

**Targeting cholesterol esterification
as a novel immune checkpoint in
viral infections and cancer**

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Doctor of Philosophy

I, Nathalie Monika Schmidt, confirm that the work presented in my thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Identifying metabolic targets that constrain tumours and viruses while boosting exhausted, dysfunctional T cells can provide novel therapeutic checkpoints. Modulating cholesterol esterification by inhibiting the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) has a direct antitumour and antiviral effect and enhances murine anti-tumour CD8⁺ T cells.

In this thesis, I showed that reduced formation of cholesterol-rich microdomains within the cell membrane (lipid rafts) was a feature of PD-1^{hi} exhausted CD8⁺ T cells. I therefore investigated the potential for rescuing exhausted human T cells by modulating cholesterol esterification and lipid raft formation.

Inhibiting ACAT enhanced the expansion of functional virus- and tumour-specific T cells from donors with chronic hepatitis B virus (HBV) infection, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, and hepatocellular carcinoma. The immune-boosting effect was not limited to circulating T cells but could also enhance the function of T cells directly *ex vivo* from the immunosuppressive liver and tumour microenvironment in the majority of donors.

ACAT inhibition led to a redistribution of intracellular cholesterol with reduced neutral lipid droplets and increased lipid raft formation, resulting in enhanced T cell receptor (TCR) signalling and T cell effector function. Additionally, ACAT inhibition induced TCR-independent bioenergetic rewiring with a skewing towards utilization of oxidative phosphorylation.

ACAT inhibition had a complementary effect with other immunotherapies, with increased responsiveness to PD-1 blockade and enhanced functional avidity of TCR-engineered T cells recognizing HBV and tumour cells.

Taken together, reduced lipid rafts are a feature of exhausted T cells and modulating cholesterol esterification by ACAT inhibition is a promising novel immunotherapeutic approach to boost exhausted antiviral and antitumour T cells in acute and chronic infection and in cancer.

Impact statement

257 million people are living with chronic hepatitis B virus (HBV) infection worldwide, causing over 2000 deaths each day, mainly due to liver cirrhosis and hepatocellular carcinoma (HCC). HCC is the third leading cause of cancer-related death with a rising incidence and ongoing high mortality. The current standard of care for HBV is a treatment with antiviral nucleos(t)ide analogues; however, they require lifelong treatment, do not modulate antiviral immune responses and do not fully prevent HCC.

The development of novel therapeutic strategies, e.g. immunotherapy, is of high importance to reduce the major health and economic burden associated with chronic HBV and HCC. Checkpoint blockade has revolutionized cancer treatment with tremendous success rates in some solid tumour; however, response rates in HBV and HCC are low and a multipronged approach targeting multiple checkpoints will be necessary to rescue the highly exhausted, dysfunctional T cells typical for HBV and HCC.

Nutrient availability, uptake and utilization play a central role in successful immune responses and targeting metabolic pathways is a promising new immunotherapeutic strategy. I have shown that a modulation of cholesterol esterification by targeting the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) boosts the function of HBV- and HCC-specific T cells. Importantly, virus- and tumour-specific T cells are rescued directly from the sites of disease, the immunosuppressive liver and tumour microenvironment.

ACAT inhibition has a complementary effect with other immunotherapeutic approaches currently explored for HBV and HCC, such as PD-1 blockade and TCR-engineered T cells used in adoptive T cell transfer, suggesting that a combination of these therapies can increase response rates in future clinical trials.

T cell exhaustion has also been described in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the finding that ACAT inhibition also boosts the function of SARS-CoV-2-specific T cells during acute infection highlights the transferability of this study to other viral infection and tumours associates with T cell exhaustion.

I have shown that T cell exhaustion is associated with reduced lipid rafts and that ACAT inhibition induces a redistribution of intracellular cholesterol, resulting in increased lipid rafts and metabolic reprogramming. Defining features of T cell exhaustion advances the scientific knowledge in the field and opens new routes that can be explored for novel therapeutic approaches.

The findings in this thesis have led to the establishment of new, interdisciplinary collaborations, the most important of which was a collaboration with Jane McKeating's group at the University of Oxford, a renowned virologist. Despite tackling the same diseases, collaborations between viral immunologists and virologists are rare and we worked together to delineate the full potential of ACAT inhibition in viral infections. The McKeating lab showed that ACAT inhibition exerts a direct antiviral effect against HBV and SARS-CoV-2, highlighting that ACAT is a unique dual viral and immunological checkpoint.

Our combined findings led to the filing of an international patent and a collaboration with a pharmaceutical company to further explore the potential of targeting cholesterol esterification as a therapeutic strategy for HBV, SARS-CoV-2, HCC and beyond.

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Abbreviations

25-HC	25-hydroxycholesterol	Bcl-2	B cell lymphoma-2
ABC	ATP-binding cassette	BID	BH-3 interacting-domain death agonist
ACAT	acyl-CoA:cholesterol acyltransferase	Bim	BCL-2-interacting mediator of cell death
ACE-2	angiotensin converting enzyme 2	BLIMP-1	B lymphocyte-induced maturation protein-1
acetyl-CoA	acetyl-Coenzyme A	BLQ	below the limit of quantification
ADP	adenosine diphosphate	BSA	bovine serum albumin
AFP	α -fetoprotein	CAR	chimeric antigen receptor
AICD	activation-induced cell death	cccDNA	covalently closed circular DNA
AK	adenylate kinase	CCL	chemokine (C-C motif) ligand
ALT	alanine aminotransferase	CCR	chemokine (C-C motif) receptor
AMP	adenosine monophosphate	CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
AMPK	adenosine monophosphate-activated protein kinase	CFSE	carboxyfluorescein succinimidyl ester
APC	antigen-presenting cell	CHB	chronic HBV infection
ARDS	acute respiratory distress syndrome	CMV	cytomegalovirus
ATP	adenosine triphosphate	CPT1a	carnitine palmitoyl transferase 1a
BAT3	HLA-B-associated transcript 3	cRPMI	complete RPMI
BAX	Bcl-2-associated X protein	cSMAC	central SMAC

CTB	cholera toxin B subunit	FOXO1	forkhead box protein O1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4	FOXP3	forkhead box P3
CXCL	chemokine (C-X-C motif) ligand	FSC-A	forward scatter area
CXCR	chemokine (C-X-C motif) receptor	FSC-H	forward scatter height
DC	dendritic cell	GAPDH	glyceraldehyde-3-phosphate dehydrogen
DDX60L	DExD/H box helicase DEAD box polypeptide 60-like	GITR	glucocorticoid-induced tumour necrosis factor receptor-related protein
DMSO	dimethyl sulfoxide	GM1	monosialotetrahexosyl-ganglioside
EBV	Epstein-Barr virus	HBcAg	HBV core antigen
ECAR	extracellular acidification rate	HBeAg	HBV e antigen
EDTA	ethylenediaminetetraacetic acid	HBsAg	HBV surface antigen
Eomes	eomesodermin	HBSS	Hanks' balanced salt solution
ER	endoplasmic reticulum	HBV	hepatitis B virus
ERK	extracellular signal-regulated kinase	HBxAg	HBV x antigen
ETC	electron transport chain	HCC	hepatocellular carcinoma
FAD⁺	flavin-adenine dinucleotide	HCV	hepatitis C virus
FAO	fatty acid oxidation	HDL	high density lipoprotein
FAS	fatty acid synthesis	HIF1α	hypoxia inducible factor 1 α
FBS	foetal bovine serum	HIV	human immunodeficiency virus
FCCP	carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone	HLA	human leucocyte antigen

HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA	LPS	lipopolysaccharide
ICAM-1	intercellular adhesion molecule-1	LXR	liver x receptor
ICOS	inducible T cell costimulator	MAGE	melanoma-associated antigen
IDO	indoleamine 2,3-dioxygenase	MAPK	mitogen-activated protein kinase
IFN	interferon	MCT	monocarboxylate transporter
IHL	intrahepatic leukocytes	MDSC	myeloid-derived suppressor cell
IL	interleukin	MEK	MAPK-ERK kinase
INSIG	insulin-induced gene	Mem	SARS-CoV-2 membrane protein
ISG	interferon-stimulated gene	MFI	mean fluorescent intensity
ITAM	immunoreceptor tyrosine-based activation motif	MHC	major histocompatibility complex
Lag-3	lymphocyte-activation gene-3	MIP	macrophage inflammatory protein
LAMP-1	lysosomal associated membrane glycoprotein-1	mTOR	mechanistic/mammalian target of rapamycin
LAT	linker for activation of T cells	mTORC1/2	mTOR complex 1/2
Lck	lymphocyte specific protein-tyrosine kinase	NAD⁺	nicotinamide adenine dinucleotide
LCMV	lymphocytic choriomeningitis virus	NASH	non-alcoholic steatohepatitis
LDL	low density lipoprotein	NF-κB	nuclear factor κb
LFA-1	lymphocyte function-associated antigen 1	NFATC1	nuclear factor of activated T cells, cytoplasmic 1

NK cell	natural killer cell	Pol	HBV polymerase
NO	nitric oxygen	PPAR	peroxisome proliferator-activated receptor
NPC	Nieman-Pick C	PPP	pentose phosphate pathway
NSCLC	non-small cell lung cancer	pSMAC	peripheral SMAC
NTCP	sodium-taurocholate cotransporter polypeptide	rcDNA	relaxed circular DNA
NUC	nucleos(t)ide analogue	RLU	relative light units
NY-ESO-1	New York oesophageal squamous cell carcinoma-1	ROS	reactive oxygen species
OCR	oxygen consumption rate	Rot/AA	rotenone and antimycin A
oxLDL	oxidized low density lipoprotein	RPMI	Roswell Park Memorial Institute 1640 Medium
OXPHOS	oxidative phosphorylation	RT	room temperature
PBMC	peripheral blood mononuclear cells	SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
PBS	phosphate-buffered saline	SCAP	SREBP cleavage-activating protein
PD-1	programmed death 1	scRNAseq	single cell RNA sequencing
PD-L1	programmed death ligand 1	SHP-2	Src homology 2 domain-containing tyrosine phosphatase 2
PGC1α	peroxisome proliferator-activated receptor γ coactivator 1 α	siRNA	small interfering RNA
PI3K	phosphoinositide 3-kinases	SLC	solute carrier
PKCθ	protein kinase C θ	SMAC	supramolecular activation cluster
PMA	phorbol 12-myristate 13-acetate	SOAT	sterol O-acyltransferase
pMHC	peptide-loaded MHC complex	SR-B1	scavenger receptor class B type I

SRC	spare respiratory capacity	TIGIT	T cell immunoglobulin with ITIM domain
SREBP	sterol regulatory element binding protein	TIL	tumour-infiltrating leukocytes
SSC-A	side scatter area	TMPRSS2	transmembrane protease serine 2
STAT	signal transducer and activator of transcription	Tim-3	T-cell immunoglobulin and mucin-domain containing-3
T-bet	T-box expressed in T cells	TLR	toll-like receptor
TAA	tumour-associated antigen	TME	tumour microenvironment
TAM	tumour-associated macrophage	TNF	tumour necrosis factor
TAP	transporter associated with antigen presentation	TOX	thymocyte selection-associated high mobility group box
TCA	tricarboxylic acid	TRAIL	TNF-related apoptosis-inducing ligand
TCF-1	T cell factor 1	T_{reg}	regulatory CD4 ⁺ T cell
TCR	T cell receptor	T_{RM}	tissue-resident memory T cell
T_{ex}^{int}	intermediate state of T cell exhaustion	VCAM-1	vascular cell adhesion molecule-1
T_{ex}^{prog}	progenitor state of T cell exhaustion	VEGF-A	vascular endothelial growth factor-A
T_{ex}^{term}	terminally differentiated exhausted T cell	VOC	variant of concern
Tfh cell	T follicular helper cell	WT	wildtype
TGF-β	transforming growth factor β	XBP-1	X-box binding protein 1
Th cell	T helper cell	ZAP-70	zeta-chain-associated protein kinase 70

1

Introduction

1.1 CD8⁺ T cells in health and disease

CD8⁺ T cells are key players in the adaptive immune response against infections and cancer, and multiple factors contribute to a successful or failed CD8⁺ T cell response.

This thesis focuses on targeting cholesterol metabolism to enhance the antiviral and antitumour function of exhausted CD8⁺ T cells. In this chapter I will therefore first discuss the process of CD8⁺ T cell activation with a focus on the role of lipid rafts (cholesterol- and sphingolipid-enriched microdomains in the cell membrane) and the immune synapse, followed by key antiviral/antitumour CD8⁺ T cell effector functions. I will then discuss features of CD8⁺ T cell exhaustion, including the expression of inhibitory checkpoints as well as transcriptional regulations.

1.1.1 The role of lipid rafts and the immunological synapse in CD8⁺ T cell activation

1.1.1.1 CD8⁺ T cell activation

Naïve CD8⁺ T cells requires three key signals during T cell activation and priming to induce proliferation, differentiation and effector function. First, antigen recognition via the T cell receptor (TCR); second, signalling through costimulatory molecules, primarily CD28; third, inflammatory cytokines (Fig. 1.1).

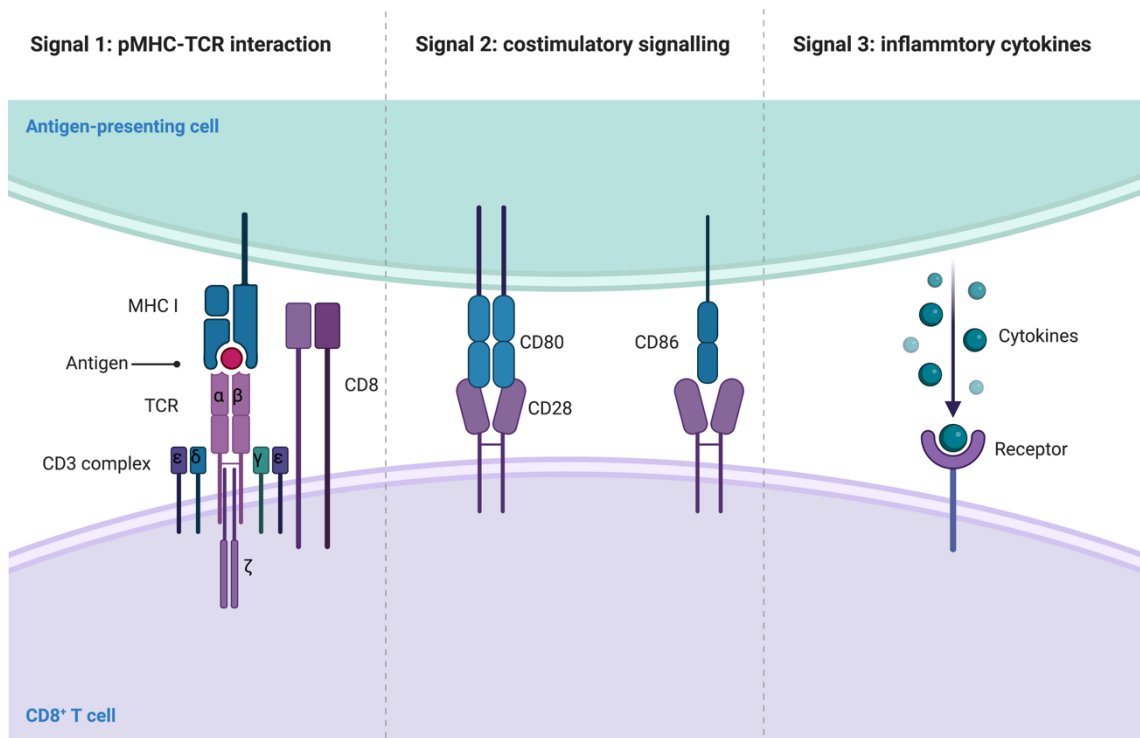


Figure 1.1 CD8⁺ T cell activation.

CD8⁺ T cells require three activation signals: TCR-pMHC interaction (left); co-stimulatory signalling (middle); pro-inflammatory cytokines (right). (p)MHC: (peptide-loaded) major histocompatibility complex; TCR: T cell receptor.

1.1.1.1.1 TCR-pMHC interaction

The antigen-specificity of a CD8⁺ T cell is determined by its unique TCR, first described in 1984 (Hedrick et al. 1984; Malissen et al. 1984; Yanagi et al. 1984). The TCR complex consists of the antigen-binding TCR $\alpha+\beta$ subunits forming a heterodimer, and the signal-transducing CD3 complex consisting of the CD3 ϵ/γ , CD3 ϵ/δ and CD3 ζ/ζ dimers (Swamy et al. 2007) (Fig. 1.1).

$\alpha\beta$ CD8⁺ T cells recognize their cognate peptide presented on major histocompatibility complex (MHC) class I molecules and the interaction between TCR and peptide-loaded MHC (pMHC) is influenced by three parameters: affinity, avidity and functional avidity (Viganò et al. 2012). Affinity is defined as the strength of interaction between a single TCR and pMHC and is usually determined by association and dissociation rates. TCR avidity measure the strength of multiple TCR-pMHC engagements. While affinity and avidity are physical parameters, functional avidity measures TCR sensitivity and determines how well a T cell responds at different peptide concentrations (Campillo-Davo et al. 2020; Viganò et al.

2012). Affinity and avidity influence the functional avidity alongside the expression of costimulatory molecules and TCR coreceptors such as CD8 binding to MHC I to stabilize the pMHC/TCR complex (Garcia et al. 1996; Viganò et al. 2012).

CD8⁺ T cells recognize pMHC class I on professional antigen-presenting cells (APC), e.g. dendritic cells (DC) or macrophages, or on infected/transformed cells (Morath and Schamel 2020). While all nucleated cells present proteasome-degraded endogenous antigens on MHC class I molecules, professional APC can acquire and present antigens in three main ways – first, direct presentation when the APC itself is infected/transformed; second, cross-presentation when the APC engulfs a transformed/infected cell by phagocytosis followed by intracellular antigen processing; third, cross-dressing when preformed pMHC are transferred from the surface of an infected/transformed cell to the APC via direct cell-cell contact (Cruz et al. 2017; Wakim and Bevan 2011).

Unbound MHC class I molecules are localised in the endoplasmic reticulum (ER) where they are assembled from heterodimers of a light and heavy chain and are stabilized by chaperon proteins such as calreticulin and tapasin (Neefjes et al. 2011). Peptide fragments are transported into the ER via transporter associated with antigen presentation (TAP). Tapasin interacts with TAP and facilitates peptide mobilization to the MHC complex. Upon peptide binding, the chaperons disengage from the pMHC complex and allow mobilisation to the cell membrane.

Exogenous peptides for cross-presentation are primarily acquired by DC via phagocytosis or receptor-mediated endocytosis (Cruz et al. 2017). Multiple pathways can be employed for cross-presentation, e.g. the phagosome-to-cytosol or cytosolic pathway and the vacuolar pathway (Joffre et al. 2012). In the cytosolic pathway, proteins are transferred from phagosomes into the cytosol where they are hydrolysed by proteasomes (Colbert et al. 2020). Degraded proteins are further transferred to the ER or a phagosome and loaded on MHC class I heterodimers as described above. In contrast, in the vacuolar pathway, exogenous peptides are both degraded by proteases and loaded on MHC class I in the phagosome. The vacuolar pathway shares similarities with the loading of MHC class II complexes in the MHC II compartment with peptides that have been degraded by proteases in the early endosome (Neefjes et al. 2011). pMHC class II complexes are essential for the priming of CD4⁺ T cells by professional APC.

Various models have been suggested for how the formation of the TCR/pMHC complex results in TCR triggering, e.g. TCR aggregation, conformational changes, and TCR segregation from inhibitory molecules and redistribution into lipid rafts (van der Merwe and Dushek 2011; Xavier et al. 1998); however, a recent study has revealed that peptide binding can trigger TCR signalling in the absence of structural rearrangement (Sušac et al. 2022). Likely a combination of various mechanisms ultimately result in phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAM) of the CD3 complex by the tyrosine kinases lymphocyte specific protein-tyrosine kinase (Lck) and Fyn (Mørch et al. 2020).

Phosphorylated ITAM serve as binding sites for the SH2 domain of the zeta-chain-associated protein kinase 70 (ZAP-70) which itself is then activated by Lck- and Fyn-mediated phosphorylation. ZAP-70 in turn phosphorylates linker for activation of T cells (LAT) and SLP-76, recruiting phospholipase C- γ (PLC- γ). PLC- γ increases cytosolic Ca²⁺ levels and activates Ras and protein kinase C by catalysing the production of inositol triphosphate and diacyl glycerol. This initiates a signalling cascade and phosphorylation of various downstream TCR signalling molecules ultimately resulting in T cell activation, proliferation and effector function (Mørch et al. 2020).

1.1.1.1.2 Costimulatory molecules and proinflammatory cytokines

Costimulatory molecules, especially CD28, are critical for establishment of the immune synapse (see chapter 1.1.1.3 for details) and for determining T cell sensitivity (Fig. 1.1).

CD28 plays a crucial role in facilitating T cell polarisation, lipid raft clustering (see chapter 1.1.1.2 for details) and establishment of the immune synapse upon TCR engagement, e.g. via actin remodelling (Raab et al. 2001) and recruitment of filamin-A to the synapse (Tavano et al. 2006). TCR signalling induces phosphorylation of CD28 that leads to a recruitment of various proteins involved in downstream TCR signalling into lipid rafts and the immune synapse, including Lck, protein kinase C θ (PKC θ) and LAT (Holdorf et al. 1999; Martin et al. 2001; Tavano et al. 2004; Yokosuka et al. 2008).

Interaction of CD28 with its ligands CD80 and CD86 on the cell surface of APC lowers the TCR threshold (Viola and Lanzavecchia 1996) and prevents the development of T cell anergy

(Harding et al. 1992) while CD3 signalling without CD28 engagement is unstable and transient (Viola et al. 1999).

Finally, inflammatory cytokines such as interleukin- (IL-) 12 and type I interferons (IFN), e.g. produced by DC, contribute to T cell activation (Mescher et al. 2006). In the absence of these third signal cytokines, TCR and CD28 signalling alone can stimulate naive CD8⁺ T cells and induce proliferation but effector function, memory formation and survival are impaired, ultimately resulting in T cell tolerance (Curtsinger and Mescher 2010). Of note, third signal cytokines are not only important during T cell priming in primary infection but also for memory recall during reinfection (Xiao et al. 2009).

1.1.1.2 The concept of lipid rafts

The concept of a fluid mosaic structure of the cell membrane was first described in 1972 (Singer and Nicolson 1972) and was followed by the observation of different membrane fractions only one year later (Yu et al. 1973). Over the years the idea of lateral heterogeneity of the cell membrane emerged and in 1997 Simons and Ikonen first discussed the concept of cholesterol- and sphingolipid-rich microdomains within the cell membrane - lipid rafts (Simons and Ikonen 1997). They observed that proteins can be selectively included or excluded from these lipid rafts, leading to the hypothesis that they can act as cellular signalling platforms. While the role of lipid rafts was further studied in the following years, findings were heterogeneous, partially due to difficulties in visualizing and observing rafts and partially due to the lack of a clear definition limiting the comparability between findings.

In 2006 a consensus definition of lipid rafts was determined: “*Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions.*” (Pike 2006). It is important to note that the term ‘lipid raft’ in itself has been discussed and other terms such as ‘membrane raft’ and ‘cholesterol-enriched microdomain’ have also been suggested. To date, there has been no clear consensus regarding terminology and in this thesis, these membrane microdomains will be referred to as ‘lipid rafts’.

While the organisation and function of lipid rafts is still under investigation, the current concept is that lipid rafts act as highly dynamic platforms for enzymes, signalling molecules and other proteins. By excluding certain proteins and facilitating the interaction between others, they are thought to play a critical role in immune signalling, host-pathogen interactions and cancer (Sezgin et al. 2017).

1.1.1.3 The immunological synapse

Recruitment of the TCR and compartmentalization of T cell signalling molecules in lipid rafts at the interface between T cells and their APC facilitate the formation of the immunological synapse – a highly dynamic structure enabling T cell/APC interaction and shaping T cell activation and effector function (Drake and Braciale 2001; Dustin 2014; Xavier et al. 1998; Zumerle et al. 2017). Three key components are necessary for the formation of the immunological synapse between a CD8⁺ T cell and APC: interaction between TCR and pMHC class I (see chapter 1.1.1.1.1), adhesion molecules and co-stimulatory molecules (see chapter 1.1.1.1.2).

The immune synapse is a highly dynamic structure, and its distinct spatial organisation is critical for its function and the regulation of T cell activation. It is considered to have a ‘bull’s eye’ structure with radial symmetry and a distinct supramolecular activation cluster (SMAC) (Potter et al. 2001). The SMAC can be further categorized into a peripheral (pSMAC) and a central (cSMAC) component.

The pSMAC primarily consists of the cytoskeletal protein talin and adhesion molecules, such as the integrin lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) and its ligand intercellular adhesion molecule-1 (ICAM-1; CD54) (Monks et al. 1998) as well as CD2 and CD58 (Demetriou et al. 2020). Upon engagement, LFA-1 arrests T cell motility and provides structural stability for sensitive antigen recognition (Dustin et al. 1997).

Most signalling proteins are located in the cSMAC, including the TCR/CD3 complex, CD28, PKC θ , Lck and ZAP-70 (Monks et al. 1998; Zumerle et al. 2017).

1.1.2 CD8⁺ T cell effector function

Upon activation, CD8⁺ T cells mediate their effector function through two main mechanisms: direct cytotoxicity and the production of cytokines such as IFN γ , macrophage inflammatory protein (MIP) 1 β and tumour necrosis factor (TNF). In this thesis, the production of antiviral/antitumour cytokines was primarily exploited to assess T cell effector function. This section will therefore give a brief overview of direct cytotoxicity and will then highlight key antiviral/antitumour cytokines relevant for this thesis, primarily focusing on IFN γ .

1.1.2.1 Direct cytotoxicity

CD8⁺ T cells mediate cytotoxicity via two main mechanisms: the release of pore-forming and cytotoxic granules, and via the Fas (CD95)-Fas ligand (FasL, CD178) pathway (Barry and Bleackley 2002).

Interaction between FasL expressed on the CD8⁺ T cell and Fas expressed on the target cell induces the binding of Fas-associated death domain protein followed by recruitment and proteolytic activation of pro-caspase-8 (Krammer 2000). Caspase-8 either directly activates caspase-3 or activates the pro-apoptotic BH-3 interacting-domain death agonist (BID). BID and the B cell lymphoma-2 (Bcl-2)-associated X protein (BAX) translocate into the mitochondrial membrane and induce cytochrome c release which activates caspase-9 (Barry and Bleackley 2002). Caspase-9 in turn activates caspase-3 ultimately inducing target cell apoptosis.

Granule-mediated cytotoxicity comprises pore-forming granules containing perforin and cytotoxic granules containing pro-apoptotic serine proteases (granzymes). Upon TCR-pMHC engagement, preformed granules are rapidly mobilized to the immunological synapse, fuse with the membrane and release their content into the synapse (Russell and Ley 2002) (also see chapter 3.9.1). Perforin polymerizes in the presence of Ca²⁺ and forms pores in the target cell membrane, facilitating granzyme entry within seconds after TCR-pMHC binding (Lopez et al. 2013; Voskoboinik et al. 2015). Granzyme B is the predominant pro-apoptotic granzyme in human T cells and induces BID-mediated target cell apoptosis (Voskoboinik et

al. 2015). Interestingly, a recent study has shown that solid tumours require repetitive exposure to sublethal damage, resulting in additive cytotoxicity, to undergo apoptosis (Weigelin et al. 2021). Multiple mechanisms protect CD8⁺ T cells from perforin-mediated membrane disruption, including high lipid membrane order preventing perforin binding, and phosphatidylserine mediated inactivation of perforin (Rudd-Schmidt et al. 2019).

1.1.2.2 Interferon γ

IFN γ was first discovered in 1965 (Wheelock 1965) as a member of a group of molecules named after their ability to interfere with the viral lifecycle – interferons (Isaacs et al. 1957). Interferons were initially divided into two groups based on their sensitivity to pH (Stewart 1980): class I interferons (pH stable) now including IFN α , β , ϵ , κ , ω ; and class II interferons (pH sensitive) with its only member IFN γ (Walter 2020). Due to genomic analysis a third group, class III interferons (IFN λ s), was later identified (Kotenko et al. 2003).

IFN γ signals through an ubiquitously expressed receptor consisting of the high affinity IFN γ R1 chain and low affinity IFN γ R2 chain (Walter 2020). Receptor binding results in dimerization of the two subunits, activates Janus kinase (JAK) 1+2 by autophosphorylation and consecutive recruitment of signal transducer and activator of transcription (STAT)-1. STAT-1 phosphorylation leads to dimerization, translocation of the STAT-1/STAT-1 homodimers into the nucleus and activation of interferon-stimulated genes (ISG) (Platanias 2005).

IFN γ is a pleiotropic cytokine with three distinct properties –antiviral, antitumour and immunomodulatory.

1.1.2.2.1 Antiviral effects of IFN γ

For a long time, it was widely believed that the antiviral function of CD8⁺ T cells was due to cytotoxic elimination of the infected target cells (see section 1.1.2.1) until landmark studies in the 1990s described the direct non-cytolytic role of CD8⁺ T cells in hepatitis B virus (HBV)

infection via the production of antiviral cytokines such as IFN γ and TNF (Guidotti et al. 1996).

IFN γ elicits antiviral function by stimulating the production of type I interferons, the prototypical antiviral interferons (Kropp et al. 2011), but also has direct antiviral properties itself. The mechanisms by which IFN γ exerts a direct antiviral effect are only incompletely understood and differ between viruses. IFN γ can limit viral entry, replication, gene expression, release, transmission and reactivation (Kang et al. 2018), and this section will highlight two examples, HBV and hepatitis C virus (HCV) infection.

Multiple seminal studies by the groups of Guidotti and Chisari have highlighted the antiviral role of IFN γ in HBV via downregulation of viral gene expression, inhibition of viral replication and destabilization of viral RNA (Franco et al. 1997; Guidotti et al. 1996, 1999). While the underlying mechanisms are not fully understood, IFN γ mediates the induction of ISG DExD/H box helicase DEAD box polypeptide 60-like (DDX60L) in hepatocytes resulting in a degradation of cytoplasmic HBV RNA (Kouwaki et al. 2016). IFN γ further suppresses HBV genome replication and viral protein translation by induction of the enzyme indoleamine 2,3-dioxygenase (IDO) (Mao et al. 2011) and induces degradation of nucleocapsid particles via altered proteasome activity (Robek et al. 2002; Xu et al. 2010). Finally, IFN γ destabilizes covalently closed circular HBV DNA (cccDNA), highly relevant for HBV persistence (also see section 4.1.1.1), via activation of nuclear APOBEC3 deaminases (Xia et al. 2016).

In HCV infection, IFN γ inhibits viral entry by downregulation and redistribution of the entry receptors claudin-1, CD81 and scavenger receptor class B type I (SR-B1) (Wei et al. 2009) and limits viral replication via induction of DDX60L as described for HBV (Grünvogel et al. 2015).

1.1.2.2.2 IFN γ and tumours

Interferons elicit a direct antitumour effect by limiting cell proliferation, promoting differentiation and inducing apoptosis (Parker et al. 2016). IFN γ inhibits tumour cell proliferation and induces caspase-mediated tumour cell apoptosis via STAT-1 signalling

pathways in a variety of tumour types (Bromberg et al. 1996; Brown et al. 1987; Guinn et al. 2017; Hao and Tang 2018; Kundu et al. 2017; Song et al. 2019). In combination with TNF, IFN γ promotes tumour senescence and dormancy, limiting angiogenesis, carcinogenesis and proliferation, e.g. mediated via IDO (Braumüller et al. 2013; Liu et al. 2017; Müller-Hermelink et al. 2008). IFN γ also alters tumour cell metabolism, e.g. by inducing ferroptosis, a cell death caused by iron-dependent accumulation of lipid peroxide (Wang et al. 2019b). Ferroptosis is mediated by IFN γ -induced downregulation of the cysteine-glutamate antiporters solute carrier family 3 member 2 (SLC3A2) and SLC7A11, resulting in an impaired uptake of cysteine and reduced efflux of glutamate.

IFN γ not only affects tumour cells directly, but also alters the function of other cells in the tumour microenvironment (TME). IFN γ induces tumour ischaemia due to regression of vascularization, reduced neovascularization, alterations of endothelial cell morphology and reduced expression of vascular endothelial growth factor-A (VEGF-A) by stromal fibroblasts (Kammertoens et al. 2017; Lu et al. 2009). By increasing the expression of the extracellular matrix protein fibronectin-1, IFN γ alters tumour architecture and limits metastatic potential (Glasner et al. 2018).

The important role of IFN γ secretion in the antitumour immune response is further highlighted by the finding that the expression of IFN γ -related genes is associated with response to immunotherapy in a variety of tumours (Ayers et al. 2017; Gao et al. 2016; Higgs et al. 2018).

Besides its antitumourigenic potential, it is important to note that IFN γ can also mediate protumourigenic effects. For example, in non-small cell lung cancer (NSCLC), low-dose IFN γ signals via the alternate ICAM1–Phosphoinositide 3-kinases (PI3K)–Akt–Notch1 pathway inducing stem-like properties and tumour progression (Song et al. 2019). In melanoma, UV-induced IFN γ can promote melanocytic cell survival and immune evasion (Zaidi et al. 2011). Additionally, IFN γ can mediate the upregulation of checkpoint molecules such as programmed death ligand 1 (PD-L1) on cancer cells (Benci et al. 2016), e.g. via the acetylation and consecutive activation of myocyte enhancer factor 2D (MEF2D) in liver cancer (hepatocellular carcinoma, HCC) (Xiang et al. 2020). As mentioned above, IFN γ

induces IDO production; while IDO has an antitumorigenic effect, it can also limit local antitumour immune responses (Puccetti and Grohmann 2007) (also see chapter 1.2.4.3.2).

1.1.2.2.3 Immunomodulatory effects of IFN γ

In addition to its direct antiviral and antitumour effects, IFN γ plays a major role in modulating immune responses.

In CD4⁺ T cells, IFN γ facilitates T helper (Th) 1 differentiation by co-localization of the IFN γ receptor with the TCR, inducing the transcription factor T-box expressed in T cells (T-bet) via STAT-1 signalling (Afkarian et al. 2002; Maldonado et al. 2004; Zhang et al. 2001). In contrast, IFN γ drives fragility of immunosuppressive, regulatory CD4⁺ T cells (T_{reg}) (Overacre-Delgoffe et al. 2017).

In CD8⁺ T cells, IFN γ directly induces proliferation in viral infection (Whitmire et al. 2005), enhances cytotoxicity and mobility (Bhat et al. 2017; Ravichandran et al. 2019) and promotes memory formation and survival (Stoycheva et al. 2015). Production of IFN γ by CD8⁺ tissue-resident memory T cells (T_{RM}) upregulates vascular cell adhesion molecule-1 (VCAM-1), facilitating recruitment of circulating memory CD8⁺ T cells and B cells (Schenkel et al. 2014). However, IFN γ plays a dual role in modulating immunity and can also limit T cell responses (Berner et al. 2007; Refaeli et al. 2002).

Besides its effects on the adaptive immune system, IFN γ also mediates immune function of innate immune cells. Synergizing with toll-like receptor (TLR) ligands, IFN γ induces a proinflammatory phenotype with enhanced proinflammatory cytokine and nitric oxygen (NO) production in macrophages (Müller et al. 2017). IFN γ also increases macrophage phagocytosis and killing of infected or transformed target cells (Jorgovanovic et al. 2020), and in the TME, IFN γ induces the development of iNOS⁺CD206⁻ macrophages that are associated with improved prognosis and promote VCAM-1 expression, T cell recruitment and vascular remodelling (Jorgovanovic et al. 2020).

By upregulation of MHC molecules, IFN γ promotes APC/T cell interactions (Jorgovanovic et al. 2020). IFN γ further induces DC maturation and modulates multiple DC functions including proinflammatory cytokine production, expression of costimulatory molecules, e.g. CD80 and CD86, and migration, while limiting anti-inflammatory features such as IL-10 production (Frasca et al. 2008; Jorgovanovic et al. 2020). However, IFN γ also limits DC survival in order to regulate persistent antigen presentation (Russell et al. 2009).

Taken together, IFN γ plays a key role in the antiviral and antitumour immune response by modulating immune function as well as eliciting direct antiviral and antitumour effects. In this thesis, IFN γ was therefore used as the main readout of antiviral and antitumour T cell function.

1.1.2.3 MIP1 β

MIP1 β , also known as chemokine (C-C motif) ligand 4 (CCL4), is another cytokine that can mediate non-cytolytic CD8⁺ T cell effector function. This has especially been highlighted in human immunodeficiency virus (HIV) infection where MIP1 β plays a major role in the antiviral effector function of CD8⁺ T cells and correlates with suppression of viral replication (Cocchi et al. 1995; Saunders et al. 2011). In HBV, high serum MIP1 β levels are associated with response to antiviral therapy and contribute to a loss of HBV surface antigen (HBsAg) (Narayanan et al. 2021).

The role of MIP1 β in cancer development is not completely understood. In some cancer types, e.g. nasopharyngeal carcinoma, MIP1 β is associated with a reduced risk (Yang et al. 2018), whereas in others, high serum MIP1 β is associated with an increased risk (Li et al. 2020a), e.g. in renal cell carcinoma where MIP1 β promotes tumour proliferation and expression of inhibitory checkpoints (Zhang et al. 2021).

In contrast, MIP1 β is also a potent chemokine and intratumoural expression of MIP1 β can contribute to T cell and natural killer (NK) cell infiltration, and induce an antitumour response (Galeano Niño et al. 2020; Luo et al. 2004). Further, MIP1 β enhances immunity by recruiting naïve CD8⁺ T cells expressing the chemokine (C-C motif) receptor 5 (CCR5) to

the site of CD4⁺/DC interaction (Castellino et al. 2006). The importance of this co-localisation is further highlighted by the finding that response to immunotherapy is associated with MIP1 β production by CD8⁺ T cells, mediating spatial tumour organization and intratumoural microclusters of CD8⁺ T cells and CD4⁺ T cells with DC (Abdulrahman et al. 2022).

1.1.2.4 TNF

TNF was first discovered in 1975 due to its tumour cell necrosis-inducing capacity (Carswell et al. 1975); however, as described for IFN γ and MIP1 β , TNF plays a dual role in carcinogenesis.

On the one hand, TNF has an anticarcinogenic effect, e.g. by promoting cancer cell apoptosis (Dace et al. 2007; Nakagawa et al. 2007) and the destruction of tumour stroma (Zhang et al. 2008) and vasculature (Hoving et al. 2006). Intratumoural injection of adenovirus expressing TNF can sensitize tumours to programmed death-1 (PD-1) blockade (Cervera-Carrascon et al. 2018) and loss of TNF sensitivity is one mechanism of tumour immune evasion (Kearney et al. 2018). On the other hand, TNF can promote tumour growth, angiogenesis and metastasis (Wang and Lin 2008).

TNF also has antiviral effects that were first described in 1986 for vesicular stomatitis virus (Mestan et al. 1986). TNF inhibits viral replication in influenza (Seo and Webster 2002), while its effect in HIV is less clear with some studies showing an inhibition of viral entry and replication (Herbein et al. 1996; Lane et al. 1999) whereas others claim a promotion of viral replication (Duh et al. 1989).

Multiple studies have highlighted the antiviral role of TNF in HBV infection. As described for IFN γ in chapter 1.1.2.1, TNF inhibits viral replication and destabilizes HBV cccDNA via activation of APOBEC3 deaminases, an effect that is additive with IFN γ (Guidotti et al. 1996; Xia et al. 2016). TNF further destabilizes viral capsids via the nuclear factor- κ B (NF- κ B) pathway (Biermer et al. 2003; Guidotti et al. 1996; Puro and Schneider 2007). Interestingly, the HBV core antigen (HBcAg) itself renders infected hepatocytes susceptible to TNF-mediated apoptosis (Jia et al. 2015a).

TNF also exerts an immunomodulatory effect, e.g. by impairing (Nie et al. 2013; Valencia et al. 2006) or enhancing (Ye et al. 2018) T_{reg} function, inducing priming, activation, proliferation and recruitment of naïve, effector and memory T cells (Calzascia et al. 2007; Mehta et al. 2018), attracting neutrophils and macrophages (Josephs et al. 2018), and promoting a proinflammatory macrophage phenotype (Kratochvill et al. 2015).

1.1.3 CD8⁺ T cell exhaustion

In acute infection, naïve CD8⁺ T cells typically differentiate into effector T cells, clonally expanded numbers of which retract after antigen clearance and resolution of infection, giving rise to long-lived memory T cells that can be activated rapidly upon reinfection. In contrast, during chronic antigen exposure, e.g. in the setting of chronic viral infections and cancer, memory induction is impaired and instead, CD8⁺ T cells progressively differentiate into an exhausted phenotype that is associated with a loss of effector function, expression of inhibitory checkpoints, metabolic alterations and a distinct transcriptional profile (Fig. 1.2).

Immune exhaustion was first described in the 1960s in studies showing that exposure to high levels of antigen can induce B cell dysfunction (Byers and Sercarz 1968; McLane et al. 2019). Later, in the 1990s, a murine model of chronic infection was established utilizing clone 13 lymphocytic choriomeningitis virus (LCMV) that is still frequently used to study T cell exhaustion. Initially, Moskophidis et al. described a loss of antigen-specific CD8⁺ T cells due to deletion (Moskophidis et al. 1993), but later studies revealed that antigen-specific CD8⁺ T cells can persist in chronic LCMV infection but are dysfunctional (Gallimore et al. 1998; Zajac et al. 1998).

While CD8⁺ T cell exhaustion was first discovered in a murine infection model, it has now been described for a variety of chronic infections, including HIV (Goepfert et al. 2000), HCV (Gruener et al. 2001) and HBV (Fiscaro et al. 2020a), as well as in multiple tumour types (Schietinger et al. 2016). Besides chronic antigen exposure, the strength of the TCR signal is a key determinant of T cell exhaustion in cancer (Shakiba et al. 2021). A variety of other factors further contribute to T cell exhaustion including tolerogenic signals, insufficient CD4⁺ T cell help and a lack of costimulatory signals. Of note, T cell exhaustion typically occurs due to chronic antigen exposure but new data are emerging describing features of T

cell exhaustion in acute infections such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Chen and Wherry 2020) and acute HCV infection (Urbani et al. 2006).

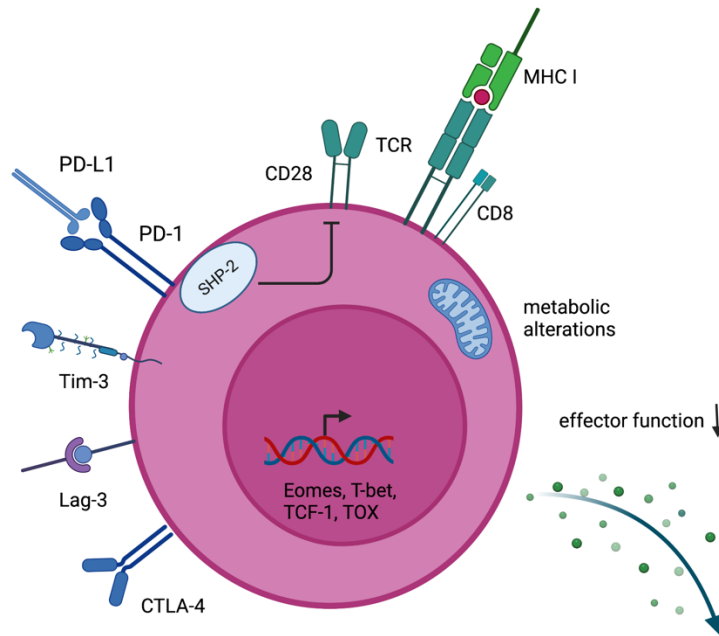


Figure 1.2 Mechanisms of CD8⁺ T cell exhaustion.

A variety of mechanisms contribute to CD8⁺ T cell exhaustion and a loss of T cell effector function: Chronic antigen exposure and strength of TCR signal; distinct profiles of the key transcription factors Eomes, T-bet, TCF-1 and TOX; metabolic alterations; expression of inhibitory checkpoint molecules including PD-1, Tim-3, Lag-3, CTLA-4. One of the mechanism by which PD-1 inhibits CD8⁺ T cell effector function is the SHP-2-mediated dephosphorylation of the costimulator molecule CD28. TCR: T cell receptor; MHC: major histocompatibility complex; PD-(L)1: programmed death (ligand) 1; Tim-3: T-cell immunoglobulin and mucin-domain containing-3; Lag-3: lymphocyte-activation gene-3; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; SHP-2: Src homology 2 domain-containing tyrosine phosphatase 2; Eomes: Eomesodermin; TOX: thymocyte selection-associated high mobility group box; TCF-1: T cell factor 1; T-bet: T-box expressed in T cells.

Although exhaustion has been extensively studied in CD8⁺ T cells, it also occurs in other immune cell subsets including CD4⁺ T cells (Crawford et al. 2014), B cells (Moir et al. 2008) and NK cells (Bi and Tian 2017).

The finding that T cell exhaustion can be reversible (Iwai et al. 2002; Leach et al. 1996) has revolutionised modern immunology and medicine, leading to the development of a new therapeutic approach with the aim of boosting exhausted T cell responses – immunotherapy (see section 6.1.2).

In this chapter, I will discuss typical features of exhausted CD8⁺ T cells including functional alterations, the expression of inhibitory checkpoint molecules and the transcriptional profile. Metabolic changes associated with T cell exhaustion will be explored in chapter 1.2.2 and disease-specific features of T cell exhaustion will be addressed in the relevant introduction chapters (HBV: chapter 4.1.1.4, SARS-CoV-2: chapter 4.1.2.1; HCC: chapter 6.1.1.3).

1.1.3.1 Functional alterations

Impaired effector function is a hierarchical, consecutive process during the development of T cell exhaustion. Loss of IL-2 is a feature of early exhaustion, followed by loss of TNF (Wherry et al. 2003). Despite its critical role in the discovery of T cell exhaustion, the inability to produce IFN γ only occurs during late stages and is associated with a terminally exhausted phenotype (Mackerness et al. 2010; Wherry et al. 2003). In contrast, the production of chemokines such as MIP1 α and MIP1 β is typically not impaired (Wherry et al. 2007).

The effect of T cell exhaustion on CD8⁺ T cell cytotoxicity is less clear with some studies showing a loss of cytotoxic capacity as early as a loss of IL-2 (Blackburn et al. 2010; Wherry et al. 2003) and others showing a preserved or even enhanced cytotoxic potential in later exhaustion stages despite lower degranulation (Paley et al. 2012).

Additional to the loss of effector function, exhausted CD8⁺ T cells are less responsive to homeostatic cytokines such as IL-7 and IL-15, that are essential for memory T cells, due to downregulation of the receptors IL-7R α and IL-2/IL15R β (Shin et al. 2007; Wherry et al. 2004).

1.1.3.2 Inhibitory checkpoints

Inhibitory checkpoints are critical for fine-tuning immune responses and preventing immunopathology. They are transiently expressed during acute infection and downregulated upon resolution of infection (McLane et al. 2019). A hallmark of T cell exhaustion is the sustained expression of inhibitory receptors on the cell surface (Fig. 1.2).

Exhausted CD8⁺ T cells express a variety of inhibitory checkpoints, including, but not limited to, PD-1, lymphocyte-activation gene-3 (Lag-3), T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). The co-expression of these molecules is associated with more profound T cell exhaustion and more severe infection in the LCMV exhaustion model (Blackburn et al. 2009).

1.1.3.2.1 PD-1

PD-1 (CD279) was first described in 1996 (Agata et al. 1996), a discovery that was awarded with the Nobel Prize in Physiology or Medicine in 2018 (The Nobel Prize 2018). PD-1 is highly expressed on exhausted CD8⁺ T cells in a variety of settings and is therefore often exploited as a phenotypic marker of T cell exhaustion (Barber et al. 2006; Boni et al. 2007; Sharpe and Pauken 2018; Urbani et al. 2006; Wherry et al. 2007). Despite its key role in T cell exhaustion, PD-1 expression is not required for the induction of exhaustion and in the absence of PD-1, terminally exhausted, cytotoxic CD8⁺ T cells accumulate in LCMV (Odorizzi et al. 2015). Instead, PD-1 is thought to maintain an exhaustion phenotype once it is established (Sharpe and Pauken 2018).

PD-1 upregulation is typically induced by TCR engagement and regulated by a variety of transcription factors including forkhead box protein O1 (FOXO1) and nuclear factor of activated T cells, cytoplasmic 1 (NFATC1) (Oestreich et al. 2008; Staron et al. 2014). PD-1 interacts with two ligands, PD-L1 (B7.H1) and PD-L2 (B7.DC), that are upregulated during inflammation (Sharpe and Pauken 2018). PD-L1 is ubiquitously expressed including expression by immune cells, endothelial cells, and tumour cells. In contrast, PD-L2 is predominantly expressed by DC, macrophages and B cells. One of the main drivers of PD-L1/2 expression are type I and II interferons, as discussed for IFN γ in chapter 1.1.2.2.2 (Benci et al. 2016).

Upon ligand engagement, PD-1 is phosphorylated and forms a microcluster with the TCR and CD28 at the cSMAC, destabilizing the immune synapse (Hui et al. 2017; Yokosuka et al. 2012). PD-1 phosphorylation recruits protein tyrosine phosphatases such as Src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2), a key mediator of the immunosuppressive function of PD-1 (Fig.1.2). It was initially thought that SHP-2 primarily inhibits TCR signalling by dephosphorylation of TCR signalling molecules (Yokosuka et al.

2012), but it has recently been demonstrated that the costimulatory molecule CD28 is the main target of SHP-2 dephosphorylation (Hui et al. 2017); however, some studies suggest that SHP-2 is dispensable for PD-1 signalling (Rota et al. 2018).

PD-1 further disrupts the interaction of CD8 with the TCR, shortens the duration of pMHC-TCR complex formation (Li et al. 2021b), increases the threshold of the number of pMHC/TCR complexes required to induce calcium flux and TCR signalling (Wei et al. 2013), and limits T cell motility (Zinselmeyer et al. 2013).

PD-1 also inhibits activation of PI3K preventing the phosphorylation and activation of Akt and limits the Ras–mitogen-activated protein kinase (MAPK) and extracellular signal–regulated kinase (ERK) kinase (MEK)– ERK signalling pathway upon TCR engagement, ultimately resulting in limited T cell proliferation due to an inhibition of cyclin-dependent phosphatases and cell cycle arrest (Parry et al. 2005; Patsoukis et al. 2012).

Additional to the mechanisms highlighted in this section, PD-1 signalling induces extensive metabolic reprogramming in CD8⁺ T cells that will be discussed in chapter 1.2.2. Therapeutic targeting of the PD-1/PD-L1 axis will be discussed in chapter 6.1.2.1.

1.1.3.2.2 Other immune checkpoints

A variety of other inhibitory immune checkpoints besides PD-1 contribute to T cell exhaustion and this section will explore the role of Lag-3 and Tim-3. CTLA-4 will be briefly discussed in chapter 4.1.1.4.4.

Lag-3 is a coinhibitory receptor with structural similarities to CD4 and inhibits CD4⁺ T cell function by binding to stable pMHC class II complexes and consecutive inhibition of intracellular signalling (Maruhashi et al. 2018). Other Lag-3 binding partners include lectins expressed on tumour cells, e.g. LSECtin (Xu et al. 2014) and galactin-3 (Kouo et al. 2015), and the liver-secreted protein fibrinogen-like protein 1 (FGL1) (Wang et al. 2019a). Interaction of Lag-3 with its ligands inhibits T cell effector function and proliferation, e.g. via the downregulation of cell cycle kinases.

Tim-3 is highly expressed on T cells in tumours and chronic viral infections, including HIV, HCV and HBV, and is considered a marker of highly dysfunctional subset of exhausted T cells (Jones et al. 2008; Ju et al. 2009; McMahan et al. 2010). Tim-3 is localized in lipid rafts and is recruited to the immune synapse upon T cell activation. In its resting form, Tim-3 is bound by human leukocyte antigen- (HLA-) B-associated transcript-3 (BAT3) (Clayton et al. 2014; Rangachari et al. 2012). Upon interaction with its ligands, e.g. galactin-9 and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), Tim-3 is phosphorylated and BAT3 dissociates, allowing recruitment of the tyrosine kinase Fyn and interaction with the phosphatases CD45 and CD148 (Clayton et al. 2014; Huang et al. 2015; van de Weyer et al. 2006). Ultimately, Tim-3 ligation induces T cell anergy and inhibition of Lck which disrupts TCR signalling and destabilizes the immune synapse (Clayton et al. 2014; Davidson et al. 2007).

1.1.3.3 Transcriptional control

CD8⁺ T cell exhaustion is regulated by a variety of different transcription factors including T-bet, Eomesodermin (Eomes), thymocyte selection-associated high mobility group box (TOX), T cell factor 1 (TCF-1), B lymphocyte-induced maturation protein-1 (BLIMP1), FOXO1 and NFAT.

This section will highlight the role of T-bet, Eomes, TOX and TCF-1 in CD8⁺ T cell exhaustion.

1.1.3.3.1 T-bet and Eomes

T-bet and Eomes are critical transcription factors for the development of effector and memory T cells (Intlekofer et al. 2005). In acute infection, T-bet and Eomes are upregulated in CD8⁺ T cells promoting effector function, including cytokine production and cytotoxicity (Pearce et al. 2003; Sullivan et al. 2003). Eomes is particularly important for memory T cell development by regulating the expression of IL15R β (Banerjee et al. 2010; Intlekofer et al. 2005; McLane et al. 2019).

While T-bet and Eomes have overlapping roles during acute infection and an effector response can be elicited in the absence of either transcription factor, both are indispensable during chronic infection, and elimination of either results in a failure to control infection (McLane et al. 2019; Paley et al. 2012). T-bet is typically expressed during early stages of T cell exhaustion, limiting PD-1 expression and sustaining virus-specific CD8⁺ T cells with proliferative capacity, whereas Eomes expression is typically associated with advanced exhaustion stages (Kao et al. 2011; Paley et al. 2012) (also see chapter 1.1.3.3.4).

1.1.3.3.2 TCF-1

TCF-1 is the key transcription factor of the Wnt signalling pathway regulating thymic T cell development, T cell fate and homeostasis (Escobar et al. 2020; Verbeek et al. 1995). TCF-1 can further modulate T cell fate via chromatin remodelling and deacetylation (Xing et al. 2016).

In chronic infections and tumours, TCF-1 expression in CD8⁺ T cells is a feature of early T cell exhaustion stages and is associated with a stem-like phenotype with self-renewal capacity, viral/tumour control and the potential to give rise to terminally differentiated T cells (also see chapter 1.1.3.3.4) (Eberhardt et al. 2021; Hudson et al. 2019; Im et al. 2016; Miller et al. 2019; Wu et al. 2016). After antigen elimination, the PD-1⁺ TCF-1⁺ CD127⁺ subset persists long-term and expands after re-infection (Wieland et al. 2017). Importantly, proliferation of TCF-1⁺ PD-1⁺ CD8⁺ T cells is induced by PD-1 blockade and is associated with treatment response (Im et al. 2016; Jadhav et al. 2019; Sade-Feldman et al. 2018; Utzschneider et al. 2016).

1.1.3.3.3 TOX

The role of TOX as a transcriptional regulator of T cell exhaustion has been described by five key studies in 2019 (Alfei et al. 2019; Khan et al. 2019; Scott et al. 2019; Seo et al. 2019; Yao et al. 2019). Expression of TOX results in epigenetic reprogramming and the expression of genes encoding other transcription factors, e.g. TCF-1, and coinhibitory receptors, e.g. PD-1, Lag-3 and Tim-3. While TOX is dispensable during acute infection and memory development, its expression is critical for the development of the TCF-1⁺ PD-1^{int} progenitor state and subsequent terminal differentiation (Mann and Kaech 2019).

TOX expression is induced by high antigen and/or chronic TCR stimulation, calcineurin, and NFAT activation (Alfei et al. 2019; Khan et al. 2019; Scott et al. 2019). In LCMV and HCV, TOX is associated with an exhausted phenotype and deletion of TOX in these settings results in a downregulation of PD-1 and induces T cell effector function (Alfei et al. 2019). In contrast, in tumours, deletion of TOX prevents an upregulation of inhibitory receptors and contributes to continuous TCF-1 expression but does not restore T cell effector function and limits T cell persistence in the TME (Scott et al. 2019).

1.1.3.3.4 Four stages of T cell exhaustion

Exhausted CD8⁺ T cells are highly heterogenous and over the years a variety of different classifications utilizing different molecules have been suggested. In this section, I will highlight a recent classification defining four stages of T cell exhaustion identified in LCMV infection and confirmed in murine and human tumours (Beltra et al. 2020) (Fig.1.3).

Beltra et al. defined two progenitor subsets (T_{ex}^{prog1} and T_{ex}^{prog2}), an intermediate subset (T_{ex}^{int}) and a fourth, terminally exhausted subset (T_{ex}^{term}).

The two progenitor subsets express TCF-1 and Ly108, a glycoprotein of the signalling lymphocytic activation molecule (SLAM) family, and can be differentiated by their expression of the transmembrane C-Type lectin protein CD69. T_{ex}^{prog1} expressing CD69 and Eomes are quiescent and tissue-restricted, whereas CD69^{neg} T_{ex}^{prog2} proliferate and have the capacity to recirculate. A recent study has now identified a CD62L⁺ subset within the T_{ex}^{prog} subsets that has stem-like potential and is regulated by the transcription factor MYB (Tsui et al. 2022). CD62L⁺ T_{ex}^{prog} retain proliferative potential and multipotency, and can maintain antiviral immunity in chronic infection.

The two progenitor subsets are interconvertible and give rise to T_{ex}^{int} , a Ly108^{neg} CD69^{neg} subset where TCF-1 expression is lost, T-bet is highly expressed, and T cells have the highest proliferative capacity. Finally, T_{ex}^{int} convert into the terminally exhausted T_{ex}^{term} subset where CD69 expression and EOMES are re-established, T-bet is antagonised by TOX, and PD-1 expression is high. T_{ex}^{term} have lost the potential to proliferate and circulate.

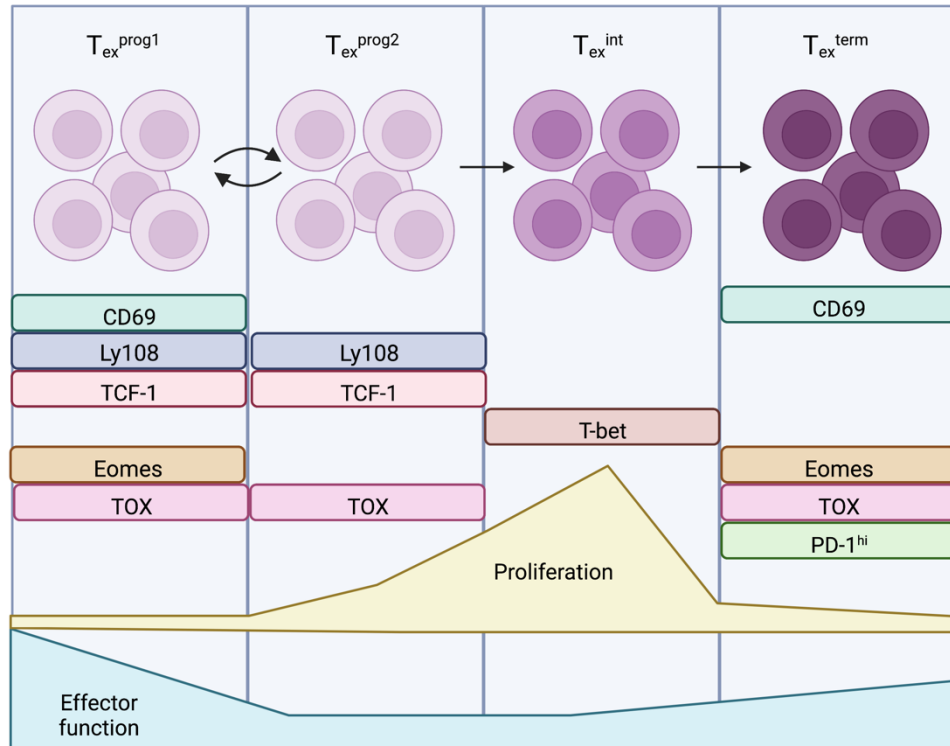


Figure 1.3 Four stages of T cell exhaustion.

Simplified schematic to highlight the four stages of CD8⁺ T cell exhaustion adapted from (Beltra et al. 2020). Eomes: Eomesodermin; TOX: thymocyte selection-associated high mobility group box; TCF-1: T cell factor 1; T-bet: T-box expressed in T cells; PD-1: programmed death 1; T_{ex} : exhausted T cells; $T_{ex}^{prog1+2}$: progenitor subsets; T_{ex}^{int} : intermediate subset; T_{ex}^{term} : terminally exhausted subset.

Transforming growth factor- β (TGF- β) has recently been defined as a regulator of T cell differentiation with a dual role depending on the differentiation state. TGF- β maintains the stem-like properties of TCF-1⁺ PD-1⁺ T cells and promotes their retention in lymphoid tissue by regulating the expression of $\alpha 4$ integrins (Hu et al. 2022; Ma et al. 2022). On the other hand, TGF- β promotes the terminal differentiation of T_{ex}^{term} (Hu et al. 2022).

Exhausted T cells can retain some capacity to produce IFN γ and degranulate, with the highest production/degranulation detected in T_{ex}^{prog1} ; however, T cell effector function is reduced compared to memory T cells (Beltra et al. 2020). Interestingly, PD-1 blockade preferentially enhances the function of CD8⁺ T cells in the T_{ex}^{prog2} and T_{ex}^{int} stages.

Taken together, CD8⁺ T cell exhaustion is a highly complex process of T cell dysfunction during chronic antigen stimulation that is orchestrated by the expression of transcription factors and inhibitory receptors.

1.2 T cell metabolism in health and disease

T cell metabolism and function are inextricably linked in health and disease. The availability and utilization of nutrients shape T cell phenotype and function, and a skewing towards different metabolic pathways depending on T cell differentiation reflects differing energy demands.

In this section, I will first discuss the major metabolic pathways in T cells, followed by alterations of T cell metabolism by PD-1, in the liver and in cancer as three examples where T cell phenotype and the local environment shape T cell metabolism. I will then highlight T cell cholesterol metabolism and the potential of targeting immunometabolism as a therapeutic approach.

1.2.1 Overview of key metabolic pathways in T cells

Six key metabolic pathways have been described in T cells: glycolysis, the tricarboxylic acid (TCA) cycle, fatty acid oxidation (FAO), fatty acid synthesis (FAS), the pentose phosphate pathway (PPP) and amino acid metabolism (O'Neill et al. 2016). These pathways are tightly regulated and highly interconnected, sharing input fuels and often requiring products from one pathway to fuel another.

Two main regulators of immunometabolism are the mechanistic/mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK). mTOR is the catalytic subunit of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) that sense amino acid availability and promote anabolic events to support cell growth, e.g. synthesis of lipids, nucleic acids and proteins, but also modulate glycolysis and oxidative phosphorylation (OXPHOS) (Bantug et al. 2018; Hukelmann et al. 2016; Weichhart et al. 2015).

AMPK is activated during nutrient starvation, promoting catabolic pathways such as FAO and OXPHOS. AMPK further inhibits mTOR via phosphorylation and therefore restricts anabolic pathways, glycolysis and cell activation (González et al. 2020).

1.2.1.1 Glycolysis

Glycolysis is the process of converting glucose into pyruvate via eight intermediate enzymatic steps (Fig.1.4). T cells import glucose from the environment via specialised membrane transporters, GLUT-1 (SLC2A1) and GLUT-3 (SLC2A3) (Hukelmann et al. 2016).

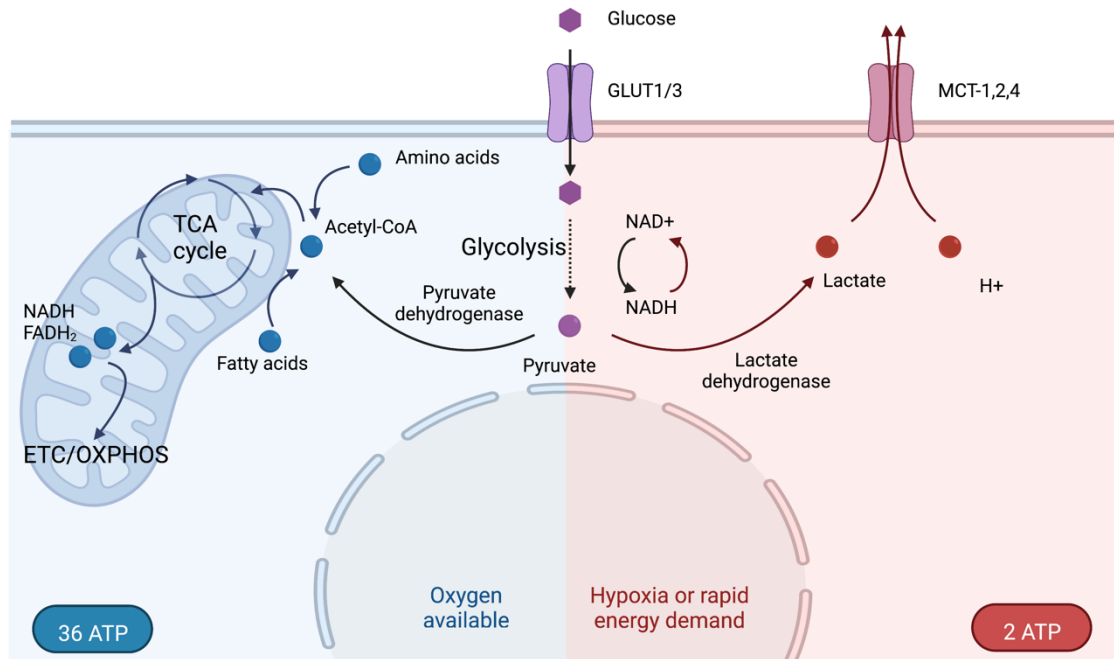


Figure 1.4 Glycolysis and TCA cycle.

Schematic of glycolysis (anaerobic and aerobic) and TCA cycle including the ATP generated from one glucose molecule. MCT: monocarboxylate transporter; ETC: electron transport chain; NAD⁺: nicotinamide adenine dinucleotide; FAD⁺: flavin-adenine dinucleotide; acetyl-CoA: acetyl-Coenzyme A; TCA: tricarboxylic acid; OXPHOS: oxidative phosphorylation; ATP: adenosine triphosphate.

Glycolysis generates 2 adenosine triphosphate (ATP) per glucose molecule and reduces nicotinamide adenine dinucleotide (NAD⁺) to NADH which acts as an enzymatic cofactor and can support anabolic pathways (O'Neill et al. 2016). Intermediate products of glycolysis can fuel other metabolic pathways, for example glucose-6-phosphate can be shunted into the PPP. The fate of pyruvate is highly dependent on oxygen availability – when oxygen is available, pyruvate is typically converted into acetyl-Coenzyme A (acetyl-CoA) and fuels the TCA whereas in hypoxic conditions, lactate dehydrogenase converts pyruvate into lactate and NADH into NAD⁺ (anaerobic glycolysis).

However, in 1924 Otto Warburg described the phenomenon that cancer cells favour glycolysis even in the presence of oxygen (aerobic glycolysis, Warburg effect) (Warburg et al. 1924) and he initially hypothesised that this was due to ‘damaged respiration’ in cancer cells (Warburg 1956).

Today, it has become clear that aerobic glycolysis is not only a feature of cancer cells, but is employed by a variety of highly proliferative cells with rapid energy demand despite functioning mitochondria (Vander Heiden et al. 2009). Glycolysis is less energy-efficient than OXPHOS (2 ATP vs 36 ATP per glucose molecule) but has two key advantages: first, ATP synthesis via glycolysis is fast whereas OXPHOS requires mitochondrial biogenesis; second, glycolysis rapidly provides not only ATP but also building blocks required for cell growth and proliferation (O’Neill et al. 2016). Interestingly a recent study has shown that aerobic glycolysis can also be exploited by tumour cells with low proliferative activity, suppressing the accumulation of reactive oxygen species (ROS) and enhancing stem cell-like properties, highlighting that the benefits of aerobic glycolysis go beyond rapid ATP production and provision of biomass (Sebastian et al. 2022).

Upon activation, T effector cells rapidly switch to glycolysis in an mTORC2-dependent manner (Gubser et al. 2013). CD28 signalling is required for this process via the activation of PI3K and Akt (Frauwirth et al. 2002). This energetic switch supports virus-specific CD8⁺ T cell effector function including the production of IFN γ (Gubser et al. 2013). An interesting mechanism by which glycolysis modulates T cell cytokine production is via glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chang et al. 2013; Gubser et al. 2013). GAPDH binds to IFN γ mRNA and prevents its translation. When glycolysis is initiated, GAPDH engages in glycolysis and dissociates from the mRNA, allowing its translation.

Glycolysis is also exploited by other activated immune subsets including macrophages (Rodríguez-Prados et al. 2010), DC (Krawczyk et al. 2010), NK cells (Donnelly et al. 2014), Th1 and Th2 CD4⁺ T cells (Michalek et al. 2011) and B cells (Doughty et al. 2006).

1.2.1.2 TCA cycle, fatty acid oxidation and OXPHOS

Pyruvate produced by glycolysis can be converted into acetyl-CoA and fuel the TCA cycle, also known as citric acid cycle or Krebs cycle (Fig.1.4) (Martínez-Reyes and Chandel 2020).

The TCA cycle is a series of nine enzymatic reactions ultimately converting acetyl-CoA to oxaloacetate. During this process, NAD^+ and flavin-adenine dinucleotide (FAD^+) are converted into NADH and FADH_2 , respectively, that are transferred to the electron transport chain (ETC) to support oxygen-dependent ATP generation via OXPHOS (see chapter 5.1.3). TCA cycle intermediates are further required as substrates for other pathways, e.g. citrate is exported to the cytosol and utilized for nucleotide and lipid synthesis (Martínez-Reyes and Chandel 2020).

Besides pyruvate, fatty acids and amino acids also contribute to the TCA cycle. Mitochondrial FAO is a catabolic process that converts long chain fatty acids into acetyl-CoA that can enter the TCA, and also creates NADH and FADH_2 that can be directly transferred to the ETC (Ma et al. 2018b).

In contrast to effector T cells that primarily rely on glycolysis, memory CD8^+ T cells preferentially use the TCA and OXPHOS to fulfil their energy demands. This is reflected by a higher spare respiratory capacity (SRC) indicating a higher energetic reserve that can be exploited upon stress (Bantug et al. 2018; van der Windt et al. 2012) (also see chapter 5.1.3). IL-15, a cytokine involved in T cell memory formation, promotes the higher SRC by inducing mitochondrial biogenesis and FAO. The enhanced utilization of OXPHOS in memory T cells is further facilitated by mitochondrial remodelling with fused mitochondria, tight cristae and closely associated ETC complexes mediated by membrane fusion protein Opa1 (Buck et al. 2016). FAO in memory T cells is fuelled by glucose and lysosomal acid lipase mobilizing fatty acids (O'Sullivan et al. 2014). The finding that an induction of mitochondrial FAO or mitochondrial membrane fusion promotes CD8^+ T cell memory development highlights that different metabolic programs directly shape T cell phenotype and function (Buck et al. 2016; van der Windt et al. 2012).

Besides memory CD8^+ T cells, T_{reg} are highly dependent on FAO and modulation of CD4^+ T cell bioenergetics can skew differentiation towards an effector or regulatory phenotype (Michalek et al. 2011).

Activated macrophages are commonly divided into two states: $\text{IFN}\gamma$ and TLR engagement induce the development of M1-like, proinflammatory macrophages, whereas IL-4 and IL-13 promote anti-inflammatory M2 differentiation (Wynn et al. 2013); however, it is important

to note that this differentiation is often considered to be simplistic and does not fully reflect the variety of macrophage subsets *in vivo*.

FAO promotes polarization towards an anti-inflammatory M2-like phenotype. CD36-mediated uptake of triacylglycerols and consecutive lysosomal lipolysis are essential for M2 activation and for the protumorigenic effect of tumour-associated macrophages (TAM) (Huang et al. 2014; Russell et al. 2019; Su et al. 2020a). Alterations of metabolic pathways, e.g. inhibiting glycolysis by knockdown of pyruvate dehydrogenase kinase 1, inhibits the activation of M1-like but enhances the activation of M2-like macrophages (Tan et al. 2015); likewise, inhibition of FAO prevents M2 activation (Vats et al. 2006). High lipid and fatty acid uptake supporting FAO also enhances the immunosuppressive function of myeloid-derived suppressor cells (MDSC) (Al-Khami et al. 2017; Hossain et al. 2015).

Importantly, many studies evaluating the role of FAO have relied on etomoxir as an inhibitor of carnitine palmitoyl transferase 1a (CPT1a), the rate limiting enzyme of FAO; however, a recent study shows that CPT1a is dispensable for memory and effector T cell as well as T_{reg} formation and that etomoxir may have off-target effects (Raud et al. 2018).

1.2.1.3 Fatty acid synthesis

Fatty acids are essential components of the cell membrane, act as signalling molecules and bioenergetic substrates, and contribute to lipids synthesis (Lee et al. 2014). Mitochondrial citrate, an intermediate product of the TCA, is exported into the cytosol and converted into acetyl-CoA that is fed into the FAS pathway. Saturated long chain fatty acids can then be further converted into more complex lipids such as cholesterol, triglycerides and phospholipids (Lochner et al. 2015). FAS is primarily regulated by the transcription factors sterol regulatory element binding protein (SREBP) 1a+c and is tightly linked with cholesterol metabolism which is discussed in chapter 1.2.5 (Shimano and Sato 2017).

De novo FAS is required for homeostatic proliferation of naïve CD8⁺ T cells and antigen-specific proliferation of effector CD8⁺ T cells without affecting differentiation (Lee et al. 2014). IL-17 producing Th17 cells rely on FAS and inhibition of acetyl-CoA carboxylase 1, a key enzyme in the FAS pathway, skews CD4⁺ differentiation towards T_{reg} in naïve cells (Berod et al. 2014) but towards a memory phenotype in parasite infection (Endo et al. 2019)

Additionally, SREBP1c-mediated induction of FAS is a key requirement for the differentiation from monocytes into macrophages and for their phagocytotic ability and inflammatory function (Ecker et al. 2010). In DC, FAS is upregulated during activation and is critical for CD8⁺ T cell priming (Everts et al. 2014).

1.2.1.4 Pentose phosphate pathway

Glucose 6-phosphate, fructose 6-phosphate and glyceraldehyde 3-phosphate, intermediate products of glycolysis, are converted into ribulose 5-phosphate and ribose 5-phosphate in the PPP. The PPP also creates NAD phosphate, provides building blocks for nucleotide and amino acid synthesis and regulates carbon homeostasis required in proliferating cells (Stincone et al. 2015). PPP is upregulated in pro-inflammatory, M1-like macrophages (Tannahill et al. 2013), fuels superoxide production by activated neutrophils (Britt et al. 2022) and regulates T_{reg} function (Liu et al. 2022).

1.2.1.5 Amino acid pathway

Besides the PPP, amino acids provide precursors for protein and nucleotide biosynthesis. Four examples of amino acid metabolism in T cells will be briefly discussed in this section: leucine, asparagine, glutamine and arginine.

Leucine, imported via the long chain amino acid transporter SLC7A5, is required for mTORC1 activation and T cell proliferation and effector function upon antigen encounter (Hayashi et al. 2013; Sinclair et al. 2013). Asparagine directly modulates T cell function by altering Lck phosphorylation and TCR signalling (Wu et al. 2021).

Glutamine, e.g. imported via the transporter SLC1A5 upon TCR and CD28 engagement, also regulates mTORC1 activation, is required for T cell effector function and proliferation, and can contribute to OXPHOS (Carr et al. 2010; Nakaya et al. 2014); however, in a recent study glutamine antagonism reduces tumour hypoxia, suppresses tumour glycolysis and enhances antitumour T cells by increasing OXPHOS and activation (Leone et al. 2019).

SLC38A9 acts as an arginine sensor for the mTORC1 pathway, activating mTORC1 in the presence of arginine (Shen and Sabatini 2018; Wang et al. 2015; Wyant et al. 2017). Arginine regulates SLC38A9-mediated transport of essential amino acids, e.g. leucine, and is further required for cell proliferation and activation of cyclin D3 and cyclin-dependent kinase 4 (Rodriguez et al. 2006; Shen and Sabatini 2018; Wang et al. 2015; Wyant et al. 2017). Upon T cell activation, intracellular arginine is rapidly converted into metabolites by mitochondrial arginase-2 (Geiger et al. 2016). High arginine availability promotes OXPHOS and gluconeogenesis while inhibiting glycolysis and induces a shift to a central memory phenotype with high survival capacity and antitumour function.

1.2.2 PD-1 and T cell metabolism

The role of PD-1 as a coinhibitory receptor in T cell exhaustion was discussed in chapter 1.1.3.2.1. One of the mechanisms by which PD-1 signalling limits T cell effector function is altering T cell metabolism.

PD-1 activation limits T cell glucose uptake, glycolysis and amino acid metabolism, and instead enhances lipolysis and the expression of CPT1 α , the rate limiting enzyme of FAO (Patsoukis et al. 2015). As discussed in chapter 1.2.1.1, glycolysis is essential during T cell activation and by inhibiting glycolysis, PD-1 limits T cell effector differentiation.

Besides glycolysis, PD-1 also represses the metabolic regulator peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1 α), inhibiting mitochondrial respiration as early as in the first week after LCMV infection (Bensch et al. 2016). The impairment of mitochondrial function is reflected in reduced mitochondrial polarization, morphological alterations such as reduction in number and length of cristae (Ogando et al. 2019) and in reduced SRC in human PD-1^{high} CD8⁺ T cells in chronic HBV infection (CHB) (Schurich et al. 2016). Interestingly, in LCMV, the metabolic alterations induced by PD-1 signalling were critical for CD8⁺ T cell memory development (Kalia et al. 2021).

In tumours, a combination of TCR signalling, PD-1 signalling and decreased mitophagy reduces mitochondrial fitness in tumour-infiltrating T cells due to accumulation of

depolarised mitochondria (Yu et al. 2020). Vice versa, induction of mitochondrial depolarisation induces epigenetic reprogramming towards an exhausted phenotype.

1.2.3 T cell metabolism in the liver

The liver constitutes a unique immunotolerant microenvironment with low oxygen levels and limited nutrient availability. T_{RM} $CD8^+$ T cells (also see chapter 4.1.1.4.1) reside in the liver long-term and are exposed to this hostile environment over a prolonged period of time, resulting in metabolic adaptations (Pallett et al. 2017, 2020; Pallett and Maini 2022).

The liver receives a high fraction of its blood supply through the portal vein carrying deoxygenised blood (Wilson et al. 2014), leading to oxygen levels as low as 30mmHg in some hepatic zones compared to 75-100mmHg in arterial blood (Ortiz-Prado et al. 2019). In low oxygen conditions, the transcription factor hypoxia inducible factor 1 α (HIF1 α) is induced and can regulate the expression of genes involved in glucose metabolism, resulting in increased GLUT-1 expression, glucose uptake and glycolysis (Finlay et al. 2012; Schurich et al. 2016) (also see chapter 1.2.4.1).

Fatty acid binding protein 1 (FABP1) is required for T_{RM} establishment in the liver (Frizzell et al. 2020) suggesting a role for exogenous lipid uptake and FAO as seen for T_{RM} in the skin (Pallett and Maini 2022; Pan et al. 2017).

$CD8^+$ T cells in the liver have depolarized mitochondria (Swadling et al. 2020). T_{RM} increase autophagy to recycle defective mitochondria in order to maintain their mitochondrial fitness and effector function in contrast to their non-resident, liver infiltrating counterparts (Swadling et al. 2020). Autophagy in T_{RM} is induced by hepatic stellate cells and the cytokine IL-15, a prototypical liver cytokine that promotes tissue residency.

Mitochondrial dysfunction is also a feature of exhausted, virus-specific circulating $CD8^+$ T cells in CHB that have likely been primed and chronically exposed to their cognate antigen in the liver. HBV-specific $CD8^+$ T cells downregulate genes encoding ETC components, mitochondrial genes and Opa1 (Fiscaro et al. 2017). These alterations are reflected in reduced mitochondrial mass, mitochondrial depolarization, increased mitochondrial ROS

levels in resting T cells and in an inability to utilize OXPHOS (Fisicaro et al. 2017; Schurich et al. 2016).

During HBV infection, MDSC accumulate in the liver, limiting local antiviral T cell responses (Pallett et al. 2015). One of the mechanisms by which MDSC regulate T cell function is the production of arginase-1, resulting in arginine deprivation. Another source of arginase in the liver are damaged hepatocytes (Ikemoto et al. 2001), e.g. in acute HBV (Sandalova et al. 2012). Arginine-deprived T cells upregulate system L amino acid transporters to take up essential nutrients (Pallett et al. 2015), a metabolic feature also found in T_{RM} $CD8^+$ T cells (Pallett et al. 2017).

1.2.4 T cell metabolism in cancer

Reprogramming of cellular metabolism is considered one of the hallmarks of cancer (Hanahan 2022). T cell metabolism in cancer is highly complex and driven by T cell exhaustion as well as interaction and competition with other components of the TME, including other immune cells, cancer cells, fibroblasts and endothelial cells. This section will focus on the metabolism of $CD8^+$ T cells in tumour-infiltrating leukocytes (TIL) and will highlight some of their metabolic constraints resulting in suppression of endogenous antitumour immune responses and metabolic barriers to immunotherapy.

Metabolic alterations in the TME can be divided into four main groups: hypoxia, acidosis, toxic and/or suppressive metabolites, and competition for nutrients (Figure 1.5); however, these alterations are not isolated but instead are highly interconnected and influence each other.

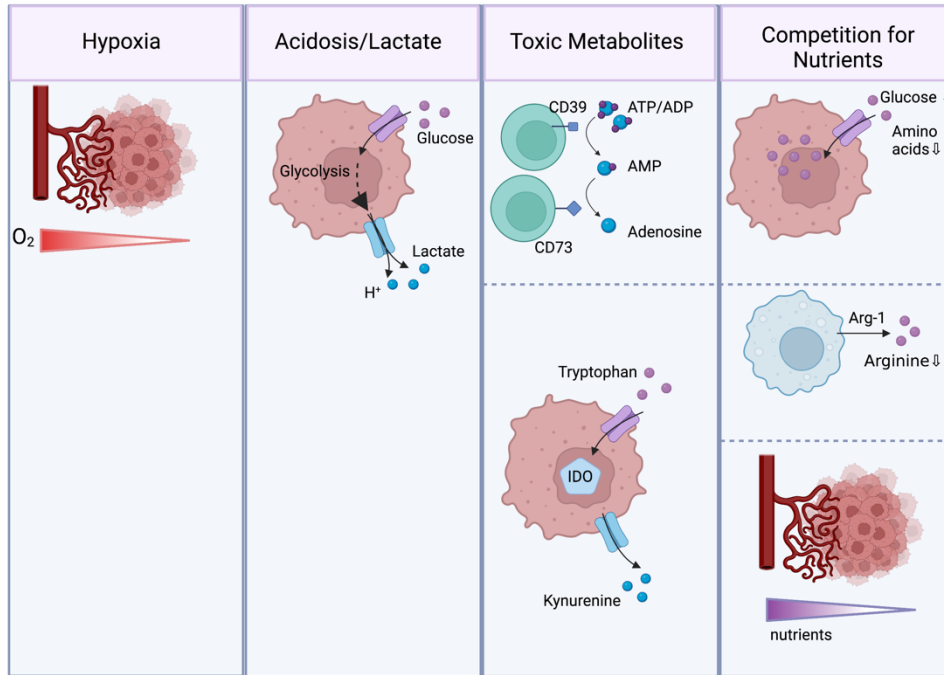


Figure 1.5 Metabolic alterations in the tumour microenvironment.

Metabolic alterations in the TME can be divided into four main groups: hypoxia, acidosis/lactate, toxic and/or suppressive metabolites (e.g. adenosine and kynurenine) and competition for nutrients (e.g. glucose and amino acids). Arg-1: arginase-1. ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; IDO: indoleamine 2,3-dioxygenase; TME: tumour microenvironment.

1.2.4.1 Hypoxia

A typical feature of cancer cells is uncontrolled proliferation due to sustained proliferative signals and evasion of growth suppressors (Hanahan 2022). This results into a rapid increase of tumour volume and progressive distancing from vasculature, limiting oxygen and nutrient supply (Pouyssegur et al. 2006). To overcome this limitation, tumours induce angiogenesis, e.g. by production of VEGF-A; however, tumour neovascularization is often exacerbated, leading to malformed vessels, chaotic blood flow and heterogenous blood supply with local regions of hypoxia. For example, oxygen levels in HCC are 0.8% compared to 4-7% in the healthy liver (Muz et al. 2015).

As described in chapter 1.2.3, hypoxia induces the transcription factor HIF1 α that modulates T cell phenotype, function and metabolism, e.g. by inducing glycolysis (Finlay et al. 2012). The role of HIF1 α and hypoxia in T cells is complex and still not fully understood.

Hypoxic conditions *in vitro* limit CD8⁺ T cell effector cytokine production and survival but can enhance cytotoxicity and activation (Caldwell et al. 2001) whereas *in vivo* activation of CD4⁺ and CD8⁺ T cells is inhibited (Ohta et al. 2011). Equally, HIF1 α induces 2-hydroxyglutarate production by CD8⁺ T cells that in turn stabilizes HIF1 α expression and limits CD8⁺ T cell effector function, proliferation and survival *in vitro* but enhances *in vivo* proliferation and effector function after adoptive transfer (Tyrakis et al. 2016).

Continuous stimulation of CD8⁺ T cells in hypoxic conditions results in T cell exhaustion and mitochondrial stress with increased ROS levels due to BLIMP-1-mediated inhibition of PGC1 α (Scharping et al. 2021). It is important to note that besides hypoxia, PGC1 α is further reduced due to chronic Akt signalling in the TME, resulting in reduced mitochondrial biogenesis (Scharping et al. 2016).

Additionally, hypoxia is associated with increased chromatin bivalency (simultaneous positive and negative histone modifications) in T_{ex}^{term} due to a loss of demethylase. Increasing oxygen levels alters the expression of bivalent genes by reducing negative histone modifications and enhances antitumour T cell activity (Ford et al. 2022).

The role of HIF1 α expression in CD4⁺ T cell differentiation is equally not fully understood. Some studies demonstrate that HIF1 α drives expression of the transcription factor forkhead box P3 (FOXP3) in CD4⁺ T cells promoting T_{reg} development and function (Ben-Shoshan et al. 2008; Clambey et al. 2012), whereas others claim that HIF1 α induces proteasomal degradation of FOXP3 and enhances Th17 development via activation of ROR γ t and mTOR and consecutive induction of glycolysis (Dang et al. 2011; Shi et al. 2011). In contrast, a recent study suggests that enhanced glycolysis and reduced OXPHOS due to HIF1 α signalling induce T_{reg} proliferation but limit T_{reg} suppressive function (Miska et al. 2019).

Hypoxia not only affects T cells but also cancer cells themselves, e.g. by inducing their production of CCL28 that promotes T_{reg} recruitment (Facciabene et al. 2011). As described for T cells, HIF1 α induces cancer cell glycolysis (Al Tameemi et al. 2019) and therefore contributes to the competition for available nutrients discussed in chapter 1.2.4.4. Additionally, hypoxia induces the expression of CD39 and CD73 in various cell types

(Eltzschig et al. 2003, 2009; Synnestvedt et al. 2002) and their role in suppressing tumour immunity will be discussed in chapter 1.2.4.3.1.

The role of oxygen availability in altering antitumour immune responses has further been highlighted by the finding that hyperoxia enhances CD8⁺ T cell infiltration, increases proinflammatory cytokines, reduces T_{reg} suppressive capacity and induces tumour regression in a murine model (Hatfield et al. 2015).

1.2.4.2 Acidosis and lactate

Increased glycolysis in the tumour tissue (Warburg effect, chapter 1.2.1.1) leads to an increase of intracellular lactate and H⁺ (Fig.1.4). Lactate is exported via monocarboxylate transporters (MCT) 1-4, that are H⁺/lactate symporters, resulting in extracellular acidosis (Payen et al. 2020). CO₂ generated by mitochondrial respiration is converted into HCO₃⁻ and H⁺ and can further contribute to the extracellular low pH (Leone and Powell 2020). Lactate can be considered a toxic/immunosuppressive metabolite but due to the tight link between lactate accumulation and acidosis, this chapter will discuss the effect of both on the antitumour immune response.

Lactate export from T cells via MCT 1, 2, 4 is gradient dependent and high extracellular lactate levels result in an intracellular accumulation (Fischer et al. 2007; Merezhinskaya et al. 2004). Lactate inhibits the proliferation, motility, cytokine production and cytotoxicity of CD8⁺ T cells, e.g. via repression of NFAT signalling (Brand et al. 2016; Fischer et al. 2007; Haas et al. 2015). CD8⁺ T cell effector function is further inhibited by lactate dehydrogenase-mediated depletion of NAD⁺ and by acidosis due to impaired phosphorylation of JNK/c-Jun and p38, downstream TCR signalling molecules involved in IFN γ production (Angelin et al. 2017; Mendler et al. 2012).

Lactate also limits CD4⁺ T cell motility via interference with glycolysis and induces skewing towards a Th17 phenotype (Haas et al. 2015). In naïve T cells, lactate inhibits autophagy via inhibition of FAK family-interacting protein of 200 kDa (FIP200), leading to mitochondrial overactivation, accumulation of ROS and induction of apoptosis (Xia et al. 2017).

In contrast, a recent study has shown that lactate can also be utilized to fuel the TCA cycle in effector CD8⁺ T cells (Kaymak et al. 2022) and further research will be necessary to fully delineate the effect of lactate on T cell effector function.

Irrespective of lactate, low extracellular pH induces T cell anergy with downregulation of the TCR, limited TCR signalling, impaired glycolysis and reduced effector function, while raising the tumour pH increases tumour immune infiltration (Calcinotto et al. 2012; Pilon-Thomas et al. 2016).

In contrast to effector T cells, T_{reg} resist lactate-induced suppression by FOXP3-mediated reduced glycolysis, enhanced OXPHOS and increased NAD⁺:NADH ratio as well as via upregulation of CD36 and consecutive peroxisome proliferator-activated receptor β (PPAR β) signalling and metabolic modulation (Angelin et al. 2017; Wang et al. 2020a; Watson et al. 2021). Instead, T_{reg} can utilize lactate as fuel to support proliferation and suppressor function (Watson et al. 2021). Another suppressive immune cell subset, MDSC, accumulate in lactate-rich environments and lactate induces arginase-1 expression in macrophages, further limiting antitumour immunity (Husain et al. 2013; Ohashi et al. 2013) (also see chapter 1.2.3).

Lactate not only inhibits antitumour T cells, but also affects other immune cell subsets. It limits the function and survival of antitumour NK cells by repressing NFAT signalling and inducing apoptosis and mitochondrial dysfunction (Brand et al. 2016; Harmon et al. 2019; Husain et al. 2013). It has further been suggested that DC activation and maturation is inhibited by lactate (Puig-Kröger et al. 2003). In contrast, acidosis improves antigen uptake and presentation by DC and enhances their capacity of priming CD8⁺ T cells (Vermeulen et al. 2004).

Lactate also has direct effects on cancer cells – it can be used as metabolic fuel, contributes to angiogenesis, e.g. by enhancing VEGF-A production, stabilizes HIF1 α , activates mTOR and consecutive protein synthesis, and facilitates DNA repair via inhibition of histone deacetylases (Payen et al. 2020).

1.2.4.3 Toxic and immunosuppressive metabolites

Besides lactate discussed in the previous section, a variety of other immunosuppressive metabolites limit antitumour immunity. This chapter will explore adenosine and kynurenine as two further examples (Fig. 1.4).

1.2.4.3.1 Adenosine

As mentioned in chapter 1.2.4.1, hypoxia induces upregulation of the ectonucleotidases CD39 and CD73. CD39 converts ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP) which is then further degraded into adenosine by CD73 (de Andrade Mello et al. 2017). A variety of cells in the TME express CD39 and/or CD73 and contribute to an accumulation of extracellular adenosine, including tumour cells (Häusler et al. 2011; Jin et al. 2010), T_{reg} (Mandapathil et al. 2009; Wang et al. 2011), Th17 (Chalmin et al. 2012; Thibaudin et al. 2016), MDSC (Ryzhov et al. 2011), endothelial cells (Wang et al. 2011), cancer-associated fibroblasts (Mediavilla-Varela et al. 2013) and mesenchymal stromal cells (Kerkelä et al. 2016). Additionally, CD39 and CD73 can be released into exosomes, further contributing to adenosine generation (Clayton et al. 2011; Smyth et al. 2013).

Extracellular adenosine is a strong immunosuppressive and protumourigenic mediator. Adenosine signals through purinergic receptors, on immune cells primarily A_{2A} and A_{2B} receptors (Leone and Powell 2020), and limits CD8⁺ and CD4⁺ T cell effector function and proliferation (Wang et al. 2011; Zarek et al. 2008), e.g. by preventing phosphorylation of TCR signalling molecules such as ZAP-70, Akt and ERK (Linnemann et al. 2009). Endothelial CD73 further inhibits CD8⁺ T cell adhesion, migration and tumour infiltration (Wang et al. 2011). Besides the effect on T cells, adenosine also limits NK cell maturation (Young et al. 2018) and DC tumour infiltration and priming capacity (Chen et al. 2020).

In contrast, adenosine enhances the function of suppressive immune cells. It induces T_{reg} differentiation and TGF- β production (Zarek et al. 2008) and promotes MDSC expansion and suppressive capacity (Ryzhov et al. 2011). Adenosine also has direct procarcinogenic effects, e.g. by enhancing tumour cell growth, inducing antiapoptotic members of the Bcl-2 family and promoting neovascularization (Ryzhov et al. 2014; Turcotte et al. 2015).

Considering its protumourigenic and immunosuppressive capacity, the adenosine signalling pathway is a promising target for immunotherapy. Antibodies blocking CD39 and CD73 stimulate DC and macrophages and enhance antitumour T cell immunity and survival in murine tumour models (Perrot et al. 2019). In line with this, A_{2A} and A_{2B} receptor blockade impairs the growth of tumour cells and cancer associated fibroblasts, suppresses metastasis, limits T_{reg} differentiation and boosts antitumour T cells and NK cells *in vitro* and in murine models (Beavis et al. 2013; Jin et al. 2010; Mandapathil et al. 2009; Mediavilla-Varela et al. 2013; Nakatsukasa et al. 2011).

1.2.4.3.2 Kynurenine

IDO is an immunoregulatory enzyme that converts tryptophane into kynurenine. This process alters immune responses by depletion of the essential amino acid tryptophane from the environment and by kynurenine binding to the aryl hydrocarbon receptor (Munn and Mellor 2013). IDO is expressed by a variety of cancer types and is associated with poor prognosis. While tumour cells themselves can produce IDO, it is also expressed by DC in the tumour-draining lymph node, for example due to interaction with T_{reg} or arginase-1 expressing MDSC, which enables DC to suppress anti-tumour T cells and induce T_{reg} differentiation (Chen et al. 2008; Fallarino et al. 2003; Mondanelli et al. 2017; Munn et al. 2004).

Depletion of tryptophan and increase of kynurenine inhibits CD8⁺ T cell function and proliferation by downregulation of the TCR ζ chain (Fallarino et al. 2006) and upregulation of PD-1 (Liu et al. 2018). In contrast, tryptophan depletion and kynurenine promote T_{reg} differentiation and suppressive function (Fallarino et al. 2006; Mezrich et al. 2010). Pharmacological reduction of IDO expression activates CD8⁺ T cell function and drives T_{reg} apoptosis (Balachandran et al. 2011).

1.2.4.4 Competition for glucose

Nutrient availability in the TME is scarce due to limited blood supply, resulting in a ‘tug-of-war’ between highly proliferative cancer cells and antitumour immune cells to fulfil their increased demand for energy supply and building blocks (Fig.1.4). This chapter will highlight

glucose as an example for how nutrient competition shapes the antitumour immune response.

The high rate of glycolysis in tumour cells, e.g. induced by PD-L1, HIF1 α or expression of the extracellular metalloproteinase inducer CD147, which modulate the Akt-mTOR axis, leads to a depletion of glucose in the TME (Al Tameemi et al. 2019; Chang et al. 2015; Li et al. 2020b). The lack of glucose results in reduced T cell mTOR activity, reduced glycolytic capacity, increased expression of PD-1 and ultimately in a dampening of antitumour effector function (Chang et al. 2015).

In CD4⁺ T cells, glucose deprivation restrains TCR-mediated Ca²⁺-NFAT signalling via sarco/ER Ca²⁺-ATPase (SERCA) which is inhibited by the glycolysis intermediate phosphoenolpyruvate when glycolysis can be utilized (Ho et al. 2015). Additionally, X-box binding protein 1 (XBP-1) upregulation in tumour-infiltrating T cells can restrict expression of glutamine transporters and therefore prevent the switch to alternative fuels for the TCA and mitochondrial respiration in low glucose environments (Song et al. 2018). Checkpoint blockade can restore glucose levels in the TME, resulting in enhanced antitumour T cell function (Chang et al. 2015).

In contrast, low glucose in the TME favours T_{reg} development as T_{reg} are less dependent on glycolysis and primarily rely on FAO as an energy source (Chang et al. 2015; Michalek et al. 2011).

However, these findings will need to be reconsidered in light of a recent study that suggests that glucose is not limited in the TME and that immune cells are the primary glucose-utilizing cell types with myeloid cells (TAM and MDSC) having the highest glucose uptake on a per cell basis followed by T cells and then cancer cells (Reinfeld et al. 2021). Instead, tumour cells favour lipid and glutamine uptake. Considering the high number of tumour cells within the tumour tissue, it was estimated that in the TME overall, tumour cells contribute to 2/3 of the glucose uptake and myeloid cells to another 1/3 whereas T cells only play a minor role in glucose consumption. Taken together, Reinfeld et al. suggest that glycolytic tumours may not inhibit antitumour T cells directly by glucose deprivation but by large-scale changes of the metabolic landscape.

1.2.5 T cell cholesterol metabolism in health and disease

Lipids, such as cholesterol, constitute an essential component of cellular plasma membranes, impacting membrane lipid order, membrane fluidity, receptor signalling and cell function. This section will discuss T cell cholesterol metabolism and its contribution to immune function in viral infections and cancer. The role of lipid rafts, cholesterol-rich microdomains within the cell membrane, for immune synapse formation and T cell activation has been discussed in chapter 1.1.

1.2.5.1 Intracellular cholesterol metabolism

There are two mechanisms for cells to acquire cholesterol: uptake from the environment or biosynthesis (Fig. 1.6).

Cholesterol in the blood is bound to clusters of lipids and proteins forming high and low density lipoprotein particles (HDL and LDL, respectively) (Bietz et al. 2017). Cholesterol uptake into the cell is mainly regulated by the LDL receptor (Cardoso and Perucha 2021). Additionally, the scavenger receptor CD36 (also known as fatty acid translocase) imports free fatty acids and oxidized lipids including oxidized LDL (oxLDL) (Xu et al. 2021) and SR-B1 facilitates the uptake of HDL (Acton et al. 1996). Besides cholesterol uptake, cholesterol can also be synthesized from acetyl-CoA via mevalonate, farnesyl pyrophosphate and squalene in a series of enzymatic steps (Cardoso and Perucha 2021).

Nieman-Pick C (NPC) proteins 1 and 2 facilitate intracellular cholesterol distribution and cholesterol is either further metabolised, e.g. into oxysterols including 25-hydroxycholesterol (25-HC) via cholesterol 25-hydroxylase, esterified, integrated into lipid bilayers, e.g. the cell membrane, or exported (Cardoso and Perucha 2021).

Due to its hydrophobic properties, free cholesterol cannot be stored intracellularly but is instead esterified to cholesteryl ester catalysed by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT; also known as sterol O-acyltransferase, SOAT) in the ER and stored in neutral lipid droplets (Leon et al. 2005). There are two isoforms of ACAT: the ubiquitously

expressed ACAT1, and ACAT2 which is primarily expressed by hepatocytes and enterocytes. The names SOAT and ACAT are used interchangeably but in most cases ACAT is used when referring to the enzyme whereas SOAT is often used when referring to the gene.

Cholesterol efflux is mediated via different receptors and transporters, primarily via ATP-binding cassette (ABC) transporters, e.g. ABCA1 and ABCG1, but it has also been suggested that SR-B1 can facilitate cholesterol efflux in some cells types and CD36 has been shown to mediate cholesterol efflux in macrophages (Bujold et al. 2009; Phillips 2014).

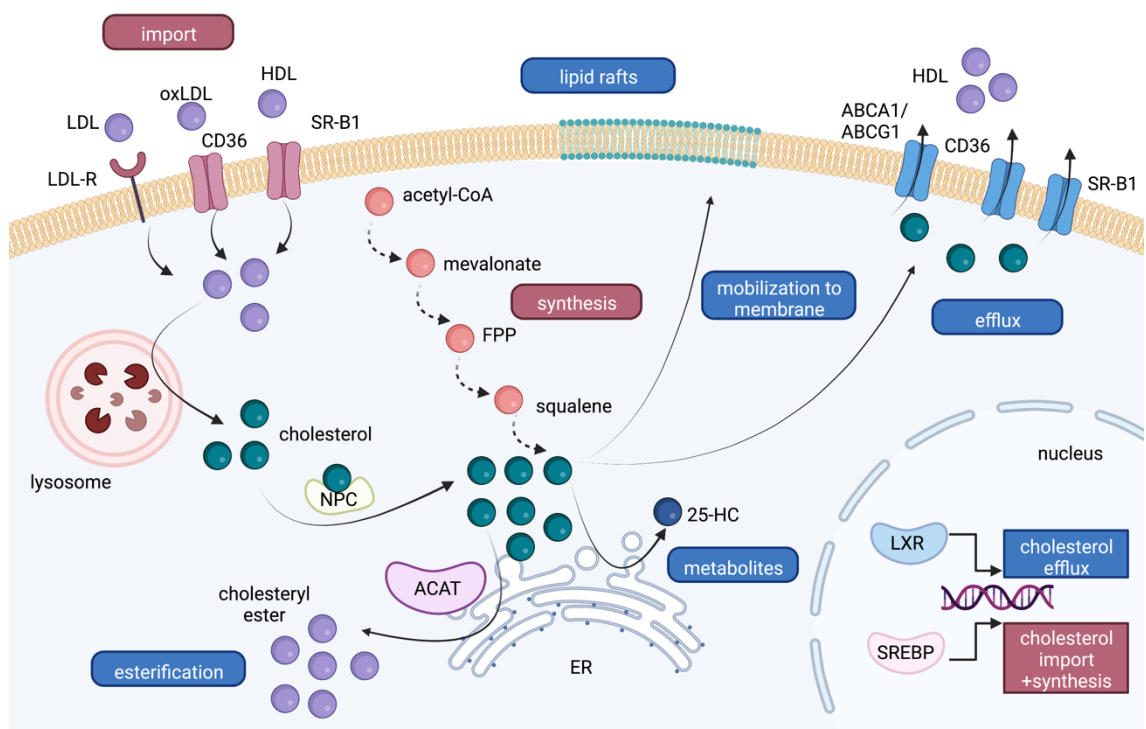


Figure 1.6 Intracellular cholesterol metabolism.

Cholesterol can be acquired by uptake from the environment or by biosynthesis from acetyl-CoA. Intracellular cholesterol is either converted into metabolites, e.g. 25-HC, integrated into cell membranes, converted into cholesteryl ester catalysed by ACAT or effluxed. Intracellular cholesterol is tightly regulated by the transcription factors SREBP and LXR. LDL(-R): low density lipoprotein (receptor); oxLDL: oxidised LDL; HDL: high density lipoprotein; SR-B1: scavenger receptor class B type I; ABC: ATP-binding cassette; NPC: Nieman-Pick C; FPP: farnesyl pyrophosphate; acetyl-CoA: acetyl-Coenzyme A; 25-HC: 25-hydroxycholesterol; ACAT: acetyl-CoA: cholesterol acyltransferase; SREBP: sterol regulatory element binding protein; LXR: liver x receptor.

1.2.5.2 Regulation of cholesterol metabolism

Cholesterol import, synthesis and efflux are tightly regulated by multiple transcription factors, mainly liver x receptor (LXR) and SREBP, to ensure intracellular homeostasis (Fig.

1.6). Out of the three SREBP isoforms, SREBP-2 controls cholesterol metabolism, SREBP-1c regulates FAS (see chapter 1.2.1.3), and SREBP-1a is involved in both pathways (Eberlé et al. 2004).

SREBP, the positive regulator of intracellular cholesterol, forms a complex with the SREBP cleavage-activating protein (SCAP) which facilitates transport from the ER to the Golgi complex where SREBP is cleaved into its activated transcription factor form that migrates to the nucleus and induces cholesterol synthesis and uptake (Radhakrishnan et al. 2008). When cholesterol levels in the ER exceed a threshold, cholesterol binds to SCAP, allowing SCAP in turn to bind to the insulin-induced gene (INSIG) which prevents mobilisation to the Golgi complex and SREBP activation (Radhakrishnan et al. 2008). Instead, high cholesterol induces LXR activation by forming a heterodimer with retinoid X receptor and binding cholesterol and 9-cis retinoic acid, promoting cholesterol efflux and limiting cholesterol uptake (Bietz et al. 2017).

1.2.5.3 Cholesterol and T cell function

Cholesterol availability and utilization are critical for T cell function. Upon T cell activation, the increased demand for cholesterol is reflected in a suppression of LXR and a rapid activation of SREBP, promoting cholesterol and fatty acid synthesis in a PI3K-mTOR pathway dependent manner (Bensinger et al. 2008; Kidani et al. 2013). Additionally, postprandial activation of mTOR stabilizes 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme for cholesterol synthesis in the mevalonate pathway (Lu et al. 2020).

LXR signalling or limited cholesterol availability reduce T cell proliferation, antigen-specific function and TCR signalling (Bensinger et al. 2008; Chyu et al. 2014). Hypercholesterolaemia induces expansion of T cells with an effector memory phenotype and reduces T_{reg} differentiation in a humanized mouse model (Proto et al. 2018). The presence of cholesterol, SREBP signalling or abrogation of the LXR pathway also induce T cell proliferation and effector function (Bensinger et al. 2008) and SREBP signalling is required for the induction of glycolysis and OXPHOS upon T cell activation (Kidani et al. 2013). Cholesterol and its metabolites are thought to be required for Th17 differentiation via ROR γ induction (Hu et

al. 2015) but it has also been shown that increased membrane cholesterol favours a Th1 phenotype (Surls et al. 2012).

Membrane cholesterol is required for the formation of lipid rafts, cholesterol-rich microdomains that are crucial for the formation of the immunological synapse and TCR signalling (for details see chapter 1.1), and a reduction in cholesterol availability reduces lipid raft formation (Chyu et al. 2014). Cholesterol can shape T cell function by altering membrane fluidity and order. CD4⁺ T cells with low membrane order and low membrane cholesterol are unresponsiveness to TCR stimulation whereas CD4⁺ T cells with high membrane order form stable synapses and have high proliferative capacity (Miguel et al. 2011). Membrane cholesterol is further required for TCR nanoclustering, enhancing the avidity of TCR-antigen interactions, and contributes to the transmembrane assembly of the TCR complex (Molnár et al. 2012; Sušac et al. 2022). In the absence of TCR-pMHC interaction, cholesterol binds to the TCR β chain and thus stabilizes the TCR in a resting conformation and prevents spontaneous switching to the active conformation (Swamy et al. 2016).

The important role of cholesterol in shaping antiviral immune responses has been further highlighted in murine models where the loss of SREBP activity due to SCAP deletion is associated with reduced proliferation and effector function of antiviral T cells during acute LCMV infection (Kidani et al. 2013) and loss of LXR signalling enhances the effector function of adenovirus-specific T cells accordingly (Bensinger et al. 2008).

Besides its role in supporting immune responses, cholesterol also has immunosuppressive potential by enhancing the function of immunosuppressive cells and by direct inhibitory effects on T cells.

The role of cholesterol in T_{reg} development is not fully understood. On the one hand, liver kinase B1 and mTOR-mediated cholesterol/lipid metabolism and activation of the mevalonate pathway promote T_{reg} proliferation and suppressive function (Timilshina et al. 2019; Zeng et al. 2013), and inhibition of cholesterol synthesis limits production of the inhibitory cytokine IL-10 in Th1 CD4⁺ T cells (Perucha et al. 2019); but on the other hand, LXR activation has also been shown to induce T_{reg} differentiation and T_{reg} were reduced in the humanized mouse model with high cholesterol diet mentioned above (Herold et al. 2017; Proto et al. 2018). Mice fed with a high cholesterol diet further upregulate the expression of

PD-L1 on B cells, regulating the motility and differentiation of T follicular helper (T_{fh}) cells (Nus et al. 2017).

Tumours are typically enriched in cholesterol and lipids that accumulate in tumour-infiltrating CD8⁺ T cells can shape antitumour T cell responses (Ma et al. 2019; Xu et al. 2021). Intratumoural CD8⁺ T cells upregulate CD36 that promotes the uptake of oxLDL (Xu et al. 2021). This is particularly profound in exhausted PD-1⁺ Tim-3⁺ CD8⁺ T cells that are further enriched for CD36. OxLDL accumulation induces lipid peroxidation and activation of MAPK, resulting in reduced antitumour effector function that can be restored by blocking lipid peroxidation or CD36 expression.

Cholesterol accumulation in tumour-infiltrating murine CD8⁺ T cells can result in T cell exhaustion associated with upregulation of inhibitory receptors including PD-1 and 2B4 via induction of the ER stress sensor XBP-1 (Ma et al. 2019). Similar observations were made in T cells from human colorectal cancer and myeloma bone marrow from two donors and these findings could be recapitulated by *in vitro* cholesterol supplementation. Additionally, adoptive transfer of cholesterol-treated CD8⁺ T cells resulted in reduced tumour control compared to untreated T cells. Cholesterol further inhibits the function of a subset of antitumour CD8⁺ T cells producing IL-9 (Ma et al. 2018a).

High-fat diet induced obesity promotes tumour growth and limits T cell and NK cell tumour infiltration in MC38 colon carcinoma and B16-F10 melanoma murine models (Dyck et al. 2022). The function and proliferation of CD8⁺ TIL is reduced in obese mice while the function and bioenergetics of splenic CD8⁺ T cells is preserved. Interestingly, this finding is dependent on tumour type and was not seen in CT26 colon carcinoma. In contrast to the findings by Ma et al. described above (Ma et al. 2019), expression of immune checkpoints including PD-1 or transcription factors associated with T cell exhaustion were not increased in TIL from obese compared to lean mice in the study by Dyck et al.. Instead, lipid droplets were associated with enhanced antitumour function and suppression of antitumour responses was associated with alterations in amino acid metabolism.

1.2.5.4 Cholesterol and other immune cell subsets

This section will give a brief overview of the role of cholesterol metabolism in other immune cell subsets besides T cells.

In B cells, membrane cholesterol and lipid rafts play a role in antigen presentation, B cell receptor signalling and interaction with CD40 expressing Tfh cells (Gupta and DeFranco 2007). As described for T cells, B cell activation induces cholesterol biosynthesis (Shimabukuro-Vornhagen et al. 2014). On the other hand, geranylgeranyl pyrophosphate, an intermediate in the cholesterol synthesis pathway, drives IL-10 production in regulatory B cells (Bibby et al. 2020).

In macrophages, lanosterol, another intermediate molecule in the cholesterol synthesis pathway, accumulates upon TLR-4 activation and reduces IFN β signalling, resulting in increased survival, reduced proinflammatory cytokine production, enhanced ROS and increased phagocytosis (Araldi et al. 2017). Type I interferons induce cholesterol and long chain fatty acid import but limit cholesterol synthesis which in turn again activates type I interferon production, ultimately resulting in an enhanced antiviral immune response (York et al. 2015).

Induction of LXR signalling, e.g. by 25-HC and 27-HC, induces an anti-inflammatory, immunosuppressive phenotype in macrophages. LXR suppresses TLR signalling and the production of inflammatory mediators such as IL-6, and instead induces expression of arginase and T cell apoptosis, ultimately limiting T cell effector function (Cardoso and Perucha 2021; Joseph et al. 2003; Ma et al. 2020). On the other hand, LXR signalling is also critical for macrophage survival and function against intracellular pathogens (Joseph et al. 2004).

In line with the predominantly anti-inflammatory effect of LXR signalling in macrophages, induction of SREBP2 via TNF promotes a pro-inflammatory phenotype (Kusnadi et al. 2019). The importance of lipids in macrophage metabolism is further highlighted in settings such as atherosclerosis where macrophages engulf high amounts of lipids, e.g. via CD36 and other scavenger receptors, resulting in intracellular lipid accumulation and foam cell development that can ultimately lead to cell death and inflammation (Nagy et al. 2012).

Cholesterol accumulation in macrophages also induces NF- κ B and the MAPK pathway which promotes TNF and IL-6 production (Li et al. 2005)

Lipids and cholesterol alter DC function in a context dependent manner. Hepatic DC can be divided into two distinct populations according to their lipid content. DC with high phospholipid content express higher levels of MHC class II molecules, secrete proinflammatory cytokines and activate T cells, whereas DC with low phospholipid content induce immune tolerance and T_{reg} (Ibrahim et al. 2012). In this study, intracellular cholesterol and cholesteryl ester were not elevated in DC with high lipid content and *in vitro* treatment with cholesterol did not alter intracellular neutral lipids (Ibrahim et al. 2012). In a murine LXRab^{-/-} model, hypercholesterolaemia induced autoimmunity with expanded population of B cells and CD11c⁺ APC (Ito et al. 2016). In this setting, CD11c⁺ APC accumulated intracellular neutral lipids primarily consisting of cholesteryl ester, had enhanced capacity of T cell priming and promoted B cell proliferation (Ito et al. 2016). In line with this, inhibition of cholesterol efflux induces autoimmunity with increased production of pro-inflammatory cytokines and enhanced T cell activation (Westerterp et al. 2017).

In contrast, in tumours, the accumulation of intracellular lipids in DC is associated with a reduced ability to stimulate T cells and present tumour-associated antigens (TAA), in some studies due to reduced capacity of antigen processing (Herber et al. 2010) and in others due to reduced pMHC complexes and resultant reduced cross-presentation (Cao et al. 2014). Tumour-produced LXR ligands limit CCR7 expression on DC, reducing migration to lymphoid organs including tumour-draining lymph nodes (Villablanca et al. 2010).

In NK cells, SREBP signalling is required for glycolysis and OXPHOS independent of its effects on lipid synthesis and SREBP-dependent shunting of citrate into the citrate-malate shuttle is required for NK cell function (Assmann et al. 2017). In obesity, PPAR signalling induces an accumulation of intracellular lipid droplets that is associated with bioenergetic impairment, inhibition of the mTOR pathway and reduced antitumour function of NK cells due to inhibited polarisation and transport of cytotoxic granules to the immune synapse (Michelet et al. 2018).

Taken together, in the last years cholesterol has emerged as a metabolic checkpoint controlling the function and phenotype of innate and adaptive immune cells. Its effects are

highly complex, context dependent and more studies are needed to fully delineate its role in shaping antiviral and antitumour immune responses.

1.2.6 Immunometabolism as a therapeutic target

The emerging role of metabolism in shaping antiviral and antitumour immune responses renders it an attractive novel target for immunotherapeutic approaches. However, targeting immunometabolism is challenging due to overlapping metabolic requirements between T cells and the tumours or infected cells they target. Limiting nutrient supply to the tumour bears the risk of starving antitumour T cells and vice versa, altering T cell metabolism to enhance their function and proliferation may also result in protumourigenic effects. This section will explore four examples of targeting metabolism as immunotherapy.

1.2.6.1 Targeting mitochondrial dysfunction in HBV

As described in chapter 1.2.3, mitochondrial dysfunction is a feature of exhausted HBV-specific CD8⁺ T cells in CHB (Fisicaro et al. 2017; Schurich et al. 2016). Therefore, Fisicaro et al. explored the immunomodulatory effect of the mitochondria-targeted antioxidants MitoQ (mitoquinone) and MitoTempo (piperidine-nitroxide) in CHB. Mitochondria-targeted antioxidants reduce CD8⁺ T cell depolarization, restore ROS levels and increase abundance of components of the ETC. These mitochondrial alterations result in a reduction of T cell apoptosis and an increased production of antiviral cytokines such as IFN γ and TNF. This study highlights the potential of targeting T cell metabolism to boost antiviral T cell effector function.

1.2.6.2 Targeting arginine metabolism

Arginine is an amino acid involved in multiple cellular processes. Tumour cells often lack argininosuccinate synthase 1 required for arginine biosynthesis and therefore rely on arginine uptake from the TME (Chen et al. 2021). Multiple preclinical and clinical trials currently explore the possibility of depleting arginine as an anticarcinogenic therapy with varying success (Chen et al. 2021). One of the mechanisms likely contributing to a limited efficiency

of this therapy is the finding that T cells also require arginine for proliferation, activation, mTOR signalling and antitumour T cell function (see chapter 1.2.1.5).

Due to the role of arginine in T cell function and its limited availability in the TME, Canale et al. developed a novel therapeutic approach where genetically engineered bacteria convert intratumoural ammonia into arginine, boosting antitumour T cell effector function (Canale et al. 2021). This approach has a complementary effect with checkpoint blockade for the induction of cancer regression. Following the same concept, arginase-1 inhibitors that limit extracellular arginine degradation due to arginase produced by MDSC are being explored in preclinical and clinical trials (Chen et al. 2021).

These two opposing therapeutic concepts – depletion or increase of available arginine – highlight the challenges faced by therapies targeting metabolism where antitumour T cells and tumours rely on the same metabolic fuels and pathways.

1.2.6.3 Targeting cholesterol metabolism

As described in chapter 1.2.5, cholesterol plays a central role in modulating immune responses. This section will explore the therapeutic potential of targeting two different enzymes involved in intracellular cholesterol metabolism: HMG-CoA reductase and ACAT (Fig. 1.7).

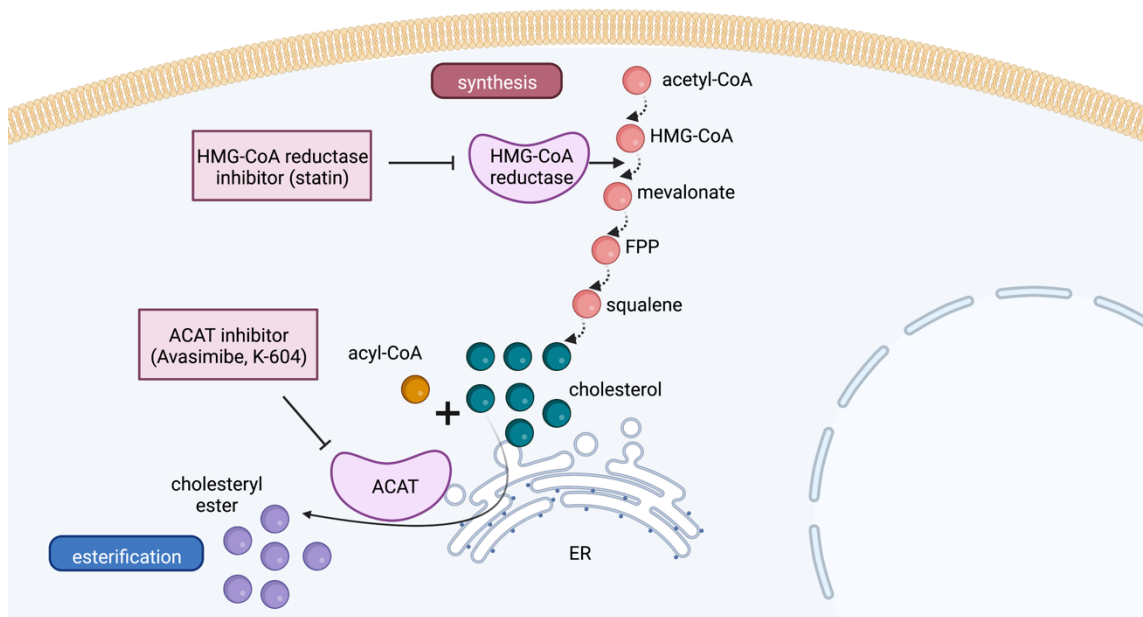


Figure 1.7 Targeting cholesterol metabolism.

Statins inhibit HMG-CoA-reductase, the rate limiting enzyme of the mevalonate pathway, that converts HMG-Co-A into mevalonate. ACAT inhibitors (e.g. Avasimibe, K-604) inhibit ACAT, the enzyme catalysing cholesterol esterification. CoA: Coenzyme A; HMG-CoA: 3-hydroxy-3-methyl-glutaryl-CoA; FPP: farnesyl pyrophosphate; ACAT: acyl-CoA: cholesterol acyltransferase; ER: endoplasmic reticulum.

1.2.6.3.1 Inhibition of HMG-CoA reductase

Inhibitors of HMG-CoA reductase (statins), the rate-limiting enzyme of the mevalonate pathway, are widely prescribed drugs to reduce cholesterol levels in hypercholesterolaemia and atherosclerosis (Blais et al. 2021). Due to the complexity of the cholesterol synthesis pathway, statins not only reduce cholesterol but also modulate other sterols, oxysterols and isoprenoid intermediates (Jain and Ridker 2005; Spann and Glass 2013).

While statins can elicit a direct anticarcinogenic effect via cholesterol-dependent and -independent mechanisms (Di Bello et al. 2020), whether statins are associated with increased or decreased cancer risk remains under constant debate, with conflicting results in different studies and a lack of prospective, randomised clinical trials (Dale et al. 2006; Feng and Qin 2021; Graaf et al. 2004; Vinogradova et al. 2011). Equally, statins have the potential to alter the course of viral infections. For example, in SARS-CoV-2 infection, statins are associated with reduced disease severity (Bergqvist et al. 2021; Gupta et al. 2021).

By blocking cholesterol synthesis, statins have the potential to alter antiviral and antitumour immune responses and may be able to reverse some of the inhibitory mechanisms of high cholesterol levels described in chapter 1.2.5. As mentioned above, statins also reduce the production of cholesterol metabolites such as oxysterols and 25-HC that can be immunomodulatory and alter interferon-mediated antiviral immune responses (Jain and Ridker 2005). For example, atorvastatin inhibits the switch from IFN γ to IL-10 production in CD4⁺ T cells (Perucha et al. 2019) and limits IL-10 production in regulatory B cells (Bibby et al. 2020).

It has also been suggested that statins could have an adjuvant potential enhancing vaccine efficacy, e.g. by inhibiting prenylation of the GTPase Rab5 in APC, resulting in enhanced antigen presentation and T cell activation in cancer vaccines (Xia et al. 2018); however, the effect of statins on clinical vaccine responses is heterogenous: in one clinical trial a combination of atorvastatin with a pneumococcal polysaccharide vaccine enhanced the antibody response (Wildes et al. 2019) whereas long-term statin use was suggested to be associated with reduced responses to influenza vaccine (Black et al. 2016).

Additionally, statins have anti-inflammatory potential, e.g. by reduction of cellular adhesion molecules and chemokines involved in recruitment of inflammatory immune cells, stabilization of endothelial leakage, and inhibition of inflammatory pathways mediated by Ras/Rho or NF κ B (Jain and Ridker 2005).

Taken together, these findings highlight that modulating cholesterol metabolism has the potential to shape immune function; however, more studies are needed to fully delineate the effect of statins on antiviral and antitumour immune responses and inflammation.

1.2.6.3.2 Targeting acyl-CoA:cholesterol acyltransferase

The conversion of intracellular cholesterol to cholesteryl ester is catalysed by the enzyme ACAT (see chapter 1.2.5.1). ACAT inhibitors were initially developed as a treatment for atherosclerosis and Avasimibe, an orally available ACAT1/2 inhibitor, was well-tolerated in clinical trials (Hiatt et al. 2004; Insull et al. 2001; Raal et al. 2003; Tardif et al. 2004).

Elevated SOAT1 and SOAT2 gene expression is associated with advanced disease, tumour aggression and poor prognosis in a variety of cancer types including breast (Huang et al. 2017c), adrenocortical (Lacombe et al. 2020), pancreatic (Li et al. 2016) and liver (Jiang et al. 2019) cancer. Lipid droplets accumulate in tumour cells, contributing to cancer cell survival and aggressiveness (Cruz et al. 2020) and inhibition of cholesterol esterification is anticarcinogenic in multiple pre-clinical cancer models (Websdale et al. 2022). The antitumour effect of ACAT inhibitors has been particularly well studied in prostate cancer where an accumulation of cholesteryl ester is associated with advanced tumour stages and metastasis (Yue et al. 2014). ACAT inhibition reduces cancer cell proliferation and metastasis directly by reduction of available cholesteryl ester (Raftopoulos et al. 2022) and via upregulation of the E2F-1 (Xiong et al. 2021) and impairment of the Wnt/b-catenin pathway (Lee et al. 2018). ACAT inhibition also has a direct antitumour effect in several HCC models, including HBV-related HCC, e.g. by inducing an accumulation of intracellular unesterified oxysterols and suppression of tumour growth (Jiang et al. 2019; Lu et al. 2013).

In 2016, Yang et al. demonstrated in a key study that inhibition of ACAT, either pharmacologically or by genetic ablation, enhances the function and proliferation of tumour-specific CD8⁺ T cells in a murine melanoma model (Yang et al. 2016). ACAT inhibition increases membrane cholesterol levels, resulting in enhanced synapse formation, TCR clustering and TCR signalling. This was the first study highlighting that ACAT inhibition can modulate immune function. In a recent study, anchoring Avasimibe onto T cells via click technology enhanced the antitumour function of TCR engineered and chimeric antigen receptor (CAR) T cells in murine tumour models (Hao et al. 2020).

In summary, targeting ACAT inhibition has a direct antitumour effect and enhances antitumour CD8⁺ T cells in murine models. To date, studies have focused on the effect of ACAT inhibition on murine CD8⁺ T cells. Yang et al. have shown in principle that ACAT inhibition also enhances the function of human CD8⁺ T cells; however, this was only assessed in peripheral blood mononuclear cells (PBMC) from three healthy donors after polyclonal stimulation. To determine the immunotherapeutic potential of ACAT inhibition it is critical to assess the effect on antigen-specific T cells from donors with cancer and viral infection which is the focus of this thesis.

2

Hypothesis & Aims

Main project hypothesis

Modulating cholesterol esterification with ACAT inhibition induces redistribution of intracellular cholesterol and enhances the antiviral and antitumour function of human T cells.

Main project aim

Evaluating the potential of ACAT as a novel immunotherapeutic target in viral infections and tumours in three pathological settings: HBV infection to exemplify a chronic infection; SARS-CoV-2 infection to exemplify an acute infection; HCC to exemplify a tumour setting.

Main project questions

- Does ACAT inhibition enhance the function of antiviral/antitumour T cells in HBV, SARS-CoV-2 and HCC?
- Does ACAT inhibition boost T cells directly *ex vivo* from the immunosuppressive liver and tumour environment?
- Does ACAT inhibition modulate the function of other immune cell subsets?
- What are the mechanisms underlying the T cell boosting effect of ACAT inhibition?
- Does ACAT inhibition have a complementary effect with other immunotherapeutic strategies?

The findings of this project will be presented in three chapters:

Chapter 4 ACAT inhibition enhances virus-specific T cells

This chapter explores the effect of ACAT inhibition on the function and proliferation of virus-specific T cells from the blood and liver of donors with CHB. The effect on other circulating immune cells subsets (T_{fh}, T_{reg}, $\gamma\delta$ T cell and NK cells) in CHB is also assessed. Additionally, this chapter explores the effect of ACAT inhibition on circulating T cells during acute SARS-CoV-2 infection as well as on memory T cells after resolved infection.

Chapter 5: Immunometabolic reprogramming upon ACAT inhibition

This chapter explores the mechanisms underlying the CD8⁺ T cell enhancing effect of ACAT inhibition, including alteration of intracellular cholesterol distribution, e.g. lipid droplets and lipid rafts, TCR signalling and TCR-independent bioenergetics. The role of lipid rafts in T cell exhaustion is also assessed.

Chapter 6: ACAT inhibition enhances the function of tumour-specific T cells and has a complementary effect with other immunotherapeutic strategies

This chapter evaluates the effect of ACAT inhibition on tumour-infiltrating T cells from HCC tumour tissue. Additionally, this chapter explores the complementary effect of ACAT inhibition with other immunotherapeutic strategies, including PD-1 blockade, proinflammatory cytokines, mitochondria-targeted antioxidants and TCR-engineered T cells.

3

Material & Methods

3.1 Ethical Approval

This study was approved by local ethics boards (Wales Research Ethics Committee 4 and UCL Biobank Ethical Review Committee: Research Ethics Committee reference number 16/WA/0289; Brighton and Sussex: Research Ethics Committee reference number 11/LO/0421) and complies with the declaration of Helsinki. The COVIDsortium cohort was approved by the ethical committee of UK National Research Ethics Service (20/SC/0149) and registered at (ClinicalTrials.gov) (NCT04318314). The Royal Free Biobank (TapB) was approved by the Wales Research Ethics Committee (16/WA/0289; 21/WA/0388; project approval reference: NC2020.11).

All study participants gave written informed consent prior to inclusion in the study and storage of all samples complied with the Human Tissue Act 2004.

3.2 Donor cohorts

The following section will provide information about the cohorts utilized in this study: HBV cohort (3.2.1), HCC cohort (3.2.2), acute SARS-CoV-2 cohort (3.2.3) and COVIDsortium cohort (3.2.3). Of note, further clinical information, e.g. fasting status, obesity/cachexia and serum cholesterol levels were not recorded (also see discussion chapter 7.2.3).

3.2.1 HBV cohort

Table 3.1 HBV cohort

Donor	Sex	Age [years]	Viral load [IU/ml]	HBeAg	ALT [IU/l]	Response to ACAT inh.
CHB1	f	32	380	negative	29	yes
CHB2	m	44	150	negative	41	yes
CHB3	m	38	BLQ	negative	32	no
CHB4	m	67	250	negative	36	yes
CHB5	m	40	3090	negative	45	no
CHB6	m	25	220	negative	38	yes
CHB7	f	22	31344	negative	37	no

Donor	Sex	Age [years]	Viral load [IU/ml]	HBeAg	ALT [IU/l]	Response to ACAT inh.
CHB8	m	34	450	negative	39	yes
CHB9	f	42	101	negative	23	yes
CHB10	f	45	1982	negative	35	no
CHB11	m	32	421	negative	58	yes
CHB12	m	44	3300	negative	97	yes
CHB13	m	74	BLQ	negative	24	yes
CHB14	m	32	9300000	positive	98	yes
CHB15	f	39	61	positive	13	yes
CHB16	f	21	7022700	positive	108	yes
CHB17	f	29	80275143	positive	36	no
CHB18	m	28	4360	negative	51	yes
CHB19	f	66	BLQ	negative	66	yes
CHB20	f	32	56	negative	14	yes
CHB21	m	25	2000	negative	26	yes
CHB22	m	47	72172	negative	405	yes
CHB23	f	52	264926	negative	66	no
CHB24	f	47	1101877	positive	42	no
CHB25	m	34	513	negative	24	yes
CHB26	f	31	60	negative	51	no
CHB27	m	32	302	negative	24	yes
CHB28	f	26	634	negative	26	yes
CHB29	m	47	BLQ	negative	35	no
CHB30	m	36	1493	negative	28	yes
CHB31	m	41	408	negative	24	yes
CHB32	f	38	BLQ	negative	21	no
CHB33	f	36	977	negative	21	no
CHB34	f	68	150	negative	22	no

Sex: male (m), female (f); viral load: below the limit of quantification (BLQ); alanine aminotransferase (ALT) normal range: 10-50 IU/L; Response to ACAT inhibition (ACAT inh.) defined as increased or *de novo* IFN γ production.

3.2.2 HCC cohort

Table 3.2 HCC cohort

Donor	Sex	Cirrhosis	Underlying liver disease	TAA assessed
EX072	m	yes	HBV	HBs
EX087	m	no	HBV	HBs, HBc
EX095	m	n/a	HBV	NY-ESO-1
EX096	m	n/a	n/a	NY-ESO-1, AFP, MAGE-A1
EX104	m	yes	HBV	HBs, HBc
EX105	f	yes	HCV	NY-ESO-1
EX118	m	yes	NASH	NY-ESO-1, AFP
EX122	m	n/a	HBV	MAGE-A1
EX125	m	yes	HCV	NY-ESO-1, AFP, MAGE-A1
EX129	m	no	HBV	HBs, HBc, NY-ESO-1, AFP
EX132	m	yes	Haemochromatosis	NY-ESO-1
EX133	f	no	n/a	NY-ESO-1, AFP, MAGE-A1
EX142	f	no	n/a	NY-ESO-1, AFP, MAGE-A1
EX145	m	yes	NASH, HBV	NY-ESO-1
EX146	n/a	n/a	n/a	NY-ESO-1
EX149	m	yes	HCV	NY-ESO-1, AFP, MAGE-A1
EX157	m	n/a	HCV	NY-ESO-1, AFP, MAGE-A1
EX170	f	no	HBV	HBs, HBc, Pol

Sex: male (m), female (f); n/a: information not available; NASH: non-alcoholic steatohepatitis.

3.2.3 SARS-CoV-2 cohorts

Peripheral blood samples were taken from unvaccinated study participants during or after SARS-CoV-2 infection during the first pandemic wave of infections in the UK (March-July 2020).

The Acute Cohort was recruited from hospitalized patients at the Royal Free Hospital, London, and SARS-CoV-2 infection was confirmed by PCR (n=22; median age 82 years; 45% female, 55% male; 73% white, 4% black, 14% Asian, 9% other).

The COVIDsortium Cohort was recruited from healthcare workers in London and SARS-CoV-2 infection was confirmed by PCR and/or serology. Samples were taken 5-6 months post infection (n=12; median age 44.5 years; 50% female, 50% male; 50% white, 8% black, 34% Asian, 8% other). More information about the cohort can be found in (Augusto et al. 2020).

3.3 PBMC isolation

PBMC from healthy donors and donors with HBV or HCC were isolated from heparinized blood by density gradient centrifugation with Ficoll-Hypaque Plus (GE Healthcare) or Pancoll (Pan Biotech). 25ml of heparinized blood were carefully layered over 12.5ml Ficoll or Pancoll and centrifuged for 20min at 800g (acceleration/deceleration 4/3) at room temperature (RT). After centrifugation, the lymphocyte layer was collected with a pasteur pipette, diluted in Roswell Park Memorial Institute 1640 Medium (RPMI; Thermo Fisher Scientific) and centrifuged (700g, 9/9, 10min, RT) to remove any remaining Ficoll/Pancoll.

The cell pellet was then resuspended in RPMI, cells were counted and viability was confirmed by trypan blue (Sigma-Aldrich) staining. PBMC were used fresh or were cryopreserved in foetal bovine serum (FBS; Sigma-Aldrich) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a concentration of 10M cells/ml and stored in liquid nitrogen prior to further use.

For samples taken during acute SARS-CoV-2 infection, PBMC were isolated from ethylenediaminetetraacetic acid (EDTA) blood using Histopaque-1077 (Sigma-Aldrich) density gradient centrifugation in Leukosep tubes (Greiner Bio One) according to the manufacturer's instructions. PBMC were cryopreserved in FBS containing 10% DMSO and stored in liquid nitrogen prior to cell culture. *PBMC isolation for acute SARS-CoV-2 samples was performed by Liã Arruda and members of the UCL Centre for Clinical Microbiology.*

For the COVIDsortium cohort, PBMC were isolated from heparinized blood using Pancoll or Histopaque-1077 Hybri-Max (Sigma-Aldrich) density gradient centrifugation in SepMate tubes (StemCell). Heparinized blood was diluted 1:1 in phosphate-buffered saline (PBS; Invitrogen) +2% FBS. 15ml of Pancoll were added into the bottom compartment of the SepMate tube and 35ml of diluted blood was layered into the top compartment. Tubes were centrifuged (1200g, 9/9, 10min, RT) resulting in a separation of red blood cells, PBMC and plasma. The plasma and PBMC layer was poured into a new falcon tube, diluted with PBS +2% FBS and centrifuged (300g, 9/9, 10min, RT). The cell pellet was resuspended in PBS +2% FBS, cells were counted and viability was confirmed by trypan blue staining. PBMC were cryopreserved in FBS containing 10% DMSO and stored in liquid nitrogen prior to cell

culture. *PBMC isolation for COVIDsortium SARS-CoV-2 samples was performed by me and other members of the COVIDsortium.*

3.4 Leukocyte isolation from liver and tumour tissue

To isolate intrahepatic leukocytes (IHL) and TIL, liver or tumour tissue was manually dissected into small pieces and incubated for 30min at 37°C in Hanks' balanced salt solution (HBSS; Thermo Fisher Scientific) with Ca^{2+} and Mg^{2+} (to support enzymatic activity), 0.01% collagenase IV (Thermo Fisher Scientific) and 0.001% DNase I (Sigma-Aldrich). Enzymatic digestion was followed by further mechanical disruption via GentleMACS (Miltenyi Biotech; one cycle of mouse spleen program m_spleen_01.01), filtration through a 70µm cell strainer (BD Bioscience) and centrifugation (700g, 9/9, 15min, RT) to remove hepatocytes.

Next, to remove any remaining parenchymal and dead cells as well as fat, cells were resuspended in 30% Percoll (GE Healthcare) with HBSS without Ca^{2+} and Mg^{2+} and centrifuged (800g, 9/9, 15min, RT). The top layer of hepatocytes/fat was removed with a pasteur pipette and the cell pellet was resuspended in RPMI. Finally, lymphocytes were isolated via density gradient centrifugation as described in section 3.3. Further details can be found in (Kucykowicz et al. 2022). IHL and TIL were always used fresh immediately after isolation.

3.5 Thawing of cryopreserved PBMC

Cryopreserved PBMC were resuspended in pre-warmed RPMI (37°C) at a concentration of 1:20, immediately centrifuged (700g, 9/9, 10min, RT) and resuspended in appropriate culture media followed by assessment of cell number and viability with trypan blue.

3.6 Cell culture

For all cell cultures, cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. All FBS used in cell cultures was heat inactivated. Post-culture viability was assessed via flow cytometry and samples with <50% viable cells were excluded from further analysis.

To assess virus-/tumour-specific T cell responses, the cytokine production in wells without peptide stimulation was subtracted to determine virus-/tumour-specific cytokine production in all summary data.

3.6.1 Cell culture media

3.6.1.1 complete RPMI

All primary cells (PBMC, IHL, TIL) were cultured in complete RPMI (cRPMI) +10% FBS. The composition of cRPMI can be found in Table 3.3. Media was replenished every 2-3d. Of note, cRPMI +10% FBS does not reflect physiological nutrient concentrations (e.g. D-glucose: <100mg/dl (serum, fasted) vs 200mg/dl (RPMI); L-glutamine 0.5-0.8mM (serum, fasted) vs 2.05mM (RPMI)) and therefore might alter the metabolic phenotype of immune cells and the effect of metabolic modulation. The exact composition of FBS alters between batches and country of origin. FBS was purchased from non-US origin and the same batch of FBS was used within experiments to limit variability.

Table 3.3 cRPMI

Reagent	Company	Dilution (in 500ml)
RPMI	Thermo Fisher Scientific	----
HEPES buffer solution	Gibco	2% (10ml)
Sodium pyruvate	Gibco	0.5% (2.5ml)
2-mercaptoethanol	Gibco	0.1% (0.5ml)
MEM non-essential amino acids	Gibco	1% (5ml)
MEM essential amino acids	Gibco	2% (10ml)
Penicillin/Streptomycin	Gibco	100U/ml (50.000 U)

3.6.1.2 HepG2 media

HepG2 hepatoma cells were cultured in HepG2 media (Table 3.4) +10% FBS. Media was replenished every 2-3d.

Table 3.4 HepG2 media

Reagent	Company	Dilution (in 500ml)
GlutaMAX DMEM	Thermo Fisher Scientific	----
Sodium pyruvate	Gibco	0.5% (2.5ml)
MEM non-essential amino acids	Gibco	1% (5ml)
Penicillin/Streptomycin	Gibco	100U/ml (50.000 U)

DMEM: Dulbecco's Modified Eagle Medium

3.6.1.3 T2 media

T2 cells were cultured in RPMI +10% FBS +100U/ml Penicillin/Streptomycin. Media was replenished every 2-3d.

3.6.2 Overnight cell culture for IHL and TIL

To examine HBV-specific T cell responses in the liver, IHL and paired PBMC were stimulated with HBV-derived overlapping peptide pools (15mers overlapping by 12 amino acids, pan-genotypic HBcAg (94 peptides), pan-genotypic HBsAg (divided into 2 pools with 84 and 82 peptides respectively), gifted by Gilead Sciences, and genotype-D derived polymerase (Pol), 15mers overlapping by 10 amino acids (165 peptides), thinkpeptides).

To examine HCC-specific T cell responses, TIL and paired IHL were stimulated with TAA-derived overlapping peptide pools (15mers overlapping by 12 amino acids; NY-ESO-1 (43 peptides), AFP (150 peptides) and MAGE-A1 (75 peptides), JPT Peptide Technologies) and IHL/TIL from HBV-related HCC were additionally stimulated with HBV-derived peptide pools as described above.

IHL, TIL and paired PBMC were stimulated with 1µg/ml peptides for 16h at 37°C in the presence of 1µg/ml Brefeldin A (Sigma-Aldrich; disassembly of Golgi complex, inhibiting protein secretion (Chardin and McCormick 1999)), in cRPMI +10% FBS, followed by

antibody staining and flow cytometric analysis. Where indicated, TIL were stimulated with 0.5µg/ml plate-bound anti-CD3 (Invitrogen) and 5µg/ml soluble anti-CD28 (Invitrogen) for 16h for polyclonal stimulation. Due to limited cell numbers, in individual cases not all peptide pools could be tested for all samples. The exact peptide pools tested for each donor are indicated in the respective figures.

Cells were treated with 0.1µM of the ACAT inhibitor K-604 (Sigma-Aldrich) or equivalent concentration of DMSO. Where sufficient cell numbers were available, experiments were performed in at least duplicates and combined prior to antibody staining.

3.6.3 Short-term cell culture

In all short-term cell cultures, experiments were performed in at least duplicates and combined prior to restimulation or antibody staining when sufficient cell numbers were available.

To examine virus-specific T cell responses in the blood, PBMC were stimulated with 1µg/ml peptide pool in cRPMI +10% FBS +20U/ml recombinant human IL-2 (PeproTech). PBMC were expanded at 37°C for 7d and 0.5µM of the ACAT inhibitors Avasimibe (Selleckchem) or K-604 as indicated in the respective figure legend or equivalent concentration of the solvent DMSO were replenished every 2d. IL-2 was replenished on d2. On d6, PBMC were restimulated with 1µg/ml peptide in the presence of Brefeldin A for 16h at 37°C, followed by antibody staining and flow cytometric analysis. Different treatment protocols were used during protocol optimization, see Fig. 4.7 for further information. Where indicated, culture media was supplemented with cholesterol (SyntheChol 1:500, Sigma-Aldrich).

To examine a complementary effect of ACAT inhibition with other immunotherapies the following additions to the above-mentioned protocol were made: PD-1 blockade: 2.5µg/ml anti-PD-L1 and 2.5µg/ml anti-PD-L2 (eBioscience) on d0. IL-12: 10ng/ml IL-12 (PeproTech) on d0. IL-15: 5ng/ml IL-15 (PeproTech) on d0, d2, d4, d6. Mitochondria-targeted antioxidants: 10µM MitoTemp (Sigma-Aldrich) on d0.

To examine SARS-CoV-2-specific T cell responses the following alterations were made: 20U/ml recombinant human IL-2 was replenished every 2d and 5µg/ml anti-CD28 was administered on d0 and d6.

The following peptides and peptide pools were used to assess virus-specific T cells in PBMC:

- HBV: pan-genotypic HBcAg peptide pool (15mers overlapping by 12 amino acids, 94 peptides).
- SARS-CoV-2: Membrane peptide pool (15mer peptides overlapping by 10 amino acids, 43 peptides) and Spike S1 peptide pool (18-20mer peptides, 18 peptides), gifted by Antonio Bertolotti, Singapore. For further information see (Reynolds et al. 2020).
- EBV: BMLF1₂₈₀₋₂₈₈ (amino acid sequence: GLCTLVAML; Immudex).
- CMV: Overlapping pp65 peptide pool (pp65; 15mer peptides overlapping by 11 amino acids, 138 peptides; JPT) or CMV pp65₄₉₅₋₅₀₃ (NLV, amino acid sequence: NLVPMVATV; ProImmune).
- Influenza A virus: Matrix protein 1₅₈₋₆₆ (amine acid sequence: GILGFVFTL; Immudex).

3.6.3.1 T follicular helper cells

To assess Tfh cells, unstimulated PBMC from donors with CHB were cultured for 7d at 37°C in cRPMI +10% FBS +20U/ml recombinant human IL-2 ±0.5µM Avasimibe or equivalent concentration of DMSO. Tfh phenotype was assessed by flow cytometry.

3.6.3.2 T_{reg}

To examine T_{reg}, PBMC from CHB donors were cultured for 24h or 7d in cRPMI +10% FBS as indicated in the respective figure legends. T_{reg} frequency and phenotype were assessed by flow cytometry.

In 24h cultures, PBMC were stimulated with 0.5µg/ml plate-bound anti-CD3 and 5µg/ml soluble anti-CD28 (where indicated in figure legend) and treated with 200U/ml recombinant human IL-2 ±0.5µM Avasimibe or equivalent concentration of DMSO.

In 7d cultures, PBMC were stimulated with 0.5µg/ml plate-bound anti-CD3 and 5µg/ml soluble anti-CD28 for 48h (where indicated in figure legend) and treated with 500U/ml recombinant human IL-2 ±0.5µM Avasimibe or equivalent concentration of DMSO. Stimulation was terminated after 48h, IL-2 and Avasimibe were replenished every 2d.

3.6.3.3 NK cells

To examine NK cells, PBMC were stimulated with 5ng/ml IL-12 (PeproTech) and 50ng/ml IL-18 (PeproTech) in cRPMI +10% FBS +20U/ml recombinant human IL-2 ±0.5µM Avasimibe or equivalent concentration of DMSO. PBMC were expanded at 37°C for 3d. On d2 cytokines and ACAT inhibitor were replenished, and the cells were treated with Brefeldin A, Monensin and anti-CD017a-APC as described in chapter 3.9.1. The cytokine production in wells without stimulation was subtracted to determine stimulation-specific cytokine production in all summary data.

3.6.3.4 $\gamma\delta$ T cells

To examine $\gamma\delta$ T cells, PBMC were stimulated with 1µg/ml plate-bound pan- $\gamma\delta$ -antibody (BioLegend) in cRPMI +10%FBS +20U/ml recombinant human IL-2 ±0.5µM of the ACAT inhibitor Avasimibe or equivalent concentration of DMSO. PBMC were expanded at 37°C for 5d and 0.5µM Avasimibe or equivalent concentration of DMSO were replenished every 2d. On d4, PBMC were restimulated with 2µg/ml plate-bound pan- $\gamma\delta$ -antibody in the presence of Brefeldin A, Monensin and anti-CD107a-APC as described in chapter 3.9.1. The cytokine production in wells without stimulation was subtracted to determine stimulation-specific cytokine production in all summary data.

3.6.4 HepG2 cell line

HepG2 hepatoma cells are an adherent cell line cultured in HepG2 media (chapter 3.6.1.2). Plates/flasks were coated with type I collagen (Sigma-Aldrich) diluted 1:40 in PBS for 30min at 37°C prior to cell culture to facilitate cell adherence.

Confluency was evaluated microscopically and HepG2 cells were split once a confluency of >80% was reached. Culture media was removed and attached cells were carefully washed with PBS to remove detached dead cells and FBS residues. This was followed by a treatment with 0.05% Trypsin-EDTA (gibco) for 5min at 37°C to dissociate attached cells (e.g. 3ml for T75 flask). Dissociation was confirmed microscopically and cells were resuspended in HepG2 media with 10% FBS to terminate enzyme activity. Cells were centrifuged (700g, 9/9, 10min, RT), resuspended in fresh media and distributed into collagen-coated flasks/plates.

3.7 TCR-engineered T cells

Human CD8⁺ T cells from healthy donors were genetically engineered to express a TCR specific for the immunodominant HBc-derived epitope HBc₁₈₋₂₇ (C18; amino acid sequence: FLPSDFFPSV) (Gehring et al. 2011) or a TCR specific for a HA-1-derived epitope (amino acid sequence: VLHDDLLEA; wildtype TCR and codon-optimized LYR-TCR as previously described) (Thomas et al. 2019).

Phoenix amphotropic packaging cells were transiently co-transfected using FuGENE (Promega) with plasmids encoding the C18 or HA1 TCR and an amphotropic envelope. Retroviral supernatants were collected for the transduction. Fresh PBMC were stimulated with 200U/ml IL-2 and 50ng/ml plate-bound anti-CD3 in cRPMI for 48h. Activated PBMC were plated on wells coated with retronectin (Takara), retroviral supernatant was added and plates were centrifuged (800g, 5/0, 90min, RT). Cells were expanded in cRPMI +10% FBS +100U/ml IL-2, 10ng/ml IL-7 (PreproTech) and 10ng/ml IL-15 (Peprotech) and transduction efficiency was confirmed after 3-4d by staining of the mouse TCR β chain (C18) or mouse CD19 (HA1) transduced simultaneously with the TCR construct. After sufficient

expansion, C18-TCR or HA-1-TCR expressing CD8⁺ T cells were sorted with a FACSAria II (BD Bioscience). *This experiment was performed in collaboration with Mariana Diniz and Oliver Amin.*

To evaluate the function of TCR-engineered CD8⁺ T cells, suitable APC (HepG2 cells for C18 TCR to mimic T cell priming in the liver; T2 cells for HA-1 TCR) were pulsed with increasing doses of the respective cognate peptide. T cells and target cells were co-cultured in an effector:target ratio of 1:1 and treated with 1µM Avasimibe or equivalent concentrations of DMSO for 16h at 37°C. HA-1 TCR T cells were additionally treated with Avasimibe for 24h prior to coculture. To determine cytokine production and degranulation by antibody staining and flow cytometric analysis, Brefeldin A, Monensin and CD107a were added for 16h as described in chapter 3.9.1.

To measure target cell lysis (see chapter 3.9.2) or immune mediator release in the supernatant, Brefeldin A, Monensin and CD107a were not added, instead supernatant was collected after 16h and stored at -20°C prior to analysis.

Immune mediators in the supernatant were measured by MAGPIX (Luminex, USA) using the human XL cytokine discovery 45-plex kit (Biotechne) according to the manufacturer's instructions. The assay was performed in three independent experiments and only soluble mediators with consistent changes in all three experiments were included in the summary data. The immune mediator concentration in wells without peptide stimulation was subtracted to determine peptide-specific immune mediator concentration in summary data. *This experiment was performed in collaboration with Oliver Amin.*

3.8 Flow cytometry

A list of antibodies and other fluorescent reagents used for flow cytometry can be found in Table 3.5. All incubations with fluorescent reagents were performed in the dark and stained samples were stored in the dark prior to flow cytometric analysis.

During analysis of flow cytometric data, viability was confirmed and samples with <50% viable cells were excluded from further analysis. Mean fluorescent intensity (MFI) and frequencies were only assessed in populations with a minimum of 50 events.

3.8.1 Surface staining

Cells were stained with saturating concentrations of surface antibodies and a fixable viability dye diluted in 1:1 PBS:Brilliant Violet Buffer (BD Bioscience) for 30min at 4°C or 15min at RT. Following surface staining, unbound antibodies were washed off with PBS (700g, 9/9, 4min, 4°C) and cells were fixed with 70µl Cytofix (BD Bioscience) at 4°C for 30min. After fixation, cells were washed with PBS and stored in PBS at 4°C until flow cytometric analysis within 24h.

3.8.2 Intracellular staining

For intracellular staining (e.g. cytokines), surface staining (section 3.8.1) was followed by fixation and permeabilization with 70µl Cytofix/Cytoperm (BD Bioscience) for 30min at 4°C. After fixation and permeabilization, cells were washed with PBS and stained in saturating concentrations of intracellular antibodies in a 0.1% saponin-based buffer (Sigma-Aldrich) for 30min at 4°C. Unbound antibodies were washed off with PBS and cells were stored in PBS at 4°C until flow cytometric analysis within 24h.

3.8.3 Intranuclear staining

For intranuclear staining (e.g. transcription factors), surface staining (section 3.8.1) was followed by fixation and permeabilization utilizing a FOXP3 buffer set (BD Biosciences). Cells were first treated with 100µl buffer A (diluted 1:10 in distilled H₂O) for 10min at RT, centrifuged (700g, 9/9, 4min, 4°C) and treated with 100µl buffer B (diluted 1:50 in diluted buffer A) for 30min at RT. Cells were then centrifuged (700g, 9/9, 4min, 4°C) followed by a staining with intranuclear antibodies in PBS for 30min at 4°C. Unbound antibodies were

washed off with PBS and cells were stored in PBS at 4°C until flow cytometric analysis within 24h.

3.8.4 Phosphoflow

After pre-treatment with ACAT inhibitors or DMSO for 7d as described in section 3.6.3, PBMC were surface stained for 15min at RT as described in section 3.8.1 followed by a stimulation with 0.5µg/ml plate-bound anti-CD3 and 5µg/ml soluble anti-CD28 for 30min at 37°C. Cells were fixed and permeabilized with PhosphoFix/Perm Buffer (BD Bioscience) for 30min at -20°C and washed with PBS followed by staining with saturated concentrations of phospho-antibodies in PBS for 30min at 4°C. Unbound antibodies were washed off with PBS and cells were stored in PBS at 4°C until further analysis on the same day.

3.8.5 Imaging cellular cholesterol by flow cytometry

To visualize neutral lipid droplets, PBMC were surface stained and fixed at 4°C as described in section 3.8.1, followed by a staining with HSC LipidTOX Neutral Lipid Stain (Thermo Fisher) diluted 1:10.000 in PBS for a minimum of 30min at RT in the dark and analysed by flow cytometry on the same day without washing off the dye. Dilution was established via careful titration of the dye to determine a suitable staining intensity for flow cytometric analysis.

To visualize lipid rafts, PBMC were surface stained at 4°C as described in section 3.8.1, followed by staining with 25µg/ml cholera toxin B subunit (CTB)-FITC (Sigma-Aldrich; binds to monosialotetrahexosylganglioside, GM1) in PBS at 4°C for 30min. Unbound CTB was washed off with PBS followed by fixation ±permeabilization as described in chapter 3.8.1 and 3.8.2. Cells were stored in PBS at 4°C until further analysis within 24h.

For the visualization of free membrane cholesterol, cells were surface stained and fixed at 4°C as described in chapter 3.8.1, followed by a staining with 50µg/ml Filipin complex

(Sigma-Aldrich) in PBS for 2h at RT. Unbound Filipin complex was washed off with PBS and cells were stored in PBS at 4°C until further analysis within 24h.

3.8.6 Assessment of cell proliferation

To assess cell proliferation, PBMC were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen) prior to the start of culture. PBMC were stained with 1µM CFDA-SE in PBS (1ml per 5M PBMC) for 10min at 37°C. Unbound dye was quenched with 10% FBS for 1min, followed by 2 washing steps with PBS to remove any residual unbound dye (700g, 9/9, 10min, RT). Intracellular CFDA-SE is converted into carboxyfluorescein succinimidyl ester (CFSE). Cell proliferation was assessed via flow cytometry by dilution of the dye, indicated by reduced MFI of CFSE and increased frequency of CFSE^{low} cells (Fig. 3.1).

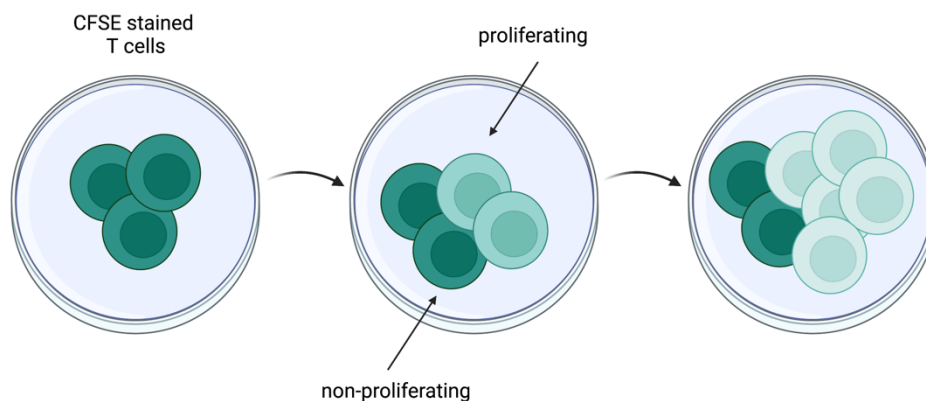


Figure 3.1 Assessment of proliferation via carboxyfluorescein succinimidyl ester (CFSE) staining.

3.9 Assessment of cytotoxicity

3.9.1 Degranulation assessed via flow cytometry

Cytotoxicity of immune cells can be indirectly assessed by flow cytometry detecting the mobilization of lysosomal associated membrane glycoprotein-1 (LAMP-1; CD107a) to the

cell surface. Cytotoxic molecules such as perforin and granzymes are stored in pre-formed granules as lysosomes in the cytoplasm for rapid release. The lysosomes are surrounded by a lipid bilayer containing various LAMP, such as CD107a. Upon immune cell activation these granules are rapidly mobilised to the cell membrane followed by membrane fusion and release of cytotoxic molecules into the immune synapse. During membrane fusion, CD107a can be detected on the cell surface as a marker of degranulation (Betts et al. 2003).

Cells were stimulated as described in chapter 3.6. and 3.7 in the presence of 1µg/ml Brefeldin A, 1:1500 Monensin (BD Biosciences; to neutralise pH of endosomal and lysosomal compartments, limiting loss of fluorescence and inhibiting protein secretion (Betts et al. 2003)) and stained with anti-CD107a-APC (BD Biosciences, 1:200 dilution) for 16h during cell culture.

3.9.2 Target cell lysis

Target cell lysis by TCR-engineered T cells (chapter 3.7) was assessed via ToxiLight Non-Destructive Cytotoxicity BioAssay (Lonza) measuring adenylate kinase (AK) released by damaged cells. 100µl AK detection reagent was added to 20µl supernatant and incubated at RT for 5min. Luminescence was measured as relative light units (RLU) on a luminometer.

To achieve 100% lysis, target cells were resuspended in 0.1% Triton X-100 (Sigma Aldrich) in PBS.

Specific target cell lysis was calculated as: $(RLU(\text{effector}+\text{target})-RLU(\text{effector})-RLU(\text{target})) / (RLU(100\% \text{ lysis})-RLU(\text{effector})-RLU(\text{target})) * 100$.

3.10 Magnetic bead isolation

Where indicated, CD8⁺ T cells were isolated via positive or negative magnetic bead isolation (Miltenyi Biotech). All magnetic bead isolation was performed in 4°C cold MACS buffer (PBS +0.5% bovine serum albumin (BSA, Sigma-Aldrich) +2mM EDTA (Sigma-Aldrich)).

For positive CD8⁺ T cell isolation, PBMC were resuspended in 70µl MACS buffer and 20µl CD8⁺ MicroBeads per 10⁷ total cells and incubated for 15min at 4°C. Cells were resuspended in 2ml MACS buffer per 10⁷ total cells and centrifuged (300g, 9/9, 10min, 4°C) to wash off any unbound antibodies. An MS or LS column was chosen depending on cell numbers, placed in the magnetic field of a MACS Separator and washed with MACS buffer. PBMC were resuspended in 1ml MACS buffer per 10⁷ total cells and loaded on the column. After the cell suspension passed through the column, the column was washed 3x. The column was then removed from the magnetic field, filled with MACS buffer and the bead-labelled CD8⁺ T cells were flushed out and collected.

For negative CD8⁺ T cell isolation, PBMC were resuspended in 40µl MACS buffer and 10µl Biotin-Antibody Cocktail per 10⁷ total cells and incubated for 5min at 4°C. After 5min, another 30µl MACS buffer and 20µl CD8⁺ T Cell MicroBead Cocktail per 10⁷ total cells were added and incubated for 10min at 4°C. An LS column was placed into the magnetic field of a MACS Separator and washed with MACS buffer. PBMC were loaded on the column and washed 3x with MACS buffer. The flowthrough was collected representing the unlabelled CD8⁺ T cell fraction.

3.11 Confocal Microscopy

PBMC from a CHB donor were stimulated with 1µg/ml of the HLA-A2-restricted immunodominant peptide CMVpp65₄₉₅₋₅₀₃ (NLV) and expanded in a short-term culture for 7d as described in chapter 3.6.3. On day 7, CD8⁺ T cells were isolated using magnetic beads from a negative CD8⁺ T cell isolation kit (see chapter 3.10).

T2 cells were pulsed with 1µg/ml NLV peptide to function as APC and labelled with Cell Tracker Deep Red (Invitrogen) diluted 1:2500 in PBS with 0.1% BSA for 30min at 37°C. Surplus dye was quenched with 5ml T2 media for 10min at 37°C. Cells were centrifuged (700g, 9/9, 10min, RT) followed by another wash with T2 media.

CD8⁺ T cells and APC were resuspended in cRPMI with 1% FBS in a 1:1 ratio and incubated on poly-L-lysine coated coverslips for 30min at 37°C. Cells were then fixed at 4°C in PBS

with 4% formaldehyde +1% BSA for 30min followed by a staining with 10 μ g/ml CTB-FITC for 30min at 4°C in cold PBS +1% BSA +0.01% NaN₃. Cells were then permeabilized with 0.1% Triton in PBS with 1% BSA. Permeabilization was followed by a staining with 10 μ g/ml anti-CD3 ϵ mAb (UCHT1, BioLegend) in PBS +1% BSA for 60min at RT and washed 3x with PBS +1% BSA. Cells were then stained with anti-mouse IgG1-AF546 followed by 3 washes with PBS +1%BSA and 1x wash with distilled H₂O. Cover slips were mounted with Prolong Gold anti-fade (ThermoFisher). Images were acquired using the DeltaVision ELITE Image Restoration Microscope (Applied Precision) coupled to an inverted Olympus IX71 microscope and a CoolSNAP HQ2 camera, deconvolved with softWoRx 5.0 and processed using Huygens Professional v4.0 and Adobe Photoshop CC 2018. *This experiment was performed in collaboration with Clare Jolly.*

3.12 Extracellular flux analysis

CD8⁺ T cells were isolated from fresh PBMC using a positive CD8⁺ T cell isolation kit (see section 3.10). Isolated CD8⁺ T cells were cultured for 16h in cRPMI +10% FBS +20U/ml human recombinant IL-2 at 37°C +1 μ M Avasimibe or equivalent concentration of DMSO. On the same day, the sensor cartridge was hydrated with Seahorse XF Calibrant (Agilent) at 37°C in a non-CO₂ incubator.

After 16h, CD8⁺ T cells were counted, viability was confirmed, and cells were plated on a Cell-Tak (Corning)-coated Seahorse XF Cell Culture Microplate (Agilent) in 180 μ l Seahorse XF media (Agilent) at a concentration of 200.000 cells/well. Cells were rested in a non-CO₂ incubator for 20 min prior to the assay.

CD8⁺ T cells were stimulated with 50ng/ml Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 500ng/ml ionomycin (Sigma-Aldrich) in Seahorse XF media (Agilent) to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real time using a Seahorse XFe96 Analyzer (Agilent). After establishment of a stable baseline OCR, 1 μ M oligomycin (ATP synthase/complex V inhibitor) was administered, followed by mitochondrial uncoupling with 1.5 μ M carbonyl cyanide-4-(trifluoromethoxy)

phenylhydrazine (FCCP) and 0.5 μ M rotenone and antimycin A (Rot/AA; complex I and III inhibitors) (all compounds from Agilent).

Calculation of basal respiration (baseline OCR minus OCR after Rot/AA), ATP production (baseline OCR minus OCR after Oligomycin), maximal respiration (OCR after FCCP minus OCR after Rot/AA) and SRC (maximal respiration minus basal respiration).

All experiments were performed in 5 replicates for each donor. For more information also see chapter 5.1.3.

3.13 Gene expression analysis

To examine the gene expression of SOAT1 (ACAT1) and SOAT2 (ACAT2) in T cells, previously published single-cell RNA sequencing data from human blood, liver and HCC tissue was probed focusing on CD8⁺ and CD4⁺CD25^{neg} T cells (available on Gene Expression Omnibus, GSE98638). *Gene expression was analysed in collaboration with Leo Swadling.*

3.14 Statistical analysis

Statistical analyses were performed with Prism 9 (GraphPad) as indicated in figure legends (Wilcoxon matched-pairs signed rank test, Mann-Whitney test, Friedman test with Dunn's multiple comparisons test, Kruskal-Wallis test with Dunn's multiple comparisons test, Fisher's exact test, Spearman correlation) with significant differences marked on all figures. In data with a sample size of >50 normality was assessed by D'Agostino-Pearson normality test and in data with a sample size of n<50 non-normal distribution was assumed and non-parametric statistical analysis was chosen. All tests were performed as two-tailed tests, and for all tests, significance levels were defined as not significant (ns) $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

3.15 Software

Prism 9 (GraphPad) was used for generation of data graphs and statistical analysis. FlowJo 10.8.1 (BD) was used for analysis of flow cytometry data. Adobe Illustrator (Adobe) was used for assembly of figures and BioRender (<https://biorender.com>) was used for generation of illustrations.

3.16 Fluorescent reagents

Table 3.5 Fluorescent reagents

Antigen or reagent	Fluorochrome	Clone	Supplier	Catalogue number	Dilution
Monoclonal antibodies					
CD45	BUV805	HI30	BD Bioscience	564914	0.5:100
CD3	BUV395	UCHT1	BD Bioscience	563546	1:100
CD3	BV711	OKT3	BioLegend	317328	1:100
CD3	PE-CF594	UCHT1	BD Bioscience	562280	1:100
CD3	BV510	OKT3	BioLegend	317332	1:100
CD3	BUV805	UCHT1	BD Bioscience	612895	1:100
CD8	AF700	RPA-T8	BioLegend	301028	1:100
CD8	FITC	RPA-T8	BioLegend	301060	1:100
CD8	BV785	RPA-T8	BioLegend	301046	1:100
CD4	APC-Cy7	RPA-T4	BD Bioscience	557871	1:100
CD4	BUV395	SK3	BD Bioscience	563550	1:100
CD4	BV510	OKT4	BD Bioscience	317444	1:100
CD56	PE/Dazzle594	QA17A16	BioLegend	392410	1:100
CD56	PE-Cy7	HCD56	BioLegend	318317	1:100
CD19	BV786	HIB19	BD Bioscience	740968	2:100
CD69	BV605	FN50	BioLegend	310938	1:100
CD69	PE/Dazzle594	FN50	BioLegend	310942	1:100
CD103	BV711	Ber-ACT8	BioLegend	350222	2:100
TCR γ/δ	PC7	IMMU510	Beckmann Coulter	B10247	2:100
TCR V δ 2	BV605	B6	BioLegend	331430	2:100
PD-1	PE	EH12.2H7	BioLegend	329906	2:100
HLA-DR	V500	G46-6	BD Bioscience	561224	1:100
2B4	FITC	C1.7	BioLegend	329505	1:100
CD38	PE-CF594	HIT2	BD Bioscience	562288	0.5:100
CD28	BB700	L293	BD Bioscience	745905	1:100
CXCR5	APC-Cy7	J252D4	BioLegend	356926	3:100
OX40	AF647	Ber-ACT35	BioLegend	350018	2:100
4-1BB	PE/Dazzle594	4B4-1	BioLegend	309825	1:100

Antigen or reagent	Fluorochrome	Clone	Supplier	Catalogue number	Dilution
ICOS	BV605	C398.4A	BioLegend	313538	2:100
CD25	APC	BC96	BioLegend	302610	2:100
CD127	V500	A019D5	BioLegend	351332	2:100
CD154	PE-Cy7	24-31	BioLegend	310832	1:100
GITR	BV711	108-17	BioLegend	371212	2:100
TIGIT	BV711	741182	BD Bioscience	747839	1:100
CD36	APC	5-271	BioLegend	336208	1:100
CD101	PE-Cy7	BB27	BioLegend	331014	2:100
GLUT1	AF700	202915	R&D Systems	FAB1418N	3:100
CD39	FITC	A1	BioLegend	328205	1:100
CD39	APC	A1	BioLegend	328209	2:100
Tim-3	PE-Cy7	F38-2E2	BioLegend	345014	1:100
SR-B1	PE-Cy7	M1B9	BioLegend	363209	1:100
CTLA-4	PE	BNI3	BD Bioscience	555853	2:100
Ki-67	FITC	MOPC-21	BD Bioscience	556026	5:100
IFN γ	V450	B27	BD Bioscience	560371	1:100
CD107a	APC	H4A3	BD Bioscience	641581	0.5:100
TNF	FITC	MAB11	BD Bioscience	554512	0.5:100
TNF	APC	MAB11	BioLegend	502912	0.5:100
TNF	APC-Cy7	MAB11	BioLegend	502944	0.5:100
TNF	PE/Dazzle594	MAB11	BioLegend	502945	0.5:100
MIP1b	PE	D21-1351	BD Bioscience	550078	1:100
IL-2	PerCP-Cy5.5	MQ1-17H12	BD Bioscience	560708	2:100
Perforin	PE/Dazzle594	dG9	BioLegend	308132	1:100
FOXP3	BV421	206D	BioLegend	320124	2:100
Eomes	PE-eFluor610	WD1928	Invitrogen	61-4877-42	2:100
T-bet	BV421	4B10	BioLegend	644816	2:100
TOX	PE	TXRX10	eBioscience	12-6502-80	2:100
pERK	PerCP-Cy5.5	6B8B69	BioLegend	369511	2:100
pS6	AF647	D57.2.2E	CellSignaling Technology	4851S	2:100
pAKT	PE	M89-61	BD Bioscience	560378	2:100
pAKT	PE-CF594	M89-61	BD Bioscience	563465	2:100
mouse TCR β	APC	H57-597	eBioscience	17-5961-82	1:100
mouse CD19	APC	1D3/CD19	BioLegend	152410	1:100
mouse IgG1	AF546	n/a	Invitrogen	A-21123	5 μ g/ml
Other fluorescent reagents					
Vybrant CFDA SE Cell Tracer Kit	n/a	n/a	Invitrogen	V12883	2.5 μ M
Cholera-toxin B (CTB)	FITC	n/a	Sigma-Aldrich	C1655-.5MG	25 μ g/ml

Antigen or reagent	Fluorochrome	Clone	Supplier	Catalogue number	Dilution
HCS LipidTOX Red Neutral Lipid Stain	n/a	n/a	Invitrogen	H34476	1:10.000
Filipin complex from Streptomyces filipinensis	n/a	n/a	Sigma-Aldrich	F9765	50µg/ml
LIVE/DEAD Fixable Blue Dead Cell Stain Kit	n/a	n/a	Invitrogen	L23105	2:1000
LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit	n/a	n/a	Invitrogen	L34976	1.5:1000
CellTracker Deep Red Dye	n/a	n/a	Invitrogen	C34565	0.4µM

n/a not applicable. FITC: fluorescein isothiocyanate; PE: phycoerythrin; AF: Alexa Fluor; APC: allophycocyanin; PerCP: peridinin chlorophyll; Cy: cyanine; BV: brilliant violet; BUV: brilliant ultra violet.

4

ACAT inhibition enhances virus-specific T cells

Modulation of cholesterol esterification by ACAT inhibition expands functional circulating and intrahepatic HBV-specific T cells in CHB as well as virus-specific CD4⁺ T cells during acute SARS-CoV-2 infection. ACAT inhibition preferentially enhances T cell function in high cholesterol environments such as the liver or after *in vitro* cholesterol supplementation. Effects on T_{reg}, $\gamma\delta$ T cells and NK cells are minor and ACAT inhibition does not induce cytotoxic antiviral T cells nor bystander immune activation.

4.1 Introduction

4.1.1 Hepatitis B virus infection

Despite available vaccines, 30% of the world's population show serological evidence of past or present HBV infection (Trépo et al. 2014). HBV is endemic in South-East Asia, China and sub-Saharan Africa and an estimated 257 million people are living with CHB worldwide (Trépo et al. 2014; World Health Organization 2017). Overall, viral hepatitis (HBV+HCV) causes 1.34 million deaths each year, mainly due to liver cirrhosis and liver cancer (World Health Organization 2017). This chapter will give an overview of HBV infection and the immune responses in HBV.

4.1.1.1 The hepatitis B virus

While the concept of an 'icterus epidemic' has been acknowledged at least since the late 19th century, in the 1960s, Baruch S. Blumberg described the 'Australia antigen', now known as HBsAg, for the first time in the blood of aboriginal Australians – a discovery that was awarded with the Nobel Prize in Physiology or Medicine in 1976 (Blumberg and Alter 1965; Gerlich 2013; The Nobel Prize 1976). The hepatitis B virion was first described by David Dane in 1970 and is therefore often referred to as the Dane particle (Dane et al. 1970). Today, it has become clear that HBV is an ancient virus circulating for at least 7000 years as the HBV genome could be isolated from neolithic and medieval skeletons found in Germany as well as 16th century Italian and Korean mummies (Kahila Bar-Gal et al. 2012; Krause-Kyora et al. 2018; Ross et al. 2018).

HBV is a non-cytopathic, partially double-stranded DNA virus from the *Hepadnaviridae* family (Seeger and Mason 2015) (Fig. 4.1). HBV is hepatotropic and enters hepatocytes via the interaction between the viral envelope protein and the sodium-taurocholate cotransporter polypeptide (NTCP) (Ni et al. 2014). The genome is remarkably small (~3kb) with a compact structure and four overlapping open reading frames encoding for seven proteins: preCore, Core (HBc), pol, X (HBx) and three envelope proteins: small (S), medium

(M, includes the preS2 domain) and large (L, includes the preS1 and preS2 domain) (Dandri and Locarnini 2012).

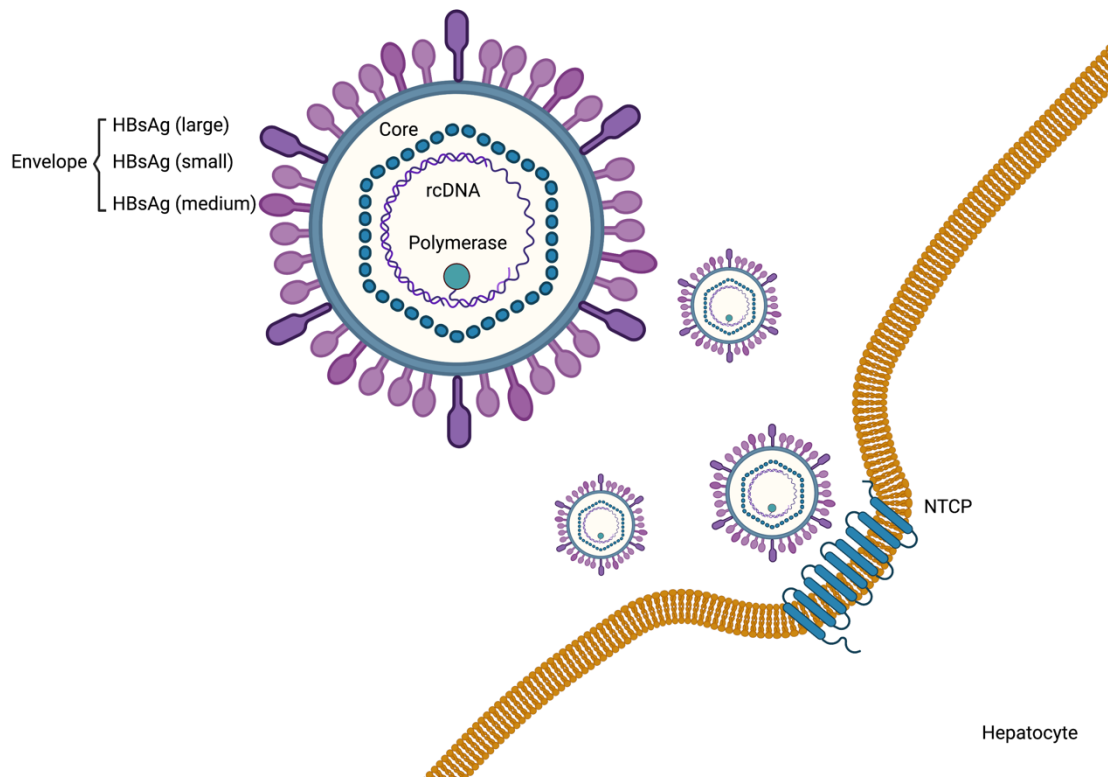


Figure 4.1 The hepatitis B virus.

The HBV DNA encodes for 7 proteins: preCore, Core, pol, X and three envelope proteins (small, medium, large). Core forms the nucleocapsid, polymerase is required for DNA synthesis and the envelope proteins facilitate viral entry via NTCP. In the hepatitis B virion, the viral DNA forms a relaxed circular DNA (rcDNA). HBV: hepatitis B virus; HBsAg: HBV surface antigen; NTCP: sodium-taurocholate cotransporter polypeptide.

HBc forms the viral nucleocapsid and the envelope proteins (also referred to as surface protein, HBs) form the viral envelope. The antigenic loop of HBs as well as the preS1 domain are required for viral entry (Li and Urban 2016). Pol encodes for the viral polymerase which has reverse transcriptase activity required for viral DNA synthesis, and the HBx protein is required for transcription of the cccDNA. The preCore protein is proteolytically processed before secretion as the HBe antigen (HBeAg). Its role in the viral life cycle remains elusive but its presence or absence in the serum are typically exploited to determine disease stage (see section 4.1.1.2) (Seeger and Mason 2015).

The viral lifecycle of HBV and other hepadnaviruses is distinct from other DNA viruses due to their unique usage of a reverse transcriptase and resembles the lifecycle of retroviruses. In the hepatitis B virion, the viral DNA forms a relaxed circular DNA (rcDNA) (Lucifora and 110

Protzer 2016). Upon target cell infection, rcDNA is converted into cccDNA that resides in the nucleus and assembles with histones to form a minichromosome. cccDNA is usually episomal (not integrated into the host genome) and acts as the template for viral RNA. cccDNA can persist in the nucleus long-term and is the major obstacle in viral clearance as even a few copies of cccDNA can re-initiate infection when viral control is lost, e.g. after termination of antiviral therapy or due to immunosuppression (Nassal 2015). The virus utilises host cell polymerase-II for transcription of cccDNA into subgenomic, pregenomic and precore RNA that are exported into the cytoplasm and translated into viral proteins. Within the nucleocapsids, pregenomic RNA is transcribed into DNA via the reverse transcriptase activity of the viral polymerase. Assembly of the nucleocapsid with the envelope proteins leads to formation of a new hepatitis B virion (Liang 2009).

Currently there are eight described HBV genotypes (A-H) and multiple subgenotypes, based on divergence from the nucleotide sequence by 8% and 4%, respectively (Tong and Revill 2016). Different genotypes are associated with differing geographic distribution, disease course and treatment response.

4.1.1.2 Natural history of HBV infection

HBV is transmitted through contact with infected blood and semen by three main routes: perinatally from infected mother to child ('vertical infection'), sexual contact, and contact with infected blood e.g. via unclean injections or transfusion of infected blood products (Trépo et al. 2014). The outcome of HBV infection is highly age-dependent: chronic infection develops in 96% when infected as neonates and 20-30% in children aged 1-5 years whereas adults only develop chronic infection in less than 5% of cases.

HBV is non-cytopathic and disease-associated liver damage is immune-mediated. Acute infections are typically asymptomatic in children whereas one third of adults develop icteric hepatitis and less than 1% present with fulminant hepatitis that is associated with high mortality (Trépo et al. 2014).

While CHB is initially typically asymptomatic, over the years it can cause fibrotic remodelling of the liver that can ultimately result in liver cirrhosis. CHB is further associated with an increased risk of HCC that often develops on the background of liver cirrhosis but can also

occur without fibrotic/cirrhotic remodelling in CHB (also see chapter 6.1.1) (Neuveut et al. 2010). CHB is typically divided into 4 phases according to liver damage (assessed by serum alanine aminotransferase, ALT), HBeAg, HBsAg and HBV DNA (Table 4.1) (European Association for the Study of the Liver 2017). A fifth phase termed ‘occult infection’ has been described with negative HBsAg and low or undetectable DNA.

Table 4.1 Phases of chronic HBV infection

	HBeAg positive		HBeAg negative	
	chronic infection	chronic hepatitis	chronic infection	chronic hepatitis
ALT	normal	elevated	normal	elevated
HBsAg	high	high/intermediate	low	intermediate
DNA	>10 ⁷ IU/ml	10 ⁴ -10 ⁷ IU/ml	<2.000 IU/ml	>2.000 IU/ml

Adapted from (European Association for the Study of the Liver 2017)

4.1.1.3 Treatment options for CHB

The current standard of care is a treatment with nucleos(t)ide analogues (NUC), such as tenofovir or entecavir, that are reverse transcriptase inhibitors targeting DNA synthesis by the HBV polymerase (Jones et al. 2013). NUC efficiently suppress viral replication, are well tolerated, and rarely induce viral resistance; however, they do not limit the activity or level of nuclear cccDNA and hence production of viral proteins like HBsAg, and do not have the capacity to modulate antiviral immune responses. Thus, NUC rarely achieve functional cure, defined as sustained loss of HBsAg, usually require lifelong treatment, and do not fully prevent the development of HCC (Lok et al. 2017; Maini and Burton 2019; Xia and Liang 2019).

The only other approved pharmacological treatment option for CHB is subcutaneous pegylated IFN α that is typically administered for 48 weeks and aims to elicit dual antiviral and immunomodulatory effects with long-term immunological control; however, pegylated IFN α is only used in highly selected patients as response rates are variable and treatment is associated with a high rate of adverse events (European Association for the Study of the Liver 2017).

For these reasons, the development of novel therapeutic strategies is a high priority to reduce disease burden and improve patient care.

4.1.1.4 Immune landscape in CHB

The critical role of antiviral immunity in viral control is highlighted by the clinical observations of HBV reactivation when patients are immunosuppressed and conversely by HBV elimination after bone marrow transplantation from donors with HBV immunity (Hui et al. 2005; Xunrong et al. 2001). Animal studies, e.g. with T cell depletion in chimpanzees, have pinned down the critical role of CD8⁺ T cells besides B cells in viral control (Thimme et al. 2003). This chapter will discuss HBV-specific CD8⁺ T cells in CHB, the main focus of this thesis, and will briefly highlight the role and function of other immune subsets that are relevant for this thesis: CD4⁺ T cells including T_{fh} and T_{reg}, $\gamma\delta$ T cells and NK cells.

4.1.1.4.1 HBV-specific CD8⁺ T cells

HBV-specific CD8⁺ T cells play an important role in HBV infection and can contribute to viral control by cytolytic and non-cytolytic mechanisms but can also cause immunopathology. CD8⁺ T cells induce lysis of infected hepatocytes via release of granules containing perforin or granzyme A+B and can mediate apoptosis via Fas/FasL interaction (Bertoletti and Ferrari 2016). While cytotoxicity clears virally infected hepatocytes, it also induces liver damage and contributes to disease pathogenesis (Thimme et al. 2003). CD8⁺ T cells can also inhibit viral replication by non-cytopathic mechanisms with minimal cell lysis via the production of the cytokines IFN γ and TNF with direct antiviral effects (see chapter 1.1.2) and via the induction of NO release from monocytes and hepatocytes (Guidotti et al. 1996, 2000; Phillips et al. 2010). Importantly, IFN γ and TNF not only inhibit viral replication but also reduce persistence of cccDNA via induction of deaminases (Xia et al. 2016). CD8⁺ T cells further control residual viral traces for decades after resolved infection (Rehermann et al. 1996).

CD8⁺ T cells specific for HBsAg, HBcAg, polymerase and HBxAg have been described in HBV (Bertoletti and Ferrari 2016). This thesis will primarily focus on HBcAg-specific T cells because HBV genotype was not evaluated in the majority of blood/tissue donors in this study and HBcAg only shows limited variation across different genotypes (Li et al. 2017).

Virus-specific CD8⁺ T cells in CHB are characterized by profound immune exhaustion and depletion with frequencies typically below 0.2% of circulating CD8⁺ T cells and impaired antiviral cytokine production compared to acute HBV infection (Bertoletti and Ferrari 2016; Boni et al. 2007; Ferrari et al. 1990; Penna et al. 1991; Schurich et al. 2013). A variety of mechanisms contribute to CD8⁺ T cell failure in CHB, including prolonged exposure to high levels of viral antigen as well as immunosuppressive regulators (Fisicaro et al. 2020b; Maini and Pallett 2018) (Fig. 4.2).

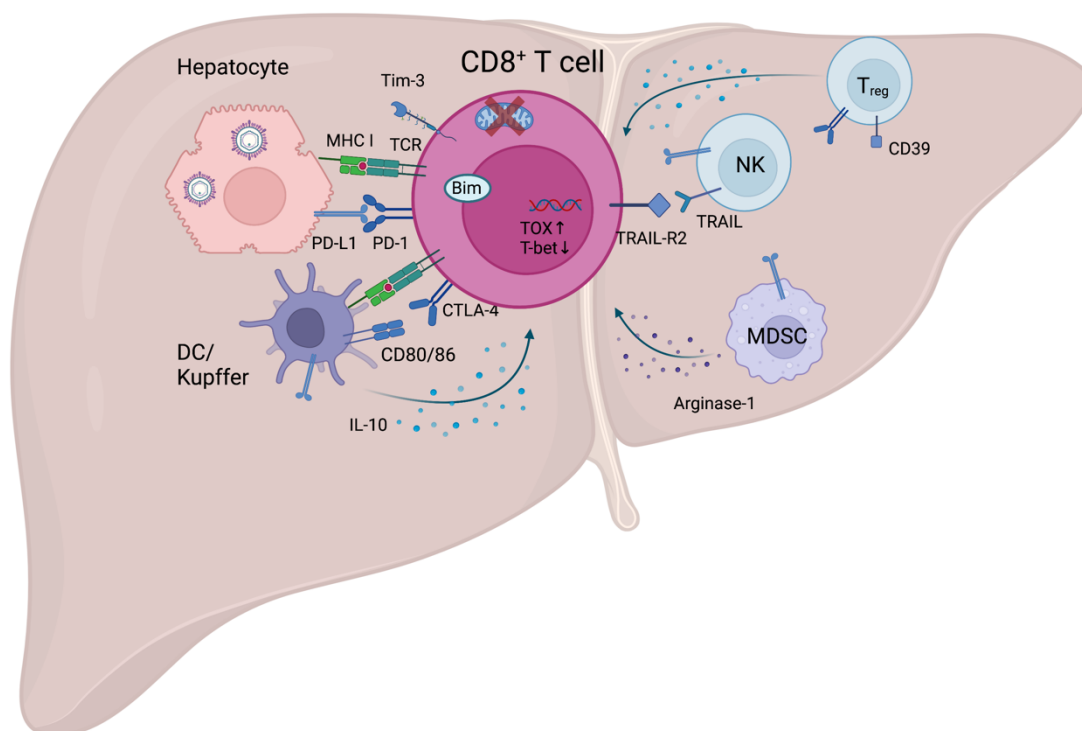


Figure 4.2 Mechanisms contributing to CD8⁺ T cell dysfunction in CHB.

A variety of mechanisms contribute to the dysfunction of HBV-specific CD8⁺ T cells. 1. Immunotolerant liver environment. 2. Impaired priming by hepatocytes. 3. APC (Kupffer cells and DC) inducing T cell tolerance and anergy. 4. Expression of inhibitory checkpoints including PD-1, CTLA-4 and Tim-3. 5. Transcriptional alterations. 6. Expression of Bim inducing apoptosis. 7. Metabolic alterations including defective mitochondria. 8. Immunosuppressive subsets including T_{reg}, MDSC and NK cells. TCR: T cell receptor; MHC: major histocompatibility complex; PD-(L)1: programmed death (ligand) 1; Tim-3: T-cell immunoglobulin and mucin-domain containing-3; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; TOX: thymocyte selection-associated high mobility group box; T-bet: T-box expressed in T cells; TRAIL(-R): TNF-related apoptosis-inducing ligand (receptor); DC: dendritic cells; NK: natural killer cell; MDSC: myeloid-derived suppressor cell; T_{reg}: regulatory T cell; Bim: Bcl-2-interacting mediator of cell death; IL: interleukin.

HBV-specific T cells are enriched in the liver (Fisicaro et al. 2010; Maini et al. 2000) and a key regulator of T cells in HBV is the fact that their priming and antiviral effector function is typically located in the liver. The liver is constantly exposed to high levels of bacteria, lipopolysaccharides (LPS) and food-derived antigens due to receiving the majority of its

blood supply from the gut via the portal vein (Pallett and Maini 2022). In order to prevent pathological inflammation, the liver constitutes a highly immunotolerant organ limiting local immune responses e.g. via its unique architecture, metabolic environment (also see chapter 1.2.3), and recruitment of immunosuppressive cells and tolerogenic APC.

In the liver, CD8⁺ T cells are either primed by professional APC (e.g. DC or liver-resident macrophages known as Kupffer cells) or by infected hepatocytes. Professional APC in the liver can induce T cell tolerance, either directly by promoting T cell deletion and anergy, or indirectly by promoting T_{reg} differentiation and recruitment (Thomson and Knolle 2010). Liver sinusoidal epithelial cells are non-myeloid tissue-resident APC that can crosspresent antigens; however, priming of CD8⁺ T cells by liver sinusoidal epithelial cells induces T cell tolerance with reduced effector cytokine production (Limmer et al. 2000). Liver stromal cells induce the differentiation of tolerogenic IL-10^{hi} IL-12^{low} DC via macrophage colony-stimulating factor and hepatocyte growth factor (Thomson and Knolle 2010).

Kupffer cells can also inhibit DC-induced T cell activation, produce IL-10 and TGF- β upon stimulation and support the immunosuppressive capacity of T_{reg} (Thomson and Knolle 2010); however, a recent study has shown that in a transgenic mouse model, priming by Kupffer cells or intrahepatic DC can yield functional HBV-specific effector T cells, whereas priming by hepatocytes induces T cells dysfunction with limited effector cytokine production and cytotoxicity (Bénéchet et al. 2019; Gehring et al. 2007). The low levels of MHC class I molecules and lack of costimulatory molecules on hepatocytes induces Bcl-2-interacting mediator of cell death (Bim)- and caspase-dependent apoptosis and clonal deletion of antigen-specific CD8⁺ T cells (Bertolino et al. 1998; Holz et al. 2008). The role of Bim-mediated deletion is further highlighted by the finding that Bim is highly expressed in HBV-specific CD8⁺ T cells (Lopes et al. 2008). T cells primed by hepatocytes have a distinct spatiotemporal localisation, a unique transcriptional profile and their function is not enhanced by PD-1 blockade (Bénéchet et al. 2019).

Another factor contributing to the profound exhaustion of virus-specific T cells in CHB is the upregulation of inhibitory receptors including PD-1, CTLA-4, Tim-3 and 2B4 on CD8⁺ T cells and the increased expression of their ligands, e.g. PD-L1 and galactin-9, on hepatocytes and Kupffer cells (Boni et al. 2007; Nebbia et al. 2012; Pallett et al. 2017; Raziorrouh et al. 2010; Schurich et al. 2011). On a transcriptional level, HBV-specific CD8⁺

T cells fail to upregulate T-bet upon antigen stimulation, limiting their antiviral cytokine production (Kurktschiev et al. 2014), and expression of TOX correlates positively with viral load and negatively with IFN γ and TNF production, proliferation and degranulation (Heim et al. 2021).

Recruitment of immunosuppressive cells to the liver during CHB further limits local immunity. Besides the recruitment of T_{reg} described above and in chapter 4.1.1.4.3, MDSC are enriched in the liver of CHB donors and produce arginase-1 that deprives intrahepatic T cells of extracellular arginine and limits their antiviral capacity (Pallett et al. 2015) (also see chapter 1.2.3). Another distinct metabolic feature of HBV-specific T cells are mitochondrial defects discussed in chapter 1.2.3 and 1.2.6.1.

Importantly, CD8⁺ T_{RM} are uniquely adapted to the adverse liver environment and can provide long-lived local protection directly at the site of disease (Pallett et al. 2017, 2020; Pallett and Maini 2022). Human T_{RM} are a distinct immune subset characterized by a prototypic transcriptional profile (T-bet^{low}, Eomes^{low}, BLIMP-1^{hi}, homolog of BLIMP-1 in T cell (Hobit)^{low}) as well as the expression of liver homing and adhesion molecules such as the C-type lectin CD69, the α chain of the integrin α E β 7 (CD103) and chemokine (C-X-C motif) receptor (CXCR) 6+3 that support tissue retention (Pallett et al. 2017). Intrahepatic T_{RM} are expanded in patients with CHB and have the potential to contribute to viral control in HBV (Pallett et al. 2017).

4.1.1.4.2 HBV-specific CD4⁺ T cells in CHB

CD4⁺ T cells in CHB have been less extensively studied than CD8⁺ T cells but they are exposed to the same immunotolerant liver environment and show signs of T cell exhaustion (Buschow and Jansen 2021).

Circulating HBV-specific CD4⁺ T cells are only detectable at low frequencies and highly express PD-1 and Lag-3 (Boni et al. 1998, 2007; Dong et al. 2019; Jacobi et al. 2019; Raziorrouh et al. 2014). In contrast to CD8⁺ T cells, other inhibitory molecules such as CTLA-4 and Tim-3 are only expressed at low levels (Raziorrouh et al. 2014). HBV-specific CD4⁺ T cells have a limited production of Th1-associated cytokines such as IFN γ and TNF and instead show a skewing towards immunosuppressive cytokine, e.g. IL-10 (Jung et al.

1995; Szkaradkiewicz et al. 2003). The important role of CD4⁺ T cells in viral control has been highlighted by the finding that HBV-specific IFN γ production by CD4⁺ T cells is associated with liver damage but also viral clearance during hepatic flares in humans (Wang et al. 2020b) and suppression of viral replication and viral clearance in murine models (Franco et al. 1997; Yang et al. 2010). On the other hand, CD4⁺ T cell depletion did not alter the course of acute HBV infection in the chimpanzee model (Thimme et al. 2003).

4.1.1.4.3 Tfh cells in CHB

Besides the production of antiviral cytokines and help for CD8⁺ T cell responses, a major role of CD4⁺ T cells in viral infections is providing help to activate and regulate B cells. Tfh cells, characterized by the expression of CXCR-5 and PD-1, are a specialized subpopulation of CD4⁺ T cells that is crucial for germinal centre formation, positive selection of B cell clones and the development of an efficient antiviral B cell response. While the majority of Tfh cells reside in lymphoid germinal centres, a subpopulation can be detected in peripheral blood (Locci et al. 2013).

Tfh express a variety of co-stimulatory molecules such as OX40, inducible T cell costimulator (ICOS) and CD40L (CD154). OX-40 is upregulated on activated Tfh, promotes Tfh differentiation and enhances help provided to B cells (Fu et al. 2021; Tahiliani et al. 2017); ICOS is a costimulatory molecule that plays an important role in Tfh differentiation and migration as well as interaction with B cells (Crotty 2019); interaction of CD40L with CD40 on B cells is required for B cell proliferation and differentiation (Crotty 2019). Besides the expression of costimulatory molecules, Tfh function via the production of cytokines such as IL-4, IL-21 and chemokine (C-X-C motif) ligand (CXCL) 13 (Crotty 2019).

In HBV, despite the finding that OX-40 is upregulated on HBV-specific CD4⁺ T cells (Jacobi et al. 2019), Tfh responses are dysregulated, promoting HBV persistence (Wang et al. 2018a). The importance of Tfh cells has further been highlighted by the finding that the frequency of circulating Tfh cells is increased in patients with NUC-induced viral clearance (Li et al. 2013).

4.1.1.4.4 CD4⁺ T_{reg} in CHB

The role of T_{reg} in CHB remains not fully understood with some studies reporting an increased frequency of circulating T_{reg} in CHB compared to healthy controls and acute or resolved HBV, contributing to disease progression (Park et al. 2016; Stoop et al. 2005; TrehanPati et al. 2011; Xu et al. 2006), whereas other studies show no alterations (Franzese et al. 2005). These discrepant findings are likely due to differences in the patient cohorts (treatment status, disease stage) and phenotypic markers used to identify T_{reg}. Overall, the majority of studies report an increased T_{reg} frequency in the periphery. The role of T_{reg} in CHB is further supported by findings in a murine HBV model where T_{reg} numbers in the liver are increased and T_{reg} depletion enhances antiviral CD8⁺ T cell function (Dietze et al. 2016).

T_{reg} limit antiviral immune responses by a variety of mechanisms including production of inhibitory cytokine such as IL-10, expression of inhibitory receptors such as CTLA-4, and expression of CD39. The molecules associated with T_{reg} activation and inhibitory potential relevant for this thesis have been briefly summarized in Table 4.2: CD69, ICOS, glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR), T cell immunoglobulin with ITIM domain (TIGIT), CTLA-4 and CD39.

Table 4.2 Molecules associated with T_{reg} immunosuppression

Molecule	Main effect on T _{reg}
CD69	T cell activation marker; associated with enhanced suppressive function of T _{reg} (Han et al. 2009; Radstake et al. 2009; Yu et al. 2018).
ICOS	Increased proliferation, survival and immunosuppressive function, e.g. IL-10 production (Li and Xiong 2020).
GITR	T _{reg} expansion, activation and immunosuppressive function, e.g. IL-10 production (Ronchetti et al. 2015); however, GITR agonist can lead to Th1 conversion and loss of suppressive function (Amoozgar et al. 2021).
TIGIT	Controls functional stability of T _{reg} (Lucca et al. 2019); enhances T _{reg} suppressive function (Joller et al. 2014); induces IL-10 production by DC through binding to CD155 (Yu et al. 2009).

Molecule	Main effect on T _{reg}
CTLA-4	High affinity for the CD28 ligands CD80 and CD86 on DC, resulting in a competition for ligands; CD80/CD86 removal via transendocytosis (Walker and Sansom 2015).
CD39	Conversion of ATP and into AMP followed by conversion into adenosine by CD73. Adenosine suppresses antitumour immune function and induces T _{reg} and MDSC (de Andrade Mello et al. 2017). Also see chapter 1.2.4.3.1

4.1.1.4.5 $\gamma\delta$ T cells

In contrast to ‘classical’ $\alpha\beta$ T cells, antigen recognition by $\gamma\delta$ T cells is not MHC restricted, which renders them an attractive novel immunotherapeutic target, especially in cancer. They have innate-like and adaptive-like features and are stimulated by a variety of ligands including, MHC class I chain related molecules A+B (MICA+B), phosphoantigens, the MHC-related protein 1 (MR1) and members of the CD1 glycoprotein family presenting lipid-based antigens (Chien et al. 2014; Le Nours et al. 2019; Van Rhijn and Le Nours 2021).

In CHB, the role of $\gamma\delta$ T cells is only partially understood. In a recent study by Chang et al., the frequency of $\gamma\delta$ T cells was not altered but $v\delta 1$ and $v\delta 2$ subsets displayed distinct transcriptional profiles during acute or chronic infection (Chang et al. 2019). Cytokine production of $v\delta 2$ $\gamma\delta$ T cells was preserved in CHB but reduced in acute HBV and inversely correlated with hepatic injury while $v\delta 1$ $\gamma\delta$ T cell function was not altered.

4.1.1.4.6 NK cells

NK cells are innate immune cells identified by the expression of CD56, the neural cell adhesion molecule (N-CAM), that play a dual role in CHB. On the one hand, they can potentially exert an antiviral effect via the production of antiviral cytokines (e.g. IFN γ) as shown in a murine HBV model (Yang et al. 2010), antibody-independent and antibody-dependent cell cytotoxicity (ADCC) e.g. by release of cytolytic molecules such as granzyme B and perforin, and by interaction with death receptors (e.g. TNF-related apoptosis-inducing ligand (TRAIL)-receptor and Fas) on infected hepatocyte; on the other hand, NK cells can

restrict antiviral T cells during natural infection and in settings of therapeutic vaccination, e.g. via upregulation of PD-L1, TRAIL and NKG2D (Diniz et al. 2022; Huang et al. 2017b; Maini and Peppas 2013). Similar to T cells, NK cells in CHB display features of exhaustion, such as impaired antiviral cytokine production and an upregulation of TOX (Marotel et al. 2021; Peppas et al. 2010; Sun et al. 2012; Tjwa et al. 2011).

4.1.2 SARS-CoV-2 infection

By August 2022, SARS-CoV-2 has caused an estimated 580 million infections and nearly 6.5 million deaths worldwide since the beginning of the coronavirus diseases 2019 (COVID-19) pandemic (World Health Organization 2022). The clinical course of acute SARS-CoV-2 infection is highly variable, ranging from asymptomatic disease to lethal acute respiratory distress syndrome (ARDS) (Lamers and Haagmans 2022). Severe disease is most common in patients with old age, male sex and obesity, and is typically associated with systemic hyperinflammation and a ‘cytokine storm’ of proinflammatory cytokines such as IL-6, IL-1 β and TNF mainly produced by endothelial cells, monocytes and macrophages (Docherty et al. 2020; Lamers and Haagmans 2022). SARS-CoV-2 is primarily a respiratory infection transmitted via respiratory aerosols and droplets but can also cause non-pulmonary disease including gastrointestinal, cardiac and neurological symptoms (Lamers and Haagmans 2022; Solomon 2021).

SARS-CoV-2 is a single-stranded RNA virus from the *Coronaviridae* family (Fig. 4.3). The viral RNA encodes for structural proteins (membrane, nucleocapsid, spike, envelope) and non-structural proteins composing the viral replication and transcription complex (V'kovski et al. 2021). SARS-CoV-2 entry is mediated via binding of the S1 subunit of the Spike glycoprotein to angiotensin converting enzyme 2 (ACE-2) and consecutive cleavage by the transmembrane protease serine 2 (TMPRSS2), facilitating the fusion of the viral and target cell membrane (Hoffmann et al. 2020). Alternatively, SARS-CoV-2 can enter the target cell via ACE-2 mediated endocytosis (Jackson et al. 2022).

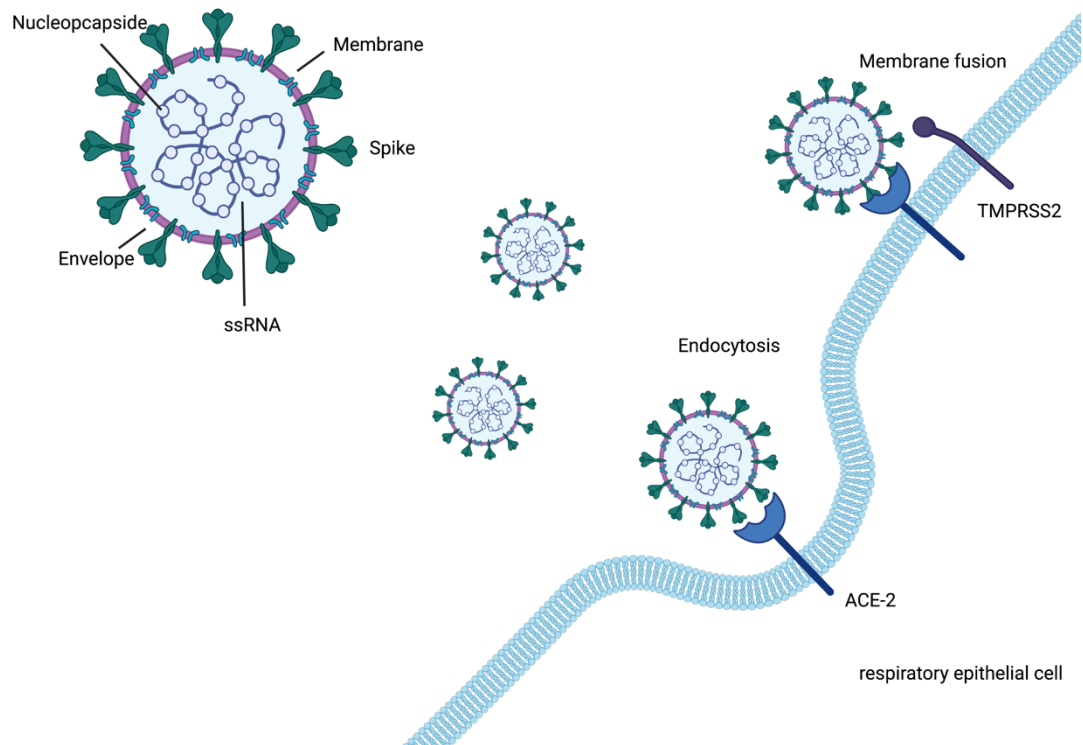


Figure 4.3 SARS-CoV-2.

SARS-CoV-2 consists of 4 structural proteins: Spike, Membrane, Envelope and Nucleocapsid. Entry into the target cell is mediated via binding of the Spike subunit S1 to ACE-2 and consecutive endocytoses or TMPRSS2-facilitated membrane fusion. ss RNA: single stranded RNA; ACE-2: angiotensin converting enzyme 2; TMPRSS2: transmembrane protease serine 2.

Preventive vaccines against SARS-CoV-2 have been developed with unprecedented speed and it has been estimated that within the first year of vaccine rollout 14.4 million deaths had been prevented (Watson et al. 2022); however, with the emergence of viral variants of concern (VOC) vaccine effectiveness has decreased (Andrews et al. 2022). Therefore, therapeutic strategies for patients with breakthrough infections or for those that are unvaccinated are urgently needed. Current therapeutic options primarily include antiviral agents such as remdesivir and ritonavir-boosted nirmatrelvir as well as SARS-CoV-2 monoclonal antibodies for specific clinical indications (NIH 2022). According to current knowledge, available antiviral agents do not have the capacity to modulate antiviral immune responses.

4.1.2.1 SARS-CoV-2-specific T cells

Antiviral CD8⁺ and CD4⁺ T cells are critical in controlling SARS-CoV-2 infection as shown in animal models (Israelow et al. 2021; McMahan et al. 2021) and highlighted by the observation in humans that an early induction of functional, IFN γ -producing SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells is associated with mild disease (Moderbacher et al. 2020; Tan et al. 2021). Conversely, a reduction of effector memory CD8⁺ T cells is a feature of severe infection (Notarbartolo et al. 2021); however, it is currently unclear whether this is cause or effect. Due to amino acid conservation between different human coronaviruses, cross-reactive T cells have been detected in pre-pandemic samples, likely induced by previous infection with common cold coronaviruses, and in donors with previous SARS-CoV-1 infection (Le Bert et al. 2020; Lineburg et al. 2021). Importantly, pre-existing, cross-reactive T cells specific for non-structural proteins expand *in vivo* and have been associated with viral clearance of subclinical SARS-CoV-2 infection without PCR positivity or antibody conversion (abortive infection), pinning down the critical role of T cells in controlling SARS-CoV-2 (Swadling et al. 2022).

CD4⁺ and CD8⁺ T cell responses are detectable against all major structural and non-structural proteins and immune responses are typically CD4⁺ dominated (Grifoni et al. 2020; Moss 2022; Peng et al. 2020; Reynolds et al. 2020; Tarke et al. 2021). SARS-CoV-2-specific memory T cells may provide protection from reinfection and are detectable for up to 300 days post infection (Jung et al. 2021). Besides the direct antiviral function of T cells, T_{fh} cells contribute to the humoral immune response and correlate with neutralising antibody levels (Boppana et al. 2021).

T cells in SARS-CoV-2 show signs of metabolic alterations, e.g. increased OXPHOS and ROS that can persist for over a month after disease onset in patients with severe disease (Bergamaschi et al. 2021). Effector memory CD4⁺ T cells in SARS-CoV-2 upregulate HIF1 α , suggesting an adaptation to hypoxia (Laing et al. 2020). Metabolomic analysis revealed a downregulation of lipid metabolism that is most profound in severe disease (Shen et al. 2020; Su et al. 2020b).

While T cell exhaustion typically occurs in settings with chronic antigen exposure, new data are emerging that suggest features of exhaustion and immune dysfunction in acute SARS-

CoV-2 infection (Chen and Wherry 2020). Marked lymphocytopenia is seen in severe disease with a disproportionate loss of IFN γ -producing CD4⁺ T cell subsets compared to Th2 T cells and T_{reg} (Laing et al. 2020). Further indications of T cell dysfunction/exhaustion in SARS-CoV-2 include a downregulation of TCR signalling molecules in effector memory CD4⁺ T cells (Laing et al. 2020) and reduced IFN γ production after polyclonal stimulation compared to healthy controls (Mazzoni et al. 2020). Additionally, impaired cytotoxicity is a feature of severe disease (Mazzoni et al. 2020).

Effector memory CD8⁺ T cells in COVID-19 are prone to apoptosis and upregulate the expression of inhibitory receptors associated with terminal differentiation and exhaustion, e.g. PD-1, Tim-3, Lag-3 and CTLA-4 (Laing et al. 2020). It is still being discussed whether this might be a feature of T cell activation instead of exhaustion as some studies claim that PD-1⁺ CD8⁺ T cells have a higher capacity of SARS-CoV-2-specific IFN γ production; however this was evaluated in the memory phase after convalescence and might not reflect the exhaustion/activation state during acute infection (Rha et al. 2021). Future studies will be necessary to fully determine the role of T cell dysfunction and exhaustion in SARS-CoV-2 pathogenesis.

4.2 Hypotheses and research questions

Hypothesis I: ACAT inhibition boosts CD8⁺ T cell function after TCR-dependent, polyclonal stimulation.

- Do human T cells in the blood and liver express SOAT1/2?
- Is the finding from Yang et al. that ACAT inhibition boosts IFN γ production by human CD8⁺ T cells reproducible in a larger cohort?
- What is the optimal concentration of Avasimibe (ACAT inhibitor) to achieve immune boosting without cell death caused by direct cytotoxic drug effects?
- What is the optimal *in vitro* treatment strategy to boost polyclonal TCR-dependent CD8⁺ T cell function?

Hypothesis II: ACAT inhibition enhances the antiviral function of T cells in CHB.

- What is the optimal *in vitro* treatment strategy to boost HBV-specific CD8⁺ T cell function?
- Does ACAT inhibition expand functional HBV-specific peripheral CD8⁺ and CD4⁺ T cells from CHB donors?
- Do donor characteristics influence treatment response?
- Does ACAT inhibition boost the function of HBV-specific CD8⁺ and CD4⁺ T cells directly *ex vivo* from the immunotolerant liver environment?

Hypothesis III: ACAT inhibition affects other peripheral immune cell subsets in CHB.

- Immunosuppressive T cells: Does ACAT inhibition modulate T_{reg} proliferation, activation and/or immunosuppressive potential?
- Non-conventional T cells: Does ACAT inhibition affect proliferation and/or function of $\gamma\delta$ T cells?
- Innate immune cells: Does ACAT inhibition affect proliferation and/or function of NK cells?

Hypothesis IV: ACAT inhibition boosts the function of SARS-CoV-2-specific T cells during acute infection in unvaccinated donors.

- What is the SARS-CoV-2-specific T cell profile during acute infection focusing on Spike- and Membrane-specific T cells?
- Does ACAT inhibition enhance the function of SARS-CoV-2-specific T cells during acute infection?
- Do donor characteristics influence treatment response?

Hypothesis V: ACAT inhibition does not trigger cytotoxic or off-target immune responses.

- Does ACAT inhibition boost CD8⁺ T cell cytotoxicity?
- Does ACAT inhibition affect unstimulated T cells?
- Does ACAT inhibition boost the function of memory T cells after acute infection?

4.3 Results

4.3.1 Identification of CD8⁺ and CD4⁺ T cells in PBMC

To investigate the effect of ACAT inhibition on human T cells, I isolated PBMC from heparinized blood by density gradient centrifugation (see chapter 3.3) and performed a multicolour fluorescent antibody staining for flow cytometry (see chapter 3.8).

Viable CD8⁺ and CD4⁺ T cells were identified by a stringent sequential gating strategy (Fig. 4.4). First, lymphocytes were identified by their size (forward scatter area, FSC-A) and granularity (side scatter area, SSC-A). Next, doublets (FSC-A vs FSC height, FSC-H) and non-viable cells (Live/Dead stain⁺) were excluded. All samples with a viability below 50% were excluded from further analysis. Next, T cells were identified by CD3 expression followed by identification of CD8⁺CD4^{neg} and CD8^{neg}CD4⁺ T cells. This gating strategy was applied in all experiments using PBMC.

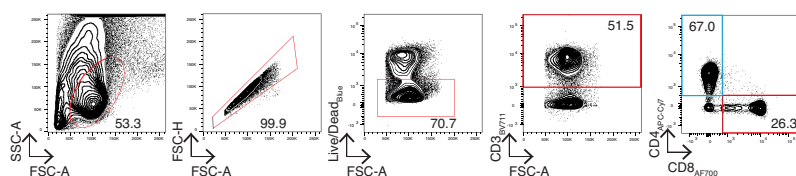


Figure 4.4 Gating strategy for PBMC.

Representative flow cytometry plots of the gating strategy to identify T cells in PBMC: lymphocytes, single cells, live cells, CD3⁺, CD4⁻/CD8⁺ (red) or CD4⁺/CD8⁻ (blue).

4.3.2 Human CD8⁺ and CD4⁺ T cells express SOAT1 in blood and liver

To test whether ACAT inhibition has the potential of affecting cholesterol esterification in human peripheral and intrahepatic T cells, I first assessed whether RNA of SOAT1 (ACAT1) and SOAT2 (ACAT2), the genes encoding the cholesterol esterification enzymes, were

present in human CD8⁺ and CD4⁺ T cells by probing a publicly available single cell RNA sequencing (scRNAseq) dataset (Zheng et al. 2017). SOAT1 RNA transcripts were detectable in an equal percentage of peripheral and intrahepatic CD8⁺ (39% and 38%) as well as CD4⁺ (both 37%) T cells (Fig. 4.5a,b) and mean RNA levels were comparable between CD8⁺ and CD4⁺ T cells and between intrahepatic and circulating T cells (Fig. 4.5c). As expected, SOAT2 was only expressed by a minority of T cells (Fig. 4.5a,b), in line with its predominant expression in hepatocytes and enterocytes (Cases et al. 1998; The Human Protein Atlas 2022).

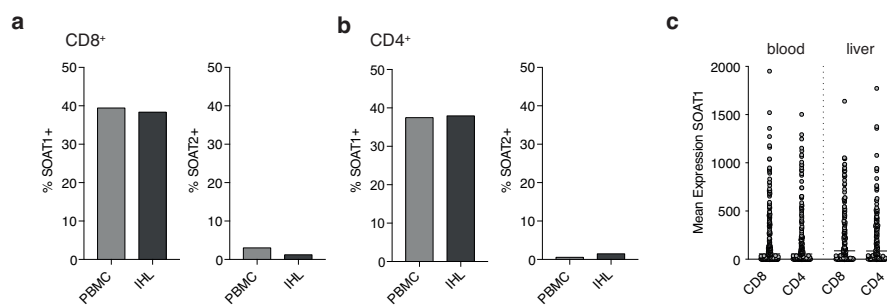


Figure 4.5 Human CD8⁺ and CD4⁺ T cells in blood and liver express SOAT1.

(a+b) Frequency of CD8⁺ **(a)** and CD4⁺ **(b)** T cells with any detectable SOAT1 and SOAT2 gene transcripts (no minimum cut off) in scRNAseq in the blood (PBMC; Range of SOAT1 transcript expression: CD8⁺: 1-1951; CD4⁺: 1-1502. Range of SOAT2 transcript expression: CD8⁺: 1-915; CD4⁺: all 1 transcript) and liver (intrahepatic leukocytes, IHL. Range of SOAT1 transcript expression: CD8⁺: 1-1639; CD4⁺: 1-1772. Range of SOAT2 transcript expression: CD8⁺: 1-203; CD4⁺: 1-370). **(c)** Mean SOAT1 expression in T cells with any detectable SOAT1 transcripts in scRNAseq (no minimum cut off; blood: CD8 n=222 and CD4 n=193; liver: CD8 n=158 and CD4 n=154). Data derived from publicly available scRNAseq data (GSE98638). Cell populations were sorted prior to sequencing based on flow cytometric identification of CD3⁺CD8⁺ (CD8⁺ T cells) and CD3⁺CD4⁺CD25⁻ (CD4⁺ T cells) expression. P values determined by Fisher's exact test **(a,b)** and Kruskal-Wallis test **(c)**. *Data analysis assisted by Leo Swadlow.*

4.3.3 Optimization of ACAT inhibition treatment strategy to boost CD8⁺ T cell function after TCR-dependent polyclonal stimulation

To study the effect of ACAT inhibition on CD8⁺ T cells, I first aimed to identify a treatment strategy that enhances T cell function without direct cytotoxic drug effects on T cells.

Yang et al. have previously shown that a 12h treatment with ACAT inhibition (5-15 μ M Avasimibe) of phytohaemagglutinin-stimulated PBMC prior to a 24h stimulation with anti-

CD3 and anti-CD28 antibodies enhances the function of human CD8⁺ T cells from three healthy donors (Yang et al. 2016). The overall aim of this project was to assess the effect of ACAT inhibition on virus-specific CD8⁺ T cells and a short-term expansion is usually necessary to study these low frequency T cells in PBMC (Boni et al. 2007; Fiscicaro et al. 2017; Schurich et al. 2013, 2016). I therefore adapted the treatment protocol by Yang et al. to mimic the short-term expansion that I planned to perform with virus-specific T cells. PBMC were stimulated with anti-CD3 and anti-CD28 antibodies, expanded for 5d and treated with increasing concentrations of the ACAT inhibitor Avasimibe (1-20 μ M) for 12h prior to restimulation (schematic Fig.4.6a; Protocol 1). In all experiments throughout this thesis, conditions without ACAT inhibitor treatment received equivalent concentrations of DMSO to rule out any effects caused by the solvent.

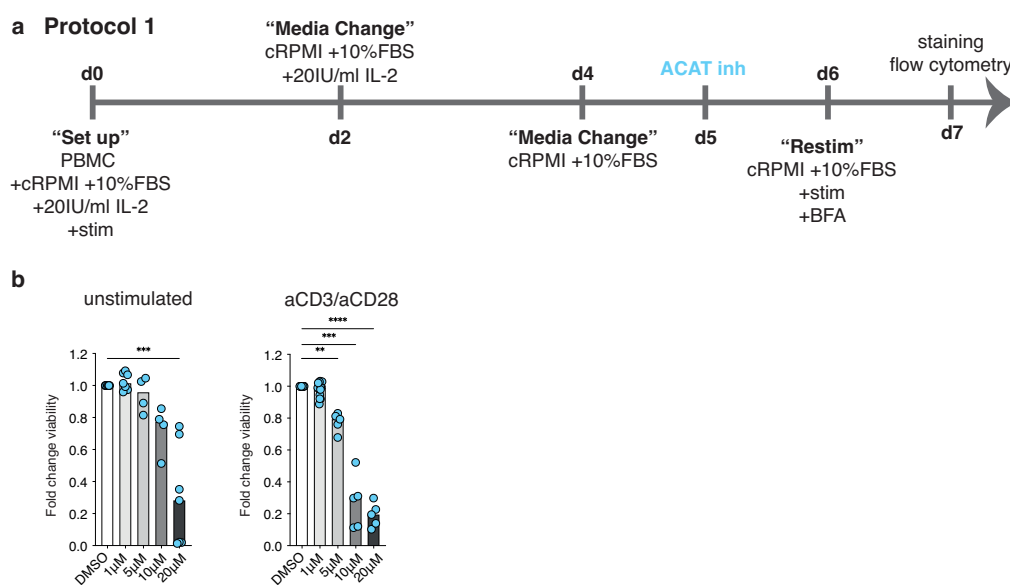


Figure 4.6 Cytotoxic effects of ACAT inhibition.

(a) Treatment strategy (Protocol 1) to evaluate direct cytotoxic effect of ACAT inhibition on PBMC. **(b)** Flow cytometric assessment of lymphocyte viability with increasing doses of the ACAT inhibitor (ACAT inh) Avasimibe (1 μ M-20 μ M) normalized to DMSO without stimulation (DMSO n=12; 1 μ M n=7; 5 μ M n=4; 10 μ M n=4; 20 μ M n=7) and after stimulation with anti-CD3/anti-CD28 antibodies (DMSO n=14; 1 μ M n=13; 5-20 μ M n=5). P values determined by Kruskal-Wallis test. Bars: mean.

Treatment with ACAT inhibition had a dose-dependent cytotoxic effect compared to DMSO that was particularly profound after anti-CD3/anti-CD28 stimulation (Fig. 4.6b). A low dose of 1 μ M Avasimibe did not induce cytotoxicity and in all further experiments 1 μ M Avasimibe or less were used as indicated in the respective methods sections.

To assess the immune boosting effect of ACAT inhibition with this lower dose of ACAT inhibitor, I focused on the production of IFN γ as a readout of CD8⁺ T cell function. Despite applying a lower dose than previously published (Yang et al. 2016), ACAT inhibition significantly enhanced the production of IFN γ by CD8⁺ T cells after TCR-dependent polyclonal stimulation when using the same stimulation protocol as described above (Fig. 4.7a; Protocol 1).

Next, I investigated the role of different treatment timepoints on the CD8⁺ T cell boosting effect of ACAT inhibition. Treatment with ACAT inhibition at the start of the experiment (d0; Fig. 4.7b; Protocol 2), at the start and 16h before restimulation (d0+d5; Fig. 4.7c; Protocol 3) or at the time of restimulation (d6; Fig. 4.7d; Protocol 4) enhanced T cell function in some donors but did not reach significance in the whole cohort. In contrast, a repetitive administration of ACAT inhibitors every 2d throughout the experiment significantly increased CD8⁺ T cell IFN γ production (Fig. 4.7e; Protocol 5).

Taken together, I identified a non-cytotoxic ACAT inhibitor concentration and two treatment protocols that boosted CD8⁺ T cell IFN γ production after TCR-dependent polyclonal stimulation.

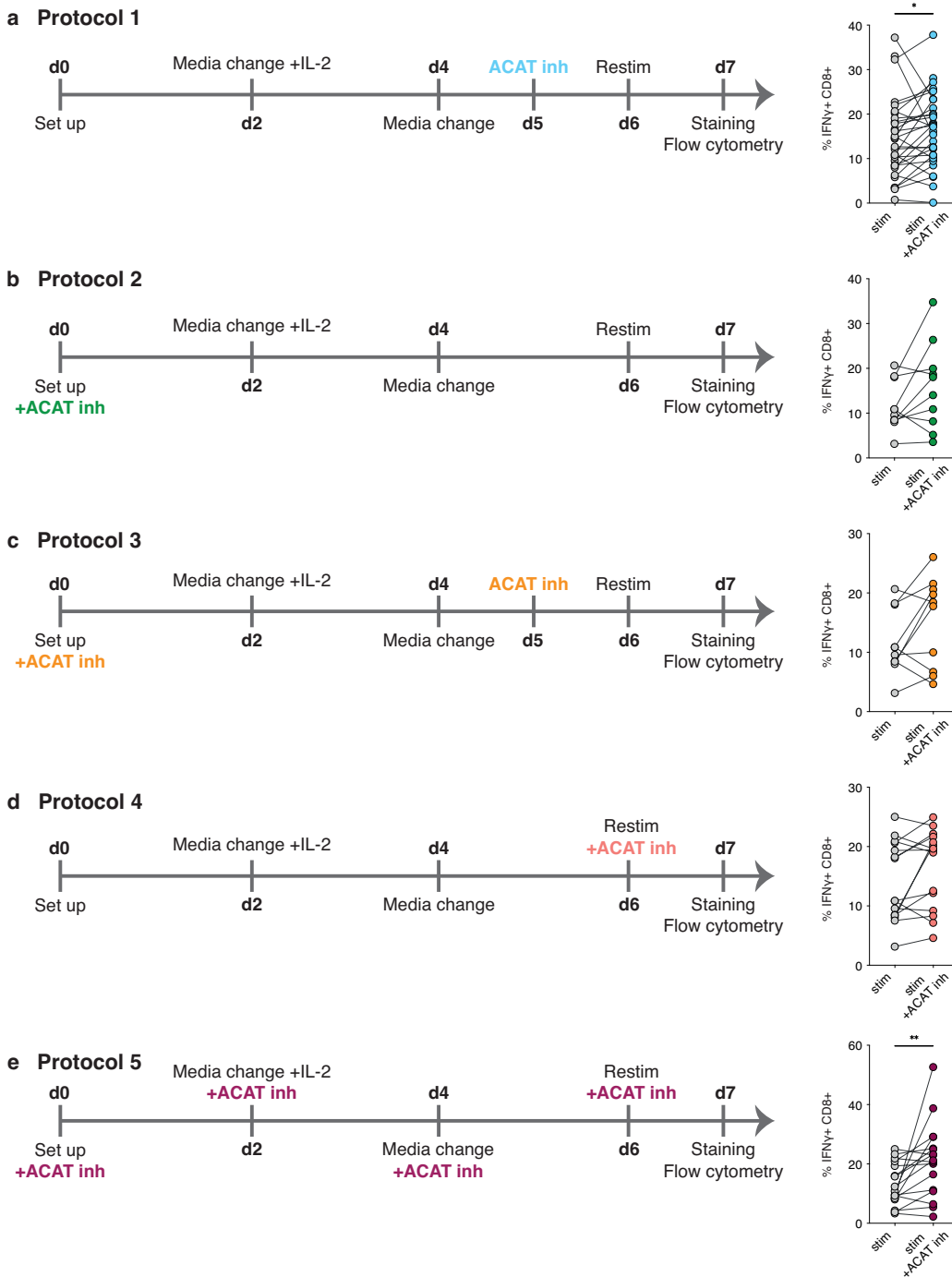


Figure 4.7 Comparison of different treatment protocols for TCR-dependent polyclonal stimulation. **(a-e)** PBMC were stimulated with anti-CD3/anti-CD28 antibodies \pm ACAT inhibitor (Avasimibe) as indicated in the figure. Assessment of IFN γ production by CD8 $^+$ T cells via flow cytometry. **(a)** Protocol 1, n=30; **(b)** Protocol 2, n=10; **(c)** Protocol 3, n=10; **(d)** Protocol 4, n=15; **(e)** Protocol 5, n=17. P values determined by Wilcoxon matched-pairs signed rank test.

4.3.4 ACAT inhibition in chronic HBV infection

4.3.4.1 Optimization of ACAT inhibition treatment strategy to boost HBV-specific CD8⁺ T cells

HBV-specific CD8⁺ T cells are highly dysfunctional and are only detectable in low frequencies in peripheral blood (Boni et al. 2007; Fiscaro et al. 2017, 2020b; Schurich et al. 2013, 2016) (see chapter 4.1.1.4.1). To study these low frequency T cells, I expanded PBMC from CHB donors antigen-specifically in a short-term culture by stimulation with an overlapping peptide pool spanning the whole amino acid sequence of the HBcAg (HBc pep; see methods chapter 3.6.3 for details) and assessed the HBV-specific IFN γ production by flow cytometry (example flow cytometry staining Fig. 4.8a).

Sequential gates to identify IFN γ -producing CD8⁺ T cells were first established on the unstimulated condition and then applied to all other samples from the same donor to ensure a consistent gating strategy. In all summary data throughout this thesis, the non-specific cytokine production measured in the unstimulated condition was subtracted from the cytokine production measured in the stimulated condition to identify the antigen/stimulation-specific cytokine production.

To establish an ACAT inhibitor treatment strategy that boosts HBV-specific CD8⁺ T cell function, I assessed the two most promising treatment strategies (protocol 1: treatment on d5; protocol 5: repetitive treatment every 2d) described in the previous chapter for polyclonally stimulated T cells.

Both strategies enhanced the HBV-specific CD8⁺ T cell function compared to a DMSO control in selected donors (Fig. 4.8a-c). The fold increase of IFN γ production (Fig. 4.8b) and the percentage of donors responding to ACAT inhibition (response defined as increased or *de novo* cytokine production; Fig. 4.8c) tended to be higher when PBMC were treated repetitively (protocol 5) but the difference did not reach statistical significance in this small cohort. Taken together, 10 out of 15 (67%) donors showed increased or *de novo* IFN γ production in at least one of the two treatment protocols.

Within the group of treatment responders, HBV-specific IFN γ production was significantly higher when ACAT inhibition was given repetitively (protocol 5; Fig. 4.8d) with 80% of donors showing the highest response with protocol 5, 10% with protocol 1 and 10% had the same frequency of IFN γ ⁺ HBV-specific CD8⁺ T cells with both protocols.

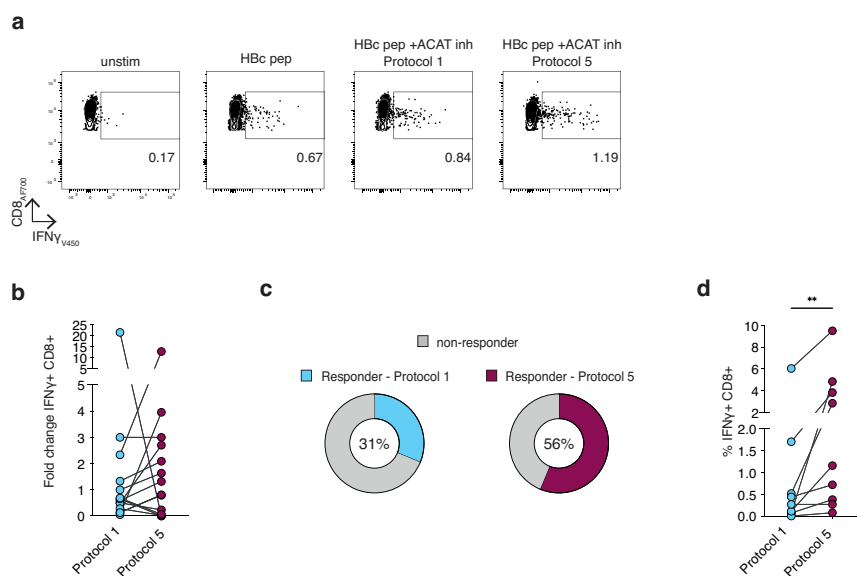


Figure 4.8 Comparison of different treatment protocols for HBV-specific CD8⁺ T cells.

(a-d) PBMC from CHB donors were stimulated with HBC peptide pool (HBC pep) \pm ACAT inhibition (Avasimibe) for 7d and IFN γ production was detected via flow cytometry. **(a)** Representative flow cytometry plot for HBV-specific IFN γ production by CD8⁺ T cells. **(b)** Fold increase of HBV-specific IFN γ production by CD8⁺ T cells with protocol 1 (ACAT inhibition on d5) and protocol 5 (ACAT inhibition every 2d) compared to DMSO (n=15). **(c)** Frequency of donors responding to ACAT inhibition in protocol 1 (blue, left) and protocol 5 (red, right). Frequency of responders indicated by coloured fraction of each doughnut chart and % in the chart centre. Response defined as increased or *de novo* IFN γ production. **(d)** HBV-specific IFN γ production by CD8⁺ T cells in donors that responded to ACAT inhibition with either protocol (n=10). P values determined by Wilcoxon matched-pairs signed rank test **(b,d)** or Fisher's exact test **(c)**.

In summary, repetitive ACAT inhibitor administration (protocol 5) resulted in the best treatment outcome (higher % of donors responding, higher cytokine production) and I applied this strategy to all further experiments assessing the effect of ACAT inhibition on PBMC unless stated otherwise in the methods or figure legend.

4.3.4.2 ACAT inhibition boosts peripheral HBV-specific CD8⁺ T cells

After establishing an optimized ACAT inhibition treatment protocol, I assessed the effect of targeting cholesterol esterification by ACAT inhibition on HBV-specific CD8⁺ T cell function in a larger cohort of CHB donors mainly focusing on IFN γ production as a key antiviral cytokine.

ACAT inhibition enhanced the HBV-specific CD8⁺ T cell IFN γ production in PBMC from the majority of CHB donors (Fig. 4.9a; responders highlighted in red in heatmap below the histogram) and induced *de novo* responses in selected donors without detectable IFN γ production when stimulated with peptide alone (Fig. 4.9b).

As shown for other *in vitro* (Bengsch et al. 2014; Fiscaro et al. 2017; Schurich et al. 2013) and *in vivo* (Gane et al. 2019) immunotherapeutic strategies, responses to ACAT inhibition were heterogeneous and in some donors HBV-specific cytokine production after ACAT inhibition was diminished. However, in the overall cohort, ACAT inhibition significantly increased the percentage and fold increase of cytokine production by HBV-specific CD8⁺ T cells (Fig. 4.9c). Of note, ACAT inhibition did not perturb cell viability in this cohort with the chosen treatment strategy (Fig. 4.9d).

Treatment with a different ACAT inhibitor (K-604; ACAT-1 specific) resulted in comparable frequency of HBV-specific IFN γ production to Avasimibe (ACAT-1+2 inhibitor) highlighting the reproducibility of my findings and confirming the immune boosting effect of inhibiting ACAT (Fig. 4.9e).

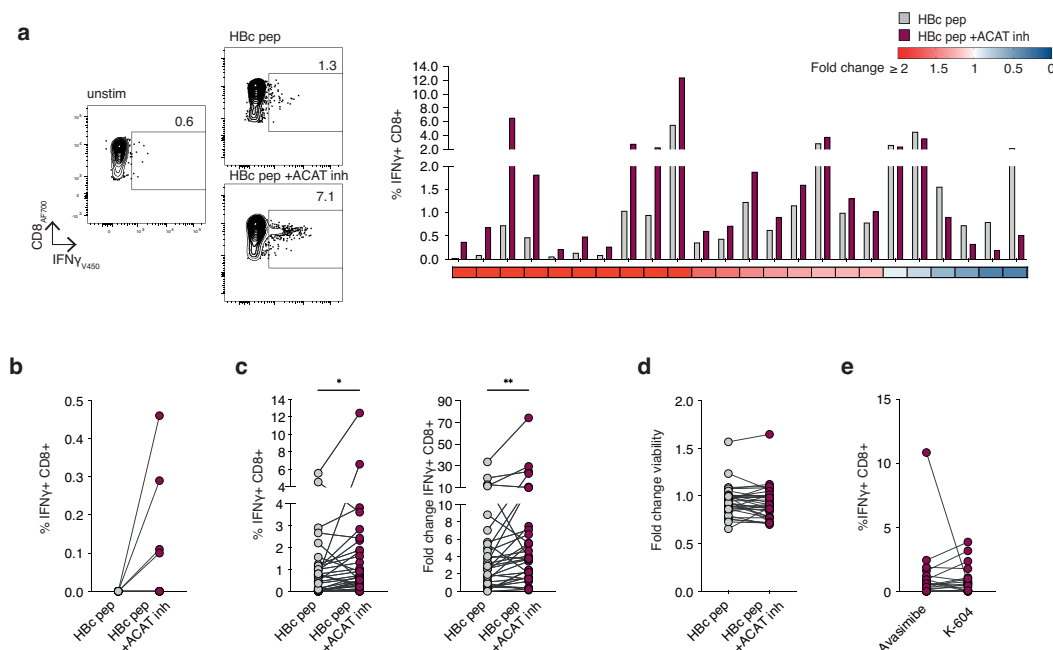


Figure 4.9 ACAT inhibition enhances IFN γ production of HBV-specific CD8⁺ T cells.

(a-d) PBMC from CHB donors were stimulated with HBc pep \pm ACAT inhibition (Avasimibe) for 7d and HBV-specific IFN γ production of CD8⁺ T cells was detected by flow cytometry. **(a)** Representative flow cytometry plot and summary data for each donor with detectable pre-existing HBc-specific CD8⁺ T cell response (n=24). Two matched bars for each donor: grey bar: IFN γ production in the presence of DMSO; red: IFN γ production in the presence of ACAT inhibition. Heatmap indicates fold change of IFN γ production with ACAT inhibition for each donor compared to DMSO. **(b)** *De novo* IFN γ production of CD8⁺ T cells in donors with no detectable baseline response to HBc pep (n=10). **(c)** Left: Summary data for HBV-specific IFN γ production by CD8⁺ T cells. Right: Fold change of IFN γ production after stimulation with HBc pep normalized to unstimulated control (n=34). **(d)** Fold change viability of PBMC stimulated with HBc pep \pm ACAT inhibition normalized to unstimulated control (n=34). **(e)** HBV-specific IFN γ production of CD8⁺ T cells with ACAT inhibition (Avasimibe or K-604; n=21). P values determined by Wilcoxon matched-pairs signed rank test.

ACAT inhibition also significantly rescued the HBV-specific production of other antiviral cytokines such as TNF (Fig. 4.10a) and the increase of TNF production correlated with the increase of IFN γ production (Fig. 4.10b).

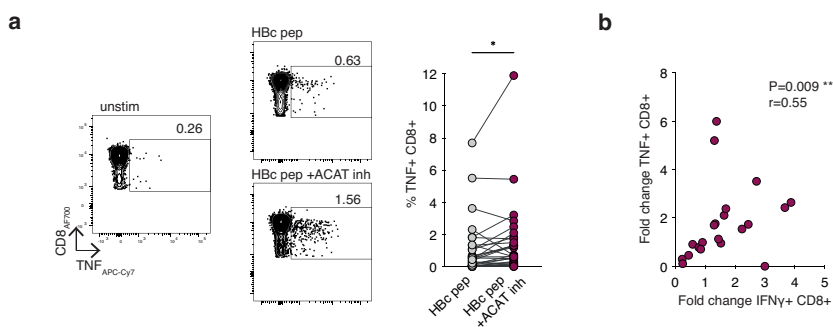


Figure 4.10 ACAT inhibition enhances TNF production by HBV-specific CD8⁺ T cells.

(a,b) PBMC from CHB donors were stimulated with HBc pep \pm ACAT inhibition (Avasimibe) for 7d and HBV-specific cytokine production by CD8⁺ T cells was detected by flow cytometry. **(a)** Representative flow cytometry plot and summary data showing the % of HBV-specific TNF production by CD8⁺ T cells. **(b)** Correlation of fold increase TNF and IFN γ production with ACAT inhibition normalised to DMSO. P values determined by Wilcoxon matched-pairs signed rank test **(a)** and Spearman correlation **(b)**.

ACAT inhibition not only recovered dysfunctional, pre-existing HBV-specific CD8⁺ T cells, but also induced a proliferation of HBV-specific CD8⁺ T cells indicated by increased CFSE dilution (Fig. 4.11a-c) (also see chapter 3.8.6). CFSE dilution was assessed in two ways: first, by frequency of CFSE^{low} HBV-specific CD8⁺ T cells (Fig. 4.11a, left). The correct gating was established on the global CD8⁺ T cell fraction in the unstimulated condition with low baseline proliferation and then applied to HBV-specific IFN γ ⁺ CD8⁺ T cells to ensure consistency between treatment conditions. Second, measurement of CFSE MFI on HBV-specific CD8⁺ T cells (Fig. 4.11b, left). The induction of proliferation was peptide-specific as IFN γ ^{neg} CD8⁺ T cells not responding to peptide stimulation did not show an increase of CFSE dilution with ACAT inhibition (Fig. 4.11a+b right). As seen for the enhancement of cytokine production, the increased proliferation was comparable between two different ACAT inhibitors (Avasimibe and K-604; Fig. 4.11c).

In line with the increased proliferation of HBV-specific CD8⁺ T cells, ACAT inhibition also increased expression of the proliferation marker Ki-67 (Gerdes et al. 1984) after TCR-dependent polyclonal stimulation in a preliminary experiment with 3 donors (Fig. 4.11d).

Taken together, ACAT inhibition enhanced the function and proliferation of peripheral HBV-specific CD8⁺ T cells.

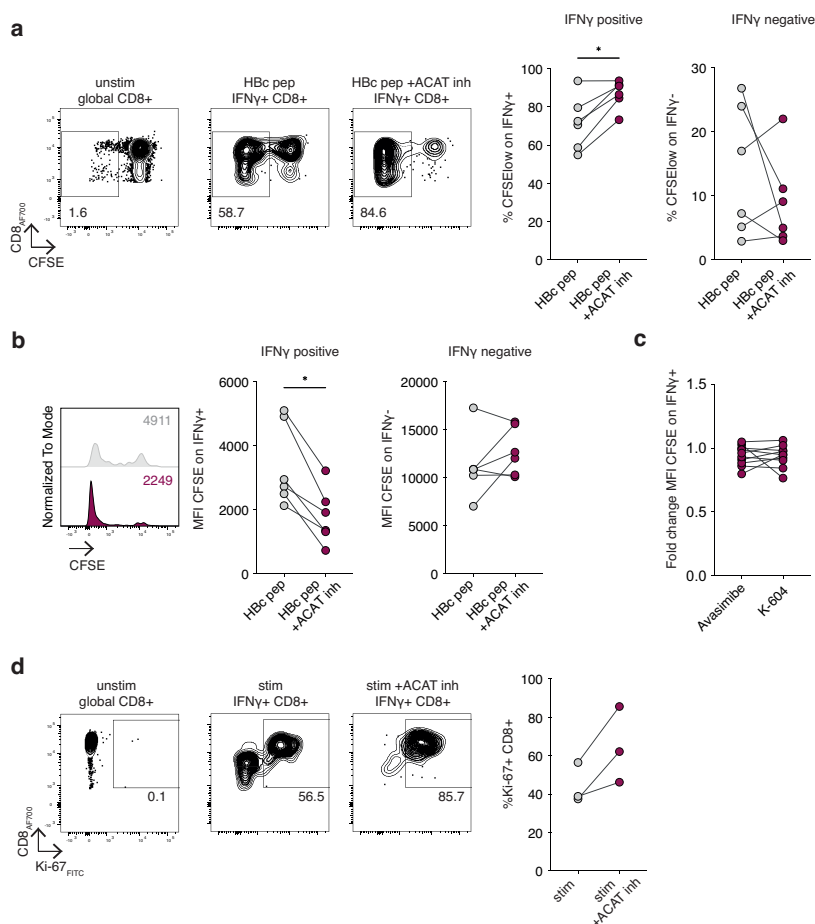


Figure 4.11 ACAT inhibition enhances CD8⁺ T cell proliferation.

(a-c) Assessment of proliferation of CD8⁺ T cells by CFSE dilution \pm ACAT inhibition (Avasimibe). **(a)** Frequency of CFSE^{low} IFN γ ⁺ (left, HBV-specific) CD8⁺ T cells (gating strategy and summary data; n=6) and frequency of CFSE^{low} IFN γ ^{neg} (right, not peptide-specific) CD8⁺ T cells. **(b)** CFSE MFI on IFN γ ⁺ CD8⁺ T cells (example histogram and summary data; n=6) and IFN γ ^{neg} CD8⁺ T cells. **(c)** Fold decrease MFI CFSE on IFN γ ⁺ CD8⁺ T cells with ACAT inhibition (Avasimibe and K-604) normalised to DMSO (n=10). **(d)** Ki-67 expression on IFN γ ⁺ CD8⁺ T cells after anti-CD3/anti-CD28 stimulation \pm ACAT inhibition (Avasimibe) (n=3). P values determined by Wilcoxon matched-pairs signed rank test.

4.3.4.3 ACAT inhibition and peripheral HBV-specific CD4⁺ T cells

As described for CD8⁺ T cells, HBV-specific CD4⁺ T cells in CHB show signs of exhaustion associated with reduced antiviral cytokine production and limited proliferative capacity (Buschow and Jansen 2021) and SOAT1 was expressed by human CD4⁺ T cells (Fig. 4.5b,c). I therefore hypothesized that ACAT inhibition could also affect the function of HBV-specific CD4⁺ T cells.

Treatment of CHB PBMC with ACAT inhibition increased the frequency and fold change of cytokine-producing HBV-specific CD4⁺ T cells in selected donors (Fig. 4.12a IFN γ ; Fig. 4.12b TNF); however, the CD4⁺ T cell boosting effect of ACAT inhibition was less consistent than for CD8⁺ T cells and was not significant in the overall cohort.

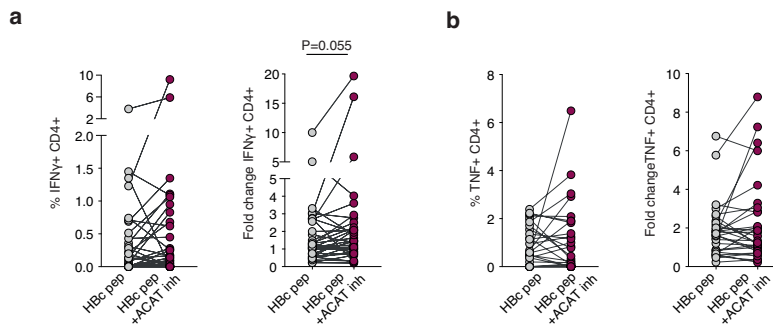


Figure 4.12 ACAT inhibition and HBV-specific CD4⁺ T cells.

(a,b) PBMC from CHB donors were stimulated with HBe pep \pm ACAT inhibition (Avasimibe) for 7d and HBV-specific cytokine production of CD4⁺ T cells was detected by flow cytometry. **(a)** Frequency of HBV-specific IFN γ ⁺ CD4⁺ T cells (left) and fold increase of IFN γ production after stimulation with HBe pep normalized to unstimulated control (right) (n=34). **(b)** Frequency of HBV-specific TNF⁺ CD4⁺ T cells (left) and fold increase of TNF production normalized to unstimulated control (right) (n=30). P values determined by Wilcoxon matched-pairs signed rank test.

4.3.4.4 ACAT inhibition and donor characteristics in HBV

Donor characteristics such as age, sex and disease stage can influence immune cell function and response to immunotherapies. In this chapter, I evaluated the effect of these factors on response to ACAT inhibition. To assess this, I calculated the fold increase of HBV-specific IFN γ production with ACAT inhibition compared to DMSO; however, it is important to note that this analysis excluded donors with *de novo* responses as a calculation of fold change from zero is mathematically impossible. To include all patients in the analysis, where suitable, I also assessed the likelihood of response irrespective of the fold increase and in these cases a response was defined as increased or *de novo* IFN γ production.

Age has a profound effect on the immune system and is associated with immune senescence, extensive alteration of immune cell function and metabolism (Akbar et al. 2016; Callender et al. 2018, 2020; Mogilenko et al. 2021). It is therefore important to consider patient age when assessing immunotherapeutic strategies. In this cohort, age was not associated with the fold change of IFN γ production by CD8⁺ or CD4⁺ T cells with ACAT inhibition (Fig. 4.13a,b).

In line with epidemiological data of CHB patients in London (majority of patients 25-44 years) (Public Health England 2019), the median age of this cohort was 37 years and age-related differences might only become apparent in an older age group.

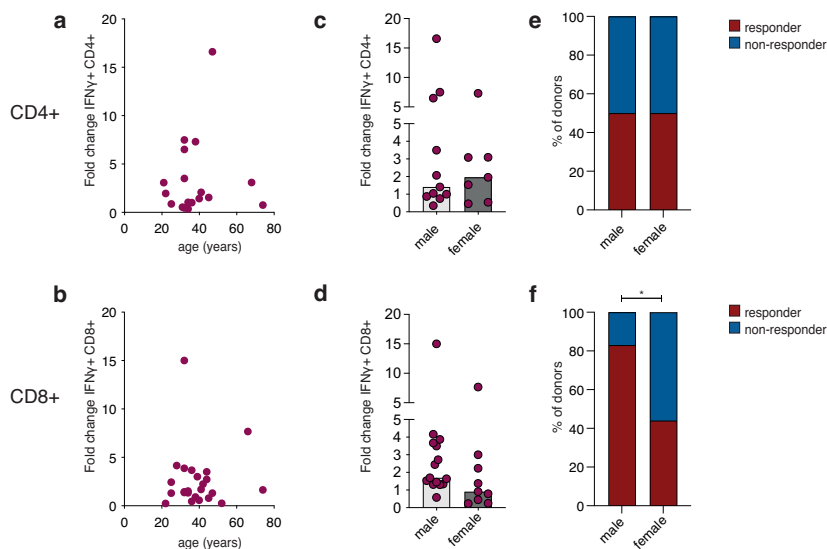


Figure 4.13 ACAT inhibition and donor characteristics in HBV.

(a+b) Fold increase of HBV-specific IFN γ ⁺ CD4⁺ **(a; n=18)** and CD8⁺ **(b; n=24)** T cells correlated with donor age. **(c+d)** Association of donor sex with fold increase of HBV-specific IFN γ ⁺ CD4⁺ **(c; male n=11; female n=7)** and CD8⁺ **(d; male n=15; female n=9)** T cells. **(e+f)** Sex difference (cohort: male n=18; female n=16) in CD4⁺ **(e)** and CD8⁺ **(f)** T cell response to ACAT inhibition. Response defined as increased or *de novo* IFN γ production. P values determined by Spearman correlation **(a+b)**, Mann-Whitney test **(c+d)** and Fisher's exact test **(e+f)**.

Donor sex is an important determinant of immune responses as well as lipid/cholesterol metabolism, with alterations driven by sex hormones such as oestrogens, progesterone and androgens (Klein and Flanagan 2016; Robinson et al. 2021). Response to ACAT inhibition in CD4⁺ T cell was not associated with donor sex (Fig. 4.13c,e); in contrast, male patients tended to have a higher fold increase of IFN γ production by CD8⁺ T cells with ACAT inhibition compared to females and were significantly more likely to respond to the treatment (Fig. 4.13d,f).

CHB is a highly variable and dynamic disease with 4 classical disease phases characterized by liver inflammation/damage (indicated by ALT levels above norm), HBV DNA levels, and HBeAg and HBsAg expression (also see chapter 4.1.1.2) (European Association for the Study of the Liver 2017). As expected in a cohort of untreated CHB donors, most donors presented with HBeAg negative chronic infection (HBeAg neg., HBe antibody pos., HBV

DNA <2000IU/ml, ALT normal). In this cohort, response to ACAT inhibition was not significantly associated with ALT levels (Fig. 4.14a,b), HBV DNA levels (Fig. 4.14c,d) or HBeAg status (Fig. 4.14e,f).

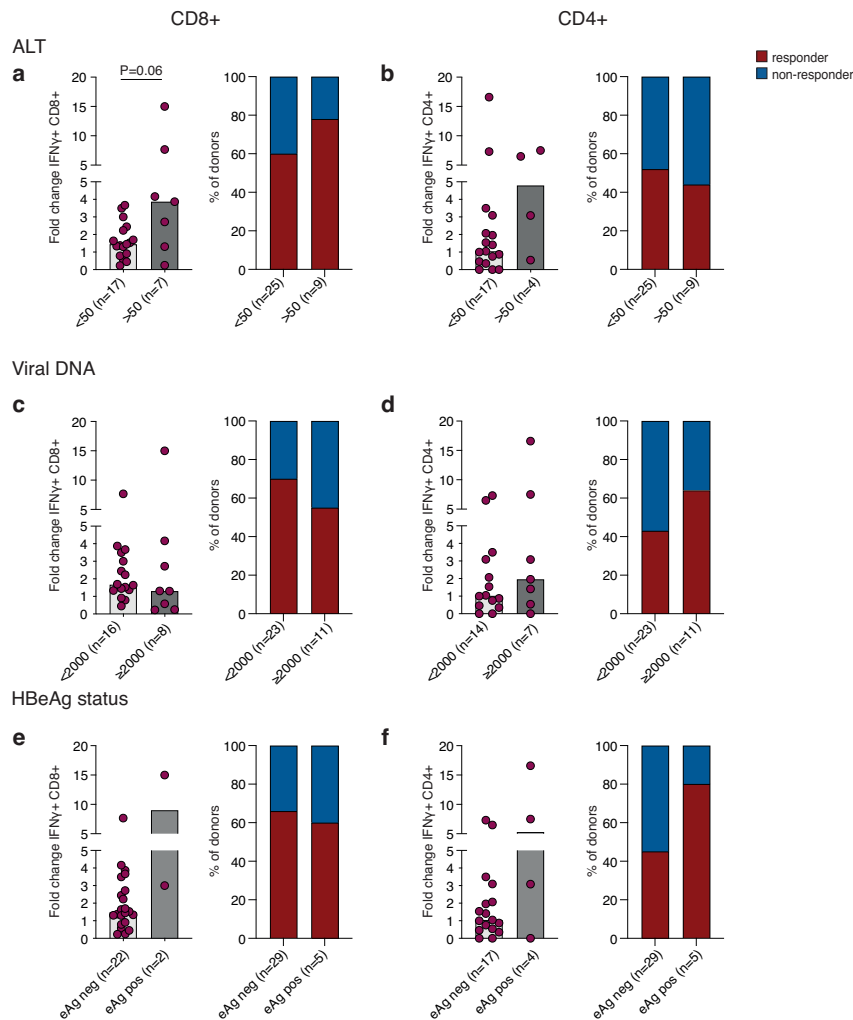


Figure 4.14 ACAT inhibition and HBV disease stage.

(a-f) Role of ALT (**a**, CD8⁺; **b**, CD4⁺), DNA levels (**c**, CD8⁺; **d**, CD4⁺) and HBeAg status (eAg; **e**, CD8⁺; **f**, CD4⁺) on fold increase of HBV-specific IFN γ production with ACAT inh (Avasimibe) compared to DMSO control (left) and response to ACAT inhibition (right; defined as increased or *de novo* IFN γ production). N numbers indicated in figure. P values determined by Mann-Whitney test (left graphs) and Fisher's exact test (right graphs).

Donors with high ALT (CHB-related chronic hepatitis) tended to have a higher CD8⁺ T cell IFN γ fold increase with ACAT inhibition compared to donors without hepatitis (CHB-related chronic infection; Fig. 4.14a) and ACAT inhibition increased HBV-specific IFN γ production by CD4⁺ T cells in 3 out of 4 HBeAg⁺ donors (Fig. 4.14f). A larger cohort with

a range of disease phases will be necessary to fully determine the effect of HBV disease phase on response to ACAT inhibition.

Overall, ACAT inhibition enhanced HBV-specific T cell responses in a variety of patients irrespective of disease stage or donor age, with males significantly more likely to respond than females.

4.3.4.5 ACAT inhibition and other peripheral immune cell subsets in HBV

To further evaluate the therapeutic potential of ACAT inhibition, I next aimed to identify the effect of ACAT inhibition on immune cells other than HBV-specific CD8⁺ and CD4⁺ effector T cells. This chapter will explore the effect of ACAT inhibition on Tfh cells, immunosuppressive cells (T_{reg}), unconventional T cells ($\gamma\delta$ T cells) and innate immune cells (NK cells).

4.3.4.5.1 ACAT inhibition and T follicular helper cells

Besides the production of antiviral cytokines, a major role of CD4⁺ T cells in viral infections is providing help to activate and regulate B cells (also see chapter 4.1.1.4.3). Tfh cells, characterized by the expression of CXCR-5 and PD-1 (Fig. 4.15a), are a specialized subpopulation of CD4⁺ T cells that is crucial for germinal centre formation, positive selection of B cell clones and the development of an efficient antiviral B cell response.

ACAT inhibition increased the frequency of Tfh in CHB PBMC (Fig. 4.15a) and increased the expression of the costimulatory molecules OX40, ICOS and CD154 (CD40L) (Fig. 4.15b-d) that are associated with Tfh activation and function, such as the induction of B cell activation, plasma cell differentiation and antiviral antibody production (also see chapter 4.1.1.4.3).

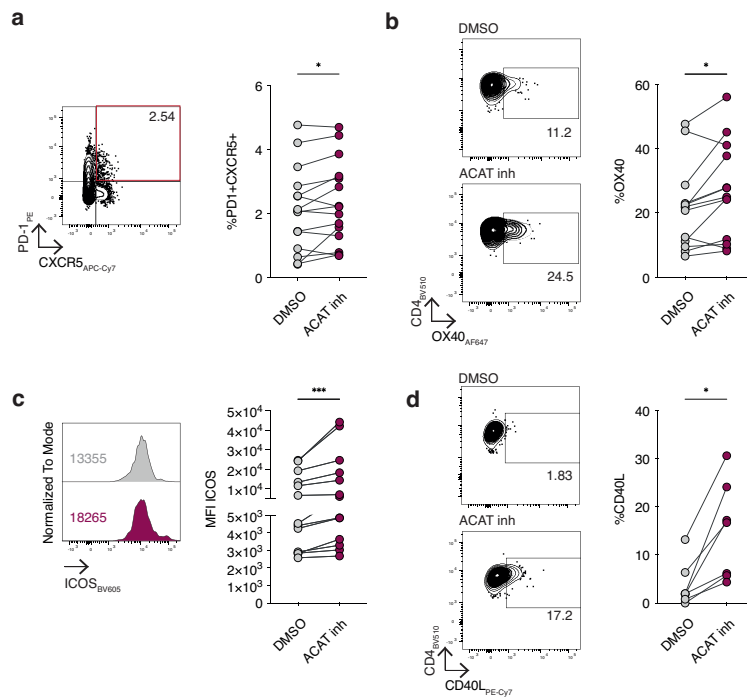


Figure 4.15 ACAT inhibition enhances Tfh cells.

(a-d) PBMC from CHB donors were treated with ACAT inhibition (Avasimibe) for 7d. **(a)** Example plot and summary data of % CXCR5⁺ PD-1⁺ Tfh cells (n=15). **(b)** Example plot and summary data of %OX40⁺ Tfh cells (n=14). **(c)** Example histogram and summary data of ICOS MFI on CXCR5⁺PD-1⁺ Tfh cells (n=14). **(d)** Example plot and summary data of %CD154⁺ (CD40L) on Tfh cells (n=6). Gates were first identified on global CD4⁺ T cells and then applied to CXCR5⁺ PD-1⁺ Tfh cells. P values determined by Wilcoxon matched-pairs signed rank test.

4.3.4.5.2 ACAT inhibition and regulatory CD4⁺ T cells

To fully understand and predict the role of immunotherapies *in vivo* it is important to consider the potential effect on immunosuppressive immune cell subsets such as CD4⁺ T_{reg} that could limit the beneficial boost of effector responses as seen for PD-1 blockade in a subset of patients (Kamada et al. 2019). CD4⁺ T_{reg} development is regulated by the transcription factor FOXP3, the expression of which is associated with high levels of IL-2 receptor chain α (IL-2R α ; CD25) (Hori et al. 2003) and reduced expression of the IL-7 receptor (CD127) typically found on effector and memory T cells (Liu et al. 2006a) (gating strategy Fig. 4.16a).

In physiological settings, T_{reg} control excessive inflammation and autoimmunity; however, they also limit antiviral/antitumour immune responses, contributing to viral and tumour immune escape. T_{reg} are metabolically distinct from conventional CD4⁺ and CD8⁺ T cells and cholesterol availability and utilization are critical for T_{reg} stability, expansion and suppressive function (Pinzon Grimaldos et al. 2022) (also see chapter 1.2). This observation

led to the hypothesis that ACAT inhibition could impact T_{reg} in CHB, either by boosting or by limiting their function, proliferation, and survival.

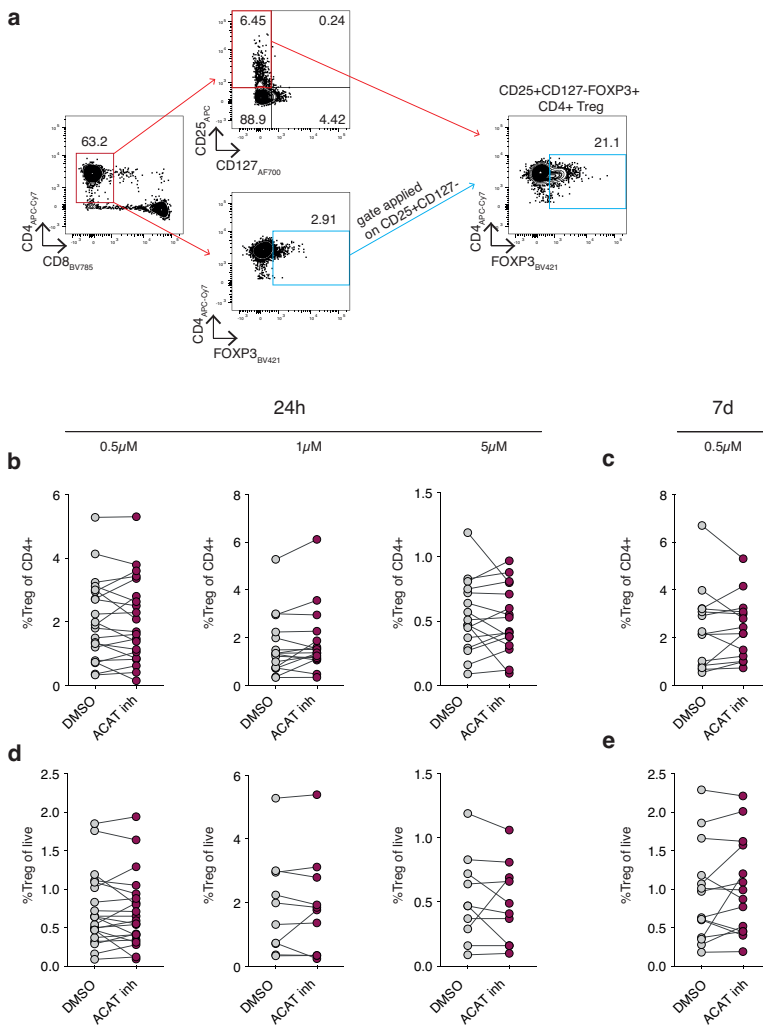


Figure 4.16 ACAT inhibition does not alter T_{reg} frequency in unstimulated PBMC.

(a) Gating strategy to identify T_{reg}: CD4⁺/CD8⁻, CD25⁺/CD127⁻, FOXP3⁺. FOXP3 gate determined on global CD4⁺ T cells and applied on CD25⁺/CD127⁻ to identify T_{reg}. **(b-e)** PBMC from CHB donors were treated with increasing concentrations of ACAT inhibitor (Avasimibe) for 24h or repetitively for 7d. **(b,c)** Frequency of T_{reg} as % of CD4⁺ T cells. **(d,e)** Frequency of T_{reg} as % of live cells. N numbers: **(b,d)** 0.5 μM: n=21; 1 μM: n=16; 5 μM: n=10; **(c,e)** n=14; P values determined by Wilcoxon matched-pairs signed rank test. *A subset of the experiments in this figure was performed and analysed by Hannah Thomsett under my direct supervision.*

ACAT inhibition for 24h or repetitively administered for 7d did not alter T_{reg} frequency in an unstimulated cell culture (% of CD4⁺ T cells: Fig. 4.16b, c; % of alive: Fig. 4.16d,e). To rule out a suboptimal dose of ACAT inhibitor, increasing concentrations (0.5 μM-5 μM) were applied in a subset of donors for 24h without any alterations of T_{reg} frequency.

ACAT inhibition also did not alter T_{reg} frequency after polyclonal TCR stimulation with anti-CD3/anti-CD28 antibodies (Fig. 4.17).

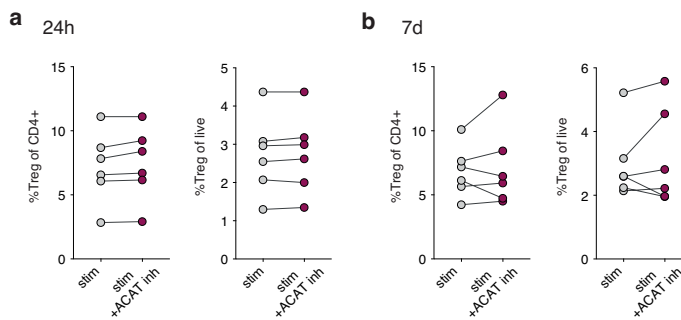


Figure 4.17 ACAT inhibition does not alter T_{reg} frequency after stimulation.

(a,b) PBMC from CHB donors ($n=6$) were stimulated with antiCD3/antiCD28 antibodies \pm ACAT inhibition ($0.5\mu\text{M}$ Avasimibe) for 24h **(a)** or 7d **(b)**. Frequency of T_{reg} as % of $CD4^+$ T cells and % of live cells. P values determined by Wilcoxon matched-pairs signed rank test.

T_{reg} utilize a multitude of immunosuppressive mechanisms to limit immune responses including metabolic disruption, direct cytotoxicity, inhibitory cytokines and inhibition of DC maturation/activation/function (Shevryev and Tereshchenko 2020; Vignali et al. 2008) (also see chapter 4.1.1.4.4). ACAT inhibition for 24h or 7d had inconsistent minor effects on the expression of a variety of molecules associated with T_{reg} activation and immunosuppressive function such as CD69, ICOS, GITR and TIGIT on unstimulated (Fig. 4.18) and stimulated T_{reg} (Fig. 4.19).

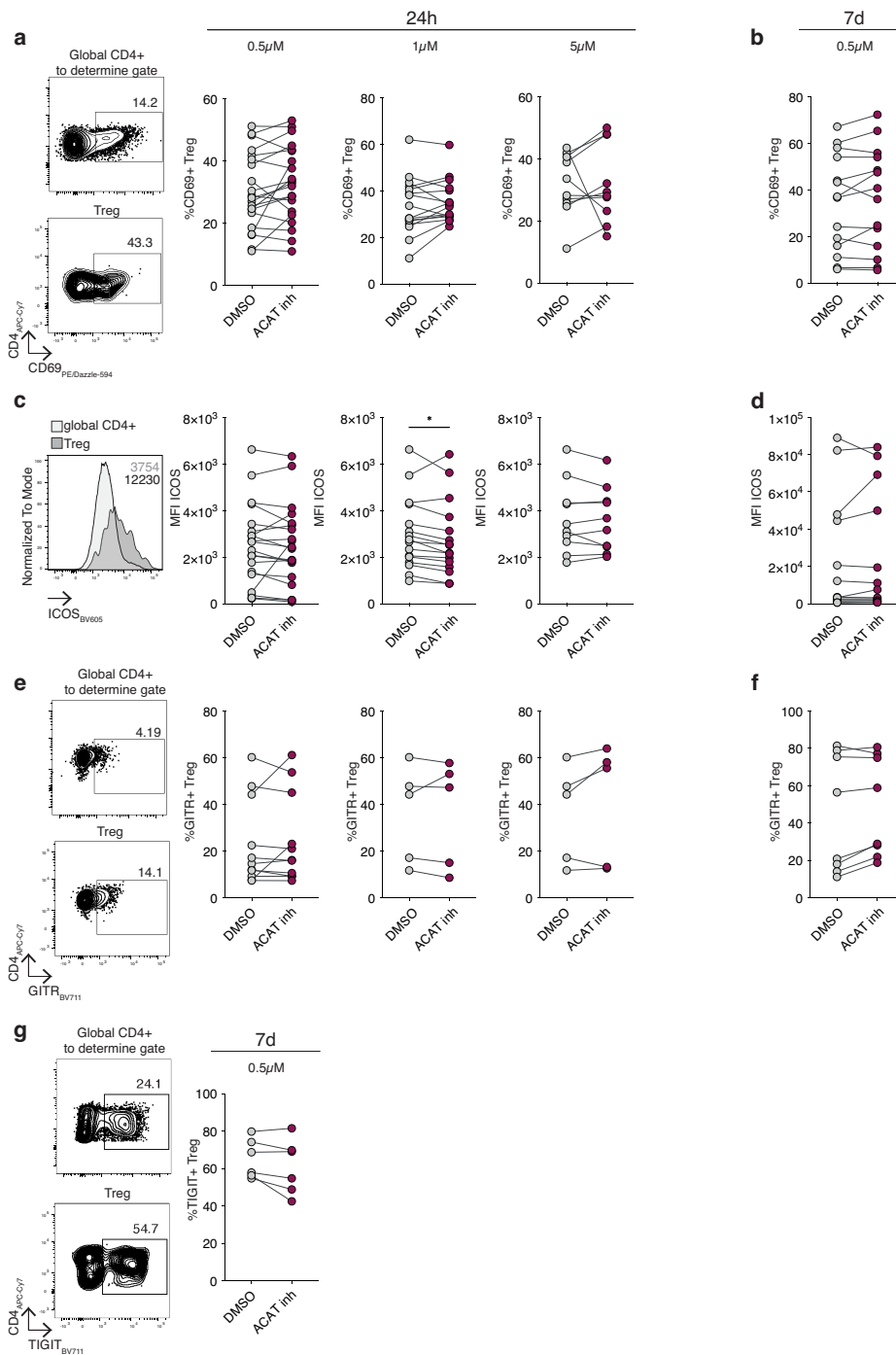


Figure 4.18 ACAT inhibition does not alter the phenotype of unstimulated T_{reg} .

(a-f) Phenotype of unstimulated T_{reg} from CHB PBMC \pm ACAT inhibition (Avasimibe) for 24h or 7d. Phenotypic markers were first identified on CD4⁺ T cells and the gate was then applied on T_{reg} . Example plot and summary data for %CD69⁺ **(a,b)**, MFI ICOS **(c,d)** and %GITR⁺ **(e,f)**. **(g)** Example plot and summary data for %TIGIT⁺ on T_{reg} \pm ACAT inhibition (Avasimibe) for 7d. N numbers: **(a,c,e)** 0.5μM: CD69 n=21; ICOS n=20; GITR n=11; 1μM: CD69/ICOS n=16; GITR n=5; 5μM: CD69/ICOS n=10; GITR n=5; **(b,d,f,g)** CD69/ICOS n=14; GITR n=8; TIGIT n=6. P values determined by Wilcoxon matched-pairs signed rank test. *A subset of the experiments in this figure was performed and analysed by Hannah Thomsett under my direct supervision.*

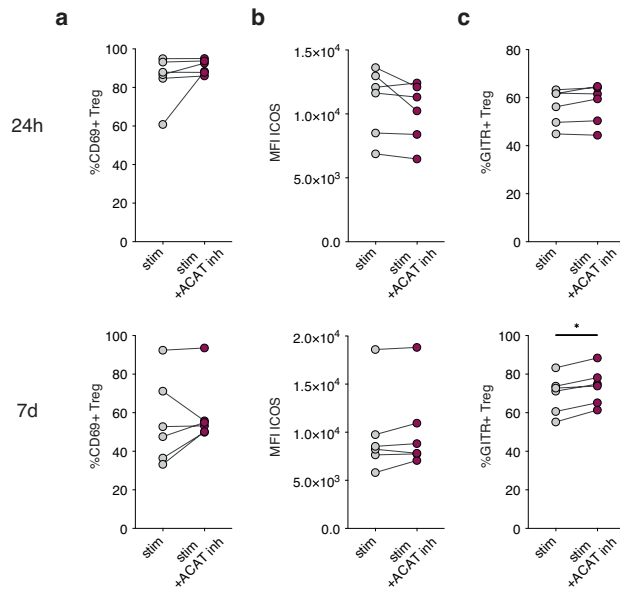


Figure 4.19 ACAT inhibition does not alter the phenotype of stimulated T_{reg} .
(a-c) Phenotype of T_{reg} from CHB PBMC ($n=6$) after stimulation with anti-CD3/anti-CD28 antibodies \pm ACAT inhibition ($0.5\mu\text{M}$ Avasimibe) for 24h (top) or 7d (bottom). **(a)** %CD69⁺ **(b)** MFI ICOS **(c)** %GITR⁺. P values determined by Wilcoxon matched-pairs signed rank test.

ACAT inhibition also did not alter the expression of CD39 or CTLA-4, molecules directly involved in the immunosuppressive function (see chapter 1.2.4.3.1 and 4.1.1.4.4), in unstimulated (Fig 4.20) or stimulated T_{reg} (Fig. 4.21).

Overall, inhibition of cholesterol esterification did not alter *in vitro* T_{reg} frequency and did not consistently affect the expression of molecules associated with enhanced T_{reg} activation/suppressive function nor CTLA-4 or CD39, directly involved in T_{reg} -modulated immunosuppression.

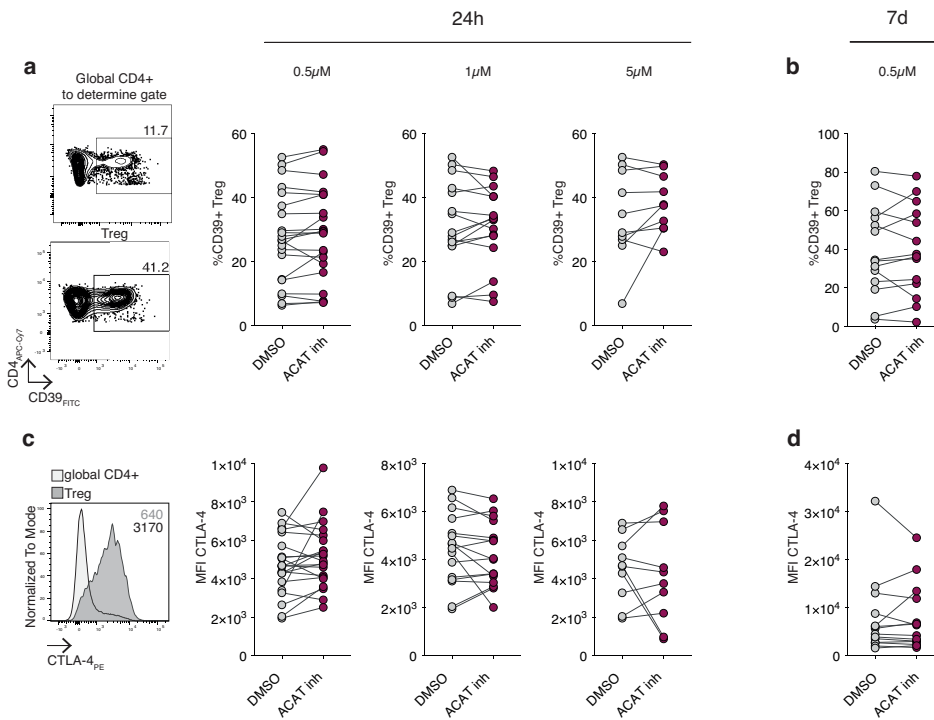


Figure 4.20 ACAT inhibition does not alter the expression of CD39 and CTLA-4 on unstimulated T_{reg}. **(a-d)** Phenotype of unstimulated T_{reg} from CHB PBMC ±ACAT inhibition (Avasimibe) for 24h **(a,c)** or 7d **(b, d)**. CD39⁺ cells were first identified on CD4⁺ T cells and the gate was then applied on T_{reg}. Example plot and summary data for % CD39⁺ **(a,b)** and MFI CTLA-4 **(c,d)**. N numbers: **(a,c)** 0.5µM: n=21; 1µM: n=16; 5µM: n=10; **(b,d)** n=14; P values determined by Wilcoxon matched-pairs signed rank test. *A subset of the experiments in this figure was performed and analysed by Hannah Thomsett under my direct supervision.*

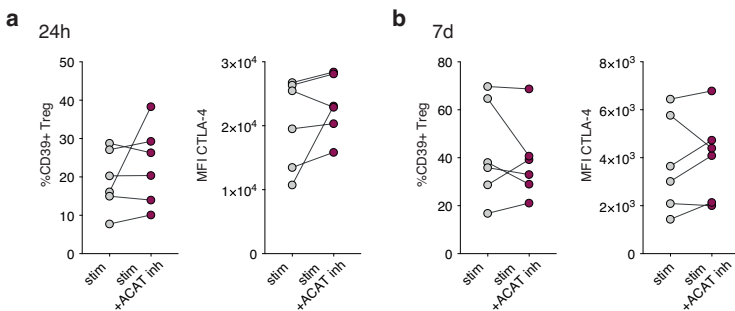


Figure 4.21 ACAT inhibition does not alter the expression of CD39 and CTLA-4 on stimulated T_{reg}. **(a)** %CD39⁺ and MFI CTLA-4 on T_{reg} from CHB PBMC (n=6) after stimulation with anti-CD3/anti-CD28 antibodies ±ACAT inhibition (0.5µM Avasimibe) for 24h **(a)** or 7d **(b)**. P values determined by Wilcoxon matched-pairs signed rank test.

4.3.4.5.3 ACAT inhibition and $\gamma\delta$ T cells

$\gamma\delta$ T cells are unconventional T cells that preferentially reside in tissue and are characterized by their expression of the heterodimeric $\gamma\delta$ TCR (Fig. 4.22a). They can be further classified by their TCR variable regions with V δ 1 $\gamma\delta$ T cells preferentially found in human tissue and V δ 2 expressed by the majority of peripheral $\gamma\delta$ T cells (Fig. 4.22c) (Chien et al. 2014; Kalyan and Kabelitz 2013; Vantourout and Hayday 2013; Zakeri et al. 2022) (also see chapter 4.1.14.5).

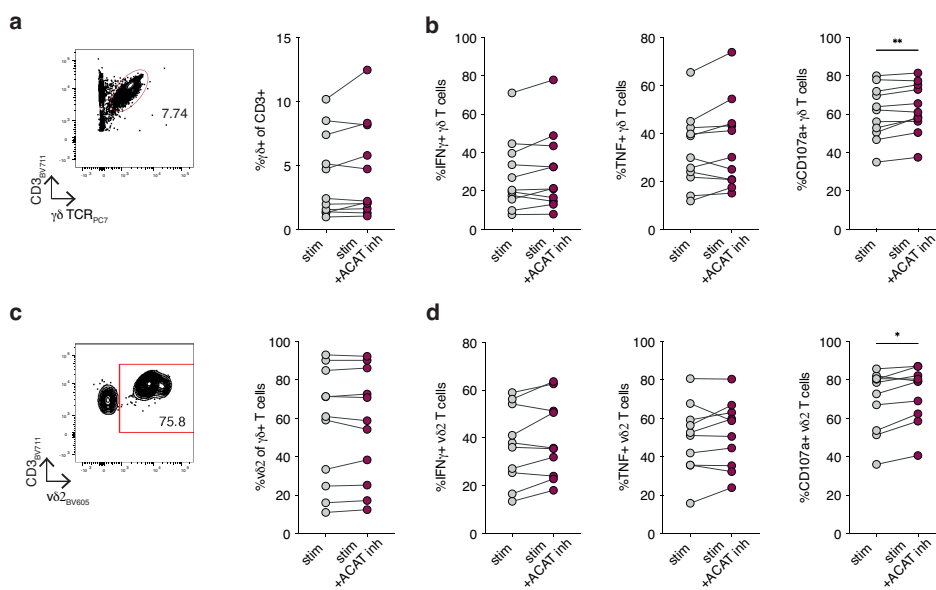


Figure 4.22 ACAT inhibition and $\gamma\delta$ T cells.

(a-d) PBMC from CHB donors were stimulated with anti-pan- $\gamma\delta$ antibody \pm ACAT inhibition (Avasimibe) for 5d (n=11). **(a)** Example gating and frequency of $\gamma\delta$ T cells of CD3⁺ T cells. **(b)** Assessment of cytokine production (IFN γ , TNF) and CD107a mobilization by pan- $\gamma\delta$ T cells via flow cytometry. **(c)** Example gating and frequency of the v δ 2 fraction within $\gamma\delta$ T cells. **(d)** Assessment of cytokine production (IFN γ , TNF) and CD107a mobilization by V δ 2 cells. P values determined by Wilcoxon matched-pairs signed rank test. *A subset of the experiments in this figure was performed by Stephanie Kucykowicz under my direct supervision.*

ACAT inhibition did not affect the frequency of global $\gamma\delta$ T cells (Fig. 4.22a) nor the production of antiviral cytokines in CHB PBMC after polyclonal stimulation with an anti- $\gamma\delta$ TCR antibody (Fig. 4.22b); however, ACAT inhibition led to a subtle but significant increase of CD107a mobilization to the cell surface as a marker of degranulation and cytotoxicity (see chapter 3.9.1). A similar pattern was seen when directly assessing the frequency and function of v δ 2 $\gamma\delta$ T cells (Fig. 4.22c,d).

4.3.4.5.4 ACAT inhibition and Natural Killer cells

NK cells, identified by the expression of CD56, have the capacity to exert antiviral effects but, similar to T cells, NK cells in CHB display features of exhaustion (Dunn et al. 2009; Marotel et al. 2021; Peppia et al. 2010; Sun et al. 2012; Tjwa et al. 2011) (also see chapter 4.1.1.4.6).

To assess the effect of ACAT inhibition on NK cells, CHB PBMC were stimulated with IL-12 and IL-18 as previously described (Dunn et al. 2009; Peppia et al. 2010) for 3d. ACAT inhibition did not alter the frequency of NK cells (Fig. 4.23a) nor did it affect the production of antiviral cytokines or the mobilization of CD107a to the cell membrane (Fig. 4.23b).

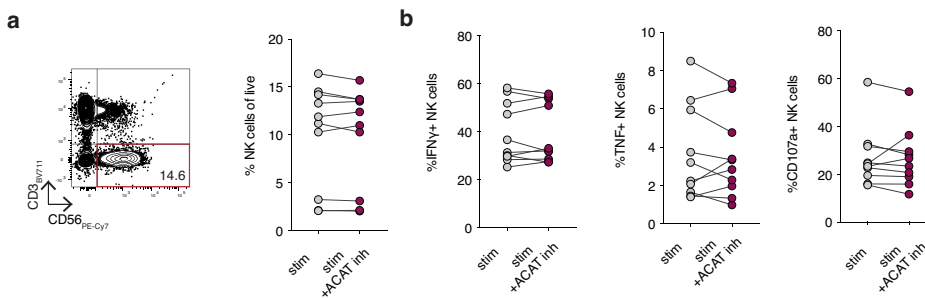


Figure 4.23 ACAT inhibition and NK cells.

(a-b) PBMC from CHB donors were stimulated with IL-12 and IL-18 \pm ACAT inhibition (Avasimibe) for 3d (n=10). **(a)** Example gating strategy and frequency of NK cells as % of viable cells. **(b)** Assessment of cytokine production (IFN γ , TNF) and CD107a mobilization via flow cytometry. P values determined by Wilcoxon matched-pairs signed rank test. *A subset of the experiments in this figure was performed by Stephanie Kucykowicz under my direct supervision.*

4.3.4.6 ACAT inhibition boosts intrahepatic HBV-specific T cells

I have shown that ACAT inhibition boosts the function of circulating HBV-specific CD8⁺ T cells from the blood of CHB donors (chapter 4.3.4.2). As HBV is a hepatotropic virus exclusively infecting and replicating in hepatocytes, any immunotherapeutic approach for CHB needs to also enhance the function of intrahepatic T cells in the highly immunotolerant liver environment (Maini and Burton 2019; Maini and Pallett 2018; Pallett and Maini 2022). SOAT1 and SOAT2 expression in T cells from intrahepatic leukocytes (IHL) was

comparable to the expression detected in blood, indicating a potential target for ACAT inhibition in IHL (Fig. 4.5).

IHL were directly isolated from human HBV-infected liver tissue and stimulated with peptide pools derived from three major HBV peptides – HBc, HBs (split into two peptide pools) and polymerase (Pol) for 16h in the presence of ACAT inhibition. CD8⁺ and CD4⁺ T cells from IHL were identified in multiparameter flow cytometry by stringent sequential gating on lymphocytes (FSC-A vs SSC-A), single cells (FSC-A vs FSC-H), viable cells, CD45⁺ leukocytes, CD3⁺CD56^{neg}CD19^{neg} T cells (excluding NK cells and B cells), CD8⁺CD4^{neg} or CD8^{neg}CD4⁺ T cells (Fig. 4.24a).

ACAT inhibition for only 16h enhanced the IFN γ production of CD8⁺ T cells specific for all three HBV-derived peptides from donors with pre-existing CD8⁺ T cell responses (Fig. 4.24b) and induced *de novo* responses in selected donors (Fig. 4.24c). Overall, ACAT inhibition significantly boosted IFN γ production by HBV-specific intrahepatic CD8⁺ T cells (Fig. 4.24d). ACAT inhibition also increased the production of TNF (Fig. 4.24e) in some donors but there was no consistent effect in the cohort overall.

Surprisingly, in contrast to my findings in PBMC, there was a highly significant increase of antiviral cytokine production by intrahepatic HBV-specific CD4⁺ T cells with ACAT inhibition (Fig 4.24f).

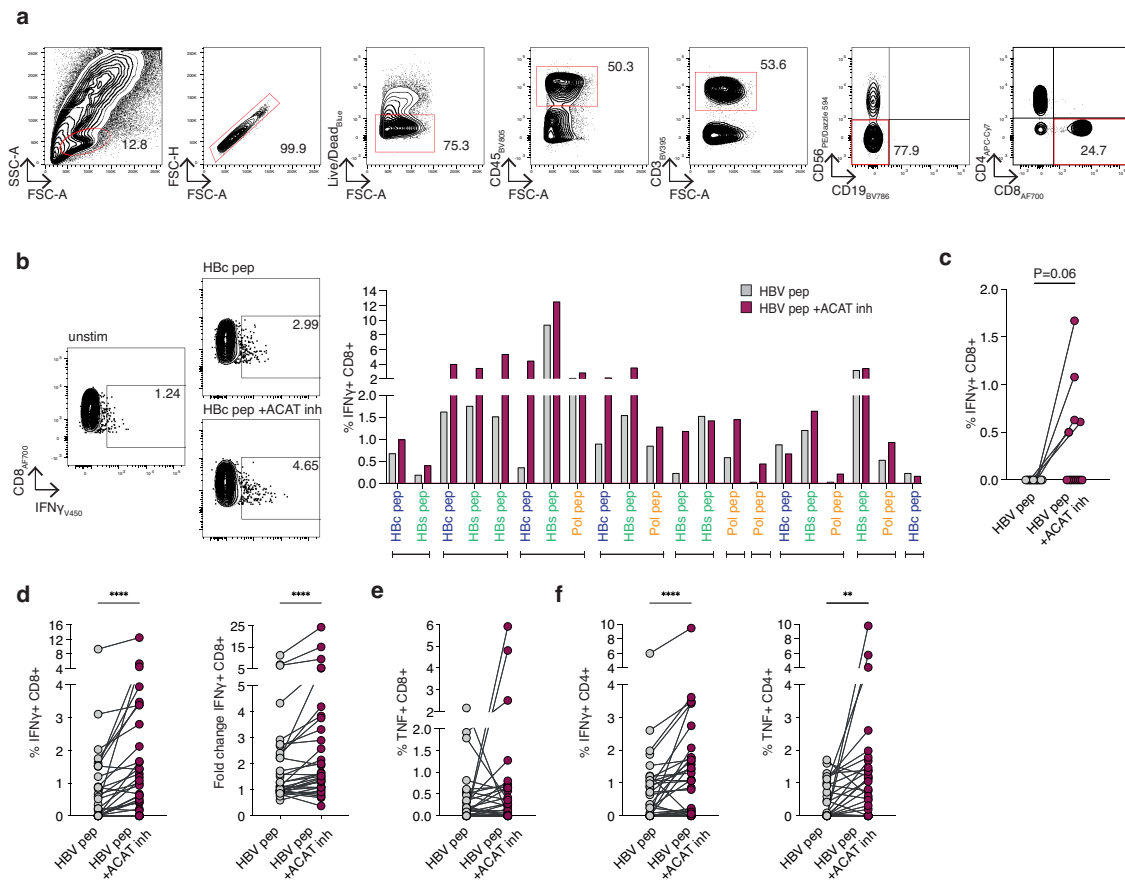


Figure 4.24 ACAT inhibition boosts intrahepatic HBV-specific T cells.

(a-f) IHL from patients with CHB were stimulated with peptide pools (pep) spanning multiple HBV peptides (HBc, HBs, Pol) \pm ACAT inhibition (K-604) for 16h. Cytokine production was detected by flow cytometry. **(a)** Representative flow cytometry plot of gating strategy for IHL and TIL: lymphocytes, single cells, live cells, CD45⁺, CD3⁺, CD56⁻/CD19⁻, CD4⁻/CD8⁺. **(b)** Representative flow cytometry plot and summary data for each individual peptide pool (n=21) and each donor (n=10) with detectable pre-existing HBV-specific IFN γ responses by CD8⁺ IHL. Brackets below the histogram indicate different pep tested in IHL from the same donor. **(c)** *De novo* IFN γ production (donors n=7; individual pep n=14) by CD8⁺ IHL. **(d)** Summary data for HBV-specific IFN γ production by CD8⁺ IHL as % of total CD8⁺ IHL (left) and fold change of IFN γ production after stimulation with HBV pep normalized to unstimulated control (right) (donors n=11; individual pep n=35). **(e)** Summary data for HBV-specific TNF production by CD8⁺ IHL. **(f)** Summary data for HBV-specific IFN γ and TNF production by CD4⁺ IHL. P values determined by Wilcoxon matched-pairs signed rank test. **(f)** A subset of the data in Fig. 4.24f was analysed by Stephanie Kucykowicz under my direct supervision.

Taken together, ACAT inhibition boosted HBV-specific T cell responses from the liver more consistently than those from the blood with only 1 out of 8 donors with paired blood and liver samples not showing increased intrahepatic IFN γ production to any HBV peptide pool tested (Fig. 4.25a). ACAT inhibition also enhanced the function of HBV-specific CD8⁺ and CD4⁺ T cells from IHL to a significantly higher extent than those from blood (Fig. 4.25b).

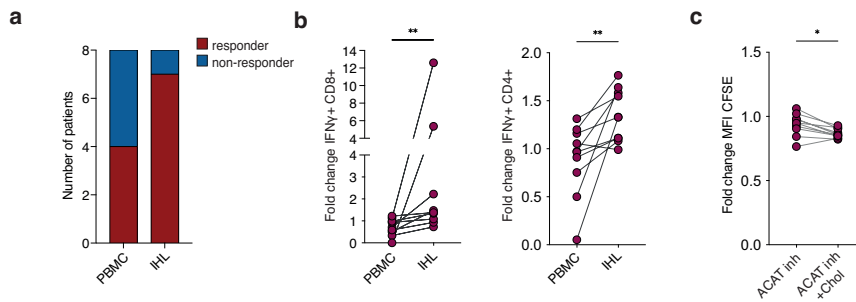


Figure 4.25 ACAT inhibition preferentially enhances intrahepatic T cells.

(a+b) IHL and PBMC from donors with CHB were stimulated with HBV peptide pools (HBV pep; HBc, HBs, Pol) \pm ACAT inhibition (K-604) for 16h. Cytokine production was detected by flow cytometry. **(a)** Response to ACAT inhibition (donors $n=8$). Response defined as increased or *de novo* IFN γ production. **(b)** Fold change of IFN γ production after stimulation with HBV pep +ACAT inhibition normalized to pep +DMSO in donors with detectable pre-existing HBV-specific IFN γ production (donors $n=5$, individual pep $n=10$). **(c)** Assessment of proliferation by CFSE dilution of anti-CD3/anti-CD28 stimulated CD8 $^{+}$ T cells from PBMC in normal or cholesterol enriched (chol) culture media \pm ACAT inhibition (K-604) for 4d ($n=10$). P values determined by Fisher's exact test **(a)** and Wilcoxon matched-pairs signed rank test **(b,c)**.

I hypothesized that the high availability of cholesterol in the liver (Chamberlain 1928), a central hub for cholesterol metabolism, could contribute to the observed increased sensitivity to ACAT inhibition in IHL. This was supported by the finding that cholesterol-enriched culture media *in vitro* further enhanced the proliferation of PBMC induced by ACAT inhibition (indicated by increased CFSE dilution; Fig. 4.25c).

Within the liver, CD8 $^{+}$ T_{RM} can provide long-lived local immune surveillance (Pallett and Maini 2022) (also see chapter 4.1.1.4.1). ACAT inhibition did not alter the expression of the tissue retention markers CD103 and CD69 (Fig. 4.26a) but significantly enhanced the function of tissue-resident (CD103 $^{+}$ CD69 $^{+}$) and non-resident, liver-infiltrating (CD103 neg CD69 neg) CD8 $^{+}$ IHL to a similar extent (Fig. 4.26b,c), highlighting the potential of boosting long-lived local immune responses directly at the site of disease.

In summary, ACAT inhibition enhanced HBV-specific CD8 $^{+}$ and CD4 $^{+}$ T cells directly *ex vivo* from human HBV-infected liver tissue.

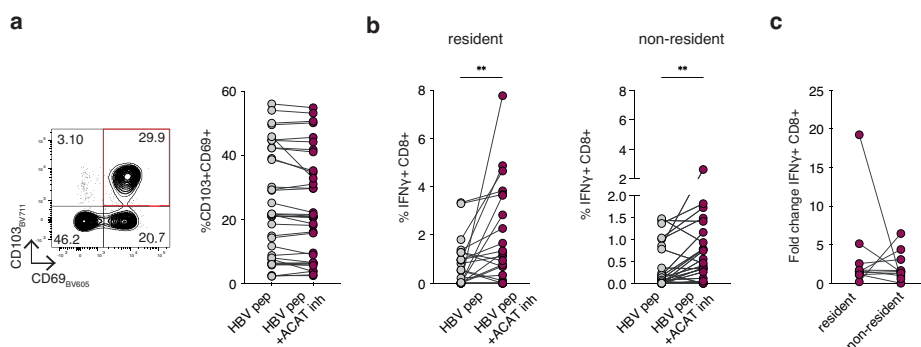


Figure 4.26 ACAT inhibition enhances liver-infiltrating and liver-resident CD8⁺ T cells.

(a-c) IHL and PBMC from CHB donors were stimulated with HBV peptide pools (pep) \pm ACAT inhibition (K-604) for 16h. Cytokine production was detected by flow cytometry. **(a)** Example plot and summary data for tissue-resident (CD103⁺CD69⁺; red box in example plot) and non-resident (CD103⁻CD69⁻) CD8⁺ IHL (n=35 pep and 11 donors). **(b,c)** IFN γ production of tissue-resident and non-resident CD8⁺ IHL. **(b)** Frequency of IFN γ ⁺ CD8⁺ IHL (n=35 peptide pools and 11 donors). **(c)** Fold increase of IFN γ ⁺ CD8⁺ IHL with ACAT inh normalized to DMSO control (n=10 pep and 5 donors). P values determined by Wilcoxon matched-pairs signed rank test.

4.3.4.7 Summary

HBV-specific T cells in CHB are highly exhausted with a poor proliferative capacity and limited antiviral function. Modulation of cholesterol esterification by ACAT inhibition boosted the expansion of functional HBV-specific CD8⁺ T cells from human blood and liver tissue including long-lived tissue-resident memory CD8⁺ T cells. ACAT inhibition also enhanced the function of T_{fh} cells as well as intrahepatic HBV-specific CD4⁺ T cells. Other immune cell subsets such as T_{reg}, $\gamma\delta$ T cells and NK cells only showed minor alterations of phenotype and function in response to ACAT inhibition. Immune boosting was irrespective of disease stage and donor age, rendering ACAT inhibition a promising new immunotherapeutic strategy for patients with CHB.

4.3.5 ACAT inhibition in SARS-CoV-2 infection

SARS-CoV-2-specific T cells in COVID-19 have been widely studied in the last years and their role in controlling infection has been highlighted in multiple studies (Moss 2022) (also see chapter 4.1.2.1). The rationale for immunotherapy in SARS-CoV-2 infection is supported by the findings that SARS-CoV-2-specific T cells in early disease are associated with disease control, viral clearance and mild symptoms as well as that pre-existing cross-reactive T cells expand in abortive infection without antibody responses (Moderbacher et al. 2020; Swadling

et al. 2022; Tan et al. 2021). Emerging data showing that SARS-CoV-2-specific T cells may be exhausted and dysfunctional (Chen and Wherry 2020) led to the hypothesis that ACAT inhibition could also boost SARS-CoV-2-specific T cells.

4.3.5.1 Characterization of SARS-CoV-2-specific T cells in acute infection

To study SARS-CoV-2-specific T cell responses, PBMC were isolated from the blood of hospitalized donors during PCR-confirmed acute SARS-CoV-2 infection. All samples were taken from unvaccinated donors during the first wave of SARS-CoV-2 infection in the UK (March-July 2020) (for details see chapter 3.2.3). PBMC were stimulated with peptide pools derived from structural SARS-CoV-2 proteins (spike, membrane) and their antiviral function was assessed after 7d via multiparameter flow cytometry (for details see 3.6.3).

During acute infection, cytokine production was significantly higher in CD4⁺ compared to CD8⁺ T cells specific for both spike (Fig. 4.27a,b) and membrane (Fig. 4.27a,c), with a positive correlation between CD4⁺ and CD8⁺ T cell responses as previously described (Habel et al. 2020; Weiskopf et al. 2020). No difference in cytokine production was detected between specificities with a positive correlation between spike- and membrane-specific CD4⁺ (Fig. 4.27a,d) and CD8⁺ (Fig. 4.27a,e) T cell function.

Taken together, the used protocol was suitable to detect SARS-CoV-2-specific CD4⁺ and CD8⁺ T cells in PBMC from donors during acute SARS-CoV-2 infection with an immune response dominated by CD4⁺ T cells.

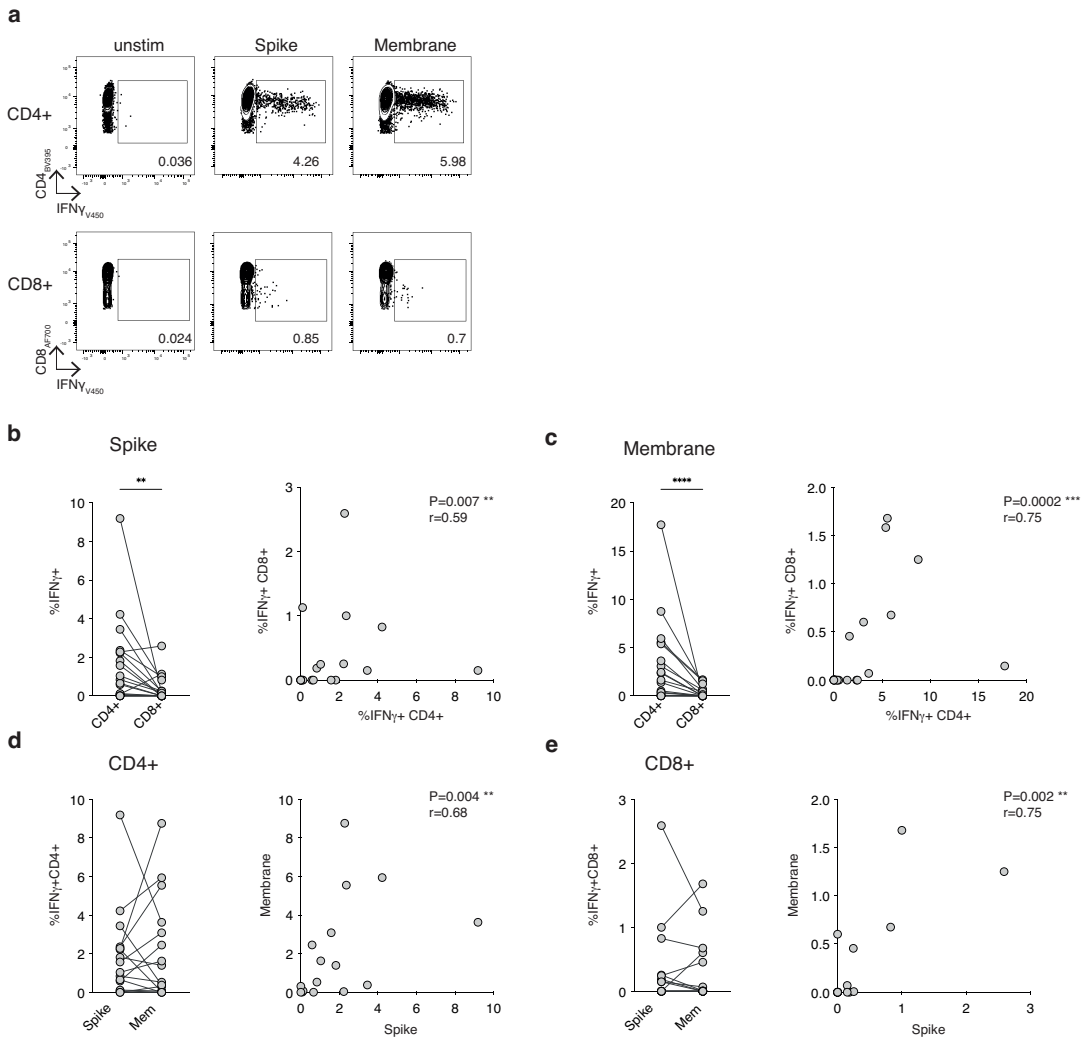


Figure 4.27 Characterization of SARS-CoV-2-specific IFN γ ⁺ T cells in acute infection. **(a-c)** PBMC from donors with acute SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (Spike and Membrane, Mem) and IFN γ production was detected via flow cytometry. **(a)** Example flow cytometry plots. **(b)** Spike-specific IFN γ production (n=19). **(c)** Membrane-specific IFN γ production (n=19). **(d)** SARS-CoV-2-specific IFN γ production by CD4⁺ T cells (n=16). **(e)** SARS-CoV-2-specific IFN γ production by CD8⁺ T cells (n=16). P values determined by Wilcoxon matched-pairs signed rank test (left) and Spearman correlation (right).

4.3.5.2 Protocol to assess effect of ACAT inhibition in SARS-CoV-2

To assess the effect of ACAT inhibition on SARS-CoV-2-specific T cells, I followed a similar treatment protocol as described for HBV-specific T cells with two minor alterations (Fig. 4.28a). IL-2 was administered repetitively during cell culture as this approach had been shown to elicit higher antiviral T cell responses by other members of the Maini lab (*data not shown*) and stimulating anti-CD28 antibody was administered simultaneously with peptide pools at d0 and d6 to provide the second co-stimulatory signal required for T cell activation (see chapter 1.1.1.1.2). These alterations allowed comparisons and continuity with other SARS-CoV-2 studies using similar protocols, e.g. (Swadling et al. 2022). Additionally, I had shown that provision of the costimulatory signal with CD28 stimulation enhanced the response rate to ACAT inhibition (see chapter 5.3.2.2).

4.3.5.3 ACAT inhibition does not affect SARS-CoV-2-specific CD8⁺ T cells in acute infection

Considering the immune-boosting effect of ACAT inhibition on HBV-specific CD8⁺ T cells described in previous chapters, I first investigated the effect of ACAT inhibition on SARS-CoV-2-specific CD8⁺ T cells in acute infection, assessing the production of the antiviral cytokines IFN γ (Fig. 4.28b), TNF (Fig. 4.28c) and MIP1 β (Fig. 4.28d). ACAT inhibition increased the production of antiviral cytokines by spike- and membrane-specific CD8⁺ T cells in individual donors but surprisingly, in contrast to HBV-specific CD8⁺ T cells in CHB (Fig. 4.9+4.10), the increase was not significant in the cohort overall.

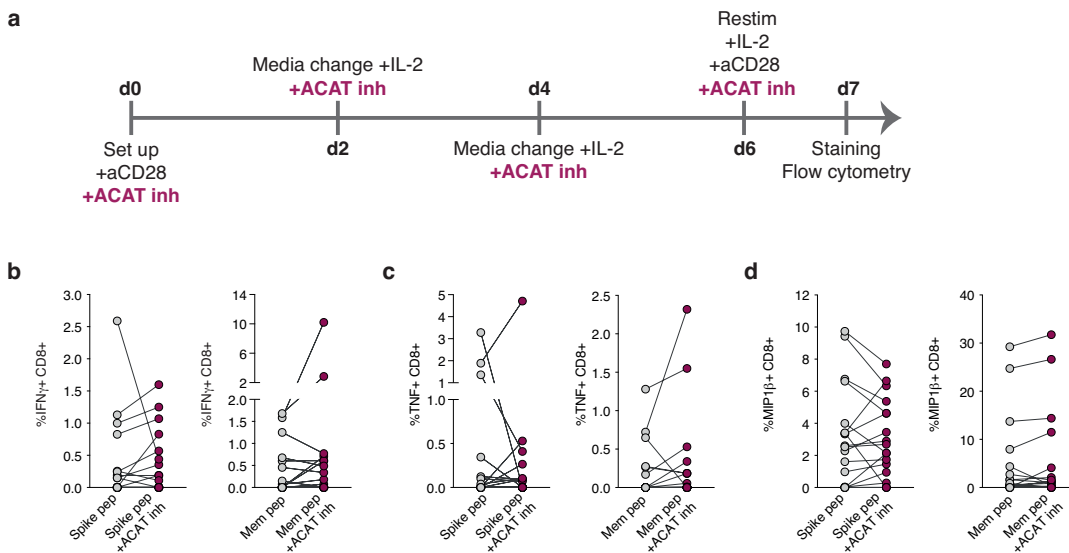


Figure 4.28 ACAT inhibition and SARS-CoV-2-specific CD8⁺ T cells in acute infection.

(a) Treatment protocol to assess the effect of ACAT inhibition on SARS-CoV-2-specific T cells. **(b-d)** PBMC from donors with acute SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (pep; Spike and Membrane, Mem) \pm ACAT inhibition (Avasimibe) for 7d and analysed via flow cytometry. Frequency of SARS-CoV-2 specific IFN γ **(b)**, TNF **(c)** and MIP1 β **(d)** production by CD8⁺ T cells (n=19). P values determined by Wilcoxon matched-pairs signed rank test.

4.3.5.4 ACAT inhibition boosts SARS-CoV-2-specific CD4⁺ T cells in acute infection

In contrast to CD8⁺ T cells, targeting cholesterol esterification by ACAT inhibition enhanced the production of antiviral cytokines by membrane- and spike-specific CD4⁺ T cells from donors with acute infection by boosting pre-existing T cell responses in some donors and eliciting *de novo* cytokine responses in other donors without any detectable responses when stimulated with peptide alone (Fig. 4.29a-c).

As shown for ACAT inhibition in CHB and for other *in vitro* and *in vivo* immunotherapies (Bensch et al. 2014; Maini and Pallett 2018; Schurich et al. 2013), responses were heterogenous with an up to 30-fold increase of IFN γ production and decreases in a minority of donors. Additional to the boosting of individual cytokines, ACAT inhibition also increased the frequency of multifunctional CD4⁺ T cells producing both IFN γ and TNF (Fig. 4.29d).

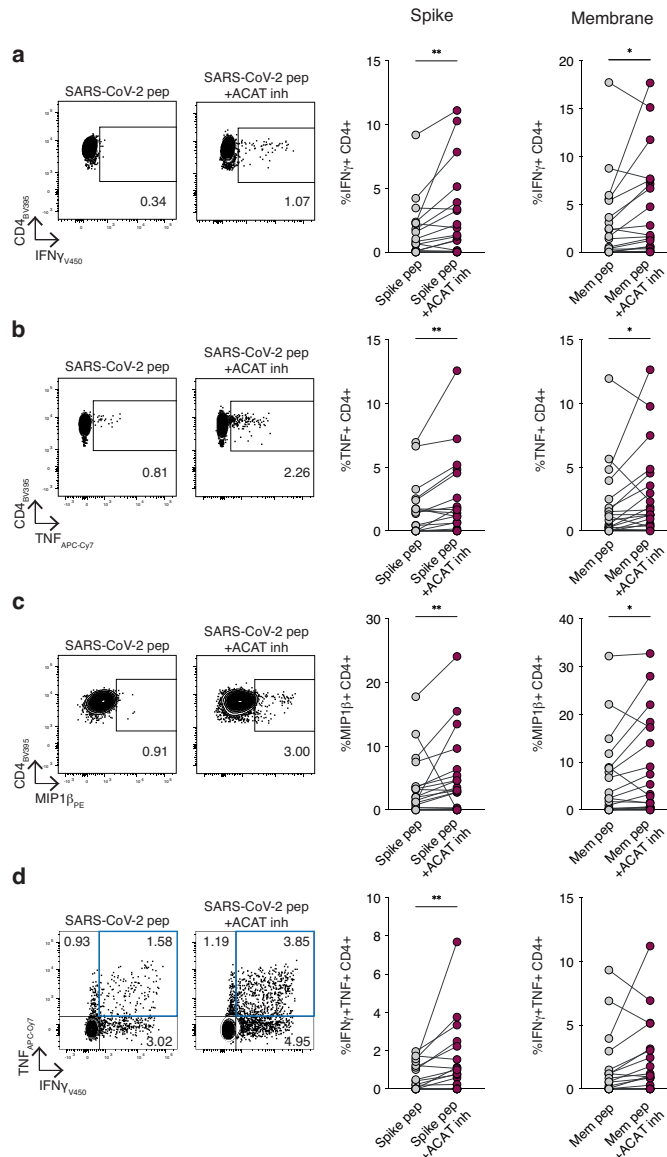


Figure 4.29 ACAT inhibition enhances SARS-CoV-2-specific cytokine production by CD4⁺ T cells in acute infection.

(a-d) PBMC from donors with acute SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (pep; Spike and Membrane, Mem) \pm ACAT inhibition (Avasimibe) for 7d and analysed via flow cytometry. Example plots and frequency of SARS-CoV-2 specific IFN γ **(a)**, TNF **(b)**, MIP1 β **(c)** and IFN γ /TNF production **(d)** by CD4⁺ T cells (n=19). P values determined by Wilcoxon matched-pairs signed rank test.

As described in chapter 4.1.1.4.3, besides the production of antiviral cytokines, CD4⁺ T cells also provide help to B cells for activation and maturation, e.g. via interaction between CD40L (CD154) on Tfh cells and CD40 on B cells. ACAT inhibition significantly increased the expression of CD154 on spike- and membrane-specific CD4⁺ T cells, indicating a potential for increased T helper function (Fig. 4.30).

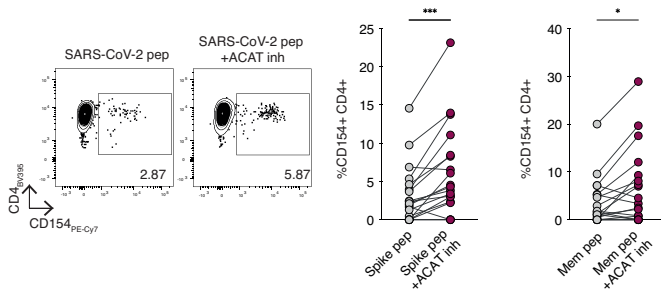


Figure 4.30 ACAT inhibition enhances SARS-CoV-2-specific CD154 expression on CD4⁺ T cells in acute infection.

PBMC from donors with acute SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (pep; Spike and Membrane, Mem) ±ACAT inhibition (Avasimibe) for 7d and CD154 (CD40L) expression was analysed via flow cytometry; n=19. P values determined by Wilcoxon matched-pairs signed rank test.

In line with the findings in CHB, ACAT inhibition not only recovered pre-existing, dysfunctional T cells, but also induced proliferation of SARS-CoV-2-specific CD4⁺ T cells (indicated by CFSE dilution; Fig. 4.31).

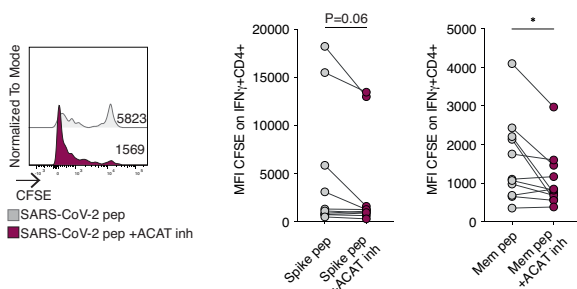


Figure 4.31 ACAT inhibition enhances SARS-CoV-2-specific CD4⁺ T cell proliferation in acute infection.

PBMC from donors with acute SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (pep; Spike and Membrane, Mem) ±ACAT inhibition (Avasimibe) for 7d. Assessment of SARS-CoV-2-specific proliferation determined by CFSE dilution gated on IFN γ ⁺ CD4⁺ T cells (Spike n=10; Mem n=11). P values determined by Wilcoxon matched-pairs signed rank test.

In summary, ACAT inhibition enhanced the expansion of functional SARS-CoV-2-specific CD4⁺ T cells in donors with acute infection.

4.3.5.5 ACAT inhibition and donor characteristics in acute SARS-CoV-2 infection

As described in chapter 4.3.4.4, donor age and sex are associated with alterations in immune function and metabolism. This is especially important in SARS-CoV-2 where increased age

(Richardson et al. 2020) and male sex (Scully et al. 2020) are determinants of increased disease severity. In line with donors being hospitalized, donors in this acute SARS-CoV-2 cohort were older than the HBV cohort with a median age of 81.5 years (range 48-97 years). Importantly, response to ACAT inhibition was not associated with donor age and the response rate did not decline in old age (Fig. 4.32a,b).

In contrast to the findings in CHB, ACAT inhibition boosted SARS-CoV-2 specific T cells irrespective of donor sex with comparable IFN γ increase and number of male and female donors responding to therapy (Fig. 4.32c,d).

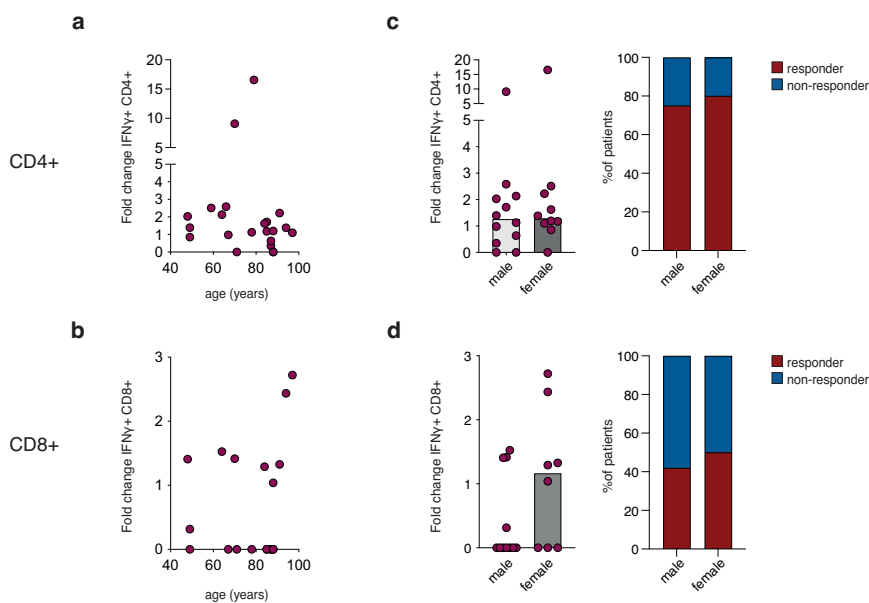


Figure 4.32 ACAT inhibition and donor characteristics in acute SARS-CoV-2 infection.

(a+b) Fold increase of SARS-CoV-2-specific (Spike+Mem) IFN γ ⁺ CD4⁺ **(a)**; n=22) and CD8⁺ **(b)**; n=19) T cells with ACAT inhibition (Avasimibe) compared to DMSO correlated with donor age. **(c+d)** Association of fold increase HBV-specific IFN γ ⁺ CD4⁺ **(c)**; male n=12; female n=10) and CD8⁺ **(d)**; male n=11; female n=8) with donor sex (left) and sex difference in response to ACAT inhibition (right). Response defined as increased or *de novo* IFN γ production. P values determined by Spearman correlation **(a+b)**, Mann-Whitney test **(c+d)**; left) and Fisher's exact test **(c+d)**; right).

4.3.5.6 Summary

ACAT inhibition enhanced the production of antiviral cytokines, proliferation and the expression of molecules associated with T_{fh} function of SARS-CoV-2 specific CD4⁺ T cells. Immune boosting was irrespective of donor age and sex, rendering modulation of cholesterol

esterification a potential treatment strategy, especially for patients with high risk of severe disease.

4.3.6 Cytotoxic and off-target immune responses

Any immunotherapy bears the risk of organ damage due to immune-mediated cytotoxicity or untargeted immune boosting. ACAT inhibition with Avasimibe did not cause cytotoxicity, organ damage or autoimmunity in a murine tumour model or in clinical atherosclerosis trials (Llaverías et al. 2003; Yang et al. 2016).

Cytotoxicity was indirectly assessed by flow cytometry detecting the mobilization of LAMP-1 (CD107a) to the cell surface (for details see chapter 3.9.1). In CHB, ACAT inhibition tended to enhance the mobilization of CD107a to the cell surface of HBV-specific CD8⁺ T cells from the periphery (Fig. 4.33a) but not from the liver (Fig. 4.33b) with no consistent, significant effect in the cohort overall, suggesting that ACAT inhibition is not likely to induce intrahepatic T cell-mediated cytotoxicity and associated liver damage. In acute SARS-CoV-2, ACAT inhibition did not significantly increase the frequency of cytotoxic T cells in the periphery, identified by CD107a on the cell surface and perforin granules in the cytoplasm (Fig. 4.33c).

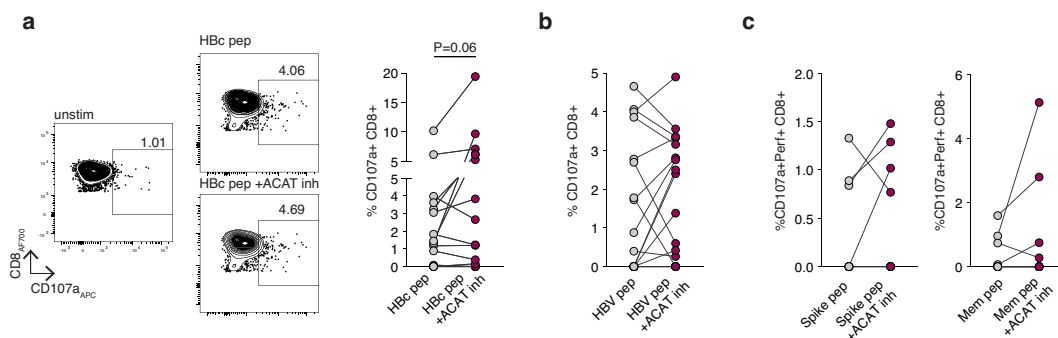


Figure 4.33 ACAT inhibition does not enhance virus-specific CD8⁺ T cell cytotoxicity.

(a) PBMC from CHB donors were stimulated with HBc pep \pm ACAT inhibition (Avasimibe) for 7d and HBV-specific CD107a mobilization on CD8⁺ T cells was detected by flow cytometry. Representative flow cytometry plot and summary data (n=17). **(b)** IHL from CHB donors were stimulated with HBV pep \pm ACAT inhibition (K-604) for 16h and HBV-specific CD107a mobilization on CD8⁺ IHL was detected by flow cytometry (n=22). **(c)** PBMC from donors with acute SARS-CoV-2 infection were stimulated with SARS-CoV-2 pep (Spike and Membrane, Mem) \pm ACAT inhibition (Avasimibe) for 7d. Assessment of SARS-CoV-2-specific CD107a mobilization and perforin co-expression on CD8⁺ T cells (n=11). P values determined by Wilcoxon matched-pairs signed rank test.

Untargeted activation and boosting of immune cells, causing inflammation and autoimmunity, are classical adverse events of immunotherapies such as checkpoint blockade (Khan and Gerber 2020). *In vitro* ACAT inhibition did not increase the background cytokine production of unstimulated CD8⁺ and CD4⁺ T cells from donors with CHB (Fig. 4.34a) and acute SARS-CoV-2 infection (Fig. 4.34b).

Additionally, ACAT inhibition did not alter the expression of activation markers including the MHC class II molecule HLA-DR and the cyclic ADP-ribose hydrolase CD38 on unstimulated PBMC from CHB donors (Fig. 4.34c), molecules typically associated with proliferation and effector function (Cesano et al. 1998; Ho et al. 1993; Hua et al. 2014; Speiser et al. 2001; Wang et al. 2018b); however, proliferation of CD8⁺ T cells without *in vitro* peptide stimulation from donors with acute SARS-CoV-2 was slightly enhanced as indicated by increased CFSE dilution (Fig. 4.34d) which may be due to activation of these cells by *in vivo* antigen exposure during acute infection.

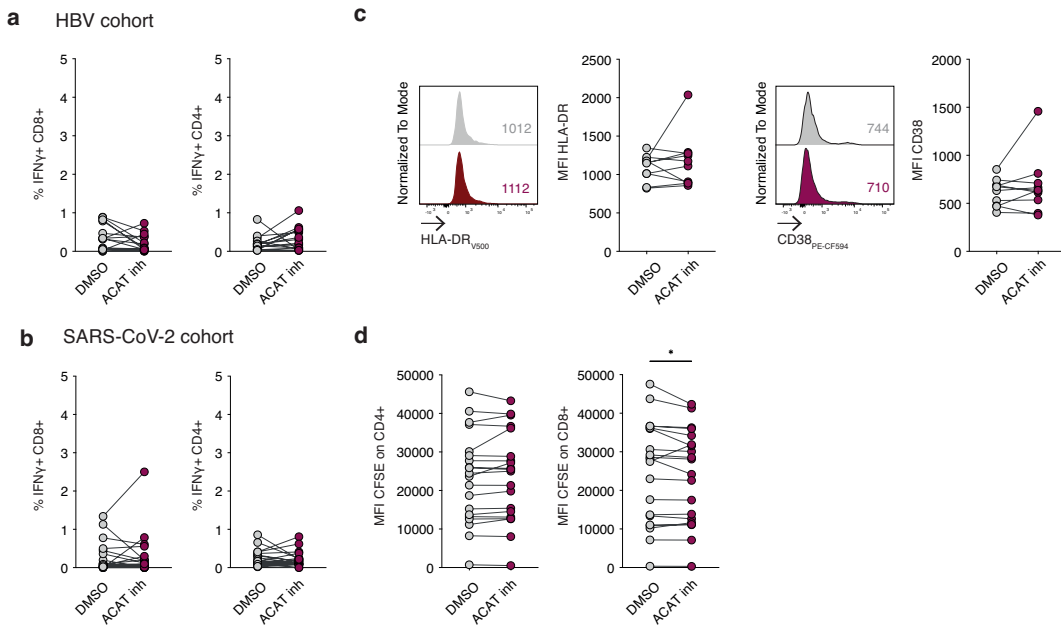


Figure 4.34 ACAT inhibition does not increase non-specific cytokine production.

(a,b) PBMC from donors with CHB **(a; n=16)** and acute SARS-CoV-2 **(b; n=22)** ±ACAT inhibition (Avasimibe) for 7d without *in vitro* peptide stimulation. Assessment of cytokine production by CD8⁺ and CD4⁺ T cells by flow cytometry. **(c)** Expression of HLA-DR and CD38 on unstimulated CD8⁺ T cells from CHB donors ±ACAT inhibition (Avasimibe) for 7d (n=10). **(d)** CFSE MFI on T cells from donors with acute SARS-CoV-2 infection ±ACAT inhibition (Avasimibe) for 7d without *in vitro* stimulation. P values determined by Wilcoxon matched-pairs signed rank test.

As shown for other immunotherapies such as IL-12 (Schurich et al. 2013), ACAT inhibition did not boost the function of highly functional memory CD8⁺ T cells in well-controlled cytomegalovirus (CMV; pp65 peptide pool and HLA-A2-restricted immunodominant epitope CMVpp65₄₉₅₋₅₀₃: NLV) infection (Fig. 4.35a) nor virus-specific CD8⁺ T cells after acute infection with influenza (Flu; Fig. 4.35b) or Epstein-Barr virus (EBV; Fig. 4.35c).

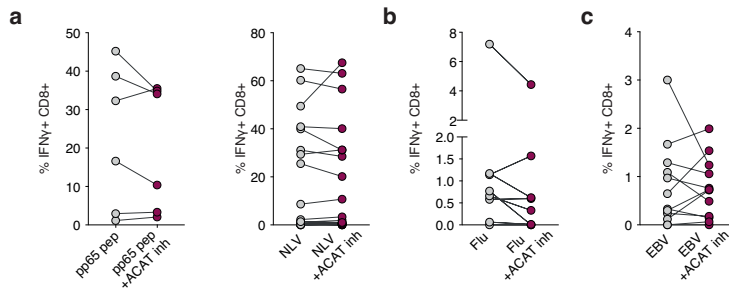


Figure 4.35 ACAT inhibition does not enhance CMV-, Influenza- and EBV-specific CD8⁺ T cells. (a-c) PBMC were stimulated with pp65 pep (a, left; n=6), NLV (a, right; n=21), Influenza (Flu; b; n=8), EBV (c; n=12) ±ACAT inhibition (Avasimibe) for 7d and IFN γ production was detected via flow cytometry. P values determined by Wilcoxon matched-pairs signed rank test.

I further confirmed this finding in a second SARS-CoV-2 cohort 6 months after PCR-confirmed SARS-CoV-2 infection during the first wave in the UK (COVIDsortium cohort). Donors were unvaccinated healthcare workers with mild disease and a median age of 44.5 years. In line with my findings for EBV- and influenza-specific memory T cells, ACAT inhibition only enhanced the production of antiviral cytokines by SARS-CoV-2-specific memory T cells in a minority of donors and the effect was inconsistent without significant change in the cohort overall (CD8⁺ T cells: Fig. 4.36; CD4⁺ T cells Fig. 4.37a-c). Similar effects were seen for the expression of CD154 on CD4⁺ T cells (Fig. 4.37d).

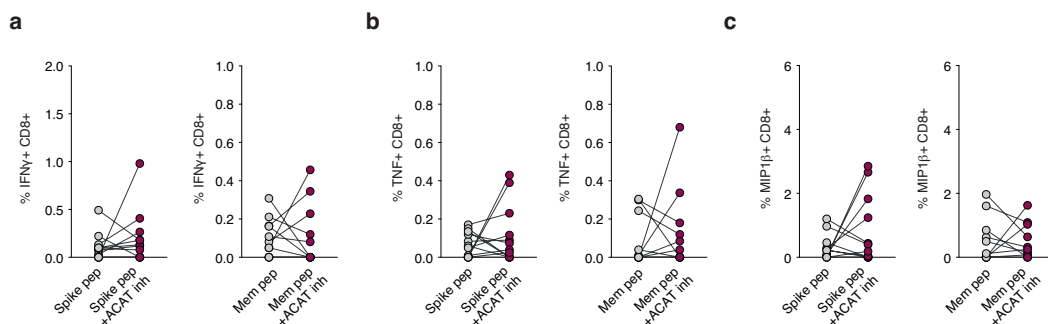


Figure 4.36 ACAT inhibition does not enhance SARS-CoV-2-specific CD8⁺ T cells 6 months post infection.

(a-c) PBMC from donors 6 months post SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (pep; Spike and Membrane, Mem) ±ACAT inhibition (Avasimibe) for 7d. SARS-CoV-2-specific cytokine production by CD8⁺ T cells was detected via flow cytometry. Summary data of SARS-CoV-2-specific IFN γ (a), TNF (b) and MIP1 β (c) production by CD8⁺ T cells (Spike n=12; Mem n=11). P values determined by Wilcoxon matched-pairs signed rank test.

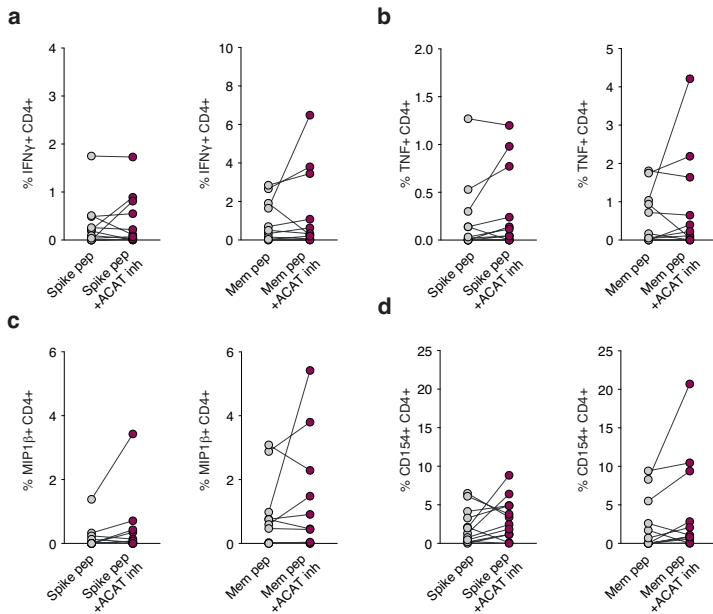


Figure 4.37 ACAT inhibition does not enhance SARS-CoV-2-specific CD4⁺ T cells 6 months post infection.

(a-d) PBMC from donors 6 months post SARS-CoV-2 infection were stimulated with SARS-CoV-2 peptide pools (pep; Spike and Membrane, Mem) ± ACAT inhibition (Avasimibe) for 7d. SARS-CoV-2-specific cytokine production by CD4⁺ T cells was detected via flow cytometry. **(a-d)** Summary data of SARS-CoV-2-specific IFN γ **(a)**, TNF **(b)**, MIP1 β **(c)** production and CD154 upregulation **(d)** by CD4⁺ T cells (Spike n=12; Mem n=11). P values determined by Wilcoxon matched-pairs signed rank test.

Taken together, ACAT inhibition did not enhance cytotoxicity in intrahepatic HBV-specific and circulating SARS-CoV-2-specific CD8⁺ T cells and did not induce non-specific activation or increased function of memory T cells.

4.4 Discussion

Nutrient availability, uptake and utilization play a central role in successful immune responses and targeting metabolic pathways is a promising new immunotherapeutic strategy (O'Sullivan et al. 2019; Pallett et al. 2019). Cholesterol is critical for antiviral T cell function (see chapter 1.2.5.3) and I have now shown for the first time that a modulation of cholesterol esterification with ACAT inhibitors boosts the expansion of functional virus-specific T cells and induces *de novo* responses in donors without detectable pre-existing responses.

After careful titration and optimisation, the chosen concentration of ACAT inhibitor was lower than previously reported to boost polyclonal CD8⁺ T cells (Yang et al. 2016), and did not cause any direct cytotoxic effects with the chosen treatment protocol.

HBV-specific T cells in CHB are highly exhausted with low frequencies and reduced antiviral effector function (see chapter 4.1.1.4). ACAT inhibition enhanced the function and proliferation of HBV-specific T cells from the blood and infected liver tissue of donors with CHB. The immune-boosting effect of ACAT inhibition was not limited to one compound but was instead reproducible using two different ACAT inhibitors, Avasimibe (inhibition of ACAT1+2) and K-604 (ACAT1-specific). Of note, only a subset of experiments was performed with both ACAT inhibitors and the direct comparison will need to be probed further in future studies.

T cell exhaustion has first been described in settings with chronic antigen exposure, such as chronic viral infections and tumours (Wherry and Kurachi 2015); however, accumulating data report features of T cell exhaustion, such as PD-1 expression, in acute SARS-CoV-2 infection, especially in severe disease (Chen and Wherry 2020), suggesting that immunotherapy might be beneficial. In line with my findings in CHB, ACAT inhibition boosted the function and expansion of SARS-CoV-2-specific T cells in hospitalized donors during acute infection. In this study, I focused on T cells specific for spike and membrane, two key structural proteins that elicit T cell responses in acute infection (Peng et al. 2020). It would be interesting to further investigate the effect of ACAT inhibition on T cells with specificity for non-structural proteins associated with abortive infection (Swadling et al. 2022).

T cells resident within non-lymphoid organs, such as the lung (Snyder and Farber 2019) and the liver (Pallett and Maini 2022), are critical to fight viral infections as they provide antiviral immunity directly at the site of disease as well as long-lasting immune memory, protecting from consecutive infections. The liver and lung are unique microenvironments with altered oxygen levels, nutrient supply and a multitude of mechanisms resulting in immune tolerance, ultimately shaping local immune responses (Pallett and Maini 2022; Traxinger et al. 2022). Both the lung and liver constitute cholesterol-rich environments with the liver being the central hub for lipid metabolism (Chamberlain 1928; Trapani et al. 2012) and cholesterol being the main neutral lipid in pulmonary surfactant (Keating et al. 2007). ACAT inhibition preferentially boosted HBV-specific CD8⁺ and CD4⁺ T cells directly *ex vivo* from the immunotolerant, cholesterol-rich liver environment, a finding that could be recapitulated by cholesterol supplementation *in vitro*. Considering the similarities between lung and liver tissue, it would be expected to see a similar enhancement of response to ACAT inhibition in SARS-CoV-2-specific T cells directly isolated from the infected lung (also see discussion section 7.2.5).

In line with the observation that SOAT1 was not only expressed by human CD8⁺ but also by CD4⁺ T cells, ACAT inhibition overall enhanced both CD8⁺ and CD4⁺ T cells. This finding is in contrast to a previously published murine study that did not detect any alterations of antitumour CD4⁺ T cell function with ACAT inhibition (Yang et al. 2016), highlighting the necessity of assessing potential drug targets in human tissue.

Interestingly, the immune boosting profile of ACAT inhibition differed between the two viral infections with antiviral CD8⁺ T cells mainly enhanced in the blood and liver of CHB donors and antiviral CD4⁺ T cells mainly enhanced in CHB liver and in SARS-CoV-2. A variety of factors could contribute to these differences and should be explored in future studies, such as differences in metabolic profile, immune dysfunction/exhaustion, T cell priming, intracellular drug target expression as well as differences in donor cohorts (age, comorbidities) and metabolic alterations during acute infection (also see discussion chapter 7.2.4). Of note, the slight alterations of the cell culture conditions (IL-2, CD28 stimulation) between the assessment of HBV- and SARS-CoV-2-specific T cells could also contribute to the detected differences.

Besides the enhancement of T cell effector function, ACAT inhibition also increased the frequency of Tfh cells and the expression of co-stimulatory molecules associated with Tfh function and activation. This study has several limitations that will need to be addressed in the future. I utilized PD-1 and CXCR-5 to identify Tfh cells; however, the expression of these molecules can be altered during culture, especially in the presence of IL-2 (Kinter et al. 2008). IL-2 further limits Tfh differentiation (Ballesteros-Tato et al. 2012) and may have masked alterations of Tfh frequency and function. I focused on the expression of costimulatory molecules and future studies should include functional analyses such as the production of the Tfh-associated cytokines IL-4, IL-21, CXCL13 (Crotty 2019) and direct functional assays, e.g, evaluating increased induction of plasma cell differentiation. Of note, this study was conducted on circulating Tfh cells and it will be crucial to assess the effect of Tfh residing in lymph nodes and spleen, the location of Tfh/B-cell interaction.

Metabolic requirements vary highly between different immune cells subsets and it is critical to also consider the effect ACAT inhibition on cells other than antiviral T cells to anticipate the effect of CHB treatment *in vivo* (also see discussion chapter 7.2.6). T_{reg} are metabolically distinct from conventional CD4⁺ and CD8⁺ T cells and especially cholesterol availability and utilization are critical for T_{reg} stability, expansion and suppressive function (Pinzon Grimaldos et al. 2022); surprisingly, ACAT inhibition did not alter T_{reg} frequency and did not consistently affect the expression of molecules associated with T_{reg} activation/suppressive function nor the expression of CTLA-4 or CD39, directly involved in T_{reg}-modulated immunosuppression, with any detected changes being inconsistent, subtle and likely not biologically relevant. This is in line with unpublished, preliminary data from our collaborators showing no effect on CTLA-4 mediated CD80/CD86 transendocytosis from APC by T_{reg} (Sansom Lab, UCL) and no alteration of T_{reg} suppressive capacity in T cell suppression assays (Gilead Biosciences). Additionally, immunomodulatory effects on $\gamma\delta$ T cells and NK cells were minor and may likely not translate into biological relevant changes *in vivo*.

It is important to note that the ACAT inhibition treatment strategy was optimised to result in immune boosting effects in HBV-specific CD8⁺ T cells. Future studies assessing the effect of ACAT inhibition on other cell subsets will need to consider alternate treatment length, drug concentrations, culture conditions and cell stimulations and, considering the important role of the tissue microenvironment discussed above, should include intrahepatic leucocytes as well as PBMC. Of note, to fully understand the effect of ACAT inhibition *in vivo*, it will

be critical to study alterations of the function of APC, and other immunosuppressive cells besides T_{reg} , e.g. MDSC (also see discussion chapter 7.2.6).

Any immunotherapy for viral infections bears the risk of excessive inflammation and organ damage due to immune-mediated cytotoxicity or untargeted immune boosting. ACAT inhibition with Avasimibe did not cause cytotoxicity, organ damage or autoimmunity in a murine tumour model or in clinical atherosclerosis trials (Llaverías et al. 2003; Yang et al. 2016) and in this study did not consistently boost T cell cytotoxicity nor the function and activation of bystander T cells or memory T cells after resolution of acute infection. However, T cell activation markers are upregulated within minutes to hours after T cell activation and their expression is only transient (Sandoval-Montes and Santos-Argumedo 2005) and therefore measuring T cell activation at earlier timepoints and over a time course would be more suitable to further address this.

It is important to note that clinical differences between the acute and memory SARS-CoV-2 cohorts in this study (age, disease severity, hospitalization) could contribute to the different efficacy of ACAT inhibition described and a longitudinal study in the same patient cohort would be of high interest to fully determine the effect of ACAT inhibition on effector and memory T cells.

Finally, for any therapy it is important to consider which patient populations are likely going to benefit from treatment to avoid unnecessary side effects and healthcare costs (also see discussion chapter 7.2.3).

CHB is a highly variable, dynamic disease with multiple non-sequential disease stages reflecting liver inflammation, antiviral immune response and viral replication (European Association for the Study of the Liver 2017). ACAT inhibition enhanced the antiviral T cell response in donors from all four main disease phases, and donors with clinical indications for therapy (liver inflammation, HBeAg positivity (European Association for the Study of the Liver 2017)) tended to have higher responses to ACAT inhibition. Future studies in larger, more diverse cohorts will be necessary to fully determine the effect of ACAT inhibition in different CHB phases.

The clinical picture of SARS-CoV-2 infection ranges from asymptomatic infection to lethal ARDS influenced by patient characteristics (e.g. age, sex, comorbidities) (Lamers and Haagmans 2022), viral variants (Fisman and Tuite 2022; Wolter et al. 2022) and vaccine status (Feikin et al. 2022; Higdon et al. 2022). This study only included unvaccinated donors hospitalised and recruited in March-July 2020 in London and therefore likely infected with the Victoria strain of SARS-CoV-2, who primarily presented with mild to moderate disease. Studies investigating the effect of ACAT inhibition in a larger cohort with a variety of clinical presentations, vaccine status, and infected with current VOC would be of high clinical interest to determine the potential of ACAT inhibition as a treatment for acute SARS-CoV-2 infection.

Besides acute symptoms, 30-70% of patients experience persistent symptoms 4 weeks or longer after acute infection, so called long COVID or post-acute sequelae of COVID-19 (PASC) (Su et al. 2022). While the mechanisms underlying long COVID have not been completely understood, emerging data suggest immune perturbation, signs of T cell exhaustion (Ryan et al. 2022; Su et al. 2022) and potential viral persistence (Brodin et al. 2022; Yong 2021). Considering the similarities between long COVID and chronic viral infections it could be speculated that ACAT inhibition could also be considered as a therapeutic approach in long COVID; however, T cells may also contribute to pathogenesis in long COVID. Future studies will need to assess immunopathology in long COVID and the potential benefits of ACAT inhibition in this cohort.

Patient sex is an important determinant of immune responses as well as lipid/cholesterol metabolism with alterations driven by sex hormones such as oestrogens, progesterone and androgens (Klein and Flanagan 2016; Robinson et al. 2021). Male sex is associated with increased severity of SARS-CoV-2 infection (Lamers and Haagmans 2022) and with an increased risk of HCC development in CHB (Liu and Liu 2014). In CHB, male donors were more likely to respond to ACAT inhibition and it is tempting to speculate that this was due to hormonal differences as donor sex did not determine treatment response in the older SARS-CoV-2 cohort where most female donors were likely postmenopausal; however future studies are necessary to fully delineate the effect of donor sex on treatment response (also see discussion chapter 7.2.3).

Finally, age has a profound effect on the immune system and is associated with immune senescence, alteration of immune cell function and metabolism (Akbar et al. 2016; Callender et al. 2018, 2020; Mogilenko et al. 2021) as well as with reduced vaccine response (Collier et al. 2021) and severe SARS-CoV-2 (Lamers and Haagmans 2022). The finding that response to ACAT inhibition did not decline with age makes it a promising treatment strategy especially for this highly vulnerable patient group.

In summary, I have shown for the first time that modulation of cholesterol esterification by ACAT inhibition has the potential to boost virus-specific T cells during chronic and acute viral infections, rendering it a promising new therapeutic approach for patients with CHB and SARS-CoV-2.

5

Immunometabolic reprogramming upon ACAT inhibition

ACAT inhibition induces redistribution of intracellular cholesterol with a reduction of cholesteryl ester stored in neutral lipid droplets. Reduced lipid rafts are a feature of exhausted PD-1^{high} CD8⁺ T cells and ACAT inhibition facilitates lipid raft formation and TCR signalling. ACAT inhibition induces extensive metabolic reprogramming and skews bioenergetics towards OXPHOS.

5.1 Introduction

5.1.1 A brief introduction to T cell exhaustion

T cell exhaustion is a distinct state associated with T cell dysfunction that is typically driven by chronic antigen exposure in settings such as chronic infections and cancer. T cell exhaustion is regulated by transcriptional changes and characterized by a hierarchical loss of T cell effector function accompanied by an upregulation of inhibitory checkpoint molecules and metabolic alterations (Wherry and Kurachi 2015).

PD-1 is the most studied inhibitory receptor and is exploited as a prototypical marker of exhausted T cells. One of the key mechanisms by which PD-1 modulates T cell function is the dephosphorylation of CD28 via SHP-2 phosphatase (Hui et al. 2017). CD28 is critical as a costimulatory molecule during T cell activation and mediates lipid raft assembly and the formation of the immunological synapse (Zumerle et al. 2017). PD-1 further induces metabolic dysfunction, including mitochondrial defects limiting the metabolic switch to OXPHOS (Schurich et al. 2016).

T cell activation and exhaustion are discussed in more detail in chapter 1.1.1 and 1.1.2, respectively. Metabolic alterations due to PD-1 signalling are discussed in chapter 1.2.2.

5.1.2 A brief introduction to T cell cholesterol metabolism

Cholesterol availability, uptake and utilization play a central role in successful immune responses. Cholesterol constitutes an essential component of cellular plasma membranes impacting membrane lipid order and membrane fluidity (Bietz et al. 2017; Ikonen 2008; Miguel et al. 2011; Sezgin et al. 2017). Cholesterol- and glycosphingolipid-enriched lipid rafts are essential for the formation of the immunological synapse between T cells and APC, and

for subsequent TCR signalling and T cell effector function (Sezgin et al. 2017; Zumerle et al. 2017).

Cholesterol import, synthesis and efflux are tightly regulated by the transcription factors LXR and SREBP to ensure intracellular homeostasis (Kidani and Bensinger 2016). Due to its hydrophobic properties, free cholesterol cannot be stored intracellularly but is either exported, mobilized into lipid bilayers, or esterified by ACAT and stored in neutral lipid droplets. Lipid accumulation constrains NK cell function (Michelet et al. 2018) as well as DC and CD8⁺ T cells in a context-dependent manner (Cao et al. 2014; Herber et al. 2010; Ibrahim et al. 2012; Xu et al. 2021).

Cholesterol metabolism is discussed in further detail in chapter 1.2.5.

5.1.3 Electron transport chain and extracellular flux analysis

Naive and memory T cells typically rely on mitochondrial OXPHOS driven by FAO for ATP synthesis (Buck et al. 2016) whereas effector T cells shift towards glycolysis to meet the rapid energy demanded upon T cell activation (Chang et al. 2013).

The ETC is located at the inner mitochondrial membrane and facilitates mitochondrial OXPHOS. It consists of five complexes and two electron carriers (coenzyme Q and cytochrome c) that oxidise NADH and FADH₂ to NAD⁺ and FAD⁺ in order to generate ATP (Yin and O'Neill 2021; Zhao et al. 2019) (Fig. 5.1).

Complex I (NADH dehydrogenase) catalyses the transfer of two electrons from NADH to coenzyme Q and exports 4 protons to the intermembrane space. Complex II (succinate dehydrogenase) directly connects the TCA cycle to mitochondrial respiration as it catalyses the oxidation of succinate into fumarate as part of the TCA cycle and in this process FAD⁺ is reduced to FADH₂. When FADH₂ is oxidised back to FAD⁺, electrons are transferred to coenzyme Q via FeS clusters. Coenzyme Q further transports electrons to complex III (cytochrome c reductase) and then via cytochrome c to complex IV (cytochrome c oxidase) where O₂ is reduced to H₂O. During this process further protons are exported via complex

III and IV. The transfer of electrons and the translocation of protons create an electrochemical gradient across the inner mitochondrial membrane that drives ATP synthesis from ADP by complex V (ATP synthase).

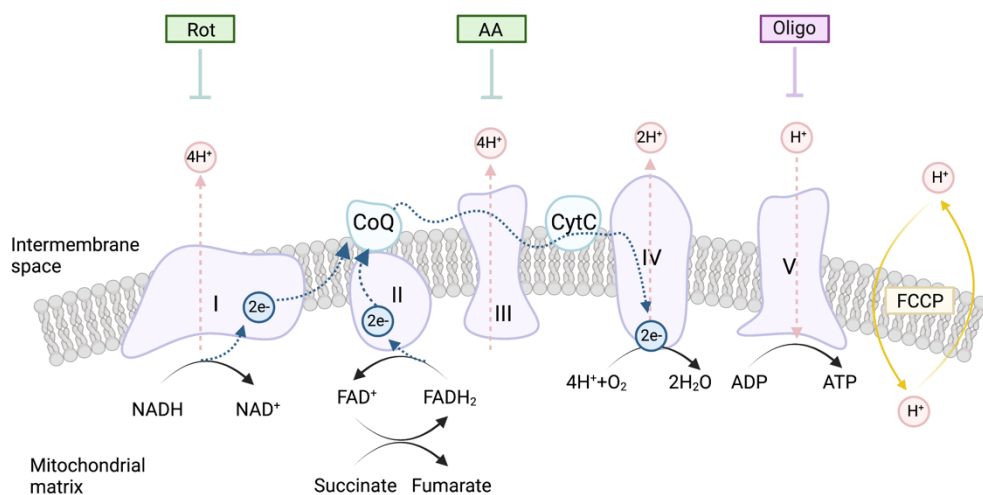


Figure 5.1 The electron transport chain.

Schematic of complex I-V of the electron transport chain adapted from (Yin and O'Neill 2021) and mechanism of action of compounds used for a mitochondrial stress test. CoQ: coenzyme Q. CytC: cytochrome C; NAD⁺: nicotinamide adenine dinucleotide; FAD⁺: flavin-adenine dinucleotide; ATP: adenosine triphosphate; ADP: adenosine diphosphate; Rot: rotenone; AA: antimycin A; Oligo: oligomycin; FCCP: carbonyl cyanide-4-trifluoromethoxyphenylhydrazone.

Extracellular flux analysis allows the assessment of OXPHOS and glycolysis in real time via the measurement of extracellular oxygen (oxygen consumption rate, OCR) and pH (extracellular acidification rate, ECAR, driven by lactic acid release), respectively (van der Windt et al. 2016). The consecutive administration of compounds targeting different parts of the ETC provides further in-depth information on mitochondrial function in a ‘mitochondrial stress test’ (Fig. 5.1 and 5.2).

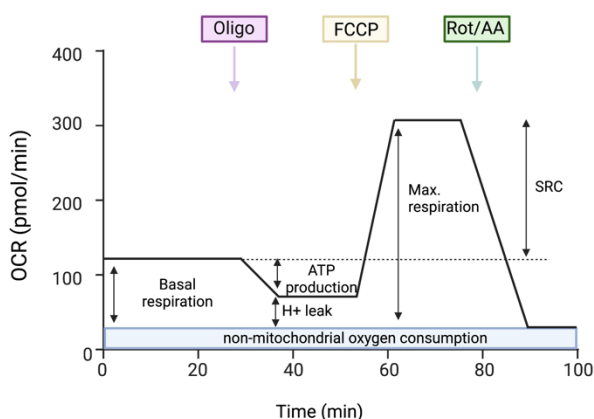


Figure 5.2 Mitochondrial stress test.

Schematic of a mitochondrial stress test to assess basal respiration, ATP production, maximal respiration and SRC. Oligo: oligomycin; FCCP: carbonyl cyanide-4-trifluoromethoxyphenylhydrazone; Rot: rotenone; AA: antimycin A; OCR: oxygen consumption rate; ATP: adenosine triphosphate; SRC: spare respiratory capacity.

After a basal measurement, the first compound injected is the complex V inhibitor Oligomycin resulting in a decrease of OCR which indicates how much of the basal oxygen consumption is due to ATP synthesis. Next, the uncoupler carbonyl cyanide-4-trifluoromethoxyphenylhydrazone (FCCP) depolarises the membrane and disrupts the proton gradient resulting in maximal ETC activity in order to re-establish the electrochemical gradient and membrane potential. Administration of FCCP allows measurement of the maximal respiration and calculation of the SRC, indicating an energy reserve that can be exploited in response to cellular stress. In a final step, rotenone (complex I inhibitor; Rot) and antimycin A (complex III inhibitor; AA) shut down the electron transport chain with any residual OCR being attributed to non-mitochondrial oxygen consumption.

5.2 Hypotheses and research questions

Hypothesis I: ACAT inhibition alters CD8⁺ T cell exhaustion phenotype reflecting enhanced T cell effector function.

- Does ACAT inhibition modulate the expression of molecules and transcription factors associated with T cell exhaustion?

Hypothesis II: T cell exhaustion is associated with reduced lipid rafts.

- Is the expression of T cell exhaustion markers associated with low lipid rafts?
- What is the relationship between CD28 expression and lipid rafts?

Hypothesis III: Inhibition of cholesterol esterification by ACAT inhibition leads to redistribution of intracellular cholesterol.

- Does ACAT inhibition reduce cholesteryl ester, the main component of intracellular neutral lipid droplets?
- Does ACAT inhibition increase membrane cholesterol levels?
- Does ACAT inhibition facilitate lipid raft formation and is this indicative of treatment response?
- Does ACAT inhibition affect cellular transporters associated with cholesterol uptake and efflux?
- Are CD28 signalling and ACAT inhibition synergistic?
- Does ACAT inhibition enhance TCR signalling?

Hypothesis IV: ACAT inhibition results in bioenergetic reprogramming.

- Does ACAT inhibition alter T cell bioenergetics (OXPHOS, glycolysis)?

5.3 Results

5.3.1 ACAT inhibition does not alter CD8⁺ T cell exhaustion phenotype

Immunotherapies have the potential to regulate the expression of inhibitory checkpoint molecules such as PD-1 and transcription factors associated with T cell function as shown for *in vitro* treatment with IL-12 (Kurktschiev et al. 2014; Schurich et al. 2013). High cholesterol in the tumour tissue can induce T cell exhaustion associated with an increase of inhibitory receptors such as PD-1 and Tim-3 (Ma et al. 2019). I therefore hypothesized that ACAT inhibition would alter the exhaustion phenotype of CD8⁺ T cells.

In the high cholesterol tumour microenvironment, PD-1 upregulation is induced as a result of ER stress and consecutive activation of XBP-1 (Ma et al. 2019); however, I found that inhibition of cholesterol esterification did not alter PD-1 nor Tim-3 expression on CD8⁺ T cells (Fig. 5.3 a,b).

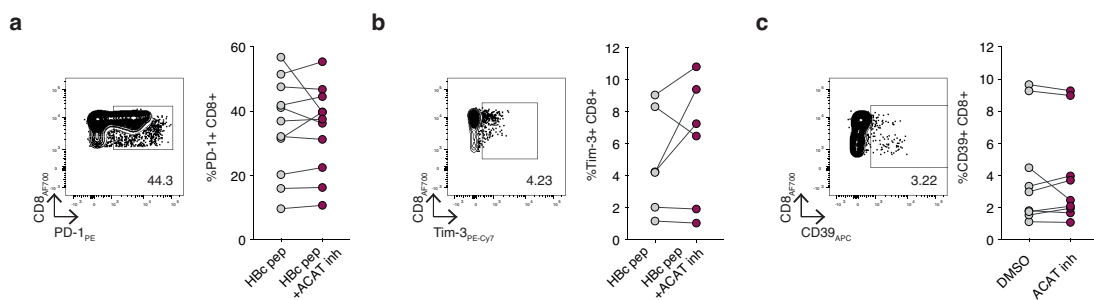


Figure 5.3 ACAT inhibition does not alter the expression of surface molecules associated with T cell exhaustion.

(a+b) PBMC were stimulated with HBe pep \pm ACAT inhibition (Avasimibe) for 7d and analysed via flow cytometry. Frequency of PD-1⁺ (**a**; n=11) and Tim-3⁺ (**b**, n=6) CD8⁺ T cells. **(c)** Frequency of CD39⁺ CD8⁺ T cells in unstimulated PBMC from CHB donors \pm ACAT inhibition (Avasimibe) for 16h (n= 9). P values determined by Wilcoxon matched-pairs signed rank test.

CD39 expression is a hallmark of T_{reg}, contributing to their immunosuppressive function (see chapter 1.2.4.3.1 and Table 4.2), but it is also a feature of terminally exhausted CD8⁺ T cells (Gupta et al. 2015). Despite CD39 being localised in lipid rafts and its lipid raft-

dependent activity (Savio et al. 2020), ACAT inhibition did not modulate CD39 expression (Fig. 5.3c).

CD8⁺ T cell fate is regulated by a variety of transcription factors including, but not limited to, TOX, T-bet and Eomes (Intlekofer et al. 2005; Mann and Kaech 2019) (also see chapter 1.1.3.3). Recently, four stages of T cell exhaustion have been defined with differential expression of T-bet and Eomes (Beltra et al. 2020). Some immunotherapies, such as PD-L1 blockade, preferentially expand the intermediate T_{EX}^{Prog2+int} subsets that don't express Eomes (Beltra et al. 2020). ACAT inhibition did not modulate the expression of TOX (Fig. 5.4a) nor did it affect the frequency of T-bet or Eomes expressing CD8⁺ T cells (Fig. 5.4b).

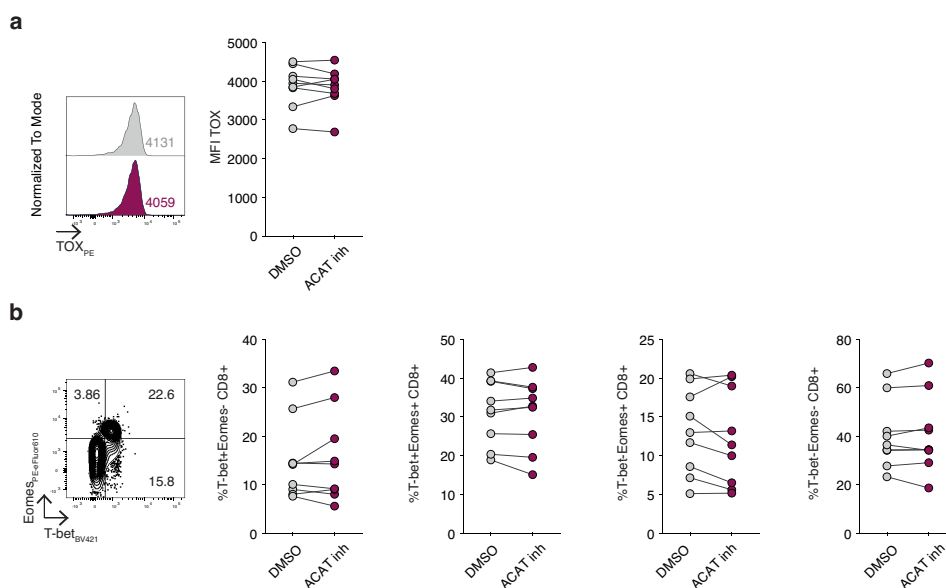


Figure 5.4 ACAT inhibition does not alter the expression of transcription factors associated with CD8⁺ T cell exhaustion.

(a+b) PBMC were treated with ACAT inhibition (Avasimibe) for 16h and analysed via flow cytometry (n=9). Example histogram/flow cytometry plot for **(a)** MFI TOX and **(b)** Eomes and T-bet co-expression on CD8⁺ T cells. P values determined by Wilcoxon matched-pairs signed rank test.

Distinct chromatin states further characterize PD-1⁺ CD8⁺ T cells in tumours, identifying a plastic and a fixed dysfunctional stage (Philip et al. 2017). The co-expression of the surface markers CD38 and CD101 (immunoglobulin superfamily member 2) is associated with a fixed dysfunctional state that cannot be rescued by immunotherapy, whereas double negative T cells are in an earlier, plastic stage that allows therapeutic reprogramming, resulting in enhanced effector function (Philip et al. 2017). ACAT inhibition did not alter co-expression

of CD101 and CD38 in PD-1⁺ CD8⁺ T cells in a small cohort of donors suggesting that it did not induce a shift of chromatin states (Fig. 5.5).

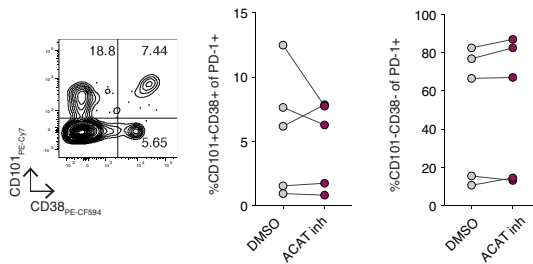


Figure 5.5 ACAT inhibition does not alter the co-expression of CD101 and CD38.

Unstimulated PBMC from CHB donors \pm ACAT inhibition (Avasimibe) for 7d and co-expression of CD38 and CD101 on PD-1⁺ CD8⁺ T cells analysed via flow cytometry (n=5). P values determined by Wilcoxon matched-pairs signed rank test.

In summary, ACAT inhibition did not alter the expression of molecules or transcription factors associated with exhaustion on global CD8⁺ T cells from CHB donors.

5.3.2 ACAT inhibition induces extensive metabolic re-wiring of CD8⁺ T cells

Since ACAT inhibition only induced minor alterations of classical T cell phenotypic features associated with T cell exhaustion, I next aimed to explore metabolic changes underlying the T cell boosting effect. Intracellular metabolic pathways are highly interconnected and are tightly controlled by regulation of nutrient uptake, biosynthesis, utilization and efflux. ACAT inhibition prevents cholesterol esterification and I hypothesized that this would shift intracellular cholesterol distribution and induce metabolic reprogramming. Considering the critical role of cholesterol and lipid rafts in T cell activation and effector function I further hypothesized that reduced lipid rafts would be a feature of T cell exhaustion.

5.3.2.1 ACAT inhibition alters intracellular cholesterol distribution

Cholesterol is a major component of the cell membrane and is crucial for T cell activation, proliferation and effector function (Bietz et al. 2017; Ikonen 2008; Pallett et al. 2019). Cholesterol uptake, efflux and synthesis are tightly regulated, and excess cholesterol is either effluxed, integrated into cell membranes or esterified by the enzyme ACAT and stored in neutral lipid droplets in the cytoplasm (see chapter 1.2.5). This section will explore the effect of ACAT inhibition on intracellular cholesterol distribution.

5.3.2.1.1 ACAT inhibition reduces neutral lipid droplets

In line with the role of ACAT in cholesterol esterification, I anticipated that ACAT inhibition would reduce neutral lipid droplets with potential direct beneficial effects on immune cell functionality. To visualize lipid droplets, I utilized a LipidTOX stain with high affinity to neutral lipid droplets which is commonly used for this application (Michelet et al. 2018; Xu et al. 2021).

As hypothesized, neutral lipid droplets in the cytoplasm of CD8⁺ T cells were reduced as early as 1h after ACAT inhibition and the reduction was sustained for 7d when ACAT inhibitors were replenished every 48h (Fig. 5.6a). Equally, ACAT inhibition reduced neutral lipid droplets in CD4⁺ T cells (Fig. 5.6b). Of note, changes of fluorescent intensity were not due to alterations of cell size (Fig. 5.6c).

High *ex vivo* neutral lipid droplets in CD8⁺ T cells correlated with the degree of recovery of HBV-specific IFN γ production after ACAT inhibition (Fig. 5.6d), highlighting lipid droplet reduction as a one of the relevant modes of action of ACAT inhibition.

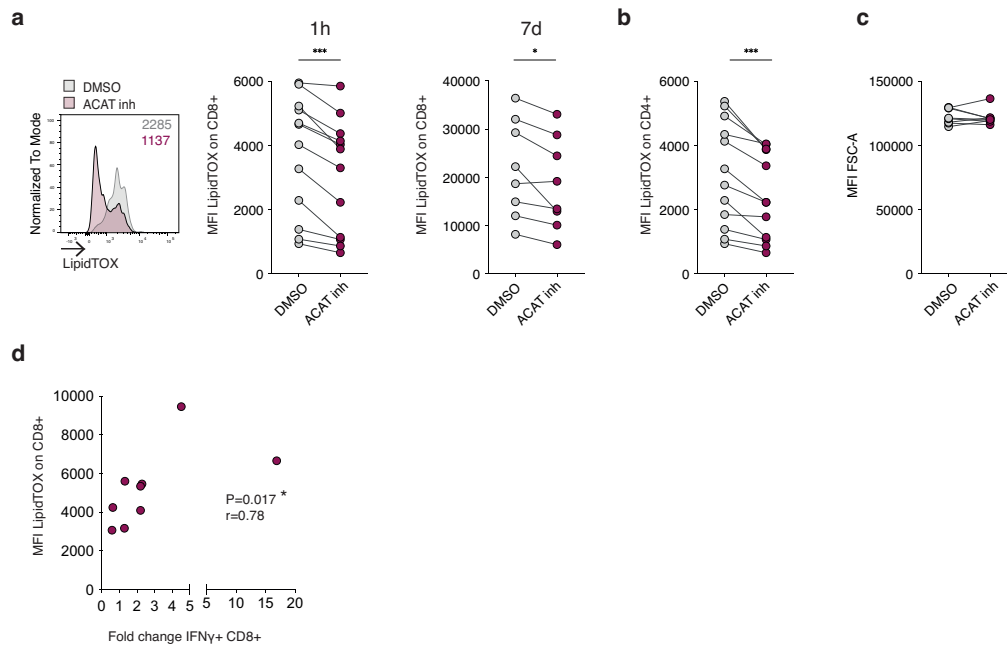


Figure 5.6 ACAT inhibition reduces neutral lipid droplets.

(a) Example histogram and summary data for neutral lipid droplets identified by LipidTOX staining of unstimulated CD8⁺ T cells \pm ACAT inhibition (K-604) for 1h (left; n=12) or repetitively for 7d (right; n=8). (b) Neutral lipid droplets in CD4⁺ T cells \pm ACAT inhibition (K-604) for 1h (n=12). (c) Cell size determined by FSC-A on unstimulated PBMC \pm ACAT inhibition (K-604) for 7d (n=8). (d) Correlation of *ex vivo* neutral lipid droplets in CD8⁺ T cells from PBMC with fold change of HBV-specific IFN γ production \pm ACAT inhibition (Avasimibe for 7d; n=9). P values were determined by Wilcoxon matched-pairs signed rank test (a,b,c) and Spearman correlation (d).

5.3.2.1.2 ACAT inhibition facilitates lipid raft formation and TCR signalling

I next hypothesized that the blockade of cholesterol esterification would lead to an increase of free cholesterol as previously shown in murine T cells (Yang et al. 2016). To visualize free, non-esterified cholesterol, I utilized Filipin complex, a naturally fluorescent antibiotic derived from *Streptomyces filipinensis*, that binds to cholesterol but not to esterified sterols and is frequently used for this purpose (Ma et al. 2019; Maxfield and Wüstner 2012; Surls et al. 2012; Yang et al. 2016).

In this *in vitro* setting, ACAT inhibition alone did not alter Filipin staining (Fig. 5.7a). As discussed in chapter 4.3.4.6, the liver is enriched in cholesterol (Chamberlain 1928) and high cholesterol levels induce ACAT activity (Gillies et al. 1990). Therefore, the impact of ACAT inhibition may be more profound in high cholesterol environments. To recapitulate the liver environment *in vitro*, additional cholesterol was added to the culture media which resulted in

a subtle but significant increase of fold change free membrane cholesterol with ACAT inhibition compared to the effect of ACAT inhibition in normal culture media (Fig. 5.7a), highlighting the enhanced efficacy of ACAT inhibitor in high cholesterol environments.

Membrane cholesterol is a key component of lipid rafts that are critical for immune synapse formation and subsequent TCR signalling (Sezgin et al. 2017; Zumerle et al. 2017) (also see chapter 1.1.). Cholera toxin B subunit (CTB) binds to monosialotetrahexosylganglioside (GM1) located in lipid rafts and is traditionally exploited to visualize lipid rafts (Janes et al. 1999; McDonald et al. 2014; Schoen and Freire 1989).

I first confirmed that fluorescently labelled CTB indeed localized in the immune synapse (Fig. 5.7b). Due to their low frequency in PBMC, HBV-specific CD8⁺ T cells are difficult to visualize by microscopy and I instead utilized CMV-specific CD8⁺ T cells for this proof-of-concept experiment. CMV-specific CD8⁺ T cells (specific for the HLA-A2 restricted epitope NLV) from an HLA-A2⁺ donor were expanded with their cognate antigen in a short-term *in vitro* culture. After staining with fluorescently-labelled CTB and anti-CD3 ϵ antibody, I visualized the immune synapse between CMV-specific CD8⁺ T cells and fluorescently labelled HLA-A2 expressing T2 cells loaded with their cognate peptide. As expected, CTB and the TCR localised in the immune synapse between T cell and APC.

I then utilized flow cytometry for a quantitative assessment of lipid rafts on a single cell level on low frequency HBV-specific CD8⁺ T cells. ACAT inhibition with two different ACAT inhibitors (Avasimibe, K-604) increased the frequency of CD8⁺ T cells with high CTB staining, indicating enhanced lipid raft formation (Fig. 5.7c).

The magnitude of increase in CTB staining tended to correlate with the increase of HBV-specific IFN γ production following ACAT inhibition (Fig. 5.7d) and donors with low *ex vivo* lipid rafts and low CD28, the co-stimulatory molecule critical for lipid raft assembly (chapter 1.1.1), were significantly more likely to respond (Fig. 5.7e), supporting the relevance of this mechanism of action. In line with the increase of lipid rafts by ACAT inhibition, treatment was associated with increased detection of doublets (two cells attached to each other) after HBc pep stimulation, potentially reflecting an increase in immune synapse formation between T cells and APC (Fig. 5.7f).

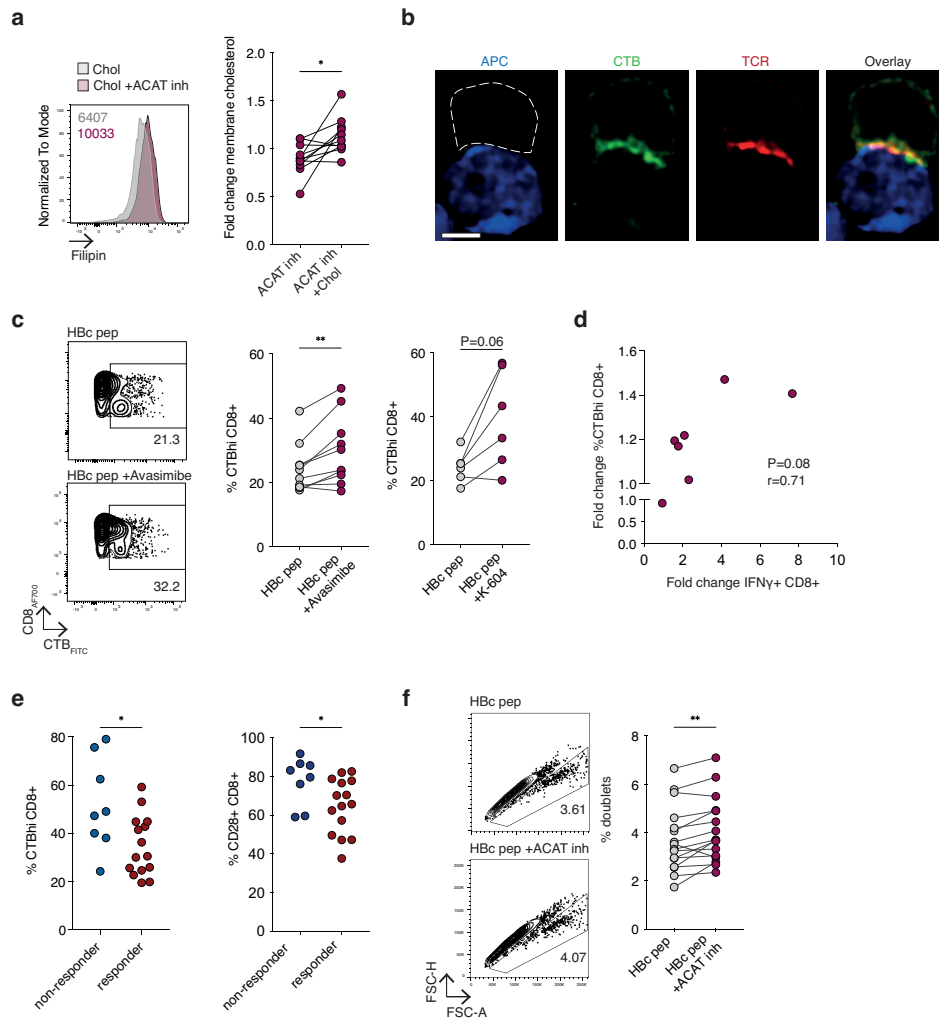


Figure 5.7 ACAT inhibition enhances lipid raft formation.

(a) Assessment of free membrane cholesterol by Filipin staining on CD8⁺ T cells. PBMC cultured in normal or cholesterol enriched (chol) culture media \pm ACAT inhibition (K-604) for 7d. Example histogram showing MFI Filipin in chol media \pm ACAT inhibition. Summary data: fold change of Filipin MFI +ACAT inhibition normalized to DMSO control (n=11). (b) Confocal microscopy visualising the immunological synapse between a CMV-specific CD8⁺ T cell (white dashed outline, identified by CTB and TCR staining) and CMV peptide pulsed T2 cell (APC, blue) with staining of lipid rafts (identified by CTB staining, green) and CD3 ϵ (red). White scale bar: 5 μ m. (c) CTB staining on PBMC CD8⁺ T cells from CHB donors after stimulation with HBc pep \pm ACAT inhibition (left: Avasimibe n=10; right: K-604 n=6) for 7d. Representative flow cytometry plot and summary data. (d) Correlation of fold change CTB staining and fold change HBV-specific IFN γ production by CD8⁺ T cells \pm ACAT inhibition (Avasimibe; n=7). (e) *Ex vivo* %CTB^{hi} CD8⁺ T cells and %CD28⁺ CD8⁺ T cells according to response to ACAT inhibition (n=23). Response defined as increased or *de novo* HBV-specific IFN γ production. (f) Doublets identified by FSC-A and FSC-H on PBMC from CHB donors after stimulation with HBc OLP \pm ACAT inhibition (Avasimibe) for 7d (n=15). P values were determined by Wilcoxon matched-pairs signed rank test (a,c,f), Spearman correlation (d) or Mann-Whitney test (e). (b) Clare Jolly assisted in performance and analysis of immune synapse visualisation by confocal microscopy in Fig. 5.7b.

I next hypothesized that the increase in lipid rafts would be reflected in enhanced TCR signalling. To assess this, I focused on the phosphorylation of three signalling molecules downstream of the TCR: ERK1/2, Akt and the S6 ribosomal protein. ERK1/2 is a MAP kinase that regulates cell proliferation, differentiation, survival and TCR-induced signal

strength via the Ras-ERK1/2-AP-1 pathway (Chang and Karin 2001; Hwang et al. 2020). Via the PI3K-Akt signalling pathway, Akt regulates cell proliferation and survival and modulates cellular metabolism by mTORC1 activation (Hwang et al. 2020; Kim and Suresh 2013). S6 ribosomal protein is a downstream phosphorylation target at the convergence of the mTOR, ERK and PI3K signalling pathways and is important for ribosomal biogenesis, cellular metabolism and T cell proliferation (Salmond et al. 2009)

In line with the critical role of lipid rafts in synapse formation and TCR signalling, ACAT inhibition enhanced TCR signalling shown by an increase of the phosphorylation of ERK (Fig. 5.8a), Akt (Fig. 5.8b) and S6 ribosomal protein (S6, Fig. 5.8c).

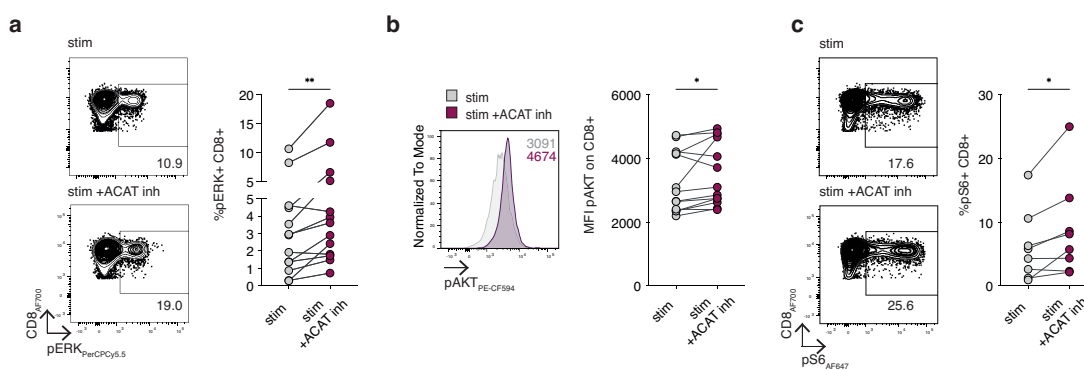


Figure 5.8 ACAT inhibition enhances TCR signalling.

(a-c) Representative phosphoflow cytometry plots/histograms and summary data to detect phosphorylated TCR signalling molecules pERK (n=13; **a**), pAkt (n=13; **b**) and pS6 (n=8; **c**) ±ACAT inhibition (Avasimibe) for 7d after stimulation with aCD3/aCD28 antibodies. P values were determined by Wilcoxon matched-pairs signed rank test.

5.3.2.1.3 ACAT inhibition and cholesterol transporters

Intracellular cholesterol levels are tightly regulated, and high free cholesterol levels result in reduced uptake and/or reduced *de novo* synthesis reflected by induction of the negative regulator LXR and a decrease of the positive regulator SREBP. It would have therefore been expected that inhibition of cholesterol esterification would reduce SREBP activity to regulate high levels of free cholesterol; however, previous studies reported a paradoxical increase of SREBP upon ACAT deletion (Kidani and Bensinger 2016; Yang et al. 2016).

In this study, ACAT inhibition subtly decreased the expression of SR-B1 involved in HDL uptake (Zheng et al. 2018) in preliminary data (Fig. 5.9a) and increased expression of CD36

responsible for long-chain fatty acid and oxLDL uptake (Wang et al. 2020a; Xu et al. 2021) (also see section 1.2.5.1) in a subtle but consistent manner in a larger cohort of donors (Fig. 5.9b). Of note, both SR-B1 and CD36 have also been reported to contribute to cholesterol efflux (Bujold et al. 2009; Phillips 2014).

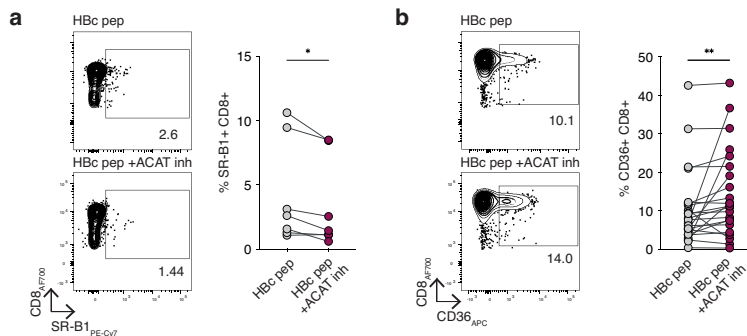


Figure 5.9 ACAT inhibition alters cholesterol transporter expression.

(a+b) PBMC were stimulated with HbC pep \pm ACAT inhibition (Avasimibe) for 7d and analysed via flow cytometry. Example plots and summary data of %SR-B1 (**a**; $n=7$) and %CD36 (**b**, $n=23$). P values were determined by Wilcoxon matched-pairs signed rank test.

5.3.2.1.4 Summary

In summary, ACAT inhibition reduced intracellular cholesteryl ester resulting in mobilization of free cholesterol to the cell membrane, formation of lipid rafts and ultimately enhanced TCR signalling and CD8⁺ T cell function. Intracellular cholesterol redistribution alters expression of cholesterol transporters involved in cholesterol import and efflux.

5.3.2.2 Synergistic effect of ACAT inhibition and CD28 co-stimulation

Considering the role of CD28 in lipid raft recruitment and synapse formation (see chapter 1.1.1), and the finding that ACAT inhibition increased lipid rafts, I hypothesized that CD28 signalling and ACAT inhibition would have a synergistic effect on CD8⁺ T cell activation.

Indeed, CD28 engagement enhanced the immune boosting effect of ACAT inhibition compared to CD3 stimulation alone (Fig. 5.10a). This could be confirmed in HBV-specific CD8⁺ T cells in preliminary data in a small cohort where co-administration of a stimulatory

anti-CD28 antibody with ACAT inhibition achieved a higher fold increase of IFN γ production in 5 out of 6 donors and fold increase of proliferating HBV-specific CD8⁺ T cells in 4 out of 5 donors compared to ACAT inhibition alone (Fig. 5.10b). These preliminary findings further highlight the importance of attenuation of lipid raft formation as a mechanism underlying the immune enhancing effect of ACAT inhibition.

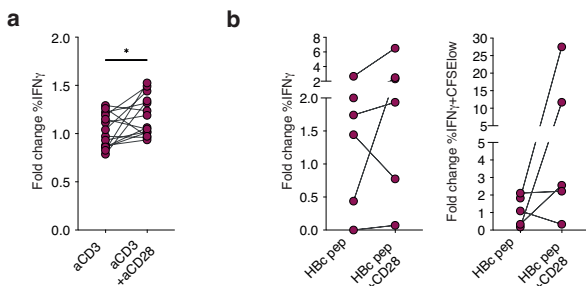


Figure 5.10 ACAT inhibition has a synergistic effect with CD28 signalling.

(a) PBMC were stimulated with anti-CD3 antibodies or anti-CD3+anti-CD28 antibodies \pm ACAT inhibition (Avasimibe) for 7d. Fold increase IFN γ ⁺ CD8⁺ T cells following ACAT inh normalized to DMSO (n=14). **(b)** CHB PBMC were stimulated with HBc pep \pm ACAT inhibition (Avasimibe) \pm aCD28 on d0+d6. Fold increase of %IFN γ ⁺ (n=6) and %IFN γ ⁺CFSE^{low} (n=5) CD8⁺ T cells with ACAT inh normalized to DMSO control. P values were determined by Wilcoxon matched-pairs signed rank test.

5.3.2.3 Reduced lipid rafts are a feature of exhausted CD8⁺ T cells

Lipid rafts are crucial for the formation of the immunological synapse between T cells and their APC and for subsequent T cell priming and TCR signalling (Sezgin et al. 2017; Zumerle et al. 2017) (see chapter 1.1). Due to the critical role of lipid rafts in T cell effector function and the preferential rescue of T cells by ACAT inhibition in settings with T cell exhaustion, I hypothesized that CD8⁺ T cell exhaustion is associated with reduced lipid rafts.

Focusing on PD-1 as a classical T cell exhaustion marker, I detected reduced CTB staining of PD-1⁺ CD8⁺ T cells *ex vivo* indicating a reduced ability to assemble lipid rafts compared to PD-1^{neg} CD8⁺ T cells (mean percentage reduction 41%; mean MFI reduction 32%; Fig. 5.11a). Similarly reduced levels in CTB staining were seen using 2B4 (CD244) as an alternative marker for exhausted CD8⁺ T cells (Waggoner and Kumar 2012) (mean percentage reduction 52%, Fig. 5.11b).

Both PD-1 and 2B4 are also expressed by early activated CD8⁺ T cells (Patsoukis et al. 2020; Waggoner and Kumar 2012) and to further address this, I co-stained PD-1 and the activation marker HLA-DR. As expected, reduced lipid rafts were a feature of exhausted (PD-1⁺ HLA-DR^{neg}) CD8⁺ T cells that was not present in recently activated (PD-1⁺ HLA-DR⁺) CD8⁺ T cells (Fig. 5.11c). I further confirmed the finding that lipid rafts are increased on activated T cells by utilizing the alternative activation marker CD38 (Fig. 5.11c).

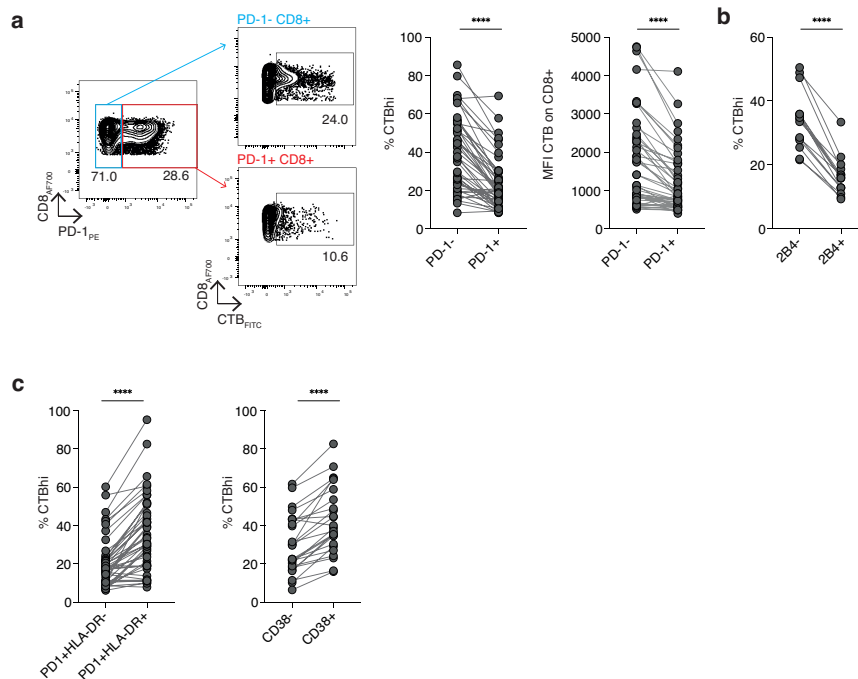


Figure 5.11 Reduced lipid rafts are a feature of PD-1⁺ exhausted CD8⁺ T cells.

(a-c) *Ex vivo* CTB staining of PBMC from CHB donors to identify lipid rafts. **(a)** Representative flow cytometry plot and summary data of %CTBhi (left) and MFI CTB (right) on PD-1⁺/⁻ CD8⁺ T cells (n=46). **(b)** %CTBhi on 2B4⁺/⁻ CD8⁺ T cells (n=15). **(c)** %CTBhi of exhausted (PD-1⁺ HLA-DR⁻) vs activated (PD-1⁺ HLA-DR⁺) CD8⁺ T cells (n=46) and of activated (CD38⁺) vs non-activated (CD38⁻) CD8⁺ T cells (n=25). P values determined by Wilcoxon matched-pairs signed rank test.

One of the mechanisms by which PD-1 signalling inhibits T cell effector function is targeting the co-stimulatory molecule CD28 (Hui et al. 2017) (see chapter 1.1.3.2.1). In line with the role of CD28 in assembling lipid rafts upon TCR engagement (Martin et al. 2001; Tavano et al. 2006; Zumerle et al. 2017), CD28⁺ CD8⁺ T cells had high CTB staining indicating high lipid rafts (Fig. 5.12a).

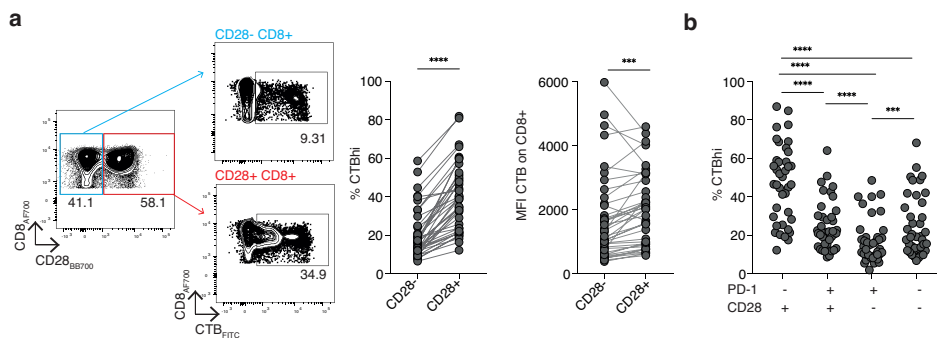


Figure 5.12 Lipid rafts and CD28.

(a,b) *Ex vivo* CTB staining of PBMC from donors with CHB to identify lipid rafts (n=39). **(a)** Representative flow cytometry plot and summary data of %CTB^{hi} (left) and MFI CTB (right) on CD28⁺/⁻ CD8⁺ T cells. **(b)** %CTB^{hi} on CD28⁺/⁻ PD-1⁺/⁻ CD8⁺ T cells. P values were determined by Wilcoxon matched-pairs signed rank test **(a)** and Friedman test with Dunn's multiple comparisons test **(b)**.

PD-1 activation results in a Shp-2 phosphatase-mediated dephosphorylation of CD28 and a subsequent inhibition of T cell function (Hui et al. 2017). Consequently, CD8⁺ T cells co-expressing PD-1 and CD28 had significantly reduced CTB staining compared to CD8⁺ T cells only expressing CD28 (Fig. 5.12b).

In summary, reduced lipid rafts were a feature of exhausted PD-1⁺ CD8⁺ T cells and may contribute to the dysfunctional immune response in exhausted T cells by limiting synapse formation.

5.3.2.4 ACAT inhibition induces TCR-independent bioenergetic reprogramming

The two major metabolic pathways fuelling T cell energy demands are OXPHOS and glycolysis (see chapter 1.2.1). I hypothesized that ACAT inhibition would alter the utilization of OXPHOS and glycolysis due to altered energy demands and the fact that fatty acids and lipids can contribute to OXPHOS via FAO.

OXPHOS and glycolysis can be assessed in real-time by extracellular flux analysis via the measurement of OCR and ECAR, respectively (van der Windt et al. 2016). The consecutive administration of compounds targeting different parts of the ETC provides further in-depth information on mitochondrial function in a 'mitochondrial stress test' (see section 5.1.3).

To interrogate CD8⁺ T cell bioenergetics independently of changes in lipid rafts and TCR engagement with consecutive mTOR signalling and metabolic alterations, I stimulated purified human CD8⁺ T cells with PMA and ionomycin bypassing the TCR signalling pathway. ACAT inhibition significantly increased both basal respiration and the portion of basal respiration that is used to drive ATP production (Fig. 5.13a). ACAT inhibition also increased the maximal respiration in 5 out of 6 and SRC in 4 out of 6 donors indicating the energy reserve that can be achieved under cellular stress (Fig. 5.13a).

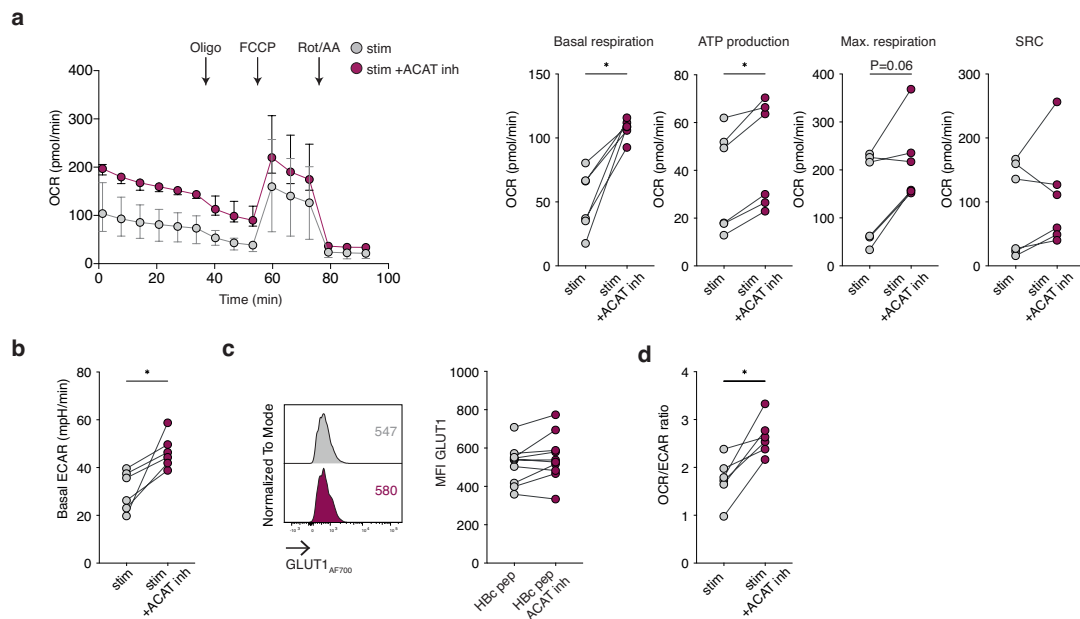


Figure 5.13 ACAT inhibition induces bioenergetic reprogramming.

(a) Time course of real-time oxygen consumption rate (OCR) of PMA/ionomycin-stimulated purified CD8⁺ T cells ±ACAT inhibition (Avasimibe) for 16h (n=6). Compounds were added as indicated (oligomycin (Oligo), FCCP, rotenone+antimycin A (Rot/AA)) to determine basal respiration, ATP production, maximal respiration and SRC. OCR time course (median and interquartile range) and summary data. **(b)** Basal extracellular acidification rate (ECAR) of PMA/ionomycin-stimulated purified CD8⁺ T cells ±ACAT inhibition (Avasimibe) for 16 (n=6). **(c)** Example histogram and summary data of GLUT1 expression on global CD8⁺ T cells from PBMC stimulated with Hbc pep ±ACAT inhibition (Avasimibe) for 7d (n=10). **(d)** Basal OCR/ECAR ratio of PMA/ionomycin-stimulated purified CD8⁺ T cells ±ACAT inhibition (Avasimibe) for 16h (n=6). P values were determined by Wilcoxon matched-pairs signed rank test.

Besides OXPHOS, ACAT inhibition also significantly increased basal glycolysis (Fig. 5.13b) with only minor effects on the expression of the glucose transporter GLUT1 (Hukelmann et al. 2016) measured by flow cytometry on peptide-stimulated CD8⁺ T cells (Fig. 5.13c).

Overall, ACAT inhibition induced extensive bioenergetic reprogramming with a skewing towards OXPHOS over glycolysis (OCR/ECAR ratio; Fig. 5.13d).

5.4 Discussion

T cell metabolism is tightly linked with T cell phenotype and function – alterations in T cell differentiation and activation result in differing energy demands and, vice versa, nutrient availability, uptake, and utilization shape immune responses. I hypothesized that the enhanced CD8⁺ T cell function after ACAT inhibition would be associated with phenotypic alterations such as a downregulation of molecules and transcription factors linked with T cell exhaustion.

Immunotherapies have the potential to alter CD8⁺ T cell phenotype, e.g. IL-12 decreases expression of PD-1 accompanied by an increase of T-bet (Schurich et al. 2013), and PD-L1 blockade preferentially expands the T-bet expressing T cell exhaustion subsets (Beltra et al. 2020). In contrast, ACAT inhibition did not alter the frequency of CD8⁺ T cells expressing inhibitory checkpoint molecules, surface markers identifying chromatin states or transcription factors associated with T cell differentiation and exhaustion on global CD8⁺ T cells from CHB donors.

HBV-specific T cells constitute only a small fraction of PBMC and alterations in their phenotype are unlikely to be reflected in the total CD8⁺ T cell pool. It would be important to assess phenotypic alterations directly on HBV-specific CD8⁺ T cells, identified either by HBV-specific cytokine production or binding of HBV-specific multimers that allow visualization of antigen-specific T cells irrespective of their effector function. Besides flow cytometric approaches, RNA sequencing would provide a detailed unbiased insight into gene expression alterations induced by ACAT inhibition and for example would allow a gene set enrichment analysis determining the alteration in genes associated with certain transcription factors, e.g. Eomes and T-bet, and exhaustion phenotypes.

A multitude of factors contribute to T cell exhaustion in chronic viral infection and tumours, one of which is the inhibitory checkpoint PD-1. Expression of PD-1 results in extensive metabolic alterations including the suppression of glycolysis and induction of FAO (Bengsch et al. 2016; Patsoukis et al. 2015) (see chapter 1.2.2). In this study, I showed that lipid rafts were reduced on PD-1⁺ exhausted CD8⁺ T cells compared to their PD-1^{neg} counterparts, defining a novel feature of T cell exhaustion.

One of the mechanisms by which PD-1 limits T cell effector function is the dephosphorylation and consecutive inactivation of CD28 (Hui et al. 2017). In line with this observation, lipid rafts were reduced on CD8⁺ T cells co-expressing CD28 and PD-1 compared to those only expressing CD28, suggesting that PD-1 inhibits the CD28-mediated lipid raft assembly and consecutive synapse formation and TCR signalling.

Visualising lipid rafts directly on HBV-specific T cells, e.g. identified by multimer staining, compared to highly functional T cells specific for other viruses, e.g. EBV or influenza, from the same donor will provide further important insight into the role of lipid rafts in T cell exhaustion; however, this is technically challenging and, in this study, I failed to simultaneously stain for CTB (used to identify lipid rafts) and HBV-specific dextramers (used to identify HBV-specific CD8⁺ T cells) likely due to interference between these two large molecules (*data not shown*). Utilization of a smaller multimers, e.g. a dimer or tetramer, might result in less interference and might be more suitable to address this question in future studies.

Lipids, such as cholesterol, constitute an essential component of cellular plasma membranes impacting membrane lipid order, membrane fluidity, receptor signalling and cell function (Bietz et al. 2017; Ikonen 2008; Miguel et al. 2011; Sezgin et al. 2017). Due to its hydrophobic properties, free cholesterol cannot be stored intracellularly but is either exported, mobilized into lipid bilayers or esterified by ACAT and stored in neutral lipid droplets. An accumulation of lipid droplets constrains the effector function of NK cells in obesity (Michelet et al. 2018) while in CD8⁺ T cells the association is less clear with some reports suggesting a relation between lipid accumulation and dysfunction (MC38 colon adenocarcinoma; (Xu et al. 2021)) and others not (B16-F10 melanoma in obese mice; (Dyck et al. 2022)) likely due to differences in the murine tumour models.

In line with the proposed mechanism of action, ACAT inhibition reduced neutral lipid droplets in the cytoplasm of human T cells by preventing cholesterol esterification. High *ex vivo* neutral lipid droplets in CD8⁺ T cells correlated with treatment response, highlighting the reduction of cholesteryl ester as one of the mechanisms by which ACAT inhibition boosts immune responses. Neutral lipid droplets, identified by LipidTOX staining, not only consist of cholesteryl ester but also contain triacylglycerol and diacylglycerol surrounded by a phospholipid layer (Bartz et al. 2007). Quantitative readouts with e.g. an AmplexRed assay

that allows the measurement of cholesterol and cholesteryl ester, or lipidomics by mass spectrometry, could be utilized to confirm that the decrease in lipid droplets is indeed due to decreased intracellular cholesteryl ester levels.

Cholesterol import, synthesis and efflux are regulated by the transcription factors LXR and SREBP to ensure intracellular homeostasis (Kidani and Bensinger 2016; Robinson et al. 2017). SREBP, the positive regulator of intracellular cholesterol, forms a complex with SCAP which facilitates transport to the Golgi complex and consecutive activation (Radhakrishnan et al. 2008). High cholesterol promotes SCAP association with INSIG, preventing mobilisation to the Golgi complex and SREBP activation (Radhakrishnan et al. 2008) and instead the negative regulator LXR is activated (Janowski et al. 1996) (also see chapter 1.2.5.2).

This highly complex process is tightly regulated, and it would have been expected that an increase of free cholesterol led to a decrease of SREBP and an increase of LXR activity. Instead, ACAT knockdown results in a paradoxical increase of SREBP-1 and SREBP-2 in murine CD8⁺ T cells (Yang et al. 2016). In this study, ACAT inhibition increased the expression of CD36 which has been shown to disrupt the interaction between SCAP and INSIG in hepatocytes, resulting in a mobilization of SREBP to the Golgi complex and consecutive activation (Zeng et al. 2022) and therefore could be one of the mechanisms by which ACAT inhibition increases SREBP activity.

Conversely, CD36 also modulates cholesterol efflux in macrophages (Bujold et al. 2009) and the increased expression after ACAT inhibition could be associated with an increase in cholesterol efflux to prevent high cytotoxic intracellular cholesterol levels which may contribute to the lack of increased free cholesterol upon ACAT inhibition in normal culture media; however, this concept is not supported by the finding that classical cholesterol efflux transporters ABCA-1 and -2 are downregulated on CD8⁺ T cells and macrophages in murine ACAT knockout models (Su et al. 2005; Yang et al. 2016).

Finally, CD36 is responsible for long-chain fatty acid and oxLDL uptake (Wang et al. 2020a). CD36 upregulation in tumour-infiltrating CD8⁺ T cells leads to intracellular lipid accumulation and T cell dysfunction (Xu et al. 2021). In my study, ACAT inhibition enhanced CD8⁺ T cell function suggesting that the described T cell dysfunction is not due

to CD36 upregulation itself and rather due to the lipid accumulation that is prevented by ACAT inhibition.

Future studies will be necessary to understand the role of CD36 expression in ACAT inhibition and can shed light on the mechanisms by which ACAT inhibition boosts antiviral T cell function, e.g. studying the effect of ACAT inhibition directly on sorted CD36^{+/neg} T cells, utilizing CD36 knockout murine models, T cells from donors with CD36 deficiency, common in Asian and African populations (Hirano et al. 2003), or blocking CD36 *in vitro*.

Besides alteration of CD36 expression, ACAT inhibition resulted in a subtle reduction of the LXR-regulated cholesterol transporters SR-B1 (Shen et al. 2018) in preliminary data. It is important to note that frequency and MFI of SR-B1 in this flow cytometric analysis were low and changes were only subtle, therefore a confirmation of these findings e.g. by western blot or RNAseq analysis will be necessary.

Utilization of labelled cholesterol, e.g. BODIPY, allows the direct visualization of cholesterol uptake and efflux in human T cells and would facilitate a better understanding of intracellular cholesterol movement during ACAT inhibition. Additionally, assessment of other cholesterol transporters (e.g. LDL receptor) as well as key genes involved in cholesterol transport and regulation will provide further insight into alterations induced by ACAT inhibition.

As mentioned above, when cholesterol esterification is inhibited, cholesterol can either be shunted out of the cell or integrated into lipophilic cell membranes as shown in a murine model (Yang et al. 2016). In this setting, I only detected an increase of free cholesterol with ACAT in a cell culture with high cholesterol levels. I have shown in chapter 4.3.4.6 that the immune boosting effect of ACAT inhibition is more profound in high cholesterol environments, likely due to an enhanced activity of the enzyme ACAT (Gillies et al. 1990). Metabolic changes are usually subtle and may be more apparent with increased activity of the target enzyme and increased substrate availability. As mentioned above, more sensitive quantitative readouts such as AmplexRed analysis or lipidomics will be needed to fully assess the redistribution of cholesterol. Additionally, metabolic alterations are time sensitive and assessment of free cholesterol at different time points after ACAT inhibition will further contribute to the understanding of cholesterol redistribution.

I show for the first time that ACAT inhibition is associated with an increase in lipid raft formation. The magnitude of lipid raft increase tended to correlate with the response to ACAT inhibition and donors with low *ex vivo* lipid rafts or CD28 expression were significantly more likely to respond, highlighting the relevance of this mechanism of action. Future studies should investigate whether ACAT inhibition also increases CD28 expression, a concept that is supported by the preliminary observation that CD28 engagement had a synergistic effect with ACAT inhibition.

Conversely, if the immune boosting effect of ACAT inhibition is due to lipid raft formation, it would be expected that inhibiting CD28 interaction with its ligands (e.g. by blocking CD80/86 antibodies or soluble CTLA-4) would reduce the efficiency of ACAT inhibition. Disruption of lipid rafts or inhibition of cholesterol transport to the membrane could shed further light on the role of lipid rafts in ACAT inhibition; however, the frequently used compounds to achieve this have limited application in this setting; methyl- β -cyclodextrin has been previously used to disrupt lipid rafts but its cholesterol depletion is only transient and not lipid raft specific (Mahammad and Parmryd 2008); U18666A is frequently used to block cholesterol transport to the membrane, but also directly inhibits ACAT (Härmälä et al. 1994).

In line with the critical role of lipid rafts in immune synapse formation and TCR signalling, ACAT inhibition enhanced TCR signalling, resulting in increased T cell effector function. Of note, future studies should assess the effect of ACAT inhibition on other TCR signalling molecules, especially those further upstream in the TCR signalling pathway, such as ZAP-70.

Increased frequency of doublets in flow cytometry potentially suggests increased cell-cell interaction and synapse formation, which is in line with previous findings (Yang et al. 2016) and should be confirmed for virus-specific T cells, e.g. by synapse visualisation via confocal microscopy (also see discussion chapter 7.2.2).

Intracellular metabolic pathways are tightly interlinked and ACAT inhibition not only altered intracellular cholesterol metabolism but also induced extensive metabolic reprogramming with an increase of OXPHOS and glycolysis, and ultimately a skewing towards OXPHOS (also see discussion chapter 7.2.2). Importantly, these findings were irrespective of TCR signalling and consecutive mTOR activation.

Mitochondrial dysfunction is a feature of T cells in SARS-CoV-2 (Liu et al. 2021; Siska et al. 2021; Thompson et al. 2021) and exhausted T cells in CHB, and is associated with a downregulation of genes encoding components of the mitochondrial ETC, reduced mitochondrial potential and reduced OXPHOS capacity (Fisicaro et al. 2017; Schurich et al. 2016). Targeting mitochondrial dysfunction can restore HBV-specific T cells (Fisicaro et al. 2017; Schurich et al. 2016), suggesting that the increased OXPHOS capacity after ACAT inhibition is a further mechanism by which virus-specific T cell function is boosted.

In this study, I could only assess bioenergetic alterations on global CD8⁺ T cells as the frequency of HBV-specific CD8⁺ T cells was low and cell numbers were limited due to ethical considerations regarding the volume of blood sampled from donors; however, it would be interesting to study altered bioenergetics directly on exhausted (e.g. PD-1⁺) or HBV-specific CD8⁺ T cells (identified by HBV-multimer staining) ±stimulation with their cognate peptide. Besides extracellular flux analysis, a novel flow cytometric assay (single-cell energetic metabolism by profiling translational inhibition, SCENITH (Argüello et al. 2020)) could provide further insight on the metabolic profile of these low frequency HBV-specific T cells on a single cell level.

Future studies should aim to assess the increase of glycolysis more thoroughly, e.g. further assessment of glycolytic capacity and glycolytic reserve via ECAR measurement during extracellular flux analysis after administration of oligomycin (inhibition of ATP synthase) and by glycolysis stress test with 2-deoxy-d-glucose, a competitive inhibitor of glucose, and glucose starvation allowing the consideration of non-glycolytic acidification. Assessing mitochondrial mass, polarisation and ROS production via flow cytometric analysis with MitoGreen, MitoTracker Deep Red and MitoSOX, respectively (Monteiro et al. 2020), or directly visualizing mitochondrial structure by transmission electron microscopy (Ogando et al. 2019) further characterize mitochondrial changes induced by ACAT inhibition. HBV-specific T cells have defect mitochondria and are not able to switch to OXPHOS in glucose deprivation (Schurich et al. 2016) and it would be interesting to assess whether this could be overcome by the OXPHOS-enhancing effects of ACAT inhibition.

In summary, ACAT inhibition caused intracellular cholesterol redistribution with reduced cholesteryl ester, increased lipid raft formation and enhanced TCR signalling. ACAT

inhibition further induced extensive metabolic reprogramming irrespective of TCR signalling with a preferential utilization of OXPHOS to fuel energy demands.

6

ACAT inhibition boosts tumour-specific T cells and has a complementary effect with other immunotherapeutic strategies

ACAT inhibition boosts the function of CD8⁺ and CD4⁺ TIL in HCC specific for a variety of different viral and non-viral TAA directly *ex vivo* from the immunosuppressive TME. ACAT inhibition has a complementary effect with PD-1 blockade and boosts the functional avidity of TCR-engineered T cells specific for HBV and HBV-related HCC.

6.1 Introduction

6.1.1 Hepatocellular carcinoma

HCC is the seventh most common cancer and the third leading cause of cancer-related death worldwide (Sung et al. 2021). HCC typically develops on the background of liver cirrhosis due to chronic liver disease, including viral hepatitis, alcoholic liver disease and non-alcoholic steatohepatitis (NASH) (McGlynn et al. 2021) (Fig. 6.1). Worldwide, CHB is the most common cause of HCC, accounting for 56% of cases (Sung et al. 2021) but the mortality due to NASH-related HCC associated with obesity and metabolic syndrome is rising and constitutes the most common cause in the United States (Brar et al. 2020; Kim et al. 2019). Less common causes include genetic disorders such as haemochromatosis and α 1 antitrypsin deficiency as well as aflatoxin B1 intoxication (McGlynn et al. 2021).

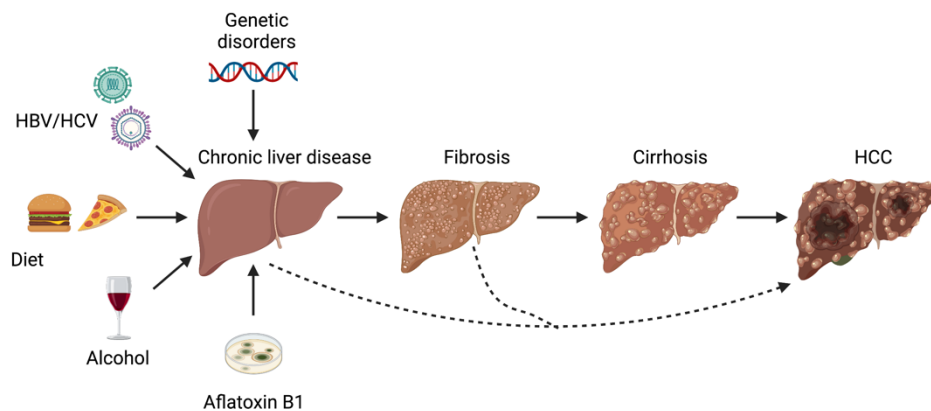


Figure 6.1 HCC development.

Risk factors contributing to HCC development: genetic disorders, chronic viral infection, diet/NASH, alcohol, aflatoxin B1. HCC usually develops on the background of chronic liver diseases, resulting in fibrosis followed by cirrhosis. In HBV and NASH, HCC can develop without fibrotic/cirrhotic remodelling. HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; NASH: non-alcoholic steatohepatitis.

Treatment options for HCC are limited and the median survival after diagnosis ranges between 6-10 months depending on aetiology (Brar et al. 2020). In early disease stages, tumour resection and radiofrequency ablation as well as liver transplantation constitute treatment options with curative potential (Reig et al. 2022). In intermediate stages,

transarterial chemoembolization (TACE) can deliver chemotherapeutic agents directly into the tumour and embolize arterial blood supply. Until recently, tyrosine kinase inhibitors such as sorafenib or lenvatinib were the only treatment option in advanced HCC; however, the treatment paradigm has been shifted and immunotherapy with atezolizumab (PD-L1 inhibitor) and bevacizumab (VEGF-A inhibitor) has become the preferred first line treatment for advanced HCC in eligible patients.

The approval of atezolizumab and bevacizumab has been a tremendous success for the treatment of advanced HCC and highlights the potential of immunotherapy in HCC; however, only 30% of patients respond to the treatment and the development of new therapeutic strategies is critical to improve patient care (Cheng et al. 2022).

HCC development in chronic liver disease is multifactorial and still not fully understood. The immune system and inflammatory signals are thought to play a critical role in HCC carcinogenesis but also in tumour surveillance. The following sections will explore the immune landscape in HCC, including the immune system as a driver of tumorigenesis, T cells and TAA, and factors contributing to a failure of antitumour immunity.

6.1.1.1 Inflammation and HCC carcinogenesis

Tumour-promoting inflammation is one of the hallmarks of cancer (Hanahan 2022). As discussed above, HCC develops on the background of chronic liver diseases that are typically associated with chronic liver inflammation. Chronic inflammation leads to cell death and increases cellular turnover and activation of non-parenchymal cells, leading to fibrotic remodelling and liver cirrhosis (Ringelhan et al. 2018). Cirrhosis, cellular stress, mitochondrial and epigenetic alterations as well as altered proliferation and survival signals result in DNA damage, genetic instability and ultimately tumorigenesis. Of note, HCC can develop in the non-cirrhotic liver, especially in highly inflammatory settings such as CHB and NASH (Desai et al. 2019).

The main mediators of inflammation in HCC carcinogenesis are proinflammatory cytokines including lymphotoxins, TNF and IL-6. Lymphotoxin- $\alpha+\beta$ are upregulated in chronic HCV and HBV infection and directly contribute to HCC development (Haybaeck et al. 2009).

TNF activates the NF- κ B signalling pathway which plays a dual role in HCC, with some studies describing a protumorigenic effect (Pikarsky et al. 2004) whereas others have shown a protective role in HBV-related HCC (Sunami et al. 2016) (also see chapter 1.1.2.4). In NASH, autoaggressive and TNF-producing CD8⁺ T cells contribute to HCC development (Dudek et al. 2021; Pfister et al. 2021).

Necrotic hepatocytes release IL-1 α that activates Kupffer cells and promotes IL-6 release (Sakurai et al. 2008). IL-6 is pro-carcinogenic in many tumour entities and induces angiogenesis, tumour cell proliferation and invasion (Taniguchi and Karin 2014). High serum IL-6 is associated with an increased risk of HCC development in patients with CHB and in atomic bomb survivors (Ohishi et al. 2014; Wong et al. 2009). Once HCC is established, IL-6 is associated with increased tumour aggressiveness (Jang et al. 2012).

Other proinflammatory signals contributing to HCC development besides cytokines include TLR signalling. TLR4 expression on tumour cells is associated with vascular invasion and early recurrence after resection (Liu et al. 2015).

6.1.1.2 T cells and tumour-associated antigens

Besides the tumour-promoting effect of inflammation, immune surveillance is critical for tumour control. HCC lesions are highly infiltrated by T cells compared to hepatic colorectal metastasis which has been attributed to increased expression of ICAM-1 and vascular adhesion protein-1 (Yoong et al. 1998). A high frequency of TIL is linked to reduced recurrence rates after HCC resection and liver transplantation (Unitt et al. 2006; Wada et al. 1998) and the presence of TAA-specific CD8⁺ T cells in the blood is associated with patient survival (Flecken et al. 2014). The role of immunological tumour surveillance is further highlighted by two case reports of HCC regression after withdrawal of immunosuppressive therapy (Kumar and Le 2016).

Antitumour T cells recognize TAA that are expressed by tumour cells but typically not, or at low levels, in healthy tissue. TAA can be divided into five groups: tumour-specific shared antigens, differentiation antigens, antigens resulting from mutations, overexpressed tumour antigens and viral antigens (Van den Eynde and van der Bruggen 1997).

Tumour-specific shared antigens are typically not expressed/presented in healthy adult tissue but are expressed in cancer cells of various aetiologies. Typical representatives of this group include cancer-testis antigens that are only expressed in placental trophoblasts and testicular germ cells without MHC class I molecules, and oncofoetal antigens that are expressed during foetal development and synthesis is terminated before or at birth (Schmidt and Thimme 2016; Van den Eynde and van der Bruggen 1997).

Differentiation antigens are associated with cell differentiation, e.g. tyrosinase in melanocytes can act as a TAA in melanoma (Nichols et al. 2007; Topalian et al. 1996). Antigens resulting from mutations include mutated proteins associated with apoptosis or proliferation, as well as chimeric proteins. e.g. bcr-abl in myeloid leukaemia due to t(9;22) translocation (Bosch et al. 1996). Overexpressed TAA are TAA that can be found in healthy tissue but are overexpressed in cancer cells, e.g. Her-2/neu in ovarian and breast cancer (Fisk et al. 1995; Van den Eynde and van der Bruggen 1997).

Viral antigens from oncogenic viruses can be expressed by tumour cells including human papilloma virus in vulval and cervical cancer (Bourgault Villada et al. 2010) as well as HBV. Indeed, in HBV-related HCC, the HBV genome is frequently integrated into the tumour cell genome and HBV antigens, e.g. HBsAg and HBcAg, can be expressed by HCC tumour cells (He et al. 2021; Liu et al. 2019; Qasim et al. 2015; Sung et al. 2012).

Besides HBV antigens, a variety of non-viral TAA have been described for HCC including the cancer-testis antigens melanoma-associated antigen (MAGE) and New York oesophageal squamous cell carcinoma-1 (NY-ESO-1), the oncofetal antigens α -fetoprotein (AFP) and glypican-3, as well as synovial carcinoma X chromosome-2 (SSX-2) and human telomerase-reverse transcriptase (hTERT).

In this thesis, I assessed the antitumour function of T cells after stimulation with HBV-derived peptides (in HBV-related HCC) and three non-viral peptides that will be discussed in the following sections: AFP, MAGE-A and NY-ESO-1.

6.1.1.2.1 α -Fetoprotein

AFP is a member of the albuminoid gene superfamily, consisting of AFP, albumin, alpha-albumin and vitamin D (Gc) protein and can act as a transporter and growth regulator (Schmidt and Thimme 2016). AFP is expressed in the liver, yolk sac and gastrointestinal tract during foetal development and expression is terminated at or shortly before birth (McLeod and Cooke 1989). It is re-expressed in up to 80% of HCC and elevated serum levels are frequently used for diagnosis and disease monitoring (Schmidt and Thimme 2016; Witkowski et al. 2011).

Various studies have described AFP-specific CD8⁺ and CD4⁺ T cells in donors with HCC and have identified multiple immunodominant epitopes (Behboudi et al. 2010; Butterfield et al. 2001, 2003; Flecken et al. 2014; Liu et al. 2006b; Meng et al. 2000; Mizukoshi et al. 2011; Thimme et al. 2008; Witkowski et al. 2011).

6.1.1.2.2 MAGE-A

MAGE-A was the first cancer-testis antigen to be identified (Simpson et al. 2005). While it was initially detected in melanoma, it is widely expressed by other tumours including up to 80% of HCC (Breous and Thimme 2011). In healthy tissue, MAGE-A gene expression is suppressed by methylation of the promoter region whereas demethylation occurs in tumour tissue and promotes gene expression (Simpson et al. 2005). The MAGE-A family consists of 12 genes (MAGE-A1-12) located on the X chromosome (De Plaen et al. 1994). MAGE-A1 modulates gene transcription by inhibition of the transcriptional regulator SKI-interacting protein and recruitment of histone deacetylase 1 (Laduron et al. 2004) and MAGE-A2 can suppress p53 (Monte et al. 2006).

MAGE-A1-, MAGE-A3- and MAGE-A10-specific CD8⁺ T cells have been detected in the blood and tumour tissue of HCC donors (Bricard et al. 2005; Flecken et al. 2014; Tauber et al. 2019; Zerbini et al. 2004).

6.1.1.2.3 NY-ESO-1

NY-ESO-1 is a highly immunogenic cancer-testis antigen that is expressed by a variety of tumour entities, including melanoma and 14-25% of HCC (Liang et al. 2013; Xu et al. 2012). NY-ESO-1 expression on HepG2 hepatoma cells is associated with increased migratory potential and it has been suggested that this is the reason for the observed correlation between NY-ESO-1 expression and relapse rate after HCC resection (Xu et al. 2012).

NY-ESO-1-specific CD8⁺ and CD4⁺ T cell responses have been particularly well studied in melanoma where multiple immunodominant epitopes have been described (Benlalam et al. 2003; Ebert et al. 2009; Jackson et al. 2006) but have also been detected in the blood of donors with HCC (Flecken et al. 2014; Gehring et al. 2009; Korangy et al. 2004; Shang et al. 2004; Tauber et al. 2019).

6.1.1.3 Failure of the antitumour immune response

While TAA-specific T cells have been detected for a variety of TAA in HCC, they are typically only present at low frequencies, highly dysfunctional and fail to provide tumour control. Even in the periphery, TAA-specific T cells are dysfunctional with limited cytokine production and proliferation (Flecken et al. 2014). In contrast, virus-specific T cells in donors with HCC are not impaired, highlighting that the loss of antitumour immunity is not due to a generalised immune defect in HCC but specific for antitumour T cells (Flecken et al. 2014).

The tumour constitutes a highly immunosuppressive environment that shapes local immune responses while at the same time the immune system also influences tumour evolution in a process called immunoediting (Dunn et al. 2004). In early stages of cancer development, the immune system has a protective effect and eliminates (pre-)malignant cells. However, if transformed cells escape this early elimination an equilibrium is reached between tumour cells and antitumour immunity. This results in selective pressure on tumour cells and favours the proliferation of cells that have developed mechanisms to avoid immune elimination, finally leading to immune escape. A variety of T cell intrinsic and extrinsic mechanisms contribute to the failure of antitumour T cell responses and bear high similarities with the immunosuppressive mechanisms in HBV (chapter 4.1.1.4).

6.1.1.3.1 T cell intrinsic mechanisms

T cells in HCC express a variety of inhibitory checkpoints including PD-1, Tim-3, CTLA-4 and Lag-3 (Chew et al. 2017), and high expression of PD-1 on TIL is associated with poor prognosis (Barsch et al. 2022). Of note, a high frequency of T_{RM} within the PD-1 positive fraction is associated with improved survival (Barsch et al. 2022). Tim-3⁺ CD8⁺ T cells are particularly enriched in non-viral HCC while TIL in HBV-related HCC exhibit a more exhausted phenotype with increased PD-1 expression and diminished antitumour cytokine production (Lim et al. 2019).

PD-1^{high} CD8⁺ T cells in HCC frequently express other inhibitory receptors and are dysfunctional with reduced antitumour cytokines, cytotoxicity and proliferation (Chew et al. 2017; Kim et al. 2018; Wu et al. 2009). They exhibit a transcriptional profile associated with T cell exhaustion with low TCF-1 expression and high frequency of TOX⁺ and Eomes⁺ T-bet^{neg} cells within the PD-1^{high} population (Barsch et al. 2022; Chew et al. 2017; Wang et al. 2019c).

CD4⁺ T cells are less extensively studied in HCC. The frequency of TAA-specific Th1 and Tfh CD4⁺ T cells is reduced and the remaining T cells are functionally impaired with reduced effector function and IL-21 production as well as impaired induction of B cell maturation (Jia et al. 2015b; Witkowski et al. 2011). Tumour-infiltration of cytotoxic CD4⁺ T cells is particularly restricted in advanced disease where effector function and proliferation are further impaired (Fu et al. 2013).

Of note, T cells specific for self-antigens, such as TAA, are frequently deleted and remaining T cells typically have low affinity TCRs (Aleksic et al. 2012; Nichols et al. 2007), further contributing to a limited antitumour T cell function.

Overall, TIL in HCC exhibit a highly exhausted phenotype with limited antitumour capacity.

6.1.1.3.2 T cell extrinsic mechanisms

Besides T cell intrinsic mechanisms, a variety of T cell extrinsic factors contribute to a failure of antitumour immunity. For example, HCC tissue is enriched in immunosuppressive immune cells, including T_{reg} and TAM (Chew et al. 2017).

T_{reg} from TIL have an activated phenotype with higher immunosuppressive capacity and increased production of immunosuppressive cytokines such as IL-10 than T_{reg} from the surrounding liver tissue (Chew et al. 2017; Tu et al. 2016). Compared to non-viral HCC, T_{reg} in HBV-related HCC are not only increased in frequency but also have increased expression of FOXP3, Lag-3 and genes involved in the IL-10 pathway, indicating a more suppressive phenotype (Lim et al. 2019). The role of T_{reg} in limiting antitumour immunity is further supported by the finding that T_{reg} infiltration correlates with poor prognosis (Shang et al. 2015). *In vitro* T_{reg} depletion restores the proliferative capacity of TAA-specific T cells but not their function (Flecken et al. 2014).

TAM are recruited into the tumour tissue via the CXCR3/CXCL10 axis and have increased immunosuppressive potential, including IL-10 production (Chew et al. 2017). TGF- β induces the expression of Tim-3 in peripheral monocytes and TAM, resulting in a skewing towards an M2-like phenotype in patients with HCC (Yan et al. 2015). Kupffer cells expressing PD-L1 are enriched in HCC tumour tissue, can inhibit the function of PD-1 expressing T cells and correlate with reduced survival (Wu et al. 2009).

MDSC accumulate in HCC patients and mouse models, have high arginase activity and not only suppress T cell effector function and proliferation but also induce the development of T_{reg} (Hoechst et al. 2008; Kapanadze et al. 2013). CXCR-2-expressing neutrophils with a protumour phenotype have further been shown to accumulate in a murine HCC model (Leslie et al. 2022).

Myeloid dendritic cells are reduced in HCC and have a decreased capacity of IL-12 secretion (Ormandy et al. 2006). The finding that the majority of TAA-specific T cells in the blood have a naïve phenotype may imply impaired priming and may be due to defects of antigen-processing and presentation described in other tumours (Leone et al. 2013; Tauber et al. 2019).

Additional to a recruitment of immunosuppressive cell subsets, tumour-intrinsic factors contribute to immune escape, including a downregulation of IFN γ receptors and Fas, preventing cytolytic and non-cytolytic antitumour effector function (Nagao et al. 1999, 2000). As discussed in chapter 1.2.4, metabolic alterations in the TME shape antitumour immunity. This has also been shown for HCC where an accumulation of the methionine metabolites 5-methylthioadenosine and S-adenosylmethionine drives T cell exhaustion (Hung et al. 2021). In non-alcoholic fatty liver disease, fatty acid accumulation in CD4⁺ T cells results in mitochondrial dysfunction and ROS production, and promotes tumourigenesis (Ma et al. 2016).

In summary, the TME constitutes an immunosuppressive environment that induces T cell exhaustion and dysfunction, limiting tumour surveillance.

6.1.1.4 Other immune cell subsets in HCC

Besides T cells and the immunosuppressive cell subsets discussed in the previous section, a variety of other immune cells shape the immune landscape in HCC and this section will briefly highlight the role of $\gamma\delta$ T cells and NK cells.

$\gamma\delta$ T cells play a dual role in carcinogenesis. On the one hand, IL-17 producing $\gamma\delta$ T cells promote HCC development in murine models via induction of CXCL-5 production and MDSC recruitment (Ma et al. 2014). On the other hand, $\gamma\delta$ T cell infiltration in human HCC is associated with reduced tumour size and favourable prognosis (Zakeri et al. 2022). However, the V γ 9V δ 2 subset is depleted in HCC (Zakeri et al. 2022) and HCC-infiltrating $\gamma\delta$ T cells are functionally impaired (Yi et al. 2013). T_{reg} suppress $\gamma\delta$ T cell function and MDSC promote a skewing towards IL-17 producing $\gamma\delta$ T cells (Ma et al. 2014; Yi et al. 2013).

NK cells can contribute to tumour surveillance, but their function is impaired in HCC with reduced cytotoxicity, antitumour cytokine production and NKG2D expression (Cai et al. 2008; Easom et al. 2018; Rennert et al. 2021). Reduced antitumour function of NK cells is caused by similar mechanisms described for T cell dysfunction, including the inhibitory effects of T_{reg} and MDSC and the upregulation of inhibitory receptors (Cai et al. 2008; Hoechst et al. 2009; Tan et al. 2020). Interestingly, NK cell suppression by MDSC is not

reliant on arginase but instead is mediated via NKp30 engagement (Hoechst et al. 2009). Downregulation of NKG2D is modulated by direct cell contact with tumour cells and can be restored by IL-15 (Easom et al. 2018).

6.1.2 Immunotherapy

Harnessing the immune system to eliminate transformed or infected cells has revolutionised modern medicine and immunology. While immunotherapy is frequently thought of as a discovery of the 21st century and cancer immunotherapy has been declared ‘Breakthrough of the Year’ by Science in 2013 (Couzin-Frankel 2013), the concept of inducing immunity and inflammation to combat cancer is much older.

Anecdotal reports of tumours vanishing after infections or high fevers are as old as ancient Egypt (Dobosz and Dzieciatkowski 2019). The first scientific description of this phenomenon was published in two independent reports by Friedrich Fehleisen and Wilhelm Busch in the late 19th century who describe tumour regression after erysipelas infection and were the first physicians to intentionally infect cancer patients with streptococcus pyogenes with variable success. In 1891, William Coley, often referred to as the ‘Father of Immunotherapy’, injected different mixtures of heat-inactivated and live *Streptococcus pyogenes* and *Serratia marcescens* (‘Coley’s toxin’) into tumours and subsequently reported over 1000 cases of tumour regression or cure (Coley 1908; Dobosz and Dzieciatkowski 2019).

Immunotherapy has come a long way since Coley’s toxin and today different therapeutic approaches either aim to induce or boost naturally occurring antiviral/antitumour immunity, e.g. via checkpoint blockade or tumour vaccination, or adoptively transfer optimised immune cells (Fig. 6.2). This section will focus on immunotherapeutic strategies relevant for this thesis: blockade of the PD-1/PD-L1 pathway, administration of stimulatory cytokines and adoptive T cell therapy. Targeting metabolic pathways has emerged as a novel immunotherapeutic strategy and is discussed in chapter 1.2.6.

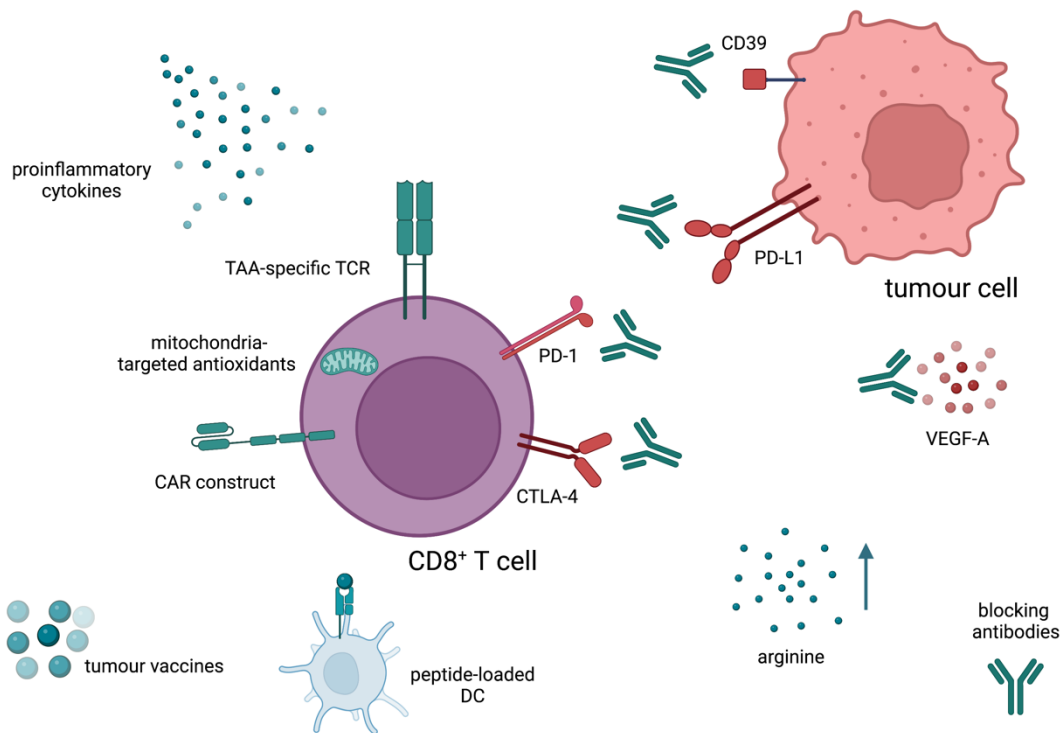


Figure 6.2 Immunotherapeutic approaches for CHB and cancer.

Examples for immunotherapeutic approaches explored for CHB and cancer. Blockade of inhibitory receptors including PD-1/PD-L1 pathway (chapter 6.1.2.1) and CTLA-4. Proinflammatory cytokines including IL-12 and IL-15 (chapter 6.1.2.2). Adoptive T cell transfer of TCR-engineered T cells and CAR T cells (chapter 6.1.2.3). Metabolic alterations including mitochondria-targeted antioxidants (chapter 1.2.6.1), increasing arginine availability (chapter 1.2.6.2) and blockade of CD39 and CD73 (chapter 1.2.4.3.1). Tumour vaccines and peptide-loaded DC. Inhibition of VEGF-A. TCR: T cell receptor; PD-(L)1: programmed death (ligand) 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; TAA: tumour-associated antigen; CAR: chimeric antigen receptor; VEGF-A: vascular endothelial growth factor-A; DC: dendritic cell.

6.1.2.1 PD-1/PD-L1 blockade

Blockade of the PD-1/PD-L1 axis has been one of the most successful immunotherapeutic strategies to date with tremendous success rates in solid tumours such as Hodgkin's lymphoma and malignant melanoma (Ribas and Wolchok 2018). In HCC, response rates are low with an objective response rate of only 20% (El-Khoueiry et al. 2017) and a potentially reduced treatment benefit in NASH-related HCC in some trials (Pfister et al. 2021). Combination therapies are now being explored to overcome treatment resistance (D'Alessio et al. 2021) and a combination of PD-L1 blockade with VEGF-A inhibition has increased the response rate to 30% (Cheng et al. 2022).

In CHB, PD-1 blockade boosts HBV-specific CD8⁺ T cells *in vitro* (Bensch et al. 2014; Boni et al. 2007; Fiscaro et al. 2010; Schurich et al. 2011) and in murine models (Maier et al. 2007), and induces viral control in woodchucks infected with woodchuck hepatitis virus (commonly used as an animal model for HBV) in combination with entecavir and therapeutic vaccination (Balsitis et al. 2018; Liu et al. 2014). In a pilot phase I study in CHB patients, single low-dose PD-1 blockade was well tolerated and achieved subtle HBsAg reduction in most patients but HBsAg loss in only one (Gane et al. 2019). Current trials are further exploring anti-PD-1/PD-L1 therapy in CHB, e.g. in combination with therapeutic vaccines (NCT05275023), interferons (NCT04638439), NUC (NCT04638439, NCT04133259) and HBV-targeted small interfering RNA (siRNA; NCT05275023) (ClinicalTrials.gov).

Factors contributing to treatment success are still being explored. In melanoma, reinvigoration of circulating exhausted T cells in relation to pre-treatment tumour burden determines treatment success (Huang et al. 2017a) whereas in HCC, a higher ratio of PD-1⁺ T_{RM}/PD-1⁺ T cells is associated with increased treatment response (Barsch et al. 2022).

Current studies aim to delineate the mechanisms by which PD-1 blockade enhances antitumour immunity. In line with the role of SHP-2-mediated CD28 dephosphorylation in the immunosuppressive function of PD-1 (Hui et al. 2017), CD28 expression is required for the rescue of CD8⁺ T cell function by PD-1 blockade (Kamphorst et al. 2017). PD-L1 blockade preferentially enhances the function of T_{ex}^{prog2} and T_{ex}^{int} subsets described in chapter 1.1.3.3.4 (Beltra et al. 2020). Importantly, PD-1 blockade reinvigorates the function of exhausted T cells but T cells become re-exhausted if antigen exposure persists and fail to develop a memory phenotype upon antigen clearance, suggesting that T cell exhaustion constitutes a distinct CD8⁺ T cell lineage (Pauken et al. 2016).

In line with PD-1 modulating T cell metabolism (see chapter 1.2.2), metabolic alterations have been suggested to play a role in the response to PD-1 blockade. A high frequency of CD8⁺ T cells with increased OXPHOS is associated with treatment failure (Li et al. 2021a) and the gut microbiome influences treatment efficacy (Gopalakrishnan et al. 2018; Matson et al. 2018; Routy et al. 2018).

Blockade of the PD-1/PD-L1 pathway induces a variety of metabolic alterations, for example, PD-L1 blockade restricts tumour glycolysis and restores glucose availability in the

TME which results in enhanced glycolysis and mTOR signalling in CD8⁺ TIL (Chang et al. 2015). However, a high expression of glycolysis-associated genes in tumour cells is associated with resistance to PD-1 blockade in melanoma (Renner et al. 2019).

Overall, the first studies in HCC and CHB are promising but a multipronged approach targeting PD-1 in combination with other strategies will likely be necessary to improve treatment response rates.

6.1.2.2 IL-12 and IL-15

Administration of immunostimulatory cytokines have been explored as immunotherapeutic approach in viral infections and cancer (Fig. 6.2).

As described in chapter 1.1.1.1, CD8⁺ T cell activation requires 3 distinct signals to induce proliferation, differentiation and effector function: First, antigen recognition via the TCR and resultant TCR signalling; second, signalling through co-stimulatory receptors, primarily CD28; third, inflammatory cytokines, predominantly IL-12 and Type I IFN, e.g. produced by DC (Curtsinger and Mescher 2010; Mescher et al. 2006).

Administration of IL-12 or IFN α in combination with peptide vaccination can induce effector and memory CD8⁺ T cell differentiation and enhance antitumour activity in murine models but is also required for antiviral CD8⁺ T cell stimulation, differentiation and function (Beadling and Slifka 2005; Berg et al. 2003; Bon et al. 2006; Keppler et al. 2012; Kolumam et al. 2005; Schmidt and Mescher 1999; Sikora et al. 2009).

Due to these properties, IL-12 administration has been explored as an immunotherapeutic approach in CHB and cancer. Early clinical trials in cancer patients failed due to high toxicity and limited tumour response but more targeted delivery approaches are currently being explored, e.g. incorporation into CAR T cell constructs (Nguyen et al. 2020). In CHB, *in vitro* IL-12 rescues the function of HBV-specific CD8⁺ T cells, inducing metabolic and mitochondrial changes (Schurich et al. 2013, 2016).

IL-15 is a proinflammatory cytokine primarily produced by macrophages, monocytes and DC as well as hepatic stellate cells, and is important for the development, function and maintenance of CD8⁺ T cells and NK cells (Swadling et al. 2020; Waldmann et al. 2020). In addition, IL-15 plays a crucial role in the development of tissue residency (Pallett et al. 2017) and provides antigen-independent stimulation to naïve CD8⁺ T cells (Alves et al. 2003).

Clinical trials with IL-15 monotherapy in cancer patients have failed due to low response rates but combination therapies, e.g. with checkpoint inhibitors, are currently being explored (Waldmann et al. 2020). In viral infections, the role of IL-15 is dependent on the model used. In LCMV infections, IL-15 plays a negligible role in effector function and memory development but is critical for the maintenance of memory CD8⁺ T cells post infection (Becker et al. 2002) whereas in vesicular stomatitis virus infection, IL-15 is required for the primary expansion of virus-specific CD8⁺ T cells as well as memory induction (Schluns et al. 2002). In CHB murine models, IL-15 fused to IL-15R α increases CD8⁺ T cell and NK cell frequency and activation (Cheng et al. 2014) and IL-15 has a complementary CD8⁺ T cell boosting effect with IFN α (Di Scala et al. 2016). *In vitro* treatment of human PBMC with IL-15 activates CMV- and EBV-specific CD8⁺ T cells but has only minor effects on influenza-specific CD8⁺ T cells (Sandalova et al. 2010).

6.1.2.3 Adoptive T cell therapy

In contrast to checkpoint blockade and cytokine treatment, adoptive T cell therapy does not aim to boost the intrinsic immune response *in vivo*, but instead provides optimised T cells recognising viral antigens or TAA. The tremendous success of CD19-specific CAR T cells for the treatment of chronic lymphocytic leukaemia (Kalos et al. 2011) has led to an exploration of genetically modified T cells specific for viral/tumour antigens in a variety of cancers and chronic viral infections.

There are two main classes of genetically modified T cells – CAR T cells and TCR-engineered T cells, briefly described in Table 6.1 (Bertoletti and Tan 2020; Guedan et al. 2022; Krebs et al. 2013; Shafer et al. 2022).

Table 6.1 Adoptive T cell therapy

	CAR T cells	TCR-engineered T cells
Construct	Single chain fragment variant (scFv) fused to intracellular signalling domain CD3 ζ and often costimulatory molecules (e.g CD28, 4-1BB)	Heterodimeric glycoprotein composed of TCR α and β chain forming a complex with endogenous CD3
MHC restriction	No	Yes
Antigen recognition	Cell surface	Any cellular compartment
Antigen density required	High	Low
Trialled in HBV	<i>In vitro</i> and in murine models	<i>In vitro</i> , in murine models and in phase I clinical trials in HBV-related HCC
Sequestered by circulating HBV antigens	Yes	No

Three of the key determinants of anti-tumour activity of TCR-engineered T cells are affinity, avidity and functional avidity (see chapter 1.1.1.1.1). One approach to enhance the function of TCR-engineered T cells is TCR affinity maturation which has shown some clinical effects but can fail to elicit effector function at low peptide concentrations (Thomas et al. 2011), can be associated with loss of antigen-specificity (Zhao et al. 2007) and with severe adverse events caused by cross-recognition (Linette et al. 2013). Additionally, high TCR affinity prevents serial triggering that is critical for T cell function, especially in setting with limited antigen presentation (Thomas et al. 2011; Valitutti et al. 1995). Another factor contributing to the efficiency of TCR-engineered T cells is the balance between immunogenic and tolerogenic APC in the TME (Hotblack et al. 2018).

New studies therefore explore the potential of enhancing TCR avidity instead of affinity, e.g. via framework engineering. Amino acid substitutions in the framework of the variable domain increases the number of TCRs expressed on low-affinity TCR-engineered T cells specific for the minor histocompatibility antigen HA1, resulting in enhanced antigen-specific proliferation and effector function (van Loenen et al. 2011; Thomas et al. 2019).

The safety and efficacy of HBV-specific TCR-engineered T cells have been shown in critical proof-of-concept case studies and small clinical phase I trials in HBV-related HCC (Meng et al. 2021; Qasim et al. 2015; Tan et al. 2019, 2022); however, only a fraction of patients

responded to the treatment and while HBsAg and HBV DNA levels were reduced, viral clearance was not achieved. Besides viral antigens, T cells specific for TAA are being explored, e.g. glypican-3-specific CAR T cells suppress tumour growth in patient-derived xenografts (Jiang et al. 2017).

6.2 Hypotheses and research questions

Hypothesis I: ACAT inhibition enhances the function of TAA-specific T cells in HCC.

- Is SOAT1 expressed in CD8⁺ and CD4⁺ TIL?
- Does ACAT inhibition boost the function of polyclonally stimulated TIL?
- Does ACAT inhibition boost the function of TAA-specific TIL?
- Does ACAT inhibition enhance the function of TAA-specific T cells from tumour-adjacent liver tissue?

Hypothesis II: ACAT inhibition has a complementary effect with other pharmacological immunotherapeutic strategies in boosting CD8⁺ T cell function.

- Does ACAT inhibition have a complementary effect with *in vitro* and *in vivo* PD-1 blockade?
- Does ACAT inhibition have a complementary effect with immunostimulatory cytokines?
- Does ACAT inhibition have a complementary effect with mitochondria-targeted antioxidants?

Hypothesis III: ACAT inhibition enhances the function of TCR-engineered T cells.

- Does ACAT inhibition increase the antitumour/antiviral cytokine production of CD8⁺ T cells engineered to express a TCR recognizing HBV-infected hepatocytes and HBV-related HCC?
- Does ACAT inhibition enhance specific target cell lysis by TCR-engineered T cells?

6.3 Results

6.3.1 ACAT inhibition boosts tumour-specific CD8⁺ and CD4⁺ T cells

Immune cells are exposed to a multitude of immunosuppressive factors in the tumour microenvironment and tumour-specific T cells are highly exhausted which is associated with expression of inhibitory checkpoint molecules and metabolic changes, ultimately leading to reduced anti-tumour function and poor prognosis (Flecken et al. 2014; Hung et al. 2021; Schmidt and Thimme 2016; Tauber et al. 2019). In both CHB and HCC, T cells are exposed to the tolerogenic liver environment and mechanisms of T cell exhaustion share similarities between chronic viral infections and tumours (see chapter 4.1.1.4 and 6.1.1.3). I therefore hypothesized that ACAT inhibition also has the potential to enhance the function of TAA-specific T cells.

I first aimed to identify functional TAA-specific CD8⁺ T cells in PBMC from HCC donors after short-term culture as described for CHB in chapter 4.3.4; however, survival of CD8⁺ T cells from HCC donors in short-term cultures was poor and, in line with my previous study (Flecken et al. 2014), I only detected functional TAA-specific CD8⁺ T cells in a small minority of donors (*data not shown*). Instead, in this chapter I therefore assessed the function of TIL directly isolated from human HCC tumour tissue.

To test whether ACAT inhibition had the potential to enhance the function of exhausted TIL, I first confirmed that SOAT1 (ACAT1) was transcribed in human CD8⁺ and CD4⁺ TIL from HCC tissue by probing a publicly available scRNAseq dataset (Zheng et al. 2017). SOAT1 was expressed by human CD8⁺ and CD4⁺ TIL at a slightly higher percentage than by peripheral T cells with no differences in mean RNA levels and the isoform SOAT2 (ACAT2) was only detected in a minority of T cells at low levels as expected (Fig. 6.3).

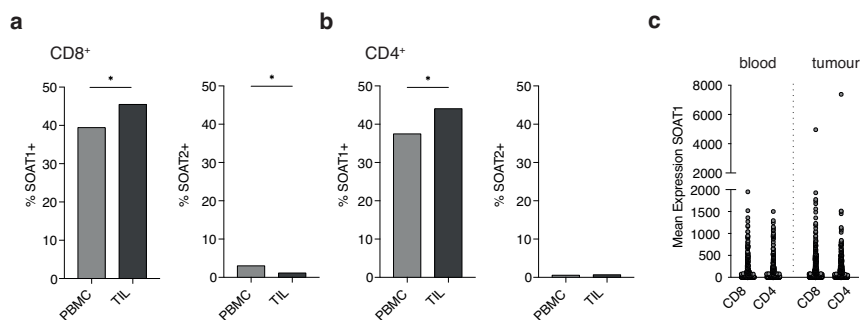


Figure 6.3 SOAT1 expression is increased in CD8⁺ and CD4⁺ TIL.

(a+b) %CD8⁺ **(a)** and CD4⁺ **(b)** T cells with any detectable SOAT1 or SOAT2 gene transcripts (no minimum cut off) by scRNA-sequencing analysis of PBMC (n=563 and n=515 respectively. Range of SOAT1 transcript expression: CD8⁺: 1-1951; CD4⁺: 1-1502. Range of SOAT2 transcript expression: CD8⁺: 1-915; CD4⁺: all 1 transcript) and TIL (n=777 and n=606 respectively. Range of SOAT1 transcript expression: CD8⁺: 1-4960; CD4⁺: 1-7383. Range of SOAT2 transcript expression: CD8⁺: 1-262; CD4⁺: 21-527). **(c)** Mean SOAT1 expression in T cells with any detectable SOAT1 transcripts in scRNAseq (no minimum cut off; blood: CD8 n=222 and CD4 n=193; tumour: CD8 n=354 and CD4 n=267). Data derived from publicly available scRNAseq data (GSE98638). Cell populations were sorted prior to sequencing based on flow cytometric identification of CD3⁺CD8⁺ (CD8⁺ T cells) and CD3⁺CD4⁺CD25⁻ (CD4⁺ T cells) expression. P values determined by Fisher's exact test **(a,b)** and Kruskal- Wallis test **(c)**. *Analysis assisted by Leo Swadling.*

I first assessed the effect of ACAT inhibition on anti-CD3/anti-CD28 stimulated CD8⁺ TIL freshly isolated from human HCC tissue. Polyclonal or PMA/ionomycin stimulation are commonly used to assess TIL (Chang et al. 2015; Dyck et al. 2022; Egelston et al. 2018; Ma et al. 2019; Yang et al. 2016) because a proportion of responding cells are likely specific for TAA due to clonal expansion of tumour-specific T cells within the tumour (Joshi et al. 2019; Woolaver et al. 2021). In line with SOAT1 expression in CD8⁺ TIL, a 16h culture in the presence of ACAT inhibition increased IFN γ production after anti-CD3/anti-CD28 stimulation of CD8⁺ TIL (Fig. 6.4a), applying the same stringent gating strategy used for IHL in CHB (Fig. 4.24a).

Next, to directly study TAA-specific CD8⁺ T cells I focused on three major, well-defined groups of TAA in HCC: global cancer-testis antigens expressed by various tumour types including HCC and melanoma (MAGE-A1 and NY-ESO-1), HCC-specific oncofoetal antigens (AFP), and, in cases with HBV-related HCC, HBV-derived viral antigens (HBc, HBs, Pol) (also see chapter 6.1.1.2).

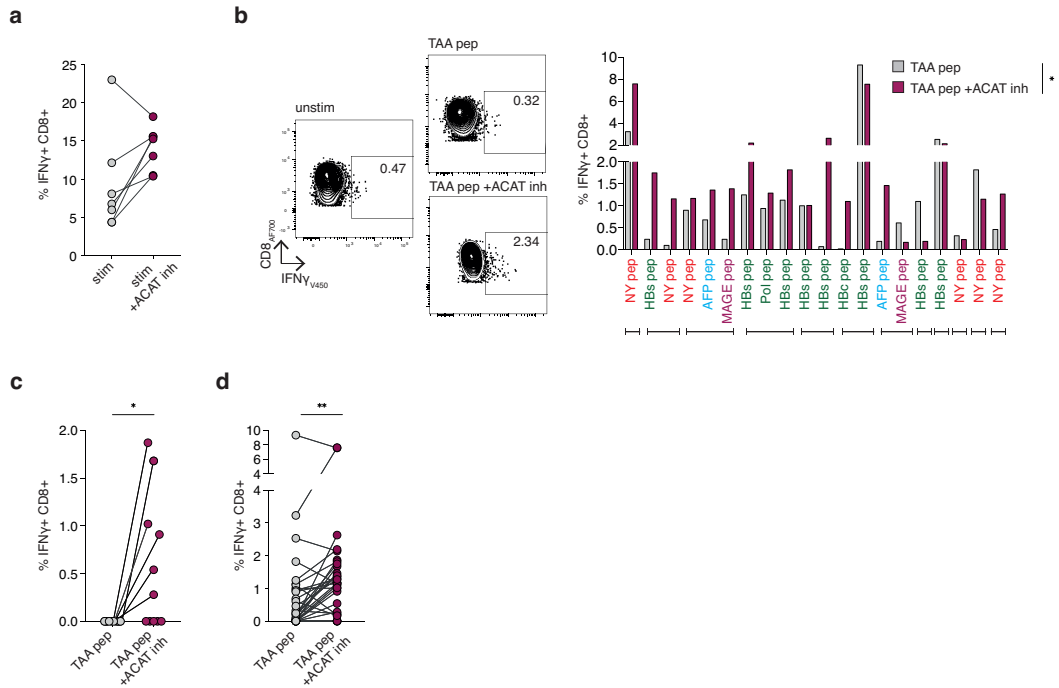


Figure 6.4 ACAT inhibition enhances TAA-specific IFN γ production by CD8⁺ TIL.

(a) TIL from donors with HCC were stimulated with aCD3/aCD28 antibody \pm ACAT inhibition (K-604; n=7) for 16h and IFN γ production of CD8⁺ T cells was assessed via flow cytometry. **(b-d)** TIL from donors with HCC were stimulated with TAA peptide pools (NY-ESO-1, MAGE-A1, AFP, HBc, HBs, Pol) \pm ACAT inhibition (K-604) for 16h and IFN γ production of CD8⁺ T cells was detected by flow cytometry. **(b)** Representative flow cytometry plot and summary data for each individual peptide pool (n=20) in donors (n=12) with detectable pre-existing TAA-specific CD8⁺ TIL responses. Brackets below the histogram indicate different TAA tested in TIL from the same donor. **(c)** *De novo* IFN γ production of CD8⁺ TIL \pm ACAT inhibition (n=10 pep pools and 6 donors). **(d)** Summary data of all donors (n=30 pep pools and 13 donors). P values determined by Wilcoxon matched-pairs signed rank test

CD8⁺ TIL stimulated with TAA-derived overlapping peptides pools and treated with ACAT inhibition for only 16h showed enhanced production of the antitumour cytokine IFN γ (Fig. 6.4b) and an induction of *de novo* responses in selected patients without detectable pre-existing antitumour responses when stimulated with the peptides alone (Fig. 6.4c).

In the cohort overall, ACAT inhibition significantly increased the frequency of TAA-specific IFN γ -producing CD8⁺ T cells with 10 out of 13 (77%) donors responding to treatment (Fig. 6.4d; response defined as increased or *de novo* IFN γ production in at least one TAA-derived peptide pool). Importantly, the increased IFN γ production was not confined to one class of TAA, with enhanced antitumour responses to TAA from all three groups.

Less consistent responses were seen with other readouts of antitumour function, e.g., the production of TNF (Fig. 6.5a), IL-2 (Fig. 6.5b) and MIP1 β (Fig. 6.5c) or the mobilization of CD107a to the cell membrane as an indicator of cytotoxic degranulation (Fig. 6.5d).

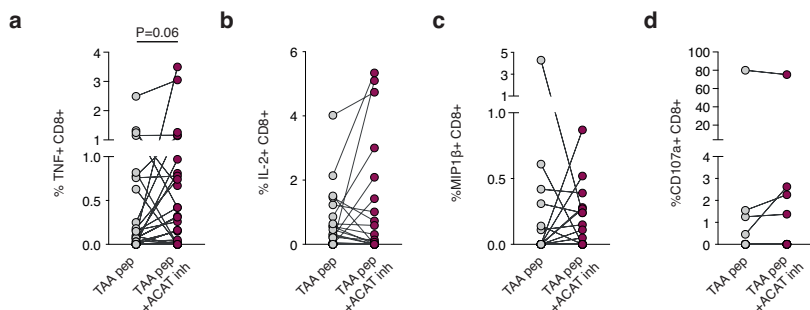


Figure 6.5 ACAT inhibition and TAA-specific cytokine production and degranulation by CD8⁺ TIL. (a-d) TIL from donors with HCC were stimulated with TAA (NY-ESO-1, MAGE-A1, AFP, HBc, HBs, Pol) peptide pools \pm ACAT inhibition (K-604) for 16h and cytokine production/CD107a mobilisation of CD8⁺ T cells was detected by flow cytometry. Frequency of TAA-specific TNF (**a**; n=33 pep pools and 14 donors), IL-2 (**b**; n=33 pep pools and 14 donors), MIP1 β (**c**; n=23 pep pools and 10 donors) production and CD107a mobilization (**d**; n=15 pep pools and 6 donors). P values determined by Wilcoxon matched-pairs signed rank test.

In line with the findings for CD8⁺ TIL, ACAT inhibition expanded IFN γ ⁺ CD4⁺ TIL (Fig. 6.6a) while the effect on other antitumour cytokines (TNF, IL-2, MIP1 β ; Fig. 6.6b-d) or degranulation (Fig. 6.6e) of CD4⁺ T cells was inconsistent.

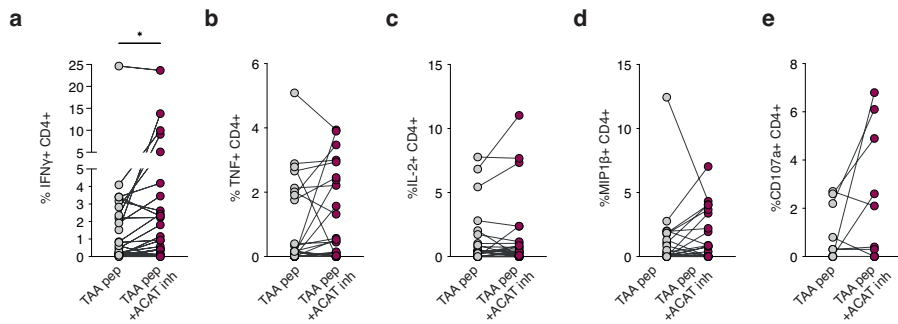


Figure 6.6 ACAT inhibition boosts TAA-specific CD4⁺ TIL.

(a-e) TIL from donors with HCC were stimulated with TAA (NY-ESO-1, MAGE-A1, AFP, HBc, HBs, Pol) peptide pools \pm ACAT inhibition (K-604) for 16h and cytokine production/CD107a mobilisation of CD4⁺ T cells was detected by flow cytometry. Frequency of TAA-specific IFN γ (**a**; n=25 pep pools and 11 donors), TNF (**b**; n=28 pep pools and 12 donors), IL-2 (**c**; n=28 pep pools and 12 donors), MIP1 β (**d**; n=20 pep pools and 12 donors) and CD107a mobilization (**e**; n=12 pep pools and 5 donors). P values determined by Wilcoxon matched-pairs signed rank test. *A subset of the data in this figure was analysed by Stephanie Kucykowicz under my direct supervision.*

Of note, ACAT inhibition did not alter the frequency of global CD3⁺, CD8⁺ and CD4⁺ TIL within live CD45⁺ TIL (Fig. 6.7).

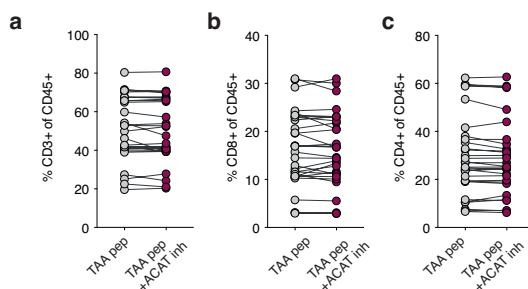


Figure 6.7 ACAT inhibition does not alter the frequency of global CD3⁺, CD8⁺ and CD4⁺ TIL.

(a-c) TIL from donors with HCC were stimulated with TAA peptide pools \pm ACAT inhibition (K-604) for 16h and frequency of CD3⁺ (**a**), CD8⁺ (**b**) and CD4⁺ (**c**) TIL was assessed via flow cytometry (n=30 pep pools and 13 donors). P values determined by Wilcoxon matched-pairs signed rank test.

Besides TAA-specific TIL, ACAT inhibition also boosted the antitumour function of TAA-specific CD8⁺ T cells in the liver tissue surrounding the tumour lesion, that have the potential to infiltrate the tumour and contribute to the local antitumour immune response (Fig. 6.8). Viral TAA-specific CD8⁺ T cells were excluded from this analysis to clearly distinguish between IHL targeting tumour antigens and those targeting viral antigens in the infected liver described in chapter 4.3.4.6.

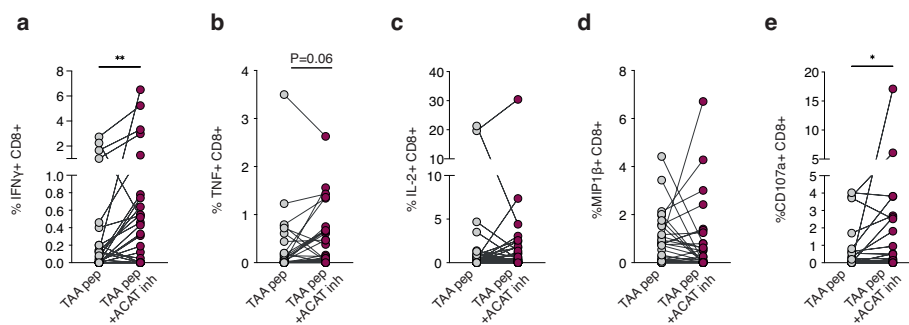


Figure 6.8 ACAT inhibition boosts TAA-specific CD8⁺ IHL from non-tumour liver tissue surrounding tumour lesions.

(a-e) IHL from donors with HCC were stimulated with non-viral TAA (NY-ESO-1, MAGE-A1, AFP) peptide pools ±ACAT inhibition (K-604) for 16h and cytokine production/CD107a mobilisation of CD8⁺ T cells was detected by flow cytometry. Frequency of TAA-specific IFN γ (**a**; n=32 pep pools and 14 donors), TNF (**b**; n=29 pep pools and 13 donors), IL-2 (**c**; n=31 pep pools and 13 donors), MIP1 β (**d**; n=32 pep pools and 14 donors) production and CD107a mobilization (**e**; n=22 pep pools and 10 donors). P values determined by Wilcoxon matched-pairs signed rank test.

Within non-lymphoid tissues, a population of long-lived CD8⁺ T_{RM} is defined by the expression of the tissue retention markers CD69 and CD103 and plays an important role in local antiviral/antitumour immune responses (Masopust and Soerens 2019; Pallett et al. 2017; Park et al. 2019).

As described for IHL (Fig. 4.26), ACAT inhibition did not alter the expression of CD69 and CD103 (Fig. 6.9a) but boosted the antitumour function of both tumour-resident TIL (CD69⁺CD103⁺) that can provide long-lived local antitumour protection and non-resident, tumour-infiltrating TIL (CD69^{neg}CD103^{neg}) that can return to the circulation and may have the potential to prevent tumour metastasis (Fig. 6.9b).

In summary, ACAT inhibition boosted the antitumour function of TAA-specific CD8⁺ and CD4⁺ T cells from human HCC tumour lesions directly *ex vivo*, a critical prerequisite for an effective immunotherapeutic approach. Additionally, ACAT inhibition enhanced the function of tumour-resident and tumour-infiltrating CD8⁺ T cells as well as TAA-specific CD8⁺ T cells in the non-tumour liver tissue surrounding the tumour contributing to tumour surveillance and potentially preventing local and distant tumour metastases.

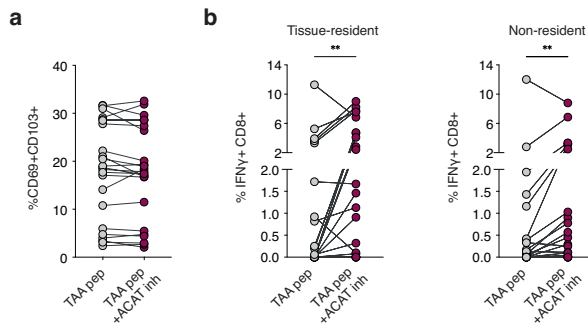


Figure 6.9 ACAT inhibition boosts tumour-resident and tumour-infiltrating TAA-specific CD8⁺ TIL. **(a-b)** TIL from donors with HCC were stimulated with TAA (NY-ESO-1, MAGE-A1, AFP, HBc, HBs, Pol) peptide pools \pm ACAT inhibition (K-604) for 16h and IFN γ production of CD8⁺ T cells was detected by flow cytometry. **(a)** Frequency of tissue-resident CD8⁺ TIL (CD69+CD103+; n=23). **(b)** TAA-specific IFN γ production by tumour-resident (CD69+CD103+) and tumour-infiltrating (CD69-CD103-) CD8⁺ TIL (n=22 peptide pools and 11 donors). P values determined by Wilcoxon matched-pairs signed rank test.

6.3.2 ACAT inhibition in combination with other immunotherapies

T cells in both CHB and HCC are highly exhausted with a multitude of different factors limiting the antiviral/antitumour immune response. Immunotherapies for CHB and HCC, especially PD-1 blockade, have been explored *in vitro*, *in vivo* and in clinical trials; however, only a minority of patients benefit from these treatments and a multipronged approach targeting multiple checkpoints and pathways simultaneously will likely be necessary to achieve higher response rates (Maini and Pallett 2018; Ozer et al. 2021) (also see chapter 6.1.2).

Here, I assessed the combination of targeting cholesterol esterification with other immunotherapies currently explored for CHB and HCC to boost functional antiviral/antitumour CD8⁺ T cells as an important first step towards combination trials *in vivo*. To address this, I mainly focused on PBMC from CHB as a model disease for T cell exhaustion.

6.3.2.1 ACAT inhibition has a complementary effect with PD-1 blockade

Blockade of the PD-1/PD-L1 axis has been one of the most successful immunotherapeutic strategies to date with tremendous success rates in some solid tumours; in HCC, response rates are low and combination therapies are explored to overcome treatment resistance (D'Alessio et al. 2021). In CHB, PD-1 blockade boosts HBV-specific CD8⁺ T cells *in vitro* and in animal models, and has been well tolerated in a pilot phase I study in CHB patients (Balsitis et al. 2018; Bengsch et al. 2014; Boni et al. 2007; Fasicaro et al. 2010; Gane et al. 2019; Liu et al. 2014; Maier et al. 2007; Schurich et al. 2011) (also see chapter 6.1.2.1). Overall, these first studies are promising but a multipronged approach targeting PD-1 in combination with other strategies will likely improve treatment responses in HCC and CHB.

As CD28^{neg} CD8⁺ T cells are a predictor of response to ACAT inhibition (Fig. 5.7) and CD28⁺ CD8⁺ T cells are the main target of anti-PD-1 therapy (Kamphorst et al. 2017), I hypothesized that a combination of the two immunomodulatory treatment strategies would target different cell populations and have an additive effect within individual patients and/or a non-redundant effect within patient cohorts.

I treated PBMC from CHB donors with PD-1 blockade, ACAT inhibition or a combination of both strategies *in vitro* and evaluated their potential to enhance HBV-specific IFN γ production by CD8⁺ T cells (Fig. 6.10a). Each set of four bars represents the HBV-specific IFN γ production for one individual donor with the respective treatment strategies, complemented by a box below the histogram indicating the treatment strategy achieving the highest IFN γ response for each individual donor.

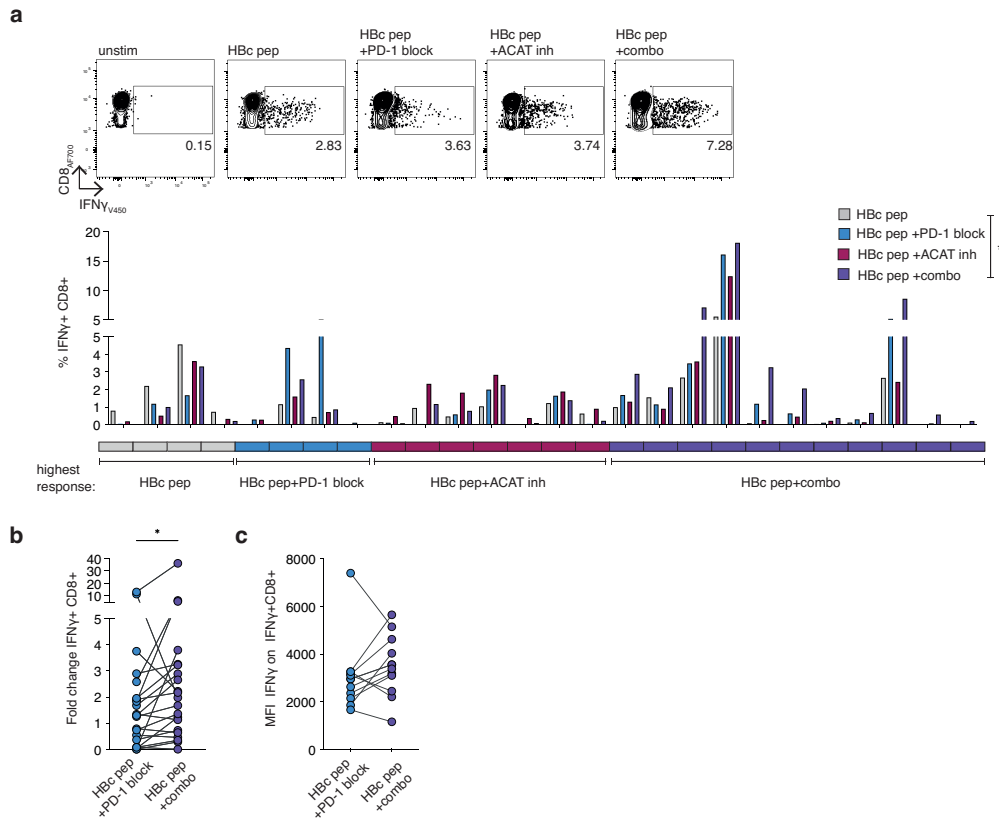


Figure 6.10 ACAT inhibition has a complementary effect with *in vitro* PD-1 blockade.

(a-c) PBMC from donors with CHB were stimulated with HBc pep in the presence of DMSO (grey), PD-L1/PD-L2 blockade (PD-1 block, blue), ACAT inhibition (Avasimibe, red) or a combination of ACAT inhibition and PD-L1/PD-L2 blockade (combo, purple) for 7d. **(a)** Representative flow cytometry plots and summary data of donors showing IFN γ production in any of the four conditions ($n=26$). Boxes below the histogram indicate treatment strategy resulting in the highest IFN γ production in the respective patients (grey: DMSO; blue: PD-1 block; red: ACAT inhibition; purple: combo). **(b)** Fold change of IFN γ production by CD8⁺ T cells normalized to unstimulated control. **(c)** MFI IFN γ assessed on IFN γ ⁺ CD8⁺ T cells ($n=12$). P values determined by Friedman test with Dunn's multiple comparisons test **(a)** and Wilcoxon matched-pairs signed rank test **(b,c)**.

As previously described, *in vitro* PD-1 blockade (blue bars) enhanced HBV-specific IFN γ production in selected donors compared to the DMSO control (grey bars) (Bensch et al. 2014; Boni et al. 2007; Schurich et al. 2011); however, PD-1 blockade achieved the highest cytokine production in only 4 out of 26 (15%) donors (blue boxed donors in key below histogram) compared to 7 donors (27%) with ACAT inhibition (red boxed donors in key below histogram), showing the non-redundant effect of both therapies within the cohort (Fig. 6.10a). A combination of both treatment strategies induced the highest IFN γ production in 11 out of 26 (42%) of donors (example flow cytometry plots and purple boxed donors in key below histogram, Fig. 6.10a) and significantly increased the fold change of

IFN γ production compared to PD-1 blockade alone, highlighting the additive effect of both immunomodulatory strategies (Fig. 6.10b).

The combination treatment not only increased the frequency of IFN γ^+ CD8 $^+$ T cells but also the IFN γ MFI on IFN γ^+ CD8 $^+$ T cells, an indicator of IFN γ production on an individual cell basis, in 8 out of 12 patients with sufficient events for analysis (Fig. 6.10c). This observation suggests that ACAT inhibition and PD-1 blockade can target the same cells; however, statistical significance was not reached in this small cohort.

In line with previous *in vitro* immunomodulatory studies (Bengsch et al. 2014; Boni et al. 2007; Maini and Pallett 2018; Nebbia et al. 2012; Schurich et al. 2011, 2013), responses were heterogenous, with a decrease of IFN γ production detected in a minority of patients, although a decline of responses tended to be less frequent after treatment with ACAT inhibition alone or in combination with PD-1 blockade compared to PD-1 blockade alone (Fig. 6.10a).

To further study the therapeutic potential of combining both treatment strategies in CHB and HCC, I utilized PBMC from a donor with HBV-related HCC who was clinically treated with anti-PD-1 therapy (Nivolumab) for two weeks. CD8 $^+$ T cell responses specific for HBc and HBs peptide pools during PD-1 therapy were strikingly increased when the *in vivo* treatment was combined with *in vitro* ACAT inhibition, including antiviral/antitumour cytokine production and degranulation/cytotoxicity (Fig. 6.11).

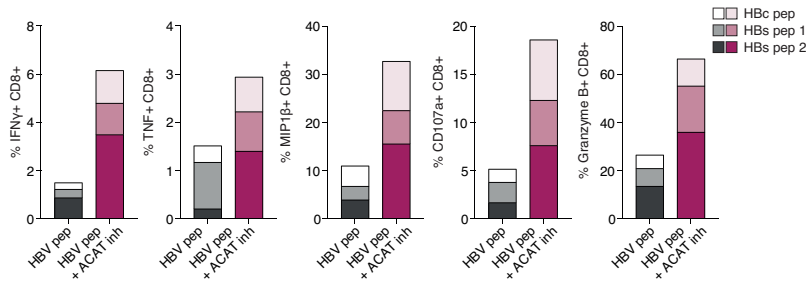


Figure 6.11 ACAT inhibition has a complementary effect with *in vivo* PD-1 blockade.

CD8⁺ T cell effector function of PBMC from a donor with HBV-related HCC 2 weeks after start of *in vivo* anti-PD-1 immunotherapy. PBMC stimulated with HBV pep \pm ACAT inhibition (Avasimibe) for 7d.

Taken together, ACAT inhibition had an additive, non-redundant effect with *in vitro* and, in one donor, *in vivo* PD-1 blockade, highlighting its immunotherapeutic potential in HBV and HCC.

6.3.2.2 ACAT inhibition and proinflammatory cytokines

Next, I examined the combination of ACAT inhibition with the proinflammatory cytokines IL-12 and IL-15 that are currently explored as an immunotherapeutic approach in chronic viral infections and cancer (see chapter 6.1.2.2).

I first explored the combination of IL-12 with inhibition of cholesterol esterification. PBMC from donors with CHB were stimulated with an HBc peptide pool with either DMSO (grey), IL-12 (blue), ACAT inhibition (red) or a combination of IL-12 and ACAT inhibition (combo; purple) and the antiviral function of CD8⁺ T cells was assessed by their capacity to produce IFN γ (Fig. 6.12a).

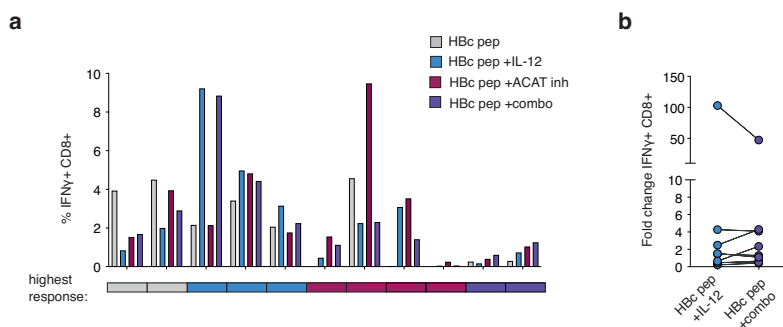


Figure 6.12 ACAT inhibition and IL-12 treatment.

(a+b) PBMC from donors with CHB (n=11) were stimulated with HBc pep in the presence of DMSO (grey), IL-12 (blue), ACAT inhibition (Avasimibe, red) or a combination of ACAT inhibition and IL-12 (combo, purple) for 7d. **(a)** %IFN γ ⁺ CD8⁺ T cells. Boxes below the histogram indicate treatment strategy resulting in the highest IFN γ production in the respective donors (grey: DMSO; blue: IL-12; red: ACAT inhibition; purple: combo). **(b)** Fold change of IFN γ production by CD8⁺ T cells normalized to unstimulated control. P values determined by Friedman test with Dunn's multiple comparisons test **(a)** and Wilcoxon matched-pairs signed rank test **(b)**.

Each set of four bars represents the HBV-specific IFN γ production for an individual donor with the respective treatment strategy and the box below the histogram indicates which treatment strategy achieved the highest IFN γ response for each donor. In this cohort, IL-12 treatment (blue bars) enhanced HBV-specific IFN γ production in 6/11 donors (55%) compared to the DMSO control (grey bars) and the combination treatment (purple bars) increased HBV-specific IFN γ in 7 donors (64%) compared to DMSO but only achieved the highest IFN γ response in 2 donors (18%) indicated by purple boxes below the histogram. Further, in a direct comparison, the fold increase of IFN γ with combination treatment did not show a significant enhancement compared to IL-12 alone (Fig. 6.12b).

I next assessed whether IL-15 had a complementary effect with ACAT inhibition using a similar approach (Fig. 6.13). IL-15 (blue bars) enhanced HBV-specific IFN γ production by CD8⁺ T cells in 8/11 (73%) donors compared to DMSO control (grey bars) (Fig. 6.13a). The combination treatment also boosted CD8⁺ T cell function in 7 donors (64%; purple bars) but only achieved the highest IFN γ response in 3 donors (27%; purple boxes below histogram) and had no significant advantage over IL-15 alone (Fig. 6.13b).

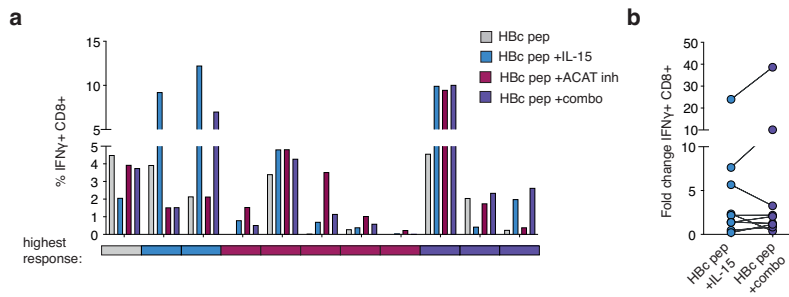


Figure 6.13 ACAT inhibition and IL-15 treatment.

(a+b) PBMC from donors with CHB were stimulated with HBc pep in the presence of DMSO (grey), IL-15 (blue), ACAT inhibition (Avasimibe, red) or a combination of ACAT inhibition and IL-15 (combo, purple) for 7d. **(a)** %IFN γ ⁺ CD8⁺ T cells. Boxes below the histogram indicate treatment strategy resulting in the highest IFN γ production in the respective donor (grey: DMSO; blue: IL-15; red: ACAT inhibition; purple: combo). **(b)** Fold change of IFN γ production by CD8⁺ T cells normalized to unstimulated control. P values determined by Friedman test with Dunn's multiple comparisons test **(a)** and Wilcoxon matched-pairs signed rank test **(b)**.

In summary, there was no indication for a complementary effect between ACAT inhibition and IL-12 or IL-15 in boosting HBV-specific CD8⁺ T cells.

6.3.2.3 ACAT inhibition and mitochondria-targeted antioxidants

HBV-specific T cells display signs of mitochondrial dysfunction and mitochondria-targeted antioxidants such as mitoquinone and piperidine-nitroxide (MitoTempo) can restore mitochondrial function and boost HBV-specific CD8⁺ T cell cytokine production from CHB PBMC (Fiscaro et al. 2017) (also see chapter 1.2.6.1). Due to my findings that ACAT inhibition skews T cell bioenergetics towards OXPHOS (chapter 5.3.2.4), I hypothesized that the two immunotherapeutic strategies could have a complementary effect.

To assess this, I used a similar protocol as previously described for MitoTempo treatment (Fiscaro et al. 2017). In this cohort MitoTempo (blue bars) only boosted HBV-specific CD8⁺ T cell function in a minority of donors compared to DMSO control (grey bars; Fig. 6.14a). The combination treatment enhanced IFN γ production in 7/11 donors (64%, purple bars) and achieved the highest IFN γ production in 4 donors (35%; purple boxes below histogram); however, there was no significant difference in the fold increase of IFN γ production compared to MitoTempo alone (Fig. 6.14b).

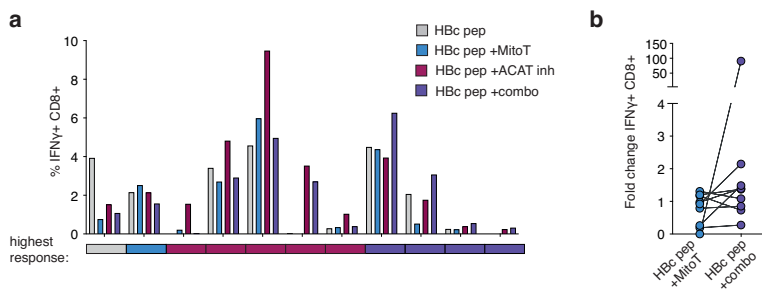


Figure 6.14 ACAT inhibition and mitochondria-targeted antioxidants.

(a+b) PBMC from donors with CHB ($n=11$) were stimulated with HBc pep in the presence of DMSO (grey), MitoTempo (MitoT, blue), ACAT inhibition (Avasimibe, red) or a combination of ACAT inhibition and MitoT (combo, purple) for 7d. **(a)** %IFN γ ⁺ CD8⁺ T cells. Boxes below the histogram indicate treatment strategy resulting in the highest IFN γ production in the respective donor (grey: DMSO; blue: MitoT; red: ACAT inhibition; purple: combo). **(b)** Fold change of IFN γ production by CD8⁺ T cells normalized to unstimulated control. P values determined by Friedman test with Dunn's multiple comparisons test **(a)** and Wilcoxon matched-pairs signed rank test **(b)**.

Taken together, there were indications of a potential complementary effect between ACAT inhibition and mitochondria-targeted antioxidants; however, the combination had no significant benefit in the cohort overall and a larger cohort will be necessary to confirm or disprove a complementary effect between the two metabolic modulators.

6.3.2.4 ACAT inhibition and TCR-engineered T cells

Besides immunotherapies aiming to boost the patient's own natural immune response, adoptive therapy is a promising novel therapeutic strategy and has shown first success in HBV-related HCC (Meng et al. 2021; Qasim et al. 2015; Tan et al. 2019, 2022) (also see chapter 6.1.2.3). Membrane cholesterol is an intricate regulator of TCR signalling – cholesterol binds to the TCR β chain and stabilizes TCR nanoclustering which results in increased TCR avidity (Molnár et al. 2012) but also keeps the TCR in an inactive, quiescent state in the absence of the cognate peptide (Swamy et al. 2016). I have shown that ACAT inhibition increased lipid rafts (chapter 5.3.2.1.2) and I therefore hypothesized that ACAT inhibition could enhance the (functional) avidity of TCR-engineered T cells.

To test this hypothesis, I transduced healthy PBMC with a TCR specific for an HLA-A2-restricted HBc-derived epitope (kindly provided by Antonio Bertoletti, Singapore; (Gehring et al. 2011)) and assessed their effector function when exposed to limited concentrations of cognate peptide. I utilized peptide-pulsed HepG2 hepatoma cells as APC in this setting to

reflect antigen-presentation by hepatocytes and tumour cells that is typically inefficient and associated with reduced T cell effector function compared to T cells primed by professional APC (Bénéchet et al. 2019; Gehring et al. 2007; Leone et al. 2013).

Indeed, ACAT inhibition increased IFN γ production by TCR-engineered T cells (increased percentage and increased MFI, Fig. 6.15a,b; increased IFN γ concentration in the supernatant measured by Luminex, Fig. 6.15c). ACAT inhibition further enhanced the production/release of a variety of other immune modulators, many of which possess antitumor capacity, e.g. TNF, MIP1 β , Fms-related tyrosine kinase 3 ligand (Flt-3L) and CD40L (all measured in the supernatant by Luminex, Fig. 6.15c; TNF, MIP1 β additionally measured by intracellular cytokine staining, Fig. 6.15d).

To further evaluate the effect of ACAT inhibition on TCR-engineered T cells, I transduced T cells to express a TCR specific for the minor histocompatibility antigen HA1 (kindly provided by Hans Stauss, UCL, and Mirjam Heemskerk, Leiden) with low cell surface expression and affinity (van Loenen et al. 2011; Thomas et al. 2019) (also see chapter 6.1.2.3). In a preliminary experiment, ACAT inhibition enhanced the functional avidity of T cells expressing the wildtype TCR (WT) at low peptide concentration comparable to the enhancement achieved by TCR framework engineering (LYR-modified TCR; see chapter 6.1.2.3) (Thomas et al. 2019) (Fig. 6.15e).

A critical feature of antitumour immunity is cytotoxicity and consecutive tumour lysis. ACAT inhibition not only increased the release of granzyme B into the supernatant (Fig. 6.15c) but also boosted specific lysis of the target tumour cell line assessed by measurement of adenylate kinase released by damaged cells in a ToxiLight assay (Fig. 6.15f).

Chapter 6 – ACAT inhibition boosts tumour-specific T cells and has a complementary effect with other immunotherapeutic strategies

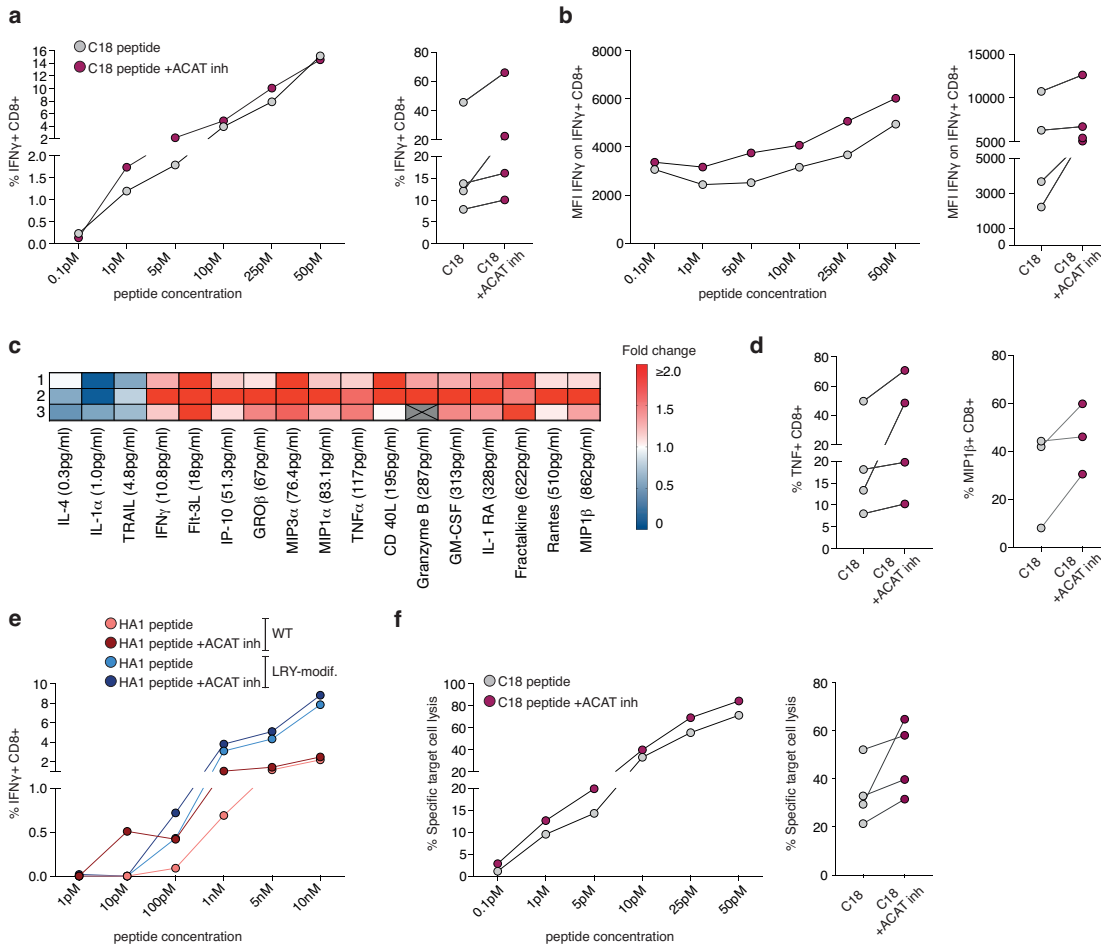


Figure 6.15 ACAT inhibition enhances TCR-engineered CD8 $^{+}$ T cells.

(a-d,f) HBcAg $_{18-27}$ -TCR-engineered CD8 $^{+}$ T cells were cocultured with HepG2 cells pulsed with C18 peptide \pm ACAT inhibition (Avasimibe) for 16h. **(a+b)** IFN γ production (**a**, %; **b**, MFI) of HBcAg $_{18-27}$ -TCR-engineered CD8 $^{+}$ T cells detected by flow cytometry. Representative data for increasing peptide concentration (0.1pM-50pM) and summary data for stimulation with 25pM peptide from 4 independent experiments. **(c)** Immune mediators in supernatant analysed by Luminex assay in 3 independent experiments (row 1-3; peptide concentration 1pM); Fold change of HBV-specific immune mediator concentration with ACAT inhibition compared to DMSO. Median HBV-specific immune mediator concentration in brackets. Grey box indicates sample with a Granzyme B concentration above the limit of detection. **(d)** TNF and MIP1 β production detected by flow cytometry \pm ACAT inhibition (peptide concentration 25pM). **(e)** IFN γ production of HA1-TCR-engineered CD8 $^{+}$ T cells with WT or LRY-modified TCR \pm ACAT inhibition (Avasimibe) for 48h cocultured with T2 cells pulsed with increasing doses of HA-1 peptide (1pM-10nM). **(f)** Specific target cell lysis by HBcAg $_{18-27}$ -TCR-engineered CD8 $^{+}$ T cells cocultured with HepG2 cells measure by Toxilight Assay. *Mariana Diniz assisted in performing TCR engineering. Oliver Amin assisted in sorting of TCR-engineered T cells and performing Luminex measurement (Fig. 6.15c).*

In summary, ACAT inhibition enhanced the functional avidity of TCR-engineered T cells, resulting in increased production of multiple antitumour/antiviral immune mediators and enhanced target cell killing.

6.4 Discussion

HCC is the seventh most common cancer and the third leading cause of cancer-related death worldwide with a rising incidence and ongoing high mortality (Bray et al. 2018). Treatment options in advanced tumour stages are palliative and only marginally improve patient survival. Immunotherapy aims to elicit *de novo* immune responses or to rescue the function of exhausted, dysfunctional antitumour immune cells. In a key study in 2016, Yang et al. demonstrated that modulation of cholesterol esterification, either by ACAT knockout or pharmacological inhibition, has the potential of enhancing antitumour CD8⁺ T cells in a murine melanoma model (Yang et al. 2016).

I now show for the first time that pharmacological ACAT inhibition could boost the function of human antitumour T cells directly *ex vivo* from HCC tumour lesions. In line with my findings for HBV and SARS-CoV-2, and in contrast to Yang et al., ACAT inhibition not only enhanced the function of antitumour CD8⁺ T cells but also of CD4⁺ T cells, highlighting the necessity of *in vitro* studies on human tissue.

The detected increase of tumour-specific IFN γ production by TIL is of high clinical relevance as multiple studies have shown an association between the increase of IFN γ during immunotherapy and treatment response in NSCLC (Boutsikou et al. 2018), melanoma (Wang et al. 2019b) and in murine tumour models (McNamara et al. 2016) as well as an association between treatment response and increased expression of IFN γ pathway genes in melanoma (Grasso et al. 2020). Conversely, a loss of IFN γ pathway genes in melanoma cancer cells is associated with treatment resistance in patients and murine models (Gao et al. 2016).

Most studies of tumour-infiltrating T cells rely on non-specific stimuli such as polyclonal stimulation with anti-CD3/anti-CD28 antibodies or even bypassing the TCR with PMA/ionomycin stimulation (Chang et al. 2015; Dyck et al. 2022; Egelston et al. 2018; Ma et al. 2019; Yang et al. 2016). The main advantages of this approach are the reliable induction of T cell cytokine production, bypassing the need for antigen presentation and the possibility to study the function of all TIL irrespective of their antigen-specificity. This is especially appealing for tumours such as HCC, where TAA expression is highly heterogenous (Breous

and Thimme 2011) and frequency of functional TAA-specific T cells is low (Flecken et al. 2014; Gehring et al. 2009; Mizukoshi et al. 2011; Tauber et al. 2019). Utilizing anti-CD3/anti-CD28 polyclonal stimulation to assess the functionality of all TIL, ACAT inhibition enhanced CD8⁺ T cell cytokine production of HCC TIL in 6 out of 7 donors, recapitulating the findings in the murine model (Yang et al. 2016); however, polyclonal stimulation has several disadvantages that need to be considered, e.g. this approach does not reflect differences in TCR affinity and avidity, determining T cell function (Shakiba et al. 2021), and assumes that all TIL are TAA-specific; however, TIL are specific for a variety of antigens, including non-tumour antigens e.g. from CMV, EBV and influenza (Simoni et al. 2018).

In contrast, by utilizing peptide pools for three well-described TAA (NY-ESO-1, AFP, MAGE-A1) and, in HBV-related HCC, viral antigens (HBc, HBs, Pol) I could directly study TAA-specific TIL and showed that the immune-enhancing effect of ACAT inhibition was not restricted to T cells for one specific TAA but increased the function of a variety of TAA-specific T cells. The finding that ACAT-inhibition enhanced the function of T cells specific for the shared TAA MAGE-A1 and NY-ESO-1, expressed by various cancer types, including melanoma and CRC, allows the speculation that the effect of ACAT inhibition is not restricted to HCC but could also be effective in other human tumours. This is supported by the finding that ACAT inhibition enhanced the function of CD8⁺ TIL in a murine melanoma model (Yang et al. 2016) but will need to be further confirmed with human TIL from different tumour entities.

ACAT inhibition increased the function of tumour-resident CD8⁺ TIL that can provide long-term local immune protections, as well as infiltrating CD8⁺ TIL that can re-enter the circulation and may have the potential to prevent distant metastasis, both of which are critical for a successful antitumour immune response (Enamorado et al. 2017). Additionally, ACAT inhibition also increased the function of TAA-specific T cells residing in the non-tumour liver tissue adjacent to the tumour, that are not directly exposed to the immunosuppressive tumour microenvironment and have the potential to infiltrate the tumour, providing antitumour immunity.

Direct cytotoxicity by T cells is critical for cancer cell elimination (Weigelin et al. 2021) and Yang et al. had shown increased CD8⁺ T cell cytotoxicity in the murine model (Yang et al. 2016); in this study, ACAT inhibition increased the mobilisation of CD107a to the T cell

surface as an indicator of degranulation and cytotoxicity in some donors but there was no overall significant effect in this small cohort. It is important to note that while CD107a is an indicator of degranulation, it does not directly reflect cytotoxicity and future studies directly measuring the release of cytotoxic molecules, e.g. granzyme B or perforin, could help to further delineate the effect of ACAT inhibition on the cytotoxic potential of anti-tumour T cells. The gold standard of assessing cytotoxicity is a killing assay directly measuring the T cells' ability to kill tumour cells; however, due to the low frequency of tumour-specific T cells and the high variability of TAA expressed in different HCC, this assay is technically challenging with primary human samples.

Blockade of the PD-1/PD-L1 axis has been one of the most successful immunotherapeutic strategies to date with tremendous success rates in solid tumours such as Hodgkin's lymphoma and malign melanoma (Ribas and Wolchok 2018); however, the response rates in phase IB (HBV) (Gane et al. 2019) and phase III (HCC) (Pinter et al. 2021) clinical trials were low and a multipronged approach targeting multiple immune checkpoints will be required to rescue these highly exhausted immune responses.

I have shown that ACAT inhibition had an additive, non-redundant effect with *in vitro* PD-1 blockade and may therefore increase the number of patients responding to this therapy. Utilizing my unique access to a donor with HBV-related HCC treated in a clinical trial with Nivolumab, an IgG4 anti-PD-1 antibody (El-Khoueiry et al. 2017), I could show that ACAT inhibition also had a complementary effect with *in vivo* PD-1 blockade.

The key question to explore the mechanism behind the complementary effect is whether both therapies target the same cells, resulting in an additive effect, or whether they target different populations and therefore overall increase the frequency of functional CD8⁺ T cells.

Considering the finding that PD-1 blockade primarily enhances the function of CD28⁺ CD8⁺ T cells (Kamphorst et al. 2017) and that CD28^{neg} CD8⁺ T cells were a predictor of response to ACAT inhibition, it could be suggested that the two immunotherapies can target different populations of CD8⁺ T cells and therefore have a complementary effect. On the other hand, the finding that the combination of ACAT inhibition and PD-1 blockade increased the IFN γ MFI in the majority of donors suggests that both strategies have the potential to target the same cell.

Further mechanistical studies will be necessary to determine how PD-1 blockade and ACAT inhibition complement each other, e.g. a first step would be assessing the effect of ACAT inhibition directly on PD-1^{+/neg} and CD28^{+/neg} cells, as well as T cell bioenergetics and metabolic profile in the combination therapy. While ACAT inhibition did not alter PD-1 expression, it did increase lipid rafts on CD8⁺ T cells and it would be interesting to assess whether this is associated with modulated SHP-2 activity or altered interaction between SHP-2 and PD-1 or CD28.

Besides the complementary effect with PD-1 blockade, in preliminary findings, ACAT inhibition also had a promising combined effect with mitochondria-targeted antioxidants that are being explored to overcome mitochondrial dysfunction and increased ROS in T cells in CHB (Fiscaro et al. 2017) and cancer (Vardhana et al. 2020; Yu et al. 2020). This might be due to synergistic effects considering that both therapeutic approaches alter mitochondrial function and a follow-up study in a larger cohort will be necessary to confirm this observation. In contrast, a combination with immunostimulatory cytokines such as IL-12 and IL-15 did not have any additive effects with ACAT inhibition.

Future studies assessing bioenergetic alterations induced by different immunotherapeutic approaches and their combination are needed to delineate why ACAT inhibition had a complementary effect with some strategies but not with others and will assist the selection of therapeutic strategies to explore as a combination in the future. Additionally, further assessment of the effect of combination strategies on other immune cell subsets besides antiviral/antitumour CD8⁺ T cells will be necessary.

Besides pharmaceutical interventions, cell-based immunotherapies such as adoptive transfer of T cells genetically engineered to target viral antigens or TAA are a promising therapeutic strategy that has already been explored for HBV-related HCC in proof-of-concept case reports and small clinical trials (Meng et al. 2021; Qasim et al. 2015; Tan et al. 2019, 2022). Due to the important role of cholesterol in TCR signalling, I explored the possibility of modulating cholesterol metabolism as a strategy to enhance the function of TCR-engineered T cells. ACAT inhibition enhanced the functional avidity of HBV/HCC-specific TCR-engineered T cells, including increased antiviral/antitumour cytokine production as well as enhanced killing of the tumour target cell line.

Of note, I confirmed this finding with T cells engineered to target a different tumour antigen, HA1, where ACAT inhibition achieved an enhancement of cytokine production comparable to that seen with framework engineering (Thomas et al. 2019) in preliminary data.

Utilizing a hepatoma cell line instead of a professional APC mirrored the liver/tumour microenvironment where T cell priming by hepatocytes or tumour cells induces immune dysfunction (Bénéchet et al. 2019; Gehring et al. 2007; Leone et al. 2013) and allows the hypothesis that ACAT inhibition will also enhance the function of HBV-specific TCR-engineered T cells when they encounter their cognate peptide in the liver or tumour.

There are two conceivable clinical treatment approaches combining pharmacological ACAT inhibition and adoptive T cell transfer – *in vitro* pre-treatment of TCR-engineered T cells prior to adoptive transfer or simultaneous adoptive transfer and *in vivo* treatment of the patient. The feasibility of *in vitro* metabolic conditioning prior to adoptive transfer has been highlighted by a recent study showing enhanced *in vivo* effector function of CD8⁺ T cells in a murine cancer model after *in vitro* glucose restriction (Klein Geltink et al. 2020).

From a clinical perspective, *in vitro* treatment would be expected to result in less side effects and less impact on the patient's quality of life, but *in vivo* treatment has the potential of boosting TCR-engineered T cells as well as autologous T cells simultaneously which might lead to superior tumour control.

From a pharmacological and immunological point of view it would be important to assess the durability of the immune boosting effect and how long metabolic and functional alterations persist after treatment termination. Alternatively, SOAT1 knockdown with siRNA can provide long-lasting ACAT inhibition, an approach that has already been successful for enhancement of CAR T cell function (Zhao et al. 2020).

In summary, ACAT inhibition boosted antitumour CD8⁺ and CD4⁺ T cells directly *ex vivo* from the immunosuppressive TME and had a complementary effect with other immunotherapeutic strategies currently under investigation for CHB and HCC.

7

Discussion

7.1 Summary

Overlapping metabolic requirements of T cells with the tumours and viruses they target can drive competition between them, contributing to a failure of an efficient antiviral/antitumour immune response due to limited nutrient availability (Andrejeva and Rathmell 2017; Kedia-Mehta and Finlay 2019). Targeting metabolic pathways is a novel immunotherapeutic strategy and I now show for the first time that inhibiting cholesterol esterification by ACAT inhibition boosted human virus- and tumour-specific T cell responses in SARS-CoV-2, CHB and HCC (Figure 7.1).

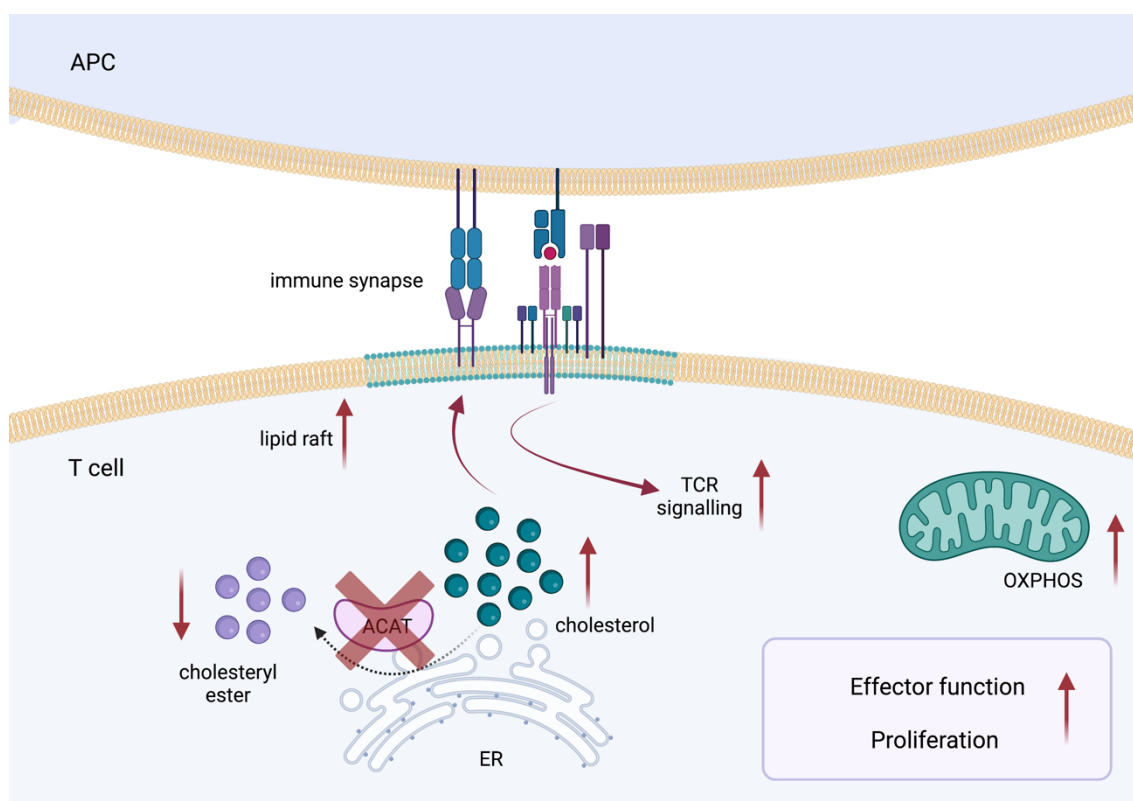


Figure 7.1 Proposed mechanisms underlying the immune boosting effect of ACAT inhibition.

APC: antigen presenting cell; TCR: T cell receptor; ER: endoplasmic reticulum; OXPHOS: oxidative phosphorylation; ACAT: acyl-CoA:cholesterol acyltransferase.

Cholesterol is a major component of cell membranes and its availability and utilization shape T cell effector function (Bietz et al. 2017; Ikonen 2008; Pallett et al. 2019). I showed that reduced lipid rafts were associated with T cell exhaustion; response to ACAT inhibition was associated with low *ex vivo* levels of lipid rafts and CD28 on CD8⁺ T cells and positively

correlated with *ex vivo* intracellular lipid droplets. ACAT inhibition reduced neutral lipid droplets in the cytoplasm and instead, the free excess cholesterol was diverted into the cell membrane and enhanced lipid raft formation, TCR signalling and T cell effector function. Independent of TCR signalling, ACAT inhibition altered T cell bioenergetics by enhancing OXPHOS and glycolysis, with a skewing towards OXPHOS.

T cells in CHB and HCC are highly exhausted and immune responses are constrained by a multitude of immunosuppressive mechanisms in the local liver and tumour microenvironment. Immunotherapeutic approaches for these diseases have only shown limited success in clinical trials (Gane et al. 2019; Pinter et al. 2021) and a multipronged therapy targeting different immune checkpoints will likely be necessary to achieve higher response rates. I have shown that ACAT inhibition had an additive, non-redundant effect with *in vitro* and, in one case *in vivo*, PD-1 blockade and may therefore increase the number of patients responding to this therapy.

In HBV and tumours, suboptimal antigen presentation and low affinity T cells further limit local immune responses (Aleksic et al. 2012; Gehring et al. 2007; Leone et al. 2013; Tauber et al. 2019). ACAT inhibition enhanced the functional avidity of T cells, overcoming these restraints, and boosted the function of TCR-engineered T cells currently in development for the treatment of CHB and HCC (Qasim et al. 2015; Tan et al. 2019).

In summary, ACAT inhibition is a promising new immunotherapeutic strategy that is not limited to SARS-CoV-2, HBV and HCC but will likely be transferrable to other tumours and viruses associated with T cell dysfunction.

7.2 Open questions and future directions

This study has highlighted the role of cholesterol as a metabolic checkpoint in T cell function and as a novel therapeutic target for immunotherapy. As discussed throughout this thesis, my findings have revealed many novel areas and questions to be explored in future studies. In this chapter, I will highlight eight key questions that remain to be fully answered to provide further insight into targeting cholesterol esterification as an immunotherapeutic strategy and the role of cholesterol in immune cell function.

7.2.1 What is the role of cholesterol and lipid rafts in T cell exhaustion?

A multitude of factors contribute to immune dysfunction and exhaustion, and in recent years the critical role of lipids and cholesterol in the tumour microenvironment has become more and more apparent. Cholesterol and lipids accumulate in CD8⁺ TIL and have been proposed to contribute to immune dysfunction (Ma et al. 2019; Xu et al. 2021), with high T cell cholesterol having the potential to induce ER stress and consecutive activation of XBP-1 regulating the expression of immune checkpoints such as PD-1 and 2B4 (Ma et al. 2019). However, while in two studies lipid accumulation was associated with immune dysfunction of CD8⁺ TIL (Ma et al. 2019; Xu et al. 2021), in another lipid droplets correlated with increased effector function (Dyck et al. 2022). This controversy highlights that immunometabolism and especially cholesterol metabolism is highly context dependent.

In this study, I have shown that T cell exhaustion was associated with low lipid rafts and that increasing intracellular free cholesterol by ACAT inhibition did not upregulate the expression of immune checkpoints such as PD-1. These findings open many interesting routes to explore in order to further delineate the role of cholesterol in T cell exhaustion. It would be interesting to assess whether the increase of free cholesterol by ACAT inhibition results in ER stress, e.g. by assessing the expression of ER-stress related genes such as XBP-1; however, the lack of increased expression of PD-1 and transcription factors associated with

T cell exhaustion does not suggest this. Alternatively, ACAT inhibition may mediate pathways that prevent XBP-1 induction or its downstream effects.

The diverging findings that PD-1 expression can be induced by high cholesterol but is associated with reduced lipid rafts suggest that there may be a disconnect between cellular cholesterol levels and lipid raft assembly in exhausted T cells. Assessing intracellular cholesterol distribution and membrane order directly on exhausted and functional virus-specific T cells as well as on T cells directly from the immunosuppressive liver and tumour environment will aid to further understand the role of cholesterol in T cell exhaustion.

7.2.2 What is the exact mechanism behind the immune-boosting effect of ACAT inhibition?

Based on my findings, I propose three mechanisms by which ACAT inhibition boosts T cell proliferation and function (Fig.7.1).

First, by targeting the enzyme catalysing the esterification of cholesterol, ACAT inhibition reduced cholesteryl ester stored in neutral lipid droplets. An accumulation of lipid droplets has been associated with reduced function of NK cells (Michelet et al. 2018) while in DC and CD8⁺ T cells the association is less clear with some reports suggesting a relation between lipid accumulation and dysfunction (Herber et al. 2010; Xu et al. 2021) and others not (Dyck et al. 2022; Ibrahim et al. 2012). The concept that reduction of lipid droplets by ACAT inhibition may directly enhance CD8⁺ T cell function is further supported by the finding that high *ex vivo* lipid droplet levels were associated with treatment response.

Second, ACAT inhibition increased free cholesterol, facilitating lipid raft formation. The findings that low lipid rafts are a feature of T cell exhaustion and are predictive of treatment response further support this mechanism of action.

Lipid rafts are critical for the formation of the immune synapse and future studies are needed to further assess the effect of ACAT inhibition on lipid rafts and the immune synapse, e.g. by measuring the effect of ACAT on the expression of molecules associated with lipid raft formation and synapse structure (e.g. CD28 (Zumerle et al. 2017), LFA1 (Cassioli et al. 2021),

CD2 (Demetriou et al. 2020)), directly visualising synapse formation by high-resolution imaging (e.g. confocal microscopy, total internal reflection fluorescence microscopy, TIRFM) and by assessing TCR recruitment to the synapse.

Furthermore, membrane order and fluidity are highly influenced by membrane cholesterol content. High membrane order is associated with immune synapse formation and T cell function and proliferation (Miguel et al. 2011), and it would be expected that ACAT inhibition increases membrane order. Membrane order can for example be assessed by di-4-ANEPPDHQ via confocal microscopy or flow cytometry (Miguel et al. 2011).

Third, ACAT inhibition induced TCR-independent bioenergetic remodelling with increased OXPHOS and glycolysis and a preferential use of OXPHOS. Future studies will need to determine the mechanisms underlying this bioenergetic change, including assessing bioenergetics after stimulation via the TCR. The findings that ACAT deletion in murine CD8⁺ T cells leads to a paradoxical increase of SREBP activity (Kidani and Bensinger 2016; Yang et al. 2016) and that SREBP signalling is required for increased glycolysis and OXPHOS upon T cell activation (Kidani et al. 2013) suggest that the bioenergetic alterations by pharmacological ACAT inhibition may be indirectly due to increased SREBP activity instead of direct effects due to inhibition of cholesterol esterification. Assessing the expression of the transcription factors SREBP and LXR in human T cells upon ACAT inhibition will be a first step to further evaluate the mechanisms underlying enhanced T cell function and metabolic rewiring.

Besides T cells in settings associated with immune dysfunction, ACAT inhibition also increased the functional avidity of TCR-engineered T cells. Assessing the similarities and differences of metabolic changes induced by ACAT inhibitors between exhausted T cells and TCR-engineered T cells will assist the definition of mechanisms contributing to the immune-boosting effect of ACAT inhibition.

It is important to note that besides cholesterol, acyl-CoA is a substrate of ACAT. Acyl-CoA derived from long-chain fatty acids is a key metabolite in a variety of metabolic pathways, including protein acetylation and synthesis of lipids such as phospholipids, sphingolipids and triglycerides, and is degraded via mitochondrial FAO (Ferreira et al. 2017; Grevengoed et al. 2014; Sandhoff and Sandhoff 2018). Considering the connection between FAO and

OXPHOS (Wang et al. 2010) and that sphingolipids are a critical component of lipid rafts (Bieberich 2018), it would be expected that the increase of acyl-CoA also contributes to the enhanced immune cell function and altered bioenergetics besides cholesterol. Blocking fatty acid synthesis by fatty acid synthase or acyl-CoA synthesis by long-chain acyl-CoA synthetase would be a first step to assess the role of acyl-CoA in the immune boosting effect of ACAT inhibition.

It would also be interesting to evaluate the contribution of FAO to the increased OXPHOS upon ACAT inhibition (e.g. by blocking CPT1a or by induction of FAO with palmitate). Finally, lipidomic and metabolomic analysis by mass spectrometry could provide further insight into the metabolic alterations induced by ACAT inhibition.

Of note, in this study I investigated the immune boosting effect of pharmacological ACAT inhibition. A limitation of pharmacological intervention is the possibility of off-target effects other than the inhibition of cholesterol esterification. In this study, I utilised two different ACAT inhibitors, Avasimibe and K-604, as indicated in the figure legends. I detected no difference when directly comparing the two ACAT inhibitors, suggesting that the described immune boosting effects were reproducible and not specific to one single drug; however, the comparison was only performed in selected experiments and will need to be further extended in future studies. Additionally, some of the immune-boosting effects seen in this study have also been shown in ACAT knockdown murine models (Yang et al. 2016), supporting the concept that my findings are indeed due to inhibition of ACAT; however, it will be necessary to confirm my findings by ACAT knockdown in human T cells in future studies.

7.2.3 Why are responses to ACAT inhibition heterogeneous and which factors contribute to treatment success?

Overall, ACAT inhibition enhanced the function of antiviral and antitumour CD8⁺ and CD4⁺ T cells; however, responses were highly heterogeneous with some donors showing striking increases of cytokine production (up to 50-fold) but also decreased cytokine production in a minority of donors. This heterogeneity is in line with previous reports for

other immunotherapeutic strategies tested *in vitro* (Bengsch et al. 2014; Fiscaro et al. 2010, 2017; Schurich et al. 2013, 2016) and reflects findings in clinical trials where usually only a fraction of patients respond to therapy (El-Khoueiry et al. 2017; Gane et al. 2019). In this study, attrition of baseline responses tended to be less common for ACAT inhibition or a combination of ACAT inhibition and PD-1 blockade compared to PD-1 blockade alone.

To further evaluate the underlying factors of response heterogeneity, it is important to differentiate between donors with stable cytokine production that is not altered by treatment and donors with a loss of cytokine production.

In cases where cytokine production is unaffected by ACAT inhibition, it will be necessary to assess whether ACAT inhibitors indeed blocked cholesterol esterification in these donors, e.g. by measuring intracellular cholesteryl ester levels or ACAT activity. Several SOAT1 (ACAT1) polymorphisms have been identified (Ohta et al. 2004; Wollmer et al. 2003) and might influence the inhibitory efficiency of different ACAT inhibitors. As discussed in chapter 4.1.1.4, multiple mechanisms restrain HBV-specific T cells, e.g. regulatory NK cells or expression of the pro-apoptotic protein Bim, that may not be overcome by ACAT inhibition in non-responders.

In cases where cytokine production is reduced, a phenomenon described as activation-induced cell death (AICD) should be investigated. T cell activation leads to a rapid clonal expansion of antigen-specific T cells; however, this process needs to be terminated, especially when TCR signalling is strong and long-lasting, to prevent excessive cytokine production (Moskophidis et al. 1993). AICD is caused by regulated, physiological apoptosis of highly activated T cells mediated via Fas/FasL (Ju et al. 1995; Saff et al. 2004) and TNF/TNFR2 (Herbein et al. 1998; Otano et al. 2020) pathways. Future studies should examine whether ACAT inhibition induces apoptosis, e.g. by measuring the binding of annexin V to phosphatidylserine on the plasma membrane of apoptotic cells (van Engeland et al. 1998) or caspase activity by fluorochrome-labelled inhibitors of caspases assay (FLICA) (Darzynkiewicz et al. 2011), and whether ACAT inhibition induces an upregulation of Fas, FasL or TNFR2 on the cell surface.

The positive correlation between increased TNF and IFN γ production after ACAT inhibition suggests that TNF-mediated AICD did not play a major role in this system;

however, TNF production was only measured after 7d by which time AICD may have already occurred. Interruption of the TNF/TNFR2-signalling pathway, e.g. by TNF inhibitors, limits *in vitro* AICD (Otano et al. 2020), enhances response to checkpoint blockade in murine models (Perez-Ruiz et al. 2019) and could be utilized to determine the role of this pathway in ACAT inhibition.

A major challenge when studying immune cell function and immunotherapeutic approaches in human tissue or in clinical trials is the vast diversity and variability of study participants, where a variety of factors can contribute to differing treatment outcomes. Identifying donors who are likely to benefit from treatment is of high clinical and scientific importance. It allows a selection of patients that are likely to respond to therapy and therefore avoids unnecessary side effects and costs for patients unlikely to respond, but it also facilitates a more thorough understanding of the mechanism behind a certain treatment. In this study, I aimed to address some of the clinical factors contributing to a treatment responses to ACAT inhibition.

I identified donor sex as a determinant of response to ACAT inhibition in CHB with male donors being more likely to respond. This is of high clinical relevance as male CHB patients have a higher risk of developing HCC compared to females (Liu and Liu 2014).

Sex differences, such as sex chromosomes and sex hormones contribute to alterations of immune function (Klein and Flanagan 2016) and lipid profile (Freedman et al. 2004). For example, females have a higher CD4⁺/CD8⁺ ratio and have a higher number of activated and proliferating T cells following *in vitro* stimulation (Klein and Flanagan 2016; Maini et al. 1996). On the other hand, males have increased frequencies of T_{reg} with increased suppressive capacity (Robinson et al. 2022). Female sex is further associated with higher HDL and lower triglyceride and LDL compared to males (Freedman et al. 2004) with LDL levels increasing and HDL decreasing during menopause (Matthews et al. 1989). Interestingly, there was no sex difference in response to ACAT inhibition in the older SARS-CoV-2 cohort where most female donors were likely postmenopausal.

While my findings are solely observational, further assessment of factors contributing to the difference between male and female donors would be interesting, e.g. by determining whether donor sex is associated with altered T cell lipid profile or bioenergetics and whether an *in vitro* or *in vivo* (e.g. contraception, hormone-replacement therapy, cross-sex hormone

treatment, androgen receptor blockers) treatment with sex hormones alters response to ACAT inhibition.

In vitro cholesterol supplementation enhanced the effect of ACAT inhibition, possibly due to the fact that high cholesterol levels increase ACAT activity (Gillies et al. 1990) and therefore the impact of ACAT inhibition on cellular cholesterol distribution might be more profound. Hyperlipidaemia also regulates ACAT activity in patients (Dallongeville et al. 1992) and it will be interesting to evaluate whether serum lipid and cholesterol levels contribute to the inter-individual variability in response to ACAT inhibition. This would be especially relevant in SARS-CoV-2 where hypercholesterolaemia and metabolic syndrome are associated with severe disease (Denson et al. 2021) and patients with these underlying health conditions will be more likely to require therapy, but where on the other hand hypolipidemia occurs during acute infection (Wei et al. 2020).

Besides serum lipids, the effect of other cholesterol-modulating drugs, e.g. statins (see chapter 1.2.6.3.1), on treatment outcome with ACAT inhibition would be of high clinical relevance as they are commonly prescribed to patients with hyperlipidaemia (Blais et al. 2021).

A variety of other patient characteristics can contribute to differing treatment responses. While the patient cohorts in this study were too small to take these variabilities into account, it will be important to address them in larger future studies, e.g. donor ethnicity, comorbidities, medication, nutritional status (e.g. obesity/cachexia as well as fasting status at the time of phlebotomy), time of phlebotomy/tissue collection to account for alteration in immune function due to circadian rhythms (Downton et al. 2020) and differences in gut microbiome that modulate response to other immunotherapies such as checkpoint blockade (Gopalakrishnan et al. 2018).

Both CHB and SARS-CoV-2 are highly variable diseases with a diverse clinical spectrum, and it would be of interest to assess the differential effects of ACAT inhibition in different stages of CHB infection as well as different disease severity, infection with different VOC and breakthrough infections in a vaccinated population in the case of SARS-CoV-2.

In addition to clinical differences between donors, I identified cellular characteristics that were associated with treatment response. In line with the proposed mechanism of action, response to ACAT inhibition was associated with reduced *ex vivo* lipid rafts and CD28 expression and high intracellular neutral lipid droplets, supporting the concept that ACAT inhibition boosted immune function by reducing lipid droplets and increasing lipid rafts, facilitating immune synapse formation.

It would be of interest to determine other cellular predictors of treatment response, e.g. by assessing the role of checkpoint molecule expression, transcriptional profile, chromatin state, activation status and *ex vivo* bioenergetics (e.g. OXPHOS and glycolysis utilization) prior to treatment and on responding peptide-specific T cells.

7.2.4 Which subsets of antiviral/antitumour T cells are enhanced by ACAT inhibition?

To further determine the mechanisms by which ACAT inhibition enhanced T cell function and why immune function is boosted in some settings but not others, it is necessary to assess which subsets of antiviral/antitumour T cells were affected.

ACAT inhibition not only improved the function of pre-existing antiviral/antitumour T cells but also induced T cell proliferation and *de novo* responses in a proportion of donors without detectable responses when stimulated with peptide alone; however, the question remains whether ACAT inhibition increased the frequency of cytokine producing T cells primarily by enhancing the proliferation of functional, but low-frequency T cells and/or whether it boosted the function of pre-existing, but exhausted T cells. Utilizing HLA/peptide multimers that allow the identification of virus-/tumour-specific T cells irrespective of their function can help to further address this question.

In this study, I showed that T cell exhaustion was associated with reduced lipid rafts and that low lipid rafts and low CD28 expression were predictive of a response to ACAT inhibition. ACAT inhibition increased lipid rafts on CD8⁺ T cells and the magnitude of increase in lipid rafts tended to correlate with the increase of HBV-specific IFN γ production. It is therefore tempting to hypothesize that ACAT inhibition preferentially enhanced the function of

CD28^{low} and/or PD-1^{high} T cells with low lipid rafts; however, this needs to be formally proven, e.g. by sorting or labelling lipid raft/CD28/PD-1^{high/low} subsets prior to treatment and assessing the antiviral/antitumour function directly on these individual subsets.

Determining whether ACAT inhibition preferentially boosted the function of cells in distinct exhaustion stages as described for checkpoint blockade, e.g. T cells in the intermediate exhaustion stages (T_{EX}^{prog2+int}) expressing T-bet (Beltra et al. 2020) and T cells with a plastic chromatin stage (Philip et al. 2017) will inform whether the frequency of these subsets prior to treatment can predict treatment response.

The finding that ACAT inhibition enhanced T cell function in CHB, acute SARS-CoV-2 and tumours, settings in which T cell exhaustion has been reported, but not in well-controlled CMV infection or after resolved acute infection (Influenza, EBV, SARS-CoV-2) further strengthens the hypothesis that ACAT inhibition primarily boosted exhausted T cells. Interestingly, mitochondrial dysfunction has been described for CHB (Fiscaro et al. 2017; Schurich et al. 2016), tumours (Scharping et al. 2016) and SARS-CoV-2 (Liu et al. 2021; Siska et al. 2021), suggesting that the enhanced OXPHOS capacity after ACAT inhibition may be another mechanism besides increased lipid rafts by which ACAT inhibition preferentially targeted exhausted T cells.

It is interesting to note that, in contrast to HBV and HCC, ACAT inhibition did not consistently boost CD8⁺ T cell function in SARS-CoV-2. Determining metabolic and phenotypic differences of CD8⁺ T cells in these settings will help to further delineate the mechanism by which ACAT inhibition enhanced T cell function and to predict other diseases in which ACAT inhibition might be beneficial.

Besides focusing on T cell exhaustion, it is also important to consider differences in T cell differentiation depending on the setting. In acute SARS-CoV-2, CHB and HCC, T cells are exposed to their cognate antigen *in vivo* at the time of cell isolation, driving a T effector phenotype, whereas T cells after resolved infection are expected to display a memory phenotype. This should be confirmed *ex vivo*, e.g. by assessing the expression of CD45RA and CD27 (Martin and Badovinac 2018).

Memory and effector T cells have distinct metabolic phenotypes reflecting their differing energy demands (Buck et al. 2016) that might render them more or less susceptible to metabolic alterations by ACAT inhibition, e.g. an increase in OXPHOS due to ACAT inhibition may have less effect on the function of memory T cells that intrinsically utilize OXPHOS even in the absence of ACAT inhibition.

Besides bioenergetic alterations, *in vitro* induced memory T cells have highly reduced cholesteryl ester levels compared to effector T cells (O’Sullivan et al. 2014) which might make them unsusceptible to ACAT inhibition, considering that reduction of cholesteryl ester stored in lipid droplets is one of the proposed mechanisms of action.

Assessing the effect of ACAT inhibition on effector T cells in acute infections not commonly associated with T cell exhaustions, as well as longitudinal studies on effector and memory T cells in the same cohort will help to further delineate the influence of T cell exhaustion and differentiation state on the response to ACAT inhibition.

7.2.5 Why does ACAT inhibition preferentially boost intrahepatic T cells?

Antiviral and antitumour immune responses are shaped by the local microenvironment and nutrient availability. The cholesterol-rich microenvironment in the liver and many tumours can contribute to T cell exhaustion and checkpoint blockade is less successful in NASH-related HCC in some trials (Chamberlain 1928; Ma et al. 2019; Pfister et al. 2021). ACAT inhibition preferentially enhanced the function of HBV-specific T cells directly *ex vivo* from the immunotolerant, cholesterol-rich liver environment compared to peripheral T cells from the same donor, a finding that could be recapitulated *in vitro* showing an enhancement of response to ACAT inhibition in high cholesterol conditions. Like the liver, the lungs are enriched in cholesterol (Chamberlain 1928), suggesting that a similar enhancement may be seen following ACAT inhibition of SARS-CoV-2-specific T cells infiltrating the infected lungs.

Besides high tissue cholesterol, the liver and tumour constitute a unique metabolic environment, and it will be important to address other metabolic changes that could contribute to the enhanced ACAT inhibitor efficacy.

CD8⁺ T_{RM} adapt to the liver environment by increasing autophagy (Swadling et al. 2020). ACAT inhibition alters cellular autophagy levels with increases (Shibuya et al. 2014, 2015) or decreases (Ogasawara et al. 2020) depending on the setting and future studies should assess the effect of ACAT inhibition on IHL and TIL autophagy, e.g. by measuring LC3-II, a marker of autophagosomes (Clarke et al. 2018).

The liver constitutes a highly hypoxic environment (Wilson et al. 2014) and hypoxia induces ACAT activity (Mukodani et al. 1990), suggesting a potential for higher efficiency of immune-boosting by ACAT inhibition in hypoxic conditions as seen in high-cholesterol environments. This hypothesis could be tested by assessing the effect of ACAT inhibition in hypoxic culture conditions *in vitro*.

Finally, dysfunctional mitochondria are a feature of liver- and tumour- infiltrating T cells (Scharping et al. 2016; Swadling et al. 2020; Vardhana et al. 2020; Yu et al. 2020) and may therefore particularly benefit from the mitochondrial rescue (indicated by increased OXPHOS) by ACAT inhibition. I have shown that ACAT inhibition induced cholesterol redistribution resulting in reduced lipid droplets and increased lipid rafts, as well as altered bioenergetics in peripheral T cells. To further understand the increased efficiency of ACAT inhibition in tissue T cells, it would be interesting to evaluate these metabolic and bioenergetic alterations directly on IHL and TIL.

7.2.6 Does ACAT inhibition affect other immune cells besides antiviral/antitumour T cells?

Metabolic requirements vary highly between different immune cells subsets, and it is critical to also consider the effect of ACAT inhibition on cells other than antiviral/antitumour T cells to anticipate the effect of treatment *in vivo*.

ACAT inhibition increased the frequency of Tfh and enhanced the expression of costimulatory molecules associated with Tfh activation and function. The finding that ACAT inhibition had the potential to enhance Tfh function in combination with preliminary data from our group suggesting a direct effect on B cells (Alice Burton, unpublished) raises the possibility that ACAT inhibition might not only enhance immune function during infection, but could also increase responses to vaccination, especially in elderly or immunosuppressed individuals with low vaccination success (Aspinall et al. 2007; Hägg and Religa 2022).

In this study, ACAT inhibition only had minor effects on T_{reg}, NK cells and $\gamma\delta$ T cells. Assessment of metabolic and bioenergetic changes in these cell types compared to effector T cells will help to shed light on why ACAT inhibition did not enhance the function in these immune cell subsets, e.g. a first step would be the confirmation that ACAT inhibition indeed blocked cholesterol esterification with the selected drug concentration in these cell subsets.

It is important to note that the protocol used in this study was optimized to boost CD8⁺ T cell responses and a thorough optimization of culture condition, drug concentration, cell stimulation and functional readouts will be necessary to rule out an effect of ACAT inhibition on these immune cell subsets. It will further be important to not only consider the antitumour/antiviral effects of NK cells and $\gamma\delta$ T cells, but to also assess NK cell-mediated killing of HBV-specific T cells (Peppas et al. 2013) as well as the pro-tumorigenic potential of IL-17-producing $\gamma\delta$ T cells that have higher lipid droplets than IFN γ ⁺ $\gamma\delta$ T cells (Lopes et al. 2021) which might render them more susceptible to ACAT inhibition.

The liver and tumour microenvironment not only shapes effector T cells but also affects T_{reg}, NK and $\gamma\delta$ T cell phenotype and function, and therefore the effect of ACAT inhibition on these immune subsets should further be assessed within IHL and TIL.

ACAT inhibition enhanced lipid rafts on T cells and TCR signalling, suggesting improved immune synapse formation. In this thesis, I have mainly focused on T cells; however, it is also crucial to assess the effect of ACAT inhibition on the other key player in the immune synapse and T cell priming – the APC. Lipids and cholesterol play a critical role in macrophage and DC function and metabolism (see chapter 1.2).

The M2-like macrophage subset is associated with FAO fuelling OXPHOS and CD36-mediated uptake of triacylglycerols (Huang et al. 2014; Russell et al. 2019). Considering the finding that ACAT inhibition increased OXPHOS and CD36 expression on CD8⁺ T cells, it will be important to assess whether this might result in the induction of an M2-like phenotype in macrophages, with the potential of limiting immune responses and promoting tumour progression. On the other hand, the increased IFN γ production by T cells in co-cultures could contribute to a skewing towards an M1-like phenotype. It will be important to study macrophages directly isolated from infected human tissue, as Kupffer cells and alveolar macrophages show enrichment of genes associated with lipid metabolism (Remmerie and Scott 2018).

In SARS-CoV-2, an excessive inflammatory response referred to as ‘cytokine storm’ is associated with severe disease. It will be critical to determine the effect of ACAT inhibition on key contributors to this phenomenon, such as IL-6 producing, airway-infiltrating macrophages recruited by high CXCL10 (Coperchini et al. 2021).

Cholesterol metabolism also plays a role in DC function and lipid droplets alter DC function in a context-dependent manner. In the liver, DC with low lipid content induce immune tolerance and T_{reg} (Ibrahim et al. 2012) whereas in tumours, the accumulation of intracellular lipids in DC is associated with a reduced ability to stimulate T cells and present TAA (Cao et al. 2014; Herber et al. 2010). In line with my findings in T cells, ACAT inhibition would be expected to reduce lipid droplets in DC and therefore alter DC function. Considering the differential effects seen in liver- and tumour-infiltrating DC, it will be important to assess the effect of ACAT inhibition in cells directly isolated from these different environments. Of note, ACAT inhibition has shown a complementary effect with DC vaccines in a murine head and neck cancer model (Chen et al. 2017), supporting the concept that ACAT inhibition can enhance DC function.

TCR-engineered T cells with known specificity and consistent production of effector cytokines would be a suitable system to study the effect of ACAT inhibition on APC priming and allows to further dissect the effect of ACAT inhibition on T cells and a variety of APC including professional (e.g. DC, macrophages and liver-resident Kupffer cells) and non-professional APC (e.g. hepatocytes) with limited capacity of antigen presentation, reflecting the impaired antigen presentation and priming in the liver and tumour.

Taken together, lipid metabolism plays an important role in many components of the innate and adaptive immune system and the effect of ACAT inhibition on different immune cell subsets needs to be further investigated to anticipate treatment responses *in vivo*.

7.2.7 Beyond immunity – what is the effect of ACAT inhibition on other cell types relevant in viral infections and tumours?

Overlapping metabolic requirements of T cells with the tumours and viruses they target are a major challenge of metabolic drug targets (Andrejeva and Rathmell 2017). Identifying metabolic checkpoints that could be targeted to enhance immune responses without simultaneously facilitating cancer progression and viral replication are critical for the development of new therapeutic strategies (O’Sullivan et al. 2019).

Elevated SOAT1 and SOAT2 expression is associated with advanced disease, tumour aggression and poor prognosis in a variety of cancer types (Huang et al. 2017c; Jiang et al. 2019; Lacombe et al. 2020; Li et al. 2016) and inhibition of cholesterol esterification has anticarcinogenic effects in multiple pre-clinical cancer models (Websdale et al. 2022). Importantly, ACAT inhibition also has a direct antitumour effect in several HCC models including tumour-xenograft mouse models of HBV-related HCC (Jiang et al. 2019; Lu et al. 2013).

Viruses are highly reliant on the host cell’s metabolism and cellular cholesterol plays an important role in multiple steps in the life cycle of a wide range of viruses including entry, replication, assembly and egress (Glitscher and Hildt 2021). Cholesteryl ester is a component of HBsAg (Gavilanes et al. 1982) and cellular cholesterol is critical for SARS-CoV-2 particle infectivity, syncytia formation and genome replication (Daniloski et al. 2021; Palacios-Rápalo et al. 2021; Sanders et al. 2021).

This, and the finding that ACAT inhibition blocks HCV assembly (Hu et al. 2017), led to the hypothesis that modulation of cholesterol esterification could also exert a direct antiviral effect against HBV and SARS-CoV-2, and I collaborated with the McKeating Lab (Oxford

University) to further address this hypothesis. Indeed, ACAT inhibition blocked HBV particle genesis to a similar extent as current standard-of-care antivirals (NUC) and, in contrast to NUC, also inhibited HBsAg release *in vitro*, a critical determinant of functional cure (Schmidt et al. 2021). ACAT inhibition also exerted a direct antiviral effect against SARS-CoV-2, including multiple VOC, by inhibiting SARS-CoV-2 entry, fusion and viral replication (*manuscript in revision*).

Taken together, ACAT inhibition constitutes a unique metabolic checkpoint enhancing antiviral/antitumour T cells while also inhibiting the tumours and viruses they target. Assessing ACAT inhibition in cocultures of antigen-specific T cells and cancer cells/virally infected cells as well as in *in vivo* studies are of high interest to further delineate the dual effect of ACAT inhibition.

Finally, in a murine fibrosis model, ACAT1 deficiency induced hepatocyte apoptosis and exaggerated liver fibrosis and inflammation (Tomita et al. 2014), highlighting the necessity of studying the effect of ACAT inhibitors on human non-immune cells in the liver, e.g. hepatocytes and pro-fibrogenic stellate cells, and suggesting a potential benefit of combining ACAT inhibition with drugs with anti-fibrogenic potential such as statins (Pose et al. 2019; Simon et al. 2015; Van Rooyen et al. 2013).

7.2.8 Does ACAT inhibition boost antitumour and antiviral T cells *in vivo*?

In vitro studies on human PBMC are critical to identify new therapeutic targets; however, they have limitations in reflecting the intricate interplay between different (immune) cell subsets and their target cells, the unique microenvironments in the infected organ or tumour, as well as drug pharmacokinetics and direct antiviral/antitumour effects. To address some of these limitations, I showed that ACAT inhibition not only enhanced circulating T cells, but also T cells directly *ex vivo* from human liver and tumour tissue and my collaborators assessed the direct effect of ACAT inhibition on virally infected cells (see section 7.2.7).

The next critical step in evaluating the therapeutic potential of ACAT inhibition will be utilizing animal models. Previous studies have shown that ACAT inhibition enhances

antitumour CD8⁺ T cells in murine melanoma (Yang et al. 2016) and it will be important to confirm these findings for HCC. Murine models for HCC include orthotopic injection of HCC tumour cells (e.g. the murine cell line Hep-53.4 (Leslie et al. 2022) or primary patient-derived tumour xenografts that allow the study of HCC with different underlying diseases (Nazzal et al. 2020)), treatment with liver carcinogens (e.g. diethylnitrosamine (Teoh et al. 2008)) or dietary alterations (e.g. American Life Style Induced Obesity Syndrome (ALIOS) to mimic NASH development (Dowman et al. 2014)).

I have shown for the first time that ACAT inhibition also boosted virus-specific T cells, and a variety of animal models could be utilized to study this effect *in vivo*.

For SARS-CoV-2, the most commonly used animal models are Golden hamsters, that can naturally be infected due to similarities in ACE-2 (Sia et al. 2020), or mice, that are not naturally susceptible to SARS-CoV-2 and need to either be genetically modified to express human ACE-2 (McCray Jr et al. 2007) or need to be infected with a mouse-adapted SARS-CoV-2 with mutations in the spike protein (Gu et al. 2020). Less commonly used animal models include ferrets (Kim et al. 2020) and nonhuman primates such as rhesus macaques (Williamson et al. 2020).

Equally, mice cannot naturally be infected with HBV due to differences in the amino acid sequence of the entry receptor NTCP compared to humans and due to other host factors yet to be determined (Lempp et al. 2017; Ni et al. 2014; Yan et al. 2012). The only animals susceptible to HBV are chimpanzees (Maynard et al. 1972), that are not routinely used due to ethical considerations, and tupaia (Köck et al. 2001), that are only permissive at low levels limiting their applicability (Protzer 2017). Thus, a variety of murine models have been established to study HBV infection, including HBV transgenic mice (Guidotti et al. 1995), HBV transfection models with adenoviral vectors (Sprinzl et al. 2004) and adeno-associated vectors (Dion et al. 2013), and hydrodynamic injection with HBVcircle (Yan et al. 2017) or HBV plasmid DNA (Huang et al. 2012). Woodchuck (Menne and Cote 2007; Summers et al. 1978) and Peking duck models (Marion et al. 2002; Mason et al. 1980) allow natural infection with non-HBV hepadnaviruses and have further been employed to mimic HBV infection including progression to HCC in woodchucks.

The ACAT inhibitors Avasimibe and K-604 have been shown to inhibit ACAT activity in mice (Yang et al. 2016; Yoshinaka et al. 2010) and hamsters (Ikenoya et al. 2007; Nicolosi et al. 1998) rendering them suitable models to assess the immune-boosting effect *in vivo*. Considering the discrepancy between my findings on human CD4⁺ T cells and a previous murine study (Yang et al. 2016), humanized mouse models may be most suitable to predict treatment outcome in patients.

7.3 A glimpse into the future of ACAT inhibition

Overlapping metabolic requirements of T cells with the tumours and viruses they target can drive competition between them, contributing to a failure of an efficient antiviral/antitumour immune response (Andrejeva and Rathmell 2017; Kedia-Mehta and Finlay 2019). Identifying metabolic checkpoints that could be targeted to restrain cancer progression and viral replication without simultaneously dampening immune responses or to boost immune cell function without enhancing cancer cell progression and viral infectivity is a major challenge in the development of new therapeutic strategies (O'Sullivan et al. 2019).

ACAT inhibitors exert direct anticarcinogenic effects restricting the growth of several cancers in pre-clinical models, including early-stage HBV-related HCC (Cruz et al. 2020; Jiang et al. 2019; Lu et al. 2013), and has a direct antiviral effect against HBV (Schmidt et al. 2021) and SARS-CoV-2. I now show for the first time that ACAT inhibition boosted human virus- and tumour-specific CD8⁺ and CD4⁺ T cell responses, supporting the observation that the enzyme ACAT may be an exception to most metabolic checkpoints, with its inhibition constraining tumours and viruses whilst conversely enhancing T cells.

Immune responses in chronic viral infections and cancer are restrained by a multitude of inhibitory mechanism and multipronged therapy targeting different immune checkpoints will likely be necessary to achieve clinical success. ACAT inhibition had a complementary effect with other immunotherapeutic strategies such as PD-1 blockade and TCR-engineered T cells used for adoptive cell transfer, suggesting that a combination of these therapeutic approach could improve response rates.

The cholesterol-rich microenvironment in the liver and many tumours can contribute to T cell exhaustion and checkpoint blockade may be less successful in NASH-related HCC (Chamberlain 1928; Ma et al. 2019; Pfister et al. 2021). My data suggest an enhanced effectivity of ACAT inhibitors in high cholesterol environments and *in vivo* animal studies have shown an accumulation of the ACAT inhibitor Avasimibe in the liver after oral administration (Burnett et al. 1999).

These findings in combination with data showing that SOAT expression is an early feature of rapidly progressing HBV-related HCC (Jiang et al. 2019), makes HBV- and NASH-related HCC compelling settings for first clinical trials. Avasimibe was well-tolerated in clinical trials for atherosclerosis (Llaverías et al. 2003); however, these trials excluded patients with abnormal liver function tests and chronic liver diseases and the safety of ACAT inhibition in this patient cohort needs to be evaluated.

In summary, ACAT is a dual metabolic checkpoint for T cells and the viruses and tumours they target. ACAT inhibition is a promising new immunotherapeutic strategy that can directly restrain tumours and viruses while boosting antitumour/antiviral T cells. This approach is not limited to HBV, SARS-CoV-2 and HCC but will likely be transferrable to other tumours and viruses associated with T cell dysfunction.

8

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