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POTENTIAL ROLE OF MAIT CELLS IN CANCER IMMUNOTHERAPY AND VIRAL INFECTION

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Potential role of MAIT cells in cancer immunotherapy and viral infection

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

Mucosa-associated invariant T (MAIT) cells are an unconventional T cell subset with specialized antimicrobial functions. In humans, MAIT cells are characterized by their effector memory phenotype, rapid cytolytic capacity, and tissue-homing properties. Given their natural enrichment in the liver and mucosal barrier tissues, this thesis aimed to explore the potential of redirecting MAIT cells for hepatitis virus-related liver cancer immunotherapy using MHC class-I restricted T cell receptors (TCRs). Additionally, we sought to explore the role of MAIT cells in the context of another front-line immune tissue, the female genital mucosa, in human immunodeficiency virus type 1 (HIV-1) infection.

In **paper I**, a MAIT cell expansion methodology was developed to generate clinically relevant quantities of human MAIT cells for immunotherapies. The MAIT cell expansion protocol was highly reproducible and resulted in MAIT cell cultures with an activated phenotype, enhanced cytolytic potential, and improved tissue homing capacity. These features make them particularly suitable for solid tumour-targeting. We tested this hypothesis in **paper II**, by engineering expanded MAIT cells to express a TCR with specificity for hepatitis B virus (HBV). Using 2D and 3D hepatoma cell models of HBV-related hepatocellular carcinoma, we compared the antiviral, cytotoxic, and tumour homing properties of the TCR-redirected MAIT cells with those of conventional T cells currently being used in the clinic. We demonstrated that the MAIT cells not only acquired a distinct polyfunctional antigen-specific profile in response to HBV, but also retained their antimicrobial properties, and readily migrated towards the tumor targets in a 3D microfluidic model. In **paper III**, we investigated the phenotype of MAIT cells in women living with chronic HIV-1 infection. We initially confirmed that circulating MAIT cells were activated and numerically depleted in the blood. However, MAIT cells were preserved in the cervical mucosa. The *TRAV1-2-TRAJ20* MAIT TCR transcript was highly expressed in the ectocervix and was significantly upregulated in the HIV-1 infected women relative to uninfected controls. This indicates that MAIT cells may be poised to recognize stimuli unique to that environment, and that HIV-1 infection may shape the MAIT cell population in the female genital tract.

In summary, the research covered by this thesis demonstrates the potential role of MAIT cells in the context of virus-related cancer immunotherapy and barrier tissue infiltration. Taken together, these findings illuminate new avenues of research in MAIT cell biology in human health and disease.

LIST OF SCIENTIFIC PAPERS

I. Parrot T, **Healy K**, Boulouis C, Sobkowiak MJ, Leeansyah E, Aleman S, Bertoletti A, Sällberg Chen M, Sandberg JK. Expansion of donor-unrestricted MAIT cells with enhanced cytolytic function suitable for TCR-redirection. *JCI Insight. 2021 Feb 9:140074*

II. **Healy K**, Pavesi A, Parrot T, Sobkowiak MJ, Reinsbach SE, Davanian H, Tan AT, Aleman S, Sandberg JK, Bertoletti A, Sällberg Chen M. Human MAIT cells endowed with HBV specificity are cytotoxic and migrate towards HBV-HCC while retaining antimicrobial functions. *JHEP Rep. 2021; 3:100318*

III. Gibbs A*, **Healy K***, Kaldhusdal V, Sundling C, Franzén-Boger M, Edfeldt G, Buggert M, Lajoie J, Fowke K, Kimani J, Kwon D, Andersson S, Sandberg JK, Broliden K, Davanian H, Sällberg Chen M, Tjernlund A. MAIT cells are preserved in the cervical mucosa of HIV infected women and exhibit a dominant usage of the Vα7.2-Jα20 TCR. *Manuscript*

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II. Svensson Akusjärvi S, Ambikan AT, Krishnan S, Gupta S, Sperk M, Végvári Á, Mikaeloff F, **Healy K**, Vesterbacka J, Nowak P, Sönnerborg A, Neogi U. Integrative proteo-transcriptomic and immunophenotyping signatures of HIV-1 Elite Control phenotype: A cross-talk between glycolysis and HIF signaling. *Accepted. iScience.*

III. Blixt L, Bogdanovic G, Buggert M, Gao Y, Hober S, **Healy K**, Johansson H, Kjellader C, Mravinacova S, Muschiol S, Nilsson P, Palma M, Pin E, Smith CIE, Stromberg O, Sällberg Chen M, Zain R, Hansson L[#], Österborg A[#]. Covid-19 in patients with chronic lymphocytic leukemia: clinical outcome and B- and T-cell immunity during 13 months in consecutive patients. *Leukemia, 2021.*

IV. Alkharaan H, Bayati S, Hellström C, Aleman S, Olsson A, Lindahl K, Bogdanovic G, **Healy K**, Tsilingaridis G, De Palma P, Hober S, Månberg A, Nilsson P, Pin E, Sällberg Chen M. Persisting Salivary IgG Against SARS-CoV-2 at 9 Months After Mild COVID-19: A Complementary Approach to Population Surveys. *J Infect Dis, 2021. 224(3): p. 407-414.*

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VI. Davanian H, Gaiser RA, Silfverberg M, Hugerth LW, Sobkowiak MJS, Lu L, **Healy K**, Sandberg JK, Näsman P, Karlsson J, Engstrand L, Sällberg Chen M. Mucosal-associated invariant T cells and oral microbiome in persistent apical periodontitis. *International Journal of Oral Science, 2019. 11(2): p. 16.*

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IX. **Healy K**, Freij U, Ellerstad M, Aulin LBS, Brückle L, Hillmering H, Sällberg Chen M, Gustafsson R. Evaluating the prevalence of Hepatitis E virus infection in a large cohort of European blood donors, 2015-2018. *Submitted manuscript.*

* Shared contribution

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LIST OF ABBREVIATIONS

ABCB1	ATP binding cassette subfamily B member 1
ACT	Adoptive cell transfer
AFP	Alpha-fetoprotein
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ART	Antiretroviral therapy
AUC	Area under the curve
CAR	Chimeric antigen receptor
CCL	Chemokine (C-C) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CRC	Colorectal cancer
Ct	Comparative threshold
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C) ligand
CXCR	C-X-C chemokine receptor
DCM	Dead cell marker
DN	Double negative
EGA	European genome-phenome archive
FSC	Forward scatter
FSW	Female sex worker
GALT	Gut-associated lymphoid tissue
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GMP	Good manufacturing practice
GPC-3	Glypican-3
Gt	Genotype
Gzm	Granzyme
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen – DR isotype
I-FABP	Intestinal fatty-acid binding protein
IFN	Interferon
IL	Interleukin
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LR	Low risk
MACS	Magnetic cell selection
MAGE-A1	Melanoma-associated antigen 1
MAIT	Mucosa-associated invariant T
MDR1	ATP-binding cassette-multi-drug efflux protein
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
mRNA	Messenger ribonucleic acid
MR1	MHC-Ib related
NIH	National Institutes of Health
NK	Natural killer
NKT	Natural killer T
NY-ESO-1	New York esophageal squamous cell carcinoma-1
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein-1
PLWH	People living with human immunodeficiency virus type-1
PLZF	Promyelocytic leukemia zinc factor

PMA	Phorbol myristate acetate
qPCR	Quantitative PCR
RORγt	Retinoid-related orphan receptor γ
sCD14	Soluble CD14
SSC	Side scatter
STI	Sexually transmitted infection
TAA	Tumour associated antigen
TAC	Tacrolimus
T-bet	T box transcription factor 21
TCR	T cell receptor
TGF-β	Transforming growth factor β
TIGIT	T cell immunoreceptor with Ig and ITIM domain
TIL	Tumour infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TNF	Tumour necrosis factor
t-SNE	t-distributed stochastic neighbor embedding
UMAP	Uniform manifold approximation and projection
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil

1 INTRODUCTION

Over the course of evolution, humans have developed defense mechanisms to protect us from invading pathogens and the virulence factors they produce. Exposure to pathogens, be they viral, bacterial, fungal, or parasitic in origin, triggers a sequence of highly coordinated immune cascades with the purpose of clearance of the infection. Additionally, the immune system can recognize self-ligands in the context of cancer, thereby protecting us from malignant transformed cells. These events are mediated by various cellular effector cells and humoral immune responses. Classically, the immune system is broadly divided into two components: the innate and adaptive systems. The innate immune system serves as the first line of defense against pathogens, with the purpose of providing rapid and potent protection in a non-specific manner, primarily through recognition of evolutionary-conserved microorganism-specific features. In contrast, the adaptive immune system is mobilized at a later phase of infection and performs a more specialized role, utilizing rearranged antigen-receptor gene segments to specifically recognize infected cells and generate immunological memory.

However, the conventional division of the human immune system into two components is now known to be exclusionary of an important subset of T cells which lie at the interface of innate and adaptive immunity: unconventional T cells, also known as innate-like T cells. Unconventional T cells are a heterogeneous group of T lymphocytes which recognize a limited range of ligands presented by monomorphic antigen-presenting molecules, through invariant or semi-invariant T cell receptors (TCRs) [1, 2]. Characterized by recurrent patterns of TCR usage in unrelated individuals and their rapid response upon encountering their cognate ligands, they play key roles in human health and disease. Unconventional T cells include γδ T cells, natural killer T (NKT) cells, major histocompatibility complex (MHC) class Ib-reactive T cells, and mucosa-associated invariant T (MAIT cells) [1]. In humans, MAIT cells are the most abundant unconventional T cell subset and comprise the focus of this thesis.

1.1 MAIT CELLS

MAIT cells were initially identified in 1999 [3], but it wasn't until 2010 that their broad responses to certain microbes were revealed [4, 5], and later in 2012 that the identity of their antigen was uncovered [6]. Since these key developments, the field of MAIT cell biology has exploded and their initial designation as sentinels for infection has expanded to potential players in autoimmunity [7, 8], metabolic disorders [9-11], and cancer [12].

1.1.1 MAIT cell activation and functional responses

Human MAIT cells express a semi-invariant αβ TCR in which a conserved TCRVα chain rearrangement (*TRAV1-2-TRAJ33/20/12*) is paired with a limited diversity of TCRβ chains (typically using *TRBV6* or *TRBV20*) [13]. The MAIT cell TCR recognizes antigens presented by the non-polymorphic MHC class I-related molecule, MR1[6]. The most potent MAIT cell activating ligands are small-molecule biosynthetic derivatives of the microbial riboflavin synthesis pathway [6, 14]. This pathway is absent in mammals but is vital for metabolic

processes in many bacteria and yeast, conferring MAIT cells with highly specialized antimicrobial recognition. The pyrimidine antigens 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) are the strongest MAIT cell agonists identified to date [14]. MR1 is not constitutively expressed on the cell surface of antigen presenting cells (APCs), and its translocation is dependent on the formation of a Schiff-base bond with a compatible ligand [15]. Interestingly, the MR1 antigen-binding cleft is not fully occupied by the currently identified ligands [6, 14, 16], indicating that there are still undiscovered antigens that MAIT cells may recognize via MR1. An *in-silico* screening of a chemical library of drugs and drug-like molecules identified that MR1 can present synthetic compounds that can modulate MAIT cell activity [17].

MAIT cells require secondary co-stimulatory signals, such as CD28 [18], cytokines [19], or toll-like receptors (TLRs) [20, 21] to fully activate. The requirement for co-stimulation is a possible tolerogenic feature to prevent MAIT cell overactivation in the presence of commensals which do not pose a threat to homeostasis [22-24]. Upon engagement of their TCR with MR1-agonist complexes, MAIT cells become activated and upregulate CD69, CD25, and the degranulation marker CD107a [25]. This is accompanied by the rapid release of proinflammatory cytokines such as TNF and IFN-γ [26], and lysis of potentially infected target cells [27]. To kill infected cells, MAIT cells release granzymes and perforin which are required for the induction of apoptosis and pore formation in target cells, respectively [28]. Resting blood MAIT cells express very little Granzyme B (GzB) and low levels of perforin, but these effector molecules are rapidly upregulated upon TCR engagement and co-stimulation [27, 29]. A small population of blood MAIT cells also produce IL-17 [26, 30], This characteristic is driven by the expression of the transcription factor retinoic acid-related orphan receptor gamma (RORγt) [26], and can be augmented in the presence of IL-7 [31]. IL-17 production is an important part of the mucosal MAIT cell response, as discussed in section *1.1.3*.

MAIT cell responses to viral infections are primarily mediated through their innate-like TCR-independent responses [19, 32]. MAIT cells express high levels of cytokine receptors for IL-12 and IL-18 [33], cytokines which can together [19], or with type I interferons [32], activate MAIT cells to produce IFN-γ and GzB. This innate-like MAIT cell phenotype is directed by the effector transcription factors promyelocytic leukemia zinc finger (PLZF) and T box transcription factor 21 (T-bet) [34]. Cytokine-mediated activation can occur synergistically with TCR-mediated responses to drive sustained MAIT cell activation [19, 35]. These responses have previously been described in viral infections including hepatitis B virus (HBV) [36], hepatitis C virus (HCV) [32], and as discussed later in section *1.3.2*, human immunodeficiency virus (HIV) [37]. In addition to viruses, TCR-independent MAIT cell activation also provides protection against riboflavin incompetent bacteria, including *E. faecalis* [38]. An overview of MAIT cell functional responses is shown in **Figure 1**.

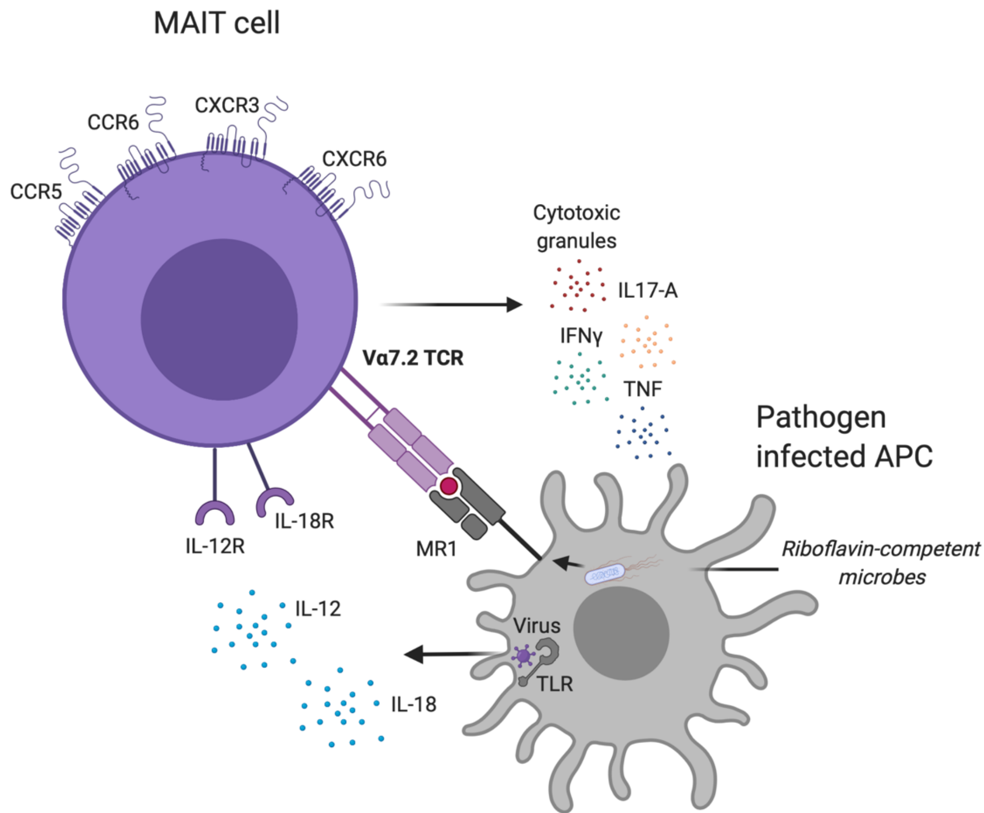

Figure 1: TCR dependent and independent MAIT cells responses to infection. Following TCR activation, MAIT cells display polyfunctional cytokine responses and release perforins and granzymes to lyse infected cells. TCR-independent activation through their IL-12/IL-18 receptors can also induce IFN-γ and GzB production. Created using Biorender.com

Subgrouping MAIT cells based on their combinational expression of CD8 and CD4, or lack thereof, also reveals distinct functional subtypes [16]. CD4-CD8+ MAIT cells comprise around 80% of blood MAIT cells and show more potent functional responses to bacteria and cytokines than CD4+ and CD8-CD4- double negative (DN) populations [39], suggesting that CD8 may play a role in MAIT TCR engagement with MR1 antigen complexes [40]. DN MAIT cells produce higher levels of IL-17 following stimulation, express higher levels of RORγt, and are more prone to apoptosis-induced cell death [39]. These subset-distinct transcriptional profiles may have implications for generating broadened antimicrobial functions in response to infection.

1.1.2 Studying MAIT cells in vitro

In flow cytometry, MAIT cells have classically been defined as CD3+TCRVα7.2+CD161^{high}, and their high expression of IL-18Rα can also be used for detection [19, 41]. Recently, MR1 tetramers loaded with 5-OP-RU have become the gold standard for MAIT cell identification and activation *in vitro* [13, 30, 42]. To determine MAIT cell functional responses, non-specific activation using phorbol myristate acetate (PMA) and ionomycin can be performed [39, 43]. However, it is known that the profile and magnitude of MAIT cell responses is dependent on the source of their stimuli [44]. Therefore, co-culture assays using microbial-fed monocytes or cell lines, such as THP-1, provide a more detailed insight into MAIT cell responses towards

specific antigens [4, 44-46]. While *E. coli* has been used as a model antigen for studying MAIT cell activation and functional responses, riboflavin competent fungal species, such as *C. albicans*, elicit distinct polyfunctional cytokine responses and with differing sensitivity [44]. This highlights the need for careful selection of microbial models depending on the experimental question to be addressed.

1.1.3 MAIT cell compartment distribution and phenotype

MAIT cells are highly abundant in the blood, where they represent up to 10% of the total T cell population [47]. Human adult blood MAIT cells display a tissue-homing effector memory phenotype, defined as $CD45RA^-$, $CD45RO^+$, $CD27^+$, $CCR7^-$, $CD44^{high}$, $CD95^{high}$, $CD62L^{low}$ [26, 48]. Circulating MAIT cells also express a unique chemokine receptor profile which mediates their migration to peripheral tissues [49]. In addition to the blood, the highest MAIT cell frequencies are found in the liver where they comprise up to 30% of intrahepatic T cells [49]. MAIT cells are known to be enriched at other key sites of microbial exposure. These include the intestine [13, 22, 50], lungs [51], skin [52], oral mucosa [43], and female genital tract [53], where they play an important role in host barrier maintenance. An overview of MAIT cell tissue distribution is show in **Figure 2**.

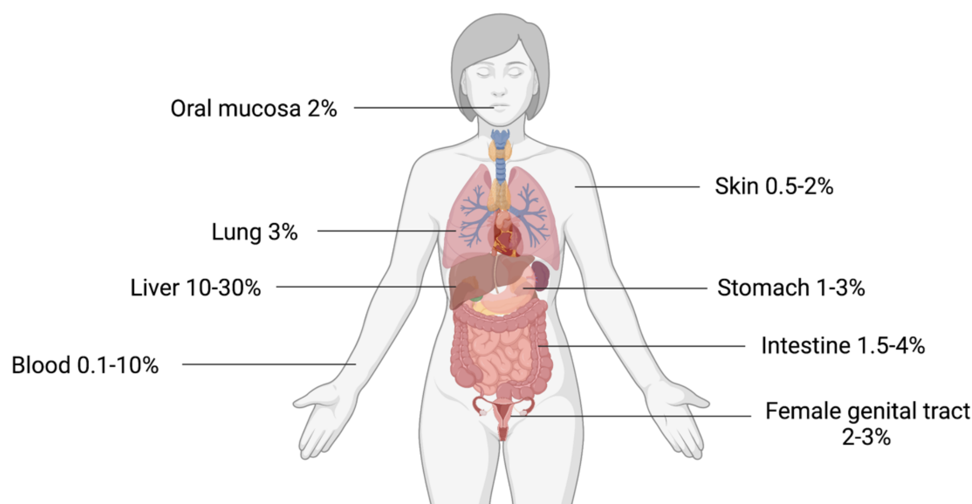

Figure 2: MAIT cell compartment distribution. Values depict mean or range values of MAIT cells out of total T cell population in that tissue. Adapted from Nel et. al. [54]. Created using Biorender.com

In situ analyses have demonstrated that MAIT cells localize below the epithelial layer in the mucosa, where they are poised to encounter microbes which may translocate across the epithelium [52, 55]. Similarly, intrahepatic MAIT cells are mainly localized to bile ducts in the portal tract [56]. In line with this proximity to microbial-derived antigens, tissue-resident MAIT cells display an inherently activated phenotype [54]. Liver MAIT cells to a large extent express the activation markers CD38, HLA-DR, and CD69 [31, 57], a pattern also observed in MAIT cells in other tissue compartments [43, 58]. Elevated expression of immunoregulatory molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), T cell immunoreceptor with Ig and ITIM domain (TIGIT), lymphocyte-activation gene 3 (LAG-3), and programmed cell death protein 1 (PD-1) is observed in gut-resident MAIT cells, even under

steady-state conditions [22]. This suggests a potential role for immune checkpoint modulation in limiting MAIT cells responses in environments rich in activating ligands.

Mucosal MAIT cells display a unique functional profile relative to their blood counterparts [53, 54, 59]. There is an uneven distribution in the DN MAIT cell populations in the mucosal tissues, where they sometimes comprise up to 80% of the total mucosal MAIT cell population [54]. While circulating MAIT cells demonstrate dominant usage of the *TRAV1-2-TRAJ33* rearrangement, a higher proportion of MAIT cells using the *TRAV1-2-TRAJ20* and *TRAV1-2-TRAJ12* TCRα chains has been observed in MAIT cells residing in the oral mucosa [43]. TCR-mediated crosstalk with local commensals in the mucosa is believed to drive a tissue-repair and barrier maintenance MAIT cell phenotype under homeostatic conditions [54, 55, 60]. This includes a functional bias towards IL-17 and IL-22 production, which strengthens epithelial tight junctions [61], and the production of tissue repair proteins such as vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) [55]. IL-17 and IL-22 also work synergistically to induce the production of antimicrobial peptides [62] and neutrophil chemoattractants [63, 64] by epithelial cells. This indicates that MAIT cells play a broad role in preserving barrier integrity. Indeed, this phenotype has been seen following *ex vivo* stimulation of MAIT cells isolated from the female genital tract [53], and oral mucosa [43]. However, in cases of barrier breach, mucosal MAIT cells respond by switching to a pro-inflammatory response with high production of IFN-γ and TNF and cytotoxic molecules [54].

In contrast to barrier tissues, liver MAIT cells do not produce IL-17 in their steady state following TCR engagement [31], and also appear to be less biased towards IL-22 production [65]. Although they comprise the primary IL-17 producing cell type in the liver (~65% of IL-17^{+} cells after *ex vivo* activation), co-stimulation with IL-7, a cytokine produced by infected hepatocytes, is necessary to unlock the full pro-inflammatory potential of intrahepatic MAIT cells [31]. As the liver is an inherently immunotolerant organ, it is logical that MAIT cell activation at this site requires licensing by TCR-independent mechanisms. Indeed, dysregulation of MAIT cell cytokine production has been implicated in liver fibrosis by driving the profibrogenic function of hepatic myofibroblasts [66]. This is at least partly mediated by IL-17 signaling which also promotes fibrosis by activation of hepatic stellate cells [67].

During steady-state conditions, circulating MAIT cells express high levels of the C-C chemokine receptors CCR5, CCR6, and varying levels of the C-X-C chemokine receptors CXCR3, CXCR4, and CXCR6 [13, 16, 26, 30]. This is in line with their reported depletion in blood and parallel enrichment at sites of inflammation such as the gut in patients with inflammatory bowel disease [7, 68, 69]. In addition to migration towards inflammation, expression of diverse CCRs also targets MAIT cells to tissues. For example, CXCL16 and CCL20 are constitutively expressed in the liver, facilitating MAIT cell homing via CCR6 and CXCR6, respectively [49, 70]. Liver MAIT cells also express high levels of CXCR3, as well as the integrins lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), and are believed to be recruited via the hepatic sinusoids [56]. The CXCR4/CXCL12

axis is also important for T cell recruitment to the liver during viral hepatitis and represents another potential mechanism of preferential MAIT cell tropism towards the liver [71].

1.1.4 MAIT cells in cancer

Given their propensity to migrate towards peripheral tissues and sites of inflammation, it is perhaps unsurprising that MAIT cells have been detected in both primary cancers and metastatic lesions [12, 72-74]. However, with their potential to generate both pro-tumour (e.g., IL-17) and anti-tumour cytokine responses (e.g., TNF and IFN-γ), it is unclear whether MAIT cells promote or protect against tumour development *in vivo*. Interestingly, how MAIT cells respond to cancers appears to be dependent on the site of the malignant lesion. For example, in mucosal-associated cancers such as colorectal cancer (CRC), MAIT cells are depleted in blood and enriched in the tumour tissue compared to the adjacent normal tissue, indicating their migration and accumulation in the malignant mucosa [12, 73, 75]. In contrast, MAIT cells occur at a significantly lower frequency in hepatocellular carcinoma (HCC) relative to neighboring healthy liver tissue and are also numerical depleted in the circulation of these patients [72, 76]. Reasons for this dichotomy are unclear, although dysregulation of the chemokine-CCR axes and intratumoral microbial factors may play a role [77, 78].
Due to the very high microbial load in the colon, the association between gut dysbiosis and CRC development has drawn a lot of attention [79], in which great interest has been focused on MAIT cells in CRC patients [73, 75, 80]. In those CRC studies, tumour-educated MAIT cells display diminished IFN-γ and TNF production, as well as an increase in IL-17, suggesting a shift towards a tissue repair phenotype [12, 73, 81]. Although wound healing functions may be important for barrier maintenance, they can have detrimental effects in the tumour microenvironment (TME) by promoting angiogenesis and tumour growth [82]. MAIT cells isolated from CRC also display an exhausted phenotype and upregulate PD-1 and CD39 [55]. The expression of CD39 appears to be induced in a TCR-mediated manner in the TME, suggesting direct involvement of local microbial species [83]. For example, *F. nucleatum*, a proposed oncopathogen [84] is frequently enriched in CRC and elicits MR1-dependent MAIT cell activation *in vitro* [83]. Because MAIT cells can also be activated in an TCR-independent manner by cytokines such as IL-12 and IL-18 [85], and they express receptors associated with NK cell function following interaction with ligands expressed on tumour cells [44], it is possible that MAIT cells also can be activated indirectly within the TME. A recent study also demonstrated that MAIT cell activation can regulate NK cell-mediated tumour immunity, indicating bystander mechanisms in the TME [86]. Similarly, for hepatotropic oncopathogens such as HBV and HCV, which are the leading causes of HCC development [87], dysfunction of circulating MAIT cells has also been described [88, 89]. MAIT cell numerical and functional impairment persists against the backdrop of these infections through to HCC progression [72]. HCC tumour-infiltrating MAIT cells express low levels of IFN-γ [56, 90] and IL-17, as well as minimal GzB and perforin [72]. They have also demonstrated a phenotypic skew towards IL-8 production [72], a pro-inflammatory cytokine which is associated with angiogenesis and is also implicated in tumour immune resistance [91].

Harnessing MAIT cells for cancer immunotherapy is an emerging field. Interestingly, MAIT cells characteristically express high levels of the ATP-binding cassette-multi-drug efflux protein 1 (MDR1), which confers upon them resistance to the cytotoxic effects of some chemotherapeutic drugs *in vitro* [92] and *in vivo* [26]. As they are highly enriched in some tumours and are known to upregulate inhibitory receptors in the TME [93], they may represent attractive targets for immune checkpoint immunotherapy. It was recently demonstrated that MAIT cells with a PD-1^{high}TIM-3^{+} terminally exhausted phenotype accumulated in colon cancer, and that their antitumour responses could partially be restored *in vitro* by PD-1 blockade [93]. Unlike conventional T cells and NK cells which have been extensively exploited for cancer immunotherapy, adoptive cell therapies involving MAIT cells are underdeveloped [16, 94, 95]. Another interesting line of research is whether MAIT cells can selectively recognize malignant cells through their endogenous TCR. Although cancer metabolite loading on MR1 has been described [96, 97], no cancer-specific antigens have been identified that can directly activate MAIT cells via their TCR [98]. However, MAIT cells have demonstrated the ability to kill cancer cell lines after pulsation with 5-OP-RU [47], indicating that they have antitumour potential in the presence of an agonist ligand.

1.2 HEPATITIS B VIRUS

HBV is a small hepatotropic DNA virus from the *Hepadnaviridae* family that causes a broad range of liver diseases including acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [99]. Despite a highly effective prophylactic vaccine, chronic HBV (CHB) represents a significant global health issue with more than 250 million people infected worldwide [100]. While 95% of unvaccinated adults who acquire the virus may clear the infection spontaneously, up to 90% of newborns develop chronic disease when they get infected [101]. HBV does not induce interferon responses, shielding it from recognition by the innate immune system [102]. Therefore, successful clearance of the virus relies on adaptive immunity. This is primarily mediated by HBV-specific T cells and neutralizing antibodies targeting the HBV envelope protein/surface antigen (HBsAg) [103]. HBV-specific T cells are believed to be vital for natural clearance of the infection [104], achieved by destruction of infected hepatocytes and mechanisms of viral control independent of liver damage [105, 106].

1.2.1 T cell failure in chronic HBV

CHB infection is characterized by a numerical and functional decline in HBV-specific T cell responses [104, 107, 108]. Persistent triggering of HBV-specific T cells in response to high antigen loads is believed to be the primary cause of T cell failure in CHB [109], although dysfunctional T cell priming by hepatocytes [110, 111], duration of the infection [112, 113], immunosuppressive cytokines [114], and regulatory T cells [115] also contribute. Chronic activation of HBV-specific T cells increases their expression of inhibitory receptors which leads to impairment in their proliferative potential and effector functions, and alters their metabolic and transcription factor profile [116]. An overview of the differing immunological landscape in self-limiting HBV and CHB is shown in **Figure 3**. BCL2-interacting mediator (Bim), a pro-apoptotic molecule, is upregulated in HBV-specific T cells in CHB infection, and is believed to contribute to their depletion [117]. Inhibitory receptors expressed in functionally exhausted HBV-specific T cells include PD-1, CTLA-4, LAG3, TIM3, and 2B4 [116]. Exhausted HBV-specific CD8 T cells possess a unique transcriptional profile including dysregulation of T-bet and eomesodermin (Eomes) [118] and the expression of thymocyte selection-associated high mobility group box (TOX) which can be used as a biomarker of HBV-specific T cell exhaustion in CHB [119]. Mitochondrion-targeting antioxidants have been shown to improve antiviral function in HBV-specific T cells, suggesting a role for mitochondrial dysregulation in T cell exhaustion [120].

Several *in vitro* studies investigating the rejuvenating effects of immune checkpoint blockade on HBV-specific T cell functions have been performed (reviewed in [121]), with PD-1 being the best characterized and the most dominant responsive receptor [122]. Interestingly, the efficacy of immune checkpoint inhibition depends on the tissue compartment within which the T cell resides. For example, intrahepatic HBV-specific T cells are more exhausted than their circulating counterparts as indicated by upregulation of PD-1 and loss of CD127 [107]. Therefore, these T cells typically require combined co-stimulation (e.g., PD-1 blockade plus activation of CD137 [123]) to restore some of their responses. A clinical trial of PD-1 blockade

in patients with CHB and advanced HCC, had no effect on the antiviral immune response and did not result in anti-HBs seroconversion in any of the patients [124]. However, in another trial [125], low-dose anti-PD-1 therapy administered to virally suppressed HBeAg negative CHB patients did demonstrate some effect in reducing HBsAg titers in 20/22 of the participants. One patient included in the study achieved HBsAg loss which was also associated with partial restoration of HBV-specific T cell responses. This indicates that targeting the PD-1/PD-L1 pathway may be benefial for boosting HBV-specific T cell responses in CHB patients.

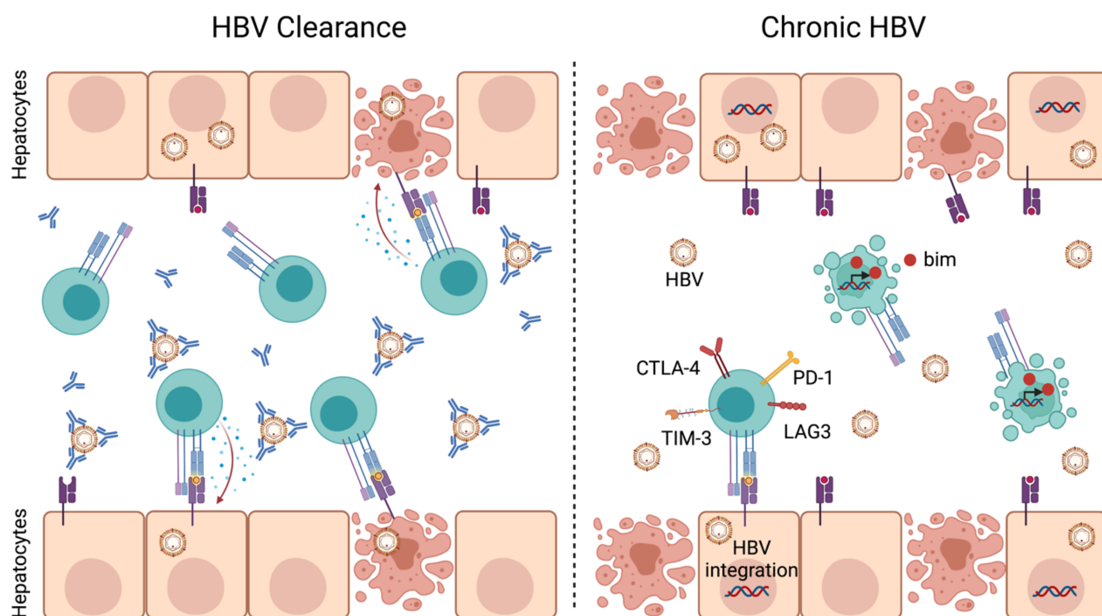

Figure 3: Schematic overview the liver environment during successful clearance and chronic HBV infection. Left panel: Neutralizing antibodies and polyfunctional T cell responses are critical for natural clearance of HBV and control the infection by cytotoxic dependent and independent mechanisms. Right panel: In CHB infection, high antigen loads result in persistent activation of HBV-specific T cell responses driving their numerical decline, exhaustion, and dysfunction. Created using Biorender.com

1.2.2 HBV-induced hepatocellular carcinoma

HCC is the primary form of liver cancer in adults and causes an estimated 800,000 deaths per year [126]. Chronic infection with viral hepatitis is the most common risk factor for HCC development and approximately 50% cases of HCC are attributed to HBV infection [127, 128]. HBV-HCC development involves an interplay between several factors including the direct oncogenic effects of the virus and the persistent inflammation, regeneration, and fibrotic processes characteristic of CHB [129]. The risk of developing HCC is also correlated to HBV genotype (gt), HBV genomic mutations, and the levels of viral replication [130, 131]. Integration of HBV into the host chromosome can lead to intracellular DNA rearrangement, chromosomal instability, and increased mutagenesis of cancer-associated genes [129, 132]. The non-structural HBV X protein is a multifunctional regulator of HBV transcription and replication and is a key player in the development of HCC through its dysregulation of different host signaling pathways [129]. These include disruption to pathways involved in cell cycle progression, hepatocyte regeneration, and host DNA repair processes [129]. Dysbiosis of the

gut microbiota is also believed to play a role in HCC development via alterations in liver antitumour immunosurveillance [133].

HBV-HCC is associated with a high mortality rate, in part due to a lack of non-invasive screening tools, and late-stage diagnosis [134]. Alpha-fetoprotein (AFP) is the only biomarker approved for HCC surveillance although its relatively low sensitivity in early HCC makes it unreliable as a single tool for early diagnostics [135]. Depending on the stage of HCC, potential treatments include liver transplantation, partial liver resection, systemic chemotherapy, or transarterial chemoembolization (TACE) [136]. The success of these therapies is highest in those with early HCC, prior to metastatic disease [136]. Advanced HCC has limited treatment options and first-line therapies such as sorafenib can only extend median survival time by approximately three months [137]. HCC recurrence following hepatic resection and liver transplantation is associated with negative survival outcomes and even more limited treatment options [138-140]. Therefore, there is an unmet clinical need for novel effective therapies to target the different aspects of HCC progression.

1.2.3 T cell immunotherapy for HBV-HCC

A prerequisite to the success of immunotherapies which boost anti-tumour immune responses is the presence of a high degree of immune infiltrate at the time of treatment (a "hot tumour"), which expresses an appropriate anti-tumour specificity, in combination with immune checkpoint molecules that can be modulated [141]. Furthermore, these therapies provide a non-specific pan activation of T cells in the patient which makes it difficult to predict both the efficacy and safety of the therapy. In patients with pre-existing inflammatory conditions, such as CHB, there is a risk for the induction of uncontrolled autoimmunity and in patients who have received a liver transplant, there is also a risk for rejection of the donor liver [142].

Adoptive T cell therapy involves the infusion of autologous or allogeneic *ex vivo* expanded T cells and holds significant promise as a cancer immunotherapy [143]. The earliest successful adoptive cell transfer (ACT) therapies involved the *ex vivo* expansion and reinfusion of tumour infiltrating lymphocytes (TILs) for the treatment of malignant melanoma [144]. When preceded by lymphodepleting chemotherapy, and supplemented with IL-2, durable clinical responses are observed in a significant proportion of the patients [144, 145]. However, isolation and expansion of TILs from HCC can be technically challenging and much attention has focused on the introduction of specificity through T cell engineering methods.

Tumour specificity was first rendered by the expression of receptors targeting classical tumour-associated antigens (TAAs) which comprise abnormally over-expressed antigens, and fetal proteins indicative of aberrant cell proliferation. Classical TAAs including AFP, glypican-3 (GPC-3), melanoma-associated antigen 1 (MAGE-A1), and New York esophageal squamous cell carcinoma-1 (NY-ESO-1) are promising targets for HCC [146]. However, given the self-derived nature of these targets, it is difficult to predict the extent of on-target off-tumour toxicities which may arise following T cell infusion. Therefore, great interest has now been directed towards neoantigens, also known as tumour-specific antigens. Neoantigens arise from

mutated tumours which carry such a high mutational burden that they become distinct from self-ligands, making them immune-reactive [147]. While targeting neoantigens carries less risk for off-tissue toxicities and has demonstrated clinical safety [148], their exploitation arises from random mutations which are different between patients and may also be heterogenous throughout the tumour itself.

Given the central role of HBV-specific T cells in elimination of HBV infected hepatocytes, and their depletion in HBV patients, there is a strong rationale for restoring these responses as an immunotherapeutic strategy for HBV-HCC [149]. Therefore, an alternative option to circumvent the issues of TAA and neoantigen-based strategies for HBV-HCC T cell immunotherapy is to target the HBV viral antigen itself. HBV chromosomal integration events are present in over 90% of HCCs and can range from complete integration of the full HBV open reading frame (ORF) to HBV fragments that generate host-chimeric proteins that can be presented to HBV-specific T cells [150]. Since HBV is a true non-self-antigen, and is exclusively expressed in liver cells, the risk of on-target events in other tissue compartments is minimal. HBV-specific T cells are deleted in HBV-HCC, indicating that immunotherapeutic strategies which replace, rather than restore, HBV-T cell responses may have greater clinical benefit in these patients [151].

The first study investigating adoptively transferred T cell immunotherapy for HBV was the development of a CAR construct specific for HBsAg, which demonstrated selective elimination of HBV-infected hepatocytes [152]. One of the main advantages of CAR-redirected T cell immunotherapies is that they are HLA unrestricted and can therefore be used to target tumours which use HLA downregulation as a mechanism of immune escape [153]. However, HBsAg concentrations in the sera of CHB patients can exceed 10-20 μg/mL (with "decoy" defective surface particles exceeding the number of virions by 10^3-10^6 fold) [154], and can neutralize the activity of envelope-specific CAR-T following systemic infusion [155, 156]. Furthermore, CAR-T cell recognition is based on engagement with conformationally intact proteins on the cell surface, which only applies to approximately 30% of all known proteins [149].

Therefore, much focus has turned to the generation of HBV-specific TCRs for their potential clinical benefit in restoring HBV-specific responses in CHB and HBV-HCC [157-161]. Classical TCRs recognize endogenously processed peptide epitopes presented via MHC, allowing them to recognize intracellular and surface-bound protein, thereby giving them a broader range of antigen coverage than CAR constructs [162]. However, a drawback of TCR redirection is their restriction to specific HLA molecules, constituting a more specialized therapy with reduced "off-the-shelf" potential. The major clinical concern for TCR redirected therapies targeting viral antigens is the inability of the TCR to discriminate between HBV-HCC and non-malignant infected hepatocytes [149]. Careful patient stratification and selection of TCR redirection methods can address some of these safety concerns and are discussed in section *1.2.4*.

1.2.4 Clinical feasibility of T cell immunotherapy for HBV-HCC

Proof-of-concept of HBV TCR-redirected T cell therapy for HBV-HCC patients was first described in 2015 in a single patient by Qasim et al. [163]. The patient presented with HCC recurrence 10 years after receiving a liver transplant for HBV-HCC and at the time of receiving the immunotherapy, had advanced disease with metastases in the lung, bones, and neck. The TCR used in this case was specific for the HLA-A0201/HBs183-91 complex [151]. While both the patient metastases and donor liver were positive for HLA-A201 expression, only the metastases were positive for HBsAg, enabling TCR selection for the extrahepatic lesions with minimal risk to the transplanted liver. This also allowed serum levels of HBsAg to be used as a surrogate marker for T cell-mediated reduction of the HCC metastases. A single infusion of autologous *ex vivo* expanded conventional T cells containing $\sim 1.2 \times 10^4$/kg retrovirally transduced HBV-specific TCR-T cells was administered. The HBV-specific T cells expanded well *in vivo* and HBsAg levels reduced by over 90% within 30 days of receiving the infusion. Importantly, no adverse events or significant hepatic inflammation was observed. This patient had very advanced disease at the time of enrollment and succumbed to the cancer within two months of receiving the therapy. However, this study highlighted that the HBV TCR-redirected T cells were well tolerated and could hold potential in immunotherapy of HBV-HCC [163].

Another clinical trial reported in 2019 [150] investigated the HBV transcript profiles of two patients with post-liver transplantation HCC recurrence and selected TCRs based on the HBV epitope encoded by tumoral HBV mRNA. This approach is of great importance because integration of the complete HBV ORF does not occur in the majority of HCCs. Therefore, this study aimed to determine whether truncated HBV integrations within the HCC genome are sufficient to generate HBV antigens which can be recognized by HBV-specific T cells. Indeed, although both patients were serologically negative for HBsAg, analysis of their tumour biopsies revealed that they were positive for HBV envelope mRNA, and encoded epitopes for which TCRs were available. These patients did not receive HLA-matched transplanted livers, which allowed selection of TCRs restricted to the host-derived HCC metastases, but not the donor liver. This study also demonstrated the clinical feasibility of using a dose-escalation treatment strategy of autologous expanded T cells which transiently expressed the TCR.

Here, the short-term HBV TCR expression facilitated by mRNA transfection improves the safety of the therapy by minimizing the risk for prolonged adverse events that may occur following T cell infusion. In contrast with retrovirally transduced HBV-specific T cells which can expand and persist *in vivo* following a single infusion [163], mRNA transfected T cells require multiple infusions and by employing a dose escalation strategy, the safety of the treatment can be carefully monitored and controlled. Like the previous study, the therapy was well-tolerated in both patients, with no cytokine release syndrome events or intracranial abnormalities observed. One of the two patients demonstrated marked volume reduction in several pulmonary metastases in a manner that correlated with drop in AFP after T cell infusion. It was suggested that this effect may have been due to induction of other anti-tumor mechanisms in the TME rather than the HBV TCR-T cells alone. Taken together, this

demonstrated a safety enhanced approach that could target a broader range of HCCs than originally anticipated and with an indication of clinical benefit in one out of the two patients investigated.

A recent phase I clinical trial explored the safety of HBV TCR-redirected conventional T cells in a cohort of eight patients who presented with advanced HCC but did not meet the criteria for liver transplantation [164]. Using the same mRNA TCR redirection method described previously [150], a dose-escalating infusion strategy with a maximum of $5x10^6$ T cells/kg was performed. Although two patients initially experienced adverse events, these normalized after the first treatment cycle and no subsequent toxicities were observed. All but one of the patients demonstrated reduction or stabilization of their circulating HBsAg and HBV DNA levels, suggesting an on-target effect, and three of the patients showed a reduction in tumour size.

Future studies in larger cohorts and with earlier-stage disease are warranted to further examine the anti-tumour potential HBV-specific T cells. Unravelling the mechanisms by which transiently transfected T cells elicit sustained anti-HBV and anti-HCC effects also holds significant importance for the broader translational potential of this approach.

1.2.5 Translational challenges of T cell immunotherapy for HBV-HCC

Although there is a strong rationale for TCR redirected T cell therapy for HBV-HCC, some challenges remain which can limit the efficacy of the treatment *in vivo*. While extensive preclinical evaluation of the functional profile and phenotype of expanded T cells can be performed *in vitro* prior to infusion into a patient, success of the therapy hinges on the ability of the T cell product to home efficiently to the tumor site. Indeed, inefficient T cell homing has been cited as a "significantly underappreciated contributor to immunotherapy resistance" [165]. Mismatch between T cell CCR expression and CC expression in HCC can impair their ability to exit the blood and traffic to the tumour site, a topic extensively reviewed by Tantalo et al. [166]. Previous studies in CAR-based therapies have shown potential improvements to T cell homing through the introduction of genes encoding specific CCRs. For example, co-transduction of CAR-T cells with the CCR2b receptor enhanced tumour infiltration in models of neuroblastoma and malignant pleural mesothelioma, improving the treatment efficacy [167, 168]. In a similar approach, introduction of CXCR1 and CXCR2 to CAR-T cells also showed augmented T cell infiltration in a mouse model of glioblastoma [169]. Another, albeit less explored strategy is to exploit integrin-based interactions to enhance tumour homing. It has been shown that upregulation of VCAM-1 (the ligand of the integrin VLA-4) can enhance the recruitment of adoptively transferred T cells to the tumour endothelium [170]. Taken together, these studies suggest that CCR and integrin profiling of T cell products should be considered as an important preclinical predictor of T cell efficacy *in vivo*.

In addition to tumour homing, another obstacle facing T cell-based immunotherapies for HCC is their ability to function within the physically and metabolically challenging HCC TME. HCC typically arises from fibrotic tissue which can be difficult for systemically infused T cells to penetrate and elicit their anti-tumour functions [171]. Hypoxia is a typical TME characteristic

[172] and has been shown to have a strong negative effect on T cell infiltration in 3D *in vitro* models of HBV-HCC [173, 174]. Furthermore, low oxygen tension has been shown to increase resistance of tumour cells to T cell-mediated killing [175] and has been shown to drive the differentiation of terminally exhausted CD8 T cells [176]. Arginase, which is released by damaged hepatocytes, is known to inhibit intrahepatic T cell function by starving the TME of arginine, an amino acid that is vital for T cell effector function [177, 178]. Immunosuppressive drugs are a vital component of the post-liver transplantation treatment regimen and are required to prevent host rejection of the donor liver. Since patients with HCC recurrence following liver transplantation represent an eligible cohort for TCR-T cell HBV-HCC immunotherapy, the effects of these drugs on T cell immunotherapy efficacy should be considered. A recent study by Hafezi et al. [179] showed that pre-treatment of TCR-T cells with individual or combinations of immunotherapeutic drugs including tacrolimus (TAC) and mycophenolate mofetil (MMF) had a severe impact on the functional and cytotoxic ability of the T cells in *in vitro* models of HBV-HCC. This impairment could be rescued by co-transfecting the T cells with mutated variants of intracellular molecules required for drug metabolism, effectively shielding them from their inhibitory effects [179].

A final, more practical consideration for the production of personalized cell-based therapies is that they require complex infrastructure and specialized personnel to facilitate cell production under good manufacturing practice (GMP) guidelines [180]. Taking into account the financial costs for implementation, these requirements make these therapies unfeasible for most patients for which these engineered are designed to treat, particularly in developing countries where chronic viral infections are endemic [181]. Therefore, careful patient screening (e.g., biomarker selection) and the optimization of the use of immunotherapeutics will be required to alleviate some of the economic burdens in both low- and middle-income countries.

1.3 HIV-1

HIV is a Lentivirus, within the family *Retroviridae*. There are two types of HIV in circulation amongst humans: HIV types 1 and 2 (HIV-1 and HIV-2, respectively). The global HIV pandemic is primarily associated with HIV-1, while HIV-2 is relatively localized to countries in the West African region. HIV-1 infection causes a broad range of pathologies including systemic immune activation, loss of CD4 T cells, and expansion of CD8 T cells [182]. The progression of HIV-1 infection can lead to acquired immunodeficiency syndrome (AIDS), although today's successful antiretroviral therapy (ART) has reduced the infection to a chronic minimal residual disease in people living with HIV (PLWH) [183]. Even so, HIV-infected individuals who are successfully treated with ART still exhibit signs of chronic immune activation and increased morbidity and mortality [184]. This indicates that pathological mechanisms independent of viral replication also contribute significantly to the accelerated immunological ageing observed in these individuals [184].

1.3.1 Microbial translocation and immune activation during HIV-1 infection

One of the earliest immunological impacts of HIV-1 infection is the preferential infection and loss of CD4 T cells. The gut-associated lymphoid tissue (GALT), in particular, is a key site involved in this immunopathogenic processes [185]. During the acute phase of HIV-1 infection, CD4 T cells residing in the gut mucosa are depleted, and high levels of viral replication drive a generalized mucosal cytokine dysregulation in other local immune sets [186]. The result is a pro-inflammatory cytokine milieu, with elevated levels of TNF, IL-1, and IL-6 [186]. This inflammation can lead to apoptosis of enterocytes and epithelial cells, and a loss of tight junctional complexes in the epithelium [187]. Coupled with the impairment of local immune cell function, this loss in barrier integrity increases susceptibility to microbial translocation from the intestinal lumen to the gut lamina propria, and eventually the systemic circulation [188]. The loss of IL-17 and IL-22 producing lymphocytes in the GALT is linked to impaired mucosal immunity which may further augment microbial translocation [189]. The composition of the gut microbiome itself can also influence this pathogenesis, as dysbiotic microbiome profiles are associated with HIV-1 disease severity [190]. Similar mechanisms have been proposed for microbial translocation across the epithelium of the female genital tract [191].

Early untreated HIV-1 infection can be characterized by high levels of inflammatory molecules in the blood, including IFN-γ, TGF-β, and high turnover of CD4+ and CD8+ T cells [192]. During progressive HIV-1 infection, CCR5+CD4+ T cells are susceptible to virally induced caspase-1-mediated cell death, leading to the detrimental CD4 T cell loss characteristic of the disease [193]. For remaining T cells, persistent activation can also drive exhaustion and reduce the overall function of the T cell compartment [194]. As HIV-1 chronicity develops, systemic immune activation is closely associated with progression of the disease [192].

Immune activation is commonly detected using cellular markers such as HLA-DR, CD38, PD-1, and CD4/CD8 ratios [195]. Elevated plasma levels of lipopolysaccharide (LPS) and soluble

CD14 (sCD14), have been described in PLWH, which are indicative of microbial-induced activation of monocytes and macrophages [187]. Other markers used for assessment of systemic microbial translocation include 16S rDNA, endotoxin core antibodies (EndoCAbs), and intestinal fatty-acid binding protein (I-FABP) [196].

Although levels of markers for microbial translocation are lower in PLWH on suppressive ART relative to viremic progressors, they are still significantly higher compared to uninfected individuals [187]. Increased plasma levels of sCD14 are correlated with increased mortality in PLWH, highlighting the importance of microbial factors in HIV-1 pathogenesis [197]. While CD4 T cell counts can be restored in successful ART therapy, those residing in the gut are not typically reconstituted to the same degree [198]. This has been attributed to high levels of collagen deposition in the GALT in response to the elevated local LPS concentrations [199]. This fibrotic environment impairs CD4+ access to IL-7 survival signals [187]. Failed immune reconstitution in the GALT, even following ART, results in persistence of microbial translocation and chronic activation of susceptible immune cells.

1.3.2 MAIT cells in HIV-1 infection

Dynamic changes in MAIT cell phenotype and function have been described over the course of HIV-1 progression, indicating that they are severely impaired by the broader immunopathogenesis associated with the disease [46, 200-202]. Growing evidence indicates that MAIT cell dysfunction is closely associated with bacterial markers in the blood, suggesting an important role for microbial translocation in driving MAIT cell failure [200, 203, 204].

Already at the peak viremic stage of acute HIV-1 infection, peripheral MAIT cells have been shown to rapidly expand and display an activated phenotype with elevated expression of HLA-DR, CD38, TIGIT, and PD-1 [200]. TCR repertoire diversification and increased plasma levels of sCD14 were also observed, indicating that even at the acute phase, microbial translocation has a direct effect on blood MAIT cell phenotype. A similar proliferative profile was mirrored in the rectal mucosa, suggestive of an early concerted MAIT cell response towards translocated microbial factors [200]. However, over time this persistent TCR-mediated MAIT cell activation eventually results in the loss and functional impairment of MAIT cells that is characteristic of chronic HIV-1 [46, 202, 203]. The potent and irreversible numerical loss of circulating MAIT cells is attributed to activation-induced apoptosis resulting from persistent TCR-stimulation [46]. There is also evidence that MAIT cells may exit the blood and migrate towards inflamed mucosal tissues, such as the GALT, which could contribute to their reduced frequency in blood [205]. Quantitatively, MAIT cells in the rectal mucosa do not differ significantly between PLWH and uninfected individuals [202], indicative of preservation of tissue resident MAIT cells or a compensatory accumulation of MAIT cells from the circulation. A proposed overview of dynamic changes in MAIT cells in the blood and GALT during HIV-1 infection is shown in **Figure 4**.

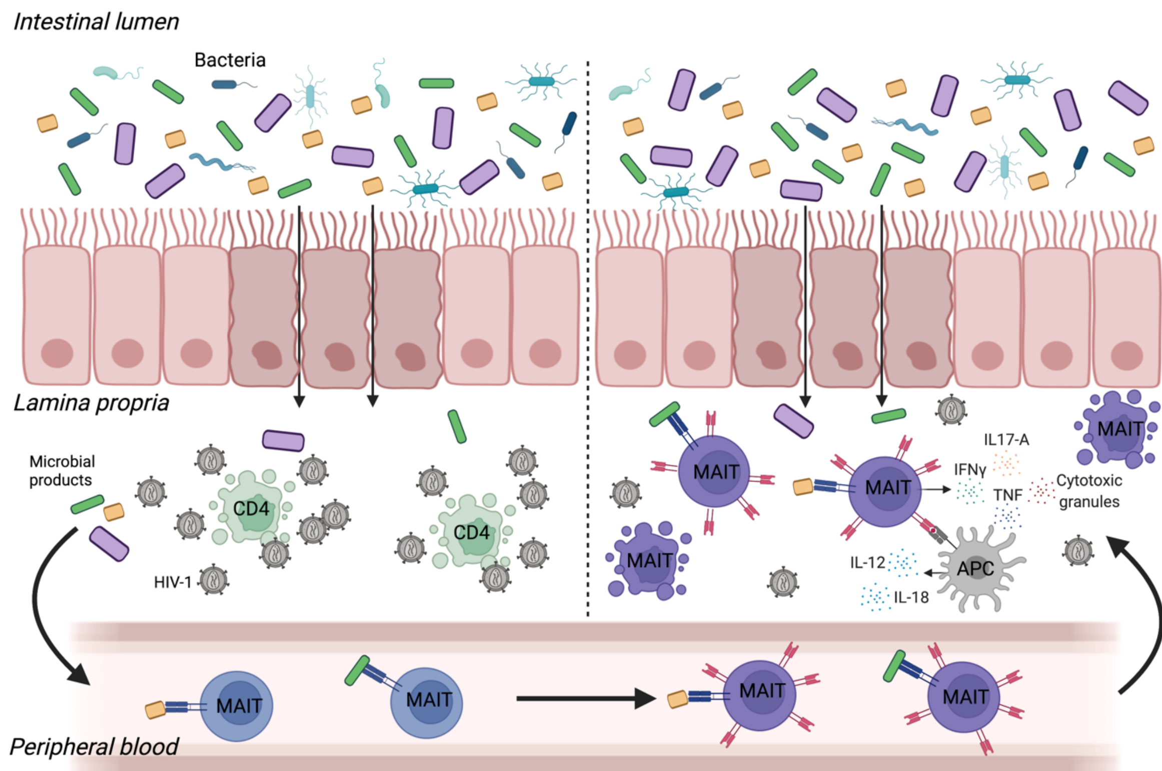

Figure 4: Schematic overview of the proposed mechanism of MAIT cell activation during HIV-1 infection. Impaired epithelial barrier integrity and weakened mucosal immunological responses results in the translocation of microbial products from the gut microbiome. Microbial products enter the lamia propria and eventually the systemic circulation, resulting in MAIT cell activation, activation-induced apoptosis, and their migration towards the inflamed GI tissue. Created using Biorender.com

Residual MAIT cells in PLWH express the activation markers HLA-DR, CD69, CD38, and PD-1 [46, 202, 204]. They also display impaired cytotoxic function with reduced ability to produce IFN-γ, TNF, and IL-17 in *in vitro E. coli* activation assays, which has been linked with abnormal T-bet and Eomes expression [204]. Partial restoration of MAIT cell function is observed in PLWH on ART, but their absolute cell counts in blood cannot be rescued [201, 206]. IL-7 has also been reported to augment MAIT cell sensitivity during HIV-1 infection and may represent an immunotherapeutic strategy to restore antimicrobial MAIT cell responses [204].

HIV-1 infection is associated with the loss and functional alteration of bronchoalveolar MAIT cells [207] although other mucosal sites such as the skin, oral mucosa, and female genital tract are underexplored. Interestingly, HIV-1 infection appears to have differential effects on distinct MAIT cells in the airway, where the CD103 expressing MAIT cell population was selectively depleted [208].

The loss of MAIT cells in PLWH is of clinical significance given the increased risk of HIV-related opportunistic infections such as *Mycobacterium tuberculosis* [207], nontyphoidal *Salmonella*, and *Streptococcus pneumoniae* [209].

2 RESEARCH AIMS

The overall aim of this thesis was to explore the feasibility of harnessing MAIT cells for cancer immunotherapy and to gain a deeper understanding of MAIT cell biology against the backdrop of viral infection.

Specific aims:

Paper I: Given their abundance in blood and rapid-response innate-like phenotype, MAIT cells represent an attractive cell type for adoptive cell transfer therapies. However, protocols to efficiently expand MAIT cells *ex vivo* are lacking. Our aim was to develop a protocol to expand MAIT cells from blood and investigate their functional properties, differentiation phenotype, and chemokine receptor profile. The aim included a proof-of-concept that tested the feasibility of MAIT cell TCR redirection in an HCV replicon hepatoma model.

Paper II: Conventional T cells are currently the mainstay of adoptive T cell transfer for cancer immunotherapy. Our aim for paper II was to compare the functional and tumour homing profile of TCR engineered conventional T and MAIT cells in an HBV-associated HCC model. This involved evaluating these parameters in 2D and 3D co-culture systems which mimic specific aspects of the tumour microenvironment, such as low oxygen tension and inflammation.

Paper III: MAIT cells in the blood are numerically and functionally impaired in people living with HIV-1. Growing evidence indicates that they are preserved at mucosal barriers, such as the gut, during infection. The aim of paper III was to investigate the circulating and cervicovaginal-resident MAIT cells in treatment-naïve HIV-infected women and their association with the local bacterial microbiome.

3 MATERIALS AND METHODS

For a comprehensive description of the methods described in this thesis, the reader is invited to consult the methods section of each paper. A summary of the main reagents and methods used in this thesis is described herein.

3.1 ETHICAL CONSIDERATIONS

All studies included in this thesis involved the use of samples from human healthy donors and patients. Ethical permits were acquired prior to the commencement of all studies, and all studies were performed in accordance with the Declaration of Helsinki. All participants provided written informed consent and samples were de-identified prior to conducting laboratory experiments.

For expansion and TCR engineering experiments using peripheral blood in **paper I** and **paper II**, we used PBMCs isolated from healthy blood donors recruited at the Blood Transfusion Clinic at Karolinska University Hospital, Huddinge. Also included in **paper I** were PBMCs from HBV-infected patients who were recruited at the Department of Infectious Diseases at Karolinska University Hospital, Huddinge.

It is noteworthy that in **paper II** we utilized a 3D microfluidic cell culture model to study T cells in an environment simulating specific aspects of the HCC microenvironment. The use of more advanced *in vitro* models addresses the 3 Rs in animal research (Replace, Reduce and Refine), which promotes humane and ethical animal research. Although not intended to be used a replacement for animal models, 3D culture systems can address questions that cannot be recapitulated in 2D settings, still in a controlled environment, and serve as a useful preclinical evaluation tool before proceeding to *in vivo* studies in animals.

In **paper III**, PBMCs and/or ectocervical biopsies were collected from women in Kenya and Sweden. The Kenyan cohort was comprised of HIV-1 seropositive female sex workers (HIV^{+}FSW), HIV-1 seronegative female sex workers (HIV^{-}FSW), and HIV-1 seronegative non-sex working women (HIV^{-}LR). These cohorts are well-characterized and have been described previously [210-213]. As some of these women represent vulnerable patient groups, additional services were put in place for their participation. This included treatment of sexually transmitted infections (STIs), access to diagnostic testing, STI prevention counselling, family planning services, contraception, medical treatment of other acute and chronic diseases, and referral for consultation specialists, as required. Since ectocervical biopsy may increase the risk of acquisition of HIV-1 infection, these women were also asked to abstain from vaginal sex for a two-week healing period after collection of the biopsy. In **paper III**, ectocervical samples from healthy premenopausal women in Sweden were recruited at Karolinska University Hospital, Stockholm and have also been previously described [214].

3.2 FLOW CYTOMETRY

Multicolour flow cytometry was used in all papers included in this thesis. This method is based on the detection of fluorescent dye-conjugated antibodies bound to specific cellular antigens and can be used to detect extracellular and intracellular molecules. The ability to generate large datasets on single-cell level makes flow cytometry a useful analytical tool in immunology. Flow cytometry was also used for the detection of TCRs using tetramer and dextramer complexes as described in **papers I and II (Figure 5)**. The MR1 tetramers described in this thesis were provided by the NIH Tetramer core facility and were loaded with the MAIT cell agonist, 5-OP-RU [13]. To detect HBV-specific TCR responses, HLA-A2-restricted dextramers loaded with 9 mer HBV_{s183} peptide were used. One of the advantages of dextramers over tetramer technology is the increased probability of binding more than one TCR on the antigen-specific T cell. This enhances the stability of the T cell: multimer interaction, making dextramers advantageous for low affinity TCRs and rare antigen-specific T cell populations [215]. Flow cytometry data was analyzed using FlowJo software (versions 9&10) or R version 4.0.5 [216]. The gating strategy used to identify MAIT cells in **paper I** is shown in **Figure 6**.

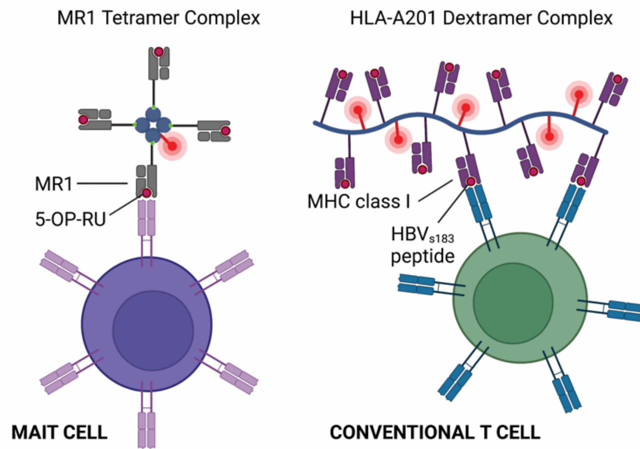

Figure 5: Schematic illustration of MR1 tetramer-bound MAIT cells (left) and dextramer-bound conventional T cells (right) as performed in paper II. Created using Biorender.com

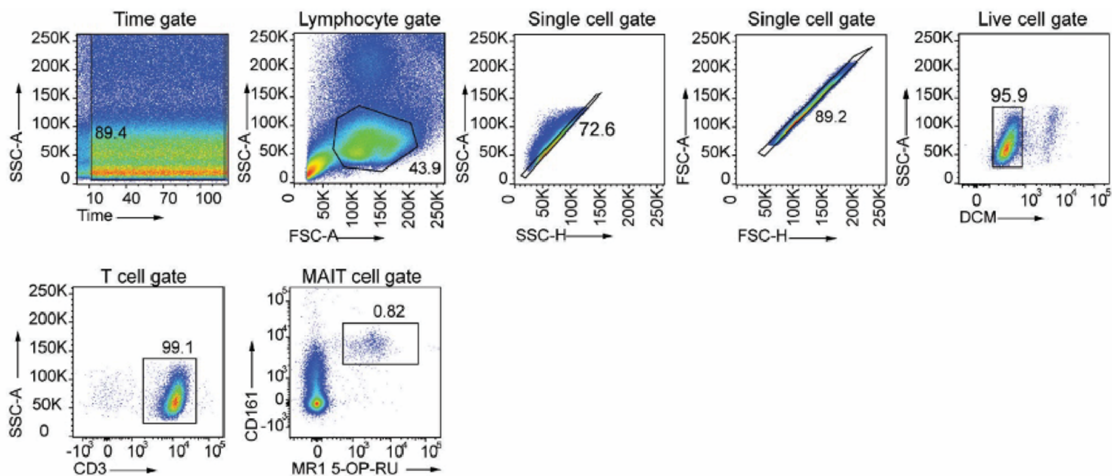

Figure 6: Representative gating strategy for the detection of MAIT cells in **paper I** using FlowJo software**.**

3.3 VISUALIZATION OF COMPLEX DATASETS

Traditional gating methods in flow cytometry analysis are useful for classifying immune cells and subpopulations of interest. However, as the number of parameters included in a flow

cytometry panel increases, so too does the complexity, which can result in a loss of information if plotted on classical single and bi-dimensional plots.

For studies of polyfunctional T cell responses which are important in the context of T cell immunotherapy, the polychromatic flow cytometry data was subjected to SPICE (Simplified Presentation of Incredibly Complex Evaluations) data mining software (Version 6). This analysis was used in **paper I and II** to visualize polyfunctional cytokine responses during functional assays [217], as depicted in **Figure 7A**. In **paper III**, t-distributed stochastic neighbor embedding (tSNE) analysis was used for post-processing flow cytometry data using R version 4.0.5. This method allows the characterization of cells based on clustering and is commonly used to identify distinct cell populations in cytometry data [218] (**Figure 7B**).

Uniform Manifold Approximation and Projection (UMAP) is the data visualization tool shown in **Figure 7C** and was used on the single-cell transcriptome data presented in **paper II**. This data was retrieved from publicly available datasets deposited by earlier published HCC studies (EGA: EGAS00001002072 and GEO: GSE98638) [76, 219]. UMAP is advantageous over tSNE when studying intercluster relationships and the larger datasets associated with transcriptome analysis [220].

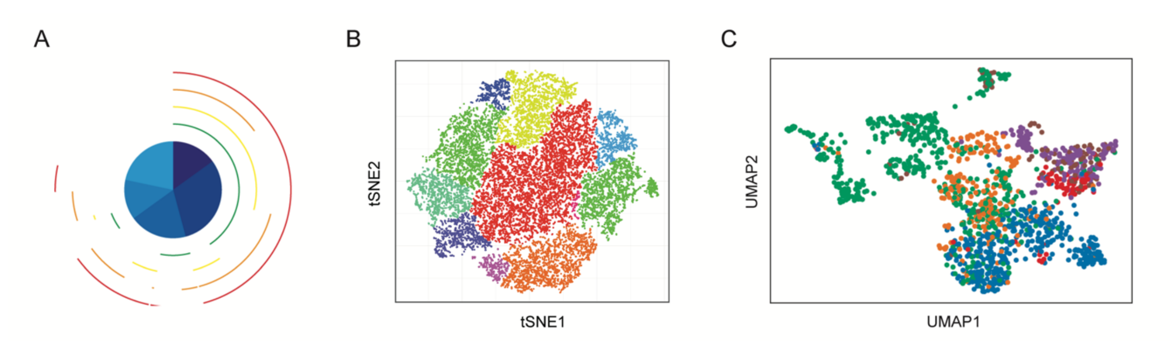

Figure 7: Representative images of the complex data visualization methods used in this thesis. (A) A SPICE plot adapted from **paper I**, (B) A tSNE plot adapted from **paper III**, and (C) A UMAP plot adapted from **paper II.**

3.4 T CELL EXPANSION AND TCR REDIRECTION PROTOCOLS

Electroporation is a transfection method where an electric current is applied to a cell solution, briefly increasing cell permeability for the introduction of nucleic acids [221]. This method is highly efficient in proliferating cells, making it particularly suitable for transfecting T cells which are undergoing an expansion protocol. Electroporation with mRNA is typically more efficient than DNA transfection and demonstrates improved viability [222]. mRNA can be generated *in vitro* using specific transcription kits on restriction enzyme linearized plasmid DNA, where the gene to be encoded lies downstream of an RNA polymerase promoter site. As the T7 RNA polymerase promoter was chosen for our plasmid constructs, the *in vitro* mRNA production in this thesis was faciliated using the mMESSAGE mMACHINE® T7 Ultra Kit (Thermo Fisher), which generates capped, poly(A)-tailed transcripts for high yield and enhanced translation.

3.4.1 Conventional T cells

Conventional human T cells for mRNA-based TCR redirection were expanded using a previously established enrichment method where total PBMCs are stimulated with IL-2 and anti-CD3 for 7-8 days, resulting in a highly pure CD3+ culture [223, 224]. TCR-redirection of conventional T cells used in **paper II** was performed by electroporation with *in vitro* transcribed mRNA as previously described [225, 226].

3.4.2 MAIT cells

Protocols for the isolation and expansion of human blood MAIT cells *in vitro* were lacking at the commencement of this PhD project, so the primary aim of **paper I** was method development. Isolation of MAIT cells from total PBMCs was performed using MR1 tetramer purification technology [227]. A MAIT cell transfection method was developed in **paper II** which employed the same TCR mRNA principle used for the conventional T cells.

3.5 FUNCTIONAL ASSAYS

The cell lines used in functional assays in **papers I and II** are shown in Table 1.

Cell Line	Cell Origin	Characteristics relevant for this thesis	Reference
THP-1	Acute monocytic leukemia	MR1+; Highly efficient at antigen presentation via MR1	[228]
293T-hMR1	Embryonic kidney	Transfected with MR1; Less prone to bacteria-induced cell death	[45, 229]
K562	Lymphoblast	MR1-; Used to study NKG21-mediated and MR1 independent cytolytic activity	[230]
P815	Mast cell neoplasm (M)	Mouse FcR+ cell line; Can be incubated with mAbs to inhibitory receptors	[231]
Hepatoma Huh-7-Lunet (HLA A2-)	Liver HCC	HLA-A2-; Transfected with a luciferase reporter replicon of HCV gt 1b	[226, 232-234]
Hepatoma Huh-7-Lunet (HLA A2+)	Liver HCC	HLA-A2+; Transfected with a luciferase reporter replicon of HCV gt 1b	[226, 232-234]
T2	Lymphoblast	HLA-A2+; Highly efficient at antigen presentation via MHC class I	[235]
HepG2	Liver HCC	HLA-A2+; Adherent HCC cell line	[236]
HepG2.2.15	Liver HCC	HepG2 + 2 head to tails stably integrated HBV gt D	[150, 237]
HepG2-PreS1-GFP	Liver HCC	HepG2 + HBV gt D preS1 covalently linked to GFP	[173, 238]

Table 1. Cell lines used for functional assays in **papers I and II**. The letter (M) after a listed primary cell type denotes that the cell line is murine in origin.

3.5.1 MAIT cell activation assays

The classical MAIT cell ligands for activation through their endogenous TCR are metabolites of the riboflavin biosynthesis pathway [6]. *E. coli* is known to utilize this pathway and is an established source of reactive antigens to induce MAIT activation *in vitro* [45]. Mildly fixed *E. coli* has previously been shown to induce similar levels of MAIT cell activation as live bacteria while also avoiding outgrowth in the culture [45]. Therefore, we used THP-1 cells loaded with fixed *E. coli* to quantify TCR-mediated activation in **papers I and II**. The dependency of activation on MR1 was explored in some experiments using anti-MR1 antibodies.

TCR independent MAIT cell activation was performed by culturing MAIT cells in 10 ng/ml IL-12p70 and 100 ng/ml IL-18 for 24h as previously described [19, 44].

3.5.2 Testing T cell functionality after TCR redirection

In **paper I**, MAIT cells were transfected with an HLA-A2-restricted TCR targeting the HCV $NS3_{1073}$ epitope. They were then tested for their ability to recognize endogenously processed HCV antigen in hepatoma Huh-7-Lunet cells replicating the HCV sub genomic RNA replicon with and without HLA-A2 expression [226, 233, 234].

In **paper II**, donor-matched MAIT cells and conventional T cells were transfected with another HLA-A2-restricted TCR targeting the $HBVs_{183\text{-}91}$ epitope [157]. The sensitivity and specificity of a T cell to recognize targets through the transiently expressed TCR was assessed by co-culturing with peptide pulsed T2 and HepG2 cells. Co-cultures with the more physiologically relevant HepG2.2.15 cell line were also performed.

The xCelligence system used in **paper II** is an impedance-based assay which can monitor the dynamics of target cell growth and death in real time. This method has previously been used in determining the functionality of engineered T cells against adherent HCC cell lines [150, 179].

3.6 MICROSCOPY

3.6.1 Live cell imaging

To visualize T cell: hepatoma cell interactions in the microfluidic device described in **paper II**, a high-throughput microscopy system was used as previously described [173, 238]. Unlike conventional 2D co-culture assays, where gravity drives the migration of T cells to their target cells, 3D systems require active migration and allow a more rigorous preclinical evaluation of TCR-redirected T cells. It has previously been demonstrated that the effects of certain aspects of the tumour microenvironment, such as hypoxia, on T cell functionality cannot be ascertained by 2D culture alone [173]. The customizable design of the device also allows the simulation of specific elements of HCC, such as the presence of inflammatory molecules [173].

3.6.2 *In situ staining*

To determine the quantification and localization of MAIT cells resident in the ectocervical mucosa described in **paper III**, *in situ* staining was performed. MAIT cells were designated as Vα7.2-IL-18RA+, in line with previous studies which examined MAIT cells in the oral and female genital mucosa [43, 53]. This definition was selected as previous experiments using CD161 and a fluorochrome-bound MR1 tetramer for *in situ* staining had been unsuccessful.

3.7 REVERSE TRANSCRIPTION AND QUANTITATIVE PCR GENE ANALYSIS

qPCR on total RNA was performed to analyze gene expression in the PBMCs and ectocervical biopsies in **paper III**. Following extraction, the RNA was subsequently reverse transcribed into complimentary DNA (cDNA) for downstream real-time qPCR analyses. For the RT-PCR results shown in **paper III**, TaqMan gene expression assays (cytokine data) and SYBR Green in combination with primers (MAIT TCR gene expression) were performed. The comparative threshold (ΔΔCt) method was used to determine the relative mRNA expression of the specific genes. For the TCR gene expression analysis, the Ct values of each sample were normalized to either the housekeeping gene GAPDH, or the Cα TCR gene (Ct target-Ct normalization gene = ΔCt). Next, the ΔΔCt (ΔCt target – ΔCt control average) was calculated, and the fold-change in mRNA expression was described using the formula $2^{(-\Delta\Delta Ct)}$.

3.8 MICROBIOME COMPOSITIONAL ANALYSIS

Cervicovaginal microbiome profiling was performed by 16S rRNA gene sequencing. DNA was extracted from cervicovaginal lavage pellets and the variable region 4 (V4) of the 16S gene was amplified and sequenced on the Illumina MiSeq (Illumina).

All bioinformatic analysis of microbiome data was performed using R version 4.0.5 [216]. Alpha-diversity was tested using the Mann-Whitney U-test, while beta-diversity was tested using the permutation and adonis tests from the Vegan package 2.5-6. Differential bacterial taxa were calculated using negative binomial models using the edgeR package and adjusted for multiple comparisons using the Benjamini-Hochberg correction.

3.9 STATISTICS

GraphPad Prism versions 8 and 9 were used for statistical analysis. To apply the correct statistical method, normality testing was initially performed on the datasets. Normally distributed data were analyzed using parametric tests including the student's t-test. Non-normally distributed data were tested for significance using the Wilcoxon matched pairs signed rank test, Mann-Whitney U-test, Kruskal-Wallis test with Dunn's post-hoc multiple comparisons, or Spearman correlation.

4 RESULTS AND DISCUSSION

4.1 ESTABLISHING A METHOD FOR HUMAN MAIT CELL EXPANSION

Human MAIT cells possess several features which make them an attractive cell type alternative for adoptive cell transfer (ACT) therapy [95, 239]. Unlike conventional T cells, MAIT cells are restricted to the non-polymorphic molecule MR1, which makes them suitable candidates for donor-unrestricted immunotherapies [240, 241]. The surface expression of MR1 on APCs is tightly regulated by antigen availability [15], minimizing the risk of off-target effects. Furthermore, their rapid innate-like effector functions and inherent tropism for tissues, mediated through their unique chemokine receptor profile, make them particularly suitable for therapies that require tissue or tumour homing and infiltration [26, 48, 242]. Therefore, in **paper I**, we sought to develop a standardized and adaptable MAIT cell expansion protocol which could generate clinically relevant MAIT cell yields for downstream ACT in the context of immunotherapy. The influence of the MAIT cell expansion on their functionality, effector memory phenotype, and tissue-homing capacity was also explored. This method formed the basis of the experimental approach used in **paper II**.

4.1.1 Purity and yield after expansion

To obtain a purified MAIT cell starting culture from human PBMCs, we performed magnetic cell selection (MACS) using a 5-OP-RU loaded MR1 tetramer (**Figure 8**). Next, we determined the optimal conditions for MAIT cell expansion *in vitro*, by activating purified MAIT cells with different cytokine combinations (IL-2, IL-15, and IL-7), cytokine concentrations (ranging from 1-500 ng/ml), co-stimulatory CD3/CD2/CD28 complexes, and autologous PBMCs or monocytes as feeder cells. Their proliferative capacity in serum-free media or media supplemented with an animal-free serum replacement was also assessed (**Paper I Suppl. Figure 1B**). The optimization tests revealed that a 19–22-day expansion initially cultured with irradiated autologous feeder cells in the presence 50 ng/ml IL-2 in and 8% serum-replacement resulted in the best conditions for MAIT cell growth (**Paper I Figure 1A**). The ability to culture MAIT cells in the absence of animal-free proteins and serums should also benefit the safety and GMP of cell production for future clinical translation.

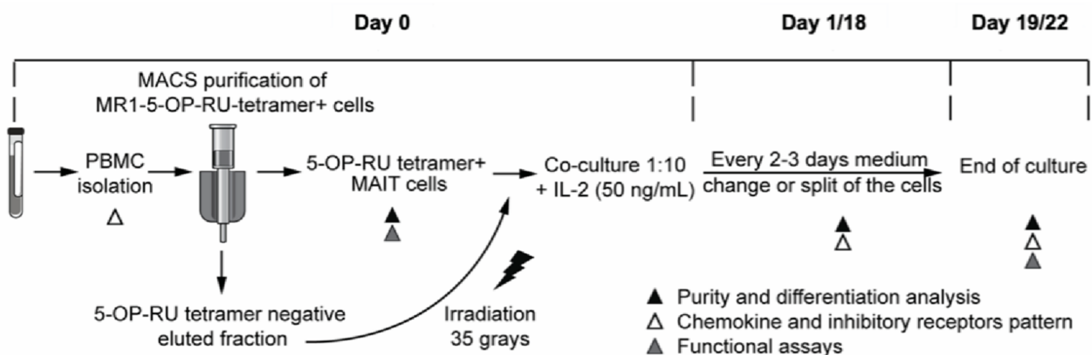

Figure 8: Schematic overview of the optimized MAIT cell expansion protocol as depicted in **paper I**.

Qualitative characterization of the MAIT cell culture at the end of the expansion showed that MAIT cell purity was over 95% (**Paper I Figure 1B&C**). The CD8 and CD4 phenotype was preserved after expansion and was comparable to the day 0 *ex vivo* MAIT cells, although a significant increase in DN MAIT cells was observed (**Paper I Figure 1D**). We hypothesized that this skew in phenotype profile was likely due to increased cellular activation in the CD8 population, as previously described [39].

MAIT cell proliferation was induced on day 7 and proceeded exponentially until the expansion culture endpoint (around day 21), reaching an average expansion fold of 258 (**Paper I Figure 1E&F)**. Furthermore, the MAIT cell viability was >85% (**Paper I Figure 1G)**. We calculated that this protocol could yield up to $1x10^9$ MAIT cells from 50 mL buffy coat (**Paper I Suppl. Table 1)**, highlighting the clinical feasibility of the method.

Importantly, given that we aimed to use these MAIT cells for adoptive T cell transfer in patients, we further assessed the efficiency of this protocol in patient derived MAIT cells. Chronic HBV patients are known to have reduced MAIT cell frequency [243-245], but application of our protocol to MAIT cells from those patients demonstrated a comparable efficiency to healthy donors (**Paper I Suppl. Figure 1C)**. Taken together, we demonstrated that our method was robust and effective at generating large, pure MAIT cell cultures both in healthy donors and CHB patients.

4.1.2 MAIT TCR-dependent and independent functions are preserved after expansion

The ability of MAIT cells to rapidly elicit effector functions in response to antigens is an important characteristic for their translation into an adoptive cell therapy. Therefore, we next aimed to determine how well these properties were preserved in the expanded MAIT cells relative to freshly sorted (D0) MAIT cells from PBMCs. To model MAIT cell responses towards bacteria-infected cells, we performed co-cultures with THP-1 cells loaded with fixed *E. coli* [45]. In parallel, we cultured MAIT cells in the presence of IL-12 and IL-18 for 24 h to induce TCR-independent activation (**Paper I Figure 2A**). Following bacterial activation, both D0 and expanded MAIT cells upregulated the proinflammatory cytokines TNF and IFN-γ, as well as intracellular production of perforin and GzB (**Paper I Figure 2B**). No marked increase in IL-17A, a characteristic MAIT cell effector cytokine, was observed in either MAIT cell type. To determine how this functional profile was associated with killing of infected target cells, we performed co-cultures with *E. coli*-fed 293T-hMR1 cells which are known to be more resistant to the direct cytopathic effects of bacterial infection [45, 229]. Despite having comparable

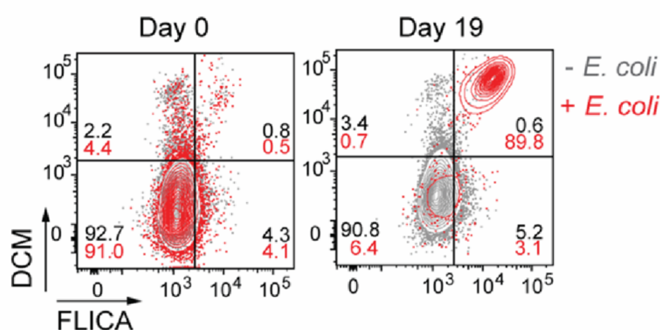

Figure 9: Expanded MAIT cells readily kill *E. coli* infected cells. Adapted from **paper III**.

degranulation marker expression, the expanded but not the D0 MAIT cells were capable of lysis of the infected targets (**Paper I Figure 2C&D; Figure 9)**. The ability to kill is an important property for immunotherapies which require clearance of infected targets such as the tumour eradication strategies described later in the thesis.

Stimulation of expanded MAIT cells with IL-12 and IL-18 resulted in potent upregulation of IFN-γ, an important TCR-independent mechanism in human MAIT cells [19] (**Paper I Figure 2A**). The preservation of this property may be an additional beneficial function in the context of viral infections, such as HBV and HCV, where IFN-γ augments antigen presentation and contributes to viral inactivation and clearance [246, 247].

4.1.3 Expanded MAIT cells are activated and retain their effector memory phenotype

MAIT cells circulating in peripheral blood display an effector memory phenotype which facilitates their ability to traffic to multiple peripheral tissues, persist in the absence of antigen activation, and rapidly launch robust functional responses [248]. The effector memory phenotype is characterized by high expression of CD95, CD45RO, CD127, CD28, CD27 and low CCR7 and CD62L expression [26, 44, 249]. At the end of the expansion culture, expression of the markers associated with this phenotype were mostly preserved, although significant downregulation of CD127 and CD27 was seen (**Paper I Figure 3A**). The loss of CD27 may be attributed to performing the expansion in IL-2, as previously described [250].

Activated T cells can upregulate the expression of immune checkpoint receptors that can positively and negatively modulate their functional responses [251, 252]. We stained D0 and expanded MAIT cells with a panel of six key inhibitory receptor markers and observed significant upregulation of all receptors except for PD-1, which was significantly downregulated (**Paper I Figure 3B; Figure 10**). We did consider the possibility that upregulation of inhibitory receptors may negatively influence MAIT cell functionality and be indicative of an exhausted phenotype. However, blockade of TIM-3 and LAG-3 did not influence expanded MAIT cell responses to *E. coli* in independent experimental approaches (**Paper I Supplemental Figure 4A**).

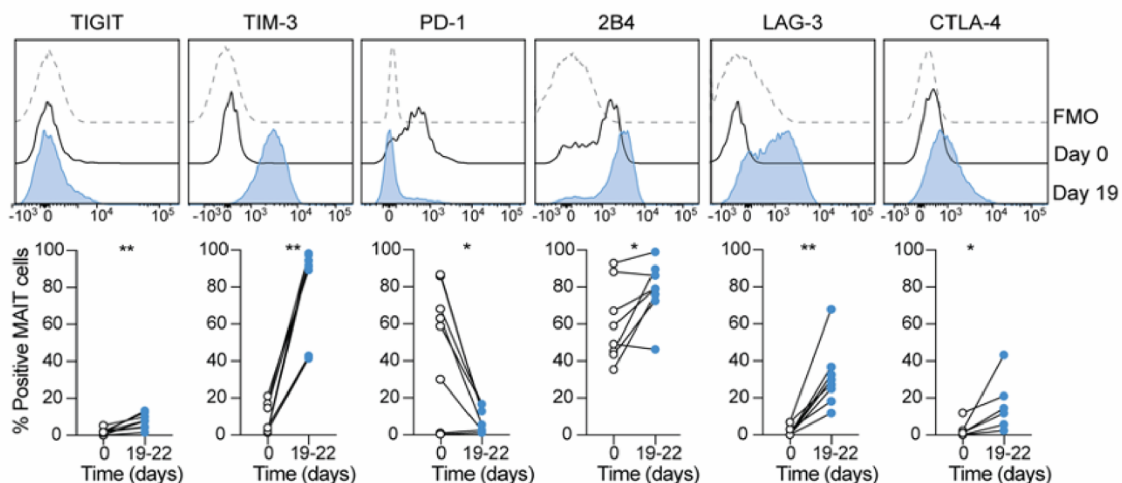

Figure 10: Acquisition of inhibitory receptors on MAIT cells after expansion. Adapted from **paper I**.

T cell infusions containing fewer senescent cells have stronger anti-tumour responses [253] so we next studied two additional markers for T cell senescence on the expanded MAIT cells: killer cell lectin-like receptor subfamily G member 1 (KLRG-1) and CD57 (**Paper I Figure 3C**). No detectable upregulation of either marker was found in the expanded cultured MAIT cells which again supported a mature, but non-terminally differentiated phenotype in the MAIT cell cultures generated using this methodology.

4.1.4 MAIT cells acquire a more diverse chemokine receptor profile after expansion

The ability of MAIT cells to migrate towards tissues is mediated by the expression of a diverse chemokine receptor profile which can sense tissue-specific chemokines at distal sites [26, 254]. Next, we described the MAIT cell chemokine receptor profile after expansion and compared it to D0 MAIT cells. After expansion, we saw that for all chemokine receptors screened, except for CXCR4, the MAIT cells retained or expressed a more diverse chemokine receptor profile (**Paper I Figure 4A**). Because liver tropism constitutes one of the main hypotheses for **Paper II**, the observation of a potentially enhanced migration via CCR5, CCR6, CXCR3, and CXCR6 was encouraging, as they have been linked to mediating liver homing [48, 49, 70, 255]. To confirm that the newly expressed chemokine receptors were functional, we performed transwell assays using single chemokines (**Paper I Figure 4B**). Strong tropism towards CCL5, and moderate migration towards CXCL10, CXCL16, CXCL9, and CXCL11, collectively indicated that expanded MAIT cells can actively sense and migrate towards liver-associated chemokines. Interestingly, despite the reduced CXCR4 expression observed after expansion, the expanded MAIT cells still readily migrated towards CXCL12, another important ligand for homing towards liver and HCC [256].

4.2 ENGINEERING MAIT CELLS TO EXPRESS HBV SPECIFIC TCRS

Numerically depleted and functionally impaired HBV-specific T cells are a hallmark of HBV-related HCC [151]. Since over 90% of HBV-HCCs contain chromosomal HBV integrations that can be presented to T cells via MHC class I, there is a strong rationale for restoring functional HBV-specific T cell responses for HCC clearance [150]. Equipped with an efficient and reproducible method for expanding MAIT cells from **paper I**, the aim of **paper II** was to engineer MAIT cells with an HBV-specific TCR and test their functional, cytotoxic, and migratory potential towards HBV-HCC (**Figure 11**). We hypothesized that the augmented cytolytic properties and liver homing potential we observed in the expanded MAIT cells make them an attractive cell type to explore for hepatic malignancies. Using 2D and 3D microfluidic co-culture models, we compared these properties with HBV TCR-redirected conventional T cells that have been described in clinical studies [150, 163].

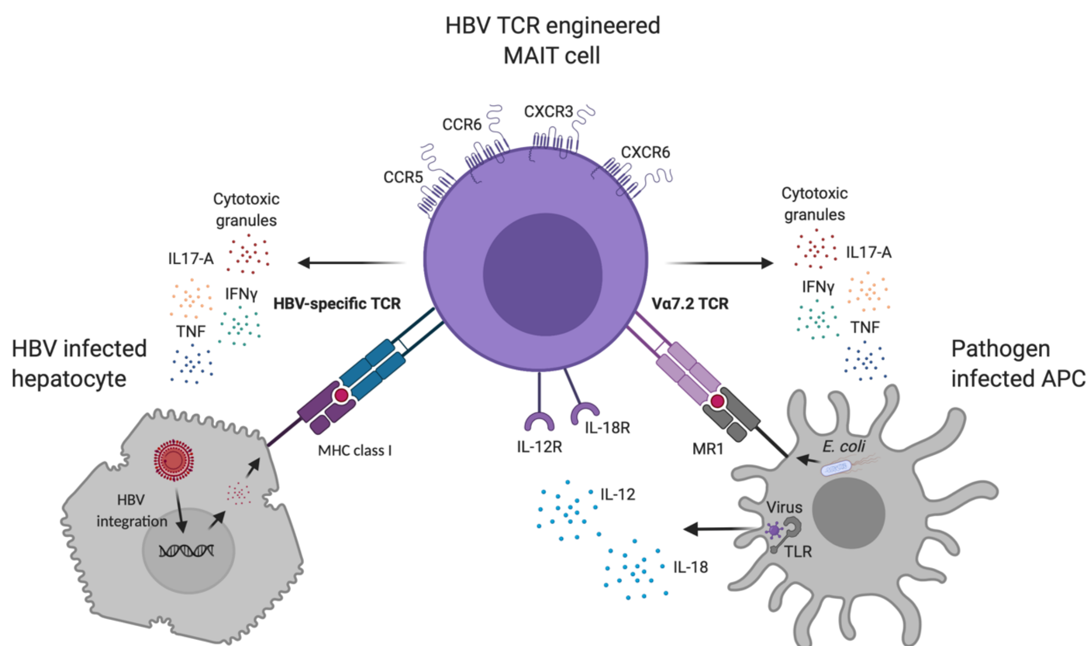

Figure 11: Illustration of the main aim of paper II: Generating HBV-specific MAIT cells through TCR-redirection. Created using Biorender.com.

4.2.1 Virus-specific MAIT cells: proof-of-concept and expression kinetics

Around the time that the MAIT cell expansion protocol was developed, we did not have access to HBV-HCC cell models to test MAIT cells responses via an HBV TCR. As an alternative, we chose to test our initial proof-of-concept in a related liver pathogen model: the HCV replicon hepatoma cell lines and corresponding HCV TCR previously evaluated in our laboratory [226, 233, 234]. We transfected MAIT cells with an HLA-A2 restricted TCR targeting the HCV $NS3_{1073}$ epitope (**paper I Figure 5**). As this TCR was generated from murine T cell hybridomas and expresses a Vβ 8.3 TCR chain [233], it was possible to confirm surface exogenous TCR expression using the murine Vβ 8.3 specific antibody. Donor-matched mock transfected MAITs (pulsed without mRNA) were used to set the baseline fluorescence to

quantify the HCV TCR-positive cells (**paper I Figure 5A**). Next, we used T2 cells loaded with the HCV_{1073} target peptide to determine the functional responses elicited through the HCV TCR (**paper I Figure 5B-D**). Comparing these functions with those mediated through *E. coli* activation shown in **paper I Figure 2A**, showed a lower expression of the degranulation marker CD107a, comparable IFN-γ production, a moderate increase in TNF, and a potent upregulation of IL-17A. It is important to note that this is a high avidity TCR which has demonstrated strong cytotoxic responses in previous experiments in activated T cells [226, 234]. Furthermore, the induction of such a differential cytokine response is in-line with what is known about MAIT cells encountering metabolites derived from different microbial sources [44].

Upon availability of the HBV cell models through our collaboration with Duke-National University Singapore, we next performed in-depth evaluations of TCR-MAIT cells in an HBV-HCC model. HBV-HCC is associated with poor prognostic outcomes, so there is a great need for therapeutic strategies [257]. For comparative purposes, we included donor-matched conventional T cells which also underwent activation and expansion. We performed TCR-redirection of conventional T cells with the HBV TCR and applying the same transfection protocol in **paper I**, we also generated HBV-specific MAIT cells. We compared their surface expression of the TCR using an HBV_{s183} loaded dextramer (**Paper II Figure 1B**). Since this TCR construct encodes a Vβ3 TCR we used antibody surface staining to track the expression kinetics over time [224]. In-line with previous kinetics studies of TCR genes introduced using mRNA transfection [223], we observed a steady decline in TCR expression over time, with almost no detectable TCR staining after 120 hours. This transient TCR expression is important from a clinical safety perspective as in cases of on target-off tissue toxicities or the induction of cytokine storm, transferred T cells will have lost their HBV specificity. The risk of non-specific targeting is particularly high in HBV-HCC where infected hepatocytes in adjacent normal liver tissue may also be selectively lysed by HBV TCR-specific T cells [149].

4.2.2 TCR-MAIT cells recognize and kill HBV targets in an antigen-specific manner

Next, we tested the functionality of the HBV TCR in both cell types. Using the same antigen presentation assay described in **paper I**, we co-cultured both cell types with T2 cells pulsed with increasing concentrations of HBV_{s183} peptide (**paper II Figure 2A**). We demonstrated that both cell types upregulated CD107a and polyfunctional cytokine responses, with a notable IL-17A production in MAIT cells which was not observed in conventional T cells. Interestingly, TCR MAIT cells responded at a log fold lower concentration of peptide than conventional T cells, which could be advantageous in HBV where MHC class I downregulation has been described [258]. However, this data is only representative of one donor, and the TCR sensitivity should be investigated further.

To test functional responses in a hepatoma cell model, we stained for degranulation marker and cytokine expression after co-culture with HepG2 cells pulsed with and without peptide. The HBV expressing cell line, HepG2.2.15 which contains a full viral genome and has been used to test HBV T cell functionality in previous experiments [150] was used as a more physiologically relevant cell model (**paper II Figure 2B&C; Figure 12**).

Both cell types were again capable of mounting potent responses towards the peptide-pulsed positive control. Relative to the conventional T cells, MAIT cells produced more IL-17 but less IFN-γ. A similar profile, albeit with a substantially lower magnitude, was observed after co-culture with the HepG2.2.15 cell line, highlighting that the functional responses are correlated to the magnitude of the TCR stimulation.

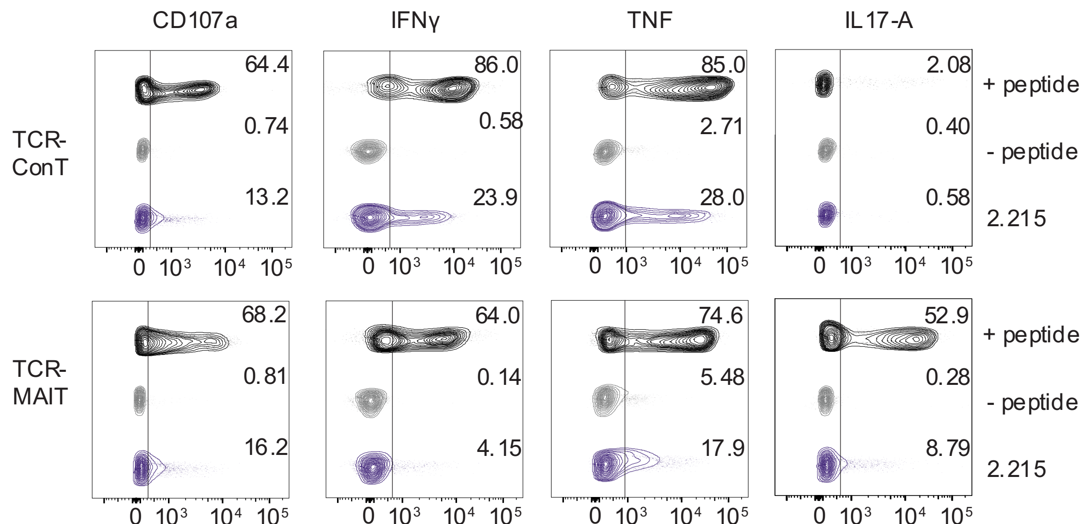

Figure 12: HBV-specific T cell responses after engineering with an HBV-specific TCR. Adapted from **paper II**.

Given the differential results in degranulation and actual killing observed in **paper I** in the expanded MAIT cells, it was important to test how well the functional responses we observed translated into HBV-HCC killing. To do this, we utilized a real-time impedance-based cytotoxicity assay. Both cell types killed the HBV expressing cell line with comparable efficiency (**paper II Figure 2D&E**), demonstrating that in 2D settings, HBV-specific MAIT cells are at least as good as HBV-specific conventional T cells at hepatoma cell killing. Adding IFN-γ to the co-cultures enhanced T cell killing by upregulating HLA-A2 expression (**paper II Figure 2F&G**).

Due to their high levels of expression of MDR1, MAIT cells have demonstrated resistance to xenobiotics, such as chemotherapeutic drugs [26, 92, 259]. In contrast, MAIT cells treated with the immunosuppressive drugs tacrolimus (TAC) and mycophenolic acid were not shielded from their anti-proliferative and cytotoxic effects *in vitro*, despite also being substrates of MDR1. Importantly, their TCR dependent and independent functions were preserved [92], which is of clinical importance given that these drugs result in severe impairment of the functionality of conventional T cells [179]. We wanted to examine MAIT cell resistance in the context of HBV-HCC immunotherapy and specifically, we explored the effects of post-liver transplantation immunosuppressive treatment regimens on HBV TCR-MAIT cell killing efficiency. In this pilot study, we treated donor-matched HBV TCR-MAIT and conventional T cells with clinically relevant concentrations of rapamycin, TAC, and TAC in combination with mycophenolate mofetil (MMF) and performed the same real-time killing assay described previously (**Figure 13**) [260].

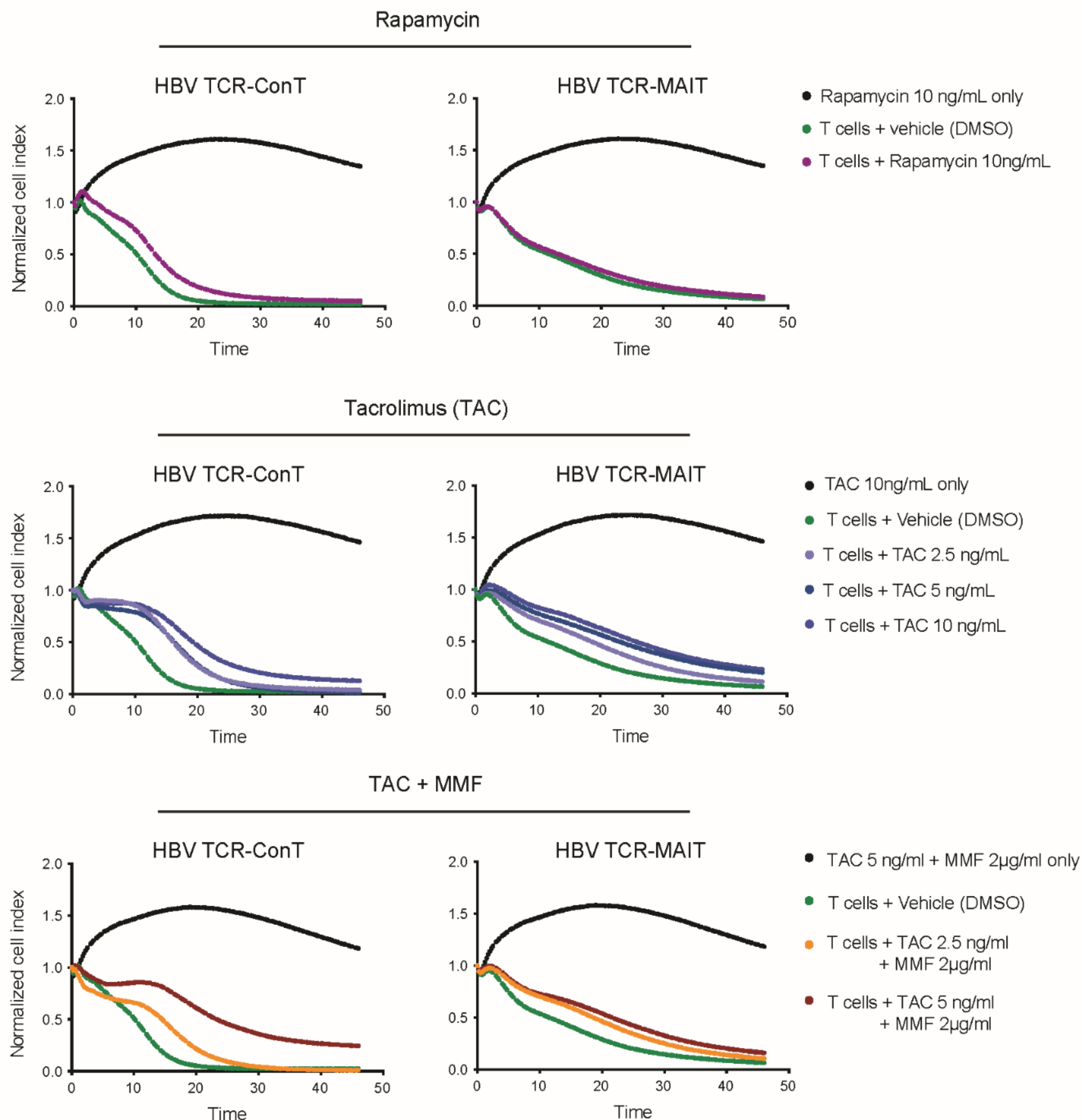

Figure 13: Effect of immunosuppressive drugs on HBV TCR-T cell killing of HepG2.2.15 cells in the real-time impedance cytotoxicity assay. Unpublished data.

TCR-redirected T cells were pre-treated for 5 hours [179] and added to HepG.2.2.15 cell at a 1:1 ratio. We observed a slight inhibition in HBV-HCC cell killing in the TCR-conventional T cells following rapamycin treatment, but TCR-MAIT cells appeared to be unaffected. It has previously been demonstrated that TAC can inhibit the efficiency of TCR-T cell killing by up to 50% in conventional T cells [179]. We observed a similar reduction in HBV-HCC killing in both cell types in the presence of the highest concentration of TAC (10 ng/mL). This indicated that MAIT cells may not be any more resistant to TAC-mediated immunosuppression than conventional T cells, and contrasts with previous observations that functionality is preserved in the presence of TAC [92]. Similarly, in the TAC + MMF combinational treatment condition, we saw a potent inhibition in T cell killing in both cell types, which is also in-line with a previous study in conventional T cells [179]. While immunosuppression is vital to prevent transplanted liver rejection, the negative effects it has on TCR-T cell functionality should be considered. It was surprising to see that the TCR-MAIT cell killing was not preserved as

previously described, especially as similar drug concentrations were used in this experiment [92]. One possible explanation is that the expansion protocol may have had an impact on expression of MDR1, resulting in suboptimal efflux of drugs, although we did not include this in our post-expansion phenotype panel. Dual electroporation with mRNA encoding HBV TCR-specific genes and mutated variants of intracellular molecules required for TAC and MMF signaling has demonstrated success in "armoring" TCR-conventional T cells from immunosuppression [179]. A similar approach could hold potential for TCR-MAIT cells.

4.2.3 TCR-MAIT cells migrate towards HBV-HCC in a 3D microfluidic model

The expanded MAIT cell chemokine receptor profile has been discussed in section *4.1.4*. However, in **paper II**, we compared it with that of TCR-redirected conventional T cells (**paper II Figure 3A**). Strikingly, CCR5, CCR6, and CXCR6 expression was dominant in the MAIT cells, while a larger proportion of conventional T cells were positive for CXCR3. From these findings, we hypothesized that while both cell types can migrate towards liver, MAIT cells may have a competitive edge through utilization of the CCR5 and CCR6 pathways. Both cell types displayed comparable expression of VLA-4, a key adhesion molecule for liver targeting [56, 261] . We also attempted to detect LFA-1, although the staining was unsuccessful, even after induced activation with PMA/ionomycin (*not shown*).

Although we were able to demonstrate chemokine receptor-mediated MAIT cell migration in transwell assays in **paper I Figure 4B**, we sought to investigate how well this translated into migration towards liver cancer cell lines. To do this, we employed a 3D microfluidic cell culture device which has previously been described to quantify the tumour homing and infiltration properties of TCR-redirected T cells [173]. The target cell used in this assay was the HepG2 cell line transfected with HBV PreS1 linked to a GFP reporter gene. We also introduced additional parameters to this assay to recapitulate specific aspects of the HCC microenvironment: low oxygen tension and inflammation [173] (**paper II Figure 5A-C**).

We observed that overall, MAIT cells migrated towards the HBV-HCC cell line more readily than conventional T cells but were less efficient at target lysis, despite normalizing for differential TCR expression. We observed large inter-donor variation, which was not observed in the 2D assays, indicating that 3D co-culture systems may be more sensitive for studying T cell interactions within the TME, as previously described [173]. Different donors were used for these experiments compared with the inhibitory receptor staining. Therefore, it is possible that differential expression of immune checkpoint molecules may have impacted the cytotoxic potential of the MAIT cells upon encountering the HBV-HCC cell line. Immune checkpoint blockade assays like those employed in **paper I** would have been useful to determine the extent to which they had an impact. It is difficult to draw conclusions on this data when such variability was observed and repeated studies with a larger number of donors are warranted.

However, this data did reinforce the enhanced liver tumour migratory potential of MAIT cells, one of the main hypotheses that underpinned this study. The combined (n=3) TCR-T cell migration data under normoxic and hypoxic conditions is shown in **Figure 14**. Interestingly, the hypoxic condition augmented TCR-MAIT cell migration which was not observed in the conventional T cells redirected with the same TCR. The preferential homing towards low oxygen tension may be particularly advantageous for successful infiltration of HCC, which can reach oxygen levels as low as 0.8% O_2 [262].

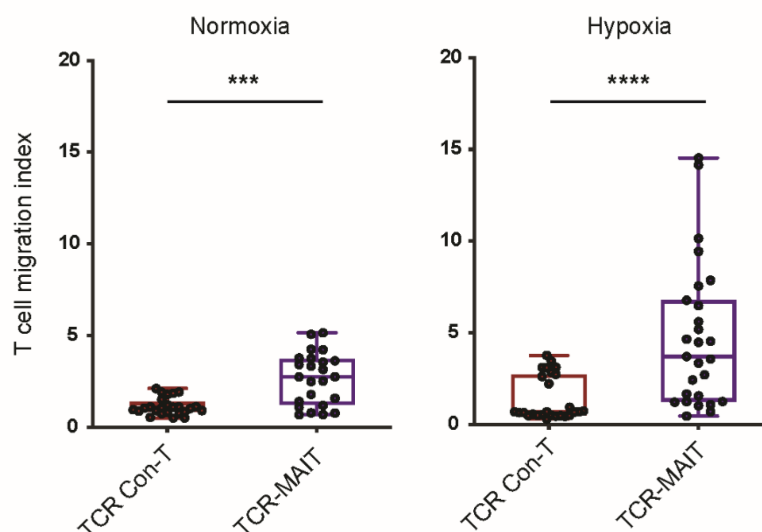

Figure 14: TCR-T cell migration towards HBV-HCC under normoxic and hypoxic conditions. Summary data from **paper II**.

4.2.4 Dual TCR expression does not inhibit endogenous MAIT cell functions

The presence of CD3 is required for stable TCR surface expression [263]. However, the introduction of exogenous TCRs can result in competition for CD3 molecules and inhibit the reactivity of one or both expressed TCRs [264]. To test the effect that HBV TCR expression had on the endogenous MAIT cell TCR, we performed 5-OP-RU-MR1 tetramer staining 24h after mRNA transfection of the exogenous TCR (corresponding to peak expression). Although we did not observe any significant difference in the % of MR1 tetramer cells before and after transfection, a significant reduction in the median fluorescence intensity (MFI) was observed in the dual-TCR population (**paper II Figure 5 A&B**). Next, we interrogated whether this decrease in TCR expression intensity translated into decreased responsiveness to a MAIT cell agonist, *E. coli*. We applied the same MAIT cell activation method described in **paper I Figure 2A** and found that they could still granulate and kill *E. coli* infected cells in a manner that was strongly dependent on MR1 (**paper II Figure 5 C-E; Figure 15)**. Importantly, IFN-γ production in response to IL-12 and IL-18 was also preserved (**paper II Figure 5&G**).

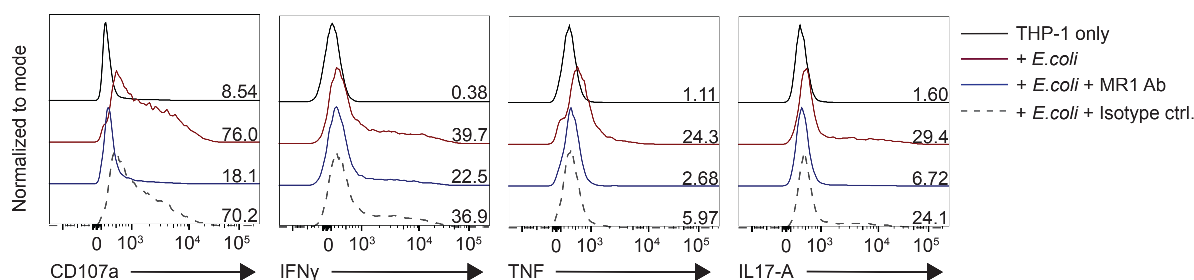

Figure 15: MR1-mediated MAIT cell responses are preserved after engineering with an HBV-specific TCR. Adapted from **paper II**.

4.3 STUDYING MAIT CELLS IN HIV-1 INFECTED WOMEN

Moving away from therapeutic strategies, the third study of this thesis aimed to address more fundamental questions about MAIT cell biology in viral infection: MAIT cell dysfunction during HIV-1. It has been reported that circulating MAIT cells are activated and proliferate during the acute phase of HIV-1 infection [200], before becoming depleted and functionally impaired as HIV-1 chronicity progresses [46, 201]. This temporally dynamic process is also observed in gut resident MAIT cells, indicating that this effect may correlated to microbial translocation at the virally compromised gut epithelium [200, 201]. However, the systemic effects of HIV-1 infection on MAIT cells residing in other tissue compartments are understudied. The female genital tract is an important site for HIV acquisition [265] and the role of the vaginal microbiome in this context is emerging [266]. MAIT cells have been detected in the female genital mucosa, but how HIV-1 influences these cells, and the extent to which they interact with the cervicovaginal microbiome, is unknown. In **paper III**, we studied circulating MAIT cells and ectocervical biopsies in a Kenyan cohort of HIV+ female sex workers (HIV+FSW), HIV- female sex workers (HIV-FSW), and HIV- low risk women (HIV-LR) to ascertain how HIV-1 infection and high-risk sex work may influence local MAIT cell composition.

4.3.1 Phenotype of circulating MAIT cells in HIV-1 infected women

In line with previous studies, circulating MAIT cells in chronic HIV-1 infected women were numerically depleted [46, 202] (**paper III, Figure 1B**). The percentage of blood MAIT cells differed significantly between the HIV+FSW, HIV-FSW, and HIV-LR groups (median: 0.14%, 0.38%, 0.95%, respectively). The majority of MAIT cells in all groups expressed an effector memory phenotype, designated as CCR7-CD45RO+, with the remaining MAIT cells showing a terminally differentiated phenotype (defined as CCR7-CD45RO-), with comparable phenotypic distributions between the groups (**paper III, Figure 1C**). However, significant loss of CD27 expression was observed in the HIV+FSW which indicated progression towards advanced maturation (**paper III, Figure 1D**).

Given that HIV-1 is associated with chronic systemic immune activation, circulating MAIT cells in all study cohorts were evaluated by flow cytometry for their expression of the activation markers CD38, HLA-DR, CD69, and PD-1. Visualization of the data using t-SNE and an unbiased cell cluster analysis identified two phenotypically different MAIT cell populations (**paper III, Figure 2A&B)**. Expression levels of activation markers distinguished non-activated MAIT cells from activated MAIT cells (**paper III, Figure 2C)**. From this data, we observed a significantly lower frequency of non-activated MAIT cells relative to activated MAIT in all three study populations (**paper III, Figure 2D)**. Next, we performed targeted analysis of MAIT cells activation markers by traditional gating methods (**paper III, Figure 3A&B**). Like previous studies, we observed significant upregulation of CD38 and HLA-DR in MAIT cells in the HIV+FSW group, although no significant differences in CD69 or PD-1 were seen. Furthermore, the percentage of circulating MAIT cells was inversely correlated to the expression of all mentioned activation markers, indicating that activation contributes to their

numerical decline in blood. The HIV+FSW group showed a significantly lower number of CD8+ MAITs relative to DN MAIT (**paper III, Figure 3C)**, a characteristic we described in **paper I** as indicative of activation, and reinforced by the observation that the percentage of DN MAITs was positively correlated to the plasma HIV load. Notably, DN MAIT cells are more susceptible to activation induced cell death [39] which may also account for the MAIT cell loss observed in the blood in this cohort (**paper III, Figure 3D)**.

4.3.2 MAIT cells are preserved in the ectocervix of HIV-1 infected women

High immune activation during HIV-1 infection is not only limited to the blood but is also seen in mucosal tissues, including the ectocervical compartment [210, 267, 268]. To ascertain whether the systemic loss of MAIT cells was also reflective of the ectocervical-resident MAIT population, we performed *in situ* staining where MAIT cells were defined as Vα7.2+IL-18+ double positive cells, as previously described [43] (**paper III, Figure 4A**). No significant differences in the number of MAIT cells residing in the ectocervical compartment was observed between the HIV+FSW and HIV-LR groups (**paper III, Figure 4B; Figure 16**). This indicates that like the rectal mucosa, MAIT cells in the ectocervical compartment appear to be less impacted quantitatively than their circulating counterparts during the chronic phase of HIV-1 infection [201]. It is worth noting that there are dynamic changes in MAIT cells over the course of HIV-1 infection [200].

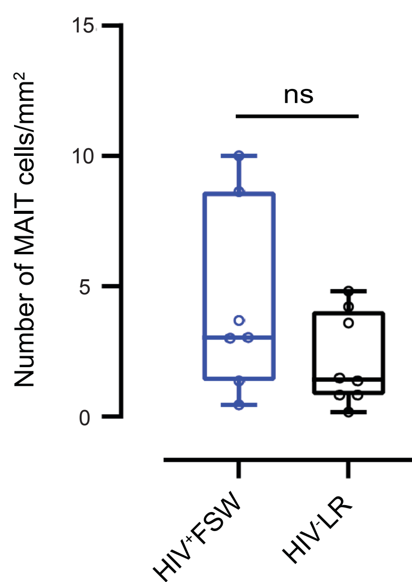

Figure 16: MAIT cells are numerically preserved in the ectocervical compartment in HIV-1 infected women relative to virus negative low-risk women. Adapted from **paper III**.

Therefore, it is possible that numerical fluctuations in ectocervical MAIT cells occur during the peak viremic phase in the early infection. However, our study design was cross-sectional, so addressing this question was outside the scope of the project. Furthermore, sample availability limited the number of individuals who could be included for the *in situ* analysis.

TCR-independent MAIT cell activation is of particular importance during viral infections and is driven by the local cytokine milieu. MAIT cells express high levels of receptors for IL-18, IL-15, and IL-7, which can independently and synergistically modulate their functional responses [19, 269]. Therefore, we performed qPCR on bulk RNA extracted from the ectocervical biopsies to quantify the expression of these cytokines (**paper III, Figure 4C)**. Interestingly, we did not observe any significant differences in cytokine expression in the HIV+ FSW and HIV- FSW groups, indicating that infection status did not impact the local inflammatory environment in female sex workers. Surprisingly, the highest levels of cytokine expression were observed in the HIV- LR group.

4.3.3 Dominant TRAV1-2-TRAJ20 TCR expression in the ectocervical compartment

MAIT cells have an invariant TCRα chain that predominantly uses *TRAV1-2* bound to *TRAJ33/20/12*. Together, these three TCR rearrangements comprise ~95% of MAIT TCR clonotypes in blood [13, 162, 270]. The *TRAV1-2-TRAJ33* rearrangement is considered the canonical MAIT TCRα chain in blood and has also been identified as the most abundant in the oral mucosa [162, 271]. It has been suggested that interactions between MAIT cells and microbes *in vivo* can shape their TCR repertoire by selective clonal expansion of more responsive MAIT clonotypes [44]. Indeed, differential MAIT cell responses towards distinct pathogens is believed to be due to expansion of MAIT cells expressing TCRs with higher functional avidity towards riboflavin metabolite antigens, rather than specialized recognition of discrete microbe-specific ligands [272]. Taken together, this indicates mechanisms of MAIT TCR adaptation to their stimuli. Therefore, we next investigated whether residency within the ectocervical compartment shaped MAIT TCR rearrangement. Due to limited sample availability, we were unable to perform single-cell TCR sequencing of MAIT cells in the ectocervical compartment and instead relied on detection of *TRAV1-2-TRAJ33/20/12* transcripts in bulk RNA extracted from ectocervical tissue (**Figure 17**). This was also performed in RNA extracted from PBMCs from the same study groups (**paper III Figure 5A**). To account for numerical differences in T cell populations between the study groups, expression of the *TRAV1-2* joining genes was normalized to the TCR Cα chain. Unlike previous studies, we did not observe a dominant proportion of the *TRAV1-2-TRAJ33* TCR rearrangement in PBMC samples, a pattern also observed in the ectocervix. No significant difference in expression of any of the three transcripts was seen in paired analysis of blood and ectocervix (**paper III Figure 5B**), indicating donor-specific, rather than compartment-specific TCR usage. This may be explained by high vascularization of the female genital tract which can result in a resident MAIT cell population with closer similarity to their circulating counterparts [273].

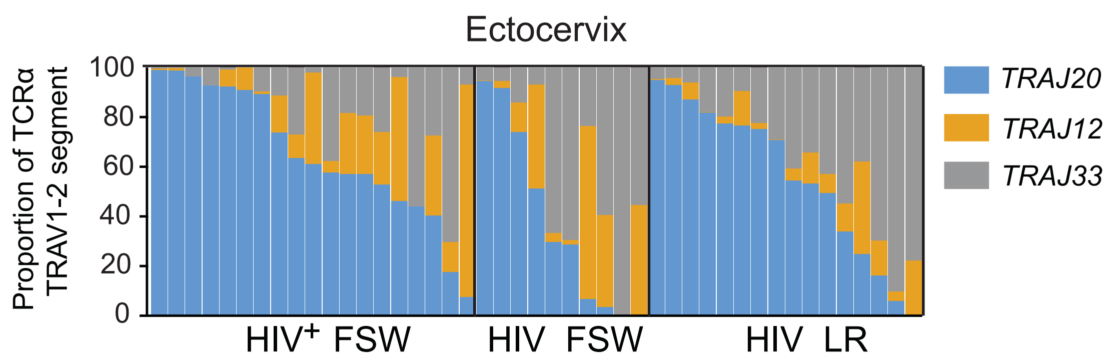

Figure 17: MAIT TCRα gene expression in the ectocervical compartment of HIV+ FSW, HIV- FSW, and HIV- LR women. Adapted from **paper III**.

To determine whether HIV-1 infection status plays a role in MAIT TCR rearrangement, expression of the *TRAV1-2-TRAJ33/20/12* transcripts was compared between the groups (**paper III Figure 5C**). No significant difference in TCR gene expression was observed in the blood-derived samples, indicating that on transcript level, viral infection does not impact

the circulating MAIT TCR *TRAV1-2-TRAJ33/20/12* composition. In contrast, normalized expression of the *TRAV1-2-TRAJ20* transcript was significantly higher in the HIV+ FSW group relative to the HIV- LR group in the ectocervical compartment, suggesting a local viral influence.

It was surprising to not see a dominance of the *TRAV1-2-TRAJ33* transcript in either compartment in any of the study groups. To determine whether there was a bias towards *TRAV1-2-TRAJ20* amplification in our experimental setup, we performed TCR expression analysis on ectocervical samples from uninfected Swedish donors and observed the classical dominant *TRAV1-2-TRAJ33* rearrangement **(paper III Supplementary Figure 2B)**. Given that the microbiome can influence the MAIT TCR profile, it is plausible that the unique *TRAV1-2-TRAJ20* rearrangement dominance may be attributed to different microbiome compositions between European and Kenyan donors. It was also interesting to observe differences in TCR expression between the study groups in the ectocervical compartment. It has previously been suggested expansion of specific MAIT clonotypes may also be influenced by the local cytokine milieu [272]. Therefore, differences in inflammation status in the ectocervical compartment between the HIV+ FSW and HIV- LR groups may perhaps explain the distinct TCR rearrangement.

4.3.4 MAIT TCR rearrangement is not associated with the vaginal bacterial microbiome

MAIT cell interactions with the local microbiome in which they reside are believed to be important for maintenance of homeostasis at barrier tissues. MAIT cells in the female genital tract are biased towards production of IL-17 and IL-22 [53], which are both important for barrier protection and tissue repair in the epithelium. In the gut, it has been suggested that this phenotype is driven by TCR interactions with metabolites produced by commensals in the intestinal lumen [52, 55]. Therefore, we next investigated whether specific MAIT TCR gene rearrangements were associated with the composition of the vaginal microbiome.

16S rRNA sequencing of cervicovaginal lavage samples from HIV+FSW and HIV-FSW detected 98 bacterial taxa at the species level, and *Lactobacilli* at the genus level (**paper III Figure 6B**). Of these, 45% were predicted to be riboflavin competent bacteria, and therefore potentially involved in TCR-mediated interaction with local MAIT cells (**paper III Figure 6C**; **Figure 18**). The healthy vaginal microbiome is typically dominated by *Lactobacilli* species which maintain homeostasis by producing bactericidal compounds and lactic acid to reduce the environmental pH [274]. Notably, both the HIV+ FSW and HIV- FSW groups displayed a more diverse vaginal microbiome with low abundance of *Lactobacillus* species which has previously been associated with increased risk of HIV acquisition [275]. Interestingly, the proportion of riboflavin competent bacteria in the vaginal microbiome was markedly different between individual donors irrespective of HIV-1 infection status, and no significant differences in alpha- and beta- diversity were observed between the groups.

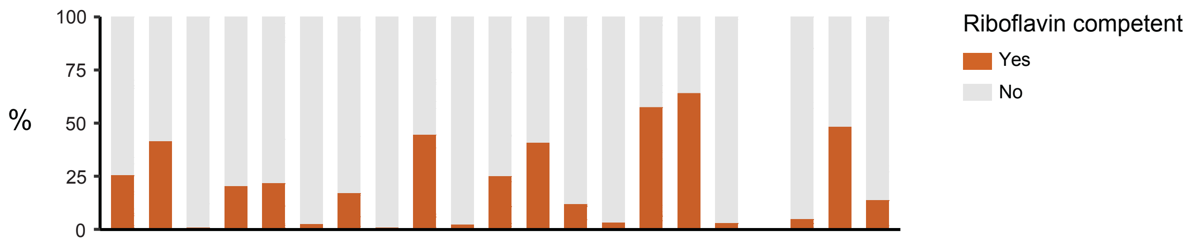

Figure 18: Composition of riboflavin bacteria in the vaginal microbiome of HIV+ FSW and HIV- FSW. Adapted from **paper III**.

One of the primary goals of bacterial microbiome analysis is to identify bacterial profiles that explain associations between study groups and outcomes [276]. However, given the complexity of microbiome analysis, multiple-hypothesis testing is required to reduce the probability of type I errors (i.e., incorrectly positive associations) [277]. In this study, we employed the Benjamini-Hochberg (BH) procedure to control for such errors and did not identify any significant associations between MAIT TCR rearrangement and any of the bacterial taxa in the microbiome analysis. However, given the small sample size, low diversity, and high rate of false negatives associated with the BH method, conservative false discovery rates were a concern. Therefore, we proceeded to present the non-corrected correlations between vaginal microbiome abundance and normalized MAIT TCR *TRAV1-2-TRAJ20/12/33* gene transcripts (**paper III Figure 6D**). We observed that the dominant *TRAV1-2-TRAJ20* expression was negatively correlated with *Lactobacillus crispatus/acidophilus* and positively correlated to *Peptostreptococcus,* of which only the latter is riboflavin competent.

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this thesis, we aimed to explore the translational feasibility of MAIT cells for T cell immunotherapy, as well as their potential role in systemic and tissue immunology during viral infection. Specifically, this involved developing a method to expand MAIT cells *in vitro*, evaluating their functionality against viral antigens encountered through an exogenously expressed MHC class I-restricted TCR, and characterizing the MAIT cell phenotype during HIV-1 infection.

As our understanding of the role of MAIT cells in health and disease grows, so too is the interest in harnessing their properties for immunotherapy. In **paper I**, we developed a protocol to generate large numbers of phenotypically enhanced MAIT cells which demonstrated potent TCR dependent and independent antimicrobial responses. Although the focus of this thesis was to use these MAIT cells for TCR-redirection strategies, the expansion protocol has great therapeutic potential as a stand-alone methodology. Given the donor unrestricted nature of their TCR interactions, expanded MAIT cells have "off-the-shelf" potential, a property cited as one of the holy grails of immunotherapy [278]. Adoptively transferred MAIT cells may hold therapeutic potential in diseases in which bacteria are driving pathogenesis, such as sepsis [279]. However, extensive preclinical evaluations for safety and efficacy under such conditions warrant future investigation. Expanded MAIT cells were poised to kill, a characteristic that we consider a benefit for our cancer immunotherapy approach, but one which may cause severe responses if they migrate to alternative sites of inflammation. This issue could be exacerbated by their effector memory phenotype which allows them to infiltrate tissues and persist. This also leads to the question of how to ensure that infused MAIT cells home to the correct tissue. In **paper I**, we presented a chemokine receptor profile that is not only involved in migration towards liver, but also potentially skin and gut. Furthermore, given that clinical translation of activated TCR-T cells for HBV-HCC has focused on targeting extrahepatic metastases in patients who have undergone HLA-mismatched liver transplantation [150, 163], their ability to home to secondary, rather than primary tumours, also warrants further investigation.

In addition to their antimicrobial properties, several questions remain to be answered regarding endogenous MAIT cell responses to cancer [241]. Given that metabolites distinct for cancer cells can be presented via the MR1 pathway [96], there is potential for TCR-mediated recognition of cancer metabolites. Although to date, this observation has been confined to a non-MAIT MR1-restricted T cell subset [96], this line of research may unlock another mechanism to explore for MAIT cell immunotherapy. It is also notable that in some cancers, such as colorectal and liver cancer, infiltration of MAIT cells is associated with negative prognostic outcomes [72, 75]. However, the precise mechanisms underlying their switch from protective to destructive in this context are not fully elucidated. Understanding the pro-tumorigenic MAIT cell phenotype has implications for their translation into the clinic.

In **paper II**, we opened up MAIT cell recognition to the vast antigenic potential of MHC class I complexed peptides. Although we specifically focused on HBV-associated peptides in this thesis, our methodology could be applied to other TCR constructs for immunotherapy such as

low avidity [234] or neoantigen-specific TCRs [280]. Furthermore, it would be of great interest to test the ability of MAIT cells to express CAR constructs and to examine how their functionality differs. Given the transient nature of mRNA TCR expression, it can be technically challenging to address multiple mechanistic questions regarding their responses via their new TCR. It is a future aim of our group to generate lentiviral transduced MAIT cell cultures to explore a wider range of questions.

One such avenue of research may be further exploration of MAIT cell behaviour in the 3D setting. It would be useful to test more donors to draw significant conclusions regarding MAIT cell homing and killing towards tumour targets. It could also be interesting to include 3D HCC spheroids [173], as well as a vascularization component [281], to study how these physical parameters affect MAIT cell interactions with the tumour. Microbial species residing in the HCC tumour microenvironment could also be included to investigate how they may directly and indirectly modulate MAIT cell function [282]. Finally, studies on combinational approaches with immune checkpoint inhibitors (e.g., MAIT cells plus PD-1 blockade) also represent a potential line of research to boost MAIT cell antitumour responses [283].

Another important consideration is the co-expression of two functional TCRs in the engineered MAIT cells which has both advantages and disadvantages. On the one hand, there is a risk for non-specific reactivities upon patient infusion. One way that this may occur is through the potential pairing of exogenous and endogenous TCR chains which can generate heterodimers with self-reactivity [284]. If it is the case that reactive TCRs are generated upon recombination, the safety risk in MAIT cells may be augmented given the limited TCR diversity which may lead to a larger proportion of mismatched TCRs recognizing the same targets. Similarly, the semi-invariant antigen specificity means that endogenous MAIT cell recognition is rather broad and raises the question of whether the MAIT cells will encounter microbial targets en route to the tumour site to which they may respond. This may decrease the efficacy of the TCR-redirected therapy, while also running the risk of toxicity at sites with a high microbial or inflammatory load. The counter argument is that it is now also evident that the gut microbiome is a key player in the tumorigenic process and can also contribute to immunotherapy resistance [285]. Therefore, it could also be proposed that introduction of a T cell which can simultaneously recognize and kill pathogen infected cells may comprise a combinational strategy for solid tumours in which bacteria play a role such as HCC [286], pancreatic cancer [287], and colorectal cancer [288].

In **paper III**, we explored the effect of HIV-1 infection on MAIT cells residing in the blood and ectocervical compartment. In line with previous studies, we found that circulating MAIT cells were activated and quantitatively reduced during HIV-1 infection [201] but were numerically preserved in the ectocervical mucosa. In contrast with MAIT cells residing in the oral mucosa in healthy donors without HIV infection [271], which display a unique TCR usage relative to their blood counterparts, ectocervical MAIT cells displayed a similar TCR gene expression profile as that observed in blood. We attributed this observation to the highly vascularized anatomy of the female genital mucosa which may result in a tissue-resident

phenotype that more closely resembles the blood [54]. One limitation of this study was sample availability, resulting in bulk rather than single-cell analysis. Isolation of MAIT cells by tetramer sorting from ectocervical tissue would enable more in-depth immunophenotyping of these cells *in vitro*. Although the phenotype of MAIT cells in the female genital tract has been described [53], it would have been interesting to examine how HIV-1 infection status and sex work impacted their activation and functional responses. For example, future studies could address tissue resident MAIT cell responses towards microbial isolates from the vaginal microbiome rather than model antigens such as *E. coli*. It may also be the case that TCR independent mechanisms dominate MAIT cell function in that tissue, allowing a heterogenous population to persist in the absence of TCR-specific antigen stimulation. Such single-cell studies require large starting samples, such as those obtained after hysterectomy, and were not feasible using the tissue samples that were available to us.

More in-depth TCR phenotyping of cervical MAIT cells would also be interesting to explore in future studies. Here, TCR phenotyping was only performed at the transcript level and only on the semi-invariant α chain. Although the α chain is vital for engagement with MR1-antigen complexes, the MAIT TCRβ chain is believed to play an important role in fine-tuning these responses and displays more heterogeneity [289]. Therefore, potentially subtle differences in MAIT TCR clonotypes were not detected from the methodology we used. It was interesting that we did not observe correlations between MAIT cell TCR usage and the local bacterial microbiome. Viral-mediated dysbiosis of the gut microbiome, including *Firmicutes*-poor and *Bacteroidetes*-rich profiles, has been shown to be significantly associated with MAIT cell function and frequency during HIV-1 infection [196]. Given that MAIT cells responses are shaped not by microbe distinct ligands, but broadly by the abundance and composition of riboflavin metabolites, riboflavin biosynthesis gene expression rather than qualitative assessment of positive and negative riboflavin competence may be a better approach for correlation in future studies. Because of the importance of commensal fungal species in the vaginal microbiome [290], MAIT cells residing in the female genital tract may be skewed towards fungal recognition. Bacteria dominate the microbiome field, but the role of the mycobiome is emerging [291]. It would be interesting in future studies to correlate MAIT TCR usage with dominant vaginal fungal flora such as *C. albicans* and compare their cytokine profile with MAIT cells derived from blood.

The fields of immunotherapy and MAIT cell biology are rapidly evolving. The main findings of this thesis demonstrate how these exciting areas can be combined and represent a contribution to basic MAIT cell biology and their potential therapeutic targeting.

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