells were purified by using magnetic beads using antibodies specific for F4/80, CD8, CD45 CD31, and ME9F1 (anti-liver sinusoidal endothelial cell marker, a gift from A. Hamman, Charite Universitaetsmedizin, Berlin, Germany). A maximum of 25% of the radioactivity was contained within this fraction, which also includes free $CD8^+$ T cells, suggesting that most of the radioactivity was not contained in non hepatocyte cells. (*B*) Hepatocytes from recipient mice were enriched by using low speed centrifugation. Hepatocytes identified by their size (top gate) were selectively enriched compared with nonhepatocytes (bottom gate). (*C*) Time course demonstrating accumulation of radioactivity in the enriched hepatocyte fraction. (*D*) Total radioactivity contained in the hepatocyte-enriched fraction after low speed centrifugation showing that up to 30% of the radioactivity in B6 recipients is contained in intact hepatocytes. (*E*) Estimated radioactivity in 10^8 hepatocytes (total number of hepatocytes in a normal mouse liver if hepatocytes were not lost or destroyed during preparation, data not shown). (*F*) Estimated percentage of intrahepatic radioactivity that would be contained in the hepatocyte fraction if the hepatocytes were not lost or destroyed during the low speed centrifugation. This percentage was calculated by using the total radioactivity in 10^8 hepatocytes calculated in *E*. Error bars represent SEM of three animals per group.

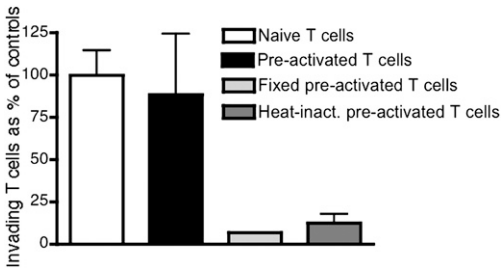


Fig. S6. Inactivated T cells did not enter hepatocytes. CFSE-labeled C57BL/6 hepatocytes were cocultured for 12 h with cell-tracker Orange-labeled naïve Des RAG T cells (white bar), live preactivated Des RAG-$1^{-/-}$ T cells (for 4 h with anti-CD3 and anti-CD28; black bar), fixed preactivated Des RAG-$1^{-/-}$ T cells (light gray bar), or heat-inactivated preactivated Des RAG-$1^{-/-}$ T cells (dark gray bar). Cocultures were analyzed by confocal microscopy, and the number of T cells inside hepatocytes counted. These results suggest that both naïve and preactivated T cells were found inside hepatocytes, but these events decreased dramatically when T cells were inactivated by heat or fixation. This result suggests that T cells were not passively phagocytosed by hepatocytes but actively invaded the liver cells.

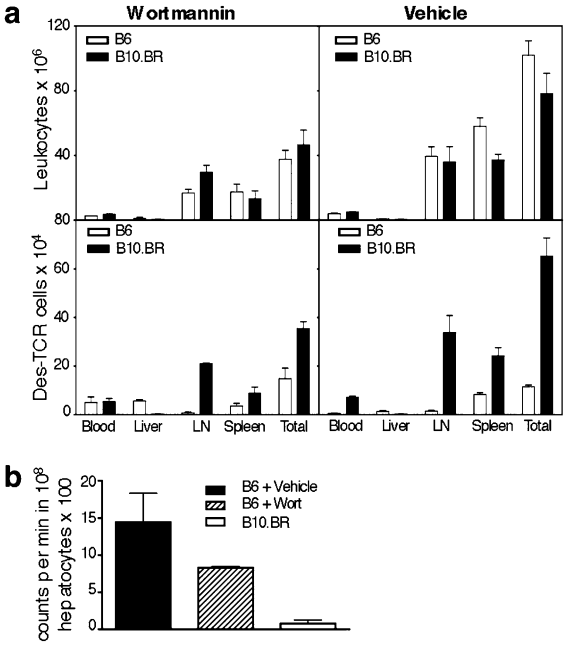
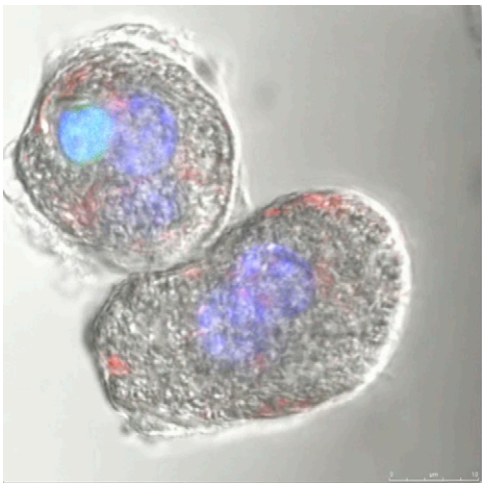
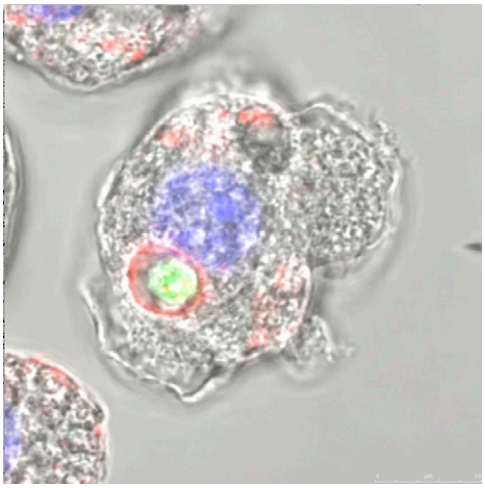


Fig. S7. Wortmannin rescues T cells in the liver by preventing invasion into hepatocytes. (*A*) Although wortmannin caused an equivalent drop in total leukocyte numbers in both B6 and B10.BR mice (probably related to its toxicity), it considerably increased the total number of CD8 Des T cells in the blood and liver of recipient B6 mice at 22 h after adoptive transfer. Error bars represent SEM of three animals per group. Data are representative of at least three independent experiments. (*B*) B6 mice adoptively transferred with ^{51}Cr-radiolabeled Des T cells were treated with wortmannin or vehicle control and hepatocytes from recipient mice were harvested after perfusion with collagenase IV. B10.BR mice adoptively transferred with Des T cells were used as controls. The radioactivity was corrected for 10^8 hepatocytes as described in Fig. S5*F*.



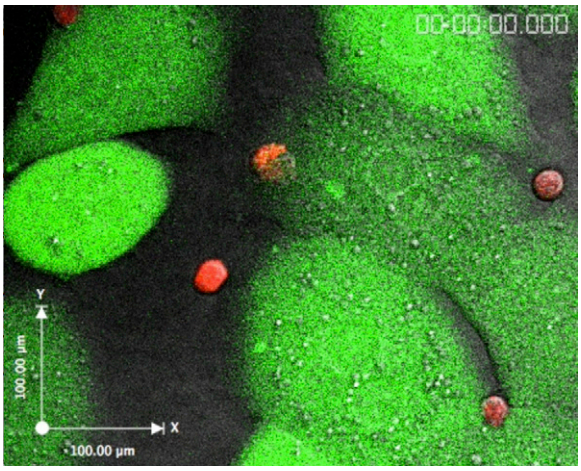
Movie S1. Movie of serial z slice confocal images of a CFSE-labeled T-cell inside an ex vivo isolated B6 hepatocyte. T cells, harvested from the LN of Des RAG-$1^{-/-}$ mice, were CFSE-labeled and adoptively transferred into B6 mice. Eight hours later, hepatocytes from recipient mice were purified, labeled with anti-LAMP-1 plus anti-mouse Alexa Fluor 647 (in red) and DAPI (blue). Detection of CFSE+ T cells was enhanced by staining with anti-FITC Alexa Fluor 488 (green cell). The movie contains two binucleated hepatocytes. The top hepatocyte contains a lymphocyte. The movie shows that the lymphocyte is contained in a vesicle inside the hepatocyte. The T-cell is DAPI+ suggesting that its nucleus is still intact. LAMP-1 is diffuse in the hepatocyte cytoplasm, but a LAMP-1 ring starts to form around the T-cell suggesting that this structure is an early step of the degradation process.

Movie S1



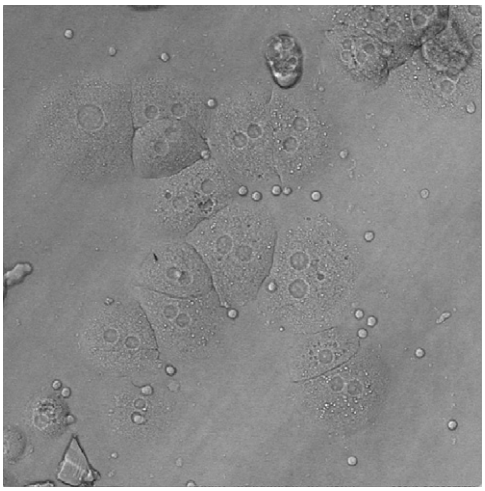
Movie S2. Movie of serial z slice confocal images of a T-cell remnant inside a LAMP-1+ vesicle within an ex vivo isolated B6 hepatocyte. Des T cells were adoptively transferred into B6 mice and recipient hepatocytes were stained as described in the Movie S1 legend. The hepatocyte shown in this movie contains a T-cell remnant (in green) that has lost its nucleus (DAPI negative). This remnant is clearly contained within a LAMP-1+ vesicle, suggesting that T cells are destroyed in this proteolytic compartment.

Movie S2



Movie S3. Movie of confocal images showing a Des RAG-1$^{-/-}$ T-cell (red) invading a B6 hepatocyte (green) after 5 h of coculture.

Movie S3



Movie S4. Movie of Des RAG-1$^{-/-}$ T-cell/B6 hepatocytes cocultures illustrating that T cells are very mobile, whereas hepatocytes are relatively inert. T cells seem to actively scan the surface of hepatocytes. In the center, a T-cell enters a hepatocyte and is contained within a vesicle. Cocultures were filmed for ≈3 h.

Movie S4