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Crosstalk between CD8$^+$ T cells and systemic bile acid metabolism controls LCMV-induced immunopathology

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Submitted by

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Declaration

I, Zsofia Keszei, am the sole author of this thesis. I received input from Andreas Bergthaler. This thesis contains one manuscript, which has been published ahead of print on bioRxiv and is included here in full, with permission of all co-authors.

Crosstalk between CD8+ T cells and systemic bile acid metabolism controls LCMV-induced immunopathology

Zsofia Keszei*, Felix C. Richter*, Henrique G. Colaço, Maximilian Baumgartner, Laura Antonio-Herrera, Magdalena Siller, Anna Hofmann, Csilla Viczenczova, Hatoon Baazim, Claudia D. Fuchs, Oleksandr Petrenko, Fabian Amman, Jakob-Wendelin Genger, Clarissa Campbell, Hanns-Ulrich Marschall, Thomas Reiberger, Michael Trauner, Andreas Bergthaler. *These authors contributed equally.

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BA metabolomic analysis was conducted in the laboratory of Hanns-Ulrich Marschall, at the Institute of Medicine at Sahlgrenska Academy, University of Gothenburg. All other experiments were performed at the CeMM Research Center of Molecular Medicine of the Austrian Academy of Sciences and at the Institute for Hygiene and Applied Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna.

I contributed to the conceptualization of the paper, performed the initial mouse breedings and experimental setups that formed the basis of the study and were used in figure 4 and 5. I designed and performed experiments, analyzed and visualized data presented in figure 1A, S4B. I analyzed and visualized data presented in figure 2A, 2B, S2A, S2B. I performed the qPCR analysis, data analysis, and interpretation using mouse liver samples provided by Henrique G. Colaço and Laura Antonio-Herrera for figure 2C, 3A, 3B, S3A, S3B, S3D, S3E, S3F. I wrote the manuscript and prepared all figures in collaboration with Felix C. Richter. I prepared the graphical summary and wrote this thesis.

Further information on authors' contributions to the research manuscript can be found in the preprint version of the manuscript included in this thesis.

The shared first author of this manuscript, Felix C. Richter agreed not to use any part of the content of this thesis for the purpose of a doctoral dissertation.

The German translation of the abstract was aided by ChatGPT-5.

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The German translation of the abstract was aided by ChatGPT-5. No other AI algorithms or generative models were used to aid the writing of this thesis.

Abstract

The interdependence of immunity and metabolism dates back millions of years of coevolution. Yet, we only started to appreciate it in the last two decades, with the emergence new discipline; immunometabolism. The liver is a special organ from the immunometabolical point of view. It acts as a key metabolic hub of the body, while also acting as the gatekeeper of molecules entering the bloodstream from the gut and preventing adverse reactions towards commensals. This also makes it an attractive place for pathogens causing chronic infection. Chronic liver inflammation poses a global health burden, which is not expected to decline. It is most commonly caused by infections and lifestyle factors, and it urgently calls for a better understanding of immune-metabolic interactions in liver disease.

Bile acids (BAs) are classically known for their role in lipid digestion. Recent discoveries have highlighted their immunomodulatory nature and revealed their role in fine-tuning immune responses by affecting multiple immune cell types including T cells. Here, we used lymphocytic choriomeningitis virus (LCMV) as a model for chronic viral hepatitis to investigate the crosstalk between BA metabolism and antiviral immunity. We found that LCMV infection markedly changed the expression of genes involved in BA synthesis and transport. Infection also increased systemic BA levels, and shifted the composition of both hepatic and circulating BAs toward host-derived conjugated species. These changes were at least partly dependent on $CD8^+$ T cells, demonstrating that adaptive immunity can influence systemic BA homeostasis.

Conversely, the infection of mice lacking the BA transporters OATP1A/1B—characterized by persistently elevated BAs—showed impaired T cell responses and reduced immunopathology.

Together, these results point to a bidirectional crosstalk between $CD8^+$ T cells and BA metabolism during viral hepatitis. They also broaden our understanding of immune-metabolic regulation during viral hepatitis, and finally, they demonstrate how systemic metabolism can influence T cells, the main effectors of antiviral and antitumor immunity.

Zusammenfassung

Haftungsausschluss: Der Autor dieser Dissertation ist der deutschen Sprache nicht mächtig. Der erste Entwurf dieser Zusammenfassung wurde mit der kostenlosen Version von ChatGPT-5 übersetzt.

Die wechselseitige Abhängigkeit von Immunität und Stoffwechsel reicht Millionen Jahre zurück, doch erst in den letzten zwei Jahrzehnten haben wir sie mit dem Aufkommen der Disziplin des Immunmetabolismus wirklich gewürdigt. Die Leber ist aus Sicht des Immunmetabolismus ein besonderes Organ: Sie ist ein zentrales metabolisches Drehkreuz und fungiert als Torwächter für Moleküle aus dem Darm, wodurch sie unseren Körper vor unerwünschten Reaktionen auf Kommensalen schützt. Dies macht sie zugleich zu einem Ziel für Krankheitserreger, die chronische Infektionen verursachen. Chronische Leberentzündungen, meist durch Infektionen oder Lebensstilfaktoren ausgelöst, stellen eine weltweite Gesundheitsbelastung dar und erfordern ein tieferes Verständnis der immun-metabolischen Wechselwirkungen.

Gallensäuren (BAs) sind klassischerweise für ihre Rolle bei der Lipidverdauung bekannt, doch neuere Entdeckungen haben ihre immunmodulatorischen Eigenschaften hervorgehoben und gezeigt, dass sie Immunantworten feinabstimmen, wobei verschiedene Zelltypen einschließlich T-Zellen betroffen sind. Wir nutzten das lymphozytäre Choriomeningitisvirus (LCMV) als Modell für chronische Virushepatitis, um den Crosstalk zwischen BA-Stoffwechsel und antiviraler Immunität zu untersuchen. LCMV-Infektion veränderte die Expression von Genen der BA-Synthese und des Transports tiefgreifend, erhöhte BA-Spiegel und verschob ihre Zusammensetzung in Richtung Wirts-konjugierter Spezies. Diese Veränderungen waren teilweise von $CD8^+$-T-Zellen abhängig, was zeigt, dass die adaptive Immunität die systemische BA-Homöostase beeinflusst. Umgekehrt beeinträchtigte anhaltend erhöhte BA-Spiegel virus-spezifische $CD8^+$-T-Zell-Antworten, während die Leberimmunpathologie begrenzt blieb, wie bei Mäusen ohne BA-Transporter OATP1A/1B.

Zusammen verdeutlichen diese Ergebnisse einen bidirektionalen Crosstalk zwischen $CD8^+$-T-Zellen und BA-Stoffwechsel während der Virushepatitis und erweitern das Verständnis der immunmetabolischen Regulation. Sie zeigen zudem, wie systemische metabolische Veränderungen $CD8^+$-zytotoxische T-Zellen beeinflussen könnten, die Schlüsselfaktoren der antiviralen und antitumoralen Immunität darstellen (OpenAI, 2025).

Publication arising from this thesis

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The manuscript is under revision for publication at a peer-reviewed journal.

Abbreviations

αDG	α-Dystroglycan
ABCB	ATP-binding cassette sub-family B
ABCC	ATP-binding cassette sub-family C
ABCG	ATP-binding cassette sub-family G
AIM2	Absent in melanoma 2
ALD	Alcohol-associated liver disease
ALT	Alanine aminotransferase
APC	Antigen presenting cell
ASBT	Apical sodium-dependent bile acid transporter
BA	Bile acid
BAAT	Bile acid-CoA: amino acid N-acyltransferase
BACS	Bile acid:CoA synthase
BAT	Brown adipose tissue
BSEP	Bile salt export pump
BSH	Bile salt hydrolase
CA	Cholic acid
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
CLR	C-type lectin receptor
CPT1	Carnitine palmitoyl transferase 1
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DCA	Deoxycholic acid
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FXR	Farnesoid X recept
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GLP-1	Glucagon like peptide-1
GLUT1	Glucose transporter type 1
GUDCA	Glycoursodeoxycholic acid
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDCA	Hyodeoxycholic acid
HIF-1α	Hypoxia-inducible factor 1-alpha
HNF4α	Hepatocyte nuclear factor 4-alpha

HSDH	Hydroxysteroid dehydrogenase
IBABP	Ileal bile acid-binding protein
IDO1	Indoleamine 2,3-dioxygenase 1
IFI16	Interferon gamma-inducible protein 16
IFN-γ	Interferon gamma
IFNAR	Interferon alpha/beta receptor
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
JNK	c-Jun N-terminal kinase
LAG-3	Lymphocyte-activation gene 3
LC-MS	Liquid chromatography–mass spectrometry
LCA	Lithocholic acid
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LRH-1	Liver receptor homolog-1
LSEC	Liver sinusoidal endothelial cell
MAPK	Mitogen-activated protein kinase
MASLD	Metabolic dysfunction-associated steatotic liver disease
MAVS	Mitochondrial antiviral-signaling protein
MBL	Mannose-binding lectin
MCA	Muricholic acid
MDA5	Melanoma differentiation-associated protein 5
MDR	Multidrug resistance protein
MHC	Major histocompatibility complex
MRP	Multidrug resistance-associated protein
mTOR	Mechanistic target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NK	Natural killer (cell)
NKT	Natural killer T (cell)
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
NTCP	Sodium taurocholate cotransporting polypeptide
OATP	Organic anion-transporting polypeptide
OSTα	Organic solute transporter alpha
OSTβ	Organic solute transporter beta
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern

PD-1	Programmed cell death protein 1
PKA	Protein kinase A
PMCA	Plasma membrane Ca^{2+}-ATPase
PPP	Pentose phosphate pathway
PRR	Pattern recognition receptor
PXR	Pregnane X receptor
RIG-I	Retinoic acid-inducible gene I
RORγ	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RXRα	Retinoid X receptor alpha
SHP	Small heterodimer partner
SLCO	Solute carrier organic anion transporter family
STAT1	Signal transducer and activator of transcription 1
STING	Stimulator of interferon genes
TBA	Total bile acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TCR	T cell receptor
TDCA	Taurodeoxycholic acid
TGF-β	Transforming growth factor beta
TGR5	Takeda G protein-coupled receptor 5 (GPBAR1)
TH17	T helper 17 (cell)
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TOX	Thymocyte selection-associated high mobility group box protein
TRAIL	TNF-related apoptosis-inducing ligand
TREM2	Triggering receptor expressed on myeloid cells 2
TRIF	TIR-domain-containing adapter-inducing interferon-β
UDCA	Ursodeoxycholic acid
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus

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Finally, I dedicate this thesis to my family, whose collective effort made it possible for me to complete it: the wonderful grandparents of Leo, and my partner András. And most of all, I thank my son Leo, for showing me how strong I am.

1. Introduction

Liver diseases pose an important threat to global health, causing one in every 25 death worldwide (Devarbhavi *et al*, 2023). The liver, as a central metabolic hub, has a huge impact on the metabolic composition of blood, by synthesizing and modifying metabolites in the circulation, both in health and disease (Lercher *et al*, 2020; Li & Chiang, 2020; Mantovani & Garlanda, 2023). Among these, bile acids stand out as metabolites with a published record of immunological impact. They were found to modulate multiple innate and adaptive immune cell types (Su *et al*, 2023). It is also known that inflammatory liver conditions frequently disrupt bile acid metabolism (Trauner *et al*, 1999). Yet, our knowledge is scarce on the immune-metabolic interactions involving bile acids in the context of viral hepatitis. Such knowledge would have the potential to enhance our understanding of disease mechanisms and help identify therapeutic targets to modulate immune responses and improve liver disease outcomes.

Lymphocytic choriomeningitis virus (LCMV), a rodent-born pathogen leading to a systemic viral infection, also infects hepatocytes and induces hepatitis (Zinkernagel *et al*, 1986; Bergthaler *et al*, 2007). Using this well-established model (Zinkernagel, 2002), we investigated the interactions of bile acids and antiviral immune responses. This thesis describes how infection with LCMV influences bile acid metabolism, which, in turn, can modulate the immune response against the virus, raising the possibility of a potential self-regulatory circuit.

The introduction will be divided into the following sub-chapters:

1.1 Burden of liver disease worldwide

Liver diseases are responsible for more than two million deaths per year. This accounts for 4% of global mortality. Acute hepatitis is only responsible for a minority of cases, while complications of chronic liver disease accounts for the majority of mortality (Devarbhavi *et al*, 2023).

A common consequence of liver diseases of different etiologies, is chronic inflammation resulting in fibrosis. The latter is characterized by the production of excessive ECM, impaired liver architecture and function. If chronic inflammation is sustained, liver fibrosis can further progress into liver cirrhosis, and ultimately, hepatocellular carcinoma (HCC) (Hammerich & Tacke, 2023). HCC is the third leading cause of cancer-related mortality worldwide (Bray *et al*, 2024).

Global efforts have been made to eliminate viral hepatitis by 2030, and yet, it is still the leading cause of liver cirrhosis(Waheed *et al*, 2018). The highest mortality is the consequence of cirrhosis and HCC complications as a result of Hepatitis B virus (HBV), followed by Hepatitis C virus (HCV) (Devarbhavi *et al*, 2023). Asia is the region most heavily burdened by viral hepatitis, followed by Africa. Strategies that overcome financial and infrastructural barriers are needed to address these diseases, the most effected countries being low- and lower-middle-income countries with considerable health-care limitations (Cooke *et al*, 2019).

Alcohol-associated liver disease (ALD) is the second most common cause of liver cirrhosis, and the global incidence of ALD is rising. Without interventions, a marked increase is expected in ALD-related mortality worldwide (Devarbhavi *et al*, 2023).

Metabolic dysfunction-associated steatotic liver disease (MASLD, also known as NAFLD) is linked to metabolic syndrome, obesity and type 2 diabetes, and is the third most common cause of liver cirrhosis. It is estimated to affect quarter of the world's adult population, and is the most rapidly increasing cause of chronic liver disease worldwide (Younossi *et al*, 2023).

1.2 Lymphocytic choriomeningitis virus

LCMV was initially described as the causative agent of the 1933 St Louis encephalitis epidemic (Armstrong & Lillie, 1934; Muckenfuss, 1934). It belongs to the Arenaviridae family. This family also includes multiple viruses causing severe hemorrhagic fever in humans, such as Lassa virus causing Lassa fever and Junin virus causing Argentine hemorrhagic fever (Oldstone, 2002; Grande-Pérez *et al*, 2016). LCMV is a rodent-borne, negative-strand RNA virus (Sevilla *et al*, 2002). Its genome is divided into large (L) and small (S) segments, each segment encoding two open reading frames (ORFs); with ambisense coding oriented in opposite directions. Both ORFs end in a central intragenic region that folds into a stable secondary structure (Taniguchi *et al*, 2020).

The L segment encodes for an RNA-dependent RNA polymerase (protein L), and a RING finger (protein Z). The latter is a structural protein essential for virion assembly and release from infected cells (Buchmeier, 2002; Perez *et al*, 2003).

ORFs on the S segment translate into a glycoprotein (GP) and a nucleoprotein (NP). The former is responsible for binding to the cell surface receptor α-dystroglycan (αDG) and cell

fusion (Cao *et al*, 1998; Spiropoulou *et al*, 2002), while the NP is the main structural element of the viral ribonucleoprotein complex. This complex encapsidates the viral RNA, and facilitates transcription through interacting with the viral RNA polymerase (Buchmeier, 2002).
Enhancing immunological insight, a number of different strains with varying virulence have been isolated from infected mice (Pfau *et al*, 1982; Ahmed *et al*, 1984). In particular, strains Armstrong 53b (Arm) and Clone 13 (Cl13), only differing by three coding point mutations, have become benchmark models for acute and chronic viral infections, respectively. A lysine-to-glutamine change at position 1,079 in the L protein is the primary driver of Cl13's persistence. A phenylalanine-to-leucine mutation at position 260 in the GP also plays a supporting role (Bergthaler *et al*, 2010; Sullivan *et al*, 2011).
Isolate WE as well as Cl13 have been found to cause T-cell mediated hepatitis in infected rodents among other pathologies (Zinkernagel *et al*, 1986; Abdel-Hakeem, 2019).
The cell surface receptor αDG is expressed in a variety of cell types across the body, including plasmacytoid dendritic cells in the spleen, that are early LCMV target cells (Macal *et al*, 2012). In the liver, Kupffer cells (KCs), the tissue-resident macrophages, are the primary targets of chronic LCMV strains. These cells initiate a strong interferon type I response to control early spread of the virus (Ou *et al*, 2001; Lang *et al*, 2010). The infection then progresses to also invade hepatocytes (Bergthaler *et al*, 2007). LCMV is a non-cytolytic virus and thus does not exert direct cytotoxic effects on infected cells (Oldstone, 2016). However, systemic infection of mice with LCMV leads to a potent $CD8^+$ T cell response, essential for pathogen clearance, also causing severe immunopathologies including the cytotoxic T cell-mediated death of infected cells including hepatocytes. Pathologies associated with LCMV are thus primarily linked to $CD8^+$ T cell-mediated immune responses (Zinkernagel *et al*, 1986; Wherry *et al*, 2003a).
Viruses were compared to the "Rosetta stone", having been clues to help humanity gain insight into how immunity works, throughout history (Abdel-Hakeem, 2019). Among them, LCMV stands out as an exceptional model organism. It had a major impact on our understanding of the immune system and played a role in the emergence of modern immunology. It facilitated discoveries such as the mechanisms of T cell memory formation (Murali-Krishna *et al*, 1998), T cell MHC class restriction (Zinkernagel & Doherty, 1975), and the phenomenon of T cell exhaustion (Moskophidis *et al*, 1993; Zajac *et al*, 1998).
Importantly, a wide range of tools have been developed within the LCMV model. These include well-characterized viral strains, transgenic T cell receptor mouse lines, defined immunodominant and subdominant epitopes for T cell receptor specificities, and cognate tetramers for *in vivo* enumeration (Pircher *et al*, 1987; Gallimore *et al*, 1998; Abdel-Hakeem, 2019). These aided the achievement of such landmark discoveries. On top, they also opened new therapeutic avenues, such as the development of immune checkpoint inhibitors. The

latter have revolutionized cancer immunotherapy (Barber *et al*, 2006; Abdel-Hakeem, 2019; Moskophidis *et al*, 1993).

1.3 Antiviral immunity

Defense against viruses is mediated by the complex network of cells, tissues and molecules that work in concert to protect the body's homeostasis from pathogens, in a series of coordinated responses known as innate and adaptive immunity.

Innate immunity provides early defense, with its components already present in the host and ready for deployment prior to infection, already protecting the host in the first hours to days of an infection. It is essential in facilitating the development of an adaptive immune response that is specific to the infection (Abbas *et al*, 2017).

In turn, adaptive immune responses need more time to develop after the infection, but they provide defenses specific to a particular pathogen. They also result in immunological memory, enabling robust secondary responses upon re-exposure to the same pathogen (Wherry *et al*, 2003b).

The innate and adaptive immune branches are interconnected at every level, affecting each other. Innate immunity provides stimulation for the adaptive branch and influences the nature of adaptive responses. It provides early danger signals and primes cells of the adaptive immune system. On the other hand, adaptive immunity can enhance and fine-tune innate responses (Clark & Kupper, 2005).

This chapter will provide an overview of antiviral immunity, first summarizing innate responses, followed by a discussion of the adaptive immune system.

1.3.1 Innate antiviral immunity

Pathogen sensing by pattern recognition receptors

Viruses are obligate intracellular pathogens that depend entirely on the host cell machinery for their life cycle, hijacking host cells for the production of viral components. Host cells identify a viral invasion by recognizing pathogen associated molecular patterns (PAMPs) by evolutionary conserved host sensors encoded withing the germline, called pattern-recognition receptors (PRRs). PPRs are not restricted to immune cells but are expressed in many cells types. They reside on the cell surface, in phagocytic vesicles and the cytosol (Abbas *et al*, 2017). Detection of viral pathogens by PPRs results in the expression of pro-inflammatory cytokines including interferons, pyroptotic cell death, the recruitment of immune cells to the site of the infection and the activation of the adaptive immune system that work in concert to curb infection (Carty *et al*, 2021). In this subchapter, I will first discuss viral recognition by PPRs, followed by a summary of the immune responses activated by these receptors.

There are five main classes of PRRs known to be involved in the recognition of viral pathogens (Abbas *et al*, 2017), see also figure 1

1. Toll-like receptors (TLRs)
2. Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs)
3. NOD-like receptors (NLRs)
4. C-type lectin receptors (CLRs)
5. Cytosolic DNA sensors

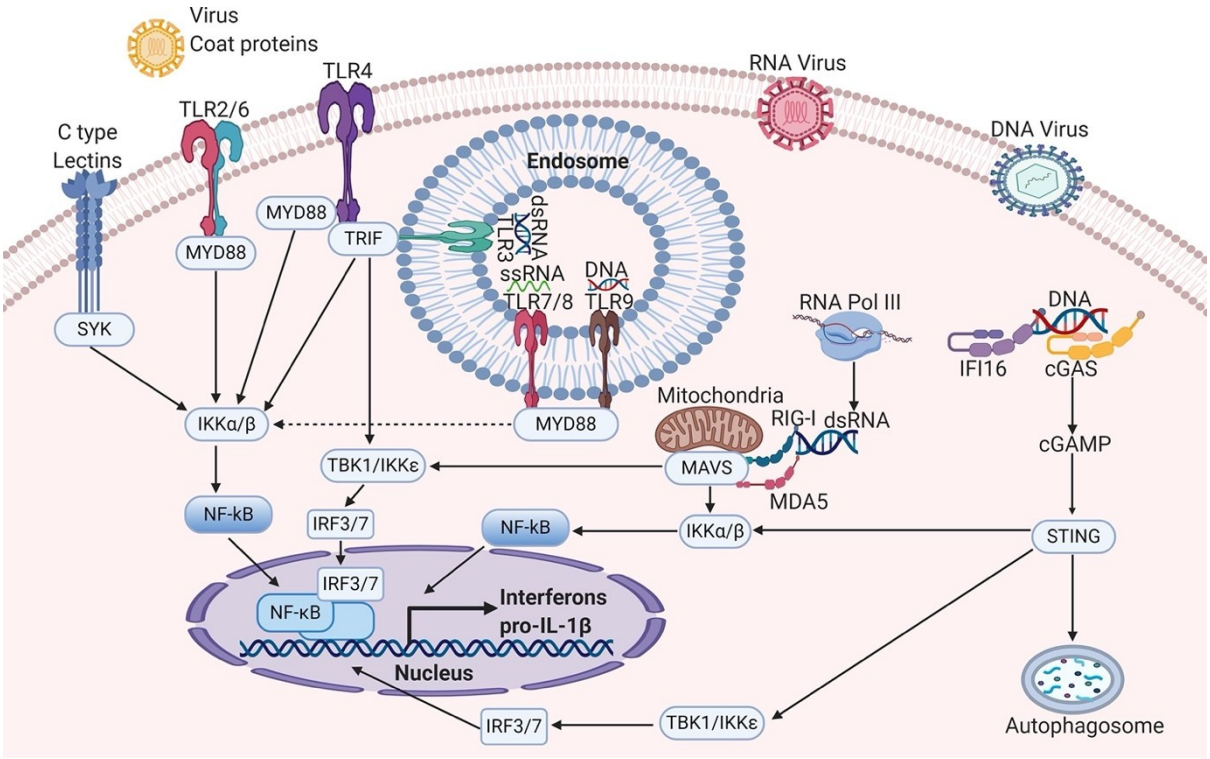

Figure 1 Detection of viruses by the immune system

Endosomal TLRs (TLR3, 7/8, 9), cytosolic RNA sensors (RIG-I, MDA5) and DNA sensors (cGAS, IFI16) detect viral nucleic acids and signal through adaptor proteins to activate transcription factors like NF-κB and IRFs. This leads to the production of proinflammatory cytokines, chemokines and interferons. Additional receptors, including C type lectins, contribute to sensing. Figure reproduced from Carty, Guy and Bowie (2021), under a Creative Commons CC-BY license.

TLRs were the first identified class of PPRs, originally described in Drosophila and found conserved across the evolutionary tree from insects to mammals (Lemaitre *et al*, 1996; Poltorak *et al*, 1998). There are 10 different types of these membrane-spanning receptors in humans. TLR1, 2, 4, 5 and 6 on the cell surface are involved in bacteria recognition, while endosomal TLRs bind nucleic acids: TLR3 recognizes double-stranded RNA, TLR7 and TLR8 bind single-stranded RNA, TLR9 detects unmethylated CpG DNA (Fitzgerald & Kagan, 2020).

Until today, several mammalian TLRs, including human TLR10, remains orphaned (Rodrigues *et al*, 2024). Ligand binding leads to receptor dimerization and the recruitment of adaptor proteins as TIR-domain-containing adapter-inducing interferon-β (TRIF) and MyD88. Most TLRs, including TLR 2, 4, 7 and 9, signal through MyD88, leading the activation of NF-kB (Nuclear factor kappa-light-chain-enhancer of activated B cells), driving the expression of pro-inflammatory cytokines and chemokines like TNF-α, IL-6, and IL-1β (Kawai & Akira, 2006; Kawasaki & Kawai, 2014).

In contrast, TLR3 signals *via* TRIF to activate regulatory factor 3 (IRF3) and IRF7 that initiates a type I interferon (IFN-I) response. In plasmacytoid dendritic cells (pDCs), known for their important role in viral infections by secreting vast amounts of IFN-I, TLR7 and TLR9 utilizes MyD88, in a signaling that phosphorylates IRF7 and induces expression of IFN-I (Kawasaki & Kawai, 2014). TLR expression is cell-type specific and reflets functional specialization. For example, pDCs express ample TLR7 and TLR9 to enable them to detect endosomal nucleic acids and play a key role in initiating antiviral responses. Other PRR classes show less cell-type specificity. This allows the broad pathogen-sensing of the body across cell types, including non-immune cells (Rodrigues *et al*, 2024).

RLRs, including RIG-I and MDA5 are cytosolic helicases sensing viral RNA in the cytosol (Carty *et al*, 2021). RIG-I detects short double-stranded RNA (dsRNA) and 5'- di- or triphosphorylated RNA. MDA5 recognizes long dsRNA, common product of viral replication (Yoneyama *et al*, 2004; Hornung *et al*, 2006; Kato *et al*, 2006; Goubau *et al*, 2014). Both signal through the mitochondrial antiviral-signaling (MAVS) protein to activate NF-κB and IRF family members (Kawai & Akira, 2006). Broad expression of RLRs enable many cell types to detect viral infections (Abbas *et al*, 2017).

NOD-like receptors (NLRs), another cytosolic family, include NLRP3 (NOD-like receptor family, pryin domain containing 3), which is an important sensor protein in the formation of inflammasomes in innate immune cells, responding to various viral infections and danger signals (Chen *et al*, 2009). Inflammasomes are cytosolic multiprotein complexes, which lead to the generation of the active form of IL-1β and IL-18. They thus contribute to antiviral immunity by recruiting immune cells, and by inducing pyroptosis, an inflammatory form of programmed cell death, which is a characteristic of macrophages and DCs (Carty *et al*, 2021).

CLRs are expressed mainly in myeloid cells (Carty *et al*, 2021). They recognize microbial carbohydrate structures, and multiple members of this family has been associated with viral detection (Geijtenbeek *et al*, 2000; Ng *et al*, 2016).

Additionally, cytosolic DNA sensors like cGAS (cyclic GMP-AMP synthase) and IFI16 (interferon gamma inducible protein 16) detect viral and self-DNA, activating STING (stimulator of interferon genes) to induce IFN-I production (Unterholzner *et al*, 2010; Sun *et al*, 2013; Carty *et al*, 2021). The cytosolic protein AIM2 (absent in melanoma 2) senses

cytoplasmic DNA. Its activation results in the forming of inflammasomes and inducing pyroptosis and IL-1b production (Hornung *et al*, 2009).

Innate immune effectors

Once a viral pathogen is detected, a series of events are initiated to contain infection.

A key pillar of viral defense is the production of IFN-I and IFN-III. While the former has a key role systemically, IFN-IIIs are important locally at the barrier surfaces where they are produced (Gewaid & Bowie, 2024). IFN-I molecules signal through interferon- alpha/beta receptor (IFNAR), a widely expressed heterodimer of IFNAR1 and IFNAR2 subunits. Canonical signaling of the receptor is initiated by the tyrosine kinases called Janus kinases (JAKs), which phosphorylate the cytoplasmatic signal transducer and activator of transcription 1 (STAT1), and STAT2. These form a heterodimer, which associate with IRF9, forming the interferon stimulated gene factor 3 (ISGF3) complex. This complex translocates to the nucleus, where it binds to IFN stimulated response elements within in the promoter region of hundreds of IFN stimulated genes (ISGs), initiating their transcription resulting in a massive transcriptional reprogramming (Schneider *et al*, 2014).

IFN-I molecules signal in a paracrine and autocrine fashion, which serves as a way to amplify the signal, and to assure neighboring cells' preparedness to infection (McNab *et al*, 2015). Many ISGs are known to interfere with viral life cycles in a multitude of different ways, others promoting further signaling, including the production of chemokines and their receptors. Some molecules of the pathway are ISGs themselves, such as STAT1 and IRF9, again serving as a positive feedback loop amplifying the signal (Schoggins, 2019). Proinflammatory cytokines secreted upon viral sensing, such as TNF-α and IL-6 are also important tools in attracting immune cells to the site of the infection (Newton *et al*, 2016).

Natural killer cells play a vital role in eliminating virus-infected cells early during an infection. They do so by inducing apoptosis through perforin and granzymes, or by death receptor pathways (Vivier *et al*, 2011). Neutrophils and macrophages are also recruited to the site of the infection. They contribute to antiviral immunity by phagocytosis, neutrophil extracellular traps, NO and ROS production, as well as proinflammatory cytokine production (Galani & Andreakos, 2015; Wang *et al*, 2024). Dendritic cells arriving at the site of the infection are the most important antigen presenting cells, acting as key mediators between the innate and adaptive arms of immunity. pDCs in particular are major producers of IFN-I, which makes them vital in antiviral immunity (Reizis, 2019).

The complement system supports antiviral immunity in multiple ways. It limits the spreading of viruses within the body by opsonizing and tagging pathogens, and enhances antigen presentation to the adaptive immune system (Stoermer & Morrison, 2011).
Additionally, the membrane attack complex, which is the terminal complex formed by complement proteins, can directly lyse enveloped viruses and virus-infected cells (Stoermer & Morrison, 2011). The system consists of over 30 different circulating proteins that are present in the plasma in an inactive form. Three general pathways can activate them in a cascade-like manner, the so called classical, alternative and lectin pathways, which all converge on C3, a central protein of the cascade. The classical pathway is mainly activated by antigen-bound antibodies of IgM and IgG isotypes (Stoermer & Morrison, 2011). The lectin pathway is initiated by PPRs, such as mannose-binding lectin (MBL) which binds carbohydrate structures present on microbial surfaces. Many viral surface proteins are glycosylated and can be target of MBL, such as HBV, HCV or SARS-CoV-2 (Takahashi & Ezekowitz, 2005; Grosche *et al*, 2023). The alternative pathway amplifies complement activation. Cleaved peptides of multiple complement proteins, called anaphylatoxins, are also important in amplifying the signal; they act as proinflammatory signals recruiting immune cells. We see that the complement system has built-in amplification mechanisms. Through these, it has a very important role in the initial activation of innate immunity. It also enhances adaptive immunity by enhancing antibody-mediated responses (Stoermer & Morrison, 2011; Ostrycharz & Hukowska-Szematowicz, 2022).

The innate immune system orchestrates an army of immune cells, cytokines, chemokines and soluble factors. These work together to mount a robust early defense, and it is also essential in initiating an adaptive immune response (Koyama *et al*, 2008).

1.3.2 Adaptive antiviral immunity

Adaptive immunity is a pathogen-specific arm of the immune system, that is able to form memory, thus having a major role in responding to reinfections with pathogens. In contrast to innate immunity, the development of adaptive immune responses is stimulated by exposure to a particular pathogen. It recognizes a very large number of antigens, usually pathogen-derived peptides, that are loaded onto major histocompatibility complex (MHC) molecules, and presented to adaptive cells, and towards which the adaptive immune system mounts specific and potent immune responses (Abbas *et al*, 2017).

Antigen presentation

Innate processes are responsible for the initiation of adaptive immune responses. Antigen-presenting cells (APCs), most commonly DCs present antigens to the two main adaptive T

cell types, $CD4^+$ and $CD8^+$ T cells, bridging innate and adaptive immunity. APCs capture viral antigens and present them to naïve T cells, which recognize these with their T cell receptors (TCR) (Guermonprez *et al*, 2002). The key to the recognition of virtually any pathogen is TCR variability; it is estimated that 4×10^{11} T cells circulate in each human, of which 10^6 to 10^8 have different specificities (Jenkins *et al*, 2010; Lythe *et al*, 2016).

Upon PAMP detection, DCs undergo maturation, migrate to lymphoid tissues, and express high levels of MHC molecules, co-stimulatory molecules and cytokines. Recognition of the peptide-MHC complex, engagement of co-stimulatory molecules, and cytokine signaling constitute the three essential signals for T cell activation (Zhu & Paul, 2008; Curtsinger & Mescher, 2010). MHC class I molecules, expressed on all nucleated cells, present endogenous peptides to $CD8^+$ T cells. MHC class II molecules, expressed predominantly on APCs, present exogenous peptides via the endocytic pathway to $CD4^+$ T cells.

T cell activation by peptides derived from virus-infected non-APCs or malignant cells is achieved through cross-presentation. This process is unique to specific DCs. In this pathway, exogenous antigens internalized by endocytosis are presented on MHC class I molecules, enabling activation of $CD8^+$ T cells (Guermonprez *et al*, 2002).

$CD4^+$ T Cells and B Cells in Antiviral Immunity

$CD4^+$ T cells orchestrate adaptive immune responses. They do so by enhancing; “helping” other immune cell types. Upon activation, they differentiate into one of four major subsets: T helper 1 (Th1), Th2, Th17, and T regulatory (Treg) cells, based on the inflammatory cytokine milieu. Each of these have specialized functions. Th1 is primarily involved in the defense against intracellular pathogens. It does so by secreting cytokines such as IFN-γ and IL-2. IFN-γ is crucial in enhancing CD8+ T cell effector function and in macrophage activation, and it enhances antigen presentation of APCs by upregulating MHC and co-stimulatory molecules. IL-2 is critical in effector and memory $CD8^+$ T cell formation (Schoenborn & Wilson, 2007; Zhu & Paul, 2008).

$CD4^+$ T cells also have a crucial role in B cell activation. B cells also need multiple signals to undergo this process, T cells providing the second signal. When a T cell’s TCR recognizes the peptide presented on the MHC class II molecule of a B cell, and this cognate interaction is further stabilized by co-stimulatory molecules, robust B cell activation, class switching and differentiation into antibody-secreting plasma cells can take place (Cyster & Allen, 2019). In addition, $CD4^+$ follicular helper cells, residing in close proximity or within the germinal center of secondary lymphoid organs, play a critical role in the germinal center reaction, facilitating the affinity maturation of B cells leading to high-affinity antibodies (Victora & Nussenzweig, 2012).

The humoral immune response mediated by B cells is important in viral clearance by neutralizing viral particles, preventing their entry into host cells, and labelling infected cells for clearance by other immune cells types (Victora & Nussenzweig, 2012; Cyster & Allen, 2019).

$CD8^+$ T cell responses

Upon activation, antigen-specific naïve $CD8^+$ T cells undergo rapid clonal expansion, giving rise to a large population of effector cells, a process characterized by major changes in gene expression, and metabolic reprogramming (Kaech & Cui, 2012). Effector T cells migrate to the site of infection. Upon recognition of their cognate MHC class I-peptide complex on target cells, they can directly eliminate virus-infected cells in peripheral tissues by forming an immunological synapse, through which they deliver cytotoxic granules (Figure 2). These contain perforin, which forms pores on the target cell membrane, enabling the delivery of granzymes; serine proteases, which induce apoptosis by activating cellular caspases (Peters *et al*, 1991). Additionally, effector T cells can also induce apoptosis extrinsically, through the involvement of death pathways. FAS Ligand (FASL), Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or TNF-α expressed by T cells bind to their corresponding receptors on target cells initiating apoptosis through caspases (Sträter & Möller, 2004; Lee *et al*, 2023).

In addition to their direct cytotoxic activity, effector $CD8^+$ T cells contribute to antiviral immunity by secreting cytokines such as the above discussed IFN-γ and TNF-α and IL-2. (Figure 2) These, along with secreted chemokines help amplify the immune response (Chang *et al*, 2014).

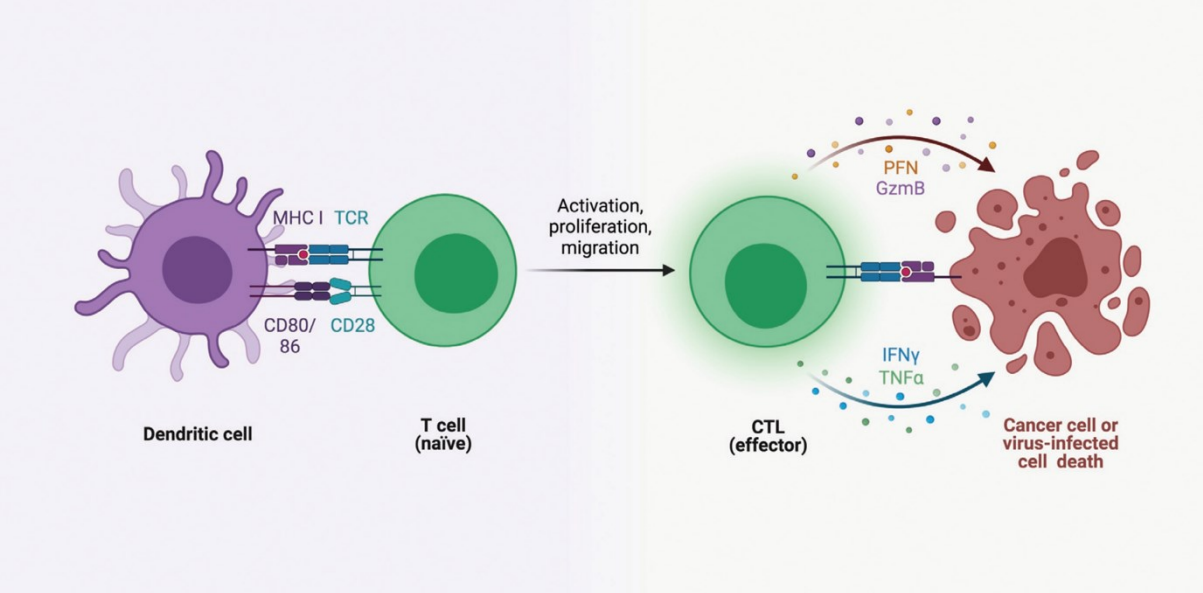

Figure 2 CD8⁺ T cell activation and effector function

When receiving the right signals from APCs, such as a recognized MHC class I – peptide complex and co-stimulatory molecules, CD8⁺ T cells get activated, undergo rapid proliferation and migrate to the site of infection. Here, they exert their effector functions by direct killing of target cells, primarily through cytotoxic granules containing perforin and granzymes, as well as cytokine secretion. Figure reproduced from (Sadeghalvad *et al*, 2022) *License number: 6146161229933*

In acute viral infections, the majority of the rapidly expanded population of antigen-specific T cells undergoes apoptosis upon viral clearance, avoiding further immunopathologies and giving space for tissue repair. A population of long-lived memory T cells persists and continues to patrol the host for recurring infections (Chang *et al*, 2014).

However, persistent antigen-exposure such as in chronic infections and in cancer, can drastically change the nature of T cell responses. On top of prolonged antigen stimulation, also other signals such as poor CD4 T cell help, signaling from inhibitory receptors, the cytokine milieu of chronic inflammation, or immunoregulatory cells can instruct T cells to gradually lose effector functions including cytokine expression and cytotoxicity, as well as proliferative capacity (Giles *et al*, 2023). This process, called T cell exhaustion can be thus mediated by all three signals required for T cell activation (Figure 3), and it is accompanied by gradual loss of antigen-specific T cells (Wherry *et al*, 2003a; McLane *et al*, 2019).

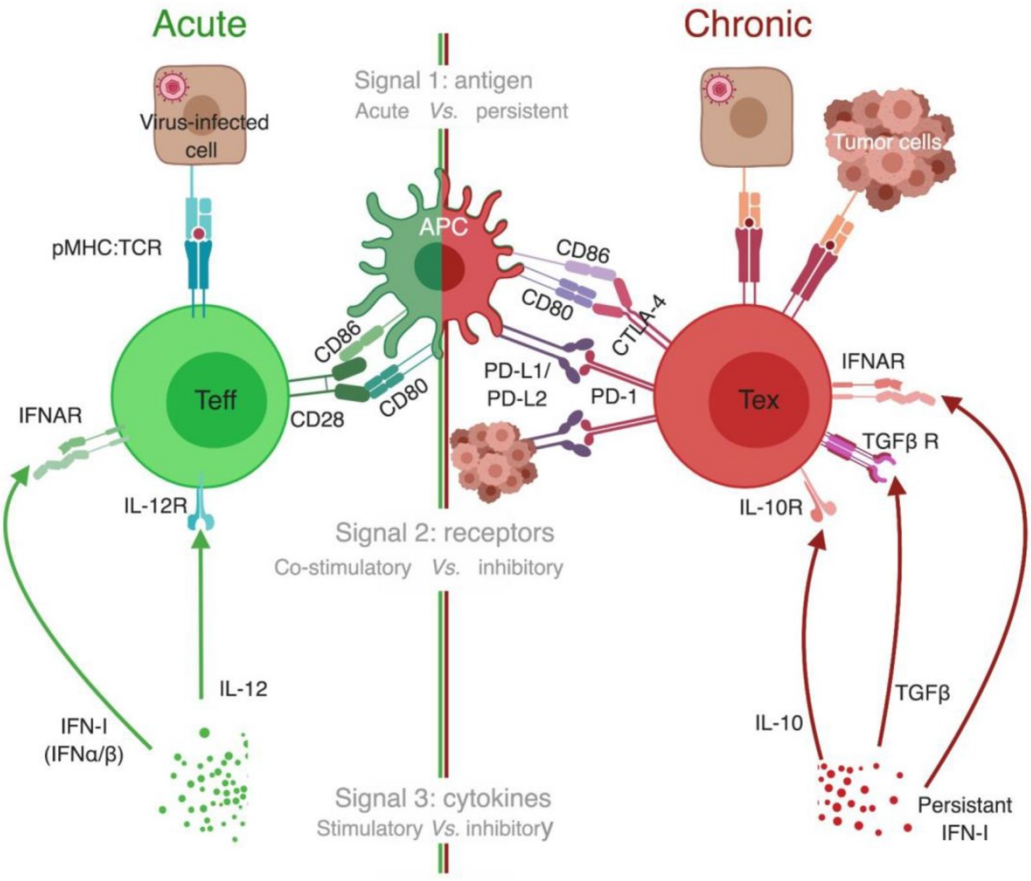

Figure 3 Signals for T cell exhaustion

Abdel-Hakeem, 2019 proposes a 3-signal model explaining the development of exhausted T cells (right half), similarly to that of effector T cells (left half).

Signal 1: Brief encounter of antigen in acute infection, while persistent stimulation in chronic disease.

Signal 2: Co-stimulatory receptors of T cells binding their ligands expressed on APCs (like CD80 and CD86) initiating the effector program. The opposite signal is communicated by inhibitory receptors and instruct T cells to enter the exhaustion program. Some of these binds to their unique ligands such as programmed cell death ligand-1 (PD-L1) or PD-L2 binging to PD-1, while others are competitive, such as CTLA4, which binds CD80/CD86, preventing binding to CD28 on the T cell surface.

Signal 3 for initiating effector T cell activation requires brief exposure to cytokines such as interleukin-12 (IL-12) and type I interferon (IFN-I). Different cytokine milieu of chronic infections contributes to exhaustion, such as persistently high levels of IFN-I and immunosuppressive cytokines like IL-10 and transforming growth factor (TGF-β).

IL-10R/ IL-12R, receptor for interleukin 10 and 12, respectively; Teff, effector T cells; Tex, exhausted T cell. Figure reproduced from: (Abdel-Hakeem, 2019) under a Creative Commons CC-BY license.

Exhaustion is progressive. Instead of complete loss of functionality, it is an adaptation of T cells to prolonged stimulation, and is important in protecting the host from immunopathology, while still enabling control over viral replication and slow viral clearance over time (Moskophidis *et al*, 1993; Wherry *et al*, 2003a).

The differentiation of exhausted T cells is epigenetically coordinated by the thymocyte selection-associated high mobility group box protein (TOX) transcription factor (Alfei *et al*, 2019; Khan *et al*, 2019; Scott *et al*, 2019), and leads to upregulation of inhibitory receptors such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), CD39 or lymphocyte-activation gene 3 (LAG-3) on the cell surface. Understanding T cell exhaustion has led to revolutionary new approaches in cancer treatment, not only immune checkpoint inhibition, but also cellular therapies such as CAR T cells, and the potential to target exhaustion even better in the future by addressing all 3 activation signals, and metabolism as a 4th signal to reprogram T cells (Giles *et al*, 2023).

1.4 Immunometabolism

The need for energy management and protection from injury and infection are two basic needs that co-evolved since the very beginning of multicellular life. The links between metabolism and immunity have been observed already as early as in 1884, when meningitis patients were noted to transiently exhibit diabetic syndrome, to the extent that it occasionally impeded the diagnoses of meningitis (Fox, 1947). Malnutrition has also been known to be immunosuppressive for a long time (Ibrahim *et al*, 2017), and Warburg described the effect named after him already in 1956 (Warburg, 1956), which was later recognized as a general metabolic rewiring enabling cell growth and proliferation, also adopted by stimulated immune cells (Frauwirth *et al*, 2002; Wang *et al*, 2011a).

It is estimated that organisms spend up to 30% of their basal metabolic rate on adaptive immune cell activation alone (Straub *et al*, 2010; Lercher *et al*, 2020). It seems obvious that immune cell activation is followed by extreme changes in cellular metabolism, however, modern immunometabolism has only started to unfold in our century, facilitated by molecular understanding of immune processes, and technological advances in quantifying a large number of different metabolites by mass spectrometry (Lercher *et al*, 2020). It is since evident that immune function and cellular metabolic changes are tightly linked, and highly regulated, by both cell intrinsic and extrinsic factors (O'Neill *et al*, 2016).

In this chapter, I will first discuss the six key metabolic pathways with great importance in supporting survival and proliferation of immune cells; glycolysis, TCA cycle, pentose phosphate pathway, fatty acid synthesis and oxidation, and amino acid pathways.

I will then briefly discuss the relevance of these pathways in myeloid and lymphoid cells, followed by an outlook on how systemic metabolism feeds in to effect immune cells, focusing on $CD8^+$ T cells.

1.4.1 Metabolic pathways

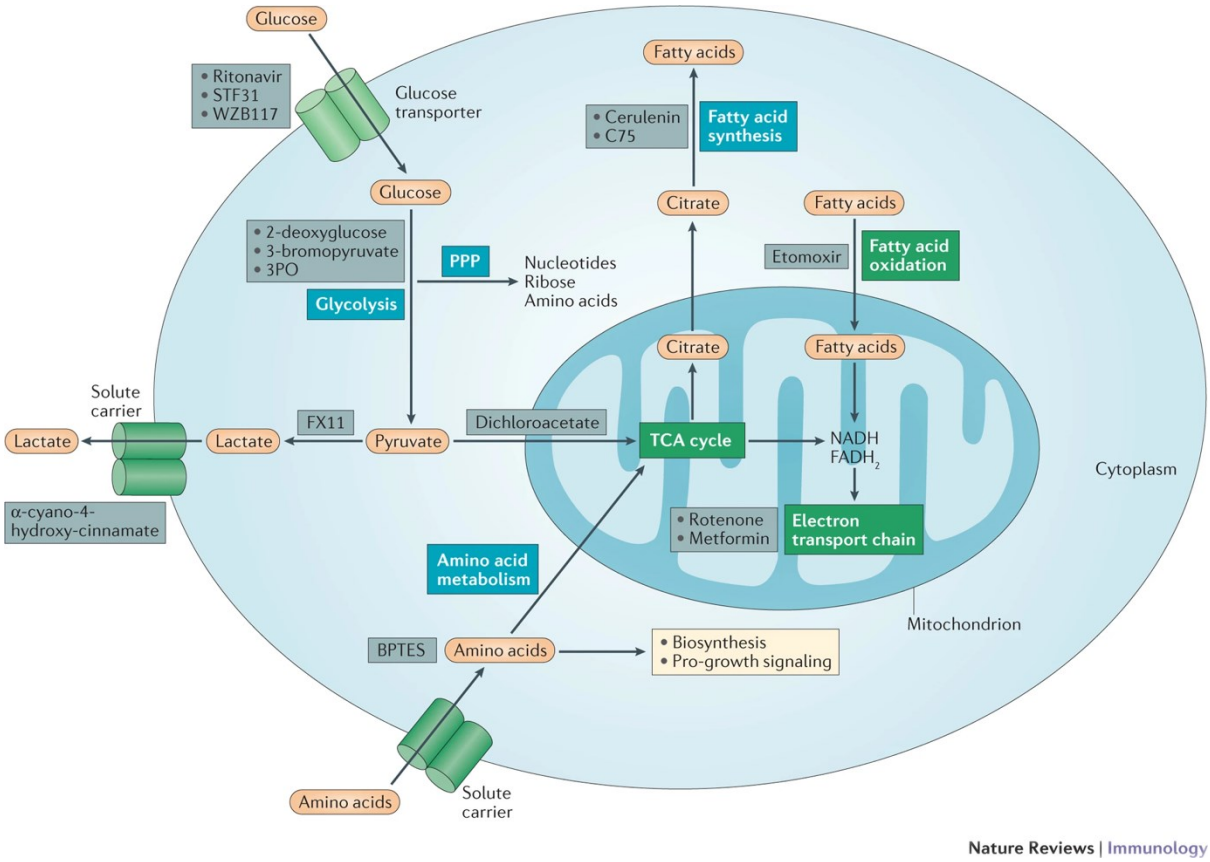

Figure 4 Overview of the six major metabolic pathways

Glycolytic intermediates can feed into the pentose phosphate pathway (PPP) and amino acid synthesis. The PPP generates ribose for nucleotide synthesis, and NADPH, crucial for fatty acid synthesis. Citrate from the TCA cycle supports fatty acid synthesis. Fatty acids can be oxidized to generate NADH and FADH2, fueling the electron transport chain.

Amino acids enter central metabolism via the TCA cycle or support anabolic processes.

Pathways requiring oxygen are marked in green and anaerobic ones in blue.

Reproduced from: (O'Neill *et al*, 2016) *License number: 6146430348125*

1.4.1.1 Glycolysis

Glycolysis is the metabolic pathway turning glucose into pyruvate, netting two molecules of ATP per glucose, in addition to the reduction of two molecules of NAD^+ to NADH. NADH provides electrons for numerous reactions as an enzyme cofactor, and thus is important for anabolic growth (O'Neill *et al*, 2016). However, sustaining the glycolytic flux needs NAD^+, and cells can reduce pyruvate to lactate to gain a NAD^+ back (Hopp *et al*, 2019).

In addition, intermediates of glycolysis feed in to biosynthetic pathways (Figure 4) such as glucose-6-phosphate into the pentose phosphate pathway for ribose supporting nucleotide synthesis, and 3-phosphoglycerate into the serine biosynthetic pathway to synthesize amino

acids. Pyruvate feeds into the TCA cycle, which can provide multiple intermediates for biosynthetic pathways such as citrate for fatty acid-, or α-ketoglutarate for amino acid synthesis (Inigo, Deja and Burgess, 2021). Glycolysis is thus of crucial importance in rapidly proliferating cells, promoted by multiple signalling pathways stimulating growth, such as phosphatidylinositol 3-kinase (PI3K) and MAPK pathways (Hu *et al*, 2016; Papa *et al*, 2019). As such, it has been shown to dominate metabolism of activated immune cells such as macrophages, DCs (van Teijlingen Bakker & Pearce, 2020), NK cells (Sohn & Cooper, 2023), B cells (Jellusova, 2020), effector and memory T cells (Huang *et al*, 2020).

1.4.1.2 TCA cycle

The TCA cycle or Krebs cycle represents a very efficient way to generate energy in the form of ATP from glucose, and is used by most quiescent cells, whose main requirement is ATP instead of biomass (O'Neill *et al*, 2016). Acetyl-CoA from glucose-derived pyruvate or from fatty acids enter the TCA cycle to produce NADH and FADH2, which feeds the electron transport chain, a very efficient way of ATP production. Alternatively, glutamate can fuel the cycle when converted to α-ketoglutarate, an intermediate of the cycle. As mentioned above, TCA cycle intermediates serve as important precursors for biosynthetic pathways, which can be exploited by proliferating cells, depleting these molecules from the cycle. To maintain cell function, these intermediates must be then replenished, a process termed anaplerosis (Akram, 2014; Inigo *et al*, 2021).

1.4.1.3 The pentose phosphate pathway

This pathway uses glucose-6-phosphate, product of the first step of glycolysis, to produce ribose 5-phosphate and NADPH and is important both for redox homeostasis and for anabolic growth of cells. Ribose 5-phosphate is an essential building block for nucleotide synthesis, while NADPH is crucial for biomass production as an electron provider, with processes such as fatty acid, cholesterol and nucleotide synthesis heavily relying on NADPH (TeSlaa *et al*, 2023). In addition, NADPH is a key electron donor for cellular antioxidant systems. Thus, this pathway is of great importance in proliferating cells, as well as upon oxidative stress. A special case of oxidative stress happens in immune cells generating ROS and NO, mainly neutrophils and macrophages, accompanied by a metabolic shift towards the pentose phosphate pathway (Azevedo *et al*, 2015; Baardman *et al*, 2018; Ghergurovich *et al*, 2020; Britt *et al*, 2022).

1.4.1.4 Fatty acid synthesis

Fatty acid synthesis takes place in the cytosol, its precursor, acetyl-CoA supplied from glycolysis or the TCA cycle through citrate. Citrate is converted to acetyl-CoA and oxalacetate in the cytosol by ATP-citrate lyase. Acetyl-CoA is then modified to generate malonyl-CoA, in

the first and rate-limiting step of fatty acid synthesis (Zhang *et al*, 2024). It is then turned into palmitate by the enzyme fatty acid synthase (FASN), in a NADPH-intensive process. Palmitate can be then further elongated, desaturated or further condensed into triacylglycerols and phospholipids, the latter crucial to support proliferation by providing the main components for plasma and organelle membranes. Fatty acid synthesis is accordingly closely coupled to cell proliferation, with mTOR signaling shown to promote it regulating key enzymes such as FASN (O'Neill *et al*, 2016; Huang *et al*, 2020).

1.4.1.5 Fatty acid oxidation (FAO)

β-Oxidation is the most common form of fatty acid breakdown, taking place in the mitochondria. It yields ample acetyl-CoA, NADH and FADH2, which can be readily used to generate energy; a single palmitate molecule has the potential to produce over 100 ATP molecules (O'Neill *et al*, 2016).

To undergo β-oxidation, fatty acids first need to be activated in the cytosol, forming fatty acid acyl-CoA. While short and medium-chain fatty acids can diffuse through the mitochondrial membrane, long-chain fatty acids need to be transported to the mitochondria conjugated to carnitine. Conjugation, mediated by carnitine palmitoyl transferase 1 (CPT1) is the rate limiting step of β-oxidation, and the enzyme is inhibited by the intermediate of lipid synthesis malonyl-CoA. This ensures efficiency by preventing β-oxidation in cells that engage in fatty acid synthesis (Longo *et al*, 2016).

While lipid synthesis is linked to immune cell activation and proliferation, quiescent and anti-inflammatory immune cells often rely on fatty acid oxidation for energy supply. These include regulatory and memory T cells, as well as anti-inflammatory M2 macrophages, relying on oxidative phosphorylation and fatty acid oxidation to generate energy (Zhang *et al*, 2024).

1.4.1.6 Amino acid metabolic pathways

Given their various distinct structures, the synthesis and breakdown pathways of amino acids are diverse. Amino acids are essential for anabolic processes, and their metabolism is linked to anabolic signaling. mTOR complexes sense amino acids levels to make sure that the requirements for growth are met (Shimobayashi & Hall, 2016). Apart from their role in building proteins, specific amino acids also link to and feed in to diverse metabolic pathways. For example, aspartate and glutamine feed in to *de novo* purine and pyrimidine synthesis (O'Neill *et al*, 2016; Mullen & Singh, 2023). They can also be used to generate energy through the TCA cycle, gluconeogenesis, or ketogenesis. For example, glutamate can be directly converted to α-Ketoglutarate to enter the TCA cycle (Ling *et al*, 2023).

1.4.2 Cell-intrinsic immunometabolism

The metabolism of immune cells is tightly regulated. Well-controlled *in vitro* experiments boosted this field in the last decades. While these models might not fully recapitulate *in vivo* settings, they did lead to crucial discoveries. They taught us about cellular metabolic reprogramming upon immune activation and “immunometabolites” with therapeutic potential (Ma *et al*, 2019; Lercher *et al*, 2020).

In a landmark paper, Vats and colleagues showed that metabolism and immune function was tightly interconnected in macrophages (Vats *et al*, 2006). They proved that metabolic shift was not just a consequence of activation, but it actively shaped the function of macrophages. Classically activated (M1) macrophages are inflammatory in nature and are activated by inflammatory stimuli such as LPS and IFNγ. The flux of glucose is then increased both into lactate and the PPP (Rodríguez-Prados *et al*, 2010; van Teijlingen Bakker & Pearce, 2020). OXPHOS, on the other hand, is diminished, and the TCA cycle is fragmented; reduced levels of isocitrate dehydrogenase result in decreased α-ketoglutarate production and accumulation of citrate (Jha *et al*, 2015). Citrate feeds into FAS and itaconate production. FAS allows the synthesis of membranes and lipid droplets (Castoldi *et al*, 2020). Itaconate, a classical immunometabolite, has multiple functions supporting macrophage activity.

Aconitate decarboxylase 1 (ACOD1, also known as IRG1) is the enzyme responsible for synthesizing itaconate from the TCA cycle metabolite aconitate, and it is induced in inflammatory macrophages. The high itaconate output (Strelko *et al*, 2011; Michelucci *et al*, 2013) further shapes macrophage phenotype. This contributes to the fragmentation of the TCA cycle through inhibition of succinate dehydrogenase, which in turn leads to succinate accumulation. Succinate acts as an inflammatory signal by stabilizing the transcription factor HIF-1α; and thus promoting IL-1β production (Tannahill *et al*, 2013). Itaconate also binds to Kelch-like ECH-associated protein 1 (KEAP1), releasing and activating NRF2, a transcription factor responsible for the expression of antioxidant and cytoprotective genes. In addition, itaconate was described to have antimicrobial properties, inhibiting bacterial enzymes (O’Neill & Artyomov, 2019).

M1 macrophages also express inducible nitric oxide synthase (iNOS), which degrades arginine to citrulline, while nitrogen monoxide (NO) is produced, a major effector molecule for killing phagocytosed pathogens (Rath *et al*, 2014).

Alternatively activated (M2) macrophages, are typically induced by IL-4 and IL-13. They are anti-inflammatory and, among others functions, play a role in tissue repair (van Teijlingen Bakker & Pearce, 2020). They express arginase1 to deplete arginine to ornithine instead of NO production (Rath *et al*, 2014). They rely on OXPHOS and FAO for energy production as opposed to glycolysis, and their TCA cycle is complete (Jha *et al*, 2015; van Teijlingen Bakker & Pearce, 2020).

In the decades that followed, it became clear that immune activation goes hand in hand with major metabolic changes across many immune cell types. Resting DCs rely on OXPHOS and FAO, which supports tolerance and antigen capture. Similarly to M1 macrophages, TLR-activated DCs undergo a rapid metabolic shift to aerobic glycolysis and lactate production, even though they do not start proliferating (Krawczyk *et al*, 2010). This shift is essential to support the migratory and secretory phenotype of activated DCs allowing them to meet the metabolic needs of intense protein and membrane synthesis (e.g. for the endoplasmic reticulum and Golgi apparatus) (Everts *et al*, 2014).

B cells also experience significant metabolic shifts during their lifecycle. Quiescent B cells reside in follicles of secondary lymphoid organs or circulate in the periphery. They rely mostly on OXPHOS (Martinis *et al*, 2025). Upon activation, they may rapidly proliferate to form extrafollicular plasmablasts, which produce early, low-affinity antibodies. Or they may enter the germinal center reaction – also characterized by rapid proliferation – where they undergo somatic hypermutation and affinity maturation before differentiating into plasma cells or memory B cells (Abbas *et al*, 2017). Glycolysis and PPP are important in activation, however, during the germinal center reaction, B cells rely primarily on OXPHOS and FAS, downregulating glycolysis, which is different compared to other proliferating cells (Waters *et al*, 2018; Weisel *et al*, 2020). When differentiating into plasma cells, they increase glucose uptake this time upregulation of glycolysis. This is how they support the energy demands of high-rate antibody production (Johnstone *et al*, 2024).

NK cells follow the same pattern, thriving on OXPHOS while resting, while mTOR is crucial for the initiation of anabolic metabolism upon activation. Glycolysis is upregulated and becomes dominant; however, NK cells also intensify OXPHOS (Donnelly *et al*, 2014). Recent evidence suggest that activated NK cells can also utilize FAO, especially under conditions of limited glucose (Delconte & Sun, 2024; Sheppard *et al*, 2024). Hypoxic conditions severely impair NK function. This have importance in tumors, where the tumor microenvironment is often limited in oxygen and nutrients (Terrén *et al*, 2019; Sohn & Cooper, 2023).

T cell immunometabolism has been a field of intense research and therapeutic interest.
As mentioned, clonal expansion of T cells upon antigen recognition and activation is an extremely energy demanding process (Straub *et al*, 2010; Lercher *et al*, 2020). TCR signaling thus drastically reprograms the cellular metabolism and proteome (Sinclair *et al*, 2013). mTOR is again essential for a metabolic shift from OXPHOS and FAO in naïve cells towards glycolysis in effector T cells, and for promoting anabolism (Chi, 2012; Werlen *et al*, 2021).

When T cells are stimulated through TCR, co-stimulation is required to engage in the metabolic programs necessary for activation; receiving signal 1 only in the form of TCR engagement results in anergy, failing to support the metabolic needs of activation (Zheng *et al*, 2009). Engagement of CD28 increases GLUT1 expression, glucose uptake and glycolysis (Frauwirth *et al*, 2002; Klein Geltink *et al*, 2017). On the contrary, engagement of inhibitory receptors PD-1 and CTLA4 can suppress this shift (Parry *et al*, 2005; Patsoukis *et al*, 2015).
Glycolysis is not only a necessary tool for proliferation, it is also intimately linked to effector function; in the lack of glycolytic flux, the glycolytic enzyme GAPDH can bind to IFN-γ mRNA, inhibiting its translation (Chang *et al*, 2013). Effector T cell development and function further depends on extracellular metabolites, including glucose and amino acids. For example, arginine is essential for T cell proliferation and function; when scarce, it can impaire TCR signaling and cytokine production, among other processes (Ma *et al*, 2024).
Metabolic programs are adapted to functional needs rather than immune cell types (Buck *et al*, 2017). Unlike effector T cells described above, memory T cells rely on OXPHOS and FAO, typically using lipids synthesized within the cell, rapidly reverting to glycolysis upon restimulation (O'Sullivan *et al*, 2014; Ma *et al*, 2024). Treg cells, similarly to memory cells, cover energetic needs by OXPHOS and FAO, but their main lipid source is exogenous lipids (Makowski *et al*, 2020).
The high metabolic demands and specific nutrient dependencies of T cells make them vulnerable to nutrient deprivation in their environment. Understanding these metabolic sensitivities offers therapeutic opportunities – not only for enhancing T cell function in solid tumors, where glucose and amino acids are often scarce, but also for dampening T cell activity in autoimmune diseases by modulating systemic or tissue metabolism (Norata *et al*, 2015; Buck *et al*, 2017; Mak *et al*, 2017).

As described above, metabolism and thus nutrient microenvironment can detrimentally influence immune cell fate and function. Thus, *in vivo* settings have flaws and they are meant to be tools to discover basic mechanisms governing immunometabolism. For example, classical M1 and M2 macrophage polarization does not recapitulate the dynamic environments found *in vivo*, where phenotypes exist along a spectrum (Martinez & Gordon, 2014; Murray *et al*, 2014). Immune cells adopt programs depending on the specific tissues they infiltrate. Advances in flow cytometry, omics and single cell techniques will help us uncover nuances of immunometabolism in tissues and organisms (Makowski *et al*, 2020), and, step by step, bring us closer to uncovering the exponentially more complex systems of systemic immune-metabolic crosstalk.

1.4.3 Systemic immunometabolism

Immune cells do not operate in isolation. They reside in tissues with specific metabolic microenvironments and circulate systemically, exposed to systemic metabolites determined by, among others – diet, circadian rhythms, age, sex and microbial communities (Lercher *et al*, 2020). In this section, I will explore how immune cells and systemic metabolism influence each other, with a particular focus on $CD8^+$ T cells.

Immunometabolism in the tumor microenvironment

Driven by tumor development, tumor microenvironments (TME) are often deprived of nutrients and characterized by excessive byproducts, which acts as a metabolic barrier for T cells. Tumor cells have a higher affinity for glucose than T cells, limiting glycolysis and IFN-γ production in T cells, which fall short in the nutrient competition (Chang *et al*, 2015; Ho *et al*, 2015). While glucose and oxygen are generally scarce, lactate is enriched due to Warburg metabolism of tumor cells (Hanahan & Weinberg, 2011). Hypoxia impairs T cell exhaustion and effector function by multiple means, such as stabilizing the transcription factor HIF 1α and inhibiting mTOR signaling (Scharping *et al*, 2016; Park *et al*, 2023). High lactate acidifies the TME, blocking T cell lactate export, undermining glycolysis, the primary source of ATP production. This in turn leads to suppressed TCR signaling, cytokine production and cytotoxicity in T cells. In addition, it promotes T cell exhaustion (Brand *et al*, 2016; Jin *et al*, 2022). Lactate is taken up by tumor-associated macrophages. In these, it promotes an M2-like, immunosuppressive phenotype. Tumor-associated macrophages also upregulate vascular endothelial growth factor (VEGF) in response to lactate, enhancing angiogenesis (Carmona-Fontaine *et al*, 2017). In addition, they upregulate arginase1, which depletes arginine in the TME further impairing T cell metabolism (Colegio *et al*, 2014; Ma *et al*, 2024). Serine and tryptophan depletion similarly limit $CD8^+$ T cells (Ma *et al*, 2017; Platten *et al*, 2019). Tryptophan is an essential amino acid that is often depleted in the TME by the enzyme indoleamine 2,3-dioxygenase 1 (IDO1). In addition, the reaction produces immune-suppressive kynurenine, a well-established suppressant of T cell proliferation and activity (Platten *et al*, 2019).

Immunometabolism in cachexia

Cancer is often accompanied by cachexia, a wasting syndrome characterized by progressive loss of adipose tissue and skeletal muscle. In cachexia, metabolic changes are promoted by inflammatory pathways, and, accordingly, it can accompany multiple diseases that feature chronic inflammation including chronic viral infections (Lercher *et al*, 2020). Specifically, the role of TNFα and IL-6 (Cawthorn & Sethi, 2008; Fearon *et al*, 2012), as well as interferons and $CD8^+$ T cells (Baazim *et al*, 2019) were shown in this metabolic rewiring. Adipose tissue is

depleted and free fatty acids are released into the circulation as part of an organism-wide redistribution of energy. In addition to serving as energy source, free fatty acids can also shape systemic metabolism by reprogramming receiving tissues such as the liver. Here they induce a shift from glucose utilization to FAO, resulting in shaping systemic glucose homeostasis and insulin sensitivity (Rosen & Spiegelman, 2006).

Immunometabolism in metabolic disease

Research in metabolic disease is not only a great example of systemic immune-metabolic crosstalk, but also a story of origin. In a landmark paper, Hotamisligil *et al.* showed that in obese and diabetic mice, adipose tissue itself secretes TNF-α, modulating systemic glucose metabolism (Hotamisligil *et al*, 1993). This was a seminal discovery that shed light on the immunological nature of metabolic disease. In addition, it also planted the seeds for the now-flourishing field of immunometabolism. Adipose tissue expansion leads to local stress and inflammation, followed by immune cell recruitment. Both stressed adipocytes and recruited immune cells secrete proinflammatory cytokines (Weisberg *et al*, 2003). Triggered by these, insulin signaling and glucose uptake are impaired in adipocytes, along with the induction of lipolysis (Hotamisligil, 2017). Expanded adipose tissue also leads to an increased production of leptin. The latter is an adipocyte-hormone suppressing appetite and promoting energy expenditure. However, obese individuals develop leptin resistance (Vilariño-García *et al*, 2024). Additionally, obesity leads to impaired $CD8^+$ T cell responses, affecting both antiviral and antitumor immunity (Kado *et al*, 2019; Ringel *et al*, 2020; Porsche *et al*, 2021).

Interestingly, TNF-α and IL-6, which are implicated in cachexia, are also major factors in the development of type 2 diabetes by interfering with insulin signaling pathways and contributing to systemic insulin resistance in multiple ways. For instance, TNF-α induces serine phosphorylation of Insulin Receptor Substrate 1, key adaptor protein in insulin signal transmission (Zatterale *et al*, 2019).

How is such cytokine-mediated crosstalk adaptive? Inflammation-induced insulin resistance, lipolysis and catabolism likely evolved as an adaptive response to infections, redirecting energy to immune cells, fueling the immense bioenergetic need of mounting a fast immune response. However, in persistent inflammation such as in cancer, obesity or chronic infection, these immune-mediated responses fuel pathological processes such as cachexia and type 2 diabetes (Buck *et al*, 2017; Hotamisligil, 2017).

Systemic immunometabolism

The serum can be regarded as an organ connecting other organs. While the importance of tissue microenvironments in immune responses is evident, circulating metabolite levels often differ from those, influenced by a multitude of factors such as age, sex, circadian rhythms,

diet, fasted/fed state, and microbial communities (Buck *et al*, 2017; Lercher *et al*, 2020). Immune cells circulating across the body and travelling to target tissues are exposed to systemic metabolite levels, which likely contribute to the quality and magnitude of their responses (Norata *et al*, 2015; Buck *et al*, 2017), as biosynthetic and bioenergetic metabolites (Geiger *et al*, 2016; Ma *et al*, 2017), or as intra- or intercellular signaling molecules (Levy *et al*, 2016; Mills *et al*, 2018).

Although the microbiome is spatially restricted within the host, a multitude of microbial metabolites enter the systemic circulation, thus surpassing the spatial restriction, and shaping physiology in the entire body, including the immune system, through their diverse effects on host metabolism (Sonnenburg & Bäckhed, 2016; Quinn *et al*, 2020). The role of these metabolites in health and disease is increasingly recognized, shaping not only enteric immunity (Belkaid & Hand, 2014; Zheng *et al*, 2020), but also the development of the immune system (Zheng *et al*, 2020), allergy (Cahenzli *et al*, 2013; Pascal *et al*, 2018), auto-immune diseases (De Luca & Shoenfeld, 2019) , and even brain function and behavior (Carabotti *et al*, 2015). In line with that, changes in the microbial composition have been correlated with health conditions such as inflammatory bowel disease, obesity, diabetes, autism, or cancer (Blumberg & Powrie, 2012; Gilbert *et al*, 2016). The role of SCFAs, secondary bile acids, polyamines and tryptophan metabolites derived from the microbiota are well established in immune signaling, effecting both innate and adaptive immune responses. T cells were as well described as targets of microbial metabolism (Rooks & Garrett, 2016). Yu *et al.* have found that mice harboring distinct microbial communities differ in colorectal cancer susceptibility due to different dynamics of T cell exhaustion (Yu *et al*, 2020). The microbiome was also proven responsible for altering T-cell receptor repertoires, conceivably through the expansion of T cells recognizing microbial epitopes (Cebula *et al*, 2013; Pearson *et al*, 2019).

A mixture of 11 commensal species were found to induce interferon-γ-producing $CD8^+$ T cells in the intestine, acting as a consortium (Tanoue *et al*, 2019). SCFAs, such as butyrate, propionate or acetate, are among the best studied microbial immunometabolites. SCFAs were found to enhance $Foxp3^+CD4^+$ T reg differentiation by multiple means (Arpaia *et al*, 2013a; Furusawa *et al*, 2013; Smith *et al*, 2013), enhance the maturation of liver-resident NK cells through inducing IL-18 production in KCs (Tian *et al*, 2023), and inhibit histone-deacetylases in $CD8^+$ T cells thus interfering with gene expression, resulting in an increase of IFN-γ and granzyme B expression (Luu *et al*, 2018) Multiple laboratories reported microbial bile acid metabolites influencing colonic $CD4^+$ T cells through multiple mechanisms (Hang *et al*, 2019; Campbell *et al*, 2020; Song *et al*, 2020) and the therapeutical bile acid NorUDCA was described to regulate $CD8^+$ T cell effector function and modulate $CD4^+$ T cell differentiation (Zhu *et al*, 2021, 2025)

Infectious diseases are also accompanied by systemic metabolite changes (Lercher *et al*, 2019; Wu *et al*, 2022; Baiges-Gaya *et al*, 2023; Shi *et al*, 2024). On top of the potential of these as biomarkers, they might influence pathogen replication and immune responses (Kosack *et al*, 2017; Lercher *et al*, 2019; Tounta *et al*, 2021; Peron & Lin, 2024), with examples across diverse classes of pathogens, including protozoa, bacteria and viruses.

Abdrabou and colleagues showed that Plasmodium falciparum infection leads to significant shifts in circulatory steroid metabolites such as pregnenolone sulfate. This shift was strongly associated with parasitemia, and proved immunomodulatory *in vitro*, suppressing T cell activation and proliferation (Abdrabou *et al*, 2021).

Marked systemic metabolite changes were reported in bacterial infections as well (Mosevoll *et al*, 2022; Li *et al*, 2024a; Bayne *et al*, 2025). In a mouse model of Staphylococcus aureus infection, Pang and colleagues found elevated plasma levels of branched chain amino acids (leucine, isoleucine, valine) and proline (Pang *et al*, 2020). Infection also upregulated arginase in various organs, outcompeting nitric oxid synthase (NOS) in their common substrate, arginine. Exogenous supplementation of the above amino acids inhibited arginase activity, shifting arginine usage towards NO production, resulting in enhanced phagocytic killing and reduced bacterial loads.

Arginase activity is also upregulated COVID-19 patients, resulting in decreased NO production, which impairs impair endothelial function and favors thrombosis, on top of reducing NO-mediated immune responses (Banoei *et al*, 2025).

COVID-19 severity also correlates with increased serum kynurenine and reduced tryptophan, accompanied by IDO1 activation, resulting in an immunomodulatory circuit. Kynurenine dampens antiviral NFκB and interferon responses through the aryl hydrocarbon receptor, suppressing NK cell and T cell activation (Shen *et al*, 2020; Uchimido *et al*, 2024). Increased glycolysis observed in severe COVID-19 patients may further enhance hyperinflammation (Banoei *et al*, 2025). Such infection-induced perturbations occur on top of pre-existing metabolic alterations in patients with obesity, insulin resistance, or diabetes. This explains the higher susceptibility to severe COVID-19 among patients with such co-morbidities; they are characterized by chronic low-grade inflammation (Batabyal *et al*, 2021).

LCMV, as a benchmark rodent model of viral infection, also proved useful in uncovering systemic immunometabolic circuits. Lercher *et al.* showed that during chronic LCMV infection, disruption of the urea cycle leads to decreased serum arginine and increased ornithine levels (Lercher *et al*, 2019). The shift impairs $CD8^+$ T cell responses ameliorating liver immunopathology. These finding pinpoint how systemic metabolic changes can directly impact immune responses. The liver, a central hub of both metabolism and immunoregulation is of particular interest in immune-metabolic crosstalk.

1.5 The liver

The liver is an essential metabolic organ that has profound influence on systemic metabolite levels (van den Berghe, 1991). It resides at the crossroads of gut-derived microbial products and blood-borne pathogens, characterized by unique immunological features (Parlar *et al*, 2023). These properties make it a key player in systemic immunometabolism. In this chapter, I will first cover liver architecture, after which I will summarize liver immunity and metabolic function.

1.5.1 Architecture of the liver

The liver is primarily built of hepatocytes, the major parenchymal cells and those responsible for its main functions. These count for approximately 80% of tissue volume, while other organ-specific cells types include liver sinusoidal endothelial cells (LSECs), liver-resident macrophages called Kupffer cells (KCs), hepatic stellate cells (HSCs), and cholangiocytes lining bile ducts (Ishibashi *et al*, 2009).

Hepatic lobules are the structural and functional unit of the organ, repeating columns that are roughly hexagonal in their cross-section (Figure 5). Blood enters the lobule at its periphery, from the so-called portal triad, which comprises a bile duct (draining bile produced by hepatocytes), a branch of the portal (delivering nutrient-rich blood from the intestine), and a branch of the hepatic artery (supplying oxygenated blood). Blood from the portal vein and hepatic artery flows radially inward, through a network of thin capillaries called sinusoids, draining at the central vein at the center of the lobule (Ben-Moshe & Itzkovitz, 2019). Hepatocytes are organized tightly around the central vein in plates, allowing space for sinusoids and the perisinusoidal space, the Space of Disse, which lies between hepatocytes and LSECs (Ben-Moshe & Itzkovitz, 2019).

Depending on their proximity to the portal triad, hepatocytes are exposed to different concentration of nutrient and oxygen, which creates distinct gene expression patterns in specific hepatocyte layers, resulting in metabolic zonation (Ben-Moshe & Itzkovitz, 2019). In a division of labor, different layers of hepatocytes are characterized by distinct gene expression patterns, with distinct cellular functions dominating in specific layers. Halpern and colleagues having recently identified 9 distinct radial layers (Halpern *et al*, 2017).

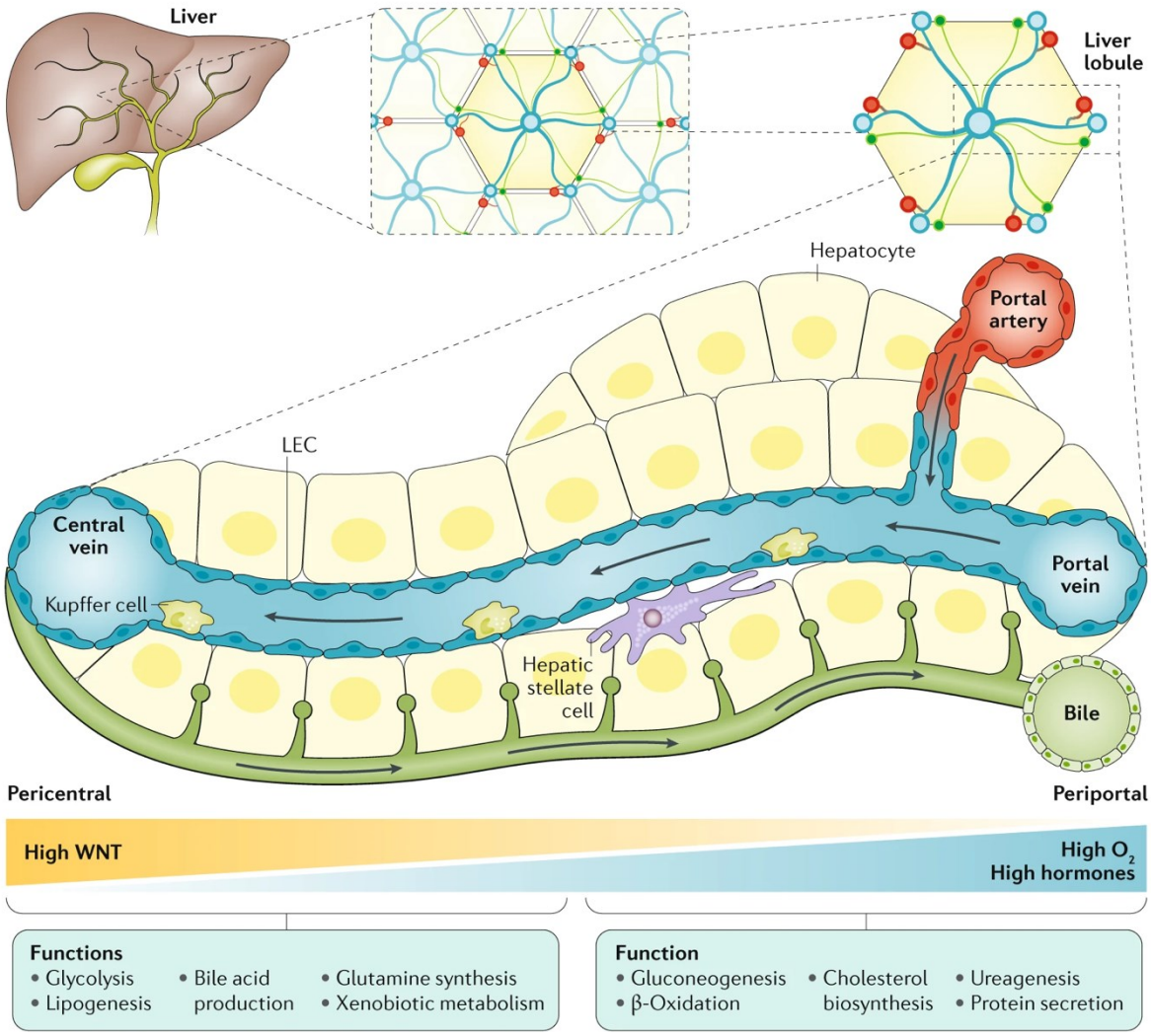

Figure 5: Architecture of the liver in homeostasis

The liver is organized into hexagonal lobules with a central vein at the core and portal triads at the periphery. A portal triad includes a hepatic artery, a portal vein, and a bile duct. Blood flows from the portal triads through sinusoids toward the central vein, establishing a nutrient and oxygen gradient resulting in the metabolic zonation of hepatocytes along the portal–central axis. Bile produced by hepatocytes flows in the opposite direction, toward the bile duct via bile canaliculi. Non-parenchymal liver cells, such as Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells, are distributed along the lobule and support hepatocyte function.

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Sinusoids are covered in LSECs, which are fenestrated and lack a basement membrane to allow the flow of substances between the blood and liver parenchyma, and intimate connections between hepatocytes and immune cells (Wisse *et al*, 1996). KCs reside intravascularly, removing larger particles from the circulation, while LSECs take up smaller particles (Ishibashi *et al*, 2009).

The Space of Disse hosts HSCs, which store fat- and vitamin A, produce ECM, and play a central role in liver fibrosis (Puche *et al*, 2013). Hepatocytes are highly polarized with distinct apical (canalicular) and basolateral (sinusoidal) membranes (Figure 6). They secrete bile into bile canaliculi – narrow intercellular channels formed by the adjoining apical membranes of neighboring hepatocytes. (Figure 6) Tight junctions between hepatocytes ensure canaliculus integrity (Arias *et al*, 1993). As a frontline filter for gut-derived antigens and microbial products, the liver also coordinates immune responses and immune tolerance.

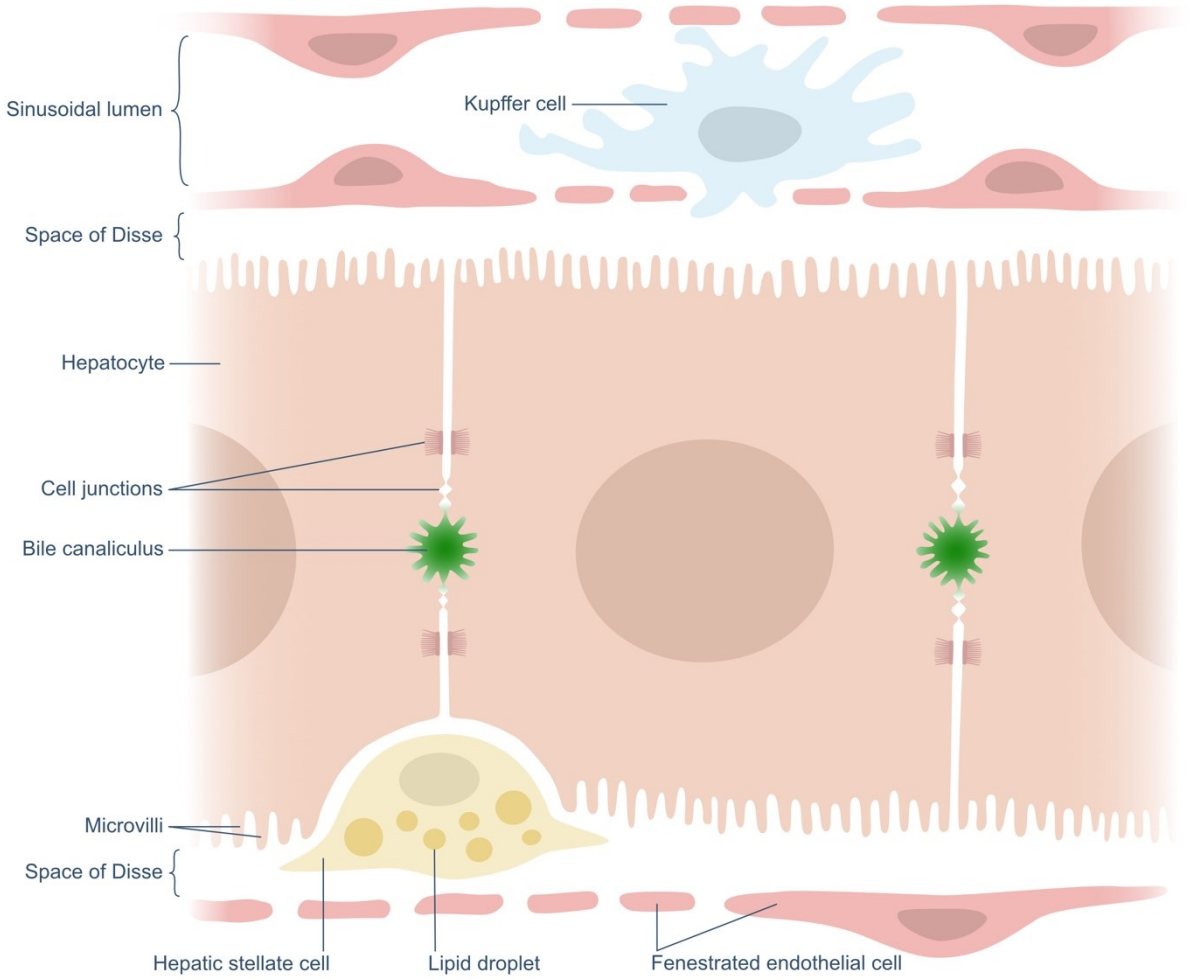

Figure 6: Liver microanatomy

Schematic of liver architecture showing hepatocytes, Kupffer and stellate cells, fenestrated endothelium, bile canaliculi, and the space of Disse.

1.5.2 Liver immunity

The liver receives the majority of its blood supply directly from the gut *via* the portal vein, and is thus target of constant exposure to antigens, PAMPs and DAMPs. As a gatekeeper, the liver has to tolerate this constant immunogenic load, while ensuring immunosurveillance for pathogenic conditions (Robinson *et al*, 2016; Parlar *et al*, 2023). The concept of a tolerogenic

liver is demonstrated by the fact that liver transplants are better tolerated than other organ transplants (Lerut & Sanchez-Fueyo, 2006). Furthermore, hepatic tolerance also induces systemic tolerance, shown by improved tolerance of a co-transplanted organ (Simpson *et al*, 2006).

The highly phagocytic sinusoidal KCs capture and break down larger particles. They express a range of PRRs, but their downstream response tends to be anti-inflammatory under homeostatic conditions, secreting anti-inflammatory IL-10 and prostaglandin E2 (Knolle *et al*, 1995). This clearance of blood from commensal- and food-derived antigens prevents excessive immune activation in the rest of the body (Robinson *et al*, 2016). One effect of IL-10 is the downregulation co-stimulatory molecules on APCs (Knolle *et al*, 1998). Indeed, hepatic DCs are also less efficient in T cell activation and produce more IL-10 compared to DCs derived from the spleen (Bamboat *et al*, 2009).

LSEC, HSCs and hepatocytes also express various PPRs and are able to present antigens to T cells. However, the local immunosuppressive cytokine milieu and the lack of co-stimulation promotes T cell anergy, exhaustion or apoptosis (Thomson & Knolle, 2010; Robinson *et al*, 2016), rendering the liver an appealing refuge for pathogens such as hepatitis viruses and *Plasmodium* spp. causing chronic infection (Protzer *et al*, 2012).

Yet, the hepatic immune system is capable of mounting strong immune responses when needed. During acute liver injury or insult, KCs undergoing MyD88-independent PRR-signaling pathways switch their secretion to pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Tu *et al*, 2008; Robinson *et al*, 2016). The pro-inflammatory milieu enables potent activation of T cells by KCs, hepatocytes and LSECs, the latter having very high scavenging activity (Knolle & Wohlleber, 2016).

KCs are among the first responders to viral infections, mounting a IFN-I response (Lang *et al*, 2010). Hepatocytes can be targeted by not only hepatotropic viruses but also systemic pathogens like LMCV. They also express a range of PPRs, through which they can mount IFN-I responses (Crispe, 2016). Priming of T cells is possible by hepatocytes but results in a more robust response when done by KCs (Bénéchet *et al*, 2019). Cytotoxic T cells arriving to the infection site reach liver cells *via* the sinusoids, actively protruding cellular extensions through the LSEC fenestrae to inspection and killing hepatocytes (Guidotti *et al*, 2015).

The liver maintains systemic metabolic homeostasis through various processes. Hepatocytes orchestrate glucose metabolism by synthesizing, metabolizing, and releasing glucose, as well as storing it in the form of glycogen. They also act as a major hub for protein and amino acid metabolism. Hepatocytes secrete most plasma proteins, including albumin, clotting factors and acute phase proteins in response to inflammation, infection, or tissue damage (Trefts *et*

al, 2017; Robinson *et al*, 2016). They act as primary sites of amino acid catabolism and the disposal of their nitrogenous waste through the urea cycle (Trefts *et al*, 2017; Mantovani & Garlanda, 2023). They store vitamins and iron, and detoxify endogenous waste products and xenobiotics (Kodicek, 1954; Anderson & Shah, 2013). Hepatocytes are also crucial in lipid and cholesterol homeostasis, and produce bile to help lipid digestion and absorption (Trefts *et al*, 2017).

1.6 Bile acids

Bile acids are cholesterol-derived amphipathic molecules synthetized by hepatocytes.
They circulate between the liver and the gut and can be modified by microbial enzymes to give rise to microbial-derived bile acids (Tyor *et al*, 1971), adding to the complexity of bile acid pool composition. While their primary function is facilitating lipid absorption, bile acids have diverse effects on various cell types throughout the body, including immune cells (Shapiro *et al*, 2018). Medical interest in bile dates back to almost three millennia, used by traditional Chinese, medicine (Wang & Carey, 2014), but their signaling properties started to be recognized in our millennium (Martinot *et al*, 2017).
In this chapter, I will first describe a simplified overview of the complex chemistry of BA synthesis, followed by microbial modification. I will then review how bile acids circulate between liver and gut and detail how synthesis is regulated. Finally, I will summarize the signaling properties of bile acids.

1.6.1 Bile Acid Synthesis and Conjugation

The machinery to turn cholesterol into bile acids also acts as a cholesterol catabolic pathway (Russell, 2009). Modifications include steroid ring hydroxylation, saturation and epimerization, as well as side-chain oxidation, chain shortening, and, finally, conjugation with glycine or taurine, achieved through 17 enzymatical steps. Many of the involved enzymes also have other functions, such as CYP27A1, catalyzing a step in vitamin D biosynthesis (Chiang, 2014; Russell, 2009). The resulting bile acid, bearing multiple hydroxyl groups and a conjugated amino acid, is amphipathic rather than hydrophobic (Figure 7), enabling its role in lipid emulsification (Fleishman & Kumar, 2024)

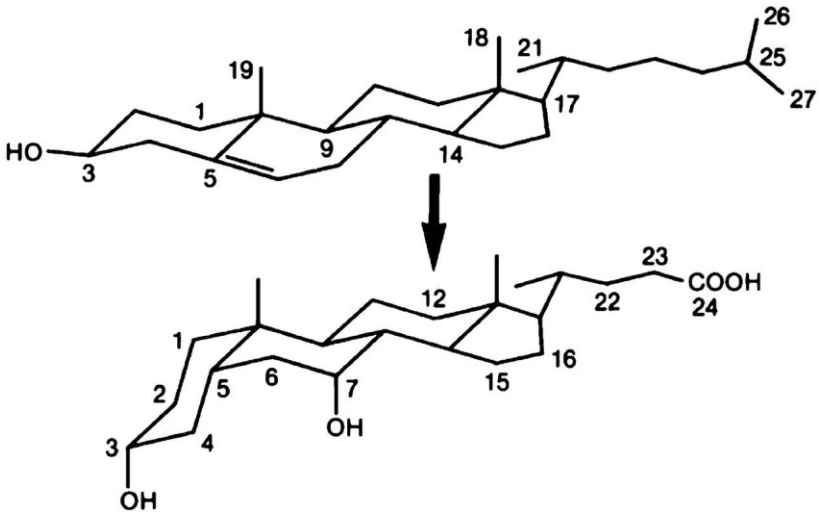

Figure 7: Chemical structure of cholesterol and CDCA

The figure shows the difference in the molecular structure of CDCA (below) compared to cholesterol (above). The resulting molecule has a hydrophobic (up) and a hydrophilic (down) side. The BA synthetic machinery catalyzes the following changes:

1) *Reduction of the double bond results in a 5β conformation and a bent ring structure*
2) *Side chain shortening and conversion of the terminal carbon to a carboxyl group.*
3) *Epimerization; a change in the orientation of the hydroxyl group from 3β to 3α.*
4) *A 7α-hydroxyl group is added.*

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The two major biosynthetic pathways are the so-called classical pathway, where steroid ring modification takes place before side-chain cleavage, and alternative (or acidic) pathway, where side-chain oxidation precedes modification of the steroid nucleus (Figure 8). The alternative pathway accounts for approximately 10% of BA production in humans, but has quantitative importance in neonates, where the CYP7B1-driven alternative pathway is essential (Setchell, 2021). Certain physiological conditions also induce CYP7B1 expression such as cold exposure (Worthmann *et al*, 2017) or liver disease (Lake *et al*, 2013). Rodents yield more substantial proportion, up to half of their BA pool from this pathway (Li & Chiang, 2020). In addition, key enzymes of the alternative pathway CYP27A1 and CYP7B1 are expressed in extrahepatic tissues such as the brain and the kidney, also representing a way to eliminate cholesterol from the brain. However, the full enzymatic machinery required for BA synthesis is only expressed in the liver, where extrahepatic oxysterols can be carried and the conversion into bile acids can be concluded (Li & Chiang, 2020; Setchell, 2021).

The classical pathway converts cholesterol into both cholic acid (CA) and chenodeoxycholic acid (CDCA) and accounts for the majority of BA production in both humans and rodents (Fleishman & Kumar, 2024). It is initiated by cholesterol 7α-hydroxylase (CYP7A1), the only

rate-limiting enzyme in bile acid biosynthesis, which hydroxylates cholesterol at position 7α to produce 7α-hydroxycholesterol (Chiang, 2013). Then, HSD3B7 (3β-hydroxy-Δ5-C27-steroid dehydrogenase) isomerizes the double bond in the sterol ring and oxidizes the hydroxyl group at carbon 3. Next, CYP8B1 (sterol 12α-hydroxylase) may catalyze a hydroxylation at carbon 12 – the functional group that distinguishes CA from CDCA, giving rise to CA. Or, if this step is skipped, the end product will be CDCA (Fleishman & Kumar, 2024). Regardless of whether 12α-hydroxylation takes place, the next steps do not differ: reduction and side-chain modifications catalyzed by AKR1D1 (Δ4-3-oxosteroid-5β-reductase), AKR1C4 (3α-hydroxysteroid dehydrogenase), and CYP27A1 (sterol 27-hydroxylase, which initiates side-chain oxidation). These modifications are completed by peroxisomal β-oxidation to shorten the 8 carbon atom side chain of cholesterol to 5 carbons (Fleishman & Kumar, 2024; Russell, 2003).

The alternative pathway starts with the hydroxylation of the side chain catalyzed by sterol 27-hydroxylase (CYP27A1) yielding 27-hydroxycholesterol, and 7α-hydroxylation is achieved by CYP7B1 (oxysterol 7α-hydroxylase). HSD3B7, then AKR1D1 and AKR1C4 continue the process similarly as in the classical pathway. Side chain cleavage is once again achieved *via* peroxisomal β-oxidation, resulting in the end product CDCA (Fleishman & Kumar, 2024).

Finally, both CA and CDCA undergo conjugation with an amino acid, which increases their ionization and solubility (Chiang, 2013). First they are activated by conjugation with CoA by the enzyme BACS (Bile acid:CoA synthase), and then, as a terminal step, glycine or taurine are conjugated to the bile acid on carbon 24, a reaction catalyzed by BAAT (bile acid-CoA: amino acid N-acyltransferase) (Russell, 2003).

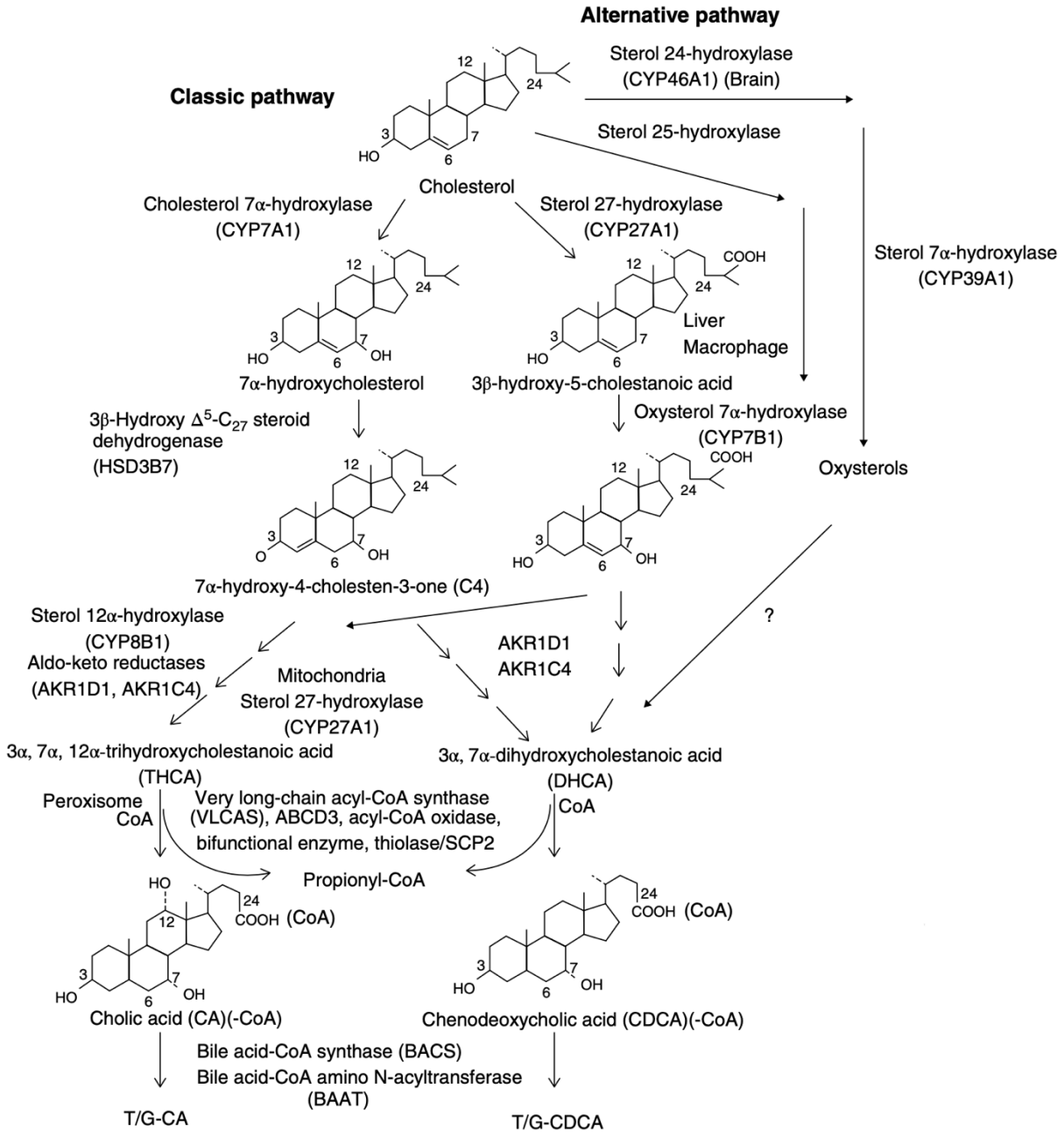

Figure 8 – Bile acid synthesis: classical and alternative pathways

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The mouse bile acid pool differs from the human in multiple aspects (Figure 9). Due to lower Cyp8b1 activity, less CA is synthesized in the mouse, and most of CDCA is readily converted into muricholic acid (MCA) by the rodent enzyme CYP2C70, adding a hydroxyl group on carbon 6, thus making the BA pool more hydrophilic. First, the intermediate α-MCA is synthesized, which is then converted to β-MCA, leaving only small amounts of α-MCA (Bhattacharya *et al*, 2023) (Figure 8). Additionally, mice bile acids are predominantly conjugated with taurine, and glycine conjugates are only detected at low levels. This contrasts

humans, where glycine is the preferred conjugation partner (Chiang, 2013). Finally, bile acids can be sulfated on both the backbone and the side chain by hepatic sulfotransferases, which increases their solubility, decreases their toxicity signaling properties, and enhances their elimination through urine and feces. In humans, sulfation most commonly occurs on C3, while murine bile acids are preferentially sulfated on C7 (Alnouti, 2009). A significant proportion of circulating BAs is sulfated in humans, a recent study identifying 27% of total serum BA to be sulfated. In contrast this number is below 1% in mice (Zheng *et al*, 2024).

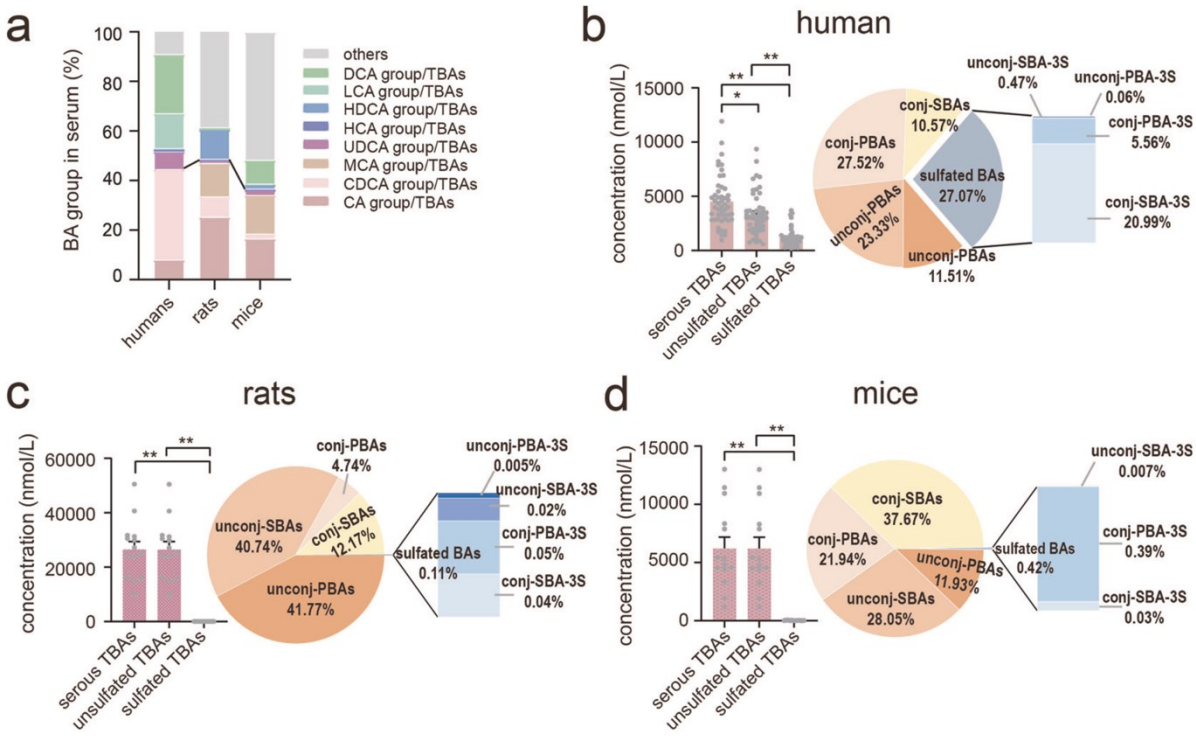

Figure 9 – The composition of serum BA in human, rats and mice

Percentage of serum BA groups, a comparison between species. Figure reproduced from: (Zheng *et al*, 2024)*, under a Creative Commons CC-BY license.*

1.6.2 Microbial modifications of bile acids

Upon entering the distal intestine, host-derived primary bile acids are exposed to a multitude of microbial enzymes catalyzing diverse chemical modifications, giving rise to microbe-derived-, also called secondary bile acids.

Deconjugation, catalyzed by bacterial bile salt hydrolases (BSH) is considered the gatekeeper reaction before further modifications can happen (Batta *et al*, 1990; Guzior & Quinn, 2021). BSH enzymes are found across all major bacterial phyla and many archaeal species, suggesting horizontal transferability of the encoding genes (Guzior & Quinn, 2021). Recent

findings uncovered bacterial reconjugation (Rimal *et al*, 2024) and acyltransfer (Guzior *et al*, 2024) of bile acids, mediated by bacterial BSH enzymes on top of deconjugation. Various amino acids can be conjugated to different BAs, resulting in an even greater variety of microbial bile acids termed microbially conjugated bile acids (MCBAs) (Rimal *et al*, 2024; Guzior *et al*, 2024; Quinn *et al*, 2020). However, Guzior *et al.* found that the acyltransferase activity of *Clostridium perfringens* BSH reached only 7% of its peak hydrolytic activity, leaving ample amounts of unconjugated bile acids available for further microbial transformation (Guzior *et al*, 2024). The latter include dihydroxylation, oxidation and epimerization of the steroid core.

7α-dehydroxylation of CA, mediated by complex multi-enzyme step, gives rise to deoxycholic acid (DCA), and the same transformation of CDCA results in lithocholic acid (LCA), as shown in Figure 10 (Guzior & Quinn, 2021). The 7α-dehydroxylation machinery is less widespread than BSH enzymes. The bile acid-inducible (*bai*) operon, which encodes the enzymatic machinery required for this transformation, is only present in a limited number of strictly anaerobic Firmicutes, primarily *Clostridium* species (Winston & Theriot, 2019).

Different bacterial hydroxysteroid dehydrogenases (HSDHs) catalyze site-specific redox transformations of BAs. These give rise to a combinatorial diversity of BA species. These reactions include epimerization, oxidation, and reduction at various hydroxyl positions (Guzior *et al*, 2024; Fleishman & Kumar, 2024). For example, both 7α-HSDH and 7β-HSDH are required to convert CDCA to ursodeoxycholic acid (UDCA) *via* 7α-to-7β epimerization (Doden & Ridlon, 2021), as shown in Figure 10. This change in stereochemistry results in markedly different hydrophobicity and toxicity, making UDCA (originally obtained from bear bile) the earliest bile acid used therapeutically (Wang & Carey, 2014). Other epimerized or oxidized bile acids have also been shown to influence immunity: 3β-hydroxydeoxycholic acid (also referred to as isoDCA, Figure 10) and 3-oxo-LCA, synthesized by 3α-HSDHs from LCA have been found to modulate T cells in the gut (Song *et al*, 2020; Hang *et al*, 2019; Campbell *et al*, 2020).

In mice, host-derived α- and β-MCAs undergoing HSDH modifications commonly result in Hyodeoxycholic acid (HDCA) and ω-MCA. Murine livers also express Cyp2a12, an enzyme human hepatocytes lack, which can convert DCA back to CA (Bhattacharya *et al*, 2023).

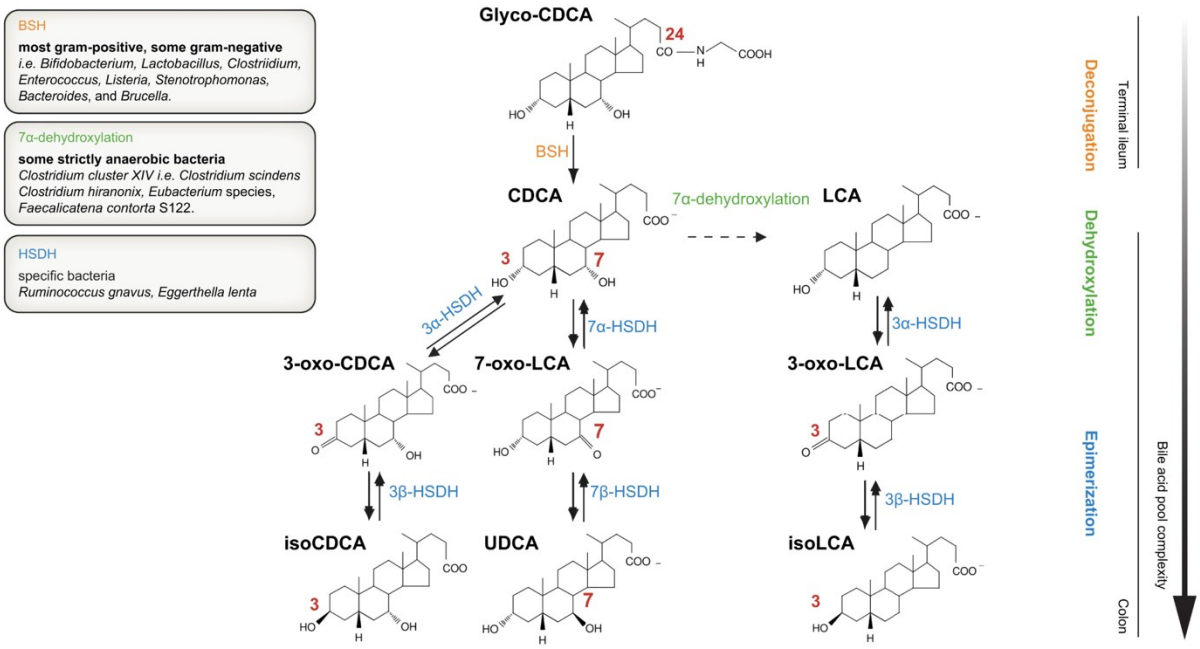

Figure 10 – Bacterial modifications of BAs

The figure illustrates key enzymatic modifications and resulting molecules through the example of Glyco-CDCA. Reproduced from: (Fuchs *et al*, 2025) *under a Creative Commons CC-BY license.*

1.6.3 Enterohepatic circulation

Bile acids are reabsorbed from the gut and recycled by the liver through enterohepatic circulation. About 95% of bile acids are recirculated this way, and hepatocytes make up for the 5% that is lost through feces, a daily amount of approximately 200-600mg in human. Bile secretion and fecal loss also represent the primary elimination route for cholesterol. (Fleishman & Kumar, 2024).

In addition to bile acids, bile contains phospholipids, cholesterol, conjugated bilirubin, electrolytes, and water, along with various detoxified end-products (Hofmann, 1999).

It is secreted from hepatocytes into bile canaliculi by multiple canalicular transporters; bile salt export pump (BSEP) encoded by the *Abcb11* (ATP-binding cassette sub-family B member 11) gene transports BAs themselves, in an ATP-dependent fashion (Kunst *et al*, 2021). Bilirubin glucuronides are transported by MRP2 (multidrug resistance-associated protein 2, gene name *Abcc2*) also transporting drug metabolites and other organic anions. MDR2 (MDR3 in humans, while both human and mouse gene is named *Abcb4/ABCB4*) transports phosphatidylcholine to the canaliculus (Kullak-Ublick *et al*, 2000), and the heterodimer of ABCG5/ABCG8 (ATP-binding cassette sub-family G member 5/8) exports cholesterol (Yu *et al*, 2005b).

Bile then travels through the biliary tree and is stored in the gall bladder, which empties postprandially (after meals). Bile then reaches the duodenum *via* the common bile duct, where

it exerts its main function in lipid and fat-soluble vitamin absorption (Fleishman & Kumar, 2024).

Bile acids are reabsorbed from the distal ileum by enterocytes expressing the Apical sodium-dependent bile acid transporter (ASBT, gene name *Slc10a2*). After uptake, bile acids traverse the cytoplasm with the help of the ileal bile acid-binding protein (IBABP, gene: *Fabp6*). Export through the basolateral membrane occurs *via* the heterodimeric transporter OSTα/OSTβ (*Slc51a/Slc51b*) to the portal blood. BAs then return to the liver *via* the portal vein, along with other intestinally absorbed molecules (Durník *et al*, 2022).

Hepatocytes reabsorb BAs at their sinusoidal (basolateral) surface using the Na^+/taurocholate-co-transporting polypeptide (NTCP, gene name *Slc10a1*), and the organic anion-transporting polypeptides (OATPs), notably OATP1A1 (*Slco1a1*), OATP1B2 (*Slco1b2*) and OATP1A4 (*Slco1a4*) in rodents (Durník *et al*, 2022; Higgins *et al*, 2014). Humans lack the exact orthologues of rodent OATPs and instead express OATP1B1 and OATP1B3 (Higgins *et al*, 2014; van de Steeg *et al*, 2010). NTCP and OATPs share substrates and are able to transport both conjugated and unconjugated BAs (Geier *et al*, 2007; Slijepcevic *et al*, 2017). However, NTCP has a higher affinity for conjugated BAs, and was shown to have a crucial role in their uptake (Slijepcevic *et al*, 2015; Kosters & Dawson, 2015), and OATPs for unconjugated BAs, critical in the uptake of these (van de Steeg *et al*, 2010). All OATPs are involved in transporting bile acids, bilirubin, steroid hormones, and various drugs from the blood into hepatocytes (van de Steeg *et al*, 2010; Durník *et al*, 2022).

Upon cholestatic conditions, increased BA sinusoidal efflux help protect hepatocytes from bile acid toxicity. This is mainly mediated by MRP3 (*Abcc3*) and MRP4 (*Abcc4*) in both human and mouse hepatocytes. Additionally, hepatocyte expression of OSTα/OSTβ (*Slc51a* and *Slc51b*) can be also induced in cholestasis aiding elimination of bile acids from hepatocytes towards the circulation (Fuchs & Trauner, 2022; Soroka *et al*, 2010)

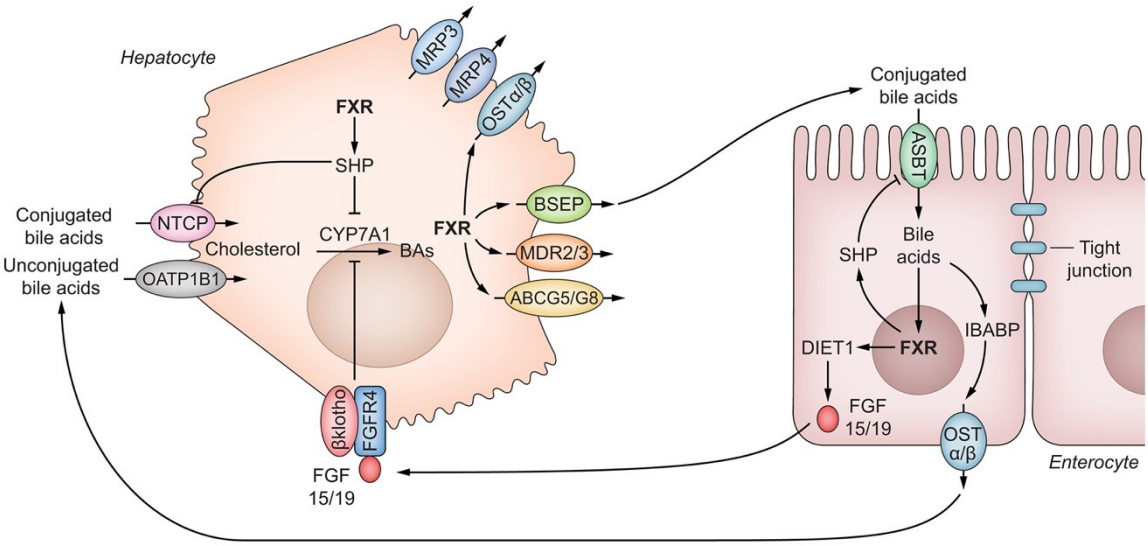

Figure 11 – Enterohepatic circulation of BAs and their regulation

BA transport, intestinal reabsorption and negative feedback regulation via FXR–FGF15/19–SHP signaling. MDR2/3 represents the mouse/human nomenclature of this protein. Figure reproduced from: (Fuchs *et al*, 2025) *under a Creative Commons CC-BY license.*

1.6.4 Regulation of Bile Acid synthesis and transport

BAs regulate their own *de novo* synthesis through a well-described negative feedback loop (Figure 11). Ileal enterocytes that take up BAs *via* ASBT activate the nuclear receptor farnesoid X receptor (FXR, gene name *Nr1h4*). Bile acids vary in their ability to activate FXR, with CDCA being the most potent. The relative potency follows the order: CDCA > DCA > LCA > CA, and conjugation to glycine or taurine generally reduces FXR activation (Jiang *et al*, 2021).

Upon BA binding, FXR translocates to the nucleus, forms a heterodimer with retinoid X receptor α (RXRα) and acts as a transcription factor, inducing genes bearing FXR response elements in their promoter regions. In hepatocytes, these include genes like BSEP, ABCB4, BAAT or SHP (small heterodimer partner) (Jonker *et al*, 2012; Shulpekova *et al*, 2022). In enterocytes, FXR induces it enhances IBABP (Hwang *et al*, 2002) and OSTα/β (Landrier *et al*, 2006) levels, as well inducing Fibroblast growth factor 15 (FGF15, FGF19 in human) expression. FGF15 secretion is promoted by the membrane protein DIET1 (Vergnes *et al*, 2013). FGF15 is secreted into the portal circulation and acts as a crucial hormone regulating BA synthesis and transport, lipid and glucose metabolism in the liver, and the feeling of satiety in the brain (Lan *et al*, 2017; Katafuchi & Makishima, 2022). Its primary target tissue is the liver, where it binds to Fibroblast growth factor receptor 4 (FGFR4) expressed on hepatocytes, β-klotho being an obligate co-receptor for FGF15 (Lin *et al*, 2007).

Both FXR and FGF15 signaling activate expression of the atypical nuclear receptor SHP lacking a DNA binding domain. Thus, FXR signaling can initiate changes in gene expression not only trough FGF15, but also directly acting on hepatocytes.
Downstream SHP functions as a transcriptional corepressor inhibiting the transcription factors HNF4α (hepatocyte nuclear factor 4-alpha) and LRH-1 (liver receptor homolog-1) (Katafuchi & Makishima, 2022). SHP expression results in the downregulation of a series of BA-related genes, mainly *Cyp7a1* and *Cyp8b1* (Wang *et al*, 2003). In addition, FGF15/19 can mediate *Cyp7a1* suppression in an SHP independent manner (Yu *et al*, 2005a; Song *et al*, 2009; Kong *et al*, 2012). Other BA synthesis and transport genes were also found affected by SHP repression, such as *Cyp7b1* and *Cyp27a1*, *Slc10a1* (NTCP) and *Slco*s (OATPs) (Wang *et al*, 2003; Stofan & Guo, 2020; Dawson *et al*, 2009). Additionally, FGF15/19 signaling in hepatocytes promotes glycogen synthesis**,** and fatty acid oxidation (FAO), suppresses lipogenesis and gluconeogenesis, and has mitogenic effects that support liver regeneration (Katafuchi & Makishima, 2022).

Inflammatory signals can also affect expression of genes related to BA-metabolism. LPS administration (Hartmann *et al*, 2002; Moseley *et al*, 1996; Roelofsen *et al*, 1995; Trauner *et al*, 1998), as well as downstream cytokines TNF-α (Tacer *et al*, 2007; Whiting *et al*, 1995), IL-6 (Bodeman *et al*, 2013; Siewert *et al*, 2004) and IL-1β (Geier *et al*, 2007; Green *et al*, 1996) were found to downregulate BA synthesis and transport genes including *Cyp7a1*, *Cyp8b1* and *Cyp27a1*, *Abcb11* (BSEP), *Slc10a1* (NTCP) and *Slco*s, among other transporters.

Cholestasis is a condition characterized by decreased bile flow from liver to intestine, resulting in the accumulation of BAs in the liver and systemically. High hepatic BA concentrations can damage hepatocytes through pro-apoptotic and pro-inflammatory pathways (Fuchs *et al*, 2025). Under cholestatic conditions, basolateral uptake receptors NTCP and OATPs are downregulated (Wagner *et al*, 2010) while alternative basolateral exporters MRP3, MRP4 and OSTα/OSTβ are upregulated to promote the efflux of BAs into the circulation (Wagner *et al*, 2010). This adaptive response is primarily regulated by FXR, but also involves overlapping contributions from other nuclear receptors of BAs including constitutive androstane receptor (CAR), vitamin D receptor (VDR) and pregnane X receptor (PXR) (Wagner *et al*, 2010).

1.6.5 Bile Acids as signaling molecules

The last subchapter covered how BAs can regulate hepatocytes to regulate their own synthesis and transport genes. However, on top of hepatocytes and enterocytes a divers set of cell types express various bile acid receptors and can be regulated by them. This section offers a brief overview.

A rebirth of interest in BAs in our century originated in the discovery of their signaling properties. In 1999, the orphan nuclear receptor FXR was the first receptor identified to be activated by bile acids ligands (Wang *et al*, 1999; Makishima *et al*, 1999; Parks *et al*, 1999). VDR was shown in early 2002 to be activated by LCA (Makishima et al., 2002), albeit with lower affinity. Later that year, TGR5 (Takeda G-Protein Receptor 5) was discovered (Kawamata et al., 2003; Maruyama et al., 2002). The latter is a G protein-coupled receptor residing on the cell surface. It is responsive to both host- and microbe-derived BAs, LCA being its most potent activator (Zangerolamo *et al*, 2025). Signaling through TGR5 induces intracellular cAMP production, leading to the activation of protein kinase A (PKA) pathways (Katsuma *et al*, 2005; Kawamata *et al*, 2003).

Other nuclear receptors are also activated by higher BA concentrations, such as PXR and the xenobiotic receptor CAR, in addition to their hormonal and xenobiotic ligands. They are important in BA detoxification under cholestatic conditions (Wagner *et al*, 2010). In addition, another G protein-coupled receptor, Sphingosine-1-Phosphate Receptor 2 (S1PR2) can be activated by high levels of conjugated BAs under certain conditions such as cholestasis (Studer *et al*, 2012), contributing to the regulation of lipid and glucose metabolism and to liver pathophysiology (Wang *et al*, 2017; Kwong *et al*, 2017).

Adipose tissue

TGR5 signaling on brown adipose tissue (BAT) enhances energy expenditure by activation of type 2 iodothyronine deiodinase (D2), which converts the thyroid hormone T4 into its active form T3. The latter enhances expression of key metabolic genes and activates thermogenesis in BAT (Zangerolamo *et al*, 2025). TGR5 is also necessary for cold-induced beiging of white adipose tissue, as its adipocyte-specific deletion prevents upregulation of markers of beige remodeling, including Uncoupling Protein 1, the key mitochondrial protein involved in thermogenesis (Velazquez-Villegas *et al*, 2018).

Adipocytes also express FXR, where its signaling inhibits adipogenesis, improves glucose sensitivity and regulates adipokine expression; reducing leptin and enhancing adiponectin expression (Rizzo *et al*, 2006; Zhou *et al*, 2025; Cariou *et al*, 2006). KO mice accordingly exhibit lower adipose tissue mass and serum leptin concentration, as well as insuline resistance and impaired glucose tolerance (Cariou *et al*, 2006). Consequently, both TGR5 and FXR are being explored as therapeutic targets for metabolic disease (Zangerolamo *et al*, 2025; Li *et al*, 2024c). Skeletal muscle cells also express TGR5, the signaling of which promotes energy expenditure, protein synthesis, hypertrophy, and regeneration. Similarly to BAT, increased D2 activity boosts local T3 production, upregulating key metabolic genes (Zangerolamo *et al*, 2025).

Intestine

Both TGR5 and FXR play a role in intestinal signaling, on top of the above-described negative feedback loop regulating BA transport and synthesis. TGR5 activation on intestinal L cells promotes the secretion of glucagon like peptide-1 (GLP-1) by enhancing mitochondrial respiration and calcium signaling, as a fast postprandial signal (Katsuma *et al*, 2005). GLP-1 acts on pancreatic cells enhancing insulin and suppressing glucagon secretion, supporting postprandial glycemic control. FXR signaling counteracts GLP-1 induction in L cells by suppressing expression (Trabelsi *et al*, 2015), which takes time to exert its effects, resulting in a rapid release and delayed inhibition (Shapiro *et al*, 2018).

Moreover, intestinal inhibition of FXR signaling was shown to have beneficial effects in mouse models of metabolic disease, improving insulin sensitivity and protecting from obesity (Xie *et al*, 2017; Li *et al*, 2013).

Brain

Interestingly, BA transporters are found in the blood-brain barrier enabling the flux of conjugated BAs, while unconjugated ones can diffuse across the barrier. In addition, while the full range of BA synthesis enzymes is only expressed in the liver, some neurons and astrocytes express low levels of some of these enzymes such as *Cyp27a1*, making central nervous system synthesis an intriguing albeit unproven possibility (Ferrell & Chiang, 2021; Monteiro-Cardoso *et al*, 2021). BAs are found in the brain in much higher concentrations than in the peripheral blood (Mano *et al*, 2004), and signal through multiple pathways influencing energy expenditure, thermogenesis, and food intake. TGR5 is expressed in various cell types and in various parts of the brain. Most importantly the hypothalamus, where it contributes to the regulation of body weight and appetite by promoting satiety after meals, and it enhances sympathetic nervous signaling to BAT, promoting thermogenesis (Zangerolamo *et al*, 2025). Diet-induced obese mice lacking TGR5 specifically in the hypothalamus gained more weight and showed increased food intake compared to controls, on top of exhibiting less thermogenesis in BAT (Castellanos-Jankiewicz *et al*, 2021).

Immune cells

A large number of immune cells have been reported to bear BA receptors, and be modulated by their signaling.

Circulating monocytes and macrophages were found to express both FXR and TGR5 (Maruyama *et al*, 2002; Kawamata *et al*, 2003; Vavassori *et al*, 2009; Mencarelli *et al*, 2009). Signaling through both of these receptors can suppresses NF-κB signaling (Vavassori *et al*, 2009; Wang *et al*, 2011b) and inhibit NLRP3 inflammasome activation (Guo *et al*, 2016; Shi *et al*, 2020; Hao *et al*, 2017), blunting the pro-inflammatory activity of these immune cells. FXR

signaling promotes the polarization of macrophages towards the M2 phenotype, an effect observed both *in vitro* and *in vivo* (Jaroonwitchawan *et al*, 2023; Biagioli *et al*, 2017). Liver-resident KCs activate SHP through FXR signaling, which leads to decreased TNFα, IL-1β, IL-6, as well as increased IL-10 expression, a pathways reported to protect rodents from liver injury (Jin *et al*, 2020; Verbeke *et al*, 2016).

Similarly, in DCs, FXR activation impairs monocyte-to-DC differentiation and reduces TNF-α production in colitis models (Gadaleta *et al*, 2011; Massafra *et al*, 2016), TGR5 activation also suppressing DC activation through NF-κB inhibition (Hu *et al*, 2021). Modulation of KCs and DCs by VDR with tolerogenic effects were also demonstrated by multiple groups (Fiorucci *et al*, 2024). However, these were induced by vitamin D or its analogs rather than bile acid ligands.

NKT cells express FXR, and the SHP-mediated inhibition of pro-inflammatory cytokine expression was reported by Mencarelli *et al*. (Mencarelli *et al*, 2009). This has resulted in reduced pro-inflammatory activity of NKT cells and attenuated liver injury in a rodent model of acute hepatitis.

Until recently, only cells of the innate immunity were known targets of BA signaling. However, in our decade, the growing interest in the microbiome and the availability high-throughput enabled the discovery of diverse processes through which bile acids – particularly microbiome-derived species – shape T cells fate and function. As for the other arm of adaptive immunity, it is yet to be shown if bile acids can directly modulate B cells.

$CD4^+$ Treg cells maintain intestinal homeostasis by their immunosuppressive activity. Foxp3 expression, known to be promoted by microbial metabolites, is crucial for their development and function (Zheng *et al*, 2010; Arpaia *et al*, 2013b). Recently, multiple studies showcased bile acids favoring Treg differentiation, through diverse mechanisms.

Hang and colleagues found two microbial BAs influencing colonic Treg /Th17 balance;
3-oxoLCA inhibiting Th17 differentiation and isoalloLCA enhancing Tregs differentiation (Hang *et al*, 2019). The same group later showed that 3-oxoLCA and isoLCA suppress Th17 differentiation by direct inhibition of the key transcription factor RORγt (Paik *et al*, 2022). IsoalloLCA exerted its effects independent of the tested BA receptors, and through enhancing OXPHOS and thus increasing mitochondrial ROS (Hang *et al*, 2019), which is required for *Foxp3* induction (Sena *et al*, 2013). Both compounds modulated Treg/ Th17 balance in mice when tested *in vivo* (Hang *et al*, 2019).

Campbell *et al*. showed that isoDCA acts on DCs, dampening their immunostimulatory activity to enhance *Foxp3* induction and thus colonic health through Treg cells (Campbell *et al*, 2020). FXR deficiency in DCs mirrored the effects of isoDCA treatment, both on the functional and transcriptional level, suggesting antagonistic effects of isoDCA on FXR-mediated transcriptional programs.

Parallelly, Song *et al.* showed that microbiota-derived bile acids promote the differentiation of colonic Treg cells in a VDR-dependent manner (Song *et al*, 2020). Transcriptomics confirmed high VDR expression of colonic RORγ$^+$ Tregs. Supplementation with BA cocktails ameliorated gut inflammation in a murine models of colitis.
Similarly, Zhu and colleagues showed that the synthetic BA 24-Nor-UDCA suppresses Th17 effector function and promotes Treg differentiation through metabolic conditioning, by inhibiting the mTORC1 (Zhu *et al*, 2025), which was previously known to control Th17 differentiation (Kurebayashi *et al*, 2012). In the small intestine, Chen *et al*. identified CAR as a transcriptional regulator enabling tissue-resident CD4$^+$ T cells to mitigate BA toxicity by upregulating the xenobiotic transporter MDR1 as well as IL-10 (Chen *et al*, 2021).

A few authors also demonstrated the modulation of CD8$^+$ T cells in response to bile acids.
Campbell and colleagues show that reduced food intake during infection limits CD8$^+$ T cell numbers in an FXR-dependent manner (Campbell *et al*, 2021). FXR thus functions as a nutrient sensor, in the lack of which T cell fitness is preserved even under nutrient scarcity. The latter leads to greater host body weight loss, showcasing a trade-off between immune activity and energy conservation.
Zhu *et al*. showed that 24-Nor-UDCA attenuates liver injury in multiple rodent models (Zhu *et al*, 2021). Mechanistically, it interfered with CD8$^+$ T cells expansion and metabolism of through mTORC1 signaling, similarly to their findings in CD4$^+$ T cells.
Varanasi and colleagues found that high concentration of conjugated BAs in hepatocellular carcinoma impaired CD8$^+$ T cell infiltration and survival, inducing oxidative and endoplasmic reticulum stress in T cells (Varanasi *et al*, 2025). Deletion of *Cyp7a1* or conjugation enzyme *Baat* shifted the BA pool resulting in increased infiltration and cytokine production, raising both a possible immune evasion mechanism and a direction to mitigate it.
Cong and colleagues found DCA elevated in fecal samples of colorectal cancer patients (Cong *et al*, 2024). They found DCA to negatively impact CD8$^+$ T cell effector function. Inhibiting the plasma membrane Ca^{2+} pump PMCA blunted the effect. Hence, DCA was found to target PMCA blocking intracellular Ca^{2+} accumulation and nuclear translocation of the key transcription factor NFAT2 (Nuclear Factor of Activated T-cells 2) to reduce effector programs and antitumor activity.
Ding *et al.* showed in a hepatitis B virus infection model that elevated BAs compromises both CD4$^+$ and CD8$^+$ T cell activation (Ding *et al*, 2022). Mechanistically, BAs disturpted Ca^{2+} homeostasis by inhibiting mitochondrial Ca^{2+} uptake, again impairing NFAT signaling.

In contrast with the immunostimulatory effects of BAs in high concentrations upon cholestasis, recent immunological findings establish BAs as potent tolerogenic and anti-inflammatory

signaling molecules acting through diverse mechanisms. In addition, the microbiota plays a central role in shaping the BA pool and its immunomodulatory functions in particular in the intestine. Bile acids and BA receptors are conserved across all vertebrates, suggesting intriguing evolutionary scenarios of host-microbe communication that balancing immunity and tolerance at key interfaces such as the liver and the gut.

Bile acids are versatile molecules with both digestive-absorptive and signaling properties, as well as well-documented immunomodulatory functions. The concentration and composition of the BA pool shows dynamic variability between species and between individuals within the same species. They also fluctuate throughout the day, and can alter significantly in response to various factors, including disease and infection. Several studies have demonstrated that such alteration can directly influence immune responses (Ladakis *et al*, 2024; Varanasi *et al*, 2025; Song *et al*, 2020), raising the intriguing question of how viral infections impact the bile acid pool and, in turn, modulate antiviral immunity.

1.7 Aims

The aims of this study were the following:

1) To determine how chronic viral infection caused by LCMV Cl13 alters systemic BA levels and BA pool composition in mice.

2) To investigate how BA metabolism-related gene expression is modulated upon chronic viral infection and identify the non-immune and immune mechanisms driving these changes.

3) To examine how systemically high BA levels influence antiviral immunity, in particular $CD8^+$ T cell responses.

2. Results

This section contains the manuscript entitled “Crosstalk between $CD8^+$ T cells and systemic bile acid metabolism controls LCMV-induced immunopathology”, which has been published ahead of print on bioRxiv and is included here in full, with permission of all co-authors.

Crosstalk between CD8$^+$ T cells and systemic bile acid metabolism controls LCMV-induced immunopathology

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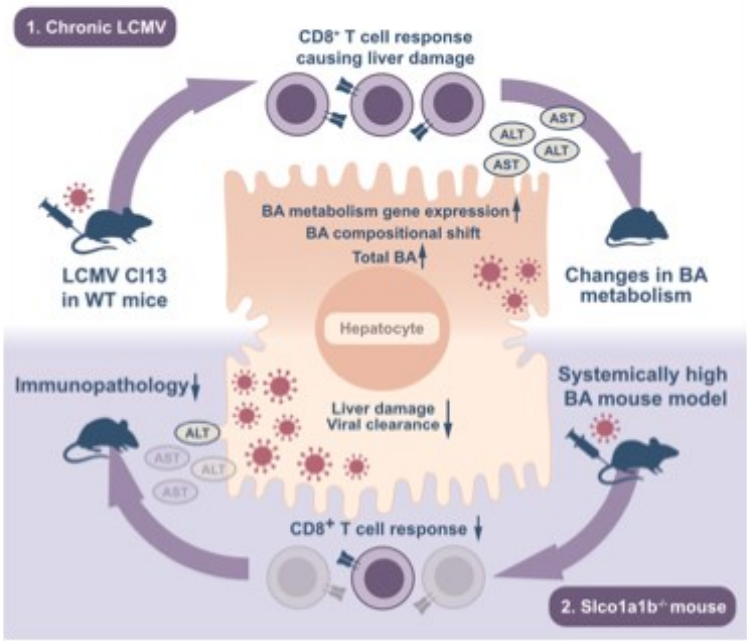
Conflict of interest

The authors have declared that no conflict of interest exists.

Abstract

Antiviral immunity profoundly impacts host metabolism, which can, in turn, modulate immune responses and influences disease pathology. The liver orchestrates systemic bile acid (BA) metabolism, a pathway disrupted in chronic liver diseases such as viral hepatitis. BAs are increasingly recognized for their immunomodulatory properties, and various BA species are being explored as therapeutic agents. Understanding the interplay between immunity and BA metabolism could unlock new therapeutic opportunities. Using lymphocytic choriomeningitis virus (LCMV) as a model, we investigated the interplay between chronic hepatotropic virus infection, BA metabolism, and immunity. Chronic LCMV infection increased BA levels and shifted circulating and liver BA composition towards host-derived, conjugated BAs. Hepatic BA transport and synthesis genes were broadly downregulated, at least partially dependent on $CD8^+$ T cells. Sustained high BA levels impacted $CD8^+$ T cell responses to chronic LCMV infection; mice with elevated circulating BAs due to the lack of BA transporters OATP1A and OATP1A, showed impaired T cell expansion and reduced liver immunopathology. These findings reveal a reciprocal interplay between $CD8^+$ T cells and BA metabolism, expanding

our understanding of adaptive immunity in viral hepatitis. They also highlight how immuno-metabolic changes in liver disease may affect the body's ability to fight infections and cancer.



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Introduction

Responsible for two million deaths annually, liver disease accounts for 4% of all global deaths and ranks as the eleventh-leading cause of mortality worldwide. Viral hepatitis still accounts for most of these cases, followed by alcoholic liver disease (ALD) and metabolic dysfunction-associated steatotic liver disease (MASLD, formerly known as NAFLD), both of which are expected to increase worldwide (1).

The liver is a critical metabolic hub, also regulating systemic immunity against a variety of pathogens (2, 3). One way it can influence systemic immunity is by modulating the levels of local and circulating metabolites (4, 5), including bile acid (BA) species with immunometabolic properties (6). BAs are amphipathic cholesterol derivatives, which circulate between the liver and the intestine. In the liver, the steroid nucleus is conjugated with taurine or glycine, which makes them impermeable to cell membranes, reducing their toxicity and increasing their solubility. This enhanced solubility promotes the formation of micelles with lipids, facilitating lipid absorption in the intestine (7). Conjugated BAs are transported to bile canaliculi by the bile acid export pump (BSEP) encoded by the *Abcb11* gene. Then, they travel through the gall bladder to the duodenum upon food uptake. Host-derived (also known as primary) BAs can undergo extensive modifications by the gut microbiota to produce microbe-derived (also known as secondary) BAs. These changes start with deconjugation catalyzed by bacterial bile salt hydrolases (BSH), a necessary step preceding subsequent modifications (8). Over 95% of BAs are reabsorbed by ileal enterocytes and then enter the portal vein blood flow back to the liver (9). In the liver, BA uptake is mediated by basolateral transporters, including the Na^+-taurocholate co-transporting polypeptide (NTCP) encoded by *Slc10a1*, and the organic anion-transporting polypeptides (OATPs), specifically OATP1b2 (*Slco1b2*), OATP1a1 (*Slco1a1*), and OATP1a4 (*Slco1a4*) in mice (10). While there is considerable overlap in substrate specificity, NTCP and OATPs each play a critical role in the uptake of conjugated and

unconjugated BAs, respectively (11–13). Genetic ablation of either NTCP/*Slc10a1* or OATPs results in altered BA pools, demonstrating the role of these transporters in influencing the serum levels of different BA species (11, 14–16). Moreover, suppression of BA uptake transporters has been described in liver inflammation, potentially protecting hepatocytes from excessive BA concentrations (17, 18).

De novo BA synthesis in hepatocytes is regulated by a well-described negative feedback loop. BAs are transported into ileal enterocytes by the apical sodium-dependent bile acid transporter (ABST/*Slc10a2*), and induce fibroblast growth factor 15 (*Fgf15*) expression via activation of the farnesoid X receptor (FXR/*Nr1h4*). FGF15 secreted into portal circulation functions as a crucial hormone regulating BA metabolism in the liver by binding to fibroblast growth factor receptor 4 (FGFR4) (19). Through the activation of the transcriptional co-repressor Small Heterodimer partner (SHP/*Nr0b2*), FGFR4 signaling initiates the downregulation of the rate-limiting BA synthesis enzyme *Cyp7a1*. In addition, hepatic FXR activation can directly induce the expression of *Shp* (20, 21). Several BA transporters were also reported to be regulated by FXR/*Nr1h4* or SHP/*Nr0b2*, contributing to hepatocellular homeostasis by limiting BA uptake. (22–26).

Liver diseases, including viral hepatitis (27–31), are commonly associated with perturbation in BA metabolism, resulting in altered BA composition (6). Such modulations of the BA pool can have a far-reaching impact on innate (32–34) and adaptive immune cell functions. Direct effects of BAs on adaptive immune cells have only recently been discovered. Multiple microbe-derived BA species modulate $CD4^+$ T cell subsets by promoting intestinal regulatory T cell formation and function (35–37). In addition, microbial-derived BAs have immunomodulatory effects on $CD8^+$ T cells by regulating both their metabolism and effector function through FXR or TCR signaling (38–41). Due to the pivotal role of $CD8^+$ T cells in combating both intracellular pathogens and cancer, understanding their modulation by BAs is of crucial

importance. However, it is not yet clear how endogenous BAs are modulated upon chronic viral infection and whether they, in turn, influence the antiviral immune response.

Thus, we set out to explore the link between liver diseases and BA dysregulation by exploiting the viral hepatitis model of LCMV Cl13. This benchmark model of viral persistence in mice (42, 43) infects hepatocytes, among other cells, and triggers a $CD8^+$ T-cell-mediated immunopathology (44–46). First, we assessed the impact of chronic viral infection on changes in BA metabolism, and second, the effects of systemically high BA levels on cytotoxic T cell-mediated immunity and host response to liver injury. We found that LCMV infection substantially elevated systemic BA levels and altered the composition of circulating BA species in wild-type mice. In parallel, we observed a downregulation of hepatic BA metabolism that was at least partially dependent on $CD8^+$ T cells. To assess the impact of systemically high BA levels, we employed a mouse model of genetic deletion of the hepatic BA transporters *Slco1a* and *Slco1b*. This model revealed that high levels of BAs reduced $CD8^+$ T cell-mediated immunity and accordingly dampened liver immunopathology during chronic LCMV infection. Thus, our data demonstrate a novel reciprocal interplay between $CD8^+$ T cells and systemic BA levels during hepatic injury.

Results

Chronic LCMV infection shifts BA levels and composition

To establish if BA metabolism is affected by viral infections, we quantified total BA species in the serum of mice infected with $2x10^6$ focus-forming units of LCMV strain clone 13 (Cl13). We observed an increase in total serum BAs, which was most pronounced 12 days post-infection (Figure 1a), confirming previous observations with another strain of LCMV (47). To validate our results and gain novel insights into the composition of BA levels during LCMV infection, we performed more sensitive analysis by targeted liquid chromatography-mass spectrometry (LC-MS) across several time points on liver and serum samples. Supporting our initial findings, we observed a profound alteration in BA profiles as the viral infection progressed (Figure 1b). This shift was marked by an enrichment of predominantly host-derived and conjugated BAs in both serum and liver, which was most pronounced 8 days post-infection (Supplementary Figure 1a, b), which coincides with the peak of $CD8^+$ T cell responses (48).

Host-derived conjugated BAs are deconjugated by microbial BA hydrolases (BSH) in the lower gastrointestinal tract. This process is pivotal for subsequent microbial modifications (8, 49). During LCMV infection, the gut microbiota undergoes a substantial compositional shift, causing gut dysbiosis (50). To examine whether the shift towards host-derived conjugated BA species may be caused by a decrease of BSH-expressing gut microbes, we performed a PICRUSt analysis (51) on published 16S rRNA sequencing data from the colon of LCMV-infected mice 8 days post-infection (50). Surprisingly, the results showed a relative increase of BSH-expressing bacteria upon viral infection. (Supplementary Figure 1c). This suggests that the accumulation of host-derived conjugated BAs in the serum and liver are likely not driven by an inability of the microbiota to process these BAs.

Alternatively, it seemed likely that this accumulation could result from a direct effect of liver damage (18). Indeed, there was a positive correlation between the liver damage marker alanine

aminotransferase (ALT) and the total concentration of BAs at day 12 post-LCMV infection as quantified by LC-MS BA profiling (Figure 1c). Thus, direct release of host-derived conjugated BAs into the bloodstream due to hepatocyte damage could explain the observed shift in circulating BA profiles.

Chronic LCMV infection decreases hepatic BA metabolism gene expression

In addition to the systemic changes in BA levels and composition, we wanted to assess the impact of LCMV infection on organismal BA metabolism. In the liver, we observed that BA transport and synthesis were substantially altered by LCMV infection on both transcriptomic and proteomic levels. KEGG pathway enrichment analysis of a published dataset (52) revealed a broad metabolic reprogramming in the liver during viral infection, with numerous metabolism-related KEGG pathways downregulated, especially on day 8 post-infection (Supplementary Figure 2a). In contrast, ample immune-related pathways were upregulated as expected (Supplementary Figure 2b). Bile secretion was among the most downregulated KEGG pathways, together with other terms related to fatty acid- and steroid metabolism (Supplementary Figure 2a). It included genes responsible for primary BA synthesis, basolateral BA transport from the blood to the hepatocyte, as well as bile secretion, as shown on the schematic (Figure 2a). Transcripts of basolateral BA uptake transporter genes (*Slc10a1*, *Slco1a1*, *Slco1a4,* and *Slco1b2*), canalicular BA export pump *Abcb11*, and BA biosynthesis (*Cyp7a1, Cyp27a1, Cyp39a1*) were all found downregulated at least in one timepoint across both transcript and protein levels (Figure 2b).

We next sought to identify the driving factors behind these transcriptional changes. Chronic LCMV infection induces anorectic behavior (53), which can significantly impact the regulation of BA metabolism and associated gene expression (19, 54). To confirm that the alterations in gene expression are not solely due to reduced food intake, we evaluated the expression of BA

transporters and rate-limiting BA synthesis enzyme *Cyp7a1* in uninfected mice that were fed an equivalent amount of food as their Cl13-infected counterparts (pair-fed mice). We observed similar expression levels in both uninfected and pair-fed mice (Figure 2c), indicating that the alterations in transcript levels are not solely influenced by reduced food intake, but likely require an inflammatory response.

The downregulation of BA metabolism in hepatocytes during chronic LCMV infection could be caused by increased FGF15 signaling *via* the FGFR4 (*Fgfr4*)-SHP (*Nr0b2*) axis (19). Alternatively, hepatic FXR (*Nr1h4*) can induce SHP (*Nr0b2*) expression (20, 21) or directly regulate BA transport and synthesis genes (22–26). To exclude that ileal FGF15 may be involved in the regulation of hepatic BA metabolism in our setting, we measured the expression of key target genes in both the ileum and liver. Ileal *Fgf15* expression was reduced, while we observed no difference in the expression of the ileal uptake transporter ASBT/*Slc10a2* (Supplementary Figure 2c), suggesting that the regulation of hepatic BA synthesis and transport genes may be independent of FGF15 signaling. In line with this, we observed that transcripts encoding for FXR (*Nr1h4*), SHP (*Nr0b2*), and the FXR-target gene *Cyp7a1* were all reduced in the liver of Cl13 infected mice at 8 days post-infection relative to naïve animals (Figure 2d). Together, our data suggest that the downregulation of BA metabolism-related genes in the liver may be independent of ileal FGF15 signaling and reduced food intake. Taking into account the correlation of liver damage with circulating BA levels, we propose that the observed perturbation may be a direct consequence of the immune activation on liver gene expression.

CD8$^+$ T cells play an important role in downregulating BA transporter expression

Infection with LCMV is characterized by an early type-I interferon (IFN-I) response, a potent innate antiviral cytokine, which can drive substantial metabolic alterations in the liver (52). To test whether IFN-I signaling controls BA transporter expression during viral infection, we

genetically ablated the Ifn receptor alpha (*Ifnar*) in the whole mouse (*Ifnar1*$^{-/-}$) or specifically in the liver (*Alb-Cre x Ifnar1*$^{fl/fl}$). Upon infection with LCMV Cl13, mice with either whole-body or liver-specific *Ifnar* deficiency exhibited similar reductions in hepatic BA transporter expression (Supplementary Figure 3a+b) compared to littermate controls. This suggests that the downregulation of BA transporters is independent of IFN-I signaling.

To address whether the adaptive immune response to Cl13 is required for the changes in BA-metabolic gene expression, we infected *Rag2*$^{-/-}$ mice which are unable to produce mature T and B lymphocytes (55) with LCMV Cl13. Interestingly, *Rag2*$^{-/-}$ mice were protected from infection-associated downregulation of all tested genes (Figure 3a). This implicates the adaptive immune response in regulating genes involved in BA transport and synthesis. To further pinpoint the key players involved, we sought to determine if CD8$^+$ T cells drive the downregulation of these transporters. Administering CD8α-depleting antibodies successfully depleted CD8$^+$ T cells from circulation (Supplementary Figure 3c), resulting in similar modulation of BA-metabolic gene expression as seen in *Rag2*$^{-/-}$ mice, albeit with slightly attenuated outcomes (Figure 3b). CD8$^+$ T cells can influence target cells through the secretion of various inflammatory cytokines. To assess the impact of CD8$^+$ T cell effector cytokines on BA transporter gene expression, we administered blocking antibodies against IL-6, TNF-α or IFN-γ every second day. None of these treatments prevented the downregulation of basolateral BA transporter expression upon LCMV infection (Supplementary Figure 3d-f). Only the downregulation of *Abcb11*, which encodes the canalicular transporter BSEP, was significantly attenuated upon IL-6 and TNF-α blockade (Supplementary Figure 3g), in line with previous reports indicating that TNF-α can regulate *Abcb11*/BSEP expression *in vitro* (56). Together, this data demonstrates the role of adaptive immunity, particularly CD8$^+$ T cells, in the transcriptional downregulation of hepatic BA transporters during LCMV Cl13 infection. These

results further support our hypothesis that immune activation drives alterations in BA levels and BA transporter expression, highlighting the central role of $CD8^+$ T cells in these changes.

Splenic $CD8^+$ T cell response is impaired in mice with genetic loss of BA transporters OATP1A/1B

Our results so far showed an increased abundance and changed composition of BAs during LCMV Cl13 infection. Considering the far-reaching immunomodulatory impact of BAs on $CD8^+$ T cells (38, 39, 41), we next sought to understand the impact of high BA levels on the $CD8^+$ T cell response during LCMV Cl13 infection. To address this, we made use of the mouse model *Slco1a1b*$^{-/-}$ (hereafter referred to as *Slco*$^{-/-}$), which maintains systemically high BA levels due to the lack of the entire locus containing the BA transporter genes *Slco1a* and *Slco1b* (11). First, we confirmed the *Slco*$^{-/-}$ genotype by verifying the deletion of genes involved in the cassette, including *Slco1a1* (Supplementary Figure 4a). As previously described (11), this deletion led to elevated levels of circulating total BAs (Supplementary Figure 4b) and bilirubin (Supplementary Figure 4c) in naïve mice. Upon infection of *Slco*$^{-/-}$ and littermate controls with LCMV (Figure 4a), we observed a decrease in virus-specific $CD8^+$ T cells in the spleens of *Slco*$^{-/-}$ compared to littermate controls (Figure 4b+c), while total splenic $CD8^+$ T cell numbers remained comparable (Supplementary Figure 4d). Interestingly, splenic $CD8^+$ T cells in *Slco*$^{-/-}$ mice exhibited reduced expression of the activation markers CD44 and PD1 (Figure 4d+e), indicating a disruption in T cell activation. In line with this, we found that the percentage of $CD8^+$ T cells expressing the cellular proliferation marker $KI67^+$ was reduced in *Slco*$^{-/-}$ mice (Supplementary Figure 4e).

To test whether splenic $CD8^+$ T cells themselves were less functional, we first restimulated total splenocytes with PMA/Ionomycin. Upon restimulation, the percentage of total $CD8^+$ T cells producing key effector cytokine IFN-γ was drastically reduced (Figure 4f). Similarly, the

percentage of $CD8^+$ T cells producing the cytolytic proteins Granzyme B (GZMB) and Perforin (PRF1) was decreased (Figure 4g). These findings align with the reduced activation and presence of virus-specific $CD8^+$ T cells in the spleen of *Slco*$^{-/-}$ mice. To assess whether virus-specific $CD8^+$ T cells show any impaired function, we next restimulated isolated lymphocytes with the immunodominant viral epitope GP_{33-41}. Interestingly, we found no differences in the production of effector molecules for either IFN-γ, TNF-α, or expression of GZMB, PRF1 among GP33-specific $CD8^+$ T cells (Supplementary Figure 4f). This suggested that virus-specific $CD8^+$ T cells in *Slco*$^{-/-}$ are functional upon encountering their cognate antigen, but suffer from impaired activation and expansion.

Once $CD8^+$ T cells are activated in secondary lymphoid organs, they migrate into target tissues, including the liver. Similar to the results found in the spleen, we found a reduction in LCMV-specific $CD8^+$ T cells in the liver (Figure 4c), which was also accompanied by a reduction in total $CD8^+$ T cells (Supplementary Figure 5a). There was no difference in the activation state of liver-resident $CD8^+$ T cells, however a reduction in total activated cells (Supplementary Figure 5b-c). Although fewer virus-specific T cells were present in the liver of mice with high serum BAs (Figure 4c), it remained unclear whether this would impact their functionality. Interestingly, we observed that once restimulated, $CD8^+$ T cells of *Slco*$^{-/-}$ mice produced cytokines and cytolytic proteins similar to their littermate controls (Supplementary Figure 5d), indicating that $CD8^+$ T cells that reach the liver are functional.

Taken together, these results indicate that perturbation in BA metabolism by *Oatp* ablation impairs $CD8^+$ T cell activation and expansion in the spleen. As a result, the decrease in virus-specific T cell numbers may impair their ability to infiltrate infected tissues, potentially leading to overall reduced effector functions.

Loss of BA transporters OATP1A/1B attenuate liver damage

LCMV is a non-cytolytic virus, and disease manifestation is primarily driven by immunopathology mediated by $CD8^+$ T cells (57). Based on the observed impairment in $CD8^+$ T cell activation in the *Slco*$^{-/-}$ mouse model, we wanted to assess its impact on pathophysiology and pathogen load upon viral infection.

Slco$^{-/-}$ mice infected with LCMV exhibited a slightly delayed onset of body weight loss compared to littermate controls (Figure 5a). Based on the reduced virus-specific T cell response, we hypothesized that viral clearance may be affected. Indeed, *Slco*$^{-/-}$ mice displayed increased viremia, and higher viral loads in the liver and spleen (Figure 5b). Consistent with the reduced T cell response and increased viremia, we found that genetic ablation of *Slco1a/1b* transporters led to reduced levels of liver damage markers, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Figure 5c). Histological assessment of liver pathology showed an increased presence of cellular infiltrates (Figure 5d), which is in line with an increased presence of virus-specific $CD8^+$ T cells in the liver of WT mice infected with LCMV Cl13 (Figure 4c). To confirm the heightened inflammatory state of the liver, we also assessed the expression of acute phase proteins and found that these were slightly reduced in *Slco*$^{-/-}$ mice (Figure 5e). Together, our data suggest that systemically high levels of BA, which is a feature of several liver diseases, including LCMV Cl13 infection, can reduce $CD8^+$ T cell activation and thereby prevent excessive $CD8^+$ T cell-driven immunopathology.

Discussion

In this study, we investigated the interplay of BA metabolism and immunity, demonstrating that $CD8^+$ T cell-mediated hepatitis leads to a downregulation of BA-metabolism genes, while also increasing systemic BA levels and shifting their composition. In turn, using mice with genetic ablation of basolateral OATP transporters as a model of sustained systemically high BA levels resulted in an impaired $CD8^+$ T cell response and a concomitant decrease in $CD8^+$ T cell-mediated immunopathology and viral clearance.

Liver diseases substantially affect systemic BA levels (29, 58–61). We found that viral hepatitis induced by LCMV Cl13 increased levels of BAs in the circulation, similarly to LCMV strain WE (47). Our in-depth BA analysis showed that viral hepatitis shifted the BA pool towards liver-abundant host-derived and conjugated BAs, a shift that was also found in patients with chronic hepatitis B infection (27). These similarities establish LCMV as a pathophysiologically relevant mouse model to study BA metabolism.

Additionally, liver diseases, such as cholestatic diseases and viral hepatitis, show sustained repression of genes involved in BA metabolism (62, 63). While the FGF15-FXR-SHP axis is the main driver of this downregulation in cholestatic diseases through the physiological feedback inhibition by BAs in the gut (62), our data indicates that this pathway is not involved in the downregulation of BA transport and synthesis genes in the context of LCMV-induced hepatitis. Instead, the adaptive immune response, specifically $CD8^+$ T cells, appears to mediate transcriptional downregulation directly.

Cholestasis is a common feature of inflammatory conditions in the liver (17, 18). Basolateral BA export pumps such as MRP3, MRP4, OSTα, and OSTβ also play an important role in cholestatic conditions, allowing hepatocytes to mitigate BA toxicity by eliminating BA back to the bloodstream (17). However, our data did not show significant regulation of *Mrp3*, *Mrp4*,

Ostα or *Ostβ* on day 8 post-infection, suggesting a differential regulation in LCMV-induced hepatitis compared to other inflammatory liver diseases.

Inflammatory cytokines such as IL-6 (64), IL-1β (17), and TNF-α (65) have been shown to control BA transporter expression. Yet, our results suggest that classical pro-inflammatory T cell-derived cytokines, including IL-6, TNF-α, and IFN-γ were dispensable for the downregulation of BA transporters, except for *Abcb11*/BSEP, during LCMV-induced hepatitis. Collectively, this highlights the importance of $CD8^+$ T cells in regulating BA metabolism either through cytotoxic effects or yet unknown mediators.

Based on our findings, we postulate that the cytotoxic activity of $CD8^+$ T cells may be a key driver of viral infection-induced changes in BA metabolism. $CD8^+$ T cell activity might be involved in altering BA pools through two distinct, possibly independent mechanisms: (1) release of host-derived BAs from damaged hepatocytes and (2) altered expression of BA transporters responsible for take-up of BAs from circulation. In line with this, Honke *et al.* showed the $CD8^+$ T cell dependence of systemic BA elevation in LCMV WE (47). Our findings solidify and establish the role of $CD8^+$ cells in driving systemic BA level elevation and the downregulation of hepatic BA metabolism, respectively. Further research will be required to elucidate whether cytotoxic T cells control BA metabolism by directly targeting hepatocytes expressing the BA metabolism machinery.

In a second line of research, we addressed the impact of systemically high BA levels on the immune response. We demonstrate that in a mouse model of systemically high BA levels, $CD8^+$ T cell activation is impaired, leading to an associated reduction of viral clearance and $CD8^+$ T cell-mediated immunopathology. We propose three plausible mechanisms based on previous findings: certain BA species could directly affect Ca^{2+} signaling (39, 66) or mTOR signaling (41) in $CD8^+$ T cells. Alternatively, BAs could indirectly modulate T cell responses by affecting dendritic cell cross-presentation (37). Another possible explanation is impaired T cell homing

to the liver; however, we observed a reduction in virus-specific T cells numbers and their activation marker expression already in the spleen, making this explanation less likely.

Using *Slco*$^{-/-}$ mice as a model of high BA levels comes with some additional effects, including elevated systemic bilirubin, altered energy metabolism, and a BA composition distinct from that observed in LCMV infection (11). In addition, some of the genes ablated in *Slco*$^{-/-}$ mice have roles in other organs, such as the brain and kidney (12), complicating the establishment of clear causal relationships in the *Slco*$^{-/-}$ model. Thus, one needs to carefully assess how the findings in *Slco*$^{-/-}$ mice may translate to LCMV and chronic hepatitis in general.

NTCP/*Slc10a1*$^{-/-}$ mice also show profound elevation of (predominantly conjugated) serum BA levels (67), but otherwise lack profound systemic BA effects. In contrast, *Slco*$^{-/-}$ mice show an elevation predominantly in unconjugated BA. Individual BA species differ in their ability to penetrate cell membranes and activate BA receptors (68). Thus, further studies should compare our findings with those from mice lacking NTCP/*Slc10a1*, which was beyond the scope of the current study.

In conclusion, LCMV-induced hepatitis shows a perturbed BA metabolism with features akin to liver diseases of various etiologies (6). Intriguingly, the induced alterations in BA metabolism raise the possibility of an immunomodulatory function of BAs to protect from excessive CD8$^+$ T cell-mediated immunopathology. These findings underscore the importance of systemic BA metabolism in shaping immunity among patients with liver diseases, which represent a global health burden. Using this explorative finding, it is reasonable to investigate the reciprocal interplay between the immune system and bile acid metabolism in liver diseases in greater depth. Such research could reveal novel therapeutic strategies modulating immune responses through metabolic interventions.

Methods

Experimental Model and Subject Details

Mice

Animals were kept and bred at the Core facility laboratory animal breeding and husbandry of the Medical University of Vienna, under specific pathogen-free (SPF) conditions. Mouse experiments were conducted in individual ventilated cages in compliance with the animal experiment licenses BMWFW-66.009/0361-WF/V/3b/2017 and 2020-0.406.011, approved by the institutional ethical committees of the Department for Biomedical Research of the Medical University of Vienna.

Wild-type mice on C57BL/6J background were bred in-house. *Slco1a1b*$^{-/-}$ mice were obtained from Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) on an FVB background. These mice were backcrossed onto C57BL/6J for 6 or more generations. For backcrossed mice, we used heterozygous or wild-type littermates as controls. Unless otherwise stated, mice were 8-15 weeks old at the start of experiments, and all mice were age- and sex-matched within experiments. Other genetically modified animals were on C57BL/6 background; *Ifnar1*$^{-/-}$ (69) (032045- JAX) and *Rag2*$^{-/-}$ (55) (008449-JAX). Pair-feeding experiments were performed on individually housed mice to ensure precise measurement of food intake. They adapted to solitary housing for three days before infection. Infected mice then received food ad libitum, while pair-fed counterparts were given the same exact amount of food consumed by infected mice.

Infections

Mice were infected intravenously with $2x10^6$ focus forming units (FFU) of Lymphocytic choriomeningitis virus Clone 13 strain (LCMV Cl13). LCMV Cl13 was grown in BHK-21 cells, and viral titers were determined with focus-forming assay (70). Mice were sacrificed at the indicated time points, and tissues were snap-frozen in liquid nitrogen to be stored at -80°C until further analysis.

Treatment with blocking antibodies

For the blocking of cytokines, animals received 0.5 mg/mouse of the following antibodies: anti-IL-6 (MP5-20F3, Rat IgG1, BioXcell #BE0046)), a- IFN-γ (XMG1, Rat IgG1, BioXcell #BE0055), a-TNF-α (XT3.110, Rat IgG1, BioXcell #BE0058) or a rat IgG1 isotype (MOPC-21, BioXcell #BE0083). The injections were given intraperitoneally every other day for seven days, with the first given one day prior to the infection with LCMV Cl13.

For blocking of CD8, animals received 0.2 mg/mouse of anti-CD8 (YTS169.4, Rat IgG2b, BioXcell #BE0117) or a rat IgG2b isotype (LTF-2, BioXcell # BE0090). The injections were given intraperitoneally two, and one day before infection with LCMV Cl13.

Blood chemistry

For serum analysis, blood samples were centrifuged at 10,000g for 5 minutes at 4°C, after which serum was stored in a new tube at -80°C until further analysis. On the day of the analysis, serum samples were diluted 1:8 in PBS. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, bilirubin, and cholesterol were measured using a Cobas C311 analyzer (Roche). Total BAs in the serum were determined by an enzymatic assay kit (Cell Biolabs #STA-63), according to the manufacturer's instructions.

RNA isolation and real-time PCR

The TissueLyser II (Qiagen) was used to homogenize the tissues, and total RNA extraction was performed using Qiazol Lysis Reagent (Qiagen, #79306) following the manufacturer's instructions. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, #K1622). Real-time PCR was performed using a Taqman Fast Universal PCR Mastermix (Thermo Fisher Scientific, #4352042), and Taqman Gene Expression Assays (Thermo Fisher Scientific) against the following mouse gene products: Slco1a1 (#Mm00649796_m1), Slco1b2 (#Mm00451510_m1), Abcb11 (#Mm00445168_m1), Slc10a1 (#Mm00441421_m1), Nr1h4 (#Mm00436425_m1), Cyp7a1 (#Mm00484150_m1), Nr0b2 (#Mm00442278_m1), Saa1 (#Mm00656927_g1), S100a9 (#Mm00656925_m1), Fgf15 (#Mm00433278_m1), Slc10a2 (#Mm00488258_m1). Ef1a was also measured by Taqman chemistry using the following primers:

5'-GCAAAAACGACCCACCAATG-3', 5'-GGCCTTGGTTCAGGATA-3', and probe: 5'-[6FAM]CACCTGAGCAGTGAAGCCAG[TAM]-3'.

Isolation of intrahepatic lymphocytes

Intrahepatic lymphocytes were assessed as previously described (71). In brief, livers were perfused via the portal hepatic vein using cold PBS. The gall bladder was removed, and the livers were collected in cold PBS. Tissue was mechanically disrupted using a 70μm cell strainer (Sarstedt) to obtain a single-cell suspension. Cells were pelleted at 400g for 5min at 4°C. Cells were then resuspended in a 42% Percoll solution (Cytiva, #17-0891-02) and centrifuged for 20min, 800g at room temperature without brake. Red blood cells were lysed by resuspending the pellet in 1 mL of RBC Lysis Buffer (Invitrogen, #00-4333-57) for 3min at room temperature. Cells were pelleted at 400g for 5min at 4°C. Finally, cells were resuspended and used flow cytometry staining.

Flow cytometry

Spleens were isolated and collected in cold PBS. Single cells were isolated by mechanical disruption of spleens through a 70μm cell strainer (Sarstedt). Cells were spun down at 400g for 5min at 4°C. Pellets were resuspended in 1mL of 1x RBC lysis buffer (Invitrogen, #00-4333-57) for 5min at room temperature. Cells were washed in PBS and spun down again at 400g, 5min at 4°C. Cells were resuspended in cold PBS and used for flow cytometry staining. For PMA/ionomycin restimulation, cells were plated in 96-well plates and then restimulated with Cell Activation Cocktail with Brefeldin A (Biolegend #423303) for 4 hours at 37°C. For viral peptide restimulation, cells were initially incubated with the peptide alone for 30min at a concentration of 1μg/mL at 37°C. Subsequently, at the same peptide concentration, Protein Transport Inhibitor Cocktail (eBioscience, #00-4980-03) was added, and cells were incubated at 37°C for 4 hours. After incubation, cells were washed and stained for surface markers and intracellular proteins.

For surface staining, cells were incubated with fluorophore-labelled antibodies, Fixable Viability Dye eFluor™ 780 (eBioscience #65-0865-14) and CD16/32 FcR-Block (Biolegend #101302). In case, virus specific tetramers (i.e., PE-labelled GP33-specific tetramers acquired from the NIH Tetramer Core Facility, US), cells were incubated for staining at room temperature for 20min. After cells were washed twice, cells were fixed in 4% PFA for 10min at room temperature. In the case of intracellular staining, cells were fixed/permeabilized using the eBioscience™ Foxp3/ Transcription Factor Staining Set (00-5523-00, Invitrogen). Cells were incubated with intracellular staining overnight at 4°C. The following antibodies were used for the staining: TCRb-PerCP-Cy5.5 (Biolegend #109227), CD8a-BV711 (Biolegend #100747), CD44-AF700 (Biolegend #103025), GranzymeB-AF700 (Biolegend #372221), Perforin-PB (Biolegend #154311), TNF-α-APC (Biolegend #506307), IFN-γ-PE-Cy7 (Biolegend #505826), PD1-BV605 (Biolegend #135219), KI67-AF488 (Biolegend #151204).

Targeted LC-MS-based metabolite measurements

Profiles of murine primary and secondary unconjugated and conjugated C24-BAs in serum (30 µl), were analyzed as published previously (72).

Bioinformatic analysis

For the analysis of bile acid LC-MS measurements, the measured BA concentrations (nmol/g) were normalized by 100*c/sum(c), c being the measured concentration. The relative BA abundances obtained this way were averaged across the replicates. BAs which were not in the top 9 BAs with respect to their peak relative abundance were aggregated into the "Other" category. The relative abundances across time were visualized using a bump plot, depicting the relative share of each BA and its rank across time.

Heatmaps were made with the ComplexHeatmap package (73), and KEGG pathway enrichments were performed using the clusterProfiler package (74) in R version 4.2.2. Relative abundance of bacterial bile salt hydrolase genes (EC:3.5.1.24) in feces of uninfected, LCMV Armstrong or LCMV Cl13 infected mice 8 days post-infection were predicted with picrust2 (75) implemented in qiime2 (76). 16S amplicon sequencing tables with ASVs generated via DADA2 of the Labarta-Bajo *et al.* study (50) have been downloaded from the qiita (77) repository (ID 11043).

Statistical analysis

The results are presented in the format of mean ± SEM and were subjected to statistical analysis as specified in the figure captions, employing GraphPad Prism. Relevant p-values were presented. Unless otherwise specified in the figure legend, data shows representative results from at least two independent experiments. For pooled data, due to the smaller individual

experimental group size, each individual experiment was presented with different shapes in the same graph, and statistical analysis was blocked for the experiment.

Study approval

Mouse experiments were conducted in individual ventilated cages in compliance with the animal experiment licenses BMWFW-66.009/0361-WF/V/3b/2017 and 2020-0.406.011, approved by the institutional ethical committees of the Department for Biomedical Research of the Medical University of Vienna.

Data availability

The metabolomics dataset supporting our findings is available as a supplementary table accompanying this publication.

Author contributions

Z.K.: Conceptualisation, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualisation. F.C.R: Conceptualisation, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualisation. H.G.C.: Investigation, Writing – Review & Editing. M.G.: Investigation, Formal analysis, Writing – Review & Editing. L.A.-H.: Investigation, Writing – Review & Editing. M.S.: Investigation, Writing – Review & Editing. A.H.: Investigation. C.V.: Investigation. H.B.: Investigation, Writing – Review & Editing. C.D.F: Investigation, Writing – Review & Editing. O.P.: Formal analysis. Fabian Amman: Formal analysis, Writing – Review & Editing. J.W.G: Formal analysis. C.C: Conceptualisation, Writing – Review & Editing. H.-U.M.: Investigation, Conceptualisation. T.R.: Conceptualisation, Writing – Review & Editing. M.T.: Conceptualisation, Writing – Review & Editing. A.B.: Supervision, Project Management, Conceptualisation, Writing – Review & Editing.

Z.K. and F.C.R contributed equally and are listed in alphabetical order.

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Supporting information

Supplementary figures 1-6

Supplementary table: BA profiling

Figure Legends

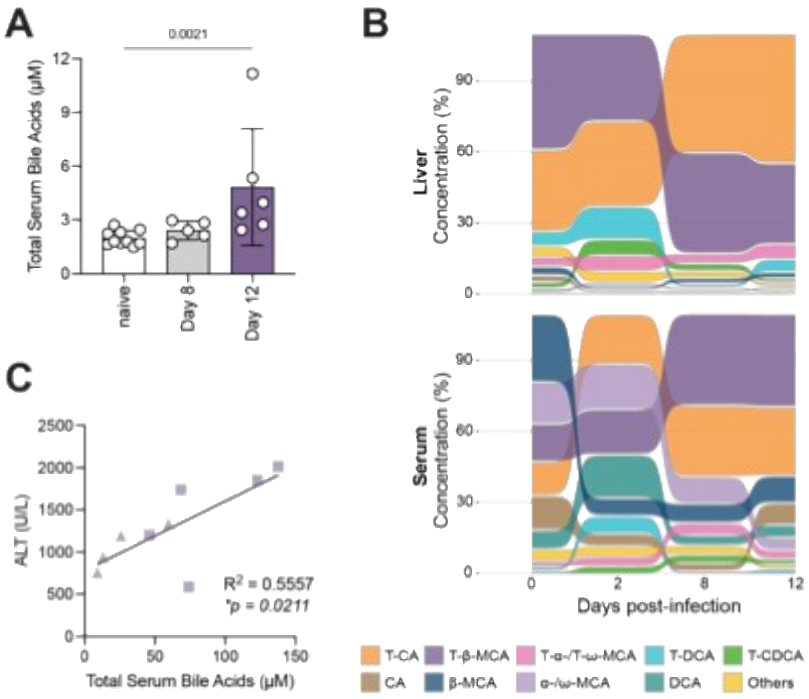

Figure 1: Viral hepatitis alters hepatic and systemic BA levels and composition. (a) Total serum BA levels of C57BL6/J mice infected with LCMV Cl13 quantified by colorimetric total BA assay. (b) Composition of serum and liver BA species in uninfected and LCMV Cl13-infected mice at 8- and 12-days post infection measured by LC-MS. The bump plot shows the relative share of each BA and its rank across time. (c) Correlation of hepatic damage marker ALT with systemic BA levels in the serum of C57BL6/J mice infected with LCMV Cl13 at day 12 post-infection measured by LC-MS.

Data presented as mean ± SEM. Data from one experiment (n = 6-10 mice/group) (a). Pooled data from two independent experiments (n = 4-5 mice/experiment) (a,b). Shapes indicate each experiment (c). Kruskal Wallis test (a). Simple linear regression (c).

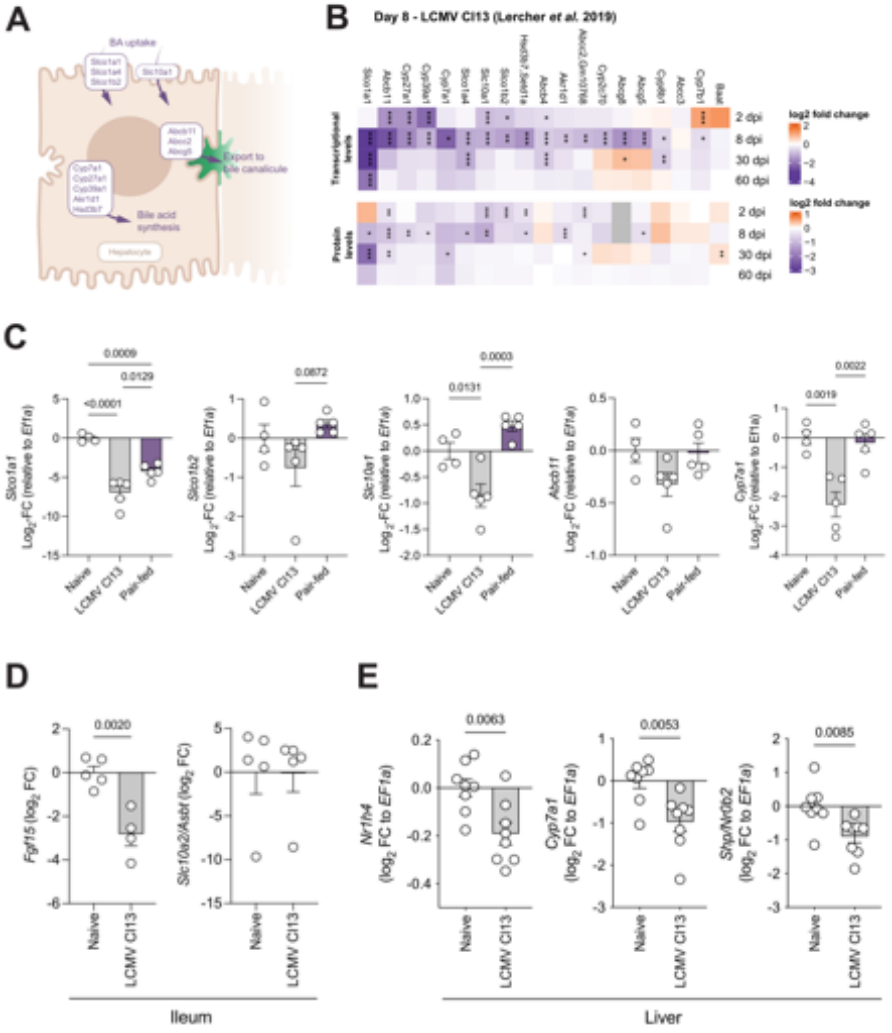

Figure 2: Chronic LCMV infection decreases the expression of genes involved in hepatic BA regulation, synthesis, and transportion. (a) Overview schematic of BA homeostasis-related genes in hepatocytes that are significantly downregulated at both the transcript and protein levels at one or more time points. (b) Expression of BA homeostasis-related genes and proteins during infection with LCMV Cl13 infection based on Lercher *et al.* (52). (c) Hepatic gene expression of BA metabolic genes in response to LCMV Cl13 infection or pair-feeding.

(d) Gene expression in ileum of uninfected mice and LCMV Cl13-infected mice at day 8 post-infection. (e) Expression of hepatic genes involved in FGF15-SHP signaling.

Data presented as mean ± SEM. Data from one experiment (n = 4-5 mice/group) (d) and pooled from two independent experiments (n = 5-8 mice/group) (c,e). (c) One-Way ANOVA. (d-e) Unpaired Student's t-test.

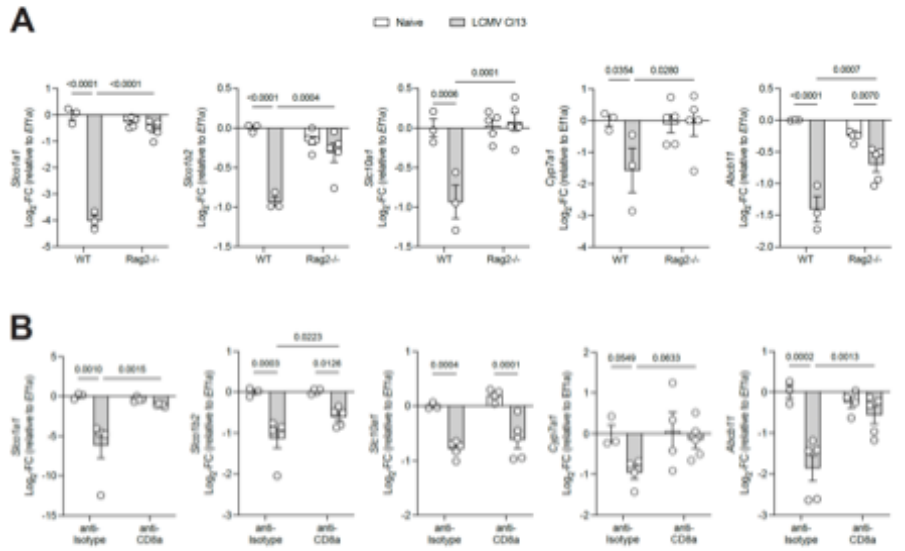

Figure 3: Downregulation of hepatic BA transporters is partially due to CD8$^+$ T cells during LCMV Cl13 infection. (a) Hepatic expression of BA metabolism genes in response to LCMV Cl13 infection in C57BL6/J and *Rag2*-deficient mice. (b) Hepatic BA metabolism gene expression in response to LCMV Cl13 infection and CD8a-depleting antibody administration.

Data presented as mean ± SEM. Representative data from two independent experiments. (a,b) Two-Way ANOVA.

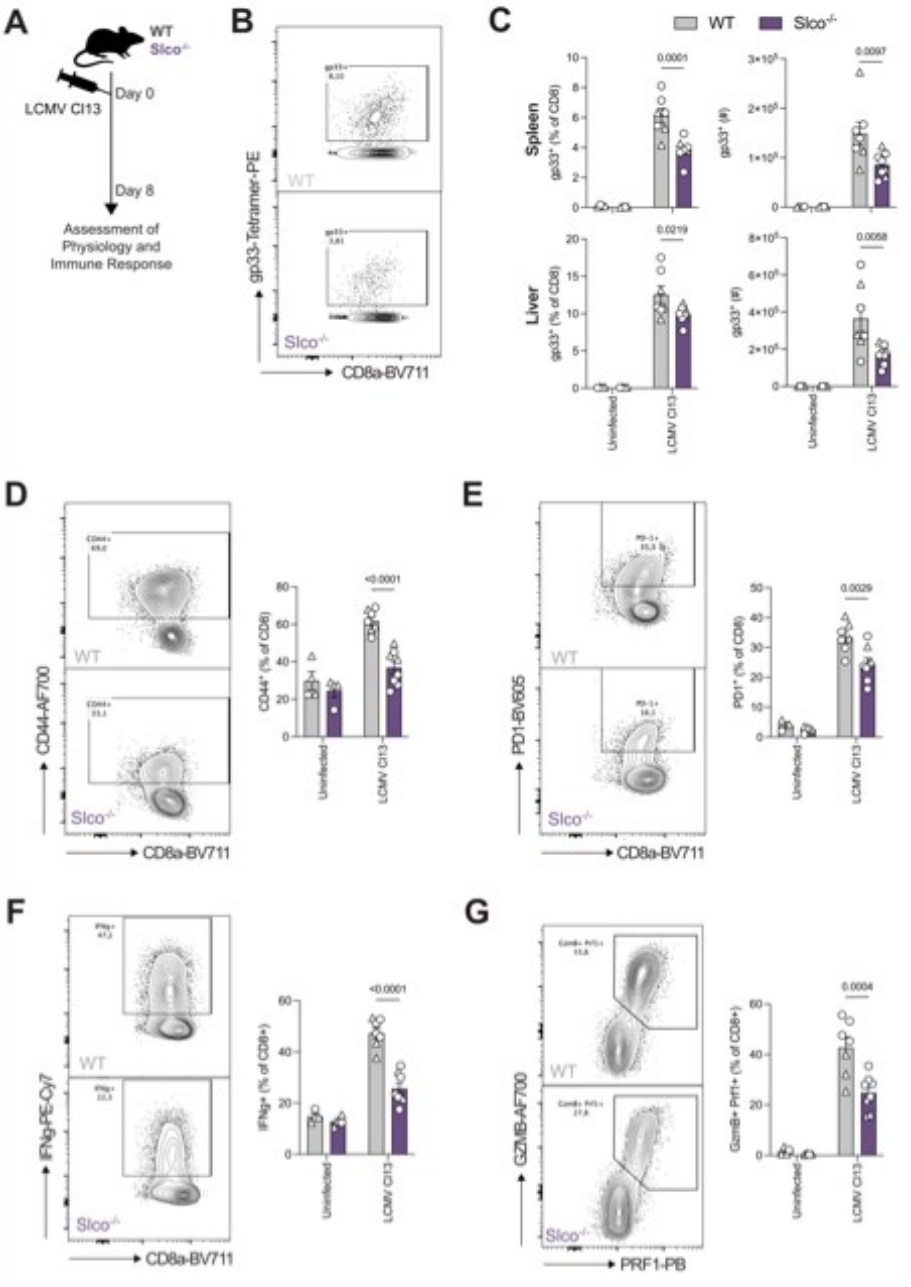

Figure 4: Impaired splenic CD8$^+$ T cell response to LCMV Cl13 infection in mice with systemically elevated BAs (a) Schematic of experimental design of LCMV infection in *Slco*-

$^{-/-}$ mice. (b) Representative flow cytometry plot of LCMV GP33-specific $CD8^+$ T cell in the spleen 8 days post LCMV Cl13 infection. (c) Frequency and total number of LCMV GP33-specific $CD8^+$ T cells in spleen and liver at day 8 post-infection. (d,e) Frequency of (d) CD44- and (e) PD1-expressing $CD8^+$ T cells in the spleen at day 8 post-LCMV Cl13 infection. (f) Frequency of IFN-γ-producing splenic $CD8^+$ T cells after 4 hours restimulation with PMA and ionomycin at 8 days post-infection. (g) Frequency of $GZMB^+$ $PRF1^+$ splenic $CD8^+$ T cells at day 8 post-LCMV Cl13 infection.

Data presented as mean ± SEM. Data pooled from two independent experiments; each experiment represented in a different shape (n = 4-8 mice/group) (c-g) Two-Way ANOVA.

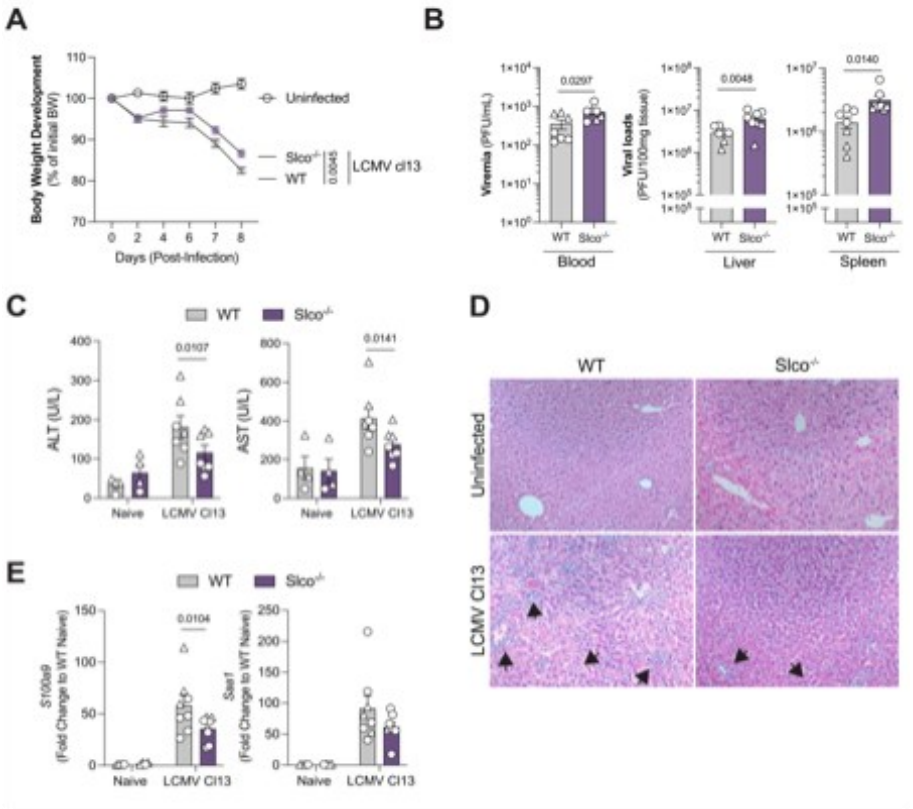

Figure 5: Loss of bile acid transporters *Slco1a/1b* reduces T cell-mediated liver damage during LCMV Cl13. (a) Body weight development of *Slco*$^{-/-}$ or littermates infected with LCMV Cl13. (b) LCMV Cl13 titers in blood, liver and spleen at 8 days post-infection in *Slco*$^{-/-}$ mice or littermate controls. (c) Serum ALT and AST levels in *Slco*$^{-/-}$ mice or littermate controls at 8 days post-infection. (d) Representative H&E staining of liver sections from WT or *Slco*$^{-/-}$ at day 8 post-infection or left uninfected. Arrows indicate cellular infiltrates. (e) Hepatic gene expression of acute phase proteins in *Slco*$^{-/-}$ mice or littermate controls at 8 days post-infection.

Data presented as mean ± SEM. Data pooled from two independent experiments; each experiment represented in a different shape (n = 4-8 mice/group). (a,c,e) Two-Way ANOVA. (b) Unpaired Student's t-test.

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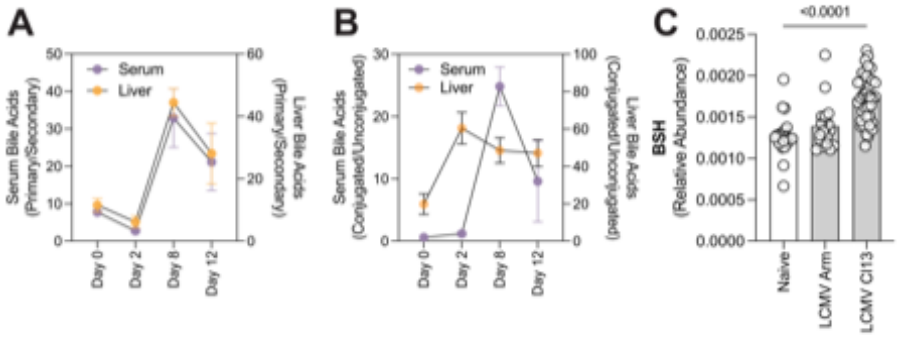
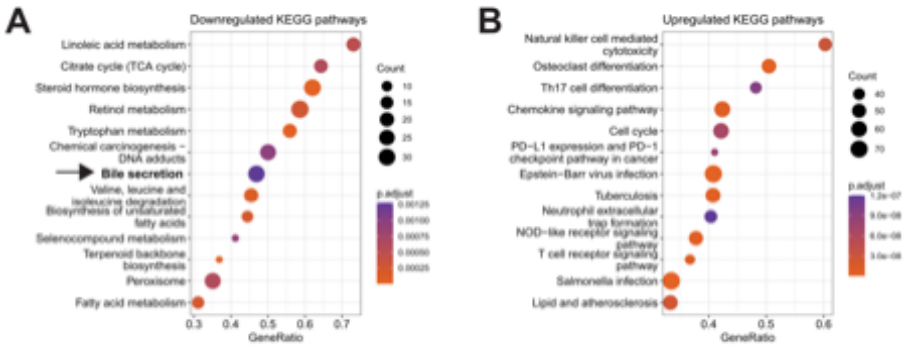
Supplementary figures

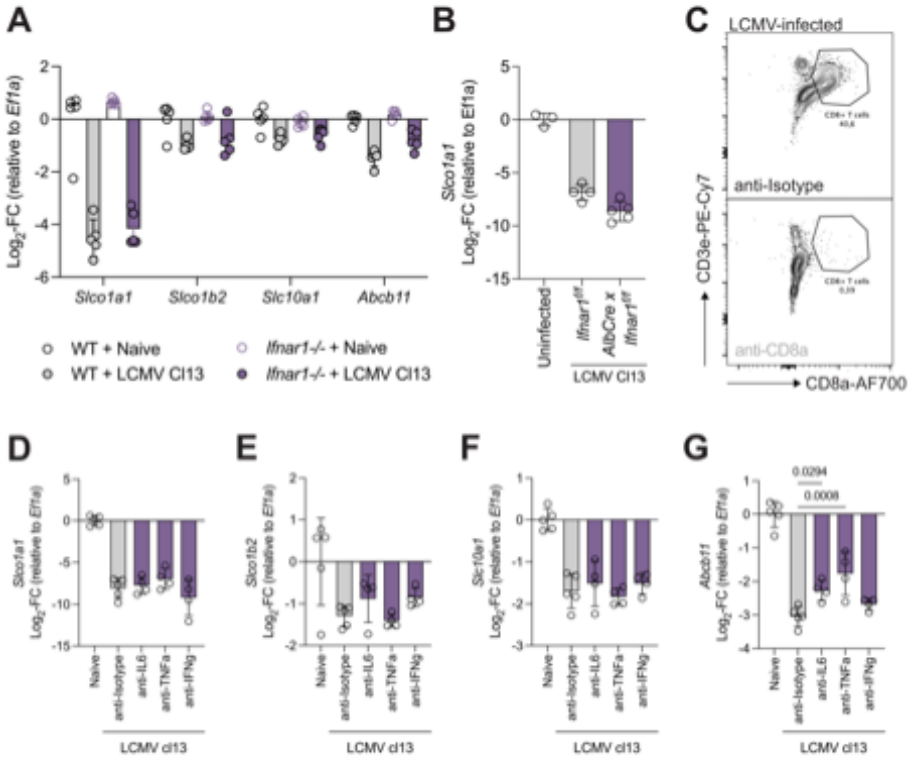


Supplementary Figure 1: Chronic LCMV infection alters BA composition, presumably independent of BSH activity. (a) Ratio of host-to-microbe-derived BA species in serum and liver over the course of LCMV infection. (b) Ratio of conjugated-to-unconjugated BA species in serum and liver over the course of LCMV infection. (c) Relative abundance of BSH-expressing microbial species in uninfected, LCMV Armstrong (Arm) or LCMV Cl13 infected mice 8 days post-infection, based on 16S RNA data from Labarta-Bajo *et al.* (1).

Data presented as mean ± SEM. Representative data from two independent experiments (n = 4 mice/time point) (a,b). Kruskal Wallis test (c)

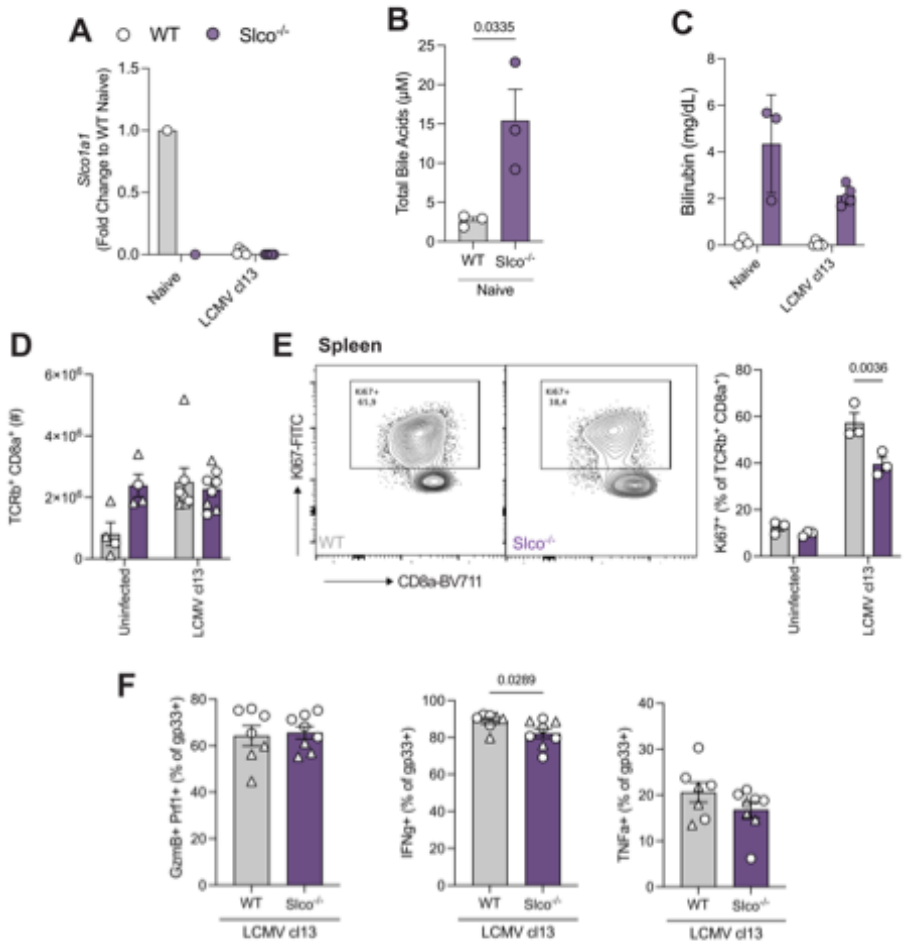


Supplementary Figure 2: Pathway enrichment analysis of livers in response to LCMV cl13 infection. (a,b) Gene set enrichment analysis of hepatic gene expression dataset (2) at day 8 post-LCMV Cl13 infection for downregulated (a) and upregulated (b) KEGG pathways.



Supplementary Figure 3: Downregulation of bile acid transporters in the liver is independent of type I signaling and largely independent IL-6, TNF-α and IFN-γ. (a) Expression of BA transporters in *Ifnar*-deficient mice at 8 days post LCMV Cl13 infection. (b) *Slco1a1* expression in liver-specific *Ifnar*-deficient mice at 8 days post LCMV Cl13 infection. (c-f) Expression of hepatic BA receptors in response to LCMV Cl13 and antibody-mediated cytokine blockade at day 8 post-infection.

Data presented as mean ± SEM. Representative data from two independent experiments (n = 3-5 mice/group) (a-b). Representative data from three independent experiments (n = 4-5 mice/group) (c-f). (c-f) One-Way ANOVA.

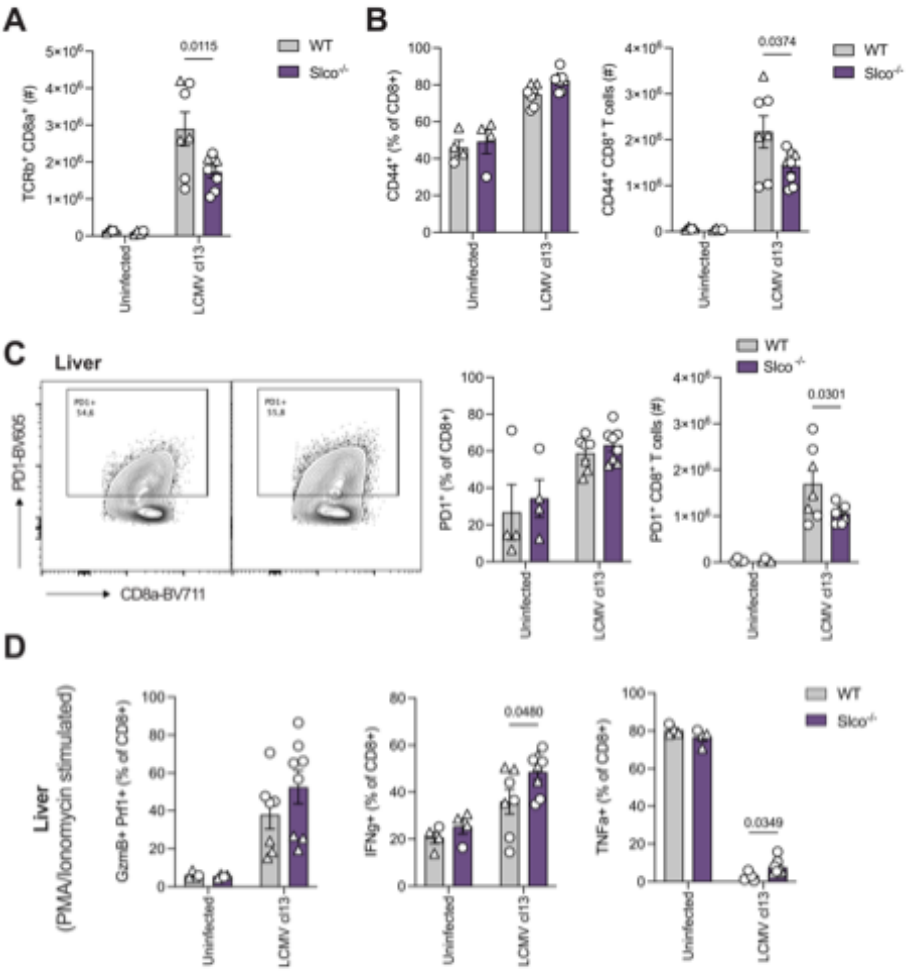


Supplementary Figure 4: Loss of BA transporter increased total BA and bilirubin levels, and is associated with changes in CD8⁺ T cell numbers and proliferation.

(a-c) Assessment of the known effects of *Slco*-deficiency in C57BL6/J background. (a) Expression of *Slco1a1* in the liver in *Slco*$^{-/-}$ and littermate controls upon infection with LCMV Cl13. (b) Total serum BA levels in uninfected *Slco*$^{-/-}$ and littermate controls. (c) Serum bilirubin levels in *Slco*$^{-/-}$ and littermate controls upon infection with LCMV Cl13. (d-e) Total number of CD8^{+} T cells in the spleen (d) of *Slco*$^{-/-}$ animals and littermate controls. (e) Representative flow cytometry plot for LCMV KI67-expression of CD8^{+} T cell in the spleen 8 days post LCMV

Cl13 infection. (f) Cytokine and cytolytic enzyme expression of $CD8^+$ T cells in spleen 8 days post LCMV Cl13 infection.

Data presented as mean ± SEM. Representative experiment from one experiment (n = 5 infected mice/group) (a), from one experiment (n = 3 mice/group) (b), from two independent experiments (n = 3-5 mice per group) (c). Data pooled from two independent experiments (n = 4-8 mice/group), each experiment represented in a different shape (d,f). Data from one experiment (n = 3 mice/group) (e). Two-Way ANOVA (d-e). Mann-Whitney test (f).



Supplementary Figure 5: CD8$^+$ T cell infiltrating in the liver of *Slco*$^{-/-}$ mice is reduced, but virus-specific CD8$^+$ T cells are functional. (a) Total number of CD8$^+$ T cells in the liver of *Slco*$^{-/-}$ animals and littermate controls. (b,c) Frequency of (b) CD44- and (c) PD1-expressing CD8$^+$ T cells in the liver at day 8 post-LCMV Cl13 infection. (d) Assessment of cytokine production in liver CD8$^+$ T cells, isolated at day 8 post LCMV Cl13 infection, upon restimulation with PMA and ionomycin for 4h.

Data presented as mean ± SEM. Data pooled from two independent experiments (n = 4-8 mice/group), each experiment represented in a different shape (a-d). Two-Way ANOVA (a-d).

References

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Supplementary table – bile acid profiling

	LCMV Liver																			
	uninfected				infected															
					Day 2				Day 8				Day 12				Day 18			
nmol/g	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
UDCA	0.1	0.2	0.2	0.7	0.0	0.2	0.2	0.1	0.0	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.4	0.0	0.5	0.3
CDCA	0.0	0.7	0.1	0.0	0.0	0.6	0.1	0.0	0.0	0.0	0.4	0.0	0.0	0.1	0.0	0.1	0.0	0.4	0.1	0.1
DCA	0.3	0.1	0.3	0.8	0.1	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.4	0.3	0.5	13.6	0.0	0.0	0.0
CA	7.3	1.9	3.9	16.0	0.3	0.2	0.5	0.3	0.2	0.2	0.4	0.2	1.6	1.0	1.7	0.9	2.0	1.1	1.5	8.7
α- + ω-MCA	2.3	2.2	2.0	6.2	1.8	0.5	0.8	0.8	0.1	0.1	0.4	0.4	1.0	0.8	0.8	1.3	1.2	0.9	3.0	3.2
β-Muri-CA	9.7	4.9	3.6	20.3	0.3	0.3	1.0	1.2	0.7	1.0	3.3	1.8	4.4	8.6	12.5	10.6	12.8	5.7	12.1	7.0
LCA	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.3	0.0	0.2
G-UDCA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
G-CDCA	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.0	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.1
G-DCA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
G-CA	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0
G-LCA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T-UDCA	7.3	8.5	7.0	20.5	5.5	3.2	3.9	6.3	1.0	1.0	3.4	1.1	3.3	3.4	3.0	3.6	18.5	5.2	12.3	14.3
T-CDCA	2.9	3.9	7.5	7.4	6.5	8.0	7.0	13.2	2.0	2.1	4.2	1.5	3.3	5.0	3.6	3.7	6.2	3.8	6.0	6.3
T-DCA	10.2	9.0	23.3	30.0	23.6	6.6	13.1	39.1	0.1	0.5	1.0	1.1	19.0	8.6	17.9	16.9	8.6	6.4	6.6	19.6
T-CA	80.4	54.6	192.3	141.2	46.9	59.8	34.6	66.7	39.4	44.2	111.2	37.2	147.3	308.9	606.1	230.5	118.1	64.4	108.8	173.6
T-LCA	0.1	0.1	0.6	0.4	0.3	0.1	0.3	0.4	0.0	0.1	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.3	0.1	0.2
T-α- + T-ω-MCA	5.8	3.0	18.8	16.8	5.1	8.1	7.7	11.4	2.2	2.9	7.9	2.3	5.1	7.3	381.2	1.7	13.1	1.0	22.1	13.7
T-β-Muri-CA	214.0	107.6	113.0	195.6	78.2	44.2	31.1	66.0	25.1	38.2	90.3	42.6	141.3	232.7	381.2	127.3	386.1	141.0	205.1	234.1
SUM	**876.5**	**733.8**	**910.9**	**995.1**	**705.0**	**669.1**	**638.3**	**744.8**	**606.9**	**627.9**	**760.7**	**627.6**	**862.7**	**1114.4**	**1546.7**	**955.8**	**1116.7**	**767.5**	**916.4**	**1020.2**
sum (µmol)	0.87652439	0.73380117	0.91089017	0.9950739	0.70504924	0.66908926	0.63831394	0.7447845	0.60680094	0.62790991	0.76068282	0.62756797	0.86267382	1.11439921	1.54671786	0.95576084	1.1167328	0.76748905	0.91643256	1.02056623

	LCMV Serum																			
	uninfected				infected															
					Day 2				Day 8				Day 12				Day 18			
nmol/L	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
UDCA	244.3	198.0	427.8	211.9	206.5	10.4	42.8	184.1	3.8	6.2	218.9	0.0	1.2	75.1	0.0	57.5	5.0	109.0	230.2	96.5
CDCA	9.9	3.6	38.7	3.5	18.6	0.0	0.0	0.0	5.1	0.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	5.4	11.1	12.6
DCA	272.8	388.8	3597.1	479.8	2113.6	209.1	486.6	3168.8	0.9	30.8	541.7	32.8	55.2	631.6	158.3	2542.0	769.0	155.3	172.8	105.2
CA	641.0	442.8	13004.8	296.6	654.2	20.9	60.9	1258.4	51.5	73.0	330.2	8.0	3.3	803.5	259.9	10127.6	132.7	607.3	1090.0	455.0
α- + ω-MCA	1469.5	1207.9	2390.2	743.2	2021.3	304.4	438.5	2775.3	5.9	41.7	2029.5	9.6	10.9	763.4	252.7	1789.8	439.9	417.4	2818.4	777.4
β-Muri-CA	3355.8	1398.3	4363.2	1102.2	814.1	27.4	173.4	1991.6	15.9	34.1	1424.1	0.0	5.3	1105.6	1538.5	9000.1	904.4	1743.4	5944.7	3705.3
LCA	15.7	12.2	66.2	8.9	17.2	10.3	14.6	61.5	11.3	6.7	5.8	7.6	5.7	1.7	6.3	6.0	10.0	5.4	15.7	3.3
G-UDCA	0.0	0.0	0.9	0.7	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.0
G-CDCA	0.0	0.0	0.0	0.0	0.3	0.4	0.0	0.5	0.0	0.0	0.0	0.1	0.0	0.0	0.0	2.6	0.8	0.4	0.0	0.0
G-DCA	0.0	0.4	0.0	0.0	0.3	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.4	0.4	0.0	0.0	0.0	0.1	0.2	0.0
G-CA	0.0	0.7	57.9	0.7	2.6	0.6	0.0	1.0	1.1	0.7	0.0	0.0	0.0	0.0	3.9	52.6	3.8	2.9	3.2	1.4
G-LCA	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1
T-UDCA	8.7	18.6	218.7	83.7	62.7	21.6	55.0	397.3	49.7	51.5	13.1	30.5	38.0	44.5	98.6	93.9	114.5	279.4	408.0	334.7
T-CDCA	12.2	14.5	258.2	39.6	91.4	34.4	78.2	782.4	124.1	127.9	7.7	41.6	81.2	46.9	124.7	188.9	98.0	134.3	258.2	236.1
T-DCA	3.3	32.3	514.4	95.7	288.4	100.3	305.0	1226.4	3.0	7.1	19.0	6.2	14.7	130.1	180.2	467.0	688.4	252.0	304.7	458.3
T-CA	85.9	300.4	12461.9	1134.1	730.6	328.5	688.7	5823.3	1240.9	1438.6	147.2	534.4	764.0	1245.6	5799.1	17144.3	4654.2	2150.1	3257.5	2782.4
T-LCA	0.0	0.0	4.9	0.6	3.5	3.2	1.2	21.4	0.0	0.4	0.2	0.0	0.0	0.2	0.3	0.3	0.5	0.3	14.1	6.6
T-α- + T-ω-M	11.5	33.2	948.5	167.6	93.9	24.0	208.5	755.0	123.2	225.4	103.8	67.9	121.3	181.6	565.2	392.0	171.7	412.7	1425.4	867.5
T-β-Muri-CA	207.8	536.8	5558.7	2070.8	906.8	363.8	545.8	4181.7	1359.6	2068.5	377.9	674.6	1309.3	1597.2	10290.5	11156.9	3782.4	5896.7	7698.4	7425.1
SUM	**12958.8**	**11208.8**	**50502.4**	**13059.9**	**14646.4**	**8069.8**	**9729.5**	**29249.0**	**9616.6**	**10733.0**	**11842.7**	**8033.7**	**9030.7**	**13247.7**	**25898.4**	**59602.0**	**18395.8**	**18786.7**	**30273.1**	**23897.9**
sum (µmol)	12.9587503	11.2088088	50.5023544	13.0598983	14.6463848	8.06975919	9.7294927	29.2490362	9.61655345	10.7330353	11.8426718	8.03371511	9.03068954	13.2476557	25.8984387	59.6020375	18.3958153	18.7867346	30.273114	23.8978557

3. Discussion

3.1 General discussion

Immune cells are metabolically rewired upon activation, which plays a critical role in their activation and function. Historically, our understanding of immune-metabolic crosstalk has been greatly shaped by knowledge gained from well-controlled *in vitro* systems (Pearce & Pearce, 2013; O'Neill *et al*, 2016), while the complexities of these processes in organisms with entire, complex and functional immune systems remained challenging to address and mechanistically dissect.

Recent years have shed light on the unique signaling and immunomodulatory functions of bile acids, a group of conserved molecules with particular variability. This variability is achieved in concert with the microbiome. BAs also circulate between the liver and the gut, and are carried in the bloodstream enabling them to reach – and crosstalk between – various organs, on top of being in the crossroads of the host and the microbiome (Fuchs *et al*, 2025).
The aim of this doctoral thesis was to investigate the interplay between immune responses and BA metabolism during chronic viral infection. We used LCMV, a benchmark model of systemic viral infection, a model which led to major discoveries in T cell biology including effector function, memory development and exhaustion (Abdel-Hakeem, 2019; Zehn & Wherry, 2015). More recently, the use of this model has been extended to unravel metabolic changes associated with infection and immunity at the organismal level (Baazim *et al*, 2019; Lercher *et al*, 2019; Campbell *et al*, 2021; Bartman *et al*, 2025)

In this work, we have demonstrated that $CD8^+$ T cell-mediated immunopathology during LCMV infection leads to a profound remodeling of bile acid (BA) metabolism, characterized by downregulation of BA-transport and synthesis genes in the liver, increased systemic bile acid levels, and shifts in bile acid pool composition. We further showed that elevated systemic BA levels, achieved by the genetic deletion of basolateral OATP transporters, lead to impaired $CD8^+$ T cell responses, consequently reducing immunopathology and viral clearance. Together these results reveal a bidirectional crosstalk between adaptive immunity and BA metabolism, highlighting the role of systemic BA metabolism in modulating immune responses in liver disease, which remain a significant global health challenge (Devarbhavi *et al*, 2023).

3.2 BA measurements in homeostasis and disease

Homeostatic BA pool size and composition and varies greatly from mammal to mammal (Zheng *et al*, 2024), and also between individuals and within individuals diurnally (Bello *et al*, 2024), and over time, affected by factors such as disease or microbiome variations (Aggarwal

et al, 2022; Fuchs *et al*, 2025). These can make the comparative profiling of BAs challenging, hence, the gold standard for reporting bile acid (BA) composition is to normalize to the total bile acid content per sample and report the relative proportions of different bile acid classes. This approach results in a consistent and comparable way to describe pool composition allowing for meaningful comparisons (Rossignol *et al*, 2024; Chiang, 2017). Zheng and colleagues reported mean serum concentrations of 4.51 ± 2.30 µM in human, and 6.22 ± 3.59 µM in mouse after overnight fasting (Zheng *et al*, 2020).

Our serum total bile acid (TBA) measurements (Manuscript figure 1a) were generated with an enzymatic cycling assay, yielding physiological values both in uninfected mice (1.99 µM ± 0.41 µM) and 12 days post-infection (dpi) (4.85 ± 3.26 µM). In contrast, the LC-MS-based quantification shown in Manuscript figure 1c reports much higher absolute BA concentrations, including values over 100 µM, which are compatible with liver disease (Summerfield *et al*, 1981; Zhang *et al*, 2012b).

LC-MS values are challenging to directly compare with enzymatic TBA measurements as the two methods measure different things. While most abundant individual BAs are characterized by LC-MS and summed up, colorimetric assays relying on 3α-hydroxysteroid dehydrogenase reportedly underestimate the highly abundant muricholic acid (MCA) species (Manuscript figure 1b) (Žížalová *et al*, 2020; Zheng *et al*, 2024). In addition, the two figures are from separate experiments, and the measured high BA values in some mice are accompanied by high ALT (alanine aminotransferase), showing severe liver damage due to immunopathology in those specific animals.

Thus, difference in the method, and inter-experimental variation likely account for the observed divergence in TBA values. Nevertheless, our results consistently demonstrate an elevation of serum TBA in infected animals, which correlates with the liver damage marker ALT (Manuscript figure 1c).

This pattern is line with another publication showing elevated serum TBA levels upon infection with LCMV WE, another LCMV strain causing significant liver damage (Honke *et al*, 2017).

We performed LC-MS on liver and serum samples collected at 0, 2, 8, and 12 days post-infection (dpi), profiling 19 different BA species (Manuscript Supplementary Table 1), including those expected to be the most abundant. It should be noted that many low-abundance BA species were not included in our panel; while they are unlikely to alter the overall picture, changes in these species may have been missed. In addition, our method could not distinguish between the rare primary α-MCA and the more abundant secondary ω-MCA (Manuscript Figure 1b).

Our data shows homeostatic dominance of β-MCA, α+ω-MCA, Tβ-MCA, TCA and CA, which is in line with expected high-abundance BAs in mouse serum (Han *et al*, 2015; Zheng *et al*,

2024). Infection resulted in a shift towards primary and conjugated BAs (Manuscript Supplementary figure 1a-b), which was largely led by the elevation of the relative share of serum Tβ-MCA and TCA to high proportions, sustained over 8-12 dpi, and accompanied by the falling share of the unconjugated β-MCA and CA (Manuscript figure 1b).

Other hepatotropic viruses are also associated with elevated serum levels of BAs. Xun and colleagues also showed elevation of total BAs in the serum of patients with chronic HBV, and LC-MS measurements revealed high serum levels of conjugated BAs, in particular TCA, TDCA, GCA, GCDCA and GDCA (Xun *et al*, 2021). In addition, they found that TCA reduced the frequency and impaired the effector function of $CD8^+$ T cells both *in vitro* and *in vivo*, limiting the efficacy of IFN-treatment in patients. While another study also reported higher proportions of primary and conjugated BAs in chronic HBV patients even without evident liver injury (Sun *et al*, 2021), Wang and colleagues found that serum TBA and primary BAs were associated with disease severity, increased in chronic HBV patients with severe liver fibrosis compared to those with mild fibrosis (Wang *et al*, 2020).

In line with the latter findings, studies of HCV patients also suggest that elevated BAs are in connection with disease severity rather than infection status (Shlomai *et al*, 2013; Khalil *et al*, 2022; Fuchs *et al*, 2025). Khalil and colleagues further pinpoint conjugated BAs in the serum of patients with previous HCV infection; when comparing serum BA profiles of patients with different stages of liver disease. They found that the elevation of TCA, GCA, GUDCA, TCDCA, GCDCA was associated with the degree of liver disease.

In VSV infection, taurine-conjugated BAs were once again found significantly increased, while unconjugated and secondary BAs were decreased in the serum compared to control mice (Li *et al*, 2024b).

Beyond viral infections, a T cell–driven graft-versus-host-disease model also demonstrated that intestinal inflammation mediated by infiltrating T cells can alter BA composition through inducing microbiome changes. Reduction in BSH-expressing microbes resulted in a shift in BA profiles (Lindner *et al*, 2024). Cholestatic diseases also tend to accumulate primary conjugated BAs; in particular, Tauro-MCAs and TCA in mice and both tauro- and glyco-conjugated CA and CDCA in humans (Straniero *et al*, 2020).

Varanasi and colleagues recently showed that tumors in both HCC patients and a mouse model of HCC exhibit an increase in conjugated BAs, mainly TCA, Tβ-MCA and TCDCA (Varanasi *et al*, 2025). In line with that, they found increased expression of *Baat* in HCC compared to non-HCC samples. They further demonstrated that tumor-specific KO of *Baat* reduced the abundance of conjugated BAs and tumor burden, through increased infiltration and functionality of T and NK cells. Curiously, they also found accumulation of conjugated BAs within T cells by LC-MS. (While the authors do not comment on this, such accumulation

would suggest the expression of a BA transporter, as conjugated BAs cannot diffuse through the cell membrane.) Additionally, through *in vitro* experiments, this study pinpointed that TCDCA, but not TCA reduced mitochondrial respiration in $CD8^+$ T cells and induced ROS leading to impaired survival (Varanasi *et al*, 2025).

In conclusion, we see a shift towards primary conjugated BAs in LCMV infected animals, which is in line with the general trend in hepatotropic viral infections, as well as several other liver diseases. Strikingly, these BAs might directly modulate $CD8^+$ T cells, although different studies pinpoint different specific BAs as key players.
Reasons behind this shift in composition are not known with certainty, however, according to speculation based on our data and literature suggestions, they might include:

- Virus-induced immunopathology leading to hepatocyte death can result in leakage of primary, conjugated BAs into the circulation, and can impair canalicular secretion altering both intrahepatic and circulating BAs.
- Differential BA synthesis enzyme expression, resulting in dysregulated BA composition.
- Altered expression of transport molecules, which might disrupt bile secretion, rerouting liver-synthesized primary BAs to the circulation. Specifically, cholestasis, frequently accompanying liver diseases results in liver retention of the synthesized BAs and their spillage to blood (Trauner *et al*, 1999).
- Level of hydrophobicity and other structural characteristics might result in the differential clearance of BAs.
- Differential microbial modifications due to dysbiosis accompanying the liver disease (Khalil *et al*, 2022)

As LCMV has been reported to induce dysbiosis and microbial shifts (Labarta-Bajo et al, 2020), we addressed the last hypothesis by analyzing the 16S rRNA sequencing dataset published by the authors of that study. Evidence from the literature also suggests that changes in BA conjugation might be driven by the microbiome; Lindner *et al.* demonstrated that the reduction in BSH genes correlates with decreased unconjugated BAs in the cecal content in a model of graft-versus-host disease (Lindner *et al*, 2024). We aimed to examine if the increase in conjugated BAs is the result of decreased microbial deconjugation in the gut. However, we found that the genome of colonic microbes in LCMV-infected mice had no less BSH-expressing bacteria, on the contrary, our analysis predicted a relative increase in taxa harboring BSH genes (Manuscript Supplementary figure 1c). Yet, this is an indirect measurement, which fails to prove gene expression or enzymatic activity. BSH activity itself has been measured by multiple groups and shown to fluctuate diurnally (Han *et al*, 2024;

Kombala *et al*, 2023). Future work would benefit from direct functional assessment, which could be performed by BSH-activatable luciferin probes, now available commercially (Khodakivskyi *et al*, 2021).

As discussed in the introduction, a series of publications recently reported direct modulation of T cell differentiation and activity by microbial BA derivatives such as DCA (Ding *et al*, 2022), 3-oxo-LCA and isoalloLCA (Hang *et al*, 2019), isoDCA (Campbell *et al*, 2020), and isoLCA (Paik *et al*, 2022) through a variety of mechanisms.
However, all of these mechanisms were reported to take place in the gut, where secondary bile acids can accumulate reaching high concentrations in the mM range (Martínez-Augustin & de Medina, 2008). Conversely, serum concentrations of niche secondary BAs remain very low. Moreover, mouse BA pools are poor in CDCA (Figure 10) and thus the amount of LCA and derivatives produced by the microbiome is minimal (Figure 9a). For instance, Zheng and colleagues measured only 2.39±1.28 nM isoLCA in mouse serum (Zheng *et al*, 2020).
Thus, immunomodulatory relevance of secondary BAs in our system is likely constrained by their low serum concentrations. An exemption is DCA, which can reach relatively high proportions in mouse serum. Nevertheless, the relative abundance of DCA in our system decreased at the peak of $CD8^+$ T cell responses, on 8 and 12 dpi (Manuscript figure 1b). How DCA levels change in our *Slco*$^{-/-}$ mouse model is not yet measured, but its reported immunomodulatory potential makes DCA an interesting candidate for further exploration.

3.3 Downregulation of BA transport and synthesis genes

The expression levels of BA related genes are often downregulated in liver diseases, however, some of the literature is contradictory. Montanari and colleagues performed transcriptomics on liver biopsies from hepatitis B virus (HBV) infected patients, and found that these samples showed downregulation of fatty and bile acid metabolic pathways compared to healthy livers, with no mention of the specific genes involved (Montanari *et al*, 2022). Sun and colleagues also found *SLC10A1* (NTCP) and *CYP7A1* downregulated in chronic HBV patients with elevated ALT (Sun *et al*, 2021). Gao *et al.* found downregulation of NTCP and multiple BA synthesis enzymes on both transcript and protein level in HBV-related hepatocellular carcinoma (Gao *et al*, 2019).
On the other hand, Oehler *et al*. used human liver chimeric mice infected with HBV to show that CYP7A1 was significantly upregulated on both the transcript and protein level, not repressed. The authors identified a viral envelop protein to be triggering this regulation through its binging to NTCP (Oehler *et al*, 2014). Importantly, NTCP has been identified as the entry receptor for HBV (Yan *et al*, 2012). Although it does not mediate uptake of hepatitis C virus (HCV), it reportedly plays a role in HCV infection as well. In HCV, it enhances viral propagation

by transporting BAs that suppress ISG expression in hepatocytes (Verrier *et al*, 2016). Vesicular stomatitis virus (VSV) infection of rodents also reportedly leads to downregulation of *Cyp7a1, Cyp27a1, Cyp8b1* and *Hsd3b7* genes (Li *et al*, 2024b).
These observations together draw the picture of a general tendency for the downregulation of BA transport and synthesis genes in diseases related to infections with hepatotropic viruses. However, it comes with caveats: observations seem to depend largely on the models, samples and controls used.
Finding appropriate controls for human liver studies is challenging. MASLD in highly prevalent in Western populations depleting the pool of healthy livers. On top, liver biopsies are almost never performed on healthy humans. Thus, including truly healthy human liver tissue as a control is close to impossible (Takyar *et al*, 2017; Minervini *et al*, 2009). *In vitro* and *in vivo* models do have their own limitations, but they provide well-defined controls for liver research.

These parallels raise the question: How do viral infections shape bile acid metabolism in the liver at the molecular level? LCMV infection induces extensive downregulation of metabolic genes in the liver. Possible explanations include limiting local viral replication by depleting resources, modulating hepatocyte-intrinsic immune mechanisms, or contributing to systemic metabolic reprogramming to support antiviral immunity, for example by redistributing resources to immune cells (Lercher *et al*, 2019). Genes involved in lipid and steroid metabolism are among the many downregulated ones. Similarly, a number of genes related to bile acid metabolism, notably BA transporters and synthesis enzymes are also downregulated (Manuscript supplementary figure 2A).
Lercher *et al.* also found that the downregulation of certain genes and pathways was absent in hepatocyte-specific *Ifnar1* knockout animals, such as genes involved in the urea cycle (Lercher *et al*, 2019). Liver diseases commonly show repression of genes involved in BA metabolism, in particular those associated with cholestasis and elevated systemic BA levels (Fuchs *et al*, 2025). The FGF15-FXR-SHP axis is key in the cholestatic downregulation of multiple genes. SHP inhibits the key liver metabolic transcription factors HNF4α and LRH-1 (Katafuchi & Makishima, 2022), resulting in the downregulation of genes such as *Cyp7a1, Cyp8b1, Cyp7b1, Cyp27a1, Slc10a1 and Slco*s (Wang *et al*, 2003; Stofan & Guo, 2020; Dawson *et al*, 2009). Inflammation-induced downregulation also plays an important part in cholestatic gene downregulation. As described in the introduction, well-established literature proves the involvement of the cytokines TNF-α, IL-6 and IL-1β (Geier et al, 2007; Green et al, 1996). This effect can even result in inflammation-induced cholestasis in patients with liver inflammation, such as in sepsis or viral hepatitis, where transporter downregulation due to inflammatory mediators can cause cholestasis, which is reversed when the underlying inflammation is resolved (Trauner *et al*, 1999).

In the light of the above knowledge on gene regulation, we assessed expression of the main regulated BA transport and synthesis genes in various experimental settings to pinpoint causal relationships. However, their downregulation was not diminished in the absence of *Ifnar1* receptor, showing a lack of causality (Manuscript supplementary figure 3a, b). Our results also show reduced Ileal *Fgf15*, as well as hepatic *Nr1h4* (FXR) and *Nr0b2* (SHP) expression upon viral infection, again failing to prove involvement of this pathway in the downregulation of genes in our setting (Manuscript figure 2d).

On top, as mentioned in the discussion of the manuscript, our setting seems to differ from the usual pattern of cholestatic gene regulation, where not only the mentioned regulatory circuit is active, with SHP exerting its effects, but also alternative transporters such as MRP3 and MRP4 are induced to alleviate hepatocyte BA toxicity.

On the other hand, we could once again show the involvement of adaptive immunity, in particular $CD8^+$ T cells (Manuscript figure 3). Hence, looking for a downstream effector, we tested the key cytokines of cytotoxic T cell. Our results only showed significant involvement of cytokines in the downregulation of *Abcb11* (BSEP) out of the tested genes, and only in the case of TNF-α and – mildly – IL-6 (Manuscript Supplementary figure 3g). These results were surprising, as, while IFN-γ has not been described to effect BA transporters, IL-6 and TNF- α have established roles in it (Geier *et al*, 2007).

It brought us to speculate that T cell cytotoxic activity might be behind this transcriptional regulation. Our results showing the correlation of serum ALT and total BA levels suggest that gene regulation might also correlate with ALT and thus be dependent on cytotoxicity.

$CD8^+$ T cells can influence target cells without the involvement of their secreted cytokines; by the delivery of cytotoxic granules containing perforin and granzymes inducing apoptosis and by extrinsic apoptotic pathways such as the FAS–FASL, or the TRAIL pathway (Lee *et al*, 2023). These options are yet to be tested, and convenient models to do so include infection of mouse models lacking perforin or granzyme B. A similar genetical, or a pharmacological intervention in the FAS-FASL and the TRAIL-TRAIL receptor interaction would allow the evaluation of the contribution of this pathway.

It is possible however, that neither perforin/granzyme-mediated killing nor extrinsic apoptotic pathways prove responsible, in which case we would be left to speculate what yet unidentified mediators or intricate immune-metabolic interplay are responsible for this effect. Possibilities include effects through other cells types, shaping of liver immune or metabolic microenvironment, or transcriptional effects of the virus.

Taken together, these findings viewed in the light of relevant literature implicate a yet unknown effector downstream of $CD8^+$ T cells in the regulation of BA-related hepatic genes, and

pinpoint the systemic immunological relevance of BA changes during LCMV infection. They expand their impact beyond the liver and propose them as direct modulators of $CD8^+$ T cell responses. Notably, our results highlighting the correlation between ALT and serum TBA, in line with Honke *et al*. having shown that the elevation of circulating BAs in LCMV depends on $CD8^+$ T cells (Honke *et al*, 2017), point towards a reciprocal interaction between immunity and BA metabolism. To dissect how systemic elevation of BAs impact immune responses, we next turned to the *Slco1a1b*$^{-/-}$ mouse model.

3.4 Systemic bile acid elevation modulates $CD8^+$ T cell responses

To directly address the consequences of the elevation of systemic TBA levels on antiviral immunity, we employed the *Slco1a1b*$^{-/-}$ (hereafter referred to as *Slco*$^{-/-}$) model, lacking the entire locus of *Slco1a* and *Slco1b* genes, where reduced hepatic clearance results in results in sustained systemic BA elevation (van de Steeg *et al*, 2010). Upon LCMV infection, we observed less $CD44^+$ $CD8^+$ T cells pointing towards impaired T cell activation in the spleen, resulting in less virus-specific T cells with drastically reduced capacity for expressing cytokines and cytolytic molecules compared to WT littermates (Manuscript figure 4). Virus-specific T cells in the liver were also reduced, however, when restimulated, they produced comparable amounts of cytokines compared to cells from control animals (Manuscript supplementary figure 4).

These results pointed towards a systemic BA modulation of T cell activation and effector function, perhaps through a specific BA species. Until today, however, the causal mediators remain unresolved. This would be supported by earlier findings already detailed in the “Systemic immunometabolism” subchapter of the introduction of this thesis, establishing BA as potent T cell modulators. Mechanistically, BAs have been shown to signal through VDR (Song *et al*, 2020) and FXR (Campbell *et al*, 2021), the inhibition of mTOR signaling (Zhu *et al*, 2021, 2025) induction of oxidative stress (Hang *et al*, 2019; Varanasi *et al*, 2025), and by interfering with Ca^{2+} homeostasis, subsequently impairing NFAT signaling (Ding *et al*, 2022; Cong *et al*, 2024). Additionally, they were reported to indirectly modulate T cells by acting on DCs, dampening their immunostimulatory activity (Campbell *et al*, 2020).

It would be an exciting follow-up to pinpoint which elevated BA species are responsible for the observed effect, such as through an *in vitro* screen by activating isolated $CD8^+$ T cells in the presence of candidate BAs and measuring their cytokine and cytolytic enzyme production. Further studies could elucidate the mechanism of action by addressing the above-described options. Involvement of BA receptors could be tested *in vitro* with employing KO cells, and *in*

vivo follow-ups would be possible with cell-type specific deletions. Pharmacological inhibitors and transcriptional assays could shed light on signaling pathway involvement. Co-cultures would also allow the testing of DC-mediated effects (Campbell *et al*, 2021).

3.5 Limitations of the *Slco*$^{-/-}$ model and alternative approaches

As demonstrated in the manuscript, the *Slco*$^{-/-}$ model is a KO of the locus containing the following genes: *Slco1a1, Slco1a4*, *Slco1a5*, *Slco1a6* and *Slco1b2* (van de Steeg *et al*, 2010). Encoding for the promiscuous OATP transporters, these are responsible for the transport of not only bile acids but also bilirubin, steroid hormones, and various drugs and xenobiotics, with overlapping substrate specificity (van de Steeg *et al*, 2010; Durník *et al*, 2022). OATP1A5 and OATP1A6 have low liver expression and are poorly characterized members of the family (Hagenbuch & Stieger, 2013). OATP1A1, OATP1A4 and OATP1B2 are expressed on the basolateral membrane of hepatocytes, lacking human orthologues; humans express OATP1B1 and OATP1B3. However, it is hard to assess individual contribution of these transporters to the functions they cover in concert, that is why the *Slco*$^{-/-}$ model has been generated (Hagenbuch & Stieger, 2013; van de Steeg *et al*, 2010). The OATP family also has numerous other members, not presented here. Some family members are also expressed in extrahepatic tissues where they mediate the uptake of a wide range of molecules including bile acids, thyroid hormones and prostaglandins (Hagenbuch & Stieger, 2013). Table 1 explains the expression pattern and substrate specificity of the transporters deleted in the *Slco*$^{-/-}$ model.

Gene name	Protein name	Main tissue expression	Endogenous substrate specificity	References
Slco1b2	Oatp1b2	Liver-specific	Bile acids, conjugated bilirubin	(Hagenbuch & Stieger, 2013; Zaher *et al*, 2008; Csanaky *et al*, 2011)
Slco1a1	Oatp1a1	Liver, kidney	Unconjugated bile acids, bilirubin conjugates, steroid conjugates	(Hagenbuch & Stieger, 2013; Zhang *et al*, 2012a)
Slco1a4	Oatp1a4	Liver, blood brain barrier	Bile acids, steroid conjugates	(Hagenbuch & Stieger, 2013; Zhang *et al*, 2013; Xu *et al*, 2024)
Slco1a5	Oatp1a5	Brain, intestine	Steroid/thyroid-related compounds	(Tani *et al*, 2008; Hagenbuch & Meier, 2004)
Slco1a6	Oatp1a6	Kidney, pancreatic islets	Bile acids, organic anions	Hagenbuch & Stieger 2013

Table 1 – Tissue expression and substrate specificity of deleted transporters in the Slco1a1b$^{-/-}$ model

OATPs are able to transport both conjugated and unconjugated BAs *in vitro* (Geier *et al*, 2007; Slijepcevic *et al*, 2017), however, the *Slco*$^{-/-}$ model showed that OATPs are critical in the

uptake of unconjugated BAs. KO animals demonstrated a 4-fold increase in plasma total BAs, almost exclusively due to unconjugated BAs, and and a 40-fold increase in total bilirubin, mostly glucuronidated. The authors also performed LC-MS measurements of 9 individual BAs, noting significant changes in each measured unconjugated BA measured, except for the low abundancy CDCA. CA, DCA and α-MCA showed robust, while UDCA showed modest elevation. The only conjugated BA to show upregulation was TCA out of the four tested conjugated BAs. (Notably, we also show a robust elevation of TCA in LCMV infection (Manuscript figure 1b)). KO animals are fertile and have a normal lifespan, a slightly increased body weight compared to WT animals, and jaundice due to hyperbilirubinemia (van de Steeg *et al*, 2010). While all hepatic OATPs likely contribute, OATP1B2 seems to be the most important hepatic basolateral transporter of unconjugated BAs based on KO studies (Csanaky *et al*, 2011).

In the light of our BA profiling and the phenotypic characteristics of *Slco*$^{-/-}$ mice, it is apparent that the model does not fully recapitulate the BA profiles, TCA being the only BA elevated in both LCMV and *Slco*$^{-/-}$ mice. However, as discussed above, TCA has been implicated in impairing the effector function of CD8^{+} T cells (Xun *et al*, 2021).
While our model proved useful in demonstrating an immunological effect of systemic TBA elevation, it did not allow us to pinpoint specific BAs and molecular mechanisms behind the observed effects.
In addition, *Slco*$^{-/-}$ mice exhibit hyperbilirubinemia – a confounding factor given its own immunomodulatory properties (Liu *et al*, 2008), as well as subtler phenotypic traits such as the slightly higher body weight.

For these reasons, I propose multiple options for the further examination of the *in vivo* effects of systemic BA perturbations.
Although *Slco10a1*-deficient mice lacking the NTCP transporter theoretically represent a model for systemic conjugated BA accumulation, in practice, most animals maintain normal BA levels through the compensatory uptake *via* OATP transporters, even though they all show decreased serum BA clearance (Slijepcevic *et al*, 2015). This observation also served as a proof that both NTCP and OATPs are involved in the uptake of conjugated BAs. Administration of Myrcludex B, an NTCP inhibitor leads to similar effects regarding systemic BA levels (Slijepcevic *et al*, 2017). However, inhibition of NTCP in *Slco*$^{-/-}$ mice led to the dramatic elevation in BAs. 5 days of Myrcludex B treatment led to concentrations reaching 1mM, mainly composed of TCA and Tβ-MCA. (Slijepcevic *et al*, 2017). Nevertheless, the confounding factors associated with the *Slco*$^{-/-}$ model would not be removed in this model.

On the other hand, bile acid sequestration would represent an elegant way to test our hypothesis on BA-mediated T cell modulation and subsequent changes in immunopathology in LCMV infection of WT mice. A chew diet containing 1-2% cholestyramine is an established model effective at significantly lowering systemic bile acid levels (Zhang & Klaassen, 2010; Nishida *et al*, 2020).

Bile duct ligation represents a common procedure to induce cholestasis accompanied by dramatic increase in systemic BAs (Zhang *et al*, 2012b). However, this method is unlike transporter KO, which traps BAs out of the hepatocyte and in the circulation; it causes extreme elevation of BA concentrations within the liver, resulting in significant, unresolvable liver damage, leading to fibrosis within three weeks (Lang *et al*, 2018). Such confounding factor would be irreconcilable with the efforts towards the dissection of changes in $CD8^+$ T cell responses and their mechanism of action due to elevations in specific BA species. Nevertheless, bile duct-ligated mice infected with LCMV were shown to develop a chronic infection due to impaired IFN-I production and $CD8^+$ T cell responses, indicating a dysfunctional antiviral immunity at very high systemic BA levels (Lang *et al*, 2018).

Similarly, BA feeding can be highly toxic. Two recent publications implicate DCA in the negative regulation of $CD8^+$ T cell effector function through disrupting Ca^{2+} homeostasis inhibiting Ca^{2+}-NFAT2 signaling (Cong *et al*, 2024; Ding *et al*, 2022). Yet, DCA feeding has been demonstrated to cause hepatotoxicity (Delzenne *et al*, 1992; Moole *et al*, 2021), while both DCA and TCA disrupts the intestinal barrier causing leaky gut (Liu *et al*, 2022). Ding *et al*. used intraperitoneal BA injections to assess immunological changes induced by systemic DCA elevation, which could be an interesting option for further exploration (Ding *et al*, 2022).

3.6 Conclusion and outlook

The work presented in this thesis provides evidence for the systemic modulation of BAs in LCMV and its interconnected relationship with immunity. Our data reveal that $CD8^+$ T cell-mediated immunopathology drives a downregulation of hepatic BA metabolism genes. Elevated BAs correlated with ALT, a proxy for $CD8^+$ T cell-mediated immunopathology, also implicating T cells in the systemic elevation of BA levels and a shift in their composition. Our results suggest multiple plausible mechanisms for $CD8^+$ T cells to drive downregulation of hepatic BA metabolism genes and contribute to elevated systemic BA level, including the release of host-derived BAs from damaged hepatocytes and the altered expression of BA transporters controlling BA uptake.

On the other hand, sustained high systemic BA levels impaired $CD8^+$ T cell activation and effector function, reduced viral clearance and immunopathology. Together, these findings suggest the existence of a novel reciprocal interplay between $CD8^+$ T cell immunity and systemic BA metabolism during viral hepatitis.

Our bile acid profiling efforts demonstrated a shift in the BA pool towards liver-abundant primary- and conjugated BAs, a pattern consistent with patients suffering from chronic HBV infection and multiple other liver diseases, successfully reinforcing the translational relevance of the LCMV model. Recent advances in bile acid-targeted therapies further highlight the clinical relevance of these findings beyond mechanistic insight. Pharmacological interventions such as BA sequestrants, FXR agonists or NTCP inhibitors demonstrate the possibility to modulate BA transport and enterohepatic circulation in patients (Simbrunner *et al*, 2021; Trauner *et al*, 2025). Such approaches could potentially be harnessed as immunomodulatory interventions to influence antiviral immunity; providing rational for future investigation.

In the same time, caution is required when extrapolating mouse findings to humans, especially in the field of BA studies, given the differences in BA composition, the enzymes involved in their synthesis and modification, as well as BA transporter expression, all having the potential to influence immunometabolic interactions.

LCMV itself also come with limitations including its systemic and relatively acute nature, as compared to human viral hepatitides, which are typically chronic in nature, often ongoing for years or decades and primarily targeting hepatocytes. The LCMV model is also accompanied by cachexia and consequent rapid and dramatic microbiome changes as confounding factors for BA pools.

Our KO mouse model is another source of confounding factors. *Slco*$^{-/-}$ mice exhibit altered BA profiles compared to WT, which may result in an altered gut microbiome. On top, their high bilirubin values and altered energy metabolism contribute additional physiological alterations that need to be considered (Staley *et al*, 2017; van de Steeg *et al*, 2010).

Our promising findings hence call for further evaluation, aiming to uncover the individual BA species involved in the observed immunometabolic circuits and their mechanism of action. These would present exciting new avenues of research and extend the potential of our work to inspire new ways to modulate $CD8^+$ T cells, the cornerstone of immunity against cancer and viral infections. Furthermore, exploring the reciprocal modulation of immunity by systemic BA levels may inspire therapeutic strategies aimed at balancing antiviral immunity with tissue protection.

Taken together, this work highlights a dynamic crosstalk between the immune system and BA metabolism in liver diseases, and showcases once again how metabolism is inseparable from understanding immunity. Further research will continue to uncover the interconnected nature of these basic functions, helping us translate insights into interventions, ultimately improving outcomes for the growing numbers of patients with liver diseases worldwide.

3.7 Graphical Summary

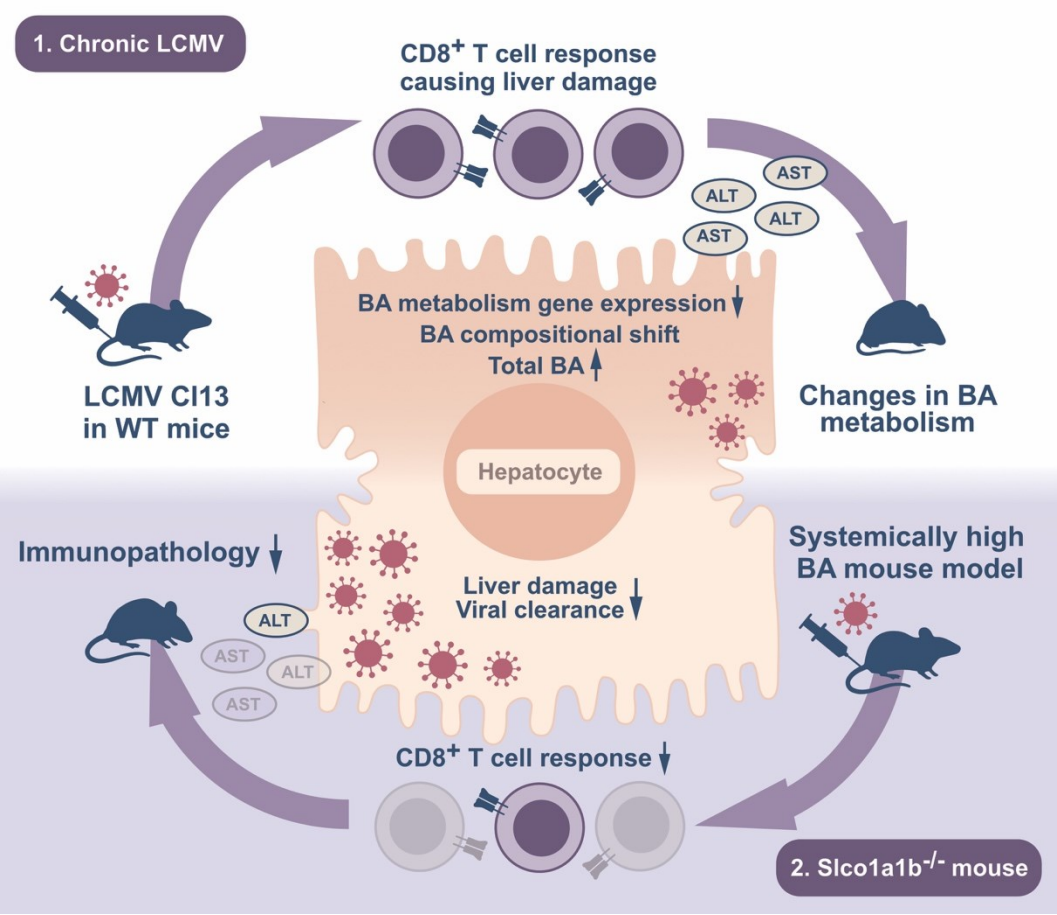

Figure 12 – Graphical summary

Figure adapter from (Keszei *et al*, 2025)*, preprint available at bioRxiv.*

4. Materials and Methods

The materials and methods associated with this doctoral work are detailed in the “Methods” section of the reprinted manuscript included in chapter 3 of this thesis.

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6. Curriculum Vitae

Zsofia Keszei

Science | Creativity | Purpose

Contact

Linkedin profile

Key skills

- Virology & immunology
- Molecular biology techniques & rodent handling
- Data analysis & reporting
- Graphic design & data visualization
- Scientific writing
- Public speaking & presentation
- Project management

Computer skills

- MS office
- Graphpad Prism
- Endnote, Mendeley, Zotero
- FlowJo
- PubMed
- Adobe Illustrator, Affinity Designer
- R for data handling & visualization

Languages

Hungarian	*Mothertongue*
English	*C1, confident*
French	*B2, conversational*
German	*A2, basic communication*
Spanish	*A2, basic communication*

Experience

PhD candidate in Immunology 2019 - 2025
CeMM Center of Molecular Medicine, Vienna

- Scientific Writing and documentation
  Writing and editing of grants, manuscripts and animal licenses. Ensuring accuracy and adherence to regulatory standards.
- Project management and collaboration
  Managed research projects and maintained clear communication and efficient timelines with collaborators.
- Data analysis and reporting, presentation of results
- Illustration, graphic design, public speaking
- Rodent handling and laboratory techniques

Internship 2022 May
Janssen Vaccines, Leiden, Netherlands

- Participated in presentations and trainings to gain insight into vaccine development and clinical research.
- Gained hands-on laboratory exposure and understanding of industry standards for documentation and quality control

Education

PhD in Immunology 2019 - present
Medical University of Vienna
Laboratory of Andreas Bergthaler
Crosstalk between immunity and liver metabolism in viral infection

MSc in Biology 2017- 2019
University of Geneva
Laboratory of Didier Picard
Localization and function of the molecular chaperone Hsp90

BSc in Biology 2014- 2017
Eötvös Loránd University, Budapest

Publications

- Keszei Z et al. bioRxiv 2025.08.17.670599; doi: https://doi.org/10.1101/2025.08.17.670599
- Keszei Z et al. Virus Res. 2020 Oct 2;287:198093; doi: https://doi.org/10.1016/j.virusres.2020.198093
- Amman F et al. Nat Biotechnol. 2022 Jul 18; doi: https://doi.org/10.1038/s41587-022-01387-y
- Popa A et al. Sci Transl Med. 2020;12(573):eabe2555; doi: https://doi.org/10.1126/scitranslmed.abe2555

Fellowships

- Excellence Master Fellowship, 2017-2019
  Awarded by the Faculty of Science, University of Geneva
- Marie Curie Fellow, 2019-2023
  Innovative Training Network "INITIATE"
  Awarded by the European Commission