SUPPLEMENTAL INFORMATION

Effective intrahepatic CD8^{+} T-cell immune responses are induced by low but not high numbers of antigen-expressing hepatocytes

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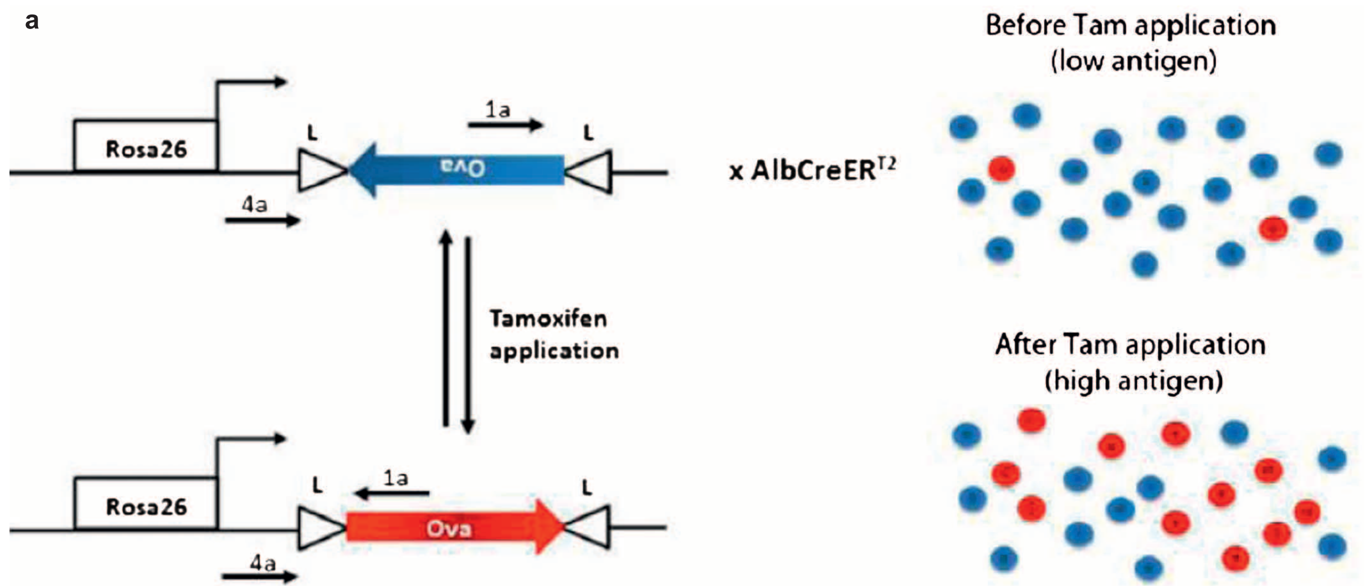
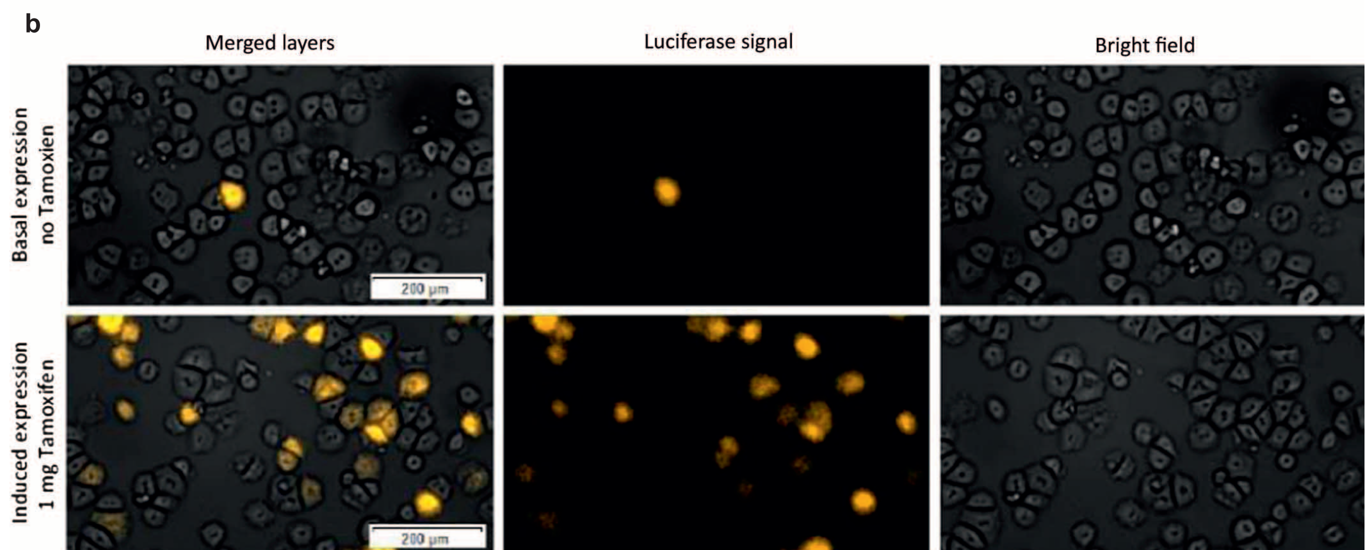

a
Rosa26
L
1a
Ova
L
4a
x AlbCreER^{T2}
Tamoxifen application
Rosa26
L
1a
Ova
L
4a
Before Tam application (low antigen)
After Tam application (high antigen)


b
Merged layers
Luciferase signal
Bright field
Basal expression no Tamoxifen
Induced expression 1 mg Tamoxifen
200 µm
200 µm

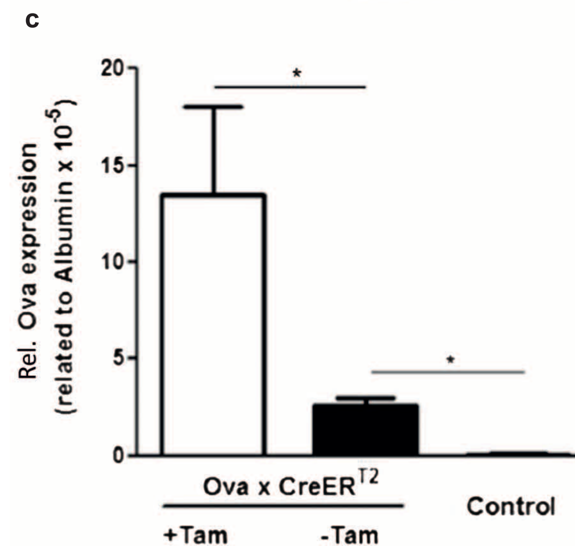

c
Rel. Ova expression (related to Albumin x 10^{-5})
20
15
10
5
0
*
*
Ova x CreER^{T2}
+Tam
-Tam
Control

Figure S1 Overall strategy and evaluation of antigen expression. (**a**) Overall strategy to achieve de novo expression of ovalbumin in hepatocytes. Ova mice with a loxP flanked ova expression cassette integrated in reverse orientation to the Rosa26 promoter are crossed to mice expressing a CreER^{T2} fusion protein under the control of the liver specific Albumin promoter (Ova x CreER^{T2}). Hepatocyte specific activation of Ova expression is achieved by Cre recombinase mediated inversion of the cassette. Cre mediated inversion of Ova is reversible in the presence of Tam, but becomes fixed upon Tam clearance. (**b**) Heterogeneous (yes/no) expression in hepatocytes upon single administration of Tam. Rosaluc x AlbCreER^{T2} mice (Sandhu et al., 2011) in which Ova is substituted by luciferase were induced with 1mg of Tamoxifen. 61 days after application, hepatocytes were isolated, purified and evaluated within 4-8 hours for luciferase expression by microscopy using the Olympus LV200 microscope. For this purpose, luciferin containing culture medium was added to the culture and light emission was measured. (**c**) Quantification of antigen expression by qRT-PCR from liver tissue of Tam-treated and non-induced Ova x CreER^{T2} mice based on primers 1a and 4a. Expression is normalized to albumin. For details of the PCR see main text. Shown is one of at least three independent experiments with $n \geqslant 3$ mice per group.

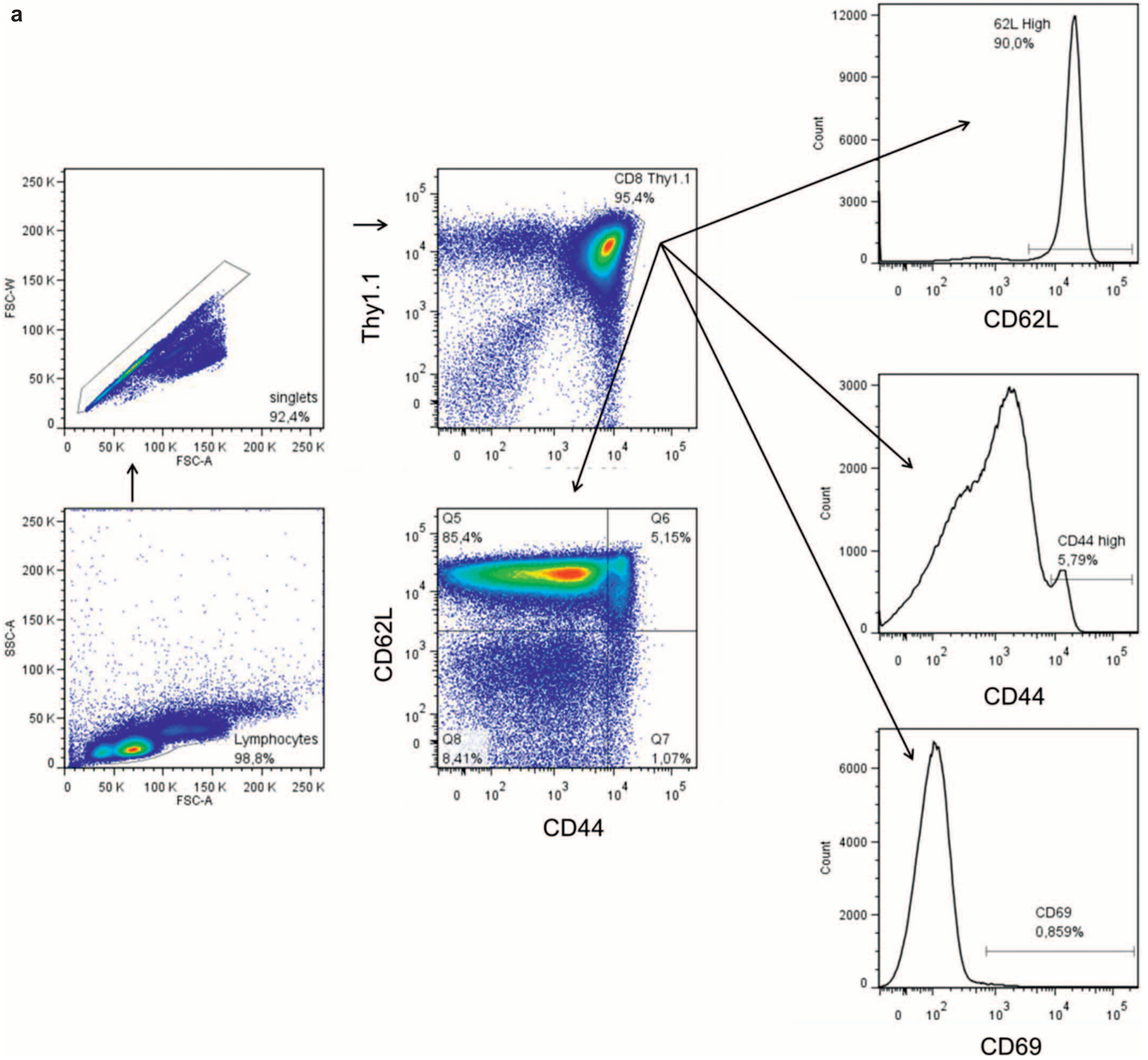


Figure S2a Purity and quality analysis of OT-I isolation. The naïve state of isolated OT-I cells was confirmed by FACS. Representative analysis of OT-I T cell phenotype after magnetic column negative isolation, as specified in the Materials and Methods section. The figure represents the gating strategy as well as staining for activation markers CD62L, CD44 and CD69. After isolation, OT-I T cells were directly used in adoptive transfer experiments as well as in coculture experiments with prior CFSE staining.

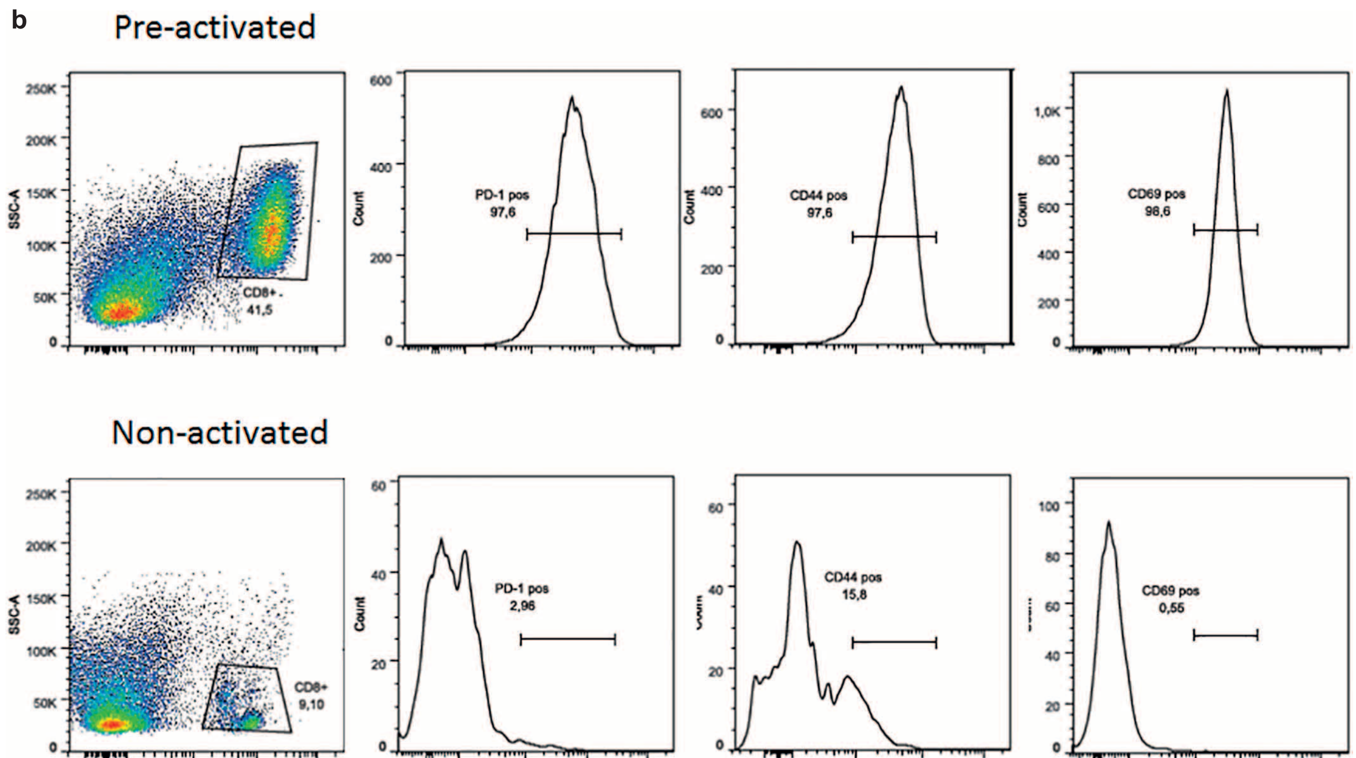


Figure S2b Analysis of the OT-I phenotype after pre-activation. For pre-activation of OT-I cells, splenic cells from OT-I mice were isolated as described above and cultured for 48 h in the presence of 3 μg/ml SIINFEKL-peptide. Afterwards OT-I cells were separated using the CD8a$^+$ T cell isolation kit (Miltenyi). FACS analysis was performed to confirm activation markers. In addition, cytolytic activity of pre-activated cells was confirmed in vivo (data not shown).

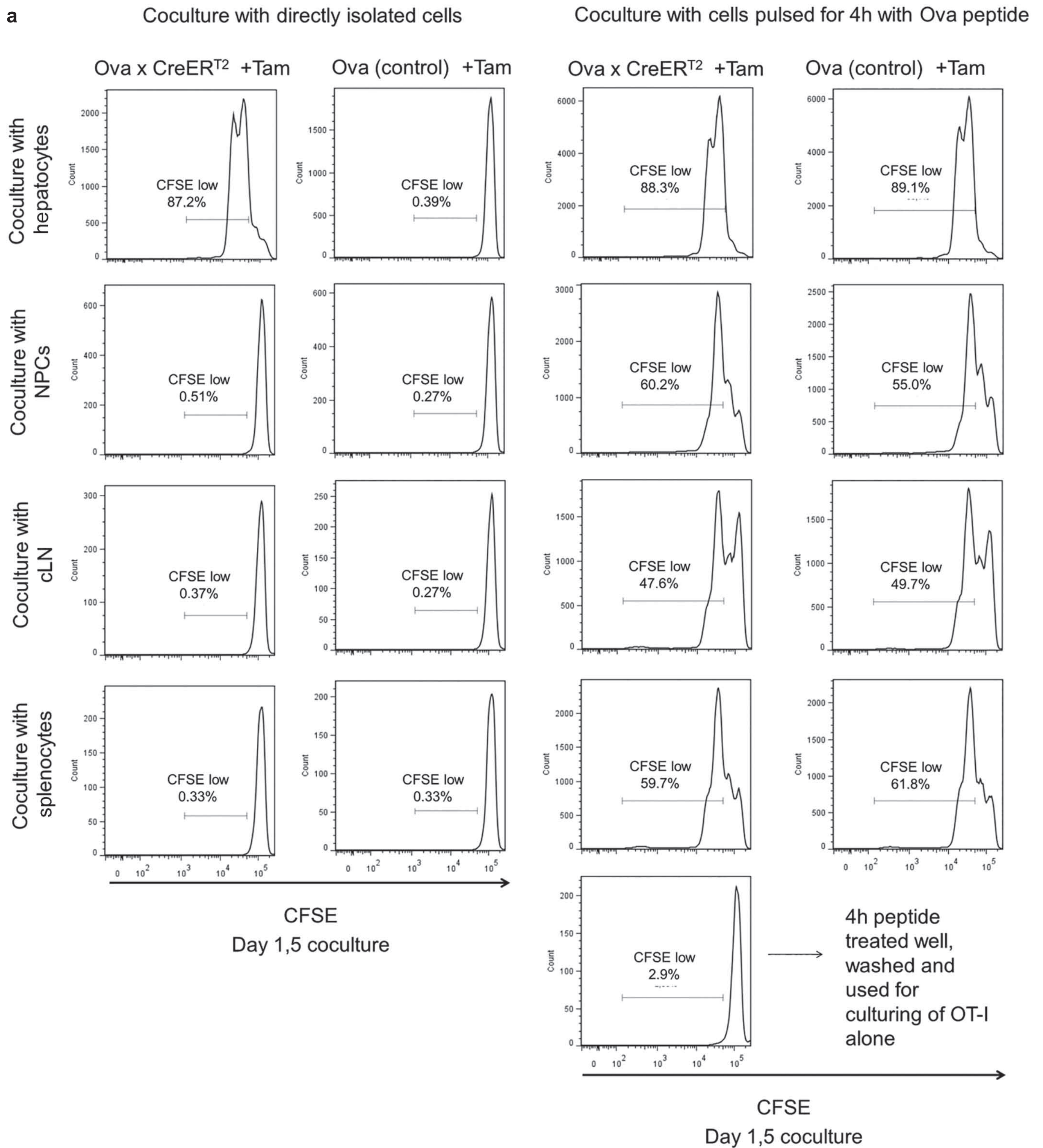


Figure S3a Lack of aberrant antigen presentation: in vitro coculture assay. In order to determine if Ova antigen in Ova x CreER^{T2} mice + Tam is expressed and presented exclusively by hepatocytes we performed a coculture assay[1] of OT-I T cells with hepatocytes, liver non-parenchymal cells (NPCs), celiac lymph node cells and splenocytes isolated from Ova x CreER^{T2} and control Ova mice lacking Ova expression. Prior cell isolation, all the animals were treated with Tam.

HEPATOCYTE ISOLATION

Primary hepatocytes were isolated using the two step perfusion protocol as described earlier[2]. In brief, narcotized mice (0.1 ml/10g body weight Ketamin/Xylazin i.p. injected) were fixed. The liver was perfused by applying 125 ml Liver Perfusion Medium (Gibco) supplemented with Heparin (2500 U/ml) (Ratiopharm) with a flow rate of 8 ml/min from portal vein to vena cava inferior. Enzymatic digestion was performed using 125 ml Liver Digest medium (Gibco) with a flow rate of 20 ml/min. Cells were shaken into a petri dish with precooled (4 °C) Dulbecco's Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Lonza), 100 U penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 2 mM glutamine (Gibco) upon resection of the digested liver and careful carving of the liver lobes. Cells were purified through a 100 μm cell strainer and centrifuged at 50 g at 4 °C for 5 min w/o break. Cells after 2 times washing were counted by using the GUAVA EASYCyte^{TM} and seeded $7{,}5 \times 10^4$ on a collagen coated well of a 48 well plate in 500μl RPMI Medium 1640 - GlutaMAX^{TM}-I (Gibco), supplemented with 10% FCS, 100 U penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 2-Mercaptoethanol. Once hepatocytes attached to the collagen (4-5 h) the medium was replaced by fresh one with or without Ova peptide (1 μg/ml).

NPCs ISOLATION

In order to isolate liver non-parenchymal cells the livers were perfused with 10ml Liver Perfusion Medium and 5 ml of Liver Digest Medium (Gibco). Isolated livers (without gall bladder) were then cut into small pieces and incubated in 10 ml of Liver Digest Medium in 37 °C for 30 min to complete the digestion. Digested tissue was minced though 100 μm cell strainer. Cell suspension was centrifuged twice at low speed ($52 \times g$) for 5 min. Each time the supernatant enriched in NPCs was collected whereas hepatocyte fractions located in pellets were discarded. Next collected NPCs fraction was further purified by centrifugation (836xg 20min, lowest acceleration and deceleration) in 33% percoll[3]. After centrifugation only pelleted fraction was collected, washed in PBS 1% FCS and treated for 5 min with erythrocyte lysis buffer with subsequent washing step.

Isolated NPCs fractions that possess antigen cross presenting potential were confirmed to comprise Kupffer cells (CD11b, F4/80 positive), liver sinusoidal endothelial cells LSECs (CD146 positive), hepatic stellate cells HSCs (specific autofluorescence induced by violet laser 405nm), dendritic cells DC (CD11c positive)(data not shown).

Isolated NPCs were counted and seeded $1{,}25x10^5$ on gelatin coated well of 96 well plate in 200μl RPMI Medium 1640 - GlutaMAX^{TM}-I (Gibco), supplemented with 10% fetal bovine serum (Biochrom), 100 U penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 2-Mercaptoethanol with or without Ova peptide (1 μg/ml). Similarly splenocytes and celiac lymph node cells were seeded (uncoated plates).

COCULTURE

After 4 h of Ova peptide pulsing (37 °C humidified cell culture incubator), hepatocytes, NPCs, splenocytes, cLN cells were washed 3 times with complete RPMI medium (as above, without Ova peptide). Next, CFSE labeled (0,5 μM) OT-I CD8 T cells were added to the washed cultures; 1×10^5 cells to 96 format well and 3×10^5 cells to 48 format well. After 36 h of coculture OT-I cells were harvested and stained with antibodies against CD8 and Thy1.1 to depict CFSE labeled OT-I T cells.

b

OT-I % of lymphocytes (OVA × Cre +TAM)

CD8 Thy1.1
15,5%

OT-I % of lymphocytes (OVA × Cre -TAM)

CD8 Thy1.1
8,14%

OT-I % of lymphocytes (OVA +TAM)

CD8 Thy1.1
1,15%

Thy1.1

CD8

OT-I % of lymphocytes (OVA × Cre +TAM)

OT-I % of lymphocytes (OVA × Cre -TAM)

OT-I % of lymphocytes (OVA +TAM)

Liver cLN spleen pLN mLN blood

% of CD69+ OT-I (OVA × Cre +TAM)

% of CD69+ OT-I (OVA × Cre -TAM)

% of CD69+ OT-I (OVA +TAM)

Liver cLN spleen pLN mLN blood

CD69 geom. MFI of OT-I (OVA +TAM)

CD69 geom. MFI of OT-I (OVA +TAM)

CD69 geom. MFI of OT-I (OVA +TAM)

Liver cLN spleen pLN M6 mLN blood

% of CD62L high + OT-I (OVA × Cre +TAM)

% of CD62L high + OT-I (OVA × Cre -TAM)

% of CD62L high + OT-I (OVA +TAM)

Liver cLN spleen pLN mLN blood

Figure S3b Lack of aberrant OVA presentation: Early activation of OT-I cells is restricted to liver. In order to exclude the possibility of initial OT-I T cells activation after adoptive transfer in organs other than liver as consequence of cross presentation or leaky and organ unspecific expression, we performed expression analysis of very early activation marker CD69 as introduced by Bertolino et al.[4] and Isogawa et al.[5] OVA X CreER^{T2} mice -/+ Tam and controls, single transgenic OVA mice + Tam were transferred with 6x10^{6} OT-I CD8 T cells (isolated by CD8 negative selection kit, Miltenyi) and sacrificed 1,5 h after adoptive transfer. Non parenchymal liver cells (NPCs) containing the lymphocyte fraction as well as lymphocytes from spleen, blood, inguinal lymph node (pLN), celiac lymph node (cLN), mesenteric lymph node (mLN) were isolated and stained with antibodies against CD8, Thy1.1 (to gate for OT-I cell) and CD 69, CD62L to determine the activation status. The upper panel shows representative dot plots indicating CD8 Thy1.1 gate on liver NPCs. The lower panels shows in turn, frequencies of OT-I cells reisolated form different organs, their CD69 expression measured as % of CD69 positive cells OT-I cells or as geometric mean fluorescence intensity. The bottom panel shows dowregulation of CD62L marker on OT-I cell. The data indicate that transferred OT-I cells accumulate and become activated within 1,5h exclusively in liver, 90-95% of OT-I T cells found in the liver express activation marker CD69, at the same time completely downregulating CD62L. Minimal increase of CD69 geom. MFI in the liver compartment of non expressing OVA mice in comparison to cell isolated from other organs was expected according to previous reports[4].

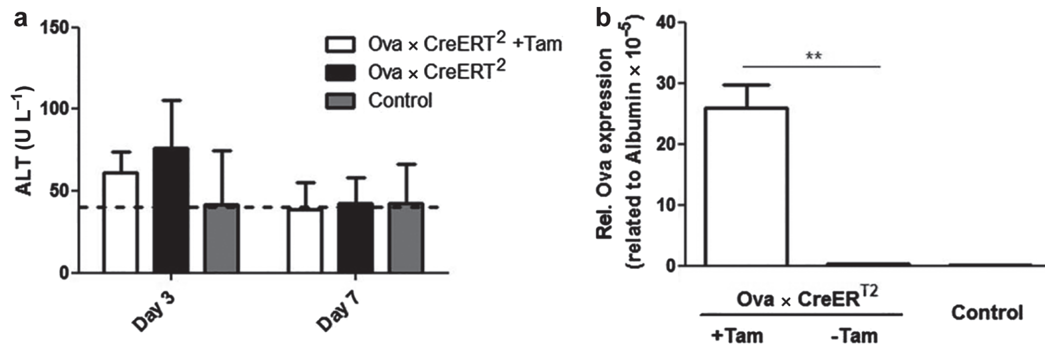


Figure S4 Pre-activated OT-I cells do not reduce high antigen load within the liver. OT-I cells were cultured in the presence of 3 μg/ml ova peptide in vitro for 2 days. 5×10^6 pre-activated OT-I cells were adoptively transferred to Tam treated or non treated Ova x $CreER^{T2}$ mice, respectively. (**a**) 3 and 7 days upon adoptive transfer serum ALT was analyzed to assess liver damage. The dashed line indicates the threshold of physiological ALT values (40 U/L). (**b**) Ova expression within the liver was determined on day 13 by qRT-PCR. n $\geqslant$ 4 mice/group ±SD. Shown is one representative experiment.

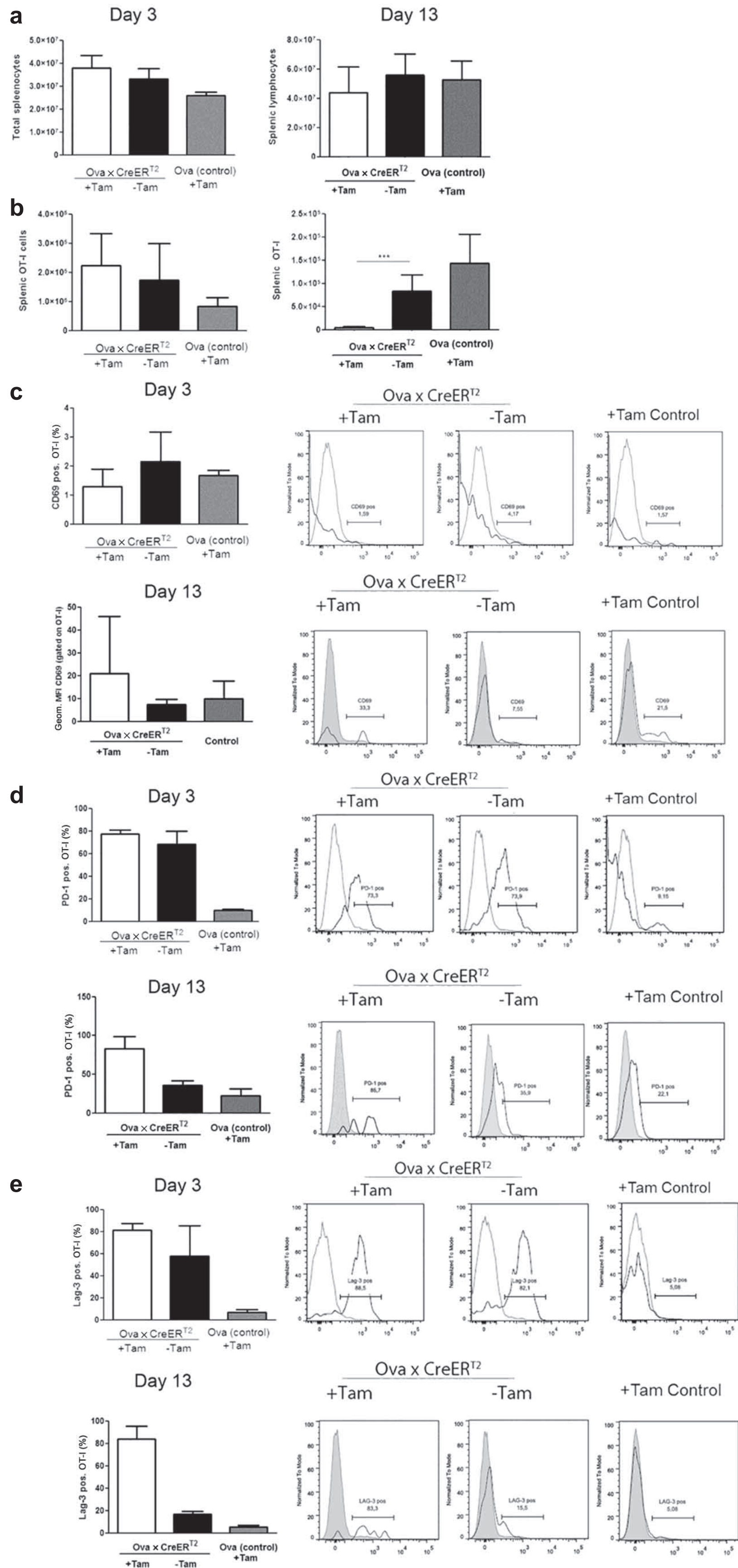

a
Day 3
Day 13
Total spleenocytes
Splenic lymphocytes
Ova x CreER^{T2}
Ova (control)
+Tam
-Tam
b
Splenic OT-I cells
Splenic OT-I
c
CD69 pos. OT-I (%)
+Tam Control
Geom. MFI CD69 (gated on OT-I)
Control
d
PD-1 pos. OT-I (%)
e
Lag-3 pos. OT-I (%)

Figure S5 High antigen load impairs the maintenance of antigen specific CD8+ T cells and induces systemic exhaustion. Total spleen resident lymphocytes (**a**) and antigen specific Thy1.1+ OT-I cells (**b**) were calculated on day 3 and 13 upon adoptive transfer. Additionally, at both time points spleen resident Thy1.1+ OT-I cells were investigated by flow cytometry for the expression of the activation marker CD69 (**c**) and the exhaustion markers PD-1 (**d**) and Lag-3 (**e**). Marker expression is given by representative histograms of each group. The marker expression of OT-I cells (black lines) was compared to unspecific CD8+ positive T cells (grey). Determination of the geometric mean fluorescent intensity from $n \geqslant 4$ mice/group was performed for quantification. This experiment was repeated three times.

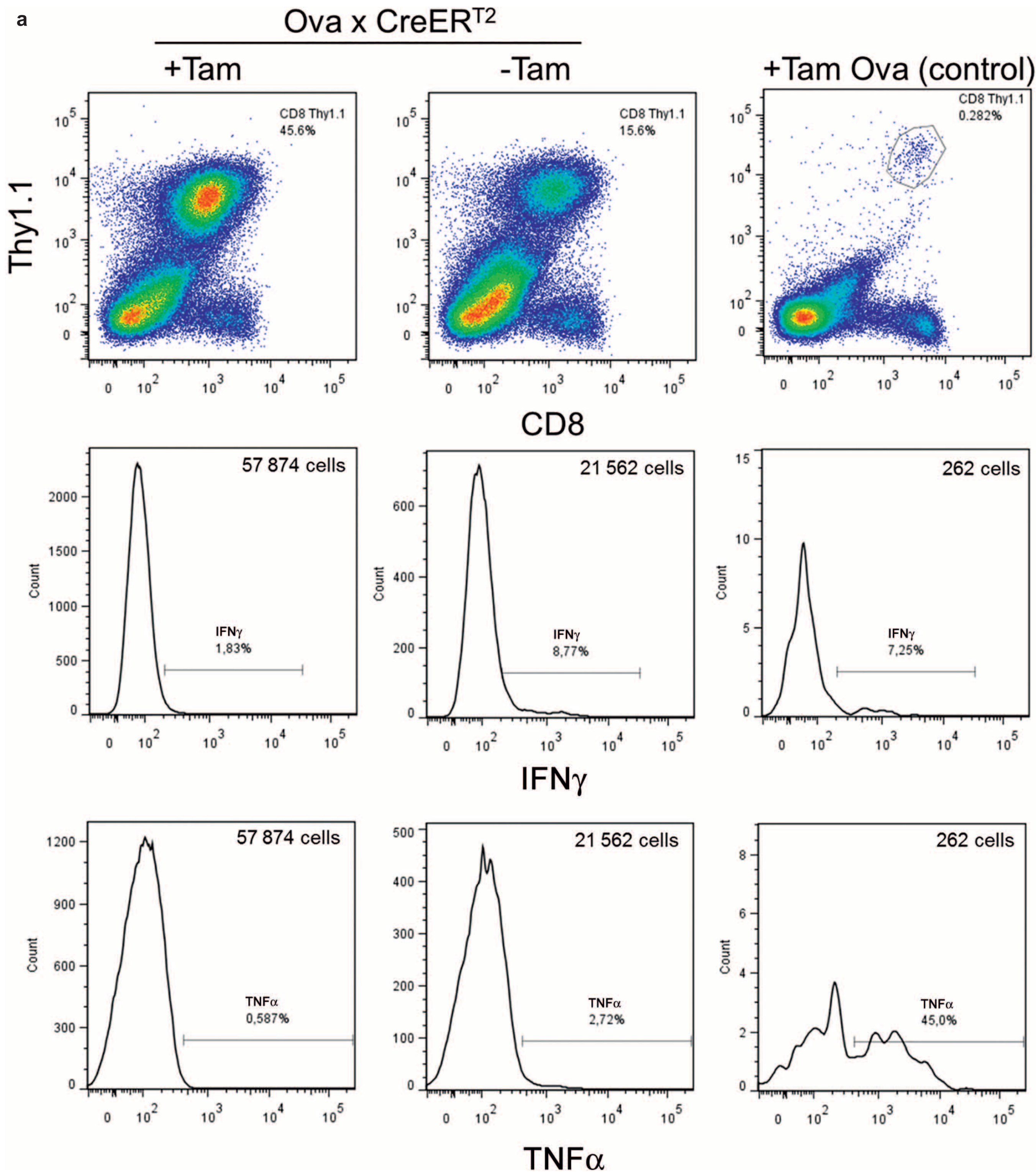


Figure S6a Representative dot plots and histograms of intracellular staining. (**a**) Representative FACS dot plots of the experiment presented in Figure 3a. Adoptively transferred OT-I cell (CD8, Thy1.1 gate) were reisolated on day 3 post adoptive transfer and stimulated in vitro with Ova peptide for 7 h. It is of note that in single transgenic Ova (control) mice which do not express Ova antigen, the OT-I T cells infiltrate the liver minimally and in an antigen unspecific manner and are still responsive to Ova stimulation. In contrast, high antigen mice have a reduced but still visible capacity to respond to Ova stimulation in vitro. Thus, the degree of the OT-I T cell responsiveness inversely correlates with the Ova antigen level, i.e. with the frequency of Ova expressing hepatocytes. In an independent experiment we titered the Tamoxifen dose to achieve gradual increase of Ova expressing hepatocyte fraction. We could show gradual loss of functionality (IFNg and TNFa expression) of OT-I T cells reisolated form livers of diversely induced mice (data not shown).

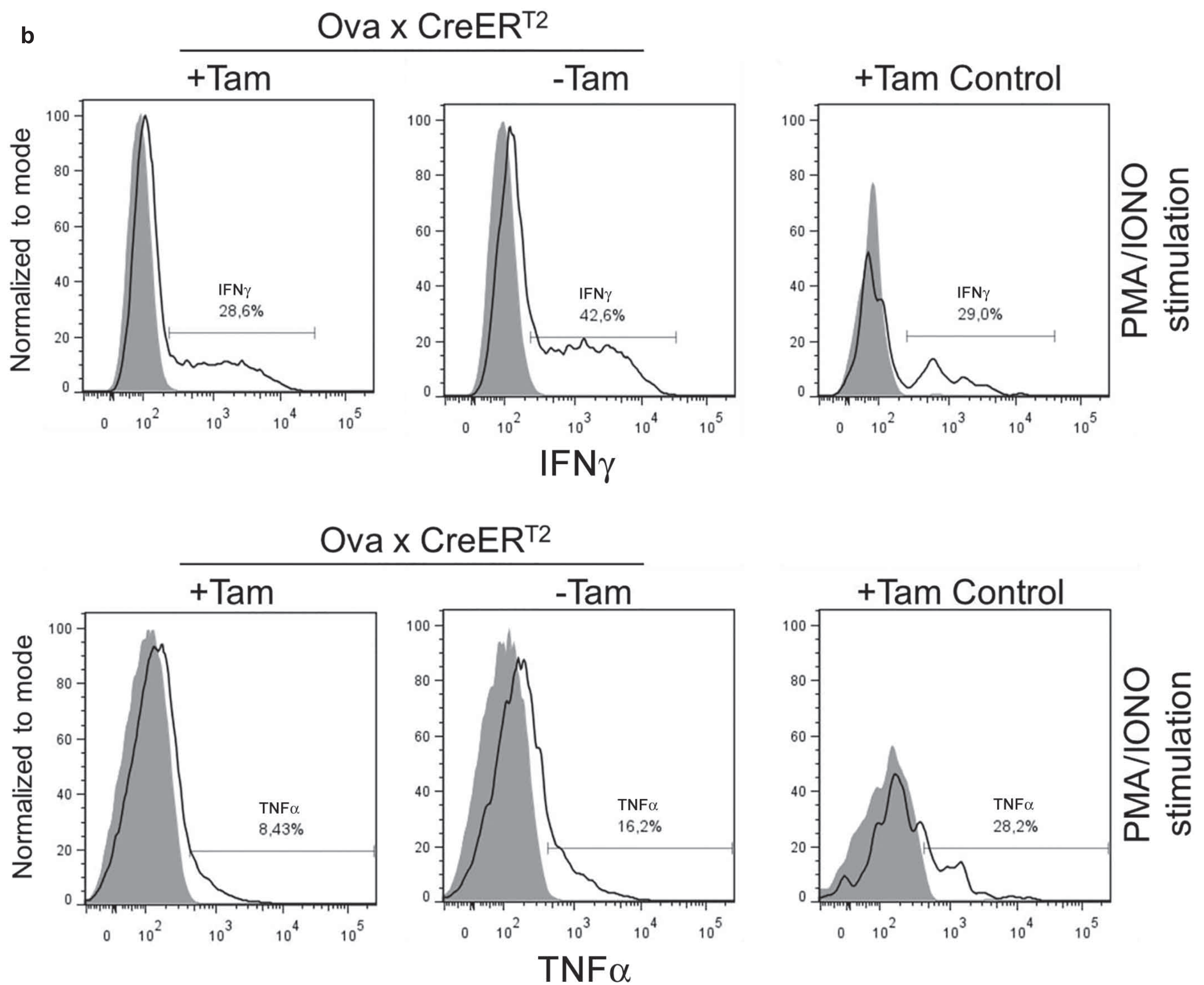


Figure S6b (**b**) As a control, OT-I T cells reisolated on day 3 post adoptive transfer were stimulated with PMA/ionomycin. Similarly to Ova stimulation we could observe antigen dose dependent impairment of OT-I T cells functionality. The representative histograms depict the OT-I T cell fraction gated for CD8, Thy1.1. Black line histograms refer to samples treated with PMA/ionomycin whereas filled histograms show same/respective samples without any stimulation.

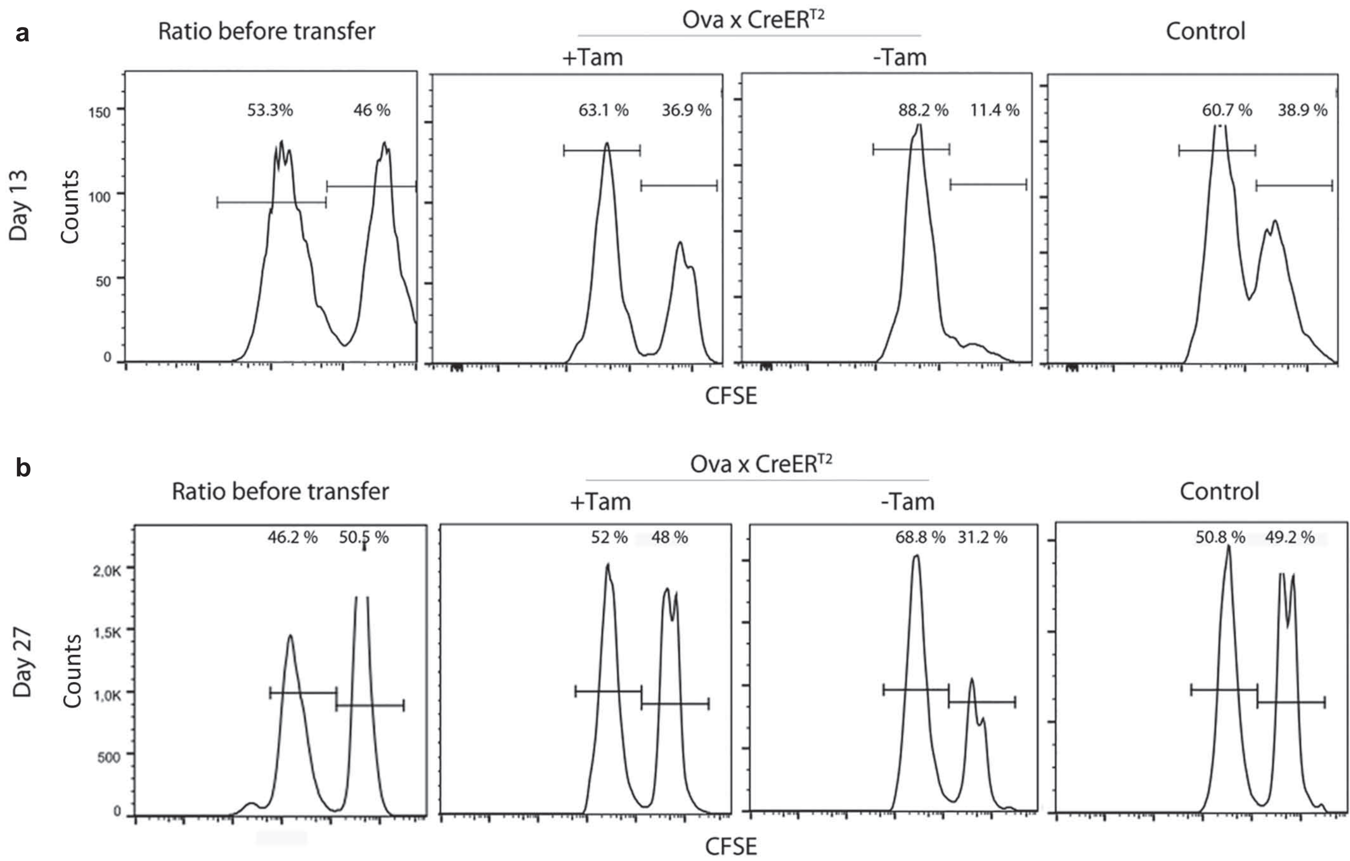


Figure S7 Efficient killing of Ova pulsed target cells in the periphery upon clearance of low antigen. Histograms are presented which are representative for the group of mice depicted in Fig 3B. The histograms depict CFSE^{hi}(Ova pulsed) and CFSE^{lo}(unpulsed) cells which were transferred into Ova X CreER^{T2} mice at day 13 (**A**) and day 27 (**B**) after OT-I transfer. The presented figure shows one out of two independent experiments.

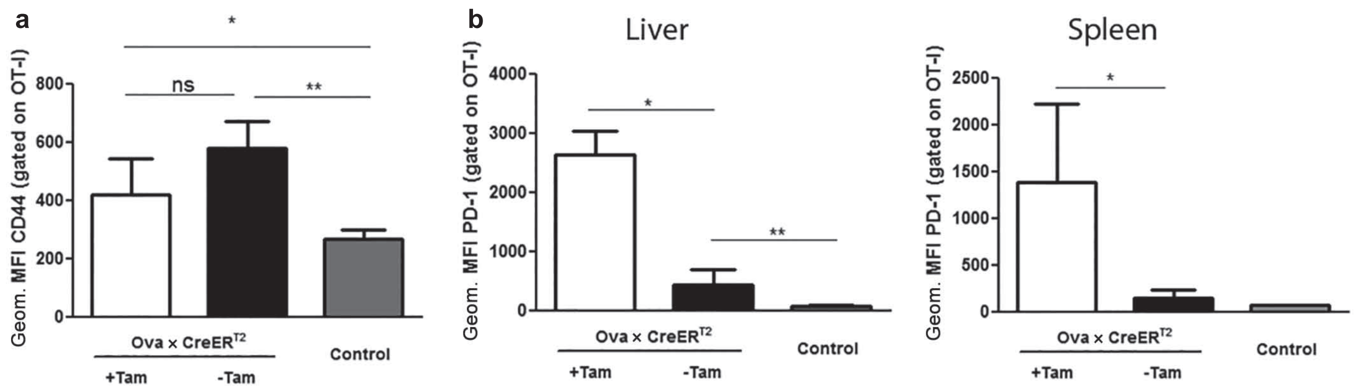


Figure S8 High intrahepatic antigen burden drives T cell exhaustion. 5×10^6 naïve OT-I cells were adoptively transferred to low antigen expressing Ova x CreER^{T2} mice. On day 13, when low antigen levels were cleared, a subset of mice was treated with 50 μg Tam to induce high intrahepatic antigen load. (**a**) 13 days upon induction of high antigen load, liver resident OT-I were analyzed for the expression of CD44, as represented by the geometric mean fluorescence intensity (MFI). (**b**) On day 13 liver and spleen localized OT-I cells were analyzed for the expression of PD-1, as represented by the geometric MFI. n $\geqslant$ 3 mice ±SD. Shown is one of two independent experiments.

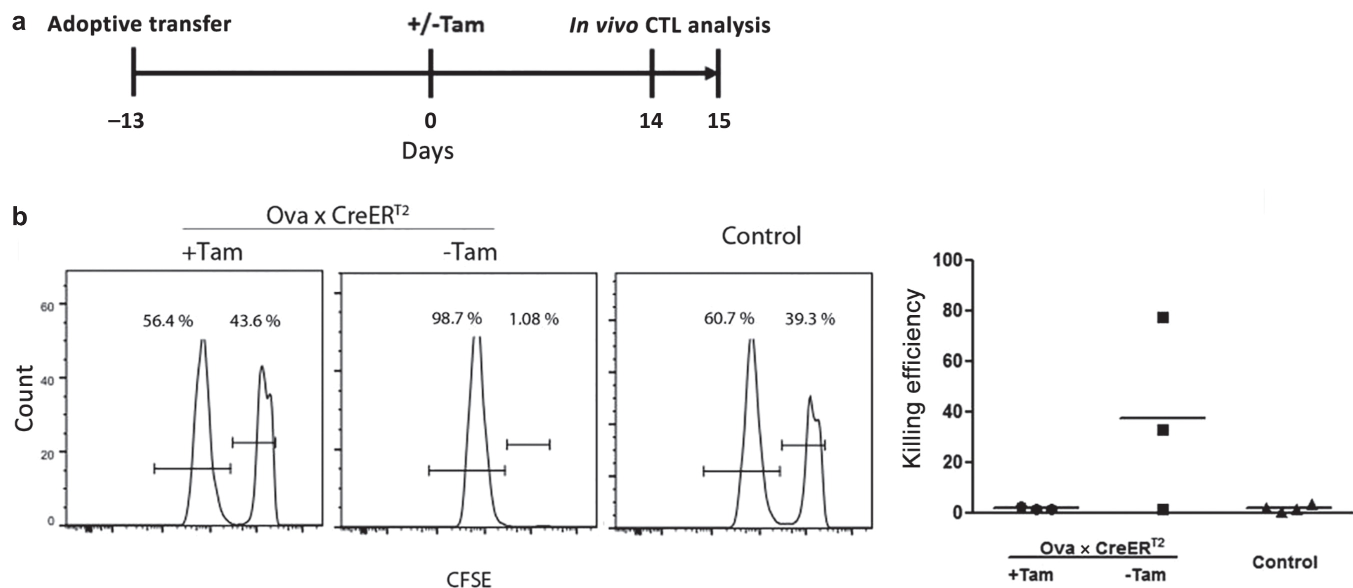


Figure S9 High antigen load induces systemic tolerance of functional effector T cells. (**a**) Experimental scheme of subsequent high antigen challenge: Ova × CreER^{T2} mice were adoptively transferred with 5×10^6 naïve OT-I cells on day -13. On day 0, mice were treated with Tam to induce high antigen expression in the liver. On day 14 recipient mice were challenged with CFSE labeled splenocytes. (**b**) Evaluation of the killing potential of OT-I cells primed by low intrahepatic antigen and exposed to high antigen load on day 0. Representative FACS plots (left panel) and quantification (right panel) of $n \geqslant 3$ mice/group is shown.

1 Ebrahimkhani, MR, Mohar, I, and Crispe, IN (2011). Cross-presentation of antigen by diverse subsets of murine liver cells. *Hepatology* **54**: 1379–1387.
2 Godoy, P, Hewitt, NJ, Albrecht, U, Andersen, ME, Ansari, N, Bhattacharya, S, *et al.* (2013). Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of toxicology* **87**: 1315–1530.
3 Kinoshita, M, Uchida, T, Sato, A, Nakashima, M, Nakashima, H, Shono, S, *et al.* (2010). Characterization of two F4/80-positive Kupffer cell subsets by their function and phenotype in mice. *Journal of hepatology* **53**: 903–910.
4 Bertolino, P, Bowen, DG, McCaughan, GW, and Fazekas de St Groth, B (2001). Antigen-specific primary activation of CD8+ T cells within the liver. *Journal of immunology* **166**: 5430–5438.
5 Isogawa, M, Chung, J, Murata, Y, Kakimi, K, and Chisari, FV (2013). CD40 activation rescues antiviral CD8(+) T cells from PD-1-mediated exhaustion. *PLoS pathogens* **9**: e1003490.