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STUDIES OF INNATE AND ADAPTIVE LYMPHOCYTES IN HUMAN LIVER DISEASES AND VIRAL INFECTIONS

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Studies of innate and adaptive lymphocytes in human liver diseases and viral infections

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„Die Erfindung ist Gegenstand der Kunst, der der Wissenschaft ist die Erkenntnis, die erstere findet oder erfindet die Tatsachen, die andere erklärt sie; die künstlerischen Ideen wurzeln in der Phantasie, die wissenschaftlichen im Verstande."

Justus von Liebig

ABSTRACT

The immune system, including innate and adaptive lymphocytes, is involved in determining the outcome of many human diseases. Two lymphocyte subsets, natural killer (NK) cells and T cells, which are present in both peripheral blood and in tissues, will be further discussed in the context of acute and chronic viral infections and inflammatory liver diseases in this thesis.

NK cells are important players in the early defense against many viral infections. To improve our understanding of human NK cell responses in acute viral infections, we here comprehensively characterized peripheral blood NK cells in patients with acute dengue virus infection, causing dengue fever, from early after symptom debut (**paper I**). In particular, less mature NK cell subsets were robustly activated, and our data further suggested an IL-18-dependent mechanism for driving the observed response. Responding NK cells exhibited a distinct chemokine receptor imprint indicative of skin homing and we could identify a corresponding NK cell subset in the skin from patients with acute infection.

In chronic viral infections, such as chronic hepatitis B (CHB), NK cells and T cells are generally dysfunctional. This dysfunction may contribute to the hosts inability to clear the infection. Nucleos(t)ide analogue (NA) therapy suppresses hepatitis B virus (HBV) replication, but rarely cures CHB. Stopping long-term NA therapy leads to viral relapse and liver inflammation but eventually to functional cure in a fraction of patients. Here, we found that structured NA treatment discontinuation in CHB patients augmented peripheral blood NK cell natural cytotoxicity responses 12 weeks following treatment cessation. This enhanced functionality was associated with liver inflammation, particularly in patients with subsequent functional cure (**paper II**). Furthermore, T cells from the CHB patients achieving a functional cure displayed a more activated phenotype. *In vitro* stimulation with HBV-specific peptides further revealed enhanced peripheral blood T cell functionality that could be boosted with PD-L1 blockade (**paper III**).

In addition to the analyses of lymphocytes in peripheral blood, we investigated the role of an unconventional T cell subset, mucosal invariant T (MAIT) cells, in peripheral blood and bile ducts of patients suffering from primary sclerosing cholangitis. The immunological mechanisms in this rare chronic progressive inflammatory disease of the biliary tract are largely unknown. While MAIT cells were enriched in the bile ducts, numbers and function of circulating MAIT cells were strongly reduced (**paper IV**).

Further analyses of the biliary tract immunological landscape revealed that TcRαβ CD8αβ effector memory T cells represented the dominant intraepithelial immune cell population. These biliary-resident T cells co-expressed gut- and liver-homing receptors and displayed a Th1/Th17 functional profile (**paper V**).

In summary, we could show a significant contribution of NK cells and T cells to the immune response in acute and chronic viral infections, whereas the characterization of biliary-resident T cells has just begun and their function in immunopathogenesis remains to be explored. Altogether, the investigation of immunological mechanisms underlying a variety of human diseases adds to our understanding of human immune cell functionality as well as presents strategies for future treatment development.

LIST OF SCIENTIFIC PAPERS

I. **Christine L. Zimmer**, Martin Cornillet, Martin A. Ivarsson, Nicole Marquardt, Lim Mei Qiu, Yee Sin Leo, David Chie Lye, Paul A. MacAry, Hans-Gustaf Ljunggren, Laura Rivino, and Niklas K. Björkström. NK cells are robustly activated and primed for skin-homing during acute dengue virus infection in humans. *Manuscript*

II. **Christine L. Zimmer***, Franziska Rinker*, Christoph Höner zu Siederdissen, Michael P. Manns, Heiner Wedemeyer, Markus Cornberg, and Niklas K. Björkström. Increased NK Cell Function After Cessation of Long-Term Nucleos(t)ide Analogue Treatment in Chronic Hepatitis B Is Associated With Liver Damage and HBsAg Loss. *J Infect Dis*. 2018; 217(10):1656–1666. *contributed equally

III. Franziska Rinker*, **Christine L. Zimmer***, Christoph Höner zu Siederdissen, Michael P. Manns, Anke R.M. Kraft, Heiner Wedemeyer, Niklas K. Björkström, and Markus Cornberg. Hepatitis B virus-specific T cell responses after stopping nucleos(t)ide analogue therapy in HBeAg-negative chronic hepatitis B. *J Hep*. 2018; 69(3):584–593. *contributed equally

IV. Erik von Seth, **Christine L. Zimmer**, Marcus Reuterwall-Hansson, Ammar Barakat, Urban Arnelo, Annika Bergquist, Martin A. Ivarsson, and Niklas K. Björkström. Primary sclerosing cholangitis leads to dysfunction and loss of MAIT cells. *Eur J Immunol*. 2018; 48(12):1997–2004.

V. **Christine L. Zimmer**, Erik von Seth, Otto Strauss, Lena Berglin, Laura Hertwig, Marcus Hansson, Urban Arnelo, Annika Bergquist, and Niklas K. Björkström. Comprehensive mapping of the human biliary tree immunological landscape in health and inflammation. *Manuscript*

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADE	Antibody-dependent enhancement
AHR	Aryl hydrocarbon receptor
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Antimicrobial peptide
APC	Antigen-presenting cell
ATF2	Activating transcription factor-2
BEC	Biliary epithelial cell
CCA	Cholangiocarcinoma
cccDNA	Covalently closed circular DNA
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CHB	Chronic hepatitis B
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T cell lymphocyte-associated molecule-4
CXCR	C-X-C chemokine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DAP	DNAX adaptor molecule
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DENV	Dengue virus
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule 1
DSS	Dengue shock syndrome
EASL	European Association for the Study of the Liver
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
Eomes	Eomesodermin
ER	Endoplasmic reticulum
ERCP	Endoscopic cholangiopancreatography

FOXO	Forkhead transcription factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBcAg	Hepatits c antigen
HBeAg	Hepatits e antigen
HBsAg	Hepatitis s antigen
HBx	Hepatitis x protein
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hepatic stellate cell
HSPG	Heperan sulfate proteoglycan
HSV	Herpes simplex virus
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KC	Kupffer cell
KIR	Killer cell immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor G1
LFA-1	Lymphocyte function-associated antigen-1
LHB	Large hepatitis B surface protein
LLT-1	Lectin-like transcript-1
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
MadCAM-1	Mucosal addressin cell adhesion molecule-1
MAIT cell	Mucosal-associated invariant T cell

MCMV	Murine cytomegalovirus
MDA-5	Melanoma differentiation-associated gene 5
MHB	Middle hepatitis B surface protein
MHC	Major histocompatibility complex
MICA/B	MHC class I polypeptide-related sequence A/B
MIP-1ß	Macrophage inflammatory protein-1beta
MR1	MHC-related protein 1
NA	Nucleos(t)ide analogue
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NLR	NOD-like receptor
NTCP	Sodium taurocholate co-transporting polypeptide
PALT	Portal tract-associated lymphoid tissue
PAMP	Pathogen-associated molecular pattern
PBC	Primary biliary cholangitis
PBMC	Peripheral blood mononuclear cell
pegIFNα	Pegylated interferon alfa
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PKB (AKT)	Protein kinase B
PLZF	Promyelocytic leukemia zinc finger
PMA	Phorbol 12-myristate-13-acetate
Pol	Polymerase
PRR	Pattern-recognition receptor
PSC	Primary sclerosing cholangitis
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RORγt	Retinoid-related orphan receptor γt
SHB	Small hepatitis B surface protein
SNE	Stochastic neighbor embedding

STAT	Signal transducer and activator of transcription
TBE	Tick-borne encephalitis
T-bet (TBX21)	T-box transcription factor 21
TBEV	Tick-borne encephalitis virus
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory RA T cell
TGF-ß	Transforming growth factor-beta
Th	T helper
TIM-3	T-cell immunoglobulin and mucin domain-containing protein-3
TLR	Toll-like receptor
T_{naive}	Naïve T cell
TNF	Tumor-necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg cell	Regulatory T cell
T_{RM}	Tissue-resident memory T cell
UC	Ulcerative colitis
ULBP	UL16 binding protein
VEGF	Vascular endothelial growth factor
YFV	Yellow fever virus

1 INTRODUCTION

1.1 THE HUMAN IMMUNE SYSTEM

The body has various defense mechanisms against infections that together build up the human immune system. The immune system protects against viruses, bacteria, fungi and parasites and is comprised of various cellular effectors (leukocytes) and the humoral arm. After birth, most leukocytes are derived from pluripotent hematopoietic stem cells of the bone marrow that give rise to a common progenitor of the lymphoid and myeloid lineage. The immune system can further be divided into the innate and the adaptive immune system. In general, the innate immune system provides immediate protection in a nonspecific way. Germline-encoded receptors recognize unique microorganism-associated conserved features. In contrast, the adaptive immune response is initiated later, performs an antigen specific-recognition mediated by re-arranged receptors from gene segments via somatic recombination, and is able to create immunological memory. The interplay of both the innate and adaptive immune system is crucial for mounting an effective immune response, but also for the discrimination of self from non-self (1-3).

1.1.1 The innate immune system

Epithelial cells, such as in the skin and the gastrointestinal tract, are considered to be the first line of defense. Epithelia are physical barriers, being able to secrete mucus and are equipped with other chemical and anti-microbial properties. Furthermore, they are populated with the common microbiota that shapes the immune system, but also competes with pathogens for space and nutrients (4). Once an infection has established, the infectious agent may be contained locally or spread throughout the body.

Microorganisms are recognized by pathogen-recognition receptors (PRRs) expressed by a variety of cells, including epithelial cells, tissue-resident mast cells, macrophages and dendritic cells (DCs). PRRs detect pathogen-associated molecular patterns (PAMPs) that are shared by several classes of microorganisms. Toll-like receptors (TLRs) are a well-characterized example. These receptors are located on the cell surface or intracellularly and recognize viral nucleic acid and bacterial products, for example lipopolysaccharide (LPS). TLR activation induces pro-inflammatory cytokine production, including tumor-necrosis factor (TNF), interleukin (IL)-1β and IL-6, which initiate inflammation. IL-1β and TNF activate the endothelium of local capillaries, allowing recruitment of leukocytes to the site of infection as well as enabling serum proteins, such as complement proteins, to enter the tissue. Activation of the complement system causes proteolytic cleavage of complement proteins, leading to the covalent attachment of the cleavage product C3b on the target cell. C3b acts as an opsonin and initiates the creation of the membrane attack complex, which facilitates a pore causing cell lysis. Cleavage products (C3a, C5a) act as anaphylatoxins and promote inflammation (3). Moreover, accumulation of tissue factor initiates the local coagulation cascade to avoid pathogen dissemination. Other PRRs are located intracellularly. These include receptors, such as NOD-like receptors (NLRs) that are involved in inflammasome activation, thereby initiating caspase-mediated activation of IL-1 family cytokines (IL-1β, IL-18, IL-33), but also sensors of double-stranded cytoplasmic viral nucleic acids, retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). The

activation of RIG-I and MDA5 causes type I interferon (IFNα, IFNβ) production and subsequent expression of IFN-inducible genes with products triggering an anti-viral state (1, 2). Local immune cells, including innate lymphoid cells (ILCs, NK cells/ILC1-ILC3), are activated early by the cytokine milieu created and shape subsequent immune responses (5). Another mechanism in innate immunity is phagocytosis, which plays an important role in pathogen killing with the most efficient cells being neutrophils and macrophages mostly fighting infections with fungi/extracellular bacteria and intracellular bacteria, respectively. Multicellular parasites get eliminated by tissue-resident mast cells releasing mediators, such as histamine, serine proteases, and various enzymes, or by eosinophils and basophils that get recruited from the circulation. DCs, which are professional antigen presenting cells (APCs), play an important role in bridging innate and adaptive immune responses. They survey peripheral tissues, detecting and phagocytosing pathogens, which are processed and may lead to DC activation and cytokine production. Activated DCs then migrate to local secondary lymphoid tissue, and initiate adaptive immune responses (1, 3).

1.1.2 The adaptive immune system

Adaptive immune responses are classically initiated in a well-coordinated fashion in secondary lymphoid tissues where naïve T cells and B cells reside in distinct areas and eventually encounter their cognate antigen (6). Peptide antigens are displayed by APCs in complex with major histocompatibility complex (MHC) class I or II (human leukocyte antigen (HLA) in humans) to T cells. Activation is followed by clonal expansion and differentiation, generating antigen-specific effector T cells and antibody secreting B cells. B cell responses are initiated in a T cell-dependent or independent fashion and accompanied by class-switch recombination in B cell follicles and somatic hypermutation in germinal centers, resulting in generation of memory B cells or long-lived plasma cells (6). T and B cells egress via efferent lymphatics before returning into the blood stream from where they can enter the site of infection. Antibodies neutralize and opsonize pathogens, which in turn promotes phagocytosis and complement activation. Fc receptor-bearing innate effector cells can recognize antibody-coated cells, release microbicidal factors or directly kill the opsonized pathogen via antibody-dependent cellular cytotoxicity (ADCC), which is mediated by the effector cells of the innate immune system, linking the innate and adaptive immune response. Effector T cells can either be cytolytic and/or release cytokines of different action, affecting the innate immune response. The resolution of an infection is followed by a contraction phase during which most effector lymphocytes die. However, a small number of memory cells persist for years, being poised for an accelerated adaptive immune response in case of subsequent encounter with the same pathogen (1, 3).

1.2 HUMAN LYMPHOCYTE IMMUNOLOGY

1.2.1 Natural killer cells

Human natural killer (NK) cells belong to the group of type 1 ILCs that commonly lack somatically recombined antigen receptors, produce IFNγ and require the transcription factor T-bet for their development (7). NK cells were discovered in 1975 by Rolf Kiessling as well as Ronald Herbermann and colleagues. NK cells were initially described as lymphocytes with naturally occurring killer activity, being able to eliminate their target cells without prior

sensitization (8-10). Closer insights into the mechanism involved in this natural killer activity were proposed by Klas Kärre *et al*. in 1986 (11, 12). He suggested that, as opposed to T cells, NK cells recognize and kill their targets in the absence of MHC class I molecules, a mechanism termed the "missing-self hypothesis" (11-13). MHC class I molecules may be lost upon cellular stress or viral infection, but are expressed by most healthy nucleated cells in steady-state conditions, ensuring tolerance to self.

Today, NK cells are well known for their important role in the early response against viral infections and malignant transformed cells. Besides their killing capacity, NK cells exhibit an immunoregulatory function by producing cytokines and chemokines (14, 15). NK cells comprise approximately 10% of all lymphocytes in peripheral blood, but are also present with varying frequencies in many peripheral tissues, such as liver, gut, and skin (16). Most studies on tissue-specific NK cells have been performed in mice. However, significant differences in NK cells between humans and mice limit the extrapolation of murine data to the human system. In detail, human NK cells are defined by the lack of CD3 and expression of CD56. NK cells are divided into two main subsets based on the differential surface expression of CD56 and the Fcγ receptor IIIA (CD16): $CD56^{bright}$ and $CD56^{dim}$ NK cells (7). Since CD56 is not expressed on murine NK cells, NK1.1 (CD161) is used instead for the identification of murine NK cells, and CD27 and CD11b for discrimination of distinct NK cell subsets in mice.

NK cell subsets differ in phenotypic and functional properties both in mice and in humans, and how they are distributed throughout the human body. Human $CD56^{dim}$ NK cells comprise roughly 90% of peripheral blood NK cells, express high levels of CD16 and are, at steady state, described to be more cytolytic and target-cell responsive (14, 15). In contrast, $CD56^{bright}$ $CD16^{dim}$ or $CD56^{bright}$ $CD16^{neg}$ NK cells are strongly responsive to inflammatory cytokine-stimulation and mainly possess immunoregulatory functions (17). However, stimulation can rapidly induce lytic properties in $CD56^{bright}$ NK cells and cytokine/chemokine-secreting functionality in $CD56^{dim}$ NK cells (14, 15).

1.2.1.1 Regulation of NK cell Functionality

NK cell activation and function is regulated by signal integration of activating and inhibitory receptors (14, 18). In this process, varying signal strength can impact the type and multiplicity of the NK cell response. Moreover, NK cell effector responses are underlying a hierarchical order. Stimulatory requirements for $CD56^{bright}$ and $CD56^{dim}$ NK cells vary based on different properties in regard to their cytokine responsiveness and receptor expression (15, 19). Numerous NK cell activating and inhibitory receptors and their respective ligands have been identified (14, 18, 20). In the following, the most relevant ones for this thesis are highlighted (**Table 1**).

Table 1: NK cell receptor-ligand pairs

Receptors	Ligands
Activating	
NKG2D	MICA, MICB, ULBP-1-4
DNAM-1	PVR (CD155), Nectin-2 (CD112)
NKp30	B7-H6, HSPG
NKp44	Viral hemagglutinin
NKp46	Viral hemagglutinin
CD16	IgG
CD94/NKG2C	HLA-E
KIR2DS1	HLA-C2
KIR2DS2	HLA-C1
KIR2DS4	HLA-A11 (some HLA-C, HLA-F)
KIR3DS1	HLA-Bw4, HLA-F
Inhibitory	
KIR2DL1	HLA-C2 (lysine at position 80)
KIR2DL2/3	HLA-C1 (asparagine at position 80)
KIR3DL1	HLA-Bw4
KIR3DL2	HLA-A3, HLA-A11, HLA-F
CD94/NKG2A	HLA-E
Siglec-7	Sialic acid
CD161	LLT-1

Human NK cells identify "self" cells via recognition of the HLA class I molecules (HLA-A, -B, -C) by killer cell immunoglobulin-like receptors (KIRs) with recent evidence of an interaction of KIRs with the non-classical HLA molecules HLA-G and -F (21-24). As mentioned above, virus-infected or transformed cells often downregulate HLA class I molecules to avoid T cell recognition, which results in the lack of the inhibitory signals suppressing the NK cell (18). The nomenclature of KIRs is based on their structure with a specified number of extracellular Ig-like domains and information about the cytoplasmic domain that can be long (L) or short (S). Inhibitory signals are transmitted via immunoreceptor tyrosine-based inhibition motifs (ITIMs) located directly in the long intracellular domain, and activating signals via adaptor molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs) that are associated with short intracellular domains. After receptor-crosslinking, ITIMs get phosphorylated and recruit tyrosine-phosphatases that in turn shut down activation by dephosphorylation of activating adaptor molecules (21). KIRs have HLA class I allotypes as ligands with the most important for this thesis being listed in Table 1. The KIR locus is highly complex, being highly polygenic and polymorphic (25), with KIR-S genes being interspersed with KIR-L genes. Based on the presence of certain genes, two KIR haplotypes have been defined as group A and B. Haplotype A only consists of inhibitory genes except *KIR2DS4*, whereas haplotype B contains combinations of inhibitory and activating genes (21, 26). Moreover, KIRs are expressed stochastically and thereby contribute to NK cell diversity (26).

NKG2-receptors are highly conserved C-type lectin receptors with seven members of this receptor family. Both NKG2A and NKG2C form heterodimers with CD94 and interact with the non-classical HLA-E molecule (27). NKG2A has an inhibitory function with an ITIM

motif in its cytoplasmatic tail (28), whereas NKG2C is an activating receptor, being associated with DAP12 containing ITAMs (29). NKG2D is another member of the same family exhibiting an activating function. As opposed to NKG2A and NKG2C, NKG2D forms a homodimer and binds to the stress-induced ligands MHC class I polypeptide-related sequence (MIC)A/B and UL16 binding protein (ULBP)1-4 (30, 31).

Similarly, another NK cell activating receptor, DNAX accessory molecule (DNAM)-1 (CD226), has been shown to bind to stress-induced ligands. DNAM-1 interacts with PVR (CD155) and Nectin-2 (CD112) (32). The expression of DNAM-1 is coordinated with the conformational change of lymphocyte function-associated antigen (LFA-1) and both DNAM-1 and LFA-1 co-localize at the immune synapse, contributing to enhanced effector functions (33).

Natural cytotoxicity receptors (NCRs) include, but are not limited to, NKp30, NKp44, and NKp46. On human NK cells at steady state, NKp30 and NKp46 are widely expressed, in contrast to NKp44 that is expressed on activated NK cells or non-NK cell ILCs. NCRs are part of the Ig superfamily and associate with different intracellular adaptor molecules (CD3ζ, DAP-12, FcεRIγ) (34). Their ligands are not well-defined, but have been suggested to be tumor-associated molecules (e.g. B7-H6) and viral hemagglutinins, the latter recognized by NKp44 and NKp46 (35, 36).

TNF-related apoptosis-inducing ligand (TRAIL) belongs to the TNF family and represents together with FasL (binding Fas/CD95) a death receptor that induces apoptosis and is upregulated upon exposure to type I IFN. TRAIL forms homodimers and binds to DR4 (TRAILR1), DR5 (TRAILR2) and soluble TRAIL receptors (decoy receptors). Apoptosis is induced via a caspase-8-dependent caspase cascade (37).

CD16 (FcγRIIIa) is a NK cell activating receptor highly expressed on $CD56^{dim}$ NK cells, mediating ADCC. CD16 signals after binding and crosslinking the Fc part of IgG antibodies via its intracellular tail that is associated with the FcεRIγ and CD3ζ adaptor molecules containing ITAMs (38).

Additionally, the cytokine environment and interactions with other immune cells such as T cells, DCs, and macrophages influence the quality of the NK cell response (39, 40). Cytokines produced by other immune and non-immune cells can directly activate NK cells. The main environmental activating cues NK cells respond to are type I IFNs, IL-2, IL-12, IL-15, and IL-18 (14). Type I IFNs have been shown to enhance cell-mediated cytotoxicity, whereas IL-12 and IL-18 enhance IFN-γ production in a synergistic manner. IL-2 and IL-15 instead primarily induce proliferation and promote survival of NK cells. In contrast to NK cell-activating pro-inflammatory cytokines, both IL-10 and transforming growth factor (TGF)-β that are present especially in the tumor environment but also healthy peripheral tissues, suppress NK cell activity and effector responses (14).

NK cell effector responses include direct killing of target cells via ADCC, death receptor mediated cytolysis (FasL- or TRAIL-mediated) or the release of cytotoxic granules (perforin and granzymes). Perforin inserts a pore into the target cell membrane, allowing granzymes to enter and cleave intracellular proteins, ultimately leading to apoptosis of the target cell (37).

ADCC allows NK cells to directly kill antibody-tagged cells, but they can also modulate the inflammation-associated immune response by directly killing APCs and T cells (14). Apart from being cytotoxic, NK cells have the capacity to release cytokines, chemokines and growth factors, including IFNγ, TNF, CC-chemokine ligand 3, 4 and 5 (macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES, respectively), and granulocyte–macrophage colony-stimulating factor (GM-CSF) (15). Hereby, effector responses follow a temporal hierarchy with degranulation and chemokine secretion being early features followed by cytokine release at later time points (15). Thus, NK cells integrate both cytokine priming and synergistic activation through receptor co-engagement, which ultimately determines the response as immune-regulators and/or direct effectors (15, 38).

1.2.1.2 NK cell differentiation and education

NK cells arise from a common lymphoid progenitor. The transcription factors inhibitor of DNA binding 2 (ID2) and E4BP4 have been proposed to specify NK cell lineage precursors, and IL-15 is known to be important for NK cell development (41). During maturation, NK cell progenitors lose CD34 and CD117 (c-kit) and acquire CD94, CD16, and KIRs that distinguishing them from other ILC family members (42). A recent attempt trying to identify a NK cell-restricted precursor that separates them from $CD127^+$ ILCs has been made (43). In fetal tissue, neonatal cord blood, and in adult tissues such a precursor ($CD34^+CD38^+CD123^-CD45RA^+CD7^+CD10^+CD127^-$) was identified lacking lineage markers for T and B cells as well as myeloid cells. These precursor NK cells have the capacity to develop into T-bet^+ Eomesodermin $(Eomes)^+$ NK cells but not $CD127^+$ ILCs *in vitro* (43). Eomes and T-bet are T-box family transcription factors known for their complementary role during NK cell development. Higher levels of Eomes are associated with less mature NK cells and higher levels of T-bet are associated with more terminally differentiated NK cells (44). Several studies indicate that $CD56^{bright}$ NK cells differentiate into $CD56^{dim}$ NK cells in a final step during maturation (45-47), a model of differentiation that is challenged by results from macaques (48). During maturation, a process termed NK cell education (or licensing), greatly impacts NK cell functionality. This process requires the recognition of HLA class I ligands by their respective receptors (KIRs or NKG2A, that are mostly inhibitory except KIR2DS1) (49-51). NK cell licensing arms the maturating cells for full functionality, whereas unlicensed NK cells are rendered hypofunctional. This preserves self-tolerance against normal cells in the missing self-setting. The inhibitory signal strength (based on the number of KIRs and the allele) during education quantitatively controls NK cell effector functions, thereby fine-tuning NK cell responses (23, 52). In addition to education, NK cells undergo a differentiation process accompanied by phenotypical changes as indicated by the altered surface expression of CD57, NKG2A, and KIRs as well as changes in the functional capacity. $CD56^{bright}$ NK cells express NKG2A, which is lost during differentiation while CD57 is expressed on more terminally differentiated NK cells. In addition, KIRs are sequentially acquired during the differentiation process, contributing to education, which occurs in parallel to differentiation. On a functional level, less differentiated NK cells respond well to cytokines and proliferate, while more differentiated NK cells gradually lose this capacity and instead become more cytotoxic (47) (**Figure 1**).

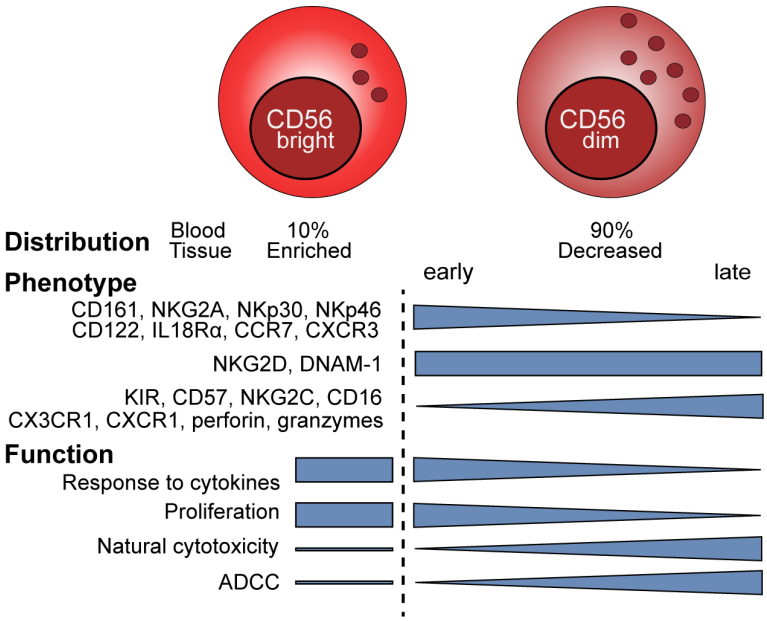

Figure 1: NK cell differentiation is associated with phenotypical and functional changes. Adapted from Björkström *et al*. (47), Long *et al*. (38), and Bernardini *et al*. (53)

1.2.2 T cells

1.2.2.1 Conventional T cells

T cells are adaptive lymphocytes that develop in the thymus. The vast majority of T cells uses the αβ chains for T cell receptor (TCR) assembly, whereas a minority uses the γδ chains (3). During maturation, T cells acquire their unique TCR via somatic V(D)J recombination using recombination-activating gene (RAG)1 and RAG2. The TCR associates with the CD3 complex (γε, δε heterodimers and the ζ homodimer chain) that contains ITAMs for signal transduction (3). The expression of a functional TCR is accompanied by the expression of the co-receptors CD4 and CD8. A selection process, termed positive selection, ensures that only MHC-restricted T cells survive that bind to the MHC molecule with sufficient affinity, but do not recognize self-antigens (negative selection). T cells become CD8 or CD4 single positive and exit the thymus. The decision which co-receptor is expressed depends on the type of MHC molecule the T cell interacts with. T cells binding to MHC class I or II become cytotoxic T cells that express CD8 or T helper cells (Th0) expressing CD4, respectively (54).

Peptides presented in complex with MHC molecules results in functional priming of T cells (signal 1). However, co-stimulatory signals (signal 2) are required for T cells to become fully activated, such as CD28 expressed on T cells binding to B7.1 (CD80) and B7.2 (CD86) expressed by APCs. Lack of co-stimulation may lead to T cell apoptosis or anergy (T cell hyporesponsiveness), a mechanism to ensure immune tolerance (55).

13-24 amino acids long peptides from extracellular pathogens are presented to naïve $CD4^+$ T helper (Th) cells in secondary lymphoid organs. Here, T cells receive cues from APCs in form of polarizing cytokines (signal 3), which direct T cell differentiation into subsets (**Figure 2**) (56). Th cells orchestrate immune responses by secreting cytokines and chemokines, provide B cell help for antibody production, and recruit other immune cells to the site of infection (56). A Th cell has a characteristic cytokine profile, resulting in a subset-

specific effector response. Th1 cells are important for cell-mediated immune responses to intracellular pathogens and mainly secrete IFNγ. IFNγ is a potent inflammatory cytokine with many functions including MHC and TLR upregulation, increased phagocytosis and macrophage activation, IgG antibody class switch, and chemokine secretion. Th2 cells are involved in the defence against multicellular parasites and contribute to the development of allergies. Th2 responses are characterized by the key cytokines IL-4, IL-5, and IL-13, which mediate antibody class switch (IgG1, IgE), DC and macrophage maturation, as well as mucus production. Th17 cells classically secrete IL-17 and IL-22 involved in the response towards extracellular bacteria and fungi by attracting neutrophils and inducing inflammatory mediators and antimicrobial peptides (AMPs). Regulatory T (Treg) cells have a regulatory function and secrete anti-inflammatory cytokines, such as IL-10 and TGF-β. More recently, Th22 and Th9 cell subsets were identified by the production of IL-22 and IL-9, respectively. Th cell subsets are induced by key cytokines that signal via signal transducer and activator of transcription (STAT) proteins. Moreover, specific transcription factors define the respective Th cell-subset (**Figure 2**) (57, 58).

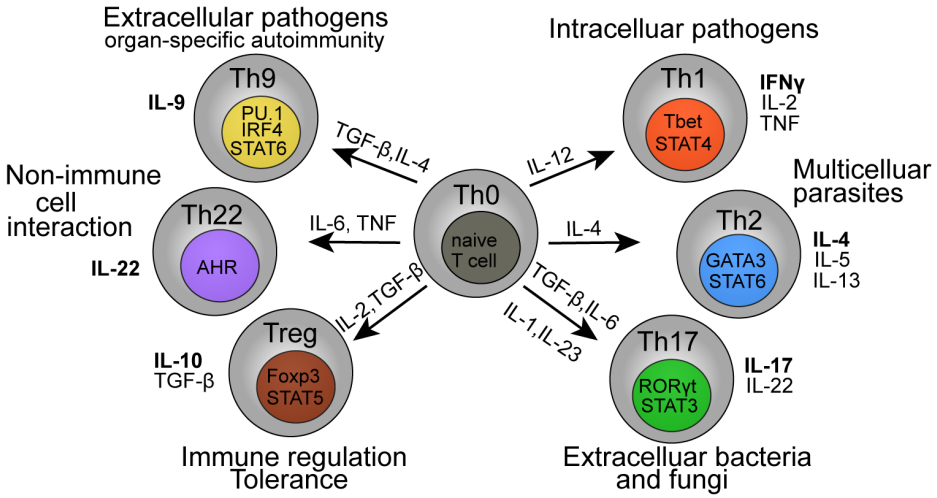

Figure 2: **T helper cell subset differentiation is driven by polarizing cues and results in subset specific effector responses**. Adapted and modified from Zhu et al. (56), Raphael et al. (57), Schmitt et al. (58)

$CD8^+$ T cells recognize 8-10 amino acid long peptides that are derived from intracellular pathogens or are cross-presented by DCs. Cross-presentation is the re-direction of exogenous antigens into the MHC class I pathway, a process for inducing immune responses against tumors and non-APC-infecting viruses. $CD4^+$ T cells may provide help to induce a robust $CD8^+$ T cell response, either indirectly via CD40-CD40L interaction for functional maturation of DCs or by a direct interaction between the $CD4^+$ and the $CD8^+$ T cell. In infections with TLR engagement that induce a strong type I IFN response, $CD4^+$ T cell help may be dispensable. Upon activation, $CD8^+$ T cells expand strongly and differentiate into cytolytic effector T cells, which can kill their targets directly via the release of cytotoxic granules (granzymes and perforin) and death receptors (TRAIL, FASL) or indirectly by the secretion of cytokines and chemokines, such as IFNγ, TNF, IL-2 and MIP-1β (59-61).

After pathogen clearance, most T cells die in the contraction phase. However, a small fraction of the T cells survives and is maintained as long-lived memory cells, a process that is

accompanied by epigenetic reprogramming and changes in the metabolism of the cell. Immunological memory is the ability to quickly mount efficient recall responses upon encountering with the same pathogen (59, 62, 63). Despite being extensively studied, the differentiation of memory T cells is not fully understood (63). Murine models demonstrated that $CD8^+$ T cell effector functions and memory formation are regulated by the transcription factors T-bet and Eomes (64, 65). Accumulating evidence suggests that the $CD8^+$ T cell long-term fate is determined by the expression ratio of the transcription factors T-bet and Eomes that drive effector functions and memory development, respectively (63). Unlike for naïve T cells, TCR signalling is largely insignificant for memory T cell homeostasis, which is mainly dependent on IL-7 and IL-15 (66). Memory T cells are divided into functionally different subsets with distinct homing capacity and effector functions: central memory (T_{CM}), effector memory (T_{EM}) (67), terminally differentiated effector memory (T_{EMRA}), and tissue-resident memory (T_{RM}) cells. T_{RM} cells are located in mucosal tissue and do not re-circulate (61, 62, 68). The expression of C-C chemokine receptor type (CCR)7 and CD45RA (67), or alternatively CD62L and CD45RO, is commonly used to distinguish between memory populations (T_{naive}: $CCR7^+$ ($CD62L^+$) $CD45RA^+$ ($CD45RO^-$), T_{CM}: $CCR7^+$ ($CD62L^+$) $CD45RA^-$ ($CD45RO^+$), T_{EM}: $CCR7^-$ ($CD62L^-$) $CD45RA^-$ ($CD45RO^+$), T_{EMRA}: $CCR7^-$ ($CD62L^-$) $CD45RA^+$ ($CD45RO^-$)). T_{CM} primarily recirculate through secondary lymphoid organs, have the greatest proliferative potential, mainly produce IL-2, and can rapidly expand and differentiate upon re-challenge due to their high sensitivity to antigen stimulation and their reduced requisite for co-stimulation (61, 69). Similar to naïve T cells, T_{CM} express CCR7 and CD27/CD28, which are important for secondary lymphoid tissue entry and co-stimulation. T_{EM}/T_{EMRA} circulate between secondary lymphoid organs and peripheral inflamed tissues with the capacity to rapidly mount effector responses. $CD8^+$ T_{EMRA} are most well equipped with perforin followed by $CD8^+$ T_{EM}. In blood, T_{CM} are predominantly $CD4^+$ and T_{EM} $CD8^+$ T cells, whereas the subset ratio in the periphery is tissue-dependent with a T_{EM} dominance in peripheral organs, such as lung, liver, and gut (61).

1.2.2.2 Mucosal associated invariant T cells

T cells that do not recognize classical peptide antigens are considered unconventional, for example CD1d-restricted NKT cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells. In contrast to conventional TCRαβ T cells, these unconventional T cells are poised for rapid effector responses with a tendency to localize in non-lymphoid tissue (70). MAIT cells are innate-like T lymphocytes that primarily contribute to anti-bacterial immunity and might play a role in sterile inflammation and cancer (71). In humans, they represent a minor fraction of total blood T cells (~5%) but are enriched at mucosal sites, particularly the liver where they account for up to 30% of all intrahepatic T cells (72-74). MAIT cells are characterized by the expression of the semi-invariant TCR (TCR-Vα7.2-Jα33 (Jα12/20)) that recognizes the non-polymorphic MHC-related protein 1 (MR1). Unlike the Vα chain, the Vβ chain can vary (but is mostly Vβ2 and Vβ13.2) and contributes to the functional outcome of the TCR-MR1 interaction (75). By flow cytometry, MAIT cells can be defined as $CD3^+$ TCR $Vα7.2^+$ $CD161^+$ ($CD4^-$) lymphocytes that are mainly $CD8^+CD4^-$ or double negative. Alternatively, MAIT cells can be identified using a fluorochrome-coupled MR1-tetramer (76). MR1 binds unstable pyrimidine intermediates derived from a biosynthetic precursor of riboflavin (vitamin B metabolites), a biosynthesis pathway that exists in many bacteria, but not in

humans (77, 78). The bacterial ligands for MAIT cells are produced by a variety of bacteria that contain the riboflavin synthetic pathway, such as *Escherichia (E.) coli*, *Salmonella*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* as well as some yeast species, for example *Candida albicans*. Human peripheral MAIT cells mostly show a T_{EM} phenotype, express the transcription factors promyelocytic leukemia zinc finger (PLZF), T-bet, retinoid-related orphan receptor (ROR) γt, high levels of IL-18Rα and the chemokine-receptors CCR2, CCR5, C-X-C chemokine receptor type (CXCR)6, CCR6, and CCR9 for tissue-homing. Besides stimulation via the TCR, the cells can also get readily activated by innate cytokines such as IL-12 and IL-18, leading to proliferation and IFNγ production (71, 72, 79). Additionally, the MAIT cell response can be potentiated by co-stimulation via CD28 (80). MAIT cells also produce other pro-inflammatory cytokines including TNF and IL-17, that may play a role in inflammatory diseases, for example inflammatory bowel disease (IBD), psoriasis, or rheumatoid arthritis (RA) (71, 79). Interestingly, human liver MAIT cells express high levels of IL-7R, and IL-7 produced in the liver potentiates TCR-dependent secretion of Th1 cytokines and IL-17 (74). Activated MAIT cells have the capacity to kill target cells via the release of cytotoxic granules containing granzyme B and perforin (81-83). Thus, MAIT cells can directly act anti-bacterially via the production of cytokines or by elimination of infected cells, but they can also recruit and stimulate other immune cells, for example neutrophils via IL-17 release (71). Notably, accumulating evidence suggests that MAIT cell effector functions vary in different tissue locations (74, 84-86) and can be heterogeneous in their response to different microbes (75).

1.3 LYMPHOCYTE HOMING TO PERIPHERAL TISSUES

The location of leukocytes in tissue plays a fundamental role in the immune responses. During inflammation, immune cells get recruited to peripheral organs via a multistep extravasation cascade involving rolling, firm adhesion, and chemotactic signals that are mediated by selectins, integrins, and chemokines, respectively. Classical examples for corresponding interaction are the inflammation-induced selectins E- and P- selectin or the interaction of LFA-1 (CD11a/CD18) binding its ligand intercellular adhesion molecule-1 (ICAM-1) (87, 88). Chemokines are chemotactic cytokines that bind to their respective receptors on leukocytes and guide them to distinct anatomical locations during homeostasis and inflammation, a process referred to as homing (89). Chemokine receptors are 7-transmembrane G-protein-coupled receptors that are differentially expressed on leukocytes (90). Chemokine binding results in a conformational change of the receptor, initiating intracellular signaling events that induce cell polarization, migration and adhesion (53). This is followed by the localization to a specific tissue-microenvironment based on a network of chemokine gradients present within the tissue (53).

As innate sentinels, NK cells are widely distributed throughout the body and $CD56^{bright}$ NK cells in particular are enriched in most human tissues. NK cells are recruited in an organ-specific manner, exhibiting tissue-specific functions (16, 91). Intriguingly, chemokine receptors are differently expressed on $CD56^{bright}$ and $CD56^{dim}$ NK cells and chemokine receptor expression is also modified throughout NK cell differentiation and during NK cell activation (47, 53). $CD56^{bright}$ NK cells express higher levels of CCR7, CXCR3, CCR5, CCR2, and CXCR4 that is also reflected by their presence in for example lymph nodes, liver, skin, and bone marrow, whereas $CD56^{dim}$ NK cells dominantly express CX3CR1 and

CXCR1 (53). Most studies addressing tissue-resident NK cells have been performed in mouse models with the help of knockout strains and blocking antibodies. In humans, studies describing chemokine-chemokine receptor interaction at inflammatory sites have been informative to understand NK cell migratory properties, but our knowledge of NK cell homing is incomplete (87) (**Table 2**).

1.4 TISSUE-RESIDENT LYMPHOCYTES

Recent studies have revealed the importance of non-circulating populations residing in peripheral tissues and highlighted some the differences between tissue-resident and circulating populations (92-99). Tissue-resident lymphocytes mediate local immune responses and include ILCs, T_{RM} cells, unconventional T cells, and intra-epithelial lymphocytes (IELs) (100-102). Phenotypically, tissue-resident cells can be identified by expression of CD69, CD49a (forms a heterodimer with β1 integrin (CD29)) that binds to type IV collagen, and CD103 (forms a heterodimer with β7 integrin) binding E-cadherin on epithelial cells (**Figure 3**). The expression of CD103 is induced by TGF-β and has been shown to be important for survival, retention, and tissue localization of lymphocytes (16, 100). Studies in mice have shown that the differentiation into tissue-resident T cells is controlled by a network of transcription factors (103). Kruppel-like factor 2 (KLF2), which drives the expression of L-selectin (CD62L) as well as S1PR1 (sphingosine 1-phosphate receptor 1) that are both involved in tissue egress, is down-regulated (104). Similarly, the expression of CD69 counteracts the expression of S1PR1 (105). Both T-bet and Eomes have been described to be down-regulated with residual T-bet expression for IL-15 responsiveness (106). Moreover, Homolog of Blimp-1 (Hobit) and Blimp-1 cooperatively repress genes for tissue-egress (107). A recent study investigating the human core transcriptional signature of T_{RM} cells in lung and spleen confirmed an up-regulation of the phenotypical marker CD69, CD49a, and CD103 in addition to PD-1 and CXCR6 and also verified the lower expression of KLF2, S1P, and CD62L (**Figure 3**). However, Hobit expression was low (99).

IELs are located at mucosal sites, where they act as sentinels, patrolling the tissue to maintain tissue integrity and provide direct (innate-like) effector functions upon local challenges (108-110). Thymic agonist selection leads to the development of natural (n)IELs expressing the CD8αα homodimer in mice, whereas the existence of nIELs in humans is still controversial (108, 109). CD8αα has been described to function as a repressor of activation, which might fine-tune the activation threshold of nIELs to self-ligands (101). Alternatively, mature T cells expressing CD4 or CD8αβ might be induced to become IELs in the periphery (101, 108, 109). Their maintenance does not depend on prolonged antigen exposure (97). Instead, IL-7 and IL-15 mediate survival and proliferation (110). TCRαβ T cells and TCRγδ T cells are common within the IEL population and are well-characterized in the gut, where they have been shown to impact mucus composition, produce growth factors and stimulate AMP secretion by epithelial cells (111). Besides their protective role in the prevention of infections, IELs can directly lyse infected cells via granzymes and perforin or FasL. Cytolysis can be activated in a TCR-dependent fashion or NKG2D/NKG2C-mediated during cellular stress. Furthermore, IELs have the capacity to secrete various cytokines, such as IFNγ, TNF, IL-2, IL-17, and IL-22, and chemokines to recruit additional leukocytes (109-111). Dysregulation of IELs, if not tightly controlled, causes loss of epithelial barrier integrity and is associated

with the susceptibility to infections and chronic inflammatory disorders, such as IBD and coeliac disease. In coeliac disease, for example, the increased production of IL-15 upregulates NKG2D/C causing cytolysis or lowers the threshold for TCR-mediated signaling without cognate antigen being present (102, 108, 110). Notably, several studies revealed that the IELs population is shaped by the tissue-dependent environmental cues (97, 98, 109, 110). Although IELs have been characterized in many organs, including the intestine, lung, skin, and liver (110, 112-115), distinct niches still remain to be explored, especially in humans, which is of importance for targeting the immunological constituent of various disorders.

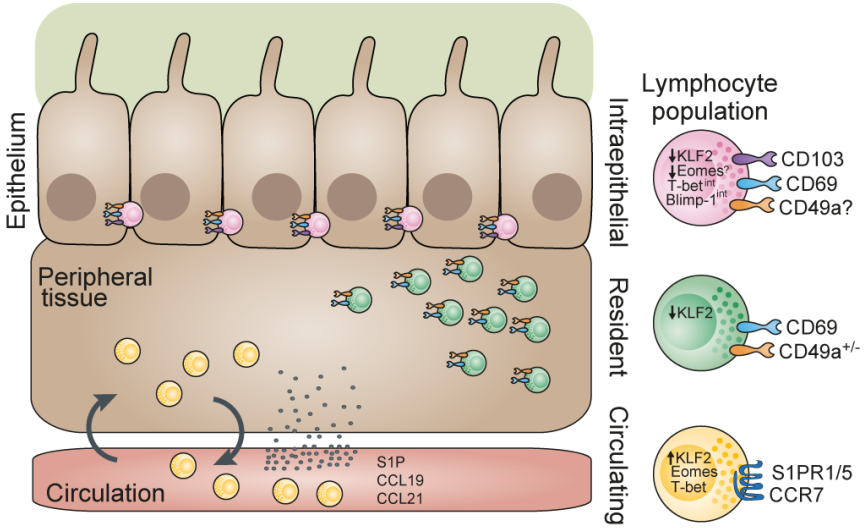

Figure 3: Mechanisms for memory T cell circulation and tissue-residency. T_{RM} cell development is associated with a specific set of transcription factors and the expression of CD69, CD49a, and CD103. Egress factors mediate tissue exit of T_{EM} cells. Adapted and modified from Schenkel *et al*. (68), Kumar *et al*. (99), Mackay *et al*. (103), and Masopust *et al*. (116).

1.5 THE LIVER AS AN IMMUNOLOGICAL ORGAN

1.5.1 Liver anatomy and cell composition

The liver is an organ important for numerous physiological processes including glucose, lipid, and protein metabolism, immune system support as well as degradation of toxic or waste products (117, 118). The liver lobules are the micro-anatomical units of the liver. Hexagonal in shape, they consist of chords of hepatocytes radiating from portal triads, which consists of the hepatic artery, portal vein and bile ducts, and converge to a central vein (**Figure 4**). The liver has a dual blood supply with oxygen-rich blood from the hepatic artery that mixes with nutrient-rich blood from the portal vein (117-119).

The structural organization of the liver is of great importance for its immune function. The liver is highly vascularized, with blood that passes through a network of sinusoids carrying approximately 10^8 lymphocytes per day (119). Minimal increase in venous pressure and a small diameter of the sinusoids facilitate prolonged contact between lymphocytes and APCs as well as lymphocyte extravasation. Furthermore, the permeable fenestrated monolayer of liver sinusoidal endothelial cells (LSECs), which lack a basement membrane facilitates direct access to the space of Dissé, where hepatic stellate cells (HSCs) reside, and to the underlying hepatocytes (**Figure 4**) (119-121).

Hepatocytes constitute about two third of all hepatic cells and are together with cholangiocytes the main parenchymal cells of the liver (117, 119). LSECs account for the majority of non-parenchymal cells and have the capacity to induce T cell immunity locally by their APC-features: receptor-mediated endocytosis/phagocytosis, antigen processing, antigen presentation via MHC class I and II, and the expression of co-stimulatory molecules. Kupffer cells (KCs) are liver-resident macrophages and account for about 20% of non-parenchymal cells. Numerous KCs line the sinusoidal vessels, are highly phagocytic and play a role in clearing the blood of microorganisms and debris that enter the liver via the portal circulation (117, 119).

In the liver, the immune cell composition differs from that in peripheral blood. NK cells are enriched and comprise 30%-50% of all human hepatic lymphocytes. Compared to peripheral blood, the frequency of $CD56^{bright}$ NK cells is increased in the liver (122, 123). Similarly, unconventional T cells, such as γδ T cells and MAIT cells are enriched. In the conventional T cell compartment, $CD8^{+}$ T cells outnumber $CD4^{+}$ T cells, and the frequency of effector and memory T cells is higher than in peripheral blood (119, 121). B cells reside in small numbers in the healthy liver. Recent evidence suggests that intrahepatic follicle-like structures similar to those of lymph nodes exist for B cell priming and maturation (124). Notably, IgA producing plasma cells lining the biliary epithelium may contribute to the protection of bile ducts from intestinal pathogens (125). Unique mechanisms in the liver ensure its tolerogenic properties, but also contribute to immune responses as described below.

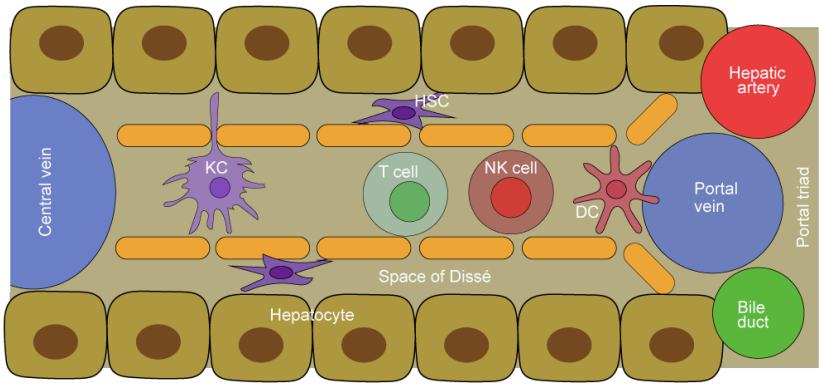

Figure 4: The liver as immunological organ. Blood passes through sinusoids from where lymphocytes can directly interact with hepatocytes via the permeable fenestrated monolayer of LSECs. Adapted from Racanelli and Rehermann (119).

1.5.2 Liver immunology at steady-state

The immunological environment in the liver is unique in that high non-self-antigen exposure from nutrients or the microbiota does not result in inflammation, a phenomenon known as tolerance. This is essential for direct multiple interactions between gut and liver ("gut-liver axis") whereby the liver gets continuously exposed to gut-derived compounds via the portal circulation including LPS (121, 126-128). Immune cells are important for maintaining liver homeostasis, but also represent key players in the initiation of liver diseases by responding to hepatic injury (119, 127, 128). Liver-resident APCs (KCs, LSECs, and DCs) line the capillary system and act as primary sensors, presenting antigens and producing cytokines and chemokines (119, 121, 129). Under homeostatic conditions, they contribute to the tolerogenic environment through various mechanisms. These include the production of anti-inflammatory

mediators (IL-10, TGF-β, arginase, prostaglandin E2), expression of indoleamine 2,3-dioxygenase and the T cell-inhibitory molecules programmed cell death 1 ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), as well as through the induction of Treg cells (117, 127). Alternative APCs, such as HSCs and hepatocytes, can also induce Treg cells or prime $CD8^+$ T cells. This priming however, results in limited proliferation and clonal deletion. Alternatively, T cell apoptosis can be induced by hepatocytes via Fas and TNF (127).

1.5.3 Breaking tolerance – liver inflammation

During infection or tissue damage, rapid immune responses need to be initiated. While PAMPs are important to detect infections, alarmins and damage-associated molecular patterns (DAMPs) classically activate the immune system upon sterile tissue injury. For example, injured hepatocytes secrete IL-33, which stimulates Th2 responses eventually promoting fibrosis or the release of the high mobility group protein B1. Also, free cholesterol, oxidized lipids, extracellular ATP, bile acids outside the biliary tree, and uric acid act as irritants. This triggers the production of pro-inflammatory mediators, such as inflammatory cytokines, including IL-1 and IL-18, chemokines (CXCL1, CXCL9-11, CCL2, CCL5), growth factors (G-CSF, GM-CSF), and adhesion molecules mediating and cellular diapedesis into tissue (121, 128). Besides classical immune cells, cholangiocytes are immunologically active cells, express TLRs, and can act as APC. They have the capacity to secrete pro- and anti-inflammatory cytokines (IL-1β, IL-6, TNF, IFNγ, TGFβ), chemokines (IL-8, CCL2, CCL25, CX3CR1) and express adhesion molecules (ICAM-1, CD40). Thereby, cholangiocytes actively contribute to immune-regulation and liver pathogenesis (130, 131).

Cellular infiltrates consist of neutrophils, macrophages, T cells, and DCs that all participate in the inflammatory response (128). KCs are important players during liver inflammation. They produce oxygen and nitrogen radicals as well as TNF, IL-1β, IL-12, and IL-18 (121). Kupffer cells also possess pro-fibrotic mechanisms, such as HSC activation leading to extracellular matrix deposition, but also participate in different processes after liver injury (121). The pro-inflammatory cytokine milieu as well as NCR ligands expressed by stressed cells activate NK cells that may contribute to liver damage and/or produce cytokines, chemokines, and growth factors, thereby also acting as regulatory cells. For example, NK cells can be activated by IL-18 and produce varying amounts of IFNγ, contributing to antiviral, antifibrotic, and antitumor effects. IFNγ release is however, modulated by the simultaneous production of IL-10, setting a baseline for pro-inflammatory immune responses (129). NK cells also indirectly regulate fibrosis by killing HSCs (121, 123). DCs in the liver are mainly located in the portal tracts. Hepatic DCs exhibit predominantly immunoregulatory rather than immunogenic functions. However, during inflammation, they switch to an immunogenic state and are found in portal tract associated lymphoid tissues (PALT), which can serve as priming sites for liver-infiltrating T cells (121). Interestingly, in mouse models of hepatitis B virus (HBV) infection, it was shown that T cells directly interact with hepatocytes via protrusion through endothelial fenestrae. This occurred in a diapedesis-independent manner by adhering to platelet aggregates in sinusoids (120). However, in liver infections, $CD8^+$ T cells show features of exhaustion, resulting in a dysfunctional immune response that may also be caused by the lack of $CD4^+$ T cell help (121, 127).

In summary, the liver creates a unique tolerogenic environment that ensures liver integrity despite continuous exposure to antigens from the gut. Multiple cell types contribute to set the threshold for the engagement of immune defenses in order to balance an inflammatory state and immunopathology causing liver damage.

1.6 LYMPHOCYTES IN HUMAN LIVER DISEASES AND VIRAL INFECTIONS

1.6.1 Dengue fever

Dengue virus (DENV) causes a major threat to global health and not only has spread wider geographically, but also increased in numbers of infections (132, 133). DENV is transmitted by *Aedes* mosquitos in tropical and subtropical regions of the world and belongs to the genus Flavivirus (*Flaviviridae* family) with four different serotypes (DENV1-DENV4). Other members of that genus are West-Nile virus, Tick-borne encephalitis virus (TBEV), Yellow fever virus (YFV), Japanese encephalitis virus, and Zika virus (134, 135). DENV consists of an enveloped spherical particle with a positive-sense, single-stranded RNA genome encoding three structural (capsid (C), precursor membrane (prM) and envelope (E)) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins. E and prM/M are part of the glycoprotein shell with the E protein being important for binding and entry (135). To date, no single receptor is yet defined to be required for viral entry, but some receptors have been suggested to play a role, for example heparin sulfate, DC-specific ICAM3-grabbing non-integrin, heat shock protein 70kDa, and mannose receptor (136). Upon receptor-mediated endocytosis and pH-mediated fusion of the viral and the endosomal membrane, the nucleocapsid is released into the cytoplasm. The viral RNA is translated at the endoplasmic reticulum (ER) into a single polyprotein that is subsequently processed by proteases. RNA synthesis begins and the new RNA is packed in C protein, buds into the ER, followed by transportation through the trans-Golgi network, where prM is cleaved off and mature viral particles are released from the cell (135).

An infection with DENV results in the clinical syndrome known as dengue fever (DF). DF affects up to 390 million individuals annually thereby causing a serious health threat and economic burden to affected areas (132). The clinical picture varies from mild to severe forms of DF, with severe forms being dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which may lead to fatal outcomes (132). Most patients recover from an either asymptomatic or self-limiting non-severe clinical course with symptoms during an acute febrile phase of 3-8 days, accompanied by skin rashes, muscle and joint pain, and headache. Some patients have symptoms affecting gut and liver, including nausea, vomiting, and eventually liver enlargement, whereas the more severe forms are characterized by plasma leakage with or without hemorrhage during the critical phase. Factors influencing the response to infection include immune status, viral serotype, host genetics, and age (137). At present, no effective anti-viral agents exist. However, supportive care by intravenous rehydration reduces the fatality enormously (138).

Interestingly, the immune system has been suggested to play a role in dengue pathogenesis. The infection starts locally in the skin where resident innate immune cells, including macrophages, Langerhans cells and mast cells reside. Langerhans cells migrate to draining lymph nodes from where the infection spreads and becomes systemic (139). Mast cells

contribute with the release of pro-inflammatory mediators, such as TNF that up-regulates E-cadherin on endothelial cells and secrete chemokines (134, 140). During primary infection with DENV, a serotype-specific adaptive immune response is mounted, whereas in secondary infection with a heterologous serotype cross-reactive plasmablasts and memory T cells can be found (134). These secondary infections with a different serotype have been claimed to be a risk factor for more disease due to antibody-dependent enhancement (ADE), a mechanism by which the infection rate is increased by binding of virus in complex with non-neutralizing antibodies to Fc receptors promoting viral uptake. This in turn has been suggested to result in increased secretion of pro-inflammatory cytokines and chemokines (135, 139). This so called "cytokine storm", induced by increased infection rate and/or cross-reactive weak-affinity T cells, includes increased levels of TNF, IFNγ, CXCR8, CXCR9-11, CCL5, vascular endothelial growth factor (VEGF), and IL-10 and is associated with the peak in symptoms after the incubation period of 4-7 days. During this phase, viral loads are rapidly decreasing, suggesting immune-mediated viral control. Disease severity correlates with the magnitude of the T cell response (135). Interestingly, Rivino *et al*. showed that T cells home to the skin during the acute phase of the infection (141). Thus, DENV-induced immune responses can both be protective, resulting in efficient control of the infection and can contribute to immunopathology, increasing disease severity.

1.6.2 NK cells in acute virus infections

NK cells are important in the response to viral infections (142). This has been demonstrated in mouse models for a diverse range of acute infections, including cytomegalovirus (CMV), herpes simplex virus (HSV)-1, influenza virus, and poxvirus (14, 142). In humans, their importance has become clearer in rare NK cell deficiencies that result in high susceptibility to herpesvirus infections including HSV, varicella zoster, and CMV (143, 144). Nevertheless, most studies that have led to advanced knowledge of NK cell responses in viral infections were conducted using murine systems (145). More recent research has revealed insights into NK cell responses during acute viral infections in a human setting (146-152). Numerous studies demonstrated that NK cells respond during natural viral infections, including hantavirus (146), TBEV (147), chikungunya virus (148), DENV (153), CMV (151), Epstein-Barr virus (EBV) (152), hepatitis C virus (HCV) (154), and human immune deficiency virus (HIV)-1 (149). However, due to difficulties in collecting human samples in a controlled way before early acute infection has passed, only few studies have longitudinally followed NK cell responses during the acute phase and beyond. In DENV infection, NK cell numbers are increased and activation markers are upregulated in patients with DF compared to patients with DHF (155, 156). In other *flavivirus* infections, such as in TBEV or after YFV vaccination, a similar activation of NK cells has been reported (147, 157). Furthermore, progress has been made during the recent years, characterizing receptor-ligand pairs and cytokine-mediated effects on NK cells during viral infections as well as infection-induced changes in the NK cell receptor repertoire (36). Besides NK cell activation seemingly driven by cytokine priming (157), previous studies have shown that certain acute infections result in expansion of distinct NK cell populations exhibiting a terminally differentiated phenotype (146, 148, 151, 158). These expansions are most likely driven by underlying human cytomegalovirus (HCMV) infection (146, 159). This phenomenon does occur in some, but not other infections, such HSV-2 or EBV infections (160, 161). Sun *et al*. described a clonal-

like NK cell expansion, contraction, and persistence upon murine cytomegalovirus (MCMV) infection, leaving a population with enhanced responses upon re-challenge, so called memory or adaptive-like NK cells (162). In humans, a population of adaptive-like NK cells highly express NKG2C, CD57, and DNAM-1, are skewed towards an increased expression of self-KIRs, and are low in NKG2A and NCR expression (158, 163). Moreover, hapten-specific as well as cytokine-induced (IL-12, IL-15, IL-18) memory cells were described with the latter ones expressing high levels of CD25, perforin, granzymes and IFNγ (164). Other receptors in direct NK cell receptor recognition of virus induced-ligands have been shown to be important, for example in influenza (NKp46) and in DENV infection (NKp44) (35, 165). Notably, epidemiologic studies have linked KIR-HLA repertoires to disease susceptibility in anti-viral immune response and therefore impact disease outcome. Such associations were shown in HCV with KIR2DL3 and HLA C1 being associated with self-resolving HCV, and in HIV showing that the KIR3DSL-HLA-B Bw80I interaction is beneficial for better HIV-1 control (166). Intriguingly, specific recognition of viral peptides has recently been shown to be a feature of NK cells binding HCMV-derived peptides presented by HLA-E or by HLA-C, activating NKG2C and KIR2DS1, respectively (167). Additionally, KIR2DS2 recognizes HLA-C-binding peptides from the NS3 helicase, which are highly conserved in *Flaviviruses* including HCV and DENV (168).

Table 2: Homing receptor-ligand pairs in infectious and inflammatory diseases

Receptor	Main ligand	Tissue	Disease	Human/mouse	Source
CCR5	CCL3	lung	Klebsiella pnemoniae	mouse	(169)
		vagina	Genital HSV-2	mouse	(170)
		liver/spleen	Toxoplasma	mouse	(171)
CCR5	CCL3, CCL4	liver	MCMV	mouse	(172)
			HIV	human	(173)
CCR2	CCL2	lung	Invasive aspergillosis	mouse	(174)
CCR4	CCL2 (CCL4, CCL5)	lung	Invasive aspergillosis	mouse	(174)
CXCR3	CXCL9-11	brain	Coronavirus	mouse	(175)
α4ß7	MadCAM-1	gut	SIV/HIV	macaques/humans	(176)
CCR5	CCL3	liver		human	(177)
		joints	RA	human	(178, 179)
		skin	Psoriasis	human	(91, 180)
CXCR6	CXCL16	liver		human	(177)
CX3CR1	CX3CL1	CNS	Multiple sclerosis	human	(181)
CXCR3	CXCL9-11	skin	Psoriasis	human	(91, 180)
CCR8	CCL1	skin	Psoriasis	human	(180)
CLA	E-selectin	skin		human	(182)
CCR7	CCL19, CCL21	lymph nodes			(183)
CCR9	CCL25	gut		human	(184)
CCR6	CCL20	skin		human	(91, 180)
CCR10	CCL27, CCL28	skin		human	(180, 182, 185)

In addition to cellular responsiveness and functional capacity, viral control requires recruitment of activated immune cells to the site of infection (142). Distinct chemokine-chemokine receptor pairs are important for NK cell recruitment to lymphoid and non-lymphoid organs during inflammation and infection. However, NK cell homing in viral infections has yet mainly been addressed in mice (**Table 2**).

Hence, little is known about temporal dynamics with regard to the NK cell response and trafficking to peripheral organs during viral infections in humans, including DENV infection.

1.6.3 Chronic Hepatitis B

Viral hepatitis is one of the leading causes for death worldwide with approximatively 250 million people being chronically infected with HBV (186). HBV is a hepatotropic, small, enveloped DNA virus that belongs to the *Hepadnaviridae* family. By itself, the virus is non-cytopathic but eventually leads to progressive liver fibrosis, cirrhosis, and an increased risk of liver cancer, in particular hepatocellular carcinoma (187, 188). Nine genotypes (A-I) and several sub-genotypes have been identified with differences in their geographic distribution (187). The most common transmission routes are mother-to-child transmission, sexual transmission, as well as needle sharing. HBV infections can be acute or chronic with low rates of chronicity in adults (5%), but very high persistence rates in neonatal infections (188). Chronic HBV (CHB) infection can be classified into different phases that are characterized by presence of varying levels of HBV DNA, hepatitis B e antigen (HBeAg), hepatitis B s antigen (HBsAg), and their respective antibodies in circulation. Some of these phases are associated with liver damage, which is measurable by alanine aminotransferase (ALT) and fibrosis markers (187).

The viruses' DNA encodes seven proteins: HBcAg (core antigen, viral capsid), HBeAg (secreted splice variant of the HBc, indicates active replication of HBV), HBV Pol (polymerase with reverse transcriptase activity), PreS1/PreS2, HBsAg (large, medium, small surface glycoproteins), and HBx (x antigen, initiation of transcription). Upon sodium taurocholate co-transporting polypeptide (NTCP)-mediated infection (189, 190), the nucleocapsid is transported to the nucleus where the HBV DNA is converted into covalently closed circular DNA (cccDNA). It serves as a template for transcription, which is followed by translation of different viral proteins. Pre-genomic RNA is reverse transcribed by the RNA polymerase into new HBV DNA. Nucleocapsids containing HBV DNA are either recycled to maintain the cccDNA reservoir or enveloped and secreted together with genome-free subviral particles, which are non-infectious, but still being produced when reverse transcription is blocked (191) (**Figure 5**). Viral genome integration of HBV occurs randomly and may lead to hepatocyte transformation (187).

The overall goal of antiviral HBV therapy is to improve survival and quality of life (187, 192). To date, treatment with pegylated interferon alfa (pegIFNα) and nucleos(t)ide analogues (NA) are the two main therapies available (187). pegIFNα elicits both anti-viral and immunomodulatory effects (193, 194) while NAs efficiently suppress HBV replication by inhibiting the viral RNA polymerase (187). NAs are well-tolerated and have a beneficial effect on disease progression (187). Thus, NAs are most commonly used nowadays. The ultimate goal of treatment is HBsAg loss with or without HBs-seroconversion (192, 195),

indicating immune control of HBV and suppression of viral replication and protein production. However, this goal is rarely achieved with current treatment options. HBsAg loss and undetectable HBV DNA, also termed functional cure, does not include complete removal of cccDNA or integrated DNA, but allows to safely stop anti-viral therapy (187, 195). NA treatment can be long-term, because the rate of HBsAg decline is low (196). In contrast, treatment with pegIFNα is given over a defined period of time with higher response rates than in NA treatment. Its goal is sustained off-treatment response with low HBV DNA levels and normal ALT levels, a marker used to determine liver injury. However, side effects during pegIFNα therapy are severe. Thus, an important clinical need is to optimize strategies to shorten the duration of antiviral therapies by augmenting HBsAg loss (187, 195). As such, a variety of studies evaluated the feasibility of stopping NA treatment in CHB patients (197-201). Despite the recurrence of HBV DNA, treatment cessation is safe and was recently introduced into the European Association for the Study of the Liver (EASL) guidelines (187). NA treatment termination is recommended for well-selected HBeAg-negative patients. Hadziyannis *et al.* highlighted the importance of this new concept by showing HBsAg loss at long-term follow up in a third of patients after NA cessation (202). In most patients, an increased viral load was followed by ALT flares (202). These results correspond with a report by Siederdissen *et al.*, who could also show an induction of inflammatory/anti-viral cytokines upon NA cessation (203). Still, the underlying immunological correlates of the observed events after NA cessation remain elusive.

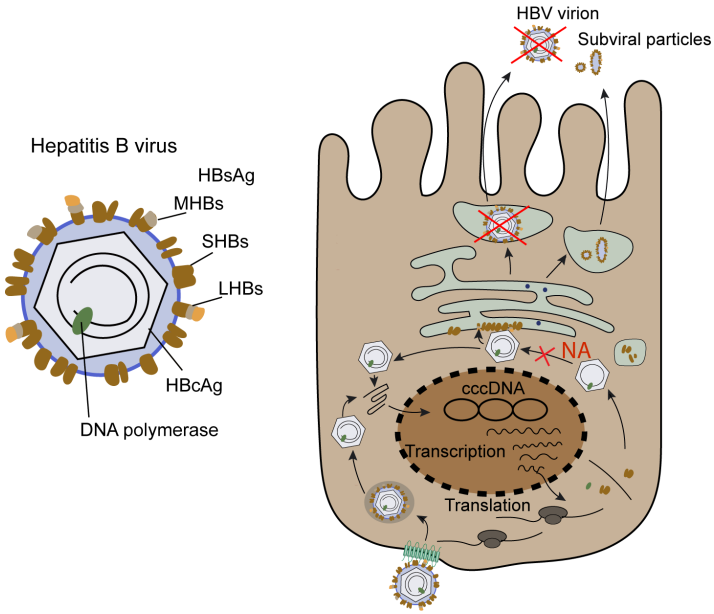

Figure 5: HBV structure and replication. HBV is an enveloped DNA virus. During viral replication cccDNA resides in the nucleus of hepatocytes and serves as template for transcription. NAs block the viral RNA polymerase and thereby block the release of infectious virions, but not subviral particles. Adapted from Yan *et al*. (189) and Seeger *et al*. (191).

Studies addressing early immune responses during natural HBV infection in humans are rare due to an asymptomatic incubation period of HBV infection (150, 204). For this reason, model systems, such as chimpanzees, have shown that HBV does not activate interferon-stimulated genes during acute infection (204). This is in line with a diminished early production of type I IFN in humans (150). Therefore, HBV has been suggested to be a

"stealth virus" (204). Moreover, HBV seems to exert several immunoregulatory mechanisms that in turn may contribute to viral persistence (205-208). In humans, acute infection was accompanied by IL-10 production and coincided with temporally impaired NK cell and T cell functionality (150). An impairment of early immunity is challenged by HBV model systems, showing non-cytolytic clearance of the virus (209, 210) as well as another report in humans showing early activation of functional NK cells (211). Generally, acute infection is described as a self-limiting inflammatory disease with potent immune responses followed by viral clearance (211, 212). Both innate and adaptive immune responses are efficient and are characterized by activated NK cells (211), robust T cell responses (213), as well as by neutralizing antibodies produced by B cells (214). The interplay between HBV replication and the host immune responses largely affects the outcome of CHB infection.

1.6.4 NK cells in chronic hepatitis B

The progression from acute to CHB results from ineffective attempts by the host immune response to clear virus (215). NK cells have been studied in the context of CHB (216). Several reports indicate an increase in $CD56^{bright}$ NK cells in CHB (216-220). A recent meta-analysis revealed that NK cells express higher levels of the activating receptors NKp46, NKp30, NKG2C and NKG2D (216), whereas Tjwa and collegues reported an increase in NKG2A and downregulation of CD16 and NKp30 (221). Furthermore, multiple studies have shown functional dichotomy in the NK cell compartment with retained cytolytic activity but suppressed cytokine production (217, 218, 222, 223). Selective defects of NK cell function may be partly due to the immunosuppressive cytokine environment created through high levels of IL-10 and TGF-β (218). Studies using transgenic mouse models have shown that liver damage is induced by immunological mechanisms rather than the virus itself (224). Although virus-specific $CD8^+$ T cells have been identified as the main effectors for controlling viral replication, in chronic infection these cells are exhausted and functionally impaired. This suggests that hepatocyte injury might be caused by non-antigen specific immune cells infiltrating the liver (225). This indicates a role for NK cells in contributing to viral persistence and liver pathology (213). In this regard, it is interesting to note that the NK cell frequency has been shown to be positively correlated with disease severity (223). In addition to retained cytotoxicity, activated TRAIL-expressing NK cells have been reported to accumulate in CHB livers (219). There, TRAIL and TRAIL receptor expression on hepatocytes are associated with elevated IFNα and IL-8 levels, respectively (219). Additionally, an increase in TRAIL-expression on $CD56^{bright}$ NK cells correlated with liver damage (223). Therefore, TRAIL-mediated death of hepatocytes was suggested to contribute to liver inflammation (219, 223, 226). This is in line with a study by Zhang *et al*. showing more pronounced cytolytic activity of hepatic NK cells as compared to NK cells from peripheral blood (227), which was associated with liver damage in HBeAg positive CHB patients (227, 228). Recently, NK cells were shown to regulate immunopathology by acting as rheostats (229) by cytolytical elimination of HBV-specific T cells (223, 226).

The HBV DNA level-decline upon NA treatment has been shown to modulate the peripheral blood NK cell phenotype (217, 221). However, contradictory results were reported for NK cell activation and cytokine-producing capacity as either unaffected (217) or restored (221) upon NA treatment. Yet another study revealed that long-term NA treatment-induced viral

suppression did not modulate frequency, phenotype, and activation of intrahepatic NK cells (230). In contrast to NA treatment, pegIFNa has been reported to induce an expansion of activated TRAIL-expressing $CD56^{bright}$ NK cells coinciding with IL-15 production (231). This was in line with the strong immunomodulatory effect observed in combination therapy of NAs and pegIFNa with higher frequency of TRAIL-expressing $CD56^{bright}$ NK cells and increased NK cell functionality in responders compared to non-responders (232-234). Without achieving HBsAg loss, however, add-on therapy is questionable. Instead, it has recently been suggested that a fraction of HBeAg-negative non-cirrhotic patients with time experience HBsAg loss after the initial viral rebound and flare of hepatitis occurring following NA interruption (202, 203). However, correlates explaining the virological and cellular events are lacking.

1.6.5 T cells in chronic hepatitis B

In contrast to self-limiting acute HBV infections, CHB is characterized by qualitatively and quantitatively impaired HBV-specific T cell responses (235-237). T cell dysfunctionality was shown to be associated to viral load (235, 237), but may also be caused by the lack of $CD4^+$ T cell help, increased Treg cells, immunosuppressive cytokines, and/or nutrient deprivation (238). In contrast to acute HBV infection, HBV-specific T cells are rarely detected in CHB (235). In this regard, Peppa and colleagues could show that TRAIL-expressing NK cells mediate deletion of TRAIL-R2 expressing HBV-specific T cells during hepatic flares (226), potentially contributing to the lack of HBV-specific T cells. Nevertheless, HBV-specific T cells are believed to be essential for viral clearance (235) and are more associated with viral control than liver damage (225). T cell exhaustion is believed to be driven by the chronic exposure to antigens and is accompanied by a hierarchical loss of anti-viral T cell functions, starting with the loss of cytotoxicity and IL-2 production, which is followed by the reduction of TNF and IFNγ and finally T cell deletion (239). Moreover, exhausted T cells lose their proliferative capacity and express a variety of inhibitory molecules (240), also termed "exhaustion markers", such as PD-1 (235), CTLA-4 (241), and TIM-3 (242). As such, PD-1 gets phosphorylated upon ligand binding (PDL-1, PDL-2), which leads to the recruitment of the tyrosine phosphatase Shp2 that dephosphorylates CD28 or the TCR complex, thereby attenuating TCR/CD28 signaling (238). Blocking these inhibitory receptors, a strategy known as checkpoint blockade, is therefore considered to be one strategy to potentially boost T cell functionality in CHB (235, 241, 242). Several inhibitory receptors can be co-expressed on exhausted cells and show a synergistic effect when blocked simultaneously (240). Additionally, recent evidence suggests that transcriptional and metabolic derangements contribute to T cell exhaustion (240). Paley and colleagues described a subset of $T\text{-}bet^{dim}$ $Eomes^{hi}$ $PD\text{-}1^{hi}$ T cells as exhausted with limited proliferative capacity, weaker cytokine production, and poor response to PD-L1 pathway blockade driven by T cell downregulation upon persistent antigen exposure (243). Indeed, a decline of T-bet (246) as well as TCF-1 (247) has been associated with dysfunctional HBV-specific T cells in CHB, whereas high T-bet expression is strongly associated with spontaneous virus control (246). T-bet and Eomes have also been shown to be associated to exhausted T cells in other viral infections, including HIV (244). In addition, exhausted T cells can co-express other inhibitory receptors, such as KLRG1, while KLRG1 and CD57 are generally described markers of terminal differentiation and/or senescence (245). Furthermore, metabolic changes causing bioenergetic deficiencies

(245) have been described to be an early driver of T cell exhaustion being more prevalent in the PD-1^{hi} subset, suggesting that the manipulation of T metabolism may be a complement to checkpoint blockade (248). To what extent HBV-specific T cell functionality is affected in the liver in still unknown. In this regard, Pallet *et al*. reported that T_{RM} cells reside in the liver and are expanded in patients with partial or complete immune control (115).

Treatment with pegIFNα and NAs results in variable outcomes in relation to HBV-specifc T cell responses. HBV-specific $CD8^+$ T cells were shown not to be affected (249) or lost (231) upon PegIFNα therapy without a restoration of T cells dysfunctionality (231, 249). As opposed to treatment with pegIFNα, reduced viral loads upon NA therapy partially restored HBV-specific T cell responses *in vitro* (250, 251), particularly in patients experiencing HBsAg loss (252). Accumulating evidence suggests that stopping NA may lead to HBsAg loss in a selective group of patients and is commonly associated with hepatic flares (202). Rivino *et al*. demonstrated that patients with sustained HBV DNA control upon NA discontinuation had increased frequencies of core and polymerase HBV-specific T cells before the treatment discontinuation. These functional HBV-specific T cells are enriched in the PD-1^+ population and were suggested as an immunological biomarker for safe therapy discontinuation (253). However, whether improved functionality of HBV-specific T cells contributes to HBsAg loss at long-term follow up remains elusive.

1.6.6 Primary sclerosing cholangitis (PSC)

Primary sclerosing cholangitis (PSC) is a rare cholestatic liver disease, which is characterised by inflammation and fibrotic scarring of intra- and extrahepatic bile ducts. The hallmark of PSC are biliary strictures and recurrent bacterial cholangitis, which ultimately leads to biliary cirrhosis and end-stage liver disease (254). 60%-80% of PSC patients simultaneously have IBD, in particular ulcerative colitis (UC). Both men and women at any age can be affected, but the classical PSC patient is male with a median age of 30-40 years at disease onset. PSC can be asymptomatic without being diagnosed early or patients may have symptoms such as itching, abdominal pain, jaundice, fevers and fatigue. For diagnostics, serum liver tests assessing for levels of alkaline phosphatase (ALP) and magnetic resonance cholangiopancreatography are used. PSC patients have an increased risk to develop cholangiocarcinoma (CCA) and colon cancer in the presence of IBD. There is no treatment that can halt disease progression. To date, liver transplantation is the only treatment option available (126, 254-256). Nevertheless, endoscopic cholangiopancreatography (ERCP) is used to treat strictures and obstruction of the large bile ducts to relieve symptoms such as itch, jaundice, and cholangitis, as well as for evaluating the characteristics of the strictures and to rule out malignancy (254). There is a need for effective treatments, yet the development of these has been impeded by a poor understanding of the pathogenesis and pathophysiology of PSC.

Several factors have been considered to contribute to the disease (254, 256). The involvement of a genetic component is supported by the fact that first-degree relatives of PSC patients have an increased risk for PSC development (257). Furthermore, genome wide association studies indicate a variety of susceptibility genes being involved in the development in PSC, identifying similarities to other autoimmune diseases, such coeliac disease and rheumatoid arthritis. These genes associate with the immune system, but also with other pathways, such

as apoptosis, cell growth/death, autophagy, and metabolism. The exact role of the majority of these genes is unclear and needs be further explored. Environmental triggers account for the total risk, with diet and smoking as factors of influence (126). The role of the gut-liver axis has been emphasized along with the "leaky gut hypothesis", suggesting that bacterial translocation in the gut increases the presence of bacterial antigens in the portal zone of the liver, thereby triggering biliary inflammation (254, 256). The presence of bacterial products and genetic associations indicate an involvement of an immune component in the PSC pathogenesis. For example, bacterial antigens present in the gut may trigger immune cell infiltration via PAMP recognition (innate) or T cell homing to the liver due to an overlap in endothelial adhesion molecules in both compartments (adaptive), highlighting the importance of the cross-talk with activated cholangiocytes (256). Furthermore, autoantibodies with unknown pathologic significance, for example anti-neutrophil cytoplasmic antibodies, are frequently detected in PSC. Both IgG and IgA classes have been found and may reflect B cell responses to gut derived antigen following sustained inflammation. Autoantibodies in PSC may also target biliary epithelial cells (BECs) directly (256, 258). Toxic bile injury caused by defects in mechanisms protecting against bile duct toxicity, for example the presence of bicarbonate (HCO_3^-) layer ("umbrella"), and disturbance in bile homeostasis have been suggested to contribute to PSC development. Interestingly, a reduction in microbial diversity has been observed, most likely also impacting bile homeostasis (254, 256, 259). Ultimately, HSCs and myofibroblast activation is involved in fibrosis development and the eventual progression to other PSC-related liver conditions, such as stricture formation, cirrhosis, and CCA (256).

1.6.7 The biliary immune system in PSC

The immune system is believed to play an important role in PSC pathogenesis (126, 256, 258). However, limited data are available that contribute to the understanding of the immunopathology in PSC that is believed to be a multi-step process (258). Histopathological findings show mixed immune cell infiltrates in portal areas and around bile ducts (258), but very little knowledge about the biliary immune system is currently available. Accumulating evidence from early studies, mainly investigating mononuclear cells in bile ducts using immunohistochemistry, suggest $CD8^+$ memory T cells to be the dominant population among IELs (260-262). Furthermore, macrophages and neutrophils reside closely to bile ducts (256). More recent genetic studies support an involvement of the immune system in the PSC pathogenesis. The strongest associations were found in the HLA complex, indicating an important role of the adaptive immune system. Nevertheless, both the innate and the adaptive immune system appear to be affected. Examples are *PRDX5*, *TGR5*, and *PSMG1* (LPS response in humans), *NFKB1*, *REL*, *PRKD2/CB* (cytokine production, proliferation), *TNFRSF14* (TNF signaling pathway), *IL2RA* (IL-2 signaling pathway), *PRKD2* (effector cytokine production), *SOCS1* and *SH2B3* (regulation of cytokine production), *CXCR1*, *CXCR2*, and *CCL20* (leukocyte homing), and *CD28*, *CD226*, *CTLA4* (co-stimulatory/inhibitory receptors) (126). In summary, various susceptibility genes may impact immune responses, particularly genes involved in T cell development and differentiation, activation and proliferation, migration, and apoptosis (126). Further studies need to shed light on their function, especially their role in immune cell cholangiocyte cross-talk. In that regard, Liaskou *et al*. showed that higher frequencies of $CD28^-$ T cells were present in livers of PSC

patients than in patients with primary biliary cirrhosis or alcoholic steatohepatitis (263). These cells localized around bile ducts and were described as being equipped with cytolytic molecules and capable of producing IFNγ and TNF, thereby contributing to BEC activation (263). Even though $CD4^+$ $CD28^-$ T cell have been linked to autoimmunity, the lack of CD28 generally identifies T cells that are antigen experienced and highly differentiated exhibiting a variety of different functions (264). The existence of disease-specific TCR repertoires may suggest the presence of disease-associated antigens. Despite a high T cell diversity in PSC, disease-associated clonotypes were identified and have therefore been suggested to be evidence for antigen-driven clonal expansions (265). On the other hand, a specific cytokine milieu, for example IL-17, has been shown to drive inflammation and fibrosis. Interestingly, Th17 as well as MAIT cells, both being IL-17 producers, are important for the defense against bacteria and fungi that are present in a majority of bile fluid samples of PSC patients. Upon pathogen stimulation, more $CD4^+$ T cells exhibit a Th1/Th17 profile in PSC patients compared to controls (266), whereas the Treg compartment appears to be dysregulated in terms of Treg frequency and function (267). Of note, cholangiocytes express increased levels of TLRs in PSC and therefore have the potential to detect PAMPs that can activate cholangiocytes causing proliferation, secretion of pro-fibrotic, and pro-inflammatory mediators (131, 268, 269). This aids tissue repair, but also promotes Th17 differentiation and the recruitment and localization of immune cells, such as T cells, expressing CXCR6, CCR6, and CCR10 binding to CXCL16, CCL20, and CCL28, respectively (131, 268-270). In that context, Eksteen *et al*. demonstrated that T cells primed for gut homing were recruited to the liver due to the aberrant expression of CCL25 and mucosal addressin cell adhesion molecule (MadCAM)-1 in PSC, binding to CCR9 and α4β7, respectively (271). Once in the liver, cholangiocyte-T cell interactions are augmented via the upregulation of adhesion molecules and antigen presentation by increased expression of MHC class I and II by activated cholangiocytes (131). In addition, and in line with antigen presentation to conventional T cells, BECs have the capacity to present bacterial antigens via MR1 to activate MAIT cells that were shown to localize in portal tracts and around bile ducts (272). In conclusion, accumulating evidence suggests an involvement of T cells in PSC pathogenesis. A detailed characterization of the biliary immune system is however missing, which is of great importance to understand bile duct-associated inflammatory diseases.

2 AIMS

The overall aim of this thesis was to characterize lymphocytes in human diseases. In particular, the work was focused on NK cell responses during an acute viral infection with dengue virus and NK cell and T cell responses after stopping nucleos(t)ide analogue treatment in a chronic infection with hepatitis B virus. Moreover, the role of MAIT cells and tissue-resident T cells was explored in primary sclerosing cholangitis.

Specific aims:

Paper I: To study the phenotype, differentiation, and education status of NK cells and the impact of DENV-infection on NK cell functionality and tissue homing properties.

Paper II and III: To investigate whether stopping NA therapy in a cohort of HBeAg negative CHB patients induces immune responses that contributes to HBsAg loss. Specifically, we aimed to characterize NK cell (**paper II**) and T cell (**paper III**) phenotypes, activation status, and functional capacity at NA treatment termination and during treatment interruption.

Paper IV: To characterize MAIT cells in peripheral blood and to assess their presence in bile ducts in patients with PSC and non-PSC controls.

Paper V: To characterize the biliary immune system during inflammation and in non-inflammatory controls. Moreover, to characterize the IEL compartment in relation to immune composition with a particular focus on biliary-resident T cells.

3 METHODOLOGICAL APPROACH

3.1 ETHICAL CONSIDERATIONS

All studies included in this thesis were performed in accordance with ethical guidelines described in the Declaration of Helsinki. All patients signed a written informed consent prior to participating in a study. The studies included were approved by the respective regional ethic committees as per the following permit numbers: DSRB 2013/00209 and DCRB 2008/00293 (**paper I**), 5908 (**paper II and III**), Dnr 2013/2285-31/3 and 2013/2084-31/2 (**paper IV and V**), and 2010/678-31/3 (**paper V**).

3.2 SAMPLE COLLECTION, PROCESSING AND IMMUNE CELL ISOLATION

Peripheral blood was collected in heparin-coated vacuum tubes (**paper I-V**). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll Hypaque, followed by the collection of the interphase. PBMCs were directly stained or cryopreserved for later analysis.

Skin blisters were induced on the forearm of DENV-infected patients and controls by applying a negative pressure (25-40 kPa) using a suction chamber for 2-4 hours until a unilocular blister was formed (273). Subsequently, the blister was covered with an adhesive dressing and the accumulated fluid was aspirated after 18-24 hours. The cellular content was pelleted and analyzed by flow cytometry (**paper I**).

Liver tissue was obtained from fresh pieces of liver after resection surgery. Sinusoidal blood was collected by flushing the liver. Liver digestion was conducted using a three-step perfusion protocol with collagenase XI (Sigma). Following digestion, the liver was cut into small pieces and subsequently filtered. The cells were then pelleted and washed twice to remove remaining collagenase. After hepatocyte removal by centrifugation, mononuclear cells were isolated by density-gradient centrifugation from the remaining supernatant (**paper V**).

Biliary brush samples were collected during ERCP, followed by an enzymatic digestion in RPMI medium supplemented with collagenase II (Sigma-Aldrich) and DNase (Roche). After the digestion was stopped, the cellular components were pelleted, washed and subsequently used for flow cytometry (**paper IV and V**).

3.3 MEASUREMENT OF PLASMA PROTEINS

Plasma concentration of proteins were measured using a magnetic luminex bead assay or by sandwich ELISA according to the manufacturer instructions (**paper I**).

3.4 KIR AND KIR-LIGAND GENOTYPING

For KIR and KIR-ligand genotype determination, genomic DNA was isolated and used for PCR amplification using KIR and HLA typing kits (Olerup SSP). The genotype was determined according to the products that were separated by gel electrophoresis (**paper I**).

3.5 IN VITRO FUNCTIONAL ASSAYS

The functional capacity of NK cells was assessed *in vitro* (**paper I and II**). PBMCs were thawed and either rested in medium or stimulated with IL-12 and IL-18 for testing NK cell responsiveness upon cytokine priming. To test natural cytotoxicity, target cells were added to the cell culture for six hours. K562 cells, a human erythroleukemia cell line, and 721.221 cells, a human B lymphoblastoid cell line, both being deficient for HLA class I expression, induce potent NK cell responses by engaging multiple NK cell activating receptors, evaluating basal levels of NK cell responsiveness. Moreover, 721.221 cells (expressing CD20) were also used to determine NK cell-mediated ADCC by adding rituximab (chimeric monoclonal antibody against CD20) to the culture. In order to detect and quantify degranulation, anti-CD107a antibody was present throughout the assay. CD107a (lysosomal-associated membrane protein-1) co-localizes with perforin in secretory lysosomes and is presented on the cell surface during degranulation upon membrane fusion of lysosomes with the cell membrane. Monensin (GolgiStop, inhibits distal Golgi function, avoiding the degradation of re-internalized proteins) and Brefeldin A (GolgiPlug, prevents exocytosis of cytokine containing vesicles) were added one hour after starting the respective assay.

To assess functionality of HBV-specific T cells (**paper III**), PBMCs were thawed and stimulated with 15-mer HBV-specific overlapping peptide pools (core, polymerase, and envelop) that were also used for re-stimulation at day 10. RhIL-2 was added to the culture on day 4 and 8 for T cell survival and expansion. On day 10, T cells were re-stimulated and Brefeldin A was added to prevent cytokine secretion. T cell restoration was evaluated with the addition of anti-PD-L1 and MitoTempo.

To evaluate the general (unspecific) functional capacity of T cells, cells from biliary brush samples and matched PBMCs were stimulated with phorbol 12-myristate-13-acetate (PMA, activates protein kinase C)/ionomycin (triggers calcium release) (**paper V**).

The functional capacity of MAIT cells was tested using thawed PBMCs that were either stimulated with the combination of IL-12 and IL-18 or *E. coli* for 24 hours. To determine which responses were MR1-dependent, anti-MR1 antibody or the IgG2a isotype control was added at the beginning of the assay to the cultures stimulated with *E. coli*. Anti-CD107a antibody was used to detect degranulation upon both stimulations. In order to measure cytokine production, Monensin and Brefeldin A were added for the final 6 hours of stimulation (**paper IV**).

3.6 FLOW CYTOMETRY

Multicolor flow cytometry

Extracellular and intracellular protein expression was detected using flow cytometry, a method that allows the binding of fluorescent dye-conjugated antibodies to their respective antigens on single cells in suspension. The fluorochromes get excited when they pass through a laser beam and emit light at a specific wavelength. The light is directed by mirrors to optical filters that provide spectral resolution and are placed in front of detectors. They restrict the wavelength range of light sensed by detectors that in turn convert it into a digital signal. In addition to fluorescently-labeled antibodies, we visualized biotinylated and purified

antibodies with fluorescent-tagged streptavidin and anti-IgM secondary antibodies, respectively. Dead cells were excluded using LIVE/DEAD cell stain kits. Before intracellular staining, cells were fixed with fixation/permeabilization buffer (eBioscience) (**paper I-V**) or Cytofix/Cytoperm kit (BD Bioscience) (**paper III**). Samples were acquired on a 16-parameter 3 laser/18-parameter 4 laser BD LSR Fortessa (BD Biosciences) (**paper I-V**).

Cell signaling analysis by phospho-flow

Flow cytometry can be used for the analyses of phosphorylated proteins at a single cell level in phenotypically distinct populations directly *ex vivo* or upon different stimulations. Thus, this technique allows the detection of cell signaling events by using phosphor-specific antibodies. Stimulation was stopped with 2% formaldehyde. Cells were stained extracellularly, permeabilized with methanol and subsequently stained for phosphor-epitopes (**paper I**).

Flow cytometry data analysis

Flow cytometry data analysis was performed using FlowJo version 9.9.4. SPICE version 5.3 (**paper I-III, V**) and R version 3.3.1 (**paper I-V**) were used for post-processing of the data. For data visualization and analyses, we used stochastic neighbor embedding analysis (SNE) that reduces a high dimensional dataset to a two-dimensional graph (274). This algorithm clusters cells with similar characteristics together, illustrating multivariate relationships between cells that otherwise may not be detectable or missed by manual gating. Furthermore, conventional gating introduces a bias that can be avoided by using SNE. The clustering is based on markers of interest and compares, using an in-house developed algorithm, two specific groups with substantial differences that are visualized as residual plot. These identified clusters can subsequently be projected onto SNE maps showing the intensity for each marker included in the analysis, allowing the reader to easily compare marker expression in the most distinguishing clusters of both groups.

3.7 MICROSCOPY

Immunohistochemistry

For analysis of protein expression in liver tissue, immunohistochemistry was used. Frozen sections were air dried, fixed with 4% paraformaldehyde and subsequently blocked in two steps, one for elimination of endogenous peroxidase activity (Bloxall) and another one for reduction of general background staining (Innovex background buster). The sections were incubated with the specific primary antibody overnight. This was followed by an incubation with the secondary anti-mouse ImmPRESS antibody that binds to the primary antibody and is coupled to peroxidase. For protein detection, the peroxidase substrate (DAB) is added, which is converted into a brown reaction product. After counter staining with hematoxylin, the tissue slides were analyzed by light microscopy (Leica DM4000B) (**paper V**).

Liver tissue clarification

To visualize protein expression in a 3D liver specimen, liver clarification was performed. In brief, samples were fixed with 2% paraformaldehyde overnight at 4°C. Samples were then sectioned using a vibratome and blocked overnight using Innovex Background Buster. The

tissue was incubated with protein-specific primary antibodies mixed in buffer containing 10% human serum and 0.1% Triton X-100 in TBS, followed by an incubation with the corresponding fluorescently-tagged isotype-specific secondary antibodies together with DAPI staining. Next, the tissue clarification was performed using Ce3D clearing media as previously described (275). After mounting, samples were analyzed with a Nikon Ti-E spinning-disk confocal microscope (20x air/ 60x oil immersion Nikon objective, Andor EM-CCD camera) (**paper V**).

3.8 STATISTICS

Graph Pad Prism version 6 and 7 were used for statistical analysis. Data were tested for normal distribution using D`Agostino-Pearson omnibus normality test. Normally distributed data were analyzed using Student´s paired or unpaired t-test, one-way ANOVA, or Pearson correlation, whereas non-normally distributed data were analyzed with Wilcoxon matched pairs signed rank test, Mann-Whitney test, Kruskal-Wallis test, or Spearman correlation.

4 RESULTS AND DISCUSSION

4.1 NK CELLS IN ACUTE DENGUE VIRUS INFECTION

NK cells have been shown to respond to a variety of viral infections in humans (146, 148, 149, 151, 152, 154) including *flaviviruses* (147, 153, 157, 276). However, few longitudinal studies characterizing NK cell responses during the very first days of acute viral infections and beyond have been performed in humans. Instead, human (live virus vaccination) and murine (infection) model systems have been used to study NK cells in this setting (145, 157, 277). We therefore set out to perform a comprehensive analysis of the very early NK cell response during acute DENV infection and followed the patients into convalescence. For this, blood samples from 25 DENV-infected patients suffering from DF and 18 community-matched controls were collected at Tan Tock Seng Hospital in Singapore (**paper I**).

4.1.1 Less mature NK cells respond during acute dengue virus infection

NK cells from patients with DENV infection were highly activated during the acute phase of the infection with a markedly increased expression of the proliferation marker Ki67, the early activation marker CD69 (**Figure 6**), as well as CD38. This activation was transient and returned to levels that were comparable to healthy controls in the convalescent phase. This response was similar to what has been reported for early NK cell responses in infections with hantavirus (146), TBEV (147), and in YFV vaccination (157). In line with this, other studies reported early NK cell activation in DENV-infected patients (155, 156) and a negative correlation between NK cell numbers and DENV infection disease severity. We could not observe any changes in NK cell frequency throughout the infection and due to only one patient being diagnosed with more severe disease (DHF), it was not possible to correlate NK cell frequency or activation with disease severity. CD69 and Ki67 were expressed in an almost mutually exclusive pattern. The highest frequency of $Ki67^{+}$ cells was observed in the $CD56^{bright}$ NK cell compartment and among the less mature cells of $CD56^{dim}$ NK cells. This seemingly mutually exclusive expression of CD69 and Ki67 was previously shown for NK cells (157) and T cells (278) during acute infections with YFV and HIV-1, respectively. These distinct subsets of either Ki67- or CD69-expressing cells could represent different populations being activated. Alternatively, NK cells might go through different stages of activation, with initial expression of CD69 as an early activation marker, subsequently followed by Ki67 indicating proliferation (157) and potential downregulation of CD69. Indeed, extensive cycling has been shown to downregulate CD69 expression in T cells (279). Notably, the magnitude of proliferation detected during acute DENV infection was stronger than in infection with TBEV and YFV vaccination (147, 157) and more in line with the NK cell response shown in hantavirus infection (146). A similar pattern with respect to CD69 expression was present when comparing response levels in acute DENV infection with other infections (147, 157). Even though CD69 is classically described as early activation marker, it has more recently been appreciated for its function in relation to regulating lymphocyte tissue-residency (16, 100), and CD69 expression could therefore indicate tissue-homing of NK cells. Homing of NK cells to peripheral tissues during acute infection could also be an explanation for the stable NK cell frequencies observed despite concurrent robust proliferation. Alternatively, ongoing proliferation and stable frequencies of cells could also indicate increased levels of apoptosis. However, we observed only a very modest decrease in

Bcl-2, and despite the lack of absolute cell counts supporting these results, this speaks against significant NK cell apoptosis during acute DENV infection.

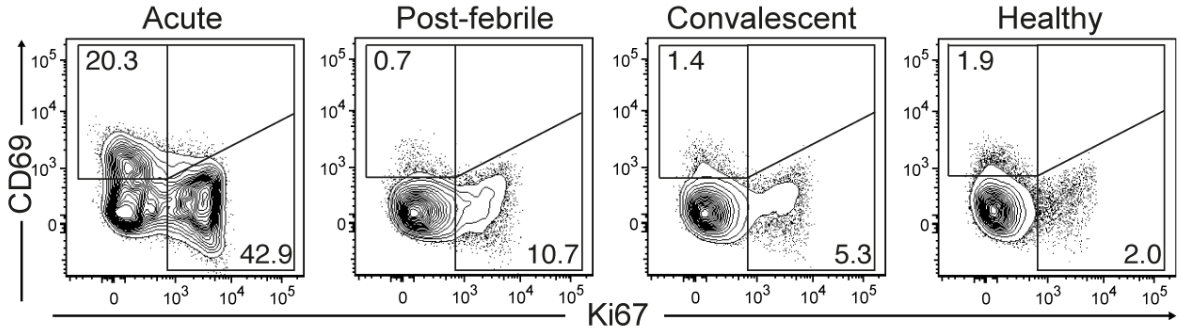

Figure 6: NK cells are highly activated during the acute phase of acute DENV infection. Both CD69 and Ki67 are upregulated during the acute phase of the infection, a response that is transient and returns to levels comparable to healthy controls.

To investigate the phenotype of responding NK cells in more detail, we took advantage of SNE, an algorithm designed for clustering high-dimensional data according to phenotypic similarities in order to display multivariate relationships as two dimensional representations. This enabled us to visualize phenotypic differences between the responding ($Ki67^+$) and non-responding ($Ki67^-$) cells in an unbiased way (280). In the SNE analysis, based on 11 phenotypic markers, we only found minor differences in the $CD56^{bright}$ NK cell compartment. However, $Ki67^+$ $CD56^{dim}$ NK cells displayed a less mature phenotype with higher expression of NKp30 and NKp46 and lower expression of CD57, NKG2C, and DNAM-1 as compared to $Ki67^-$ cells during acute DENV infection. This phenotype argues against an expansion of memory-like NK cells with high expression of NKG2C and CD57 (mature phenotype) that has been shown to expand in some infections (146, 148, 151, 158) but not in others (160, 161), including TBE (147). Nevertheless, the recent association of NK cells with memory-like recall responses during infections raises the questions about (a) which NK cell subsets are activated during secondary DENV infection and (b) whether a new population of NK cells is recruited during each subsequent infection. For our studied cohort, no information on primary/secondary/tertiary infection was available, and the respective analysis could therefore not be performed. Instead, the activation of primarily immature NK cell subsets in our study indicates a cytokine-mediated activation (157, 281) that could potentially occur in lymph nodes, where NK cells reside more frequently during inflammation and might be primed by activated DCs (139, 277).

4.1.2 IL-18-induced signaling plays a role in NK cell activation and is uncoupled from NK cell education

In order to get further insights into the mechanisms involved in the NK cell response observed, we addressed the role of KIR-mediated education and the possible involvement of cytokine-priming. KIR acquisition and education is a process occurring in parallel to maturation, and although our results showed the response to be dominated by less mature NK cells, the role of education is less clear in infectious conditions in humans. Moreover, *flaviviruses* have been shown to upregulate MHC class I expression in infected cells, which might enable evasion from NK cell-mediated recognition via inhibitory KIRs (282, 283) or activation in the context of KIR2DS2 and HLA-C (168). An experimental mouse model with

MCMV revealed the NK cell response to be dominated by uneducated cells, whereas human CMV infection drives the expansion of educated NK cells (160, 163). In other human model systems of acute viral infection, however, the degree of NK cell proliferation was independent of the education status, indicating that cytokine responsiveness is uncoupled from education (157, 281). This is in line with our results, showing that both educated and uneducated NK cells contributed to a similar extent to the NK cell response during acute DENV infection.

Next, we wanted to explore the role of NK cell activating cytokines in the preferential activation of less mature NK cells during acute DENV infection. To this end, we analyzed soluble factors in plasma from the acute phase and found that the NK cell-activating cytokines IL-12, IL-15, and IFNα were significantly increased, albeit present only at low levels. In contrast, IL-18 levels were high and significantly elevated during the acute phase of the infection. NK cell priming with IL-18, together with IL-12, is important to drive IFNγ production (14, 284). In experimental model systems, the combination of IL-12 and IL-18 was shown to drive NK cell activation/proliferation (285-288) and to be critical in the early stage of viral infections, including MCMV, influenza, and vaccinia virus (285, 287, 289). Interestingly, we found the IL-18Rα receptor to be highly expressed on $CD56^{bright}$ NK cells and lower on $CD56^{dim}$ NK cells. Within the $CD56^{dim}$ NK cell subset, IL-18Rα expression was highest on less mature cells ($NKG2A^{+}$ $CD57^{-}$) as compared to terminally differentiated ($NKG2A^{-}CD57^{+}$) NK cells. Furthermore, we studied phosphorylation of IL-18 signaling components known to regulate the cell cycle and survival (290-293). Phosphorylation of nuclear factor-κB (NF-κB), AKT (known as protein kinase B), activating transcription factor-2 (ATF2), and the forkhead transcription factor FOXO3A was substantially increased in NK cells *ex vivo* in patients during acute DENV infection. ATF2 promotes proteins involved in driving the cell cycle, such as cyclins and anti-apoptotic molecules (290). FOXO3A, located downstream of AKT, inhibits proliferation in its unphosphorylated form and translocates, if phosphorylated, into the cytoplasm for degradation (291). NF-κB is multifunctional and induces pro-inflammatory cytokines, adhesion molecules, anti-apoptotic proteins, chemokine receptors, and activation markers, including CD69 (292). This is in line with the induced activation and proliferation in NK cells we observed during the acute phase of the infection. However, it is unlikely that IL-18 is the sole mediator of NK cell activation and most likely acts in concert with other NK cell activating cytokines, such as IL-12, IL-15, and IFNα, which are produced upon activation of the initial targets of DENV, including DCs (294) and macrophages (139). These cytokines may activate NK cells that in turn increase phagocytosis and DC maturation by secreting IFNγ (145). IFNγ production also contributes to Th1 polarization of $CD4^{+}$ T cells that are potent inducers of antigen-specific $CD8^{+}$ T cell responses (295). DCs have also been shown to produce TNF, IL-6, and IL-10 during DENV infection (294), which were all, including IFNγ, found to be elevated in patient plasma during acute infection. This is in line with what has been observed during acute infection with hantavirus (Puumala). Hantavirus is another virus that can cause hemorrhagic fever, where the above-mentioned cytokines, in addition to IL-18, can be elevated (personal communication with K. Maleki). Interestingly, DENV has been reported to interfere with the production of IFNα, which may explain the relatively low, but still significantly increased IFNα levels detected. Moreover, elevated levels of IFNα together with sTRAIL are

associated with mild dengue disease (296). Nevertheless, in the context of DENV infection a combination of IFNα and TNF or IL-18 was shown to activate NK cells (297) or γδ T cells (298), respectively. Moreover, for NK cells this response occurs in a contact-dependent manner (297). In this regard, it is noteworthy that IL-15 is rarely secreted and mainly trans-presented while being bound to IL-15Rα (299). Transpresented IL-15 has been shown to drive activation and proliferation of NK cells in hantavirus infection (300). Despite the potential role of other NK cell activating cytokines, high levels of IL-18 are produced in skin compared to other tissues (GTEx Multi Gene Query database). IL-18 levels were even further increased in skin blisters from DENV-infected patients compared to healthy controls (own results, data not shown), suggesting that cytokine levels may differ at the site of infection as compared to patient plasma.

4.1.3 NK cells potentially home to the skin during acute dengue infection

Besides rapid recruitment of immune cells to target organs (142), NK cell functionality is important for viral control. We found NK cells to be fully functional in response to cytokine-priming as well as to target cell stimulation during acute DENV infection. This was somewhat surprising since NK cell functionality has been shown to be affected during the acute phase in other viral infections. During acute TBEV infection, $CD56^{dim}$ NK cells were functionally impaired upon target cell stimulation but responded well to cytokine stimulation (147). However, contrary to TBEV infection, NK cell functionality was boosted after YFV vaccination, indicating cytokine priming of NK cells *in vivo* (157).

Next, we sought to evaluate whether these responding and functional NK cells were primed for homing to those peripheral tissues that DENV-infected patients have disease manifestations in, such as skin, liver, and gut (138). In this regard, the presence of maculopapular skin rashes is associated with milder disease, which suggests an involvement of anti-viral immune responses (301). Our results revealed a high and increased expression of CLA, CCR5, CXCR3, and CXCR6 in the responding ($Ki67^{+}$) compared to the non-responding ($Ki67^{-}$) $CD56^{bright}$ NK cell compartment. Furthermore, we detected a modest expression of CCR2 and CCR6, as well as a significant increase, but still low expression of CCR9 and CX3CR1, whereas CCR7 was down-regulated. Responding $CD56^{dim}$ NK cells had a similar profile but generally expressed lower levels of homing molecules, especially for CLA, CXCR3, and CCR7. As compared to Ki67-expressing NK cells, $CD69^{+}$ $CD56^{bright}$ NK cells presented with a partly deviating adhesion molecule profile with high expression of CCR5 and CXCR6, intermediate expression of CCR6, but very low expression of CLA.

The high expression of CLA indicated that responding ($Ki67^{+}$) $CD56^{bright}$ NK cells may be primed for skin homing during acute DENV infection. E-cadherin, a ligand for CLA, has been reported to be upregulated during inflammation in the skin (182). Furthermore, $CD56^{bright}$ NK cells have been shown to accumulate in psoriatic skin driven by the migration towards CXCL10 and CCL5, binding to CXCR3 and CCR5, respectively (180). In the same study, the authors could also demonstrate modest expression of CCR6 and low levels of CCR2, CCR4, and CCR7, which is in line with the phenotype we observed. Interestingly, keratinocytes respond to (and produce) IL-18 by producing CXCL10 that might attract $CXCR3^{+}$ NK cells or T cells (141, 302) that in turn produce IFNγ, which potentiates keratinocyte activation (91). As compared to skin-homing NK cells, CCL27-mediated

attraction via CCR10 has been reported for CLA^+ T cells (185). Of note, Rivino *et al.* could recently demonstrate that DENV-specific T cells migrate to the skin during acute infection, with a phenotype mirroring the phenotype of responding $CD56^{bright}$ NK cells we observed in our study, expressing homing receptors including high expression of CLA, CCR5, and CXCR3 (141). Indeed, we could also detect these NK cells being present in skin blister fluid from acute DENV-infected patients with NK cells highly expressing CLA and CD69. CXCR6-expressing NK cells have been shown to be highly enriched in the liver (303). In this regard, increased CXCR6 expression on NK cells during acute DENV infection could be indicative of potential liver homing by these cells. Hudspeth *et al.* demonstrated the presence of $CXCR6^+CCR5^+$ $CD56^{bright}$ NK cells binding to CXCL16 and CCL3 in the liver (177). Staining of liver biopsies identified LSECs and KCs as targets of DENV (304). Interestingly, in a mouse model, infiltrating NK cells were shown to cause liver damage at early stages of the infection (305). However, the role for human NK cells possibility infiltrating liver in severe DENV infection still remains to be explored.

4.1.4 Concluding remarks on NK cells in acute DENV infection

To conclude, we found less mature NK cells to be highly activated and proliferating during the acute phase of the infection. We further identified a potential role for IL-18 in driving this response. NK cells retained their functional capacity throughout infection and exhibited a homing receptor profile indicative for skin homing. Future experiments are needed to address the role of NK cells in disease severity with larger patient cohorts with DF and DHF patients. Furthermore, it would be interesting to evaluate differences between primary and secondary infection in regards to potential NK cell subsets being activated. Finally, *in vitro* infection assays may reveal further insights into the activation of NK cells upon DENV infection. To further investigate tissue homing properties, tissues from different organs including skin and liver should be collected to investigate the presence of an increased NK cell infiltration as well as their homing receptor profile.

4.2 NK CELL AND HBV-SPECIFIC T CELL RESPONSES AFTER STOPPING NA THERAPY IN CHB PATIENTS

The goal of CHB therapy is to achieve a functional cure, which is defined as undetectable HBsAg and HBV DNA in serum with or without HBsAg seroconversion, and to maintain lifelong viral control without anti-viral therapy (187). Despite NA treatment, less than 1% of CHB patients achieve functional cure each year (201) and it has been estimated that the median time until HBsAg clearance is 52.2 years of treatment (196). Therefore, new strategies are needed to achieve a higher degree of HBsAg loss (215). As such, NA cessation was shown to accelerate HBsAg loss at long-term follow up (197, 199, 202).

In order to gain further insights into the underlying immunological mechanisms, we investigated peripheral blood NK cell and T cell responses at treatment termination as well as at week 4, 8, and 12 following NA cessation (**paper II and III**). To this end, 15 HBeAg negative non-cirrhotic CHB patients, who had all been on long-term NA treatment, were included in this prospective study (**paper II and III**). Furthermore, 10 healthy individuals (**paper II and III**) and 4 CHB patients with continuous NA treatment sampled longitudinally (**paper III**) were included as controls. A detailed description of the cohort and the virological

outcome was reported by Siederdissen and colleagues (203). Thirteen out of 15 patients experienced a virological relapse starting at week 4 after stopping treatment, which was followed by a biochemical relapse, peaking at week 8 and week 12, respectively. Most patients were re-treated at week 12. HBsAg levels were stable before and throughout short-term follow up with three patients clearing HBsAg at long-term follow up (**Figure 7**) (203).

4.2.1 Phenotypic imprint of CHB and NA cessation on NK cells and T cells

A previous report exploring early events after stopping NA treatment in the same cohort revealed a potent induction of cytokines and chemokines (203). Hence, re-occurrence of viral replication may affect immune responses. At baseline, however, no changes in peripheral blood NK cells (**paper II**) or T cells (**paper III**) could be detected after long-term NA treatment as compared to healthy controls. This is in contrast to what has been reported for untreated CHB patients, showing increased levels of $CD56^{bright}$ NK cells (216-218) and was more in line with the unaltered distribution of NK cells when treated with NAs (216, 230). Moreover, this is also in contrast to the increased frequency of $CD56^{bright}$ NK cells (231-233) during pegIFNα therapy, which is accompanied by a loss of $CD8^+$ T cells (231).

Next, we investigated the potential effect of stopping NA therapy on the NK cell and T cell phenotype. Using unsupervised SNE analysis, our data revealed major differences between healthy controls and CHB patients before stopping NA treatment (**Figure 7**), which indicates a general imprint of HBV infection on immune cells (**paper II and III**) (208). NK cells (**paper II**) exhibited an increased expression of CD57 and pan-KIR and lower expression of NKG2A. Lower NKG2A and higher CD57, and KIR expression indicates the presence of a more differentiated NK cell compartment (47). As opposed to our results, a meta-analysis of NK cells in CHB demonstrated increased expression of activating receptors. Moreover, we observed lower expression of CD16 and NKp30, which is in line with previous reports (221) on NA-treated patients. Furthermore, we detected lower expression of Siglec-7. The lack of Siglec-7 was associated with a dysfunctional NK subset in HCV (306), which might suggest some degree of dysfunctionality in the NK cell compartment also in CHB. Additionally, a report by Boni *et al.* showed that NK cells in naïve CHB patients have an activated/inflammatory phenotype ($TRAIL^+$, $Ki67^+$, $CD38^+$) that normalized upon NA treatment (217). Thus, our results are more in line with this latter quiescent phenotype. IFNα has been reported to be induced during natural flares of HBV (219) and was shown to initiate, together with concomitant IL-8, NK cell-mediated killing of hepatocytes and/or HBV-specific T cells via TRAIL (219, 226). Tan *et al*. (307) demonstrated, more in line with our results, that IL-8 and CXCL9-10 were the only inflammatory mediators being upregulated without inducing TRAIL expression in flares upon stopping NA treatment. We also reported on the absence of the early activation marker CD69 and TRAIL. Both markers are induced by IFNα (231, 232), hence, the lack of IFNα detected in our cohort (data not shown) would explain this phenotype. Lack of IFNα is more in line with the cytokine pattern observed during acute HBV infection (150). This suggests that we might have missed NK cell activation occurring at a time point earlier than 4 weeks after stopping therapy. Alternatively, IL-10 that was shown to be upregulated at week 4 after treatment discontinuation (203), might have dampened NK cell activation (218).

When analyzing T cells via SNE analysis, our data revealed an imprint of CHB infection (**paper III**) with down-regulation of CCR7 and CD45RA and, hence, an increased frequency of T_{CM} and T_{EM} cells within both $CD4^+$ and $CD8^+$ T cells as well as more T_{EMRA} cells among $CD8^+$ T cells. Additionally, we confirmed a more differentiated phenotype for $CD8^+$ T cells, indicated by higher expression of CD57 and KLRG1. Furthermore, PD-1 expression was increased for both $CD4^+$ and $CD8^+$ T cells, which has been described as an inhibitory molecule typically expressed on exhausted T cells in CHB (235).

Following NA termination, only minor phenotypical changes could be observed for NK cells (**paper II**), whereas T cell markers associated with exhaustion were slightly altered (**paper III**), including an upregulation of PD-1 and down-regulation of TCF-1. $TCF\text{-}1^+$ T cells have been associated with sustained viral control during chronic infections and were described to give rise to TCF^- T cells exhibiting an increased effector potential (247). Our results showed that TCF-1 down-regulation occurred at week 4 when HBV DNA levels increased. Next, we compared the patients who cleared HBsAg at long-term follow up to the remaining cohort. In this analysis, we observed an increased expression of the activation marker CD38 on NK cells at week 12 (**paper II**). Furthermore, the frequencies of both $KLRG1^+PD1^+$ $CD4^+$ and $CD8^+$ T cells of patients who cleared HBsAg were below the mean frequency of the cohort at baseline and thereafter. Moreover, the patients who achieved functional cure also had higher levels of $CD38^+$ $Ki67^+$ T cells that correlated with the HBsAg fold decline (week 48 compared to baseline) (**paper III**). Together, these results show only minor phenotypic alterations of NK cells and T cells (**paper II and III**) and indicate that T cells from patients with subsequent HBsAg loss display a less exhausted and more activated phenotype (**paper III**).

4.2.2 Increased natural cytotoxicity responses are temporally correlated with liver damage and HBsAg loss

Several studies have demonstrated a functional impairment of the NK cell compartment in CHB (217, 218, 221, 222, 227) with retained cytotoxicity, but a decreased capacity for cytokine secretion (218, 221, 222). During anti-viral therapy, this functional dichotomy was reported to be unaffected (217) or improved (221). In order to investigate NK cell functionality after long-term NA treatment at baseline and after stopping NA therapy, we stimulated NK cells with different target cells (K562 cells, 721.221 cells, and rituximab-coated 721.221 cells) to test their capacity for performing natural cytotoxicity and ADCC, and evaluated NK cell functionality upon cytokine stimulation (IL-12+IL-18) (**paper II**). A simultaneous evaluation of five functional readouts (CD107a, IFNγ, TNF, MIP-1β, and GM-CSF) revealed only minor differences when comparing NK cell responses at baseline with healthy controls, with reduced functionality upon cytokine priming, particularly in the $CD56^{dim}$ NK cell compartment. Stopping NA therapy however, significantly boosted NK cell natural cytotoxicity responses, degranulation, and IFNγ, TNF, and GM-CSF production, which reached significance for $CD56^{dim}$ NK cells (**Figure 7**). Hence, NA treatment cessation boosted NK cell multifunctionality at week 12. Interestingly, stopping NA treatment also induced cytokine and chemokine production in this cohort at the time of virological relapse (203), including IL-12 that is known as an NK cell-stimulating cytokine. On the other hand,

simultaneous induction of IL-10 (203) may impact NK cell functionality as previously shown by Peppa and colleagues (218).

Interestingly, we detected positive correlations between natural cytotoxicity-induced functional responses upon stimulation with K562 cells for $CD56^{dim}$ NK cells and ALT-levels at week 8 and week 12 (**Figure 7**), the time points of the peak of HBV DNA and ALT, respectively. Interestingly, high ALT after treatment cessation was associated with HBsAg loss at long-term follow up (203), which was in line with other studies (201, 202), including a study evaluating HBsAg loss in more than 5.400 CHB patients receiving NA treatment (201). Very few correlations were found for other time points or between NK cell phenotypic and clinical parameters. Together, this may indicate a contribution of NK cells to the liver damage following viral relapse as shown for patients with CHB (222) and in contrast to natural flares or IFNα therapy. Natural flares and IFNα therapy are both associated with an upregulation of TRAIL (219, 233, 308) and reduced functionality (233, 308), but also with potential TRAIL-mediated killing of hepatocytes (219). However, our findings were based on prototypic target cell lines and need to be confirmed in a more physiological setting.

When comparing donors who cleared HBsAg to the remaining donors in the cohort, NK cells from the functionally cured group displayed increased NK cell responses towards K562 cell stimulation in particular in the $CD56^{dim}$ NK cell subset. The latter also expressed high levels of CD38 as shown for the patients experiencing HBsAg loss.

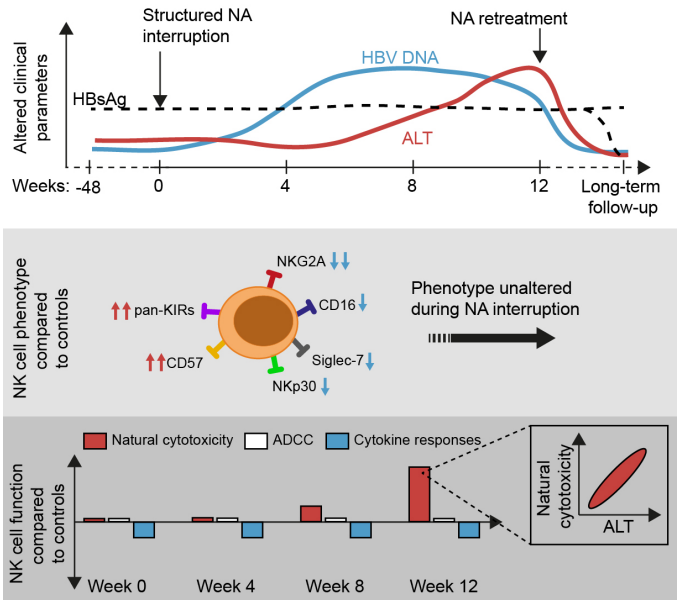

Figure 7: Graphical summary of NK cell responses during NA cessation in CHB. Clinical characteristics of CHB patients undergoing structured NA interruption (top). Phenotypical changes of NK cell at baseline (middle) and increased natural cytotoxicity responses at week 12 after treatment cessation (bottom).

4.2.3 Increased HBV-specific T cell responses upon NA cessation

HBV-specific T cells are believed to be important for viral clearance (235), but are functionally impaired (235-237) and rarely detectable in CHB patients (235). Therefore, therapy aims at boosting the magnitude and quality of virus-specific immune responses (215). Since T cell function is affected by the viral load (235, 237), and NA therapy partially restores HBV-specific T cell responses *in vitro* (250, 251), we set out to explore HBV-specific T cell functionality in patients after stopping NA therapy (**paper III**). HBV-specific T cell responses were evaluated upon stimulation with core-, polymerase-, or envelop-specific overlapping peptides. Our data revealed that HBV-specific $CD4^{+}$ T cells significantly

increased IFNγ production starting at week 4 after treatment cessation and peaked at week 12. Furthermore, an increased production of MIP-1β was detected at weeks 8 and 12. HBV-specific $CD8^+$ T cells showed an increase in IFNγ production at week 12 following treatment discontinuation. Increased IFNγ production was also reported in an early study by Tan *et al*., who however, only observed functional improvement in one out of five patients after NA withdrawal (307). In addition, our results revealed improved multifunctionality at 8 and 12 weeks following treatment cessation with an increased T cell population exhibiting 2 or 3 functions including degranulation and cytokine production simultaneously for both HBV-specific $CD4^+$ and $CD8^+$ T cells. This functional boost was only detected upon stimulation with core-specific, but not for polymerase- or envelop-specific peptides.

Importantly, some degree of heterogeneity in HBV-specific T cell responses was observed on an individual patient basis. Considering responsiveness to different epitopes being present in four different core-specific peptide pools, some patients had a low or strong fluctuation in their T cell response. Nevertheless, stopping NA treatment increased IFNγ production of HBV-functional T cells at least 3-fold for 50%-70% of the patients at week 4 to week 12. Notably, patients who subsequently lost HBsAg did not have the strongest HBV-specific T cell responses, and no correlations were found between the T cell response and the clinical data or HBsAg loss. As opposed to our results, Boni *et al.* could show that the magnitude of the HBV-specific T cell response correlated with HBV DNA in flares of HBeAg positive patients (235). In line with this concept, T cell responses seemed to be associated with the viral rebound in most patients, but also decreased while HBV DNA was continuously increasing. This indicates that increasing HBV DNA levels promote T cell responses by peptides being presented by newly infected hepatocytes together with a modified cytokine milieu (203, 309). Alternatively, increasing HBV DNA levels might induce T cell exhaustion by chronic exposure to antigen (235). In this study, however, early re-treatment might have masked this association. In order to rule out spontaneous fluctuations, and considering the presence of only one baseline sample per patient, four CHB patients with continuous NA treatment were recruited. These patients showed barely detectable HBV-specific T cell responses and only minimal fluctuations over time.

Exhausted HBV-specific T cells have been shown to express the inhibitory receptor PD-1 (235). PD-1/PD-L1 blockade is considered a potential strategy to boost T cell functionality in diverse clinical settings including HBV treatment (235, 241, 242). Indeed, we could show that a stimulation with core-specific peptides together with blocking PD-L1 significantly boosted $CD4^+$ and $CD8^+$ T cell responses, reaching a 2-fold increased IFNγ production in around 50% of the patients at week 8 following treatment discontinuation. In contrast to polymerase-specific T cell responses, PD-L1 blockade also induced a significant increase in envelop-specific T cell responses at week 12 as well as significantly increased multifunctionality upon stimulation with core-specific peptides at all time points studied. PD-1 expression may however be a mechanism to prevent excessive liver inflammation, and PD-L1 blockade could potentially cause T cell-mediated adverse events. Interestingly, Rivino and colleagues demonstrated that patients without flare after NA withdrawal had more vigorous T cell responses during therapy and that these cells were mainly found within the $PD\text{-}1^+$ T cell fraction (253). This suggests a role of PD-1 in preventing over-stimulation of HBV-specific T cells. However, in the study by Rivino *et al*, results were not evaluated in regard to immune

function after stopping therapy or treatment outcome. Furthermore, mitochondrial dysfunction has been linked to be driving T cell exhaustion with an improvement of T cell functionality by using mitochondria-targeted antioxidants, such as MitoTempo (310). Intriguingly, we could show that the addition of MitoTempo improved T cell functionality *in vitro* in two patients at a level comparable to PD-L1 blockade.

4.2.4 Conclusion and future perspectives on immunological events after NA discontinuation

The magnitude and function of the immune response seem to direct the outcome of HBV infections towards either an acute resolving infection or CHB. Thus, in order to establish long-term control of the virus, the aim of immunotherapy is to restore HBV immunity (215).

Here, we reported augmented NK cell natural cytotoxicity responses in HBeAg negative CHB patients at week 12 following treatment cessation that were associated with liver damage that was more pronounced in patients achieving HBsAg loss (**paper II**). Furthermore, we demonstrated that HBV core-specific T cell responses were boosted upon *in vitro* peptide stimulation after treatment discontinuation, a response that was further augmented using PD-L1 blockade (**paper III**). However, we could not find an association between HBsAg loss and HBV-specific T cell functionality and detailed correlations are not possible to perform since only three patients subsequently lost HBsAg. Therefore, our findings need further confirmation using larger cohorts. Nevertheless, we can speculate that the quantity of the response is less important than the quality. Also, our study design restricted NK cell and T cell analysis to only week 4, 8, and 12 after stopping treatment (**paper II and III**). HBsAg loss, however, occurred at long-term follow up and it would therefore be important to study immune responses at later time points. Additionally, early retreatment may have limited further induction of immune responses and/or avoided T cell exhaustion. To gain additional insights into immune responses occurring after stopping NA therapy, a direct analysis of immune cells in the intrahepatic environment is needed to understand the mechanisms underlying HBV control. In that regard, Pallet *et al*. showed that $CD8^+$ T_{RM} cells were enriched in HBV-infected livers compared to healthy livers and expressed significantly more PD-1. Interestingly, the frequency of intrahepatic $CD8^+$ T_{RM} cells was inversely associated to viral load with the highest frequency in patients with well-controlled infection. These cells were maintained and functional in patients who had achieved functional cure (115). Additionally, the intrahepatic $CD8^+$ T_{RM} cells constituted a major fraction of HBV-specific T cells in HBV infected livers with the capacity to produce IFNγ, TNF, and IL-2 (115) with IL-2 being absent or low upon peptide stimulation of peripheral blood derived T cells (252). Moreover, restoration of T cell functionality upon PD-1 pathway blockade has been shown to be more efficient in the intrahepatic compartment as compared to peripheral blood (236).

From an NK cell perspective, HBV infection is known to affect NK cell receptor ligands on hepatocytes (207, 226) and HBV-specific T cells (226). Despite potential viral escape from NK cell recognition, NK cell activation has been shown to inversely correlate to HBsAg levels in CHB patients (311). Alternative treatment approaches are currently being tested including targeting mitochondrial dysfunction, therapeutic vaccination, or combination therapies (215). Furthermore, experimental approaches using methods allowing for a broad

analysis of immune-related markers, such as RNA sequencing, will aid the biomarker discovery. In conclusion, stopping NA therapy is a favorable treatment option for a selective group of patients and may be combined with other treatment strategies to boost HBV-specific immunity.

4.3 ASSESSMENT OF MAIT CELLS IN PSC

MAIT cells are highly enriched in the liver (312) and are well known for their anti-microbial functions and role in inflammatory conditions (72). Interestingly, MAIT cells have been shown to localize around bile ducts in portal tracts (272). Together, this suggests MAIT cells to possess an important function in liver immune surveillance (313). For this reason, we studied MAIT cells in PSC, a chronic inflammatory disease of the bile ducts that is often associated with concomitant IBD (**paper IV**). We used a cohort of 28 PSC patients of whom 23 had PSC and IBD, and five patients with PSC only. Twelve healthy subjects were recruited as controls. Additionally, seven IBD patients and eight patients with primary biliary cholangitis (PBC), another autoimmune cholestatic disease of the small intrahepatic bile ducts, were included as diseased controls. To investigate the presence of MAIT cells in the biliary epithelium, we used cells acquired from biliary brush samples collected during ERCP of PSC patients and as controls, patients undergoing ERCP for other reasons with no suspicion of biliary inflammation (**paper IV and paper V**).

4.3.1 MAIT cell frequencies are decreased in blood but retained in bile ducts

In order to gain further insights into the role of MAIT cells in PSC, we first investigated their presence in peripheral blood in PSC patients and controls. MAIT cells were severely reduced in PSC patients and disease controls as compared to healthy controls. This is in line with other studies that reported a selective loss of MAIT cells in chronic inflammatory liver diseases, such as chronic HCV (314), non-alcoholic steatohepatitis (NASH) (315), alcoholic liver disease (316), PSC, and PBC (315). This suggests that the loss of MAIT cells is not specific for biliary inflammatory diseases. Indeed, a severe reduction of MAIT cells has also been shown for patients with, among other chronic inflammatory diseases, systemic lupus erythematosus (317), type 2 diabetes (318), and IBD (84, 319). Interestingly, MAIT cells have been shown to home to the inflamed intestine in IBD (84, 319) and were also shown to infiltrate the portal tracts of the liver in chronic inflammatory liver disease (272). To test whether MAIT cells are present in the biliary epithelium, we analyzed cells acquired from biliary brush samples. As compared to peripheral blood, MAIT cells were found to be four times increased in bile ducts. The frequency and absolute number of biliary MAIT cells in PSC did not differ as compared to non-PSC controls, suggesting that MAIT cells are retained at the site of inflammation in PSC.

4.3.2 Peripheral blood MAIT cells are phenotypically activated, but dysfunctional in PSC

To investigate the potential activation of MAIT cells in PSC, we evaluated the phenotype using SNE analysis and found an increased expression of CD69, CD56, and NKG2D and decreased expression of CD28, CD127, and CXCR6. Some of these findings could be recapitulated with manual gating. Conventional gating revealed an increase in CD39, an ectonucleotidase being associated with terminally exhausted T cells (320), and PD-1, a

marker of exhausted/recently activated cells (320). CD69 is classically seen as an activation marker in peripheral blood, whereas CD56 was associated with a subset of MAIT cells with high expression of IL-12R, IL-18R, Eomes, and T-bet (75). CD28 and CD127 may get down-regulated upon MAIT cell activation or after cytokine stimulation (66, 321). A decrease of CXCR6, which is associated with migration to the liver, could indicate that MAIT cells have homed to the liver. Interestingly, this activated phenotype was not unique for PSC and also seen in IBD and PBC patients, suggesting that this is a common MAIT cell phenotype in chronic inflammatory diseases. Indeed, an activated ("exhausted") phenotype was reported in other chronic inflammatory diseases, including autoimmune hepatitis, NASH (315), alcoholic liver disease (316), type 2 diabetes (318), HIV-1 (322), and HCV (314).

Finally, we set out to test the function of the remaining MAIT cell population in blood upon stimulation with *E. coli* or cytokines (IL-12+IL-18). Circulating MAIT cells were functionally impaired upon *E. coli* stimulation with a reduction of CD107a, TNF, IFNγ, and CD69, which was MR1-dependent, whereas almost no differences were detected upon cytokine stimulation, except TNF that was found to be reduced. These results reflect what has been shown in chronic HCV (314) as well as the functional impairment of MAIT cells in HIV (322). Böttcher and colleagues recently reported functional exhaustion of MAIT cells in autoimmune liver diseases, including PSC (315). As opposed to our results, they showed that MAIT cells retained their capacity to produce TNF and IL-17. In this regard, HSC-MAIT cell co-cultures resulted in activation of HSC with increased expression of pro-fibrotic genes, suggesting a contribution of IL-17 produced by MAIT cells to liver fibrosis (315).

Low frequencies of circulating MAIT cells in chronic inflammatory diseases including PSC (84, 314-319) together with the activated phenotype suggest reduced survival due to a possible common mechanism. Chronic stimulation *in vivo* has been proposed to cause MAIT cell loss due to activation-induced cell death. This would be in line with an activated phenotype that we and others have found, followed by dysfunctionality and finally apoptosis. Loss of gut integrity causing increased translocation of intestinal bacteria, bacterial antigens, or metabolites was suggested to be the driving force (316, 323). Other diseases, such as HCV (314) and alcoholic liver disease (316) are associated with a so called "leaky" gut, which could also occur in PSC, considering the high frequency of patients with concomitant IBD. To test this hypothesis, markers for microbial translocation can be assessed, including sCD14 or sCD163 that indicate activation of macrophages, or markers for damage of the intestinal epithelium, such as fatty acid binding protein (324). No associations between the loss of MAIT cells and clinical characteristics could be found in our cohort. One explanation for the missing association might be that all patients had mild disease and larger cohorts with a substantial number of patients with more terminal disease would be required. Also, age and gender impact MAIT cell numbers of individuals (325) with a possible effect on MAIT cell loss, especially in the slight gender/age unmatched PBC group in our study. Hedge *et al.* suggested that MAIT cells themselves drive pro-inflammatory properties of hepatic myofibroblasts and monocyte-derived macrophages that in turn secrete IL-6 and IL-8, thereby exhibiting a pro-fibrotic function (326). Activated monocytes or macrophages in turn may drive MAIT cell activation through secretion of IL-12 and IL-18. Interestingly, Böttcher *et al*. could show that long-term stimulation with IL-12 and IL-18 drives MAIT cell exhaustion (315). We, however, did not determine the levels of cytokines in our cohort.

Nevertheless, both IL-12 and IL-18 are known to be increased in autoimmune liver diseases (315). To further investigate whether MAIT cells are truly exhausted, transcription factors can be assessed. Indeed, downregulation of Eomes, T-bet, and RORγt has been associated with the loss of effector functions of MAIT cells (83, 316).

In conclusion, we found a severe reduction of circulating MAIT cells in PSC. Remaining MAIT cells exhibited an activated phenotype and impaired MR1-dependent functionality. It is yet unclear whether circulating MAIT cells die or home to peripheral tissues in chronic inflammatory diseases in humans. Also, it remains to be determined whether MAIT cells are protective or harmful, eventually contributing to the development of fibrotic strictures at the site of inflammation.

4.4 EXPLORATION OF THE IMMUNE SYSTEM IN THE BILIARY TREE

The biliary epithelium is affected by various liver diseases, including PSC (254). BECs serve as a mucosal barrier and can sense and respond to pathogens. Furthermore, the bile duct is in continuity with the gut flora (121, 268). However, very little is known about the immune system within the bile duct. In order to characterize the biliary immune landscape during inflammation and at steady state, we developed a new method to assess cells isolated from biliary brush samples collected during ERCP and compared those to matched peripheral blood (**paper V**). Samples were taken from inflamed bile ducts of 62 PSC patients and 41 non-PSC controls. The latter underwent ERCP due to other indications, including bile duct stone disease, chronic pancreatitis, malignant stricture, papillary adenoma, post-operative stricture, or an unknown stricture.

4.4.1 Biliary inflammation is associated with cellular infiltration of neutrophils

Tissue inflammation is generally characterized by infiltration of leukocytes, including neutrophils (327). In line with this, we found a five-fold increase of leukocytes and a considerably increased number of neutrophils, making up to 30% of the total cellular infiltrate, in inflamed (PSC patients) as compared to non-inflamed bile ducts. Since very few neutrophils were detected in the non-inflamed control samples, this validates the integrity of this group as a control cohort. An increased presence of neutrophils in the inflamed samples is not surprising since neutrophils are known to be rapidly recruited during liver inflammation, attracted by chemokines or ATP, which is released by necrotic cells (327). BECs themselves have been shown to be immunologically active cells and to play a role in infectious but also non-infectious hepatobiliary diseases (131, 268). As such, they have the capacity to induce inflammation by secreting pro-inflammatory cytokines, including IL-1β, IL-6, and TNF, but also CXCL8 (268) that is known for attracting neutrophils. Indeed, CXCL8 has been shown to be upregulated in patients with cholestatic diseases, which could indicate a general involvement of this pathway in biliary pathologies (126).

4.4.2 Conventional $CD8^+$ effector memory T cells are the dominant tissue-resident population in bile ducts

Next, we set out to explore the biliary immune system with regard to the presence of IELs. IELs have been described to reside and patrol epithelial surfaces in the intestine, lung, and skin, where they play an important role in tissue homeostasis and disease (109, 110, 114,

328). Therefore, we evaluated the expression of the tissue-residency markers CD69, CD49a, and CD103 (100). All markers were expressed by cells from the biliary epithelium with a substantial fraction of $CD69^+CD103^+$ cells that were highly prevalent among cells isolated from bile ducts. Interestingly, these cells were less frequent in the liver parenchyma and/or in sinusoidal blood and very low in peripheral blood (**Figure 8**). This was in line with our observation that intra- and extrahepatic BECs expressed high levels of E-cadherin, the ligand of CD103 integrin, whereas only low expression could be detected on hepatocytes. Indeed, $CD69^+CD103^+$ IELs were shown to reside in barrier tissues, and were recently also reported to be present in the liver (115, 329). We detected the highest frequency of these cells in samples taken from the biliary epithelium, which suggests that intrahepatic $CD69^+CD103^+$ IELs may be located in proximity to the bile ducts. This further suggests that different intrahepatic tissue-resident lymphocyte populations might inhabit distinct niches within the liver and that this is regulated by the different tissue-residency markers.

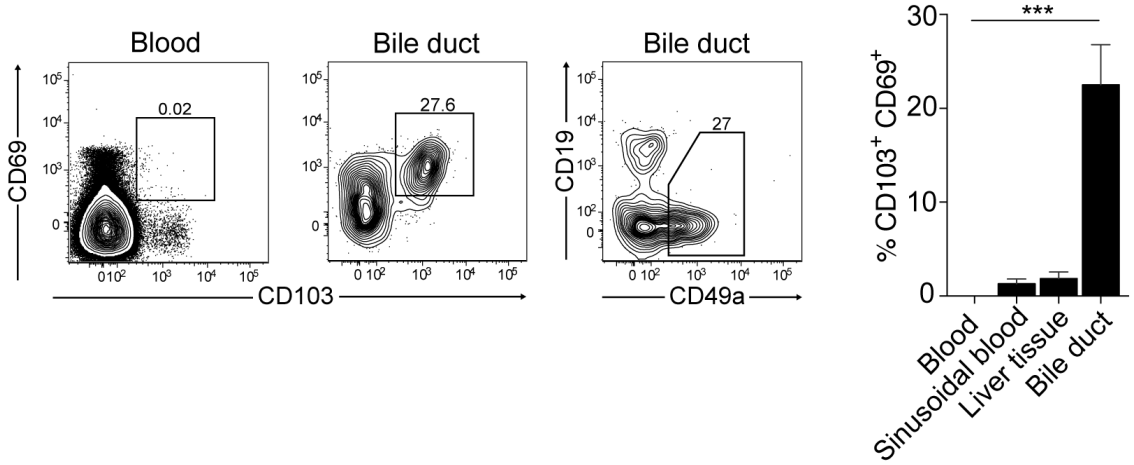

Figure 8: Cells isolated from the biliary epithelium express tissue-residency markers. Tissue-resident cells are detected in proximity to bile ducts. Shown is the frequency of $CD103^+CD69^+$ cells out of live $CD45^+$ leukocytes.

In order to unravel the composition of the $CD69^+CD103^+$ cells compared to circulating ($CD69^-CD103^-$) cells from bile ducts, a SNE algorithm was used that reduces high dimensionality of complex data but keeps multivariate relationships without introducing a bias from conventional gating (280). The SNE analysis revealed expression of the tissue-residency markers investigated with a dominant co-expression pattern of CD69 and CD103. Furthermore, the data revealed $CD8^+$ T cells to be the main subset being present within this population of tissue-resident cells (**Figure 9**). This was also accompanied with a $CD4^+/CD8^+$ T cells ratio shift as compared to peripheral blood towards more CD8 T cells. This is in line with what has been reported for IELs in other tissues, including intestine, skin, and lung (103). Besides changes in the T cell compartment, we also found minor changes among other subsets, including a lower frequency of NK cells and monocytes/macrophages in bile ducts as compared to peripheral blood. These findings could be recapitulated with conventional gating and were applicable for both inflamed and non-inflamed bile ducts.

Next, we investigated the nature of the biliary T cell compartment in more detail. Different T cell memory subsets can be identified using CCR7 and CD45RA (67). Our results revealed that the majority of biliary-resident T cells within both the $CD4^+$ and the $CD8^+$ T cell

compartment had a T_{EM} phenotype. The non-tissue-resident T cell population from bile duct samples showed a profile similar to peripheral blood with a fraction of naïve T cells, some T_{EM}, and a population of T_{CM} within the $CD4^+$ T cell compartment. Generally, $CD8^+$ T cells contained a larger population of T_{EMRA} as compared to $CD4^+$ T cells. IELs from the small intestine are well-known to contain a major population of TCRαβ CD8αβ effector memory T cells (109, 110). Future experiments using microscopy shall address the location of these cells in liver tissue. IELs in mice often comprise a population of TCRαβ CD8αα-expressing T cells (108, 109). As opposed to mice, our results revealed that CD8αα co-receptor usage was extremely rare among the CD8 T cells in bile ducts, which supports the literature with respect to the controversial existence of a CD8αα population in humans (109). Based on differences in the tissue microenvironment, however, TCRαβ CD8αα T cells may still exist among IELs at a different location or very low frequency in humans.

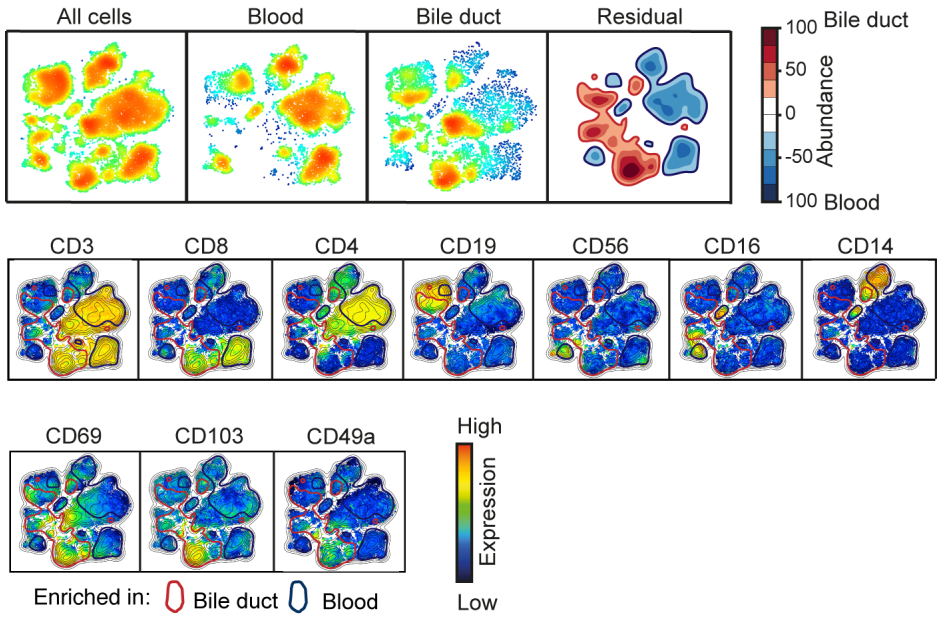

Figure 9: $CD8^+$ T cells are the main population within tissue-resident cells in bile ducts. SNE analysis showing the abundance of cells from bile ducts (red) and blood (blue). Relative expression intensities of single markers with phenotypes being more common in bile ducts and blood encircled in red and blue, respectively.

Unconventional T cells are known to contribute to the pool of IELs (101). In our analysis, MAIT cells were enriched and γδ T cells slightly increased among tissue-resident cells in bile ducts as compared to the circulating population and peripheral blood. MAIT cells are known for being enriched in the gut (71, 84), but were also found in other tissues (72, 73, 85, 330), including a population expressing CD103 (86, 330). IELs contain a substantial population of γδ T cells in humans (101). In bile ducts however, they may not be selectively enriched. Nevertheless, we generally found higher frequencies of γδ T cells in PSC patients than in controls. This is in line with earlier reports showing an increase in γδ T cells in PSC patients in blood and portal tracts (331). Interestingly, γδ T cells were also reported to be associated with disease severity in patients with IBD (332). Considering the relatively low frequency of unconventional tissue-resident T cells, our results revealed that the main population in bile ducts was comprised of conventional TCRαβ CD8αβ memory effector T cells.

4.4.3 Tissue-resident T cells exhibit a distinct chemokine receptor and functional profile

Cellular migration is guided by environmental cues that direct immune cells to target tissues (89, 333). Due to the direct connection with the intestine, we hypothesized that the homing receptor expression of tissue-resident and circulating $CD8^+$ T cells from inflamed and non-inflamed bile ducts might have shared features between liver (CXCR6) and gut (CCR9, α4β7). We also evaluated CCR6 and CXCR3 expression, known to be important for homing to mucosal or inflamed tissues, respectively (271, 333-335). Bile duct-resident T cells displayed a distinct homing receptor profile with high expression of CXCR6 and α4β7 (**Figure 10**). Expression of CCR6 and CCR9 was modest but still increased in biliary-resident cells as compared to circulating T cells from bile ducts or blood and mostly expressed on cells co-expressing CXCR6 or α4β7. The overall homing receptor profile of circulating T cells from bile ducts was more similar to the one in peripheral blood as compared to the profile of the tissue-resident population (**Figure 10**). $CD8^+$ T cells from non-inflamed bile ducts exhibited a similar profile to the inflamed bile duct with a trend towards higher expression of CXCR3 and lower expression of CCR9 as compared to samples from PSC patients. Interestingly, not only LSECs but also BECs are known to express membrane-bound CXCL16 and also secrete CCL20, which indicates that both CXCR6 and CCR6 may play a role in the localization of lymphocytes in the liver (115, 131, 270, 333). Eksteen *et al.* could show that only gut-derived DCs, but not liver DCs, had the capacity to selectively prime T cells for gut-homing via CCR9 binding to CCL25 (335). In regard to PSC pathogenesis, it was hypothesized that gut-primed T cells home to the liver due to an inflammation-driven aberrant expression of CCL25 and MadCAM-1 in addition to liver homing molecules (271, 336). Indeed, we observed a tendency towards higher CCR9 expression in inflamed bile ducts from PSC patients than in non-inflamed bile ducts from controls, substantiating such a hypothesis. During inflammation, the CXCR3 ligands CXCL9-CXCL11 are produced by hepatocytes, cholangiocytes, and HSC (337) and were shown to promote recruitment of CXCR3-expressing cells to the liver in mice (270). High CXCR3 expression was also confirmed to a tissue-resident T cell population in human liver (115). Likewise, the importance of CXCR3 expression for tissue-localization was also emphasized in other organs, such as skin and lung (114, 328). In conclusion, biliary-resident T cells co-express a gut and liver homing receptor profile.

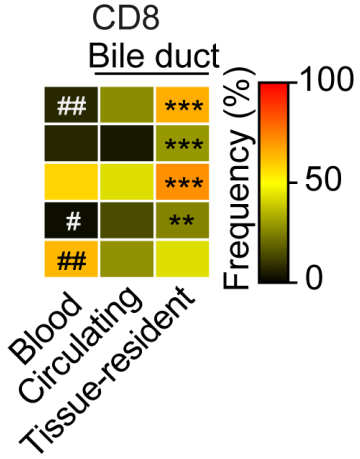

Figure 10: $CD8^+$ biliary-resident T cells express a gut and liver homing receptor profile. Heat map summarizing the frequency of expression. Stars and hashes indicate statistical difference for the tissue-resident vs. circulating population and tissue-resident vs. blood, respectively.

IELs are essential for epithelial barrier integrity and protection. Upon excessive activation, however, they may contribute to disease (110). We compared the production of cytokines upon stimulation with PMA/ionomycin of $CD8^+$ tissue-resident ($CD103^+$) T cells to the circulating population ($CD103^-$) from bile ducts as well as to $CD8^+$ T_{EM} cells from peripheral blood. Biliary-resident cells produced all investigated cytokines, with the highest production of TNF and IFNγ. TNF and IFNγ were produced by circulating and tissue-resident $CD8^+$ T cells to a similar level as well as by peripheral blood $CD8^+$ T_{EM} cells (**Figure 11**). During infection, TNF and IFNγ induce upregulation of TLRs, HLA class I and II, as well as adhesion molecule expression on BECs (268). This results in increased cytokine production and T cell-cholangiocyte cross-talk. Both cytokines also induce chemokine secretion, including CCL2, CXCR8 (IL-1β, TNF), and CXCL9-CXCL11 (IFNγ), thereby attracting other immune cells. The production of TNF and IFNγ may also play a role in disease pathology by causing epithelial barrier disruption via the induction of apoptosis or reduction of tight junction proteins (110, 335).

We found an increased production of IL-2, IL-17, and IL-22 in the biliary-resident T cell population compared to the circulating one from bile ducts or blood (**Figure 11**). Increased production of IL-2 is in line with an earlier report by Pallet *et al.* (115) and may indicate the requirement of IL-2 in an environment with little presence of $CD4^+$ T cells. Also, *IL-2RA* is one of the PSC-associated SNPs although the exact mechanism of action for this SNP to potentially contribute to disease is unknown (126). Th17 cell responses have been linked to responses to microbes in PSC (266). Both IL-17 and IL-22 are involved in the recruitment of neutrophils and the production of anti-microbial peptides (111, 338-340). Generally, IL-17 is considered to induce an inflammatory response by secreting cytokines and chemokines, including the production of IL-6, GM-CSF, CCL2, CCL20, and CXCL8, leading to the chemo-attraction of macrophages, neutrophils, and CCR6-expressing T cells. IL-22 is known for its regenerative capacity. However, if IL-17 and IL-22 are not tightly controlled, both cytokines can contribute to autoimmune or chronic inflammatory processes (338). In summary, biliary-resident T cells are functional with a Th1/Th17/Th22-like profile.

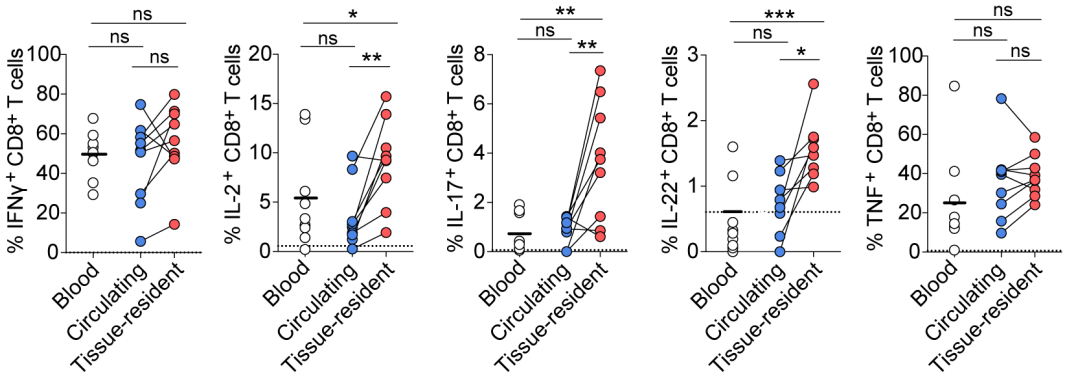

Figure 11: Functional profile of $CD8^+$ T cells from blood and bile ducts. Cytokine production from effector memory $CD8^+$ T cells from peripheral blood (line indicates the mean) was compared to T cells from bile ducts. The dotted line indicates the background of the respective cytokines detected in the unstimulated controls.

Bile duct injury and chronic inflammation in PSC is known to contribute to concentric fibrosis (131, 256). Dysregulated myofibroblasts drive fibrosis with both HCS and portal

fibroblasts being engaged in biliary fibrosis initiation (268). Upon injury, cholangiocytes themselves promote worsening of liver damage by responding with proliferation, immune cell recruitment, and fibrosis initiation, a process termed ductular reaction. IL-6 is produced by activated BECs and drives proliferation in an autocrine fashion (131, 268). Additionally, cholangiocytes actively contribute to the recruitment of KCs/macrophages that in turn secrete pro-inflammatory cytokines and are producers of TGF-β, which is a potent activator of HSC (327). Another cytokine implicated in the development of liver fibrosis is IL-17 (327, 341), potentially derived from highly functional biliary-resident T cells. IL-17 activates KCs to increase cytokine production, including IL-6, IL-1β, TNF, and TGF-β. Moreover, IL-17 promotes fibrogenic myofibroblast transformation as well as the production of collagen (341) and can directly affect cholangiocytes (342). Cholangiocytes in turn augment the production of IL-17 by initiating Th17 cell development and recruitment by cytokine and chemokine secretion (342). Thus, complex immunologic pathways contribute to fibrosis development with both cholangiocytes and recruited immune cells including biliary-resident T cells, actively contributing to dysregulation of myofibroblasts.

4.4.4 Conclusions and future perspectives on biliary-resident T cells

Using immune cells isolated from brush samples, we established a new method allowing for a high resolution single cell analysis of the biliary immune system. We found increased cellular infiltration of neutrophils as well as a substantial population of biliary-resident TCRαβ CD8αβ effector memory T cells with a distinct homing receptor and functional profile. In this study, certain limitations should be considered. Due to low cell numbers, we could not evaluate functionality of biliary T cells from non-inflamed controls. However, methods other than flow cytometry might be applicable to gain deeper insights into the nature of the cells residing in the biliary tree, for example single cell sequencing. Such data could indicate an origin of these cells based on TCR sequencing, enabling the investigation of overlapping clonotypes that may also indicate antigen-driven expansions (265). Moreover, sequencing data may be used for pathway analysis or a comparison to reference datasets on T cells residing in other organs, such as the gut. We did not get the possibility to collect matched samples from inflamed and non-inflamed areas of the biliary tree and/or the gut to be able to link these results with clinical data. Future studies shall address immunological correlates with clinical characteristics of PSC patients in order to get further insights into the PSC pathogenesis. This knowledge may aid treatment development for PSC.

5 CONCLUDING REMARKS

Lymphocytes, including NK cells and T cells, are important players in the human immune system. Relying on experimental model systems is not sufficient in order to understand immunological mechanisms in multifaceted human disease settings. Therefore, studying lymphocytes in human diseases will provide further insights. A summary of the key findings presented in this thesis are listed below:

- NK cells are highly activated and proliferate in acute DENV infection, a response that is mainly confined to immature NK cell subsets, most likely driven by cytokines and uncoupled from NK cell education (**paper I**)
- Responding NK cells exhibit a homing receptor profile indicating potential migration to peripheral tissues in acute DENV infection, particularly the skin (**paper I**)
- As compared to healthy controls, NK cells and T cells exhibit phenotypical changes upon long-term NA treatment in HBeAg neative CHB patients with only minor phenotypical changes upon treatment cessation (**paper II and III**)
- Stopping NA therapy in HBeAg negative CHB patients augments NK cell natural cytotoxicty responses, which coincides with liver damage and functional cure in a subset of patients (**paper II**)
- Structured NA discontinuation improves the exhausted HBV-specifc T cell functionality in CHB, and this can be even further boosted upon PD-L1 blockade (**paper III**)
- The MAIT cell compartment is affected in PSC patients with reduced frequencies and functional impairment in circulation, whereas levels of MAIT cells are retained within bile ducts, the tissue affected by PSC (**paper IV**)
- The biliary immune system contains a substantial population of biliary-resident cells expressing CD69 and CD103 that differ in the immune compositon as compared to $CD69^{-}CD103^{-}$ cells from bile ducts and immune cells from peripheral blood (**paper V**)
- The main population of tissue-resident immune cells within bile ducts are TCRαβ CD8αβ effector memory T cells that co-express gut and liver homing receptors and possess a Th1/Th17 functional profile (**paper V**)

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