UNIVERSITY OF CALIFORNIA SAN DIEGO

# Defining the requirement for peroxisomes during $CD8^+$ T cell response to viral infection

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The thesis of Natalia Avina Ochoa is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

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# DEDICATION

To my family, friends, and mentors

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ABSTRACT OF THE THESIS

Defining the requirement for peroxisomes during $CD8^+$ T cell response to viral infection

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The transition from naïve to effector $CD8^+$ T cells is marked by metabolic reprograming that requires the interplay of different organelles to maintain cellular homeostasis amid changing intracellular demands and extracellular conditions. Previous studies have demonstrated that peroxisomes have important functions in fatty acid oxidation, regulating reactive oxygen species, and propagating type I interferon signals. However, up to this point, little is known about the importance of this organelle in $CD8^+$ T cells. Thus, this thesis addresses this gap in our understanding of peroxisomes in $CD8^+$ T cell development and function.

To address this, we generated a mouse model of peroxisome deficient $CD8^+$ T cells and analyzed their development both in-vitro and during acute and chronic lymphocytic chronic meningitis virus (LCMV) infection. Peroxisome-deficient $CD8^+$ T cells behaved identically to wild-type controls in-vitro. However, in-vivo, the lack of this organelle resulted in a significant impairment of their ability to survive beyond day four post-infection. Subsisting peroxisome-deficient $CD8^+$ T cells retained their ability to undergo normal proliferation, function, and differentiation into effector and memory lineages. These results show that peroxisomes are required for viability in-vivo. Further research needs to be conducted to uncover the mechanisms by which peroxisome deficiency is detrimental to $CD8^+$ T cell survival.

## Introduction

The immune system is composed of two branches: innate and adaptive, which together mediate rapid and long-term responses to infectious pathogens. Pathogens first trigger an innate response, wherein macrophages, neutrophils, dendritic cells, and other innate cell types are recruited to induce inflammation at the site of infection and promote activation of the adaptive immune response, which is orchestrated by T and B cells (Medzhitov, 2007). In response to viral infections, cytotoxic T cells expressing cluster of differentiation 8 (CD8), are activated to eliminate infected cells and halt viral propagation. T cell activation is a tightly regulated, multistep process consisting of three sequential signals. The primary signal is the recognition of an antigen-major histocompatibility complex (MHC) by the cognate T cell receptor (TCR), the second is provided by binding of co-stimulatory molecule CD80 or CD86 on antigen presenting cells to CD28 on T cells, and the last consists of the exposure to fate-determining cytokines (Guerder & Flavell, 1995). In the context of an acute infection, activated $CD8^+$ T cells undergo a rapid expansion and differentiate into effector cells, which mediate elimination of infected cells through the release of cytotoxic molecules such as perforin and granzyme B. Following antigen clearance, effector $CD8^+$ T cells undergo a contraction phase and a small fraction of the population differentiates into long-lived memory T cells to protect the host against reinfection (Kaech & Cui, 2012).

In addition to a myriad of signaling events, $CD8^+$ T cell differentiation is also marked by metabolic reprograming to meet the bioenergetic demands associated with each state (Peng et al., 2021; Zhao et al., 2021). Naïve T cells in a quiescent state with low anabolic needs mainly generate energy for homeostasis through mitochondrial oxidative phosphorylation (OXPHOS) (Menk et al., 2018). Upon activation, T cells transition into a highly anabolic state by increasing

the import of glucose through the GLUT1 transporter to fuel glycolysis and the citric acid (TCA) cycle to provide biomass for rapid expansion (Frauwirth et al., 2002). This in turn produces byproducts such as reactive oxygen species (ROS) which further bolster activation signals (Sena et al., 2013). As the antigen is cleared, a subset of activated T cells differentiates into a memory lineage, wherein metabolism is again rewired as anabolic demands decrease and a shift towards energy production for long-term homeostasis occurs. Under these conditions, fatty acid oxidation (FAO) becomes a major source of energy as established by studies using pharmacological inhibition of the rate limiting enzyme Carnitine Palmitoyltransferase 1 (CPT1) (Pearce et al., 2010; van der Windt et al., 2012), which facilitates fatty acid transport into the mitochondrial matrix to undergo oxidation. However, the requirement for CPT1-dependent mitochondrial FAO in memory T cells remains inconclusive (Raud et al., 2018), raising the possibility of other unrecognized contributions to FAO in T cells. For example, medium-chain and short-chain fatty acids can enter the mitochondrial matrix independently of CPT1 to fuel FAO (Knottnerus et al., 2018). Moreover, the potential contribution to FAO by other organelles in T cells has not been thoroughly examined.

Peroxisomes are ubiquitous, single-membrane bound organelles with numerous roles in cellular metabolism and regulating oxidative stress **(Fig. 1)**. Peroxisomes can perform both alpha- and beta-oxidation of fatty acids depending on the structure of the fatty acid (R. J.A. Wanders et al., 2010). Of note, peroxisomes are required to initially catabolize very long chain fatty acids (VLCFAs) to lengths suitable for undergoing complete oxidation in the mitochondria (Tahri-Joutey et al., 2021). In addition, peroxisomes can oxidize medium- and long-chain fatty acids in the absence of functional mitochondrial FAO (Violante et al., 2019), suggesting that they may support T cell metabolism at various stages of differentiation. Additionally,

peroxisomes in conjunction with the lysosome are required for cholesterol metabolism and are also essential for the breakdown of pro-inflammatory lipids such as arachidonic acid (Chu et al., 2015; Ferreira et al., 2019). A significant byproduct of these metabolic processes is hydrogen peroxide ($H_2O_2$), from which peroxisomes were named. As a result, peroxisomes also contain neutralizing antioxidant enzymes, such as catalase, to regulate cellular levels of ROS (Forrester et al., 2018). In addition to these catabolic processes, peroxisomes also serve as the site of ether lipid synthesis, which are required for membrane synthesis and cell differentiation (Lodhi & Semenkovich, 2014).

In humans, genetic mutation of core peroxisomal genes results in a family of peroxisomal biogenesis disorders that commonly manifest with severe neuronal pathology (Waterham et al., 2016). Thus, the contribution of peroxisomes to cellular metabolism and function have been largely characterized in the brain and metabolic tissues such as the liver (Dirkx et al., 2005; Faust et al., 2005). However, peroxisomes have also recently been demonstrated to be essential in multiple innate immune cell types. For example, knockout of the peroxisomal ether lipid synthesis enzyme Peroxisome Reductase Activating PPARγ (PexRAP) results in neutrophil apoptosis and neutropenia (Lodhi et al., 2015). Likewise, knockout of the peroxisomal ether lipid synthesis enzyme Glyceronephosphate O-acyltransferase (GNPAT) impairs production of endogenous lipid ligands for the CD1d receptor, resulting in less proliferation and impaired maturation of invariant natural killer T (iNKT) cells (Facciotti et al., 2012). Peroxisomes associate with ATP-dependent motor proteins to traffic throughout cells (Rapp et al., 1996), and have been shown localize to the phagosome to facilitate its function; genetic inhibition of peroxisomal biogenesis results in impaired bacterial engulfment by macrophages, leading to increased susceptibility to infection (Di Cara et al., 2017). Yet, despite recent advances in our

understanding of the peroxisome in innate immunity, its importance in T cells has remained a mystery. Thus, given the myriad of metabolic reactions associated to peroxisomes and their described importance in innate immune cells, we hypothesized that peroxisomes are required for CD8+ T cell development and function.

## Materials and Methods

### Mice

Mice were housed in a pathogen-free facility under Institutional Animal Care and Use Committees (IACUC) of the Salk Institute for Biological Studies guidelines. Wild type C57BL/6J (B6) mice were purchased from Jackson Laboratory. LCMV GP 33-41 TCR-transgenic P14 ($P14^{+}$) mice were used for the adoptive transfer experiments. The congenic markers Thy1.1 and Thy1.2 were used to differentiate wild-type and Pex5KO.

### Generation of $Pex5^{fl/fl}$ GranzymeB-Cre LCMV GP 33-41 TCR-transgenic P14 mice

Mice were generated by interbreeding of GranzymeB-Cre $P14^{+}$ Thy1.1/1.1 mouse with a Pex5 fl/fl strain on a C57BL/6J background (strain #031665) purchased from Jackson Laboratory. The loxP sites are located between exon 11 and 14, which encodes a domain crucial for the gene function of this component of the peroxisomal protein import system.

### Splenocyte Isolation

Spleens were resected from mice and processed by filtering them through a 70uM nylon cell strainer with RPMI (Gibco), followed by treatment with Ammonium-Chloride-Potassium lysing buffer (ACK; Fischer Scientific) to eliminate red blood cells.

### CD8+ T cell enrichment

The cells were incubated in MACS Buffer (500ml 1x DPBS, 1mM EDTA, and 2% BFA) and normal Rat Serum (Jackson Immunology). The following biotinylated antibodies were added: anti-CD4, anti-CD11b, anti-CD11c, anti-CD49b, anti-Ter119 (all from Biolegend). CD8+

T cells were isolated using MojoSort Streptavidin Nanobeads (Biolegend) and an EasySep Magnet (Stemcell Technologies).

**T cell Culture**

Cells were cultured in T cell Media (500ml RPMI, Thermo Fischer Scientific; 100mM Sodium Pyruvate, Thermo Scientific; 25mM HEPES, Thermo Scientific; 5ml Penicillin-Streptomycin, Sigma Aldrich; and 500ul Beta Mercaptoethanol, BIORAD) and 1ug/ml of recombinant murine IL-2 (Prepo Tech).

**Cell staining and flow cytometry**

Cells were labeled with CellTrace Violet (Thermo Fisher Scientific) before adoptive transfer for the cell proliferation assay. Upon tissue collection cells were stimulated for 4hrs with 10ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1mg/ml ionomycin (Cell Signaling Technology), and 5mg/ml Brefeldin A (BFA; BioLegend). Cell viability was assessed using Zombie Aqua Fixable Viability Kit (BioLegend). Cells were stained with PE anti-mouse CD44, BUV395 anti-mouse Cd62L, PE anti-mouse CD69, PE-Cy7 anti-mouse CD25, BUV737 anti-mouse CD8a, PerCP-Cy 5.5 anti-mouse Thy1.2, PE-Cy7 anti-mouse Thy1.1, PE anti-mouse CD127, FITC anti-mouse klrg1, and BV510 anti-mouse PD1 (BioLegend) antibodies. Staining was performed in a 1.2% FBS in PBS solution for 1hr. The cells were fixed and permeabilized by resuspending them in a Fixation/Perm diluent and Fixation/Permeabilization Concentrate (Invitrogen eBioscience) solution for 30min. Intracellular staining using BV711 anti-mouse IFN-y (Biolegend), BV785 anti-mouse TNFa (Biolegend), and AF647 anti-mouse PMP70 (Thermo Scientific) in 1X Perm/Wash Buffer (BD Biosciences). Cells were maintained in staining buffer

(2% FBS in 1X Dulbecco's Phosphate Buffered Saline (DPBS)) until flow cytometry analysis. Compensations were calculated using UltraComp eBeads Compensation beads stained with matching single fluorophores (Invitrogen).

**Western Blotting**

Whole-cell lysates of the Pex5KO and WT CD8+ T cells were obtained using Pierce RIPA buffer (Thermo Scientific) supplemented with 1X cOmplete EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich). The supernatant was collected and quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were prepared using 4X LDS Sample Buffer (Invitrogen by Thermo Fischer Scientific), and heated to 95$^{o}$C for 5 minutes. The samples were loaded into a 4-15% precast gel (Biorad) along with a Precision Plus Protein Dual Color Standards (Biorad) and run in 1X running buffer (10x = 0.25M Tris base, 1.9M Glycine, and 0.035M SDS). The gel was transferred into a membrane for protein blotting (Biorad) using 1x Transfer buffer (10x = 0.25M Tris base and 1.9M Glycine). The membrane was blocked with a 5% Bovine serum albumin (BSA; Sigma Aldrich) in 1% Tween-20 solution in DPBS. The blot was posteriorly washed with a 0.11% Tween-20 solution in 1X PBS and incubated overnight with a solution made of 1:1000 anti-Pex5 Rabbit (Proteintech) or S6 (Cell Signaling) in 1% BSA in 0.1% Tween-20 solution in PBS. The gel was then incubated with a 1:10,000 solution of Goat anti-Rabbit IgG Secondary Antibody (Invitrogen) in PBS. The blot was developed using Super Signal West Pico Plus Chemiluminescent Substrate Kit (Thermo Scientific), and imaged on a Biorad imager.

**CD8+ T cells adoptive transfer and LCMV infection**

Recipient C57BL/6J Thy1.2/1.2 mice were injected retro-orbitally with 50,000 total P14+ naïve CD8+ T cells in a 1:1 ratio (WT:Pex5KO) for day 4, 5, and 8 timepoints. For the CTV experiment, 2 million total naïve $P14^+$ $CD8^+$ T cells were injected retro-orbitally in a 1:1 ratio (WT:Pex5KO) into a B6 Thy1.2/1.2 mice. The mice were injected intravenously with $2x10^5$ plaque forming units (pfu) LCMV Armstrong 24 hours after the adoptive transfer.

For the in-vitro activated experiment, $P14^+$ $CD8^+$ T cells of each genotype (WT or Pex5KO) were activated with gp33 and IL2 in-vitro and maintained in culture for 3 days. 200,000 total cells were then injected retro-orbitally into a recipient B6 Thy1.2/1.2 mice in a 1:1 ratio. The mice were injected intraperitoneally with $2x10^5$ plaque forming-units (pfu) of LCMV Armstrong virus the same day.

**CD8+ T cells adoptive transfer and Clone 13 infection**

5,000 total naïve CD8+ T cells were injected retro-orbitally into a recipient B6 Thy1.2/1.2 mice in 1:1 ratio (WT:Pex5KO). The mice were infected retro-orbitally with $2x10^6$ focus forming units (ffu) the following day.

**Data Analysis**

Flow cytometry data was analyzed using FlowJo v.9.9.4 (TreeStar) and statistical analyses were performed using Prism 7 (GraphPad Software INC)

## Results

### Peroxisomal content is increased upon activation in CD8+ T cells

To determine the importance of peroxisomes in $CD8^+$ T cell development, we first assessed how activation influences peroxisomal content. We isolated and cultured wildtype (WT) CD8+ T cells in the naïve state or activated with anti-CD3/CD28 and IL-2 in-vitro. The samples were analyzed after 48 hours, and CD44 expression was used to verify the naïve or activated status of the cells. To assess peroxisomal content, we utilized the peroxisomal membrane protein PMP70 or ABCD3, a transporter present in the peroxisomal membrane (Imanaka et al., 2000), as a marker. We observed that peroxisomal content increases upon $CD8^+$ T cell activation **(Fig. 3A)**. This is consistent with proteomics analysis by the Immunological Proteome Resource (ImmPRes; http://immpres.co.uk), which identified elevated protein content of genes involved in peroxisome biogenesis (PEX5, PEX13, PEX14, PEX6, and PEX26), peroxisome fatty acid transport (ABCD1 and ABCD3), and peroxisome metabolism (ACOX1, GNPAT, and AGPS) in activated CD8+ T cells as compared to naïve **(Fig. 3B)**. Conversely, protein content of L-selectin (CD62L) and the interleukin 7 receptor (IL7R) decreases following TCR activation **(Fig. 3B)**, as has been previously reported (Chao et al., 1997; Xue et al., 2002). Thus, peroxisome content increases in $CD8^+$ T cells shortly after activation.

### Pex5 deletion does not affect $CD8^+$ T cell activation and differentiation in-vitro

Given that peroxisomal content is increased upon activation in-vitro, we next sought to determine the effects of peroxisome deficiency in $CD8^+$ T cells. To create a model that interferes with peroxisome biogenesis, we utilized a conditional knockout mouse model of the peroxisomal importer Peroxin 5 (PEX5). PEX5 transports newly synthesized peroxisomal proteins containing

a peroxisomal targeting signal 1 (PTS1), which encompasses ninety-percent of peroxisomal enzymes (Wimmer et al., 1998), to the peroxisome lumen **(Fig. 2)**. Consequently, ablation of PEX5 results in the lack of functional peroxisomes (Baes et al., 1997; Baumgart et al., 2001; Meinecke et al., 2016). To utilize this model as an investigational tool of peroxisomal function in $CD8^+$ T cell differentiation and function, we crossed to mice expressing Cre-recombinase under the control of the Granzyme B promoter (Jacob & Baltimore, 1999) to achieve deletion of Pex5 in activated $CD8^+$ T cells, hereinafter referred to as Pex5KO. We additionally crossed in a transgenic T cell receptor against the LCMV antigen, gp33-41, referred to as P14 (Pircher et al., 1987) to study antigen-specific responses. Analysis of protein levels from P14 Pex5KO $CD8^+$ T cells 5 days after in-vitro activation with gp33 peptide and IL-2 revealed a significant ablation of Pex5 expression when compared to control **(Fig. 3C)**, confirming that our model successfully knocks out the *Pex5* gene upon activation.

To understand the role of peroxisome deficiency in $CD8^+$ T cell development, we activated WT and Pex5KO cells with anti-CD3/CD28 antibodies and IL-2 in-vitro. The frequency of live Pex5KO $CD8^+$ T cells was similar to WT at day 2 and 4 post activation **(Fig 3E)**, indicating that peroxisomes are not essential for viability in-vitro. Additionally, proliferative capacity and the expression of activation markers such as CD44, CD25, and CD69 were unaffected by the loss of Pex5, nor the frequencies of effectors ($CD44^+$ $CD62L^-$) and memory precursors ($CD44^+$ $CD62L^+$) between the two genotypes (Gerberick et al., 1997) **(Fig. 3D, 3F, and 3G)**. To evaluate how peroxisomes affect $CD8^+$ T cell function, activated $CD8^+$ cells were activated and cultured in the presence of 10ng/ml of IL-2 for 72hrs. The cells were later cultured with 10ng/ml IL-2 alone or in combination with 50ng/ml of IL-12 for 48hrs, as IL-12 is a potent cytokine for promoting IFNy production (Henry et al., 2008). The cells where then stimulated

with 20 ng/mL PMA and 1 ug/mL ionomycin for 4hrs. Frequency of interferon gamma (IFN-y) and tumor necrosis factor alpha (TNF-a) producing cells were comparable to WT cells regardless of the cytokines present in culture, as well as total cytokine production **(Fig. 3H and 3I)**. These results demonstrate that peroxisomes are dispensable for $CD8^+$ T cell activation and function in-vitro.

**Peroxisomes are essential for antigen-specific $CD8^+$ T cell survival during acute infection**

Previous studies have shown that $CD8^+$ T cells activated in-vitro utilize glycolytic metabolism to a greater degree than those activated in-vivo (Ma et al., 2019). Thus, we hypothesized that Pex5 deficiency in the context of an in-vivo infection would have more deleterious effects. To test this, we transferred naïve $P14^+$ WT and Pex5KO $CD8^+$ T cells in a 1:1 ratio **(Fig. 4B)** into B6 mice and infected them with LCMV Armstrong to generate an acute infection; spleens were subsequently collected at day 4, 5, and 8 post infection **(Fig. 4A).** At day 4 post infection, Pex5KO $CD8^+$ T cells were present at similar frequencies to WT cells. However, Pex5KO $CD8^+$ T cells showed a striking decrease in frequency by day 8 post infection **(Fig. 4C)** as compared to WT, suggesting that peroxisome deficiency is detrimental to $CD8^+$ T cells in-vivo**.**

We next investigated whether the decrease in Pex5KO $CD8^+$ T cells was caused by an impairment in expansion following activation. To test this, naïve $P14^+$ WT and Pex5KO $CD8^+$ T cells were labeled with Cell Trace Violet (CTV) and transferred into B6 mice infected with LCMV Armstrong. No impairment in proliferation was observed at day 4 post-infection, and CTV dye was completely diluted out in both WT and Pex5KO $CD8^+$ T cells by day 5 post-infection **(Fig. 4D)**, when the decrease in Pex5KO frequency was first observable **(Fig. 4C)**.

Thus, we conclude that Pex5 KO cells undergo a similar initial proliferative burst as WT cells following activation, but fail to persist in-vivo**.** Despite the negative effect on survival**,** Pex5KO cells were able to differentiate normally, as the expression of CD44 and CD62L were similar between genotypes despite the disparity in overall cell numbers at day 8 post-infection **(Fig. 4E)**.

Similar to in-vitro, peroxisome deficiency did not suppress $CD8^+$ T cell function, as no impairment to cytokine production was observed in the remaining Pex5KO cells at day 8 post-infection **(Fig. 4F).** Thus, this data demonstrates peroxisome are not required for early proliferation following an acute infection, however, they are essential for $CD8^+$ T cell survival in-vivo.

**Survival defect in Pex5KO $CD8^+$ T cells follows proliferative burst**

Given that we observed similar viability between Pex5KO and WT CD8+ T cells following in-vitro activation, we hypothesized that Pex5KO $CD8^+$ T cells expanded in an in-vitro environment may persist in-vivo. To test this hypothesis, WT and Pex5KO $CD8^+$ T cells were activated with gp33 peptide in-vitro and expanded in culture for 3 days in media supplemented with IL-2. The cells were then transferred into B6 mice that were subsequently infected with LCMV Armstrong **(Fig. 5A)**. Although a greater number of Pex5KO $CD8^+$ T cells were initially transferred **(Fig. 5B),** these cells comprised only a minor frequency of total $CD8^+$ T cells at day 7 post infection **(Fig. 5C)**. However, no defect in differentiation was observed in the surviving Pex5KO $CD8^+$ T cells **(Fig. 5D).** This suggests the survival defect is caused by an event that occurs after activation and does not interfere with the initial proliferation outburst.

**Peroxisomes are dispensable for memory formation in CD8+ T cells**

$CD8^+$ T cell memory formation is accompanied by metabolic rewiring towards greater dependency on mitochondrial oxidative metabolism (van der Windt et al., 2012). As peroxisomes are an important counterpart to mitochondrial oxidative metabolism (Wanders et al., 2016), we examined whether Pex5 KO $CD8^+$ T cells are able to differentiate into memory cells. Although the formation of memory precursors ($CD44^+$ $CD62L^+$) $CD8^+$ T cells appeared normal in mice at day 8 post-infection **(Fig. 4E)**, we reasoned that because peroxisomal deficiency perturbs mitochondrial function there may be defects in memory formation at later time points (Peeters et al., 2015). To assess this, naïve or pre-activated WT and Pex5KO $CD8^+$ T cells were co-injected into mice that were infected with LCMV Armstrong, and splenocytes were isolate and analyzed over 40 days post-infection **(Fig. 6A)**. As in the effector timepoint, Pex5KO $CD8^+$ T cells were significantly reduced by the memory timepoint in comparison to WT **(Fig. 6B).** However, the remaining peroxisome-deficient cells displayed no defect in memory differentiation as measured by the frequency of $CD44^+$ $CD127^+$ cells (Joshi et al., 2011) **(Fig. 6C).** As in the effector timepoint, pre-activation of the cells in-vitro prior to adoptive transfer had no impact on their ability to survive after the contraction phase as shown by the decrease in the frequency of Pex5KO $CD8^+$ T cells in this scenario **(Fig. 6D).** Normal memory differentiation was also observed with pre-activated cells after accounting for the ratio of each genotype in the initial transfer mixture **(Fig. 6E).** These results suggest that although Pex5 is required for survival of antigen-specific $CD8^+$ T cells following an acute infection, memory formation itself is not impaired by peroxisomal deficiency.

**Peroxisome deficiency does not impair survival of antigen specific CD8+ T cells in chronic viral infections**

To assess the effect of peroxisome deficiency in $CD8^+$ T cell survival during chronic infection, we isolated naïve $P14^+$ WT and Pex5KO $CD8^+$ T cells and injected them in a 1:1 ratio into a B6 mouse infected with LCMV clone 13, which establishes a chronic infection (Ahmed et al., 1984). Splenocytes were isolate and analyzed at day 7 and 38 post infection **(Fig. 7A)**. Pex5KO $CD8^+$ T cells activated and expanded similarly to WT cells during the initial stages of infection, but eventually showed a defect in survival in chronic infection at day 38 **(Fig. 7B).** Nevertheless, cells showed normal differentiation into short lived effectors defined as $CD127^-$ $Klrg1^+$ (Herndler-Brandstetter et al., 2018) **(Fig. 7C)**. Additionally, peroxisome deficiency resulted in no alterations to the normal cell exhaustion exhibited as a result of clone 13 infection (Hudson et al., 2019), as similar frequencies of progenitor and transitory ($PD1^+$ $CD44^+$ $SlamF6^+$ and $PD1^+$ $CD44^+$ $CX3CR1^+$), as well as terminally exhausted T cells ($PD1^+$ $CD44^+$ $Tim3^+$ and $PD1^+$ $CD44^+$ $CD101^+$) were observed **(Fig. 7D**). These results indicate that peroxisomes are required for survival in both chronic and acute infection.

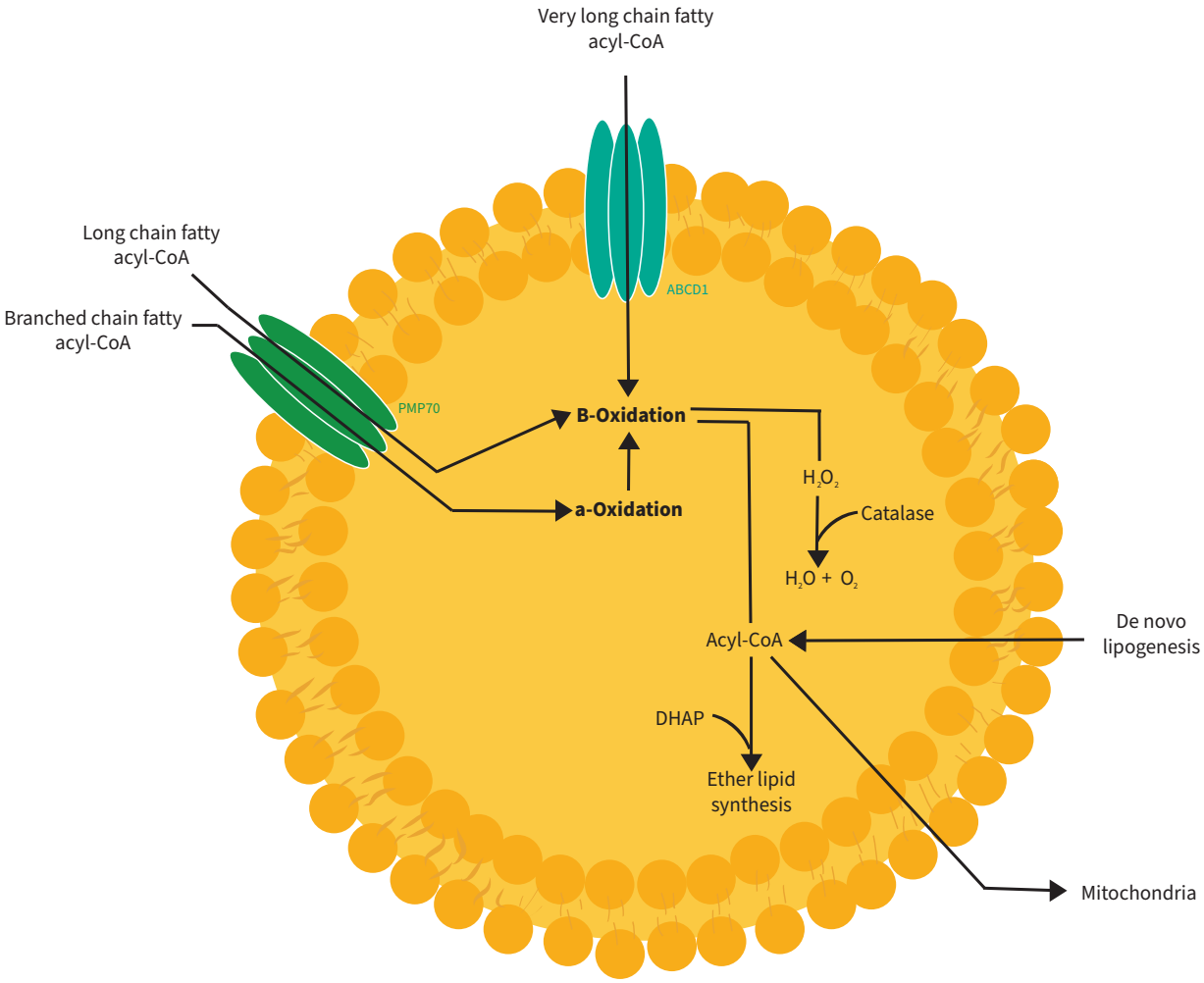


**Figure 1. Peroxisomes are complex metabolic organelles**
The peroxisome metabolizes long, very long, and branched chain fatty acids though alpha- and beta-oxidation. These fatty acids are frequently incompletely catabolized and instead shunted into peroxisomal de-novo lipogenesis of various lipids, including ether lipids, or shuttled to the mitochondria to undergo complete oxidation. The peroxisome is also well-equipped with enzymes to catalyze harmful reactive oxygen species (e.g., $H_2O_2$) generated by the peroxisome itself and other organelles.

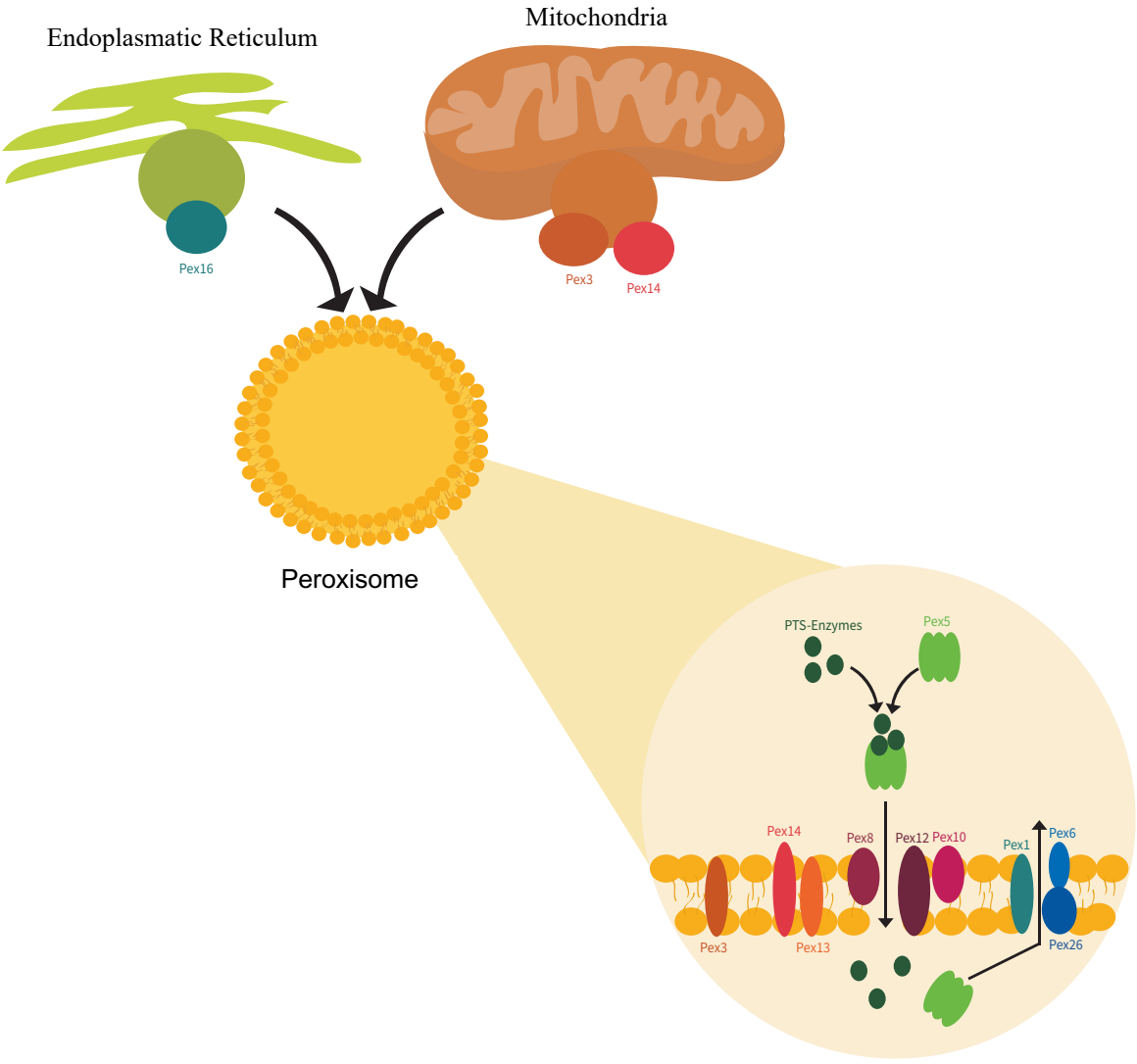


**Figure 2. Peroxisomes biogenesis is tightly coordinated by Peroxin family proteins**
Peroxisomes are formed by the fusion of vesicles derived from the endoplasmatic reticulum and the mitochondria. Peroxins (Pex) encode a functionally diverse family of proteins important for the biogenesis of peroxisomes. Membrane biogenesis is initiated predominantly by Pex3, which interacts with newly-synthesized peroxisomal membrane proteins in the cytosol. Nearly all peroxisomal proteins are synthesized in the cytosol and imported into the peroxisomal lumen by a Pex5-mediated system through interactions with Pex13 and Pex14. After the proteins have been imported, Pex5 is released from the peroxisome, though a process in part mediated by Pex1 and Pex6, to restart this transport cycle or for degradation.

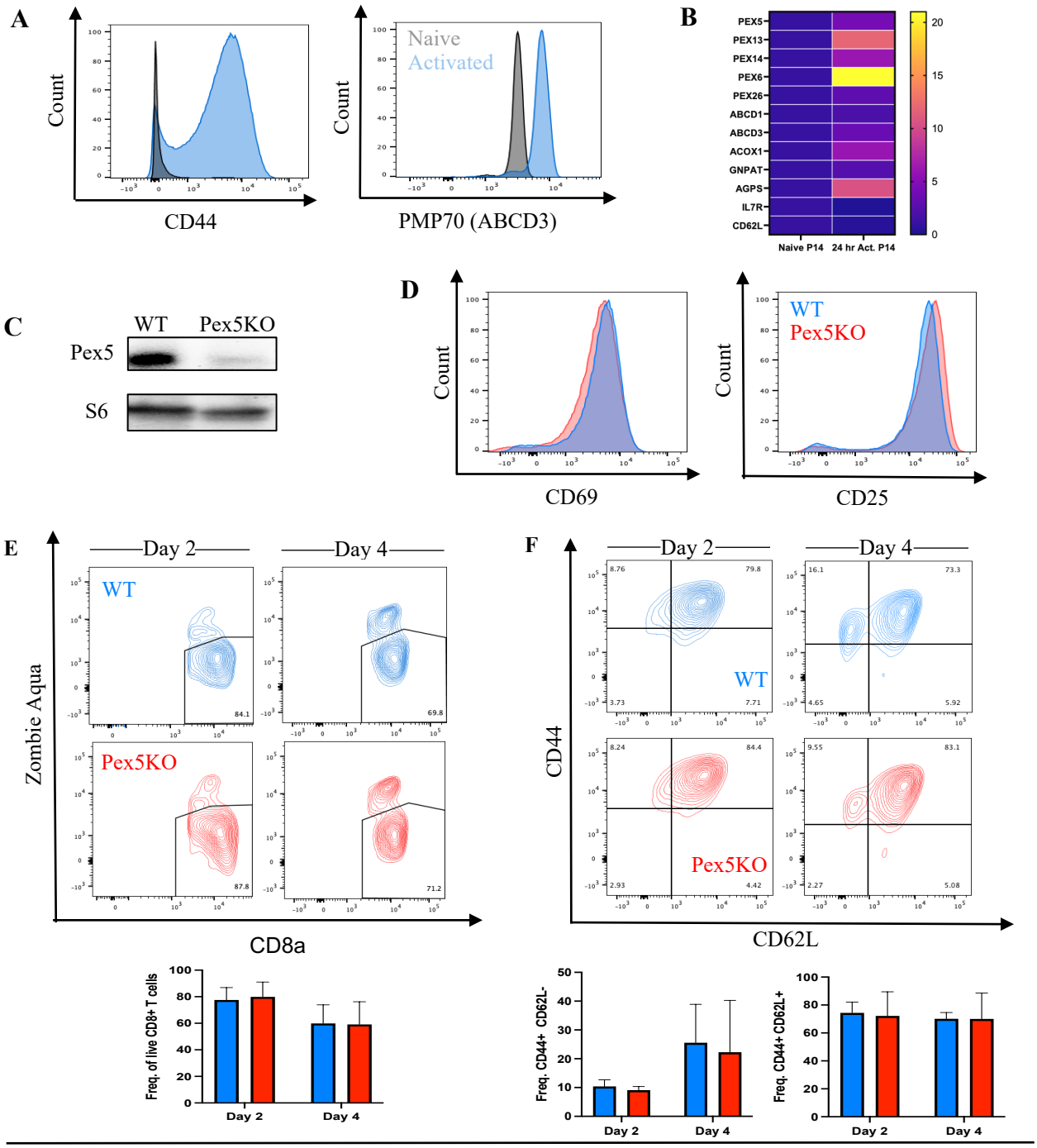


**Figure 3. Peroxisomes are dispensable for CD8$^+$ T cell survival in-vitro**
CD8$^+$ T cells were isolated from the spleen of a WT mouse and kept in-culture alone (naïve) or with anti-CD23/CD28 and IL2 (activated) **(A)** Expression of CD44 and PMP70 was assessed after 2 days in-culture. **(B)** Proteomic analysis of naïve and gp33 peptide activated P14$^+$ CD8$^+$ T cells, activated cells were cultured with IL-2 for 24 hours. CD8$^+$ T cells were isolated from spleen of WT and Pex5KO mice and activated with gp33 peptide. Cells were kept in culture supplemented with IL-2 for 5 days and **(C)** Pex5 deletion was validated by western blot. CD8$^+$ T cells were activated in the presence of anti-CD3/CD28 and IL-2 for 5 days. **(D)** Expression of the activation markers CD69 and CD25 was assessed at day 2 post-activation. **(E)** Viability and **(F)** differentiation were evaluated at day 2 and 4 post-activation (n = 2).

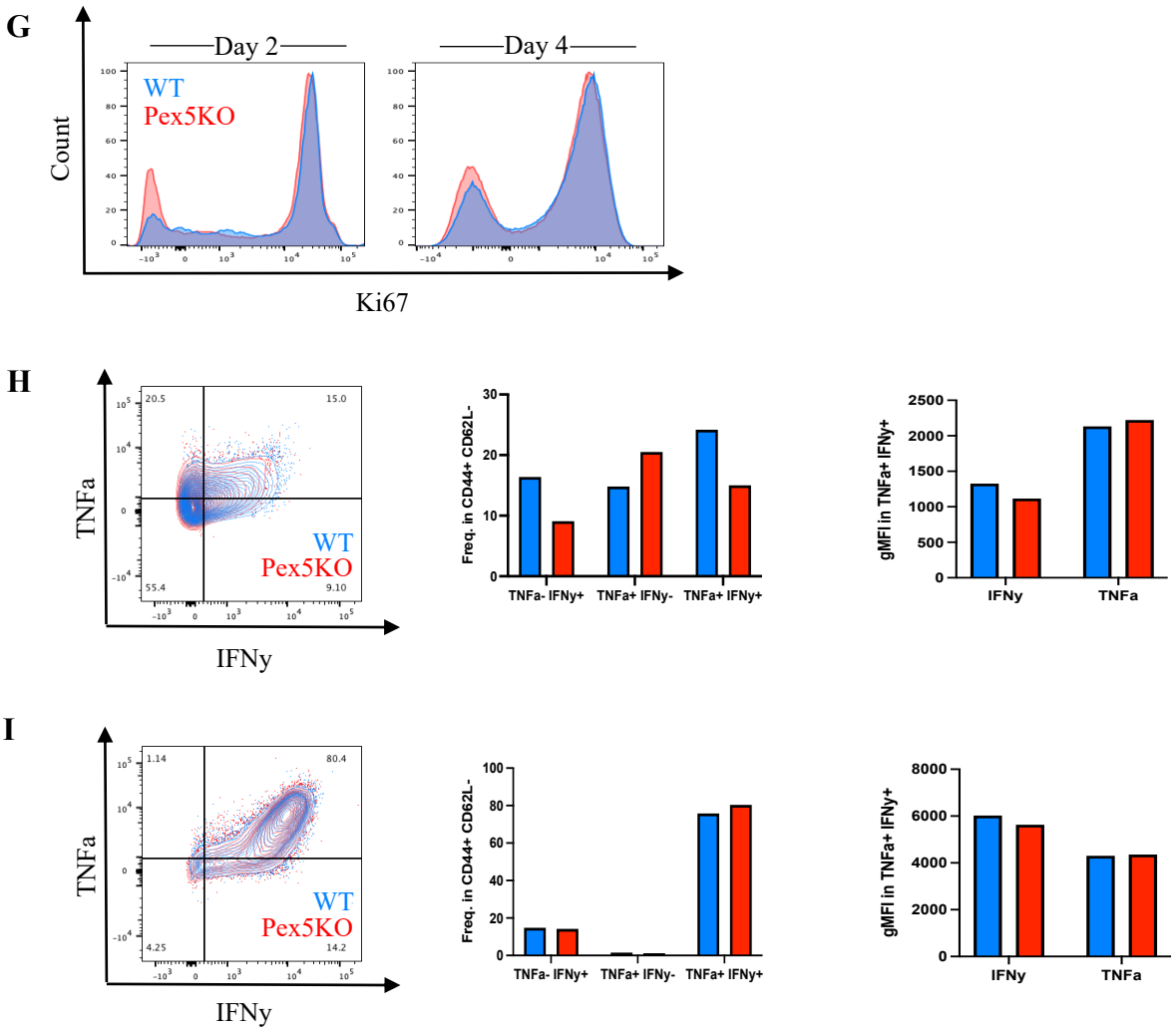


**Figure 3. Continued**

**(H)** Proliferation was assessed at day 2 and 4 post-activation. $CD8^+$ T cells were activated in the presence of anti-CD3/CD28 antibodies and cultured with IL2 for 3 days, then with IL2 **(G)** or combination of IL2 and IL12 **(H)** for 2 days. Cells were stimulated (PMA and ionomycin) for 4hrs, stained, and analyzed at day 5 by flow cytometry (n = 2). Geometric Mean Fluorescence Intensity (gMFI) obtained from the $CD8^+$ $IFNy^+$ $TNFa^+$ T cell population

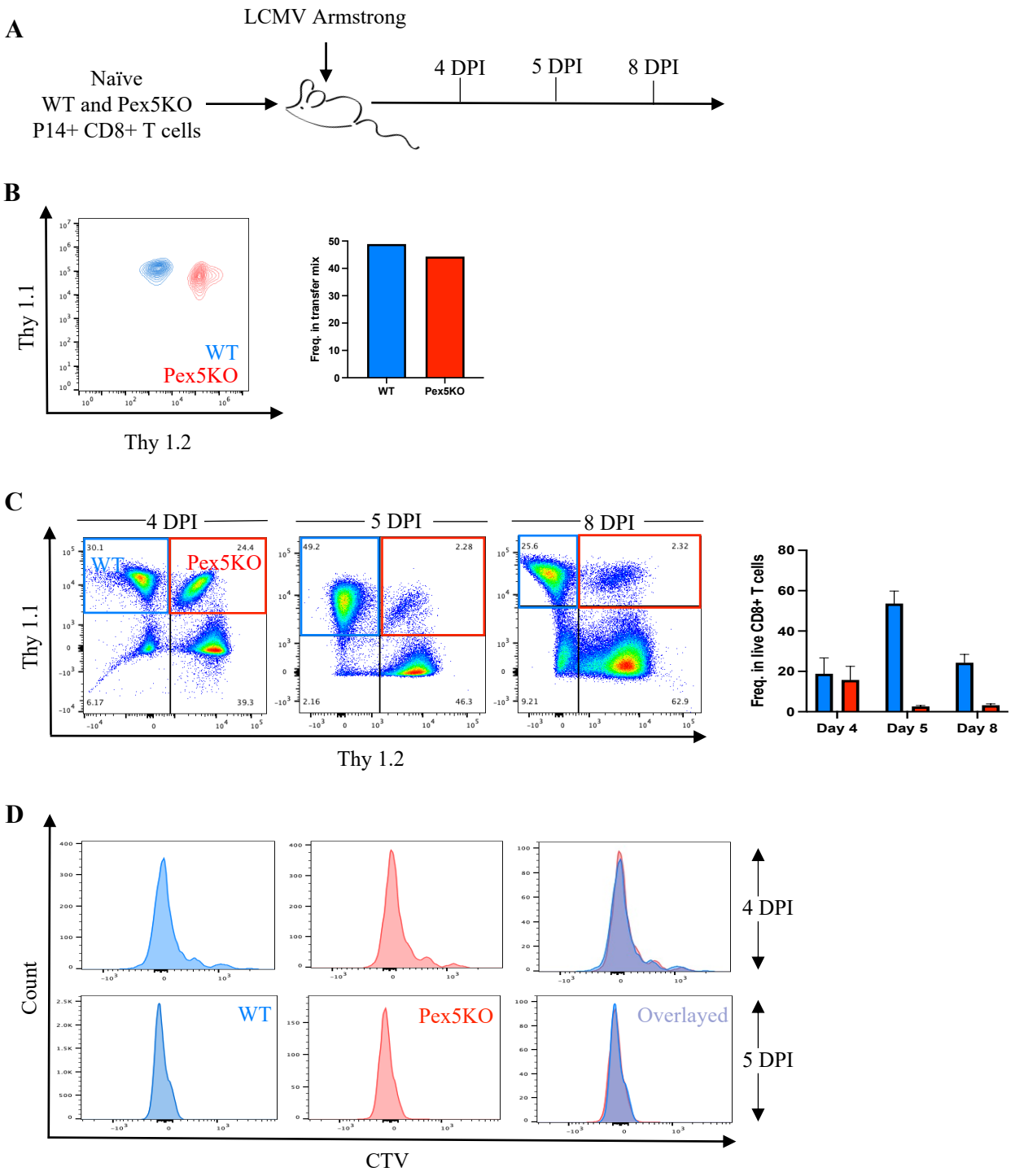


**Figure 4. Peroxisomes are required for antigen-specific CD8$^+$ T cell survival during acute infection**
**(A)** Experimental design for naïve CD8+ T cells in-vivo transfer during acute infection. **(B)** Frequency of WT and Pex5KO CD8$^+$ T cell in the mixture used for adoptive transfer (Plots representative of day 4 experiment). **(C)** Viability of CD8$^+$ T cells was assessed using Zombie Aqua at days 4, 5 and 8 post infection (Plot representative of day 8) (n= 4, 3, and 5, respectively). **(D)** Proliferation was assessed by Cell Trace Violet (CTV), samples were analyzed by flow cytometry at day 4 and 5 post infection.

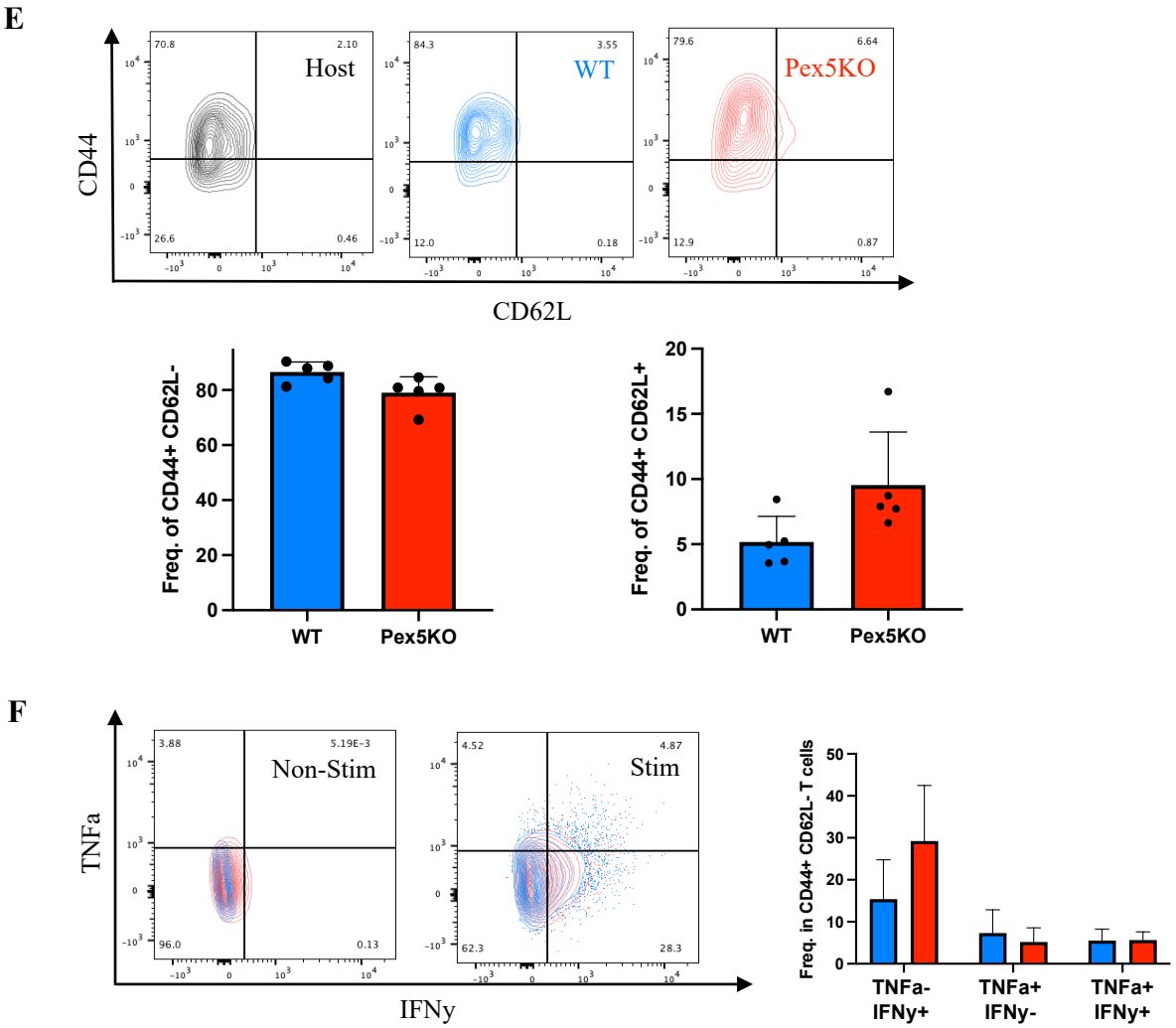


**Figure 4. Continued**

**(E)** CD8$^+$ T cell differentiation was assessed at day 8 post infection (n= 5). **(F)** CD8$^+$ T cells were stimulated (PMA and ionomycin) for 4hrs, stained, and analyzed at day 8 by flow cytometry (Plots representative of day 8) (n = 5)

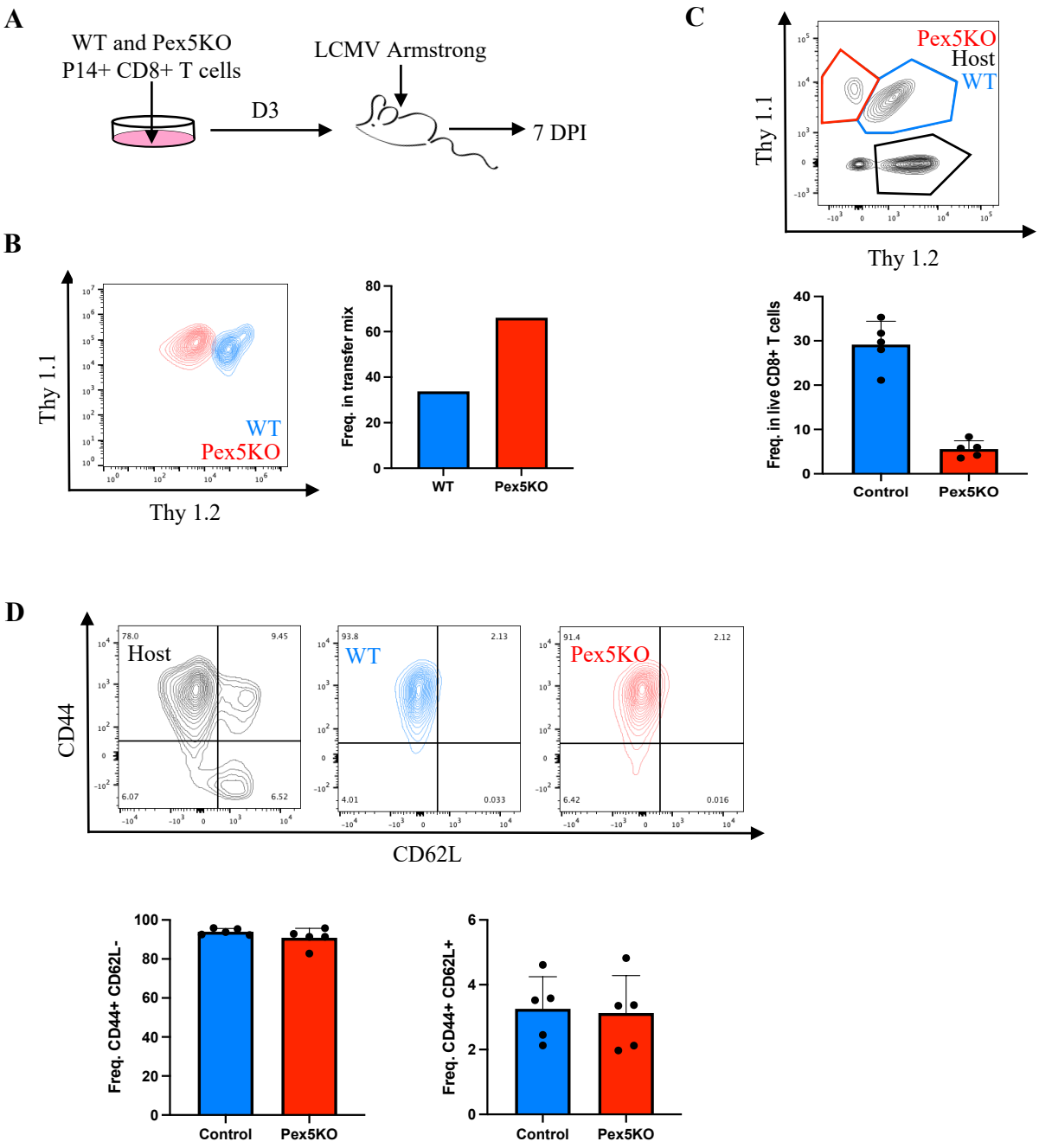


**Figure 5. In-vitro activation fails to rescue survival of Pex5KO CD8⁺ T cells in-vivo**
**(A)** Experimental design for adoptive transfer of activated CD8+ T cells in acute LCMV infection. **(B)** Frequency of WT and Pex5KO CD8⁺ T cell in the mixture used for adoptive transfer. **(C)** Viability of CD8⁺ T cells was assessed using Zombie Aqua at day 7 post infection (n= 5). **(D)** CD8+ T cell differentiation was assessed at days 7 post infection (n= 5)

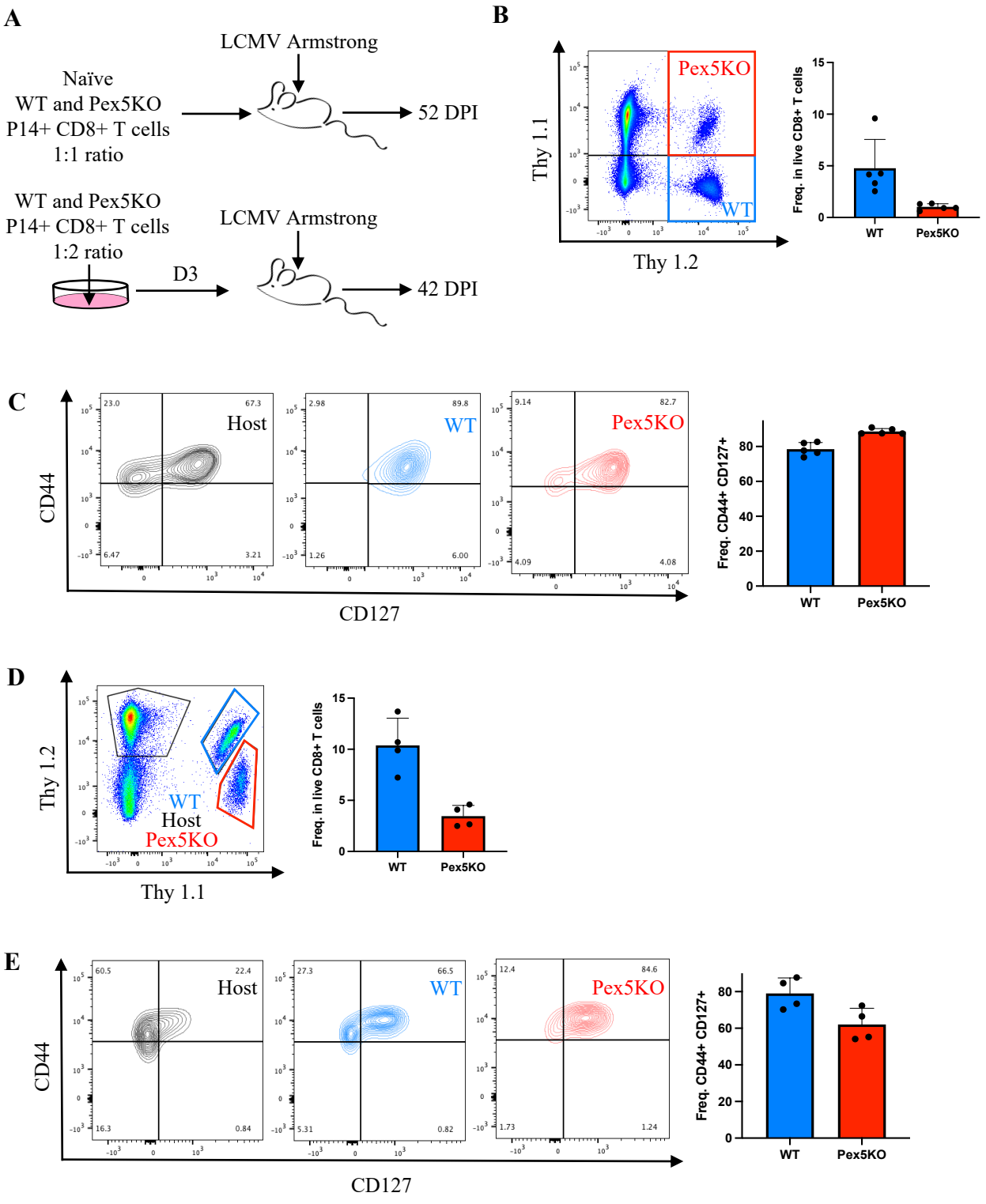


**Figure 6. Remaining Pex5KO CD8⁺ T cells differentiate into memory cells**
**(A)** Experimental design for naïve and activated CD8+ T cells in-vivo transfer during acute infection. **(B)** Frequency of naïve WT and Pex5KO CD8⁺ T cell at day 52 post-infection (n = 4). **(C)** CD8⁺ T cell differentiation was assessed at days 52 post infection (n= 4). **(D)** Frequency of in-vitro activated WT and Pex5KO CD8⁺ T cell at day 42 post-infection (n = 4). **(E)** CD8⁺ T cell differentiation was assessed at days 42 post infection (n= 4)

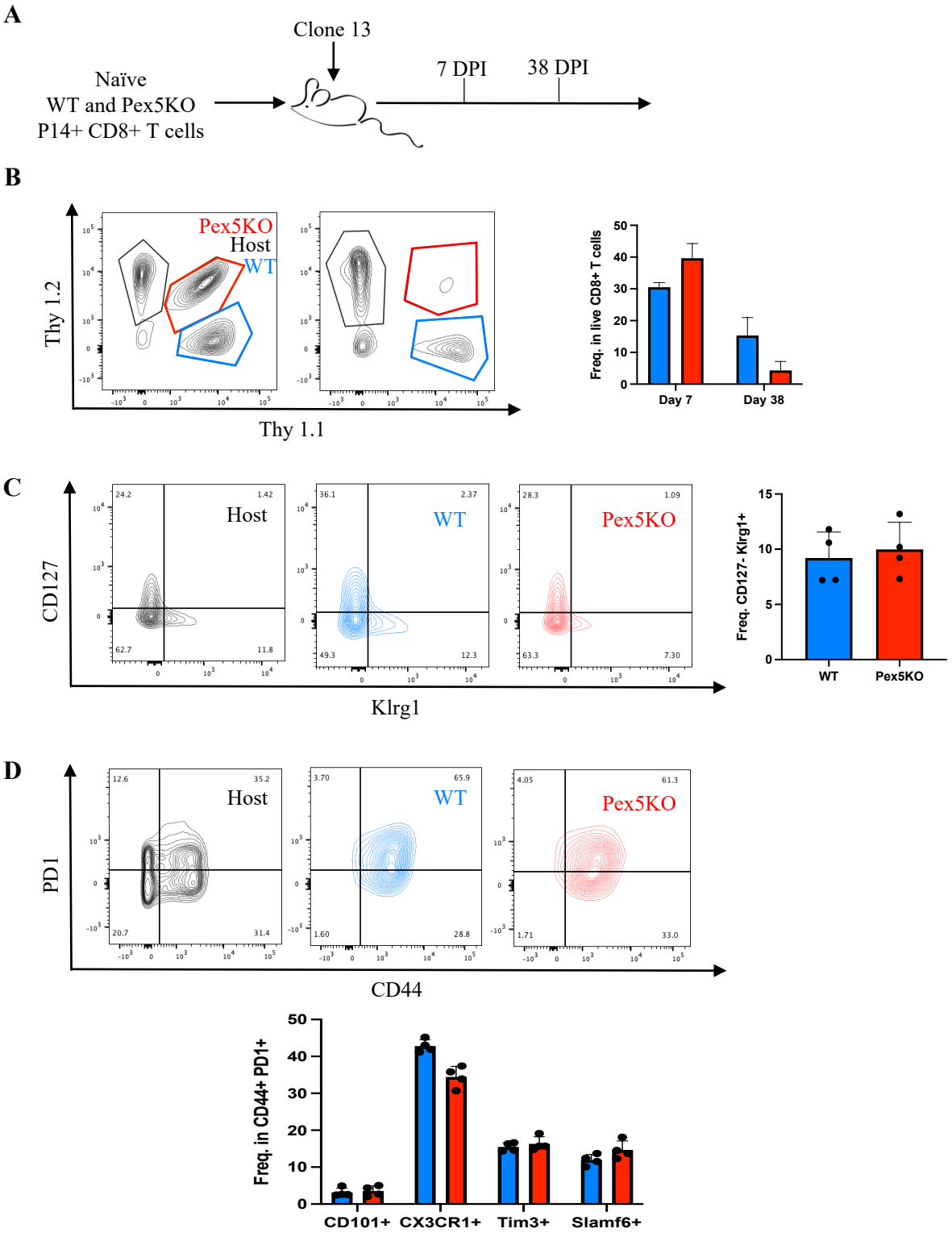


**Figure 7. Pex5KO CD8$^+$ T cells persist during chronic LCMV infection**
**(A)** Experimental design for naïve CD8+ T cells in-vivo transfer during chronic infection. **(B)** Viability of CD8$^+$ T cells was assessed using Zombie Aqua at day 7 (left) and day 38 (right) post infection (n= 4). **(C)** CD8$^+$ T cell differentiation was assessed at day 7 post infection (n= 4). **(D)** Expression of exhaustion markers was assessed at day 38 post infection (n= 4)

## Discussion

In this study, we investigated the requirement for peroxisomes in $CD8^+$ T cell activation and differentiation. Previous findings have shown peroxisomes are essential for fatty acid oxidation and innate immune signaling (Dixit et al., 2010). However, it remained unclear how peroxisomes affect $CD8^+$ T cell function and development. To address this question, we studied the function of peroxisomes in the context of viral infections using a genetic model of peroxisomal deficiency. We observed that following activation, peroxisome-deficient $CD8^+$ T cells proliferated, differentiated, and functioned normally in-vitro. The same was observed in-vivo, albeit several days post-infection, Pex5KO $CD8^+$ T cells rapidly decreased in abundance in both acute and chronic infections, suggesting that peroxisomes are essential for $CD8^+$ T cell survival in-vivo.

A recent study by Muri et al. reported peroxisomes are dispensable for $CD8^+$ T cells in response to infection (Muri et al., 2022), but we identified two differences in our respective approaches that may account for this discrepancy. First, Muri et al. generated a CD4-Cre driven Pex5 deletion, whereas we utilized a Granzyme-B-Cre. Cre recombinase driven by the granzyme-B promoter is expressed upon antigen stimulation, and thus the deletion occurs after T cell activation (Cgg et al., 1999). Conversely, Cre recombinase expression driven by the CD4 promoter is turned on during the double-positive phase of T lymphocyte development in the thymus (Sawada et al., 1994). Therefore, we hypothesize that those cells experiencing Pex5 deletion early in development, before committing to the $CD8^+$ lineage, have time to adapt to the lack of this organelle. This is in contrast to our model, in which peroxisome deficiency occurs abruptly after activation, resulting in the sudden disruption of T cell homeostasis during a rapid metabolic rewiring state (Frauwirth et al., 2002), which could limit the time or ability to engage

compensatory mechanisms. Another possibility is that peroxisome deficiency occurs during a period where cells either differentiate towards a certain lineage or are eliminated by the lack of fitness to commit to a program resulting in cell death.

An important remaining question is why Pex5KO $CD8^+$ T cells significantly decrease in number over the course of an infection in our model. One possible hypothesis to explain this event is that lack of peroxisomes results in a defect in type I interferon signaling. Type I interferon is essential for a substantial $CD8^+$ T cell expansion following infection (Kolumam et al., 2005) and cells with an impaired ability to respond to IFN I stimulation are eliminated by a natural killing (NK) cell-mediated mechanism (Crouse et al., 2014). This would explain why we observe a minor frequency of Pex5KO $CD8^+$ T cells starting at day five post-infection which is similar to the reported time frame that this phenomenon is known to occur. A possible way to test this would be to conduct an experiment where the infected animals are treated with an NK cell depletion antibody, which would discern whether the elimination of peroxisome deficient cells is caused by this phenomenon. Furthermore, peroxisomes are important for the rapid expression of interferon stimulated genes (ISGs) independently of Type I interferon (Dixit et al., 2010). This would suggest that even though peroxisome deficient cells are partially desensitized to respond to interferon signaling, they still function as they express genes involved in inhibition of viral replication. To validate this, it would be useful to monitor viral titer over the course of infection.

Another question that remains is regarding the residual Pex5KO $CD8^+$ T cells that persist as memory cells following infection. Increased mitochondrial mass enables memory $CD8^+$ T cells to undergo a strong secondary expansion upon activation compared to naïve cells (Van Der Windt et al., 2013), but whether peroxisomal content matters is unknown. One possible scenario

is that the mitochondria compensate for the lack of peroxisome, or vice versa (Peeters et al., 2015; Violante et al., 2019); the knockout of mitochondrial fatty acid oxidation genes in Pex5KO could help rule out this possibility. Furthermore, a dual knockout model could determine the requirement of fatty acid oxidation to memory formation maintenance.

Along these lines, it is unknown whether peroxisome deficient cells that differentiate into memory have an intact recall response to antigen re-challenge. It is important to consider that the function of peroxisomes and mitochondria are tightly linked; in fact, lack of peroxisomes results in mitochondrial fragmentation (Tanaka et al., 2019) and impaired respiration (Peeters et al., 2015). Given that mitochondrial metabolism has been shown to be essential for memory, it is possible that even though these cells exist they are unable to respond to a re-challenge (van der Windt et al., 2012). Future experiments should aim to study the function of peroxisome-deficient memory T cells in an antigen re-challenge model.

Additionally, while we have focused on central memory T cells (Tcm) residing in lymphoid tissues in our studies, tissue-resident memory (Trm) $CD8^+$ T cells that reside in non-lymphoid tissues comprise a major arm of long-term adaptive immune responses (Steinert et al., 2015). Phenotypically, $CD8^+$ Trm T cells utilize exogenous fatty acids to a greater extent than Tcm cells (Steinert et al., 2015). An important class of molecules to this phenotype is the family of fatty acid-binding proteins (FABP), which facilitate fatty acid uptake and intracellular trafficking of lipids (Reina-Campos et al., 2021). Moreover, it has been shown that $CD8^+$ Trm cells adopt distinct fatty acid binding protein expression depending on the tissue they reside in (Frizzell et al., 2020). It has previously been shown in other tissues, such as liver, that activation of the peroxisome proliferator activated receptors (PPARs) simultaneously induces the expression of FABPs and peroxisomal beta-oxidation genes (Katoh et al., 1985). Additionally,

some isoforms of FABP have been shown to physically interact with the peroxisome (Antonenkov et al., 2006) suggesting that $CD8^+$ Trm cells may be specifically affected by peroxisome deficiency in some, but not all, tissues. These lines of investigation will be pursued in future studies.

Lastly, our preliminary results in a model of a chronic viral infection suggest that peroxisomal deficiency is also detrimental in this context. However, the decay of Pex5KO cells follows different a kinetic, suggesting that the observed phenotype is in part model-dependent. Nevertheless, it is important to highlight that even though the infections follow a different timeline of survival, peroxisome deficient cells do not show increased, nor reduced levels of exhaustion as compared to wildtype. Therefore, the alterations observed by the lack of this organelle do not appear to influence the process of T cell exhaustion.

In conclusion, we demonstrate that peroxisomes are not essential for proliferation, differentiation, and function of $CD8^+$ T cells during chronic and acute infection. However, they are required for survival following the initial clonal expansion triggered by antigen exposure. These results support a scenario in which the abrupt loss of peroxisomes renders $CD8^+$ T cells susceptible to elimination or cell death during viral infection.

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