MAIT cells in the liver environment



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Abstract

Mucosal associated invariant T (MAIT) cells are an important innate T cell population capable of recognising riboflavin-derived ligands presented by the evolutionarily conserved MHC-related 1 (MR1) molecule. The ability to activate MAIT cells either through their T cell receptor or cytokines alone enables them to respond to both bacterial and viral infections. The liver is a common site of bacterial and viral infection, due partly to its anatomical setting and partly its tolerogenic environment. MAIT cell enrichment and function in the liver and their impairment in liver and non-liver diseases is not fully defined/understood. In this project we investigated MAIT cell activation by liver resident cells and looked closely at hepatocyte specific influence on MAIT cells to understand the role of liver-activated MAIT cells in liver protection or pathogenesis. Using liver cell lines and primary MAIT cell co-cultures and flow cytometry I investigated the ability of liver resident cells to influence primary MAIT cells derived from healthy donors. I have identified that MAIT cells could be activated by liver endothelial cells using both MR1 and cytokine pathways in response to bacterial stimuli which was not seen with hepatocytes or hepatic stellate cells. MAIT cells were suppressed indirectly in a contact-independent manner, by a small, soluble, cyclooxygenase-dependent, hepatocyte specific mediator/s. Strong suppression of MAIT IFN-γ by prostaglandins and a partial recovery with cyclooxygenase inhibitor treatment of hepatocytes strongly indicate the role of prostaglandins as well as other factors in controlling MAIT cell activation and function. Prostaglandins can be produced by several liver cell types, are primarily involved in liver homeostasis and regeneration but can also impact MAIT cell function by potently suppressing their pro-inflammatory and cytotoxic capacity. The role of MAIT cells in liver disease was analysed by high dimensional, multiparametric spectral flow cytometry. MAIT cell numbers declined significantly in cirrhosis and liver tumor, displayed an exhausted phenotype and were highly dysfunctional. Unsupervised analysis of the same cohort reveals exhaustion and loss of several MAIT cell-related populations which had reduced cytotoxic and inflammatory potential. Overall our data identifies the potential for MAIT cell activation by liver resident cells which can be altered by paracrine signals from neighbouring cells. Furthermore, the loss and functional defects in MAIT cells results in reduced liver protection.

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"The gift of knowledge is the highest gift in the world" - Swami Vivekananda.

"The more one knows, the luckier he is, for knowledge is the greatest gift in life."
L Frank Baum.

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Abbreviations

18RAP	IL-18 receptor alpha protein
3-FSA	3-formylsalicyclic acid
5-AR-U	5-amino-6-d-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formyl pterin
ADP	Adenosine diphosphate
AFP	Alpha feto protein
AIH	Autoimmune hepatitis
AILD	Autoimmune liver disease
ALD	Alcoholic liver disease
AMP	Adenosine monophosphate
APC	Antigen presenting cell
ARC	Alcohol related liver cirrhosis
AS	Ankylosing spondylitis
ASH	Alcoholic steatohepatitis
ATCC	American Type Culture collection
ATP	Adenosine tri-phosphate
BAX	Bcl-2-assoicated X protein
BCL	B cell line
Blimp1	B lymphocyte-induced maturation protein 1
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CDR	complementary determining region
CIA	collagen induced arthritis
COX	Cyclooxygenase
CREB	cAMP response element binding protein
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C Motif Chemokine receptor
CYP450	Cytochrome P450
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP1	Prostaglandin D2 receptor 1
DP2	Prostaglandin D2 receptor 2
E. coli	*Escherichia coli*

EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECACC	European Collection of Authenticated Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic acid
ELISA	Enzyme-linked immunosorbent assay
EOMES	Eomesodermin
EP1	Prostaglandin E2 receptor 1
EP2	Prostaglandin E2 receptor 2
EP3	Prostaglandin E2 receptor 3
EP4	Prostaglandin E2 receptor 4
FACS	Fluorescence activated cell sorting
FASL	Fas-Ligand
FASR	Fas-receptor
FBP1	Fructose-Bisphosphatase 1
FCS	Fetal calf serum
FGF	Fibroblast growth factors
FLAP	5-lipoxygenase–activating protein
FO	7,8-didemethyl-8-hydorxy-5-deazariboflavin
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA binding protein 3
GDF-15	Growth/differentiation factor-15
GIPR	Gastric Inhibitory Polypeptide Receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gMFI	geometric mean fluorescence intensity
GPCR	G protein-coupled receptors
Gr	Granzyme
GSA	Gene specific analysis
GSEA	gene set enrichment analysis
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HSC	Hepatic stellate cell
ICAM	Intracellular adhesion molecule
ICOS	Inducible T cell costimulator
IDO	Indoleamine
IFI27	Interferon alpha inducible protein 27
IFIT1	Interferon Induced Protein with Tetratricopeptide Repeats 1
IFN	interferon
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IGF1	Insulin growth factor 1
IHL	intrahepatic lymphocytes
IL-x	Interleukin-x
IP10	Interferon gamma induced protein 10
IP3	Inositol triphosphate
IS	Immunological synapse
IV	Intravenous
KC	Kupffer cell
KIR	Killer cell immunoglobulin-like receptors
KLRB1	Killer Cell Lectin Like Receptor B1
KO	Knock out
LAG3	Lymphocyte Activating gene 3
LAMP3	Lysosomal Associated Membrane Protein 3
LCMV	Lymphocytic choriomeningitis virus
LEC	Liver endothelial cell
LFA-1	Lymphocyte function-associated antigen 1
LILR	leukocyte immunoglobulin-like receptors
LN	Lymph node
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LEC	Liver endothelial cells
LSEC	Liver sinusoidal endothelial cell
LT	Leukotriene
LXA_4	Lipoxin A4
MAIT	Mucosal associated invariant T cell
MDDC	Myeloid derived dendritic cell
MHC-I	Major histocompatibility complex-I
MHC-II	Major histocompatibility complex-II
MIP	Macrophage Inflammatory Protein
MM	Multiple myeloma
MR1	Major histocompatibility complex class I-related gene protein
mRNA	messenger ribonucleic acid
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NFKB1	Nuclear Factor Kappa B Subunit 1
NK	Natural killer cell
NKG2D	Natural killer cell receptor
NKT	Natural killer T cells
NLRP3	NLR family pyrin domain containing 3
NOD2	Nucleotide-binding oligomerization domain-containing protein 2

NOS3	Nitric Oxide Synthase 3
NSAIDS	Non-steroidal anti-inflammatory drugs
PALM	paralemmin protein
PAMP	Pathogen associated molecular patterns
PBC	Primary biliary cholangitis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	programmed cell death protein 1
PDE	Phosphodiesterase
PDGF	Prostaglandin F
PFA	Paraformaldehyde
PG	Prostaglandin
PGD_2	Prostaglandin D2
PGDS	Prostaglandin D synthase
PGE_2	Prostaglandin E2
PGES	Prostaglandin E synthase
PGF_{2a}	Prostaglandin F2a
PGH2	Prostaglandin H2
PGI_2	Prostaglandin I2
PGJ_2	Prostaglandin J2
PKA	Protein kinase A
PLA_2	Phospholipase A2
PLI	Photolumazine I
PLIII	Photolumazine III
PLZF	Promyelocytic leukaemia zinc finger
PMA	Phorbol 12-mystrate 13-acetate
PPR	Pathogen recognition receptors
PSC	Primary sclerosing cholangitis
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RORyt	RAR-related orphan nuclear receptor
S.E.M	Standard error of the mean
SLE	Systemic lupus erythematosus
SN	Supernatant
SOCS	Suppressors of cytokine signalling
ST2	IL-33 receptor
STAT	Signal Transducer and Activator of Transcription
Stim	Stimulated
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TIGIT	T cell immunoreceptor with Ig and ITIM domains

TIM3	T-cell immunoglobulin and mucin-domain containing-3
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll like receptors
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TXAS	Thromboxane A synthase
TXBS	Thromboxane B synthase
Unstim	Unstimulated
VCAM	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WT	Wild type
αGalACer	α-galacturonosylceramide
β2M	β-2-microglobulin

Thesis contributions

Philipp Hackstein – PGD_2 ELISA – Chapter 4 – figure 4.15b

CHAPTER 1 - INTRODUCTION

1.1 Innate and adaptive immunity

The immune system forms one of the most important systems in the human body and is requisite to protect us from infections. It is divided into two major segments; innate and adaptive immunity both of which are supported by the complement system (Murphy and Weaver 2016a).

Innate immunity comprises of cells that rapidly (within hours) respond to invading pathogens after recognition of pathogen associated molecular patterns (PAMPs) (Telser 2002). PAMPs are molecules that are unique to microbes and are absent from human cells thus allowing the discrepancy between self and harmful foreign material. Some examples of PAMPs include lipopolysaccharide (LPS) found on gram-negative bacterial cell wall, peptidoglycans on gram-positive bacterial cell wall, flagellin, viral and bacterial nucleic acids like CpG and single stranded viral RNA (Mogensen 2009). Innate immune cells recognise PAMPs through pattern recognition receptors (PRRs), membrane bound PRRs include the family of toll like receptors (TLRs) and C-type lectin receptors (CLRs). The cytoplasmic PRRs include the NOD-like and RIG-I like receptors (Mogensen 2009). After PRRs recognise and bind their ligands they initiate activation of innate immune cells that can phagocytose pathogens/infected cells and secrete cytokines to activate other immune cells (Murphy and Weaver 2016a). Innate cytokines, particularly the type I interferons are very important for successful antiviral responses and are involved in the activation of many innate and adaptive immune cells (Akira, Uematsu, and Takeuchi 2006). Cellular components of the innate immune system include macrophages, dendritic cells (DC), natural killer (NK) cells and neutrophils all of which can be found

throughout the body, particularly at the site of infection[2,5]. Some innate cells possess cytotoxic activity like the natural killer (NK) cells which can directly kill their targets (Uzman 2001). Most innate cells will be activated through PPRs and cytokines but the NK cells are unique as they have activating (killer immunoglobulin-like receptors (KIRs), NKG2D, NKp46 DNAM-1, etc.) and inhibitory receptors (KIRs and leukocyte immunoglobulin-like receptors (LILRs)). The balance between the activating and inhibitory receptors determine the activation of NK cells or their inhibition (Vivier et al. 2011). The strong binding of NK cells to the major histocompatibility complex class-I (MHC-I) molecule on cells provides an inhibitory signal thus preventing damage to normal healthy cells and allows them to differentiate between self and non-self (Murphy and Weaver 2016b).

The complement system consists of several plasma proteins and as the name suggests complements innate immunity by labelling pathogens for optimal recognition - this process is called opsonization (Rus, Cudrici, and Niculescu 2005). Other functions of complement proteins include chemoattractant activity and structural destruction of pathogens (Dunkelberger and Song 2010).

The adaptive immune system is the second line of defence should innate immunity fail to contain the pathogen. Adaptive immunity is activated several days post infection and requires the help of innate cells like DCs that have specialised molecules to activate T and B cells (Murphy and Weaver 2016b). Pathogen specific epitopes/antigens are presented to T and B cells. In the case of T cells this done by molecules like major histocompatibility complex (MHC) MHC-I, MHC-II and major histocompatibility class-1-related protein (MR1) (Germain 1994), and, in combination with cytokines like

interleukin (IL) -2 (IL-2), IL-12, IL-4, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and many others, they induce differentiation and activation of T cells (David D. Chaplin 2010; Flies n.d.). T and B cells have somatically rearranged receptors thus represent a diverse pool and have the potential to recognise pathogens with high specificity (David D. Chaplin 2010). A major feature of the adaptive immune system is the formation of memory cells; these can be activated quickly to respond to pathogens that have been encountered before, consequently allowing rapid clearance of the infectious agents (Murphy and Weaver 2016b).

The innate and adaptive immunity is bridged by antigen presenting cells (APC) like DCs and unconventional T cells that have characteristics and functions of both the innate and adaptive immune cells, they are also termed innate-like T cells (Gao and Williams 2015). Innate like T cells comprise of invariant natural killer cells (iNKT), gamma delta T cells (γδ T cells) and mucosal associated invariant T cells (MAIT) (Godfrey et al. 2015); a detailed description of antigen specificity and their function is described below.

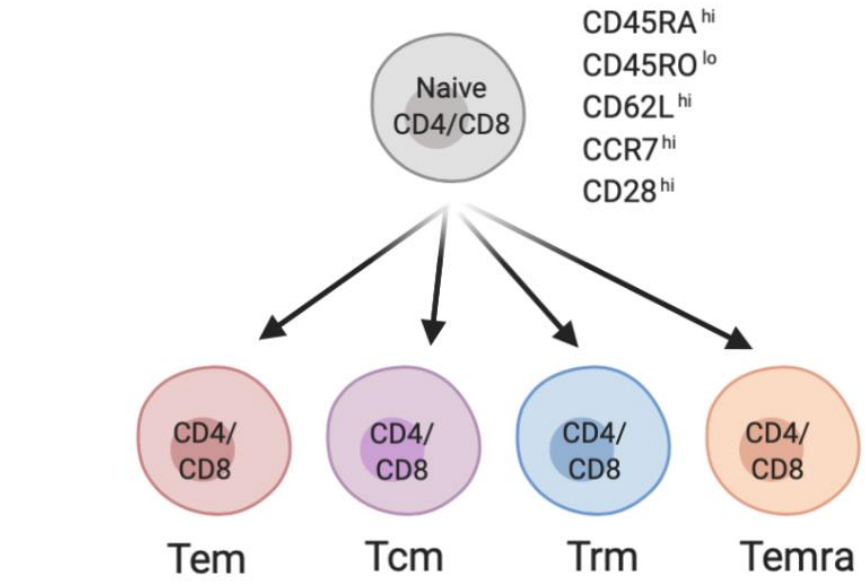
1.2 Conventional T cells

Conventional T lymphocytes express the cluster of differentiation (CD), CD4 or CD8 co-receptor and the αβ T cell receptor (TCR) which is responsible for recognising antigen fragments (Germain 1994). T cells form a diverse group of cells determined by their TCRs which are highly specific for individual antigens, TCR diversity is generated by genetic recombination of the DNA-encoded segments by a process known as somatic V(D)J recombination (Murphy and Weaver 2016b). T cells recognise peptide antigens

presented in the context of MHC-I and MHC-II and respond to intracellular/extracellular pathogens and to malignant cells (David D. Chaplin 2010; Murphy and Weaver 2016b). T cells develop in the thymus, exit as naïve T cells that can be activated in secondary lymphoid organs after they encounter their antigen presented by APCs, a process known as T cell priming (Blair and Dustin 2013). The recognition of antigens by T cells triggers their differentiation into effector cells and initiates clonal expansion (David D. Chaplin 2010). Chemokines such as (C-X-C motif) ligand (CXCL)18 (CXCL18) (lungs), CXCL16 (liver) (C-C motif chemokine) ligand (CCL)25 (CCL25) (intestine) and CCL27 (skin) direct activated T cells to the site of infection where they are able secrete cytokines and kill the infected cells by specialised processes (Turner et al. 2014), for example, cell lysis by cytolytic molecules like granzymes and perforin or through receptor mediated killing by Fas-Ligand (FASL) binding to Fas receptor (FASR) on the target cell (Golstein and Griffiths 2018). Once the infection is cleared the T cells will contract and few of them will develop into long-lived memory T cells (Murphy and Weaver 2016b). Memory T cells can be divided into several subtypes based on their effector function, ability to expand and the site at which they reside (Golubovskaya and Wu 2016) (figure 1.1).

The two major groups of T cells are the T helper cells (CD4), that typically recognise extracellular antigens presented on MHC Class II molecules, and cytotoxic (CD8) T cells, that recognise typically cytosolic antigen presented on MHC Class I molecules (Murphy and Weaver 2016b). T cells differentiate into different subsets by cytokine polarization and are also classified based on their cytokine profiles and unique expression of transcription factors (Bluestone et al. 2009). Common T cell subsets, their cytokine profiles, transcription factors and functions are outlined in figure 2.2.

Regulatory T cells (Tregs) are a subset of T cells defined by their ability to modulate immune responses usually by suppressing activated T cells and APCs (Vignali, Collison, and Workman 2008). Regulatory T cells are important to maintain peripheral tolerance known as immune homeostasis and in preventing autoimmunity (Romano et al. 2019). Tregs express the FOXP3 transcription factor, express CD25 (IL-2 receptor) and secrete regulatory cytokines like IL-10, TGF-β and adenosine (Hori 2003). Tregs formed in the thymus are termed natural Tregs, they protect against damage to healthy cells. Whereas, Tregs formed in the periphery are termed induced/adaptive Tregs and they mediate tolerance to foreign antigen (Dhamne et al. 2013). Tregs are particularly important in the liver (discussed below) where they work with other hepatic cells to maintain a tolerogenic environment to prevent unnecessary damage (Jeffery, Braitch, et al. 2016).



	Tem	Tcm	Trm	Temra
CD45RA	low	low	low	high
CD45RO	high	high	high	low
CD62L	low	high	low	low
CCR7	low	high	low	low
CD28	high	high	-	low
CD103	-	-	high	-
CD69	-	-	high	-

Figure 1.1 - Memory T cell subtypes - Memory T cells can be subdivided into four major groups, the Tem=effector memory, Tcm=central memory, Trm=resident memory and Temra=terminally differentiated CD45RA+. The different subsets are defined by the expression of distinctive receptors (left) on their surface with CD45RO being the main marker defining memory while others like C-C chemokine receptor (CCR)7 (CCR7) define central memory or CD103 and CD69 markers that identify resident memory cells. Image created using BioRender.

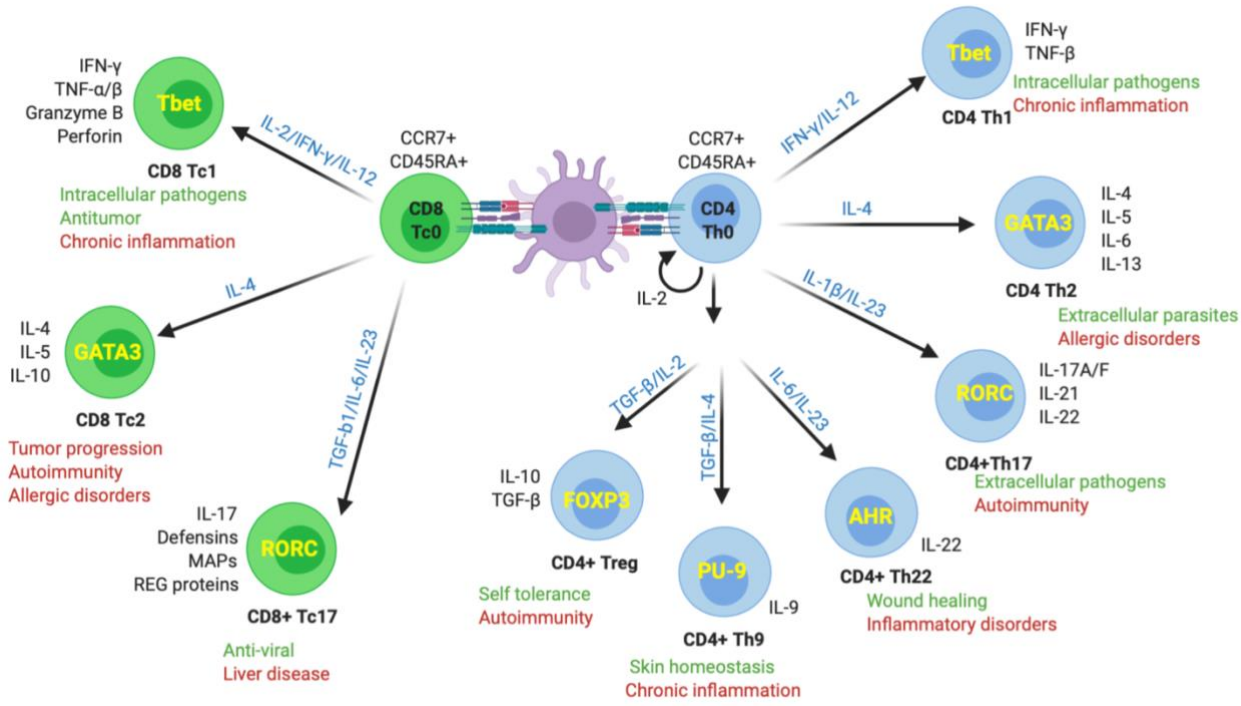


Figure 1.2 - Conventional T cell subsets, transcription factors, cytokine profiles and associated functions. Naïve T cells (Th0/Tc0) differentiate to several subsets (Blue/green cells) by the direction of cytokines (blue text above the arrows) and acquire transcription factors (Yellow text-centre) that define their phenotype and function based on the antigen stimuli (labelled adjacent to subsets in green-protective and red-pathogenic roles). Image created using BioRender.

1.3 T cell activation

T cell activation occurs in secondary lymphoid organs and can also occur throughout the body when T cells come into contact with APCs like DCs (Germain 1994) or, as described below, by cytokines alone for innate cells. During T cell activation the point of contact between the T cell and APC is known as the immunological synapse (IS) (Dustin 2014). The IS is formed by adhesion molecules (lectins and glycans) and contains CD3 and its co-receptors CD4/CD8, cytokine receptors, co-stimulatory/co-inhibitory molecules, and most importantly the antigen presenting molecules, e.g. MHC-I, MHC-II, MR1 and CD1d (Dustin 2014). Three signals are required for efficient T cell activation which results in proliferation and effector function or T cell anergy and death (Goral 2011). Several factors will determine the intensity of the immunological synapse and consequently different thresholds define the outcome. The first signal requires interaction between the TCR and the antigen loaded MHC complex forming the central structure of the immune synapse which is surrounded by a ring of adhesion molecules (Dustin 2014). The initial recognition of antigen-MHC complex by the TCR is accompanied by the binding of CD4/CD8 to stabilize the interaction (David D. Chaplin 2010). Signal two is mediated by engagement of co-stimulatory molecules like CD28-CD80/CD86 (particularly important for helper T cell activation) (Sharpe and Abbas 2006). In the later stages of an immune response co-inhibitory molecules like cytotoxic T lymphocyte-associated protein 4 (CTLA-4) will compete with CD28 to control and shut down the response once the infection is cleared. Survival signals, e.g. inducible T cell costimulatory (ICOS), 4-1BB, and OX40, also form part of signal two and are upregulated

if there is strong recognition of foreign antigen but are not constitutively expressed like CD28 (Chen and Flies 2013). Lastly, signal three is mediated by cytokines and the type of cytokine involved determines the polarization of T cells and its effector response (Curtsinger and Mescher 2010). For example, helper T cell exposure to IL-12 leads to Th1 differentiation and IL-4 to Th2 differentiation (Otagiri et al. 2016). Likewise, other cytokines drive polarization to different T helper subsets or cytotoxic T lymphocytes (CTLs), refer to figure 1.2. T cell activation signals can be modulated by molecules other than immune cytokines, for example lipid metabolites like prostaglandins (Harris et al. 2002) (*the effects of immune modulation by prostaglandins with relevance to chapter 5 and liver immunology is discussed in more detail in chapter 5*).

1.4 Innate like T cells

Innate-like T cells also known as unconventional T cells recognise non-peptide antigens presented by non-polymorphic antigen presenting molecules like CD1a/d and MR1 (Pellicci, Koay, and Berzins 2020). In contrast to conventional T cells, unconventional T cells have a limited TCR repertoire hence recognise a reduced number of conserved antigens (Godfrey et al. 2015). Unlike conventional T cells, innate-like T cells share a common transcription factor, promyelocytic leukaemia zinc finger (PLZF) and display a mature phenotype prior to leaving the thymus, enhancing their ability to respond quicker than conventional T cells (Salou et al. 2019). Unconventional T cells comprise a diverse group of T cells of which γδ, iNKT and MAITs have been studied extensively. These innate like T cells are generally found at mucosal surfaces and their combined

innate/adaptive properties make them efficient cells for first line of defence in barrier tissue (Garner, Klenerman, and Provine 2018).

1.4.1 γδ T cells

γδ T cells make up to 0.5-5% of the peripheral lymphocytes in humans and mice (Dantzler, Parte, and Jagannathan 2019). γδ T cell subsets are defined by their TCR variable segment usage; these are dominated by the TCRδ in humans and TCRγ in mice. Vδ2 and Vδ1 are the two main populations found in humans in the blood (Vγ9Vδ2) and skin, respectively, and can be found at other sites like the lungs, intestine and liver (Lawand, Déchanet-Merville, and Dieu-Nosjean 2017). γδ T cells detect a diverse range of antigens such as the cellular ligands like phosphoantigens (HMB-PP) which are also produced by pathogens like *E*. coli, *Listeria* and *M tuberculosis*, stress induced proteins and even lipid antigens presented by CD1d (Vantourout and Hayday 2013). γδ T cells are governed by the transcription factor PLZF which defines their innate features. Like other innate cells γδ T cells can be stimulated independently of their TCR by cytokines alone or through the TCR and have the ability to function as professional APCs (Brandes et al. 2009). Activation of γδ T cells leads to the production of pro-inflammatory cytokines like IFN-γ, TNF-α and IL-17 and chemokines like RANTES, IP-10 and lymphotactin (Ribeiro, Ribot, and Silva-Santos 2015). Additionally, γδ T cells can exert cytotoxic activity through activation of cell surface receptors like NKG2D causing the release of granzyme B (GrB) and perforin (Ribeiro et al. 2015), or by releasing granulysin and through activation by cell surface ligands such as Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) and CD95L inducing apoptosis of target cells (Dantzler et al. 2019). Such properties of γδ T cells allow them to function efficiently as

antimicrobial and antitumour cells but, in some cases, have been implicated in autoimmunity (functions of γδ T cells have been reviewed in more detail in (Lawand et al. 2017).

1.4.2 iNKT cells

iNKT T cells are a distinct subset of innate-like lymphocytes that are broadly divided into two groups; the type I iNKT T cells and type II iNKT cells based on their antigen specificity and TCR composition (Krovi and Gapin 2018). iNKT cells are composed of an invariant TCR combined with expression of NK cell receptors (NK1.1) allowing them to function with properties of innate and adaptive immunity as well as natural killer cell functions (Coquet et al. 2008). iNKT cells are restricted by the CD1d molecule and recognise self and pathogenic lipid antigens like α-galactosylceramide (αGalcer) (Kumar and Delovitch 2014). Like other innate-like T cell populations iNKT cells have a restricted TCR repertoire which in humans is comprised of Vα24Jα18 TCRα chain that preferentially couples with Vβ11 TCRβ chain and in mice consists of Vα14Jα18Vβ2 (Godfrey et al. 2015). Development of iNKT T cell parallels with MAIT cells (discussed in detail in (Garner et al. 2018)) and both are regulated by the innate-transcription factor promyelocytic leukaemia zinc finger (PLZF) and like the MAIT cells, iNKT T cells acquire an effector memory phenotype prior to existing the thymus (Garner et al. 2018). iNKT cells form a very small percentage in human blood, comprising of less than 1% in peripheral blood mononuclear cells (PBMCs) however they are abundant in the liver and lung (Krovi and Gapin 2018). iNKT cells were initially divided into subsets by their expression or absence of the NK1.1 receptors (Benlagha 2002; Pellicci et al. 2002), but later, subsets were attributed based on expression of transcription factors and cytokine profiles (Kumar and

Delovitch 2014). Based on this theory, three functional subsets were identified, iNKT1 cells that produce large amount of IFN-γ, iNKT2 cells that produce large amounts of IL-4 and iNKT17 cells that secretes high amounts of IL-17 (Kumar and Delovitch 2014). iNKT cell functions can be further classified through expression of CD4 and CD8, where CD4 iNKT T cells have an immunoregulatory role and the DN and CD8 iNKT T cells have a stronger cytolytic function (Gumperz et al. 2002; Seino and Taniguchi 2005). iNKT T cells are activated through their TCR but are also activated independently of TCR by cytokines released from microbial activated DCs, in particular by IL-12 (Reilly, Wands, and Brossay 2010). Similarly, two other reports have shown IL-18, in addition to IL-12, as a critical cytokine for iNKT cell activation during viral and LPS stimulation with minimal dependence of CD1d involvement in triggering iNKT cells (Nagarajan and Kronenberg 2007; Tyznik et al. 2008). Upon activation, iNKT T cells are capable of producing both IFN-γ and IL-4, eliciting type 1 and type 2 immunity (Kumar and Delovitch 2014). The multifunctional properties of iNKT cells permits them to respond competently to tumour and microbes (Mandal and Viswanathan 2015) as well as limiting autoimmunity (Van Kaer and Wu 2018). Despite the protective functions of iNKT T cells they may also become pathogenic and aggravate conditions like allergy (Iwamura and Nakayama 2018). The dual roles of iNKT T cell is visible in tumour settings where iNKT T cell derived IFN-γ has been shown to induce anti-tumour responses, but their overactivation can lead to tumour progression facilitated by T cell anergy and skewing of the response towards an anti-inflammatory - Treg and Th2 mediated (Krijgsman, Hokland, and Kuppen 2018).

1.5 MAIT cells

1.5.1 MAIT cell discovery and TCR

Mucosal associated invariant T cells are an innate-like T cell subset first identified in 1993 by Porcelli et al. Their research analysing TCR repertoires in humans revealed two distinct TCRα chains; the Vα24Jα18 that defines iNKT cells and another Vα7.2Jα33 chain (Porcelli et al. 1993). The Vα7.2Jα33 chain was later attributed to MAIT cells after identification of these in human and mice by Lantz and colleagues in mucosal tissue. In mice, MAIT cells express the Vα19Jα33 chain (Tilloy et al. 1999). In both humans and mice these TCR α-chains are combined with restricted TCR-β chains (Gapin 2014) (humans: Vβ2 and Vβ13, mice: Vβ6 and Vβ8). Unlike the invariant α chain, the β chain of the MAIT TCR is variable but still important for the recognition of MR1 and activation of MAIT cells (Dias, Leeansyah, and Sandberg 2017). For the inhibitory MAIT ligand, 6-FP, MAIT cell activation was limited by preventing the CDR3β interaction with MR1 (Reantragoon et al. 2013); thus, having equal importance for MR1 recognition and can contribute to ligand discrimination (Lopez-Sagaseta et al. 2013; Patel et al. 2013). These T cells were found enriched at mucosal locations like the gut in humans and lamina propria in mice and thus were named MAIT cells (Treiner et al. 2003). T cells expressing such conserved TCR repertoires recognise non-peptide antigens presented by the MHC-I-like molecule, MR1 are often described as MR1 restricted T cells (Gherardin et al. 2016).

1.5.2 MAIT cell antigens

MAIT cell antigens remained ambiguous for a long time after their discovery – however, more recently several antigens have been discovered. Studies from 2010 showed the ability to activate MAIT cells by a wide range of bacteria, yeast and fungi species. These studies also demonstrate a lack of MAIT cell response to some microbes. A couple of years later Kjer-Nielsen et al demonstrated the presentation of vitamin B metabolites by MR1 to MAIT cells. This finding was correlated with microbial organisms that possess the riboflavin synthesis pathway that could activate MAIT cells, whereas those that lacked the pathway were not able to activate MAIT cells in an MR1 dependent manner. Not all vitamin B metabolites can activate MAITs, for example 6-FP, a vitamin B9 metabolite, binds to MR1 and increases its cell surface expression, but does activate MAIT cells. On the other hand, riboflavin metabolites, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) derived from the 5-amino-6-D-ribitylaminouracil (5-A-RU) intermediate are potent stimulators of MAIT cells. MAIT cell antigens are not restricted to vitamin B metabolites as pharmacological agents like diclofenac and derivatives of methotrexate are also able to activate MAIT cells, but are weaker compared to 5-OP-RU. Derivatives of pharmacological drugs like 3-formylsalicyclic acid (3-FSA) and 2,4-diamino-6-formylpteridine (2,4-DA-6-FP) function as competitive ligands of MR1 inhibiting MAIT cell activation. More recently even more MR1 binding antigens have been identified, these include photolumazine (PL) I and PLIII (riboflavin derived) and FO (7,8-didemethyl-8-hydorxy-5-deazariboflavin), a riboflavin analog. Recent studies show MAIT cell activation by non-riboflavin derived microbial antigens, and some from tumour cells,

but, these ligands are yet to be fully defined. Furthermore, some studies demonstrated the incomplete occupancy of the MR1 binding grove and hence it is thought that a diverse range of MR1 ligands exists.

1.5.3 MAIT cell development

MAIT cell development occurs in the thymus by the initiation of random rearrangement of TCR genes and is similar to the development to iNKT (Garner et al. 2018). Unlike conventional T cells that require positive selection by MHC expressed on thymus epithelial cells, MAIT cell development occurs by TCR expressing cells interacting with MR1 on double positive cells thymocytes (CD4+CD8+) (Seach et al. 2013). MAIT cell development has been described as a three-stage sequence in humans and mice with the initial identification of immature MAIT cells in the thymus using MR1 tetramers (Koay et al. 2016). In the second stage, MAIT cell development is dependent on MR1 interactions and in stage three MAIT cells start to express their classical markers like IL-18R and also display a mature phenotype before they egress the thymus(Martin et al. 2009), each of these stages are defined by specific markers as shown in figure 1.3. Some human stage 2 MAIT cells can be found in the periphery and their transition to stage 3 is dependent on the PLZF transcription factor and other factors like MR1, IL-18 and microRNA-181a/b-1(Winter et al. 2019). A difference between human and mice MAIT development is the absence of MAIT cell discrimination into type 1 and type 17 MAIT cells in human but is visible in mice MAIT cells at stage 3 (Koay et al. 2016). In the periphery MAIT cells undergo further maturation and proliferation increasing the pool of MAIT cells localized in the blood which is much higher compared to the thymus at any age(Novak et al. 2014). MAIT cell frequencies in human decrease with age and usually

reduces to less than 1% in adults over the age of 70, with highest numbers of MAIT cells detected around the age of 25(Novak et al. 2014). A higher frequency of CD8αα as compared CD8αβ MAIT cells were identified in microbe challenged mice and were absent in the periphery of germ free mice highlighting two key findings, firstly the progressive transition of CD8αβ to CD8αα MAIT cell as a feature of peripheral MAIT cell maturation and secondly the microbe mediated development of MAIT cells which is different to thymic development of MAIT cells(Chen et al. 2016; Leeansyah et al. 2014).

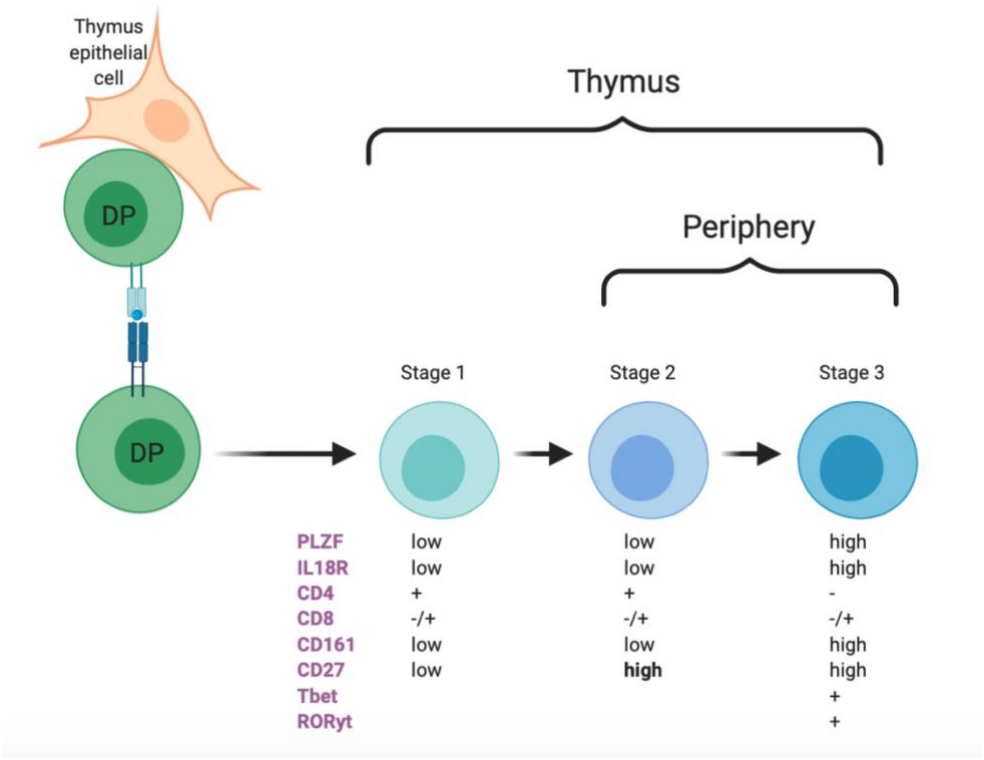


Figure 1.3 – MAIT cell developmental stages and markers – MAIT cell development starts in the thymus with the contact of double positive (DP) thymocytes interacting with epithelial cells (left). MAIT cell development occurs in three stages (right) with all stages identified in the thymus whereas stage 2 and 3 can be found in the periphery. The three stages are defined by the expression of different transcription factors and surface markers (table below).

1.5.4 MAIT cell transcription factors

MAIT cells have many effector functions that are directed by different transcription factors. The expression of T-box expressed in T cells (T-bet) allows them to induce type 1 immunity, retinoic-acid-receptor-related orphan gamma (RORγt) and signal transducer and activator of transcription 3 (STAT3) are responsible for the type 17 features, cytotoxicity is governed by eomesodermin (EOMES) and B lymphocyte-induced maturation protein-1 (Blimp-1), and innate functionality of MAIT cells is associated with PLZF(Godfrey et al. 2019; Provine and Klenerman 2020). The co-expression of these receptors in humans makes them a homogenous populations which is different to mice where two distinct populations are defined on either T-bet expression or RORγt expression(Hinks et al. 2019; Rahimpour et al. 2015). The conventional CD8 T cell associated type 1 (IFN-γ) and CTL function (GrB and perforin) in MAIT cells is a result of the co-expression of T-bet, EOMES and Blimp-1(Provine and Klenerman 2020). Together with these transcription factors and high expression of the IL-12Rβ1 MAIT cells induce type 1 immunity like that of conventional CTLs. IL-17 and IL-22 production by MAIT cells is controlled by the RORγt transcription factor however this is tightly regulated as induction of IL-17 from MAIT cells requires strong stimulation such as combined cytokine signalling with IL-1β or IL-7 or sustained TCR signalling(Gibbs et al. 2017; Leeansyah et al. 2015). IL-17 production by MAIT cells is more prominent in tissue resident MAIT cells whereas blood MAIT cells produce significantly lower levels(Dusseaux et al. 2011). The expression of PLZF by MAIT cells enables their innate functions through expression of IL-18Rα, IL-18RAP and IL-12-Rβ1 and by cytokine triggering, independent of TCR signals(David D Chaplin 2010).

1.5.5 MAIT cell distribution

MAIT cells are found mainly in peripheral tissue with lower numbers detected in secondary lymphoid organs. In 2003, trainer et al showed the enrichment of Vα7.2Jα33 transcript in the gut compared to skin(Treiner et al. 2003) and later studies have identified MAIT cells in different peripheral tissues using the MR1 tetramer and surrogate markers(Gherardin, Souter, et al. 2018). MAIT cells are present in the blood and comprise up to 10% of the total T cells but this percentage varies considerably between individuals(Gherardin, Souter, et al. 2018; Novak et al. 2014). MAIT cells express many tissue homing makers like the gut associated α4β7 integrin(Serriari et al. 2014), liver specific CXCR6, CCR6(Toubal and Lehuen 2016) and intestine related CCR9, CCR2(Dusseaux et al. 2011); tissue homing markers traffic MAITs cell to peripheral tissues and have been particularly useful in differentiating between tissue resident and infiltrating MAIT cells. In humans MAIT cells are highly enriched in the liver and on average 6-15% is detected in healthy liver with higher numbers found in diseased liver making up to 50% of the total CD8 T cells(Kurioka et al. 2016). The other organs that consist of a high percentage of MAIT cells is the lungs (~4%), stomach (~4%) and oral mucosa (between 0.1-7%)(Provine and Klenerman 2020). MAIT cells have also been detected in the female genital tract and the uterus and on average make up to 1% of the total lymphocytes(Gibbs et al. 2017). Within the gut, MAIT cells are found at several anatomical sites at varying quantities, the colon has between 2-12.4% MAIT cells, an average 1.7% in the duodenum(Booth et al. 2015) and in one particular individual MAIT cells comprised 60% of the total lymphocytes in the jejunum(Reantragoon et al. 2013). MAIT cells are not only detected at different sites but are also considered to re-

distribute, for example circulating MAIT cells can infiltrate into tumours(Li et al. 2020; Ling et al. 2016). However a parabiosis study in mice shows limited tissue redistribution which results from thymic defined transcriptomic programming of MAIT cells(Salou et al. 2019).

1.5.6 MAIT cell subsets

Using the T cell co-receptors CD4 and CD8, MAIT cells can be divided into 5 groups, double positive (DP)-CD4+CD8+, double negative (DN)-CD4-CD8-, CD4+CD8-, CD4-CD8$\alpha\beta$+ and CD4-CD8$\alpha\alpha$+(Godfrey et al. 2019). A large proportion of MAIT cells in the human body are CD8$\alpha\alpha$+/CD8$\alpha\beta$+ with a significant DN population and a much smaller percentage of CD4 MAIT cells (Kurioka et al. 2017). Most MAIT cell subsets are able to produce similar levels of IFN-γ, TNF-α, IL-17A and IL-2, it is thought CD8+ MAIT cells are more pro-inflammatory and the DN MAITs are more cytotoxic(Gherardin, Souter, et al. 2018; Magalhaes, Solders, and Kaipe 2020; Provine and Klenerman 2020). CD4 MAIT cells identified by MR1-tetramers produced more IL-2 than other subsets(Gherardin, Souter, et al. 2018) and those identified by TRAV1-2 and CD161+ produced type 2 cytokines like IL-4 and IL-13 but these cells may not be MAIT cells as they could not be stained with MR1 tetramers(Kurioka et al. 2017). MAIT cells are sometime characterised by their cytokine profiles, those that make type 1 cytokines are defined as MAIT-1 and those that make IL-17 are defined as MAIT-17(Rahimpour et al. 2015). Likewise, MAIT cell subset are described according to their TCR β chain usage(Koay et al. 2019) and are even defined by their expression of NK cell related receptors, a feature associated with enhanced MAIT response to cytokines IL-12 and IL-18 (Kurioka, Klenerman, and Willberg 2018). A distinct difference in MAIT cells in the blood and tissue is the greater level of

activation displayed by tissue resident MAIT cells that generally produce more IL-17A whereas blood MAIT cells are less activated (Dusseaux et al. 2011). MAIT cells are more commonly defined by MR1 tetramers and the TRAV1-2+Jα33+ TCR α chain. However the analysis of MR1 tetramer positive human T cells identified other TRAJ gene usage by these MR1 restricted T cells, a similar occurrence was identified for mice thus showing a more diverse group of MR1 restricted T cells exist based on TRAJ genes(Gherardin et al. 2016).

1.5.7 MAIT cell activation

MAIT cells can be activated by two methods, one through their TCR and the other by cytokines alone and the combined TCR and cytokine signalling leads to optimal MAIT cell activation(Magalhaes et al. 2020). T cells require TCR signalling for their activation likewise MAIT cells can be activated through the TCR by MR1 ligands produced by both pathogenic bacteria and commensal bacteria(Tastan et al. 2018). TCR mediated activation of MAIT cells leads to the production of cytokines, cytolytic molecules, initiates migration and proliferation(Provine and Klenerman 2020). As MAIT cells in the periphery have effector memory function MAIT cells are readily activated in primary infections(Nakanishi and Daniel W Rosenberg 2013) and do not require priming, however such TCR signalling is tightly regulated and contrasts to conventional T cell signalling whereby persistent TCR signalling in MAIT cells prevents proliferation and cytokine production(Slichter et al. 2016). This feature is particularly useful in preventing unnecessary activation of MAIT cells especially because MR1 is expressed ubiquitously in cells and tissues(Riegert, Wanner, and Bahram 1998). The indispensable role of MAIT

TCR signalling is highlighted by studies showing MAIT cell mediated killing of bacterial infected epithelial cells(Le Bourhis et al. 2013) and protection of mice from legionella infection through the antigen bound MR1 recognition(Nakanishi and Daniel W Rosenberg 2013).

The TCR independent triggering of MAIT cells involves the activation of MAIT cells by cytokines requiring signals from a minimum of two cytokines(Ussher, Klenerman, and Willberg 2014). Cytokine signalling is feature of the innate function of these cells and IL-12 and IL-18 are commonly used to stimulate MAIT cells independently of the TCR(Ussher, Bilton, Attwod, Shadwell, Richardson, de Lara, et al. 2014). Other cytokines like, type 1 interferons, IL-15, IL-7 and TNF-α also mediate TCR independent activation of MAIT cells(van Wilgenburg et al. 2016). The combination of cytokines for activation determines the cytokine profile of MAIT cells for example IL-12 and IL-18 induces IFN-γ and GrB(Ussher, Bilton, Attwod, Shadwell, Richardson, de Lara, et al. 2014). Another cytokine mediated activation pathway for MAIT cells involves IL-15 dependent IL-18 induction from monocytes that can synergise to activate MAIT cells(Sattler et al. 2015), or IFN-γ mediated TNF-α induction from monocytes (Provine et al. 2019). Cytokine activation of MAIT cells induces limited activation as compared with TCR mediated stimulation and does not stimulate IL-17 production. Nonetheless, IL-17A production from MAIT cells is enhanced in the presence of IL-1β and IL-7 co-stimulation(Böttcher et al. 2018; Lamichhane et al. 2019; Tang et al. 2013). TNF-α is one such cytokine that is robustly induced by TCR mediated signalling but is not induced significantly by cytokine mediated activation in MAIT cells(Ussher, Bilton, Attwod, Shadwell, Richardson, de Lara, et al. 2014). In many settings both these pathways are active together resulting in

potent, comprehensive stimulation of MAIT cells. More importantly MAIT cell activation is tightly regulated and this is evident from our understanding that commensal bacteria can actively produce the MAIT ligand but even then MAIT cells are not always activated(Tastan et al. 2018). This can be explained by the threshold theory where sufficient ligand and co-stimulation is required to trigger MAIT cells(Slichter et al. 2016; Turtle et al. 2011). MAIT cell activation in the absence of antigen is also possible, for example in inflammatory settings where TLR ligands produce the necessary cytokines for TCR-independent activation of MAIT cells(Chen et al. 2016; Jo et al. 2014; Kurioka, van Wilgenburg, et al. 2018).

1.5.8 Role of MAIT cells in health and disease

MAIT cells have been studied extensively in multiple disease settings both in human and mice. Several MAIT cell models have been developed and used alongside patient samples to determine the role of MAIT cells in various disease settings(Godfrey et al. 2019; Provine and Klenerman 2020). From the early discovery studies of MAIT cells and through the identification of the microbial riboflavin derived metabolite - 5-OP-RU, as a potent stimulator of MAIT cells; the antimicrobial function of MAIT cells was the first to be defined(Le Bourhis et al. 2010; Corbett et al. 2014). Later studies have contributed to our understanding of the additional and diverse functions of MAIT cells, which includes their anti-viral activity(Wilgenburg et al. 2016), helper function(Toubal et al. 2019) and their role in non-infectious diseases like cancer(Ling et al. 2016) and autoimmune disorders(Chiba, Murayama, and Miyake 2018). MAIT cells have also been shown to augment immune process and more recently they have been shown to possess tissue

repair function(Constantinides et al. 2019; Leng et al. 2019; Salou and Lantz 2019). Since MAIT cells can be activated by two pathways, individually and synergistically, the outcomes can be analysed by the specific pathway that is active, as described in the review; MAIT cells in health and disease (Provine and Klenerman 2020).

The first significance of MAIT cells in disease is seen through the loss of MAIT cells in the blood which is sometimes associated with the accumulation at the site of infection, as seen for M. tuberculosis, where MAIT cells accumulated in the lungs of infected mice(Gold et al. 2010; Gold, Napier, and Lewinsohn 2015). Salerno-Goncalves and colleagues have also confirmed tissue homing properties of MAIT cells in *Salmonella enterica subsp. enterica serovar typhimurium* infected patients where gut-homing receptors CRR9 and CCR6 were upregulated on MAIT cells(Salerno-Goncalves et al. 2017). However, the reduction of MAIT cells in the blood is not always associated with disease outcome and this trend is visible even when MAIT cells are pathogenic (Duan et al. 2019). For HIV infection, MAIT cells are lost early in disease and this loss is irreversible(Cosgrove et al. 2013), however such a loss of MAIT cell does not define disease progression thus MAIT cell quantification is not a reliable factor to judge disease outcome. Though many studies show loss of MAIT cells, how MAIT cell numbers decline is unclear but as the remaining MAIT cells display a highly activated phenotype, it is speculated that they may be lost by activation induced cell death(Magalhaes et al. 2020; Provine and Klenerman 2020). In metabolic diseases like diabetes, MAIT cell decline was attributed to increased glucose concentrations that lead to higher MAIT cell apoptosis(Touch et al. 2018). In contrast higher frequencies of circulating MAIT cells amongst other innate like T lymphocytes is found to provide protection against bacterial sepsis(Grimaldi et al. 2014). Furthermore, proliferation of MAIT cells in infected tissue

has been shown for non-human primates that received vaccination for M. Tuberculosis therefore MAIT cell numbers may increase at the site of infection rather than migrate to the site(Greene et al. 2017).

Some important aspects of MAIT cell biology are derived from in-vitro studies, for example, lung epithelial cell mediated MR-1 dependent activation of MAIT cells could produce IFN-γ and TNF-α to control the infection, possibly by activating autophagy driven by IFN-γ (Le Bourhis et al. 2010; Gold et al. 2010). The importance of MAIT cell driven antimicrobial effector function is demonstrated in MAIT cell deficient models where the lack of MAIT cells accounted for bacterial overload with reduced survival in *Legionella longbeachae* and *Klebsiella* infection(Nakanishi and Daniel W Rosenberg 2013)(Hartmann et al. 2018). MR1 dependent activation of MAIT cells is also crucial for its cytolytic effector function, and the ability to directly kill *E*. coli and *Shigella flexneri* infected epithelial cells has been shown by Le Bourhis et al(Le Bourhis et al. 2013). Despite the strong antimicrobial capacity of MAIT cells, they are not always protective and are sometimes involved in the pathogenesis as seen in Helicobacter pylori infection(D'Souza et al. 2018). Mice deficient for MAIT cells showed limited pathology in the form of gastritis and were protected against H. pylori induced inflammation, this is thought to be due to reduced IFN-γ production by MAIT cells(Thalmaier et al. 2002). Likewise, MAIT cell activation by super antigens is strongly connected to toxic shock syndrome(Shaler, Choi, et al. 2017).

The helper capacity of MAIT cells has been confirmed for many lymphocytes; in a pulmonary infection model of *F. tularensis,* MAIT cells were required to recruit and promote pro-inflammatory monocyte differentiation by producing GM-CSF(Meierovics

and Cowley 2016). In the following year, Salio M et al, showed how ligand activated MAIT cells could signal to DCs and monocytes through the CD40-CD40L axis to induce their maturation as measured by the expression of TNF-α, IL-12p40 and co-stimulatory molecules(Salio et al. 2017). Moreover MAIT cells have been shown to induce human neutrophil differentiation into APCs by soluble factors, providing nonspecific help but ultimately enhanced proliferation and activation of CD4 and CD8 T cells(Davey et al. 2014). Despite the latter finding, it seems MAIT cell possess opposing roles as they can also kill neutrophils through a TNF-α dependent mechanism after activation by 5-A-RU(Schneider et al. 2020). In addition to DC, monocyte and neutrophil regulation, MAIT cells have the capacity to enhance memory B cell differentiation to plasma blasts and produce the immunoglobulins (Ig) IgA, IgG and IgM(Bennett et al. 2017).

A more recently described role for MAIT cells is their tissue repair function, studies of NOD mice that are deficient for MAIT cells - the $MR1^{-/-}$ NOD mice show loss of intestinal integrity(Provine and Klenerman 2020; Rouxel et al. 2017). Intestinal integrity was measured by increased bacterial translocation and accumulation of islet specific T cells in the lymph nodes and pancreas(Rouxel et al. 2017). Similarly MAIT cell deficient mice lacked survival following bone marrow transplant, this was again a result of damaged barrier integrity and donor T cell accumulation in the colon(Varelias et al. 2018). RNA-sequencing data of MAIT cells stimulated by different methods highlighted the TCR dependent upregulation of tissue repair genes(Hinks et al. 2019; Leng et al. 2019; Salou and Lantz 2019). The wound repair capacity was demonstrated by activated MAIT cell derived supernatant treatment of physically damaged epithelial monolayer which resulted in regrowth and gradual closure of the wound(Leng et al. 2019). Tissue repair

function of MAIT cells highlights a homeostatic role and also protects/repairs disease specific damage making them essential for the upkeep of the gastrointestinal tract.

The protective antimicrobial role for MAIT cells is largely MR1-dependent however the response to viral infections, *F tularensis* and *M bovis* is also dependent on cytokines as the loss of IL-12p40 resulted in impaired MAIT cell responses to these bacteria(Chua et al. 2012; Meierovics, Yankelevich, and Cowley 2013). IL-12 and IL-18 cytokine receptors are highly expressed on MAIT cells thus their TCR independent activation by these cytokines, produced by TLR triggering, is not unexpected and has been shown in-vitro. However the cytokines don't always seem to be important as IL-18 deficient mice were still able to induce MAIT cell IFN-γ to infection with *Francisella* species similar to WT mice in vivo, but not in-vitro(Jesteadt et al. 2018). In viral infections like HCV, dengue and influenza, MAIT cell activation is dependent on IL-18 which maybe acting in synergy with cytokines like type I interferons, IL-12 and IL-15(Wilgenburg et al. 2016). Ex-vivo analysis of MAIT cells from acute influenza and dengue infection patients show that MAIT cells express activation and exhaustion markers despite this similarity the circulating MAIT cells in dengue do not differ from healthy controls but are decreased in influenza patients(van Wilgenburg et al. 2016). The IL-12 and IL-18 signalling and activation of MAIT cells in chronic viral infections remains intact despite their inability to respond to ligand mediated stimulation(Barathan et al. 2016; Boeijen et al. 2017; Eberhard et al. 2016; Fernandez et al. 2015; Ussher, Bilton, Attwod, Shadwell, Richardson, de Lara, et al. 2014). In contrast to this finding, there are reports that show the opposite effect where MAIT cells are functionally impaired(Hengst et al. 2016), nevertheless, impaired MAIT cells can be restored by treatment with IL-7 making them responsive to *E*. coli and cytokines(Sortino et al. 2018). MAIT cell derived cytokines were

also important for promoting adenovirus vaccine stimulated CD8 T cell responses thus acting as an immune boosters(Provine et al. 2019). IL-7 can synergise with IL-12 to stimulation MAIT cells and such exposure to IL-7 augments the production of IL-17 from MAIT cells in the absence of TCR signalling(Tang et al. 2013; Wallington et al. 2018). MAIT cell derived IL-17 has been implicated in the pathogenesis of inflammatory diseases like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and ankylosing spondylitis (AS)(Chiba et al. 2018; Murayama et al. 2019; Toussirot et al. 2018). The in-vivo model of collagen induced arthritis underlines the pathogenic role of MAIT cells which was not visible in $MR1^{-/-}$ mice(Chiba et al. 2012). Such pathogenesis is caused by MAIT cell accumulation in the synovial joints and by high TNF-α levels promoting upregulation of adhesion molecules(Kim et al. 2017). This accumulation of MAIT cells in the synovial joint is associated with a highly activated state that produced high amounts of IL-17 compared to blood-MAIT cells(Kim et al. 2017). Amongst the MAIT cell-cytokine driven pathologies, the pro-fibrogenic and tumourigenic roles of MAIT cells are discussed in more detail below and in chapter 6.

MAIT cells have been detected in multiple cancers and therefore are speculated to play a role in anti-tumour responses. A large bioinformatics study in 2015 by Gentles analysed 39 malignancies which revealed the Killer cell lectin-like receptor subfamily B member 1 (KLRB1) gene encoding for CD161 to be the most significantly and highly correlated with better prognosis of tumours(Gentles et al. 2015). MAIT cell TCR transcripts and MR1 have been detected in various cancers including brain, kidney, glioma and uveal melanoma tumour, both before and after the identification of MAIT cells(Peterfalvi et al. 2008)(Godfrey et al. 2019). Although MAIT cells are detected in tumours their numbers are significantly lower in the blood than in healthy controls and

in some tumours like colorectal cancer MAIT cells are found to be infiltrating the tumour lesions(Ling et al. 2016; Zabijak et al. 2015). Interestingly the loss of MAIT cells from the blood was noticed in lung, gastric, colon and cervical cancer which are mucosal tumours (Won et al. 2016), whereas this was not the case for liver, breast and thyroid cancers (Duan et al. 2019; Huang et al. 2019; Won et al. 2016). In-vitro stimulation of breast duct-derived MAIT cells responded to bacterial stimulation of breast carcinoma cell line by producing IL-17A only, but when stimulated with PMA/ionomycin they were able to make IFN-γ and IL-17A(Zumwalde et al. 2018). IL-17 is also produced by blood MAIT cells from colorectal cancer patients with reduced ability to produce IFN-γ (Ling et al. 2016). IL-17 is a cytokine with pro-tumour activity therefore IL-17 producing MAIT cells are thought to be detrimental in tumours, this negative correlation has been reported for colorectal cancer(Zabijak et al. 2015). In contrast, MAIT cells are largely enriched in the HCC microenvironment compared to mucosal tissue and secreted significantly less IFN-γ, TNF-α, GrB and perforin, moreover, HCC-MAIT cells upregulated multiple exhaustion markers, namely PD-1, CTLA-4 and TIM-3(Duan et al. 2019). This exhausted and impaired pro-inflammatory profile of MAIT cells from HCC is accompanied by pro-tumour reprogramming linked with IL-8, CXCL12 and HAVCR2 gene expression(Duan et al. 2019). MAIT cells in liver colorectal metastasis within the tumour and the peritumour region are dysfunctional in comparison to the normal liver tissue(Ling et al. 2016; Shaler, Tun-Abraham, et al. 2017). For both colorectal cancer and HCC, MAIT cell infiltration in these tumours is associated with poor prognosis(Duan et al. 2019; Zabijak et al. 2015). In multiple myeloma MAIT cells were low in the blood compared to healthy adult controls but similar to healthy, elderly individuals with reduced capacity to make IFN-γ in newly diagnosed patients but not in relapsed and refractory patients (Gherardin, Loh, et al.

2018). The findings suggest the importance of functional MAIT cells in MM but the reduced numbers might be an age related occurrence. Lastly, differing with our assumptions of MAIT cells as effective anti-tumour cells, an experimental murine lung metastasis study has revealed MR1 driven tumour growth and spread which was absent in MR1 KO mice(Yan et al. 2020). IL-17 is also reported in this model to be a tumour promoting factor whereby MAIT cells suppress NK cell effector function via MR1(Yan et al. 2020), hence MR1 can be a potential therapeutic target for the treatment of MR1-dependent cancers.

A significant proportion of MAIT cell research has focused on MAIT cells in liver disease and this has been discussed separately, in greater detail in chapter 6, where we aimed to understand the phenotype and function of MAIT cells in liver cirrhosis and liver tumour.

1.6 Liver immunology

1.6.1 Liver structure

The liver is recognised for its fundamental role in metabolism, synthesis/storage of nutrients and detoxification of blood derived products. The detoxification process requires help from immune cells that are able to quickly recognise and remove harmful material present in the nutrient rich blood arriving from the gut(Robinson, Harmon, O'Farrelly, et al. 2016). The liver is largely composed of hepatocytes and various non-parenchymal cells, like the liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSC) and include a large repertoire of innate and adaptive immune cells(Wang and Zhang 2019). The highly vascularized liver receives bloods from the circulation and the intestine with 80% of the hepatic blood supply coming from the gut via the portal vein(Robinson, Harmon, O'Farrelly, et al. 2016). The arterial blood and venous blood mix in the small vessels lined by specialised thin, fenestrated cells known as LSECs(Shetty, Lalor, and Adams 2018). The slow blood flow in the liver allows cells to interact with the sinusoidal endothelial cells and other liver resident populations like Kupffer cells. The fenestrae is a sieve like, structural feature in the sinusoidal endothelial cells which allow cells within the sinusoids and the liver infiltrating cells to interact with hepatocytes which are separated from the circulating blood by a layer of endothelial cells (DeLeve and Maretti-Mira 2017). HSCs also known as Ito cells are found in the perisinusoidal space between the hepatocytes and LSECs and account for approximately 5% of the non-parenchymal population in the liver(Yin et al. 2013). HSCs function as fat and vitamin A storing cells but are also involved in regulating other liver and immune cells(Senoo,

Kojima, and Sato 2007). Like hepatocytes, HSCs interact with the cellular components in the sinusoids though the LSEC fenestrations and are implicated as the major effector cell in fibrosis(Fujita and Narumiya 2016). Kupffer cells are the largest organ resident macrophage population, their location in the hepatic sinusoids allows them to recognise, phagocytose and remove pathogens from the blood, making them an important cell type involved in the first line of defence to harmful substances(Dixon et al. 2013).

1.6.2 Liver immune cell composition and functions

The immune cell composition of the liver is different to other organs and though it contains numerous important populations like DCs, NK cells, αβ T cells, the liver has a significantly higher percentage of innate like T cell populations, namely the γδ T cells, iNKT cells and MAIT cells thus it is also referred to as an innate immune organ(Gao, Jeong, and Tian 2007).

1.6.2.1 Liver dendritic cells

In both human and murine liver, DCs are found around the portal tracts and rarely in the parenchymal space(Eckert et al. 2015). Of the two major subsets, the myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), the pDC dominate in the murine liver microenvironment but are less prevalent in the human liver(Kelly et al. 2014; Thomson and Knolle 2010). Like DCs in the periphery, liver DCs can capture, process and transfer antigens to lymphoid tissues, however, isolated hepatic DC's display an immature phenotype, expressing limited co-stimulatory molecules and are less immunogenic compared to their spleen and blood counterparts(Pillarisetty et al. 2004). In the murine

liver DCs are densely located under the liver capsule and exhibit zonal heterogeneity(Soysa, Wu, and Crispe 2017). mDCs express high levels of CD11c and can be subdivided into two groups the CD1c or CD141+ DCs whereas pDCs are CD303+ and can also be further subdivided into two groups, the CD2+ or CD2-(Strauss 2014). 95% of blood mDCs are CD1c+ and 5% are CD141+, this ratio changes in the liver with about 30% DC's being CD141+ and the remainder are CD1c+(Kelly et al. 2014). The CD141+ mDC population is enriched within the liver and is also unique in its ability to cross present antigens from necrotic cells(Jongbloed et al. 2010) while the pDCs in the liver are important for the type 1 interferon alpha (IFN-α) responses to viral antigens(Koda et al. 2019). DCs, like other lymphoid and non-lymphoid populations in the liver can contribute to liver tolerance by producing anti-inflammatory mediators(Lau 2003a).

1.6.2.2 Liver NK cells

NK cells make up to 40% of the liver lymphocytes and are highly enriched in the liver compared to the spleen and circulation (Tian, Chen, and Gao 2013). Subsets of NK cells have been defined in mice where the liver and spleen subsets differ in their expression of CD49a and DX5 (Yokoyama et al. 2013). Furthermore the liver retains large quantities of NK cells that express high levels of TRAIL but lack maturation makers whereas the splenic NK cells decline after birth (Takeda et al. 2005). NK cells from human liver are less cytotoxic with lower expression of CD16, inhibitory KIRs and CD62L and express high CD69 in comparison to the blood NK cells(Burt et al. 2009). A special feature of liver NK cells but not blood-NK cells is their ability to mediate memory like responses to contact hypersensitivity which was dependent on CXCR6 a receptor highly expressed on murine CD49a+DX5- hepatic NK cells(Paust et al. 2010). Hepatic NK cells make large amounts of

IFN-γ in response to IL-12 and one mechanism driving their cytotoxic function in the liver is dependent on Kupffer cell released IL-18(Tu et al. 2008). NK cell cytotoxicity can also be activated through TRAIL engagement with death receptors induced by viruses on hepatocytes(Chen et al. 2010). High expression of TRAIL on liver-NK cells is also involved in the elimination of antiviral T cells in viral infections of the liver and this has been shown to result in negative regulation of immune responses to viruses (Peppa et al. 2013). The greater reactivity in the liver-NK cells is advantageous for anti-tumour and anti-viral functions, yet they this heightened activation can contribute to hepatic damage. Whether hepatic NK cells play a role in liver tolerance is undefined but their phenotypic difference to blood-NK cells is distinctly adapted for the liver microenvironment.

1.6.2.3 Liver iNKT cells

Canonical and non-canonical iNKT cells represent a large population of T cells in both humans and mice in the liver (Huang et al. 2018). iNKT cells express both T cell and NK cell markers, they recognise glycolipid antigens presented by CD1d and can also be activated by PRRs expressed on APCs or iNKT cells themselves(Bandyopadhyay, Marrero, and Kumar 2016). The large numbers of iNKT cells in the liver maybe attributed to their potential to respond to cell wall components of the intestinal bacteria amongst others, which may also be the reason for the activation markers detected on these cells in healthy liver(Emoto and Kaufmann 2003). Upon activation liver iNKT cells can produce pro-inflammatory and anti-inflammatory cytokines and chemokines. iNKT cells can be activated in a cytokine dependent manner by APC-secreted type I interferons, IL-12 or IL-18(Bandyopadhyay et al. 2016). Moreover, iNKT cells are able to produce Th1,

Th2 and Th17 cytokines to different stimuli in the liver, for example, Type 1 iNKT cells predominantly make IFN-γ in response to ischaemic injury and can secrete IL-4 and IL-17 in response to α-Galcer (Kumar 2013).

1.6.2.4 γδ T cells

γδ T cells are an unconventional T cell subset accounting for around 15-25% of the total T cells in the liver compared to only 1-5% in the periphery and 2-3% in secondary lymphoid organs (Hammerich and Tacke 2014). γδ T cell derived IL-17A was shown to drive the innate immune responses in the early stages of *Listeria monocytogenes* infection by Hamada and colleagues (Hamada et al. 2008). However, γδ T cells seem to protect the liver from injury in an IL-17A independent manner and may also prevent liver failure by inhibiting iNKT cells (N. Zhao et al. 2011). Contradictory to their protective features, a higher proportion of γδ T cells were found in fibrotic liver, in patient samples with chronic hepatitis and several models of chronic liver injury(Hammerich and Tacke 2014) therefore can be destructive for the liver. In humans, in HCV infection, activated γδ1 T cells contribute to HCV-mediated immunopathology with increased IFN-γ production(Agrati et al. 2001). On the other hand, CD56+CD161+, IFN-γ, TNF-α and IL-2+ γδ1 T cells exhibit a protective role in liver metastasis of colon cancer with effective cytotoxic capacity to tumour cell lines in-vitro(KENNA 2004).

1.6.2.5 Liver MAIT cells

MAIT cells are specifically enriched in the liver and make up to 20-50% of the total intrahepatic T cells in the liver, they are able to recognise bacterial ligands that are abundant in the liver due to the large inflow of antigen rich blood from the gut (Kurioka

et al. 2016). In the steady state liver, various features of intrahepatic MAIT cells make them a functional population capable of regulating other liver cell types through their capacity to make pro-inflammatory cytokines including, IL-17 (Toubal and Lehuen 2016). Intrahepatic MAIT cells are an important effector population in the liver, as they, along with NK cells possess the ability to make IFN-γ in response to IL-12/IL-18 derived from TLR8 stimulated monocytes(Ussher, Bilton, Attwod, Shadwell, Richardson, Lara, et al. 2014). The liver sinusoidal cells are particularly sensitive to the TLR8 agonist and thus intra-sinusoidal populations, comprising MAIT cells are the likely population responding to viral and bacterial RNA (Ussher, Willberg, and Klenerman 2018). Intrahepatic MAIT cells also display a highly activated phenotype defined by their high expression of CD69, HLA-DR and CD38, which would allow them to easily detect and respond to gut derived antigens (Jeffery, Van Wilgenburg, et al. 2016). Though liver MAIT cells are less inclined to make IL-17 compared with those from the gut, they are capable of producing IL-17 in a TCR-dependent manner during liver infection if licensed by IL-7 (Tang et al. 2013). Nonetheless intrahepatic MAIT cells do not seem to be IL-17 skewed like in the intestine and female genital tract in response to bacterial stimuli (Gibbs et al. 2017; Serriari et al. 2014). Our current understanding of MAIT cells in liver disease has been reviewed in more detail in chapter 6.

1.7 Aims of the thesis

My thesis will try to address the role of MAIT cells in the liver microenvironment by studying MAIT cells in several settings. Firstly, we will look at the potential for MAIT cell activation by liver resident cells. These initial findings will be used to understand how activation in the liver microenvironment can affect the function of intrahepatic and liver infiltrating MAIT cells. We will parallel these findings with the phenotypic and functional properties of MAIT cells from liver disease enabling us to get a broader understanding of the role of MAIT cells in the liver microenvironment. To determine the role of MAIT cells in the liver, our aims were to

1. Assess the ability of liver resident cells to activate MAIT cells in response in bacteria and virus
2. Investigate the influence of neighbouring cells on MAIT cells activation and function
3. Study the effects of lipid metabolites on MAIT cell activation and function
4. To compare the function and phenotype of MAIT cells from liver-tumour and cirrhosis to healthy liver-MAIT cells.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Media and buffers

2.1.1 R10

RPMI 1640 (Sigma Aldrich) + 10% heat-inactivated fetal calf serum (FCS; Gibco) + 2mM L-Glutamine (Gibco) + 1X Penicillin-Streptomycin solution (Gibco).

2.1.2 D10

Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) + 10% heat-inactivated fetal calf serum (FCS; Gibco) + 2mM L-Glutamine (Gibco) + 1X Penicillin-Streptomycin solution (Gibco).

2.1.3 Freezing medium

90% heat inactivated FCS (Gibco) + 10% dimethyl sulfoxide (DMSO; Sigma Aldrich).

2.1.4 MACS buffer

Phosphate buffered saline (PBS) + 2mM ethylenediaminetetraacetic acid (EDTA; Sigma Aldrich) + 0.5% heat inactivated FCS (Gibco).

2.1.5 Hepatocyte isolation media

HBSS (life Technologies) + 0.5% BSA (Sigma Aldrich) + 0.05% collagenase IV (Sigma Aldrich) +10nM $CaCL_2$ (Sigma Aldrich).

2.1.6 Primary hepatocyte culture medium

William's E medium (Gibco) + 1% Penicillin-Streptomycin solution (Gibco) + 1% Glutamax (life Technologies), + 0.5% BSA (Sigma Aldrich) + 2% human serum (Sigma Aldrich) + 100nM insulin (Life Technologies) and 100nM dexamethasone (Life Technologies).

2.2 Cells

2.2.1 Cell lines

THP-1 cells - THP-1 cells are a monocytic cell line derived from an acute monocytic leukaemia patient. These were bought from ECACC, UK and maintained in R10 media at a maximum confluency of 1x106/ml, with an initial seeding of $2x10^5$/ml. THP-1 cell have been as an alternate to blood derived monocytes as they represent the most suitable cell line with antigen presenting capacity.

Raji cells (B cell line) are primary B cells acquired from a healthy donor that were Epstein-Barr virus (EBV)-transformed to generate B cell lines (BCL). Raji cells were maintained at $5x10^5$ cells/ml in R10 media.

2.2.2 Liver derived and non-liver derived cells lines

a) HepG2 cells, originally acquired from ATCC and other batches acquired within the group or from the Mckeating group (Nuffield Department of Medicine, University of Oxford)
b) Huh7 cells – were originally purchased from ATCC but acquired within the group
c) HHL12 cells– were acquired from Dr. Arvind Patel, (University of Glasgow)
d) Liver endothelial cells were kindly provided by Dr Naoya Kobayashi (Department of surgery, Okayama University, Japan)
e) Hepatic stellate cells were kindly provided by Dr Naoya Kobayashi (Department of surgery, Okayama University, Japan)
f) HeLa cells– were originally purchased from ATCC and provided within the group

g) Caco-2 cells – were originally bought for ATCC and kindly provided by the Uhlig group (Translation gastroenterology, Oxford).

Cell lines a-g were maintained in D10 medium and split at 90% confluence, usually on $3^{rd}/4^{th}$ day of culture.

2.2.3 Primary cells

Murine Hepatocytes - isolated from WT C57BL/6 mice - detailed protocol outlined in Chapter 4.

Peripheral blood samples - healthy donor whole blood leukocytes cones were acquired from NHS blood and transplant, UK.

Liver samples – Colon metastasis, alcoholic liver disease and HCC tumour samples were kindly provided by Professor Georg Lauer at the Harvard Medical school. Healthy liver in our cohort of samples refers to the excess unaffected tissue surrounding colon metastasis of the liver. Lymphocytes were for some patients were collected from the tumour and the peri-tumor region, stored separately for tumour and peri-tumour analysis. Liver samples were collected under the IRB protocol # 1999P004983 not requiring patient consent as only clinically deidentified tissue was collected. Liver tissue samples were from patients with non-viral pathologies, like alcohol associated cirrhosis, diagnosis and other details for individual donors provided in supplementary table 2.

2.3 Cell isolations

2.3.1 Peripheral blood mononuclear cells (PBMC) isolation from whole blood

PBMCs were isolated from human whole blood leukocyte cones acquired from NHS Blood and Transplant, UK. Blood was diluted in 50mls of sterile PBS and then layered on Lymphoprep (STEMCELL Technologies). Layered blood was centrifuged at 2222rpm for 22 minutes at 22 ^{O}C, with slow deceleration. The buffy coat was extracted in another falcon containing R10 medium and washed (1500rpm for 10minutes, quick acceleration and deceleration), washing in R10 was repeated trice before resuspending PBMCs in freezing medium between 20-50x10^{6}/ml and frozen in two stages; initially frozen at -80 ^{O}C for 24 hours and then transferred to liquid nitrogen for long term storage.

2.3.2 Thawing process

PBMCs or cell lines were removed from liquid nitrogen on ice and partially thawed in a water bath. The ice cold PBMCs resuspended gently in cold R10 in falcon tubes, centrifuged at 1600rpm for 4 minutes to wash out excess DMSO. Cells are then resuspended in R10, counted and used immediately or stored for short periods at 37°C prior to use.

2.3.3 CD8 T cell and CD14+ cell isolations

CD8+ and CD14+ cells were positively selected from healthy donor PBMCs following the Miltenyi isolation protocol using magnetic microbeads and MS columns, achieving purities above >90%.

2.4 Bacteria and virus

2.4.1 DH5-a *E*. coli

E. coli was acquired from Thermo Fisher Scientific.

2.4.2 *E*. coli preparation

DH5-a *E*. coli was grown on Luria Bertani (LB) agar plates for 24 hours at 37°C and stored at 4°C for up to 6 months. A single *E*. coli colony was submerged in 10mls of LB broth using a sterile pipette tip and cultured for 20 hours in a shaking incubator. *E*. coli cultures were transferred to v bottom falcon tubes and pelleted by centrifugation. *E*. coli pellets were gently resuspended in PBS and washed twice in sterile PBS. Next, *E*. coli was fixed in 2% paraformaldehyde (PFA) for 20 minutes and again washed twice with PBS, before resuspending in a small volume and storing at 4°C. A negative control with no *E*. coli was prepared in parallel with the identical steps, the negative control was used to determine any potential contaminations to the *E*. coli cultures that may have occurred during the preparation.

2.4.3 *E*. coli quantification

A small volume of *E*. coli was retained for quantification prior to washing and fixing the *E*. coli. Dilutions between 10^4-10^9 were prepared in duplicate by diluting in sterile PBS. Each dilution was plated on LB agar plates and spread using beads. Beads were removed from the plates and cultured in an incubator overnight before counting colonies on plates where colonies counts were between 30-100.

2.4.4 Calculation for *E*. coli stock (CFU/ml)

1000/vol. plated (µls) x no. of colonies x dilution factor x initial culture volume = total CFU

Fixed *E*. coli was resuspended at a final concentration of 10^7M CFUs/ml and stored at 4°C.

2.4.5 HBV and HCV

HBV and HCV viral lysates were kindly provided Dr Narayan Ramamurthy. Briefly, HBV, genotype D, was grown in HepG2.2.15 cell line following the protocol mentioned in (Gripon et al. 2002) using the clone detailed in (Sells, Chen, and Acs 1987). HCV, genotype 2a, accession no. DQ437509, was grown in Huh7.5 cell line following the protocol in (Pietschmann et al. 2006).

2.5 Functional assays

2.5.1 T cell/MAIT cell activation

PBMCs - $2.5x10^6$/ml PBMCs were stimulated with 50CFU/cell *E*. coli or for 16 hours in R10 medium with Brefeldin A (BFA) (eBioscience) and monensin (BioLegend) added for the last 4 hours prior to staining.

CD8 T cells - $1x10^5$ THP-1/CD14+ primary monocytes*/B cells/ liver endothelial cells (LECs)** and HSCs** were co-cultured with $2x10^5$ isolated CD8 T cells -/+ 30CFU/APC *E*. coli in a final volume of 200μls R10 for 16 hours, with BFA and monensin added for the last 4 hours.

**were cultured in 96 well flat bottom plat, **48 well flat bottom plate*

2.5.2 Cytokine stimulation assays

$5x10^5$ healthy donor PBMCs or $2x10^5$ isolated CD8 T cells were added to 96 wells plates with 50ng/ml of IL-12 and IL-18 in a final volume of 200μls R10. Cells were stimulated for 16 hours with BFA and monensin added for the final 4 hours.

2.5.3 PMA/Ionomycin stimulation assays

$5x10^5$ healthy donor PBMCs or $2x10^5$ isolated CD8 T cells were added to 96 wells plates with 250ng/ml PMA (Sigma Aldrich) and 250ng/ml Ionomycin (Sigma Aldrich) in a final volume of 200μls R10. Cells were stimulated for a total of 5 hours with BFA and monensin added for the final 4 hours.

2.5.4 TCR stimulation assays

96 well flat bottom plates were coated with 50μls /well of α-CD3 antibodies (BioLegend) in sterile PBS at a concentration of 2.5ug/ml, sealed and incubated at 37°C for 2 hours or at 4°C overnight prior to use. Wells were washed twice with sterile PBS and once with R10 medium. $5x10^5$ PBMCs or $2x10^5$ CD8 T cells were plated with 1ug/ml of α-CD28 antibodies (BioLegend) supplemented with IL-12 or IL-18 (*as specified in the figures*) in a final volume of 200μls /well and cultured overnight for 16 hours. BFA and monensin was added for the last 4 hours of the stimulation.

2.5.4 Blocking assays

Blocking assays were set up according to stimulation described above in section 2.5.1 with the addition of blocking agents (outlined in table 2.1) before adding *E*. coli /stimuli. Cells were cultured overnight (16 hours) with BFA and monensin added for the last 4 hours of the stimulation.

Blocking antibodies	Concentration used	Supplier
α-MR1	5ug/ml	BioLegend
α-IL-12	5ug/ml	BioLegend
α-IL-18	5ug/ml	MBL
α-IL-10	5ug/ml	BioLegend
α-TGF-β	5ug/ml	BioLegend

Table 2.1 – Blocking antibodies and concentrations used in the blocking assays

2.6 Flow cytometry

2.6.1 Extracellular staining

Cells were washed twice with PBS and stained with 50μls /well of 1/300 dilution of Live dead dye for 10 minutes. Live dead dye was washed off using excess PBS and cell were then resuspended by gently tapping the plate. Extracellular antibody master mix was made up in PBS according to dilutions in table 2.2 and added to each well (50μls /well) for 15 minutes. Excess unbound antibody was washed with PBS twice and then fixed in 4% PFA solution for 10 minutes. PFA was removed and cells were washed with PBS once more before resuspending them 100μls of R10 to acquire o on the MACSQuant.

2.6.2 Intracellular staining

Intracellular staining was continued after fixation of cells, instead of washing with PBS cell were permeabilised with 200μls of Perm buffer for a further 10 minutes. Intracellular antibody master mixes were made up in perm buffer according to dilutions in the table 2.2 And added to each well (50μls /well). Cells were stained for 30 minutes then washed twice with PBS to remove excess unbound antibody and then resuspended in 100μls of PBS. Cell were either acquired immediately on the MACSQuant or stored at $4^{O}C$.

2.6.3 Data acquisition and analysis

Flow cytometry data shown in chapter 3-5 was acquired on MACSQuant (Miltenyi Biotech) and analysed using FlowJo v8-10.

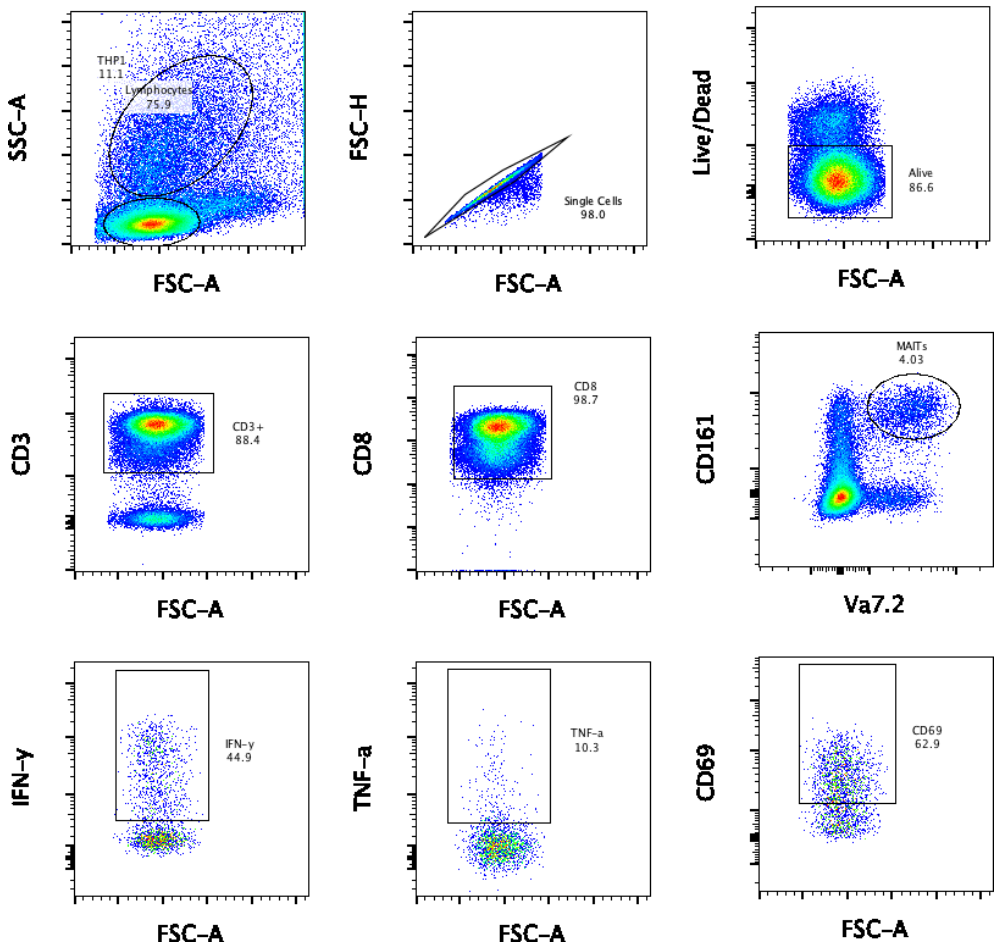


Figure 2.1 – Gating strategy

Representative flow plots for 8 color flow cytometry analysis of experiments in chapters 3-5, the above gating strategy was used to gate on MAIT cells and activation induced markers. First lymphocytes are gated on the FSC-a vs SSC-a plot, followed by doublet exclusion, then live cells were selected by gating on dye negative cells. Next, CD8 T cells were identified by gating on CD3 single positive cells followed by gating on CD8 positive cells, MAIT cells were identified by CD161 bright and Vα7.2 expression as double positive cells. IFN-γ, TNF-α and CD69 along with other markers were individually analsyed and dye positive cells were quantified as a percentage of the total CD8+ MAIT cells.

2.6.4 Flow cytometry antibodies

Flow cytometry antibodies	Fluorochrome	Clone	Company	Dilution
CD3	eFluor450	UCHT1	eBioscience	1:100
	FITC	UCHT1	BioLegend	1:100
	PerCp-Cy5.5	UCHT1	BD Biosciences	1:100
	PE-Cy7	UCHT1	BioLegend	1:100
CD4	PerCP-Cy5.5	OKT4	eBioscience	1:100
	PE-Cy7	SK3	BD Biosciences	1:100
	BV650	SK3	BD Bioscience	1:100
	VioGreen	VIT4	Miltenyi Biotec	1:100
CD8	PerCP-Cy5.5	RPA-T8	eBioscience	1:100
	Alexa Fluor 700	RPA-T8	BD Biosciences	1:100
	VioGreen	BW135/80	Miltenyi Biotec	1:100
	eFluor450	OKT8	eBioscience	1:100
	AF532	RPA-T8	Thermofisher	1:100
CD56	APC	AF12-7H3	Miltenyi Biotec	1:50
	BV421	HCD56	BioLegend	1:50
	FITC	NCAM16.2	BD Biosciences	1:50
	BV480	NCAM16.2	BD Bioscience	1:50
CD161	PE	191B8	Miltenyi Biotec	1:100
	APC	191B8	Miltenyi Biotec	1:100
	PE-vio770	91bb	Miltenyi Biotec	1:50
Vα7.2	PE	3C10	BioLegend	1:50
	APC	3C10	BioLegend	1:50
	PE-Cy7	3C10	BioLegend	1:50
	FITC	3C10	BioLegend	1:50
MR1-tetramer	PE	H4A3	BD Bioscience	1:1000
IFN-γ	FITC	45-15	Miltenyi Biotec	1:50
	PE	45-15	Miltenyi Biotec	1:50
	PerCP-Cy5.5	4S.B3	eBioscience	1:50
	Alexa Fluor 700	B27	BD Biosciences	1:50
	BV750	B27	BD Bioscience	1:50
TNF-α	PE-Cy7	MAb11	BioLegend	1:100
	PerCP-Cy5.5	MAb11	BioLegend	1:50
	eF450	MP6-XT22	Thermofisher	1:50
	PE	MAb11	BioLegend	1:50
IL-17A	PE	eBio64CAP17	eBioscience	1:25
	Alexa Fluor 488	BL168	BioLegend	1:50
	FITC	BL168	BioLegend	1:100

IL-17F	PE	SHLR17	Invitrogen	1:100
	PeCy7	SHLR17	Invitrogen	1:100
	AF647	O33-782	BD Bioscience	1:100
	PerCP-Cy5.5	SHLR17	Invitrogen	1:100
IL-2	PerCP-Cy5.5	MQ1-17H12	BD Biosciences	1:100
	PECF594	5344.111	BD Bioscience	1:50
	FITC	REA689	Miltenyi Biotec	1:50
Granzyme A	PerCP-Cy5.5	CB9	BioLegend	1:100
Granzyme B	APC	GB11	Invitrogen	1:50
	Alexa Fluor 700	GB11	BD Biosciences	1:50
	AF700	GB12	Invitrogen	1:50
Granzyme K	FITC	24C3	Immunotools	1:25
Perforin	Pacific blue	B-D48	BioLegend	1:100
	PerCP-ef710	dG9 (delta G9)	Thermofisher	1.100
PD-1	BV421	EH12.2H7	BioLegend	1:50
LAG-3	BV711	11C3C65	BioLegend	1:100
CD39	BB515/FITC channel	TU66	BD Bioscience	1:50
TIGIT	BV605	A15153G	BioLegend	1:50
TIM3	SB436	F38-2E2	Thermofisher	1:50
CXCR6 (CD186)	BV786	13B 1E5	BD Bioscience	1:50
CD103	BV510	Ber-ACT8	BD Bioscience	1:50
CD25	PE	BC96	BioLegend	1:50
	PECY5	V T-072	BioLegend	1:50
	FITC	M-A251	BioLegend	1:50
CD38	AF700	HIT2	BD Bioscience	1:100
CD69	FITC	FN50	eBioscience	1:50
	APCFIRE750	FN50	BioLegend	1:50
	efluro450	FN50	BioLegend	1:50
IL-1β	PE	CRM56	eBioscience	1:50
HLA-ABC	PerCP-e710	W6/32	eBioscience	1:50
	APC	W6/32	eBioscience	1:50
MHC-class I	FITC	W6/32	Abcam	1:50
HLA-DRQ	BV570	L243	BioLegend	1:50
	AF488	L243	BioLegend	1:50
	BV421	L243	BioLegend	1:50
	PeCy7	L243	eBioscience	1:50
	APC	L243	BioLegend	1:50
CD40	PeCy7	5C3	BD Bioscience	1:50
	Bv421	5C3	BioLegend	1:50
CD80	PE	2D10	BioLegend	1:50
	PE	REA661	Miltenyi Biotec	1:50

CD86	PE-Vio770(TM)	REA968	Miltenyi Biotec	1:50
CD83	bv421	HB15e	BioLegend	1:50
Live/dead	near IR	n/a	Invitrogen	1:500
	Live dead blue	n/a	Invitrogen	1:300

Table 2.2 – Flow cytometry antibodies used for staining – the use of these numerous antibodies and different combinations of antibodies with different fluorochrome conjugates depended on panel design to accommodate multiple markers and multiple panels per assay. Most panels were 8 colour panels whereas the liver samples were stained with a 24 colour panel. NB- since some markers are not available on all fluorochromes therefore the other common antibodies, e.g. CD3-e450, had to be replaced by CD3-PerCP-cy5.5 to accommodate the use rare antibody conjugates.

2.7 ELISA

2.7.1 IL-12 and IL-18

IL-12 and IL-18 concentrations from supernatant treated, *E*. coli stimulated THP-1 cells was were measured by Nicholas Provine using the Human IL-12p70 Quantikine ELSIA kit (R&D Systems) and IL-18 Human ELISA kit (MBL international), respectively, according the manuals. Samples were run in duplicate and compared to the unstimulated THP-1 cells.

2.7.2 PGD_2

PGD_2 concentrations in cell culture mediums were quantified from 4-day standard liver and non-liver cell line cultures using the PGD_2 competitive ELSIA kit (BioVision) following the supplier's instructions.

2.7.3 PGE_2

PGE_2 concentrations in cell culture mediums were quantified from 4-day standard liver and non-liver cell line cultures using the PGE_2 ELSIA kit (Enzo Lifesciences) following the supplier's instructions.

2.7.4 IFN- α/β ELSIA

IFN- α/β ELSIA concentrations from THP-1 cells stimulated with *E*. coli and treated with HepG2 supernatants in an overnight assay, were measured using separate Human IFN Alpha or Human IFN Beta ELSIA kits (PBL Assay Science) following the manufacturers guidelines.

2.8 Reverse Transcriptase-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed on THP-1 cells stimulated with or without *E*. coli and treated with or without cell culture supernatants. THP-1 cells were centrifuged, the culture supernatant was removed and then the cells were washed twice with PBS. Washed THP-1 cells were frozen in 200μls of RLT buffer (Qiagen) at -20°C for RNA extraction. RNA was extracted using Qiagen RNase mini kit using the manufacturer's protocol. RNA was then quantified on the nanodrop and cDNA was synthesised using Superscript III reverse transcriptase synthesis kit (Invitrogen) using the manufacturers guidelines. To assess gene expression the TaqMan gene expression assay was performed with primers to the gene of interest (listed in table 2.3) combined with primer specific probes in the TaqMan master mix (Applied Biosystems) according to the manufacturer's guidelines. RT-PCR reactions were performed on the Roche Lightcycler 480 machine. Samples were run in triplicates with gene expression levels quantified relative to the GAPDH mRNA expression using the ΔCT method(Ramamurthy et al. 2016).

Gene	Forward primer	Reverse primer
MR1	CCATTGTCCTTGTCATCGTG	GGCTCCATTTTGCTCTCG
HLA-A	GCGGCTACTACAACCAGAGC	CCAGGTAGGCTCTCAACTGC
HLA-DRA	TTTCCGCAAGTTCCACTATCTCCC	ATAATGATGCCCACCAGACCCAC
IL-12	GAATGTTCCCATGCCTTCAC	AGTTTGTCTGGCCTTCTGGA
IL-18	CAGATCAAGGACTGCCAATC	TAGGCCCTGGGTTCCTGT
IL-1β	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTTGGGATCT
TNF-α	GTCCAGGCTTGTCCTGTCTAC	AGTCCTGAGGCCGTGTTTTG
NLRP3	CACCTGTTGTGCAATCTGAAG	GCAAGCTCCTGACAACGTGC
COX1	TCCATGTTGGTGGACTATGG	GTGGTGGTCCATGTTCCTG
COX2	CTTCAGCATCAGTTTTTCAAG	TCACCGTAAATATGATTTAAGTCCAC

Table 2.3 Primers used in the RT-qPCR assays – Primers were designed using the Roche universal probe library finder. Primer sequences for HLA-A2 were acquired from GenBank ID - Z46633.1 and HLA-DRA used from NCBI database ID NM_019111.4.

2.9 Cytokine blot assays

2.9.1 Samples

Batch 1 (THP-1)	Batch 2 (Primary monocytes)
a) THP-1 cells + *E*. coli	e) Primary monocytes + *E*. coli
b) THP-1 cells, untreated	f) HepG2 supernatant
c) THP-1 cells + HepG2 cell culture supernatant	g) Primary monocytes, untreated
d) THP-1 cells + *E*. coli + HepG2 cell culture supernatant	h) Primary monocytes + *E*. coli + HepG2 cell culture supernatant

Table 2.4 – Samples processed for cytokine blot assay in two batches for two cell types, THP-1 (left) and primary monocytes (right). Cells were stimulated with or without *E*. coli and were left untreated or treated with HepG2 cell culture supernatant.

2.9.2 Cytokine blot assay

Supernatants were collected by a brief centrifugation, 90% of the supernatant was transferred to Eppendorf's and frozen at -80 ^{O}C. Supernatants were analysed for protein expression using cytokine blots (MBL) according to the manufacturer's protocol. Blots were then developed on LAS4000 and analysed for spot density using the image quant software.

2.10 RNA-Sequencing

2.10.1 THP-1 samples

6 samples were sequenced; replicates of three samples per condition were obtained from 3 individual experiments from three different passages of THP-1 cells.

X3 *E*. coli stimulated THP-1 cells

X3 *E*. coli stimulated THP-1 cell treated with HepG2 Supernatant

$2x10^5$ THP-1 cells were cultured with 30CFU/APC *E*. coli and treated with or without HepG2 cell culture supernatant for 16hrs. Cell were pelleted and frozen at -20oC in RLT buffer for RNA extraction.

2.10.2 RNA-extraction

RNA was extracted from $5x10^5$ THP-1 cells using the Qiagen RNeasy mini kit (Qiagen) following the manufacturer's protocol.

2.10.3 RNA quantification and quality test

Total RNA was quantified using Qubit™ using the RNA broad range Assay kit (Fisher Scientific using the manufacturer's protocol. RNA quality was measured using the Agilent 2100 Bioanalyzer, samples were prepared according to the manufacturer's protocol using the Agilent high sensitivity RNA kit.

2.10.4 Poly A selection of mRNA fragments and Library preparation

Sequencing Libraries were prepared with 200ng of total RNA with RNA integrity (RIN) values of >8. mRNA was selected using the NEBNext® Poly(A) mRNA Magnetic isolation

module and then sequencing libraries were synthesised using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®, Ampure XP (Beckman Coulter) purification beads were bought separately to the main kit and used for clean-up and size selection.

2.10.5 Library quantification and size analysis

Sequencing libraries were checked for size and quality using the Agilent HS DNA kit, samples were prepared according the manufacturer's protocol. Libraries were then quantified using the NEBNext library quantification kit by following the manufacturer's protocol. Sample were run on Roche Light cycler 480, absolute values quantified using LC480 software. Samples were run in triplicate and average absolute values were plugged into the NeBioCalculator online to determine the library concentrations.

2.10.6 Sequencing

Libraries were pooled and denatured according to the Illumina denaturing protocol. Pooled libraries were sequenced immediately after denaturing on Illumina, NextSeq500 using the mid-output kit allowing for paired end sequencing for 75 base pair read length.

2.10.7 Data analysis (Partek)

Illumina generated, index trimmed FASTQ files were loaded into Partek and analysed using the sequential steps outlined in the flow chart below.

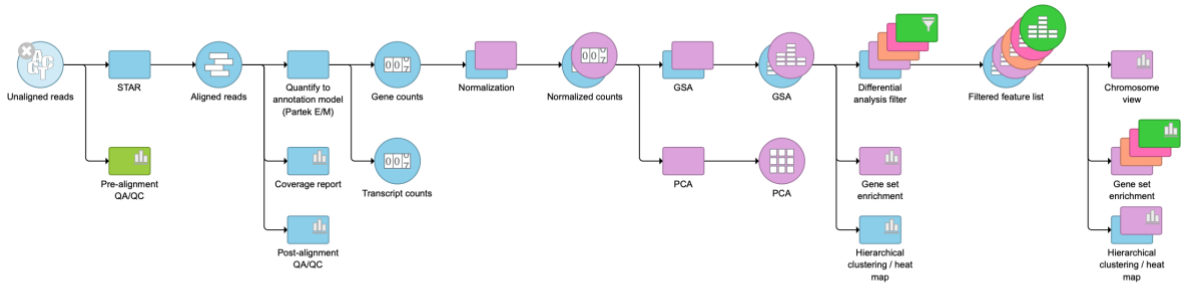


Figure 2.2 – Example flow chart for bulk RNA-sequencing data analysis. This is flow chart has been acquired from the Partek flow software to illustrate the different analyses performed on Illumina NextSeq500 generated FASTQ files.

First, unaligned reads were aligned to the human genome using the star aligner and checked for quality. Next the aligned reads were quantified using the hg-19-Ensembl transcripts release 75 and were subjected to a coverage report and pre-alignment QA/AC. After acquiring the genes and transcripts, they were counted and then normalized as counts per million (CPM). Then the gene lists were processed for differential expression (DE) analysis using the gene specific analysis (GSA), whereby the supernatant treated and supernatant untreated THP-1 cells were selected for comparison. The lists generated by DE analysis were filtered for a minimum number of counts >10, P value <0.05, and fold change between -2<->2. Using the visualization features offered in Partek we were able to create principal component analysis graphs (PCA), heat maps and hierarchical clustering maps. Finally, significantly upregulated and

downregulated genes were analysed by gene set enrichment analysis to determine their specific involvement in biological processes.

FDR filtering was not applied to our data as it obliterated the signal however prior to further analysis we checked the relevance of the significantly upregulated and downregulated genes to the biology our project and previous findings.

2.11 Statistical analysis

Graph Pad Prism, Version 7/8, was used to perform all statistical analysis. Data is presented as the standard error of the mean (SEM) and where it differs, is outlined in the figure legends along with the statistical significance specified by the test description and P values.

Different multiple comparisons tests have been used and have been noted in the individual figure legends. Bonferroni has been used to compare every mean with every other mean whereas the Sidak's test has been used compare specific treatment conditions with the control or for comparison between the treated groups. The Dunnett's test was used to compare the mean of each treatment condition with the mean of the control (*in most cases the stimulated, supernatant untreated or supernatant treated)*. Lastly, the t-test has been used to make pairwise comparisons where only two groups/conditions were assessed, i.e. stimulated vs treated.

CHAPTER 3 - MAIT CELL ACTIVATION BY LIVER RESIDENT CELLS

3.1 Introduction

3.1.1 Liver specific antigen presentation, T cell activation and tolerance

The liver has a unique immunological environment that favours tolerance and this was first recognised during porcine liver transplant, the organ was accepted with minimal hepatic damage and functioned efficiently unlike other transplants(CALNE et al. 1969). Moreover, the liver transplants also allowed better acceptance of subsequent transplant of other organs (Calne 2000). Several unique mechanisms and cellular phenotypes contribute to liver tolerance, which are significantly different to the composition of cells in the lymphoid organs (Racanelli and Rehermann 2006). The CD8 to CD4 ratio in the liver is higher which includes a high proportion of DN T cells. Both liver DC's and Kupffer cells display an immature phenotype and make a greater amount of IL-10 compared to IL-12 (Goddard et al. 2004; Robinson, Harmon, and O'Farrelly 2016). Regulatory T cells (Tregs) also contribute to the liver IL-10 pool and if depleted, results in loss of tolerance and leads to liver damage (Ma et al. 2007; Oo and Adams 2014). Like, Treg derived IL-10, other tolerogenic compounds, such as retinoic acid (RA), TGF-β and lipid metabolites like, PGE_2, (*discussed in detail in chapter 5.1*) are secreted by almost all liver resident cells and contribute to liver tolerance (Horst et al. 2016a). Antigen presentation and T cell activation in the liver is feasible in many situations however the primary function of liver APCs is to maintain tolerance and prevent liver damage. Various cell specific features of both professional and non-professional antigen presenting cells within the liver have the capacity to control responses to maintain liver tolerance, these are discussed in more detail below.

3.1.2 Liver DCs

Liver dendritic cells form a small but highly specialized APC found in the portal tracts and rarely in the parenchyma (Rahman and Aloman 2013). Both the classical DCs (cDCs) and plasmacytoid DCs (pDCs) are found in the liver, with pDCs being the dominant DC population in the liver (Eckert et al. 2015). The cDCs express higher levels of MHC-II, functioning efficiently to capture and present antigen whereas, their counterparts, the pDCs are less efficient at presenting antigen but are effective against virus due their capacity to produce IFNs (Thomson and Knolle 2010). Liver dendritic cells were specifically differentiated from skin-DCs by their secretion of IL-10, which is a feature of tolerance in liver induced by liver-DCs (Goddard et al. 2004). Moreover, both liver and skin DCs failed to secrete IL-12p70 after stimulation, in fact liver-DC stimulated T cells produced IL-4 and IL-10 in comparison to T cells stimulated by skin DCs that produced IFN-γ and IL-4 (Goddard et al. 2004). Liver-DCs display an immature phenotype with low expression of MHC-II, barely detectable co-stimulatory molecules (Thomson and Knolle 2010), limited production of IL-12 (Eckert et al. 2015), and produce immunosuppressive compounds like PGE_2, indoleamine 2,3-deoxygenase (IDO) and IL-10 (Calne 2000; Racanelli and Rehermann 2006). These properties of liver-DCs result in the induction of IL-10+ Tregs. Nucleotide-binding oligomerization domain containing protein-2 (NOD2) signalling is dominant in pDCs which are redundant in the liver, moreover, NOD2 signalling down-modulates secretion of pro-inflammatory cytokines but enhances the expression of PD-L1 therefore preventing T cell proliferation (Castellaneta et al. 2009). Other cell-cell contact dependent mechanism limiting liver-DC maturation include CTLA-4 and LFA-1 interactions, these mechanisms are operational in combination with DC

maturating cytokines. This immunoregulatory feature was demonstrated by Chen *et al* in a mouse HCC model (Chen, Du, and Huang 2012). All these mechanisms show reduced capacity of liver DCs to activate T cells and also shows enhanced functional potential of Treg induction by liver DCs.

3.1.3 Kupffer cells

KCs are the major phagocyte population in the liver and are the body's largest resident macrophage population, KCs play an important role in bacterial phagocytosis and elimination (Dixon et al. 2013). Steady state KCs are thought to be M2 polarized and mediate immune tolerance by secreting many immunosuppressive mediators like, IL-10, PGE_2, TGF-β and nitric oxide (NO) but can also make pro-inflammatory cytokines, notably IL-1β, IL-6 and TNF-α (Horst et al. 2016a). The constant exposure of these cells to LPS in the liver promotes the M2-like polarization, thus enabling endotoxin tolerance to prevent liver injury (Li et al. 2015). The ability of M2 polarized KCs to selectively apoptose M1-polarized KCs in an arginase and IL-10 dependent manner and thereby inducing tolerance has been shown for alcoholic steatohepatitis (ASH)(Wan et al. 2014). Heymann *et al*, has shown that KCs can induce local as well as systemic tolerance by arresting CD4 T cells and through the secretion of IL-10 induce expansion of Foxp3+ Tregs (Heymann et al. 2015). Like LSECs, KCs also express PD-L1 and PD-1, allowing them to modulate immune responses through these molecules in liver disease as shown in the context of HCC (Wu et al. 2009), autoimmune hepatitis (Oikawa et al. 2007) and HBV infection (Xie et al. 2009). For HBV infection, PD-L1 and PD-1 expression correlated with the inflammatory state and during chronic infection, PD-L1 was reduced which enhanced anti-viral properties of CD8 T cells and prevented effector cell exhaustion (Xie

et al. 2009). The importance of KC mediated immune tolerance in the liver is also highlighted through liver transplant studies, in-vitro experiments done with graft KCs showed that KCs could modulate liver tolerance by upregulating Fas-L, hence controlling IL-10 and TGF-β production by allogenic lymphocytes (Sun et al. 2001). Furthermore, liver tolerance by KC and Treg cell derived IL-10 was vital to protect from liver damage in the Concanavalin A hepatitis model demonstrating a crucial role for KCs in liver tolerance to prevent damage from autoimmunity (Erhardt et al. 2007).

3.1.4 Hepatocytes

Hepatocytes possess unique antigen presenting capacity, they express low levels of MHC-I and MHC-II under homeostasis and are capable of activating both CD8 and CD4 T cells respectively (Franco et al. 1988). Hepatocytes from inflamed liver were able to induce IL-10+CD4+ T cells through notch activation(Burghardt et al. 2013), thus contributing to liver tolerance through induction of Tregs. CD8 T cell priming during primary hepatic immune responses requires low antigen whereas high antigen load upregulates PD-1 on naïve T cells resulting in anergy and exhaustion (Tay et al. 2014). Bertolino et al performed a study which looked at survival of CD8 T cells primed by hepatocytes in the absence of co-stimulation, exogenous cytokine supplement and CD4 T cell help. In comparison to DCs, hepatocyte-primed CD8 T cells were activated and functional but they failed to survive beyond three days(Bertolino, Trescol-Biémont, and Rabourdin-Combe 1998). Hepatocytes can also limit immune responses by a process called emperipolesis, where hepatocytes engulf autoreactive cells which are directed to the endosomal and lysosomal compartment where they are degraded(Benseler et al. 2011). In contrast to these studies, hepatocytes were able to induce protective T cell

responses to malaria parasite but only to low parasite load (Corradin and Levitskaya 2014).

3.1.5 Liver sinusoidal endothelial cells

Liver sinusoidal endothelial cells (LSECs) are the first cell type encountered by circulating lymphoid cells, they have the best scavenging capability allowing them to efficiently capture antigen (Knolle and Wohlleber 2016; Sørensen et al. 2012a). A fascinating study by Jessica Mates and colleagues showed the uptake of 100 million HIV-like particles from the blood by murine LSECs in just 1 minute, highlighting their efficient endocytic capacity (Mates et al. 2017). They also recruit cells to the liver through their constitutive expression of ICAM-1, VCAM-1, CD44 and hyaluronan(Knolle and Wohlleber 2016). LPS mediated TLR4 signalling in LSECs under homeostasis results in accumulation and elimination of activated CD8 T cells(John and Crispe 2005). LSECs make IL-10 and also express PD-L1, which differentiates naïve T, cells into IL-4 producing Th2 cells instead of inducing type 1 immunity (Carambia et al. 2013; Klugewitz et al. 2002). The abundance of IL-10, a regulatory cytokine, reduces expression of MHC-II and co-stimulatory molecules on non-professional APCs and DCs (Knolle et al. 1998). Furthermore, LSEC primed CD4 T cells develop into IL-10 secreting Tregs instead of Th1 cells due the absence of IL-12 during priming (Knolle et al. 1999). The LSEC mediated induction of Tregs has been shown to protect mice from experimental autoimmune encephalomyelitis (EAE) even in developed disease, thereby showing that tolerogenic mechanisms of LSECs are beneficial and needed to protect from autoimmunity (Carambia et al. 2015). Unlike for CD4 T cells, LSEC interaction with CD8 T cells leads to deletion or differentiation of the naïve CD8 T cells towards a central memory T cell like

phenotype(Horst et al. 2016a). Antigen loads also play a role in inducing tolerance, low level antigen during non-inflammatory states causes LSEC primed CD8 T cells to expand in the initial phase but later renders them refractory (*unable to make IFN-γ*) (Kaczmarek et al. 2014; Limmer et al. 2005; Schurich, Berg, Stabenow, Böttcher, Kern, Schild, Kurts, Schuette, Burgdorf, Diehl, Limmer, and Percy A. Knolle 2010). Additionally, the co-inhibitory receptor, PD-L1 signalling inhibits IL-2 production by CD8 T cells which could not be prevented with additional CD28 co-stimulation (Schurich, Berg, Stabenow, Böttcher, Kern, Schild, Kurts, Schuette, Burgdorf, Diehl, Limmer, and Percy A Knolle 2010). On the other hand, high antigen load could induce CTLs, this may be attributed to strong TCR signalling with high IL-2 concentrations (Schurich, Berg, Stabenow, Böttcher, Kern, Schild, Kurts, Schuette, Burgdorf, Diehl, Limmer, and Percy A Knolle 2010), in addition, these LSEC primed CD8 T cells acquire a memory phenotype and express CD62L (Böttcher et al. 2014). If reactivated, such as during an infection they can still function efficiently, as they are able to produce IFN-γ and can eliminate pathogens(Wong et al. 2015) . LSECs mediate tolerance in the liver by regulating not just CD4 and CD8 T cells but also DCs and adjacent cells (Schildberg et al. 2008), and indeed, Schildberg et al showed that LSECs can limit DC cell ability to make IL-12 which is necessary for the induction of effector cells(Schildberg et al. 2008).

3.1.6 Hepatic stellate cells

The role of HSC cells in immune tolerance is also becoming clearer. HSCs can be activated by HCV infection which can cause HSC differentiation into extracellular matrix producing myofibroblasts following the conversation of vitamin A to RA thus exerting fibrogenic function (Weiskirchen and Tacke 2014). Expression of antigen presenting molecules

MHC-I/MHC-II and CD1c/CD1d and co-stimulatory molecules like CD86 on HSCs has been demonstrated. However they are not efficient APCs compared with conventional and non-professional APCs of the liver (Winau et al. 2007). HSCs are termed as immunological bystanders as they produce RA and TGF-β to induce Tregs (Dunham et al. 2013; Ichikawa et al. 2011). Murine and human HSCs can veto CD8 T cell activation by DCs in a cell contact dependent manner through ICAM-1, high expression of ICAM-1 prevented IL-2 mediated signalling to T cells which could be overcome by exogenous IL-2 (Schildberg et al. 2011). Despite these tolerogenic features, it seem that HSCs can indirectly present their antigens by a process known as trogocytosis (exchange of MHC-I molecules between cells), trogocytosis between HSCs and LSECs has been shown in two different studies, this mechanism would allow HSC antigens to become visible to infiltrating and sinusoidal resident CD8 T cells(Horst et al. 2016b). Another group also demonstrated that cross presentation of soluble antigens by transfer of MHC-I molecules to LSEC was able to induce effective CTL immunity but this resulted in immune mediated hepatitis (Schölzel et al. 2014).

T cell activation by liver resident cells is not as straightforward and the findings from studies highlighted above demonstrate an inclination towards tolerance. Furthermore, features such as antigen threshold become more important in the liver as well the type of secondary signal. A significant number of these findings are derived from conventional T cells however as the liver contains a large number of innate like T cells, especially MAIT cells, we therefore wanted to investigate the ability of liver resident cells to activate MAIT cells in response to bacterial and viral exposure. Additionally, we

studied the pathways important for MAIT cell activation by liver derived cells lines and compared the activation capacity of liver resident cells to conventional APCs.

3.2 Aims

1. Investigate the ability of liver resident cells to activate MAIT cells
2. To determine the pathways used by liver derived cells to activate MAIT cells
3. Compare the efficiency of liver derived cells to conventional antigen presenting cells
4. Test the ability of virus exposed liver resident cell lines to activate MAIT cells

3.3 Materials and methods

3.3.1 Cell detachment from culture flasks/plates

Adherent cells were lifted from the surface of culture flasks by enzymatic digestion using trypsin EDTA (Sigma Aldrich). Old culture medium was removed, cells were then washed using PBS. PBS is left on the cells for a minute before replacing with Trypsin EDTA for 2 minutes, trypsin is added in sufficient quantity to cover the flask but not in excess (*approximately 3mls for T125 flask*).

3.3.2 MAIT cell activation by liver and non-liver derived cell lines

Liver derived, HepG2, Huh7, HHL12, LEC, and HSCs and non-liver derived, HeLa and Caco-2 cells lines were seeded in flat bottom plates at $2.5x10^5$/ml in a final volume of 200µls/well with *E*. coli (CFU/APC mentioned in figure legends) and $1x10^6$/ml ($0.2x10^6$/well) isolated CD8 T cells. Cells were cultured for 16 hours with BFA/Monensin added for the last 4 hours before analysing by flow cytometry.

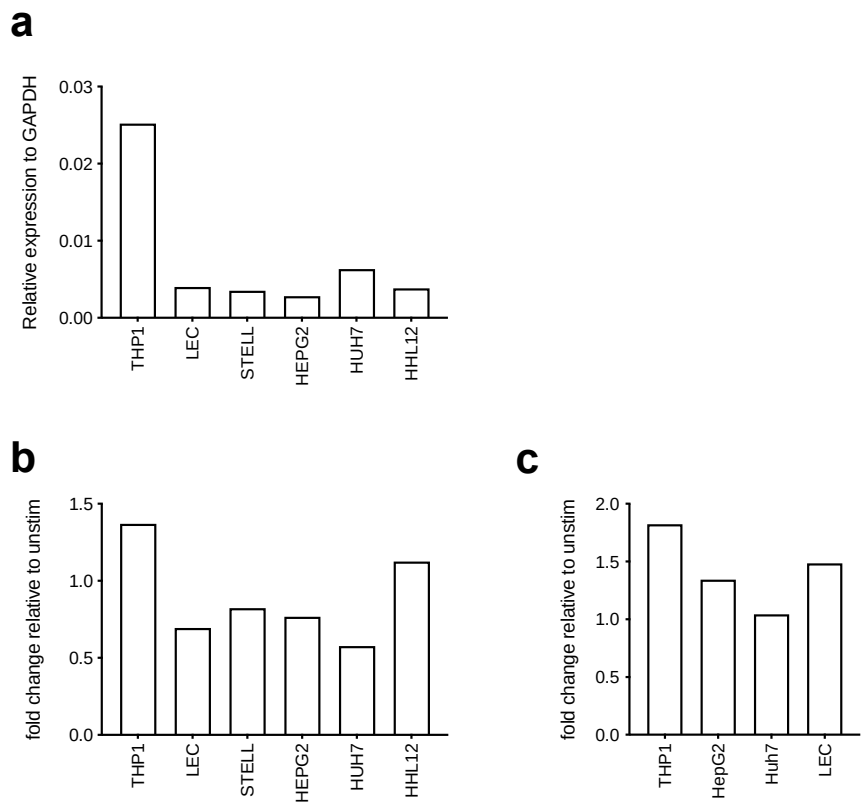


Figure 3.1 - MR1 gene expression in unstimulated and *E*. coli stimulated Liver derived cell lines

MR1 gene expression from liver derived cell lines, liver endothelial cells (LEC), HSCs, hepatoma cell lines - HepG2, Huh7, and HHL12 and THP-1 cell lines was measured by RT-PCR. THP-1 cell line serves as a control. a) MR1 gene expression measured from THP-1 and liver derived cell line cultures. b) Cell lines were stimulated with *E*. coli (x30CFU/cell) for 24 hours before measuring gene expression. c) Cell lines stimulated with high dose *E*. coli (x100CFU/cell) for 24 hours prior to measuring gene expression. Data in b and c shown as fold change relative to unstimulated cell lines.

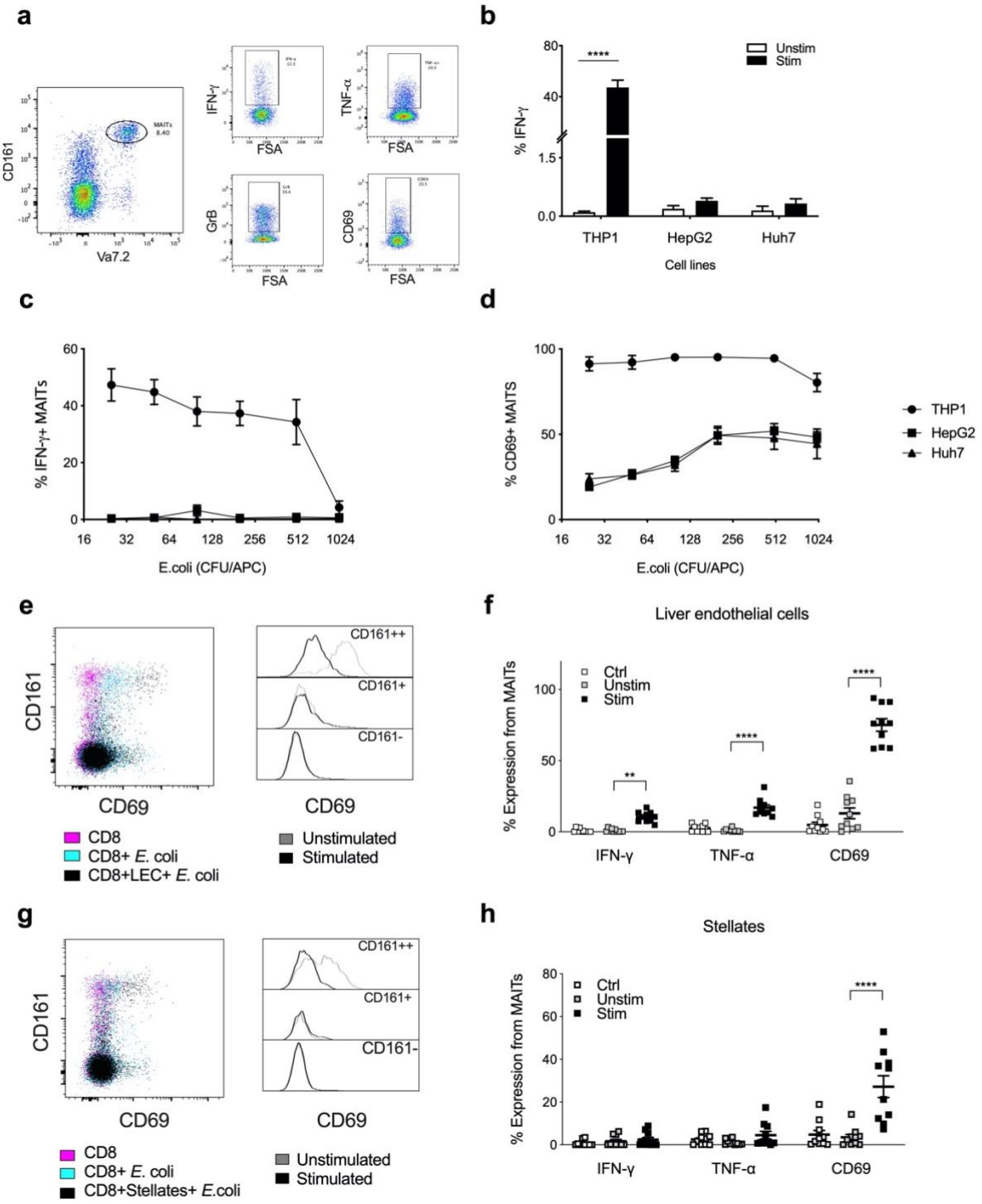


Figure 3.2 - **MAIT cell activation by *E*. coli exposed liver derived cell lines**

Isolated CD8 T cells were co-cultured with *E*. coli exposed THP-1 or liver derived cell lines, HepG2, Huh7, Liver endothelial cells (LEC) or HSCs overnight (16 hours) to activate MAIT cells, before analysing by flow cytometry. a) Representative plots showing gating for MAIT cells and their cytokine profile. All experiments were analysed with this method of gating,

(n=4), Statistical significance - Sidak's multiple comparisons test, ****P<0.0001. b) IFN-γ expression from CD8+CD161++Vα7.2+ MAITs activated by *E*. coli stimulated THP-1, HepG2 and Huh7 cell lines. c) IFN-γ and d) CD69 expression from CD8 MAITs stimulated by *E*. coli exposed THP-1, HepG2 and Huh7 cell lines. *(n=4)*. MAIT cell activation by *E*. coli exposed e) LEC and f) HSC, *(n=10)*. Representative FACS plots and histograms for CD69 expression from CD8+CD161++, CD8+CD161+ and CD8+CD161- populations stimulated with f) LECs and h) HSCs. Statistical significance - Dunnett's multiple comparisons test, **P<0.005, ****P<0.0001.

MAIT cells are found at many sites in the human body, however, they are most abundant in the blood and the liver. Numerous studies have quantified MAIT cell frequency and investigated their function and phenotype in disease however there is minimal research on the activation of MAIT cells in the liver. In the initial part of this project we explored the ability of liver derived cells to activate MAIT cells, the pathways used to activate MAIT cells and their efficiency to activate MAIT cells in comparison to conventional antigen presenting cells.

3.4.1 Hepatoma cell lines failed to activate MAIT cells in response to *E*. coli exposure

Previous studies have outlined the ability of multiple liver specific cells to function as APCs with the ability to activate T cells in response to various stimuli in both antigen dependent and independent modes (Crispe 2011; Racanelli and Rehermann 2006). More recently Jeffery et al showed that MAIT cells could be activated by *E*. coli exposed liver biliary epithelial and B cells in an MR1 dependent, cytokine independent manner

(Jeffery, Van Wilgenburg, et al. 2016). To study the capacity of other liver cell subtypes to activate MAIT cells, we used hepatocyte-like cells; HepG2, Huh7 (hepatoma lines) and HHL12 cell lines, liver endothelial cell (LEC) and HSC lines exposed to *E*. coli. Hepatoma cell lines did not induce any IFN-γ when stimulated with low doses of *E*. coli (30 CFUs/APC), (Fig. 3.2b). The ability of MR1 transfected HeLa cells to activate MAIT cells and induce CD69, CD25 and IL-2 has been demonstrated by two groups(Jeffery, Van Wilgenburg, et al. 2016). Le Bourhis et al had shown that MAIT cell activation by MR1 transfected HeLa cells required high bacterial loads to induce CD25 and CD69 expression from MAIT cells(Le Bourhis et al. 2013). We explored the ability of HepG2 and Huh7 cells lines to activate healthy blood derived CD8 MAIT cells by exposing the hepatoma cell lines to *E*. coli loads between 25-1000 CFUs/APC. Similar to the previous findings we also saw a dose dependent increase in CD69 expression on MAIT cells, with CD69 expression peaking at 200 CFUs/APC when stimulated with *E*. coli exposed HepG2 and Huh7 cells (Fig. 3.2d). The higher *E*. coli loads still failed to induce the same levels of CD69 expression from MAITs stimulated with *E*. coli exposed THP-1 cells, in fact, the maximum CD69 expression by *E*. coli exposed HepG2 and Huh7 cells was only 50% of the expression induced by THP-1 cells thus making these cells less efficient compared to THP-1 cells (Fig. 3.2d).

3.4.2 MAIT cells can be activated by *E*. coli exposed LEC but not by HSCs

In contrast to hepatoma cell lines, stimulation of healthy blood derived CD8 MAIT cells by *E*. coli exposed liver endothelial and HSCs did activate and induce CD69 expression, however the expression of proinflammatory cytokines like IFN-γ and TNF-α was different for the two liver cell types. Like the hepatoma cell lines, both the LEC and HSCs lines

required higher bacterial loads to activate MAIT cells (Fig. 3.3); consistent with the higher thresholds necessary to activate T cells in the liver (Tay et al. 2014). Although these cells required higher bacterial loads, they were able to activate MAIT cells in terms of CD69 expression with half the amount of *E*. coli as compared to HepG2 and Huh7 cells (Fig. 3.3). *E*. coli exposed LECs were able to induce MAIT cell IFN-γ and TNF-α, whereas the HSCs could not induce any IFN-γ or TNF-α from MAIT cells. The average CD69 expression induced by HSCs was less than 40% and with LECs around 60%, (Fig. 3.2f/h), therefore demonstrating that LECs are more efficient than the HSCs for MAIT cell activation in response to *E*. coli infection.

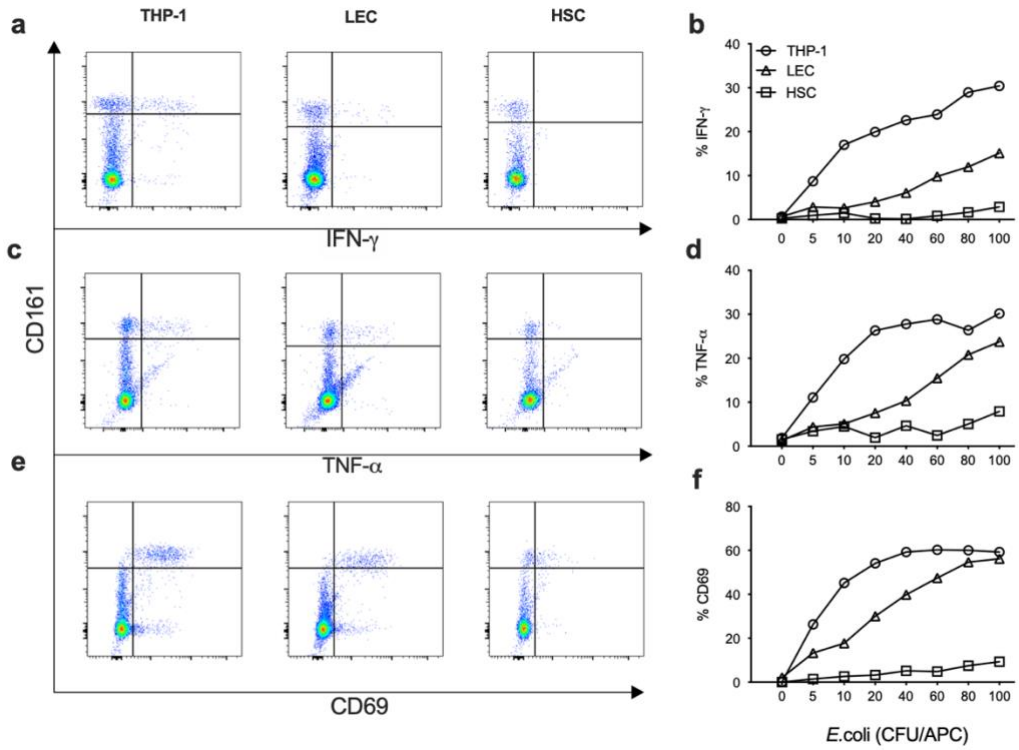


Figure 3.3 - Dose dependent MAIT cell activation by *E*. coli exposed LECs and HSCs. Isolated CD8 T cells were co-cultured with THP-1, LEC or HSCs and *E*. coli was titrated into the cultures. All three cell line assays were set-up in parallel using CD8 T cells from

the same four donors, cultured overnight and treated with BFA/monensin in the last 4 hours, prior to ICS staining and analysed by flow cytometry. Representative FACS plots for THP-1, LEC and HSC induced a) IFN-γ, b) TNF-α and c) CD69 expression from MAIT cells. Quantification of d) IFN-γ, e) TNF-α and f) CD69 expression from CD8 MAIT cells at different *E*. coli doses *(n=4)*.

3.4.3 MAIT cell activation by LECs is not as efficient compared to the THP-1 cells

Though liver resident cells can function as APCs, the unique immunology in the liver results in altered stimulatory capabilities suited to the liver microenvironment (Crispe and Smith 2009). In addition, there is significant differences in the expression of co-stimulatory molecules and the ability to make proinflammatory cytokine by liver resident cells in comparison to conventional APCs (Lau 2003b), suggesting differences in the T cell stimulatory potential of liver resident APCs. To study the stimulatory capacity of hepatoma, LECs and HSCs we set up *E*. coli titration cultures conducted in parallel alongside the THP-1 cell line (*which represents the conventional APC*) to investigate the most efficient liver resident APC for MAIT cell activation. LECs and HSCs were studied separately to hepatoma cells due the differences in bacterial loads required for MAIT cell activation. All cell lines upregulated CD69 expression on MAIT cells in a dose dependent manner where the HepG2 and Huh7 cells induced only 50% of the CD69 expression induced by *E*. coli exposed THP-1 cells (Fig. 3.2c/d and Fig. 3.3f). Unlike the hepatoma and HSCs, the LECs induced IFN-γ expression in a dose dependent manner but never achieved the same levels of IFN-γ expression from MAIT cells stimulated with THP-1 cells (Fig. 3.3.b). On the other hand TNF-α expression levels by *E*. coli exposed LECs

were similar to those induced by *E*. coli exposed THP-1 cells at a bacterial load of 100CFUs/APC with the potential to induce more TNF-α at higher bacterial concentration whereas TNF-α induction by *E*. coli exposed THP-1 cells had reached the maximum expression at an *E*. coli concentration of 60CFUs/APC. Maximum CD69 expression from MAIT cells was achieved at 40CFUs/APC by *E*. coli exposed THP-1 cells and at 100 CFUs/APC by LECs, again showing the dose dependent increase in CD69 expression by LECs that could reach the same levels induced by the THP-1 cell line (Fig. 3.3c). The HSC line failed to induce substantial levels of IFN-γ, TNF-α and CD69 even at high bacterial loads making them the least efficient cell type for MAIT cell activation in response to *E*. coli infection (Fig. 3.3). LECs were the most efficient liver resident APC capable of inducing multiple activation makers, with the ability to induce similar levels of TNF-α and CD69 as the THP-1 cell line. Hepatoma cell lines according to our model are also considered inefficient MAIT cell activators as they only induced CD69 expression which is an early activation marker easily activated in response to numerous stimuli including toxic mediators produced by cell death and high bacterial loads.

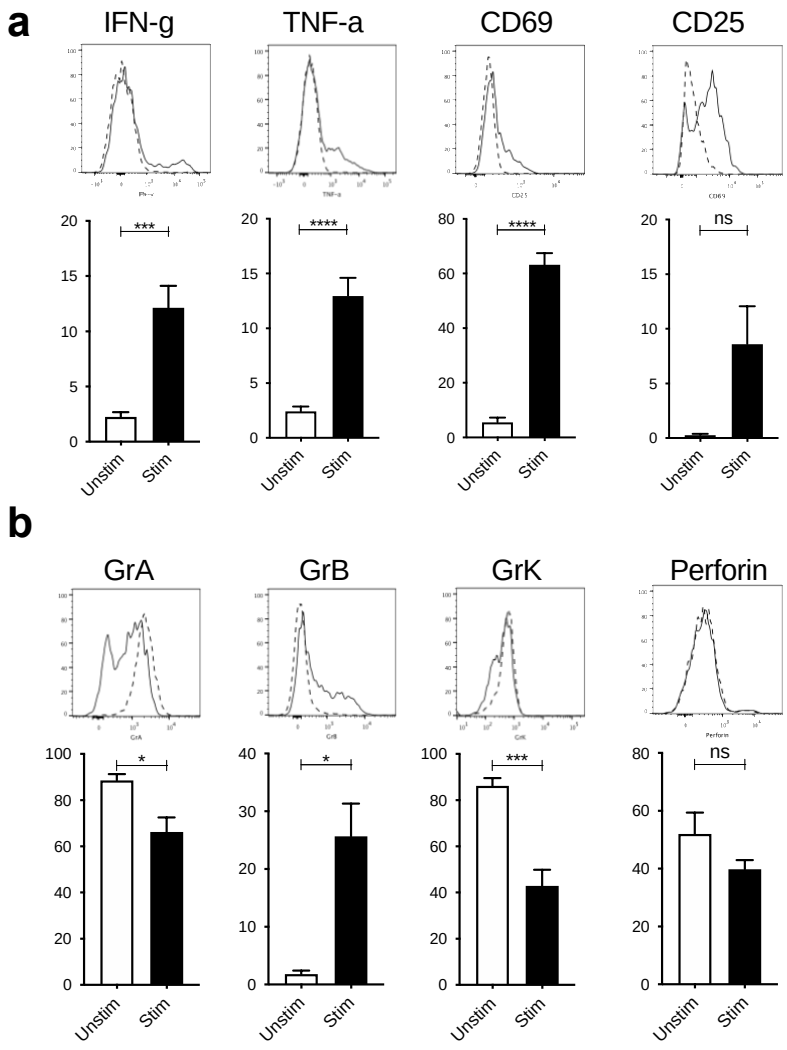


Figure 3.4 - *E*. coli exposed LECs can induce expression of multiple cytokines, surface receptors and cytotoxic granules from blood derived CD8 MAIT cells.

LECs and isolated CD8 T cells were co-cultured overnight in the presence of 100CFU/APC *E*. coli and analyzed by flow cytometry. CD8+CD161++Vα7.2+ MAIT cells were phenotyped for a) activation – IFN-γ, TNF-α, CD69, CD25 *(n=13)* and b) Cytotoxicity - GrA, GrB, GrK and perforin *(n=9)*. Statistical significance - Two tailed paired t test, *P<0.0193, ***P=0.0001, ****P<0.0001, ns=non-significant.

3.4.4 *E*. coli exposed LECs were able to activate MAIT cells comprehensively

As the LECs were able to induce significant CD69 expression from MAIT cells we investigated the capacity of LECs to upregulate other activation markers and the ability to induce cytolytic molecules to *E*. coli exposure. We used the optimal concentration of 100CFUs/APC of *E*. coli that induced maximum CD69 expression to look at the expression of CD25, GrA, GrB, GrK and perforin expression from MAIT cells. *E*. coli exposed LECs are capable of inducing significant expression of IFN-γ, TNF-α and CD69 expression but induced limited CD25 expression from MAIT cells which was non-significant (Fig. 3.4a). GrA and GrK was high in unstimulated CD8 MAIT cells compared to GrB. When stimulated with *E*. coli exposed LECs, GrB expression in MAIT cells increased significantly and both GrA and GrK levels decreased with a 50% and 20% reduction in GrK and GrA, respectively (Fig. 3.4b). Perforin expression was higher in unstimulated MAIT cells and decreased very slightly after stimulation with *E*. coli exposed LECs (Fig. 3.4b). These finding indicate that LECs can activate MAIT cells comprehensively compared to hepatoma and HSCs.

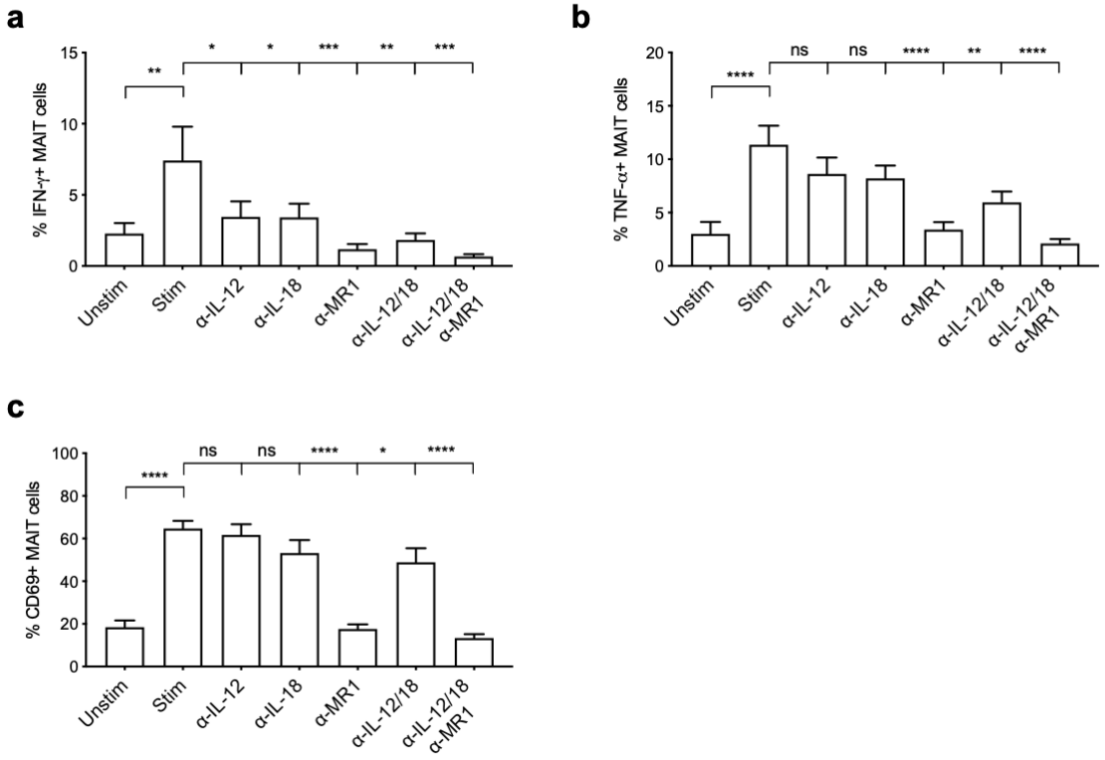


Figure 3.5 - Blockade of MR1 and cytokine pathways for MAIT cell activation by *E*. coli exposed LECs

LECs and isolated CD8 T cells were co-cultured with *E*. coli in the presence of blocking antibodies to MR1, IL-12 and IL-18 individually or in combination. CD8+CD161++Vα7.2+ MAIT cells were analyzed by flow cytometry to measure a) IFN-γ, b) TNF-α and c) CD69 expression, (*n=13*). Statistical significance - Holm-Sidak's multiple comparisons test, *P<0.025, **P<0.0045, ***P<0.0003, ****P<0.0001.

3.4.5 LECs can use both the cytokine and MR1 signalling pathways to activate MAIT cells

To identify the pathways used by LECs to activate MAIT cells we blocked IL-12, IL-18 and MR1 signalling individually or in combination. MAIT cell activation by MR1 was more prominent than cytokine mediated activation, but more importantly, LECs utilised both pathways and in combination account for optimal activation of MAIT cells. Blocking the MR1 signalling pathway alone resulted in a greater reduction in MAIT IFN-γ, TNF-α and CD69 expression from MAIT cells in comparison to the individual and combined blockade of IL-12 or IL-18 (Fig. 3.5). CD69 expression was unaffected by the individual blockade of IL-12/IL-18 and showed minimal reduction when IL-12 and IL-18 signalling was blocked in combination. Likewise, TNF-α expression was not reduced by the individual inhibition of these cytokines and there was minimal effect on TNF-α expression when blocked in combination (Fig. 3.5b/c). In contrast to the latter, IFN-γ expression was significantly reduced by the combined and individual blockade of IL-12 and IL-18 (Fig. 3.5a). Blocking all three signalling pathways in combination results in potent reduction of all three markers, matching the expression levels in the unstimulated MAIT cells (Fig. 3.5). It is not possible to determine whether IL-12 or IL-18 provides better co-stimulation for the TCR signalling in our model or if both are necessary for TCR stimulation. The ability of cytokines other than IL-12 and IL-18 has been shown to support TCR signalling enhancing MAIT cell activation. Thus examining the role of other cytokines produced by LECs to activate MAIT cells is necessary to determine the best combination of TCR+cytokine signalling to MAIT cells in the liver.

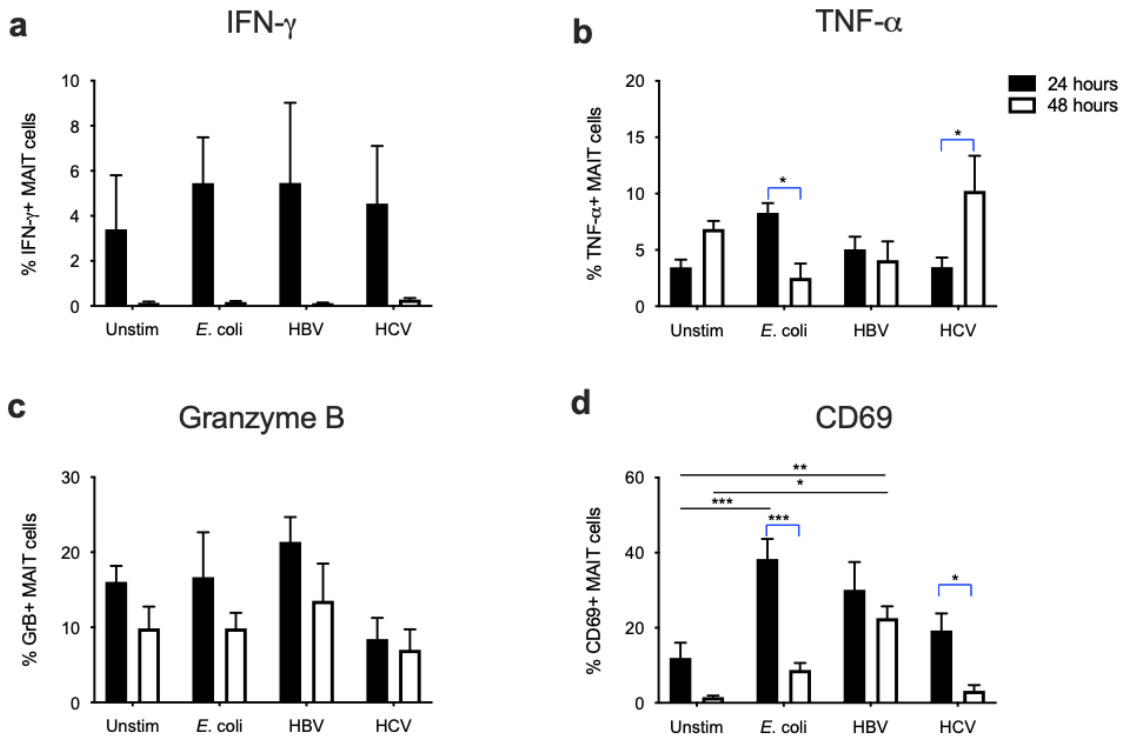


Figure 3.6 - Healthy donor MAIT cell activation by viral exposed Liver endothelial cell lines

Isolated CD8 T cells were co-cultured with *E*. coli, HBV, or HCV exposed LECs. Response was assessed for 24 (black bars) and 48 (white bars) hours. a) IFN-γ, B) TNF-α, c) GrB and d) CD69 expression from CD8+CD161++Vα7.2+ MAIT cells, *(n=4)*. Statistical significance - Sidak's multiple comparisons tests, 1st test (black) - expression from stimulated cells is compared to unstimulated cells, 2nd test (blue) compares the difference in expression at 24 vs 48 hours. *P<0.01, **P<0.004, ***P<0.0004.

3.4.6 HBV and HCV exposed LECs failed to induce inflammatory cytokines from MAIT cells and show limited activation of MAIT cells

Previous work from our group has shown MAIT cell activation by virus infected macrophages that use cytokine signalling to activate MAIT cells. Liver resident cells have the capacity to make inflammatory cytokines that can activate innate cell populations like the NK cells and T cells, therefore we tested the potential for LECs to activate MAIT cells in response to HBV and HCV in 24 and 48 hour stimulations. There was no significant increase in IFN-γ, TNF-α or GrB by HBV and HCV stimuli. CD69 expression was detected in the first 24 hours by HCV and diminished by 48 hours whereas for HBV stimulation, CD69 expression was higher at 24 hours and could be detected at 48 hours, but to a lesser extent. GrB expression did not change much compared to the unstimulated MAIT cells with lower levels of expression at 48 hours. There is significant difference in TNF-α expression at 24 vs 48 hours for MAIT cells activated by HCV exposed LECs, the same pattern of expression was observed in the unstimulated group (non-significant) however there is no significant difference in TNF-α expression between unstimulated and HCV stimulated MAITs, thus indicating that TNF-α expression increases by an unidentified mechanism as a result of the interactions between LECs and CD8 T cells.

3.5 Discussion

The liver consists of a huge number of innate cells, NK cells, and innate like T cells such as the iNKT, γδ and MAIT cells. MAIT cells are most abundant in the liver, they are located close to biliary epithelial cells (Jeffery, Van Wilgenburg, et al. 2016) and are found throughout the liver parenchyma (unpublished data). Like other lymphoid cells, MAIT cells infiltrating the liver enter through the sinusoidal lumen. The lumen is lined by fenestrated endothelial cells which allow hepatocytes and HSCs to communicate with cells in the sinusoidal lumen. Also, the presence of MAIT cells in the parenchyma strongly implies their interaction with hepatocytes and thus the potential to be activated by hepatocytes.

T cell activation in the liver is a complex process, different to other organs. Liver immunity is skewed towards tolerance and T cell activation in the liver is limited by active regulatory mechanisms needed to limit unnecessary responses to harmless antigens originating from the gut. Nevertheless, upon pathogenic invasion, the liver has the ability to clear the infection. Both parenchymal and non-parenchymal cells have been shown to function as antigen presenting cells with the capacity to activate T cells.

Despite the close proximity of MAIT cells and hepatocytes, our results show limited MAIT cell activation by *E*. coli exposed HepG2 and Huh7 cell lines. Even though hepatocytes lack expression of co-stimulatory molecules CD80 and CD86, they were able to induce activation and effective proliferation of specific naive CD8+ T cells in the absence of exogenously added cytokines(Bertolino et al. 1998), the absence of such co-

stimulatory signalling may be a limiting factor for direct hepatocyte-specific MAIT cell activation. MAIT cell activation by MR1 transfected HeLa cells required extremely high bacterial loads to induce CD25 and CD69, therefore we assumed that as hepatocytes are an epithelial cell they would stimulate MAIT cells if exposed to high bacterial loads. Indeed, CD69 expression, the only activation marker induced by *E*. coli exposed hepatoma cell lines was upregulated at high bacterial loads. T cell activation in the liver is regulated by antigen thresholds which determine activation or tolerance. For hepatocyte-T cell interactions, low antigen load results in T activation, expansion and effector function while high antigen loads induce T cell anergy and exhaustion. We did not observe any activation of MAIT cells at low bacterial doses and for higher bacterial loads, we could only detect limited CD69 expression.

Healthy liver hepatocytes express low levels of MHC-I/II on the surface compared to conventional antigen presenting cells however MR1 expression could not be detected on hepatoma cell lines by staining. MR1 is present endogenously in vesicles and is trafficked to the surface once bound to its ligand. In order to detect surface MR1 expression we exposed liver cell lines to fixed *E*. coli, even with *E*. coli exposure we could not detect MR1 on the surface. Similar to the staining data, MR1 quantification by RT-PCR showed minimal expression in liver cell lines compared to the THP-1 cell line (Fig. 3.1). As MR1 could not be detected on hepatoma cell lines, it is possible that hepatocytes lack the capacity to activate MAIT cells in a TCR dependent manner. However, in contrast to cell lines, MR1 was detectable on primary hepatocytes, biliary epithelial cells, HSCs and LSECs (unpublished data). MAIT cell activation by *E.* coli exposed hepatoma cells resulted in limited MAIT cell activation. Furthermore, CD69 expression in MAIT cells was

induced only at very high bacterial loads, suggesting that hepatoma cells may be less efficient at phagocytosing and presenting antigen to MAIT cells. Moreover, the lack of co-stimulatory molecules on hepatocytes provide inadequate signalling to MAIT cells resulting in incomplete activation and subsequently T cell anergy. However the latter requires investigation. In contrast to hepatocyte-like cells, primary healthy liver derived hepatocytes were the most efficient MAIT cell activators (unpublished data). Primary hepatocyte exposure to *E*. coli lysate resulted in MR-1 dependent MAIT cell activation, inducing IFN-γ expression, upregulation of activation markers like CD69, CD137 and PD-1 and also resulted in degranulation of MAIT cells as assessed by CD107a staining. This robust MAIT activation by primary hepatocytes was also confirmed using pure synthetic MAIT-ligand, 5-OP-RU, which produced very similar results (unpublished data). MR1-dependent MAIT cell activation was also possible by primary LSECs, BECs and HSCs, with the latter producing a fibrogenic response when stimulated with antigen. Although HepG2 and Huh7 are hepatocyte-like cell lines, they are derived from tumours therefore termed hepatoma cells and are significantly different to primary hepatocytes from healthy liver(Clayton et al. 2005; Wilkening, Stahl, and Bader 2003), this difference may explain their inability to activate MAIT cells. This finding also shows that cell lines should be used with caution and that the effects observed with the use of cell lines may differ significantly to primary derived cells.

The ability of HSCs to function as efficient antigen presenting cells has been demonstrated previously where HSCs were able process and present peptides to CD4+ and CD8+ T cells in a *Listeria monocytogenes* model eliciting protection against infection (Winau et al. 2007). Furthermore, HSCs possess the capacity to induce vigorous iNKT cell

responses both in vitro and vivo. Such studies confirm HSC interactions and the capacity to activate immune cells. MAIT cell activation by HSCs was weak and limited compared to LECs, this may be due the presence of co-regulatory/inhibitory molecules instead of, or lacking co-stimulatory molecules on the surface. HSCs in the healthy liver are generally quiescent but in chronic liver injury can be activated and act as major drivers of liver fibrosis. Liver fibrosis is driven by type 3 cytokines like IL-17A which can be expressed by MAIT cells, more so by tissue resident MAIT cells. HSCs have been implicated in liver fibrosis by the induction of cytokines like TNF-α and IL-17 and because both these cytokines can be produced by MAIT cells thus it is highly likely that HSCs stimulate fibrosis promoting cytokines in MAIT cells. Though MAIT cells have been described as a profibrogenic population in the liver (Hegde et al. 2018), a more comprehensive analysis of MAIT cells stimulated by HSCs is needed to determine if HSCs promote profibrogenic MAIT cells. Furthermore, the production of TGF-β from fibrogenic HSCs is likely to suppress T cell activation because TGF-β is a regulatory cytokine and has been shown to dampen inflammatory cytokine production by T cells (Batlle and Massagué 2019).

MAIT cells entering the liver from the circulation are bound to come into contact with liver sinusoidal cells hence understanding the LSEC-MAIT cell interaction is important. Unlike the hepatoma and HSC lines, the LEC line could induce comprehensive MAIT cell activation. Our results demonstrate that LECs can present antigen to MAIT cells and are able to efficiently activate MAIT cells in an MR1 and cytokine dependent manner. In line with previous findings, MAIT cell activation by LECs was dependent on antigen load as MAIT cells could only be activated in the presence of higher bacterial loads compared to

THP-1 cells. MAIT cells activated by *E*. coli exposed LECs upregulated cytolytic granules and type 1 cytokines giving them the capacity to clear infected cells. Nonetheless, LECs were less efficient at activating MAIT cells compared to conventional APCs, this may be due to a combination of stimulatory and inhibitory signals provide by LECs, such as PD-L1 expression on LECs which is a common feature of the immunoregulatory liver environment. This could be a possible mechanism affecting MAIT cell activation as MAIT cells can express PD-1 on their surface however we have not investigated this yet. In contrast, MAIT cells did not respond well to virus exposed LECs. As viruses do not produce the ligand necessary for TCR mediated MAIT cell activation, MAIT cells can only be activated by virus induced cytokines from LECs. Another possibility would be the inability of LECs to express the appropriate cytokines necessary for MAIT cell activation upon virus encounter. Our virus exposure assay is limited to LEC activation through PRRs and is not an ideal model to study viral infection associated MAIT cell activation as the LECs are not permissive to these viruses. Whether HBV and HCV exposed LECs can make MAIT cell activating viruses needs to be assessed before probing the pathways that maybe involved in this model.

Cell lines provide endless supplies of primary-like cells but they are largely tumour derived and are transformed cells hence the results observed may differ to the activation by primary cells. This is also true for MAIT cell activation by hepatoma cells, as primary hepatocytes were able to activate MAIT cells whereas the hepatoma cell lines failed to induce functional activation of MAIT cells (unpublished data). Moreover, these two-cell co-culture experiments provide a basic insight in to the actual response that is otherwise occurring in a multicellular environment. The multicellular environment of

organs means that several cell types may be activated simultaneously and would be exerting similar or opposing effects on the effector cell of interest, thus tuning of the response is subject to signals from many cells. These signals maybe contact dependent as identified for HSCs which are capable of suppressing T cell activation by up-regulating ICAM-1 (Schildberg et al. 2011) or mediated by locally present soluble factors, such as IL-10, TGF-β and prostanoids (Horst et al. 2016b). The paracrine impact of hepatoma cells on MAIT cell activation has been investigated further in the next chapter and demonstrates the suppressive paracrine effect by hepatoma cells on MAIT cells activated by conventional APCs and LECs.

3.6 Summary

1. *E*. coli exposed hepatoma cell lines are not able to activate healthy donor blood derived MAIT cells.
2. MAIT cells could be activated by *E*. coli exposed LECs but not by HSCs.
3. LECs require higher bacterial loads to activate MAIT cells thus are less efficient compared to conventional APCs.
4. MAIT cell activation by *E*. coli exposed LECs required both MR1 and cytokine pathways for optimal activation.
5. MAIT cell response to virus exposed LECs was non-significant.

CHAPTER 4 - HEPATOCYTE INFLUENCE ON MAIT CELL ACTIVATION AND FUNCTION

4.1 Introduction

Hepatocytes are the most abundant cell type of the liver, accounting for up to 80% of the liver mass. Despite being protected from direct contact with blood components by liver sinusoidal cells (Sørensen et al. 2012b), hepatocyte cells can directly interact with the blood via cellular protrusion through LSEC fenestrae or can receive signals from the blood by the process of transcytosis (Poisson et al. 2017). After receiving signals, the hepatocytes may respond by contact dependent mechanisms or by secreting soluble factors and thereby directly or indirectly influence neighbouring cells. The focus of this chapter is to explore the influence of hepatocytes on T cells, particularly MAIT cells, in the liver microenvironment. As contact dependent mechanisms of hepatocyte regulation of immune responses have been discussed in the previous chapter, in this chapter we will explore soluble factors generated by hepatocytes in homeostasis or disease and will discuss how these contribute to immunity and their potential influence on immune responses in the liver.

Hepatocytes are one of the most active cell types of the body and are enriched with organelles like mitochondria, endoplasmic reticulum and lysosomes(Schulze et al. 2019). These organelles support the metabolic functions, protein synthesis, detoxification and immunological roles hepatocytes play. Through these processes hepatocytes produce a variety of compounds. Many soluble factors modified and released by hepatocytes, e.g. ammonia detoxified to urea, are harmless and excreted from the liver whilst some compounds are produced to facilitate homeostasis, others to assist immunity and some are responsible for liver pathology.

While investigating the immunological properties of the liver, the role of "Hepatocytes as immunological agents" has become more evident(Crispe 2016). This is demonstrated by numerous studies analysing the immunological features of both innate and adaptive immunity associated with hepatocytes. Innate immunity is activated by hepatocytes in response to infectious agents and induces production of many soluble factors like bactericidal proteins, LPS signalling regulators, opsonin's, complement proteins, fibrinogen, transferrin, proteinase inhibitors and cytokines(Gao et al. 2007). Complement proteins released by hepatocytes are involved in direct killing of pathogens while other proteins, for examples the c-reactive protein (CRP) modulates inflammation by activating other cells types or by inducing pro-inflammatory/anti-inflammatory cytokines(Du Clos and Mold 2004). CRP synthesis and secretion by hepatocytes is one mechanism of paracrine modulation of immune responses by hepatocytes. In response to infection, tissue injury or other forms of cellular stress, hepatocytes increase CRP synthesis. Elevated CRP levels augment upregulation of adhesion molecules on endothelial cells thereby recruiting immune cells into the liver. CRP also enhances the production of pro-inflammatory cytokines like IL-6, TNF-α, IL-1β and IL-18(Sproston and Ashworth 2018). In contrast to the pro-inflammatory properties of CRP, the anti-inflammatory roles of CRP include IL-10 induction, desensitising neutrophils to IL-6 and inhibition of superoxide synthesis(Du Clos and Mold 2004).

The liver is described as an immunologically tolerant organ and this is maintained partly by the secretion of regulatory compounds like IL-10, TGF-β and PGE_2, these compounds are able to modulate other cells in a paracrine manner and can impact the activation

and effector functions of T cells(Horst et al. 2016a). IL-10 production is not limited to a particular cell type and in addition to hepatocytes is made by liver dendritic cells, Kupffer cells and LSECs(Heymann and Tacke 2016). IL-10 secretion by hepatocytes forms another mechanism for paracrine modulation of immune cells by hepatocytes. A major role for IL-10 in the liver is the modulation of APC function by limiting their antigen presenting capacity and reducing IL-12 secretion (Crispe 2014). Moreover, IL-10 is able to induce regulatory cells, thus dampening the immune response by enhancing regulatory T cell function (Heymann and Tacke 2016). Other than the liver tolerance effect of hepatocyte derived IL-10, hepatocyte-IL-10 is crucial for the control of chronic inflammation and associated immunopathology as shown in the *T congolense* infection model (Stijlemans et al. 2020). Along with IL-10 production by hematopoietic cells, hepatocytes derived IL-10 was key in protecting against uncontrolled inflammation which could cause anaemia, tissue injury and hepatosplenomegaly in *T congolense* infection.

Whether hepatocytes can actually produce TGF-β1 is controversial. Hepatocytes have been described to store the latent form and therefore are able to secrete TGF-β1 to various stimulations (Roth, Michel, and Gressner 1998). In response to infection with HCV, HepG2 cells have been shown to increase TGF-β1 gene expression (Kim, Lee, and Lee 2016; Shin et al. 2005). TGF-β1 is a pleiotropic cytokine with anti-inflammatory and inflammatory capacity and can modulate immune cells (Schon and Weiskirchen 2014). The potential for hepatocytes to store, synthesize and secrete TGF-β1 identifies an additional paracrine mediated regulation by hepatocytes in the liver microenvironment. Furthermore, hepatocytes have been shown to synthesise both prostaglandins and leukotrienes (Mion, Jasuja, and Johnston 1995; Titos et al. 2000). PGE_2 is widely

implicated in the liver as a tolerance inducing compound and for liver regeneration (Hossain et al. 2006). The immunosuppressive functions of PGE_2 are demonstrated by many in-vitro studies where PGE_2 is shown to directly impact APCs by inhibiting pro-inflammatory cytokine production, polarizing macrophages to a type 2-like phenotype, limiting antigen presentation and also suppresses effector cell responsiveness by downregulating cytokine receptors (Harris et al. 2002).

In addition to bile synthesis hepatocytes are heavily involved in substrate homeostasis and play a key role in regulating their levels. Carbohydrates, lipids and proteins are metabolized by hepatocytes to produce substrates like amino acids, cholesterol, glucose and fatty acids(Nagarajan et al. 2019). Hepatocytes are particularly important in glucose homeostasis and a significant amount of glucose is removed from the circulation by the liver where it is converted to ATP, stored as glycogen or converted to fatty acids and amino acids (Klover and Mooney 2004). Any dysregulation in the metabolic function of hepatocytes leads to altered liver function which can be detrimental as described for fatty liver disease(Postic and Girard 2008). Immediate sources of fatty acids include dietary triglycerides and cellular phospholipids. In non-alcoholic fatty liver disease (NAFLD), triglycerides accumulate in hepatocytes(Brunt et al. 2015). High levels of triglycerides give rise to fatty acids and augment production of lipid metabolites e.g. leukotrienes, prostaglandins, epoxyeicosatrienoic acid (EET) and many others(Alves-Bezerra and Cohen 2017). Lipid metabolites are not only involved in liver homeostasis but are increasingly recognised as immunological agents as described above for PGE_2. While some are involved in liver tolerance an imbalance in these metabolites may contribute to liver pathology such as tumorigenesis(Zang et al. 2017). Altered lipid metabolic function has been reported between primary hepatocytes and cells lines.

However, such altered lipid profiles have only been used to demonstrate suitability of cell lines for liver metabolism studies(Cassim et al. 2017). A comprehensive analysis of the differences in lipid metabolite profiles between primary hepatocytes and hepatocyte cell lines and how it affects immunity would be useful.

These are a few of the widely studied proteins and immunoregulatory compounds that are produced by hepatocytes in homeostasis and in response to stimuli. There are likely to be many more compounds that are produced by hepatocytes and have the potential to positively regulate or suppress immune responses. In addition to these well studied effector molecules, various studies suggest that hepatocytes have the potential to produce many more compounds with immunomodulatory properties. Taken together, these findings also demonstrate that hepatocytes have a prominent role in liver immunity and are able to influence T cell activation and function through paracrine signals.

In the previous chapter we described the direct activation of MAIT cells by liver resident cells, which was limited to LECs. In this chapter we aimed to understand how hepatocytes influence MAIT cell activation by conventional antigen presenting cells. Can hepatocytes enhance MAIT cell activation by conventional APCs or do they deliver immunosuppressive signals like those described above? If so, which effector molecules are involved?

4.2 Aims

1. Investigate hepatocyte influence on MAIT cell activation
2. Identify whether hepatoma cell-mediated suppression is contact dependent or independent
3. To define the mechanism for MAIT cell suppression by hepatoma cell derived mediator/s
4. To determine if MAIT cells are being suppressed directly by hepatoma cell derived mediator/s or indirectly, through other cells.
5. Explore the impact of inhibiting lipid metabolite synthesis in hepatocytes.

4.3 Materials and methods

4.3.1 Primary hepatocyte isolation from liver resection surplus and culture

Human liver tissue was collected from patients undergoing surgery to remove metastasis of colorectal cancer or hepatocellular carcinoma. Samples were obtained with written consent through the Oxford Gastrointestinal Illnesses Biobank (NHS REC 16/YH/0247) for the use of excess tissue (resection surplus) for research.

Liver tissue resections were collected on ice and dispensed in D10 media during transport. Liver tissue was minced to 1-3mm cubes in a petri dish in D10 media. A single cell suspension was collected in ice cold D10 media, achieved by mechanical disruption of tissue by gentle pressure to pass the tissue through a 100μM cell strainer using the end of a syringe. The single cell suspension was washed twice in D10 at high-speed centrifugation of 500g. The cells were resuspended in D10 media and isolated from the non-parenchymal fraction by low speed centrifugation for 10 minutes. Isolated cells were subjected to three rounds of centrifugation with increased speeds, 30xg, 40xg and 50xg to increase the purity of the collection. Cells were seeded in collagen coated flasks (Corning™ BioCoat™ Collagen I vented culture flask) at varying densities and allowed to adhere for an hour before replacing the medium to remove floating/dead cells. Cells were cultured for 4 days and culture medium was collected and stored at 4°C.

4.3.2 Primary murine hepatocyte isolation

The whole liver was removed from the abdominal cavities of WT C57BL/6 mice, stored in PBS during transport and processed immediately. Liver was transferred to a petri dish in ice cold hepatocyte isolation medium, minced into small 5mm cubes and mechanically disrupted through a 100μM cell strainer using minimal pressure. Primary hepatocyte culture medium is used to collect singlets in falcon tubes and to wash the cell strainer during disruption. Cells were pelleted with gentle centrifugation at 1500rpm for 5 minutes, medium was removed with a stripette and the cells were then washed again in primary hepatocyte culture medium. Cells were then resuspended in hepatocyte culture medium, counted and transferred to precoated collagen flasks (Corning™ BioCoat™ Collagen I vented culture flask).

4.3.3 Culture and collection of primary murine hepatocyte derived culture medium

Cells were allowed to settle and adhere to the surface for an hour at 37°C in primary hepatocyte culture media (chapter 2.1.6) before changing the medium to remove any dead cells. Flasks with approximately 90% coverage were kept for culture for 4 days before collecting the cell culture medium. Cell culture medium was used immediately or stored at 4°C for future experiments.

4.3.4 Cell culture supernatant preparation, collection, and storage

Supernatant was collected from liver derived, HepG2, Huh7, HHL12, LEC and HSCs (HSC) or HeLa and Caco-2 cells from cells cultured in D10 medium for approximately 4 days or until 90% confluent. Supernatants were transferred to falcon tubes by straining through 40μM Minisart syringe filters (Fischer Scientific) and stored at 4°C for further use.

Further details of cell line treatments with various inhibitors and supernatants collected at different time points are provided below.

4.3.5 Fractionating hepatoma cell culture supernatant

Hepatoma cells were cultured for 4 days as explained in chapter 2. Culture supernatant was collected and filtered and then transferred to Amicon ultra filters (Merck Millipore) with a molecular weight cut off of 3kDa. Hepatoma culture supernatants were centrifuged at for 20 minutes at 4000g according to the manufacturer's guidelines. The smaller fraction was left inside the falcon, capped and stored at $4^{O}C$. The larger fraction was resuspended in PBS (same volume as the initial volume used to fractionate), transferred to a sterile falcon and stored at $4^{O}C$ for use in supernatant assays.

4.3.6 Heat treatment of HepG2 cell culture supernatants

1ml of the pre-collected HepG2 culture supernatant was transferred to an Eppendorf and heated at $60^{O}C$ in a water bath for an hour; cooled on ice and used immediately or stored in the fridge.

4.3.7 TLR stimulation of HepG2 cells

HepG2 cells were seeded at a density of 1.5 $x10^{5}$/ml in 12 well plates in 2mls of D10 medium. Cells were cultured for 24 hours before replacing media and adding TLR1-9 agonist (Human TLR kit (Invitrogen)). TLR treated HepG2 cells were cultured for a further 24 hours before extracting the supernatant. Supernatants were filtered and used immediately.

4.3.8 Cytokine stimulation so HepG2 cells

HepG2 cells were seeded at a density of 1.5 x10^5/ml in 12 well plates in 2mls of D10 medium. Cells were cultured for 24 hours before replacing media and adding cytokines (Table 4.2). Cytokine treated HepG2 cells were cultured for a further 24 hours before extracting the supernatant. Supernatants were filtered and used immediately.

4.3.9 Hepatoma cells treated with enzyme inhibitors

HepG2 cells were seeded at a density of 3x10^5/ml in 6 well plates in D10 medium. Cells were cultured for two days, allowing cells to reach a confluency of 70%. Inhibitors were added individually or in combinations as specified in table 4.1. Cells were cultured for a further 24 hours before extracting the cell culture supernatants. Hepatoma cell culture supernatants were used immediately in supernatants assays or stored at 4^OC.

Enzyme	Inhibitor	Concentration used	Supplier
PLA_2	Bromo enol-lactone	1μM	CAYMAN chemicals
COX-1	Indomethacin	1μM	Sigma Aldrich co.
COX-2	Celecoxib	1μM	Cambridge Bioscience Ltd
LOX-5	CJ-13610	1μM	CAYMAN chemicals
CYTP450	1-Aminobenzotriazole	10μM	Tocris bioscience
TXAS	Carbethoxylhexyl Imidazole	1μM	Insight Biotechnology Ltd
PGDS	HQL-79	100nM	Insight Biotechnology Ltd

Table 4.1 – Enzyme inhibitors used to treat HepG2 cells

4.3.10 Successive treatment of HepG2 cells with COX-1 and COX-2 inhibitors

Multiple HepG2 cell cultures were set-up in parallel, in a 6 well plate at $1x10^5$/ml in a final volume of 3mls/well. Cells were left untreated or treated with COX-1 (Indomethacin) or COX-2 (Celecoxib) inhibitors. Cultures supernatant was collected from a single well at 24-hour intervals and the remainder cultures retreated with the same inhibitors at the same time. Collected supernatant was filtered and stored as described above and used in supernatant assays. Cultures were examined daily under the microscope to detect changes in morphology or potential death of cells (detached floating cells) caused by successive drug treatment.

4.3.11 Collection of HepG2 supernatants at different time points

HepG2 cells were seeded at 10^5/ml in 6 well plates in a final volume of 3mls. 4 wells worth of HepG2 cells were cultured in parallel to prevent reductions in the volume of the original culture and to the concentrations of any proteins. Cell culture medium was collected at 24, 48, and 72 and 96 hours from separate wells, collected medium was stored at 4^OC for future use.

4.3.12 Supernatant assays

Supernatants were added to MAIT cell activation assays at a ratio of 1:1 at the beginning along with the stimuli. $1x10^5$ THP-1 +30CFU/APC *E*. coli + $2x10^5$ CD8 T cells were cultured in 96 well U bottom plates in a volume of 100μls of R10, followed by the addition of 100μls of R10 (Control) or liver cell culture supernatant and incubated for 16 hours, BFA and monensin was added for the last 4 hours.

The same method was applied for cytokine (96 u bottom plate) or TCR+cytokine (flat bottom plate) stimulation of MAIT cells treated with supernatants. Stimulation assays

were set-up in 100μls of R10 and then supplemented with cell line culture supernatant, supernatants from treated cell lines, or supernatants collected at different time points.

4.3.13 Cytokine priming of purified CD8 T cells

Isolated CD8 T cells were treated with cytokines listed in table 4.2 for 24 hours before proceeding with the set-up of supernatant assays. Cytokine treated cells were centrifuged and their supernatants removed, cells were washed twice with sterile PBS to removing any remaining traces of exogenous cytokines. Cells were resuspended in fresh room temperature R10 and added to *E*. coli stimulated THP-1 cells and treated with or without HepG2 cell culture supernatant.

Cytokines	Concentration used	Company
IL-2	50ng/ml or titration	Miltenyi Biotec
IFN-γ	50ng/ml	Invitrogen Gibco
IFN-α	2000units/ml	Miltenyi Biotec
IFN-β	50ng/ml	R&D
IL-7	50ng/ml	BioLegend Inc
IL-15	50ng/ml	Miltenyi Biotec
IL-12	50ng/ml	Miltenyi Biotec
IL-18	50ng/ml	MBL international

Table 4.2 - Cytokines and concentrations used in stimulation assays and supplement assays

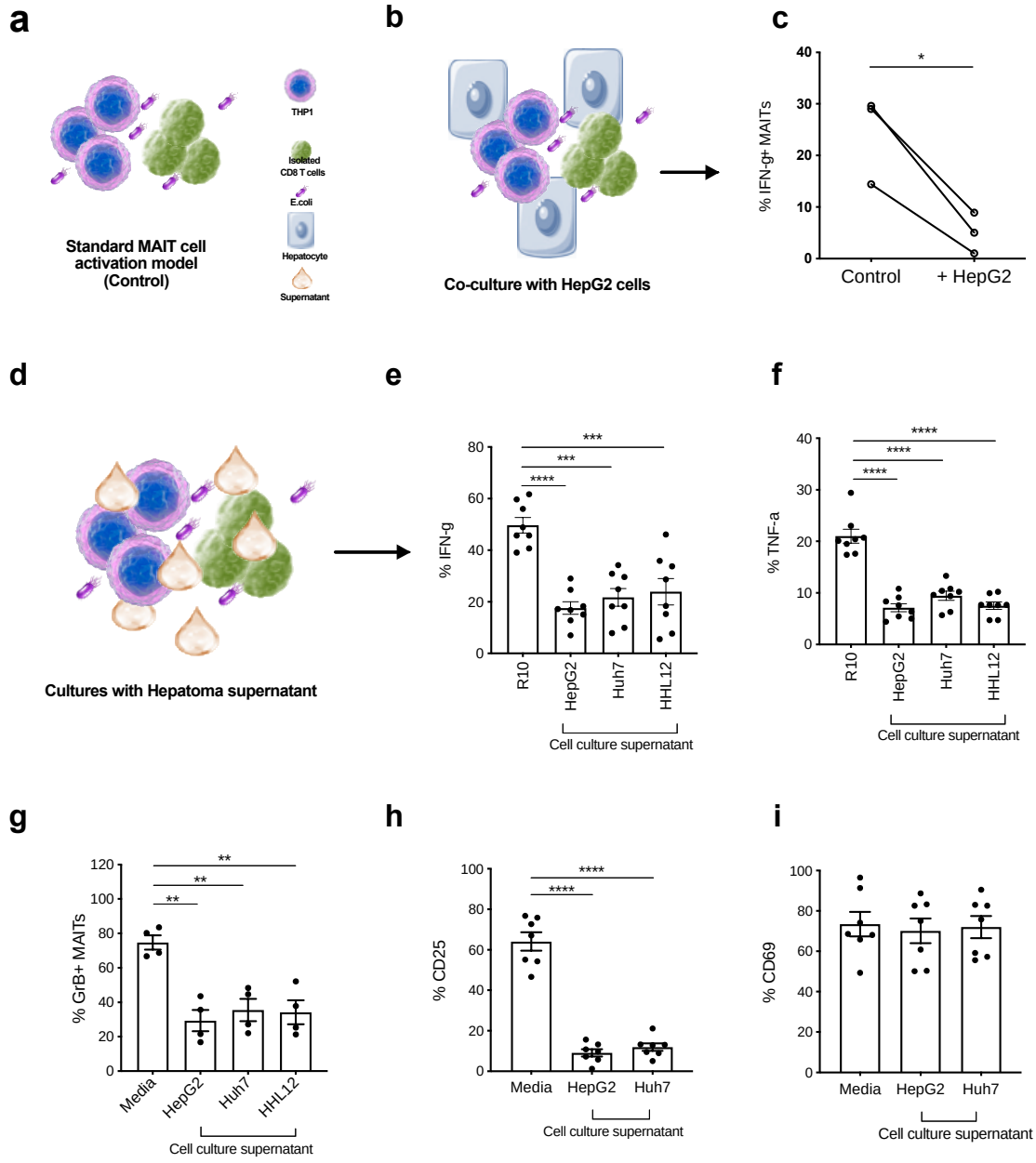


Figure 4.1 - Hepatoma cell culture medium potently suppressed MAIT cells activated by *E. coli* stimulated THP-1 cells.

All experiments were performed for 16 hours, analysed by flow cytometry and all read outs are for CD8+CD161++Vα7.2+ MAITs acquired from Healthy donor blood. a) Schematic representation of the standard MAIT cell activation assay - Isolated CD8 T cells cocultured

with THP-1+ *E*. coli. b) Schematic representation of Hepatoma cell addition to the standard MAIT cell activation assay. c) IFN-γ expression from MAIT cells activated by *E*. coli stimulated THP-1 cell in the presence of Hepatoma cells, *(n=3)*. d) Schematic representation of Hepatoma cell culture supernatant addition to the standard MAIT cell activation assay. e) IFN-γ and f) TNF-α were measured from MAIT cells activated with THP-1+ *E*. coli and treated with R10 (control) or HepG2, Huh7, or HHL12 cell culture supernatants, *(n=7)*. g) GrB, *(n=4)*, h) CD25, *(n=6)*, i) CD69, *(n=6)* expression from MAIT cells stimulated with THP-1+ *E*. coli with Hepatoma cell culture supernatant or R10 treatment. Statistical significance - Bonferroni's multiple comparisons test, ***P<0.0002, ****P<0.0001.

4.4.1 MAIT cell activation and cytotoxicity was suppressed by hepatoma cell culture derived soluble molecules in a contact independent manner

The liver is composed of many cells types that can influence each other through contact dependent or paracrine interactions; therefore, we investigated how hepatoma cells may impact MAIT cell activation. We performed multi-cell co-culture experiments where MAIT cell activation by *E*. coli stimulated THP-1 cells served as the positive control and was also selected as the standard MAIT cell activation assay to study the impact of hepatoma cells or cell culture supernatants on MAIT cell activation. The addition of HepG2 cells to the standard MAIT cell activation assay led to the strong inhibition of MAIT cell IFN-γ, suggesting a possible suppressive effect of hepatoma cells on MAIT cell activation (Fig. 4.1a-c).

Next, we examined which mechanisms were behind this suppression and whether it was contact dependent or independent. To investigate this, we extracted supernatants from hepatoma cell cultures with a confluency of 90%, filtered out any floating cells and then added them to the standard MAIT cell activation assay. Just like the HepG2 cells, supernatants acquired from hepatoma cell lines, HepG2 and Huh7 and the hepatocyte cell line, HHL12, potently suppressed MAIT cell IFN-γ (Fig. 1d/e). HepG2, Huh7 and HHL12 cell culture supernatants also suppressed MAIT cell TNF-α, CD25 and GrB expression to a similar extent. However, they did not inhibit CD69 expression on MAIT cells (Fig. 4.1f-i).

It is important to mention that we have used multiple controls such as cell free, cultured and uncultured D10 medium, PBS and R10 (stimulated (positive control)) to validate that the compound is cell specific and not a result of the different medium used to culture the hepatoma cells or the alterations to the media in culture (Supplementary Fig. 1). Moreover, hepatoma derived supernatants did not compromise the viability of MAIT cells (Supplementary Fig. 2). Thus, taken together these results strongly indicate MAIT cell suppression by hepatoma cell derived soluble compound/s.

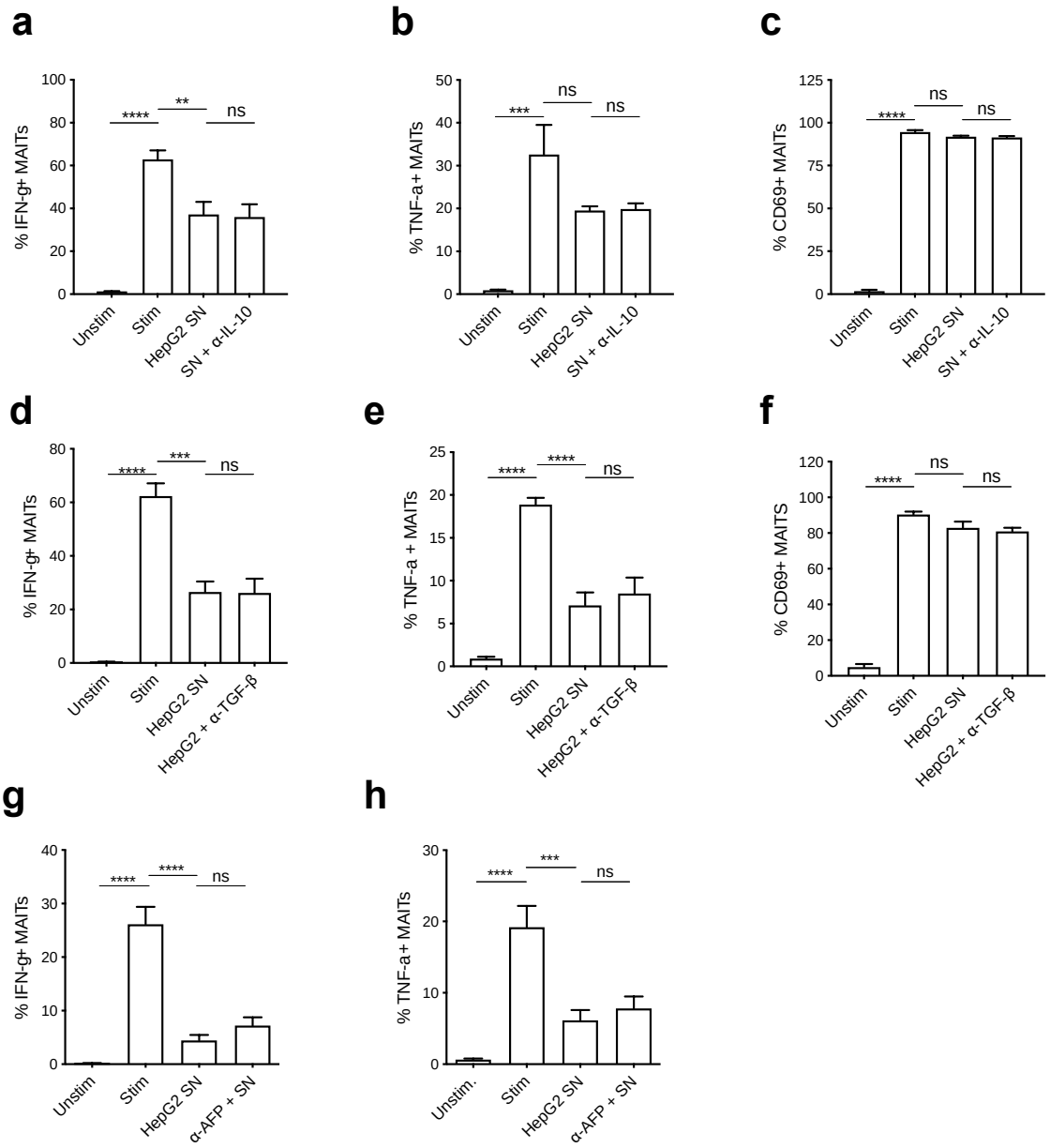


Figure 4.2 - Inhibition of tumour derived and regulatory factors in HepG2 supernatant assays. HepG2 cell culture supernatant treated THP-1+ *E*. coli activated CD8 T cells were incubated with α-IL-10, α-TGF-β and α-AFP, neutralizing antibodies and analyzed by flow cytometry. a) IFN-γ, b) TNF-α and c) CD69 expression from CD8+CD161++Vα7.2+ MAIT cell supernatant cultures treated with IL-10 neutralizing antibodies. d) IFN-γ, e) TNF-α and f) CD69 expression from CD8+CD161++Vα7.2+ MAIT cell supernatant cultures treated with TGF-β neutralizing antibodies. g) IFN-γ and h) TNF-α expression from CD8+CD161++Vα7.2+

MAIT cell supernatant cultures treated with AFP neutralizing antibodies, *(n=4)*. SN=supernatant. Statistical significance - Sidak's multiple comparisons test - **P<0.0061, ***P<0.0003, ****P<0.0001, ns=non-significant.

4.4.2 A small heat stable compound/s released specifically from hepatocyte-derived cells suppresses MAIT cell activation in a concentration dependent manner

To identify the possible suppressive molecule and mechanism of MAIT cell inhibition by hepatoma culture supernatant, we inhibited the common regulatory compounds that are produced by hepatocytes under homeostatic conditions in healthy liver (Horst et al. 2016a). We blocked IL-10 and TGF-β signalling using IL-10 and TGF-β neutralizing antibodies added to supernatant treated MAIT cell activation cultures. Neutralizing antibodies were added at the beginning just before adding the supernatants. There was no difference in HepG2 cell culture supernatant mediated suppression of MAIT cell IFN-γ or TNF-α in the presence of IL-10 and TGF-β neutralising antibodies (Fig. 4.2a-f), suggesting these cytokines were not present or that they do not contribute to MAIT cell suppression by hepatoma cell culture supernatants.

Another protein we blocked is Alpha fetoprotein (AFP). AFP is a protein which is present at high levels in fetal liver and at low levels in adult livers but is found to be increased liver tumours (Bialecki and Di Bisceglie 2005). AFP is produced by hepatoma cell lines such as HepG2 and huh7 cell lines(Kawai 2001), and has been shown to inhibit NK cell responses(Yamamoto et al. 2011), hence we speculated that hepatoma cell derived AFP may be able to suppress MAIT cell activation. To test if AFP could be the suppressive factor present in the HepG2 cell culture supernatants and if it has the potential to

suppress MAIT cells we used antibodies against AFP to block its signalling in the supernatant treated MAIT cell activation model. Similar to IL-10 and TGF-β , α-AFP did not prevent suppression by hepatoma cell culture supernatant (Fig. 4.2 g/h). We have also tried removing AFP from supernatants prior to its addition to the MAIT cell activation model; however, there was no difference in suppression in AFP+ and AFP depleted supernatants (Supplementary Fig. 3).

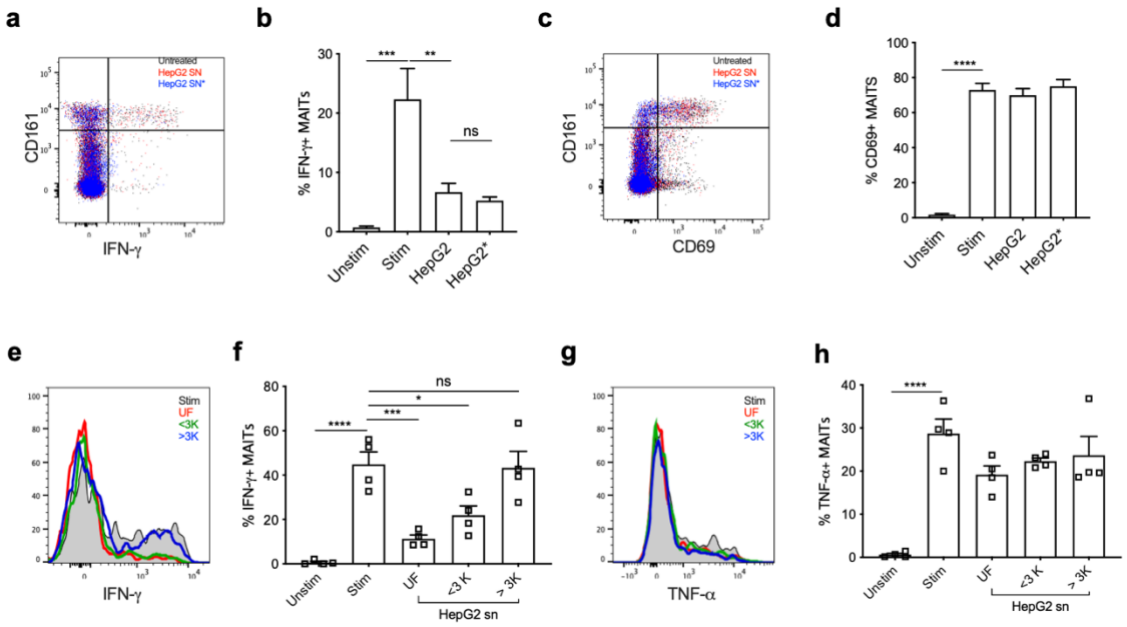


Figure 4.3 - Hepatoma culture supernatant is heat stable and the suppressive compound is smaller than 3kDa

THP-1+ *E*. coli stimulated MAIT cells were treated with HepG2 cell supernatant or HepG2*-cell culture supernatant heated at 60°C for 1 hour. a) Representative IFN-γ FACs plot b) IFN-γ quantification c) representative CD69 FACS plot and d) CD69 quantification from MAIT cells. MAIT cells activated with *E*. coli stimulated THP-1 cells were treated with unfractionated (UF) or fractionated HepG2 cell culture supernatant. e) Representative IFN-

γ histogram, f) IFN-γ quantification, g) representative TNF-α histogram and h) TNF-α quantification from MAIT cells treated with fractionated HepG2 culture supernatant, *(n=4)*. Statistical significance - Sidak's multiple comparisons test, *P<0.01, **P<0.004, ***P<0.0006, ****P<0.0001.

In addition to blocking the signalling of common regulatory cytokines and tumour specific factors, we heat-treated the supernatants to test the stability of the suppressive compounds present in HepG2 cell culture supernatants. A small volume (1ml) of HepG2 culture supernatant was heated at 60°C for an hour, cooled on ice and then added to the standard MAIT cell activation model, there was no difference in the suppression of MAIT cell activation between heat treated (HepG2*) and untreated HepG2 cell culture supernatants (Fig. 4.3a-d). We repeated the heat treatment of HepG2 cell culture supernatants and were able to show that both batches responded almost identically with no effect on the suppressive capacity of the supernatants (Supplementary Fig. 4). Hepatoma cell culture supernatants generated in large quantities were frozen at -80°C for more than six months and reused in many experiments, and similar to the heat treated supernatants, freeze thawing of the supernatants did not change their suppressive capacity. Taken together, this indicates that the suppressive compound/s generated by the HepG2 cell line are extremely stable.

To identify the size of the suppressive compound released by HepG2 cells, we fractionated the supernatants into various sizes ranging from <3kDa - 50kDa and compared the suppressive capacity of the fractionated supernatants to the

unfractionated supernatant. We found that the smaller fraction of <3kDa still suppressed MAIT cell IFN-γ, but albeit slightly less than the unfractionated supernatant (Fig. 4.3f). The larger fractions were never suppressive and the levels of MAIT cell IFN-γ for the larger fractions of HepG2 SN was similar to the positive control containing R10 (Fig. 4.3f & Supplementary Fig. 5).

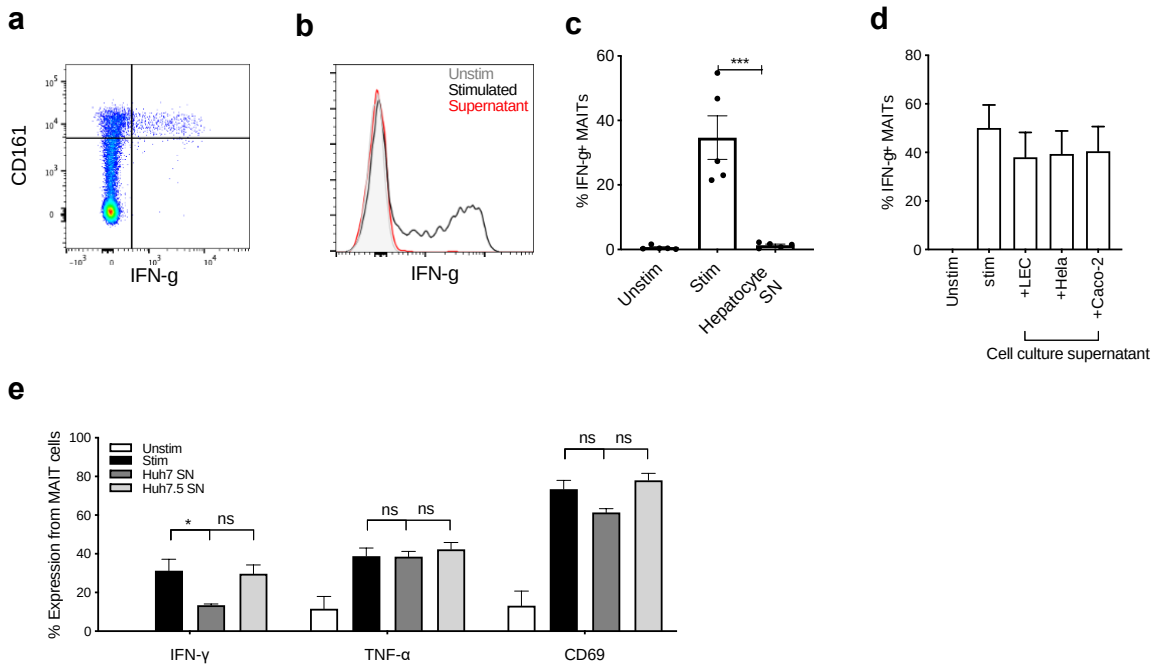


Figure 4.4 - MAIT cell suppression is both liver and hepatocyte specific

All experiments were performed for 16 hours, analysed by flow cytometry and all read outs are for CD8+CD161++Vα7.2+ MAITs acquired from Healthy donor blood. MAIT cells were activated with THP-1+ *E*. coli and treated with different cell line derived cell culture supernatant. a) Representative IFN-γ histogram and b) quantification of IFN-γ expression from activated MAIT cells treated with primary hepatocyte cell culture supernatants from C57BL6 mice, *(n=5)*. Statistical significance - Bonferroni's multiple comparisons test, ***P<0.0002. c) Representative IFN-γ FACS plot and d) IFN-γ expression from activated

MAIT cells treated with LEC, HeLa or Caco-2 cell culture supernatant, *(n=4). e)* IFN-γ, TNF-α and CD69 expression from MAIT cells treated with Huh7 and Huh7.5 cell culture supernatants *(n=4)*. Statistical significance - Dunnett's multiple comparisons test, *P<0.016. HepG2 and Huh7 cells are immortalized hepatic tumour derived cell lines, thus having significant differences to normal, healthy liver derived hepatocytes(Wilkening et al. 2003). Therefore, we compared the suppressive capacity of hepatoma cells to primary hepatocytes and then to other non-parenchymal liver resident cells (cell lines). Firstly, we isolated hepatocytes from murine liver, cultured them over four days, collected the supernatants, filtered them to eliminate cells and then added it the MAIT cell activation assays. Primary murine hepatocyte derived cell culture supernatant resulted in inhibition of MAIT cells which was even more potent than that of hepatoma cell line culture supernatants with 100% suppression of MAIT cell IFN-γ (Fig. 4.4a-c). In contrast to the HepG2 and Huh7 cell line supernatants, supernatants from a modified version of the Huh7 cell line, Huh7.5, did not suppress MAIT cell activation and there was no difference in the levels of IFN-γ, TNF-α and CD69 from MAIT cells treated with Huh7.5 cell culture supernatant (Fig. 4.4e). The difference in the supernatants of Huh7 and Huh7.5 cells requires further investigation, however for the purpose of this thesis we continued to define how unmodified hepatoma cell line (mainly HepG2 cells) derived supernatants impact MAIT cell activation.

To determine if the suppression was liver specific we tested cell culture supernatants from liver derived, LEC line and non-liver derived, HeLa and Caco-2 cell lines. None of these cell line supernatants suppressed MAIT cell IFN-γ to the same extent as the liver hepatocyte cell lines did. Although there is a small, non-significant reduction in MAIT cell

IFN-γ expression (Fig. 4.4d), this may be attributed to the lack of nutrients in the supernatant assays.

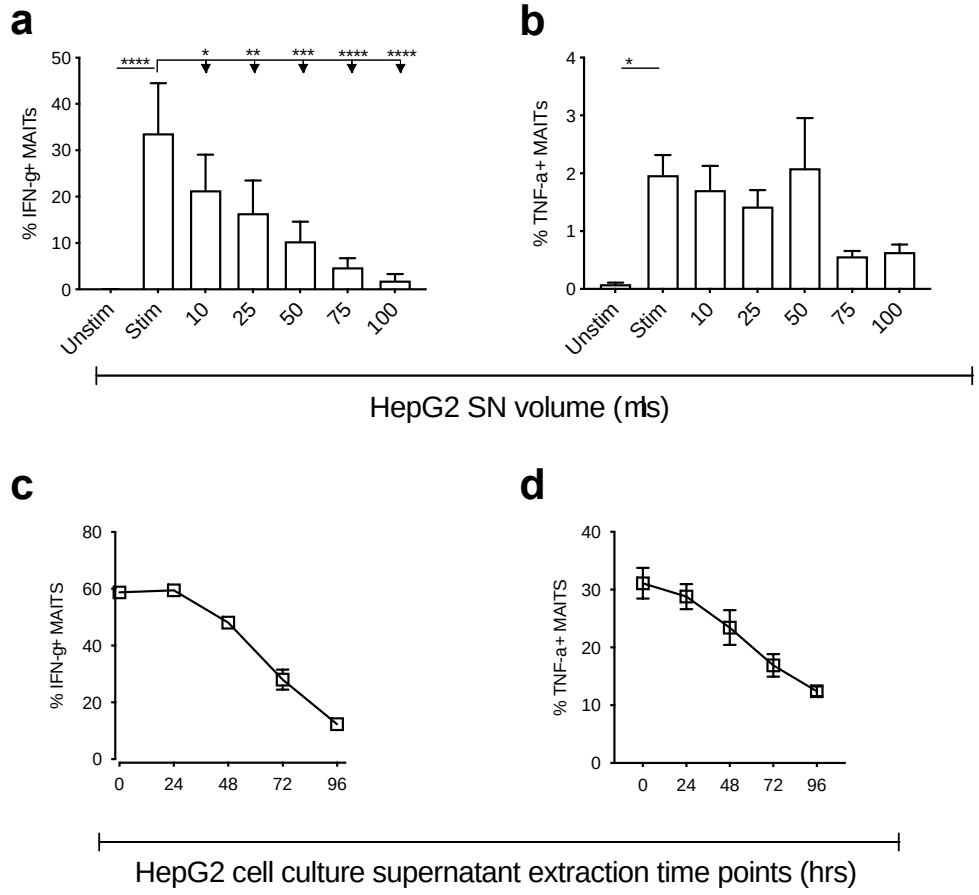


Figure 4.5 - Concentration dependent suppression of MAIT cell activation by hepatoma HepG2 cell culture supernatant.

a) IFN-γ, b) TNF-α and c) CD69 expression measured from CD8 MAIT cells activated by *E*. coli stimulated THP-1 treated with different volumes of HepG2 supernatant (*n=4*). d) IFN-γ, e) TNF-α and f) CD69 expression measured from CD8 MAIT cells activated by *E*. coli stimulated THP-1 treated with HepG2 supernatant collected at 24 hour intervals from a four day HepG2 cell culture (*n=4*).

Despite not being able to identify the suppressive compound, we were able to show that HepG2 cell culture supernatants suppressed MAIT cell IFN-γ and TNF-α in a dose dependent manner. We were able to confirm the dose dependent suppression of MAIT cell activation by two methods, one where we used different volumes of HepG2 cell culture supernatant and second by collecting supernatants at different time points from HepG2 cell cultures. In most supernatant assays we used 100μls of HepG2 culture supernatant as a standard (1:1 ratio of culture supernatant to R10 media), but even then suppression could be demonstrated with the use of smaller volumes as seen by the supernatant titration assay (Fig. 4.5a/b).

Supernatants acquired from 96-hour cultures were most potent at suppressing MAIT cell IFN-γ and TNF-α, with IFN-γ being suppressed by more than 90% compared to supernatants acquired from 24-hour cultures, which did not suppress IFN-γ (Fig. 4.5c/d). Furthermore, by testing supernatants collected successively from parallel HepG2 cultures and the increasing suppressive potency from lengthier cultures, we can conclude that HepG2 cells continuously produced the suppressive compound/s which could be proportionate to the cell numbers or accumulates with time.

4.4.3 Hepatoma cell culture supernatants influence MAIT cell activation by altering the phenotype of antigen presenting cells

As described above, to test the impact of hepatoma derived supernatants we used a standard MAIT cell activation model consisting of *E*. coli stimulated THP-1 cells and purified CD8 T cells; therefore our assay contains two cell types that could be influenced directly, indirectly or sequentially. First, we tested whether HepG2 derived supernatants specifically suppressed monocytes or whether other professional and non-professional antigen presenting cells could also be affected. We set up assays exactly like the standard MAIT cell activation assay and replaced the THP-1 cells for healthy blood derived CD14+ cells, B cells (*cell line*) or LECs. We found that all cell types could induce MAIT cell activation as measured by the increased expression of IFN-γ and TNF-α from CD8+CD161++Vα7.2+ cells (Fig. 4.6a-c). LECs induced more IFN-γ than primary CD14+ cells and the B cells whereas B cells induced maximum TNF-α and both LECs and primary CD14+ cells produced similar amounts of TNF-α but to a lesser extent to B cells (Fig. 4.6a-c). HepG2, Huh7 and HHL12 derived supernatants were able to suppress both IFN-γ and TNF-α from MAIT cells activated using either primary cells or the LEC and B cell line, thus indicating the action of the supernatants was not specific to monocytes but had an impact on a range of antigen presenting cell types. However, an effect of the supernatant directly on MAIT cells could not be ruled out (Fig. 4.6a-c).

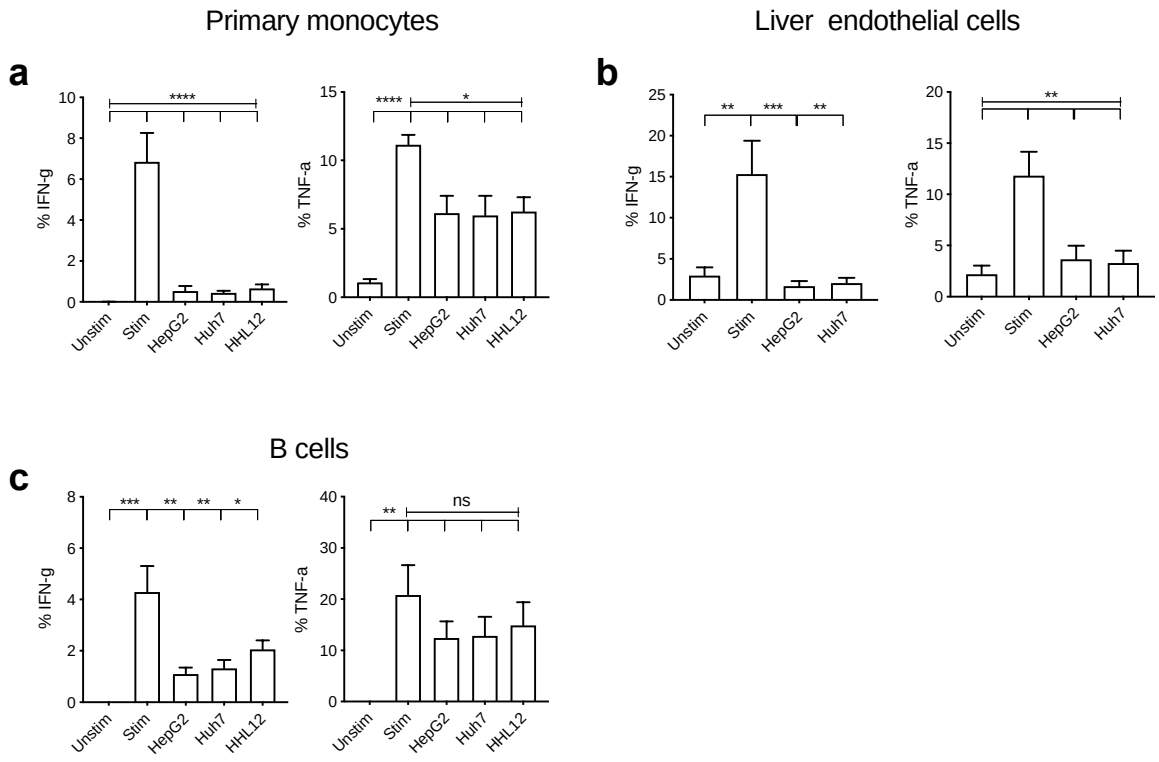


Figure 4.6 - Effect of hepatoma cell culture supernatant on MAIT cells activated with *E*. coli **stimulated primary monocytes, liver endothelial and B cells.**

E. coli stimulated a) Primary isolated CD14+ monocytes, b) LECs or c) B cells were used to activate MAIT cells and treated with HepG2, Huh7 and HHL12 cell line culture supernatants. IFN-γ and TNF-α expression from CD8+CD161+Vα7.2+ MAIT cells was measured by flow cytometry (*n=4*). Statistical significance - Dunnett's multiple comparisons test, all groups compared to the stimulated group, *P<0.02. **P<0.007, ***P<0.0009, ****P<0.0001.

In support of this finding, the evaluation of supernatant treated and untreated *E*. coli stimulated THP-1 cells by RNA-sequencing, revealed significant differences (Fig. 4.7b) in THP-1 cells associated with loss of cytokine signalling and increased cyclase activity. The data show a significant downregulation of interferon related genes such as interferon alpha inducible (IFI) protein 27 (IFI27), interferon-induced protein with tetratricopeptide 1 (IFIT1), insulin like growth factor 1 (IGF1) and the chemokines, C-X-C motif chemokine (CXCL)11 (CXCL11), CXCL14 and CXCL10 (Fig.4.7a). Lysosome associated membrane protein 3 (LAMP3) is a marker present in maturing dendritic cells (De Saint-Vis et al. 1998), the downregulation of LAMP3 in THP-1 cells suggests the supernatants prevent the maturation of monocytes; thus, limiting T cell activation, a similar effect is seen for MAIT cells in our model. Gene set enrichment analyses of downregulated genes showed that cytokine mediated signalling, defence responses to viruses and external stimuli, interferon-gamma-mediated signalling were impacted along with type 1 interferon signalling (Fig. 4.7c). On the other hand, the significant upregulation of genes like gastric polypeptide inhibitory receptor (GIPR), nitric oxide synthase 3 (NOS3) and paralemmin (PALM) associate with an increase in cyclase activity, regulation of lyases and nucleotide metabolic pathways with significant changes to the membrane potential as a result of changes in calcium signalling, (Fig. 4.7).

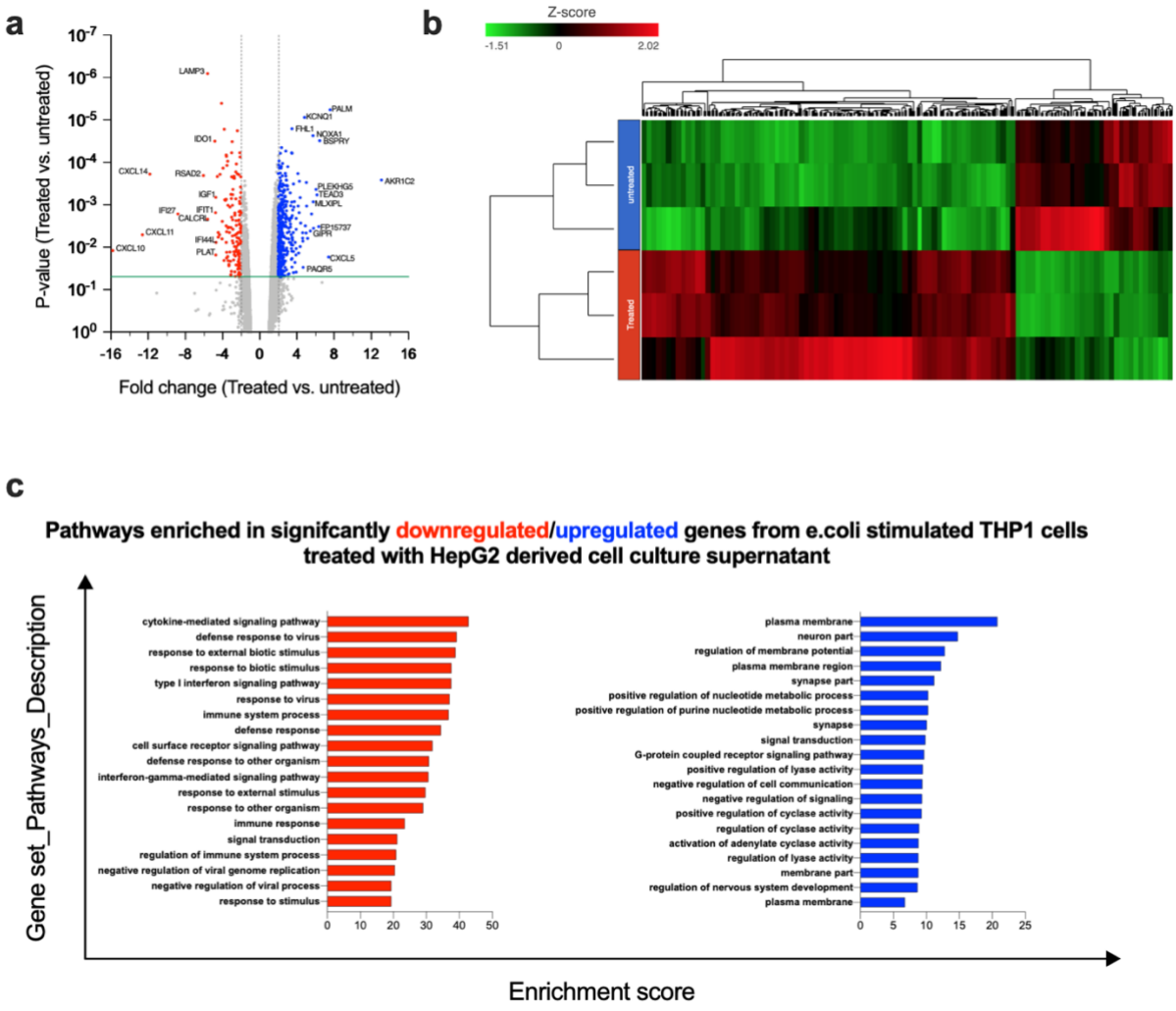
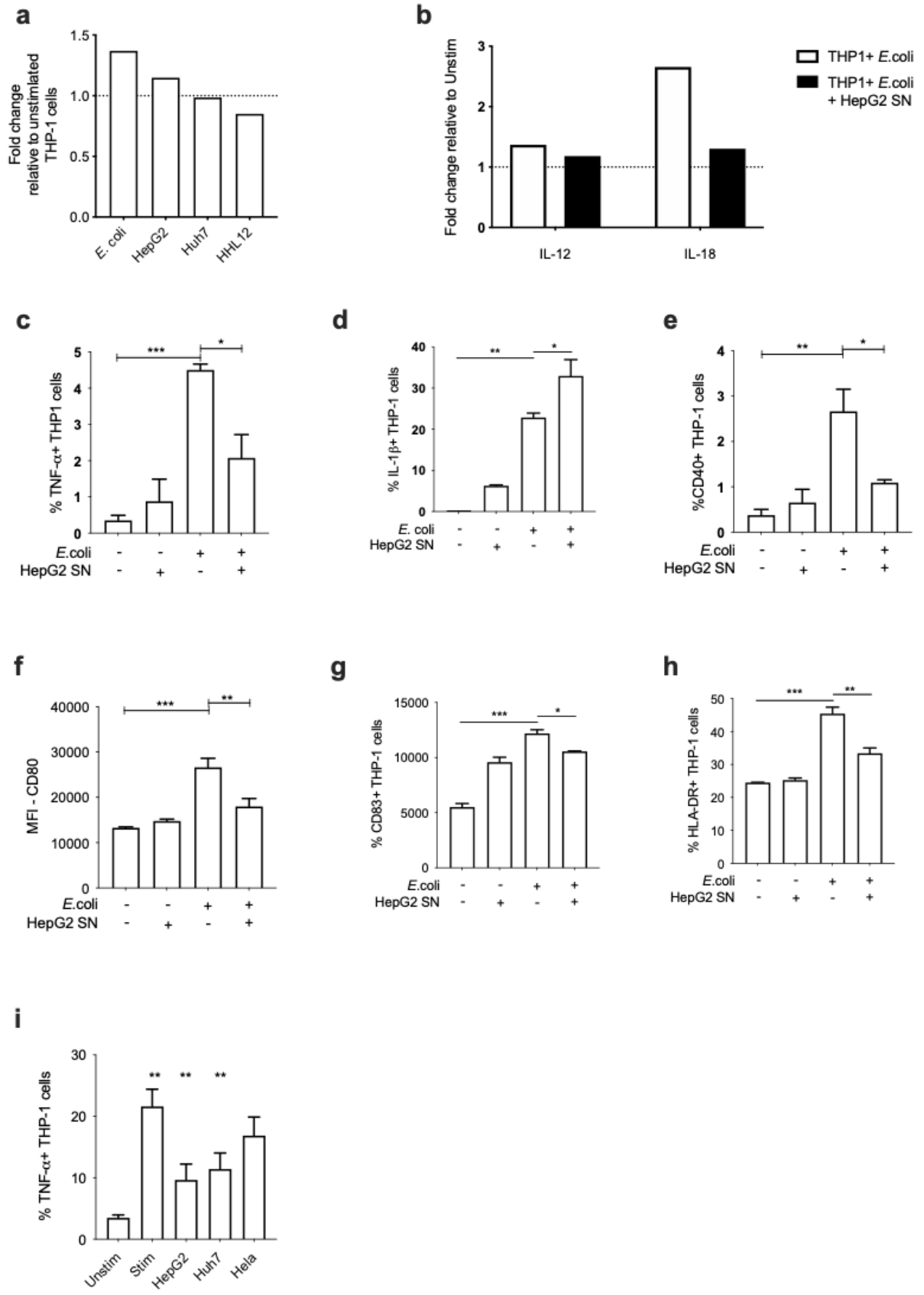


Figure 4.7 - Gene expression analysis of *E*. coli stimulated THP-1 cells treated with HepG2 cell culture supernatant.

RNA sequencing was used to analyse gene expression from THP-1 cell lines stimulated with *E*. coli, and treated with or without HepG2 cell culture supernatant. a) Volcano plot showing up regulated (blue) or down regulated (red) genes expressed from *E*. coli stimulated THP-1 cell treated with HepG2 supernatant relative to *E*. coli stimulated, supernatant untreated THP-1 cells. b) Heat map of differentially expressed (DE) genes from THP-1 cell stimulated with *E*. coli (blue row) or *E*. coli stimulated THP-1 cells treated with HepG2 supernatants (red row). Red indicates high expression, while blue denotes low expression, c) pathways enriched in significantly down regulated and up regulated genes from *E*. coli stimulated THP-

1 cells treated with HepG2 cell culture supernatant. Data in a and b is generated from the 443 significantly and DE genes based on counts <10, P value of 0.05 and fold change of < -2 or > 2. Gene set pathways are determined from significantly DE, 20 most upregulated and 20 most downregulated genes. For this specific dataset we did not filter by FDR to prevent of signal obliteration, nonetheless the genes were studied to determine biological significance prior to further analysis.

To check the impact of HepG2 cell culture supernatants on the proinflammatory capacity of supernatant treated, *E*. coli stimulated and unstimulated THP-1 cells, we investigated changes to the expression of TNF-α, IL-1β, MHC-II, CD40, CD80, CD83 by flow cytometry and MR1, IL-12 and IL-18 gene expression by RT-PCR. MR1 expression was lower in *E*. coli stimulated THP-1 cells treated with cell culture supernatants (Fig. 4.8a). Similar to MR1 we saw a decrease in IL-18 expression from *E*. coli stimulated THP-1 cells treated with HepG2 cell culture supernatant, the same result was observed by IL-18 ELISA. IL-12 was not detectable by ELISA and by RT-PCR, we detected a minimal increase in IL-12 gene expression in THP-1 cells stimulated by *E*. coli, which remained unchanged with HepG2 cell culture supernatant treatment (Fig. 4.8b). Expression of TNF-α, CD40, CD80, CD83 and MHC-II was reduced from the surface of *E*. coli stimulated THP-1 cells treated with HepG2 cell culture supernatant (Fig.4.8c-h). In contrast IL-1β expression from *E*. coli stimulated THP-1 cells was enhanced by HepG2 cell culture supernatant (Fig. 4.8d). Of note, though non-significant there is a small but visible increase in TNF-α, CD40, CD83 expression in HepG2 cell culture supernatant treated, unstimulated THP-1 cells compared to unstimulated, untreated THP-1 cells, the opposite effect is seen with HepG2 supernatant treatment of *E*. coli stimulated THP-1 cells.


a
Fold change relative to unstimlated THP-1 cells
1.5
1.0
0.5
0.0
E. coli
HepG2
Huh7
HHL12
b
Fold change relative to Unstim
3
2
1
0
IL-12
IL-18
THP1+ E.coli
THP1+ E.coli + HepG2 SN
c
% TNF-α+ THP1 cells
***
*
E.coli
HepG2 SN
d
% IL-1β+ THP-1 cells
**
*
E. coli
HepG2 SN
e
%CD40+ THP-1 cells
**
*
E.coli
HepG2 SN
f
MFI - CD80
***
**
E.coli
HepG2 SN
g
% CD83+ THP-1 cells
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E.coli
HepG2 SN
h
% HLA-DR+ THP-1 cells
***
**
E.coli
HepG2 SN
i
% TNF-α+ THP-1 cells
**
**
**
Unstim
Stim
HepG2
Huh7
Hela

Figure 4.8 - Hepatoma supernatants suppresses pro-inflammatory cytokine gene expression from *E*. coli stimulated THP-1 cells

Unstimulated and *E*. coli stimulated THP-1 cells were incubated with and without Hepatoma cell culture supernatant for 16 hours and analysed by RT-PCR and flow cytometry. a) MR1 expression from hepatoma supernatant treated THP-1 cells relative to untreated THP-1 cells. b) IL-12 and IL-18 gene expression from HepG2 treated *E*. coli stimulated THP-1 cells relative to unstimulated THP-1 cells. c) TNF-α, d) IL-1β, e) CD40, f) CD80, g) CD83 and h) MHC-II expression from THP-1 cells measured by flow cytometry *(n=4)*. Statistical significance – Sidak's multiple comparisons test, *P<0.05, **P<0.003, ***P<0.0007. i) TNF-α expression from *E*. coli stimulated THP-1 cells treated with hepatoma cell culture supernatant from the standard MAIT cell activation assay, *(n=4)*. Statistical significance – Dunnett's multiple comparisons test *P<0.02, **P<0.006.

A significant reduction in TNF-α expression from *E*. coli stimulated THP-1 cells was also replicated in the complete model consisting of CD8 T cells in addition to THP-1 cells, *E*. coli and HepG2 cell culture supernatant (Fig. 4.8i). The TNF-α production by THP-1 cells in the complete model was higher than from THP-1 cells cultured in the absence of CD8 T cells signifying stronger paracrine induction of TNF-α (Supplementary Fig. 6) which was significantly suppressed by HepG2 and Huh7 cell culture supernatants but not by HeLa cell culture supernatants, this finding correlated well with the suppressive and non-suppressive supernatant impact on MAIT cell activation (Fig. 4.8i). The latter highlighting a mechanism for TNF-α induction by MAIT cells and the subsequent importance of TNF-α signalling to MAIT cells necessary for efficient/enhanced activation.

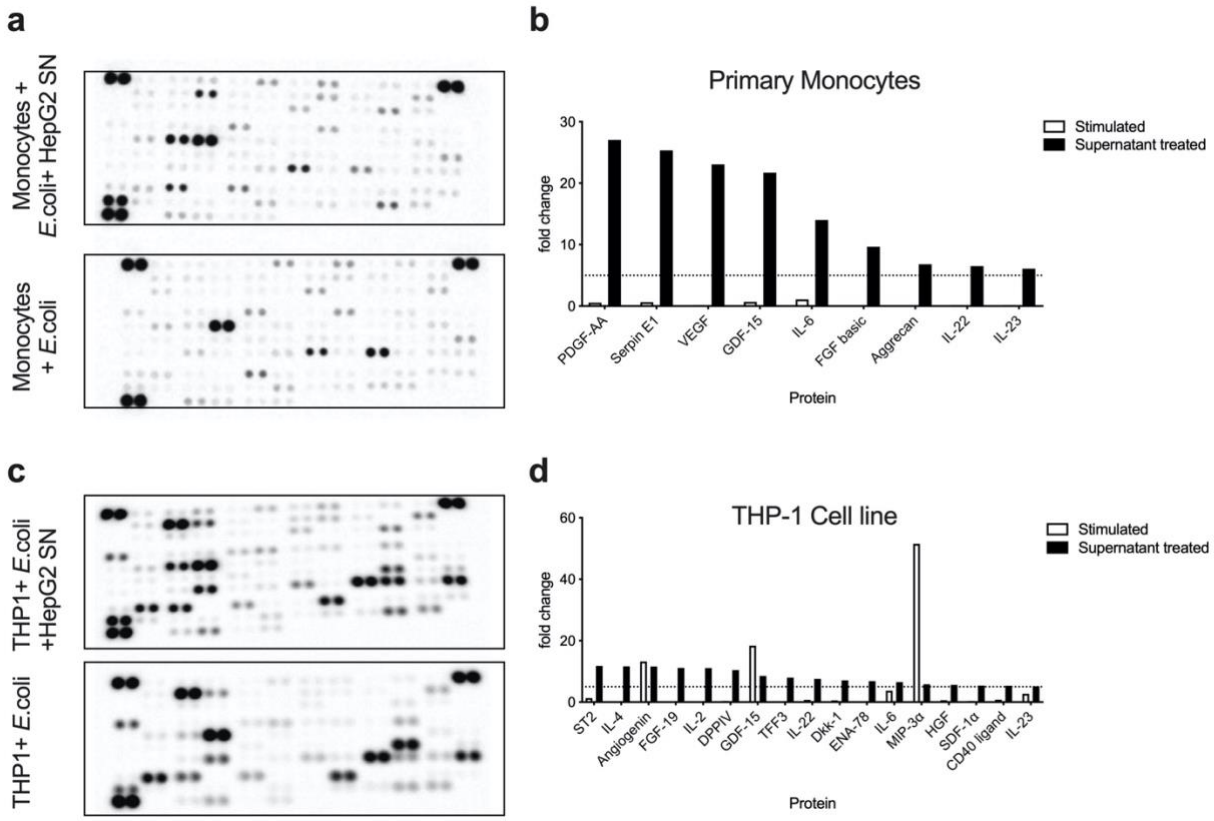


Figure 4.9 - Protein analysis of *E*. coli stimulated antigen presenting cells treated with HepG2 cell culture supernatant.

Protein profiles of Primary monocytes and THP-1 cells stimulated with *E*. coli and treated with HepG2 cell culture were analysed by cytokine blot assays. Cytokine blots of a) Primary monocyte, and c) THP-1 cell. Quantification of fold change differences in protein expression between HepG2 cell culture supernatant treated and untreated *E*. coli stimulated b) Primary monocytes and d) THP-1 cells. The spot density was quantified using the image quant software, followed by background removal in excel and the raw values were then used to determine differential expression of proteins by comparison of fold change (>5) between supernatant treated and untreated *E.* coli stimulated THP-1 cells.

To further address the impact of hepatoma cell culture supernatant on APC phenotype we used cytokine blot assays to detect changes at the protein level, the cytokine blot assays allowed us to measure 105 proteins from *E*. coli stimulated THP-1 and primary monocyte cells, and from HepG2 cell culture supernatant treated, *E*. coli stimulated THP-1 or primary monocytes (CD14+ cells). A fold change of greater than or equal to 5, from supernatant treated groups for both cells types, was used to determine the total number of proteins significantly increased or decreased, compared to stimulated but supernatant untreated cells. A greater number of proteins showed differences in expression after HepG2 supernatant treatment of *E*. coli stimulated THP-1 cells than for the primary monocytes (Fig. 4.9), 17 and 9 proteins, respectively. HepG2 supernatant generally increased expression of T cell inhibitory proteins like platelet derived growth factor-AA (PDGF-aa), growth differentiation factor-15 (GDF-15), fibroblast growth factor -19 (FGF-19) and IL-6. Likewise, type 2 and type 17 polarizing cytokines and protein expression was higher in supernatant treated cells. Of the changes, we saw increased expression of IL-22, IL-23 and IL-6 cytokines for both cells types treated with HepG2 supernatant. GDF-15 expression increased by manifold with HepG2 supernatant treatment of primary monocytes (Fig. 4.9a) but was reduced in *E*. coli stimulated THP-1 cells treated with supernatant (Fig. 4.9b). Type 2 polarizing proteins, interleukin 1 receptor like-1 (IL1R1/ST2) and IL-4 were higher in supernatant treated THP-1 cells compared to supernatant untreated *E.* coli stimulated THP-1 cells (Fig. 4.9b). HepG2 supernatant also increased IL-2 expression by 11-fold from THP-1 cells and caused a 45-fold decrease in expression (Fig. 4.9b). Overall, these results demonstrate that hepatoma cell culture supernatant inhibits the pro-inflammatory phenotype of *E*. coli stimulated antigen presenting cells.

4.4.4 Hepatoma cell culture supernatants had no effect on TCR signalling but delayed cytokine signalling required for MAIT cell activation

Firstly, to determine if hepatoma culture supernatant inhibited antigen presentation in our standard MAIT cell activation model we treated unstimulated THP-1 cells and *E*. coli stimulated THP-1 cells with supernatants for 24 hours, or stimulated THP-1 cells with *E*. coli for 24 hours first and then treated these cells with supernatant prior to culturing them with purified CD8 T cells for a further 5 or 16 hours (Fig. 4.10a). In comparison to cultures where supernatant was present from the beginning and not removed, there was potent suppression of MAIT IFN-γ and TNF-α for 5 hour and 16 hour stimulated MAIT cells (Fig. 4.10b/c). On the other hand, HepG2 supernatant pre-treatment of unstimulated and *E*. coli stimulated THP-1 cells, followed by the removal of the supernatant prior to co-culture with CD8 MAIT cells had no impact on MAIT cell activation; indicating that the supernatant needed to be present to mediate suppression of MAIT cell activation. Where supernatant was replaced for fresh HepG2 supernatant, MAIT cell activation was still suppressed compared to MAIT cells activated by *E*. coli stimulated THP-1 cell for which R10 was replaced for fresh R10 (Fig. 4.10).

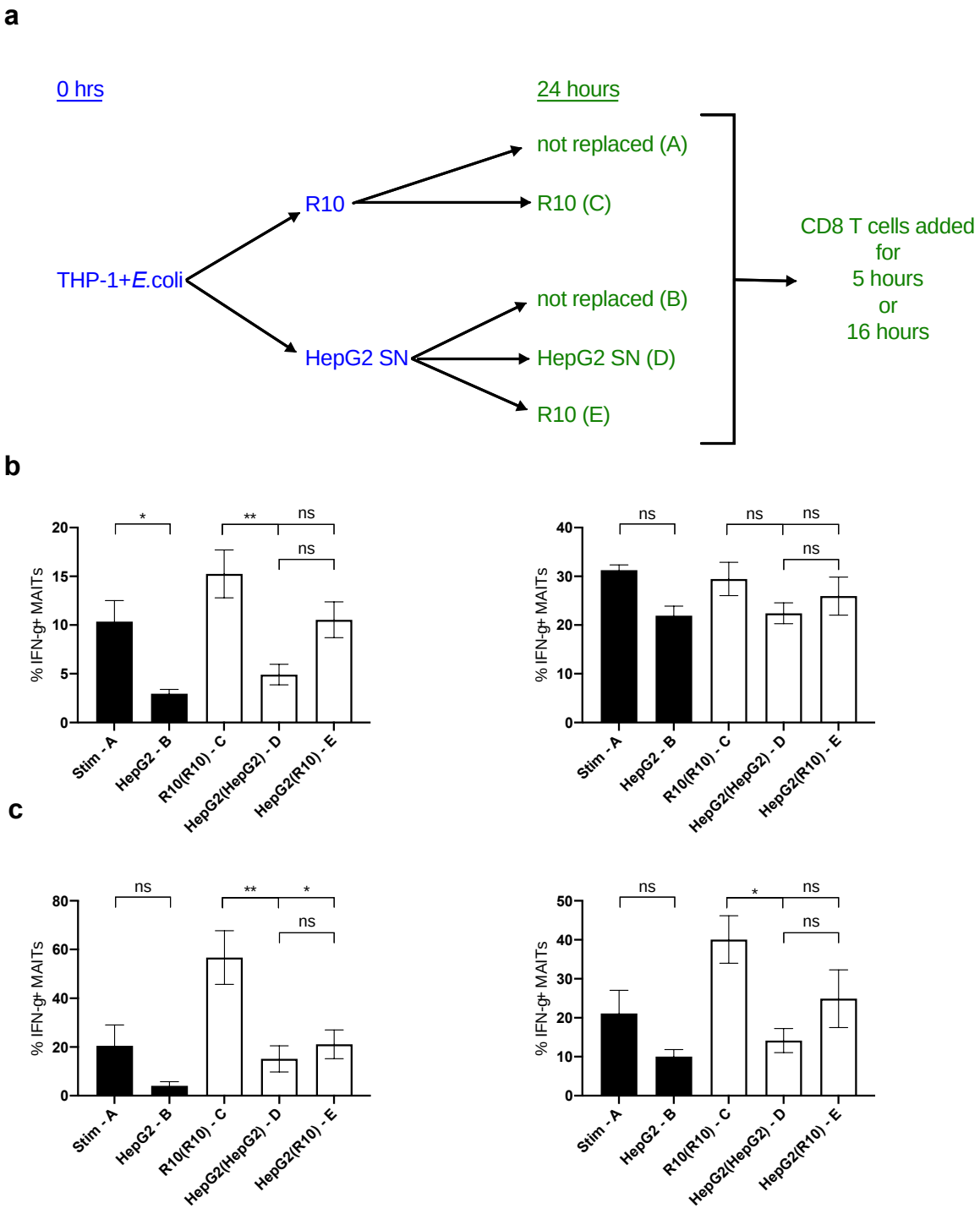


Figure 4.10 – MAIT cell activation is suppressed only in the presence of Hepatoma supernatant

THP-1 cells were stimulated with *E.* coli in the presence of R10 (control media) or HepG2 SN for 24 hours before replacing culture supernatants with R10, or fresh HepG2 supernatant or were left unchanged. Purified CD8 T cells were added to the cultures and incubated for 5

hours or 16 hours and analysed by flow cytometry. a) Schematic of the supernatant replacement assay. b) IFN-γ and TNF-α expression from MAIT cells stimulated for 5 hours. c) IFN-γ and TNF-α expression from MAIT cells stimulated for 16 hours, (*n=4*). Statistical significance – Sidak's multiple comparisons test, *P<0.0370, **P<0.0041, ns=non-significant.

The early activation of MAIT cells is predominately TCR mediated but the later phase is cytokine dominated. MAIT cells can be activated by cytokines alone or through the TCR. Therefore, we analysed the effects of hepatoma supernatants on TCR and cytokine dependent MAIT cell activation. We examined the ability of HepG2 culture supernatant to suppress cytokine activated MAIT cells. CD8 T cells were incubated with IL-12 + IL-18 with or without hepatoma supernatant, and IFN-γ expression was measured from CD8+ MAIT cells. HepG2 cell culture supernatant did not suppress IFN-γ expression from cytokine activated MAIT cells (Fig. 4.11).

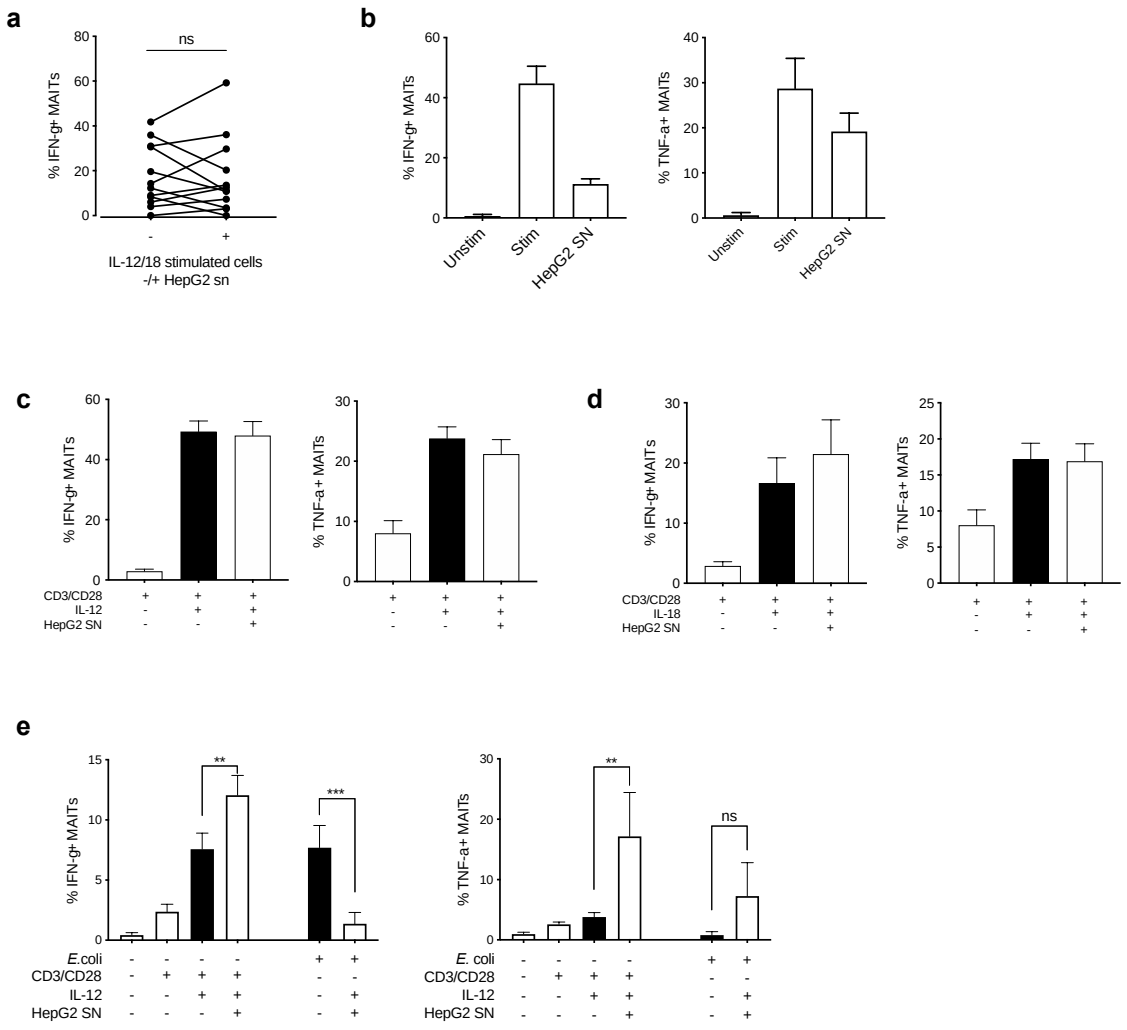


Figure 4.11 - Effect of HepG2 cell culture supernatant on cytokine, antigen, and TCR stimulated MAIT cells a) IFN-γ expression from cytokine (IL-12+IL-18) stimulated MAIT cells, *(n=12)*. Statistical significance – Paired T test, ns = P>0.05. b) IFN-γ and c) TNF-α expression from antigen stimulated, hepatoma culture supernatant treated MAIT cells. d) IFN-γ and TNF-α expression from TCR+IL-12 activated MAIT cells +/- HepG2 supernatant. e) IFN-γ and TNF-α expression from TCR+IL-18 stimulated activated MAIT cells +/- HepG2 supernatant, *(n=4)*. f) IFN-γ and g) TNF-α expression in CD8+ MAITs from TCR+ IL-12 stimulated PBMCs +/- HepG2 culture supernatant, (*n=5)*. Statistical significance – Sidak's multiple comparisons test, **P<0.008, ***P<0.0002.

Next, we examined the suppressive effect of hepatoma supernatants on the TCR pathway. For this we stimulated MAIT cells in a TCR dependent manner using α-CD3/CD28 antibodies with cytokines IL-12 or IL-18. It is important to note that MAIT cells cannot respond to TCR signalling in the absence of additional cytokine co-stimulation, the most potent being IL-12. In contrast to the suppressive effect of HepG2 supernatant on antigen stimulated MAIT cells (Fig. 4.11b), hepatoma supernatants did not suppress IFN-γ or TNF-α from TCR (CD3+CD28) + cytokine (IL-12 or IL-18) stimulated MAIT cells (Fig. 4.11c/d). We tested the effect of hepatoma supernatant on TCR+cytokine stimulation of MAIT cells within PBMCs (Fig. 4.11e). Interestingly, the suppressive impact of hepatoma culture supernatant differed for TCR+cytokine stimulated MAIT cells from both isolated CD8 T cell and within PBMCs. For TCR+cytokine stimulated MAIT cells within PBMCs, HepG2 culture supernatant treatment resulted in even higher levels of IFN-γ and TNF-α expression from MAIT cells in comparison to the supernatant untreated TCR+IL-12 stimulated MAIT cell population present in purified CD8 T cells (Fig. 4.11e). This suggests that other cells other than antigen presenting cells, e.g. Helper T cells augment MAIT cell activation.

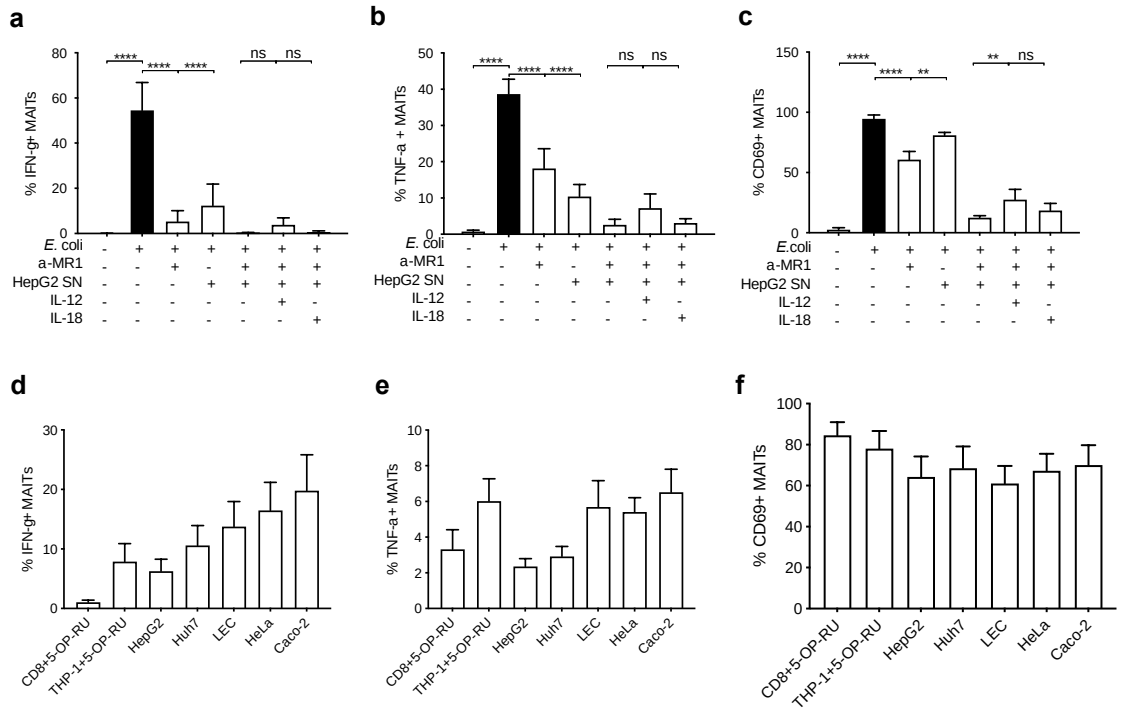


Figure 4.12 - **Cytokines cannot rescue hepatoma mediated MAIT cell suppression in the absence of MR1 signaling**

Liver and non-liver resident cell derived culture supernatant treatment of MAIT cells activated with 5-OP-RU stimulated THP-1 cells. a) IFN-γ, b) TNF-α and c) CD69 expression from CD8+CD161++Vα7.2+ MAIT cells, *(n=4)*. THP-1+ *E.* coli stimulated MAIT cells were incubated with HepG2 supernatant with a-MR1 blocking antibodies and supplemented with exogenous IL-12 or IL-18 for 16 hours before staining and analyzing by flow cytometry. c) IFN-γ, d) TNF-α and e) CD69 expression from CD8+CD161++Vα7.2+ MAIT cells, *(n=4)*. Statistical significance – Sidak's multiple comparisons test, **P<0.004, ****P<0.0001.

As 5-OP-RU is a MR1 ligand and potent activator of MAIT cells, we assessed the effect of hepatoma culture supernatants on 5-OP-RU stimulated MAIT cells within PBMCs hepatoma culture supernatant suppressed TNF-α, but did not suppress IFN-γ from 5-OP-RU stimulated MAIT cells. In contrast to hepatoma culture supernatant (HepG2 and Huh7) (Fig. 4.12a-c), supernatants from LEC, HeLa and Caco-2 cell line cultures enhanced IFN-γ (Fig. 4.12a) and though they did not enhance TNF-α, they did not suppress it either (Fig. 4.12b). In relation to the findings discussed above it is likely that non-suppressive supernatants contain cytokines that boost TCR-dependent stimulation. Additionally, we show that TNF-α like other cytokines also enhances MAIT cell activation through a feedback loop involving activated MAIT cells (Diagram 4.1). Activated MAIT cells signal to THP-1 cells, subsequently inducing TNF-α production by the APC thereby boosting MAIT cell stimulation, as shown in the diagram below and supplementary figure 6. The inhibition of TNF-α by hepatoma culture supernatants in this model (Fig. 4.12a/c) shows inhibition of activated MAIT cell dependent, paracrine TNF-α signalling to MAIT cells.

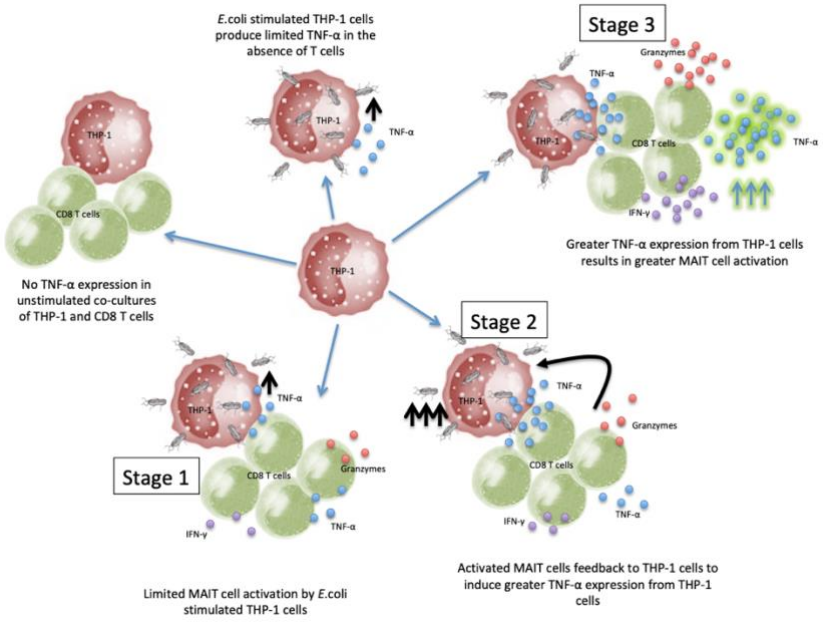


Diagram 4.1 – Illustration of TNF-α induction in THP-1 cells by activated MAIT cells resulting in augmented MAIT cell stimulation. *E.* coli stimulated THP-1 cells produce little to no TNF-α, likewise, THP-1 cells co-cultured with CD8 T cells without antigen results in no TNF-α induction from THP-1 cells. In the initial stage E.coli stimulated THP-1 cells weakly activate MAIT cells. In stage 2, activated MAIT cells feedback to THP-1 cells, this increases TNF-α production by THP-1 cells which then results in enhanced MAIT cell activation as shown in stage 3.

Antigen presentation is dependent on the APC processing and presenting antigen to a T cell. As hepatoma supernatants has no effect directly on MAIT cells, but did in the context of an APC it can be assumed that APC mediated stimulation is inhibited by the supernatants. This is further supported by data in figure 4.9 showing the suppression of pro-inflammatory cytokine expression in *E.* coli stimulated monocytes by HepG2 supernatant. Firstly, we tested the hypothesis that the monocyte antigen presentation pathway was not targeted, but the ability of the monocytes to express cytokines was. Therefore we tried to rescue MAIT cell activation by supplementing supernatant treated cells with cytokines. The addition of IL-12 was able to restore activation of MAIT cells (Fig. 4.13a-d). However, it remained unclear if MR1 signalling was active or whether IL-12, jointly with other cytokines in the supernatant, activated MAIT cells in a TCR independent manner. Therefore, we repeated the previous assay with the addition of a blocking MR1 antibody, here IL-12 was not able to rescue MAIT cell activation in the absence of MR1 signalling, making MR1 indispensable and also proving that hepatoma supernatant does have an effect on TCR signalling (Fig. 4.12d-f). We expanded this to show that it was not just IL-12 and IL-18 that could restore MAIT cell activation, but also other cytokines. Addition of various concentrations of IL-2 could also rescue MAIT cells in a dose dependent manner (Fig. 4.13e/f). 50ng/ml of IL-2 restored MAIT cell IFN-γ completely and the lower concentrations of IL-2, as low 0.5ng/ml could restore some of the IFN-γ suppressed by the HepG2 culture supernatant (Fig. 4.13e/f).

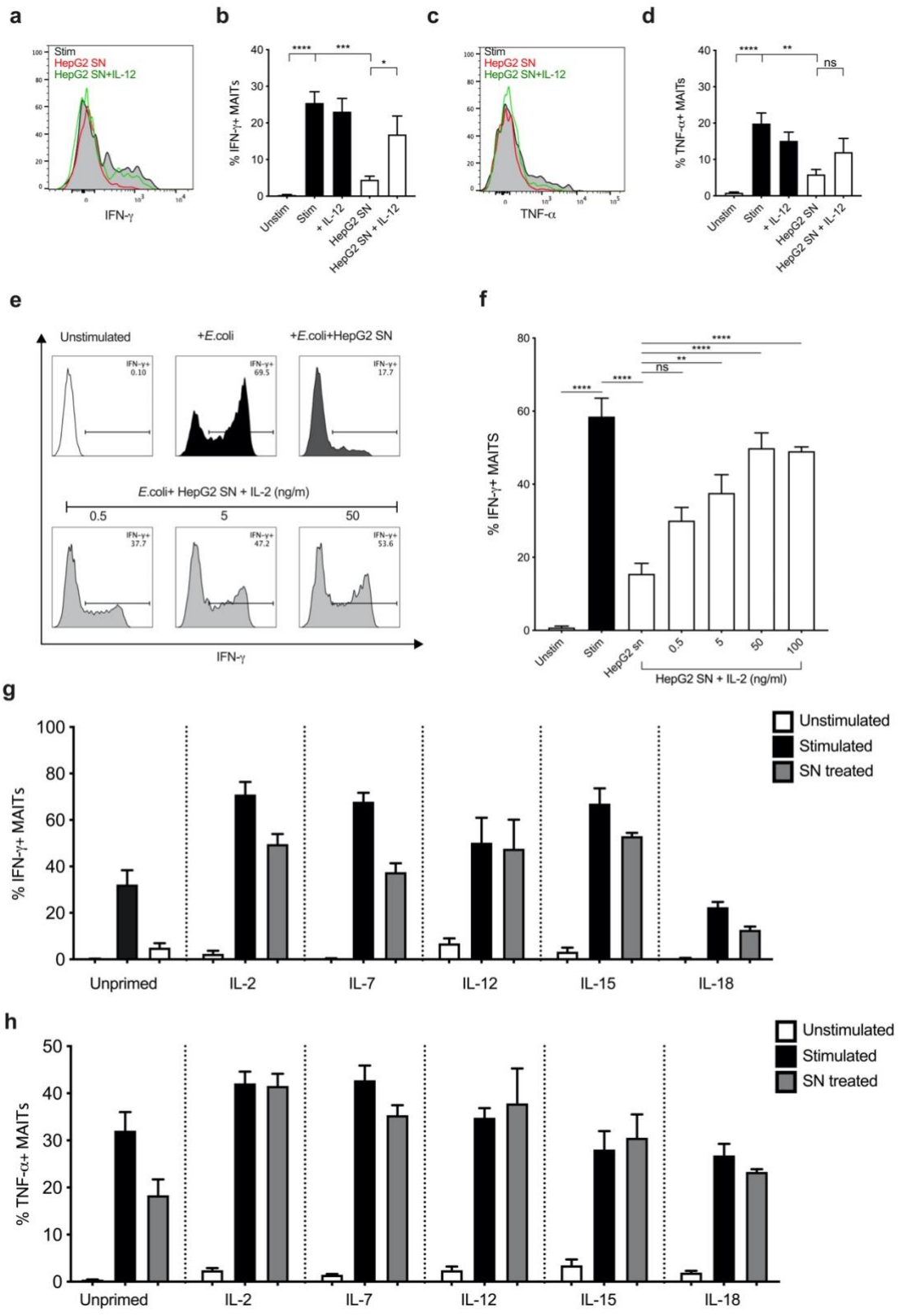


Figure 4.13 - HepG2 culture supernatant did not suppress activation of cytokine primed and exogenous cytokine supplemented, antigen stimulated MAITs cells

Isolated CD8 T cells were activated by *E*. coli stimulated THP-1 cells, treated with HepG2 culture supernatant and supplemented with cytokines in an overnight culture before analyzing MAIT cell activation by flow cytometry. a/b) IFN-γ, c/d) TNF-α and expression from IL-12 supplemented antigen stimulated MAITs treated with HepG2 supernatants, *(n=4)*, Statistical significance – Sidak's multiple comparisons test, *P<0.0383, **P<0.0019, ***P<0.0003, ****P<0.0001. d) representative IFN-γ expression histograms and e) IFN-γ quantification from antigen activated MAIT cells treated with HepG2 culture supernatant with the addition of various concentrations of IL-2, *(n=4)*, Statistical significance – Sidak's multiple comparisons test - **P<0.0018, ****P<0.0001. f) IFN-γ and g) TNF-α expression from cytokine primed, supernatant treated, antigen activated MAIT cells, *(n=4)*, Statistical significance – Sidak's multiple comparisons test, **P<0.0018, ****P<0.0001.

In addition to cytokine supplement experiments, we analysed the effect of hepatoma culture supernatants on antigen inexperienced, but cytokine primed MAIT cells. To verify the effects of HepG2 culture supernatant on cytokine primed MAIT cells we treated isolated CD8 T cells with IL-2, IL-12, IL-18, IL-7 and IL-15 for 24 hours, then removed the cytokines by thoroughly washing the cells before adding the cytokine primed CD8 T cells to the supernatant assays. Surprisingly the supernatants could no longer suppress MAIT cell activation, in comparison to the suppression of un-primed MAIT cells (Fig. 4.13g/h). Single cytokine primed MAIT cells were able to express IFN-γ stimulated in an antigen-dependent manner in the presence of hepatoma culture supernatant; thus, indicating that cytokine primed-MAIT cells are able to respond to TCR signalling even in the absence of cytokine co-stimulation of TCR signalling from the APC.

In our antigen-dependent activation model, cytokines required to prime MAIT cells are induced by TLR recognition of PAMPs from *E.* coli (Ussher, Bilton, Attwod, Shadwell, Richardson, de Lara, et al. 2014). Therefore, it is possible that the hepatoma culture supernatants suppress TLR signalling, or suppress downstream pathways, preventing cytokine expression.

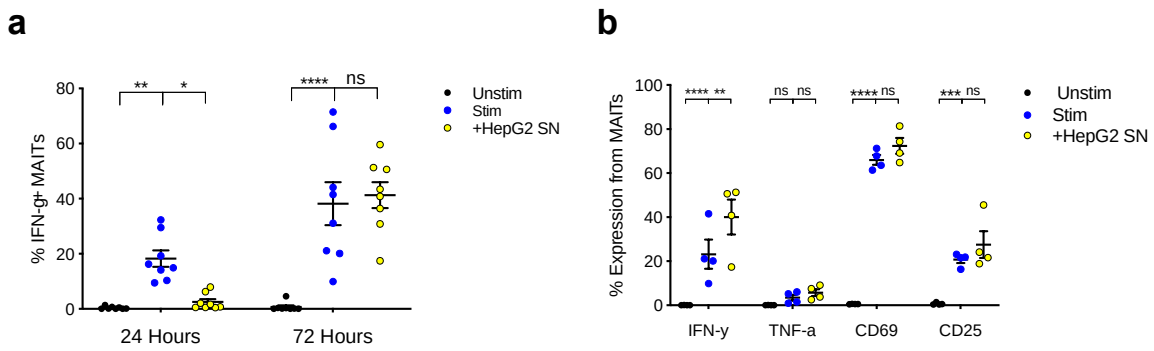


Figure 4.14 - Hepatoma cell culture supernatant mediated suppression is active in the initial phase of stimulation and diminishes by 72 hours

MAIT cells were activated by THP-1+ *E*. coli and treated with or without HepG2 cell culture supernatants for 24 hours and 72 hours. CD8+CD161++Vα7.2+ MAIT cell response was analysed by flow cytometry. a) IFN-γ expression from MAIT cells at 24 hours and 72 hours, *(n=8)*. b) IFN-γ, TNF-α, CD40 and CD25 expression from MAIT cells after 72 hours of stimulation -/+ HepG2 SN, *(n=4)*. Statistical significance - Sidak's multiple comparisons test, *P<0.01, **P<0.002, ***P<0.0003, ****P<0.0001.

Having established the suppression of cytokines from antigen presenting cells by hepatoma culture supernatant we tested their capacity to suppress antigen dependent MAIT cell activation over 72 hours, which is cytokine dominated in the later phase. MAIT cells were no longer suppressed at 72 hours and, in fact, higher IFN-γ expression was detected from supernatant treated MAIT cells than the supernatant untreated cells at

72 hours. Other MAIT cell markers like TNF-α and CD25 were also unaffected by the supernatant at 72 hours (Fig. 4.14).

4.4.5 Inhibition of lipid metabolite synthesis pathways in hepatoma cells partially blocks MAIT cell suppression

One of the extensively studied lipid metabolite, PGE_2, is produced by many cells of the liver (Heymann and Tacke 2016), its function in liver homeostasis was established early and its role in regulating immune responses has been widely reviewed(Lone and Taskén 2013). Higher levels of PGE_2 have also been reported in various cancers resulting in immune evasion and receptor specific signalling related resistance to immunotherapy(Wang, Honn, and Nie 2007).

Considering the production of PGE_2 by many liver cell types and higher levels in certain disease conditions, we speculated PGE_2 as a likely candidate suppressing MAIT cell activation. We first confirmed whether PGE_2 was present in the supernatants of liver derived, HepG2, Huh7, Huh7.5, HHL12, LECs and primary murine hepatocyte cells, and non-liver derived cell lines, the HeLa and Caco-2 cells. The data confirms the presence of PGE_2 present in large quantities in Huh7 cells lines as well HepG2 and primary murine hepatocytes (Fig. 4.15a). Unlike PGE_2, PGD_2 was barely detectable in any of the supernatants we examined (Fig. 4.15b). Other liver derived cell lines, Huh7.5, HHL12 and LECs produced very little to no PGE_2, similarly HeLa and Caco-2 cells did not make much PGE_2 either. The levels of PGE_2 from cell lines correlated with their suppressive impact on MAIT cell activation, with the exception of the HHL12 cell line, where HHL12 cell culture supernatants suppressed MAIT cell activation but did not show high expression of PGE_2 (Fig. 4.15).

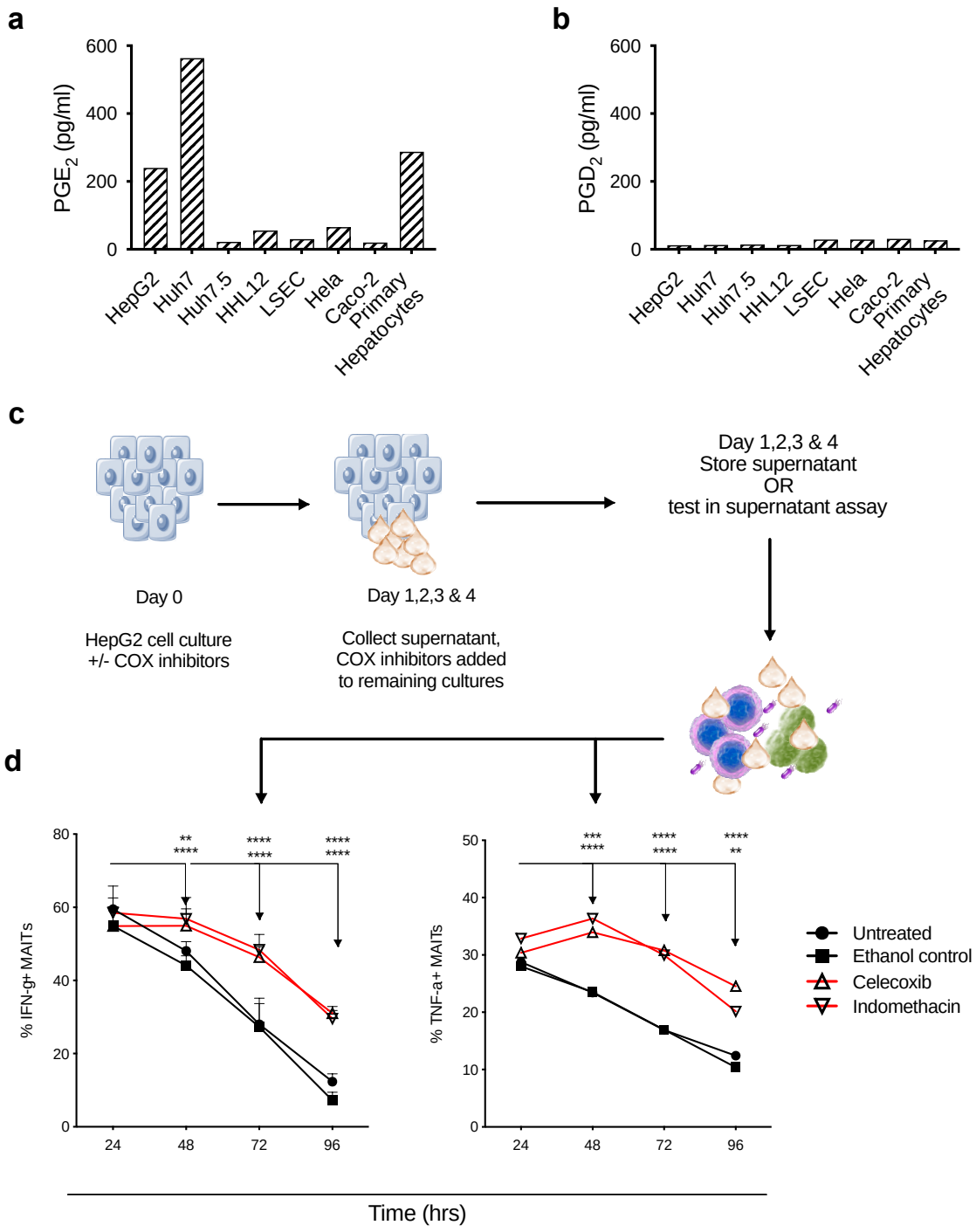


Figure 4.15 - Prostaglandin quantification from hepatoma cells and inhibiting of COX signaling HepG2 cell lines

a) PGE_2 and b) PGD_2 concentrations quantified by ELISA from four-day cell culture supernatants of various liver and non-liver cell lines (*n=1*). c) Time scale and summary of COX inhibition assay. d) IFN-γ and TNF-α expression from CD8 MAIT cells activated by *E*. coli

stimulated THP-1 cells in the presence of COX inhibitor treated HepG2 supernatants, acquired at 24-hour intervals over four days, *(n=8)*. Statistical significance – Tukey's multiple comparisons test, the impact of COX inhibitor treated HepG2 cell derived supernatant was compared to untreated HepG2 cell derived supernatant at each time point, **P<0.006, ***P<0.0006, ****P<0.0001.

After confirming the presence of PGE_2 in the supernatants of hepatoma cells lines we investigated the effect of blocking PGE_2 production from the hepatoma cells. We blocked COX-1 and COX-2 enzymes involved in PGE_2 synthesis, over four days, as this was the optimal period for cultures to reach 90% confluency, which matched the durations of the initial supernatant batches. Since PGE_2 is produced constantly by a positive feedback loop between PGE_2 and the COX enzymes, and because COX-enzyme inhibitors are short lived, we treated the cultures daily with selective COX-1 or COX-2 inhibitors without replacing the medium (Fig. 4.15c). The inhibition of PGE_2 production from hepatoma cells showed reduced suppression of MAIT cell IFN-γ and TNF-α as compared to the untreated hepatoma cell culture supernatants (Fig. 4.15d). There was no suppression of MAIT cell IFN-γ by COX-inhibitor treated and untreated HepG2 cell culture supernatant at 24 hours, suppression increased over the next 72 hours with maximum suppression at 96 hours for both COX- inhibitor treated and untreated HepG2 culture supernatants. Even though suppression of MAIT cell IFN-γ and TNF-α was reduced by COX inhibitor treatment of HepG2 cells, this did not completely prevent supernatant mediated suppression of MAIT cell activation (Fig. 4.15d).

As it was challenging to completely block the production of PGE_2 due the short half-life of the COX inhibitors in cell cultures, we tried blocking PGE_2 receptors in the supernatant assays. Hepatoma supernatant mediated suppression could not be reversed by PGE_2 receptor antagonist. However, in the controls (red bars Fig. 4.16), PGE_2 signalling could be blocked by combined EP2 and EP4 receptor antagonism, but only EP4 blocking prevented PGE_2 mediated suppression of MAIT cell IFN-γ when used individually (Fig. 4.16a-c). Using antagonists against EP1 and EP3 receptors had no effect on MAIT cell IFN-γ levels in either the controls or the supernatant treated cultures. Similarly, blocking DP1 receptor signalling in supernatant untreated but PGD_2 treated cultures resulted in lower suppression of MAIT cell IFN-γ but this did not prevent suppression of MAIT cell IFN-γ completely (Fig. 4.16d/e). We did not try combined DP1 and DP2 blockade, as DP2 antagonist did not show any effect on the levels of MAIT cell IFN-γ. Moreover, gene expression data shows no expression of the DP2 receptor genes in either THP-1 or MAIT cells and the DP1 receptor gene was only expressed in MAIT cells (Supplementary Fig. 9). The failure to prevent the supernatant mediated suppression of MAIT cells using PGE_2 and PGD_2 receptor antagonists lead to further questions. Firstly, if the concentration of PGE_2/PGD_2 in HepG2 cell culture supernatant is sufficient to block MAIT activation, like that used in the positive controls, i.e. >100nM (chapter 5, figure 5.1). Secondly, if there were other COX-dependent suppressive compounds or whether suppression of MAIT cells was mediated by multiple molecules. Indeed, PGE_2 has a potent dose dependent suppressive effect on MAIT cell activation as shown in the next chapter, however PGE_2 receptor blockade in supernatant assays could not prevent HepG2 supernatant mediated suppression of MAIT cell IFN-γ.

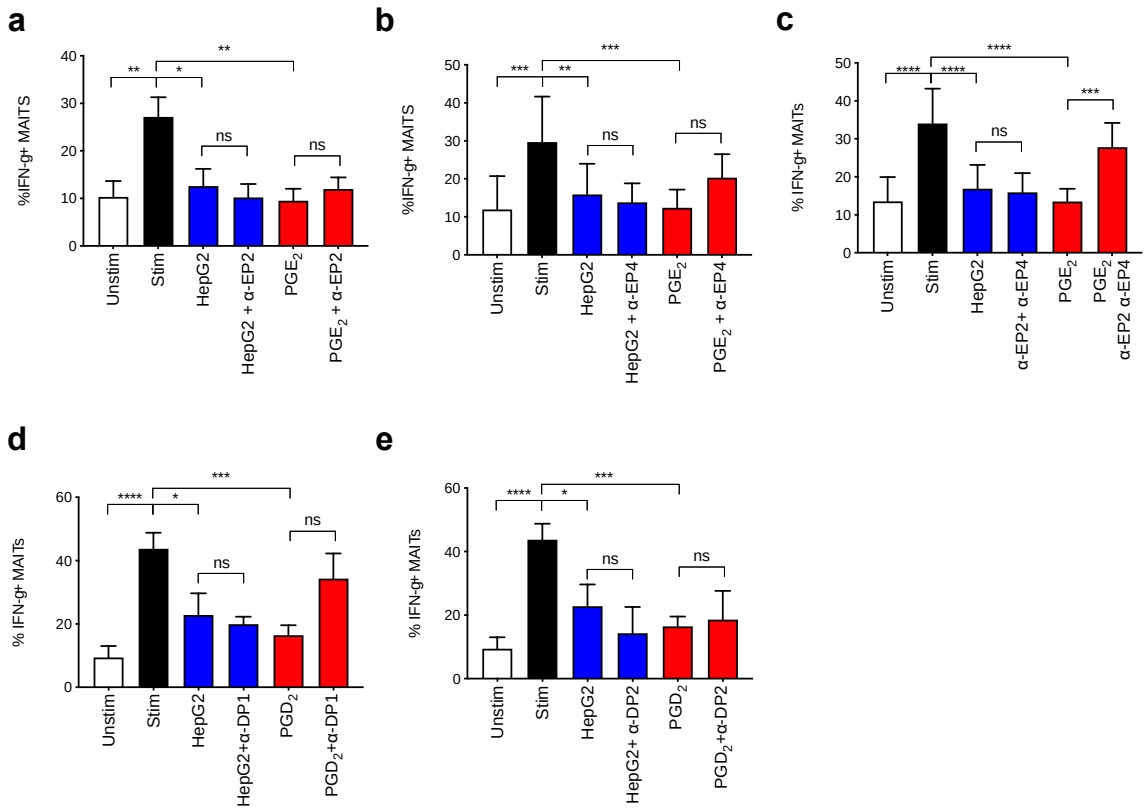


Figure 4.16 – Blockade of PGE_2 and PGD_2 signaling in HepG2 supernatant treated antigen stimulated MAITs cells

MAIT cells were activated by THP-1+ *E*. coli and treated with or without HepG2 cell culture supernatants, with or with EP1-4 and DP1/2 receptors antagonist or with PGE_2/PGD_2 alone, or with HepG2 supernatant + EP1-4 and DP1/2 receptors antagonist. CD8+CD161++Vα7.2+ MAIT cell responses were analysed by flow cytometry. IFN-γ expression from MAIT cells treated with a) EP2 receptor antagonist b) EP4 receptor antagonist, c) EP2+EP4 receptor antagonist, d) DP1 receptor antagonist and e) DP2 receptor antagonist, *(n=8)*. *P<0.0354, **P<0.0096, ***P<0.0007, ****P<0.0001.

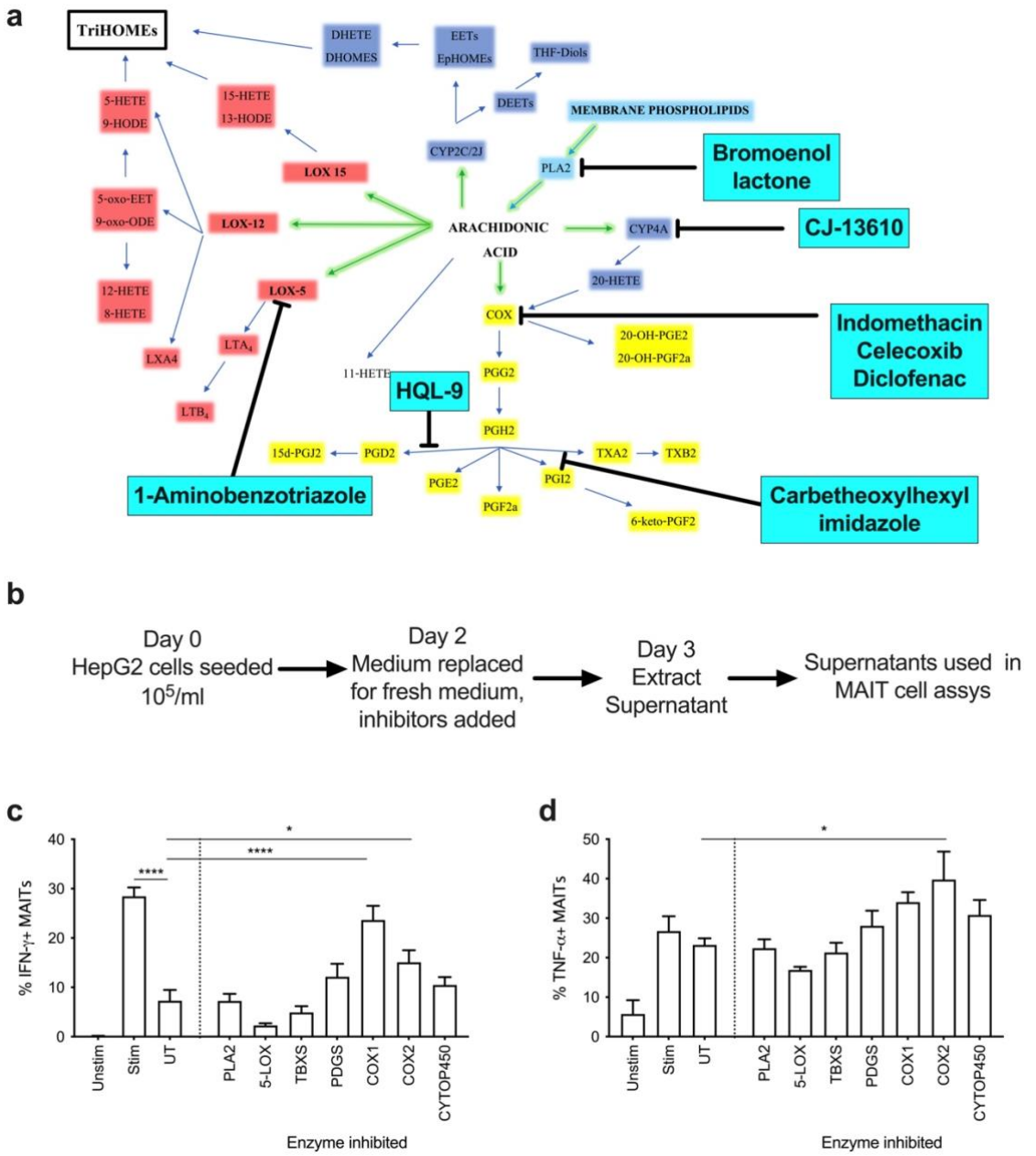


Figure 4.17 - Inhibition of different lipid synthesis pathways in HepG2 cells to block HepG2 supernatant mediated suppression of MAIT cell activation

HepG2 cells were treated with various enzyme inhibitors blocking specific lipid synthesis pathways. Inhibitor treated HepG2 supernatants were then used in supernatant assays. a) Inhibitors, cyan colored boxes, used to block different pathways of the lipid metabolite synthesis pathways. b) Summary of the protocol used to test inhibition of lipid metabolite synthesis enzymes. c) IFN-γ measured from CD8 MAIT cells activated by *E*. coli stimulated

THP-1 cells, treated with supernatants acquired from HepG2 cells treated with individual enzyme inhibitors compared to untreated HepG2 supernatant (UT), *(n=4)*. d) TNF-α measured from CD8 MAIT cells activated by *E*. coli stimulated THP-1 cells and treated with supernatants acquired from HepG2 cells treated with a combination of enzyme inhibitors, *(n=4)*. Statistical significance – Dunnett's multiple comparisons test, *P<0.03, **** P<0.0001.

Lipid metabolism generates various metabolites as shown in figure 4.17a and a single metabolite can have multiple receptor subtypes(Wei and Gronert 2019). Some of the metabolites shown in this figure 4.17a have been studied in the context of disease or for their general function on immune cells. The contradictory results observed with PGE_2 receptor blockade and use of COX inhibitors on HepG2 cells led us to the idea of testing the probability of other lipid mediators that may be produced by HepG2 cells which may contribute to the suppression of MAIT cells. Similar to COX-inhibitor treatment of HepG2 cells we blocked the phospholipase A2 (PLA2), 5-lipoxygenase (5-LOX), cytochrome P450 (CYTOP450) pathways and the prostaglandin D synthase (PGDS) and thromboxane synthase (TXBS) enzymes. Inhibiting COX-1 using indomethacin alone was able to prevent suppression of MAIT cell activation in one of the batches but this could not be reproduced. Celecoxib reduced MAIT cell suppression by 50% (Fig. 4.17c/d) and similar results were produced by the combined inhibition of celecoxib and indomethacin (Supplementary Fig. 8). Inhibition of CYTOP450, PLA2, 5-LOX or TXBS did not have any effect on the suppressive capacity of treated HepG2 derived supernatant (Fig. 4.17c). HQL9 is a PGDS inhibitor, PGDS is involved in the production of PGD_2 that can suppress MAIT cell activation (*detailed in the next chapter*), the inhibition of PGDS by HQL9

reduced MAIT cell suppression by approximately 40% (Fig. 4.17c). These results are strongly indicating the involvement of COX and prostaglandin synthase derived lipid mediators; however, it is possible that they produce mediators that have yet to be identified. Supernatants acquired from the inhibition of the 5-LOX enzyme was more suppressive than supernatants acquired from untreated HepG2 cells. The inhibition of COX enzymes augments the LOX pathway and vice versa hence blocking the 5-LOX enzyme may have boosted the COX pathway resulting in higher production of the suppressive metabolite and thus the 5-LOX treated supernatant being more suppressive than the supernatant of untreated HepG2 cells. Diclofenac is a COX inhibitor which also reduced MAIT cell suppression by the HepG2 supernatant. However, whether this was an effect of inhibiting COX or enhancing MAIT cell activation cannot be defined because diclofenac acts as an MR1 ligand thus complicating the analysis of this outcome. These results indicate more than one molecule, produced by hepatoma cells, via the COX pathway being responsible for the suppression however what it exactly is remains ambiguous.

4.5 Discussion

In the previous chapter we investigated the ability of liver resident parenchymal and non-parenchymal cells to activate MAIT cells. The results indicated a lack of MAIT cell stimulation by hepatoma and HSCs whereas LECs were able to stimulate MAIT cells, albeit at higher bacterial loads. The hepatocytes may not be able to directly activate MAIT cells. However, they constitute a major cell type in the liver and, though separated by LSECs from the sinusoidal lumen, their ability to interact with immune cells in the sinusoidal lumen through fenestrations is well described(Warren et al. 2006). Additionally, hepatocytes can make anti-inflammatory cytokines like IL-10 and also induce IL-10 expressing T cells thus able to influence T cells directly and indirectly(Stijlemans et al. 2020). The presence of HepG2 cells in the standard MAIT cell activation assay resulted in MAIT cell suppression (Fig. 4.1c). Since hepatocytes can express PD-L1 it could be assumed that hepatocytes may have prevented MAIT cell activation by PD-L1 ligation to PD-1 on MAIT cells in a contact dependent manner(Liang, Kwota, and Sun 2016; Yong et al. 2018). We did not test this mechanism, but it is worth checking PD-L1 mediated suppression of MAIT cells by hepatocytes, nonetheless we focused on the potent inhibition of MAIT cell activation by hepatocyte derived soluble mediators.

To study and identify the suppressive mechanism we extracted supernatants and added it to our standard MAIT cell activation assay. In-vivo, it is likely that both soluble and contact dependent mechanisms are operational and thus may be able to facilitate a more potent suppression. Furthermore, this model is limited as it does not include CD4

T cells, other lymphoid and myeloid cells which are influenced by hepatocytes that are capable of inducing Tregs and immunoregulatory factors(Crispe 2016). These all in turn may negatively regulate MAIT cell activation. One possible method to improve the current assays would be to use organoids constructed from multiple cell types. Of note, though we refer to the suppression as being hepatocyte specific, the culture supernatants were acquired from hepatoma cell lines, mainly HepG2 cells which are acquired from a tumour therefore these cells are transcriptionally and functionally different from healthy hepatocytes (Wilkening et al. 2003). Since supernatant acquired from primary murine hepatocytes from non-diseased mice also inhibited MAIT cell activation is it likely this suppressive mechanism maybe a result of hepatocyte specific liver tolerance.

Hepatoma derived supernatant suppressed multiple features of activated MAIT cells preventing MAIT cells from recruiting/activating other immune cells as well killing infected cells. TNF-α suppression by hepatocytes could be important to prevent TNF-α mediated liver damage as described for chronic liver inflammation,(Yoon Mee Yang, Ph.D. and Ekihiro Seki, M.D. 2016) but the suppression of IFN-γ may result in suppressed immunity to infection. Likewise, the suppression of MAIT cell cytotoxic function by hepatocytes may protect from unwanted liver damage but in the context of infection may prove detrimental.

Hepatoma supernatants did not suppress CD69 expression from MAIT cells, and as CD69 induction in our model is dependent on TCR signalling, it shows that TCR signalling is not affected by hepatoma supernatant (Fig. 4.1i). Furthermore, hepatoma derived culture

supernatants did not suppress cytokine primed MAIT cells. This suggests that MAIT cells primed for example in the periphery by cytokines, during infection, may be resistant to hepatocyte-mediated suppression. Moreover, since MAIT cell activation could be rescued by cytokine supplement, it shows that TCR signalling is still intact but there is a lack of cytokine co-stimulation. Additionally, in the absence of TCR signalling, cytokines were not able to prevent hepatoma supernatant mediated suppression of MAIT cell activation. This also indicates that hepatoma supernatants do not prevent antigen presentation to MAIT cells but may impact other pathways such as the recognition of PAMPs or PRR induced effector molecule synthesis. Efficient MAIT cell stimulation requires both TCR and cytokine signalling and more than one cytokine is required to stimulate MAIT cells in a TCR-independent manner (Slichter et al. 2016; Ussher, Bilton, Attwod, Shadwell, Richardson, Lara, et al. 2014). In our TCR stimulation model we use a single cytokine to co-stimulate TCR signalling, therefore it is not possible for the MAIT cells to be activated without an active TCR signal or independent of the TCR. More importantly, this model highlights the absence of stimulatory cytokines in hepatoma culture supernatants that may combine with IL-12/IL-18 to stimulate MAIT cells in the absence/inhibited TCR signal. Also this implies that basal cytokine production by hepatocytes do not support MAIT cell activation. It can be assumed that TLR ligands may induce cytokines from hepatocytes supportive of MAIT cell activation, but even the supernatants from TLR stimulated HepG2 supernatants were suppressive rather than stimulatory for MAIT cells (Supplementary Fig. 7).

Hepatocytes produce anti-inflammatory molecules like IL-10 and PGE_2 which are components of liver homeostasis and the tumour microenvironment(Kim and Bae

2016). PGE_2 has been shown to suppress the cytotoxic effector function of innate like cells especially the NK cells(Agard, Asakrah, and Morici 2013). Since NK cells and MAIT cells are innate cells, sharing many features of activation and function, it could be assumed that PGE_2 may suppress the cytotoxic effector function of MAIT cells in similar manner to NK cells. However, blocking the signalling of PGE_2 in the supernatant treated MAIT cell activation assays did not prevent suppression hence the results identify a strong suppressive impact on MAIT cells by a yet unknown hepatocyte derived soluble mediator/s.

Hepatoma supernatants suppressed the initial activation of MAIT cells but did not suppress MAIT cells at 72 hours. There are several possibilities for the lack of suppression at later stages of MAIT cell stimulation, firstly the supernatants may contain a suppressive molecule which is catabolized by either MAIT cells or the antigen presenting cell. Secondly the hepatoma derived suppressive compound maybe activating SOCS proteins that would suppress the production of pro-inflammatory cytokines necessary for MAIT cell activation. However, SOCS proteins regulated by a feedback loop results in diminished activation of SOCS at later time points that would override the suppression of pro-inflammatory cytokines. Again, it should be noted that in-vivo the hepatocytes would be producing the suppressive compound constantly. Therefore, the availability, and hence ability to suppress MAIT cell activation would remain operational until other signals become active, possibly during infection that can override the production/actions of this suppressive compound released from hepatocytes. Further investigation of the involvement of SOCS proteins in the delayed

cytokine response from hepatoma supernatant treated APCs could be tested by using SOCS protein siRNA or SOCS knockout cell lines.

Upregulated genes in supernatant treated THP-1 cells were associated with pathways positively regulating cyclase activity. Enhanced cyclase activity results in increased synthesis of cAMP which in turn suppresses the production of pro-inflammatory cytokines from DCs as well promoting the release of anti-inflammatory cytokines(Koga et al. 2009). As the hepatoma supernatants increase cyclase activity, and thus cAMP, this may lead to increased extracellular cAMP. cAMP is broken down by cell surface phosphodiesterase's (PDEs) and the resulting compounds, adenosine diphosphate (ADP) and adenosine monophosphate (AMP), could bind to purinergic receptors P2Y1/P2Y12 and A2A respectively, facilitating T cell suppression rather than T cell activation (Shinohara and Tsukimoto 2018; Silva-Vilches, Ring, and Mahnke 2018). Since we could prevent MAIT cell suppression partially by treating HepG2 cells with COX inhibitors it is possible that cAMP levels were elevated by PGE_2 which is an arachidonic acid (AA) metabolite produced by COX. PGE_2 is potent inducer of cAMP by its signalling through EP2 and EP4 receptor (Reader, Holt, and Fulton 2011). However, as we could not prevent MAIT cell suppression by EP2 and EP4 receptor antagonism this pathway does not seem to be the pathway involved in MAIT cell suppression. Thus, MAIT cell suppression seems to be mediated by a different compound mimicking the actions of PGE_2 to suppress MAIT cells.

So far, our data suggests that the suppression of MAIT cell activation occurs through the modulation of APC cytokine expression by COX-specific compound/s secreted into

hepatoma cell culture supernatants. Common immunoregulatory, COX-derived mediators such prostaglandins could be detected from hepatoma culture supernatant, but their activity could not be blocked by receptor antagonist suggesting that other COX-derived mediators are responsible for the suppression. Altered lipid metabolism in ovarian cancer has been shown to modulate T cell function (Wefers et al. 2018) and, as hepatocytes are major lipid metabolising cells, they are highly likely to produce diverse lipid metabolites that can then interfere with the activation of immune cells. To identify the unknown COX-derived mediators, screening the differences in the lipid metabolite profiles of suppressive and non-suppressive cell culture supernatants could be determined by liquid chromatography-mass spectrometry (LC-MS). Indeed, our initial LC-MS experiment has demonstrated a difference in the metabolite profiles between culture media and HepG2 supernatant (Supplementary Fig. 10). However, this result has not been consistent between two batches and requires further investigation. The identification of these suppressive compound(s) would make it easier to quantify and, importantly, correlate within disease settings. This would also help to identify unknown, novel compounds associated with hepatocytes that are undefined but important to liver biology.

4.6 Summary

1. Hepatoma cell culture supernatants have the capacity to potently suppress MAIT cell activation.
2. MAIT cell suppression by culture supernatant is liver and hepatocyte specific.
3. MAIT cell suppression by hepatoma supernatant is caused partly by a small COX dependent lipid metabolite/s.
4. Suppressive hepatoma cell culture supernatants expressed the COX-derived PGE_2, but this was not the suppressive metabolite because PGER antagonists failed to block suppression.
5. More than one compound is likely to be responsible for MAIT activation and may involve other lipid metabolite pathways.
6. Hepatoma supernatants mediate MAIT cell suppression by altering the phenotype of APCs and limit their ability to induce pro-inflammatory cytokines necessary for MAIT activation.

CHAPTER 5 – IMPACT OF LIPID METABOLITES ON MAIT CELL ACTIVATION AND FUNCTION

5.1 Introduction

Prostaglandins (PG) and Leukotrienes (LT) are bioactive lipid signalling molecules of the eicosanoid family. They are largely synthesised by the action of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes on the AA precursor or other precursors like eicosapentaenoic acid and linolenic acid (Tallima and El Ridi 2018). Both prostaglandins and leukotrienes have been studied extensively and are involved in various physiological processes like, platelet aggregation, vascular permeability, regulation of smooth muscle tone, control of hormone synthesis of the reproductive system and are important regulators of immune processes(Funk 2001). Additionally, the high expression of prostaglandins and leukotrienes during inflammation and in chronic inflammation has allowed us to learn more about the functions of these eicosanoids and their roles in pathophysiological processes, disease, autoimmunity and cancer(Dennis and Norris 2015). The major difference between prostaglandins and leukotrienes is that the former is made by many cells types, whereas leukotriene production is largely made by leukocytes(Kilmer 2010; Schmidt et al. 2011). Moreover, the actions of different prostaglandins and leukotrienes are highly context dependent and receptor specific, as discussed in more detail below.

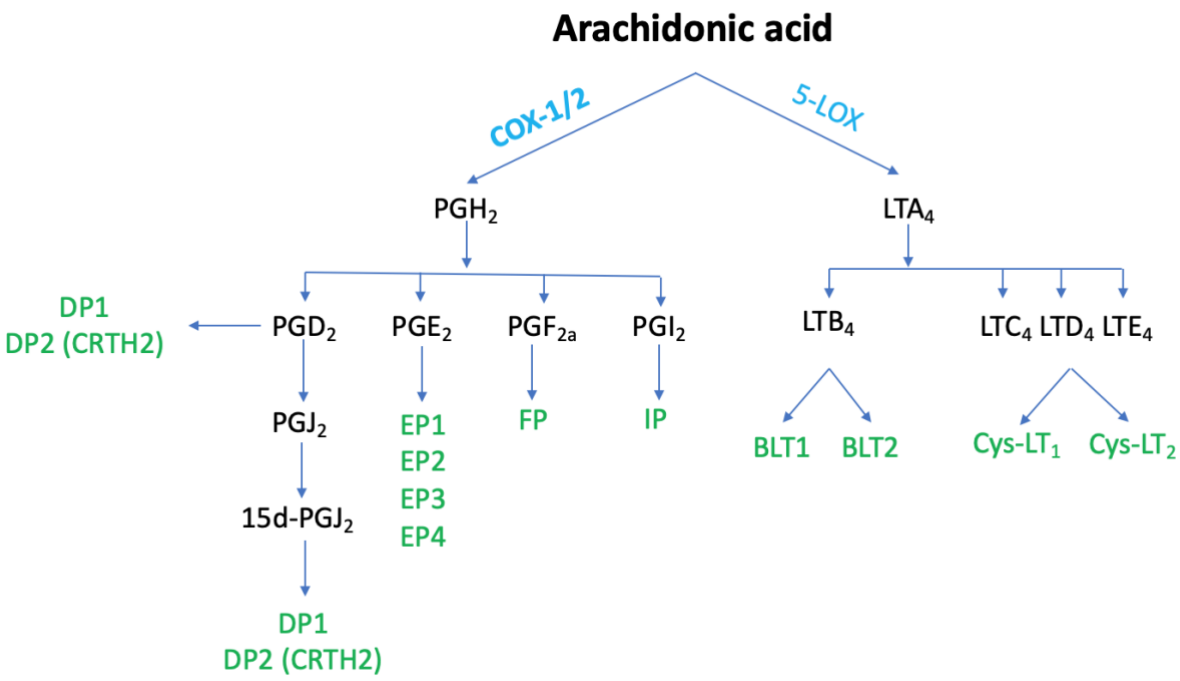


Diagram 5.1 – Overview of prostaglandin and leukotriene synthesis and their respective receptors. Prostaglandins are biosynthesised by COX enzymes and signal through their corresponding receptors. Leukotrienes are biosynthesised by 5-LOX and signal through their subtype specific receptors.

5.1.1 Prostaglandin biosynthesis, receptors and signalling pathways

The four primary prostaglandins PGD_2, PGE_2, PGF_{2a} and PGI_2 are synthesised by the sequential action of multiple enzymes. First, phospholipase A2 liberates AA from membrane phospholipids, which is then catalysed by COX to form the unstable intermediate PGH_2. PGH_2 is converted to the different prostaglandins by their respective synthases(Funk 2001). PGJ_2, a cyclopentanone PG, is a secondary prostaglandin formed by the dehydration of PGD_2 forming PGJ_2, which is then converted to 15-deoxy-Δ12,14-prostaglandin J2 (15d- PGJ_2)(Guyton et al. 2001). The PGE_2 receptor, EP3, has 8 isoforms

and the PGF_{2a} receptor has 2 isoforms[7]. PGE_2 receptors are also present in the cytosol and on nuclear membranes allowing them to regulate transcription of genes like inducible nitric oxide synthase (iNOS)[8].

PGD_2 and PGE_2 signal through more than one receptor whereas only a single receptor has been identified for PGF_{2a} and PGI_2 signalling (Diagram 5.1). In contrast to the primary prostaglandins, no specific receptor has been identified to date for PGJ_2 and 15d- PGJ_2. However, it is thought they use the PGD_2 receptors to signal (Bhattacharya et al. 1999; Hata and Breyer 2004). All prostaglandin receptors are G-protein coupled receptors (GPCR) with the DP2 receptors being unique as it functions as a chemoattractant and therefore belongs to the family of chemokine receptors (Tait Wojno et al. 2015). Prostaglandin-signalling through the DP1, EP2, EP4 and IP receptors increase production of cAMP, cAMP stimulates PKA signalling, thereby inducing the influx of Ca2+. Prostaglandin-signalling through DP2, EP1, EP3 and the FP receptor induce intracellular Ca2+ mobilization through the activation of the inositol triphosphate (IP3)(Hata and Breyer 2004).

5.1.2 Prostaglandins in inflammation

Both primary and secondary PG's have immunomodulatory roles with PGE_2, PGD_2 being the most extensively studied. Additionally, several knockout and overexpression experiments have outlined the receptor specific roles which are discussed briefly below.

PGE_2 - PGE_2 is the most widely studied prostanoid. Knockout studies have revealed both pro- and anti-inflammatory effects of PGE_2 with the ability of PGE_2 to modulate T cells directly or through APCs. Many studies and reviews highlight PGE_2 ability to skew T-cell

responses towards either Th2 or Th17(Gagliardi et al. 2010; Nakanishi and Daniel W. Rosenberg 2013), while at the same time, some also demonstrated induction of Th1 responses(Yao et al. 2009, 2013). A few PGE_2-specific effects include, EP1 mediated hyperalgesia(Sarkar et al. 2003), EP2 mediated neuronal injury(Liu et al. 2019), tumour growth caused by a loss of EP3 signalling in colon cancer(Shoji 2004), and EP4 mediated suppression of microglial inflammation in Alzheimer's-associated inflammation(Woodling et al. 2014). PGE_2 further exerts it effects through EP2 and EP4 receptors to modulate dendritic cell maturation and migration(Krause et al. 2007; Legler et al. 2006) but can also inhibit full maturation of DCs by inducing IDO1(Trabanelli et al. 2015). The presence of PGE_2 during antigen priming of DCs limits their ability to produce IL-12 and instead induces high IL-10 production(Kaliński et al. 1997). In addition, PGE_2 primed DCs induce the differentiation of naïve T cells into IL-4 producing Th2 cells(Snijdewint et al. 1993). As bone marrow dendritic cells can make PGE_2, PGE_2 can also modulate DC function in an autocrine fashion, enhancing IL-23 production which would promote differentiation of Th17 cells(Sheibanie et al. 2004). Similar to its effects on DCs, PGE_2 was shown to polarize macrophages towards an M2-like phenotype through cAMP and CREB dependent signalling(Luan et al. 2015; Yamamichi et al. 2017). PGE_2 can also suppress the bacterial killing by macrophages via the inhibition of NADPH oxidase, as shown in alveolar macrophages(Serezani et al. 2007), and regulates cytokine production (TNF-α, IL-1β, IL-18) by activated macrophages, moreover, it was shown to reduce the expression of IL-12R(Gagliardi et al. 2010). In both DCs and macrophages, LPS stimulation induces considerable PGE_2 production and is enhanced through a positive feedback loop which identifies a role for autocrine regulation by PGE_2(Tang et al. 2017). As described for DCs in certain infections PGE_2 can polarize M1 macrophages

by augmenting the activation of the inflammasome(Sheppe et al. 2018; Wu et al. 2020). PGE_2 also regulates neutrophils by promoting an anti-inflammatory phenotype and causes neutrophils to migrate out of the site of inflammation. PGE_2 activates lipoxygenase resulting in the production of LXA_4 which governors neutrophil migration, resulting in inflammation resolution(Loynes et al. 2018). For B cells PGE_2 potentiates proliferation in a CD40 dependent manner but suppresses their capacity to secrete immunoglobulins(Garrone et al. 1994) and in another model, PGE_2 inhibited B cell proliferation through EP4 signalling(Murn et al. 2008). The PGE_2 mediated effects on DCs, indirectly impact T cells by skewing the immune response towards Th2 and Th17, whereas direct modulation of T cells by PGE_2 inhibits their proliferation, induces apoptosis and alters cytokine production. PGE_2 limits T cell proliferation by suppressing IL-2 production and is also capable of inhibiting their activation(He and Stuart 1999; Vercammen and Ceuppens 1987). In general, PGE_2 has dual roles and can promote both inflammation as well as produce anti-inflammatory effects that can be positive for the resolution of inflammation but can be detrimental when it inhibits inflammation that is requisite for infection clearance.

PGD_2 – PGD_2 is part of the D series of prostaglandins, and is synthesised by two enzymes, the haemopoietic-PGDS (H-PGDS) and the lipocalin-PGDS (L-PGDS). The dehydration of PGD_2 forms the members of the J series of prostaglandins, including 15d- PGJ_2(Joo and Sadikot 2012) which along with PGD_2 is also involved in regulating biological process and immune responses as discussed later. Initially, PGD_2 was recognised for its role in the central nervous system and has been shown to regulate sleep and nociception(Urade and Hayaishi 2010). In addition, PGD_2 is involved in the regulation of immune responses

and others functions of PGD_2 have been identified from its involvement in atopic conditions(Rittchen and Heinemann 2019). In the airways, PGD_2 is mainly made by mast cells which triggers and IgE mediated allergic response(Satoh et al. 2006). A rapid and sharp rise in PGD_2 levels has been detected in the bronchoalveolar lavage fluid of asthmatic patients indicating PGD_2 mediated airway hyperactivity (Fajt et al. 2013). In support of this, PGD_2 challenge resulted in bronchoconstriction and airway eosinophil infiltration(Arima and Fukuda 2011) which are common events seen in asthma. These proinflammatory effects of PGD_2 are prominent in the airways but in other settings PGD_2 can produce anti-inflammatory effects(Joo and Sadikot 2012). Similar to PGE_2, PGD_2 also modulates the function of dendritic cells and suppresses their ability to produce IL-12 leading to a Th2 polarized immune response(Xue et al. 2005). In contrast to the former though, PGD_2 did not increase IL-10 production and instead decreased IL-10 production by DCs. Pre-treatment of mature DC cells with PGD_2 also lead to the time dependent upregulation of IL-4 but the downregulation of IFN-γ by T cells(Theiner, Gessner, and Lutz 2006). Furthermore, PGD_2 is capable of delaying the maturation of DCs as shown in a mouse model of neonatal respiratory infection(Werder et al. 2018). A similar study has identified an age-related increase in PGD_2 related to impaired respiratory DC migration which resulted in diminished virus specific T cell response(J. Zhao et al. 2011). For PGD_2, the pro-inflammatory effects can be exerted by both DP1 and DP2 receptor signalling with PGD_2 binding to both receptors with similar affinity(Pettipher 2008). DP1 mediated airway inflammation involves the stimulation of chemokines and cytokines which recruits immune cells to the airway inducing airway hypersensitivity in asthma(Kupczyk and Kuna 2017). Another feature of DP1 signalling prevents eosinophil apoptosis which may exacerbate airway responses to allergen with a potential to cause

damage(Gervais et al. 2001). One of the anti-inflammatory roles of PGD_2 was identified by its signalling through DP1 receptor which inhibited inflammasome activation in a neurotropic coronavirus model(Vijay et al. 2017). DP2 receptor signalling functions more as a chemoattract, recruiting cells to the site of inflammation. Werder et al showed that DP2 signalling can promote severe viral bronchiolitis by inhibiting IFN-α production(Steiman and Gern 2018). In this model they also report the dynamic PGD_2 signalling where DP1 agonism and DP2 blockade was necessary to protect from viral bronchiolitis. The latter findings highlight that dual signalling, rather than receptor specific signalling can occur during inflammation, in the case of PGD_2 and by enhancing/suppressing receptor specific signalling resolution can be achieved(Steiman and Gern 2018).

Interestingly prostaglandins have the ability to regulate each other's activity as shown by Lalier et al. Their study demonstrates a mechanism where PGE_2 binds to Bcl-2-assoicated X protein (BAX) and by changing its conformations triggers apoptosis of HeLa, LN-18 and HCT116 cell lines(Lalier et al. 2011). The addition of PGD_2 was able to alleviate PGE_2 mediated BAX activation in a concentration dependent manner, thus the balance between different prostaglandins during inflammation can shape the outcome by regulating one another.

5.1.3 Cyclooxygenases in inflammation:

There are several COX enzyme isoforms, however the most studied isoforms are COX-1 and COX-2. COX-1 is constitutively expressed whereas COX-2 is highly inducible to stimuli like inflammatory cytokines (IL-1β and TNF-α), LPS, hormones and various growth

factors(Zarghi and Arfaei 2011). This difference in expression also identifies COX-1 as a driver of the initial phase of acute inflammation while COX-2 is upregulated in a delayed fashion(Ricciotti and FitzGerald 2011). Pharmacologically, COX enzymes can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDS), for example, aspirin, ibuprofen, diclofenac and the COXIBS, which prevent the generation of prostanoids (Brune and Patrignani 2015). Overexpression of COX-2 and high levels of PGE_2 in the tumour microenvironment have been reported in multiple studies. Accordingly, the use of COX-2 specific inhibitors results in tumour regression by altering the balance between IL-10 to IL-12, which is vital to restore the anti-tumour response(Stolina et al. 2000). In contrast, in a murine arthritis model, the amplification of COX-2 activity and PGE_2 production was necessary to attenuate inflammation and restore homeostasis(Chan and Moore 2010). Such studies demonstrate that direct regulation of the COX-2 enzyme is critical for the resolution of many COX-2 related pathologies.

5.1.4 Leukotriene biosynthesis, receptors and signalling pathways

Leukotrienes are divided in to two groups; the conventional leukotriene, LTB_4, and the cysteinyl LTs, LTE_4, LTC_4, LTD_4(OGAWA and CALHOUN 2006). LTB_4 is mainly produced by neutrophils, whereas the cysteinyl class of leukotrienes are largely produced by mast cells, basophils and eosinophils(Peters-Golden et al. 2005). Leukotrienes are also synthesized from AA by the action of 5-LOX enzyme with the assistance of 5-Lipoxygenase activating protein (FLAP), which produces the LTA_4 intermediate that is then converted to LTB_4, LTC_4, LTD_4 and LTE_4 by LT hydrolase or leukotriene synthases(Peters-Golden and Brock 2003). Like the prostaglandins, leukotrienes also

exert their effect by binding to GPCRs, the activation of which leads to an increase in Ca2+ but a decrease in intracellular cAMP levels(Funk 2001).

5.1.5 Leukotrienes in inflammation

Leukotrienes, like prostaglandins are potent bioactive lipid mediators that have been associated with pathology in asthma, arthritis, ischaemic reperfusion injury, ulcerative colitis and psoriasis(Di Gennaro and Haeggström 2012; Henderson 1994; Roberts et al. 1997). The most-studied leukotriene, LTB_4, functions as a chemoattractant, attracting neutrophils and eosinophils, causes their degranulation and is able to induce reactive oxygen species(Ohnishi, Miyahara, and Gelfand 2008). On the other hand, the main functions of CysLTs are vasodilation, bronchoconstriction and mucus production(Okunishi and Peters-Golden 2011). LTB_4 mediates its pro-inflammatory effects primarily through the BLT1 receptor which is expressed mainly on leukocytes. Its second receptor, BLT2, is expressed ubiquitously, especially on epithelial cells, where signalling through BLT2 is associated with epithelial homeostasis and wound healing(Saeki and Yokomizo 2017). The dual role of LTB_4 has been demonstrated by studies that have shown its ability to recruitment CTL to the tumour and on the one hand, it also promotes tumour growth by activating neutrophils(Jala et al. 2017). LTB_4 induced the generation of B regulatory cells (Bregs) in a murine breast cancer model which facilitated cancer metastasis(Wejksza et al. 2013). Increased levels of LTs in the airway are detrimental and exacerbate airway inflammation, especially in asthma but also play a protective role by signalling through the BLT2 receptor(Matsunaga et al. 2013; OGAWA and CALHOUN 2006). They are also involved in the pathogenesis of rheumatoid arthritis where LTB_4-BLT1 signalling made the condition worse whereas

BLT1-/- mice were protected from RA development(Mathis et al. 2010; Mathis, Jala, and Haribabu 2007). Furthermore, LTE_4 is implicated in inflammatory bowel disease, significantly higher levels of LTE_4 was detected in the urine of patients with active disease in comparison to remission patients (Hof et al. 2012).

In addition to the roles of leukotrienes in disease, LTs can modulate APCs. LTB_4 enhanced the generation of pro-inflammatory microRNAs which in turn promote MyD88 dependent macrophage activation by suppressing suppressor of cytokine signalling 1 (SOCS1)(Wang et al. 2014). LTs have also been shown to augment cytotoxic activity and enhance production of factors like IL-1β, TNF-α and hydrogen peroxide from macrophages thereby boosting macrophage ability to clear pathogens (Gagnon et al. 1989).

Dendritic cells have been reported to possess the ability to make leukotrienes, due to their expression of the 5-LOX enzyme during differentiation from CD34+ progenitors and are found be the predominant cell type expressing 5-LOX in the epidermis in humans(Spanbroek et al. 2000). Cytokines like TGF-β1 enhance the expression of 5-LOX whereas IL-4 and IL-10 signalling result in its downregulation, thereby respectively increasing or decreasing the production of LTs (Spanbroek et al. 2001; Steinhilber, Radmark, and Samuelsson 1993).

LTs can regulate DC migration to the lymph node as shown by Robbiani et al, but can also influence cytokine production by dendritic cells(Robbiani et al. 2000). In one study LTD_4 was shown to increase IL-10 production, in contrast to this LTB_4 does not affect the production of IL-10 or IL-12p70 from DCs(Machida et al. 2004). In another study the blockade of CysLT1 receptor resulted in lower expression of IL-10 and IL-12p70 from DCs when stimulated in-vitro with LPS(Jozefowski et al. 2005). Leukotriene modulation of

dendritic cell cytokines has an effect on T cell differentiation and similar to PG's, leukotrienes also polarize towards type 2 responses (Jozefowski et al. 2005).

LTs are also able to impact T cells directly. LTB_4 has been shown to induce BLT1 receptor expression on TCR stimulated, CD4 and CD8 T cells via triggering intracellular Ca^{2+} mobilization(Romaniuk 2008). Furthermore, the BLT1 receptor was expressed substantially on effector cells compared to naïve and memory cells, and signalling through the BLT1 receptor functions as a chemoattractant, recruiting T cells to the site of inflammation(Goodarzi et al. 2003). In severe eosinophilic asthma, LTE_4 synergises with PGD_2 to enhance recruitment and activation of Tc2 cells in a TCR independent manner(Hilvering et al. 2018). Likewise, LTE_4 and PGD_2 synergy has been shown to drive Th2 functions and also induced the production of diverse nonclassical Th2 inflammatory mediators like IL-22, IL-8 and GM-CSF(Xue et al. 2015). These studies show that leukotriene mediated effects differ depending on the site of infection, receptors it signals through, synergy with other lipid mediators and can generate both anti-inflammatory and pro-inflammatory responses.

5.1.6 Lipid mediators in the liver

Prostaglandins are produced by various liver cell types in homeostasis and also have been detected in the inflamed liver(Kuiper et al. 1988). The importance of PGE_2 has also been evaluated in a murine model of non-alcoholic steatohepatitis (NASH), in which microsomal PGE synthase (mPGES-1) deficient mice have augmented liver inflammation compared to WT mice(Henkel et al. 2018) . The ability of the liver to regenerate after surgery has been linked to increased PG release and in combination with other factors like nitric oxide (NO) triggers liver regeneration(Schoen Smith and Lautt 2005) .

Furthermore, PGs can play a protective role in ischemic reperfusion injury by limiting the production of reactive oxygen species and improving hepatic lipid and insulin metabolism(Hossain et al. 2006) . Another protective role of PGE_2 in the liver involves the induction of HSC apoptosis to limit liver fibrosis by downregulating microRNAs(Brea et al. 2018) . Conversely, prostaglandins also drive liver cancer development, one mechanism of PGE_2 driven liver cancer involves the upregulation of fuse binding protein 1 (FBP1) expression which increases cancer cell viability(Ma et al. 2013) . Hepatic PGE_2 levels in decompensated cirrhosis patients increase by seven times compared to healthy patients and are partly responsible for the cirrhosis-mediated immune suppression(O'Brien et al. 2014) .

In addition to LT production by liver-resident cells, CysLT can be taken up from the circulating blood and are shown to accumulate in the liver. Moreover, liver infiltrating inflammatory cells can synthesis LTs adding to the liver LT reservoir(Keppler et al. 1985). There is limited information on how leukotrienes impact T cells in the liver; however, their ability to modulate dendritic cells and phagocytes would have consequences for naïve T cell differentiation as described in other settings(Lone and Taskén 2013) . None the less, more broader effects of leukotrienes are defined through blocking experiments in rat models of liver injury (*induced by bile duct ligation*). These experiments showed significant improvement of histopathological changes and also suppressed vascular endothelial growth factor (VEGF), transforming growth factor b (TGF-β), tissue inhibitor of metalloproteinase (TIMP-1) and nuclear factor kappa b (NF-$\kappa\beta$) expression when LT function was abrogated (EL-Swefy and Hassanen 2009) .

The negative effects of leukotrienes in acute liver failure have been demonstrated in a study that showed the blockade of the 5-LOX pathway, this attenuated acute liver failure

by inhibiting macrophage activation(Li et al. 2014). Like, PGs, LTB_4 is also involved in the initial stages of liver regeneration and inhibition of the 5-LOX enzyme resulted in the delayed activation of the initial liver regeneration events, indicating a protective function of LTB_4 in the liver(Lorenzetti et al. 2019) . In general leukotrienes in the liver negatively impact liver disease outcomes; although, in some instances, facilitate liver protection. More research is required to understand the impact of leukotrienes on different immune cell populations of the liver.

Despite our knowledge of eicosanoids in multiples diseases, including some liver diseases there is no understanding of how eicosanoids impact liver T cell populations. Moreover, as both leukotrienes and prostaglandins are synthesised by multiple cell types in the liver, it is important to understand how they influence MAIT cells, an important population enriched in the liver. We aimed to investigate the impact of PGs and LTs on MAIT cell activation and function.

5.2 Aims

1. To study the effect of exogenous prostaglandins and leukotrienes on MAIT cell activation
2. Identify receptors used by PGD_2 and PGE_2 to signal to MAIT cells
3. Define the outcome of PGE_2 and PGD_2 on different MAIT cell activation pathways
4. To investigate whether prostaglandin mediated inhibition is specific to MAIT cells or impacts multiple cell types
5. To determine if PGE_2 and PGD_2 suppress MAIT cells directly or indirectly

5.3 Materials and methods

5.3.1 Prostaglandin and leukotriene treatment in MAIT cell activation assays

MAIT cells were activated with various stimuli (THP-1+*E*. coli, IL-12+IL-18 or TCR+cytokine) as described in chapter 2.5.1/2.5.2 and incubated with PG's or LTs. 10-fold dilutions (*prepared in PBS*) of concentrations between 10^{-5}-10^{1}M or concentrations for PGs and LTs mentioned in table 5.1 were added to activation assays. Cells were then incubated at 37°C for 16 hours with BFA and monensin added for the last 4 hours and stained for flow cytometry analysis.

	Concentration used	**Supplier**
Leukotrienes		
LTB_4	Titration (Fig.5.1b)	Santa Cruz Biotechnology
LTE_4	Titration (Fig. 5.1b)	Santa Cruz Biotechnology
Prostaglandins		
PGD_2	1μM/titration	Chem Cruz
PGE_2	100nM/titration	Tocris bioscience
PGF_{2a}	Titration or 1μM	Sigma Aldrich co.
PGJ_2	Titration or 1μM	Cayman chemicals
PGI_2	Titration or 1μM	Cambridge Bioscience Ltd

Table 5.1- Leukotrienes and prostaglandins used with the concentrations used in assays other than the titration assays.

5.3.2 Receptor blockade experiments

MAIT cell activation assays with THP-1+ *E*. coli were set up as described in chapter 2.5.1, with the addition of receptors antagonist (concentrations mentioned in table 5.2 individually or in combination (*as specified on graphs axis in figure 5.2*) for 30 minutes before adding 100nM PGE_2 or 1µM PGD_2.

Receptor	Chemical name	concentration used	Supplier
DP1	BW868c	5µM	Cambridge Bioscience Ltd
DP2	OC000459	5µM	Cambridge Bioscience Ltd
EP1	SC 51089	5µM	Tocris Bioscience
EP2	PF04418948	5µM	Tocris Bioscience
EP3	L-798,106	5µM	Cambridge Bioscience Ltd
EP4	ONO AE3 208	5µM	Tocris Bioscience

Table 5.2 – PGD_2 and PGE_2 receptor antagonist and the concentration used in receptor blockade assays.

5.4 Results

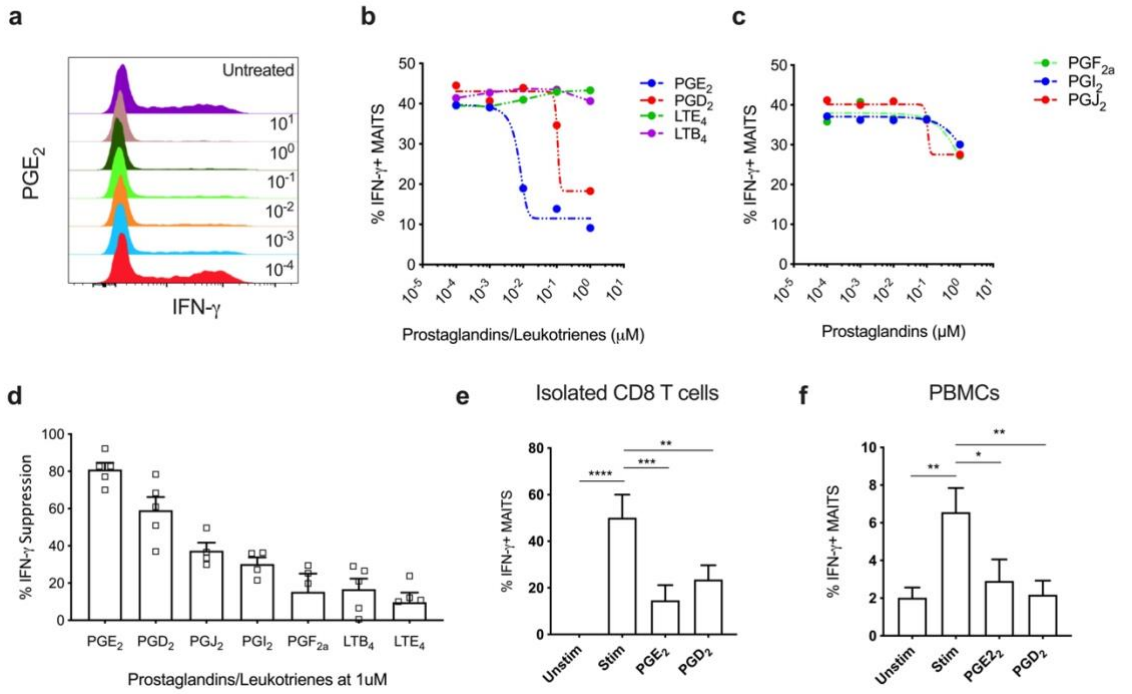


Figure 5.1 - Effect of lipid metabolites on MAIT activation.

a) Representative histogram of PGE_2 titration on *E.* coli stimulated THP-1 activated CD8 MAIT cells. b) Prostaglandins, PGD_2, PGE_2, leukotrienes, LTB_4, LTE_4, and c) prostaglandins PGI_2, PGJ_2 and PGF_{2a}, were titrated onto THP-1+ *E*. coli activated MAIT cells in a 16-hour assay. IFN-γ expression was measured from CD8+CD161++Vα7.2+ MAIT cells by flow cytometry, *(n=8)*. d) % IFN-γ suppression from THP-1+ *E*. coli stimulated MAIT cells treated with 1µM of prostaglandins and leukotrienes. e) *E.* coli stimulated healthy donor PBMCs -/+ prostaglandins, IFN-γ expression measured from CD8+CD161++Vα7.2+ MAITs, *(n=4)*. f) Stimulation of healthy donor isolated CD8 T cells by THP-1+ *E*. coli. IFN-γ expression measured from CD8+CD161++Vα7.2+ MAITs, *(n=4)*. Statistical significance - Dunnett's multiple comparisons test, all groups compared to the stimulation group, *P<0.03, **P<0.009, ***P<0.0004, ****P<0.0001.

5.4.1 MAIT cell activation is suppressed in a dose dependent manner by prostaglandins PGE_2 and PGD_2 but not by leukotrienes

Since lipid metabolites are produced by multiple cell types in the liver, we tested the impact of prostaglandins and leukotrienes on MAIT cell activation and phenotype to various modes of stimulation. For MAIT cells activated with *E. coli* stimulated THP-1 cells we observed a dose dependent suppression of IFN-γ by prostaglandins but not by leukotrienes (Fig. 5.1a-c). PGE_2 suppression of MAIT cell IFN-γ was more potent compared to PGD_2 and to other prostaglandins/leukotrienes that were tested (Fig. 5.1d). The presence of multiple cell types in the PBMC model (where PBMCs were stimulated with *E. coli* instead of isolated CD8 T cells) did not change the suppressive capacity of PGE_2 and PGD_2; however, the suppression was greatest when MAIT cells, from isolated CD8 T cells, were activated with *E. coli* stimulated THP-1 cells (Fig. 5.1 e/f). This suggests that PGE_2 and PGD_2 potently suppresses many cells types possibly by suppressing both the cytokine and MR1 pathways needed to stimulate MAIT cells.

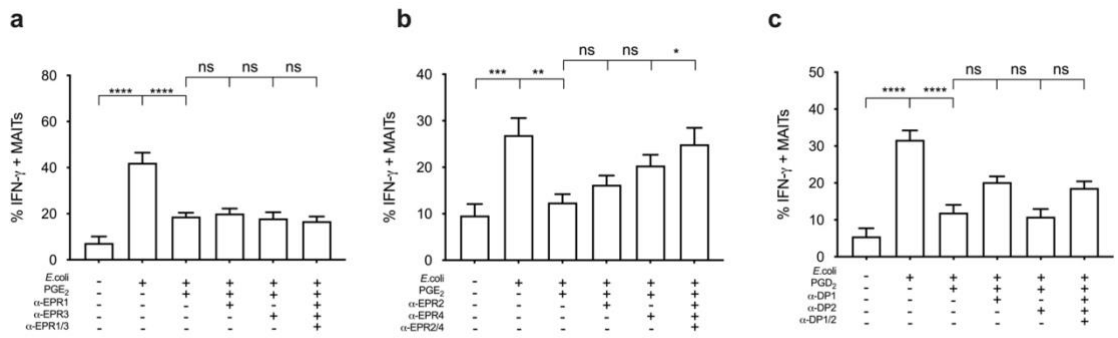


Figure 5.2 - Prostaglandin receptor antagonists used to determine receptor subtypes used by PGE_2 and PGD_2 for signaling.

MAIT cells were stimulated with THP-1+ *E*. coli and treated with synthetic PGE_2 and PGD_2 analogs. Receptor antagonists were added 30 minutes prior to prostaglandin treatments. THP-1+ *E*. coli activated MAIT cells treated with PGE_2/PGD_2 +/- receptor antagonist for EP1-4/DP1-2 individually or in combination. a) IFN-γ expression from stimulated MAITs cells treated with EP1/EP3 receptor antagonists, b) IFN-γ expression from stimulated MAITs cells treated with EP2/EP4 receptor antagonists, *(n=8)* and c) IFN-γ expression from stimulated MAITs cells treated with DP1/DP2 receptor antagonists, *(n=11)*. Statistical significance - Sidak's multiple comparisons test, *P<0.0165, **P<0.0038, ***P<0.0004, ****P<0.0001, ns = non-significant.

5.4.2 MAIT cell suppression by PGE_2 and PGD_2 is mediated though the EP2, EP4 and DP1 receptors, respectively

Prostaglandins can have multiple receptor subtypes, thus allowing for differential effects by the same prostaglandin. To determine the receptors used by PGD_2 and PGE_2 for signalling, we used receptor antagonist for DP1/2 and EP1-4, respectively. Receptor antagonists were added individually and in combination to MAIT cell activation assays 60 minutes prior to adding *E. coli*. PGE_2 uses two receptor subtypes, EP2 and EP4 to signal, and PGD_2 uses only DP1 to signal (Fig. 5.2). Receptor antagonist for EP1, EP3 and DP2 did not prevent suppression of MAIT IFN-γ (Fig 5.2b).

The use of combined receptor antagonist for DP1 and DP2 failed to block PGD_2 signalling completely. This may be a result of signalling through an alternative receptor/s. Similarly, the use of EP2 and EP4 receptor antagonist individually, were inadequate in preventing PGE_2 signalling; however, PGE_2 signalling was completely blocked with the combination of EP2 and EP4 receptor antagonism (Figure 5.2).

To specify further which cell types each of these receptor subtypes were present on, we used preliminary data for gene expression analysis of RNA-seq data, generated in Chapter 4 for THP-1 cells and for MAIT cells sorted from healthy donor blood (Leng et al. 2019). PGE_2 receptors, EP2 and EP4 were present on both MAIT cells and the THP-1 cell line. The PGD_2 receptor DP1 gene expression was only found in MAIT cells (Supplementary Fig. 8 and Supplementary table 1).

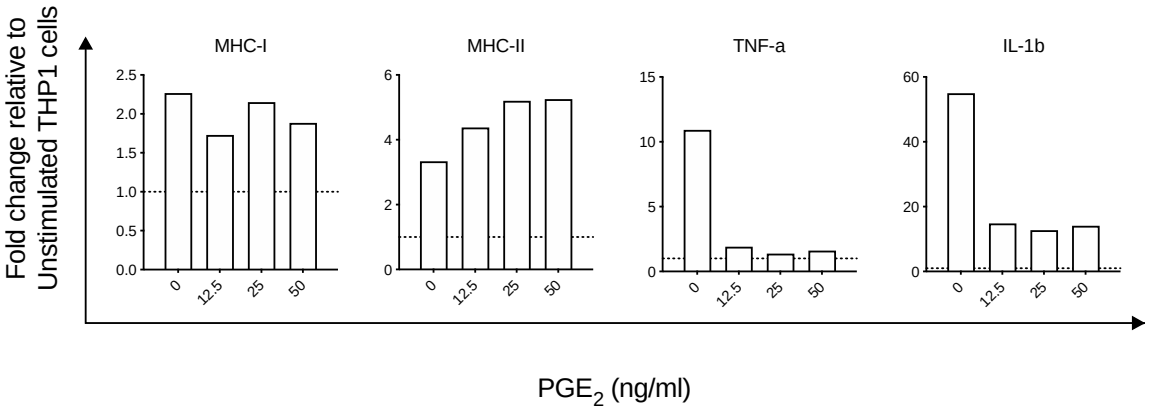


Figure 5.3 - Phenotype of *E.* coli stimulated THP-1 cells treated with PGE_2.

MHC-I, MHC-II, TNF-α and IL-1β gene expression was measured by qPCR. Bar charts show fold change difference in genes relative to the expression in unstimulated and untreated THP-1 cells.

5.4.3 PGE_2 suppresses the proinflammatory capacity of *E. coli* stimulated THP-1 cells, polarizing them towards an M2 phenotype

As discussed above, prostaglandins and their receptors are found on many cell types, including T cells and antigen presenting cells. Thus, giving prostaglandins the ability to mediate its effects directly on T cells as well as indirectly through antigen presenting cells. We studied the effect of different concentrations of PGE_2 on the pro-inflammatory phenotype of *E. coli* activated THP-1 cells. Low concentrations of PGE_2 were able to strongly suppress the ability of THP-1 cells to express TNF-α and IL-1β. These cytokines are potent pro-inflammatory cytokines associated with an M1 macrophage phenotype. PGE_2 also inhibits the expression of the MHC-I antigen presenting molecule, but

enhanced the expression of MHC-II. Together, this suggests that PGE_2 skews *E. coli* stimulated THP-1 cells away from an M1, proinflammatory, phenotype and towards a more M2-like phenotype.

5.4.4 PGE_2 and PGD_2 suppressed antigen specific cytokine production, resulting in a failure to prime T cells and provide co-stimulatory support for TCR signalling

MAIT cells can be activated by cytokines alone or through ligands presented by the MR1 molecule present on antigen presenting cells. We used different modes of stimulation to investigate whether the TCR or cytokine signalling was affected by PGE_2 and PGD_2. We found that PGE_2 and PGD_2 were able to significantly suppress the expression of IFN-γ, TNF-α and GrB expression from MAIT cells activated by *E. coli* stimulated THP-1 cells (Fig. 5.4 a, b, d). Similarly, both these prostaglandins suppressed IFN-γ and TNF-α, but not GrB expression from α-CD3 α-CD28 +IL-12/IL-18 stimulated MAIT cells (Fig. 5.6). In contrast to the latter two methods of stimulation, PGE_2 and PGD_2 was not able to suppress any of these markers in IL-12+IL-18 stimulated MAIT cells (Fig 5.5). These results suggest that prostaglandins maybe suppressing TLR induced cytokine production as would be expected with LPS from *E. coli,* which signals via TLR4 to induce IL-12. Cytokines are necessary for the co-stimulation of the TCR signalling as well as priming of T cells. Thus, the loss of these key cytokines would limit MAIT cell activation.

CD69 expression was not suppressed by PGE_2 or PGD_2 in any of the stimulations and instead was enhanced on cytokine activated MAIT cells by PGE_2 (Fig. 5.5c). As CD69 expression on antigen activated MAIT cells is mainly TCR induced, and since it is not inhibited by PGE_2 or PGD_2, this strongly indicates that the TCR signalling is not affected.

Another indication that the TCR signalling is not affected by PGE_2 and PGD_2 is their inability to suppress CD69 and GrB from MAIT cells activated by α-CD3+CD28 + IL-12/IL-18 (Fig 5.6). Taken together, the differences in PGE_2 and PGD_2 mediated suppression on different stimulations strongly implies the inhibition of cytokine production by antigen presenting cells, with a high possibility to induce anti-inflammatory cytokines that can repress MAIT cell activation both directly and indirectly.

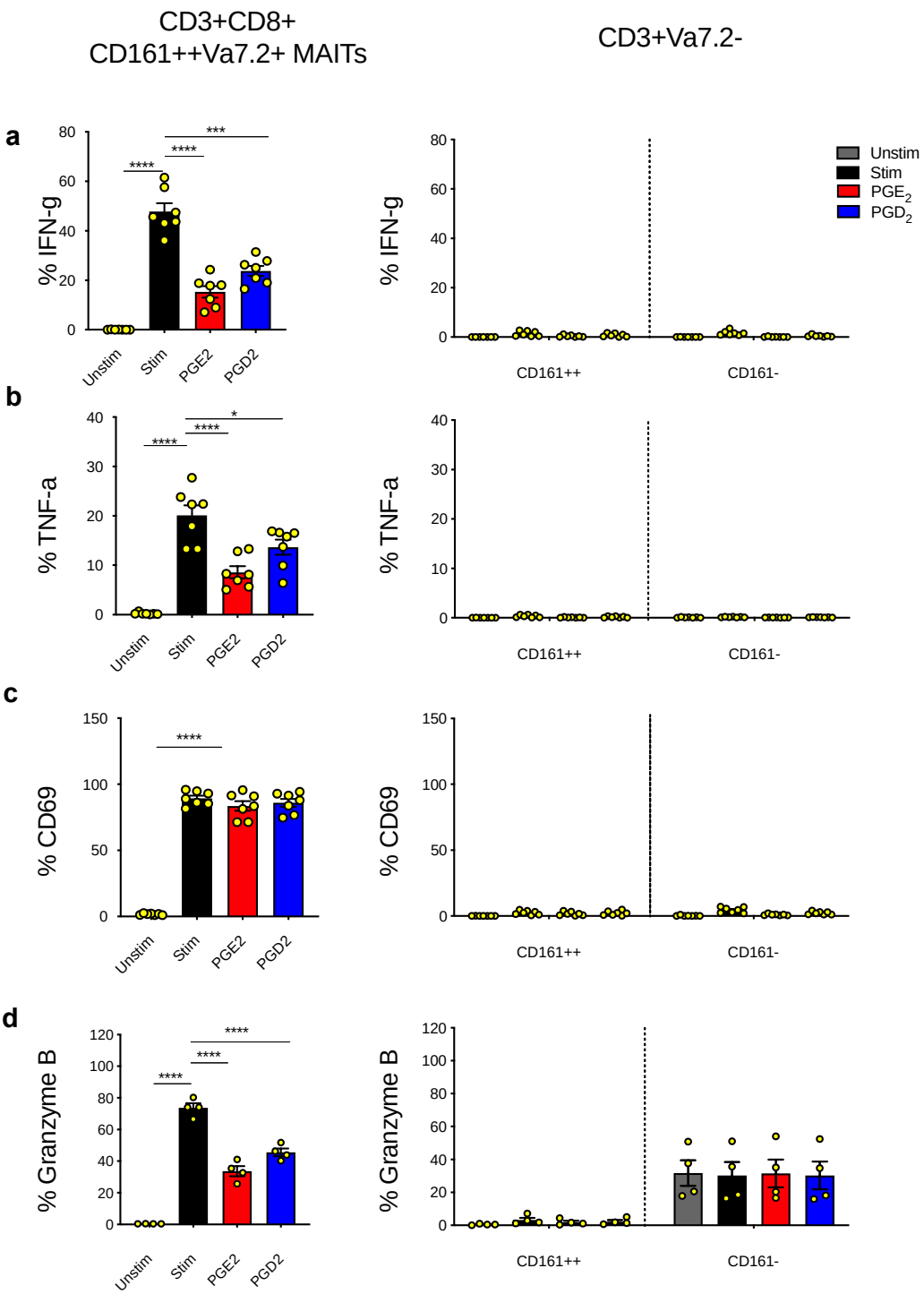
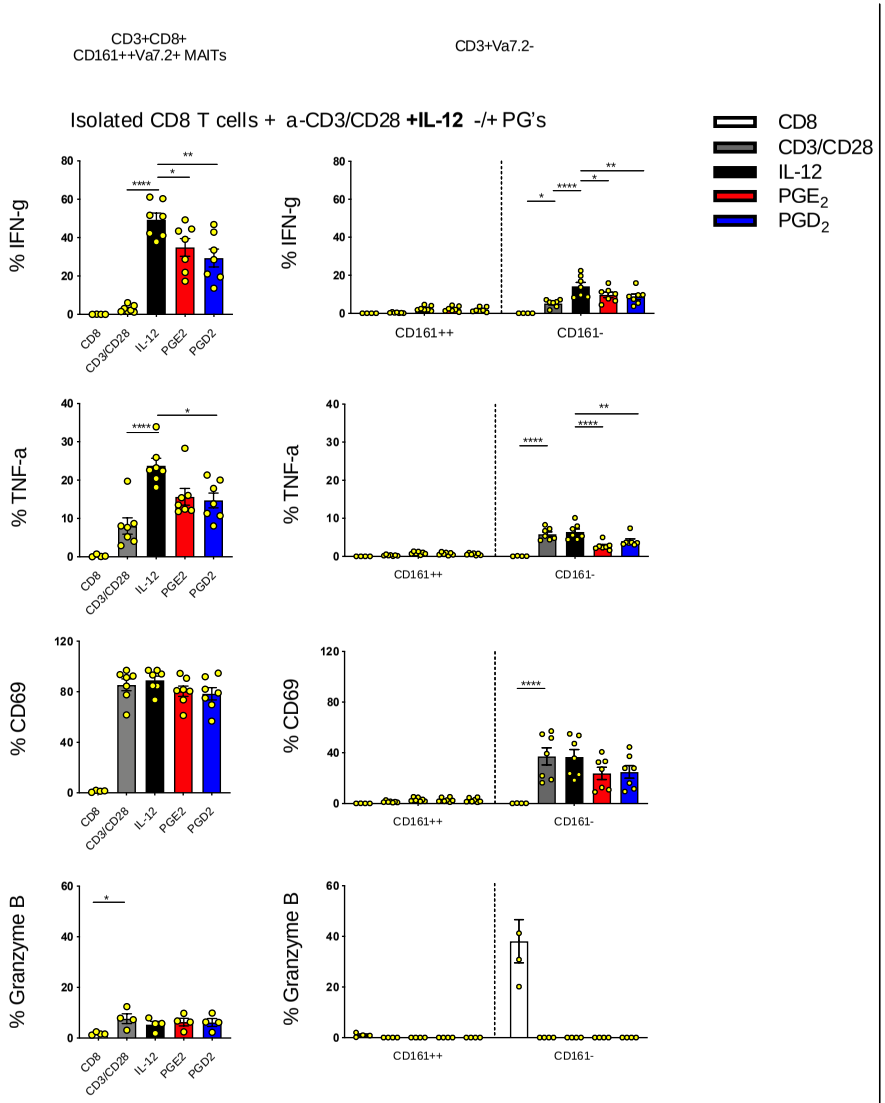


Figure 5.4 - Impact of PGD_2 and PGE_2 on THP-1+ *E.* coli stimulated CD8 MAIT cells and Vα7.2- T cells.

CD8 T cells isolated from healthy donor blood were stimulated with THP-1+ *E*. coli and treated with PGD_2 or PGE_2 for 16 hours before analyzing by flow cytometry. a) IFN-γ, b) TNF-α, c) CD69, *(n=7)* and d) GrB expression, *(n=4)*, was measured from CD8+CD161++Vα7.2+

MAITs and CD3+Vα7.2- CD161++/CD161- T cells. Statistical significance - Dunnett's multiple comparisons test, all groups compared to the stimulated group. *P<0.04, **P<0.003, ***P<0.0007, ****P<0.0001.


CD3+CD8+
CD161++Va7.2+ MAITs
CD3+Va7.2-
Isolated CD8 T cells + a-CD3/CD28 +IL-12 -/+ PG's
CD8
CD3/CD28
IL-12
PGE2
PGD2
% IFN-g
% TNF-a
% CD69
% Granzyme B
CD161++
CD161-

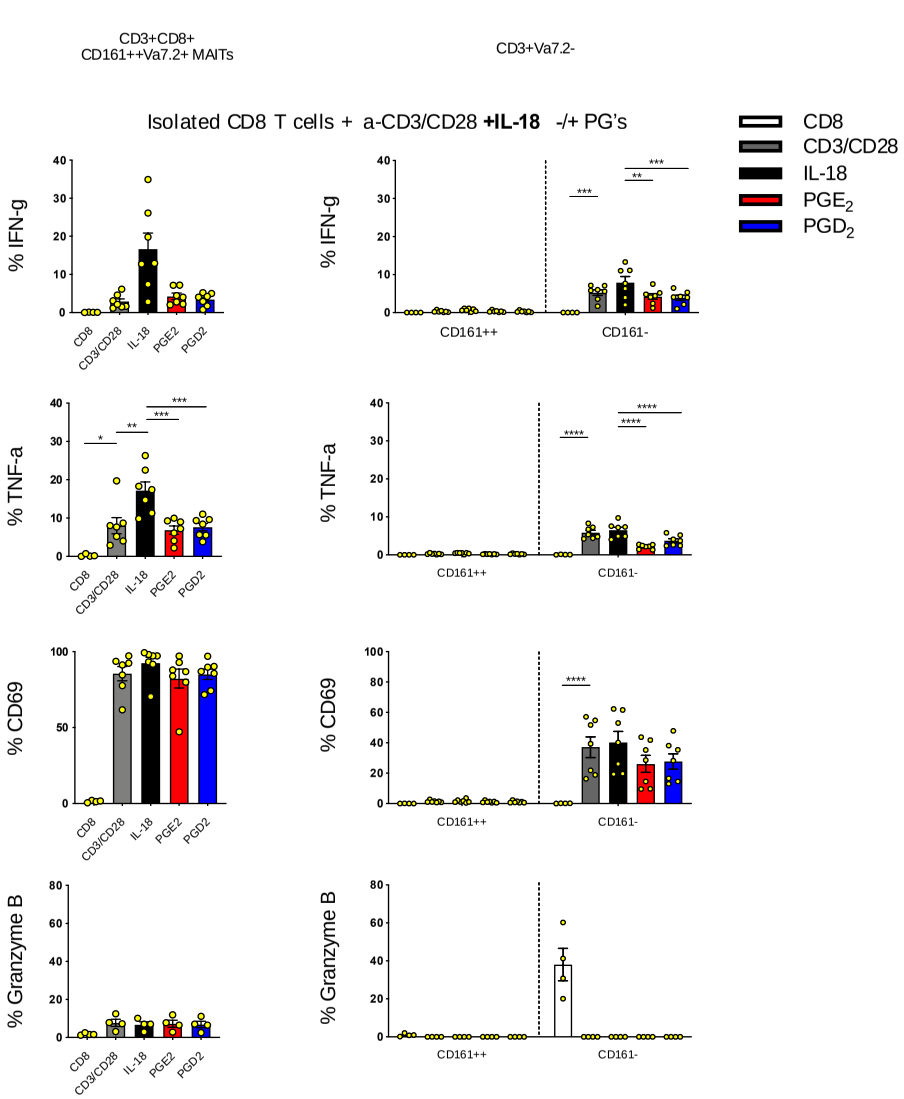

CD3+CD8+
CD161++Va7.2+ MAITs
CD3+Va7.2-
Isolated CD8 T cells + a-CD3/CD28 +IL-18 -/+ PG's
CD8
CD3/CD28
IL-18
PGE2
PGD2
% IFN-g
% TNF-a
% CD69
% Granzyme B
CD161++
CD161-

Figure 5.5 - Impact of PGE_2 and PGD_2 on TCR pathway.

CD8 T cells isolated from healthy donor blood were stimulated with α-CD3/CD28 +IL-12 (left) / +IL-18 (right) and treated with PGD_2 or PGE_2 for 16 hours before analyzing by flow cytometry. a) IFN-γ, b) TNF-α, c) CD69, *(n=7)*, and d) GrB, *(n=4)*, expression was measured from CD8+CD161++Vα7.2+ MAITs and CD3+Vα7.2- CD161++/CD161- T cells. Statistical significance - Dunnett's multiple comparisons test, all groups compared to the stimulated group. *P<0.0439, **P<0.005, ***P<0.0009, ****P<0.0001.

5.4.5 Suppressive effects of PGE_2 and PGD_2 are significant for MAIT cells in comparison to MAIT-related and CD161- populations

To explore the effect of PGE_2 and PGD_2 on MAIT-related and unrelated, conventional T cells, we analysed responses from Vα7.2-, CD161 bright and CD161 negative cells. We found that these cells responded similarly to MAIT cells in most conditions, with a few differences. Firstly, the Vα7.2-CD161++ cells were not able to make GrB in response to any of the three stimulation conditions used. Vα7.2-CD161++ cells only produced TNF-α in responses to *E. coli* stimulated THP-1 (Fig. 5.4b). We observed limited TNF-α expression in the Vα7.2-CD161- cells stimulated with cytokines or *E. coli* stimulated THP-1 cells, on the other hand TCR stimulation in combination with IL-12/IL-18 was able to induce higher amounts of TNF-α (Fig. 5.4/5.6). Vα7.2-CD161++ cell did not respond to TCR+cytokine stimulation, but did express IFN-γ, TNF-α and CD69 expression in response to THP-1+*E. coli* and cytokine stimulation. Despite differences in the responsive capacity of these Vα7.2- CD161++ and CD161- cells to different stimulations we noted a similar suppressive effect exerted by PGE_2 and PGD_2. We saw a significant suppression of IFN-γ expression in the CD161- compartment of the Vα7.2- T cells stimulated with α-CD3/CD28 +IL-12/18 (Fig.5.5). Unlike the MAIT cells, PGE_2 and PGD_2 suppressed CD69 expression from the CD161- compartment but not the CD161++ Vα7.2- T cells (Figs. 5.4-5.6) for both the THP-1+*E. coli* stimulation as well as the TCR+cytokine stimulation, and enhanced the expression in both the bright and negative compartment for IL-12+IL-18 stimulated Vα7.2- T cells (Fig. 5.5c). For TCR+cytokine stimulated Vα7.2- T cells, IFN-γ and TNF-α expression was suppressed significantly in the CD161- compartment (Fig. 5.5).

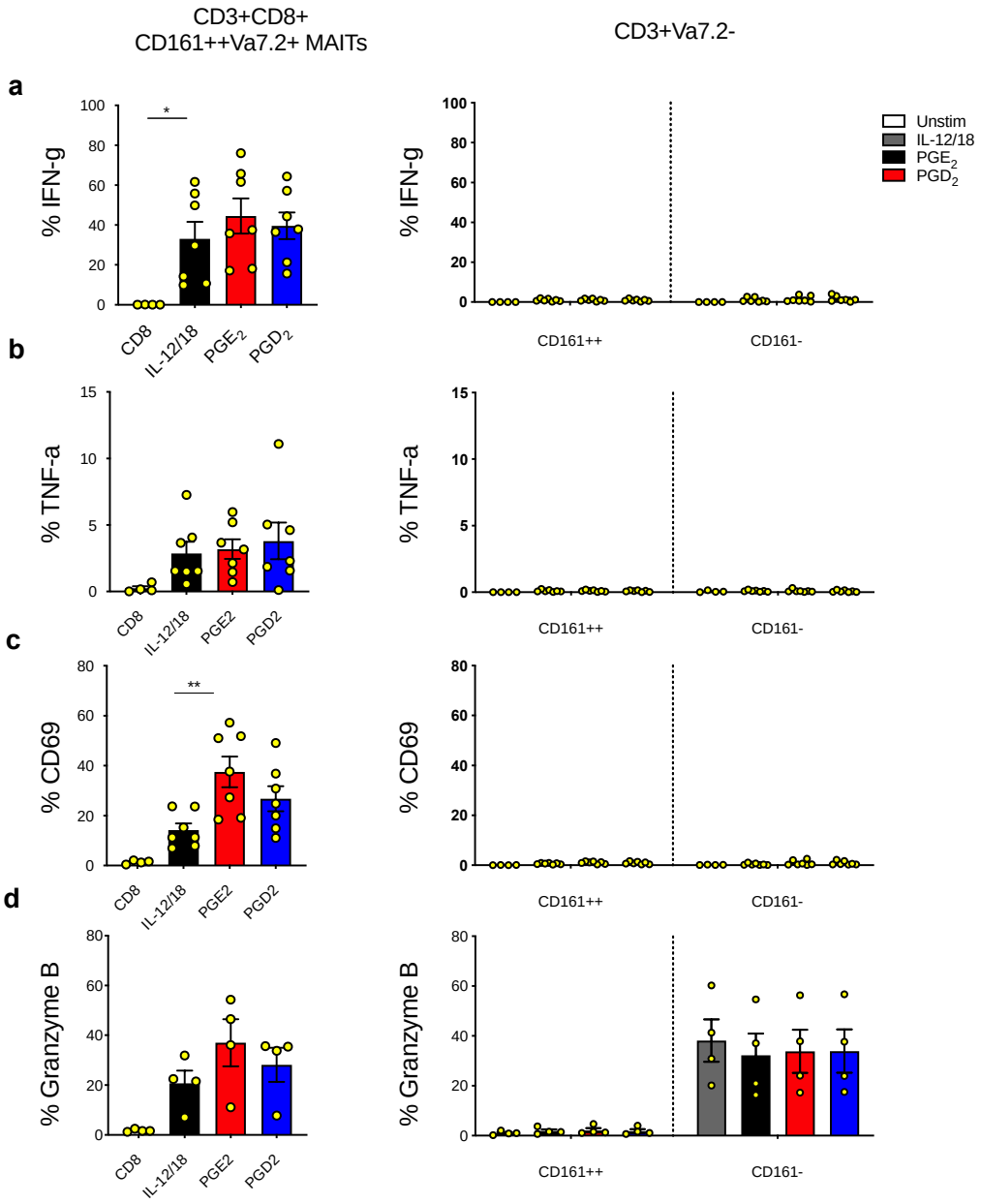


Figure 5.6 - Effect of PGE_2 and PGD_2 on cytokine (IL-12+IL-18) stimulated CD8 MAIT cells and Vα7.2- T cells.

CD8 T cells isolated from healthy donor blood were stimulated with IL-12+IL-18 and treated with PGD_2 or PGE_2 for 16 hours before analyzing by flow cytometry. a) IFN-γ, b) TNF-α, c) CD69, *(n=7)* and d) GrB expression, *(n=4)*, was measured from CD8+CD161++Vα7.2+ MAITs and CD3+Vα7.2- CD161++/CD161- T cells. Statistical significance - Dunnett's multiple comparisons test, all groups compared to the stimulated group. *P<0.04, **P<0.004

5.4.6 Prostaglandins can inhibit MAIT cell IFN-γ and TNF-α directly.

The expression of prostaglandin receptors on both antigen presenting cells and T cells, allows them to regulate the cell directly on each cell type. To study the direct impact of PGE_2 and PGD_2 on MAIT cells we stimulated purified CD8 T cells with CD3/CD28 antibodies + IL-12 or IL-18 in the absence of THP-1 cells. We found that PGE_2 and PGD_2 could still suppress MAIT cell IFN-γ and TNF-α expression (Fig. 5.6) indicating the ability of these prostanoids to modulate MAIT cells directly. We investigated PGE_2 and PGD_2 receptor gene expression on both THP-1 and MAIT cells using RNA-sequencing data, indeed MAIT cells and THP-1 cells express EP2 and EP4 whereas the DP1 receptor was only expressed on MAIT cells (Supplementary Fig. 8 and table 1). Moreover, the ability of PGE_2 to enhance CD69 and GrB in cytokine activated MAIT cells, in the absence of TCR signalling /APCs, also shows direct modulation of MAIT cells by PGE_2. Unlike the suppressive effect of PGE_2 on antigen and TCR activated MAIT cells, PGE_2 enhanced CD69 and GrB expression in cytokine stimulated MAIT cells, therefore enhancing the function of activated MAIT cells but inhibiting antigen specific activation of MAIT cells.

5.5 Discussion

In the previous chapter we showed that hepatoma cell lines can make prostaglandins; furthermore, we showed the ability of supernatants from these lines to suppress MAIT cell activation by *E. coli* stimulated THP-1 cells. Suppression could be inhibited by treating hepatoma cells with COX-1/2 inhibitors. Initially hepatocyte derived prostaglandins were the obvious candidates. However, blockade of PGE_2 receptors in the supernatant assays could not prevent suppression. Nonetheless, the fact that hepatoma cell lines make prostaglandins demanded the need to investigate the impact of lipid metabolites on MAIT cell activation and function.

PGE_2 is a widely studied lipid metabolite involved in many physiological processes and plays an important role in regulating immune responses(Sreeramkumar, Fresno, and Cuesta 2012). While the effects of PGD_2 are established from brain studies(Ogorochi et al. 1984) and colonic disease(Le Loupp et al. 2015) as well as in the airway(Domingo et al. 2018). PGE_2 production and its role in liver homeostasis, inflammation and immune regulation are described in various studies. PGE_2 was originally defined as an anti-inflammatory mediator and more recent studies have suggested it can also exert pro-inflammatory effects. Prostaglandin mediated effects are highly context dependent and further controlled by specific receptor signalling.

Our results show a dose dependent suppression of MAIT cell activation by all five prostaglandins tested, but not by LTB_4 and LTE_4. Identifying prostaglandins, as direct modulators of MAIT cell activation and function. This was not surprising as PGE_2 has

been shown to suppress effector function of γδ T cells(Martinet et al. 2010); another innate like T cell abundant in the liver. It is important to note that the suppressive concentration of PGE_2 and PGD_2 correlates with levels found in tumour settings and a high concentration of PGE_2 in the portal veins of colorectal cancer patients has been shown to suppress liver associated immunity, whereas physiological levels (pg/ml) of PGE_2(Chen et al. 2018) do not have a suppressive effect on MAIT cells. This also means that PGE_2 produced under homeostatic conditions in the liver is unlikely to prevent MAIT cell activation, but in hepatocellular carcinoma or other liver diseases where PGE_2 concentrations increase significantly(Henkel et al. 2018; Loo et al. 2017; Peltehian et al. 1996), suggests PGE_2 could suppress MAIT cell activation and function. The loss and exhaustion of MAIT cells in tumours has been associated with poor outcome(Toubal et al. 2019) which may be attributed to increased PGE_2 levels in the tumour microenvironment.

PGE_2 and PGD_2 do not prevent DC cell maturation. In fact, both prostanoids enhance endocytic and antigen presenting properties, but impair the ability of MDDC to produce pro-inflammatory cytokines, skewing T cell differentiation toward Th2 and Th17 cells(Gosset et al. 2005; Harizi et al. 2003). IFN-γ expression by MAIT cells is highly dependent on pro-inflammatory cytokines like IL-12 and IL-18 secreted from APCs. PGE_2 is known to suppress production of inflammatory factors like IL-12 and TNF-α by monocytes, macrophages and DCs(Smyth et al. 2009). Thus, can indirectly inhibit MAIT cell expression of IFN-γ by suppressing pro-inflammatory cytokine production by *E. coli* stimulated THP-1 cells. This indirect mechanism of MAIT cell suppression by PGE_2 via the APC is supported by our data. We have shown the suppression of TNF-α and IL-1β gene

expression from THP-1 cells stimulated in the presence of PGE_2. Though not measured, it highly likely that both IL-12 and IL-18 secretion by THP-1 cells is inhibited by PGE_2, resulting in lower IFN-γ expression from antigen stimulated MAIT cells in the presence of PGE_2 and PGD_2. In addition, PGE_2 and PGD_2 could not supress activation of cytokine primed MAIT cells. Therefore, cytokine signalling to MAIT cells must be limited by PGE_2 and PGD_2 in the antigen stimulation model. Moreover, PGE_2 treatment of *E. coli* activated THP-1 cells resulted in increased MHC-II but reduced MHC-I; thus, favouring antigen presentation to CD4 T cells instead of CD8 T cells in the presence of PGE_2. In vivo, PGE_2 stimulated CD4 T cells differentiate into regulatory T cells that are able to suppress CD8 T cells(Baratelli et al. 2005). Though we studied the effects of PGE_2 and PGD_2 in a two-cell model, their effect in PBMCs did not change. The latter suggesting that in vivo, PGE_2 induced regulatory T cell mediated modulation may also be functional and thus may regulate MAIT cell function.

Both PGE_2 and PGD_2 were able to suppress MAIT cell IFN-γ and TNF-α from TCR+cytokine activated MAIT cells, but not CD69 and GrB. Prostaglandin suppression of MAIT cells in the absence of APCs demonstrates direct modulation of MAIT cells. EP2 and EP4 signalling has been associated with increased exhaustion in CTLs during chronic viral infection and cancer(Sander, O'Neill, and Pohl 2017; Wang and Dubois 2006). Higher levels of PGE_2, and upregulation of EP2 and EP4, on virus-specific CTLs in a mice chronic lymphocytic choriomeningitis virus (LCMV) infection model was directly linked to the suppression of CTL survival and function(Chen et al. 2015). The expression of EP2, EP4 and DP1 receptors on MAIT cells make this possible where signalling through these receptors enhances cAMP levels(Ricciotti, A, and Fitzgerald 1998). The increased cAMP

level are able to potently suppress TCR mediated activation and function of effector T cells(Brudvik and Taskén 2012). Likewise, MAIT cell IFN-γ and TNF-α is most likely suppressed by this mechanism in our models. Lastly, Walker *et al* showed that PGE_2 can directly inhibit T cell IL-2 production and T cell responsiveness to IL-2, thus preventing expansion of antigen specific T cells(Vercammen and Ceuppens 1987). The reduced sensitivity to IL-2 could be an active mechanism limiting MAIT cell expansion during infection in the presence of prostanoids.

These results signify a net suppressive effect of prostaglandins on MAIT cell activation and function. Understanding the impact of prostaglandins on MAIT cell activation and function is important especially within the liver environment where MAIT cells are abundant. The liver microenvironment has significant levels of PGE_2 under homeostatic conditions, as well as during inflammation. It is produced by various liver cell types, like hepatocytes, Kupffer cells and liver DC's. However, neither PGE_2 nor PGD_2 were able to suppress cytokine primed MAIT cell activation and cytotoxic function . This finding could prove useful for therapy where cytokine primed MAIT/CD8 cells are used to overcome prostanoid mediated suppression. Overall, we demonstrated for the first time, that MAIT cells can be regulated by lipid metabolites.

5.6 Summary

1. Prostaglandins were capable of suppressing the pro-inflammatory and cytotoxic molecules from antigen stimulated MAIT cells
2. PGE_2 and PGD_2 were the most suppressive prostaglandins whereas leukotrienes did not have a significant suppressive effect on MAIT cell activation
3. Prostaglandins can skew the APC towards an M2 like phenotype thereby suppressing MAIT cells
4. The prostaglandin mediated suppression impacted mainly the MAIT cell population and not the non-MAIT CD161++ or negative populations

CHAPTER 6 – MAIT CELLS IN LIVER DISEASE

6.1 Introduction

T cells play an important role in the adaptive immune system, there are several types of T cells of which MAIT cells consist a significant population in the liver with unique properties of the innate immune system(Kurioka et al. 2016). The innateness of MAIT cells is characterized by their ability to respond rapidly to cytokine stimulation in a TCR independent manner (Jo et al. 2014; Ussher, Bilton, Attwod, Shadwell, Richardson, Lara, et al. 2014). MAIT cells respond to antigens presented by MR1, such as 5-OP-RU; a metabolite of the riboflavin synthesis pathway(Corbett et al. 2014; Kjer-Nielsen et al. 2012). As MAIT cells can produce pro-inflammatory cytokines and cytolytic effector molecules they are highly effective at responding to invading pathogens by clearing away infected cells and activating other immune cells(Godfrey et al. 2019; Magalhaes et al. 2020; Provine and Klenerman 2020). As the name suggests, MAIT cells are largely found at mucosal barriers but are also highly enriched in the liver, lungs and intestine(Kurioka et al. 2016; Treiner et al. 2003). The abundance of MAIT cells in the liver may not be surprising due to the large presence of innate and innate like cells in the liver; however, since MAIT cells make up to 20-50% of the total T cells in the liver, MAIT cells are believed to have a crucial role in the liver(Gao 2016; Kurioka et al. 2016). In 2016, Jeffery et al had shown that intrahepatic MAIT cells could be activated by bacteria exposed BECs and liver B cells, highlighting a potential for intrahepatic MAIT cell activation during liver infection(Jeffery, Van Wilgenburg, et al. 2016). Their importance has been reinforced by the extensive analysis of MAIT cells in liver disease(Magalhaes et al. 2020; Provine and Klenerman 2020). MAIT cells are involved in a broad range of infections where they have been shown to successfully resolve many

bacterial, fungal and parasitic infections but are also implicated in non-infectious disease such as autoimmune conditions and cancer(Chiba et al. 2018; Duan et al. 2019; Ling et al. 2016; Wong, Ndung'u, and Kasprowicz 2017; Yan et al. 2020). More recently, MAIT cells have been shown to possess the potential to exert tissue repair function, a feature that is particularly interesting in a highly regenerative organ like the liver(Hinks et al. 2019; Leng et al. 2019; Salou and Lantz 2019).

A significant amount of research on MAIT cells in liver disease has outlined both protective and pathological roles of MAIT cells in the liver(Atif, Warner, and Oo 2018; Bolte and Rehermann 2018; Duan et al. 2019; Li et al. 2018; Moa Sa, Sojeong Kim, Won Kim 2017; Toubal and Lehuen 2016; Zhang, Kong, and Wang 2020). Studies looking at MAIT cells in liver specific diseases have quantified MAIT cell numbers in the blood, showing a decline in the number of circulating MAIT cells. Some studies that have quantified MAIT cells in both the tissue and blood show loss of MAIT cells from the liver as well. However, in a study of MAIT cells from autoimmune hepatitis (AIH), specifically primary biliary cholangitis (PBC), are an exception to this as MAIT cell numbers declined in the circulation, but were higher in the liver compared to healthy controls(Jiang et al. 2018). Jiang et al report an increase in IL-7 levels in PBC, which promotes MAIT cell inflammatory cytokines and GrB through the induction of STAT5. In another study Setsu et al compared MAIT cells in PBC to MAIT cells from chronic viral hepatitis. They reported a persistent reduction in MAITs in PBC in both the blood and liver. MAIT cells from the blood of PBC patients expressed lower levels of IL-7 receptor and failed to produce TNF-α after IL-7 stimulation in-vitro. Furthermore, their study reports the inability to recover MAIT cells to normal levels with ursodeoxycholic acid despite the biochemical responses (Setsu et al. 2018). In primary sclerosing cholangitis (PSC) circulating MAIT cells had

declined but were localized and retained in the bile ducts. Similar to MAIT cells in other liver conditions, their functional capacity to *E*. coli and cytokine stimulation was also reduced in PSC(Seth et al. 2018).

Several studies looking at MAIT cells in alcohol related liver disease report a loss of MAIT cells which is associated with compromised anti-bacterial immunity (Li et al. 2019; Riva, V. Patel, et al. 2018). MAIT cells in the blood and liver of alcohol-related liver disease produced similar levels of cytokines like IFN-γ and TNF-α but produced significantly high levels of IL-17A in chronic alcoholics only (Marrero et al. 2020). In 2015, Hartmann reviewed the cross talk between the gut microbiome and the liver in alcoholic liver disease (ALD) which outlined both quantitative and qualitative changes in the intestinal microbiome which was later used to investigate how a leaky gut would impact MAIT cell function in ALD (Hartmann, Seebauer, and Schnabl 2015). The loss of MAIT cells and limited antimicrobial potency in alcoholic liver disease has been linked to a leaky gut. The leaky gut results in excessive contact with microbial products which render MAIT cells dysfunctional hence leading to increased bacterial infections and demonstrate the importance of retaining MAIT cells in order to prevent secondary infections(Riva, V. Patel, et al. 2018). A further study on MAIT cells in ALD shows a similar decline in MAIT cell numbers, higher plasma levels of LPS, CD14 and cytokines relevant for MAIT cell activation. The declining numbers of MAIT cells correlated with high expression of GrB, HLA-DR, CD69, CD38, and PD-1, this highly activated but exhausted state of MAIT cells decreased with alcohol abstinence but the numbers of MAIT cells did not fully normalize. The latter demonstrating that alcohol abuse can result in significant changes to MAIT cell frequencies which may be irreversible (Li et al. 2019).

In contrast, MAIT cells from non-alcoholic liver disease shows that blood-MAIT cells produce IL-4 and IL-10 and lower levels of IFN-γ and TNF-α compared to controls(Li et al. 2018). In non-alcoholic fatty liver disease (NAFLD), MAIT cells were shown to improve the condition by polarizing macrophages to an M2 phenotype. In contrast, mice deficient in MAIT cells exhibited severe hepatic steatosis and inflammation in response to methionine and choline deficient diet (MCD)(Li et al. 2018).

In autoimmune hepatitis, an enhanced MAIT cell cytotoxic function is observed, shown by increased GrB production by blood and tissue MAIT cells(Renand et al. 2018). IL-17 levels were also increased in blood-MAIT cells, but IFN-γ and TNF-α levels only increased in response to TCR stimulation(Böttcher et al. 2018). Autoimmune liver diseases are characterised by fibrosis and cirrhosis as a result of immune damage in the liver and MAIT cells are found to drive liver fibrosis by activating HSCs, this is in contrast to γδ T cells of the liver that induce HSC apoptosis thus protecting from liver fibrosis(Böttcher et al. 2018; Hegde et al. 2018). Two studies have demonstrated the capacity of MAIT cells to promote liver fibrosis, Hedge et al used patient samples in their study and confirmed a reduction in circulating MAIT cells. Additionally, they use a mouse model to highlight the accumulation of MAIT cells and fibrogenic cells in liver fibrotic septa(Hegde et al. 2018). Böttcher et al showed a reduction in MAIT cells in the periphery and the tissue of autoimmune liver disease (AILD) that is accompanied by impaired function and high ex-vivo expression of exhaustion markers(Böttcher et al. 2018). They also demonstrate the exhaustion of MAIT cells, likely induced by repetitive cytokine stimulation and, interestingly, this repetitive stimulation with IL-12 resulted in the

upregulation of profibrogenic IL-17A(Böttcher et al. 2018). This study also investigated the interactions between MAIT cells and HSCs in-vitro, and showed that MAIT cells induced activation and profibrogenic activity of HSCs in an IL-17 dependent manner(Böttcher et al. 2018).

To date, there are two studies conducted on MAIT cells in viral hepatitis with distinct differences in the MAIT cell responses and numbers. MAIT cells in chronic HBV patients were more activated as measured by the CD38 expression but were not depleted like in HIV or HCV infected individuals(Boeijen et al. 2017). In-vitro activation of MAITs from HBV patients reveals that they produce more GrB in response to *E*. coli stimulation in comparison to healthy controls and the highly activated MAIT cells could be normalized with nucleoside treatment. On the other hand, MAIT cell numbers in the blood were lower in patients with HCV infection with an inverse correlation between the frequency of intrahepatic MAIT cells. MAIT cells were also highly activated with high expression of PD-1 and TIM-3 indicating high exhaustion. The study also demonstrates the requirement for monocyte derived IL-18 to activate MAIT cells that were responsive to cytokines, but not to TCR mediated stimulation. Moreover, the intrahepatic MAIT cell frequency had increased after anti-viral therapy, however, anti-viral therapy did not improve TCR mediated MAIT cell stimulation(Bolte et al. 2017).

The role of MAIT cells in HCC has been assessed by a large study of 841 HCC samples obtained from surgical resections from 4 independent cohorts that were collected between 2006-2015. The study revealed that compared to normal hepatic tissue, MAIT cells are less frequent within the tumour, but like in other liver conditions are

dysfunctional as they produced significantly less IFN-γ, IL-17, very low amounts of GrB and perforin. Moreover MAIT cells in HCC express high levels of PD-1, TIM-3 and CTLA4, this exhaustive phenotype was accompanied by tumour producing cytokines such as IL-8. Transcriptional analysis of tumour-derived MAIT cells also suggests reprogramming of MAIT cells toward tumour promoting function with significant downregulation of NFKB1 and STAT5B. MAIT cell density in HCC correlated with unfavourable outcomes thereby indicating MAIT cell related pathology in HCC as a result of impaired anti-tumour but tumour promoting activity of MAIT cells (Duan et al. 2019).

Our focus has been to understand the role of MAIT cells in liver disease by analysing the function of MAIT cells in cirrhosis, HCC, and healthy liver. In addition to quantifying MAIT cells in healthy and diseased liver samples we phenotyped MAIT cells for exhaustion prior to investigating their functional capacity in vitro, to *E*. coli and cytokine stimulation. Unlike previous studies, we have also examined MAIT cells and MAIT-related populations in liver disease and further used the same dataset to identify novel disease specific populations based on spectral flow cytometry generated data, probing 25 parameters.

6.2 Aims

1. Quantify MAIT cells in healthy and liver disease
2. Determine MAIT cell phenotype in liver disease
3. Analyse liver-MAIT cell responses to antigen and cytokine stimulation
4. Compare MAIT cell responses to other related populations in HCC and CL disease
5. Identify disease specific populations using unsupervised analysis

6.3 Materials and methods

6.3.1 Samples - Healthy donor blood was acquired from NHS as described in chapter 2.3.1 and liver samples were kindly provided Professor Georg Lauer at the Harvard Medical School. Liver samples were collected under the IRB protocol #1999P004983 not requiring patient consent as only clinically deidentified tissue was collected. Liver tissue samples were from patients with non-viral pathologies, like alcohol-associated cirrhosis, diagnosis and other details for individual donors provided in supplementary table 2.

6.3.2 Sample processing/stimulation – Isolated blood and liver lymphocyte samples were processed by thawing in a 37°C water bath and dispensed immediately in R10 containing DNase I (Sigma Aldrich). Cells were then washed twice and resuspended in the same media at the desired concentration, plated and stimulated immediately with 30CFUs/cell *E*. coli or 50ng/ml of IL-12+IL-18 for 16 hours or 250ng/ml PMA/Ionomycin for 5 hours. BFA and monensin was added in for the last 4 hours.

6.3.3 Staining liver samples – Staining was performed in 96 well u bottom plates and all markers were stained in 50μls/well in specified buffers. First, cells were washed twice in PBS and stained with live dead blue dye (Invitrogen) in PBS for 15 minutes. Excess dye was removed by adding 200μls of PBS, centrifuging and removing the PBS; this process is used throughout to wash unstained antibodies. Cells were resuspended and then stained with MR1-Tetramer in PBS containing 1% FCS, for 20 minutes in the dark at RT. Unbound tetramer was washed with excess PBS and cells were then stained for extracellular markers in PBS for 20 minutes in the dark. After washing the unbound

antibodies, cells were fixed in 4% PFA for 10 minutes in the dark. PFA was washed out after 10 minutes and the cells were then permeabilised with perm solution for 20 minutes prior to intracellular staining. Cells were stained intracellularly for 30 minutes in perm buffer. Unbound antibodies were washed twice with PBS and then resuspended in 200μls of PBS and run immediately on the Aurora.

6.3.4 Single colour stains were prepared by incubating fluorescently labelled antibodies with anti-mouse IgG (R&D) and anti-RAT IgG (R&D) compensation beads for 10 minutes in PBS just before acquiring them. Samples were washed twice with PBS and resuspended in 100μls of PBS prior to acquiring. 10000 events were recorded for each dye.

6.3.5 Live dead samples were prepared by incubating 0.5M PBMCs or 0.1M liver lymphocytes in 200μls of R10 at 60oC for 10 minutes, followed by 1-minute incubation on ice. The sample was split in half, leaving some dead cell unstained and other half stained for live dead dye in PBS for 10 minutes. A sample proportion of cells from each sample was left unstained as a control for autofluorescence correction.

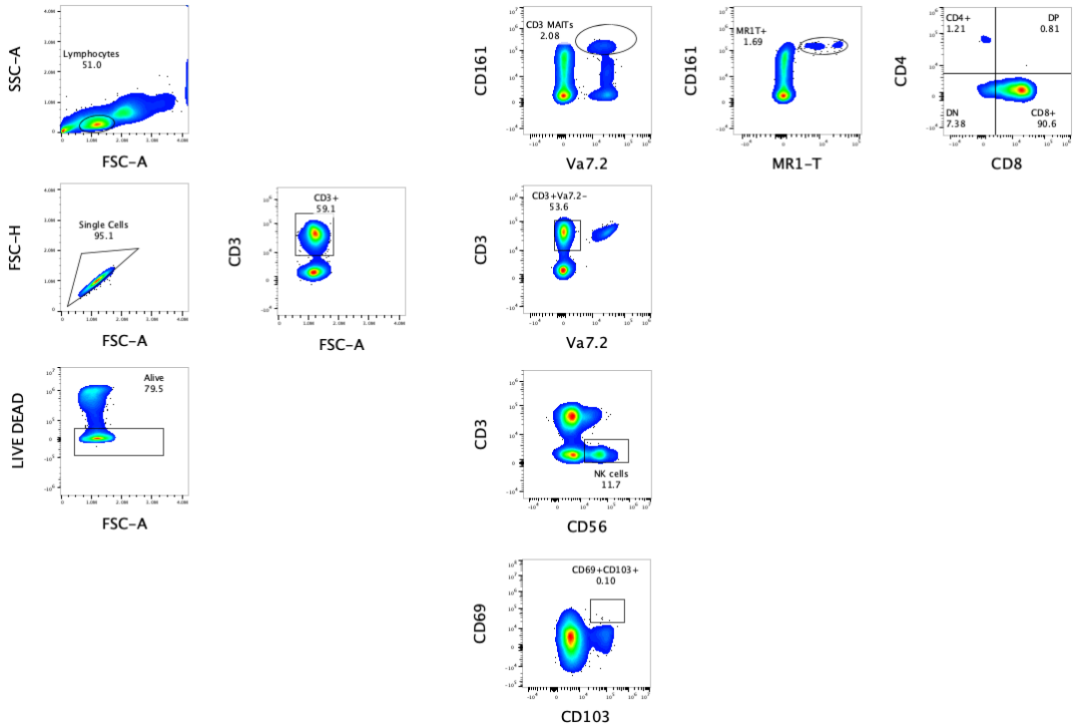
6.3.6 Acquisition on Cytek™ Aurora – Single colour stains were run for each individual run on the same day before the samples were acquired and unmixing of the data was done using those single colour stains only.

6.3.7 Analysis – Samples were acquired on Cytek™ Aurora with SpectrolFlo® software. The unmixing was done using single colour stains on SpectroFlo and then transferred to

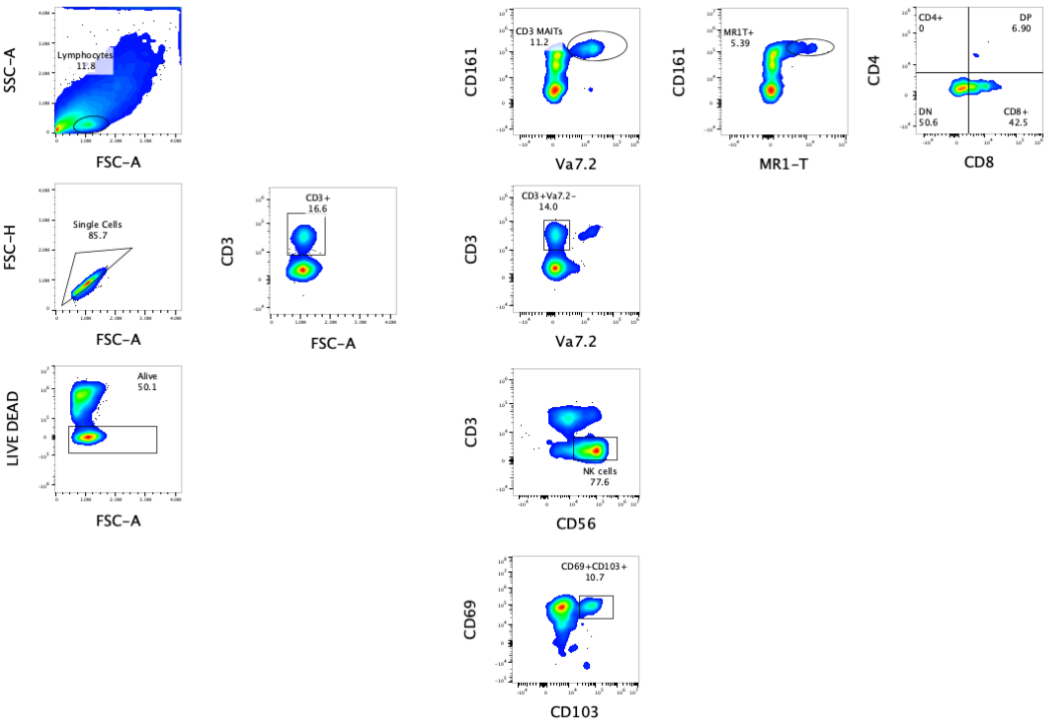
FlowJo for further compensation corrections and complete analysis of various T cell populations.

6.4 Results

Healthy PBMCs



Healthy liver lymphocytes



Liver tumour lymphocytes

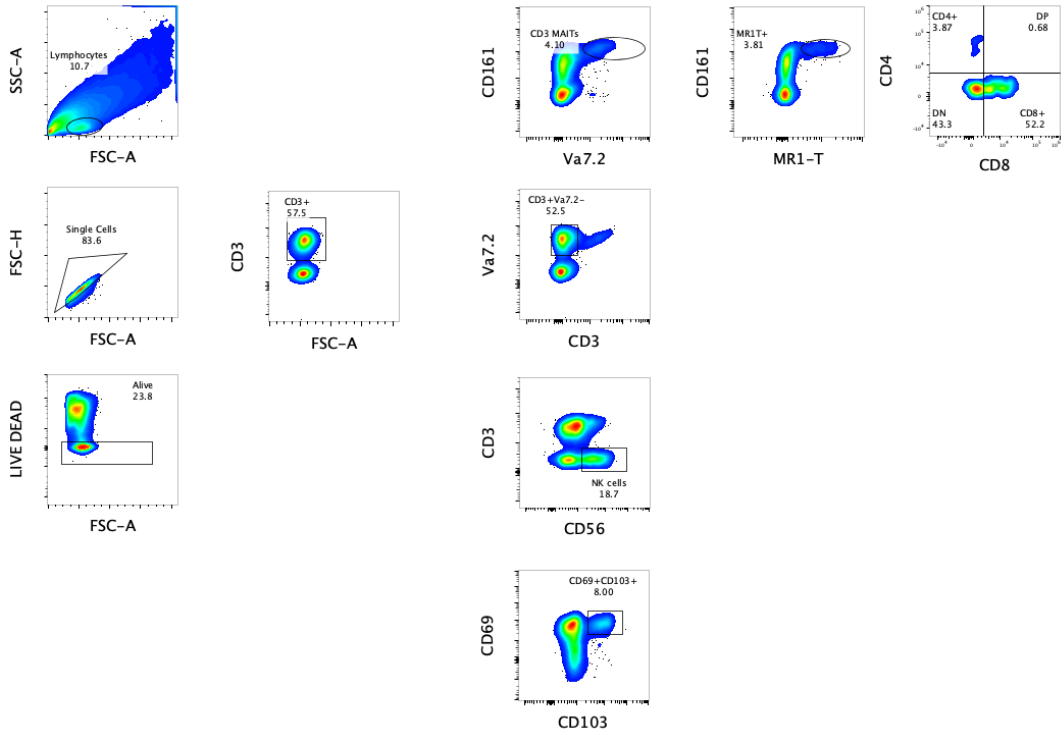


Figure 6.1 – Gating strategy applied for spectral flow cytometry data. MAIT cells and related populations were gated for each condition - healthy blood, healthy liver and liver disease. First all events were gated on FSC-A vs SSC-a to determine lymphocytes, followed by doublet elimination on FSC-A vs FSC-H plot. Events negative for the live dead dye were gated to define viable cells prior to gating CD3+ positive cells. MAIT cells (CD161++Vα7.2+/CD161++MR1tetramer+), CD3+ Vα7.2+CD161++, NK cells (CD3-CD56+), and resident T cells (CD103+CD69+) were gated before gating the CD4, CD8 and DN subsets within the parent T cell population. This gating strategy has been applied to all samples before studying their ex vivo phenotype and functional capacity to antigen and cytokine stimulation.

To determine the clinical relevance of MAIT cells in liver disease we investigated the phenotype and function of MAIT cells from alcohol defined liver disease. We acquired cirrhotic liver (CL) and liver tumour (LT) samples and compared their pro-inflammatory, cytotoxic potential and exhaustion profiles to healthy liver (HL) (*peri-tumour tissue from colon metastasis in the liver*) and healthy blood (HB). Though MAIT cells have been investigated in HCC recently (Duan et al. 2019), our analysis involves the use of the newer, multicolor spectral flow cytometry technique allowing for the broad analysis of numerous parameters and multiple populations in our technically difficult samples (low number and low viability), all in a single procedure. Spectral flow cytometry enabled us to analyse more parameters by combining existing fluorochromes that could not be used in the same panel for conventional flow cytometry due to large spillover. Current specifications allow detection across 64 channels with 30 color panels successfully demonstrated by Cytek. To our knowledge this has not be done before for MAIT cells in liver disease. Using this technique, we demonstrate the phenotype and function of MAIT cells, Vα7.2-CD161++ MAIT-related, CD103+CD69-liver resident, iNKT-like and NK cells in cirrhosis and HCC. Additionally, our study is one of the few that uses unsupervised analysis of flow cytometry data to identify novel populations allowing us to define changes to several predetermined and undetermined populations of interest.

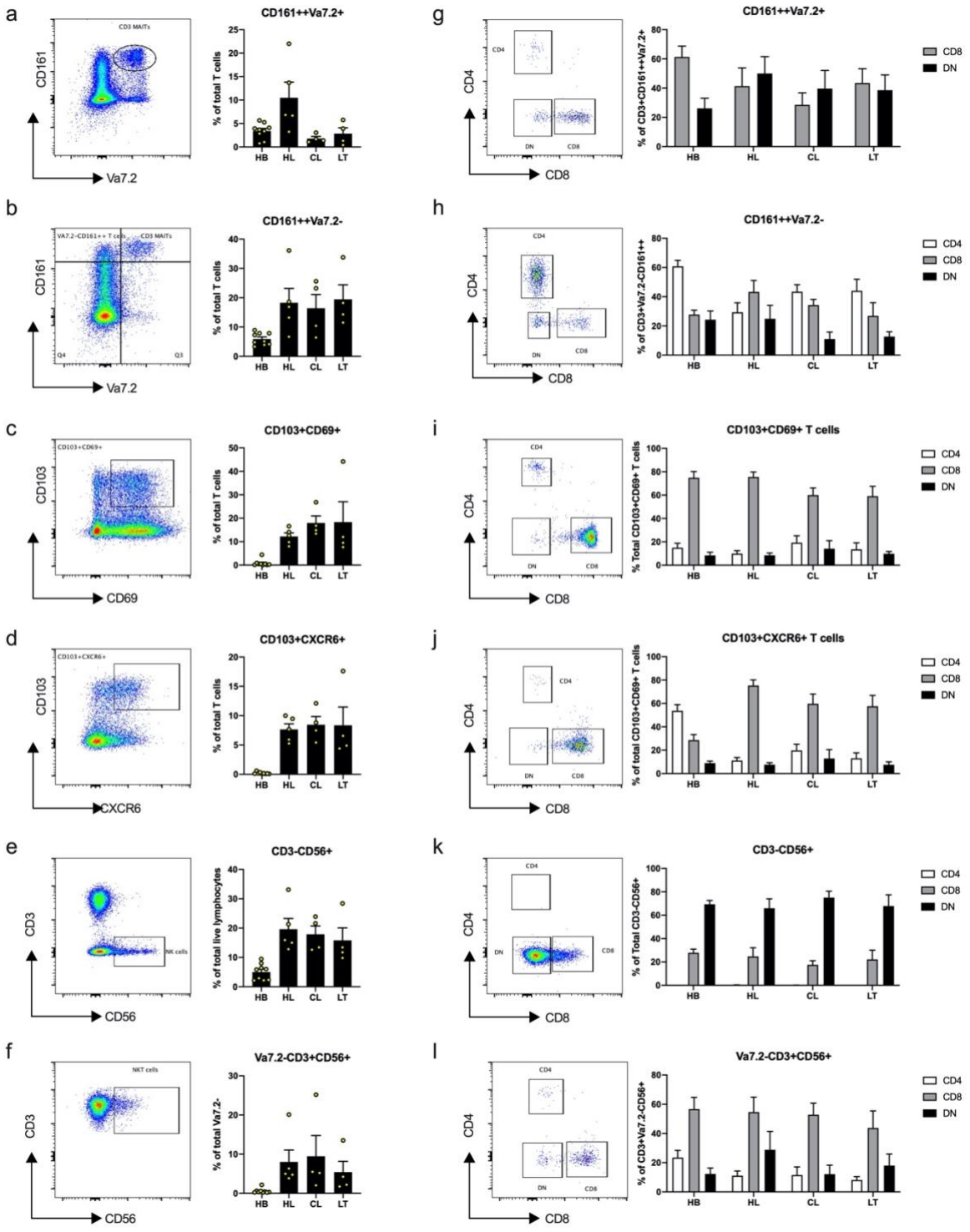


Figure 6.2 - Quantification of cell populations and CD4, CD8 and DN subsets in healthy blood and healthy/diseased liver samples.

Representative flow plots for different cell population gating and corresponding bar charts showing frequencies in healthy blood (HB), (n=9), healthy liver (HL), (n=5), cirrhotic Liver

(CL), (n=4) and liver tumour (LT), (n=5) samples (*unmatched*) of a) CD3 MAIT cells (CD161++ Vα7.2+), b) CD3+ Vα7.2-CD161++ T cells c) CD103+CD69+ Trms, d) CD103+CXCR6+ T cells, e) CD3-CD56+ NK cells, and f) CD3+ Vα7.2-CD56+ iNKT cell percentages in blood and liver. Quantification of CD4, CD8 and DN subsets within different T cells and NK cell populations in Healthy blood (HB), Healthy liver (HL), Cirrhotic Liver (CL) and Liver tumour (LT) samples; g) CD3 MAITs, h) CD3+ Vα7.2-CD161++, i) CD103+CD69+ Trms, j) CD103+CXCR6+ cells, k) CD3-CD56+ NK cells l) .CD3+ Vα7.2-CD56+ iNKT-like cells

6.4.1 MAIT cell numbers are significantly reduced in CL and LT compared to HL and to other MAIT/liver related populations

Firstly, we quantified MAIT cells and MAIT-related (Vα7.2-CD161++, iNKT-like cells (CD3+CD56+ Vα7.2-) and liver NK cells and resident CD103+CD69+ populations as a percentage of the total CD3+ population and then quantified the CD4/CD8/DN subpopulations within the parent population. We identified MAIT cells using surrogate markers Vα7.2 and CD161++ as well as MR1 tetramers and since the percentage of MAIT cells identified by either method does not differ much (Fig. 6.1), we based our functional and phenotypic analysis on CD161++ Vα7.2+ cells. NK cells were identified as CD3-CD56+ cells, whereas iNKT-like cells were gated by excluding Vα7.2+ cells and then gating on CD3+CD56+ cells. In agreement with previous findings, we found that MAIT cells are present at a higher frequency in the liver compared the blood(Jeffery, Van Wilgenburg, et al. 2016) (Fig. 6.2a). When comparing MAIT cells in liver disease vs healthy liver, we found that MAIT cells were significantly reduced in both CL and LT as compared to healthy liver (Fig. 6.2a). MAIT cells in the blood are mostly CD8+, whereas the liver consists of almost equal proportions of CD8 and DN MAIT cells (Fig. 6.2g). NK-like T cells are also present at a higher frequency in the liver compared to blood. The numbers of NK-like T cells do not change in cirrhosis but are reduced in LT when compared to numbers in healthy liver (Fig. 6.2f). Even though CD8+ iNKT-like cells are dominant in the liver, the proportion of CD4, CD8 and DN subpopulations of iNKT cells remains very similar in the blood, liver and liver disease (Fig. 6.2l). CD8+ T cells dominate the liver microenvironment; this is also confirmed in other populations where they form a high percentage of the parent population as compared to CD4 and DN cells. The frequency

of the remainder of the populations analysed did not change as a result of disease but in general, were enriched in the liver with the NK cells being the most abundant cell type in the liver (Fig. 6.2).

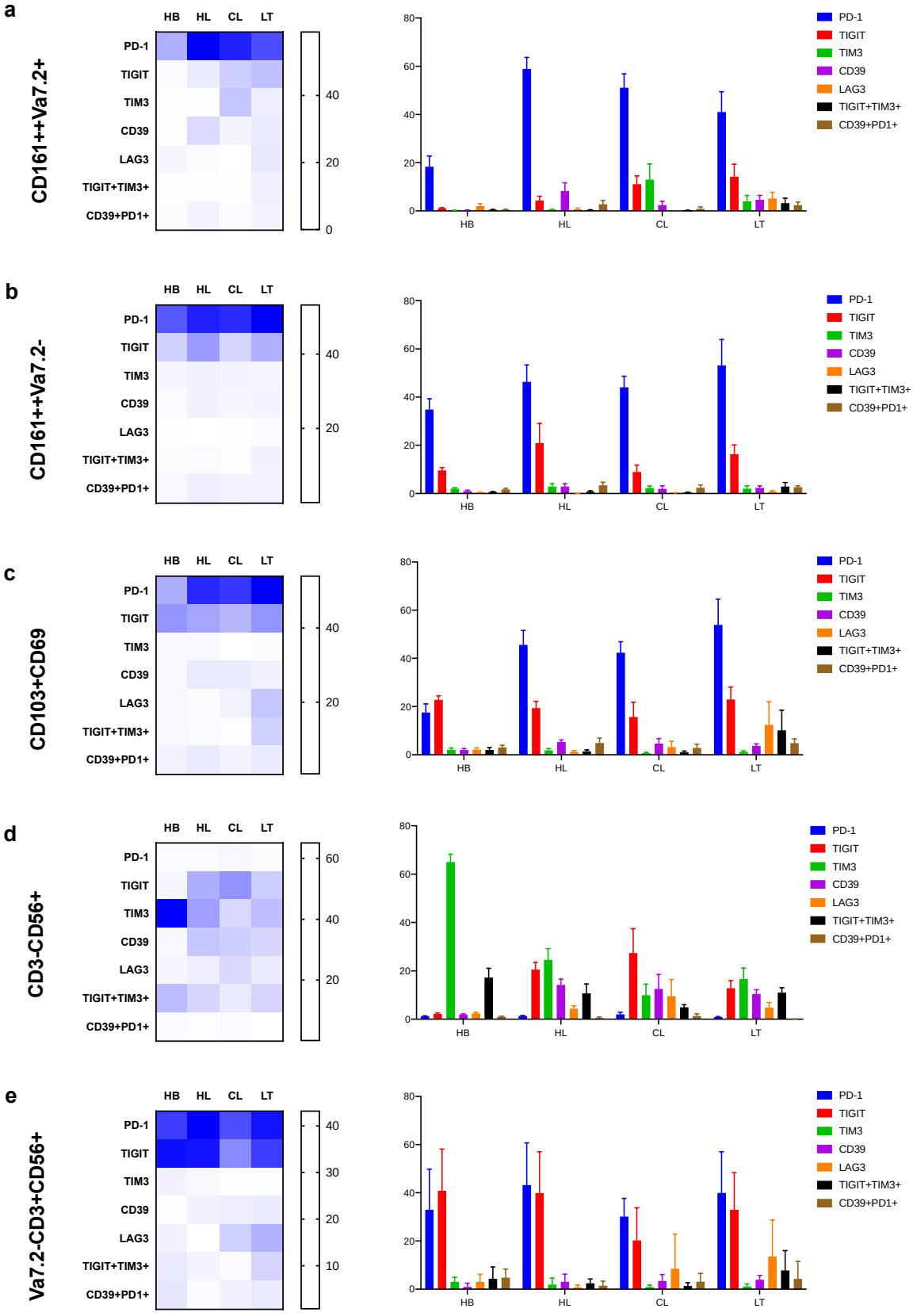

a
CD161++Va7.2+
b
CD161++Va7.2-
c
CD103+CD69
d
CD3-CD56+
e
Va7.2-CD3+CD56+
HB
HL
CL
LT
PD-1
TIGIT
TIM3
CD39
LAG3
TIGIT+TIM3+
CD39+PD1+

Figure 6.3 – Ex vivo exhaustion profile of MAIT cells and MAIT cell related populations from liver disease compared to healthy liver and blood. Healthy donor PBMCs or liver lymphocytes ($2.5x10^6$/ml) were thawed and cultured in R10 overnight for 16 hours in either, *E*. coli, IL-12+IL-18 or PMA/ionomycin. BFA and monensin were added for the last 4 hours of the assay prior to staining cells for spectral flow cytometry analysis on Cytek Aurora. Heat maps and bar charts show exhaustion markers expressed on unstimulated cells in four condition; HB=healthy blood (*n*=9), Healthy liver (HL) (*n=5*), cirrhotic liver (CL) (*n=4*) and liver tumour (LT) (*n=5*) from 5 different populations; a) MAIT cells (CD161++ Vα7.2+), b) CD3+ Vα7.2+CD161++, c) resident T cells (CD103+CD69+), d) NK cells (CD3-CD56+), and e) iNKT-like (Vα7.2-CD3+CD56+) cells.

6.4.2 MAIT cells in CL and LT show enrichment for exhaustion markers

As shown in other liver specific diseases and in cancers (Magalhaes et al. 2020), MAIT cells express exhaustion markers associated with T cell dysfunction. We investigated the expression of exhaustion markers, PD-1, TIM-3, LAG3, TIGIT and CD39 in CL and LT. In general, we found that populations from CL and LT showed higher expression of these markers compared to HB and HL (Fig. 6.3). Our data indicate expression of PD-1 on unstimulated MAIT cells and in all settings regardless of them being in the blood or in the liver. TIGIT expression is also visible on MAIT cells to a very limited extent, which was slightly increased in HL-MAIT cells. A higher level of TIGIT expression is observed on CL and LT-MAIT cells (Fig. 6.3a). LT-MAIT cells expressed low levels of most exhaustion markers, TIM3, CD39 and LAG3, including a smaller percentage of TIGIT and TIM3 or PD-1 and CD39 double positive MAIT cells. All cell populations investigated from LT tissue was positive for two or more exhaustion markers and cells derived from LT displayed greater exhaustion compared to CL or HL tissue (Fig. 6.3). CD103+CD69+ (Trms) cells expressed twice as much PD-1 compared to those found in the circulation and Trms from LT expressed highest levels of PD-1 compared to resident cells from HL (Fig. 6.3c). TIGIT was expressed on CD103+CD69+ cells present in HB, HL and liver disease, all at a similar percentage in each specific condition (Fig. 6.3c). The same trend for TIGIT expression in the blood and liver was seen for Vα7.2-CD161++ and iNKT-like cells but not for NK cells (Fig. 6.3b, e). Instead, we noted high expression of TIM3, greater than 60% on NK cells from HB, which was lower in HL and decreased even further in CL and LT (Fig. 6.3d). TIM-3 expression in all other populations was low in all four settings. The CD39 marker was

expressed mainly on NK cells but not on MAIT cells or iNKT-like cells and LAG3 expression was limited to liver disease (Fig. 6.3).

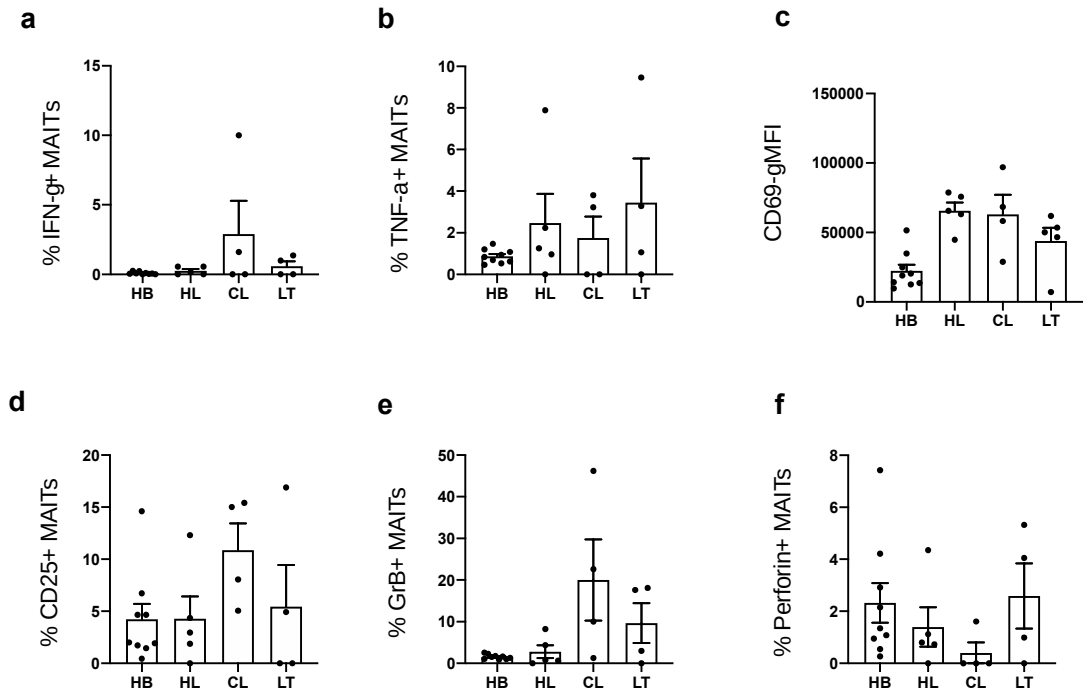


Figure 6.4 – Ex-vivo pro-inflammatory and cytotoxic profile of unstimulated MAIT cells from HB, HL, CL and LT. Unstimulated, lymphocytes ($2.5x10^6$/ml) from blood and liver samples were rested for 16 hours overnight in R10 with BFA and monensin added for the last 4 fours of the assay. MAIT cells were stained for extracellular and intracellular markers of activation and cytotoxicity and analysed by spectral flow cytometry. Inflammatory cytokines a) IFN-γ, b) TNF-α, activation markers c) CD69 and d) CD25 and cytotoxicity makers e) GrB and f) Perforin expression from unstimulated CD161++ Vα7.2+ MAIT cells from HB=healthy blood (*n*=9), Healthy liver (HL) (*n=5*), cirrhotic liver (CL) (*n=4*) and liver tumour (LT) (*n=5*).

6.4.3 MAIT cells from CL and LT patients are activated compared to MAIT cells in HB and HL tissue

Before analyzing the MAIT cell response to stimulation we checked the phenotype of unstimulated MAIT cells that were rested for 16 hours in conditioned RPMI medium. MAIT cells from the liver are more activated than those in the blood and the activated state of MAIT cells is more prominent in liver disease (Fig. 6.4). IFN-γ expression was not seen in HL or HB-MAIT cells (Fig. 6.4a), whereas TNF-α expression was detected in HL and LT MAIT cells (Fig. 6.4b). All liver MAIT cells expressed high levels of CD69, for this reason CD69 could not be used as an activation marker. Different to our expectation, CD69 levels were high on blood-MAIT cells although slightly less than MAIT cells from the liver that were almost 100% positive for CD69 (Fig. 6.4c). On the other hand, we note an increased CD25 expression on CL-MAIT cells, which was twice as much as the average CD25 expression on MAIT cells in all other settings (Fig. 6.4d). Ex-vivo analysis of GrB and perforin expression in MAIT cells reveals increased GrB expression in CL and LT, however this was highly variable between donors. On average, 20% of MAIT cells were GrB positive in liver disease and little to no GrB was detected from HB and HL MAIT cells (Fig. 6.4e). In contrast to GrB, baseline perforin expression was low, but it was detectable in all settings expect for CL-MAIT cells (Fig. 6.4f). These findings show that MAIT cells from liver disease are more activated compared to HB and HL. Unlike MAIT cells, iNKT-like cells from the liver had a higher baseline IFN-γ expression and a few donors in all settings had greater than 5% TNF-α expression from iNKT-like cells (Supplementary Fig. 11/12). Both NK cells and iNKT-like cells from the blood have high baseline expression of GrB and perforin. GrB expression in liver-NK cells was 5 times

lower than that of blood-NK cells, but was similar across the three liver settings. Whereas, iNKT-like cells from liver disease expressed higher levels of GrB than in HL, with expression levels in liver disease being very similar to GrB expression of blood-iNKT-like cells (Supplementary Fig. 15). HL-iNKT cells did not have a baseline expression of perforin but NK cells in the liver have between 20-50% baseline perforin expression and for both cell types in the blood, perforin expression is higher and highly variable for iNKT cells (Supplementary Fig. 16). Baseline CD25 expression on NK cells and iNKT-like cells across all settings is twice as much as that for MAIT cells; however, like MAIT cells, NK cells from CL and iNKT-like cells from liver disease patients, had the highest levels of CD25 expression ex-vivo, average expression of 30% and 50%, respectively (Supplementary Fig. 14). Overall, cell populations are activated in the liver and even more so in the context of liver disease.

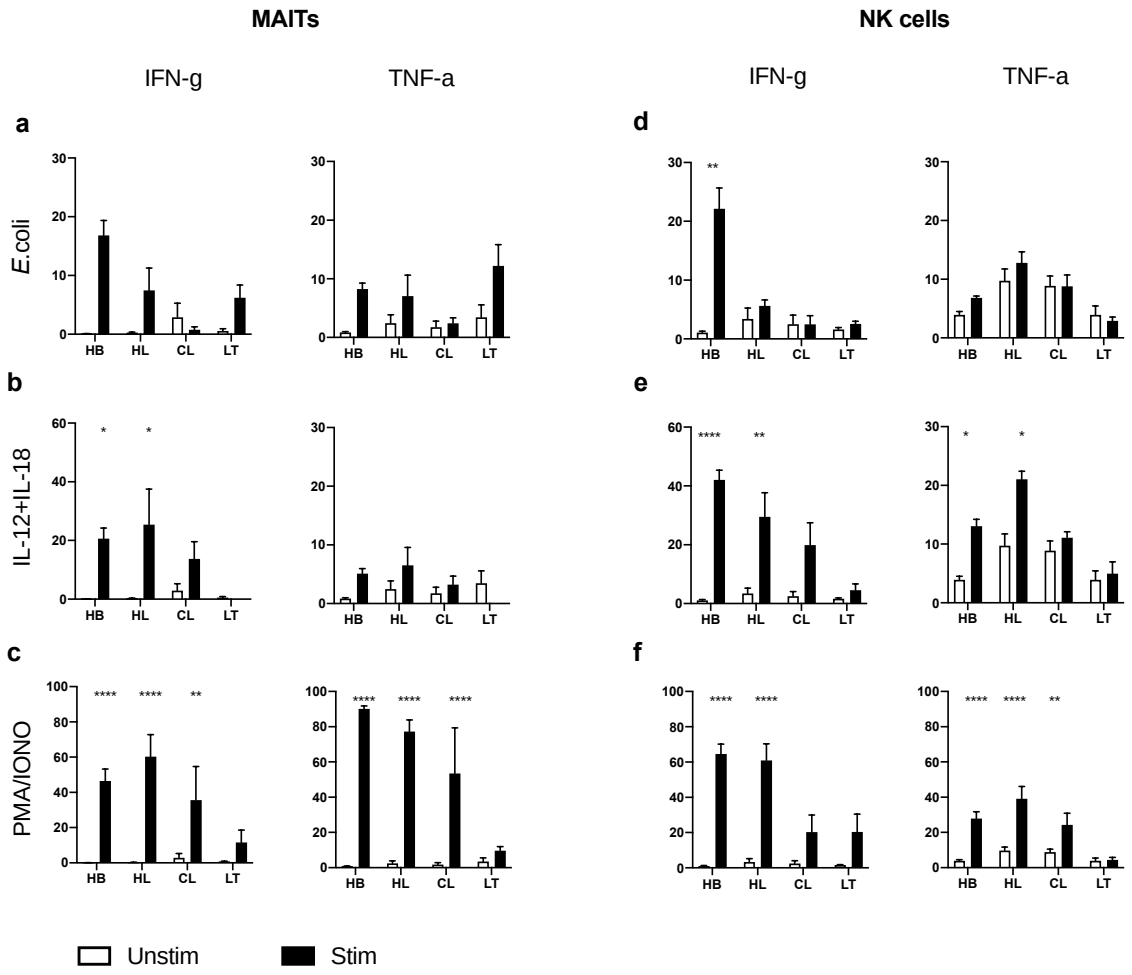


Figure 6.5 – Pro-inflammatory cytokine expression from stimulated MAIT and NK cells from healthy and diseased liver. Healthy donor PBMCs or liver lymphocytes ($2.5x10^6$/ml) were thawed and cultured in R10 overnight for 16 hours with or without either, *E*. coli, IL-12+IL-18 or PMA/ionomycin. BFA and monensin were added for the last 4 hours of the assay prior to staining and spectral flow cytometry analysis on Cytek Aurora. IFN-γ and TNF-α expression from a-c) MAIT cells and d-f) NK cells in response *E*. coli, IL-12+IL-18 or PMA/Ionomycin stimulation (rows) from four conditions (*x-axis*); HB=healthy blood (*n*=9), Healthy liver (HL) (*n=5*), cirrhotic liver (CL) (*n=4*) and LT (LT) (*n=5*). White bars = unstimulated cells and black bars = stimulated cells. Statistical significance – Dunnett's multiple comparisons test - *P<0.0028, **P<0.008, ****P<0.0001.

6.4.4 MAIT cells and MAIT-related populations from the liver have a reduced pro-inflammatory potential compared to their blood counterparts

To assess the pro-inflammatory capacity of MAIT cells from HL and liver disease we stimulated cells with *E*. coli or IL-12+IL-18 allowing us to assess both the TCR and cytokine mediated activation of MAIT cells and related populations. We found that MAIT cells in HB and HL could be activated by cytokine stimulation (Fig. 6.5b). Despite a visible IFN-γ and TNF-α response from MAIT cells to *E*. coli stimulation, the activation is not significant (Fig. 6.5a). Nonetheless, by using PMA/IONO stimulation we can see that MAIT cells from HB, HL and CL have the potential to induce pro-inflammatory cytokines in response to stimulation (Fig. 6.5c). In contrast, MAIT cells from LT failed to induce significant levels of IFN-γ to any of the three stimulations. However, MAIT cells from LT could produce TNF-α when stimulated with *E*. coli, but not when stimulated by cytokines or PMA/IONO (Fig. 6.5a-c). It is also worth noting that IFN-γ and TNF-α expression in response to stimulation is mainly seen in MAIT cells from disease free settings, but in liver disease, ex-vivo MAIT cells have low pro-inflammatory potential even after being stimulated (Fig. 6.5a-c). If we compare the NK cell response, we observe that NK cells from the liver do not respond to *E*. coli stimulation but could induce significant levels of IFN-γ and TNF-α to IL-12+IL-18 stimulation. Liver disease NK cells were not able to induce IFN-γ and TNF-α. However, CL-NK cells have pro-inflammatory potential. In general liver NK cells were less pro-inflammatory than their blood counterparts. Though HL-NK cells had greater TNF-α expression than blood-NK cells, they were still less pro-inflammatory compared to NK cells in the blood (Fig. 6.5d-f). A similar pattern was observed for all 5 populations assessed where MAIT cells from disease free settings show better pro-

inflammatory potential to cytokine stimulation compared to populations from CL and LT. Though HB and HL iNKT-like cells expressed high amounts of IFN-γ in response to cytokine stimulation (Supplementary Fig. 11), iNKT cells could not produce TNF-α when stimulated with IL-12+IL-18, whereas NK cells from HB and HL could produce TNF-α in response to cytokine stimulation (Supplementary Fig. 12).

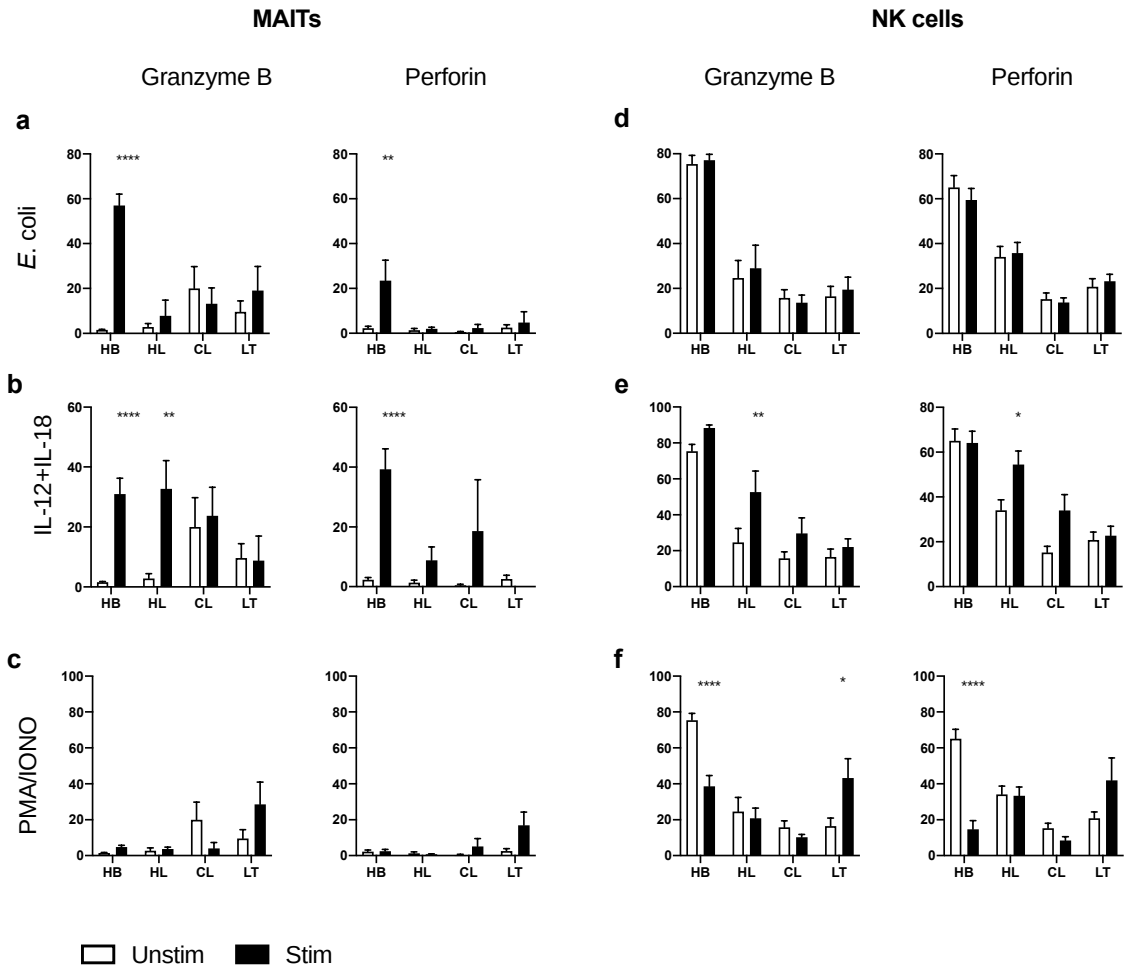


Figure 6.6 – Cytotoxic potential of MAIT cells and NK cells from liver disease. Healthy donor PBMCs or liver lymphocytes ($2.5x10^6$/ml) were thawed and cultured in R10 overnight for 16 hours with or without either, *E*. coli, IL-12+IL-18 or PMA/ionomycin. BFA and monensin were added for the last 4 hours of the assay prior to staining cells for spectral flow cytometry analysis on Cytek Aurora. GrB and perforin expression from a-c) MAIT cells and d-f) NK cells in response *E*. coli, cytokine or PMA/Ionomycin stimulation (rows) from four conditions (*x-axis*); HB=healthy blood (*n*=9), Healthy liver (HL) (*n=5*), cirrhotic liver (CL) (*n=4*) and liver tumour (LT) (*n=5*). White bars=unstimulated and black bars = stimulated cells Statistical significance - Dunnett's multiple comparisons test - *P<0.0249, **P<0.067, ****P<0.0001.

6.4.5 MAIT cells in healthy/diseased liver are less cytotoxic compared to MAIT cells in the blood

To test the cytotoxic potential of MAIT cells and related populations within the liver we stimulated blood and liver lymphocytes with *E*. coli, IL-12+IL-18 or PMA/IONO and compared it to unstimulated cells within the same setting or to stimulated cells in other settings. MAIT cells from the blood responded well to *E*. coli stimulation and produced about 60% GrB, which was twice as much, compared to GrB induction by cytokine stimulated MAIT cells. Higher GrB expression from *E*. coli stimulated MAIT cells is likely due to combined TCR and cytokine signalling (Fig. 6.6 a/b). MAIT cells from liver disease failed to induce GrB in response to either stimulation, and perforin expression was only seen in MAIT cells stimulated with IL-12+IL-18, with significant levels induced from blood-MAIT cells but not by liver-MAIT cells (Fig. 6.6a-c). There is no significant difference in GrB and perforin expression from NK and iNKT-like cells in the blood. However there was a significant induction of GrB and perforin from NK cells from HL stimulated with IL-12+IL-18, but not to *E*. coli stimulation (Fig. 6.6d/e and Supplementary Fig. 15/16). PMA/IONO stimulation causes strong degranulation of NK and iNKT-like cells, however, only the LT-NK cells had the potential to express GrB, which was not seen with IL-12+IL-18 or *E*. coli stimulation of NK cells and for other populations from LT samples (Supplementary Fig. 15/16). The general trend across all populations is the reduced cytotoxic potential of populations in healthy and diseased liver compared to populations from HB. Moreover, populations from healthy and diseased liver had limited capacity to produce cytotoxic molecules in response to antigen/cytokine stimulation (Supplementary Fig. 15/16).

6.4.6 LT-MAITs are dysfunctional and do not respond to stimulation

The analysis of five pre-defined populations revealed a striking dysfunction in all the populations from LT. Although populations from LT had some baseline expression of TNF-α they did not produce TNF-α after stimulation (Fig. 6.5 and Supplementary Fig. 11). Likewise expression of activation markers like CD25 and CD38 did not change after stimulation (Supplementary Fig. 13/16). Populations from LT samples also lost their ability to make pro-inflammatory cytokines and cytotoxic molecules even to stimulation with PMA/IONO (Supplementary Fig. 10-11, 14-15), whereas, populations from CL could respond to PMA/IONO stimulation and were able significantly upregulate IFN-γ, TNF-α and IL-2 to PMA/IONO stimulation but not to *E*. coli or cytokine stimulation. The activated states of NK and iNKT-like cells from LT remained unchanged after stimulation but the MAIT cells from LT could not be activated by any of the methods. Since MAIT cells from LT did not respond to PMA/IONO stimulation they are likely to be functionally defective, however they may express pro-tumour cytokines, but we have not checked for this yet (Supplementary Fig. 10-17). As LT populations express various exhaustion markers, the ability to revive them functionally may also be limited or their localization in the liver may have reprogrammed the cells to match the tolerogenic requirements of the liver.

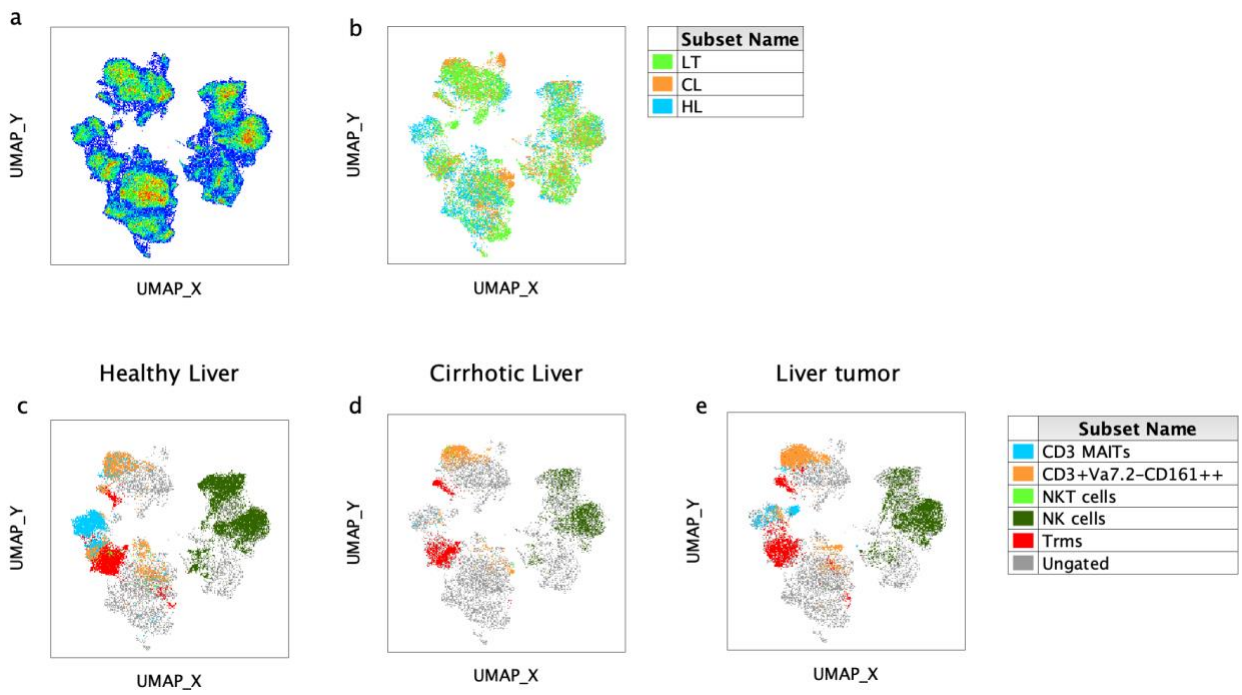


Figure 6.7 –Visualization of pre-defined manually gated populations in healthy vs liver disease by manual gating and overlay.

a) Clusters formed by UMAP algorithm from a total 116056 events, *(65583-HL, 11670-CL, 33803-LT*). b) Condition specific contribution of events to different clusters. Manually gated populations are overlaid in each of the three conditions investigated, **c)** Healthy liver lymphocytes (HL), d) Cirrhotic liver lymphocytes (CL) and e) Liver tumour lymphocytes (LT).

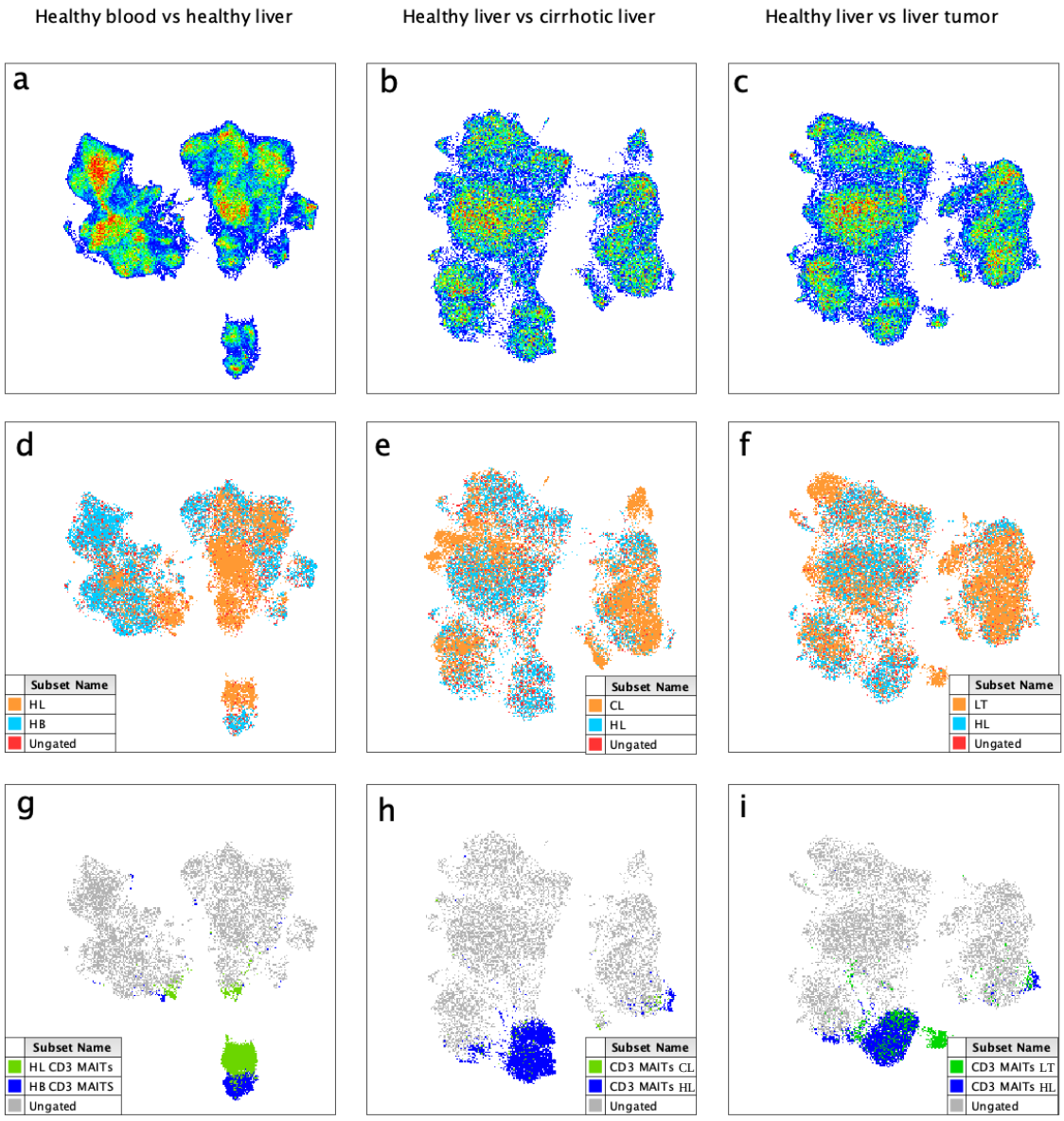
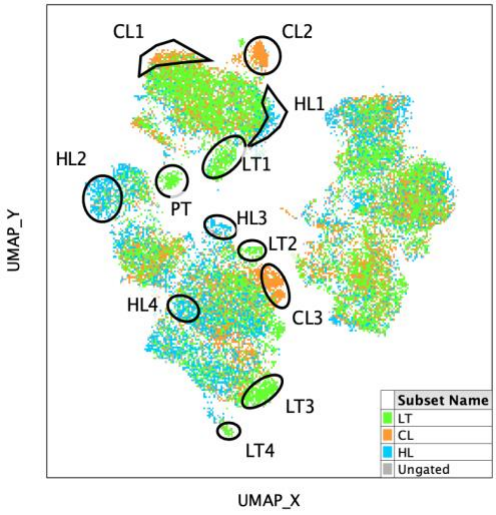


Figure 6.8 - UMAP analysis of T cells from HB vs HL (left), HL vs CL (middle) and HL vs LT (right). a-c) cluster formations from events of two conditions (stated above columns). d-f) conditions specific contribution to different clusters and g-i) Manually gated MAIT cell populations overlaid to differentiate and compare MAIT cells from disease conditions compared to MAIT cells derived from HB or HL compartments, blue is healthy liver or blood and green CL or LT MAITs. Total events per condition = Healthy blood-900000, healthy liver-42190, cirrhotic liver-9575 and liver tumour-21190



HL1	CD4 T cells*
HL2	Liver CD8/DN MAITs, CD161++ Vα7.2+ CD38+ PD-1^{lo}
HL3	Liver resident T cells DN, CD161++ CD38+ PD-1^{lo}
HL4	CD8+ T cells, Vα7.2^{lo} GrB^{lo} TIGIT^{hi}
CL1	Liver resident cytotoxic CD4 T cells, CD161++
CL2	CD38 expressing cytotoxic CD4 T cells
CL3	iNKT-like cells, CD8+ CD56+CD161+GrB+CD38+
LT1	Liver resident CD4 Tregs, PD-1+CD25+CD39+TIGIT++
LT2	Cytotoxic CD8 T cells, TIGIT^{hi} CD38+TIM3^{lo}, LAG3^{lo}
LT3	Cytotoxic Vα7.2+ CD8 T cells, CD38+ TIM3^{lo}LAG3^{lo}
LT4	Exhausted DN T cells, CD161++
PT	DN MAITs, TIM3+PD-1+ CD38+

Figure 6.9 – Unsupervised analysis of healthy and liver disease samples UMAP (left) constructed from viable liver lymphocytes (total events =116056) from three condition - HL=Healthy liver (Cyan), CL-Cirrhotic liver (orange), LT-Liver tumour (green) and PT= peritumour (green). Samples within each group were combined and then overlaid to show condition specific contribution of events to different islands defined by colour. Right - table with population specific markers identified by manual gating of distinct populations and markers identified by manual reading from histograms. *negative for all markers except live dead, CD3 and CD4.

6.4.7 Unsupervised analysis of lymphocyte populations from HL, CL and LT show complete loss of MAIT cells and related populations in CL

In addition to analysing the pre-determined populations (MAIT cells, Vα7.2-CD161++, Trms, NK cells and iNKT cells) for which we had designed our panel, we searched for populations that were unique to the liver, CL or LT, using the same panel. To do this, we analysed our samples by UMAP; prior to applying the strategy we cleaned the data by first excluding non-viable samples, i.e. removal of samples that did not have at least 1000 viable lymphocytes. Next, we randomly selected 3000 viable events from each of the blood healthy donor samples but used all the events from the liver samples because the viable cell numbers were already low but still met the minimum requirement of 1000 events per sample. Our results confirmed our quantitative analysis of the pre-determined populations. By manually overlaying combined samples from each condition we could see an equal contribution by events towards the approximately 32 islands generated by the UMAP algorithm, therefore we could be confident that no specific donor or condition dominated the analysis (Fig. 6.7a). Furthermore, the UMAP nicely separates out the T cells, further segregating them into distinct CD4, CD8 and DN populations (Supplementary Fig. 18). Then, by overlaying the five pre-defined manually gated populations onto three individual UMAPs that are specific to the disease status (HL, CL and LT) we were able to see visible, disease specific differences in our 5 populations of interest. We note a complete loss of MAIT cells from CL and a significant reduction in the MAIT cell population in LT. iNKT-like cells formed a very small population compared to the NK cells that were also visibly reduced in liver disease compared to healthy liver NK cells (Fig. 6.7c).

For a more precise analysis of MAIT cells we performed a similar type of UMAP analysis by gating on viable T cells prior to generating the UMAP. Again, events from all settings contributed equally to the islands. The number of islands change between the three groups due to the different disease settings being compared (Fig. 6.8a-c). In another layer of analysis we overlaid manually gated MAIT cell populations from two settings; the UMAP algorithm efficiently isolated out the MAIT cells from other T cells populations, with majority of the MAIT cells being CD8+ in all settings, (Fig. 6.8g-l*; the blue and green colours refer to the disease settings the events are from*). This analysis also highlighted a population of MAIT cells specific to tumour bearing liver, figure 6.8i, these are the double negative MAIT cells. Despite the DN MAIT cells in LT being a noticeable population, all the events were from a single donor. Therefore, to determine if such a large population of DN MAIT cells are present in HCC or if this was a rare occurrence, that would need to be determined by assessing a larger group of LT samples. Lastly, we performed an unsupervised analysis of our samples using the UMAP algorithm to identify unique populations. Our analysis successfully forms genuine islands based on T cell, resident, cytotoxicity and exhaustion markers, where T cells where separated from other lymphocytes with a further clear separation between CD4 and CD8 T cell populations (Supplementary Fig. 22). The unsupervised analysis showed at least three unique liver condition specific populations with some populations present in healthy liver that were absent in CL and LT (Fig. 6.9). Furthermore many more disease specific populations were noticeable that were originally not known or defined during the experimental design for the same dataset. On the downside, due a low sample number some of the disease specific populations were formed by events from a single donor and, therefore, significance could not be determined. Nonetheless, our data imply vast

heterogeneity in populations between donors in the same disease setting. Interestingly, we also found an exclusive population in HL samples, which are the CD4 T cells. However, these cells were negative for all downstream markers we stained for; thus, showing that such populations may be important, but can be easily missed when a limited number of pre-defined parameters are assessed.

LT has 5 distinct populations that were mainly exhausted, including a CD4 Treg population that had high PD-1 and TIGIT expression along with CD39 and CD25 expression. CD38 expression was also prominent in 3/5 of the LT specific populations (LT2-LT4). There were two cytotoxic CD8 T cell populations, one that was Vα7.2, but did not express CD161 (LT4), and the other CD8 population did not express either Vα7.2 or CD161. However, this population did express high levels of TIGIT (LT3), which was absent in the Vα7.2+ cytotoxic population. Both populations expressed low levels of TIM-3 and LAG3 ex-vivo. A large DN CD161++ exhausted MAIT cell population (PT) is prominent in the LT samples, which corresponds to cells in the peri-tumour region from one donor.

In the CL samples, two cytotoxic CD4 T cells populations were found, CL1 and CL2. The CD4 cytotoxic T cell population expressing high CD161 also expressed the tissue resident markers CD103, CD69 and CXCR6 (CL1), whereas these markers were not detected in the other population, which were CD38 positive (CL2). The third CL specific population CL3, were the CD8+ iNKT-like cells that were positive for CD38+ and GrB and expressed medium levels of CD161.

In the HL samples we identified four different populations, a CD8+ population, a DN population, CD8+ and DN MAIT cells and a CD4 population. The HL-MAIT cells (HL2) did not express GrB ex-vivo but had low PD-1 and high CD38 expression. The CD4 population, HL1, was negative for all the markers we tested thus identifying a potential

important population that is lost in chronic liver disease. The DN population, HL3 expressed the liver homing marker, CXCR6 and tissue resident CD103 and CD69 markers and they are CD161 bright cells that have low PD-1 expression and high CD38 expression. The fourth is a CD8 population, HL4, had high TIGIT expression but low GrB expression and were marginally positive for Vα7.2.

6.5 Discussion

A large number of studies have investigated MAIT cells in disease and have highlighted the disease-associated loss of MAIT cells, increased vulnerability to subsequent infections and poor prognosis. We wanted to understand if MAIT cells were lost from liver disease as shown in previous studies and to determine the functional capacity of the remaining MAITs. Though MAIT cells have been studied in liver disease, the studies do not always look at MAIT cells from the tissue and those that do look at both blood and liver MAIT cells, have reported data based on limited parameters. In many studies MAIT cells are identified using the surrogate markers, CD161 and Vα7.2, but it is important to study MAITs using MR1 tetramers as some non-MAIT cells exist within the CD161++ Vα7.2+ population and MAIT cells can downregulate CD161 upon activation. While our analysis so far indicates that the surrogate approach and tetramer staining lead to equal results, subsequent samples will be studied with MR1 tetramers and the same approach should be applied by other groups to make studies more accurate and comparable.

We have investigated numerous parameters and studied MAIT cells from the blood and liver. However, as these samples were not matched, our results can only provide a general overview of how MAIT cells from the disease site behave in relation to their blood counterparts. Analysis of MAIT cells and related populations from two liver disease settings, cirrhosis and LT, caused by alcohol consumption in most of the cases has revealed the loss of MAIT cells in these two chronic liver disease conditions with the remaining MAIT cells showing high exhaustion. A study by Hedge et al in 2018 looked at MAIT cells in the blood and tissue of alcohol related liver cirrhosis (ARC), also reporting

a loss of MAIT cells in the circulation and from the liver(Hegde et al. 2018). Similar to their study, we detected TIGIT expression in addition to the PD-1 and TIM-3 expression on liver MAIT cells from cirrhosis, indicating that MAIT cells express additional exhaustion markers than previously reported. Furthermore, we show that MAIT cells from liver cirrhosis have reduced pro-inflammatory and cytotoxic potential ex-vivo. However, we did not stain for IL-17 and, therefore, could not determine the pro-fibrogenic potential of MAIT cells from our cohort. Nevertheless, since the data from their group and our group are very similar it is highly likely that MAIT cells could behave in a pro-fibrogenic manner in cirrhosis.

In contrast to MAIT cells in CL, our study shows that MAIT cells from LT are highly dysfunctional and do not respond to TCR and cytokine stimulation in-vitro. Similar results were reported by Duan et al in 2019. This study reported that dysfunctional MAIT cells were highly activated, but exhausted and contributed to the progression of HCC. They also showed the increased production of IL-10, IL-4, IL-22 and IL-8 from tumour-MAIT cells that suggested skewing of the response towards a regulatory, type-2 response and pro-tumour like. We did not test for these cytokines yet, but as seen in their analysis, we can confirm high expression of CD38 on our MAIT cells and related populations of the liver. However, in contrast to their findings, we did not see a difference in HLA-DR expression. To determine why MAIT cells from LT are dysfunctional further studies need to be done to investigate changes at the transcriptional level and to investigate the possible loss of co-stimulatory/cytokine receptors (IL-12R and IL-18R) on MAIT cells.

CD38 is a marker used to study T and B cell activation and differentiation. Expression of CD38 on mature T cells is associated with reduced ability of T cells to proliferate, but

these cells have improved potential to produce cytokines(Sandoval-Montes and Santos-Argumedo 2005). We, like other groups found high CD38 expression on MAIT cells as well as MAIT cell-related populations in the liver, but not in the blood. CD38 expression did not change between unstimulated and stimulated populations, with the exception of MAIT cells from HB where CD38 expression had increased significantly in response to *E*. coli stimulation (Supplementary Fig. 17). However, CD38+ MAIT cells and related populations from the liver did not have improved capacity to produce cytokines. Though we didn't test the proliferative potential of the CD38+ MAIT cells, it can be assumed that they will be less proliferative as a result of high CD38 expression like that shown in Sandoval-Montes study(Sandoval-Montes and Santos-Argumedo 2005).

As yet no study has compared the functional capacity of MAIT-related populations in liver disease, but by investigating such populations in our study we found that many populations in LT are dysfunctional, in particular, MAIT cells and the CD161++ populations were most affected. The KLRB1 gene encodes for CD161, the loss of this gene in multiple malignancies has been correlated with poor outcome and because MAIT cells and MAIT cell-related populations express high levels of CD161, the loss of these CD161+ populations and their dysfunction is not surprising, but also highlights the importance of these cells in controlling tumours. We also noted that MAIT cells, iNKT-like cells and the Vα7.2- CD161++ cells from cirrhosis retain their pro-inflammatory and cytotoxic potential. Therefore, sequencing MAIT cells from cirrhosis and LT samples would show how these cells differ transcriptionally in the two settings and may help to identify strategies to rescue MAIT cells in LT.

The study of MAIT cells in other tumour settings such as in advanced colorectal cancer patients shows MAIT cell numbers in the circulation decline but increase in the tumour,

this was accompanied by reduced IFN-γ and higher IL-17 production(Ling et al. 2016). In a mouse lung tumour model MAIT cells were also found to produce higher levels of IL-17A and the increased production of IL-17A was associated with suppression of NK cell function thus demonstrating MAIT cell can support tumour initiation, growth and metastasis(Yan et al. 2020). Despite not being able to confirm MAIT cell infiltration in LT and loss from the circulation, we could confirm the loss of IFN-γ expression from LT-MAIT cells thus making them less pro-inflammatory. Whether LT MAIT cells make IL-17 and tumour-promoting cytokines remains to be investigated in our study. In contrast to the lung tumour model, the number of NK cells did not decline in our LT samples suggesting that MAIT cells may not promote tumour in the liver by inhibiting NK cells and have a different role, possibly a protective one. Further studies, especially those using in-vivo LT models, combined with longitudinal studies would be required to fully understand the function of MAIT cells in LT.

One of the most interesting findings from our study has been the identification of unique populations within HL, CL and LT using the same dataset designed to understand MAIT cells in liver disease. These unique populations share markers, but are still exclusive and can also be negative for all the markers, like the HL1 population that we found in our cohort. Since the HL1 population was not limited to a single donor and was formed by multiple donors from the pool of healthy liver samples, a genuine CD4 population exists in healthy liver that requires further investigation. Another feature visible from the UMAP analysis is the exhaustion of CD8 T cells, but not the CD4 populations with the exception of CD4 Tregs that expressed PD-1 and TIGIT. Though PD-1 and TIGIT are known as exhaustions markers these were found on many populations of the liver, hence they may function differently, possibly contributing to the liver tolerance. Due to limited

sample numbers it has been difficult to assess the functional capacity of the un-determined populations in response to stimulation, nonetheless, analysis of many more populations than anticipated has already provided valuable insights, showing important differences at the cellular level. Inclusively, our data shows loss of MAIT cells in cirrhosis and LT and those that remain, have a reduced pro-inflammatory and cytolytic function. MAIT cells from CL have some potential to respond to stimulation, but those in LT are highly dysfunctional. Strategies to rescue dysfunctional MAIT cells from LT could improve the outcome for LT patients. Further studies are need to identify methods that augment proliferation of MAIT cells with tissue repair capacity that may be able to reverse liver damage that occurs in cirrhosis.

Major caveats – Samples were acquired from a cohort where matched blood samples were not collected; therefore we were not able to make adequate comparisons between liver and blood. Therefore, the comparisons made between the liver and blood are cross sectional. Despite the availability of matched PT and LT samples from two donors we could not extract parallel information, as the cells from one donor were not viable and the other sample had a very low cell count making it difficult to analyse. Liver disease samples, especially the CL samples were technically very difficult to process with some samples having extremely low viability thus limiting the sample size. Originally our study design involved processing 8-9 samples per condition, but this has reduced by half due to the viability of samples. The sample viability was beyond our control as we did not directly process the liver, but as liver damage is already quite high in cirrhosis and HCC, low sample viability would be expected. Following the initial analysis we had anticipated the arrival of further samples to supplement the existing data, but due to

the COVID pandemic this has been delayed, the remaining analysis will be completed in the near future.

6.6 Summary

1. MAIT cell numbers are significantly reduced in liver disease
2. Liver disease MAIT cells are highly dysfunctional
3. MAIT cells from CL and LT display an exhausted phenotype combined with impaired pro-inflammatory and cytotoxic capacity
4. LT-MAIT cells do not respond to stimulation and have reduced potential to express inflammatory cytokines
5. Several T cells population, in particular MAIT cells and MAIT cell related populations are altered or lost in liver disease

CHAPTER 7 – DISCUSSION

7.1 Discussion

MAIT cell research has increased exponentially and allowed us to appreciate both beneficial and pathological roles of this innate like T cell population. MAIT cells are abundant in humans, especially in the liver and blood. MAIT cells have been studied extensively in several disease settings including liver disease, but their exact function and regulation in the healthy liver is still not fully understood. The aim of this thesis was to explore the potential for MAIT cell activation in the liver, define their liver specific function and to investigate how changes to the MAIT cell phenotype and function in liver disease affects liver function.

To understand the role of MAIT cells in the liver microenvironment we used cell lines of hepatic origin to first activate MAIT cells and then explored how liver resident cells could influence activation of MAIT cells by conventional antigen presenting cells. Our data shows that MAIT cell activation in the liver was limited to LECs and they that could activate MAIT cells comprehensively but required higher antigen loads compared to conventional APCs. In contrast to LECs, HSCs and hepatoma cell lines failed to induce cytokine and cytolytic molecules from MAIT cells in response to *E*. coli exposure; however, MAIT cells could be activated by primary liver hepatocytes (*unpublished data*). The use of cell lines for such investigations should be considered carefully and findings should be paralleled with primary cell work. However, because primary cell processing can also alter the function of cells it would be ideal to investigate MAIT cell activation within the liver using an in-vivo model. Our data has explored the pro-inflammatory and cytolytic potential of MAIT cells activated by liver resident cells to *E*. coli exposure, but

whether liver resident cell activated MAIT cells can actually kill infected target cells and if this process plays a significant role *in vivo* requires further investigation.

MAIT cells from the blood and liver differ significantly. As our data uses healthy donor intrahepatic resident MAIT cells. Nonetheless our data provide a valuable insight into liver-MAIT cell activation, especially because MAIT cells can redistribute from the blood to liver. To fully evaluate activation and function of liver resident MAIT cells, our initial findings need to be confirmed using MAIT cells from healthy and diseased liver.

Kupffer cells are the largest organ resident macrophage population and together with liver dendritic cells are important for antigen uptake and presentation in the liver. Therefore, it would be valuable to determine MAIT cell activation by Kupffer cells and liver-DCs, which is still unexplored. Furthermore, MAIT cell activation needs to be assessed in the presence of various cell types as the liver is a multicell organ, where neighbouring cells can influence the response. As the liver is constantly exposed to large quantities of antigen from the gut, the long-term antigen exposure to MAIT cells in the liver requires investigation. Likewise, additional studies are need to explore the activation of MAIT cells by liver resident cells exposed to different pathogens, especially the MAIT cell response to viral infection as liver disease and HCC is often caused by chronic viral infection with HBV and HCV.

Previous studies investigating T cell activation in the liver and liver tolerance have highlighted both direct and indirect modulation of T cells by liver resident cells. We also explored the effect of hepatoma cells on MAIT cell activation by conventional and liver APCs. Data from chapter four shows that the presence of hepatoma cells in the MAIT cell activation assay can potently inhibit MAIT cell proinflammatory cytokines and GrB,

an effect also observed in assays using only hepatoma cell culture supernatant. This finding identifies a potential paracrine mechanism of MAIT cell suppression by hepatocyte derived soluble mediators and, though not explored in this project, there may also be a direct, contact dependent mechanisms for MAIT cell suppression by hepatoma cells. Data from chapter four identifies a small soluble COX-derived molecule that can alter pro-inflammatory cytokine production by conventional antigen presenting cells to suppress MAIT cell activation. Liver resident cells are already known to produce various other soluble compounds with suppressive activity, including IL-10, PGE_2 and TGF-β . After much investigation it has been difficult to fully identify the suppressive compound. Nevertheless, the initial investigation of suppressive and non-suppressive cell culture supernatants show liver and hepatocyte specific suppression. There is also a significant difference in the lipid metabolite profiles of the suppressive and non-suppressive supernatants. Indeed, altered lipid metabolite concentrations have been shown in obese mice with alcohol administration and in plasma of NASH patients(Caussy et al. 2020; Puri et al. 2016) and in another study of ovarian cancer, altered lipidome was associated with negative regulation of T cell responses(Wefers et al. 2018). A third study has revealed significant differences in the metabolomics and eicosanoids, including the identification of several prostaglandins and leukotrienes that are not only involved in HBV-cirrhosis and HCC, but also allows differentiation between the two liver diseases (Gong et al. 2017). Whether the hepatoma cell derived soluble mediator/s is disease specific or part of the liver tolerance mechanisms is not clear but with further investigation to identify the compound it would be possible to establish its function and quantify the compound in uncompromised liver as well as in liver disease. Our data demonstrate the dual regulation of MAIT cell function by hepatocytes. Despite the

ability of primary hepatocytes to robustly activate MAIT cells, hepatocytes could also suppress MAIT cell activation by paracrine modulation of APCs.

A key metabolite generated by the COX pathway is PGE_2, which is expressed by many liver resident cells(Horst et al. 2016b), and is reported to contribute to liver tolerance by dampening the inflammatory capacity of immune cells in the liver. Moreover, elevated levels of PGE_2 is one of the hallmarks of the tumour microenvironment(Wang 2006). Therefore, we examined the impact of PGE_2 on MAIT cell activation and function.

In chapter five we explored the effect of five prostaglandins and two common leukotrienes. Prostaglandins, particularly PGE_2 and PGD_2, but not leukotrienes, were able to potently suppress pro-inflammatory cytokine and GrB expression from MAITs. This inhibitory effect was similar to the hepatoma supernatants however prostaglandin receptor antagonists did not prevent MAIT cell suppression in hepatoma supernatant assays. Nonetheless, prostaglandins could suppress MAIT cells and mediated their suppressive effect both directly on MAIT cells and indirectly. The indirect suppressive mechanism of prostaglandins involves the inhibition of pro-inflammatory cytokine production by APCs. This effect was only observed at very high concentrations similar to those found in tumour microenvironment. Therefore, in homeostasis, in the liver, PGE_2 would not suppress MAIT cells but it is highly likely that liver tumour- PGE_2 will inhibit the activation and pro-inflammatory function of MAIT cells. Though low concentrations of PGE_2 did not suppress MAIT cells in-vitro, physiological concentrations of PGE_2 in the liver maybe able to suppress MAIT cells indirectly by inducing Tregs, this effect has been shown in a lung cancer model where PGE_2 induced Tregs were able to supress anti-tumour responses(Sharma et al. 2005). For a comprehensive understanding of how PGE_2 impacts MAIT cells it would be ideal to use murine models like the PGE_2 receptor

knockout models to determine the signalling pathways and receptors involved. The liver disease models will help to identify the PGE_2 mediated alteration to MAIT cells and to check if PGE_2 inhibitors may help to improve MAIT cell function in liver tumour or other PGE_2 mediated liver diseases.

Lastly to investigate MAIT cells in liver disease we used high-dimensional flow cytometry data to evaluate the phenotype of MAIT cells from cirrhosis and HCC. Similar to previous studies investigating MAIT cells in HCC, we found that MAIT cells were significantly reduced and displayed an activated and exhausted phenotype ex-vivo. Stimulation of MAIT cells from cirrhosis and HCC confirmed dysfunction of MAIT cells in tumour whereas cirrhosis-MAIT cells had the potential to respond but failed to respond optimally to *E*. coli and IL-12+IL-18 stimulation. Cirrhosis is the scarring process associated with chronic liver conditions, limiting resolution, this could, possibly be prevented by MAIT cells as they have tissue repair capacity(Leng et al. 2019). Whether liver-MAIT cells have tissue repair capacity needs to be explored and requires further investigation to determine when and what causes the loss of the tissue repair function of MAIT cells in disease. Single cell sequencing will be a useful next step in this study to understand heterogeneity and changes at the transcriptional level to both MAIT cells and MAIT cell related populations.

7.2 Future prospects

The findings from this thesis highlight some very important and previously unexplored aspects of MAIT cells in the liver environment which can be progressed in many directions. An essential experiment would be to determine the differences in primary hepatocytes and hepatocyte cell lines as only primary hepatocytes were able to activate healthy donor blood-MAIT cells. Since MAIT cells can be activated by virus induced cytokines it would interesting to the test the capacity for ~~MAIT activating~~ viruses to induce the necessary MAIT cell activating cytokines from liver resident cells and whether the viruses can replicate in liver resident cells other than hepatocytes. Since LECs were able to induce cytotoxic molecules from MAIT cells the next step would be to test the capacity of LEC-activated MAIT cells to kill infected cells.

While we have already shown a strong impact of hepatoma cells on MAIT cell activation by conventional APCs, further investigation to identify the inhibitory molecule involved is required. Blocking different lipid synthesis pathways suggest an essential role for the COX pathway. However, complete inhibition of the COX enzymes is difficult, limiting our analysis. Thus, a better approach would be to test hepatocyte cell culture supernatants from COX knockout mice or even engineer COX knock hepatocyte cell lines using the CRISP-Cas9 technology. Initial mass spectrometry results show significant enrichment of an unknown lipid metabolite, however the results from two different runs were not completely consistent. Nevertheless, it is worth probing further with GC-MS, a technique suitable for smaller and thermally stable metabolites and to fragment the sample for specific molecular masses that can then be analysed in detail by mass spectrometry. The Huh7.5 cell line is a variant of the Huh7 cell line and unlike the Huh7 culture supernatant, the Huh7.5 cell line culture supernatant did not suppress MAIT

cells. Therefore, investigating the differences of these two cell lines at the transcriptional and metabolic levels may shed light on the possible compounds or pathways responsible for the suppressive activity of hepatoma cell line derived supernatants. Numerous factors are likely to be responsible for the suppression therefore we need to block multiple compounds/pathways instead of a single enzyme, receptor or compound to match the physiological settings where various compounds and pathways are functional. The latter will reduce redundancy in our supernatant assays and narrow the remaining factor/s we are looking for.

Prostaglandins, especially PGE_2, is a pleiotropic effector molecule and is able to strongly inhibit the pro-inflammatory and cytotoxic capacity of MAIT cells. Analysing the phenotype and functional potential of MAIT cells in prostaglandin driven diseases, such as cancers(Wang and Dubois 2006), will help us to understand if MAIT cells are contributing to or are protective in the disease. Furthermore, as PGE_2 has been shown to skew T cells towards a type-17 response, whether PGE_2 can induce IL-17 from MAIT cells needs to be explored. If PGE_2 can induce IL-17 from MAIT cells, then it may be likely involved notably in the regulation of the development of liver fibrosis. If so, this effect should be investigated in a liver fibrosis model where blocking PGE_2 may prevent IL-17 induction by MAIT cells and thus support liver fibrosis resolution. In the liver, MAIT cells are likely to be exposed consistently to low levels of PGE_2 and therefore, how MAIT cells function after long term exposure to PGE_2 is also an important aspect which requires investigation. Another question worth investigating is the whether constant or excessive levels of exposure to PGE_2 induces an exhaustive phenotype in MAIT cells or permanently modifies the phenotype of MAIT cells to a more regulatory type.

The current data on MAIT cells in HCC and cirrhosis is derived from alcohol defined liver disease and indicates MAIT cell dysfunction as well as the decline in the MAIT cell numbers, which coincides with an exhausted phenotype. We plan to look at MAIT cells from viral defined HCC and cirrhosis and will compare differences in these two cohorts. Ideally MAIT cell identification is best done with MR1 tetramers. Therefore, the next data set will include MR1-tetramer staining, plus CD161 and Vα7.2. Additionally, single cell RNA sequencing of liver samples from HCC and cirrhosis will be used to compare our cohorts to existing cohorts and assess more features and populations associated to liver disease. Through the single cell RNA sequencing we expect to acquire a broader overview of the transcriptional changes to MAIT cells and other populations in liver disease.

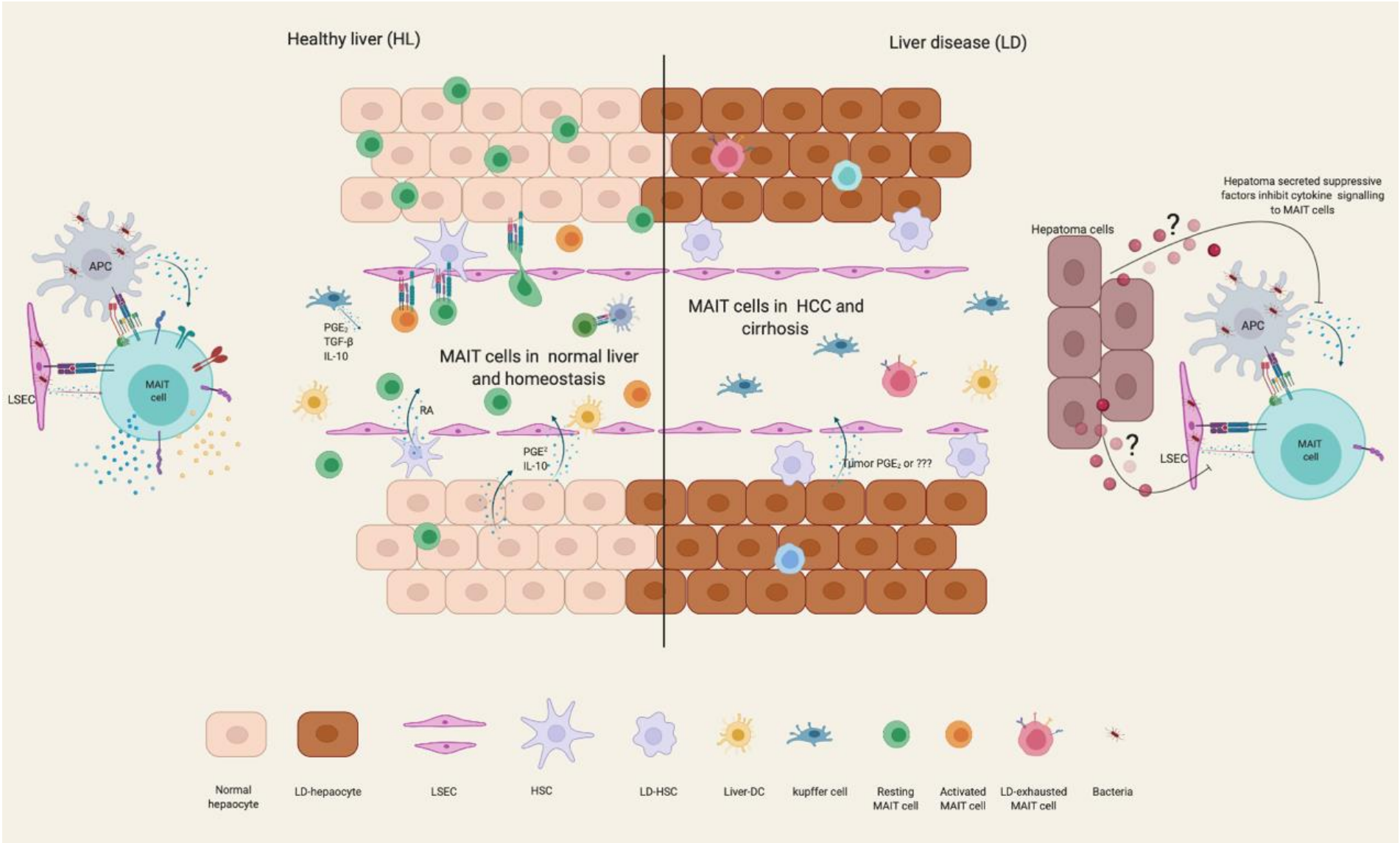

Healthy liver (HL)
Liver disease (LD)
APC
MAIT cell
LSEC
PGE2
TGF-β
IL-10
MAIT cells in normal liver and homeostasis
RA
PGE2
IL-10
MAIT cells in HCC and cirrhosis
Tumor PGE2 or ???
Hepatoma cells
Hepatoma secreted suppressive factors inhibit cytokine signalling to MAIT cells
?
?
APC
MAIT cell
LSEC
Normal hepaocyte
LD-hepaocyte
LSEC
HSC
LD-HSC
Liver-DC
kupffer cell
Resting MAIT cell
Activated MAIT cell
LD-exhausted MAIT cell
Bacteria

Diagram 7.1 – MAIT cell activation, phenotype and modulation by liver residents in healthy vs liver disease. Left section illustrates enrichment of MAIT cells in the healthy liver, their localisation in the sinusoidal lumen and within the parenchymal space and their potential interaction with professional and non-professional antigen presenting cells. To the far left is a schematic of MAIT cell activation by conventional APC's and LECs in response to bacterial stimulation in the liver microenvironment. Right section represents liver disease where MAIT cell numbers are much lower and are phenotypically and functionally different MAIT cell in normal liver. To the far right of the diagram is an illustration of MAIT cell modulation by paracrine signals derived from hepatoma cells in liver disease. Image created using BioRender.

7.3 Conclusion

The dynamics of T cell activation in the liver is tightly regulated to maintain tolerance and prevent damage. However, this also means that T cell activation does not occur readily, and many thresholds have to be overcome to reach activation. Though liver resident cells have antigen presenting capacity, they don't have all the optimal components for T cell activation. Further, they could interfere with T cell activation by the expression of co-inhibitory receptors like PD-L1 and the production of immunoregulatory compounds. Hence, MAIT cell activation in the liver is possible but can be impacted on multiple levels. This may be due to the tolerogenic properties of the liver and the constant secretion of immunoregulatory compounds in homeostasis. The enrichment of MAIT cells in the liver seems beneficial as they would be able to respond rapidly due to their innate characteristics, support the regeneration of the liver through their tissue repair capacity and could provide targeted therapy as a result of their MR1 restriction. However, in some circumstances MAIT cells fail to mount efficient immune responses or become overactive, in both situations leading to chronic liver damage. As yet, our understanding of MAIT cells in the liver is limited and requires further examination to help us design efficient MAIT cell-based therapies that can be beneficial for the resolution of liver diseases.

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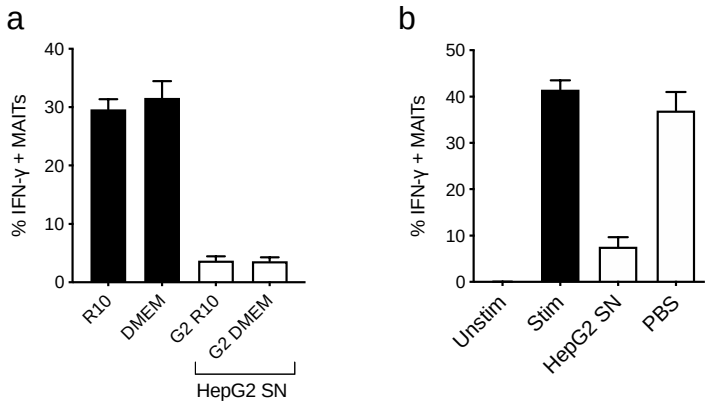
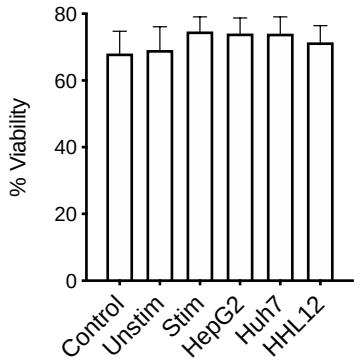
APPENDIX

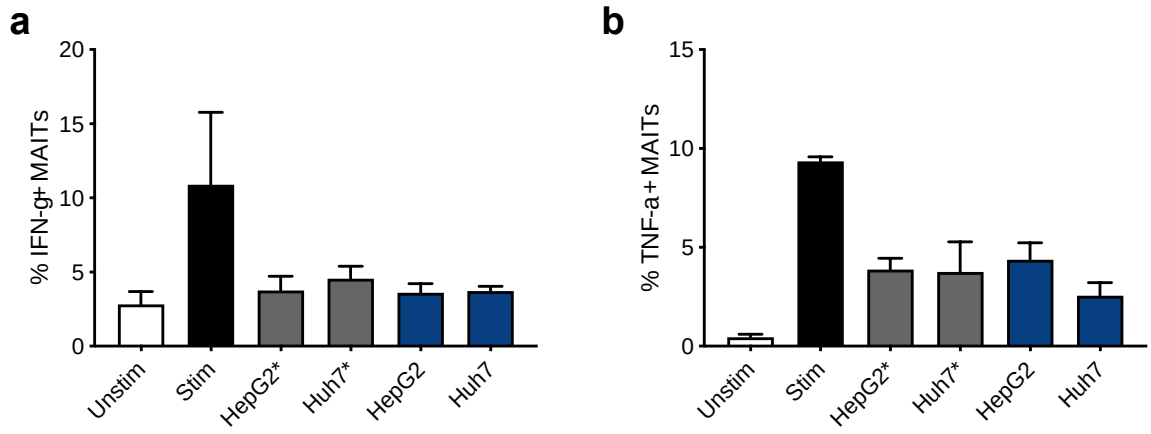
Supplementary figures



Supplementary figure 1 – Culture media does affect MAIT cell suppression in supernatant assays. Isolated CD8 T cells were activated with *E.* coli stimulated THP-1 cells for 16 hours in the presence of R10 medium, DMEM or PBS. Cells were stained and analysed by flow cytometry, IFN-γ expression from CD8+Vα7.2+CD161++ MAIT cells, (n=4).

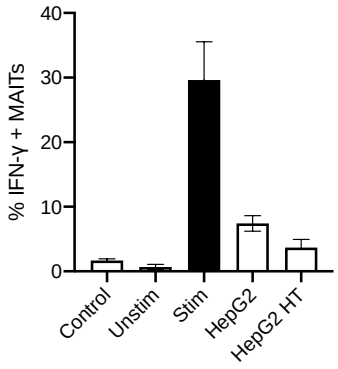


Supplementary figure 2 – Hepatoma supernatant does not affect viability of MAIT cells in supernatant treated assays. Isolated CD8 T cells were activated with *E.* coli stimulated THP-1 cells +/- Hepatoma cell culture supernatant. Viability of MAIT cells was determined by flow cytometry using near IR Live dead stain, (n=4).

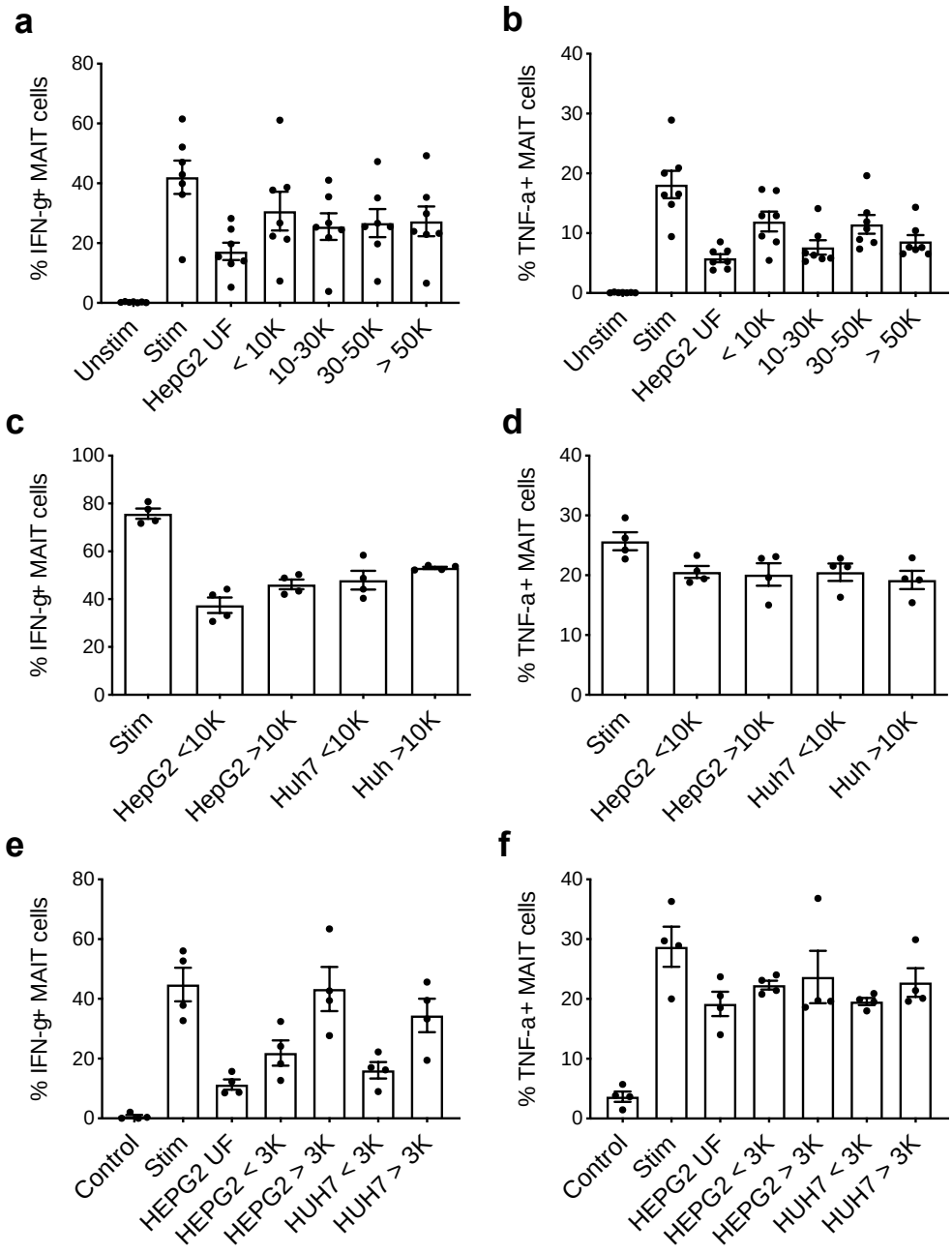


Supplementary figure 3– AFP depletion from hepatoma cell derived culture supernatant does not prevent suppression of MAIT cell IFN-γ and TNF-α

AFP depleted HepG2 and Huh7 culture supernatant was added to supernatant assays, incubated overnight and analyzed for IFN-γ and TNF-α expression by flow cytometry analysis. a) IFN-γ and b) TNF-α expression from CD8+CD161++Vα7.2+ MAIT cells, (n=3). Bars: black=stimulated, white=unstimulated, grey*=AFP depleted supernatant, blue=standard hepatoma culture supernatant.

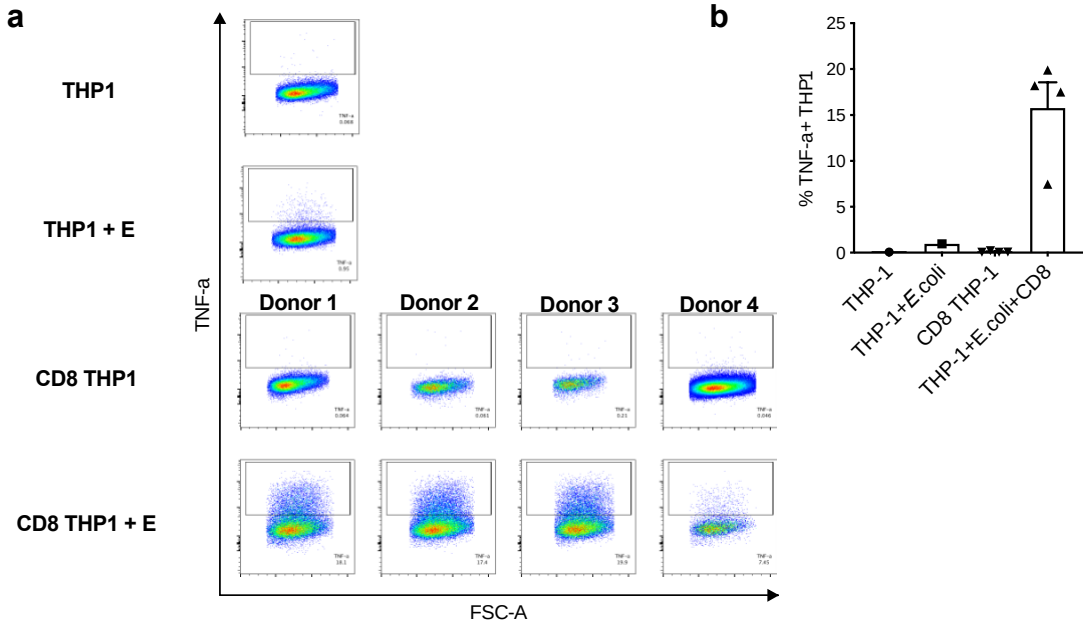


Supplementary figure 4 - Batch test for heat denatured HepG2 culture supernatant. Isolated CD8 T cells were activated with *E.* coli stimulated THP-1 cells for 16 hours with standard HepG2 supernatant or heat treated HepG2 supernatant. Cells were stained and analysed by flow cytometry, IFN-γ expression from CD8+Vα7.2+CD161++ MAIT cells, (n=4). HT=heat treated.

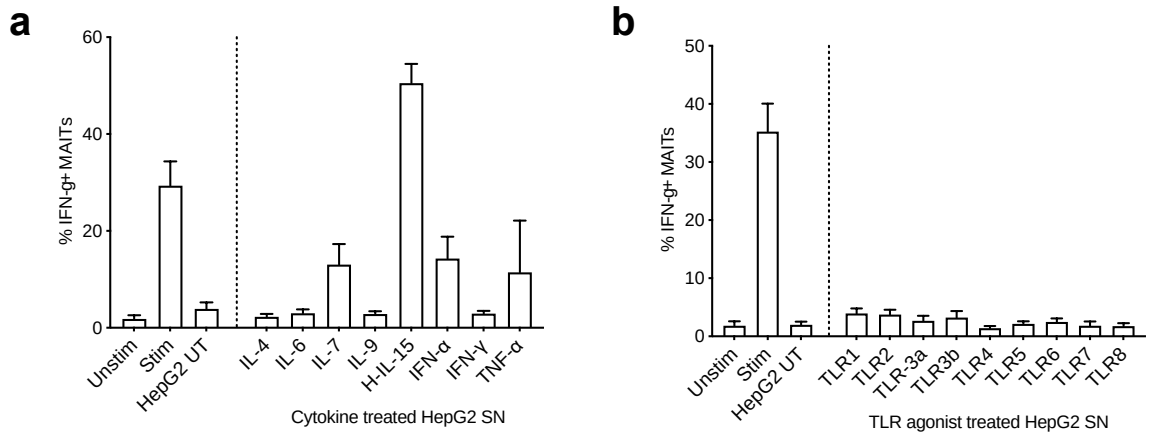


Supplementary figure 5 – Hepatoma cell culture medium fractioning to determine size of inhibitory factor/s. Fractionated HepG2 and Huh7 cell line supernatants were added to supernatant assays, incubated overnight and analyzed by flow cytometry. HepG2 culture supernatant was subject to serial fractionation using 3 filters, 10, 30 and 50 kDa before adding to the supernatant assays. a-b) IFN-γ and TNF-α expression from CD8+CD161++Vα7.2+ MAIT cells, (n=7). c-d) IFN-γ and TNF-α expression from *E.* coli

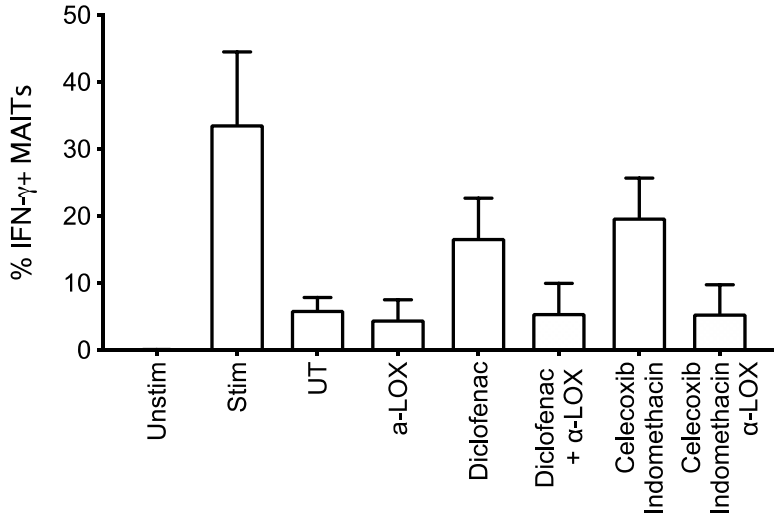
stimulated MAIT cells treated with HepG2 and Huh7 culture supernatants fractionated with 10 kDa filter, (n=4). e-f) IFN-γ and TNF-α expression from *E.* coli stimulated MAIT cells treated with HepG2 and Huh7 culture supernatants fractionated with 3 kDa filter, (n=4).



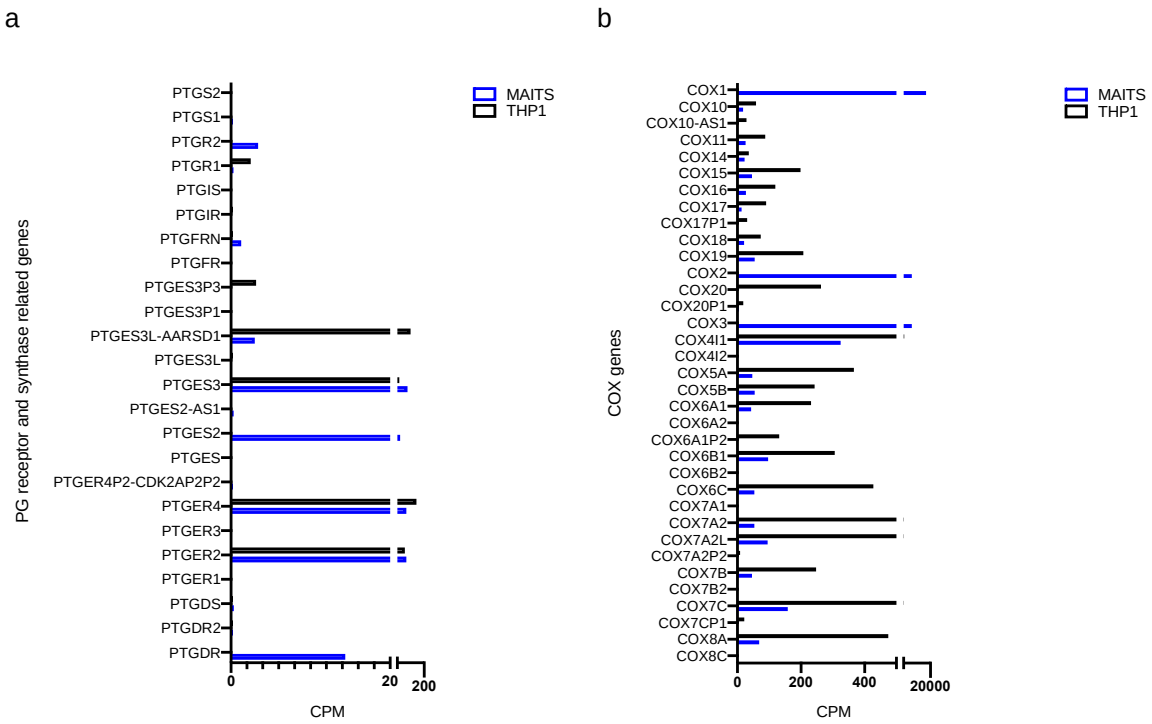
Supplementary figure 6 - TNF-α expression from THP-1 cells is induced by paracrine feedback from activated MAIT cells. *E.* coli stimulated and unstimulated THP-1 cells were cultured with and without CD8 T cells to determine MAIT cell mediated paracrine feedback to THP-1 cells, TNF-α expression from THP-1 cell was measured by flow cytometry analysis. a) Flow cytometry plots showing TNF-α expression from single positive THP-1 cells cultured without and with CD8 T cells from 4 individual donors. b) Quantification of TNF-α expression from THP-1 cells.



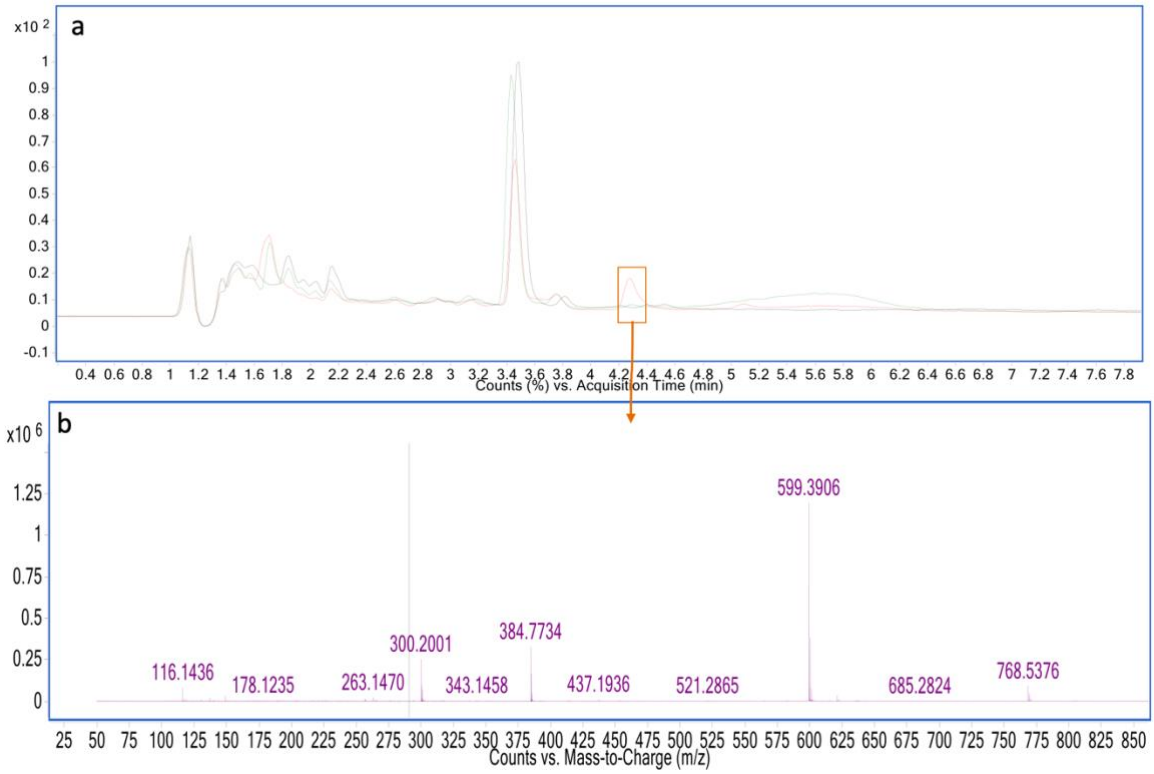
Supplementary figure 7 – Effect of cytokine and TLR stimulated HepG2 derived supernatant on MAIT cell IFN-γ expression. HepG2 cells were stimulated with cytokines and TLRs for 48 hours, supernatants were extracted and added to supernatant assays for 16 hours prior to measuring IFN-γ expression by flow cytometry, (n=4).



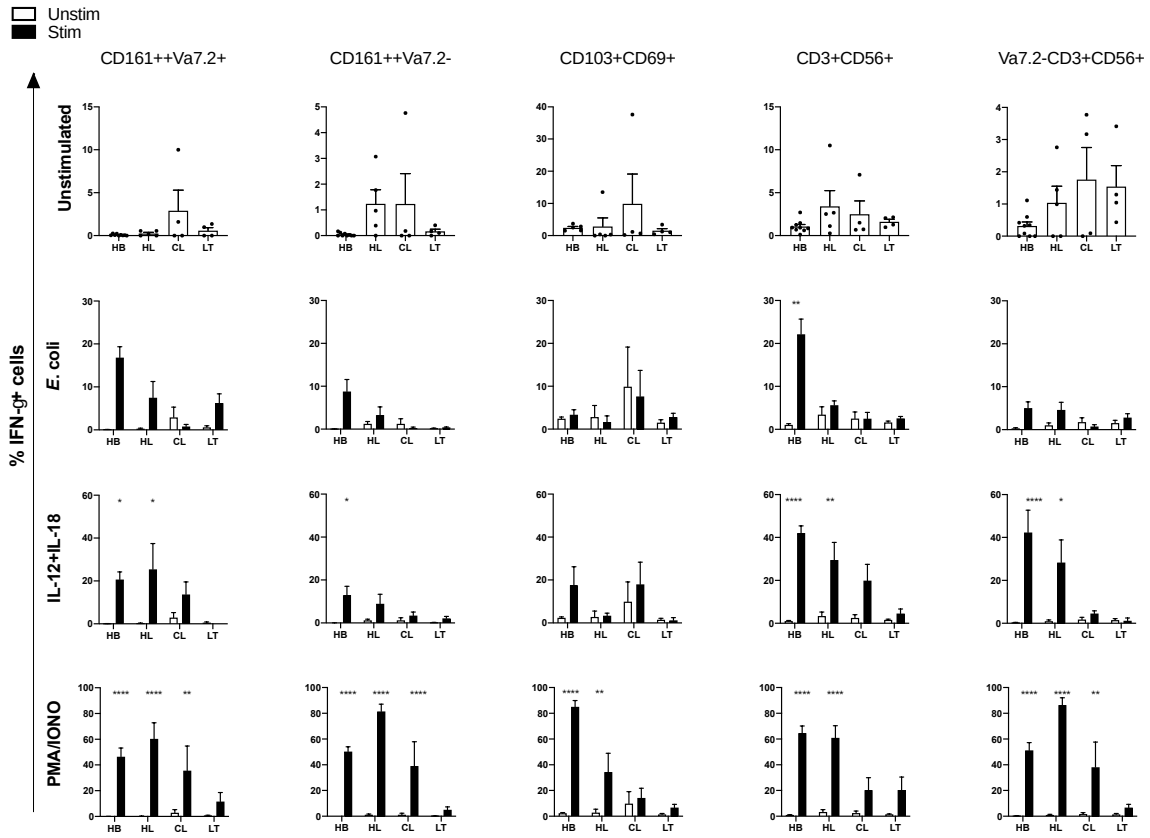
Supplementary figure 8 – HepG2 cells treated with diclofenac and combination of inhibitors of COX and LOX enzymes. MAIT cells were activated with *E.* coli stimulated THP-1 cells for 16 hours + R10 (stim) or COX/LOX inhibitor treated HepG2 culture supernatant. IFN-γ expression from MAIT cells measured by flow cytometry, (n=4).



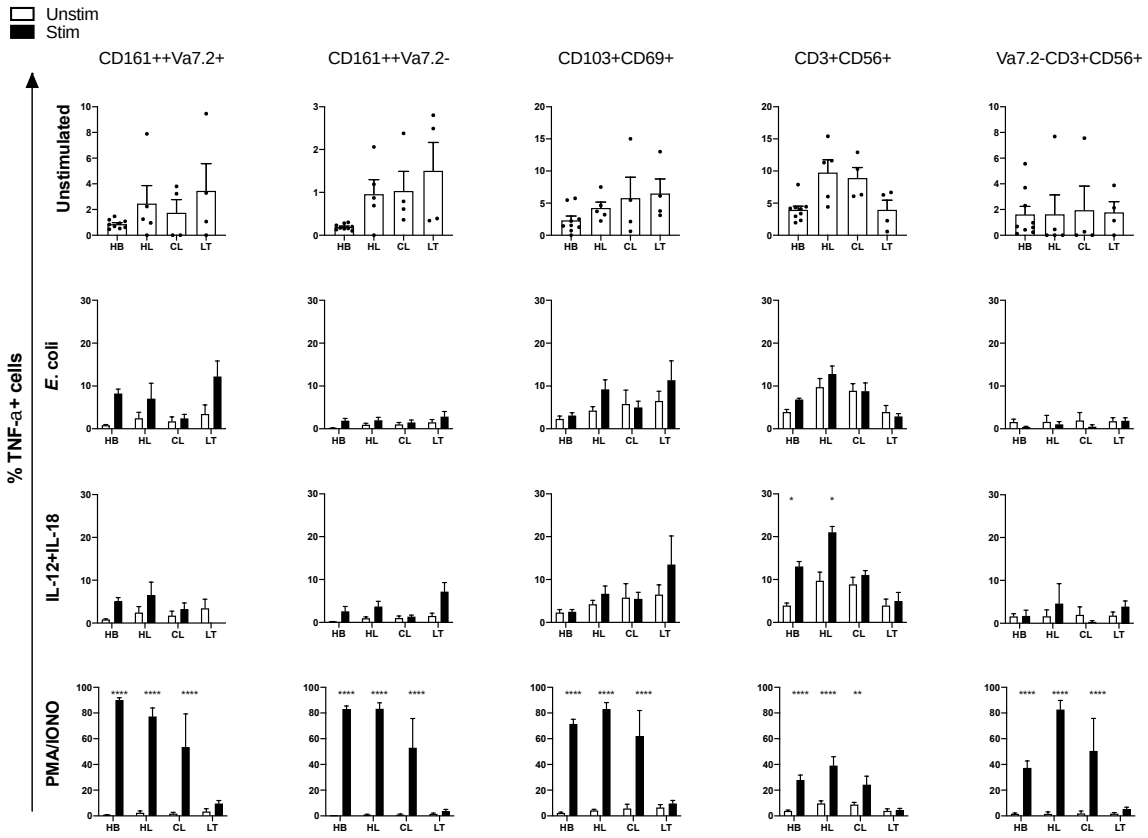
Supplementary figure 9 – Prostaglandin and cyclooxygenase gene expression from MAIT cells and THP-1 cells. Gene expression from unstimulated sorted MAIT cells (blue) and untreated THP-1 cells (black). a) prostaglandin and related genes and b) cyclooxygenase genes, data shows raw counts expressed as counts per million (CPM).



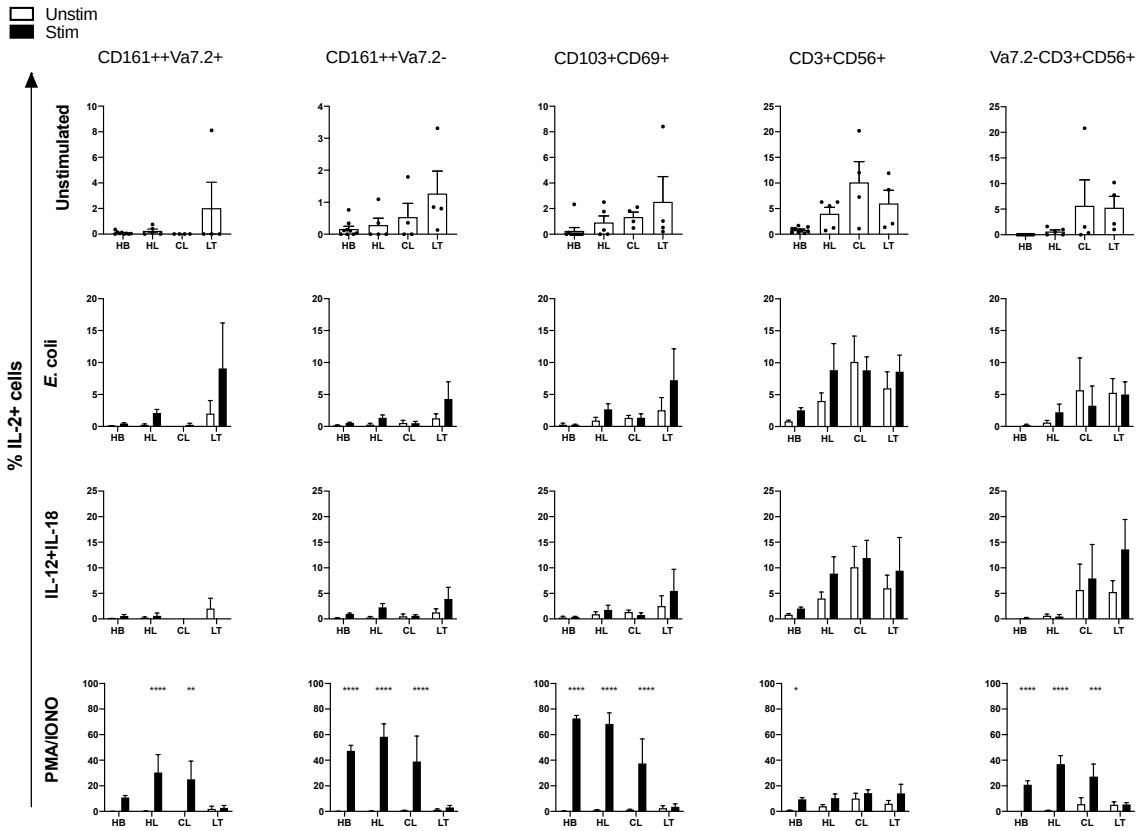
Supplementary figure 10 – Mass spectrometry analysis of HepG2 culture supernatant. Culture Medium (black), HepG2 (red) and HeLa cell culture supernatants were screened for small lipid metabolites by liquid chromatography-mass spectrometry (LC-MS) on 6560 ion mobility QTOF LC/MS mass spectrometer (Agilent Technologies). **a)** Chromatogram of metabolites detected in the total sample over approximately 8 minutes. Individual samples overlaid to show differential expression of metabolites indicated by peaks. **b)** Abundance and mass-to-charge ratio of metabolites within the HepG2 peak (orange rectangle). *(n=3)*.



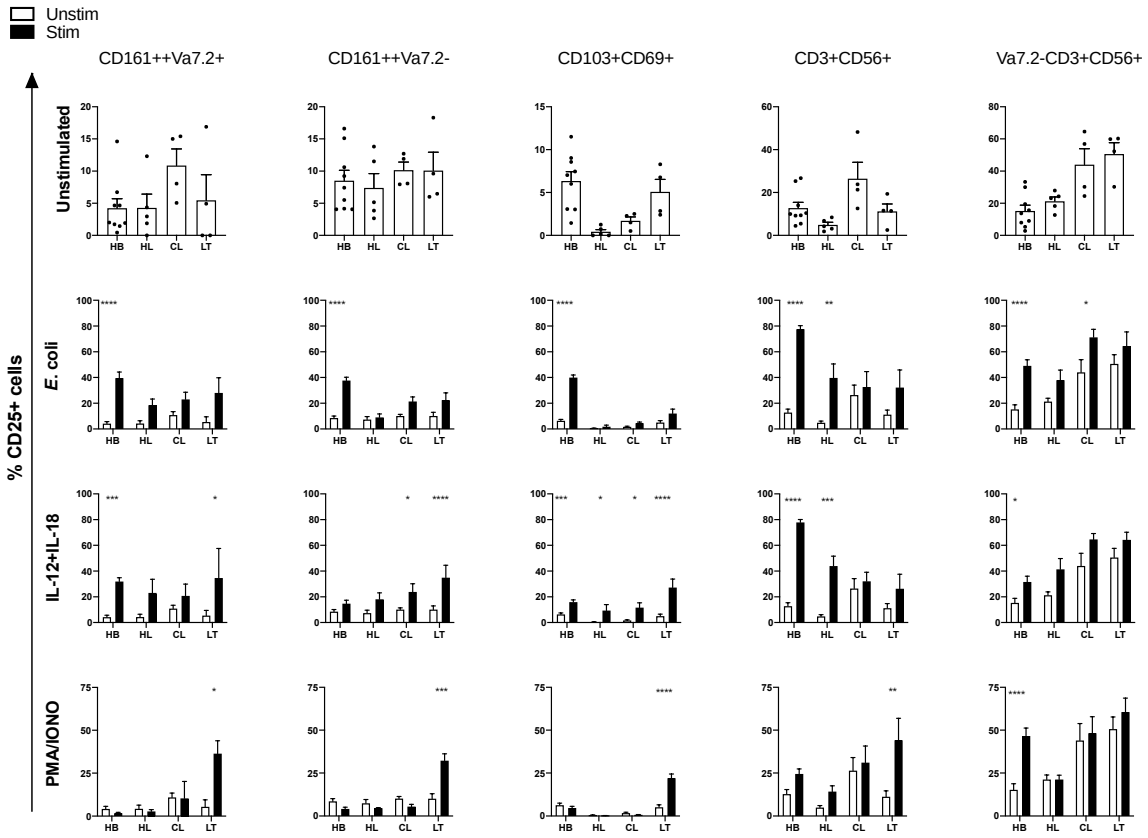
Supplementary figure 11 – IFN-γ expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++ Vα7.2+(MAITs), CD161++ Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0332, **P<0.0051, ****P<0.0001.



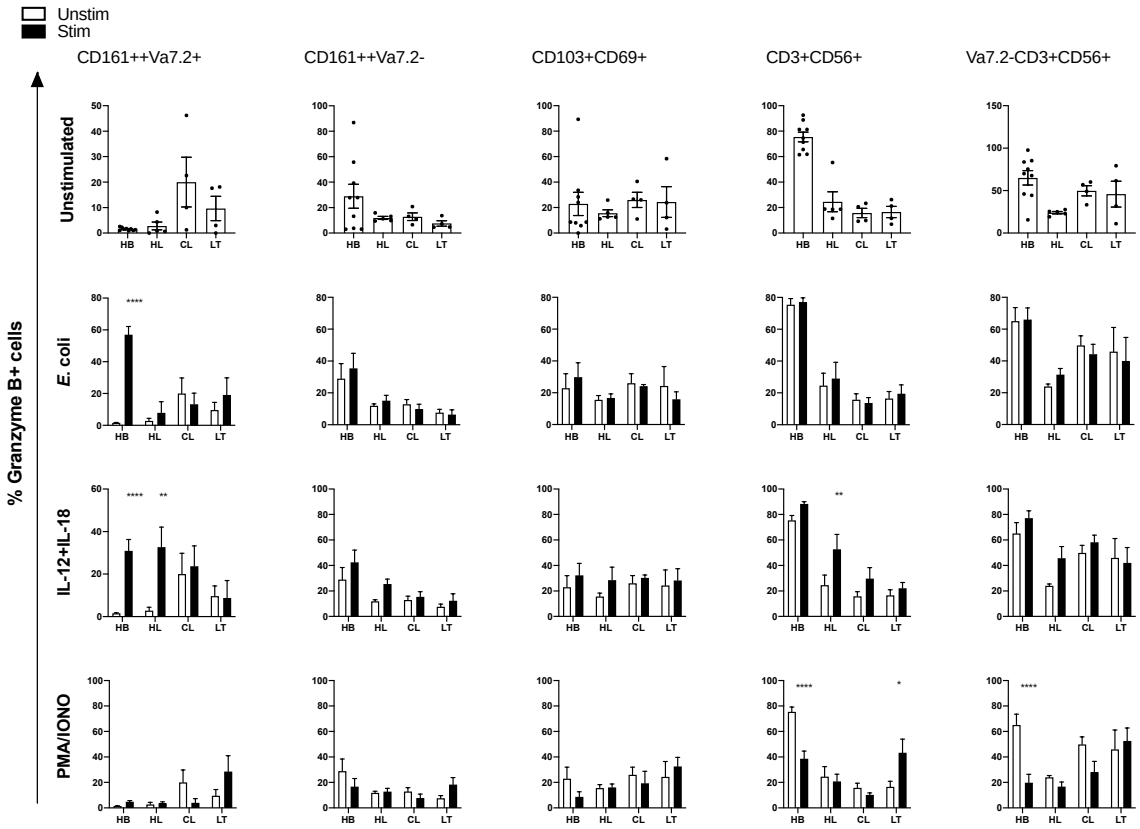
Supplementary figure 12 - TNF-α expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++ Vα7.2+(MAITs), CD161++ Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0215, **P<0.0039, ****P<0.0001.



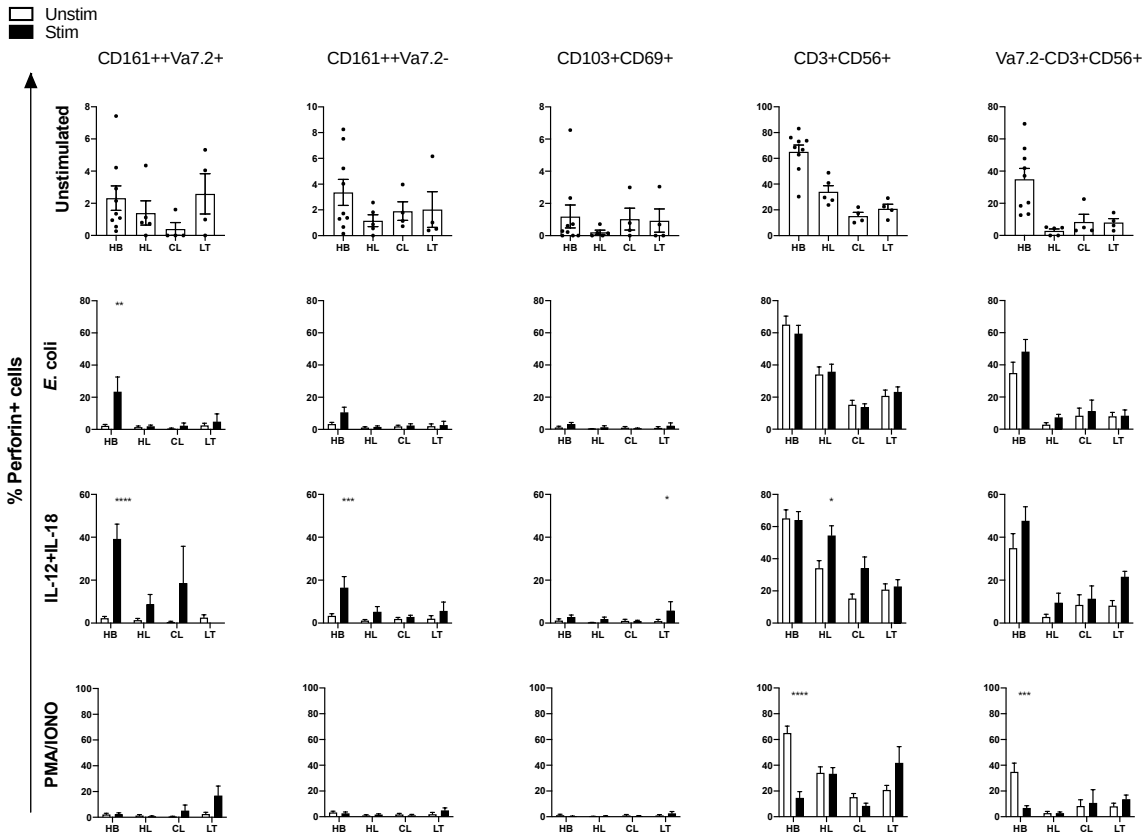
Supplementary figure 13 – IL-2 expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++ Vα7.2+(MAITs), CD161++ Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0106, **P<0.0021, ***P<0.0005, ****P<0.0001.



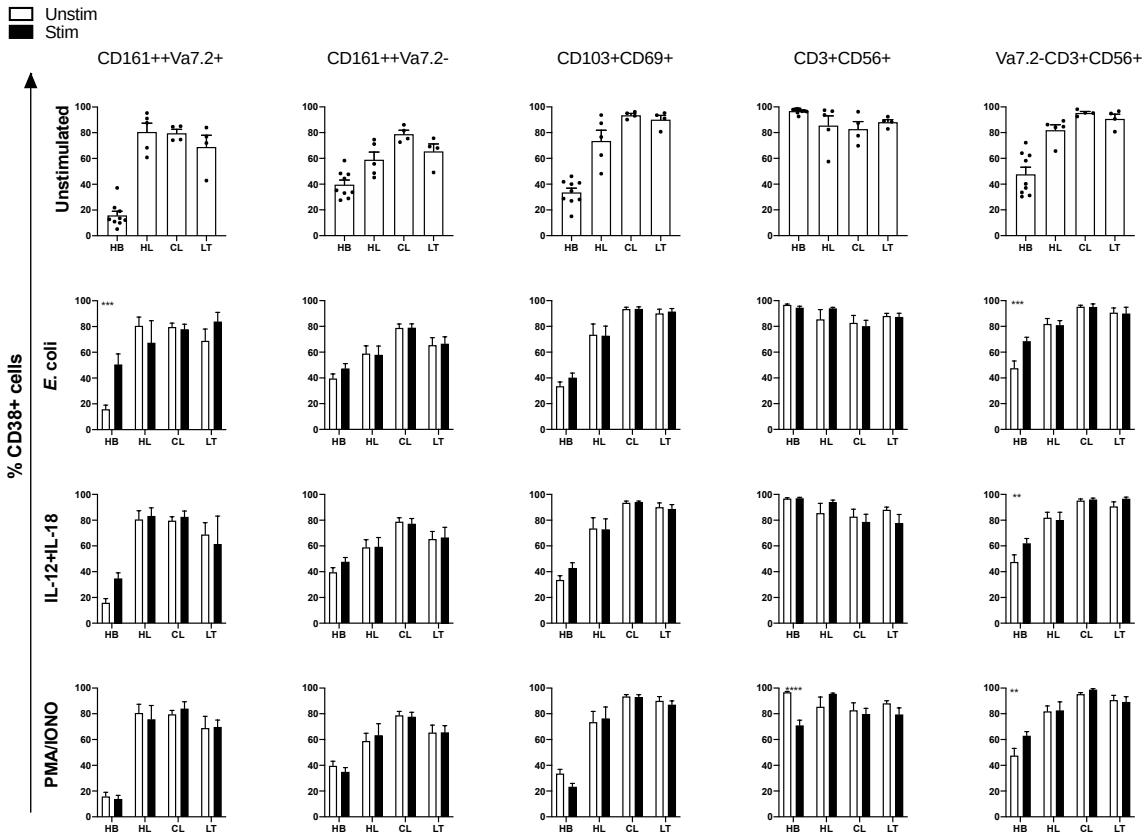
Supplementary figure 14 – CD25 expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++ Vα7.2+(MAITs), CD161++ Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0451, **P<0.0069 ***P<0.0008, ****P<0.0001.



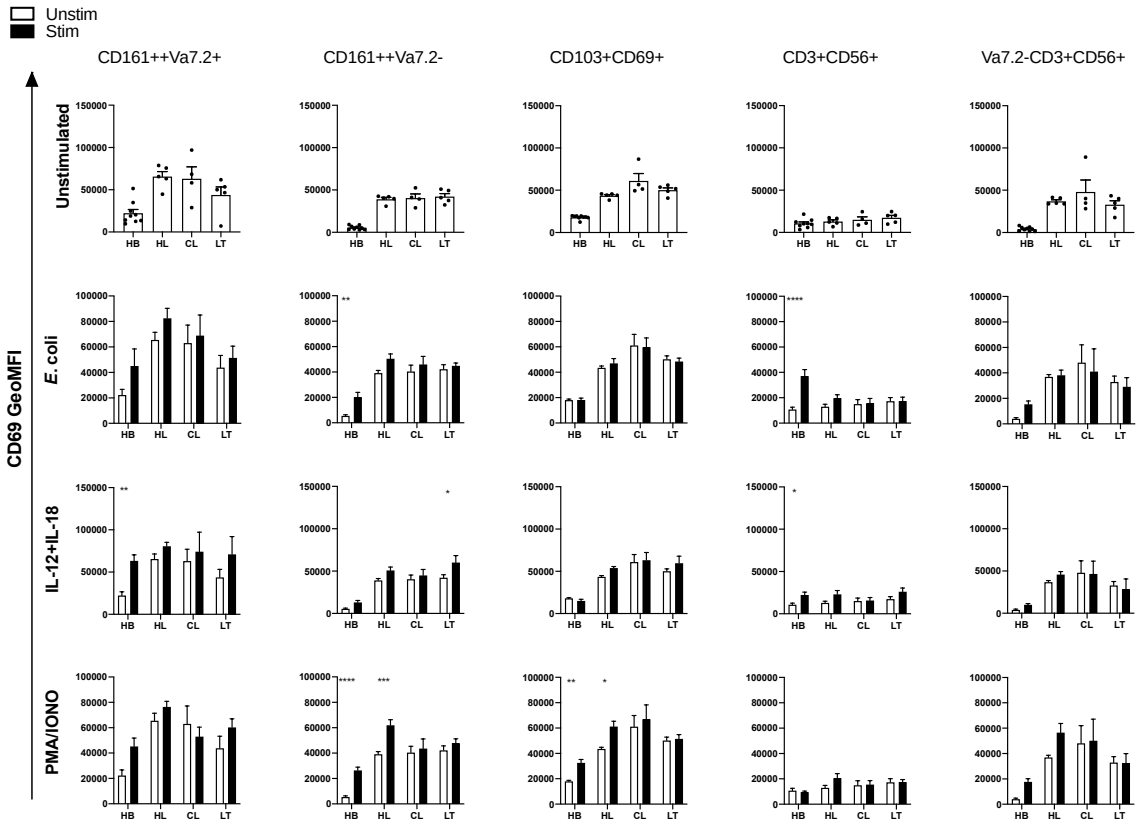
Supplementary figure 15 – GrB expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++ Vα7.2+(MAITs), CD161++ Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0249, **P<0.0067, ****P<0.0001.



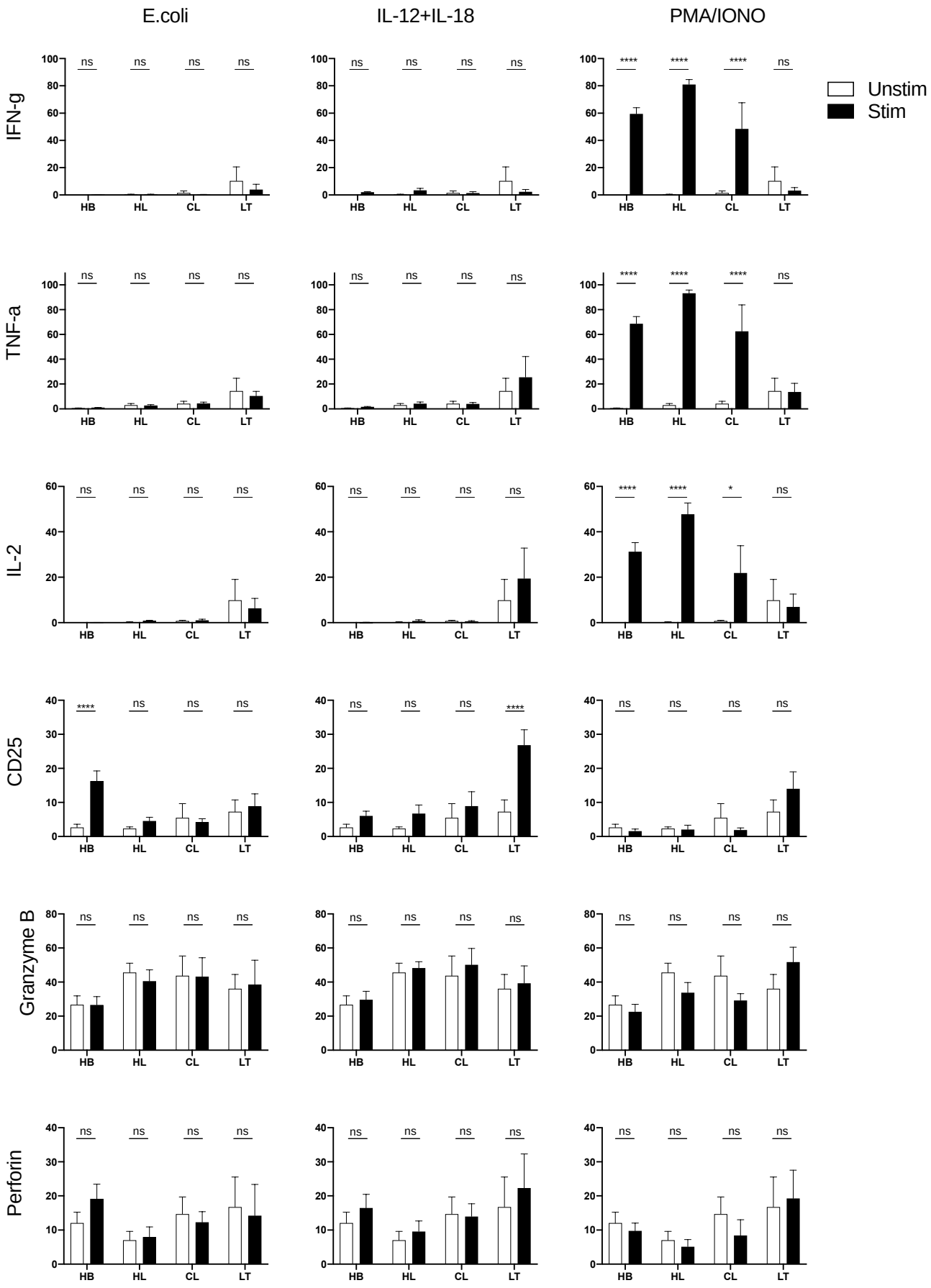
Supplementary figure 16 - Perforin expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++Vα7.2+(MAITs), CD161++Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0468, **P<0.0066, ***P<0.0003, ****P<0.0001.

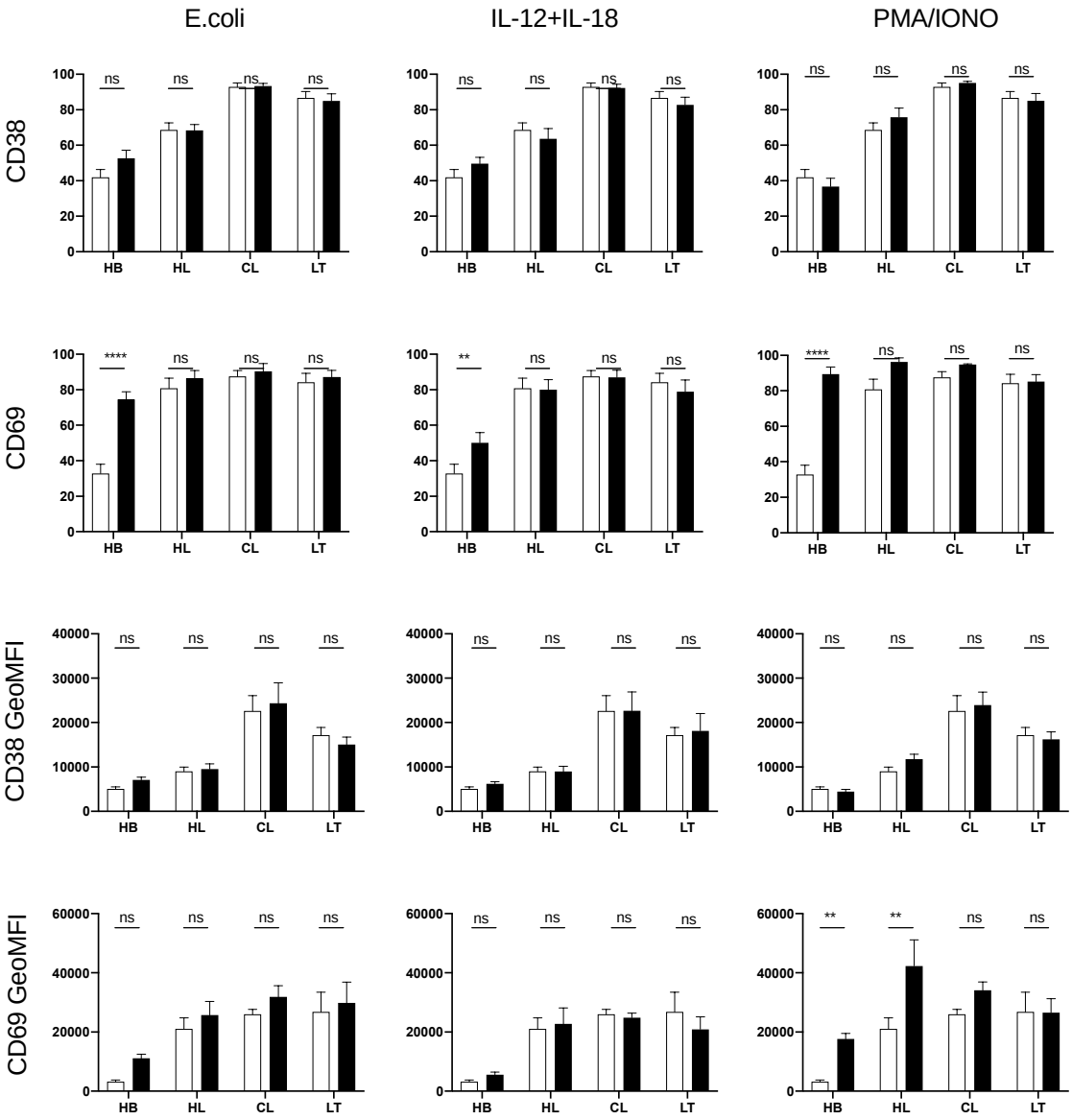


Supplementary figure 17 – CD38 expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++Vα7.2+(MAITs), CD161++Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, **P<0.0095, ***P<0.0006, ****P<0.0001.

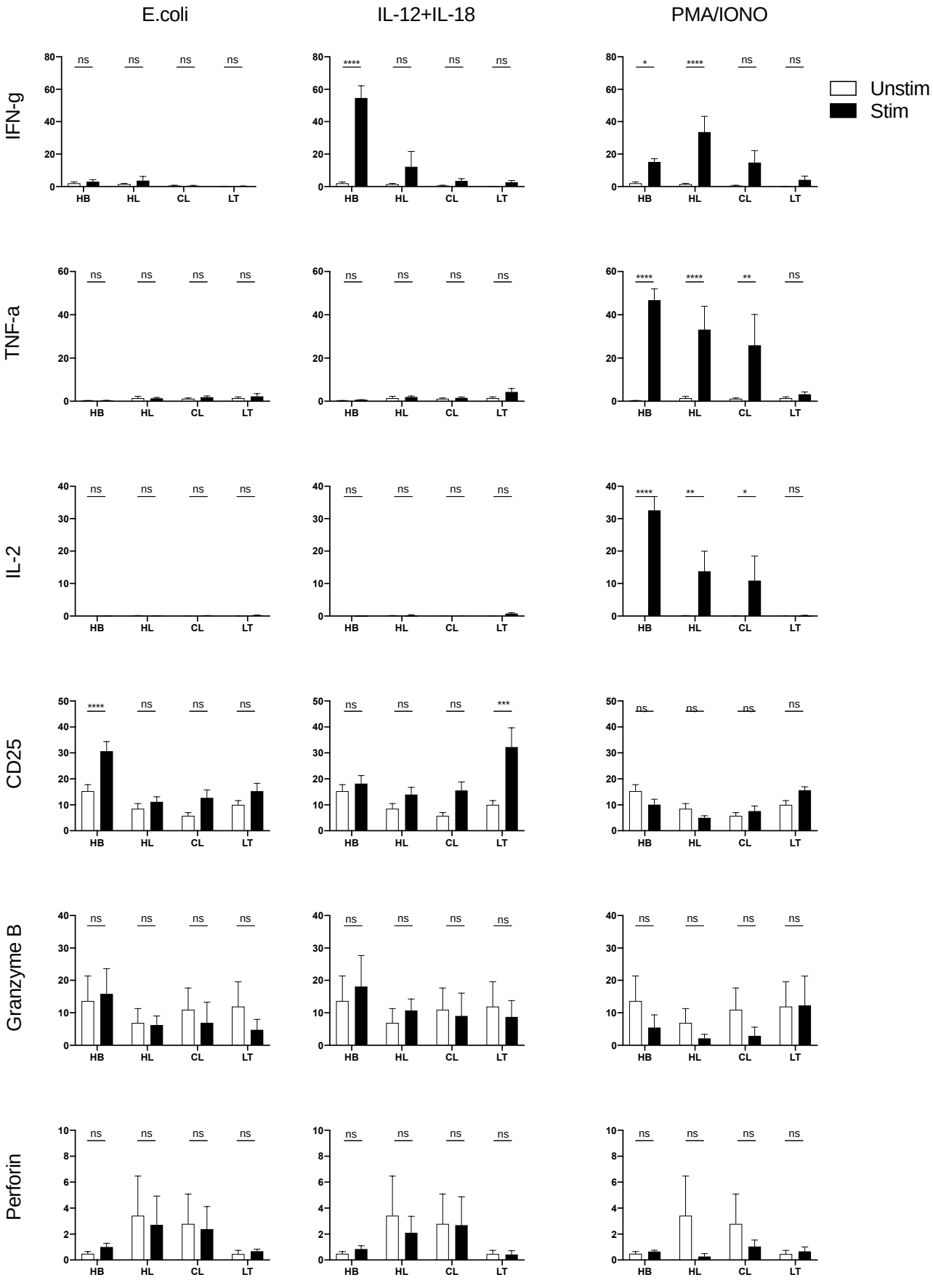


Supplementary figure 18 – CD69 expression from various populations from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different populations, left to right; CD161++Vα7.2+(MAITs), CD161++Vα7.2-, CD103+CD69+ (Trm), CD3-CD56+ (NK cells) and Vα7.2-CD3+CD56+ (NKT-like cells). Rows represent different stimulations. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells. Statistical significance = Dunnett's multiple comparisons test, *P<0.0183, **P<0.0085, ***P<0.0008, ****P<0.0001.


E.coli
IL-12+IL-18
PMA/IONO
Unstim
Stim
IFN-g
TNF-a
IL-2
CD25
Granzyme B
Perforin
HB
HL
CL
LT
ns
****
*



Supplementary figure 19 – Phenotype and functional capacity of bulk CD8 population from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different stimulations. Rows represent activation markers, cytokines a or cytotoxic molecules. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells.


E.coli
IL-12+IL-18
PMA/IONO
Unstim
Stim
IFN-g
TNF-a
IL-2
CD25
Granzyme B
Perforin
HB
HL
CL
LT
ns
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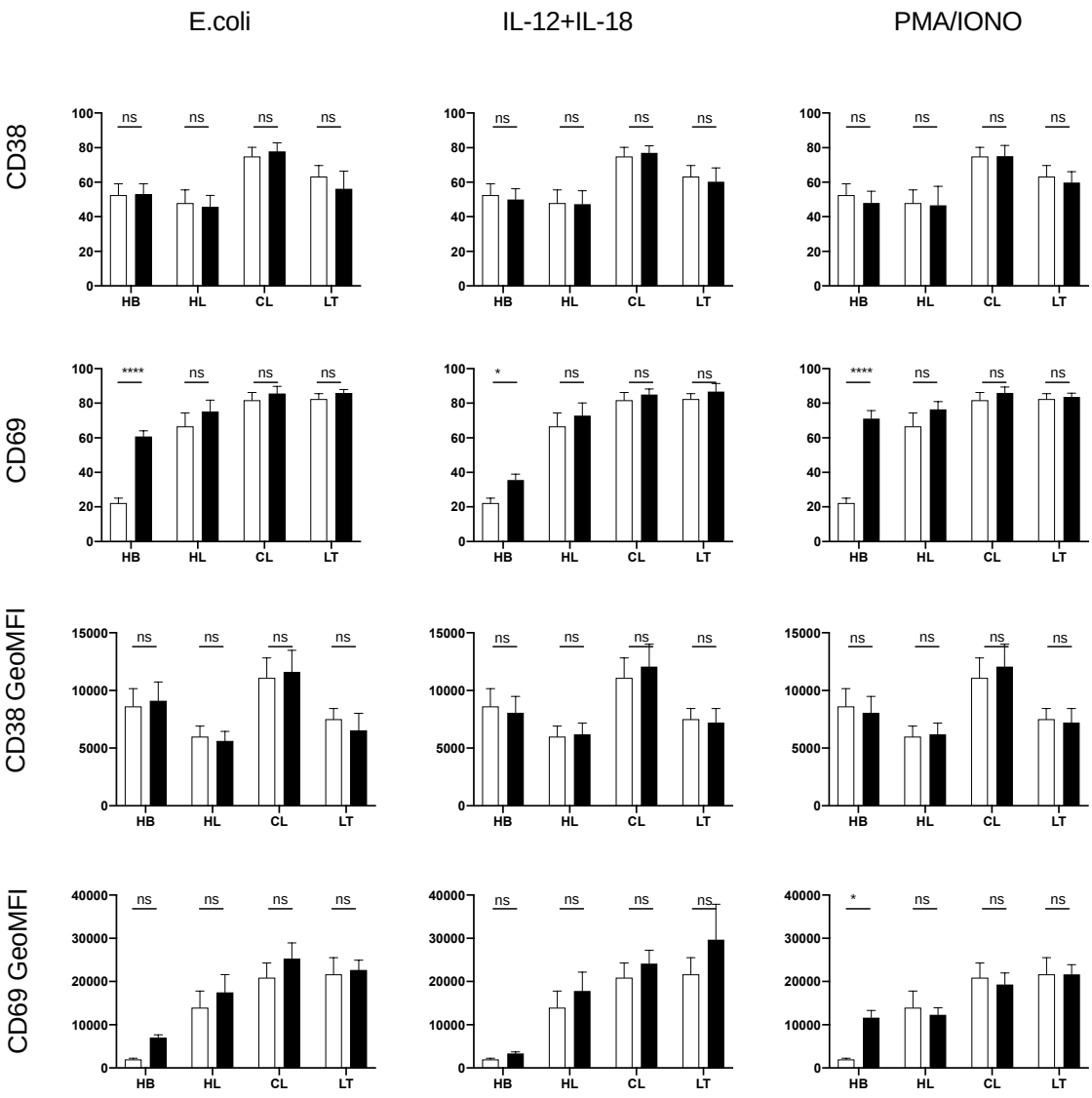
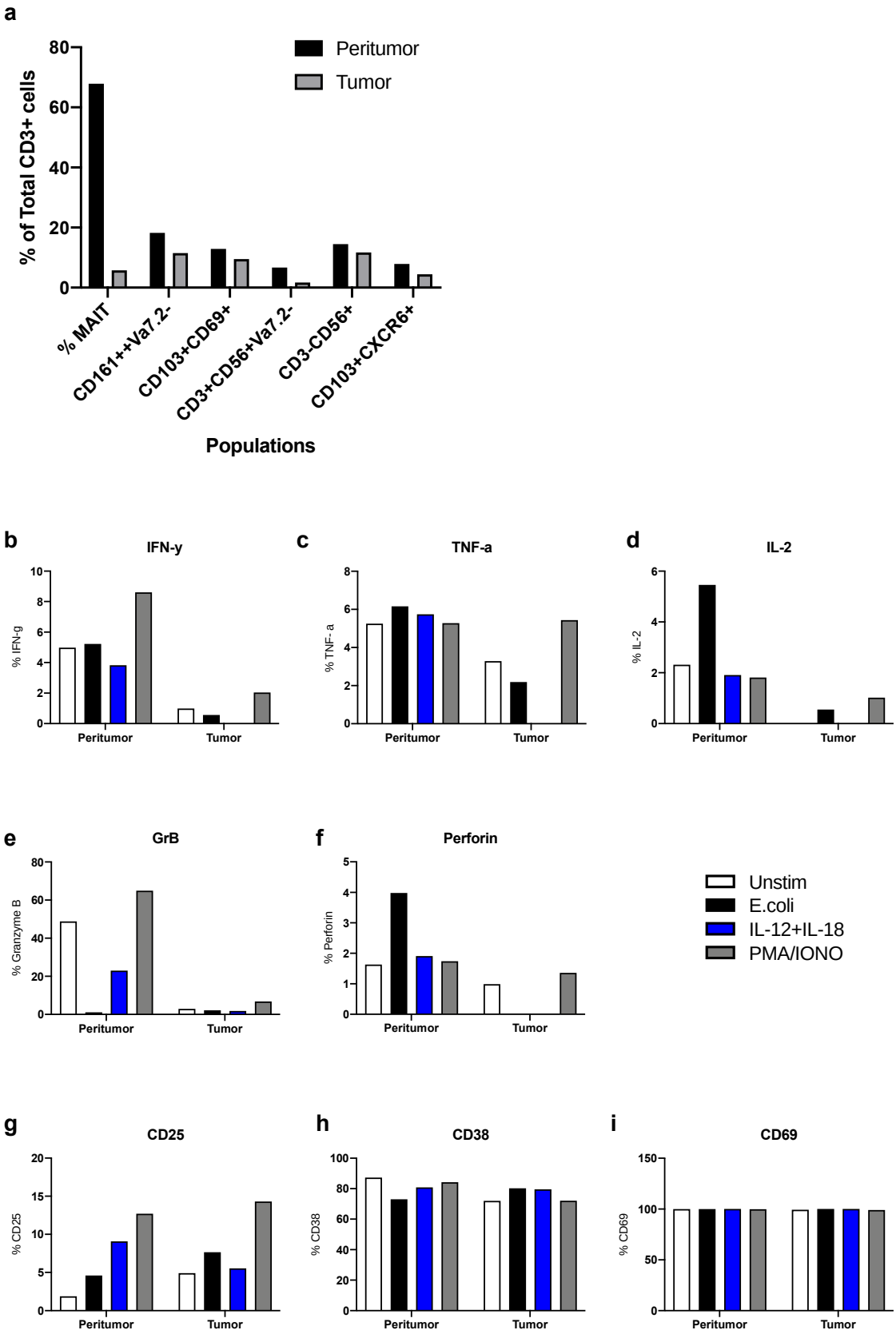
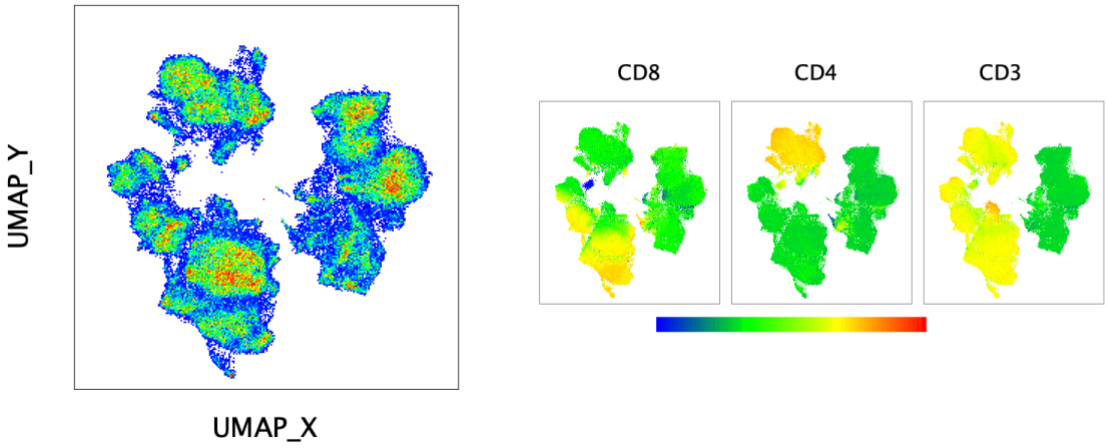


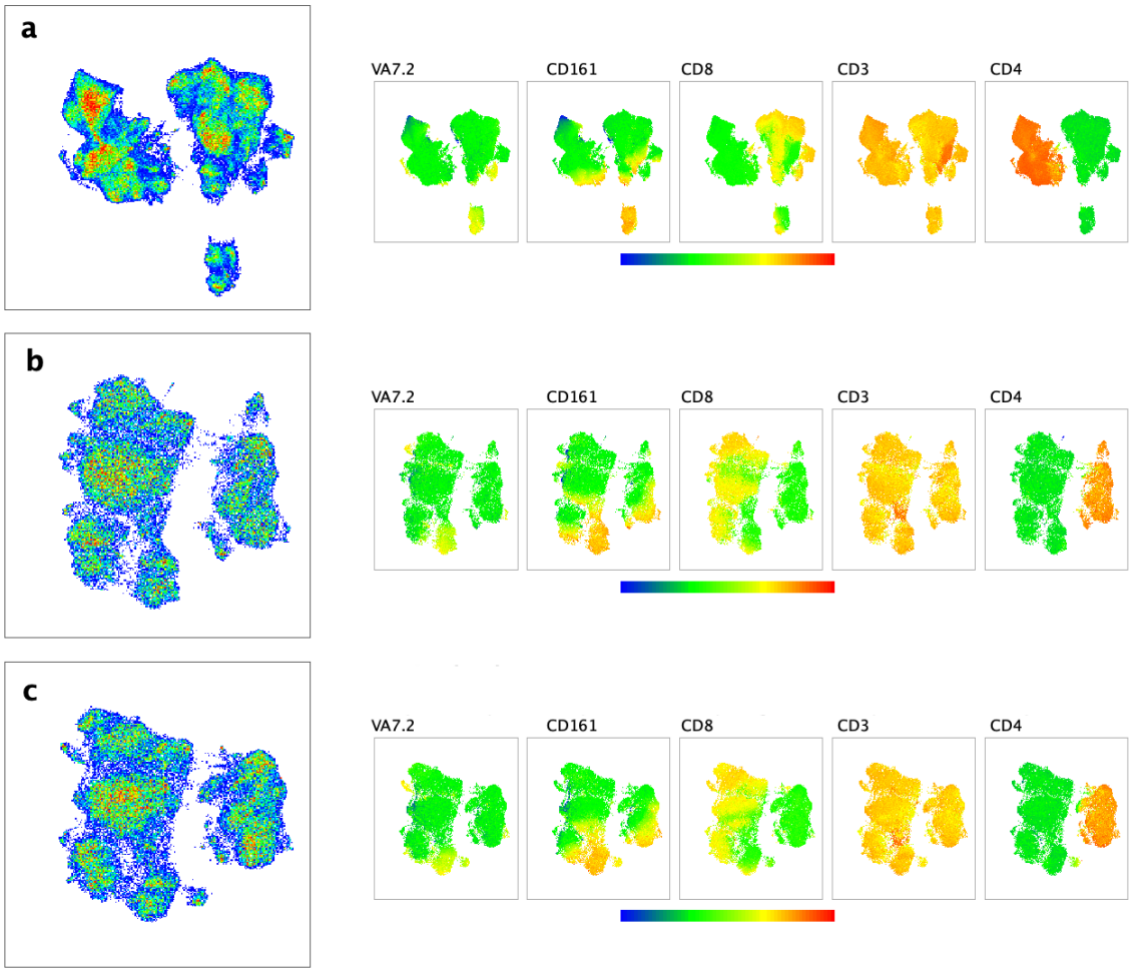
Supplementary figure 20 - Phenotype and functional capacity of bulk CD4 population from healthy blood, liver and liver disease. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. Columns represent different stimulations. Rows represent activation markers, cytokines a or cytotoxic molecules. HB=healthy blood, HL=healthy liver, CL= cirrhotic liver and LT=liver tumor. White bars indicate unstimulated/baseline readings and black bars represent stimulated cells.


a
Peritumor
Tumor
% of Total CD3+ cells
80
60
40
20
0
% MAIT
CD161++Va7.2-
CD103+CD69+
CD3+CD56+Va7.2-
CD3-CD56+
CD103+CXCR6+
Populations
b
IFN-y
% IFN-g
Peritumor
Tumor
c
TNF-a
% TNF- a
d
IL-2
% IL-2
e
GrB
% Granzyme B
f
Perforin
% Perforin
Unstim
E.coli
IL-12+IL-18
PMA/IONO
g
CD25
% CD25
h
CD38
% CD38
i
CD69
% CD69

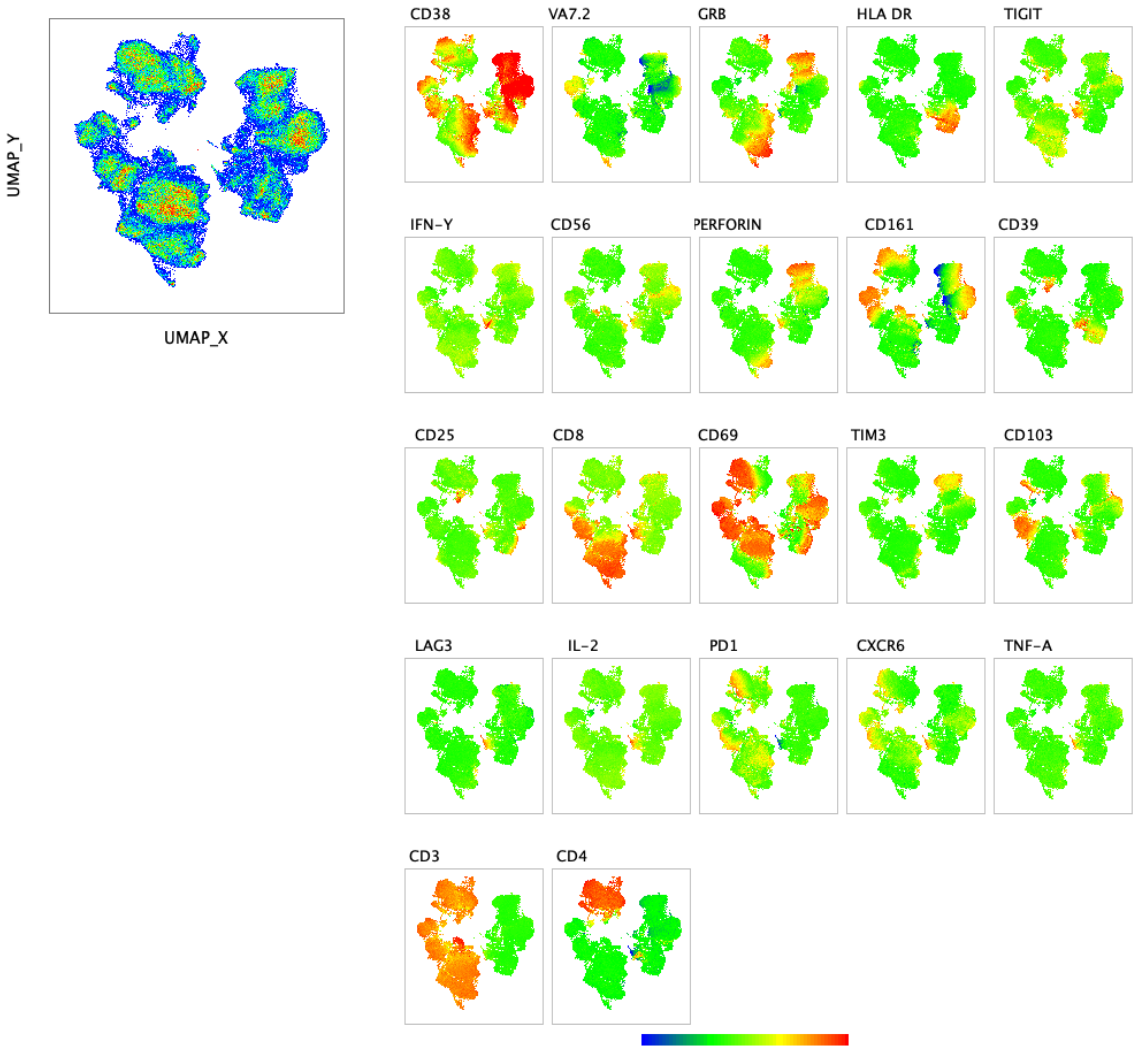
Supplementary figure 21 – Analysis of a single matched peritumor and tumor sample. Blood or liver lymphocytes were stimulated with *E*. coli and IL-12+IL-18 for 16 hours or PMA/IONO for 6 hours prior to staining and spectral cytometry analysis. a) Percent of MAIT and MAIT related populations in tumor and peritumor of donor Y. b - d) Cytokine potential of MAIT cells from tumor vs peritumor in response to stimulations. e-f) GrB and perforin expression from *E.* coli and cytokine stimulated MAIT cells from peritumor and tumor MAIT cells. g-i) Activation markers expressed by *E.* coli and cytokine stimulated MAIT cells from peritumor and tumor MAIT cells.



Supplementary figure 22 – UMAP generated from viable lymphocytes from healthy liver, cirrhotic and liver tumor samples (left) and individual marker specific identification of islands for CD3, CD4 and CD8 (right).



Supplementary figure 23 – UMAP plots created from viable T cells and individual plots (right) show T cell and MAIT cells associated marker contribution to the UMAP. UMAP created from viable T cells of a) Healthy blood and healthy liver, b) healthy liver and cirrhotic liver and c) healthy liver and liver tumor.



Supplementary figure 24 – UMAP plot created from viable lymphocytes from healthy liver, cirrhotic liver and liver tumor. Individual plots to the right of the main plot show 23-marker specific contribution to specific islands in the main UMAP plot (left).

Supplementary tables

	MAITS	THP-1
PTGDR	14.38	
PTGDR2	0.23	0.23
PTGDS	0.36	0.18
PTGER1	0.05	0.03
PTGER2	80.61	71.00
PTGER3	0.01	0.05
PTGER4	81.00	148.83
PTGER4P2-CDK2AP2P2	0.15	
PTGES	0.05	0.08
PTGES2	39.63	0.03
PTGES2-AS1	0.30	
PTGES3	88.32	33.82
PTGES3L		0.15
PTGES3L-AARSD1		108.50
PTGES3P1		0.01
PTGES3P3		3.18
PTGFR	0.01	0.05
PTGFRN	1.28	0.14
PTGIR	0.03	0.14
PTGIS	0.03	
PTGR1	0.27	2.51
PTGR2	3.43	0.10
PTGS1	0.19	0.01
PTGS2	0.01	

	MAITS	THP-1
COX1	16768.05	
COX10	17.79	58.93
COX10-AS1		28.62
COX11	26.02	87.89
COX14	23.17	36.04
COX15	46.57	198.65
COX16	26.92	119.31
COX17	13.39	90.30
COX17P1		30.57
COX18	21.68	73.61
COX19	54.24	207.43
COX2	6382.95	
COX20	1.76	263.09
COX20P1	2.28	18.52
COX3	6400.10	
COX4I1	325.00	1083.30
COX4I2	0.03	
COX5A	47.33	366.47
COX5B	54.35	242.81
COX6A1	43.19	231.58
COX6A2	0.01	
COX6A1P2		131.55
COX6B1	96.87	306.01
COX6B2	0.16	
COX6C	53.50	427.84
COX7A1	0.05	
COX7A2	53.57	676.68
COX7A2L	94.86	594.25
COX7A2P2		8.23
COX7B	45.75	247.98
COX7B2	0.01	
COX7C	158.18	537.56
COX7CP1		22.26
COX8A	68.83	474.69
COX8C	0.01	

Supplementary table 1 – Prostaglandin and COX genes - Raw counts of Prostaglandin and COX genes from bulk RNA-sequencing of unstimulated sorted MAIT cells and unstimulated THP-1 cells, data expressed as counts per million (CPM), blank spaces indicate the gene was not detected in one of the cell types.

Donor ID	Age	Gender	Dx
Metastasis			
11-025	69	F	Colon ca
12-134	54	M	Colon ca
12-136	64	M	Colon ca
13-069	65	F	Colon ca
13-084			Colon ca
Cirrhosis No tumor			
11-012	43	F	ETOH
11-049	70	M	ETOH
13-007	47	M	ETOH
13-055	54	M	ETOH
Cirrhosis with tumor			
12-166	56	M	ETOH/HCC
14-126	60	F	PBC/HCC
14-137	53	M	ETOH/HCC
12-115 L	69	M	HCC
12-115 T			HCC

Supplementary table 2 – Liver samples

Cell type dependent activation of Mucosal associated invariant T cells in the Liver



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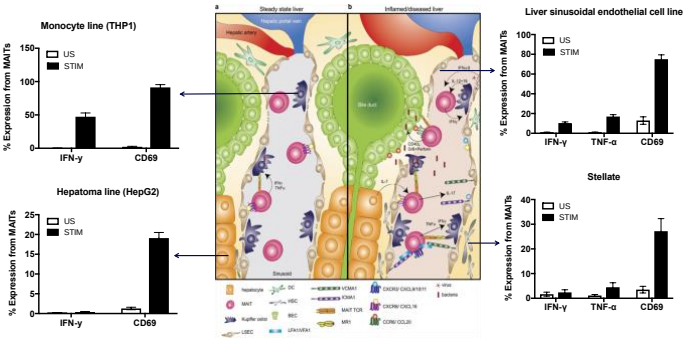
[1]Peter Medawar Building for Pathogen Research, University of Oxford, Oxford, Oxfordshire, United Kingdom
[2]Oxford NIHR Biomedical Research Centre, The John Radcliffe Hospital, Oxford, United Kingdom

Introduction

The liver has a number of immunological roles including the synthesis of immunomodulators such as chemokines and cytokines. Furthermore, the liver acts as a firewall to bacteria that cross the mucosal barrier within the gut [1]. As part of its unique immunological role, the liver is populated by a number of innate-like T cell populations, including Mucosal associated invariant T (MAIT) cells, defined by high CD161 expression and the semi-invariant T cell receptor (Va7.2Ja33). MAIT cells are restricted to the highly conserved MHC class I related protein 1 (MR1) and can be activated through the T cell receptor, or by cytokines alone, allowing them to respond to many types of pathogens [2]. MAIT cells are enriched in the liver in comparison to the blood and increase to approximately 50% of total CD8 T cells in liver disease; thus, making them a highly important hepatic T cell subset to study. To date, the ability of MAIT cells to interact directly with liver cells, such as hepatocytes, liver sinusoidal endothelial (LSE), stellate, and Kupffer cells, has not been studied. Therefore, we aimed investigate the capacity of different hepatic cell lines to activate MAIT cells in response to both bacteria and viruses.

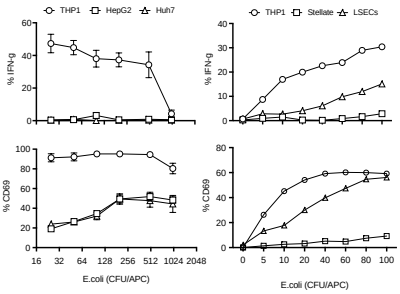
Results



Modified from Kurioka A, et. al. Clinical & translational immunology. 2017 Feb;6(2):e132.

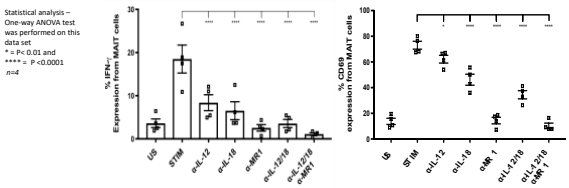
Only LSECs, but not hepatocyte-derived cell lines or stellate cells, could activate MAIT cells in response to *E.coli* infection.

Liver cells lines were co-cultured with isolated CD8 T cells in the presence or absence of *E.coli* (DH5-α) overnight and analysed by flow cytometry. Graphs showing IFN-/TNF-α/CD69 expression from MAIT cells (CD8+CD161++Va7.2+).
Titration of E.coli onto the cell lines confirmed that even at high CFU/APC only the liver sinusoidal cells (LSECs) and THP1 cells could induce IFN-γ from MAIT cells in response to bacteria. However, all cell lines were able to induce CD69 expression on MAIT cells upon stimulation.

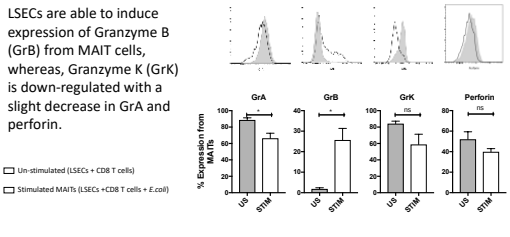


MAIT cell activation by LSECs is predominately MR1 dependent, but requires cytokine co-stimulation for optimal activation

Both IFN-γ and CD69 expression in MAIT cells is MR1 dependent, but we also noted a significant reduction in their expression when blocking IL-12 and IL-18 alone or in combination.

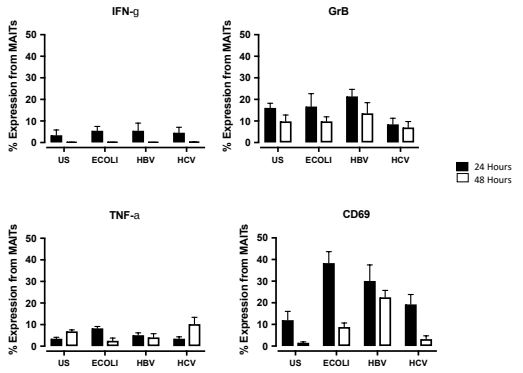


LSECs are able to induce expression of Granzyme B (GrB) from MAIT cells, whereas, Granzyme K (GrK) is down-regulated with a slight decrease in GrA and perforin.



Statistics = Two-tail, paired end , parametric T test was used to analyse this data where *n = 4* and ** P<0.05*.

Virus sensing by Liver sinusoidal endothelial cells induces GrB and CD69 expression on MAITs cell but no IFN-γ or TNF-α



Conclusion

Hepatocytes were inefficient at activating MAIT cells in response to both bacterial and viral infection however, of the non-parenchymal cells only the LSECs were capable of inducing MAIT cell activation. LSEC stimulation of MAIT cells was predominantly MR1 dependent, but required the alternate cytokine pathway to induce full MAIT cell activation. In contrast to stellate cells, the LSECs were able to induce limited IFN-γ and high granzyme B and CD69 expression from MAITs at higher bacterial loads. Minimal activation was observed when the viruses HBV and HCV were used to stimulate MAITs with LSECs. Overall, LSECs are able to stimulate MAIT cells in response to bacteria and virus albeit not to the same extent as conventional antigen presenting cells.

P.B4.02.11

Reference:
[1] – Robinson MW, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. Cellular & molecular immunology. 2016 May;13(3):267.
[2] - Kurioka A, Walker LJ, Klenerman P, Willberg CB. MAIT cells: new guardians of the liver. Clinical & translational immunology. 2017 Feb;6(2):e132.

Mechanisms of MAIT cell regulation in the liver

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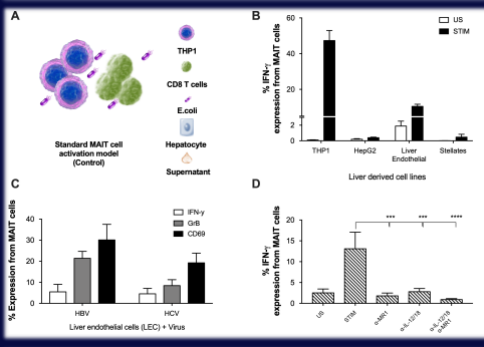
[2]Oxford NIHR Biomedical Research Centre, The John Radcliffe Hospital, Oxford, United Kingdom

Introduction

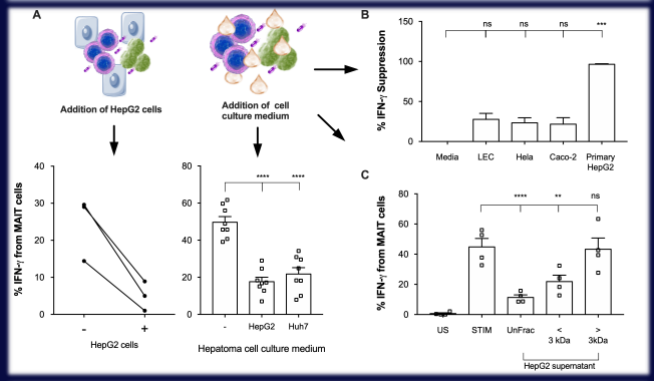
The liver has a number of immunological roles including the synthesis of potent local immunomodulators establishing a "tolerogenic" environment. As part of its unique immunological role, the liver is populated by a number of innate-like T cell populations, including Mucosal associated invariant T (MAIT) cells, defined by high CD161 expression and the semi-invariant T cell receptor (Vα7.2Jα33). MAIT cells are restricted to the highly conserved MHC class I related protein 1 (MR1) and can be activated through the T cell receptor, or by cytokines alone, allowing them to respond to many types of pathogens. The cells with which they interact within the liver environment and the local signals which control their activation are not yet known. Therefore we aimed to investigate the capacity of different hepatic cell lines to activate MAIT cells and explored the mechanism used by hepatocytes to regulate MAIT cell activation. Specifically, prostaglandins are small lipid molecules involved in immunoregulation and we tested their ability to impact on MAIT cell activation..

Figure 1: MAIT cells can be activated by bacterial and virus exposed Liver endothelial cells



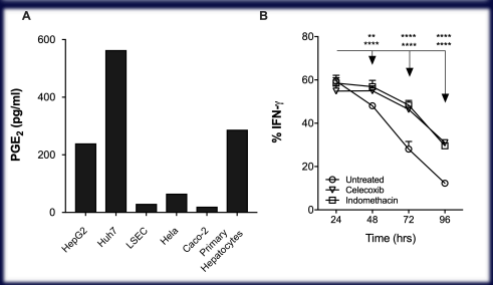
A- Representative MAIT cell activation model showing THP1, CD8 T cells and E.coli co-culture. All experiments were analysed by Flow cytometry (MACSquant). B- Liver derived cell lines used to activate MAIT cells, only LECs were able to activate MAIT although to a limited extent. C- Virus exposed LECs can induce a limited amount IFN-γ, Granzyme B (GrB) and CD69 in CD8+CD161++Vα 7.2+ MAIT cells. D- E.coli exposed LEC stimulation is inhibited by blocking the MR1 and IL-12.18 signalling pathways. Holm-Sidak's multiple comparisons test. ***$p \leq 0.0002$, ****$p \leq 0.0001$.

Figure 2: MAIT cell IFN-γ is suppressed by a small and soluble hepatoma cell derived molecule



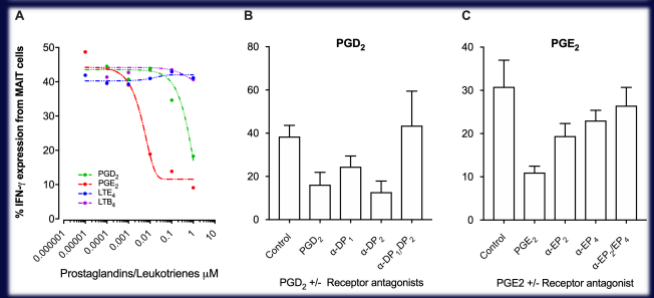
A- HepG2 cells or B- HepG2 cell culture medium is added to the MAIT cell activation model. In both cases we observed suppressed MAIT cell IFN-γ. C- Cell culture media from primary hepatocytes were potent at suppressing MAIT cell IFN-γ whereas all other cell lines did not suppress MAIT-IFN-γ. D- Testing of fractionated HepG2 culture medium showed suppression mediated by a molecule smaller than 3kDa. Dunnett's multiple comparisons test. **$p \leq 0.02$, ***$p \leq 0.0005$, ****$p \leq 0.0001$, $ns = p \leq 0.1$.

Figure 3: Cyclooxygenase (COX) inhibitors partially recover IFN-γ suppression by hepatoma supernatants



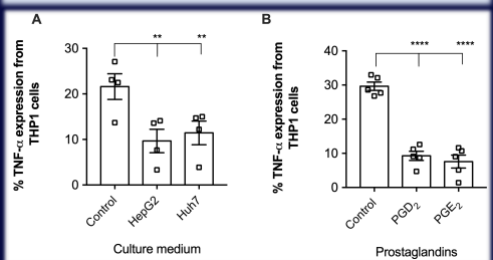
A- PGE_2 ELISA on primary hepatocytes and cell lines show PGE_2 production by liver cells only. B - HepG2 cultures were treated with indomethacin (COX-1/2) celecoxib (COX-2) cyclooxygenase inhibitors every 24 hours for 4 days. COX inhibitor treated HepG2 culture medium showed reduced ability to suppress MAIT cell IFN-γ. Tukey's multiple comparisons test **$p \leq 0.0012$, ****$p \leq 0.0001$

Figure 4: Prostaglandins can modulate IFN-γ production by MAIT cells



A- Prostaglandins and leukotrienes were titrated onto MAIT cells activated by E.coli exposed THP1 cells in an overnight assay. Flow cytometry analysis shows a dose dependent suppression of MAIT-IFN-γ by PGE_2 and PGD_2 where PGE_2 is more potent. B/C- Receptor antagonism showed that PGD_2 used DP_1 and CRTH2 (DP_2) and PGE_2 requires EP_2 and EP_4 to signal in the MAIT cell activation model.

Figure 5: Hepatocyte culture medium and PGD_2/PGE_2 suppresses THP1-TNF-α



A- Hepatocyte cell culture media and B- PGE_2 and PGD_2 both suppressed TNF-α expression from THP1 cells present in the MAIT cell activation model. Dunnett's multiple comparisons test, **$p \leq 0.0034$, ****$p \leq 0.0001$.

Conclusion

- MAIT cell activation in the liver is limited to the endothelial cells which induced limited activation of MAIT cells, figure 1.
- Hepatocytes showed a suppressive effect on MAIT cell IFN-γ that was mediated through a small soluble molecule, figure 2.
- As the hepatocyte dependent suppression was not observed by non-liver derived cell culture medium, this effect is specific to the liver and in particular hepatocytes.
- Only the primary hepatocytes and hepatoma cells produced PGE_2, figure 3A, which potently suppressed MAIT-IFN-γ (figure 4) that could be partially recovered by treating hepatocytes with COX inhibitors, figure 3B.
- Similar to PGE_2, PGD_2 was also able to suppress MAIT cell IFN-γ in a dose dependent manner. Both prostaglandins used two receptors for signaling which could be completely reversed by blocking the receptors in combination, figure 4.
- Prostaglandins have the capacity to influence MAIT cell activation by altering the pro-inflammatory phenotype and cytokine secretion profile of the antigen presenting cell as shown in figure 4 and 5.

Overall MAIT cells could be activated by the liver endothelial cells but appear to be subject to suppression by the hepatocytes which is -at least in part- likely a result of small lipid metabolites.

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In preparation

1. High concentrations of prostaglandins suppress the inflammatory and cytotoxic function of antigen stimulated MAIT cells.

   Hema Mehta, Christian Willberg, Paul Klenerman.

2. Modulation of MAIT cell function in the liver microenvironment by hepatocyte derived soluble factors.

   Hema Mehta, Christian Willberg, Paul Klenerman.

3. High dimensional analysis of MAIT cells and related populations in healthy liver, cirrhosis and liver cancer.

   Hema Mehta, Paul Klenerman.

Submitted and under review

Robust MAIT cell activation by primary human liver cells: Implications for liver physiology and disease

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