PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF CD4+ T CELLS IN THE HUMAN LIVER

by

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A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

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October 2018

Abstract

The liver has a unique connection with the immune system; harbouring vast numbers of lymphocytes, able to instigate secondary lymphoid organ-independent naive T cell activation, and promoting potent immune tolerance. We set out to determine the effect of this unique microenvironment on the biology of $CD4^+$ T cells at three key interaction points: following migration into the parenchyma, after short-term hepatocyte contact, and at long-term tissue-residency. Modelling transmigration through hepatocytes revealed intrinsic, disease-specific cytokine responses in blood-derived $CD4^+$ T cells, not discernible through static co-culture. However, short-term co-culture did induce activation-independent CD69 upregulation, reliant upon cell-cell contact. This phenotype mimicked the similar hepatic $CD4^+$ $CD69^{INT}$ cells that we discovered in liver tissue. Unlike $CD69^{HI}$ cells which represented the tissue-resident memory T cells (T_{RM}) of the liver, $CD69^{INT}$ cells were the most activated population, likely able to migrate to many liver and gut niches, and singularly able to produce IL-4 and IL-10. By contrast, $CD69^{HI}$ T_{RM} displayed a resting phenotype, marked for more restricted movement, and produced the best multifunctional T_H1 responses following stimulation. These data demonstrate the importance of studying migration, and provide detailed characterisation of $CD69^{HI}$ T_{RM} and novel $CD69^{INT}$ cells, along with their proposed roles and generation pathways.

Dedication

For Kassie, my biggest fan and most invaluable supporter.

And Sophie, who could be a better scientist than I ever will.

Acknowledgements

I would first like to thank Emily Li for providing immeasurable help with the project. Her hard work, helpful discussions, and good humour honestly meant so much to me, I could not imagine how I would have made it without her. Next to Scott Davies who has been like a brother as we've gone through this journey together. Susan Liu and Kostas Aliazis too have been such great fun to work with and incredibly important in helping me stay sane. Additional thanks go to Helen McGettrick for her help and guidance with collagen gel experiments, and to John Curnow for lots of project feedback and constantly pushing me to improve. Last, but by no means least, Zania, who has been a great mentor and friend in and out of science, and without whom I would never be the Immunologist I am. I am very grateful for all her guidance, support, crazy plant-like ideas, and challenging me to be the best I can be.

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Cell Types & Anatomical Features
APC – Antigen-presenting cell
BEC – Biliary epithelial cell
CNS – Central nervous system
cTEC – Cortical thymic epithelial cell
DC – Dendritic cell
DN – Double negative ($CD8^-CD4^-$)
DP – Double positive ($CD8^+CD4^+$)
FRT – Female reproductive tract
GALT – Gut-associated lymphoid tissue
GC – Germinal centre
HEV – High endothelial venule
HSC – Hepatic stellate cell
HSEC – Hepatic sinusoidal endothelial cell
iMATES – Intrahepatic myeloid cell aggregates for T cell clonal expansion
KC – Kupffer cell
LIMC – Liver-infiltrating mononuclear cell
LN – Lymph node
mDC – Myeloid Dendritic cell
MDSC - Myeloid-derived suppressor cell
MLC – Memory lymphocyte cluster
MPEC – Memory precursor effector cell
mTEC – Medullary thymic epithelial cell
NK – Natural killer cell
NKT – Natural killer T cell
NLT – Non-lymphoid tissue
PBMC - Peripheral blood mononuclear cell
pDC – Plasmacytoid Dendritic cell
PHH – Primary human hepatocytes
pT_{REG} – Peripheral regulatory T cell
SLEC – Short-lived effector cell
SLO – Secondary lymphoid organ
SP – Single positive
T_C – T cytotoxic cell type
T_{CM} – Central memory T cell
T_{EFF} – Effector T cell
T_{EM} – Effector memory T cell
T_{EMRA} – T effector memory cell RA
T_{FH} – T follicular helper cell
T_H – T helper cell type
TIL – Tumour-infiltrating lymphocyte
TLO – Tertiary lymphoid organ
T_{MM} – T migratory memory cell
T_N – Naïve T cell
T_R1 – Type-1 regulatory cell
T_{REG} – Regulatory T cell
T_{RM} – Tissue-resident memory T cell
TSPs – Thymic seeding progenitor cells

tT_{REG} – Natural regulatory T cell
VAT – Visceral Adipose Tissue

Molecules

AHR – Aryl hydrocarbon receptor
AIM – Activation inducer molecule (CD69)
AP-1 – Activator protein-1
Bcl-6 – B-cell lymphoma 6
Blimp-1 – B-lymphocyte-induced maturation protein 1
c-MAF - Cellular homolog of the avian virus oncogene musculoaponeurotic fibrosarcoma
CCR – CC chemokine receptor
CD – Cluster of Differentiation
CD40L – CD40 ligand
CLA – Cutaneous lymphocyte antigen
CLEVER-1 - common lymphatic endothelial and vascular endothelial receptor-1
CTLA-4 – Cytotoxic T-lymphocyte-associated protein 4
CX3CR – CX3 chemokine receptor
CXCR CXC chemokine receptor
DAMP – Damage-associated molecular pattern
EGF – Epidermal growth factor
Eomes – Eomesodermin
ERG-1 – Erythroblast transformation-specific related gene-1
Fas – First apoptosis signal receptor
FasL – First apoptosis receptor ligand
FoxP3 – Forkhead box P3
GATA-3 – GATA binding protein 3
HGF – Hepatocyte growth factor
HIF-1α - Hypoxia inducible factor, alpha subunit
Hobit – Homolog of Blimp-1 in T cells
ICAM – Intercellular cell adhesion molecule
ICOS – Inducible T-cell costimulator
IFN- Interferon
Ig – immunoglobulin
IL- Interleukin
IRF4 – Interferon regulatory factor 4
JAK – Janus kinase
KLF2 – Krüppel-like factor 2
KLRG-1 – Killer cell lectin-like receptor G1
L-Arg – L-arginine
L-SIGN - Liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
LAG-3 – Lymphocyte activation gene-3
LAT1 – Large neutral amino acid transporter-1
LFA – Lymphocyte function-associated antigen
LPS – Lipopolysaccharide
LYVE-1 - Lymphatic vessel endothelial hyaluronan receptor-1
MAdCAM-1 – Mucosal vascular addresin cell adhesion molecule-1
MHC – Major histocompatibility complex
MR1 – MHC-related 1

mTGF-β - Membrane-bound TGF-β
mTOR - Mechanistic target of rapamycin
NF- κB – Nuclear factor kappa B
NO – Nitric oxide
NR4A1 - Nuclear receptor subfamily group A member 1
PD-1 – Programmed cell death protein 1
PD-L – Programmed death ligand
PDC-E2 – Pyruvate dehydrogenase complex, E2 subunit
PGE2 – Prostaglandin E2
PKC – Protein kinase C
PNAd – Peripheral lymph node addressin
PRR – Pattern recognition receptor
PU.1 – Purine-rich box 1
RAG – Recombination activating gene
RORc – RAR-related orphan receptor C
S1P – Sphingosine-1-phosphate
S1PR1 - Sphingosine-1-phosphate receptor-1
S1PR5 - Sphingosine-1-phosphate receptor-5
STAT - Signal transducer and activator of transcription proteins
T-bet – T box expressed in T cells
TCF-1 – T-cell factor-1
TCR – T cell receptor
TF – Transcription factor
TGF-β - Transforming growth factor-β
Tim-3 - T-cell immunoglobulin and mucin-domain containing-3
TLR – Toll-like receptor
TNF- Tumour necrosis factor
VAP-1 – Vascular adhesion protein-1
VCAM-1 – Vascular cell adhesion protein-1
VEGF – Vascular endothelial growth factor

<u>Pathological Conditions, Agents & Models</u>
AIH – Autoimmune hepatitis
ALD – Alcoholic liver disease
BCS – Budd-Chiari syndrome
CTCL – Cutaneous T-cell lymphoma
GvHD – Graft versus host disease
HBV – Hepatitis B virus
HCV – Hepatitis C virus
HFE – Haemochromatosis
HSV – Herpes simplex virus
IBD – Inflammatory bowel disease
L-CTCL – Leukaemic cutaneous T-cell lymphoma
LCMV – Lymphocytic choriomeningitis virus
MF – Myosis fungoides
MS – Multiple sclerosis
Mtb – Mycobacterium tuberculosis
NAFLD – Non-alcoholic fatty liver disease
NASH – Non-alcoholic steatohepatitis
NCPH – Non-cirrhotic portal hypertension

PBC – Primary biliary cholangitis
PLD – Polycystic liver disease
PSC – Primary sclerosing cholangitis
RSV – Respiratory syncytial virus
SBC – Secondary biliary cholangitis
SLE – Systemic lupus erythematosus

Procedural & Technical Nomenclature
Ab - Antibody
AF - Alexafluor
APC - Allophycocyanin
BV – Brilliant Violet
CoA – Certificate of analysis
Cy - Cyanine
DMEM - Dulbecco's Modified Eagle Medium
EDTA – Ethylenediaminetetraacetic acid
FBS – Foetal Bovine serum
FITC – Fluorescein isothiocyanate
hTC – hepatoma-cultured T cells
IMC – Isotype-matched control
IQR – Interquartile range
MFI – Median fluorescence intensity
NaN_3 – Sodium azide
NaOH – Sodium hydroxide
NEAA – Non-essential amino acids
PBS – Phosphate-buffered saline
PE - Phycoerythrin
PerCP – Peridinin chlorophyll protein complex
PHA-M – Phytohaemagglutinin-M
PMA - Phorbol 12-myristate 13-acetate
RPMI – Roswell Park Memorial Institute (media)
RT – Room Temperature
T-SNE – t-Distributed Stochastic Neighbour Embedding
TBS – Tris-buffered saline
Tresp – T responder cells
tTC – T cells cultured alone
V450 – Violet 450
ZA – Zombie Aqua™ live/dead dye
ZNIR – Zombie near infa-red™ live/dead dye

Chapter 1 – Introduction

1.1 The Biology of T cell responses

Human immunity has evolved as a fluid, dynamic, multifaceted system to cope with a seemingly endless array of diverse pathogens[1, 2]. With effective defence against pathogens so intimately linked to survival, development of a highly functional and efficient immune system was a critical selection factor. Constant improvements and upgrades have been added over the millennia as we co-evolve with pathogens in the constant battle for supremacy[3].

In principle, immunity of all jawed vertebrates can be split into two main parts: the innate and adaptive immune systems. The innate system exists as a series of simple barriers; antimicrobial proteins; and cells that recognise conserved pathogenic motifs that will lead to the engulfment (phagocytosis) of the pathogen, direct attack of the invader, and/or recruitment of further immune cells through the initiation of inflammation. Within this response, pathogens are taken up, degraded and transported to lymphoid organs where adaptive immunity can be triggered. Adaptive immune cells comprise T and B lymphocytes, with the astounding ability to recognise almost any imaginable constituent of pathogens in a highly specific manner. Rare antigen-specific T and B cells will respond, expand, and traffic to infected areas to provide a potent, focussed response. B cells produce antibodies that both mark pathogens for destruction and neutralize toxins; while T cells can kill pathogens directly and augment many other vital arms of the immune response. Crucially, only adaptive lymphocytes can generate long-term immune memory that provides much faster and stronger responses upon future pathogen reencounter, the main goal of vaccination.

This study focusses on the T cell arm of the immune response, specifically examining $CD4^+$ T cells – the so-called orchestrators of adaptive immunity. In the following sections, the development of T cells will be briefly discussed, followed by walkthroughs of primary and secondary immune responses, alongside the stringent regulatory steps put in place at each and every stage.

1.1.1 T cell Development

Following haematopoiesis in the bone marrow, thymic seeding progenitor cells (TSPs) traffic to the thymus to begin the maturation process. These multipotent precursors express neither CD8 nor CD4 (CD – cluster of differentiation), and are thus referred to as double negative (DN). DN cells destined to become conventional T cells upregulate recombination-activating genes (RAG) -1 and -2 to allow rearrangements of the T cell receptor (TCR)-β chain as they migrate through the thymic cortex[4]. Productive rearrangements are then tested with an invariant TCR-α partner and CD3 signalling machinery. Passing this test allows a T cell to survive, proliferate and the daughter cells to upregulate both CD8 and CD4 to become double positive (DP) cells. Here T cells use the RAG enzymes to rearrange their TCRα genes. DP cells can then test their fully rearranged TCR through interactions with cortical thymic epithelial cells (cTECs) expressing self-peptide held in major histocompatibility complex (MHC) molecules, in what is called the positive selection process[4, 5]. The ability of a DP cell to successfully recognise antigen-MHC complexes leads to simultaneous downregulation of either CD4 or CD8 to become a single positive cell (SP). DP cells that recognise antigen held in MHC class I become CD8 SP cells; while those that recognize antigen-MHC class II complexes become CD4 SP cells[6, 7]. All cells that fail to bind peptide-MHC die by apoptosis. Surviving

SP cells then enter the final stage of thymocyte development – negative selection. This process occurs in both the cortex, and medulla, where cTEC, medullary thymic epithelial cells (mTEC), as well as dendritic cell (DC) populations display a vast array of self-peptides (derived from proteins that are expressed all over the body) held in MHC complexes. Any SP cells with too high an affinity for self-antigen are either deleted, or differentiate into thymus-derived regulatory T cells (tT_{REGS}), a vital inhibitory population[7, 8]. This mechanism is termed central tolerance, and is vital for preventing the release of potentially autoreactive T cell clones into the periphery. All remaining, now mature, naïve T cells then leave the thymus and continually recirculate between the blood and lymphoid tissue in search of cognate antigen.

1.1.2 Naïve T cell Activation & Differentiation

Naïve T cell activation (T cell priming) takes place in secondary lymphoid organs (SLOs), such as lymph nodes (LNs) and the spleen. Here naïve T cells interact with DCs that have engulfed and processed pathogens in the periphery, and display resulting antigens on their surface, held in MHC molecules. $CD8^+$ and $CD4^+$ T cells recognise antigens in the context of MHC class I, and class II molecules respectively. In order for the linked processes of activation, proliferation, and differentiation to occur, a naïve T cell must receive three signals - antigen recognition, co-stimulation and cytokine modulation (Fig. 1.1.1.). Importantly, the nature of the three signal types is principally dependent on the type of infection present. All signals are integrated to determine not only the level of activation, but the differentiation pathway chosen. This ensures that the output effector cell population is best suited to eliminate the pathogen[9-13]. Following priming, activated effector T cells (T_{EFF}) migrate into the periphery and traffic to the site of infection where

they either kill the pathogen directly, or use their arsenal of effector cytokines to augment other arms of the immune response.

Signal 1 describes the specific recognition of cognate peptide-MHC complexes by the TCR. Alongside providing precise specificity to the response, TCR signal strength is also an important initial determinant of the magnitude of the response, and the eventual differentiation path chosen[14, 15]. ***Signal 2*** comes from the binding of T cell co-stimulatory receptors to co-stimulatory ligands on the DC. CD28 ligation with CD80 and/or CD86 is essential in the activation process[11, 16-18]; yet ligation of many other co-stimulatory receptors can also supplement T cell signalling, promote T cell activation and imbue differentiation pathway preferences. These include CD2, CD27, and inducible T-cell costimulator (ICOS)[9, 19]. Conversely, negative co-stimulation (co-inhibition) exists to regulate these responses. Co-inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), or lymphocyte activation gene 3 (LAG-3) work to dampen or prevent the activation process. It is the net sum of these positive costimulatory and negative co-inhibitory signals that determines whether a T cell becomes activated or not, and how this balance changes over time affects the length and quality of the activation event[9]. Antigen recognition in the absence of sufficient co-stimulation leads to either cell death, or T cell anergy – a functional and prolonged state of hyporesponsiveness that requires a supernormal stimulus to reverse[20]. Because only DCs that have encountered pathogens (through innate pattern recognition receptors - PRRs) should have upregulated co-stimulatory ligands, signal 2 also acts as a barrier to autoantigen responses, and anergy reduces the likelihood of autoreactive T cells being activated in future. Finally, ***signal 3*** is provided by the cytokine milieu. DC-derived cytokines not only promote T cell survival and proliferation, but are instrumental

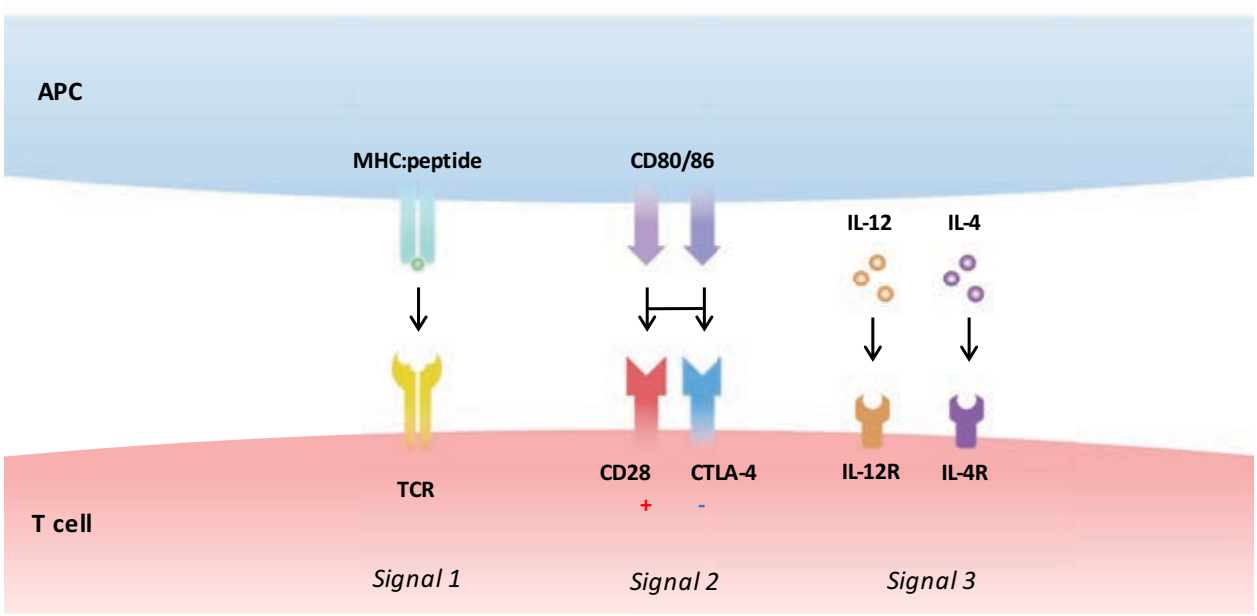


Figure 1.1.1 - The three signal model of T cell activation. In order for full activation and differentiation to occur, T cells must receive three signals from the antigen-presenting cell (APC). The first is received through specific recognition of cognate peptide held in MHC molecules (MHC class I for $CD8^+$ T cells, class II for $CD4^+$ T cells). The second is through co-stimulatory molecule binding. The example here shows CD80 and/or CD86 binding CD28 to give a positive stimulatory signal (+); but many other costimulatory ligand-receptor pairs exist, including CD70 - CD27, ICOS ligand - ICOS, and CD58 - CD2. Co-inhibitory molecules serve to inhibit T cell activation (-) - shown here by CTLA-4 competing for CD80/86 ligation (other ligand-receptor pairs include PD-L1/2 - PD-1, and putatively MHC class II binding LAG-3[9]). The balance of these positive and negative signals determines whether a T cell is activated, or enters a state of hyporesponsiveness known as anergy. Last, signal 3 is through cytokine ligation. Cytokines enable T cell survival and proliferation (IL-2), or the skewing towards different lineages (see Fig. 1.1.2). IL-12 and IL-4 promote differentiation into T_H1 and T_H2 cells respectively. IL-12R - IL-12 receptor, IL-4R - IL-4 receptor.

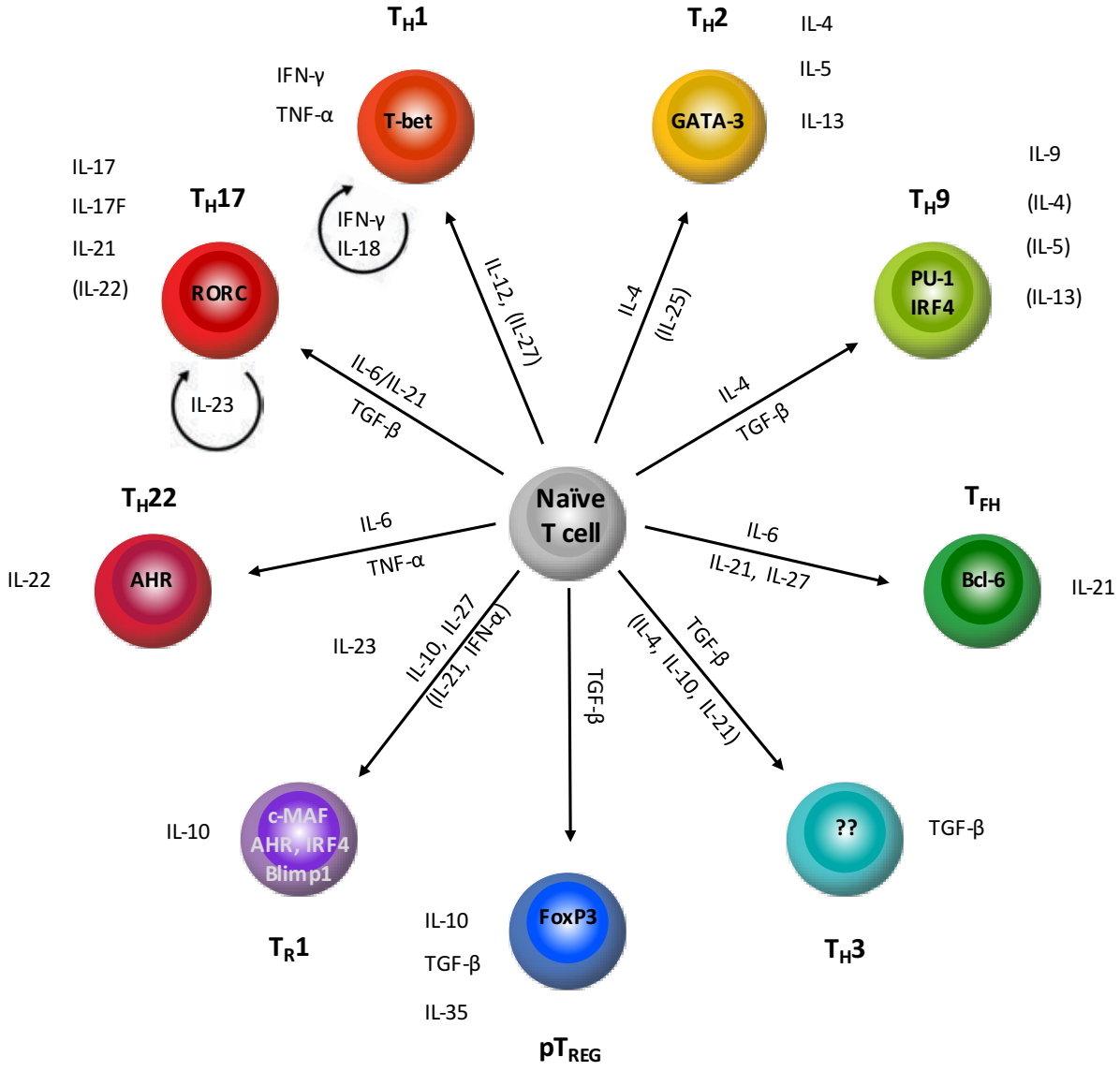
in providing instructional differentiation cues[21]. Alongside the three signals, priming location plays a key role in instructing the final effector cell phenotype, a concept discussed further in section 3.1.

Having received sufficient activation signals, T cells proliferate extensively, such that a single clone can produce tens of thousands of heterogenous progeny[22, 23]. Concomitantly, T cells undergo characteristic changes in their phenotype, reflective of their activated status. Many such activation markers have been described, and among the most commonly used are CD69, CD25, CD38 and Human leukocyte antigen – antigen D related (HLA-DR)[24-26]. CD69 is the earliest known activation marker – upregulated within 2 hours of stimulation[27, 28]. This molecule is not only likely involved in the inhibition of further, excessive, activation[29, 30], but in modulation of the LN dwell time in order to allow the T cell more time to become fully activated[31]. CCL19 and CCL21 attract $CCR7^+$ naïve T cells to SLOs, whereas sphingosine-1-phosphate (S1P) concentration gradients draw these cells back out to the blood through their sphingosine-1-phosphate receptor 1 (S1PR1) expressions[23, 32]. Receptor desensitisation of both CCR7 and S1PR1 ensures the continued recirculation of naïve T cells through blood and SLOs in the steady state. However, this equilibrium is skewed towards longer SLO dwell time by CD69, which directly causes the degradation of S1PR1[31, 33, 34]. T cell antigen affinity increases with longer LN dwell times, as the higher affinity T cells outcompete the rest to remain in the LN for the longest[35]. With CD69 so critical to our studies, this molecule is discussed in far more detail later (sections 1.2, 4.1, & 5.1). Meanwhile, expression of intermediate-term activation marker CD25 is seen later – typically beginning at 6-12 hours post-stimulation[24, 36]. CD25 is the high-affinity α-chain of the IL (interleukin)-2 receptor. CD25 expression endows the activated T cell with enhanced sensitivity to IL-2, a cytokine key in T cell

survival and proliferation[37]. CD38 is an interesting multifunctional activation marker, upregulated in response to both antigenic and cytokine stimulation. Also expressed in the intermediate timeframe[25], this ectoenzyme also has roles in T cell migration, proliferation and regulatory function [reviewed in ref [38]]. Last, HLA-DR is an MHC class II molecule expressed late in the T cell activation process[24]. This enables T cells to present antigen to other T cells, though interestingly some data suggests the outcome of this is tolerance in the form of anergy or deletion[39].

$CD4^+$ T cells (helper T cells) are often named the master orchestrators of the immune system due to their ability to amplify and regulate many different aspects of the immune response. This is made possible by the multiple T-helper (T_H) subsets within the $CD4^+$ T cell compartment, each with specific impacts on immunity (Fig. 1.1.2). T_H1 cells are induced by IL-12 that promotes the expression of lineage-specific transcription factor (TF) T-box expressed in T cells (T-bet). T_H1 cells are defined by production of Interferon (IFN)-γ and tumour necrosis factor (TNF)-α that combat intracellular pathogens by increasing macrophage activation and inhibiting viral replication[11, 12, 15]. The T_H2 differentiation programme is controlled by GATA-binding protein 3 (GATA-3); which is both promoted by, and drives expression of, the T_H2 prototypic cytokine IL-4. These cells can also secrete IL-5 and IL-13. T_H2 cells promote antibody class switching (to immunoglobulin [Ig] E and IgA) in B cells; and activate granular mast cells, eosinophils and basophils. This is important in the clearance of parasites and other extracellular pathogens, but also in allergic and atopic responses[11]. Highly pro-inflammatory T_H17 cells and anti-inflammatory peripheral T_{REGS} (pT_{REGS}) – induced from conventional T cells in the periphery as opposed to generated in the thymus) seem diametrically opposed in function, yet share inducing signals. IL-6 alone induces RAR-related orphan receptor C

A



B

Subset	Reported role(s)	Refs.
T_H1	Intracellular pathogens, autoimmunity	11
T_H2	Parasites, allergy/atopy, fibrosis	11
T_H17	Extracellular pathogens, autoimmunity, allergy, tumour suppression	11, 13, 15
T_{FH}	B cell class switching/affinity maturation: extracellular pathogens	11, 15
T_H9	Parasites, allergy/atopy, mucosal autoimmune disease, tumour suppression	11, 15, 40, 41
T_H22	Barrier tissue immunity & autoimmunity, allergy	42
iT_{REG}	General immune suppression, tumour progression	11, 43
T_R1	Local immune regulation, tumour progression	44
T_H3	General immune suppression (specifically oral tolerance)	45, 46

(legend on next page)

Figure 1.1.2 - CD4+ T cell subsets: differentiation requirements and roles in immunity. A - Differentiation requirements for each identified CD4+ T cell subset. Cytokines (shown along arrows) in the SLO environment direct a naïve CD4+ T cell towards different fates. Cytokines in parentheses are thought to be secondary/less important in each differentiation pathway, or have not been heavily reported in the literature. Each lineage is defined by a master regulator - a transcription factor (shown within each differentiated cell) that is crucial for driving development and re-enforcement of each subset. For many of the subsets, multiple transcription factors cooperate to regulate subset differentiation and stability[14], so only the main one is shown here (except T_R1 cells where a main factor is not currently known, but c-MAF, AHR, IRF4 and Blimp1 are the best candidates[44]; and T_H3 cells that do not yet have an identified master regulator). Cytokines shown in looped arrows are not required for initial formation of each subset, but are important in propagation of the phenotype/additional acquisition of lineage-specific effector functions. Each subset is defined by its function - the complement of effector cytokines it produces that will feed into the immune response. The main prototypic cytokines for each subset are shown adjacent to the cells (in parentheses are non-prototypic cytokines, but that are commonly associated with the subset(s)). Part A influenced from similar figures in sources [11, 14, 47], while additional information came from refs [10, 12, 13, 44, 45, 48-54]. AHR - Aryl hydrocarbon receptor, Bcl-6 - B-cell lymphoma 6, Blimp1 - B-lymphocyte-induced maturation protein 1, c-MAF - cellular homolog of the avian virus oncogene musculoaponeurotic fibrosarcoma (c-MAF), IRF4 - interferon regulatory factor 4, PU-1 - purine-rich box 1). **B -** Table showing reported general roles for each subset in pathogen clearance, autoimmune/autoinflammatory disorders, allergies and cancer. Extracellular pathogens include extracellular bacteria, fungi; intracellular pathogens include intracellular bacteria, viruses.

(RORc) expression and T_H17 lineage differentiation, while IL-6 and transforming growth factor-β (TGF-β) together instead favour Forkhead box P3 (FoxP3) expression and pT_{REG} generation[13]. T_H17 cells produce IL-17A, IL-17F, IL-21, and IL-22, and are involved in neutrophil activation, recruitment, and clearance of extracellular bacterial and fungal infections[11, 13]; whereas T_{REGS} produce anti-inflammatory cytokines such as IL-10 and TGF-β, and express high levels of inhibitory molecules like CTLA-4 in order to suppress excessive immune responses[55]. Other $CD4^+$ T cell types also exist, including follicular helper T cells (T_{FH}) that are essential for B cell affinity maturation in germinal centre (GC) reactions; T_H9 cells that produce IL-9; T_H22 cells that produce IL-22; and T_H3 and type-1 regulatory (T_R1) regulatory cell types that produce TGF-β and IL-10 respectively (Fig. 1.1.2)[14, 42, 44, 48, 52, 56-58].

Briefly, $CD8^+$ T cells, also known as cytotoxic T cells (T_C) due do their direct killing ability, are generally assumed to have a more linear differentiation path than their $CD4^+$ counterparts. IL-12 together with type-1 IFNs promotes differentiation into effector T cells with high cytotoxic potential[11]. However, like $CD4^+$ T cells, polarising cytokines can drive different $CD8^+$ T cell fates too. These T_C designations (T_C1, T_C2, T_C17 etc.) broadly correspond to T-helper subsets in both induction requirements, and output cytokine profiles [Reviewed in ref [59]].

T cell differentiation is a plastic and dynamic process. Differentiation into a certain subset is not necessarily final or irreversible, adding flexibility into immune responses. Numerous instances of interconversion between various subsets have been documented in both mice and humans; and these range from simple acquisition of one effector cytokine associated with a different lineage (as evidenced by the debate as to whether T_H9 cells

as a separate lineage, or simply an IL-9$^+$ T_H2 cell) to full-blown adoption of a new, unrecognisable cellular identity[15, 47, 60]. The stability of a subset is currently thought to depend on cytokine and co-stimulatory conditions upon secondary antigen encounter, TCR signal strength, TF networks, T cell clonality, and epigenetic modifications [reviewed in refs [15, 60]]. Based on these factors, a stability hierarchy appears to exist with pT_{REGS} and T_H17 cells more plastic than T_H1 and T_H2 subsets [reviewed in ref [60]]. Benefits of plasticity include the ability of effector cells to adapt to pathogens that have moved into a different niche, skewing to a less inflammatory subset (immune deviation) to prevent tissue damage, and upregulation of effector cell-specific TFs and associated homing molecules in T_{REGS} that allows each subset to be closely followed and regulated[61, 62]. Conversely, a dangerous situation could be envisaged where self-specific T_{REGS} convert to pathogenic T_{EFF} cells, as has been shown in mice[63-68]. Therefore, whatever the implications of plasticity, the CD4$^+$ T cell compartment can be viewed as a fluid and malleable multi-dimensional spectrum allowing for highly complex adaptations to best eliminate pathogens.

1.1.3 T cell memory

Following the effector response and clearance of pathogen comes a contraction phase where most effector T cells die by apoptosis. A small proportion survive and become long lived memory cells that are capable of dealing with pathogen re-exposure with heightened response times and effector functions[69]. The generation of these cells is the basis of vaccination strategies, and accordingly is a major cornerstone of much immunological research.

Memory cells can be broadly split into three subsets – central memory (T_{CM}), effector memory (T_{EM}) and tissue-resident memory (T_{RM}) T cells. By convention, T_{CM} recirculate throughout blood and lymphoid organs, have a high proliferative potential, and produce abundant IL-2; but do not exhibit high effector functions (Fig. 1.1.3). By contrast, T_{EM} mostly recirculate through blood and non-lymphoid tissues (NLTs), are less proliferative and produce little IL-2; but are highly responsive with robust effector function[70]. Contrastingly, T_{RM} only reside within NLTs, providing robust frontline effector functions (see section 1.2 for extensive T_{RM} discussion). It is clear that a division of labour exists between these three subsets, with T_{EM} and T_{RM} important in limiting the spread of pathogens in infected tissues, while T_{CM} provide delayed back up – responding to antigen in SLOs and differentiating into a second wave of new, effector-ready T_{EM}[31, 71].

For $CD8^+$ T cells, the required cellular phenotypes and factors involved in the conversion to memory are relatively well understood. Memory precursor effector cells (MPECs) have been identified and show several key differences in surface phenotype, transcriptional activity, and metabolic requirements from short-lived effector cells (SLECs)[72, 73]. Moreover, the integration of combined signal strength from signals 1-3 in T cell priming impacts these fate decisions; with lower strength stimulations (beyond a certain activation threshold) favouring memory cell generation, and higher strength stimulations preferentially inducing SLEC cells; even if the precise precursor-product relationships have not yet been defined[72]. Conversely, $CD4^+$ memory T cell generation appears to be a more flexible process. $CD4^+$ equivalents of SLEC and MPECs have not been defined, with evidence instead suggesting that memory cells can form from multiple precursors[73]. As such, specific antigen signal strengths (again beyond a certain initial survival threshold); co-stimulation inputs; and signals from cytokines such as IL-2,

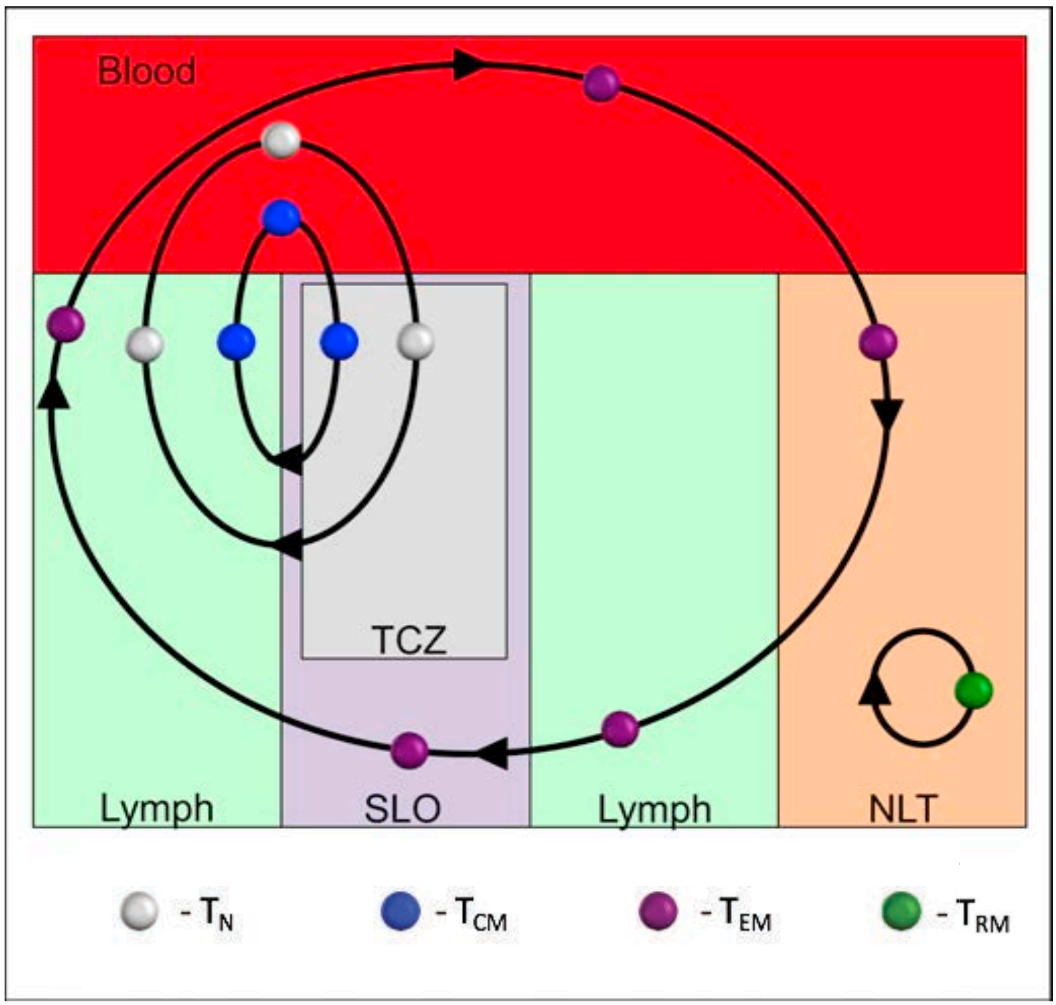


Figure 1.1.3 - Recirculation patterns of naïve and memory T cell subsets. Naïve and T_{CM} cells recirculate through blood and T cell zones (TCZ) of secondary lymphoid organs (SLOs) via the lymphatic vessels. T_{EM} are mainly found in blood and non-lymphoid tissues (NLTs) but also recirculate through the lymphatics (via sinuses of SLOs) to drain back into the blood. T_{RM} are confined to individual NLTs. Placing these migratory restraints on different subsets allows for an effective division of labour system whereby T_{EM} and T_{RM} patrol frontline tissues for immediate responses to re-encountered pathogens, while T_{CM} with their increased proliferative potential, provide back up later on from their SLO garrisons. Image adapted from Schenkel & Masopust, 2014 – ref [71].

IL-12, and IL-21 are not absolutely required for $CD4^+$ T cell memory cell generation; although modifying these parameters does lead to variations in the efficiency of memory cell production[69, 73, 74]. However, exposure to the homeostatic cytokine IL-7, may be an absolute requirement for memory generation and maintenance[69, 74-76].

Just like their primary $CD4^+$ effector cell counterparts, memory $CD4^+$ T cells exhibit remarkable heterogeneity and plasticity. T_{EM} cells can become T_{CM} in order to generate more stable long-lived memory cells[77]; or switch to different secondary effector types, for instance allowing a memory cell generated from a T_H1 effector precursor to switch to a T_H2 type in the correct conditions[73, 78-81]. These changes are imparted by different tissue niches, which are in turn informed by the nature of the infectious agent[73]. Thus, the secondary $CD4^+$ T cell immune response allows the flexibility to adjust and adapt to returning pathogens as required, and promote immune tolerance by only unleashing effector function when and where instructed.

Most studies on human memory T cells have been carried out using peripheral blood samples, which do not accurately reflect the rich array of distinct tissue-specific memory cell phenotypes present in peripheral organs[82]. Additionally, generation and maintenance mechanisms may be completely different for tissue T cells when compared to those isolated from blood and lymph[83]. Key to furthering our understanding of T cell memory are T_{RM} cells. The existence and introduction of T_{RM} as such a major component of T cell memory has already begun to make researchers aware of the increased complexity and heterogeneity of the memory pool; as well as the importance of tissue-specific immunity.

1.2 Tissue Resident Memory T cells

In the old, two-subset model of anamnestic immune responses, central memory cells garrison the forts (SLOs), providing back up to the frontline where needed, while effector memory cells patrol through the blood and tissues constantly searching for antigen and responding with robust effector function. While a logical system, what if the body could supercharge this response by providing specialised local troops to every site of the body, ready to ambush any invading pathogen as soon as they infiltrate? Well, it turns out that evolution has already provided, arming us with a third memory T cell type – tissue resident memory T cells (T_{RM}).

As their name suggests, T_{RM} are retained in peripheral tissues, providing rapid frontline recall immunity. Developing within the infected/inflamed tissue during effector responses, T_{RM} will take up residence in peripheral niches and remain for what seemingly can be the lifetime of the host[84]. Their location makes them ideal for rapid responses to pathogens as there is no need for antigen trafficking to nearby lymphoid organs[85]. Furthermore, not only have they been shown to be crucial players in mouse models of infection, but recent data is also revealing their importance in multiple human inflammatory diseases[23, 86]. These properties have earned T_{RM} their place at the forefront of immunology research. One recent study used novel quantitative immunofluorescence microscopy to adduce that there are between 50- and 70-fold more $CD8^+$ TRM in mouse peripheral tissues than previously appreciated, and as many as 91% of total mouse T cells are actually tissue-resident[87]! Similarly, human T_{RM} have been shown to outnumber circulating memory cells[88] – the lungs contain approximately 10 billion T cells, the same as in the entire circulation, while the skin surface contains double this number[23, 89]. Coupled with the fact that it is thought the vast majority of tissue-associated cells are resident[87, 88, 90, 91], it is

astounding that these cells were only recently discovered (Box 1.1). Finally, it is important to note that the majority of studies in mice and humans have only investigated $CD8^+$ TRM residence, leaving $CD4^+$ TRM as a relatively unknown quantity. This section will discuss the characterisation, development and maintenance, function of T_{RM} in mice and humans (examining the $CD8^+$ lineage unless otherwise specified); before focussing on $CD4^+$ T_{RM} specifically.

Discovery & History of T_{RM}

Before the idea of T_{RM} cells was postulated, the dogma stated that following resolution of an infection, remaining effector T cells in NLTs either returned to the blood and SLO, or underwent apoptosis[86]. Moreover, in the absence of infection, it was thought that any T cells found in NLTs must be T_{EM} cells trafficking through the organ as part of continuous recirculation.

These ideas began to be questioned when researchers noticed antigen-specific $CD8^+$ T cell populations persisted long-term following viral infection of the lungs and brain in mouse models[92-96]. The pioneering work of David Masopust and his team uncovered long-lived $CD8^+$ T cells within a whole array of different NLTs in mice, and crucially, first hypothesised that these cells are permanent tissue-residents[97]. The next key discovery came from Gebhardt et al., who showed that 'passenger' lymphocytes carried in sensory ganglia grafts stayed resident in the donor graft[98]. In proving this, the authors officially† coined the term 'tissue-resident memory T cells'[98]. The final nail in the coffin of the old memory T cell dogma came via numerous parabiosis experiments that definitively proved tissue-associated T cell populations did not equilibrate between surgically conjoined congenic mice, instead remaining at their original tissue sites[99-102].

True T_{RM} have proved more difficult to identify in humans (see Box 1.2). Studies began in human skin. Grafting skin from psoriasis patients into immunodeficient mice revealed the presence of a resident, disease-linked T cell population[103]. These findings were built upon by the work of Rachel Clark – a key player in human T_{RM} biology. Her body of work uncovered the vast reservoir of skin-associated T cells, with distinct homing receptor phenotypes and diverse TCR repertoires that heavily implied at tissue-residence[89, 104, 105].

† - Similar nomenclature has been used in the literature long before this, with resident memory[106], resident-memory T cells[107], and tissue-resident memory[93] all evident. The self-explanatory nature of these terms make it difficult to pinpoint the first researchers to use these terms.

Box 1.1 – The discovery and history of tissue-resident memory T cells

1.2.1 Characterising Tissue Resident Memory T cells

Tissue resident memory cells represent a completely distinct population to their circulating effector memory counterparts. Although T_{RM} are principally defined by their long term peripheral location; differences in surface phenotype[108, 109], TF expression[84, 110], maintenance requirements[111], functionality[98, 100, 102, 112], sensitivity to stimuli[113], and even metabolism[114] have all been described. Mouse T_{RM} have now been characterised in a plethora of different NLTs[85, 100-102, 109, 110, 115-130]. Breakthroughs in mouse T_{RM} characterisation subsequently led to the discovery of human T_{RM} in the gut[88, 90], lungs[131-135], stomach[136], peritoneum[137], pancreas[138, 139], adipose tissue[140], female genital mucosa[141-145], salivary glands[91], conjunctiva[146], bone marrow[147], and liver[82, 148, 149]; as well as in the skin[143, 150-152].

Here, I will first discuss the different phenotypic signatures associated with T_{RM} in mice, before moving onto the characterisation of human T_{RM}.

1.2.1.1 Characterising Mouse T_{RM}

Just as T_{RM} are defined by their location, the markers used to characterise them are largely associated with tissue retention. The expression of surface markers CD69, integrin α1 (CD49a), integrin αe (CD103); alongside the lack the of lymph node homing markers CCR7 and CD62L, as well as tissue egress marker S1PR1 all act to keep T cells localised in the tissue. Each of these putative T_{RM} markers is discussed in detail below.

The first phenotypic criteria that a T_{RM} cell must meet is to be CD45RA$^-$, CCR7$^-$, and CD62L$^-$. The absence of expression of the latter two molecules prevents T_{RM} returning to SLOs[23].

CD69 is the closest surface molecule to a universal T_{RM} marker[109, 153, 154]. To date, T_{RM} from all NLTs studied expressed CD69[71, 92, 109, 110, 153, 155-157], and genetic deletion of CD69 abolished T_{RM} populations in skin and lungs[110, 156, 158, 159]. Despite also being an activation marker, CD69 clearly has another role in tissue retention as most T_{RM} in tissues have not been recently activated by antigen[109], although maintenance of CD69 expression may be cytokine-mediated[23]. The dominant role of CD69 in tissue retention is thought to be due to its reciprocal interaction with S1PR1, whereby CD69 directly downregulates S1PR1[153, 158, 160], and thus prevents migration towards S1P that encourages tissue egress[23, 32, 161-164]. Fittingly, forced S1PR1 expression led to a failure to establish T_{RM}[153], emphasising the importance of this marker pair for T_{RM} biology. Lastly it is worth noting that despite its merits, CD69 is not the perfect murine T_{RM} marker as some T_{RM} in the pancreas, salivary tract, and female reproductive tract (FRT) were CD69$^-$ [87]; while some CD69$^+$ cells in the thymus were not truly resident[130].

CD103 expression has been strongly linked to T_{RM} in a number of NLTs where it plays important roles in T_{RM} maintenance post-seeding[109, 110, 115, 156, 165]. CD103 upregulation appears to depend on upstream TCR signalling in a tissue-dependent manner[115, 121]. CD103 makes up half of the $\alpha e\beta 7$ integrin complex. This integrin is able to bind E-cadherin on epithelial cells expressed in barrier tissues[166]. Therefore, it has been speculated that physical tethering to epithelial cells directly contributes to tissue retention[31, 101, 109, 167]. Yet, not all T_{RM} cells express CD103[109, 157]. Furthermore, this

interaction is not strictly required for tissue residence as CD103$^+$ T cells have been found in the dermis[168], and in the brains of mice[115], despite the absence of E-cadherin in these locations[169]. Additionally, CD103 is a poor marker of CD4$^+$ T_{RM} (see 1.2.4). Instead, CD103 may act as a differentiation marker for T_{RM}[23], and may promote survival in NLTs[110, 115].

Similarly, CD49a is highly expressed on T_{RM} from skin, small intestine, lung and brain[98, 110, 133, 170, 171], but like CD103, its expression is not an absolute requirement for tissue residency in all organs[172]. CD49a forms half of integrin $\alpha1\beta1$ and may promote retention by binding tissue laminins and collagens[71]. However, $\alpha1\beta1$ integrin expression was required for maintenance in the lungs[173].

Alongside retention markers, some other common T_{RM} characteristics include elevated expression of co-inhibitory markers such as PD-1 and CTLA-4[109, 174], and enhanced expression of quiescence marker nuclear receptor subfamily group A member 1 (NR4A1)[170, 174-176]. Downregulation of the tissue egress receptor sphingosine-1-phosphate receptor-5 (S1PR5) may also be important at some sites[71], while the aryl hydrocarbon receptor (AHR) is seemingly important in T_{RM} persistence in the skin and gut[177]. There are undoubtedly many tissue-specific adaptations of T_{RM}, beyond the scope of this report, although expression of CXCR3 and CXCR6 on T_{RM} is of particular importance in many tissues[128, 174, 178-181].

Multiple studies have shown differential TF changes in murine T_{RM} cells (Fig. 1.2.1)[109-111, 130, 153, 170, 174]. The seminal work of Mackay et al. identified a core transcriptional signature for murine T_{RM} cells comprising of 37 key transcripts and their products that distinguish

T_{RM} from both traditional circulating memory subsets[110]. In a follow up study, the TF Homolog of B lymphocyte-induced maturation protein 1 (Blimp1) in T cells (Hobit) was identified from these transcripts as a key transcriptional regulator of T_{RM} cells[174]. Hobit operates in conjunction with Blimp1 itself to precisely coordinate the T_{RM} differentiation programme[84, 174]. Blimp1 is a transcriptional repressor broadly expressed in many effector cell lineages, promoting T cell effector function and terminal differentiation pathways[174, 182-184]. By contrast, Hobit is specifically upregulated in $CD8^+$ T cells from different NLTs including the skin, gut, kidneys and liver[174]. Deletion of either factor led to a reduction in resident $CD8^+$ T cell numbers, but knockout of both led to a complete block of residency across multiple organs[174]. Hobit-1 and Blimp-1 together repressed the genes for CCR7, S1PR1 and Krüppel-like factor 2 (KLF-2), as well as T-cell factor-1 (TCF-1 - a key determinant of circulating memory cells)[174]. Thus, these two master regulators promote the residency programme while simultaneously repressing other fates[84]. Interestingly, both Blimp-1 and Hobit bind many of the same target sites, but yet both are required for the full residency programme. Mackay and Kallies suggest that perhaps there is a division of labour amongst the two TFs whereby Blimp-1 is induced early and then Hobit induced later allowing sequential gene regulation, and/or different tissue environments rely more on either factor due to differential induction of upstream Blimp-1 or Hobit binding partners[84].

Other TFs such as KLF-2 are downregulated in multiple tissue-resident cell types[130, 153, 174]. KLF-2 directly promotes S1PR1 expression, prevention of which is critical for T_{RM} formation; and indirectly supports CD69 expression through the lack of competition with S1PR1[153, 158]. TFs T-bet and eomesodermin (Eomes) are also repressed in skin, gut, lung and brain-resident T cells[110, 170]. T-bet and Eomes are expressed in short-lived effector

cells, and long-lived memory cells respectively[72]. Downregulation of the pair in T_{RM} promotes responsiveness to T_{RM}-promoting cytokines, and allows Hobit expression to emerge[84, 111, 178].

1.2.1.2 Characterising human T_{RM}

Many of the surface markers used to characterise mouse T_{RM} are transferrable to humans. CD69 is widely considered the best marker of human T_{RM}, having been observed on memory $CD8^+$ and $CD4^+$ T cells from every peripheral tissue studied[86, 88, 90, 131, 136, 185, 186]. In support of this, it was very recently shown that CD69 expression alone delineates cells with transcriptionally distinct tissue-resident programmes that share core features with mouse T_{RM} cells[91]. Additionally, CD103 is often associated with human $CD8^+$ T_{RM}, particularly in mucosal sites and skin[91, 132, 133, 136, 147, 152, 185, 187], while CD49a has been heavily associated with lung and skin T_{RM}[131, 151, 188]. As in mouse, these two molecules are not absolutely required for residence. For instance, many describe $CD103^-$ cells as a separate resident subset – distinct from $CD103^+$ cells in their anatomical location, function, phenotype, and transcriptional profiles[91, 132, 146, 152, 187]. Similar suggestions have now been made about CD49a as a T_{RM} subset discriminator rather than a bone-fide universal marker[82, 188].

A number of excellent studies coming out of the Farber lab and others have assessed tissue residency profiles across a number of lymphoid, and non-lymphoid tissues[82, 88, 90, 91]. These types of studies provide the field with invaluable maps of body-wide T cell trafficking patterns, phenotypes and function. For resident T cell biology, these investigations have recently been taken one step further – through the transcriptomic

profiling of resident T cells from lung and spleen[91, 134]. First off, T_{RM} populations from different tissues consistently showed more genetic similarities to each other, than to circulating T_{EM} cells[91, 134]. T_{RM} show a strong association with the genes for CD49a and CD103, as well the downregulation of the genes coding for tissue egress receptors S1PR1 (and its associated TF KLF-2), and CD62L[91, 134]. Other key hallmarks of T_{RM} included an upregulation of mRNA for inhibitory receptors such as PD-1, CTLA-4, LAG-3, CD39, NKG2A, and CD101; and the chemokine receptors CXCR6 and CX3CR1, which showed up-, and down-regulation respectively[91, 134]. Hombrink et al. reported additional changes in metabolic pathways, including an enhanced glucose-deprivation signature, and increases in hypoxia-related genes[134]. Importantly, Kumar et al. demonstrated the majority of these properties are similar to previously identified mouse T_{RM} transcriptional profiles, helping to validate mouse models in the study of T_{RM}[91, 110, 170, 174]. An obvious caveat of these studies is that the only NLT investigated in both was lung tissue. However, Kumar et al. did show that spleen and lung NLT cells were very similar genetically; and went on to verify many of the key identified molecules that associated with lung/spleen T_{RM} across intestine, LN and tonsil tissue at the protein level[91]. Together these profiles go towards identifying a universal tissue programme that clearly has a lot of shared features across different tissue types.

Within these studies, clues into the transcriptional regulation of human T_{RM} were investigated. Like their mouse counterparts, human T_{RM} appear to have diminished expression of the genes coding for T-bet and Eomes[134, 149]. However, unlike mouse T_{RM}, Hobit is not a key differentiator of human T_{RM}[134], as it can be expressed following both $CD8^+$ and $CD4^+$ T cell activation[189, 190]. Furthermore, Hobit is not appreciably expressed in T_{RM} from liver, lung, or spleen[91, 148]. Thus, although a great many characteristics of

mouse T_{RM} translate well to human T_{RM}, a master TF for the human lineage has yet to be identified.

1.2.2 Development and Maintenance of Tissue Resident Memory T cells

The development of T_{RM} cell populations requires a combination of intrinsic cellular and tissue specific factors. This process requires multiple checkpoints including tissue entry, antigen recognition and sensitivity to specific tissue cues[71]. The great majority of our knowledge on this topic comes from mouse models, again discussed first.

1.2.2.1 Mouse T_{RM} development & maintenance

Given the phenotypic similarities of T_{RM} and T_{EM}, it was initially assumed that the former developed from the latter. However, largely only effector, not memory, cells can enter NLTs in mice[115-117, 191]. So, it is likely T_{RM} develop directly from effector cells in the tissues following an immune response[71]. Second, T_{RM} develop from killer cell lectin-like receptor G1 (KLRG-1)-negative precursors[110, 116, 157]. This is akin to circulating long lived $CD8^+$ memory T cells that develop from $CD127^{hi}KLRG\text{-}1^-$ cells, as opposed to short-lived effector populations that arise from the $CD127^{lo}KLRG\text{-}1^+$ pool[192, 193]. KLRG-1 downregulation may also be caused or increased by TGF-β signalling[194], and may be important in allowing CD103-mediated adhesion as both KLRG-1 and CD103 can bind to E-cadherin[108, 195]. Further, Gaide et al. demonstrated that the generation of skin T_{RM} led to parallel generation of T_{CM} in lymph nodes, suggestive of a shared common precursor[196]. The T_{CM} pool could even replenish the T_{RM} population following activation[196]. Additionally, like T_{CM} cells, T_{RM} are more quiescent than T_{EM}[31], and are not terminally

differentiated, as adoptive transfer of T_{RM} to the circulation caused switching to the T_{CM} phenotype[155]. Lastly, the mechanistic target of rapamycin (mTOR) signalling pathway may be important in this cell fate decision, as mTOR signalling pushes precursor cells down the T_{RM} route[197]. Collating all the available evidence, it is likely that T_{RM} develop from a common central memory precursor due to shared longevity properties, but following differentiation adopt a more effector memory-like phenotype with the ability to rapidly carry out effector functions following stimulation.

The first important step in becoming a T_{RM} cell is tissue entry. Effector cells upregulate homing receptors upon activation in SLO, and these determine specific tissue migration capabilities (see 3.1). Following entry, the tissue microenvironment imprints susceptible T cells with a residence phenotype as key T_{RM} signatures such as CD69 and CD103 are only acquired following tissue entry[116, 155]. $CD4^+$ T cells can aid the entry of $CD8^+$ T_{RM} into the skin, lungs and FRT through IFN-γ production, and Laidlaw et al. demonstrated $CD4^+$ T cell help is essential for $CD8^+$ T_{RM} formation in the lungs[178, 198, 199]. Therefore, just as the process of circulating memory $CD8^+$ T cell formation depends on $CD4^+$ T cell help[200], so too might the generation of $CD8^+$ T_{RM}[108], at least at certain tissue sites.

Intriguingly, it appears that T_{RM} do not absolutely require antigen recognition to form. Antigen-independent T_{RM} development has been observed in the mouse airways, FRT, and skin epithelium[100, 121, 124, 201, 202]. However, brain T_{RM} formation was antigen-dependent[109, 121]. Perhaps then non-cognate inflammatory signals are sufficient to drive T_{RM} production in many tissues? Casey at al. dispute even this hypothesis, demonstrating that naïve T cells transferred into lymphopaenic hosts seed many NLTs and are able to adopt the phenotype of T_{RM} cells in the absence of either antigen or inflammation[109].

However, while it appears often neither antigen-mediated or inflammatory signals are absolutely required, the combination of both has shown to be necessary for optimum T_{RM} generation as illustrated particularly elegantly by so-called 'prime and pull' vaccination strategies (immunisation followed by local chemokine application to 'pull' effector T cells into desired tissue)[121, 124, 202, 203]. Notably, repeated antigen exposures increased T_{RM} dissemination throughout the entire skin surface or whole FRT, far beyond the local infected area[100, 124, 202]. Interestingly, it now seems that weaker TCR stimulations are better at establishing fully functional T_{RM} in brain tissue[204].

Having established T_{RM} do not always require antigen to form, others asked whether T_{RM} maintenance depends on cognate signals. It appears that while resident T cells in the brain and dorsal root ganglia require antigen stimulation for persistence[109, 115, 121, 129], the general consensus is that T_{RM} in most other NLTs seem to be antigen independent[109, 110, 117, 121, 126, 127, 176], albeit with some contradicting evidence in lung tissue[156]. Moreover, chronic antigen stimulation actually skewed cells away from the T_{RM} phenotype[109, 171]. This could represent a similar situation to the prevailing model in circulating memory $CD8^+$ T cell differentiation where increased antigen signal strength preferentially generates SLECs[72].

Once T_{RM} progenitors have entered the tissue, the cytokine milieu appears to have a profound impact on the development and maintenance of the tissue residency programme (Fig. 1.2.1). The cytokine most implicated in this process is TGF-β, consistently being associated with T_{RM} development at a variety of sites, including skin, gut, and lungs[109-111, 156, 157, 171, 205]. TGF-β promotes the development of T_{RM} cells through four known mechanisms. First, TGF-β signalling promotes CD103 upregulation. These

findings fit nicely with the model of KLRG-1$^-$ cells being T_{RM} precursors, as KLRG-1$^-$ cells express more TGF-βRII than their KLRG-1$^+$ counterparts[110, 157], and TGF-β is only able to induce CD103 upregulation on effector, not resting memory cells[109]. In addition, CD49a expression can be driven by TGF-β[171]. Third, TGF-β signalling enabled KLF-2 downregulation and the acquisition of the residency programme, effects that were augmented by the cumulative addition of first IL-33, then TNF-α[153]. Lastly, the competitive interaction between TGF-β signalling and T-bet promotes residence. TGF-β drives downregulation of both Eomes and T-bet, which indirectly enhances surface TGF-β receptor expression, creating a positive feedback loop, as both these TFs are negative regulators of TGF-β receptor gene expressions[111, 178]. However, residual T-bet is needed to promote CD122 expression (the β-subunit shared by IL-2 and IL-15 receptors) that allows subsequent IL-15 signalling[111]. IL-15 is another cytokine commonly cited in T_{RM} development[110], and long term T_{RM} survival[111, 206]. IL-15 is also thought important in the upregulation of CD103[110, 126]. Whereas T_{RM} regulator Hobit is induced following tissue entry in a T-bet and IL-15 –dependent manner[174], Blimp-1 does not require either factor, instead being induced in the early effector phase by IL-2 and IL-12 signalling[207]. This supports the theory that first Blimp-1, then Hobit are induced in T_{RM} development, playing different temporal roles[84]. Lastly, CD4$^+$ T cell-derived IFN-γ has also been implicated in the establishment of residence via suppressing T-bet on CD8$^+$ T cells, and promoting subsequent CD103 expression and settling in lung tissue[178].

Although we have come far in understanding a lot of the cytokine signals that trigger T_{RM} development and maintenance, there is no one factor that is ubiquitously required for this programme. For example, some studies have reported a TGF-β independent development programme in the gut, and nasal epithelium[201, 208], while others showed it

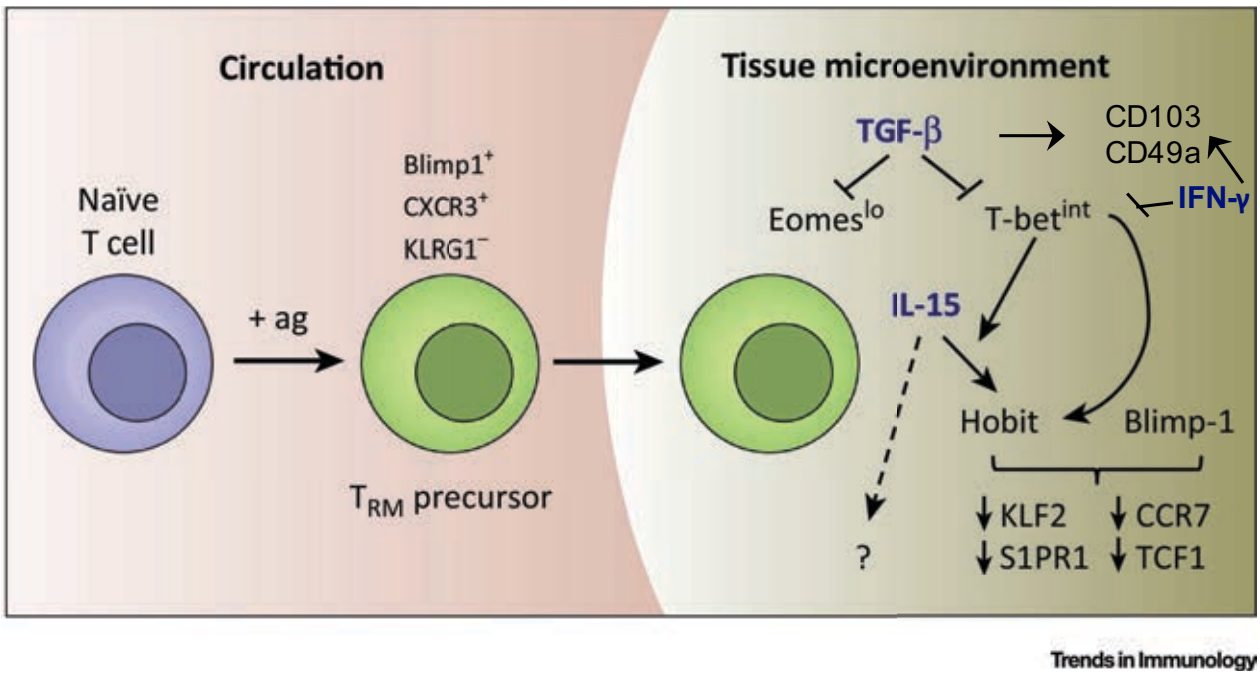


Figure 1.2.1 - Signals and transcription factors required for the development of T_{RM} in mice. Following activation and migration into NLTs, T_{RM} precursors that are putatively KLRG-1^{-} Blimp1^{+} CXCR3^{+} can develop into T_{RM} due to influences from the tissue microenvironment. The two cytokines most implicated are TGF-β and IL-15. TGF-β can promote expressions of T_{RM}-associated integrins CD49a and CD103; and repress both Eomes and T-bet - resulting in enhanced sensitivity to further TGF-β. Retention of low/intermediate T-bet levels is required for IL-15 responsiveness however. IL-15 can also promote CD103 upregulation (not shown); and induce Hobit expression alongside residual T-bet. Together, Hobit and Blimp1 coordinate the downregulation of genes associated with tissue egress such as KLF2, S1PR1, CCR7 and TCF1. KLF2 and S1PR1 allows the upregulation of CD69 (not shown) and establishment of residency. Additionally IFN-γ can contribute to this circuit, through its suppression of T-bet, and promotion of CD103 expression.

Mechanisms shown summarise current knowledge on CD8^{+} T cells across mouse NLTs. While this is a general paradigm, T_{RM} formation in some tissues has shown to be TGF-β or IL-15-independent[208, 209], suggestive of tissue-specific influences. Figure adapted from Mackay & Kallies 2016 - ref [84]. Additional references: [110, 111, 153, 171, 174, 178].

was not required for long-term population maintenance in the salivary glands[126]. Comparably, although skin and lung $CD8^+$ T_{RM} depend on IL-15 for their maintenance, resident cells in many other tissue sites such as gut and FRT, are not reliant on it to persist[125]. Furthermore, different subsets of T_{RM} require different signals to form. Bergsbaken et al. found that $CD103^-$ T_{RM} in the small intestines of mice require pro-inflammatory IL-12 and IFN-β, which suppresses TGF-β-mediated CD103 expression required by the more appreciated $CD103^+$ subset[210]. It is therefore likely that different NLTs impose their own residency requirements based on their distinct cytokine microenvironments, and favoured 'in-house' cell subtype(s) they choose to dwell there. Illustrating this, some have found a universal requirement for IL-33 for maintenance of all lymphocyte tissue residency in adipose tissues[84, 211, 212].

Finally, Notch1 signalling may contribute to the maintenance of T_{RM}. Hombrink et al. showed that Notch1 signalling was important in the persistence of lung $CD8^+CD103^+$ T_{RM} cells, promoting metabolic programmes that allow tissue retention. Notch1 signalling interconnects with the TGF-β signalling pathway through common downstream signalling mediators, and the induction of Notch1 ligand expression in epithelial cells; thus providing an integrated mechanism to maintain T_{RM} cells, at least in lung tissue[134].

1.2.2.2 Human T_{RM} development & maintenance

There is precious little data on human T_{RM} development so many of our assumptions must come from mouse models (see Box 1.2 for problems associated with studying human T_{RM}). Human lung T_{RM} have been found to express low KLRG-1 mRNA[134]. One likely possibility is that, akin to mouse, human $CD8^+$ T_{RM} also share a $KLRG\text{-}1^-$ T_{CM}

Complications in the study of human T_{RM}

Tools for studying mouse T_{RM} such as parabiosis, *in vivo* imaging, and tissue grafting are generally not possible in humans. Furthermore, determining phenotype and function of target cells through multi-parametric flow cytometry requires tissue digestion and cell isolation which introduces a number of potential problems. These include the admixing of different tissue microenvironments; sampling bias due to inefficient removal of blood contaminants, inefficient tissue-associated cell extraction (neither mechanical nor enzymatic digestion can remove 100% of tissue lymphocytes), or the inclusion of tissue-associated lymphoid structures; and this methodology gives no architectural information[71]. On top of this, lymphocyte yields from NLTs are often low, thus investigators require a large amount of starting material, and T_{RM} are not thought to survive well in culture[71, 170]. Even in mouse models, total body T_{RM} were vastly underestimated in number until recent *in vivo* imaging corrected this[87]. It is worth noting these complications because as the human T_{RM} field explodes, so too will the need for scrutiny and careful research. This is not to say that excellent studies do not exist. As a positive, CD69 has proven to be a robust marker and key distinguisher of T_{RM} in every human tissue studied[91, 186], although not perfect in mice[87, 213], perhaps due to the specific pathogen free conditions in which they are housed[214]. Until new technologies develop for improved isolation, or visualisation of *ex vivo* T_{RM}, the field must take great care to minimise the associated risks with studying human T_{RM}.

Box 1.2 – Complications in the study of human T_{RM}

precursor that maintains low KLRG-1$^-$ expression once differentiated into a resident cell type. Alternatively, the tissue environment could directly downregulate KLRG-1 expression, as has been shown for TGF-β[194]. Furthermore, clues to the long-term persistence of human T_{RM} may lie in their phenotype, with the quiescent, inhibited, resting phenotype of these cells being shown in multiple studies[91, 134, 140, 175].

Cytokines are indisputably important in human T_{RM} cell generation too. To our knowledge, Pallett et al. were the first to generate CD8$^+$ T cells with T_{RM} phenotypes (CD103$^+$CD69$^+$) from peripheral blood mononuclear cells (PBMCs). Optimal T_{RM} induction was achieved through either IL-15, or anti-CD3 stimulation; followed by TGF-β exposure[148]. IL-15 was also implicated in retention in the human tonsil through downregulating KLF2 and

S1PR1[187]. Thus, while studies of human development are still in their infancy, key similarities to mice gives confidence that in the future, clear hypothesis-driven research can be tested effectively.

1.2.2.3 Longevity of mouse and human T_{RM}

Having discussed known maintenance requirements for T_{RM}, an important question is how long can these cells persist? Unfortunately, data is lacking on the longevity of human memory T cells, even in peripheral blood[71]. However, studies in mice and rhesus monkeys noted T_{RM} persistence for up to 700 days[97, 100, 215]. This is complemented by circumstantial evidence of human skin T cell numbers remaining stable even in patients in their 90s[86, 216]. Nonetheless, none of these studies exclude the possibility that circulating memory T cells are constantly replenishing the T_{RM} pool, a phenomenon observed for $CD8^+$ T cells in murine lung tissue, but not skin[100, 159, 217]. The situation is likely even more complex, as murine dermal-dwelling $CD4^+$ T_{RM} were far more transiently resident compared to $CD8^+$ T_{RM} retained in the epidermis[218]. Finally, competition for space, antigen, and other local signals can shape the T_{RM} repertoire[108, 177, 219]. So, it appears that the cytokine signals, cumulative antigen exposure history, and tissue niche properties all control the length of stay of T_{RM}.

An alternative explanation suggests tissue residence is a continuum - ranging from true T_{EM} that merely pass through the tissue, up to true life-long resident memory T cells, and every possibility in between. Research over the coming decade, including both longitudinal studies and advanced phenotypic analyses will help us address this possibility.

1.2.3 Function of Tissue Resident Memory T cells

T_{RM} are superior to their circulating counterparts in terms of cytokine responses, and anamnestic immunity. T_{RM} from many different mouse NLTs been proven to be better at clearance of a variety of infections, including those in the lungs[102, 112, 113, 201, 220-223], FRT[98, 99, 124, 218, 224, 225], and skin[100, 121, 199, 226-228]. A pertinent example of this superior protection includes the 99.99% impairment in vaccinia virus clearance in the absence of T_{RM} in mice[100]. Crucially, T_{RM}-mediated protection is often critical for pathogen clearance[99, 102, 121, 128, 220, 229]. Analogous findings have since been made for human T_{RM}. For example, respiratory syncytial virus-specific T_{RM} numbers correlate strongly with reduced symptoms and reduced viral load[230]. Similarly, lung T_{RM} cells contain more influenza-specific clones, and respond better than blood T cells to this pathogen[131, 132]. Mouse, followed by human, T_{RM} functions are covered below.

1.2.3.1 Mouse T_{RM} function

T_{RM} have a number of key advantages over circulating memory T cell subsets, allowing them to assert functional superiority. T_{RM} respond rapidly to antigen, and can do so entirely within the tissue microenvironment[85, 113, 115, 226]. Furthermore, T_{RM} appear to need only peptide recognition, not costimulatory signals, to become activated[231]; and may actually function better after receiving lower strength TCR stimulation[204]. The specific types of antigen-presenting cells (APCs) needed to prime T_{RM} in the tissues are now starting to be uncovered[232, 233]. Third, resting skin T_{RM} in mice appear to adopt a dendritic morphology; and move in slow, deliberate Lévy walks, both of which enhance their

antigen scanning capabilities[31, 108, 177, 181, 218, 226, 234]. Fourth, T_{RM} respond to antigen with robust cytokine responses and direct cytolysis action[97, 155, 231]. These are often, but not limited to, type 1 and/or type 17 responses, and T_{RM} can frequently produce multiple cytokines simultaneously[100, 113, 128, 224, 235-237].

Schenkel et al. and others illustrated that T_{RM} act as immune sentinels as well as direct effectors. Through rapid production of pro-inflammatory cytokines following antigen recognition, T_{RM} induce an antiviral state in surrounding cells including CXCL9, CXCL10 and vascular cell adhesion protein-1 (VCAM-1) upregulation on nearby endothelium. This leads to the recruitment of a whole host of other immune cells, including circulating memory $CD8^+$ T cells, B cells and inflammatory monocytes; activation of natural killer (NK) cells; and maturation of DCs[71, 129, 224, 231, 238, 239]. The whole process depended on a combination of T_{RM}-derived IFN-γ, TNF-α, and IL-2[224]. Thus, T_{RM} act as both adaptive and innate-type immune 'alarm' cells in their own right. T_{RM}-mediated immunity and the tissue-wide state of alert induced has shown to be so powerful that it can provide bystander protection against antigenically distinct pathogens to those immunised with in mouse models[71, 220, 221, 238]. Furthermore, local T_{RM} induction can lead to more far reaching protection. Vaccinia immunisation at just one site led to T_{RM} seeding throughout the entire skin[100], while skin infection with the same virus led to the generation of lung-tropic T_{RM} that protected against pulmonary challenge[227]. Importantly, T_{RM} in the central nervous system (CNS) can mediate clearance of lymphocytic choriomeningitis virus (LCMV) without causing overt immunopathology and tissue destruction, a feat not matched by circulating T cells[129].

1.2.3.2 Human T_{RM} function

T_{RM} also play important protective roles in humans. In the skin, HSV-specific $CD8^+$ T_{RM} persist long-term, and the density of these cells correlated directly with suppression of viral reactivation at sites of previous lesions[143]. Lung T_{RM} are enriched for influenza-specific clones[131-133], while cervical T_{RM} are enriched for herpes simplex virus (HSV)-2-specific cells[240]. The same is found for hepatitis B virus (HBV)-specific clones within liver T_{RM} that provide the best protection against the disease[148].

Robust cytokine responses are a key facet of human T_{RM} biology. Like mouse T_{RM}, type-1 and type-17 responses have been most often documented. Two studies showed $CD8^+$ T_{RM} from the lungs produce more IFN-γ, TNF-α, and IL-2 than T_{EM}, as well as comprising more multifunctional cells that produce all three[131, 132]. Such multifunctional cells have been best associated with protective immunity[241-243]. Interestingly, lung $CD8^+$ T_{RM} had a high abundance of mRNA for IFN-γ and TNF-α at rest, allowing rapid cytokine responses following stimulation[134]. In another study, resident cells in the lung have been linked to better IL-2, IL-10, and IL-17 production than non-residents. This same study showed that the former two cytokines were part of a body-wide $CD4^+$ and $CD8^+$ T_{RM} signature[91]. IL-10 expression at rest complements the constitutive self-regulatory programme seen in T_{RM}[91]. In the liver, elevated IL-2 has been reported in resident $CD8^+$ T_{RM} cells[148]; while in the stomach, T_{RM} make more IFN-γ and IL-17 than T_{EM} counterparts (although no multifunctional cytokine advantage was noted in this study)[136]. In the skin, T_{RM} mostly secrete IFN-γ and IL-2[89], have a lower threshold for cognate activation than blood T cells[244], and were able to single-handedly protect against cutaneous infections in the absence of circulating T cell subsets[150]. Cheuk et al. has demonstrated how CD49a can

be used as a functional discriminator of skin T_{RM} cells – showing that CD49a$^+$ and CD49a$^-$ cells preferentially express IFN-γ and IL-17 respectively[188]. On this theme, Watanabe et al. showed CD103$^+$ T_{RM} in skin produced more IFN-γ, TNF-α, and IL-22; but diminished proliferative capacity compared to the CD103$^-$ subset[152]. Lastly, lung CD8$^+$ T_{RM} have shown more cytotoxic potential than circulating T cells[132, 134], although this was not paralleled in their liver equivalents[148, 149].

1.2.3.3 Implications for vaccines

With these prolific immune properties in mind, T_{RM} sound like the ideal guardians of the body. Able to respond rapidly, and robustly not only directly to antigen, but to alarm and recruit the rest of the immune system; able to provide heterosubtypic protection; and able to confer protection at anatomically distinct sites without causing excessive tissue destruction. T_{RM} sound like the perfect cell type to manipulate for disease intervention. Indeed, in mouse models inducing local tissue resident skin cells through skin scarification with vaccinia virus has led to up to 100,000 times better prevention against viral reinfection, than when immunised through the subcutaneous, subdermal, and intramuscular routes[228]. Similarly, live-attenuated influenza virus (a common licenced influenza vaccine) administered intranasally, generated T_{RM} that were able to provide cross-strain protection, properties that were not seen with standard subcutaneous injection[221]. Indeed, in a respiratory syncytial virus (RSV) infection model, immunisation through the intramuscular route actually increased pathogenesis, whereas intranasal immunisation induced T_{RM}-mediated protection[222]. Moreover, the efficacy of a recently developed malaria vaccine, PfSPZ, has been pinned on resident lymphocytes in the liver of rhesus monkeys[245]. Additional data has suggested T_{RM} generation improves responses

in vaccinations against mycobacterium tuberculosis[186], and even drastically improves HIV responses in mouse models [246, 247].

Such promising studies have led to many leading immunologists in the field proposing that new vaccines should be optimised to prioritise T_{RM} generation[23, 86, 108, 186, 248]. This would hypothetically principally involve immunisation at sites relevant to protection, such as therapies directed to the gut, or synovium administered through endoscope or arthroscope respectively[23]. The 'prime and pull' strategy also has promise. Coupling standard subcutaneous immunisation with local chemokine application has been successful in optimising anti-HSV-2 responses in the murine FRT[124]. However, in certain situations the efficacy of T_{RM} is also their undoing as these cells are starting to emerge as key players in various autoimmune and inflammatory diseases.

1.2.4 $CD4^+$ Tissue Resident Memory T cells

Tissue-resident $CD4^+$ T cells have been investigated far less than $CD8^+$ T_{RM}. This is likely in part due to the infection context in which T_{RM} are generally studied - viral infections of mice generate a far more robust $CD8^+$ T cell response compared to $CD4^+$ T cells[23]. This is unfortunate, as $CD4^+$ T cells are the dominant T cell population in the skin, mucosal tissues and lungs of mice and humans[88-91, 133, 152, 186, 213, 218, 249]. Similar to $CD8^+$ T cells, the majority of memory $CD4^+$ T cells in these human tissues are phenotypically tissue resident[88]. Thus, resident $CD4^+$ T cells represent an immunological goldmine of unanswered questions waiting to be addressed.

In mice, $CD4^+$ T_{RM} have been described in the skin[199, 218], lungs[99, 102, 112, 133, 229, 250], FRT[99], intestines[237], salivary glands[126], LNs[251], and bone marrow[118]. In humans, analogous findings have been made in the skin[82, 89, 103, 151, 152], FRT [144, 240], stomach[136], conjunctiva[146], salivary glands[91], lungs[82, 88, 90, 91, 131, 135, 252], gut[82, 88, 90, 91, 253], and liver[82]. $CD4^+$ T cells are often located in the parenchymal regions of these NLTs rather than at epithelial sites where $CD8^+$ T cells tend to predominate[108]. For example, $CD4^+$ T cells favour the dermis of the skin, and the underlying lamina propria of the FRT[99, 151, 218].

For both mouse and man, the majority of the same markers used for $CD8^+$ T_{RM} can be applied to their $CD4^+$ counterparts (Fig. 1.2.3)[91]. $CD4^+$ T_{RM} display the effector memory phenotype ($CD45RA^-CCR7^-$), express CD69 and downregulate KLF-2 and S1PR1[88, 91, 102, 133, 186]. Just as for $CD8^+$ T_{RM}, CD69 is widely accepted as the best surface marker of resident $CD4^+$ T cells[91, 186]. Again, similar to $CD8^+$ T_{RM}, the expression of CD49a, and an increased density of CD11a expression, was also favoured by different tissue-resident $CD4^+$ T cells in the lungs[102, 131, 254], although the association with CD49a was stronger in $CD8^+$ T cells[91]. However, CD103 has repeatedly proved a very poor marker for $CD4^+$ T_{RM}[23, 88, 91, 102, 126, 186, 213, 218, 255]. Perhaps this is due to less reliance on epithelial tethering compared to $CD8^+$ T_{RM}, as in human skin $CD103^+$ $CD4^+$ T_{RM} were only found in the epidermis, not the dermis[152]. Additionally, as part of the core gene expression signature that both human $CD4^+$ and $CD8^+$ T_{RM} share, Kumar et al. identified upregulation of PD-1, CXCR6, and the downregulation of CX3CR1[91]. Last, Wong et al. recently stratified human $CD4^+$ T_{RM} into four main clusters based on dimensionality reduction algorithms. They describe two subsets elevated in skin ($CLA^+HLA\text{-}DR^-CD25^-$, $CLA^+HLA\text{-}DR^+CD25^+$), and two lung-enriched subsets ($CCR5^+CD27^-$, $CCR5^+CD27^-$) (CLA – cutaneous lymphocyte antigen)[82].

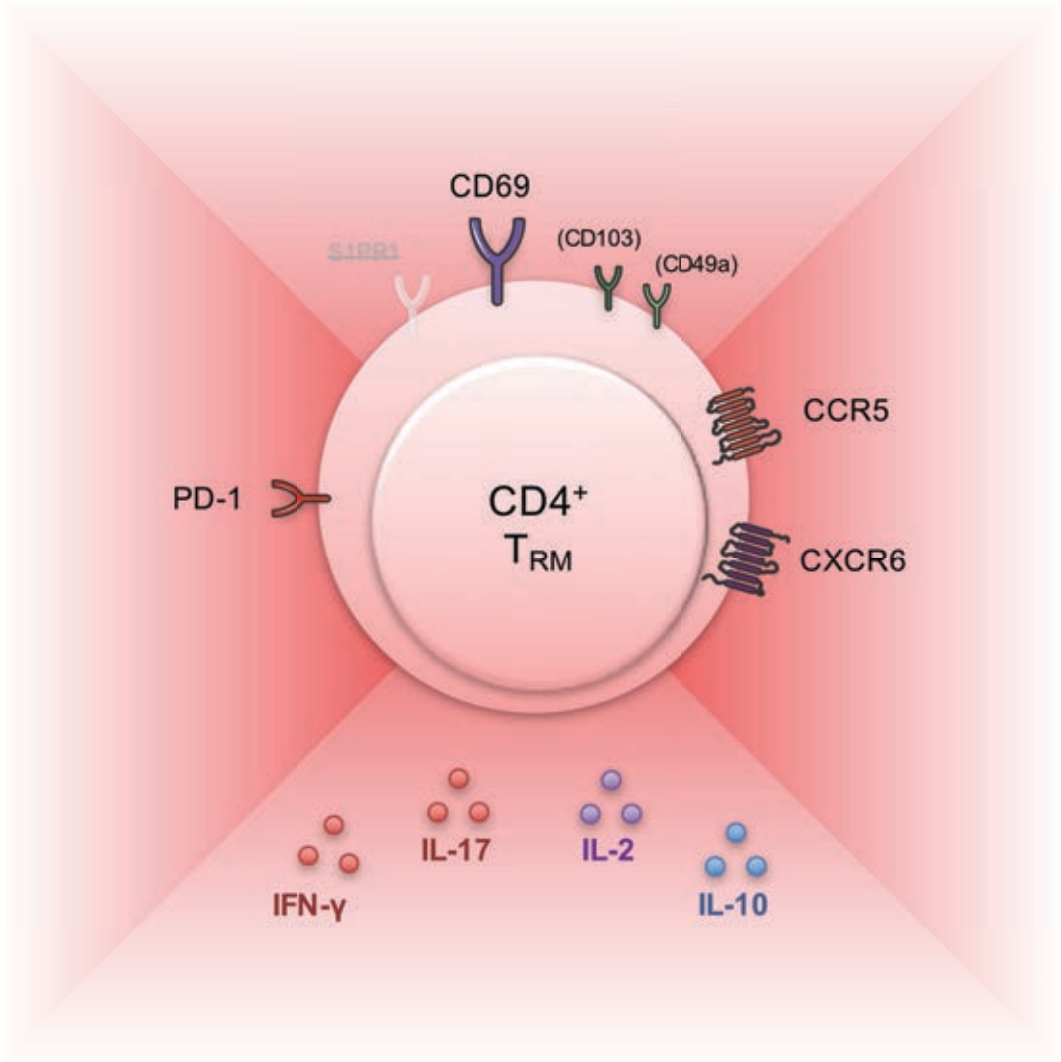


Figure 1.2.2 - Common characteristics of human $CD4^+$ T_{RM} cells. Current knowledge on Shared phenotypic and functional features of human $CD4^+$ T_{RM} from different tissues. Markers associated with tissue retention, homing, functional inhibition; and commonly enhanced cytokines are shown in the top, right, left, and bottom quadrants respectively. All markers/cytokines shown have been reported on $CD4^+$ T_{RM} from at least two different NLTs, and in two independent studies. Tissue residence in $CD4^+$ T_{RM} (like $CD8^+$ T_{RM}) is associated with the loss of tissue egress receptor S1PR1 (shown in grey with strikethrough font). CD103 and CD49a have been repeatedly associated with $CD4^+$ T_{RM}, but are often not a discriminator but markers of further subsets, hence their inclusion in parentheses. Figure concept drawn from Kumar et al. (ref [91]). For additional information, see text.

Recent work by Oja et al. investigated the transcriptional profile of $CD4^+$ T_{RM} in the lungs, partnering work performed by Hombrink et al. for lung-resident $CD8^+$ T cells[134, 135]. This study revealed that lung $CD4^+CD103^+$ T_{RM} express low levels of the tissue egress receptor S1PR1 and associated TF KLF2; transcriptional regulators T-bet and Eomes; and high CXCR3, CXCR6 and CCR5. Interestingly, although Hobit was upregulated at message level, no protein expression was seen, once again arguing against its use as a T_{RM} differentiator in humans. The authors speculate it could be involved in lung $CD4^+$ T_{RM} development, and later dispensable in their maintenance. $CD4^+$ T_{RM} expressed high levels of effector cytokine mRNA, including IFN-γ, but also high levels of inhibitory receptors such as CTLA-4, PD-1 and 2B4 to limit excessive T_{RM} responses[135]. Interestingly, the lung $CD8^+$ T_{RM} described in the partner study by Hombrink et al. were extremely similar to the lung $CD4^+$ T_{RM} described by Oja et al., suggesting the residency signature supersedes differences in T cell lineage. These similarities included a reliance on the Notch1 signalling pathway for T_{RM} presence in the lungs, as confirmed by knockout mouse models[134, 135].

Other than data on the reliance on Notch-1 for human lung $CD4^+$ T_{RM}[135], limited research has been conducted on the development and maintenance requirements for $CD4^+$ T_{RM}, and the few existing studies are somewhat conflicted. Some have suggested that $CD4^+$ T_{RM} are more dependent on antigen for their development than $CD8^+$ T_{RM}, as this is the case in the salivary glands and FRT of mice[99, 126]. Perhaps antigen is not needed for maintenance in all tissues however, as Teijaro et al found that T_{RM} can persist in the murine lung without cognate stimuli[102]. In the FRT of mice, $CD4^+$ T_{RM} organise into memory lymphocyte clusters (MLCs) along with $CD8^+$ T cells, DCs, and macrophages[99].

These MLCs were distinct from tertiary lymphoid organs (TLOs) as they had no lymphatic architecture, or GCs; and persisted in the absence of infection and inflammation[256]. These structures have been proposed to maintain $CD4^+$ T_{RM} through CCL5 secreted by macrophages within the cluster. This constitutive chemokine secretion was in turn due to low level $CD4^+$ T_{RM}-derived IFN-γ caused by cognate signals, suggestive of a requirement for antigen for $CD4^+$ T_{RM} maintenance [256]. Similar CCL5-dependent $CD4^+$ T_{RM} association in MLCs have since been observed in the dermis around hair follicles[213].

TGF-β is often vital for $CD8^+$ T_{RM} development and maintenance, acting partly through upregulation of CD103. Perhaps then as CD103 upregulation is a less important aspect of $CD4^+$ T_{RM} biology, TGF-β is consequently less important in their development. Some studies in the mouse salivary glands and intestines agree with this hypothesis[126, 208], however in the kidney TGF-β is required for the development of $CD103^-$ $CD4^+$ T_{RM} cells[257]. It is plausible that other pro-inflammatory cytokines such as IL-12 and IFN-β could aid in the differentiation and persistence of $CD103^-$ $CD4^+$ T_{RM}, as has been shown recently for mouse intestinal $CD103^-$ $CD8^+$ T_{RM}[210]. It may be the case that like circulating memory $CD4^+$ T cells, $CD4^+$ T_{RM} depend on IL-2 also[69, 73, 74, 258]. This has been shown for both allergic asthma- and LCMV-reactive T_{RM} development in the lungs of mouse models[259, 260]. Interestingly, both T_H1 and T_H2 type T_{RM} cells required IL-2 for their generation, in a process where B cells inhibit the early formation of T_H1 T_{RM}, but are critical for their long-term maintenance[260]. In sum, it can clearly be seen that despite the confusion created by these early studies that have so far investigated different species, tissues and T_{RM} induction regimens; a combination of different tissue-specific cognate and non-cognate signals is likely needed to generate and maintain $CD4^+$ T_{RM}, and these signals are likely subtly distinct from those regulating $CD8^+$ T cell residence.

Due to different tissue niche preferences, $CD4^+$ T_{RM} show distinct migration and morphology patterns to $CD8^+$ resident T cells. Whilst resident $CD8^+$ T cells in the epidermis display a resting dendritic morphology and slow velocities, $CD4^+$ T_{RM} retain more rounded shapes and are much more motile in the dermis, travelling greater distances within the skin[218]. Likewise, $CD4^+$ T_{RM} have been found spread out within the vaginal lamina propria, as well as aggregated in MLCs[256]. In the lung, while $CD8^+$ T_{RM} predominantly localise to areas of tissue injury, $CD4^+$ T_{RM} are thought to cluster in bronchus-associated lymphoid tissue structures[135, 261].

Some have postulated the increased motility of $CD4^+$ T cells in tissue sites may also point to more transient tissue residency compared to $CD8^+$ T cells. Collins et al. showed in parabiotic mice that the majority of $CD4^+$ T cells are in equilibrium with the circulation of infection-naïve mice, and that CD69 and CD103 were modulated on entry and exit, suggesting transient residency. However, consistent with other studies in mouse models, and human skin, a long-term resident $CD4^+$ T cell population formed after infection[89, 152, 213, 262]. Thus, $CD4^+$ T cells in tissue could comprise a more heterogeneous population than $CD8^+$ T cells, including recirculating cells, transient residents, and long-term resident memory cells that together mediate optimum immunosurveillance.

$CD4^+$ T_{RM} are superior to circulating $CD4^+$ T cells in responses to many infections. In mouse models, this has been seen following infections of the lungs[102, 112, 229, 263-265], skin[199], FRT[99, 124], and gut[237, 266]. Just like other $CD4^+$ T cell lineages, the resident population controls infection efficiently through rapid recruitment and activation of other aspects of the immune system. In the skin, $CD4^+$ T_{RM} recruit and activate inflammatory

monocytes[239], while in bone marrow resident CD4+ T cells provide B cell help through CD40-CD40L interactions[118]. In the FRT following HSV-2 infection, circulating CD4+ T cells take 2 days to enter the tissue, and a further 3 days to start secreting IFN-γ, whereas CD4+ T_{RM} are constantly secreting IFN-γ at low levels and this can be enhanced extremely rapidly upon antigen encounter[99]. Furthermore, CD4+ T_{RM} are sufficient to protect against HSV-2, even in the absence of CD8+ T_{RM}[267]. As for CD8+ T_{RM}, CD49a may act as a functional distinguisher of CD4+ T_{RM}, In the murine lungs, CD49a+ cells had a far more robust IFN-γ response and increased survival when compared to those lacking this molecule[254].

Human CD4+ T_{RM} tend to be better cytokine producers than circulating CD4+ T_{EM}. This is best illustrated through the study by Oja et al. where lung CD4+CD103+ T_{RM} IFN-γ, IL-2, and TNF-α responses not only more rapid than circulating memory equivalents, but of a better quality too. A great many more multifunctional responders were also present in the T_{RM} pool[135]. In addition, an upregulation of both IL-2 and IL-10 have both been considered as hallmarks of T_{RM} biology[91]. Watanabe et al. revealed T_{RM} in human skin produce type-1, type-2 and type-17 cytokines more efficiently than recirculating cells[152], while Booth showed gastric CD4+ T_{RM} displayed significantly higher IFN-γ, TNF-α, and CCL4, as well as a higher CD107a expression marking enhanced degranulation and cytotoxicity[136]. Lung and spleen CD69+ T_{RM} also express more IL-17 and IFN-γ mRNA than their CD69- counterparts[91]. Comparing CD103+, and CD103- T_{RM} in the skin, the former were the best cytokine-producers, but had a more limited proliferative potential[152]. CD4+ T_{RM} in the lung are thought to be highly type-1-multifunctional, and enriched for influenza- and mycobacterium tuberculosis (Mtb)-specific clones[131, 252]. Finally, CD4+ T_{RM} are also extremely likely to be involved in human autoimmune pathology. These cells influence

disease recurrence in psoriasis[103], in a mechanism that likely involves long-term resident T_H22 cells[151]. Furthermore, adoptive transfer of gut-resident $CD4^+$ T_{RM} into an immunodeficient host caused disease in inflammatory bowel disease (IBD) mouse models[268], and fittingly may also be involved in the human disease[253].

Together, $CD4^+$ T_{RM} appear to be just as potent as $CD8^+$ T_{RM} in protection from infection, due to rapid activation and robust cytokine responses. Despite the limited data, their abundance in tissues, and proximity to barrier surfaces makes it almost undeniable that they are highly involved in protective responses, and by extension, organ-specific inflammatory disease. There may be a division of labour between $CD4^+$ and $CD8^+$ resident T cells, due to their distinct tissue niches, migration preferences, maintenance requirements, and inherent functions[152, 186, 218]. The specific contributions and collaborations between $CD4^+$ and $CD8^+$ T_{RM}, $CD4^+$ T_{RM} longevity properties, and their exact roles in local immune responses in different tissues are all key questions still to be answered.

1.3 The Liver as an Immune Organ

1.3.1 Biology of the Human Liver

The liver is a remarkable organ. It performs over 500 functions, including vital roles in digestion and nutrient storage, plasma protein synthesis, blood detoxification, and immune homeostasis. The importance of the liver in survival is emphasized by its size, being the largest internal organ; its receival of 25% of the cardiac output – more than any

other organ; and its unique ability to regenerate that likely evolved out of the necessity to protect these functions [reviewed in ref [269]].

The liver produces and excretes bile, allowing the emulsification and subsequent breakdown of fats and fat-soluble vitamins[270]. It is involved in the metabolism and storage of proteins, carbohydrates, lipids, vitamins and trace elements. This allows for control of blood glucose levels; the storage of iron for erythrocyte production and the production of plasma proteins and hormones such as albumin, clotting factors, complement proteins and thrombopoetin; as well as many other roles. A wide range of endogenous proteins (urea, bilirubin etc.) as well as the majority of drugs are also metabolised in the liver, preventing build-up of toxic metabolites[271-274]. It is likely due to the large frequency of such potentially damaging insults the liver receives, as well as toxins from the gut, that it developed the ability to regenerate[269]. On top of these functions, the liver is considered an immune organ – containing a large immune component, acting as a second line of defence to pathogen entry through the digestive tract, and playing non-redundant roles in systemic immune tolerance[275-277].

One helpful way to visualise the liver architecture (Fig. 1.3.1) is through imagining its stepwise construction, beginning with the liver vasculature. The liver receives blood from two sources. Approximately 80% comes from both the portal vein which drains from the splanchnic circulation, carrying nutrients and microbial products from the gut. The remaining 20% arrives from the hepatic artery, which drains from the coeliac trunk[278-280]. Blood from both percolates as vessels branch further into liver sinusoids. Sinusoids are lined by hepatic sinusoidal endothelial cells (HSEC), unusual due to their large (50-

180nm) fenestrations that allow solute access to hepatocytes[271]. Blood exits the liver via central veins that then drain into the hepatic veins[280].

Hepatocytes, the parenchymal cell of the liver, comprise between 60-80% of liver cells (and around 78% of tissue volume), and carry out all the main metabolic, protein synthesis, detoxification and storage functions discussed above[279, 281-284]. Hepatocytes are arranged in chords that radiate towards the centre of hexagonal hepatic lobules, running alongside the sinusoids. At lobule intersections are the portal areas which consist of portal venules and hepatic arterioles that carry blood to the liver, bile ducts, lymphatic vessels, and vagus nerve branches. Central veins are positioned at the centre of each lobule[279, 285]. Hepatocytes can be classified as zone 1, 2, or 3, ascending depending on the proximity to blood input from the portal areas. Hepatocytes in these different zones differ in their metabolic capacities, a phenomenon known as metabolic zonation[271]. A large proportion of resident macrophages called Kupffer cells, and liver-associated NK cells called Pit cells are located within the sinusoids; whereas in the space of Disse (the space between the abluminal side of the sinusoids and the basolateral surface of hepatocytes) hepatic stellate cells (HSCs) that are involved in storage and synthesis of vitamin A, and synthesis of extracellular matrix proteins can be found[279, 284, 286]. Of note, as well as the hepatic lobules, liver functional units can be described in terms of liver acini (that centre on input portal venules and hepatic arterioles), or portal lobules (that centre on portal areas)[285].

Finally, the biliary tree must be integrated into our representation. Hepatocytes produce bile that is secreted into bile canaliculi, formed by tiny passages formed between apical

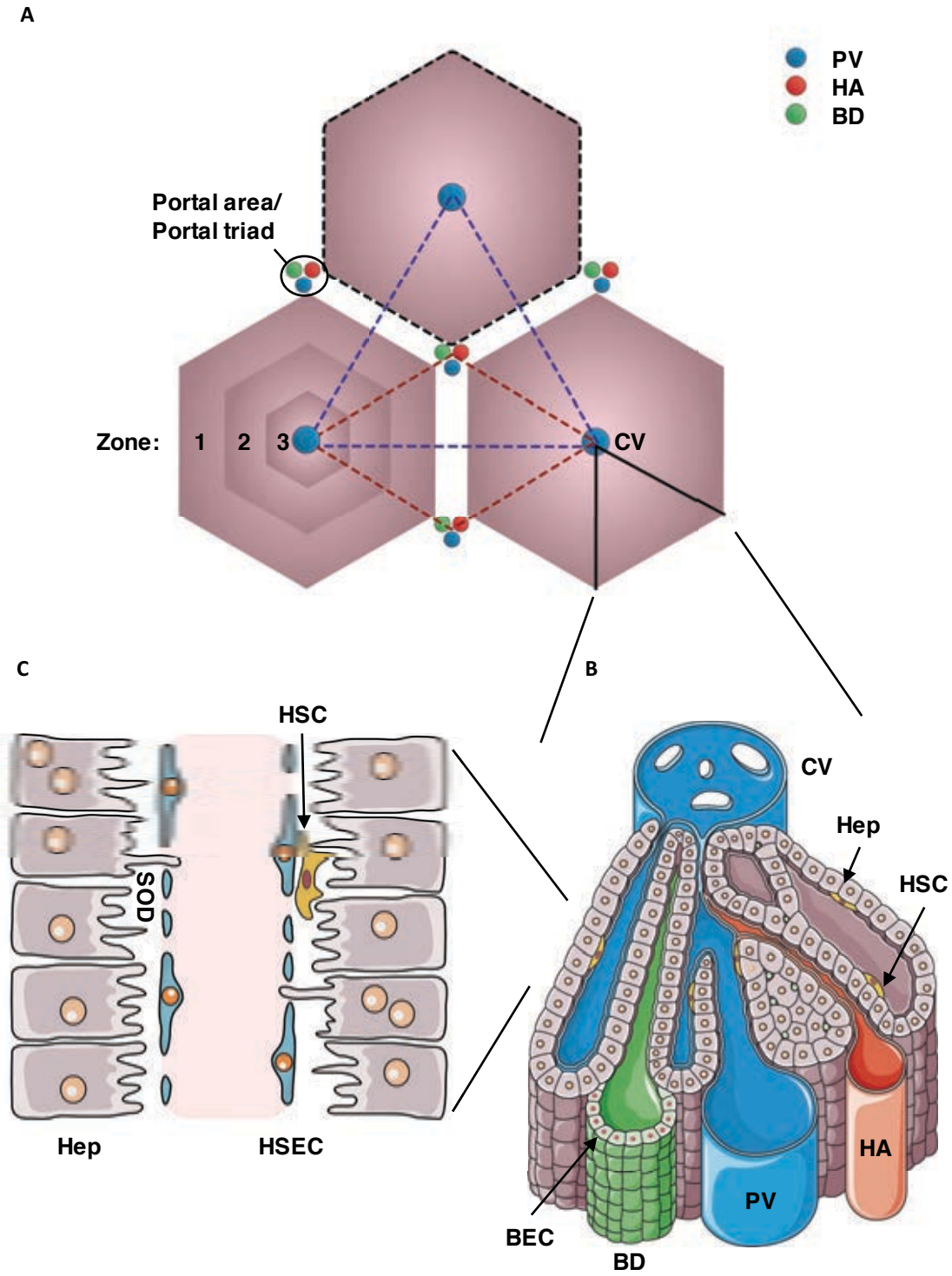


Figure 1.3.1 - Liver microarchitecture. A - Liver functional units can be described in terms of liver lobules (within black dashed area), acini (red dashed area), or portal lobules (blue dashed area). The liver lobule are hexagonal areas comprised of hepatocyte chords that radiate from portal areas (or portal triads) where blood enters at the hexagon vertices, towards central veins (CV) in the middle. A liver acinus shows a diamond-shaped area centred on portal vessels (that run between liver lobules - not shown) and shows the tissue area supplied by these branches. Contrastingly, a portal lobule centres on a portal triad and thus shows the area supplied (and drained by) by this triad. **B -** A portion of a liver lobule. Blood flows from portal veins (PV) and hepatic arteries (HA), mixes in the sinusoids, and exits the liver via the central veins. Hepatocytes (Heps) produce bile that travels along canaliculi towards bile ducts (BD) comprised of biliary epithelial cells (BEC) in the portal triad. **C -** Close up representation of a hepatic sinusoid. Hepatocytes lay underneath hepatic sinusoidal endothelial cells (HSEC) that themselves form the fenestrated sinusoidal endothelium. The space of Disse (SOD) is the gap between these two cell types, where hepatic stellate cells (HSCs) are located. Servier medical art utilised as the basis of part B. References: [271, 279, 285].

surfaces of neighbouring hepatocytes. From here bile drains towards the portal areas where it enters bile ducts, both lined by biliary epithelial cells (BEC)[279, 285].

1.3.2 Immunological properties of the Liver

As well as being a key driver of innate immunity through complement and acute-phase proteins, cytokines and chemokines, the liver possesses an exceptionally large immune component[279, 281]. Kupffer cells are the largest resident macrophage population in the human body, representing 80-90% of all tissue macrophages[281, 287]. These cells play vital roles in the phagocytosis of microbes and debris, toxin clearance, and erythrocyte recycling[288]. Both antigen-presenting myeloid DCs (mDCs), as well as proinflammatory plasmacytoid DCs (pDCs) are also found in the liver under resting conditions[289], primarily in portal areas in order to monitor the portal circulation[290]. Approximately 10^{10} lymphocytes are contained within an average healthy human liver, containing both resident and recirculating cells[281, 291]. Particularly interesting is the large and unusual complement of innate lymphocytes. NK cells make up 30-50% of liver lymphocytes[287]. Whereas the two main NK cell subsets, $CD56^{LO}$ and $CD56^{HI}$, are present at approximately a 9 to 1 ratio in the periphery, these subsets are similarly abundant in the liver[289]. Natural killer T (NKT) cells and $\gamma\delta$-T cells are also markedly enriched in the liver compared to blood[275]. However, conventional, $\alpha\beta$-T cells are still the dominant lymphocyte population in the liver, just like in blood[275, 281, 289]; but the CD4:CD8 ratio is reversed (2:1 and 1:3.5 in blood and liver respectively)[292]. Of note, mucosal-associated invariant T (MAIT) cells (an innate invariant population that recognises the MHC class I analog MHC-related 1 [MR1] that presents bacterial antigens) represents around 30% of T cells in the liver –

making them the largest innate T cell population in humans[289]. Lastly, B cells comprise a minor lymphocyte population, around 6% of liver lymphocytes[281].

Other features make the liver unique from an immunological standpoint. First, fenestrated endothelium and the lack of a substantial basement membrane allow T cells direct access to the underlying hepatocytes[286, 293, 294]. Such parenchymal access is not possible in other organs[295]. Second, contact time between T cells and liver APCs is increased by low blood flow rates caused by small sinusoid diameters, and perturbations due to physical occlusions by Kupffer cells[281, 296]. Third, HSEC possess phagocytic and antigen presentation capabilities, and express scavenger receptors and other markers more traditionally associated with lymphatic endothelium cells than vascular endothelium[297]. Fourth, the liver is the primary site of lymphocyte development in the foetus, which may have some evolutionary hangover effects to the adult liver environment[298-300]. Fifth, the liver is the only organ that can facilitate direct antigen presentation and activation of $CD8^+$ [294, 301, 302], as well as $CD4^+$ T cells[303]. However, the outcome of these interactions is normally immune tolerance, the promotion of which is a defining aspect of liver immunobiology[294, 296].

1.3.3 Immune Tolerance in the Liver

Regulation of T cell immunity must occur at every stage in order to prevent autoreactivity. Central tolerance in the thymus prevents the escape of potentially autoreactive clones. Peripheral tolerance has many aspects; including anergy or apoptosis in response to insufficient co-stimulation, and direct inhibition by T_{REGS}. Intrinsic regulatory mechanisms exist, limiting T cell response times or magnitude; and inherent plasticity allows skewing

to different, less pathogenic, subsets (immune deviation), or egress from site of tissue damage. Although generally considered detrimental, immunosenescence and exhaustion (decline in cellular function that is age-associated, or due to chronic antigen stimulation respectively) both also suppress the immune response. Last, immune privilege applies to organs like the eye or the brain, where immune cell tissue access is limited[304, 305].

The liver is a highly tolerogenic organ, employing many of these mechanisms to limit immunity. It is likely that the necessity to promote tolerance over immunity evolved to prevent unnecessary responses to the multitude of food and microbial antigens arriving from the gut, as well as the numerous neo-antigens created by liver metabolism[306]. Hepatic immune tolerance was first observed when pig liver transplants were tolerated even across full MHC mismatch, and the paradigm subsequently strengthened when co-transplant of other organs that were normally rejected alone, were accepted along with donor liver[307, 308]. Similarly, human allogenic liver transplants are tolerated better than other organs, even following removal of immunosuppressive regimens[287]. The liver is also responsible for the maintenance of oral tolerance[277, 309]. Last, tumours often metastasize to the liver, and the tolerance effect is misappropriated by certain pathogens such as HBV and hepatitis C virus (HCV)[306].

Multiple cell types can present antigen to $CD8^+$ and $CD4^+$ T cells in the liver; including hepatocytes, HSEC, Kupffer cells (KCs), resident DCs, and HSCs. All of these are involved in the liver tolerance effect. $CD8^+$ T cells are often deleted, or left functionally impaired by these interactions; while $CD4^+$ T cells preferentially undergo switching into more tolerogenic (T_H2 and T_{REG}) types, as well as suffering their own dampening of function (Fig. 1.3.2)[284, 291, 306, 310, 311].

Immature mDC types (with low MHC and costimulatory molecule expressions) predominate in the liver, and interactions with these mDCs inhibits proliferation, and promotes anergy of $CD8^+$ T cells; and can skew towards T_H2 and T_{REG} induction in $CD4^+$ T cells[281, 306, 310, 312, 313]. Similarly, HSEC presentation to $CD4^+$ T cells results in the dampening of T_H1 functionality, but the enhancement of T_H2 cytokine production[310, 314]. $CD8^+$ T cells that recognise antigen displayed on HSEC are unable to proliferate or mount substantial cytokine responses, and undergo early apoptosis[281, 306, 315, 316]. KCs secrete inhibitory prostanoids and nitric oxide, and were vital in the development of systemic tolerance to portal antigen injection in mouse models. Furthermore, KCs and HSEC also constitutively express immunoregulatory cytokines IL-10 and TGF-β, that promote immature DC phenotypes and T_{REG} induction[277, 279, 306, 310, 311]. Interestingly, it may be the constant exposure to the low levels of lipolysaccharide (LPS) (up to 1ng/ml in the portal circulation) that results in a state of 'LPS-resistance' among KCs and HSEC that in turn causes high IL-10 release in the liver and an overall dominant tolerance effect[277, 279, 291, 317].

HSCs are an additional major source of TGF-β, and they too have been directly implicated in the generation of pT_{REGS} through a retinoic acid-dependent mechanism in mice[306, 318, 319]. Finally, many liver-resident cells (hepatocytes, HSEC, mDCs, pDCs and KCs) express inhibitory ligands such as programmed death-ligand (PD-L)1 and/or 2, or express first apoptosis receptor (Fas) ligand that directly promotes effector cell apoptosis; but do not express co-stimulatory ligands such as CD80 or CD86[294, 310, 311, 320]. To date, no evidence supports a role for BEC in modulating T cell responses through presentation[284, 291, 321].

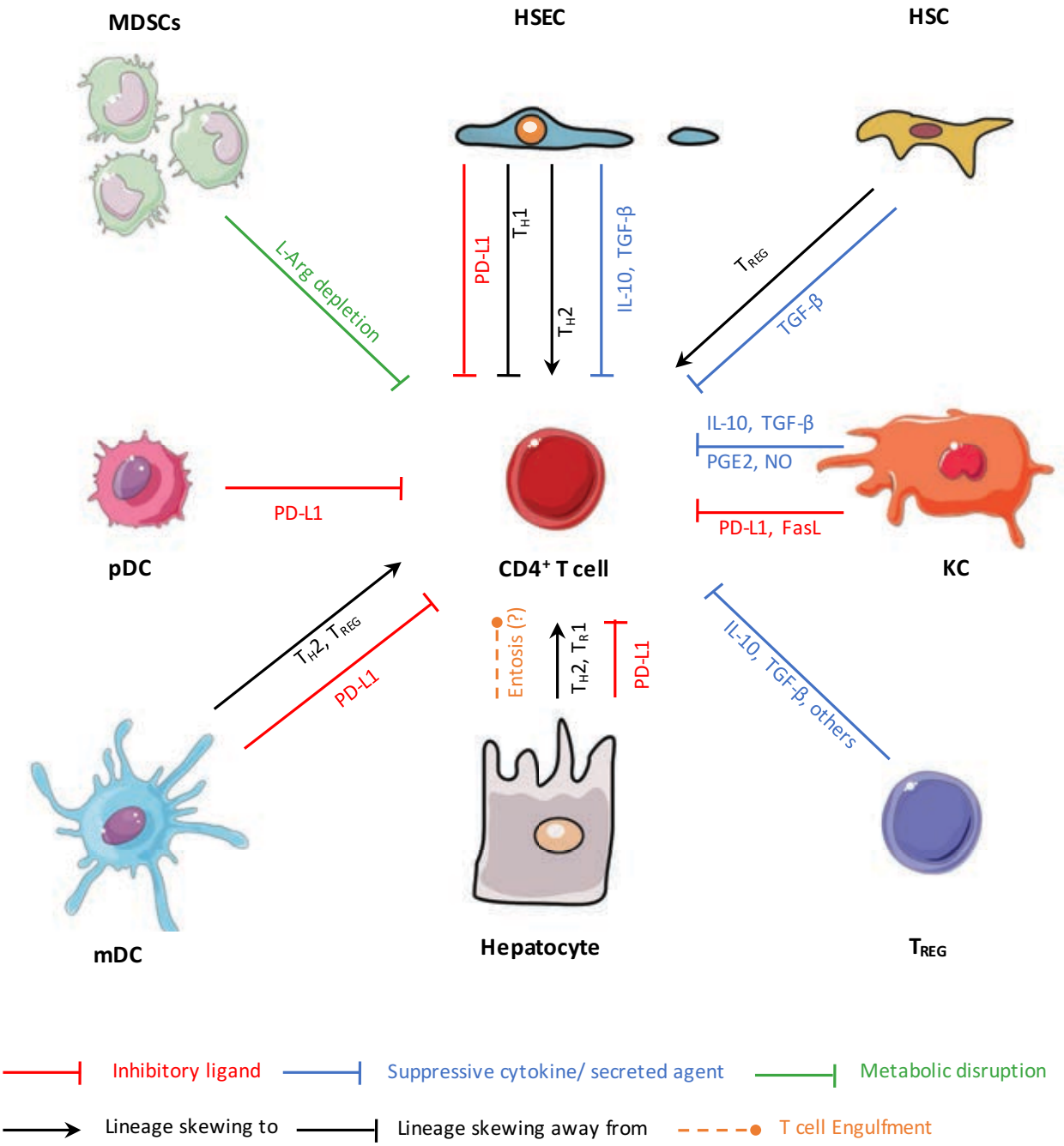


Figure 1.3.2 - Immune tolerance mechanisms acting on hepatic CD4$^+$ T cells. Illustrated are the various ways that different hepatic cell types can promote tolerance in CD4$^+$ T cells. Many cells directly inhibit T cell activation or cause apoptosis through inhibitory ligands PD-L1 and/or first apoptosis receptor (FasL). Additionally, direct inhibition through inhibitory cytokine production has been shown for HSECs, KCs, intrahepatic T_{REGS}, and HSCs. KCs can additionally produce nitric oxide (NO) and inhibitory prostanoids such as prostaglandin E2 (PGE2). Many different liver cells can present antigen to CD4s, either through constitutive or induced MHC class II expression; but such interactions often skew to the T_H2 (HSEC, mDC, hepatocyte), or T_{REG} lineages (mDC, HSC). Hepatocytes may also be able to induce T_R1 type cells in mice[322]; while HSECs can dampen T_H1 function[314]. MDSCs can suppress CD4$^+$ T cells through L-Arginine (L-Arg) depletion[323]. Finally timely live cell engulfment (entosis) and release may act as a suppression mechanism by preferentially releasing T_{REGS} when required, although this has not been experimentally

verified (hence the ? [Z.Stamataki, unpublished data]). For further details and additional cellular interactions, see text body. (*Legend cont. on next page*)
Lineage skewing is shown by normal arrows (where lineage listed along the arrow is being skewed towards), and by inhibition arrows (where lineage skewed away from is listed). Figure inspired by design from Crispe, 2014 (ref [311]). Additional references used - [277, 294, 306, 310, 312, 319].

Therefore, non-parenchymal cells from the liver are extremely good at preventing full T cell activation. Interestingly, other liver subsets control T cell responsiveness. These include myeloid-derived suppressor cells (MDSCs) that constrain T cell activation[323, 324]; and intrahepatic myeloid cell aggregates for T cell clonal expansion (iMATES) that promote $CD8^+$ T cell activation[311, 325]. Finally, innate lymphocytes such as NK and NKT cells also alter the balance of T cell immunity vs. tolerance in the liver[326-330; 331].

Hepatocytes also contribute to immune tolerance. Hepatocytes only express MHC class I constitutively, though MHC class II and costimulatory molecules can be upregulated by inflammatory signals – a unique feature amongst all parenchymal cells[277, 332, 333]. Therefore, most work on hepatocyte - T cell interactions has focussed on $CD8^+$ T cells. In contrast to when antigen is presented conventionally in lymph nodes, exclusive antigen expression on hepatocytes leads to dysfunctional $CD8^+$ responses in transgenic mouse models[296, 334-337]. Benseler et al. more recently demonstrated three waves of hepatocyte-mediated $CD8^+$ T cell tolerance as a result of these interactions. First around 80% of the cells are deleted by suicidal emperipolesis (an antigen-specific active T cell invasion into hepatocytes, followed by their lysosomal degradation), while most of the remaining cells are inhibited through PD-1-PD-L1 interactions. Any leftover cells display low TCR expression levels and are functionally unresponsive[294, 338]. Because of the massive deletion of activated $CD8^+$ T cells in the liver, the liver has been described as a T cell 'graveyard' in the past, referring to the idea that the liver is where many activated systemic T cells go to die[339-341]. It has long been known that liver $CD4^+$ and $CD8^+$ T cells are more

activated than cells in the blood[314, 342], and influenza-specific T cells can be found in the liver where they cause bystander damage, despite no intrahepatic virus presence[343]. However, it is important to point out that by no means all cells that travel to the liver are deleted; effective immune responses can still be achieved in the liver; and the liver is a hospitable environment for memory cells[277, 296].

Meanwhile, forced MHCII expression on hepatocytes results in cognate $CD4^+$ T cell activation, but insufficient to cause hepatitis in mice[332]. Furthermore, murine $CD4^+$ T cells that encountered antigen on hepatocytes were skewed towards T_H2 functionality, and were less able to provide help to virus-specific $CD8^+$ T cells[344]. Indeed, hepatocytes are such powerful agents of immune tolerance that when Lüth et al. expressed myelin basic protein in hepatocytes, mice were protected from otherwise lethal autoimmune neuroinflammation in an MS model in a T_{REG}-dependent manner[345]. Others suggest mouse hepatocytes are able to induce inhibitory T_R1 cells [322]. *In vitro* studies have shown human hepatocytes modulate mDCs to inhibit $CD4^+$ T cell activation through IL-10[346], and are thought to be able to directly cause apoptosis in T cells through PD-L1 expression[347]. Furthermore, our lab have demonstrated human hepatocytes can engulf, store, and excrete $CD4^+$ T cells, particularly conventional T_{REGS} [unpublished observations]. However, whether this promotes tolerance through the timely release of stored T_{REGS}, or prevents excessive inhibition through regulatory T cell sequestration, is still under investigation.

Despite these multiple regulatory measures, the liver can promote immunity to certain pathogens such as hepatitis A virus; or in individuals that spontaneously clear HBV or HCV infection. Overriding liver tolerance can be caused by proinflammatory signals such

as type I IFNs, or toll-like receptor (TLR) ligands; or due to cross-presentation by certain conventional APCs in the liver, or in SLOs[281, 294, 295, 325, 348-350]. Third, antigen density plays a key role, with lower densities of antigen favouring full activation (as lower antigen density presumably promotes only the activation of strongest affinity T cells and limits T cell exhaustion)[294, 302]. Therapeutically, checkpoint blockade agents that target inhibitory molecules such as PD-1 or its ligands have shown some efficacy in the reversal of T cell exhaustion and the restoration of protective immunity[294, 351, 352]. Unfortunately though, T cell responses are compromised in many chronic liver diseases, as discussed below (section 1.3.5).

1.3.4 T cell migration into the Liver

Homing molecules are the ligands and receptors involved in the chemotaxis, localisation and retention of leukocyte subsets. Broadly they can be parsed into chemokines and adhesion receptors. Chemokines (chemotactic cytokines) are small proteins that specifically attract leukocytes that express their apposite receptors. Chemokines are named based on the amino acid composition at the first two cysteine residues (CC, CXC, and CX3C chemokines possess 0, 1, and 3 amino acid residues between these cysteines respectively; while the non-conformist XC chemokines lack two adjacent cysteine residues)[353]. Chemokines are promiscuous, often biding multiple receptors with differing affinities, and causing subtly different responses in different cell types; allowing for extremely detailed control of leukocyte migration[354]. Adhesion receptors include integrins and selectins that allow physical tethering and retention of immune cells; as well as extravasation into tissues. Together these homing receptors allow for precise control of

locomotion, directing immunity and homeostasis through tissue-specific programmes (see 3.1).

The process of leukocyte migration across endothelium and into tissue can dogmatically be broken down into four steps. First is the capture phase where weak leukocyte interactions, often mediated by endothelial selectins, continually form and break, slowing immune cell migration and producing characteristic rolling. Next, chemokines presented on the endothelial glycocalyx are detected by the leukocyte, resulting in conformational activation of integrin molecules that in turn mediate firm adhesion, the third stage. Finally, leukocytes extravasate into the tissue, following chemokine signals to the focus of inflammation[286, 355].

While this process is followed in most vascular beds, migration into the liver displays a number of stark differences. First, most immune cells enter the liver parenchyma through the sinusoids, whereas in most other organs, tissue access is largely restricted to post-capillary venules[356, 357]. Second, HSEC express very low levels of selectin molecules constitutively, and the leukocyte rolling phase does not occur in the liver[356, 358-361]. The slow/intermittent flow in sinusoids may negate the need for this step, with cells skipping straight to firm adhesion instead; or other molecules such as Intracellular adhesion molecule (ICAM)-1 or vascular adhesion protein-1 (VAP-1) take up this role in the hepatic sinusoids[286, 360, 362]. HSEC constitutively express ICAM-1 and ICAM-2 at a higher level than other endothelial cells, as well as low levels of VCAM-1; and upregulate all three further following inflammation[286, 363]. Both ICAM-1 and VCAM-1 have proved important in optimum adhesion to HSEC *in vitro*[362, 364], as well as Stabilin-2 – an integrin $\alpha_M\beta_2$ ligand also constitutively expressed by HSEC[365]. Third, the extravasation process is distinct as

HSEC lack tight junctions and consequently express low levels of cell junctional molecule and extravasation-linked CD31, which is strongly expressed in other vascular endothelium[355, 360, 366]. Fourth, HSEC can express many molecules more typical of lymphatic than vascular endothelium. These include scavenger receptors such as CD36, liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN), lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1); and transmembrane glycoprotein VAP-1[297, 356, 367]. CLEVER-1 and VAP-1 are of particular importance in recruitment to the human liver. CLEVER-1, constitutively expressed on HSEC but upregulated further in inflammation, promotes the transmigration of T_{REGS} preferentially[368, 369]. VAP-1 is constitutively expressed on resting HSEC and lymphatic endothelium, but not any other vascular beds[360]. VAP-1 mediates firm adhesion and transmigration[364, 370-372], and through its unusual monoamine oxidase activity promotes the upregulation of ICAM-1, VCAM-1, MAdCAM-1, and selectins; and binding of gut-tropic $\alpha_4\beta_7$ lymphocytes[373-376]. Membrane-bound VAP-1 levels are increased in steatohepatitis; and the levels of soluble VAP-1 and its enzymatic activity correlate with disease activity in chronic liver, but not other inflammatory diseases[376-381]. For summary, and a comparison with portal vascular endothelium homing molecule requirements, see Fig. 1.3.3.

Certain homing molecule combinations endow T cells with specific tissue-tropism. For example, integrin $\alpha4\beta7$ and CCR9 expression; and combinations of CCR4 and CCR10 promote homing to the gut and skin respectively[116]. However, to date no tissue specific phenotype has been attributed to the liver[360, 367]. T cells expressing CCR1, CCR5, CXCR3, or CXCR6 are all preferentially recruited into inflamed liver tissue; but all of these

receptors are associated with general inflammatory infiltrates rather than specific for liver[353, 367, 382, 383]. CCR5 ligands CCL3-5 are expressed at portal endothelium, and consequently intrahepatic CCR5$^+$ T cell frequencies are particularly high[353, 360, 362, 367, 382, 384]. CXCR3 ligands CXCL9, CXCL10, and CXCL11 are all produced by inflamed HSEC, as well as hepatocytes, stellate cells, KCs, other infiltrating leukocytes, and damaged BEC in primary biliary cirrhosis (PBC)[353, 356, 385-387]. T_H1, T_H17, and effector CD8$^+$ T cells all express CXCR3 at increased levels in inflamed human livers, allowing their recruitment through sinusoidal endothelium and into the hepatic parenchyma[353, 367]. Other chemokine receptors are then likely responsible for subsequent positioning, such as CCR6 in T_H17 migration to bile ducts[388]. Interestingly, CXCR3 is also used by T_{REGS} in liver migration, in order to co-localise with their T_{EFF} counterparts[353, 367]. Upon reaching the liver, T_{REGS} then use CCR10 or CCR4 to locate near cholangiocytes or intrahepatic DCs respectively[389, 390]. CXCL16 is mostly expressed on inflamed BEC, but also hepatocytes and HSEC[391]; and so attracts T cells expressing its apposite receptor CXCR6[353]. CXCR6 expression has been shown to be essential for the retention of T cells in the liver[148, 180, 392, 393]; and the same can be applied for CXCR6 expression on

NK, and NKT cells[394, 395]; arguing of its importance as a liver retention marker. The immune cell possession of other chemokine receptors have also been linked to homing to particular areas of the liver. For example, CXCR1$^+$ T cells have been shown to be associated with inflamed hepatocytes[396], and CCR6, CXCR4, and CX3CR1 have all also been associated with infiltration of bile duct areas[353, 388, 389, 397-399]. Intrahepatic CCR7$^+$CD62L$^-$ T cells have also been described, leading to suggestions these cells are trafficking to draining lymph nodes via afferent lymphatics, or associated with tertiary

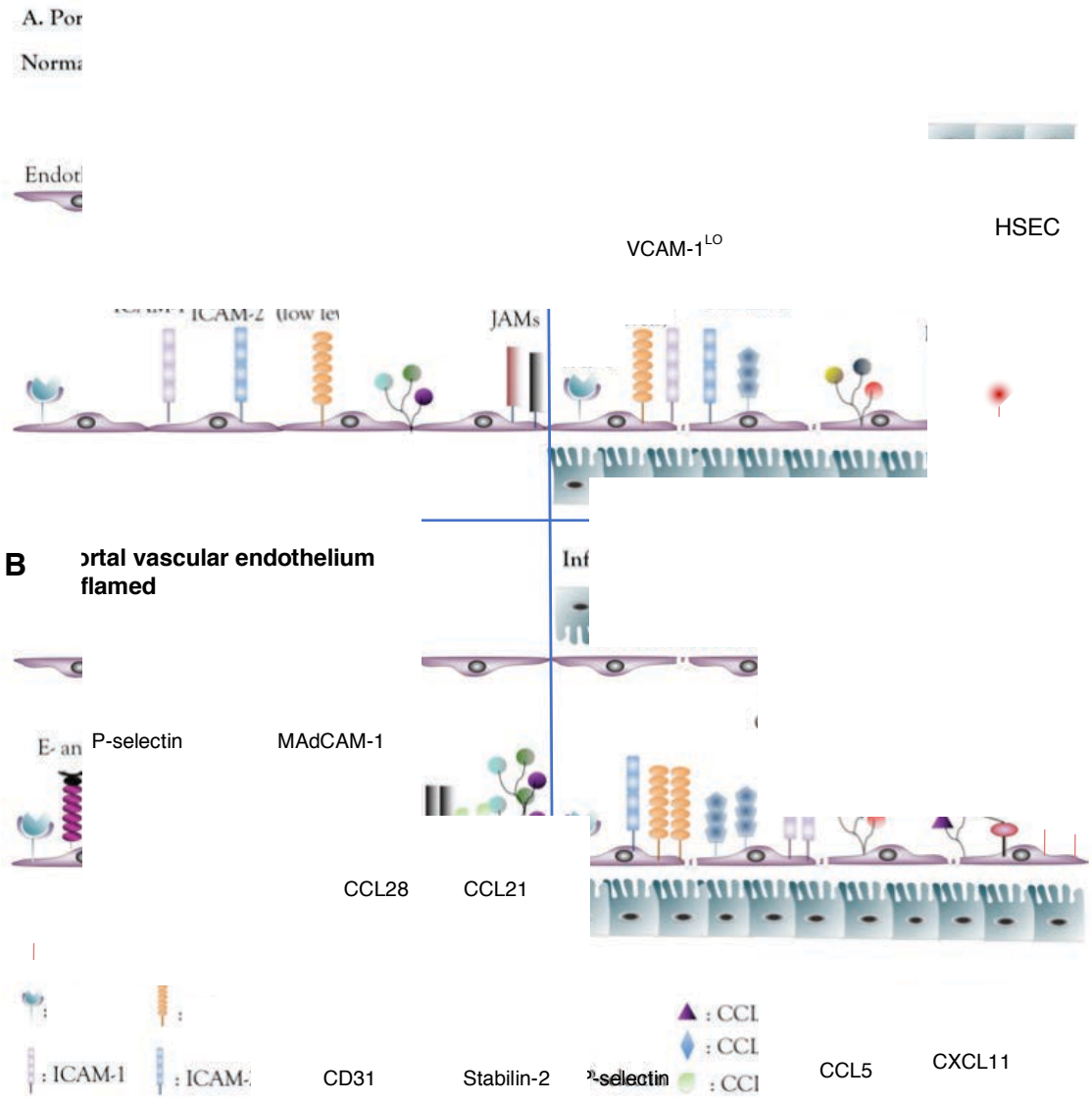


Figure 1.3.3 - Homing molecules in lymphocyte recruitment to the inflamed and non-inflamed human liver. A - Non-inflamed portal vasculature constitutively expresses ICAM-1, ICAM-2, and VCAM-1. VAP-1 is also present alongside junctional adhesional molecules (JAMs) and CD31. Low levels of portal associated cytokines CCL3, CCL4 and CCL5 are also presented at rest. **B -** Following inflammatory signals, upregulation of E-, and P-selectins is observed; as well as CCL25 and MAdCAM-1 (ligands for gut-associated T cell homing receptors CCR9 and integrin α4β7 respectively). CCL28 and CCL21 upregulation can also be observed (CCL21 upregulation has been associated with the formation of portal-associated lymphoid tissue[400]). **C -** HSEC also constitutively express VAP-1, and both ICAM molecules, as well as CLEVER-1 and Stabilin-2. Levels of JAMs and CD31 are low, perhaps owing to the lack of tight junctions between HSEC. HSEC express low levels of CXCR3 ligands (CXCL9-11), and CXCR6 ligand CXCL16; and production of all of these is increased in inflammation, as well as gut-tropic ligands CCL25 and MAdCAM-1(**D**). Image adapted from reference [363], which was itself an adaptation from reference [401].

lymphoid structures that are present in many chronic liver diseases[353, 362, 392]. Last, evidence from an overlap between the gut and liver immune system comes from data showing that the chronically inflamed hepatic endothelium in IBD-associated livers expresses CCL25 and mucosal vascular adressin cell adhesion molecule-1 (MAdCAM-1) that attract T cells expressing the gut-tropic homing molecules CCR9 and integrin $\alpha_4\beta_7$[401-404]. Moreover, in mouse livers, HSEC can directly imprint this gut-tropic phenotype independent of intestinal DCs[405].

A recent study uncovered a novel intrahepatic T cell migration mechanism. Guidotti et al. utilised multiple advanced imaging approaches to suggest that surprisingly, early $CD8^+$ effector T cell trafficking in transgenic mouse livers was independent of α_4 and β_2 integrins, VAP-1, chemokine receptors and antigen recognition. Instead, for many T_{EFF}, arrest depended on docking to endothelium-bound platelet aggregates. This allowed subsequent crawling along the endothelium, probing underlying hepatocytes through fenestrae, and even exerting of cytotoxic effector functions on target hepatocytes, all from within the sinusoids[406, 407]. Although compelling, this study only accounts for the early migration of 50% of $CD8^+$ T_{EFF} cells in mice – finding the others to be independent of all these adhesion mechanisms! Of note, another study found murine naive $CD8^+$ T cells to rely on ICAM-1 - $\alpha_L\beta_2$ integrin interactions for antigen-dependent trapping in the liver[408], indicative of disparate context and cell-type dependent mechanisms. As discussed above, in humans VAP-1, integrin binding, and chemokine receptor expression are all undoubtedly important for the adhesion and migration of many T cell types[356, 360, 385, 388, 390]; but nevertheless, it will be interesting for future studies to determine whether platelet docking can account for a proportion of human liver T cell trafficking phenomena, and whether this can also be applied to $CD4^+$ T cells.

The fate of effector T cells following migration into the parenchyma has been far less studied than requirements for entry. Conceivably, these cells would either apoptose, egress back to the circulation, or remain resident once they have carried out their effector functions. Excitingly, immunologists are beginning to investigate liver resident T cell populations, a topic covered fully in section 5.1.

1.3.5 T cells in Liver health and pathology

Despite the remarkable ability of the liver to cope with most insults, chronic liver injury is a major worldwide healthcare problem. Most commonly, causes include repeated abuse with alcohol or poor diet, hepatotropic viruses, or autoimmune attack. Regardless of underlying cause, progression to chronic liver disease displays the same hallmarks – persistent inflammation that leads to fibrosis, cirrhosis and increased risk of liver cancer[409]. This section will briefly cover roles and phenotypes of $CD4^+$ T cells in each liver disease aetiology used in this study.

1.3.5.1 The Healthy Liver

Data from recent tissue mapping studies investigated T cell phenotypes and function in healthy livers. Of relevance to our work, Wong et al. has demonstrated intrahepatic $CD4^+$ T cells tend towards CXCR3, CXCR4, and CCR7 expression, and like most other tissues, T_H1 cells comprised the biggest population[82]. Although analyses of bulk populations may not reveal distinct expression patterns, donor numbers were low, and portal flush methodology was used instead of tissue digestion; this study still provides a good

introduction to intrahepatic CD4+ T cell phenotypes[82]. Otherwise, data on CD4+ T cells in the human liver is surprisingly scarce. Some studies have gathered limited data on healthy intrahepatic CD4+ T cells as controls for their investigations into disease populations; showing for instance that both T_{REGS}[390, 410, 411], and T_H17 cells[412, 413], are scarce in the healthy human liver. Other studies have investigated CD8+ T cells in the healthy liver in much more depth[148], but it remains to be seen whether liver CD4+ T cells display similar alterations to their better studied CD8+ counterparts.

1.3.5.2 Autoimmune Liver Disease

Autoimmune hepatitis (AIH), PBC, and primary sclerosing cholangitis (PSC) comprise the three main autoimmune liver conditions. AIH is characterised by interface hepatitis and autoimmune-mediated destruction of hepatocytes; while the targeted cells in PBC and PSC are the BEC of small interlobular and medium hepatic bile ducts respectively[414]. The exact aetiology behind these diseases is unknown, suffice to say that all three develop due to complex genetic and environmental interactions. Hepatocyte-derived autoantigens stimulate T cell activation and damage in AIH, possibly through molecular mimicry or failed central tolerance mechanisms; and resultant self-perpetuating multi-cellular immunity ensues[414, 415]. Mitochondrial constituent pyruvate dehydrogenase complex, E2 subunit (PDC-E2) is a major autoantigen in PBC, and forms the basis of one pathway to injury. Modified PDC-E2 can be found on the surface of apoptotic cells. However, apoptotic cholangiocyte PDC-E2 remains unmodified, acting as an immunogenic apotope (apoptotic epitope)[284, 416-418]. This stimulates immune responses in susceptible individuals; leading to further biliary cell destruction, neoantigen generation and increased antigen presentation in the inflamed area[414]. Interestingly, molecular mimicry may also

be at play as anti-PDC-E2 antibodies cross react with several bacterial species including *E. coli*, and a member of the *Sphingomonas* genus; and several commonly used xenobiotics can modify PDC-E2 to increase immunogenicity[414,419-421]. Finally, PSC is thought to develop from excessive responses to microbes, or innocuous antigens that propagates further injury, biliary inflammation and again, neoantigen formation[414]. Importantly, if left untreated, all three diseases progress to liver failure.

T cells form major components of liver autoimmunity. T cells are highly represented in inflammatory infiltrates, and in AIH these are predominantly $CD4^+$ T cells[414]. Typical of autoimmune pathologies, T_H1 responses are believed to predominate, as demonstrated by studies on human blood in AIH and PBC[422;423, 424]; as well as the liver in PBC and PSC[425-427;428]. However pathogenic T_H17 cells may also play key roles. In AIH, preliminary studies have demonstrated expansions of circulating and intrahepatic T_H17 cells and associated cytokines IL-17, IL-23, IL-1β and IL-6. Furthermore, IL-17 stimulated the further release of IL-6 from hepatocytes, positing the idea of positive feedback to generate more T_H17 cells[413]. In PBC, circulating T_H17 cells can be found in greater number[429], and liver-infiltrating IL-17^+ lymphocytes are also increased in abundance[412]. The importance of T_H17 cells in PSC pathogenesis is highlighted by the association of genes involved in T_H17 differentiation and signal transduction with disease in genetic linkage studies[414, 430]. Accordingly, bacteria in portal ducts in PSC has shown to stimulate T_H17 responses from PBMCs *in vitro*[431].

T_{REG} alterations have also been documented in liver autoimmunity, although results have often been contradictory (seemingly largely due to variations in T_{REG} markers used). Depletions in the number and suppressive capacities of T_{REGS} in AIH patient blood have

been documented[432, 433]; but more recent data using a more complete marker set ($FoxP3^{+}CD25^{HI}CD127^{LO}$ versus $CD25^{+}$) argues against defects in either parameter, and actually argues for increased T_{REG} frequencies in active disease[434]. Furthermore, no numerical T_{REG} deficit is observed in AIH livers[390, 435]. Similarly, arguments over the existence of T_{REG} depletion in PBC patient blood continue[410, 411, 429], though it is generally agreed that the frequency of liver-infiltrating T_{REGS} in PBC is significantly lowered[411]. Thankfully, no data yet contradicts the finding that T_{REG} frequency and function is decreased in both liver and blood of PSC patients[436, 437].

Comprehensive characterisation of liver-infiltrating human T cells is required to further identify disease-linked culprit populations and/or pathways relevant to the pathogenesis of these cells. Sadly though, such studies are largely lacking. One in-depth study by Liaskou et al. illustrated a 10-fold enrichment of $CD28^{-}$ $CD4^{+}$ T cells in PSC livers. These cells were predominantly T_{EM}; expressed CXCR6, CX3CR1, and CCR10 that are associated with biliary tract infiltration; and possessed high cytotoxic potential. Interestingly, proinflammatory TNF-α induced $CD4^{+}CD28^{-}$ T cell differentiation *in vitro*, while active vitamin D inhibited it[437]. This may relate to an older study that noted impaired proliferation and cytotoxicity of liver infiltrating lymphocytes in PSC, an effect that too was partially dependent on TNF-α[428]. Similarly, $CD28^{-}$ T cells may be important in PBC too. Isse et al. showed elevated $CD4^{+}CD28^{-}$ T cell numbers in damaged bile ducts in PBC[438]; while Tsuda et al. found an enriched population of $CD28^{-}$ $CD57^{+}$ $CD8^{+}$ T_{EM} that preferentially expressed integrin-$\alpha_4\beta_7$ and secreted increased levels of IFN-γ and IL-5 in PBC patient blood, alongside evidence that $CD8^{+}CD57^{+}$ cells infiltrate into portal areas in the disease[439].

1.3.5.3 Dietary Liver Injury

Alcoholic liver disease (ALD) and non-alcoholic steatohepatitis (NASH) are very common liver diseases in the western world. As their names suggest, these conditions are principally due to chronic excess alcohol consumption, and sustained excess caloric intake respectively (though clearly stratification into one or the other group is not always straightforward). Non-alcoholic fatty liver disease (NAFLD) is commonly used to refer to a less advanced dietary liver injury that is not associated with inflammatory and fibrotic damage.

ALD is an inflammatory disease. Toxic by-products of ethanol metabolism not only cause hepatocyte apoptosis directly, but lead to excess radical formation and related adducts that stimulate inflammation through acting as damage-associated molecular patterns (DAMPs) and/or B and T cell immunogens[440, 441]. Chronic ethanol exposure itself also activates Kupffer and NKT cells, upregulates TLR expressions, promotes hepatocyte proinflammatory cytokine production[442]. Continuing damage often appears to lead to the development of associated autoimmunity, against cytochrome P450 enzymes, or oxidised phospholipids for example[442]. Inflammatory processes also drive the pathogenesis of NASH. In this condition, hepatocyte cell death is caused by lipotoxicity and oxidative injury. Hepatocyte death leads to DAMP formation, stimulation of TLRs, activation of innate immune cells and subsequent adaptive cell recruitment which continues the inflammatory cycle. Pro-inflammatory adipokines from adipose tissue, and bacterial endotoxin leakage into the circulation can both also contribute to this progression[443, 444]. Like ALD, adaptive immune responses against lipid peroxidation by-products are frequently detected in NASH patients[445].

ALD liver infiltrates contain both $CD4^+$ and $CD8^+$ T cells[441]. However, in alcoholic patient blood, one study reported a decrease in all T cell numbers, especially within the $CD8^+$ T cell compartment[446]. TNF-α concentrations are raised in the plasma of alcoholic patients, prompting vague speculation that T_H1 responses are important to pathology[447]. More peripheral blood data shows elevations of activated $CD57^+$ $CD4^+$ T cells in ALD, but these activated cells are seen in many inflammatory conditions[448]. The only study found that investigated intrahepatic T cells in ALD demonstrated a clear increase in T_H17 cells (assumed from $CD3^+$IL-17^+ staining), the frequency of which correlated with patient fibrosis scores[449].

T cells are thought to be involved in the progression to NASH in patients and different mouse models of disease[444, 445, 450-452]. In one mouse model, injury was driven by increased representation of activated T_H1 cells that promoted proinflammatory macrophage activation. Interestingly, both T and B cell responses against a lipid peroxidation adduct were detected[450]. This same adduct is also detected in many NASH patients[450]. A recent study reported significantly fewer intrahepatic $CD4^+$ T cells in mice models of NASH that was driven by cell-type-specific free fatty acid-mediated oxidative processes. They also showed very low numbers of intrahepatic $CD4^+$ T cells in human livers, reduced compared to viral disease, though comparable to healthy controls[453]. In human studies, Inzaugarat et al. documented increases in circulating human $CD4^+$ T cells, specifically in memory $CD4^+$ T cells that were producing IFN-γ[454]. Rau et al. provides data that show human peripheral $CD4^+$ T cells make more IFN-γ too[455]. Data from Boujedidi et al. indicate a disease specific alteration in chemotaxis – showing peripheral $CD4^+$ T cells from NASH patients migrate better to CXCL12 than control subjects[456].

Mouse models heavily implicate T_H17 cells in the pathogenesis of NASH[457-460], whereas T_H22 cells may be protective[458]. Furthermore, IL-17 promotes steatosis and pro-inflammatory IL-6 production from the human hepatocyte cell line HepG2, suggestive of a mechanism[457]. In human livers, elevated levels of IFN-γ[461]; as well increases in IL-17, IL-17-expressing T cells, and other T_H17-related genes have been detected[412, 455, 457, 462]. The study by Rau et al. is the best of its kind to date to phenotype intrahepatic $CD4^+$ T cells in NASH. It concludes they are more activated and more capable of IFN-γ, IL-4, IL-17, and IL-21 production than healthy liver controls, perhaps not a surprising finding given the inflammatory nature of the condition. However, they did demonstrate that progression to NASH correlated with intrahepatic T_H17 frequencies[455], demonstrating early signs of their importance in human NASH pathogenesis[451]. The authors also put forward a decrease in naïve T_{REG} frequency in the blood, although minimal changes were seen in activated/memory T_{REGS}[455]. Other than this study, it is unclear whether T_{REG} phenotype or function is affected in NASH; with studies using mouse models currently conflicted about whether there is a relative reduction of these cells within livers[463], or not[453].

1.3.5.4 Viral Liver Disease

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two most significant hepatotropic viruses; with chronic infection respectively affecting approximately 350, and 170 million people worldwide[464]. HBV infections are usually self-limiting, with 95% of those infected spontaneously clearing the virus. However, HCV infection progresses to chronicity in the majority (~70%)[465]. An efficient prophylactic vaccine only currently exists for HBV infection, although a promising HCV vaccine is currently in phase II clinical trial[464, 466]. Effective treatment options consist of type 1 IFNs for both chronic infections,

nucleoside/nucleotide inhibitors for chronic HBV, and ribivarin and new direct acting antivirals for chronic HCV infection[464, 467].

Many similarities exist in the immunopathology of both diseases. $CD4^+$ and $CD8^+$ T cell responses are vital in the clearance of HBV and HCV[465 468-470], although neutralising antibodies are only currently thought to be critical in controlling HBV infection[464, 471]. T cell responses against both viruses must be robust, sustained, and of broad specificity in order to mediate complete spontaneous clearance[465,464]. Accordingly, progression to chronicity is associated with T cell exhaustion, and substantial clonal deletion in HCV and HBV alike. HCV- and HBV-specific $CD4^+$ T cells from the liver as well as peripheral blood of patients often express high levels of exhaustion markers such as PD-1[472;473], and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3)[474;475]; and substantial impairments in proliferation and cytokine production[476-484;485]. Likewise, similar exhausted phenotypic and functional alterations have been observed on peripheral and intrahepatic $CD8^+$ T cells in patients with chronic HCV[351, 474, 486-490], as well as chronic HBV infection[473, 475, 491-498]. The better studied exhausted antiviral $CD8^+$ populations can also present with high levels of KLRG-1, 2B4, and CTLA-4; and low expression of CD127[192, 488, 489, 494;493, 499-501]. Notably, the most exhausted cells in both patient groups present at an intermediate ($CD27^+$ T_{EM}) and not late (T_{EMRA}) differentiation stage[488;499]. Partial reversal of the exhaustion phenotype has been observed following blockade of one or more of these inhibitory receptors *in vitro*[351, 489, 494, 502-504;473, 475, 485]. However, even blocking multiple inhibitory receptors did not have any effect on some patient T cells[505], and PD-1 blockade has so far had limited therapeutic efficacy in patients with chronic HCV[352].

The reasons behind the weak T cell responses in chronic HBV and HCV can be blamed on the interlinked events of viral persistence, lack of sufficient $CD4^+$ T cell help, and excess immunoregulation[465]. In addition, viral escape is thought to play a big role in subverting T cell responses in HCV[464, 465]. Interestingly, progression to chronicity in HBV is thought to be worsened when antigen-specific $CD8^+$ T cell responses are inadequate to control infection, and so ongoing inflammatory processes recruit non-specific 'bystander' T cells that cause inflammatory damage without the benefit of specifically targeting infected cells[496, 506]. T_H1 responses are correlate with clearance in both diseases[482, 507;491, 508, 509], while conflicting data on T_H17 responses means their exact role in clearance is still unclear[504, 510-513;514, 515]. A recent study has also implicated inducible T-cell costimulator $(ICOS)^+$ T_{FH} cells in the clearance of HCV[516]. By contrast, increases in numbers and regulatory capacity of T_{REGS} has been documented in the blood and liver of HCV patients[504, 517-522]. Analogously, the field is in agreement that T_{REG} numbers are increased in chronic HBV blood, but some dispute whether their ability to inhibit effector T cells is affected[523-525]; although intrahepatic T_{REGS} are thought to be both increased in numbers and function[526]. Additionally, granulocytic MDSCs have associated with preventing robust anti-HBV T-cell responses through an L-arginine depletion mechanism[323], whereas NK cells have been shown to directly kill antiviral intrahepatic $CD8^+$ T cells in chronic HBV[328]. Finally, a role for MDSCs in promoting HCV persistence has also been hinted at, with MDSCs suppressing $CD8^+$ T cells through the production of reactive oxygen species[527].

As mentioned above, dissecting immune responses within the human liver is key to understanding and altering the disease course. Alongside the studies discussed, recent

work involving resident T cell subsets in HBV infection has deepened our knowledge on the contribution of this crucial subset to pathogenesis[148, 149] (see 5.1).

1.3.5.5 Other Liver Diseases

The sections above cover the vast majority of the liver aetiologies used in this study. The remaining samples were acquired from patients with polycystic liver disease (PLD), non-cirrhotic portal hypertension (NCPH) Budd-Chiari syndrome (BCS), and cryptogenic liver disease. One sample came from a sufferer of secondary biliary cirrhosis (SBC), while the majority of control peripheral blood used was obtained from patients with haemochromatosis (HFE).

PLD is a genetic disorder culminating in the growth of cysts throughout the liver, which can infrequently obstruct portal or hepatic vein blood flow leading to liver failure[528]. NCPH is a broad spectrum of diseases encompassing any cirrhosis-independent process that substantially increases portal venous blood pressure. Such factors include prothrombotic conditions, infections and immune disorders, or exposure to certain medications[529]. BCS is a form of NCPH where hepatic venous outflow is specifically obstructed[530]. SBC on the other hand, describes bile duct obstruction that is not caused by autoimmune attack of BEC. HFE is another genetic disorder, this time affecting the regulation of iron levels in the bloodstream. Sufferers absorb, and deposit excess iron within hepatocytes, which can lead to liver damage and eventual cirrhosis[531]. Lastly, as the name suggests, cryptogenic liver disease is defined by unknown aetiology.

The broad (or unknown) nature of most of these diseases makes immunology research largely inane. There does not appear to be any studies on altered T cell responses in PLD, but some differences within HFE patients do occur. Relatively low numbers of $CD8^+$ T cells in blood and liver have been reported, but most studies show that $CD4^+$ T cell numbers are unaffected[532-536].

1.3.6 Tissue Resident Memory cells in the Liver

Like many other organs, T_{RM} have also been identified in the human liver[82, 148, 149], and have already been shown to be important in HBV pathogenesis[148]. A full and detailed discussion on the current knowledge on liver T_{RM} is covered in section 5.1, and so will not be repeated here.

1.4 Investigation aims and hypotheses

Given the immunological properties of the liver in health, and the need to further identify relevant populations in liver pathology; the liver is an attractive organ for further study. From the above discussion, it is evident that there are a number of gaps in our understanding. First and foremost, $CD4^+$ T cells, a population of such importance to global immunity, are drastically understudied in the human liver. Second, while the migration of T cells into the liver has been well characterised; the consequences once they reach the parenchyma are still debated. How long do cells stay, and what characteristics and new abilities might the hepatocytes imprint them with? This links us nicely to the immensely important T_{RM} population. The paucity of $CD4^+$ T cell knowledge extends to the T_{RM} field, and especially the hepatic compartment where no study has yet

characterised substantial CD4+ T_{RM} populations[82]. Relatively simple questions concerning CD4+ T_{RM} remain, such as what role are these cells playing in different human pathologies? How long can these cells remain in tissues? How are these cells generated? Is there a spectrum of cells with different dwell times? Sufficient access to human tissue samples has thwarted the answering of many of these questions, and limited progress in understanding the role of intrahepatic T_{RM} in human disease besides.

In these studies, I attempted to answer many of these questions by investigating the phenotypes and functions of CD4+ T cells at three key interaction points within the liver. First the migration of peripheral-blood derived CD4+ T cells into the hepatic parenchyma was modelled (chapter 3). This allowed the consequences of parenchymal, as opposed to endothelial, migration to be documented; and crucially offers a way to study disease-relevant CD4+ T cells that would migrate into the liver in autoimmune and viral liver diseases without using liver tissue. Second, the fate of CD4+ T cells following short term *in vitro* contact with hepatocyte cell lines was investigated (chapter 4). These experiments illustrated the impact hepatocytes could have on these cells, and hinted at what roles the T cells could be taking up. These studies both link to the investigation into a human hepatic CD4+ T_{RM} type (chapter 5). Using both healthy liver samples, and liver samples from multiple different aetiologies allows for comparison of any findings across disease groups, with the potential to highlight alterations in disease. Furthermore, collating data with the *in vitro* hepatocyte co-culture work may provide answers on the generation, and longevity properties of hepatic CD4+ T_{RM}. Finally, the identification and in-depth characterisation of these cells will allow for their placement in the ever-expanding matrix of human T_{RM} from different tissues.

Chapter 2 – Materials and Methods

2.1 Isolation of PBMCs and PBMC-derived CD4+ T cells

2.1.1 Isolation of Peripheral blood mononuclear cells

Venous blood samples were obtained with written informed consent from patients with haemochromatosis (HFE), HCV, or PBC; while matched liver patient peripheral blood was taken shortly before liver transplant surgery. All samples obtained from the Liver unit at the Queen Elizabeth Hospital, Birmingham, UK (South Birmingham ethical approval reference 06/Q2708/11). HFE blood was considered healthy for the purposes of this study. PBMCs were isolated from whole blood by standard density centrifugation: 35mls whole blood was carefully layered on 15mls Lympholyte-H media (Cederlane, Canada), and centrifuged for 20 minutes (270 × *g*, brake 1). PBMCs were then harvested from the central layers (between plasma and Lympholyte), washed (centrifuged for 5 minutes, 270 × *g* , full brake; supernatant removed, resuspended in new solution) twice in phosphate-buffered saline (PBS - Gibco, UK; or in-house) and counted using a haemocytometer. For large volumes (<140mls) of starting whole blood, a prior 20-minute centrifugation step was carried out (270 × *g* , brake 1), the buffy coats harvested, diluted in PBS, and this was then layered on Lympholyte.

2.1.2 CD4+ T cell isolation

CD4+ T cells were isolated from blood derived PBMCs using the CD4+ EasySep™ human CD4+ T cell enrichment kit (Stemcell Technologies, UK). This involved resuspending PBMCs in T cell isolation media (Table 2.1) at 5 x 10^7 cells/ml in a 15ml Falcon tube, then adding 50μl/ml of antibody cocktail for 10 minutes, followed by 100μl/ml vortexed magnetic particles for a further 5 minutes. The magnetic particles bind to the antibodies

that themselves bind to unwanted immune cells and erythrocytes. After topping up the tube to 10ml with T cell isolation media (or 5ml for <1 x 10^8 starting PBMCs) the tube was placed into an EasySep™ magnet ('The Big Easy', Stemcell Technologies, UK) to allow the withholding of unwanted cells. Desired cells were then poured off into a new tube, washed and counted. $CD4^+$ purity was consistently over 98% as tested with anti- CD4 staining and flow cytometry (Fig. 2.1).

Naïve and Memory $CD4^+$ T cells were isolated using EasySep™ human naïve $CD4^+$ T cell enrichment, and EasySep™ human memory $CD4^+$ T cell enrichment kits respectively (both Stemcell Technologies, UK), according to manufacturer's instructions. Naïve T cell isolation required a prior 15 minute incubation step with anti-CD45RO antibodies, while the antibody cocktail in the memory $CD4^+$ T cell isolation kit contains anti-CD45RA antibodies that remove unwanted naïve T cells. The rest of the protocol is principally the same as for total $CD4^+$ T cell isolation described above.

2.2 Cell culture assays

2.2.1 Simple culture

Hepatic epithelial cell lines (Huh-7, HepG2, Hep3B) and non-hepatic epithelial cell lines (A549, AdAh, Caco-2, HeLa) were cultured in complete DMEM; LX2 cells were cultured in DMEM – 2% FBS; primary BEC in BEC media; and primary HSEC in HSEC media (see Table 2.1 for all media details). Huh-7, HepG2, Hep3B, HeLa; AdAh; A549; and Caco-2 were kind gifts from Prof. Jane McKeating; Dr Claire Shannon-Lowe; Dr Dalan

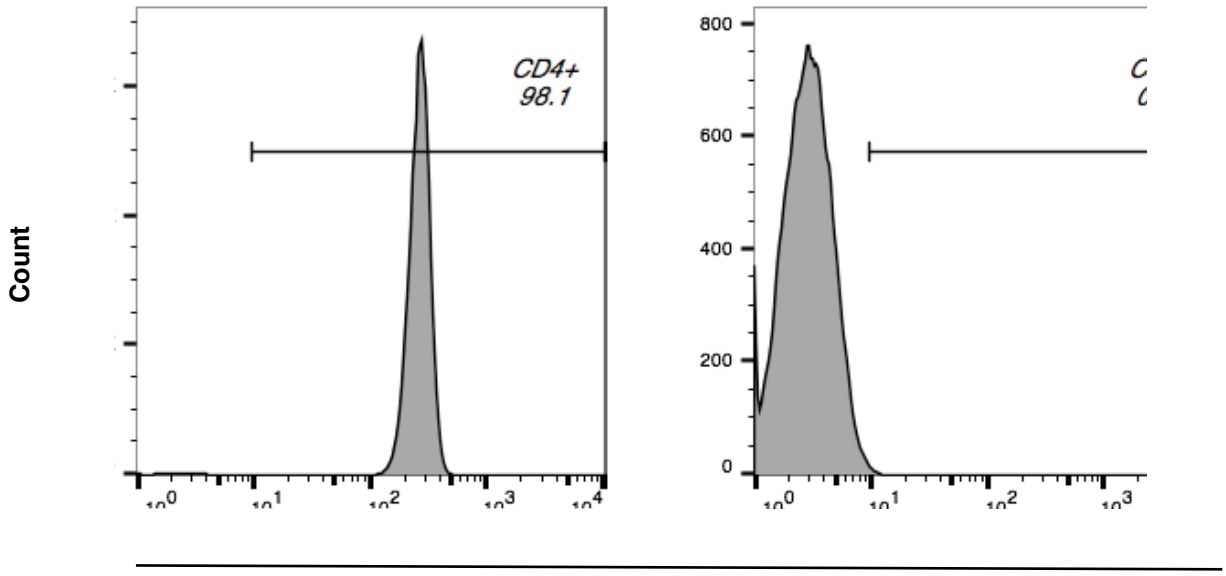


Figure 2.1 - CD4+ T cell purity following isolation from PBMC. Demonstration of highly pure CD4+ population by surface staining and flow cytometry following isolation of PBMC-derived $CD4^+$ T cells. Histogram is representative of 3 independent experiments.

Bailey; and Dr Emma Shepherd (all University of Birmingham) respectively. All cell lines were routinely mycoplasma tested every 3 months. Pre-experimentation, all cell types were cultured in flasks, and these were coated with 40μg/ml type-1 rat-tail collagen solution (Sigma Aldrich, UK) for HSEC and BEC cell culture. Primary BEC and HSEC were kindly isolated and provided by Gill Muirhead, University of Birmingham. Briefly, this involved collagenase digestion and harvest of non-parenchymal cells through density centrifugation, followed by immunomagnetic isolation using epithelial glycoprotein HEA-125 and CD31 for BEC and HSEC respectively[371, 388, 537]. Previous work has shown BEC and HSEC purity to be >95% and >85% respectively[538, 539], and both were used within 5 passages. All cell culture incubation was at 37°C, 5% CO_2.

Adherent cells were harvested by trypsinisation (with 0.05% Trypsin-EDTA solution – ThermoFisher Scientific, UK). Cells were then resuspended in the specified media, and plated out in 24-well plates (1ml cell suspension/well) in order to achieve 100% confluence. Seeding density was found using surface area calculations and the following formula:

$$Volume\ of\ suspension\ for\ 1\ well\ coculture = \frac{200mm^2}{7500mm^2} \times 5000\mu l \times \left(\frac{1}{x/100}\right)$$

Where x= % starting confluence of cell line. This formula assumes that cells begin in T75 flasks (surface area of 7500mm^2), are resuspended in 5mls media and are being plated into 24-well plates (200mm^2 surface area/well). Other flask/well sizes were adjusted for accordingly.

Media	Details
T cell isolation media	PBS + 2% FBS (Sigma Aldrich, UK), 1mM EDTA (ThermoFisher Scientific, UK)
Complete DMEM	DMEM (ThermoFisher Scientific, UK) + 10% FBS, 100U/ml Penicillin, 100μg/ml streptomycin, 1% L-Glutamine, 1% NEAA (all four ThermoFisher Scientific, UK).
DMEM – 2% FBS	DMEM + 2% FBS, 100U/ml Penicillin, 100μg/ml streptomycin, 1% L-Glutamine, 1% non-essential amino acids.
BEC media	1:1 Ham's F12 media (ThermoFisher Scientific, UK) & DMEM + 10% heat-inactivated human serum (HD supplies, UK), 2mM Penicillin/Streptomycin, 10μg/L EGF, 10μg/L HGF (both Peprotech, UK), 124IU/L Insulin, 20μg/L Hydrocortisone (both Queen Elizabeth Hospital Pharmacy, Birmingham, UK), 10μg/L Cholera Toxin, 0.2nM Tri-iodothyronine (both Sigma Aldrich, UK).
HSEC media	Human Endothelial Serum-free media (ThermoFisher Scientific, UK) + 10% heat-inactivated human serum, 2mM Penicillin/Streptomycin 10μg/L HGF, 10μg/L VEGF (Peprotech, UK).
Complete RPMI	RPMI (ThermoFisher Scientific, UK) + 10% FBS, 100U/ml Penicillin, 100μg/ml streptomycin, 1% L-Glutamine, 1% NEAA
FACS Buffer	PBS + 1% FBS, 2mM EDTA, 0.01 NaN_3 (Sigma Aldrich, UK)

Table 2.1 - List of media used. Media types listed in order of appearance in text. Companies listed only first time reagent is used. DMEM - Dulbecco's Modified Eagle Medium; EDTA - Ethylenediaminetetraacetic acid; EGF - Epidermal growth factor; FBS – Foetal bovine serum; HGF – hepatocyte growth factor; NaN_3 – sodium azide; NEAA – Non-essential amino acids; PBS – phosphate buffered saline; RPMI – Roswell Park Memorial Institute (media); VEGF – Vascular endothelial cell growth factor.

In the case of mixed liver cell cultures (Fig. 4.1.6), each cells' media made an equal contribution to the overall solution. Once confluence was reached, the required number of isolated $CD4^+$ T cells were carefully added to the wells in 100μl complete RPMI (Table 2.1). Following culture period of choice, $CD4^+$ T cells were harvested gently to minimise non-immune cell disturbances, and used in downstream applications. Included T cell only controls started with 1ml complete DMEM, before T cells (in complete RPMI) were added.

2.2.2 Simple culture variants

2.2.2.1 T cell stimulation and proliferation assays

Three different T cell stimulants were used. Anti CD3/CD28 stimulation beads (Dynabeads™ Human T-activator CD3/CD28 – ThermoFisher Scientific, UK) were washed and prepared per manufacturer's instructions, and added to T cells at a concentration of 25μl beads/10^6 cells to give a 1:1 cell-to-bead ratio. Protein Kinase C (PKC) activator Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore Ionomycin were used together at concentrations of 50ng/ml and 1μM respectively (both Sigma Aldrich, UK). T cell mitogen Phytohaemagglutinin-M (PHA-M) was added to cells at 50μg/ml (Sigma Aldrich, UK).

Monitoring cytokine production involved the culturing of T cells with the above stimulants in the presence of protein transport inhibitor Brefeldin A (10μg/ml; Sigma Aldrich, UK). T cells were resuspended in complete RPMI containing the stimulants and Brefeldin A for the specified time period (37°C, 5% CO_2), before the stimulants washed off and intracellular staining and flow cytometry performed (see section 2.5).

Assessment of proliferation in response to these stimuli was done using CellTraceViolet™ proliferation dye (ThermoFisher Scientific, UK). Prior to the culture/co-culture period, T cells were first washed three times in PBS, then suspended at 1 x 10^7 cells/ml in 5μM CellTraceViolet solution (diluted in PBS) for 20 minutes in the dark. Dye was quenched in serum-containing complete RPMI for 5 minutes, cells were washed twice more, and then used in downstream applications.

Additionally, cytokine stimulations were performed (Fig. 3.1.5). T cells were cultured in complete RPMI containing either the pro-inflammatory cocktail (IL-1β, IL-6, IL-22, TNF-α, IFN-γ - all Peprotech, UK), or each of its constituents at a cell concentration of 1 x 10^7

cells/ml for 24 hours before adding into downstream assays. All cytokines used at a concentration of 10ng/ml.

2.2.2.2 Assessment of T-cell contact dependence

Where the influence of direct T cell-hepatocyte contact on CD69 upregulation was under investigation (Fig. 4.3.3), 0.4μm pore transwell inserts (Scientific Lab Supplies, UK) were used. At this pore size, it is not possible for T cells to transmigrate. Huh-7 cells were simply plated out in the bottom of wells as described, allowed to settle and then such transwell inserts were added to the wells along with 150μl complete DMEM on top. $CD4^+$ T cells were then added to the top of the inserts, in 100μl complete RPMI. After overnight co-culture, T cells were harvested, analysed, and compared with standard co-culture conditions.

2.2.2.3 Huh-7 fixation

For Huh-7 fixation (Fig. 4.3.4) three different fixatives were used as follows. For both 100% methanol and 1% formaldehyde (both Sigma Aldrich, UK), media was first removed from the confluent Huh-7 cells, the cells were carefully washed twice with PBS. The fixative was added to the confluent Huh-7 monolayers (200μl/well) for 20 minutes, washed off 4 times with PBS and complete DMEM restored, before T cells were added. For 0.025% glutaraldehyde (Sigma Aldrich, UK) fixation, the Huh-7 cells were first trypsinised, washed in PBS and resuspended in 1ml fixative for 3 minutes. Cells were thoroughly washed (2x centrifugation wash) and plated into new 24 well plates in complete DMEM before T cells were added.

2.2.2.4 Modulating ICAM-1 – LFA-1 interactions

When assessing the influence of ICAM-1 or LFA-1 on CD69 upregulation (Fig. 4.3.5), the following protocols were used. To block ICAM-1 on the Huh-7 cells, anti-ICAM-1 (clone BBIG-I1, R&D Systems, UK) was added to each well of the confluent Huh-7 cells at 10μg/ml in 200μl complete DMEM for 20 minutes, washed off three times with PBS, and the media restored. Blocking or stimulating LFA-1 components on the T cells involved a 20-minute pre-treatment step in 200μl complete RPMI (per million T cells) before washing twice with PBS and adding to the Huh-7 cells. Blocking CD18 – clone 7E4, 10μg/ml (Beckmann Coulter, UK); stimulating CD18 – clone MEM-48, 5μg/ml (Abcam, UK); stimulating CD11a – clone MEM-83, 5μg/ml (Abcam, UK). Recombinant human ICAM-1 (ThermoFisher, UK) was added at 12.5μg/ml (in 200μl distilled water) to wells of a 48-well plate for an hour to coat, before washing 3 times and adding T cells once the plate was dry. Isotype controls for these experiments were mouse IgG1 (Biolegend, UK & ThermoFisher Scientific, UK).

2.2.3 Migration assays

2.2.3.1 Transwell migration assays

In addition to co-culture conditions described above, cell lines were seeded onto the 3μm pore, high density transwell inserts (Scientific Lab Supplies, UK) placed in complete DMEM-containing 24-well plates (1ml/well), at the same density as static co-culture wells (150μl/insert). Once 100% confluence was reached, 1 x 10^6 $CD4^+$ T cells were added to

each insert (100μl/insert) and plates cultured for specified time period. Cells were gently harvested from the top of the transwell insert (non-migrated), the insert discarded, and then harvested from the bottom of the well (migrated). T cells were then used in downstream applications. If manual cell counts were required (Fig. 3.3.1), small aliquots of the migrated cells were taken, diluted 1:1 with dead cell marker Trypan Blue (Sigma Aldrich, UK), and counted with a haemocytometer. Migrated cell counts were compared to counts from aliquots of the corresponding T cell only controls. This allowed for calculation of % migration when compared to the input population size. When inserts and/or wells were coated with type-1 rat tail collagen (Fig. 3.1.4), this was done using a 40μg/ml solution that was added for a few seconds then removed, before plates were allowed to dry prior to the assay(s). If dual hepatocyte monolayers were required (Fig. 4.3.2), transwell inserts were inverted, 150μl cell line solution added to the top, inserts cultured for 3-4 hours (or until cell adherence), and then the insert turned over and placed in complete DMEM in 24-well plates. The second (now top) layer could then be seeded as normal in the well of the transwell insert.

2.2.3.2 Gel culture and 'Trans-gel' migration assays

Each ml of collagen gel was formed by mixing 830μl rat-tail type 1 collagen dissolved in acetic acid (2.15mg/ml; First Link Ltd, UK), with 160μl FBS and 170μl 10x media 199 (ThermoFisher Scientific, UK). The pH of the solution was neutralised with careful addition of 1N NaOH (Sigma Aldrich, UK). All reagents were kept ice-cold to allow an even solidifying of the gel. 300μl gel was carefully seeded per well and incubated (37°C, 5% CO_2) for 15-30 mins, or until set. Huh-7 cells above gels were simply seeded on top of set gel. Huh-7 cells below gel were seeded first (in only 200μl completed DMEM) then

gel was set on top and 800μl complete DMEM added (drip wise). For the Huh-7 within gel condition, gel was made and equilibrated without FBS component, then the T cells were incorporated within the FBS fraction. Gel could then be set in the incubator and complete DMEM added on top.

For 'trans-gel' assays, T cells were added to Huh-7 cells above gels overnight. Non-migrated cells were harvested carefully from the gel top after the addition of 500μl warm EDTA for 2 minutes. Migrated cells were harvested after a gel digest with 300μl/well 2x collagenase (Sigma Aldrich, UK).

2.3.4 Suppression assays

Suppression assays were based around established methodology[540]. Briefly, candidate suppressor cells were cultured with CellTraceViolet™-labelled responder T cells (Tresp) in flat-bottom 96-well plates, at different ratios with anti-CD3/CD28 stimulation beads (25μl/million cells), 1μg/ml anti-CD3 and anti-CD28 antibodies (both ThermoFisher Scientific, UK), or anti-CD3 and anti-CD28 antibody tetramers (Immunocult™ Human CD3/CD28 T cell Activator, Stemcell Technologies, UK; 25μl/million T cells). Candidate suppressor T cells were generated either from overnight Huh-7 co-culture (hTCs), or from overnight culture alone (tTCs). TGF-β was used as a positive control (1μg/ml; Peprotech, UK). For percentage suppression and cell surface phenotype outputs, cells were cultured together for 5 days; for cytokine analyses, cells were cultured together overnight. Percentage suppression, as determined by flow cytometric analysis, was calculated as follows:

$$\frac{\%\ divison\ (Tresp\ alone) - \%\ division\ (Tresp\ with\ hTC)}{\%\ division\ (Tresp\ alone)} \times 100$$

A diagram of this set up, along with explanations of both standard and constant suppression assays can be found in Fig. 4.2.1 and section 4.3.

2.3 Isolation of immune cells from liver and lymph nodes

Donor liver tissue was obtained from the Queen Elizabeth Hospital, Birmingham, UK with written informed consent (South Birmingham ethical approval reference 06/Q2702/61). Liver tissue was taken from various end-stage liver disease patients, or 'healthy' donors that were unsuitable/not used in time for transplantation (see Table 2.2 for breakdown). Lymph nodes were identified and excised from healthy donor tissue.

	N=	% FEMALE	MEDIAN AGE (IQR)
DONOR	8	29*	51** (19.5)
ALD	20	15	59 (14.25)
NASH	8	50	60.5 (9.3)
PBC	7	71	44 (11.5)
PSC	12	25	35.5 (12.3)
AIH	1	0	21 (N/A)
HCV	2	0	59 (6)
HBV	1	0	42 (N/A)
CRYPTOGENIC	2	50	53.5 (10.5)
BCS	1	0	31 (N/A)
PLD	3	100	61 (7)
NCPH	1	0	63 (N/A)
SBC	1	100	58 (N/A)

Table 2.2 – Liver donor patient information. Numbers of livers of each disease group used, along with gender and age summaries. Grey text indicates donor numbers <6. Matched lymph nodes (n=3) all taken from healthy donors. Matched blood (n=6): 2 ALD, 2 NASH, 1 PLD, 1 Cryptogenic. AIH, Autoimmune Hepatitis; ALD, Alcoholic Liver Disease; BCS, Budd-Chiari syndrome; CRYPTOGENIC, Cryptogenic Liver Disease; DONOR – Healthy Donor Liver; HBV, Hepatitis B; HCV, Hepatitis C; NASH, Non-Alcoholic Steatohepatitis; NCPH, Non-Cirrhotic Portal Hypertension; PBC, Primary Biliary Cholangitis; PLD, Polycystic Liver Disease; PSC, Primary Sclerosing Cholangitis; SBC, Secondary Biliary Cholangitis. * One patient sex information missing so data compiled from the remaining 7 donors. ** One patient age missing so data compiled from the remaining 7 donors.

Liver-infiltrating mononuclear cells (LIMCs) were isolated by mechanical digestion and density centrifugation. Liver tissue was finely chopped then rinsed with ice-cold PBS until the supernatant ran clear to minimise blood cell contamination. Tissue was macerated further using a stomacher machine (Seward, UK) for 6 minutes at 260rpm. Tissue homogenate was ground through a fine mesh and transferred to 50ml falcon tubes. Liver samples were washed multiple times until the supernatant became clear (an optional extra 10-minute, 55 × *g* centrifugation step (brake 0) followed by harvest of the supernatant was used for particularly debris-rich livers). The samples were then layered on Lympholyte-H media, and then centrifuged (20 mins, 270 × *g*, brake 1), harvested and counted as described for PBMCs above.

To isolate lymph node mononuclear cells, lymph nodes were first roughly chopped and then transferred to gentleMACS™ C tubes (Miltenyi Biotec, UK) with 10mls non-supplemented RPMI. Homogenisation was carried out using a gentleMACS™ dissociator (Miltenyi Biotec, UK). Homogenate was then pushed through a fine mesh, washed and cells isolated by density centrifugation as for LIMCs.

2.4 LIMC cultures and liver slice methodology

For analysis of cytokine production by different CD69-expressing subsets (section 5.6), LIMCs were first stained for CD69, CD4, CD56, and γδ-TCR (see Table 2.3 for list of antibodies and section 2.5 for antibody staining protocols), before 3 washes and stimulation with PMA & Ionomycin, anti-CD3/CD28 beads, or PHA-M for 2 or 5 hours (concentrations as in section 2.2.2.1), all in the presence of protein transport inhibitor Brefeldin A. Cytokine production was measured by intracellular staining and flow cytometry.

Liver slices used in section 2.6 were cut to a thickness of 2mm with a TruSlice tissue slicer (CellPath, UK). These uniform pieces were then placed into 48-well plates in 500μl DMEM – 2% FBS per well. Autologous PBMCs were added on top in complete RPMI (100μl/well), cultures incubated for 5 hours, and then the PBMCs harvested for downstream analysis.

2.5 Flow cytometric analyses

2.5.1 Surface staining

Following 2 PBS washes to remove media, cells to be stained were incubated (room temperature - RT, 20 mins, dark) with antibodies of interest or isotype-matched controls (IMCs) in FACS buffer (Table 2.1). 50μl of antibody solution was added per 1-2 million cells of interest. Cells were then washed twice in FACS buffer (centrifuged 270 × *g*, 5 mins), and resuspended in 300μl/tube 1% formaldehyde solution (Sigma Aldrich, UK). Samples were either then run directly or stored for up to 7 days at 4°C before running on the flow cytometer. Antibody dilutions were all determined individually through titration experiments. All antibodies used, their fluorochromes, dilutions and IMCs are listed in Table 2.3.

Compensation beads (OneComp eBeads™; ThermoFisher Scientific, UK) were used to allow correction for spectral overlap in multicolour experiments. 1 drop of beads was added to each tube containing 100μl FACS buffer. One matched antibody of each colour (fluorochrome) was added to each tube at the same test concentration as added to the cells. Incubation was for 20 mins (RT, dark), then beads were washed twice and

resuspended in 600μl PBS ready for running on the cytometer. Beads could be pre-made and used for experiments for up to 2 weeks.

If live/dead staining was required, this was incorporated into the staining protocol immediately before surface antibody addition. Following PBS washes, live/dead dyes Zombie Aqua, or Zombie NIR (1/400 and 1/1000 dilutions in PBS respectively; both Biolegend, UK) were added at 50μl per million cells for 10 minutes at RT in the dark. Antibody solutions were then added directly to the cells without the need for washing.

Last, counting beads (AccuCheck counting beads; ThermoFisher Scientific, UK) were used in section 3.4 according to manufacturer's instructions. 5μl thoroughly resuspended beads were added to a 300μl sample before acquisition. The bead population was gated on and used to calculate the absolute cell number in the beginning sample as follows:

$$No.of\ cells\ in\ sample = \frac{No.of\ beads\ added}{No.of\ beads\ counted} \times No.of\ cells\ in\ gate\ of\ interest$$

The number of beads added was calculated by multiplying the number of beads/μl (from each lot's certificate of analysis [CoA]) by 5 (as 5μl added/sample). Accuracy was verified by internal control – two bead populations were separated by fluorescence and the proportion of each matched to the CoA before using generated counts.

2.5.2 Cytokine & Intracellular staining

Following surface staining and fixation with 1% formaldehyde or 3% paraformaldehyde (15 mins, RT; Sigma Aldrich, UK), or cell stimulation for cytokine detection and fixation;

cells were resuspended in 0.1% saponin (Sigma Aldrich, UK) for 10 minutes. After one wash in 0.1% saponin (270g, 5 mins), cells were resuspended in intracellular antibody cocktails (or IMCs) in 0.1% saponin solution for 30 minutes, before 2 final washes and resuspension in 300μl/tube FACS buffer. All antibodies used listed in Table 2.3.

For Hobit and FoxP3 staining, we instead used the FoxP3 Transcription factor staining set (ThermoFisher Scientific, UK). Hobit staining involved fixation with the fixation/permeabilisation solution (50μl/tube) for 30 minutes on ice, centrifugation and removal of the supernatant (270g, 5 mins) and addition of the anti-Hobit antibody (see Table 2.3) diluted in the permeabilisation component (50μl/tube) for 30 mins (on ice, kept dark). Next, cells were washed twice with permeabilisation buffer (500μl each) and the fluorescently-labelled secondary anti-human IgM antibody was added in permeabilisation buffer (30 mins, ice, dark). After 1 final wash, cells were resuspended in FACS buffer ready for analysis. For FoxP3 staining the same protocol was followed, but without need for a secondary antibody step.

2.5.3 Flow cytometry

Samples were acquired with a 9-colour ADP CyAnTM flow cytometer running Summit v4.3 software (both Beckmann Coulter, UK). Offline analyses (compensation, gating, generation of plots and population statistics) were carried out with FlowJo v.10 (Flowo LLC, USA).

Antigen	Fluorochrome	Dilution	Company (Ref.)	IMC
CCR10	PE	1/50	Biolegend (341504)	A-Hm IgG (Biolegend 400908)
CCR5	PE	1/50	Biolegend (359106)	Rt IgG2b (Biolegend 4006363)
CCR6	AF488	1/50	Biolegend (353414)	Ms IgG2b (Biolegend 400329)
CCR7	PE-Cy7	1/50	Biolegend (353226)	Ms IgG2a (Biolegend 400232)
CCR9	PerCP-Cy5.5	1/20	Biolegend (358906)	Ms IgG2a (Biolegend 400252)
CD103	APC	1/40	BD Biosciences (563883)	Ms IgG1 (BD Biosciences 550854)
CD127	BV510	1/40	Biolegend (351332)	Ms IgG1 (Biolegend 400172)
CD25	PE	1/40	BD Biosciences (555432)	Ms IgG1 (Biolegend 400112)
CD25	PE-Cy5	1/50	Biolegend (302608)	Ms IgG1 (Biolegend 400118)
CD27	FITC	1/50	Biolegend (356404)	Ms IgG1 (Biolegend 400108)
CD27	APC-Vio770	1/25	Miltenyi Biotec (130-098-605)	Ms IgG1 (Miltenyi Biotec 130-096-653)
CD28	PE	1/50	Biolegend (302908)	Ms IgG1 (Biolegend 400112)
CD3	APC Fire750	1/200	Biolegend (300470)	Not required
CD3	BV510	1/100	Biolegend (317332)	Ms IgG2a (Biolegend 400267)
CD38	APC-Vio770	1/100	Miltenyi Biotec (130-099-151)	Ms IgG2a (Miltenyi Biotec 130-096-638)
CD4	APC	1/100	BD Biosciences (555349)	Ms IgG1 (Biolegend 400120)
CD4	BV510	1/100	Biolegend (344634)	Ms IgG1 (Biolegend 400172)
CD4	FITC	1/200	Biolegend (317408)	Ms IgG2b Biolegnd 400310)
CD45RA	BV421	1/50	BD Biosciences (562885)	Ms IgG2b (BD Biosciences 562748)
CD45RA	V450	1/50	BD Biosciences (560363)	Ms IgG2b (BD Biosciences 560374)
CD49a	PE	1/50	Biolegend (328304)	Ms IgG1 (Biolegend 400112)
CD49d	BV421	1/50	Biolegend (304322)	Ms IgG1 (Biolegend 400158)
CD56	APC-Vio770	1/50	Miltenyi Biotec (130-100-690)	Ms IgG1 (Miltenyi Biotec 130-104-618)
CD69	APC	1/50	Biolegend (310910)	Ms IgG1 (Biolegend 400120)
CD69	FITC	1/25	Biolegend (310904)	Ms IgG1 (Biolegend 400108)
CD69	PE-Dazzle594	1/50	Biolegend (310942)	Ms IgG1 (Biolegend 400176)
CD8	PE-Cy5	1/200	Biolegend (301010)	Ms IgG1 (Biolegend 400118)
CD80	APC	1/400	Biolegend (305220)	Ms IgG1 (Biolegend 400120)
CD86	BV421	1/50	Biolegend (305426)	Ms IgG2b (Biolegend 400342)
CTLA-4*	PE-Dazzle594	1/25	Biolegend (349922)	Ms IgG1 (Biolegend 400176)
CX3CR1	PE-Cy7	1/100	Biolegend (341612)	Rt IgG2b (Biolegend 400618)
CXCR1	PE-Cy7	1/40	Biolegend (320620)	Ms IgG2b (Biolegend 400326)
CXCR3	AF488	1/100	Biolegend (353710)	Ms IgG1 (Biolegend 400129)
CXCR6	APC	1/50	Biolegend (356005)	Ms IgG2a (Biolegend 400219)
CXCR6	PerCP-Cy5.5	1/50	Biolegend (356010)	Ms IgG2a (Biolegend 400252)
FoxP3*	PE	1/20	eBioscience (12-4776-42)	Rt IgG2a (eBioscience 12-4321-42)
Fas	APC	1/100	Biolegend (305612)	Ms IgG1 (Biolegend 400120)
HLA-DR	FITC	1/50	BD Biosciences (555811)	Ms IgG2a (Biolegend 400208)
HLA-DR	BV421	1/50	Biolegend (307636)	Ms IgG2a (Biolegend 400260)
Hobit*	-	1/10	Gift from Prof. Van Lier, Sanquin Institure, Amsterdam, NL	Ms IgM (Biolegend 401604)
IFN-γ*	APC	1/167	BD Biosciences (554702)	Ms IgG1 (BD Biosciences 550854)
IL-10*	BV421	1/33	Biolegend (501421)	Rt IgG1 (Biolegend 400429)
IL-10*	PE	1/25	Biolegend (501404)	Rt IgG1 (Biolegend 400408)
IL-17A*	APC-Cy7	1/50	Biolegend (512320)	Ms IgG1 (Biolegend 400128)
IL-17A*	PerCP-Cy5.5	1/50	Biolegend (512313)	Ms IgG1 (Biolegend 400150)
IL-2*	PE	1/167	eBioscience (12-7029-82)	Rt IgG2a (eBioscience 12-4321-42)
IL-4*	PE-Cy7	1/50	Biolegend (500824)	Rt IgG1 (Biolegend 400416)
IL-6*	PE	1/33	Biolegend (501107)	Rt IgG1 (Biolegend 400408)
Integrin β7	PE	1/100	Biolegend (321204)	Rt IgG2a (eBioscience 12-4321-42)
Ki67*	PB	1/25	Biolegend (350512)	Ms IgG1 (Biolegend 400151)
KLRG-1	PE	1/100	Biolegend (367712)	Ms IgG2a (Biolegend 400214)
PD-1	PE	1/50	Biolegend (329906)	Ms IgG1 (Biolegend 400112)
S1PR1	eFluor660	1/50	eBioscience (50-3639-41)	Ms IgG1 (eBioscience 50-4714-80)
TGF-β*	APC	1/25	Novus Biologicals (IC420A)	Ms IgG1 (Biolegend 400120)
TNF-α*	eFluor450	1/50	eBioscience (48-7349-41)	Ms IgG1 (eBioscience 48-4714-82)
γδ-TCR	APC-Vio770	1/50	Miltenyi Biotec (130-109-360)	Ms IgG1 (Miltenyi Biotec 130-104-618)
Mouse IgM*†	PE	1/50	Biolegend (408608)	Not required (secondary antibody)

Table 2.3 – List of antibodies used for flow cytometric-based detection. All antibodies used in these studies along with fluorochrome information, final dilutions used, and company and isotype-matched control details. Fluorochrome abbreviations: AF – AlexaFluor; APC - Allophycocyanin; BV – Brilliant Violet; Cy – Cyanine Cy7; FITC - Fluorescein isothiocyanate; PE – Phycoerythrin; PerCP - Peridinin Chlorophyll Protein Complex; V450 - Violet 450. Antibody host species abbreviations: A-Hm – Armenian Hamster; Ms – Mouse; Rt – Rat. eBioscience is now part of ThermoFisher Scientific. All products ordered from UK subsidiaries. * - indicates antibody used for intracellular staining. † - indicates used as a secondary antibody.

2.6 Immunofluorescence

Immunofluorescence staining was carried out on 3μm-thick formalin-fixed paraffin embedded sections. Sections were first dewaxed and rehydrated by being put through 3 xylene, 2 ethanol, and 2 water washes for 3 minutes each. Slides were microwaved for 30 mins in pre-warmed high pH antigen unmasking solution (Vector Labs, UK) and subsequently cooled. Next, slides were placed in Harris' haematoxylin (Leica, UK) for 60 seconds, to quench background autofluorescence, and washed off twice in water for 3 minutes each. Wash buffer (TBS [Tris-buffered saline]-Tween; Agilent, UK) was added for 5 minutes, followed by 2% Casein solution (in distilled water; Vector Labs, UK) for 10 minutes. Primary antibodies or IMCs were then diluted in wash buffer and added overnight. The following day, secondary antibodies (in wash buffer) were added for 1 hour following 3 washes with wash buffer. After a final wash buffer addition and placement into water, slides were mounted with prolong gold mountant (ThermoFisher Scientific, UK). All wash buffer steps and antibody incubations involved the addition of 200μl volume; and were carried out in the dark, on a low speed rocker. All antibodies used are detailed in Table 2.4. Both primary and secondary antibodies were individually titrated to determine the optimum test concentration before use together.

Imaging was carried out using a Zeiss LSM 880 Laser scanning microscope, running Zeiss Zen software (both Zeiss, UK). Offline analysis, image correction and presentation also used Zeiss Zen software.

Target Antigen	Species/Isotype	Fluorochrome	Dilution	Company (Ref.)
CXCR6	Rabbit IgG	-	1/50	Abcam, UK (ab8023)
CX3CR1	Rabbit IgG	-	1/50	ThermoFisher Scientific, UK (711353)
CD69	Mouse IgG2a	-	1/25	R&D Systems, UK (MAB23591)
CD4	Mouse IgG1	-	1/50	Leica, UK (CD4-368-L-CE-H)
NKp46	Mouse IgG2b	-	1/50	R&D Systems, UK (MAB1850-100)
(IMC)	Rabbit IgG	-	1/100	Biolegend, UK (910801)
(IMC)	Mouse IgG2a	-	1/25	Biolegend, UK (401504)
(IMC)	Mouse IgG1	-	1/62.5	Biolegend, UK (401404)
(IMC)	Mouse IgG2b	-	1/50	Biolegend, UK (401212)
Rabbit IgG†	Goat IgG	AlexaFluor594	1/1000	ThermoFisher Scientific, UK (A-11012)
Mouse IgG2a†	Goat IgG	AlexaFluor647	1/200	ThermoFisher Scientific, UK (A-21241)
Mouse IgG1†	Goat IgG	AlexaFluor350	1/500	ThermoFisher Scientific, UK (A-21120)
Mouse IgG2b†	Goat IgG	AlexaFluor488	1/500	ThermoFisher Scientific, UK (A-21141)

Table 2.4 - Antibodies used for immunofluorescence detection. In descending order, antibodies shown are primary test antibodies, primary isotope-matched controls, and secondary antibodies. Secondary antibodies are marked with †.

2.7 Data analysis, Data presentation & Statistics

Data was presented graphically and tested for statistical significance using GraphPad Prism v6 software (Graphpad, US). Additional calculations were performed using Microsoft Excel (Microsoft, US). Diagrams and other artworks were created using a combination of Microsoft Powerpoint (Microsoft, US), Gravit designer (Gravit GmbH, DE), and Servier medical art (Les Laboratoires servier, SAS, FR). All combined data displayed as median with non-parametric descriptions unless otherwise specified. Statistical tests used were as follows: Wilcoxon matched-pairs signed rank tests were used to compare two paired groups; Freidman tests with Dunn's multiple comparison tests compared three or more paired groups; and Kruskal-Wallis tests with Dunn's multiple comparisons tests were used to compare three or more unpaired groups. Statistical difference representations follow the standard convention (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Individual figure legends contain donor number information for each experiment. All work carried out is my own unless specifically stated in the figure legend. Dominik Niesen (D.N) contributed substantially to data collection in sections 3.5, while Fras Ahmad (F.A) made small contributions to data collection in section 4.2. Emily

(Xiaoyan) Li (X.L) provided major help with LIMC preparation and staining throughout chapter 5.

Chapter 3 – Migration through hepatic epithelia reveals intrinsic, liver disease-specific cytokine profiles in peripheral blood $CD4^+$ T cells

3.1 Introduction – Migration as a key component of T cell function & chronic liver disease-specific alterations in peripheral blood

T cell function and migration are intrinsically connected[31]. Both parameters can be used to subset cells: function determined by cytokine production is used to place cells into helper subsets (T_H1, T_H2, T_{REG}), whereas differential homing capabilities allow for the branching of memory cells into three main groups (T_{CM}, T_{EM}, T_{RM}). The homing abilities of a T cell are tightly regulated throughout its life cycle, with differential modulation of adhesion and chemokine receptors observed with changes in T cell activation, differentiation, and tissue tropism[360]. Naïve T cells must efficiently traffic to the T cell zones of SLOs in order to encounter cognate antigen presented on APCs. Following activation, T cells upregulate specific homing receptors, the exact combinations of which depend on the nature of infecting pathogen, and site of priming[541]. For example, priming in skin draining LN promotes upregulation of E- and P-selectin ligands, CCR4 and/or CCR10 promoting entry to the skin, while in GALT, CCR9 and integrin $\alpha4\beta7$ expression is preferentially induced, allowing T cell homing to the gut[31, 70, 542]. Furthermore, T helper subsets often show preferential chemokine receptor associations: T_H1 cells express CXCR3, CCR5 and CXCR6, T_H2 cells express CCR4 and CCR8. T_H17 cells express a combination of both the above – CXCR3, CXCR6, and CCR4; as well as CCR6[353]. T_{REGS} are generally thought to express CCR4 and CXCR3[353], but can also specifically upregulate the same chemokine receptors as their effector counterparts to follow them to effector sites and monitor their activities there[543]. Once in NLTs, $CD8^+$ T cells must directly contact infected target cells in order to mediate targeted cytotoxicity, whereas $CD4^+$ T cell pro-inflammatory cytokine production must also be localised to the infected site to tightly direct communication to the target cells and/or minimise bystander tissue damage. The resolution stage of inflammation also relies on efficient migration, through the prevention

of further pro-inflammatory cell influx, egress of surviving cells to the blood and lymph, and entry of immune regulatory cells[544, 545]. Lastly, the efficient division of labour between memory cells that allows T_{CM} to repopulate the memory pool from SLOs, while T_{EM} and T_{RM} perform immunosurveillance and effector responses in NLTs, is also possible through specific homing patterns[70]. Thus, control of migration is a key aspect of T cell biology at every stage, and should be considered a vital component of their function.

$CD4^+$ T cells play key roles in driving the pathology of viral and autoimmune liver pathologies[414, 465, 546]. Most studies on human T cell populations implicated in disease pathogenesis have been conducted using peripheral blood[410, 465, 547]. For HCV, studies comparing peripheral T cell populations from resolved acute, and chronically-infected patients have concluded that robust, sustained, and broadly focussed $CD4^+$ T cell responses are crucial for viral clearance[477, 480-483, 546, 548]. In progression to chronicity, HCV-specific $CD4^+$ numbers dwindle dramatically[476, 479, 482, 484, 549, 550]. Similar to $CD8^+$ T cells[486-488, 551], remaining $CD4^+$ T cell populations show hallmarks of exhaustion – high expressions of inhibitory markers such as PD-1; and impaired proliferation, IFN-γ and IL-2 responses to HCV antigen stimulation[472, 474, 476-479, 482, 504]. Additionally, while strong T_H1 responses are correlated with clearance, skewing towards T_H2, or T_{REG}, has been implicated as part of the chronic disease course[504, 507, 552]. Accordingly, conventional T_{REG} numbers are higher in the blood of chronically infected compared to acute resolved or healthy patients[517], and are superior at inhibiting $CD8^+$ proliferation and cytokine responses compared to these control groups[504, 519, 520]. In sum, $CD4^+$ T cells in chronic HCV can largely be defined by functional inactivation, and shifts towards more regulatory subsets.

In contrast to HCV, the number of studies on $CD4^+$ T cells in PBC pathogenesis is surprisingly poor. Normal total $CD4^+$ numbers in blood have been reported, though elevation in HLA-DR expression was seen compared to healthy controls[553]. Alterations in T_{REGS} have been observed, with some suggesting that PBC patient blood contains a reduced frequency of T_{REGS}[410, 429]; while others claim this is only evident in the liver, accompanied by increased T_H17 responses[411]. Basic cytokine profiling of patient sera seems to point towards T_H1 and/or T_H17 immunity primarily[424, 429], whereas experiments in liver additionally implicate IL-5[412, 425, 426, 439]. Finally, recent data suggests the frequency of circulating T_{FH} is increased in PBC patient blood, alongside enhancements in their function (measured by IL-21 production and promotion of B cell maturation)[554]. Therefore, it can be seen that data on disease-specific alterations $CD4^+$ T cell phenotype and function in PBC are still severely lacking.

Ideally, diseased liver tissue would be used to advance our understanding of viral and autoimmune liver disease immunopathology. However, access to liver tissue is relatively scarce, and unfortunately the use of blood is not an ideal alternative as we are currently limited in our ability to detect relevant effector T cells in the circulation[547, 555]. Furthermore, although MHC-tetramer based approaches have been effective at studying hepatic virus-specific cells in peripheral blood[192, 488, 556]; and autoreactive cells in PBC[557]; this does not give information on the total $CD4^+$ T cell pool entering the inflamed liver, and cannot be applied to autoimmune diseases like PSC where driving autoantigen(s) are still unknown[558]. We sought a way to circumvent the tissue access problem, and simultaneously pinpoint peripheral blood T cells that may be involved in pathogenesis. Hypothesising that the most responsive T cells would be those with the greatest capacity to migrate into liver tissue; we modelled T cell migration into the parenchyma using

transwell migration assays. Armed with preliminary data from our lab that first suggested $CD4^+$ T cells that migrated through hepatoma monolayers increased IFN-γ production following stimulation, and second raised the possibility of the cytokine profiles revealed by transmigration assays being distinct in chronic HCV patients, we composed the following aims:

1. Investigate the cytokine expression profiles of circulating T cells before and after migration through hepatic epithelia
2. Characterise migrated and non-migrated T cells for cell surface antigen expression
3. Determine whether the act of migration through hepatic epithelia induces these phenotypic and functional changes
4. Establish if T cell cytokine expression following migration can reveal disease-specific restrictions in patients with liver disease of autoimmune or viral aetiology

3.2 Migration of $CD4^+$ T cells through hepatic epithelia reveals differences in the production of TNF-α, IFN-γ, and IL-4

In order to determine if $CD4^+$ T cell cytokine production potential is different pre-, and post-migration; we carried out transwell migration assays and monitored the production of T_H1 cytokines IL-2, TNF-α, and IFN-γ; T_H2 cytokine IL-4; and T_H17 cytokine IL-17 following stimulation. Briefly, these assays involved culturing Huh-7 hepatoma cells to 100% confluence on a transwell insert and allowing the overnight migration of PBMC-derived $CD4^+$ T cells (Fig. 3.1.1A). Huh-7 cells were chosen principally for their ability to reliably form complete continuous monolayers (Zania Stamataki - personal

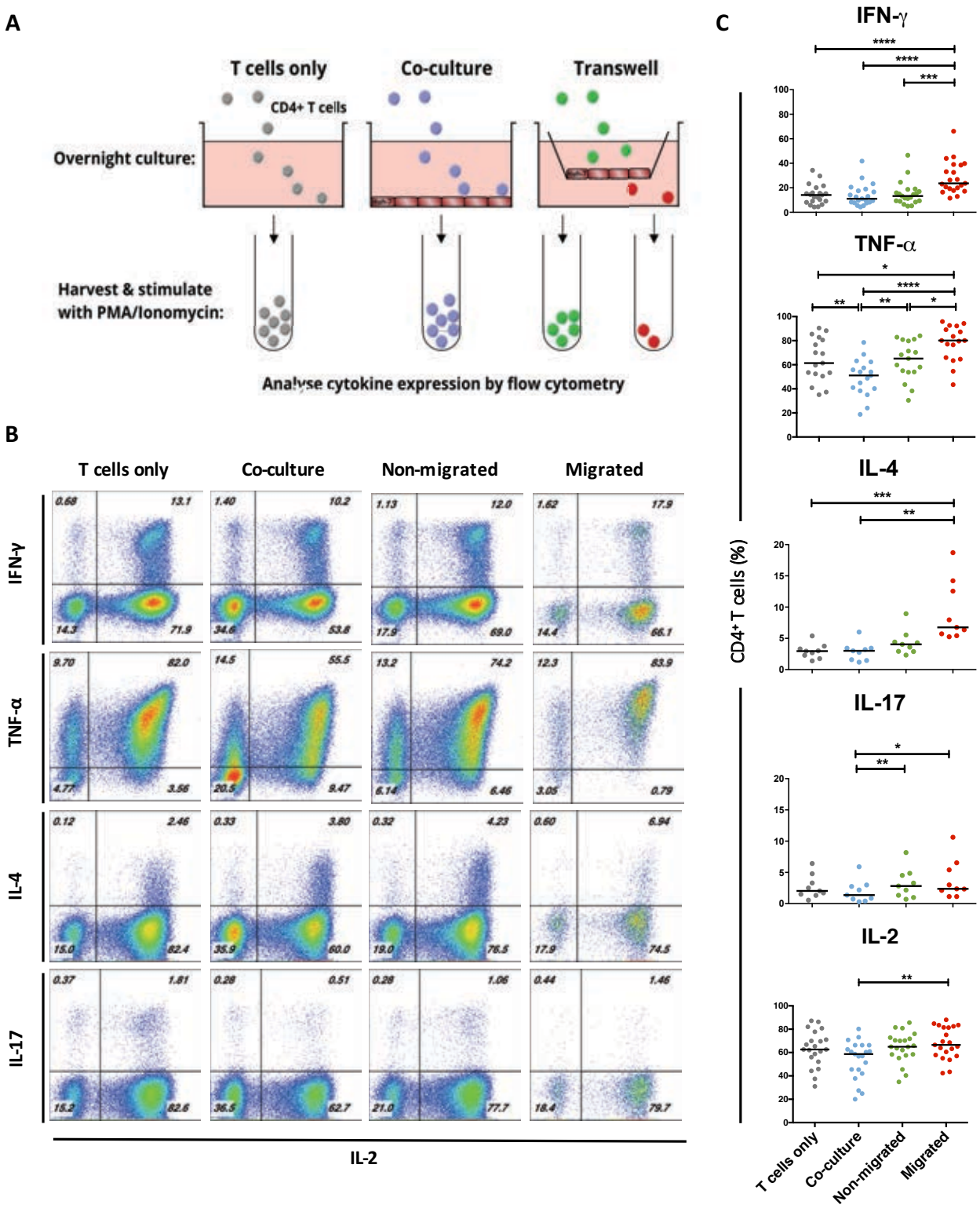


Figure 3.1.1 - Migration of CD4$^+$ T cells through hepatic epithelia reveals increased IFN-γ, TNF-α, and IL-4 responses following stimulation. A – Transwell migration assay set up. Huh-7 cells were cultured to 100% confluence in 24 well plates – either at the bottom of the well, or on high density, 3µm pore transwell inserts. Isolated PBMC-derived CD4$^+$ T cells were then added to both conditions as well as to media alone (a T cells only control) and cultured overnight. T cells were harvested from above (non-migrated) and below (migrated) the transwell insert, as well as from static co-culture and T cells only; stimulated with PMA/Ionomycin with Brefeldin A, and intracellular cytokine production detected through intracellular staining and flow cytometry. **B –** Representative flow cytometry plots showing IL-2, IFN-γ, TNF-α, IL-4, and IL-17 production in the CD4$^+$ T cell subsets. *(Legend cont. on next page)*

C – Combined % $CD4^+$ T cell expression data from all healthy donors collected for all cytokines studied. Graphs show all data points + median values. N=9-21, 1 donor assayed for for IL-2, IFN-γ and TNF-α staining by D.N.

communication). Non-migrated and migrated fractions were then harvested, stimulated with PMA/Ionomycin (and Brefeldin A) for 5 hours, and cytokine detected by intracellular staining and flow cytometry. T cells alone, and T cells cultured on confluent Huh-7 layers on the bottom of 24 well plates (referred to as co-culture) were included as assay controls. Migrated cells clearly contained greater percentages of cells that were able to produce IFN-γ and TNF-α, as well as IL-4 (Fig. 3.1.1B,C). Migrated IL-2 and IL-17 showed no clear differences from T cells only controls. Interestingly, although there was a significant decrease in the fraction of TNF-α^+ cells in co-culture, the vast majority of comparisons revealed static co-culture did not affect cytokine production. Previous work by others in the lab has demonstrated that these differences, in TNF-α and IFN-γ at least, were not dependent on the prior activation state of a T cell, or blood versus liver origin, as both blood $CD4^+$ T cells previously activated with CD3/CD28 stimulation, and liver-derived $CD4^+$ T cells demonstrated the same cytokine production patterns [D. Niesen – unpublished observations & Fig. 3.4.1]. Similarly, migration through first HSEC and then Huh-7 together (mimicking the architecture of the liver) did not affect the upregulation of cytokine$^+$ cells in the migrated pool [D. Niesen – unpublished observations]. Together these data reveal differences in both type-1 and type-2 cytokine potential following migration through hepatic epithelia that is not evident through simple co-culture systems. For ease, we designate this enrichment of cytokine-producing cells in the migrated fraction compared to the other conditions, the *transwell migration effect*.

Eager to understand if the *transwell migration effect* was restricted to the Huh-7 hepatoma cell line, we stained for IL-2, TNF-α and IFN-γ following migration through two additional hepatocyte cell lines – HepG2 and Hep3B. Preliminary data showed broadly similar enhancements in cytokine production were seen regardless of hepatoma cell line used,

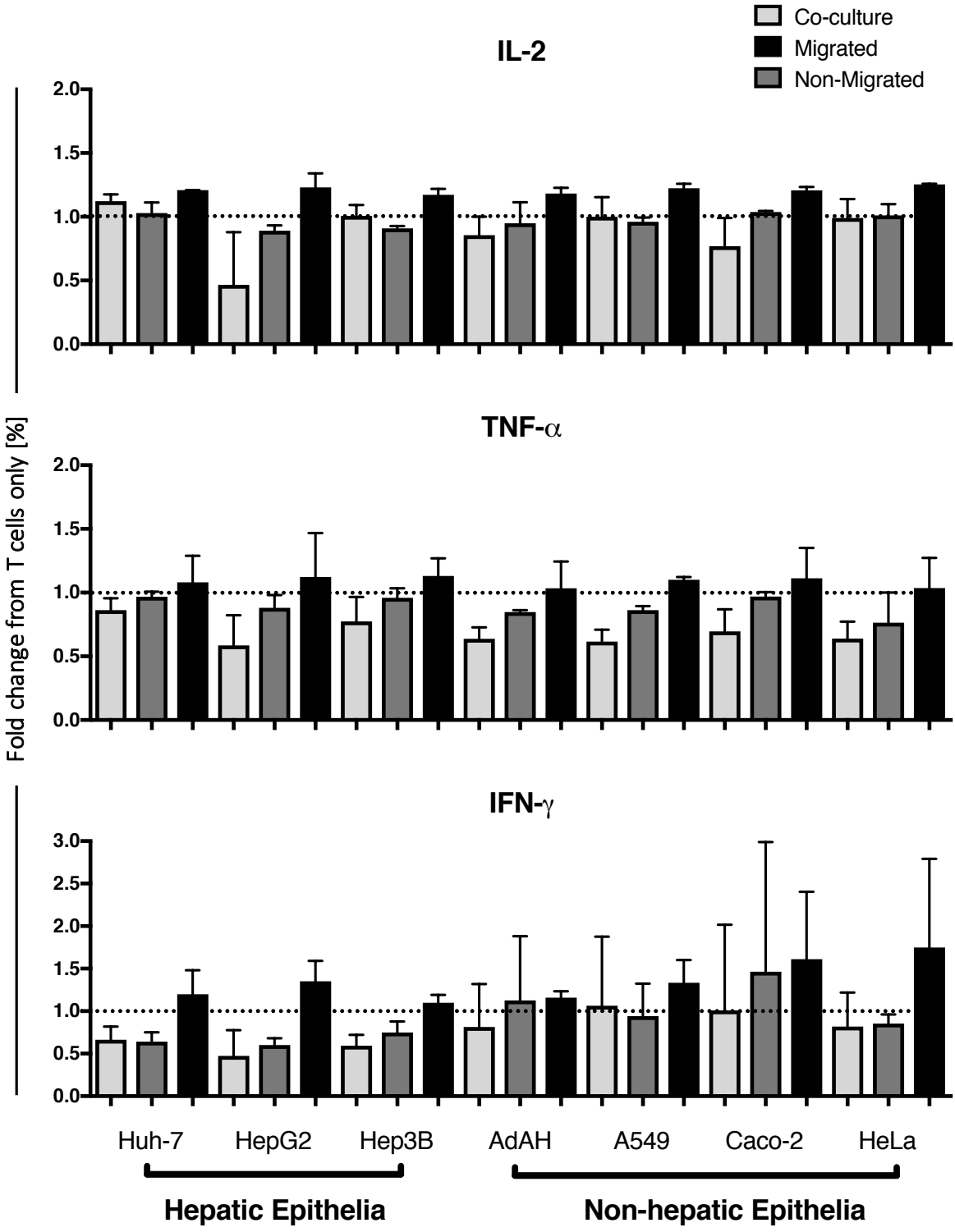
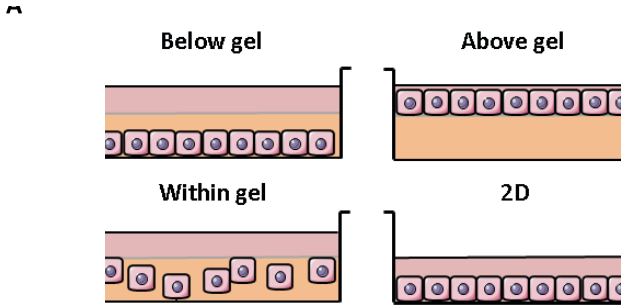
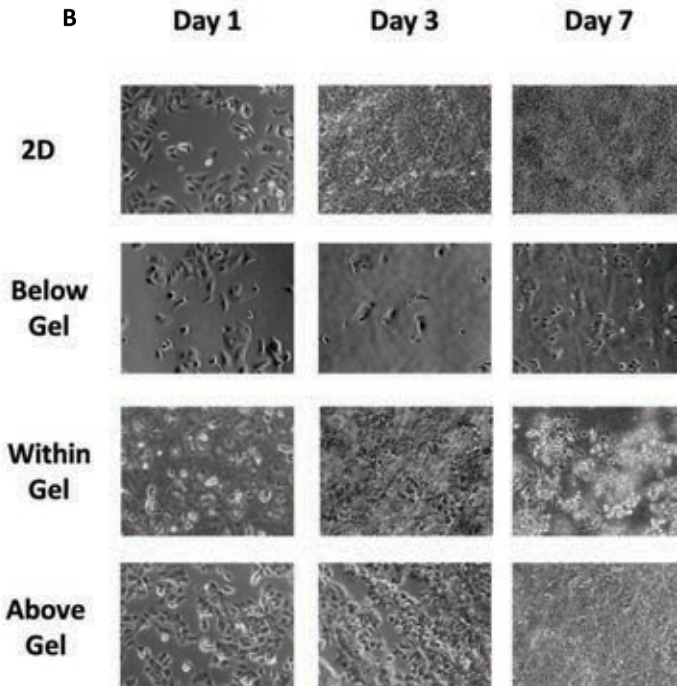


Figure 3.1.2 – Migration through multiple epithelial cell types reveals increases in T_H1 cytokine production among $CD4^+$ T cells. Transwell migration experiments were carried out as in Fig. 3.1.1, but using additional hepatic (HepG2, Hep3B), and non-hepatic epithelial cell lines (AdAh, A549, Caco-2, HeLa). Percentages of $CD4^+$ T cells expressing IL-2, TNF-α, or IFN-γ in co-culture, non-migrated, and migrated conditions were analysed and presented as fold changes from T cells only controls in the bar charts above. N=3, median + IQR displayed.

although IFN-γ production following migration through Hep3B cells was less clear-cut (Fig. 3.1.2). To determine whether we were seeing a hepatocyte specific, or general epithelial effect, we used a variety of cell lines generated from epithelia of other tissues (AdAh – nasopharyngeal, A549 – alveolar, Caco-2 – colorectal, HeLa – cervical). It appeared migration through all epithelial cell lines resulted in the transwell migration effect to a similar degree, at least for IL-2 and IFN-γ, though more data is needed to confirm this statistically.

Aiming to extend our system beyond transwell migration assays, we adapted gel-migration assay methodology currently used by collaborators[559]. Huh-7 cells were cultured in three conformations – below, within and above type-1 rat tail collagen gels; as well as in a standard 24-well plate (2D) as a control (Fig. 3.1.3A). Hepatocyte growth and morphology was monitored over time to determine the best conformation to take forward and compare with transwell assays. Huh-7 cells cultured below the gels appeared to grow slowly and adopted more dendritic morphology (Fig. 3.1.3B). Cells within the gel displayed a more classic 'box-like' morphology but failed to reach confluence even at greatly enhanced seeding densities and extended culture periods. However, this conformation was the most promising for generating 3-dimensional cultures with more polarised hepatocytes (despite unsuccessful attempts to assess hepatocyte polarity – data not shown), another initial aim of these experiments. Cells cultured above the gel were the clear choice to take forward into migration assays due to favourable morphology and growth characteristics in this condition. We therefore directly compared the cytokine production from $CD4^+$ T cells migrated through Huh-7 on transwells, with cytokine production from the same cells migrated through Huh-7 on collagen gels. This latter assay will be termed the '*trans-gel*' assay through lack of discernible wit.





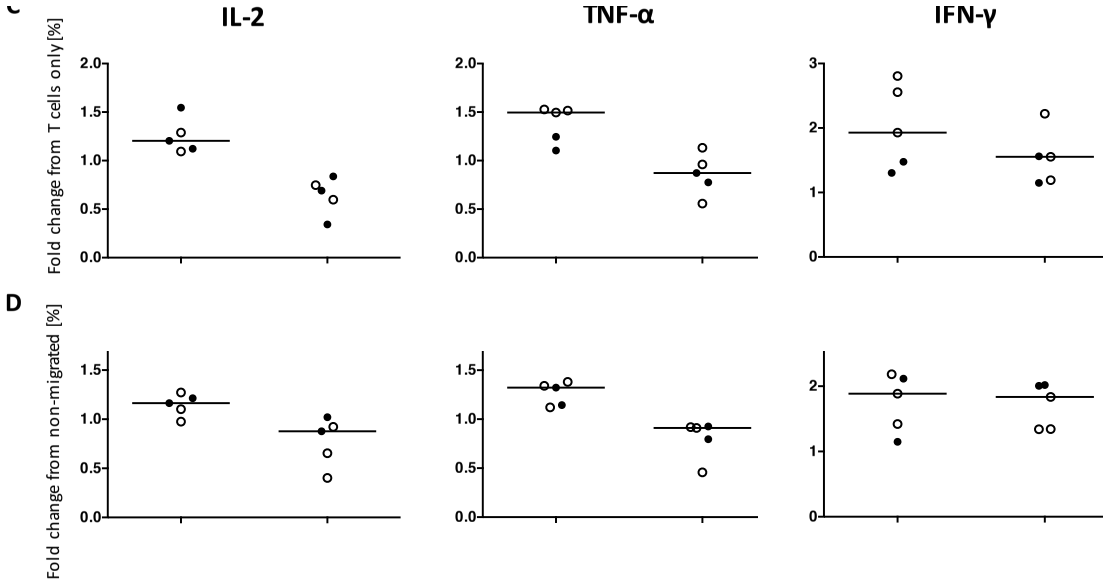


Figure 3.1.3 – Using collagen gel migration systems reveals different alterations in cytoki production. A, B – Gel culture assay set-ups. Huh-7 cells were cultured either below, above, within gels; or alone in normal 24-well plates. Light microscopy images were taken at 10x objecti at different time points to monitor hepatocyte growth and morphology in the different cultu conditions. **C, D –** Cytokine production (% of $CD4^+$ T cells) in the transwell assays compared to t 'trans-gel' assays. Data is shown as fold change from T cells only (**C**), and fold change from the nc migrated conditions. Open circles represent non-activated input $CD4^+$ T cells, while closed circl show $CD4^+$ T cells have been pre-activated with anti-CD3/anti-CD28 ligation + 500IU/ml IL-2.

Comparing first the differences in % cytokine production compared to T cells only, trans-gel assays allowed a similar IFN-γ enhancement following migration; but, although no statistical differences were seen, no increases in TNF-α were apparent in the trans-gel assay; and IL-2 production actually seemed decreased in the gel-migrated pool (Fig. 3.1.3C). To remove contact with the gel as a variable, migrated cells were also compared directly with their non-migrated cells from the same conditions (Fig. 3.1.3D). Similar patterns were observed – IFN-γ % increases were apparent no matter the migration system, whereas no clear increases in IL-2 or TNF-α were seen in trans-gel assays. Wondering whether these differences between the two migration systems were due to some immunomodulatory effect of the collagen, we performed transwell assays where Huh-7 cells were cultured on type-1 rat tail collagen-coated plates. No differences were seen in any cytokine with or without collagen, even when migrated T cells came in contact with collagen directly post-migration (Fig. 3.1.4). Thus, the migration system used may affect divergences in IL-2 and TNF-α production, but not IFN-γ.

Last, we asked whether a proinflammatory environment that would be seen following recruitment into inflamed livers *in vivo* affected this *transmigration effect*. Preliminary data indicated that a pro-inflammatory cytokine cocktail skewed the non-migrated cells towards IFN-γ-producing, at the expense of the migrated fraction [D. Niesen – unpublished data]. Aiming to replicate this 'reversal effect', and discern which specific cytokine(s) were responsible, we pre-treated PBMC-derived CD4+ T cells with pro-inflammatory cytokines (IL-1β, IL-6, IL-22, TNF-α, IFN-γ) for 24 hours either alone in combination and performed transwell migration assays with Huh-7 cells as before. However, using 6 donors, we saw no differences in cytokine production profiles in the pro-inflammatory cocktail treated cells, regardless of condition (Fig. 3.1.5A). Similarly,

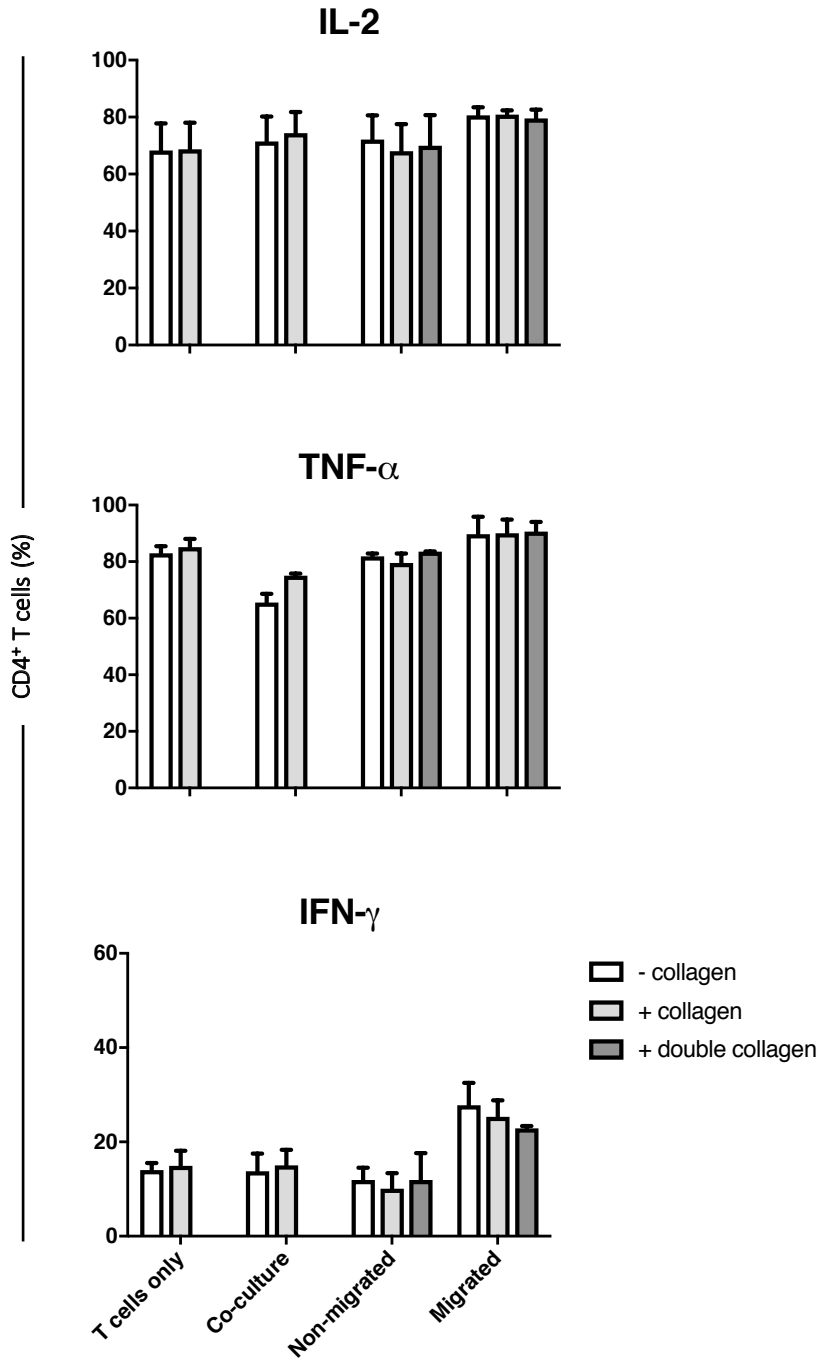


Figure 3.1.4 – The Presence of collagen has no impact on cytokine skewing following transwell migration assays. Blood-derived $CD4^+$ T cells were subjected to standard transwell assay (Fig. 3.1.1, - collagen, white bars), or transwell assay conditions with Huh-7 cells cultured on collagen (+ collagen, light grey bars). For the + collagen T cell only and co-culture conditions this meant collagen coated 24 well plates, whereas for non-migrated and migrated + collagen conditions this meant collagen coated transwell inserts. Finally a + double condition (dark grey bars) was set up for the non-migrated and migrated cells where collagen coating was performed on the transwell insert as well as the plate bottom. Bars show % cytokine expression as median values (+95% CI) from 2 donors.

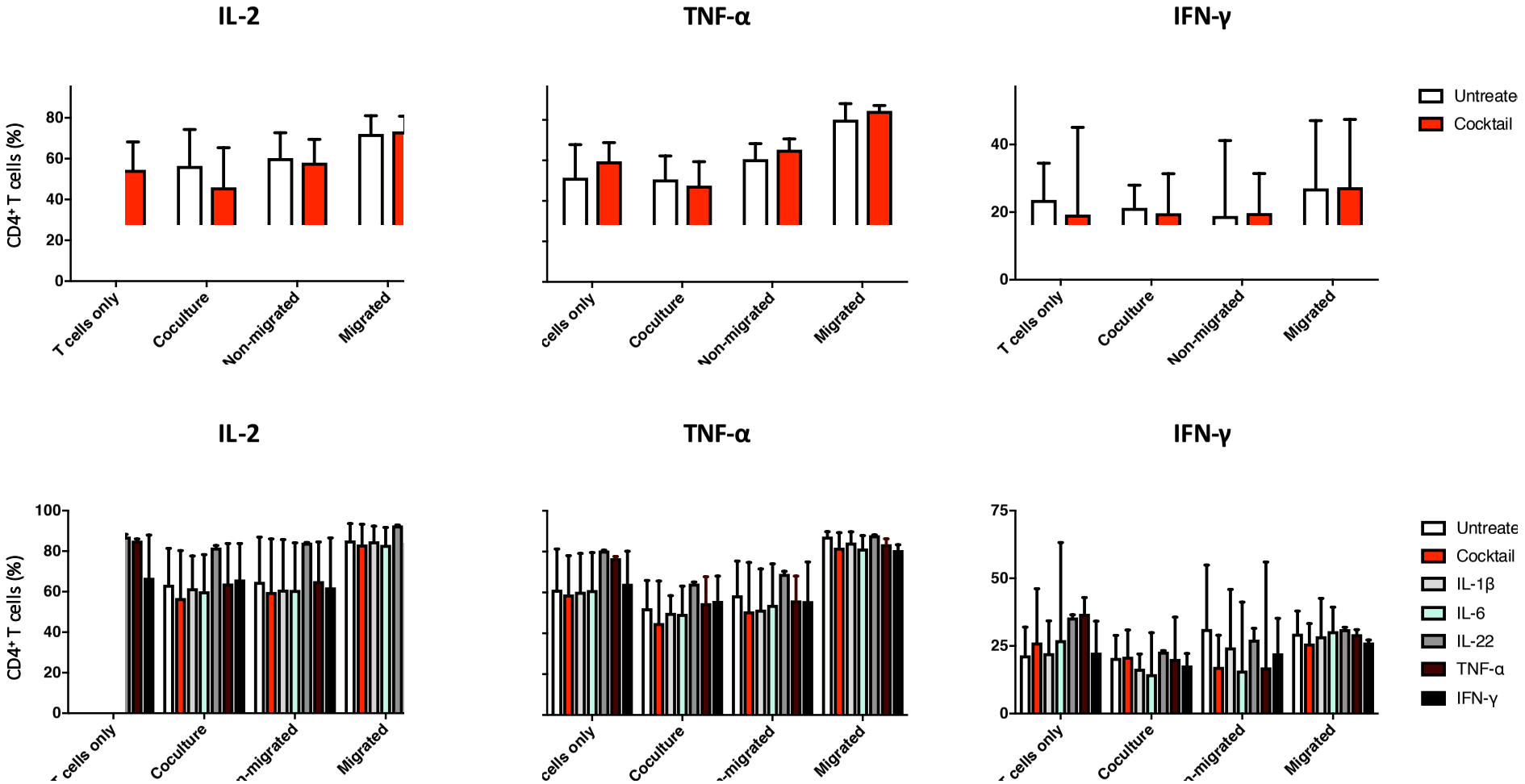


Figure 3.1.5 – Pro-inflammatory cytokine pre-treatment does not affect the transwell migration effect. Isolated $CD4^+$ T cells were pre-treated for 24h wit a cocktail of pro-inflammatory cytokines (IL-1β, IL-6, IL-22, TNF-α, IFN-γ) (**A**), or each cytokine individually (**B**) before transwell assays were carried out and % cytokines produced following PMA/Ionomycin stimulation were ascertained. In part A: 8 untreated donors (5 pre-activated with anti-CD3/CD28 stimulation), treated (3 pre-activated with anti-CD3/CD28 stimulation). In part B, n=2 (both pre-activated with anti-CD3/CD28 stimulation). All bars show median values + IQF

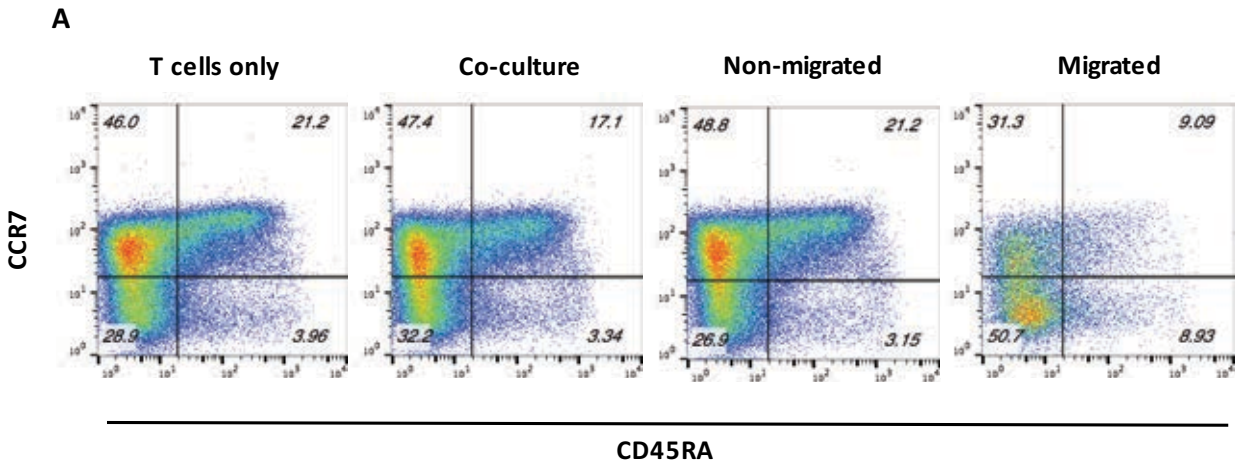
when assessing individual cytokines, no cytokine alone made any difference to TNF-α or IFN-γ expression patterns (Fig. 3.1.5B). The only cytokine that appeared to have an effect was IL-22, which increased the percentage of $CD4^+$ T cells producing IL-2 in every culture condition.

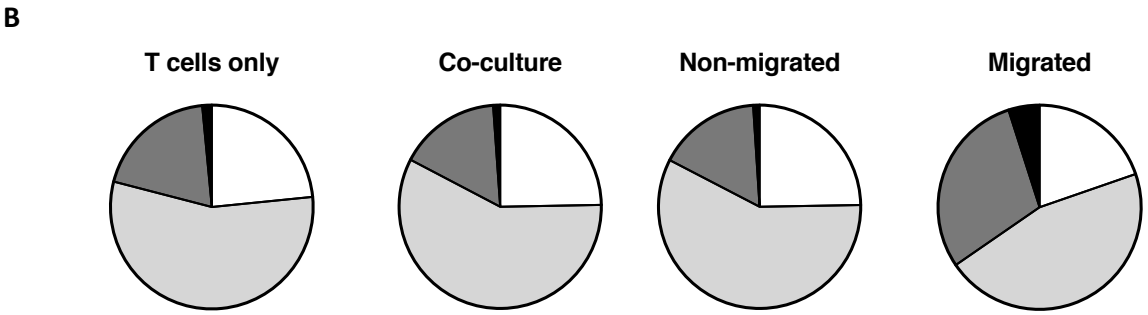
In sum, these experiments show that following migration through hepatic and non-hepatic epithelia in transwell systems, the proportion of cells expressing TNF-α and IFN-γ was enhanced; a finding that can be also applied to IL-4 production following Huh-7 migration. This *transwell migration effect* was only partially applicable to gel-culture, with alterations in IL-2 and TNF-α; and pro-inflammatory cytokine pre-treatments did not affect the phenomenon.

3.3 $CD4^+$ T cells display an altered memory phenotype and distinct activation profile following migration through hepatic epithelia

To see whether an alteration in the cytokine-production capacity of migrated T cells was accompanied by a change in cell surface phenotype, we first looked at naïve and memory representation. Migrated cells displayed preferences for T_{EM} and T_{EMRA} cell types, in keeping with the enhanced migratory capacity of these cells in peripheral tissues (Fig. 3.2.1). By contrast, T cells only, co-culture, and non-migrated conditions all presented with very similar naïve/memory make-up.

Assessment of activation and inhibitory marker expression revealed some interesting patterns. The percentage of CD25, HLA-DR, PD-1 and Fas-expressing T cells were all enhanced in the migrated pool (Fig.3.2.2A). Notwithstanding, proportions of $CD4^+$ T cells





Population	Key	Significant?						IQR (%)			
		T vs. C	T vs. NM	T vs. M	C vs. NM	C vs. M	NM vs. M	T	C	NM	M
Naive		ns	ns	ns	ns	ns	ns	28.1	30.6	22.8	11.8
Central Memory		ns	ns	ns	ns	ns	ns	22.6	29.3	21.8	17.3
Effector Memory		ns	ns	ns	ns	**	***	6.90	3.61	4.99	14.0
TEMRA		ns	*	ns	ns	***	****	0.99	0.80	0.73	7.96

Figure 3.2.1 – Increased T_{EM} and T_{EMRA} representation in migrated $CD4^+$ T cells. Percentages of different naïve and memory $CD4^+$ T cell populations assessed following transwell migration assay. **A -** Representative flow cytometry staining in the four transwell assay conditions. **B -** Combined percentage data from 12 healthy donors, one pie chart per transwell condition. Statistical comparisons were made within each naïve/memory designation, across transwell conditions (T - T cells only, C - Co-culture, NM - Non-migrated, M - Migrated). Table shows this data as well as IQR values for all conditions. Huh-7 cells used in these experiments. 1 of 12 donors assayed by D.N.

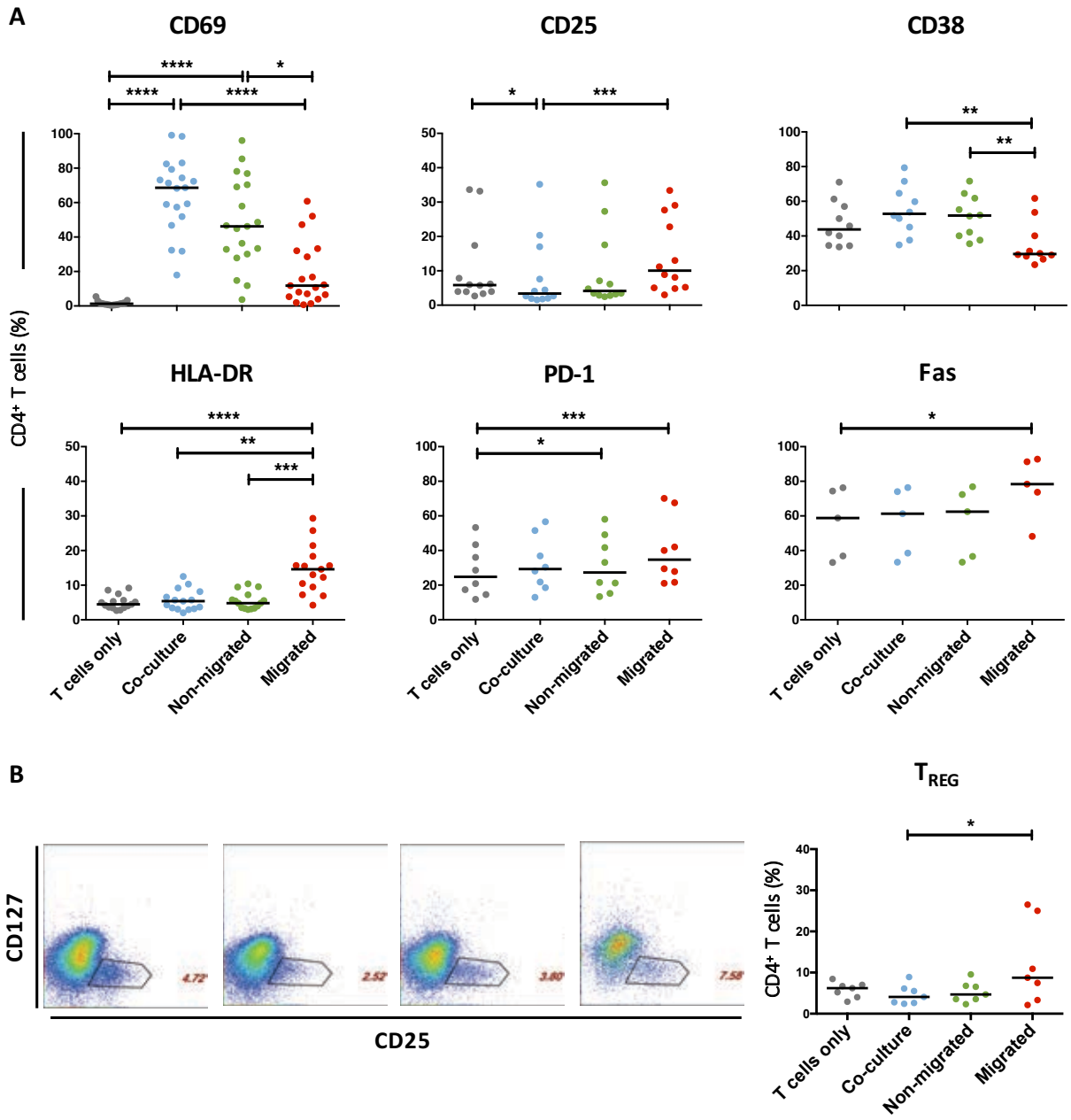


Figure 3.2.2 – Altered activation state following migration through hepatic epithelia. Expression of the activation markers CD69, CD25, CD38, HLA-DR, PD-1 and Fas (**A**), as well as conventional regulatory T cells ($CD25^{HI}CD127^{LO}$ - **B**) were assessed following transwell migration assays through Huh-7 cells. Lines show median values.

expressing CD38 were decreased, while early activation marker CD69 was not significantly increased following migration when compared to T cells only. The CD69 expression patterns in these experiments were of particular interest, showing robust and consistent upregulation in co-culture, followed to a slightly lesser degree in the non-migrated condition. This finding forms the basis for chapter 4, where it will be discussed in length. Additionally, we assessed expression of CD127 alongside CD25 to determine T_{REG} contributions in each condition. We found a marginally increased conventional T_{REG} percentage following migration, but this was only statistically significant compared to co-culture (Fig. 3.2.2B). Therefore, together these results determined that the migrated pool was enriched for effector memory and T_{EMRA} cells, with distinct activation phenotypes.

3.4 Migration involves selective recruitment of cytokine-responder CD4$^+$ T cells through the monolayer, not *de novo* generation through the act of migration

Based on the above data, we proposed two hypotheses in order to explain the changes in the migrated population. First, the act of migration through hepatic epithelia is imprinting the CD4$^+$ T cells with an effector phenotype and enhanced cytokine production capacity. This could be due to physical and/or mechanical pressures exerted on a T cell during the migration process, or adhesion molecule interactions which have an activatory effect on the cell. Alternatively, migration is preferentially separating out CD4$^+$ T cells that possess these effector phenotypes and are better able to respond to stimuli.

To test these hypotheses, first it was important to determine the proportion of cells that migrated through the monolayer, as failing to account for this could lead to misinterpretation of data that shows apparently large changes in the migrated fraction, yet very minor/no changes in the non-migrated pool. We observed the proportion of CD4$^+$

T cells that migrated through the Huh-7 monolayers was small (Fig. 3.3.1A). An average of just 4.6% of unactivated cells migrated through the monolayers, rising to 19.4% amongst activated $CD4^+$ T cells. However, the presence of Huh-7 monolayers augmented the proportion of T cell able to migrate, as seen when comparing with migration through transwell inserts alone. This effect was observed both with and without prior $CD4^+$ T cell activation.

Having seen only a small proportion of input T cells migrate through monolayers, we next asked whether we could see a clear induction of cytokine producing ability in greater numbers of T cells, as well as the previously observed greater percentages. To do this we incorporated counting beads as part of the flow cytometry protocol to determine how many $cytokine^+$ and $cytokine^-$ $CD4^+$ T cells there were in each transwell condition (co-culture excluded as irrelevant in this case). Combining both non-migrated and migrated cells into one data display, it was obvious across all three donors that there were no increases in the numbers of T cells able to make IL-2, or IFN-γ compared to the input population (T cells only, Fig. 3.3.1B). Additionally, combining our cytokine stains with stains for proliferation marker Ki-67 confirmed that migrated cells did not proliferate any more than their non-migrated counterparts, ruling out any enhancements in T cell numbers by cell division (Fig. 3.3.2).

Similarly, we isolated naïve $CD4^+$ T cells to prove that no *de novo* induction of memory cells was occurring with migration. After confirming the high purity of isolated naive T cells (Fig. 3.3.3A), we show that when naïve T cells are put through Huh-7 transwell migration assays no real switch to memory was seen (Fig. 3.3.3B). Following migration, there was

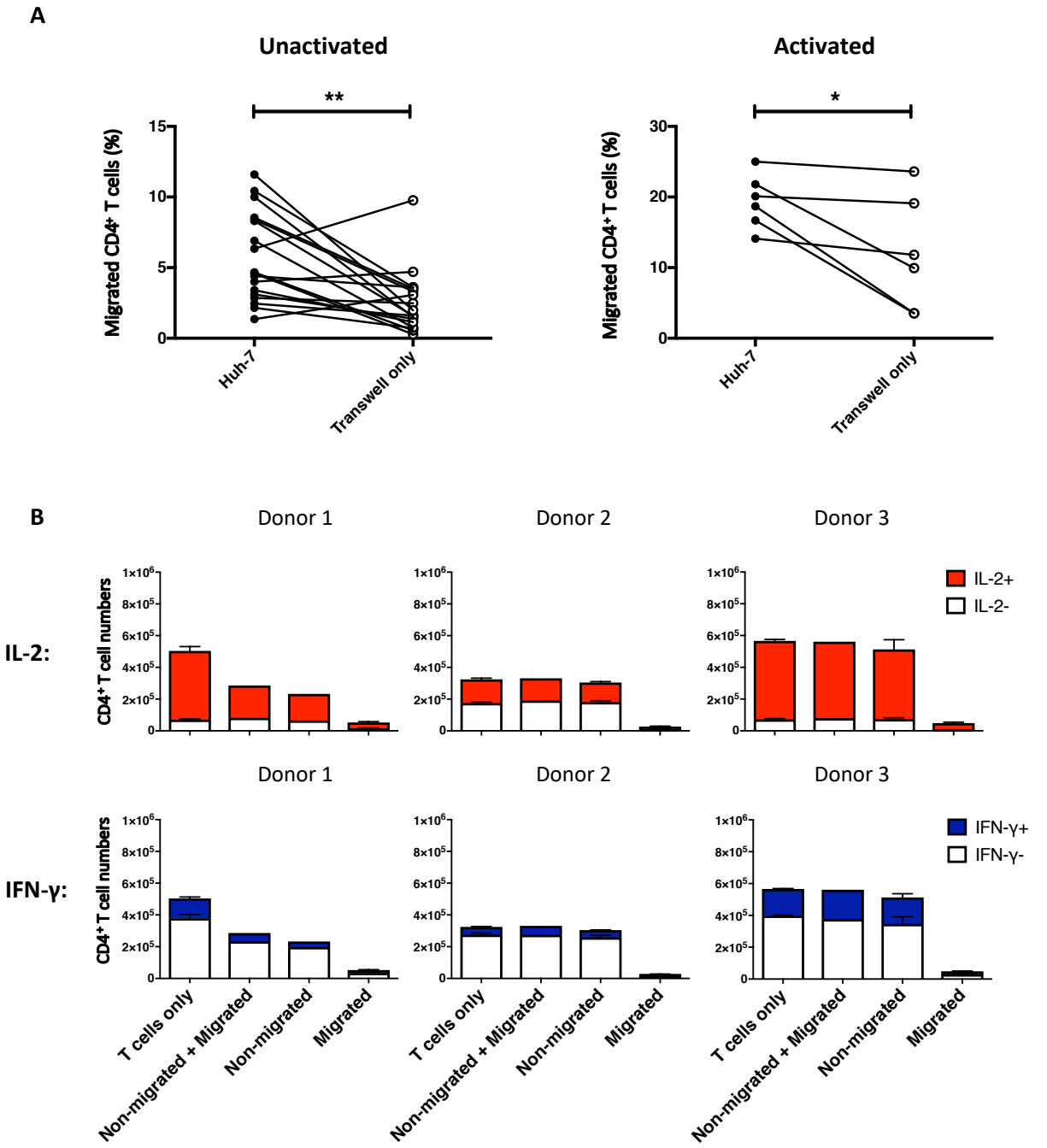


Figure 3.3.1 - Migration through hepatomas provides an enhanced selection of CD4$^+$ T cells with high functional potential. A - Both unactivated, and activated (with anti-CD3/CD28) CD4$^+$ T cells were migrated through Huh-7 cells on a transwell insert, or a transwell insert alone, and the number of migrated cells was counted manually. Percentages of migrated cells were calculated by comparing with T cells only control numbers. Migration percentages in transwell assays with and without Huh-7 cells were compared. **B -** Counting beads were used to determine the absolute numbers of cytokine$^+$ CD4$^+$ T cells (red - IL-2, blue - IFN-γ) and cytokine$^-$ cells (white) in transwell assays with Huh-7 cells. 3 donors displayed separately. Data presented as stacked bars, and non-migrated + migrated combined data included to illustrate the lack of appreciable new cytokine-producing cell generation. Each donor data is comprised of two technical replicates, bar heights and error bars show median and range of these replicates.

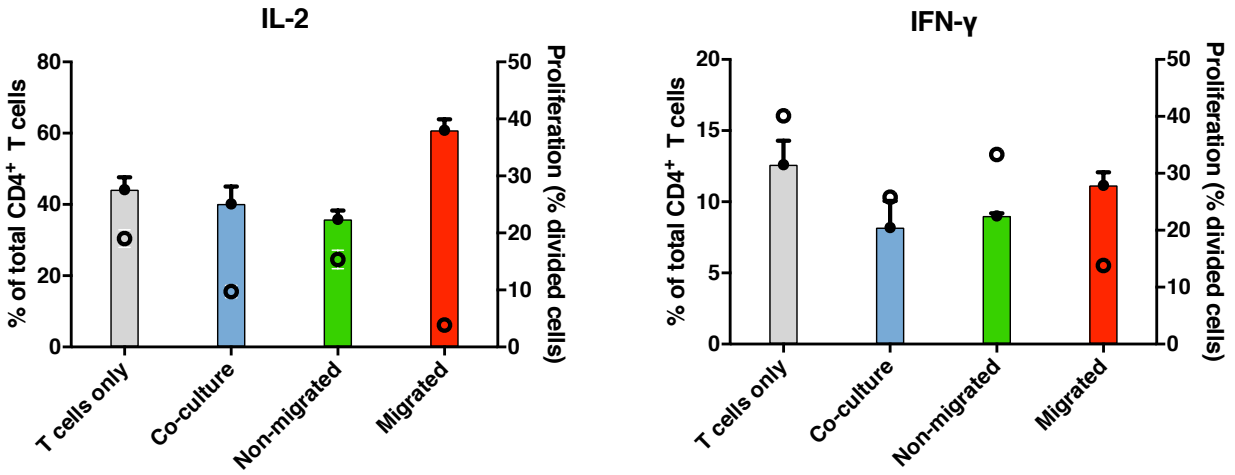


Figure 3.3.2 - Migrated cells do not proliferate any more than their non-migrated counterparts. Shown are IL-2 and IFN-γ % expression (coloured bars) following transwell migration assay and PMA/Ionomycin stimulation in each condition. Overlaid are % divided cells, as measured by Ki-67 staining (open circles). N=1, 2 technical replicates for each staining procedure.

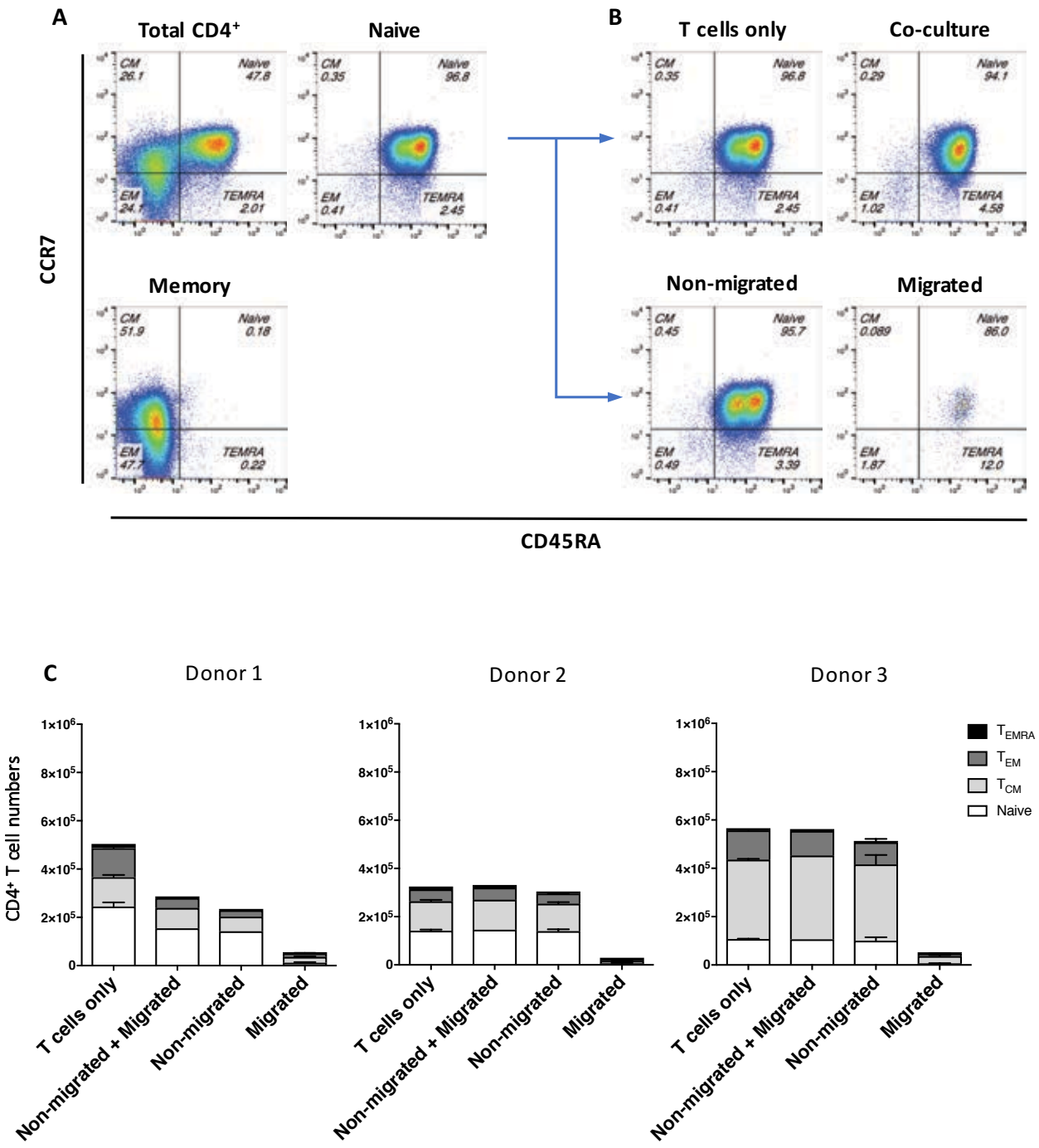


Figure 3.3.3 - No memory conversion accompanies transwell migration through hepatic epithelia. Total, naïve, and memory $CD4^+$ T cells from 2 healthy blood donors were isolated before being subjected to transwell migration assays through Huh-7 cells. **A -** Representative flow plots showing highly efficient naïve and memory isolation protocols. **B** - Proportion of isolated naïve T cells in each transwell condition that were given each naïve/memory designation as defined by CD45RA and CCR7 staining. **C -** Total $CD4^+$ T cells from 3 donors were put through transwell assays with Huh-7 and absolute numbers determined with counting beads, similar to Fig. 3.3.1 (2 technical replicates per donor, median and range shown).

a small population of cells in the T_{EMRA} gate that was not present in the other conditions. We did not think this represented a real conversion from naive into T_{EMRA} as numbers of naïve cells that migrated were very small and so more prone to signal artefacts, and of course T_{EMRA} need long-term persistent antigen stimulation to form which is not possible in our transwell assays as Huh-7 do not express MHC class II molecules (data not shown). Nonetheless to resolve this issue, we put total $CD4^+$ T cells through migration assays and used counting beads to prove no appreciable memory T cell formation occurred (Fig. 3.3.3C).

These data disprove the phenotypic induction hypothesis, due to no appreciable increase in IL-2^+ or IFN-γ^+ numbers following migration, and no conversion from naïve to memory subsets. Instead they support an '*enhanced selection*' phenomenon whereby the hepatoma monolayer specifically selects more active, effector type $CD4^+$ T cells; at an enhanced rate from that seen due to the act of migration alone.

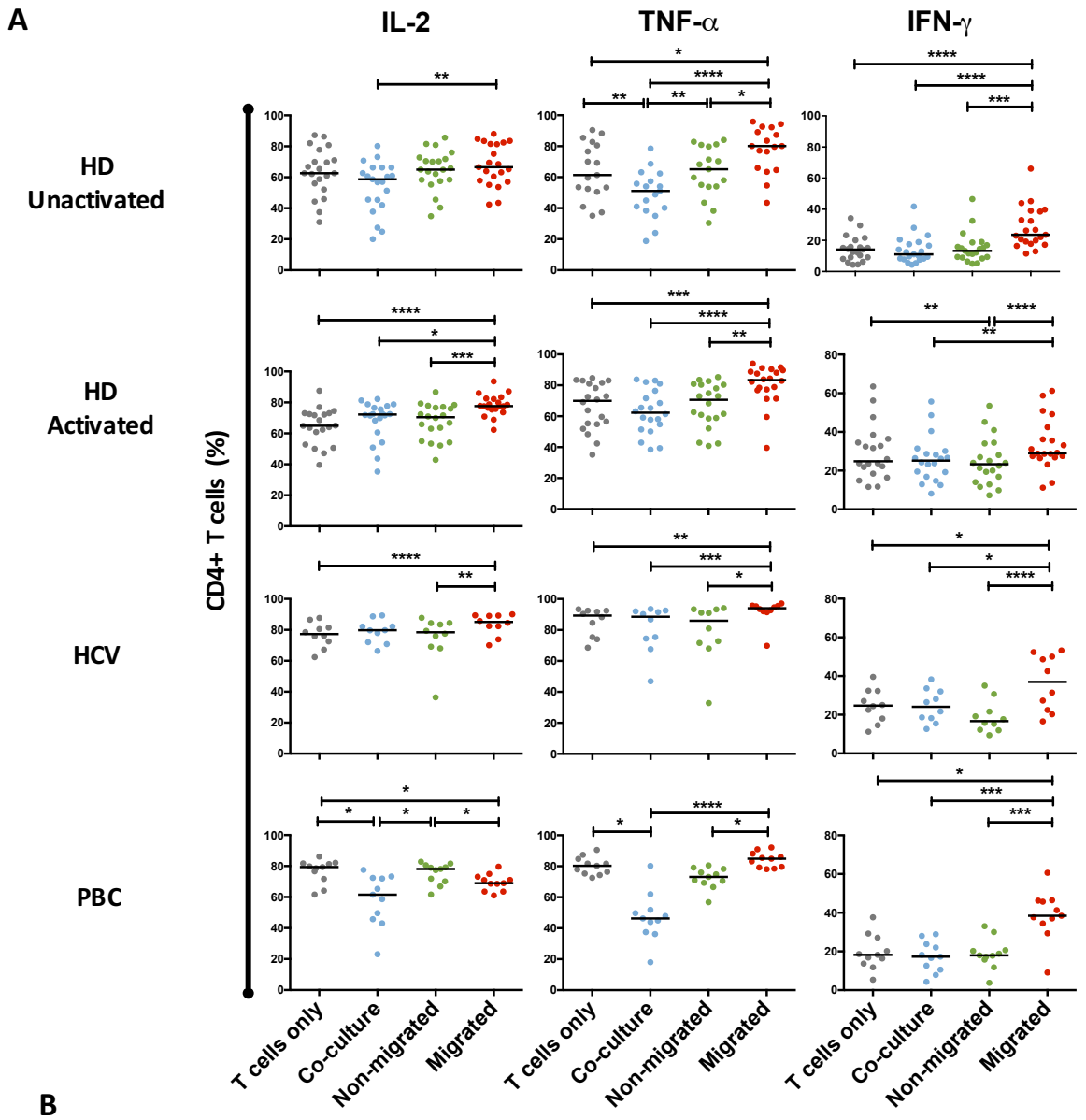
3.5 Transwell migration assays reveal cytokine profiles that discriminate healthy donors, and patients with chronic PBC or HCV infection

Thus far we have determined that migration through hepatic epithelia is able to enrich $CD4^+$ T cells with the potential to produce cytokines from the total blood population. Previous work has shown that autoimmune and viral liver diseases have different $CD4^+$ T cell components and linked functional abilities from healthy controls[410, 412, 414, 437, 465, 476, 518, 560]. Furthermore, many studies on the immune responses to viral or autoimmune liver diseases have been carried out on peripheral blood cell populations. This is as liver tissue is more difficult to access. However, researchers run the risk of missing immune phenotypes and characteristics that are only present in the liver tissue, at the site of

inflammation. We wondered whether by assaying blood T cells that can migrate into the liver parenchyma, we would be able to sidestep this limitation. Therefore, using blood from HCV and PBC patients, we utilised transwell migration assays with hepatic epithelia as a tool, in the hope of revealing intrinsic differences not possible to discern from bulk peripheral T cells, and to investigate any distinctions between disease groups.

As discussed in section 3.2, unactivated $CD4^+$ T cells from healthy donors presented with increased TNF-α and IFN-γ fractions following migration, and diminished TNF-α in co-culture. (Fig. 3.4.1A, also Fig. 3.1.1). Activated healthy donor $CD4^+$ T cells that migrated showed an additional increase in the percentage of IL-2^+ cells, but the decrease in TNF-α in co-culture was lost; and the increase in migrated IFN-γ was not as pronounced. This last aspect of the data was likely due to higher starting (T cells only) IFN-γ responsiveness masking any increases to some extent. Nonetheless, in HCV patient blood alone, the fraction of migrated cells producing all three cytokines was significantly higher following migration, despite the relatively high starting values of these cytokines. The cytokine profile showed by PBC patients was the most distinct however. Despite increased IFN-γ in the migrated cells, TNF-α showed no change from T cells only and migrated IL-2 proportions were actually less than T cell only controls. In addition, diminished IL-2 and TNF-α capacity was seen in co-culture.

These results are summarized in Fig. 3.4.1B, where significant differences are colour-coded to form a heat-map. Using this visualisation method, it was evident that once again very few distinctions could be made using static co-culture (3/13 statistical differences).



B

	Cytokine expression relative to T cells alone								
	Co-culture			Non-migrated			Migrated		
	IL-2	TNF-α	IFN-γ	IL-2	TNF-α	IFN-γ	IL-2	TNF-α	IFN-γ
HD-resting									
HD-stimulated									
HCV									
PBC									

-4	-3	-2	-1	0	+1	+2	+3	+4

Figure 3.4.1 - Transwell migration assays allow the discrimination of different chronic liver diseases according to CD4$^+$ T cell function. A - Transwell migration assays were carried out using CD4$^+$ T cells from healthy (HD) blood donors (n=21; 1 performed by D.N), previously activated (anti-CD3/CD28) healthy blood donors (n=20; 11 performed by D.N), HCV blood donors (n=10; 8 performed by D.N), and PBC blood donors (n=11; 1 performed by D.N). Following harvest and 5h PMA/Ionomycin stimulation; percentage expression of IL-2, TNF-α, and IFN-γ in each transwell condition were determined by as shown in the plots (medians + all data points). **B -** Data from **A** represented as a heat-map where statistically significant differences compared to T cells alone corresponded to intensity of colour. Standard statistical difference p value representations *, **, ***, and **** denoted 1, 2, 3, and 4 on the scale. Red and green indicated negative and positive displacements respectively.

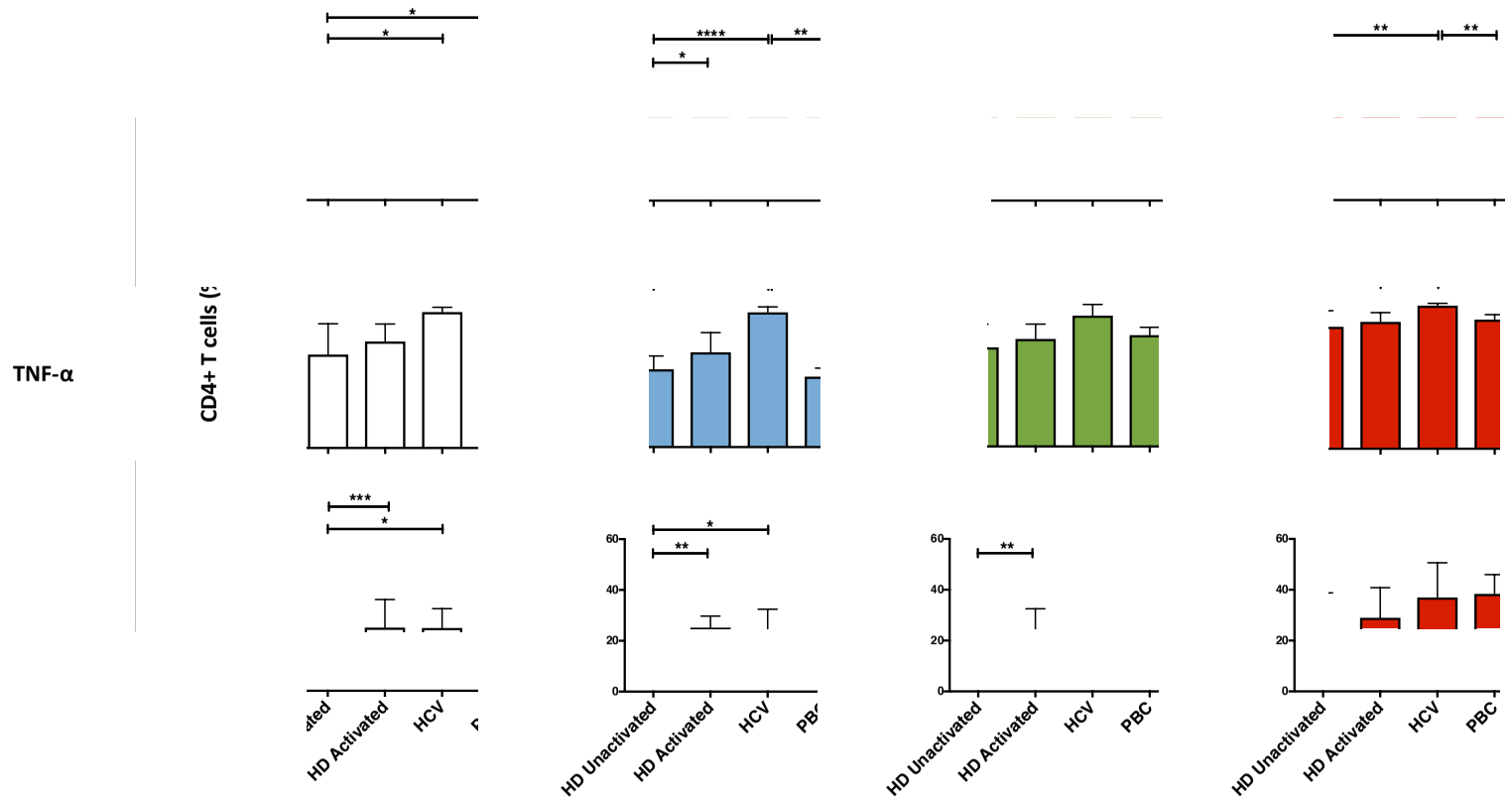


Fig. 3.4.2 - Comparison of CD4$^+$ T cell cytokine responsiveness amongst chronic liver disease patients. Shown are the
of CD4$^+$ T cells in each disease group that produced the cytokines IL-2, TNF- TNF-α, and IFN-γ. Columns from left to right sho
T cells only, Co-culture, Non-migrated, and Migrated conditions. Combined data are the same as from Fig. 3.4.1. Bar heights sho
median values, error bars - IQR.

Instead, transwell migration assays were much more efficient at separating T cells on the basis of their functionality (9/13 differences). In conjunction with these data, we compared cytokine-expressing T cells across the different healthy donor and disease groups (Fig. 3.4.2). Interestingly, HCV patient $CD4^+$ T cells were often the best producers of all three cytokines, with T cells only, co-culture, and migrated conditions all demonstrating this trend.

Combined, these data illustrate that transwell migration assays can reveal intrinsic, disease-specific differences in cytokine responses that are not perceptible through static co-culture assays alone. Reciprocally, it is possible that the magnitude and direction of these cytokine shifts may be of use to determine disease aetiology in this patient cohort.

3.6 Discussion

Addressing the three aims of this chapter, we first demonstrated that migration through hepatic epithelia in transwell assays led to increased IFN-γ, TNF-α, and IL-4 responses in the migrated $CD4^+$ T cell compartment, and an enrichment for distinct activated, T_{EM} and T_{EMRA} phenotypes. We then showed the act of trans-hepatocyte migration was not inducing this phenotype. Instead the transwell migration effect was due to an *enhanced selection* mechanism, where hepatomas preferentially allowed more activated, stimuli-responsive T cells to migrate through. Last, we identified cytokine patterns that distinguish peripheral blood $CD4^+$ T cells from viral, and autoimmune liver diseases that were only revealed through transwell migration assays (Box 3.1).

Chapter 3 – Core Findings:

- Following migration through hepatic epithelia, $CD4^+$ T cells displayed proportional increases in IFN-γ, TNF-α, and IL-4 in response to stimuli
- Migrated cells also contained greater T_{EM} frequencies and a predominantly activated phenotype
- These differences between migrated and non-migrated cells were due to preferential migration of activated, stimuli responsive T_{EM}, at an enhanced rate when compared to migration through transwell insert alone (*enhanced selection)*
- Migration assays revealed distinct cytokine production patterns that are specific to autoimmune and viral liver diseases, and healthy controls

Box 3 3.1 – Core findings from chapter 3

Effector memory and T_{EMRA} migration in preference to naïve and T_{CM} was expected as these cell types possess a greater array of homing receptors required for entry in the tissue, as befits the central versus effector memory homing paradigm[70]. We also documented an increased cell surface expression of some (CD25, HLA-DR, PD-1, Fas), but not all (CD69, CD38) activation-associated markers in migrated cells. This broadly fits with our model of more activated T cells being better able to migrate. HLA-DR is a late marker of activation[24, 26, 561], indicative of previous cognate interactions (as opposed to induced by cytokines and other non-cognate mediators)[26, 562, 563]. PD-1 and Fas, are also associated with exhaustion, and activation-induced cell death respectively; both also requiring substantial antigen contact[564, 565]. Therefore, it is possible to go even further and suggest that the specific migration-associated activation phenotype is likely due to previous antigen-induced contact, as opposed to due to exposure to non-cognate stimuli that could explain CD69 and CD38 expression [refs [29, 562, 566], and our own unpublished data]. CD25 can also be induced by both cognate and bystander interactions[567], and is also proportionally increased in the migrated pool, complicating interpretations; although perhaps antigen-triggered CD25 upregulation dominates here. The cognate-interaction hypothesis could be tested through directly comparing CD3/CD28 stimulated with

cytokine-stimulated cells for their capacity to migrate through transwells, and accompanying phenotypic marker analysis.

The fact that not all activation markers were elevated in the migrated pool is intriguing. Any increases in early activation marker CD69 in the migrated cell pool may have been dwarfed by those induced by static co-culture and the non-migrated condition (see chapter 4), but $CD38^+$ migrated cells were proportionally decreased. CD38 has interesting biology beyond a marker of activation; acting as a key regulator of endothelial transmigration, a multifunctional ectoenzyme, and a correlate of immunosuppressive function in T_{REGS}[38]. Whether any of these aspects of CD38 expression link to the decreased levels following hepatoma transmigration may constitute worthwhile avenues for further investigation. Future work adding new markers, and combining them with those already assessed will allow further discrimination of this migration-competent cell type. Therefore, migration assays revealed distinct, activated profiles that could be specifically suggestive of previous antigen encounter.

Interestingly, we found the proportion of both T_H1 (TNF-α, IFN-γ), and T_H2 (IL-4) cytokine-producing cells to be increased in the migrated T cell pool. This indicates that the hepatomas we used were not specifically selecting a particular T-helper phenotype, but rather a generally activated, effector cytokine-competent cell type. Additionally, if the general paradigms are to be believed, it would argue that neither preferential chemokine expression on T_H1 (CXCR3, CXCR6, CCR5), nor on T_H2 (CCR4, CCR8) cells are vital for migration through the monolayer[353]. Therefore, other homing molecules could be encouraging cells to migrate, such as ICAM-1 binding through LFA-1, and CXCR4-CXCL12 interactions, as myself and others in our lab have started to show [Z. Stamataki

- unpublished observations, and data not shown]. Future chemokine receptor blocking experiments and chemokine-directed chemotaxis experiments using the same transwell assay system could be performed to test these hypotheses.

The transmigration effect was not just Huh-7 specific: different hepatic and non-hepatic epithelial cell lines replicated this effect. This could mean that T cells that have migrated through epithelial cells of many organs share these cytokine profiles, although only T_H1 cytokines were tested in these experiments, and primary, non-cancerous cells would be needed to determine if this migration profile would be a feature of transmigration through non-neoplastic epithelia. This phenomenon is likely epithelial-specific, as lymphocytes do not migrate through non-activated liver endothelial cells directly[364, 568]. Our data suggest that the act of migration alone separates functionally activatable subsets – migration through bare transwell inserts resulted in similar increases in IFN-γ and TNF-α percentages (data not shown).

Using gel migration assays, we uncovered deviations from the *transwell migration effect*, with both IL-2$^+$ and TNF-α^+ migrated cells unchanged in representation. This difference was not due to the presence of collagen in the gel. Perhaps the differences were due to an effect of slower T cell velocities in the gel, or different mechanical pressures[569]. Nonetheless, attempting to replicate these findings with collagen-free gels such as matrigel would be an interesting comparison[570]. Although the transwell migration assays allowed efficient separation of disease-linked cytokine profiles, and as such are recommended for continuing use for this purpose, a better understanding of physiological T cell responses to migration through the liver parenchyma may be achieved through other models. This was the initial aim of the collagen gel system, including attempting to

polarise the hepatocyte cell lines to a more *in vivo* representative cell type as has been seen in other gel systems[570, 571]. Taking these experiments forward we could migrate autologous donor $CD4^+$ T cells through precision-cut liver slices (cut using a Krumdiek slicer). Although the liver architecture would remain intact and viable for up to 48h[572], tissue damage factors may contribute, and autologous patient blood is difficult to acquire. Alternatively, 'liver chips', biomimetics which incorporate multiple liver cell types into physiologically representative complex 3-dimensional architecture with modifiable fluid flow dynamics, may provide an attractive alternative in the future[573]. Some such systems now support the monitoring of immune cell trafficking[574]. Finally, perfusing labelled autologous T cells into liver wedges is another option being explored in our department[575]. This system would allow us to utilise unreconstructed liver components in their most natural state. Although using wedges would not specifically allow us to see the effect of hepatocytes on a T cell, T cells could be harvested from the wedge tissue and vasculature by mechanical digestion and perfusion respectively, allowing general tissue specific influences on T cell biology to be studied.

Initially, we wondered whether the transwell migration effect was due to the hepatic epithelia altering the biology of the T cells. This was a reasonable assumption based on the fact that T cells only, co-culture, and non-migrated conditions all displayed a similar phenotype, with migrated cells being the only changed population. Logically, we would expect reflective changes in the non-migrated population if a selection effect was responsible. Also, it is possible that hepatocytes could have had made non-cognate impressions on the function and phenotype of a T cell, as shown by others in the past[347, 576, 577]. However, problems in this interpretation arose when trying to explain the shift towards memory phenotypes as antigen recognition was not occurring in our system, and

our assays were all under 24h in duration. Assessing the percentages of T cells that migrated, and looking at absolute T cell numbers was key in settling this dispute. Observing very few T cells migrate at resting conditions, we were able to explain the lack of discernible corresponding changes in non-migrated populations as this population was far greater in numbers. Data showing no new appearance of cytokine^{+}, T_{EM}, or T_{EMRA} numbers in the migrated pool also served to confirm this. Therefore, we demonstrated that specific T cell migration through Huh-7 cells was a selection mechanism, not an induction phenomenon. We termed this an *enhanced selection* due to the fact that migration efficiency was increased in the presence of Huh-7 cells. It is noteworthy that *enhanced selection* was not observed for B cells, which required CXCL12 to migrate through Huh-7 monolayers [Z. Stamataki – unpublished observations]. T cell-specific *enhanced selection* is interesting as Huh-7 cells represent an additional physical barrier, so perhaps are encouraging migration through adhesion receptor expression and/or providing cytokine signals to the T cell migration candidates. Both possibilities could be tested using blocking experiments as mentioned above. Overall, recognising that hepatic epithelia select particular activated T cells, rather than induce phenotypic changes, will allow us to further understand the influences of a hepatocyte on a T cell's biology.

We next profiled the cytokine responses of migrated cells from HCV and PBC patient blood. Relatively little is known about functional alterations of intrahepatic T cells in these diseases, despite T cells being central to the pathology of both[414, 465]. Issues with the use of liver tissue include poor availability, often insufficient quantity, and low accessibility of tissue at different disease stages[506, 547, 555]. Mouse models are often poor substitutes for human chronic liver disease, and the use of chimpanzees has significant ethical and financial considerations[547, 578]. Although the use of human peripheral blood sidesteps

many of these issues, the main challenge is reliably identifying disease-linked populations. MHC-tetramers have been incredibly important tools in aiding the profiling antigen-specific T cells in both diseases[481, 486, 550, 556, 557], but such studies rely on prior knowledge of the antigens and MHC haplotypes involved[555]. Furthermore, the often extremely small frequencies of viral- or autoantigen specific cells in peripheral blood makes detection difficult or even impossible[555, 556, 579], requiring enrichment steps that may alter native cell biology[555, 580]. The most advanced advance on these techniques are the use of activation markers such as CD25 and CD40L as indicators of response to viral antigen stimulation of T cells from chronic HCV-infected blood[479, 484, 549]. These activation markers are thought to emerge even if no proliferative or cytokine responses are seen, and therefore are well suited to studying the exhausted cell populations seen in HCV[555]. In our study, we offer an alternative on this theme. We exploit the inherent activated status of migration-capable $CD4^+$ T cells to investigate not only antigen-specific T cells, but the whole pool of activated T cells that may enter the site of inflammation, the liver parenchyma, in HCV and PBC.

We observed that $CD4^+$ T cells from different chronic liver diseases revealed different cytokine response patterns when put through transwell migration assays. These differences were not evident with static co-culture, reinforcing the usefulness of transwell migration as a tool to study activated T cells in peripheral blood. Moreover, the cytokine profiles revealed by transwell assays appeared distinct in each disease, which offers potential for their use in disease determination, and the further study of migrated populations that could be important in disease progression and outcome.

While these data alone do not prove migrated cells are involved in pathogenesis, it provides links to their role in the disease process that can be tested further. Similar to the activation marker studies discussed in the previous paragraph, we have interrogated an activated population with the ability to enter the liver parenchyma, so we are likely to be enriching for disease-specific cells. Interestingly, the profiles as revealed by transwell assays appeared to be disease-specific, raising the possibility of the specific involvement of migrated cells in disease pathogenesis. Such a role for non-viral specific intrahepatic $CD8^+$ T cells has been proposed previously in chronic HBV infection. Failure to establish an efficient initial T cell response in some patients appeared to lead to inflammation-driven recruitment of non-specific cells that contribute to liver damage but not viral clearance[496, 506]. Alternatively, transwell migration assays could be used for disease determination, potentially increasing the readouts for the efficacy of therapeutic interventions in liver disease patients. In contrast to other studies that showed weak antigen-specific type 1 $CD4^+$ T cell responses in chronic HCV infection[476, 479, 482], we observed more robust responses in HCV infection than in healthy blood. Differences could be down to the presence of many non-HCV specific clones that are not functionally inhibited in the migrated pool (that could still be involved in HCV damage through bystander activation or other mechanisms), or our use of mitogens to coax responses from cells, overriding the exhausted or anergic phenotype. Future address of type 2, type 17, circulating T_{FH}, and regulatory responses would allow determination of any skewing of helper subsets in these diseases, as has been suggested in HCV and PBC[411, 504, 510, 552, 554]. Therefore, we hypothesise that by modelling migration into the parenchyma, we are picking out disease relevant T cells from the blood, without need to sample liver tissue.

It would be interesting to examine whether other chronic liver diseases also showed distinct patterns. We could start by using other viral and autoimmune patient donors, HBV and PSC, before moving onto dietary injury liver diseases such as ALD and NASH. Further assay of the migrated populations in each disease would also help determine if this pool was relevant to the disease pathology. For example, as a first step, harvested migrated T cells from HCV patients could be stimulated with HCV viral antigens in antigen presentation assays in order to measure antigen-specific responses. Similar experiments could be carried out with PBC patient T cells, using PDC-E2 autoantigens[414, 557]. If the migrated population is found to be disease-specific we could address outstanding questions such as what is the relevance of the depleted IL-2 production capacity in migrated PBC blood T cells? Could this reflect an exhausted T cell phenotype, no longer able to support survival and self-replication; or perhaps a reduced ability to promote T_{REG} maintenance, resulting in a less controlled pro-inflammatory response? Similarly, does the increase in all cytokines in migrated HCV patient blood T cells suggest elevated bystander activation in this population, like that described in HBV infection[496]? Thus, future work investigating antigen specificity, *in vitro* impacts on accessory cells, and integrated phenotypic and functional characterisation would help address disease-specific alterations in chronic liver diseases.

To increase biological relevance, we aim to use primary human hepatocytes (PHH) in future studies in order to remove any potential tumour effects. Modelling parenchymal T cell migration need not stop there as any or all of precision cut slices, microchips, or liver wedge systems could be used as more realistic models of the liver microenvironment, as discussed above. The importance of multi-parametric staining and bioinformatics tools would also be advantageous. Lastly, as mentioned, combining cytokine stains to look at

multifunctionality may help distinguish migrated T cells from different diseases further; while combining multiple activation markers would allow for the discrimination of precise phenotypes that are able to migrate into the liver parenchyma.

In conclusion, we have used transwell migration assays as a tool to separate out specific activated effector type CD4+ T cells from peripheral blood with high cytokine producing potential, and clear consistent variations on this theme were apparent across different liver diseases. These data led us to propose that the migrated populations are important to the specific disease processes. Further work with primary hepatocytes, additional disease groups, and antigen responsiveness of the migrated cells; will help us to determine physiological relevance, applicable breadth, and disease relevance respectively. Determining these factors will help us understand how migration into the parenchyma changes T cells on a population level, which in turn may help to understand intricate mechanisms behind pathology in different chronic liver diseases.

Chapter 4 – Hepatocyte co-culture induces an activation-independent $CD69^{INT}$ phenotype in CD4+ T cells

4.1 Introduction – CD69

CD69 (also historically called activation inducer molecule [AIM]) is a homodimeric transmembrane glycoprotein and member of the type II C-type lectin family[29, 581]. Located in the NK gene cluster on chromosome 12 (chromosome 6 in mice), CD69 shares structural, but not functional homology with NK activatory and inhibitory receptors CD94, and NKG2 molecules[29]; Its expression is controlled by multiple transcription factors including nuclear factor kappa B (NF-κB), erythroblast transformation-specific related gene-1 (ERG-1), activator protein-1 (AP-1), and hypoxia inducible factor 1α (HIF-1α)[30, 582]. CD69 is best known as an early activation marker, upregulated on all leukocytes and platelets following stimulation[28]. However, this molecule has a number of other reported roles; most notably in the control of leukocyte migration and tissue residence, propagating regulatory cell development, and in the overall regulation of immune responses[29]. The importance of CD69 as a key marker of T_{RM} has already been discussed in detail (section 1.2), but its other roles are covered here.

CD69 is particularly important in controlling the migration of T cells. By antagonising tissue egress receptor S1PR1, CD69 prevents entry to the efferent blood and lymph, thereby increasing dwell time in both SLOs and NLTs[71]. This is not only important for precisely controlling the length of stay in SLOs, which in turn allows naive T cells time to receive optimum stimulation and differentiation cues[31]; but is also vital for the development and maintenance of T_{RM} cells as discussed in section 1.2 [110]. CD69 also affects migration through control of chemokine expression patterns and associated chemokine responsivity as demonstrated in CD69-deficient mice[583]. Additionally, CD69 is important in both thymic and peripheral T_{REG} development. tTregs derive specifically from $CD69^{HI}$ progenitors[584], and CD69 also appears important in mediating efficient pT_{REG}

induction, and enhancement of T_{REG} function[585, 586]. This process is likely through both CD69-triggered TGF-β production, that is needed for T_{REG} polarisation[581, 587]; and the triggering of the the JAK3/STAT5 (JAK – Janus kinase; STAT – signal transducer and activator of transcription proteins) signalling pathway downstream of CD69 that promotes T_{REG} development while simultaneously inhibiting effector T_H1 and T_H17 differentiation[30, 588].

In its best-known role, CD69 is the earliest T cell activation marker. Not expressed on resting T cells, CD69 expression occurs as early as 2 hours following cellular activation, peaks between 18 and 24 hours, then declines[27, 28]. This is in contrast to other activation markers CD25, CD38 and HLA-DR that arise progressively later after the initial activation event[24-26, 561], a difference that has been attributed to the presence of pre-formed intracellular CD69 protein stores in resting lymphocytes[589]. CD69 is not only upregulated through cognate antigen recognition; but also via cytokines such as type I interferons, TGF-β, and TNF-α; presence of local glucocorticoids; hypoxia; and interactions with the microbiota[29, 30, 171, 582, 585, 590]. Functionally, the role of CD69 is less clear – is this molecule simply a consequence of cellular activation, or is it contributing to it*?* In the absence of a known ligand for CD69, early studies used antibody-mediated CD69 ligation to study the CD69 signalling pathway. *In vitro* application of these stimulatory antibodies induced proliferation, generation of the pro-inflammatory cytokines IL-2 and TNF-α, increased glycolysis, and increased calcium flux[27, 581, 591-594]; but also, TGF-β synthesis[595, 596]. CD69 was also required for T cells to stimulate macrophages[597]. Therefore, from these data it appeared CD69 was generally acting as a pro-inflammatory, activatory receptor.

However, *in vivo* data paint a different picture, indicating CD69 does not act as a costimulatory molecule[581, 598]. Constitutive T cell CD69 expression did not lead to enhanced pro-inflammatory responses[581, 599, 600], and knocking out CD69 demonstrated no impairment of T cell activation or proliferation[598, 601]. However, CD69-deficient mice often show dysregulated and elevated pro-inflammatory immune responses, as evident in mouse models of asthma[602], arthritis[595], IBD[583, 585], myocarditis[603], bacterial clearance[604], and cancer[596, 605]. However, examples to the contrary have also been less frequently reported[606-608]. These discrepancies could be down to differences in mouse models used, that in turn are differentially more dependent on different immune cellular and molecular components, but in any case, these studies combined highlight the great potential of CD69 as a complex, multipurpose regulator of adaptive immune responses. Furthermore, the arguments for CD69 acting as an immune regulator are not limited to mouse models. Anti-CD69 autoantibodies have been detected in a subset of human RA patients, and the presence of these directly correlates with disease severity[609]. Therefore *in vivo* prevailing theory dictates that CD69 is acting as a regulator of inflammation.

Recent data has uncovered candidate endogenous ligands for the CD69 molecule that begin to help explain some of these properties at a molecular level. Two candidate CD69 ligands have been proposed – Galectin-1, and Calprotectin (S100A8/S100A9 complex)[610, 611]. Expressed on DCs and macrophages, Galectin-1 (through this interaction with CD69) promotes JAK3/STAT5 activation and T_{REG} development, concomitant skewing away from T_H17 lineages, and an increased T cell IL-10 production through the AHR pathway[610, 612]. Calprotectin is a calcium-binding protein expressed by various myeloid cells. Its ligation of CD69 in human PBMCs also resulted in a skewing towards T_{REG} cells, accompanied by enhanced TGF-β production[611]. In addition, CD69

can form lateral (cis) interactions with the system L1 amino acid transporter complex LAT1-CD98, as well as S1PR1[613, 614]. CD69 association with LAT1-CD98 increases uptake of tryptophan and other amino acids, promoting mTOR pathway activation and HIF-1α activation, as well as activity of AHR[30, 613, 615]. Intense mTOR signalling is required for T_H1, T_H2, and T_H17 differentiation[30], while low intensity signalling is necessary for T_{REG} development[616]. Conversely, AHR signalling promotes T_H17 differentiation and HIF-1α downregulation[613, 617]. Moreover, S1PR1 directly represses T_{REG} lineage commitment, enhancing signalling through the mTOR pathway[618]. Perhaps CD69-mediated mTOR activation through LAT1-CD98 is sufficient for the low level mTOR signalling necessary for T_{REG} promotion, whilst CD69-mediated S1PR1 downregulation prevents this signal becoming too intense and promoting effector cell differentiation. Combined, it is becoming clear that CD69 can precisely tune immune responses through complex signalling pathway interactions we are just beginning to grasp; promoting or repressing effector cell differentiation based on cellular context, putative ligand availability, oxygen content, prevailing cytokine signals and the metabolite milieu [reviewed in ref [30]].

In addition to aiding the differentiation of conventional T_{REG} cells, CD69 also marks a novel regulatory T cell subset in its own right[29]. In a mouse model of systemic lupus erythematosus (SLE), $CD69^+$ $CD4^+$ T cells suppressed their $CD69^-$ counterparts[619]. Han et al. built on this work, demonstrating these $CD69^+$ suppressive $CD4^+$ cells are induced by liver tumours in mice, but do not express FoxP3, and suppress through membrane-bound TGF-β (mTGF-β)[620]. Similar $CD69^+$mTGF-$\beta^+$$FoxP3^-$ $CD4^+$ T cells have since been detected in human blood, with suppression *in vitro* shown to be TGF-β and IL-10 dependent[621]. Furthermore, $CD69^+$ T cells were present in HCC patient blood and liver where they were associated with worsening disease progression, whereas the frequency

of these cells correlated with lower risk of developing graft-versus host disease post-transplantation[622-624]. Crucially, Han et al. showed these cells were induced by liver tumours and suppress normal anti-tumour responses via mTGF-β[623]. Finally, recent data have indicated it may be possible to distinguish regulatory from recently activated T cells. Stable long-term expression of CD69 controlled by non-canonical NFκB signalling is thought to mark regulatory or tissue-resident phenotypes, whereas the classic transient CD69 expression pattern mediated by the canonical NFκB signalling pathway is a clear giveaway of recently activated cells[29, 625, 626].

Thus, CD69 is a multifaceted molecule, with the ability to act as a marker of activation, tissue residency, and of regulatory cell types. In this chapter, we will systematically address the activation, and regulatory angles to determine what CD69 upregulation following hepatic epithelia contact means for the T cell. The possibility of co-culture-induced CD69 upregulation representing an aspect of tissue residence formation will be discussed further in Chapter 5. With this in mind, we concocted the following aims:

i. Establish whether CD69 upregulation on $CD4^+$ T cells by hepatic epithelia represents an activation event
ii. Determine if these $CD69^+$ $CD4^+$ T cells acquire linked suppressive functions
iii. Identify the possible mechanisms behind hepatocyte-induced CD69 upregulation

4.2 Co-culture with hepatic epithelia promotes an activation-independent CD69 upregulation on $CD4^+$ T cells

Building on previous work (section 3.3), we first showed that overnight co-culture with Huh-7 cells imbued blood-derived $CD4^+$ T cells with CD69 expression (Fig. 4.1.1A,B). This was consistent across all 35 healthy blood donors. While $CD4^+$ T cells cultured alone possessed an average of only 1.2% CD69 expression (range 0.3 – 5.5%), this was increased to a median of 60% (range 11.4 – 99.1%), corresponding to an average fold increase of 51.8 times (range 5.8x – 143x). Importantly though, this upregulation was always to an intermediate expression level (Fig. 4.1.1A). Comparable induction of CD69 expression was observed not only with different hepatocyte cell lines (HepG2, Hep3B), but also in non-hepatic epithelia (A549, AdAh, Caco-2, HeLa) (Fig. 4.1.1C). Thus, we demonstrated a robust and consistent upregulation of intermediate-level CD69 expression is triggered on $CD4^+$ T cells following contact with both hepatic and non-hepatic epithelia.

We next asked what this CD69 upregulation represented. CD69 is best known as a marker of early activation[581], so we decided to investigate this possibility first. Therefore, we compared CD69 upregulation by Huh-7 cells with conventional T cell activation stimuli anti-CD3/CD28, PMA/Ionomycin, and PHA. We also looked at sequentially later markers of activation CD25, CD38, and HLA-DR, for up to 7 days in culture. Although the percentage of cells expressing CD69 in Huh-7 co-culture was similar to the activation stimuli; the expression levels per cell were not increased from the few $CD69^+$ T cells cultured alone (Fig. 4.1.2). This was in stark contrast to the high median fluorescence intensity (MFI) seen following T cell stimulant addition. Furthermore, expressions of

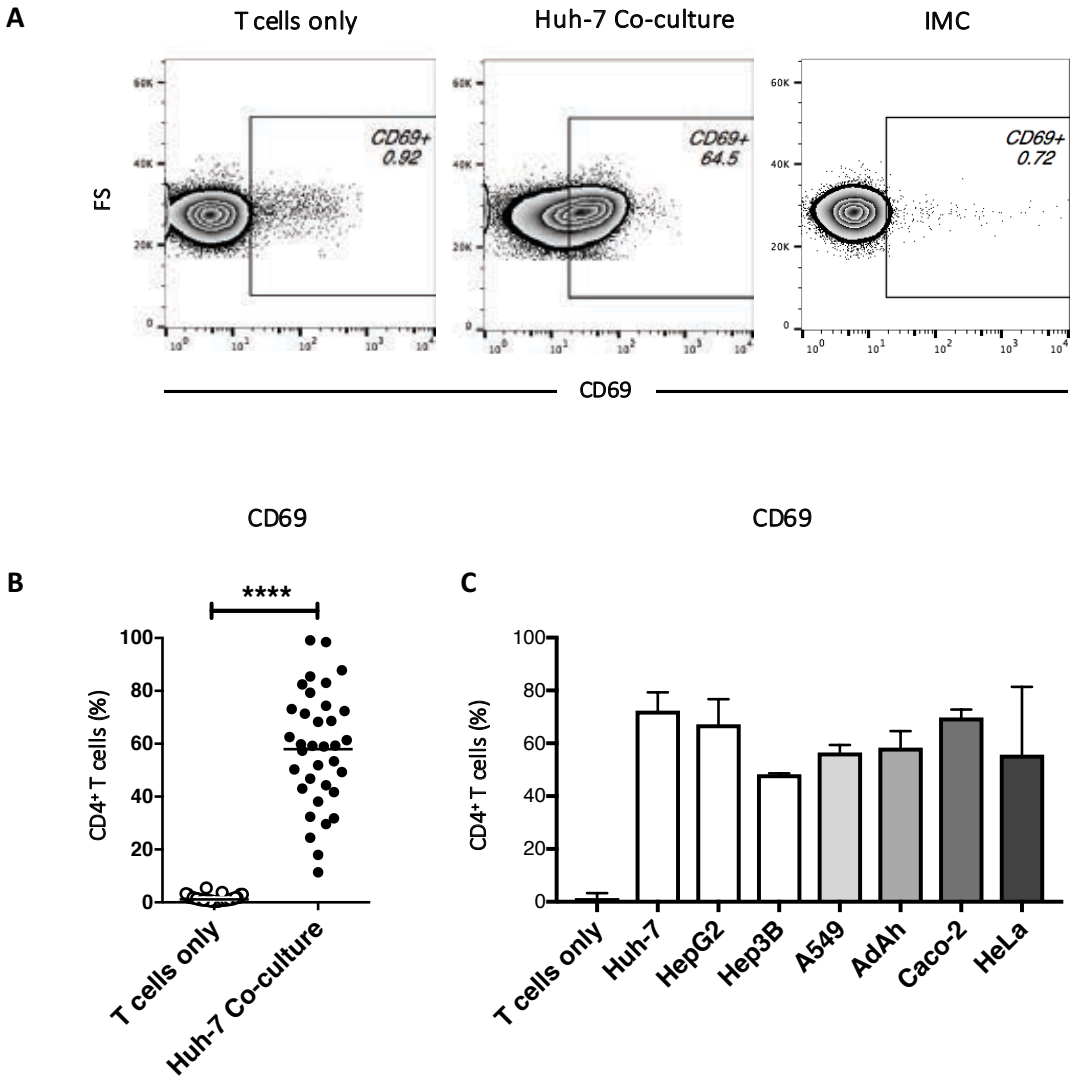


Figure 4.1.1 - CD69 expression induced on blood-derived CD4$^+$ T cells co-cultured overnight with both hepatic and non-hepatic epithelial cell lines. CD4$^+$ T cells were purified from healthy donor peripheral blood, and cultured with hepatic/non-hepatic epithelia overnight, before CD69 determination by surface staining and flow cytometry. **A -** Representative flow cytometry plots showing CD69 expression following co-culture with Huh-7 cells, but not in T cells cultured alone (IMC control also shown). **B -** Combined CD69% expression data and median of CD4$^+$ T cells from 35 donors cultured +/- Huh-7 cells. **C -** Median (+IQR) % CD69 expression data following overnight culture with hepatic (white bars), or non-hepatic epithelia (grey bars). Epithelial cell lines same as used in Fig. 3.1.2. N=3 for all cell lines (except Hep3B - n=2) 2/3 experiments for part C carried out by F.A..

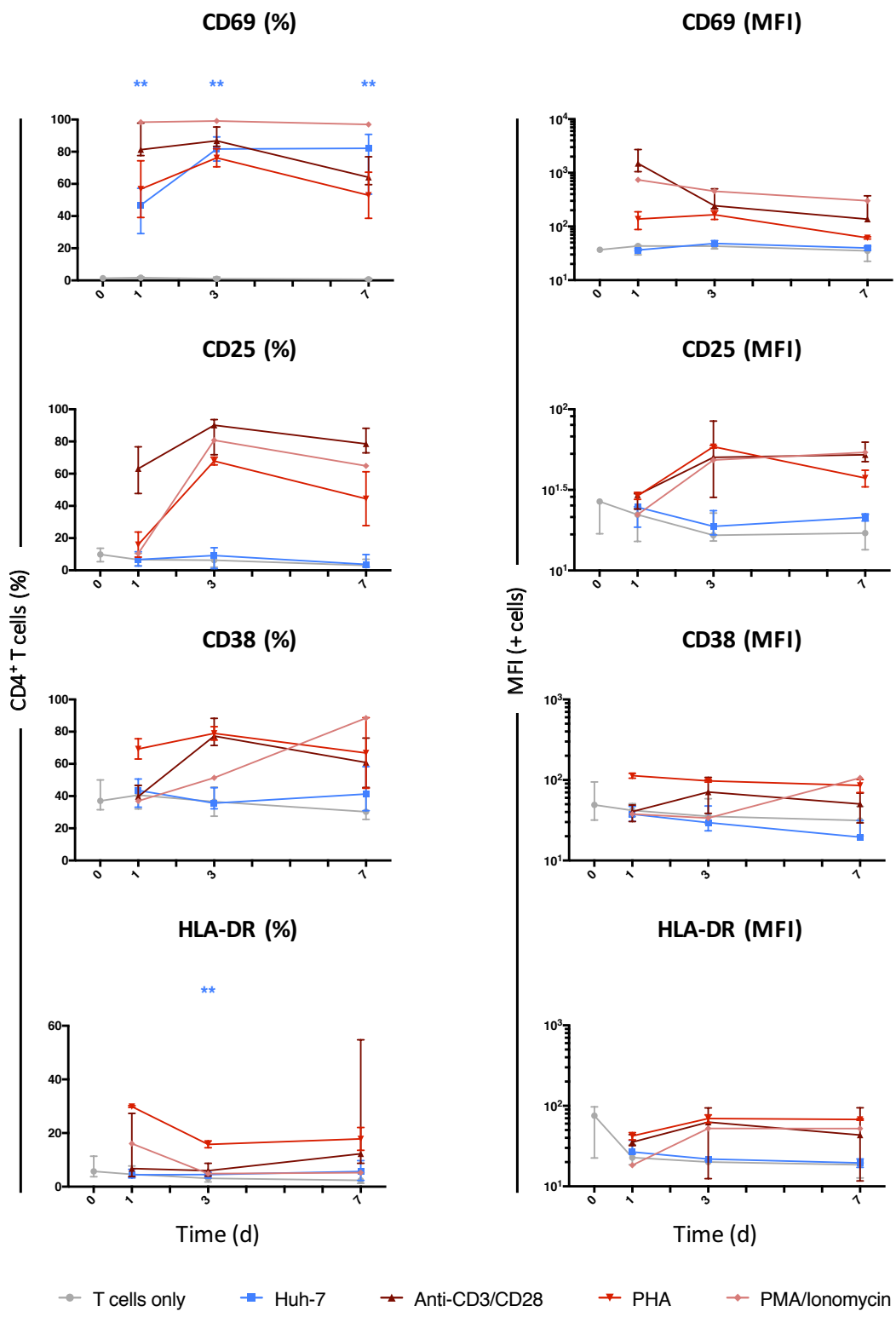


Figure 4.1.2 - Co-culture-induced CD69 upregulation is distinct from classical T cell activation. $CD4^+$ T cells were cultured alone; with Huh-7 cells; or with classical activation stimuli (anti-CD3/CD28 beads, PHA, or PMA & Ionomycin) for 7 days. Expression patterns of CD69, CD25, CD38, and HLA-DR (top to bottom panels) were assessed by staining and flow cytometric analysis at 4 different time points as shown. Both percentage marker expression (left panels) and median fluorescence intensity of the marker-positive cells (right panels were shown). Only T cells only and Huh-7 conditions were statistically compared at each time point due to donor number considerations. T cells only, Huh-7 - n=8-10 for % data, n=3-4 for MFI data; anti-CD3/CD28 beads - n=4-5 for % data, n=3-4 MFI data; PHA - n=2 all data; PMA & Ionomycin - n=1 all data. 1 donor time-course (for CD69, CD25 and HLA-DR) carried out by F.A.

CD25, CD38, and to a lesser extent HLA-DR were only increased with the three activation stimuli, by both percentage and MFI. Intriguingly, combining co-culture and activation stimuli together actually decreased the % CD69 upregulation from Huh-7 co-culture alone, but only with PHA and PMA/Ionomycin, not anti-CD3/CD28 stimulation (Fig. 4.1.3). Similarly, all activation stimuli combined with Huh-7 co-culture decreased CD38% expression, and no CD25 upregulation was seen when PMA/Ionomycin and Huh-7 cells were used together. Therefore, these data argue against Huh-7-induced CD69 expression representing conventional T cell activation, due to the relatively low CD69 expression levels, and failure to induce other activation markers compared to conventional T cell activation stimuli.

We also observed that Huh-7 co-cultured $CD4^+$ T cells did not proliferate appreciably over 7 days, whereas as expected, activation stimuli triggered robust proliferation (Fig. 4.1.4A). The small amount of apparent cell division that was noted in the co-cultured cells was mostly due to the $CD69^-$, not the $CD69^+$ subset (Fig. 4.1.4B). In any case, it appears that this was an artefact of staining that was only seen in co-cultured conditions because cells did not halve their fluorescence with each division. Rather some appeared to not have taken up the dye efficiently as the cells were all at the negative end of the divided gate (Fig. 4.1.4C). We next illustrated that $CD69^+$ T cells generated by Huh-7 co-culture did not preferentially apoptose compared to their $CD69^-$ counterparts (Fig. 4.1.4D). Although quite a lot of apoptosis was observed by day 7, this can be accounted for by the lack of IL-2 added to the cultures alongside the metabolically active Huh-7 cells depleting nutrients from the media. In order to compare CD69 upregulation in co-culture with that associated with conventional activation, we were also interested in further study of the dynamics of CD69 as an activation marker following conventional activation stimuli. We

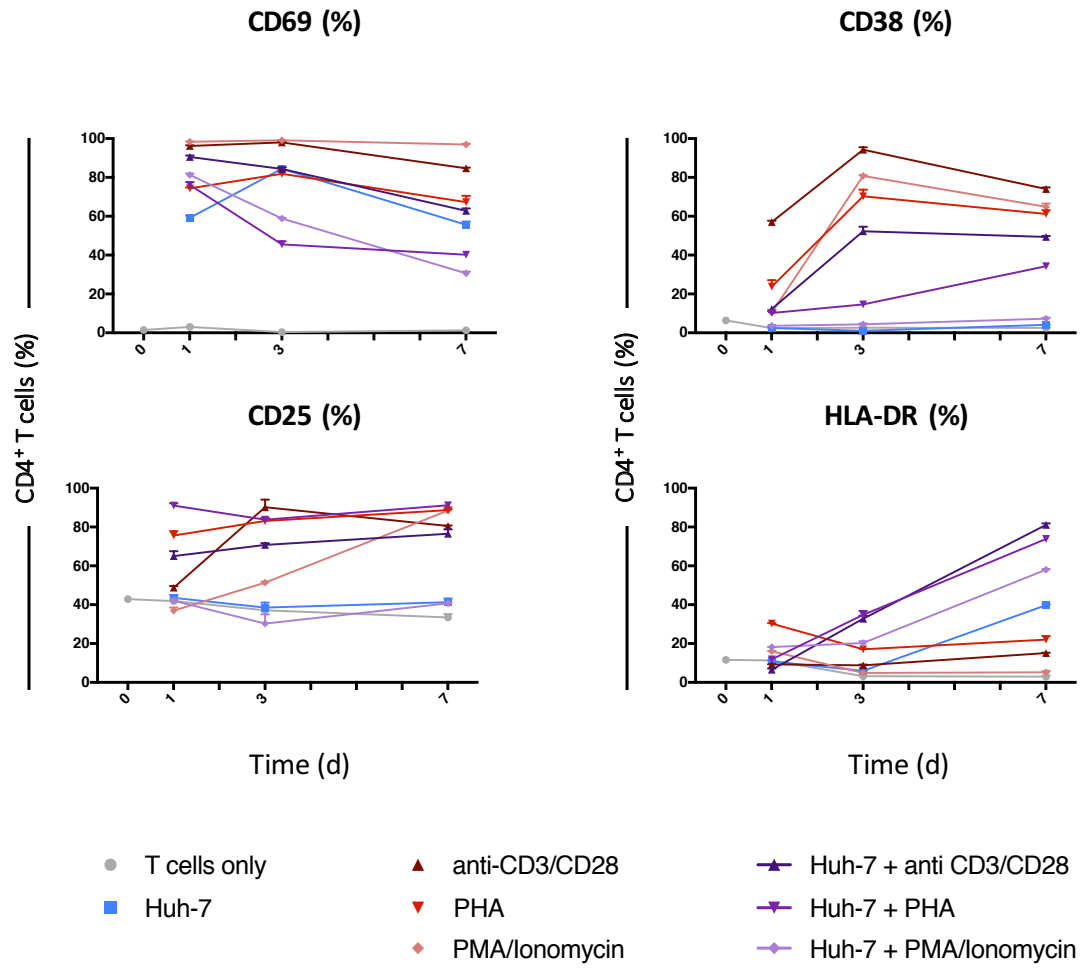
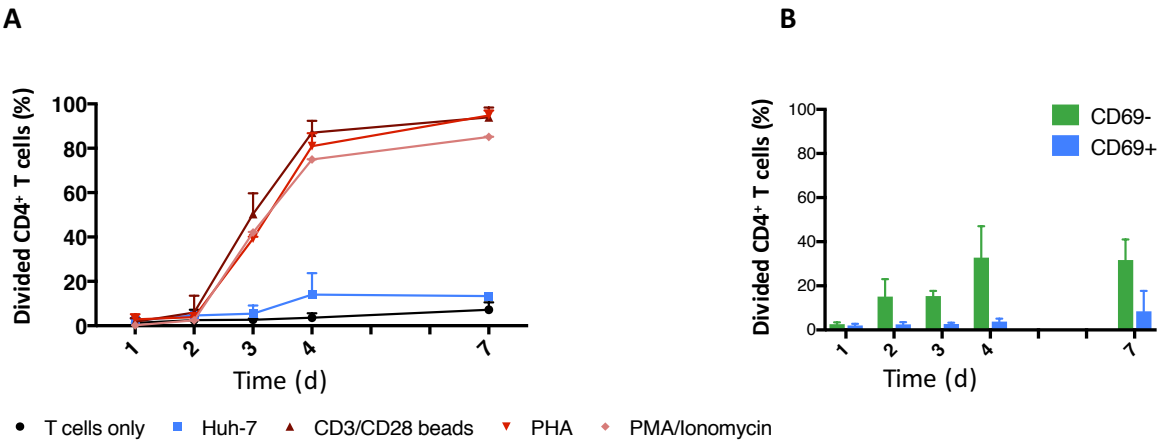
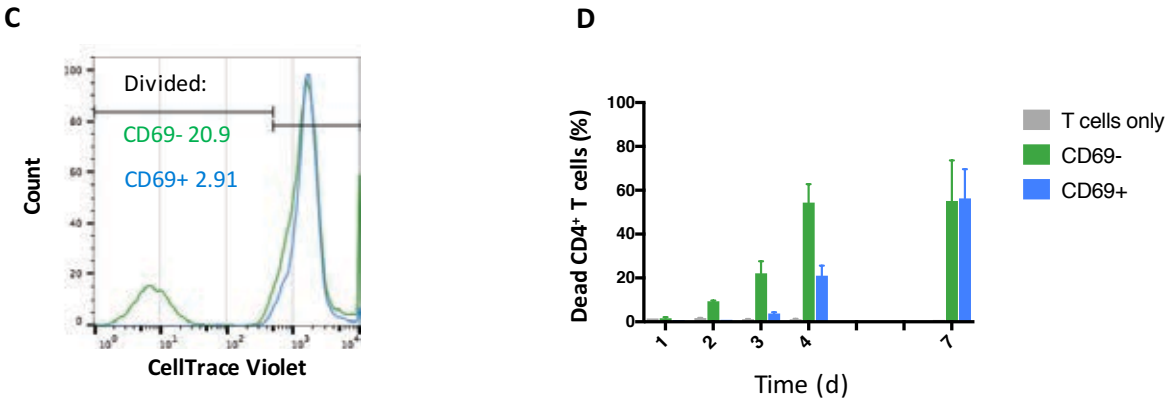


Figure 4.1.3 - Combining hepatocyte co-culture and activation stimuli has unique effects on T cell activation status. Experiments designed and results presented as in Fig. 4.1.2, but with activation stimuli and co-culture combinations also included. All graphs show % marker expression, n=1 (2 technical replicates).





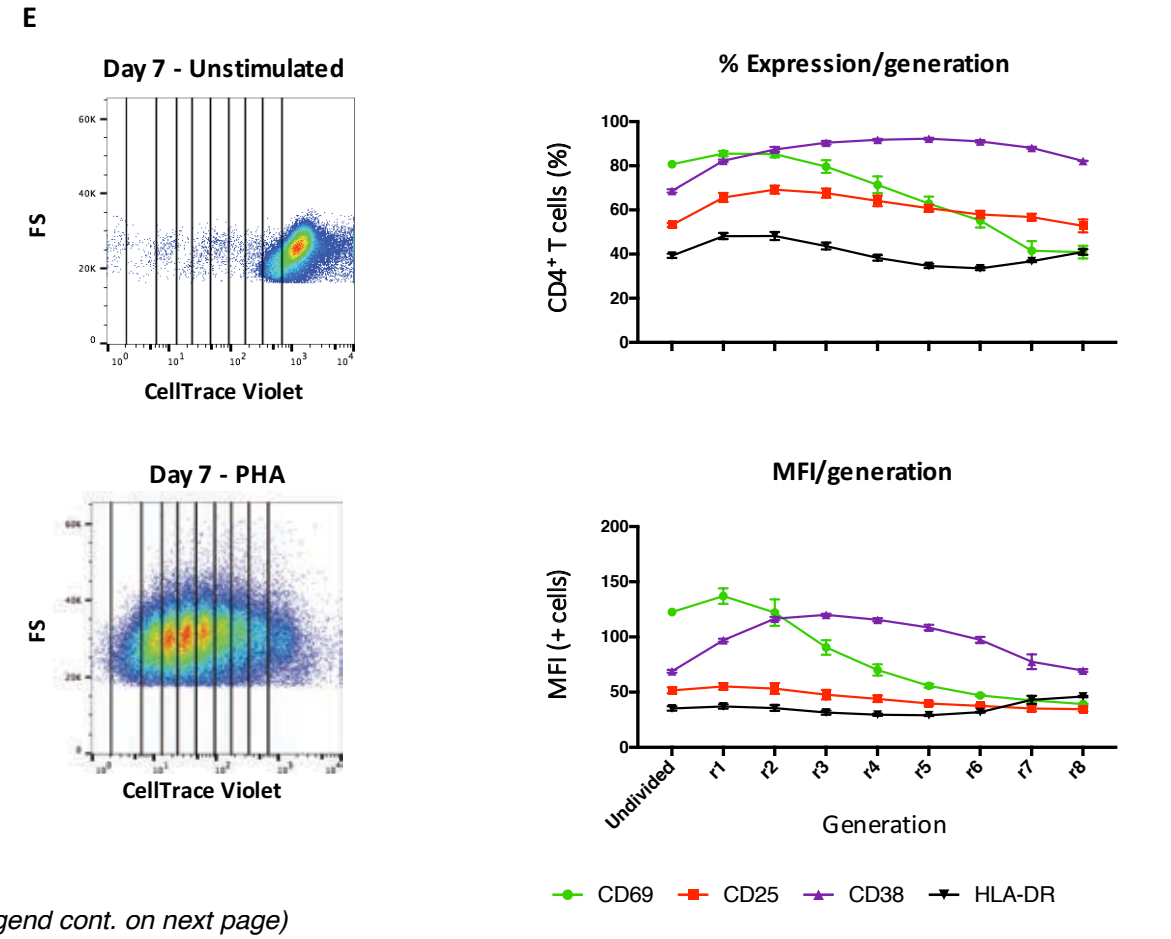


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Figure 4.1.4 - Co-cultured CD69$^+$ T cells do not proliferate or apoptose more than their CD69$^-$ counterparts. A - % Proliferation over time as assessed by CellTrace Violet staining following CD4$^+$ T cell culture alone, with Huh-7 hepatomas, or with the different activation stimuli (n=2, median and range shown). **B -** % division amongst CD69$^+$ and CD69$^-$ cells in co-culture (n=2, median + IQR). **C -** Example flow cytometry plot showing staining artefact in some Huh-7 co-cultured T cells when assessing proliferation (day 4 Huh-7 co-culture example shown). **D -** % of dead CD4$^+$ T cells over time as assessed by Zombie Aqua staining. T cells only, Huh-7 co-cultured CD69$^+$, and Huh-7 CD69$^-$ cells shown (n=1 [2 technical replicates], median + 95% CI). **E -** Activation marker expression per divided generation. Flow cytometry plots show examples of cell division at day 7 with or without PHA. Each division shown in a different gate. Adjacent line graphs show how expression of each activation marker changes with each generation in both % (top) and MFI (bottom). N=1 (2 technical replicates), median + range.

chose PHA as our model proliferation stimulus as it generated the clearest and the most divisions over 7-day culture. We noted that CD69 expression gradually weakened with each generation after the first division (Fig. 4.1.4E). CD25 took until division 3 to start declining, while CD38 took until division 4-5. HLA-DR declined at division 2, but at division 7 started to rise again. Lastly, we investigated the stability of hepatocyte-induced CD69. Removing $CD4^+$ T cells from co-culture, we noted that even after 72h there was no appreciable drop in CD69% expression, hinting at stable imprinting (Fig. 4.1.5). These data combined also suggest that Huh-7-induced CD69 upregulation was not part of an activation programme as no increases in proliferation or apoptosis were seen, and no drop in T cell CD69 expression was observed following removal of the 'stimulatory' agent (i.e. the hepatocyte).

So far we have demonstrated that CD69 upregulation occurs with epithelial cell lines. However, this falls short of demonstrating physiological significance with a primary cell type. Further, we wondered whether other liver cell types were capable of inducing the same phenotypic change. Therefore, we used primary BEC and HSEC cells, as well as the hepatic stellate cell line LX2 as our potential inducers. No CD69 upregulation was seen following co-culture with HSEC (Fig. 4.1.6A). Following BEC co-culture, little upregulation was seen overnight, but by day 3 a clear substantial induction was evident. LX2 co-culture resulted in no upregulation at day 1, but a minor change at day 3 onwards. Notably, all cell types that produced an upregulation, did so to an intermediate expression level, as evidenced by comparison to anti-CD3/CD28 stimulation where the upregulation was high. Finally, in an effort to lay groundwork for future studies, we performed mixed cultures of combinations of these cell types. These data would give us an idea of how a T cell would regulate CD69 expression following contact with multiple cell types as it

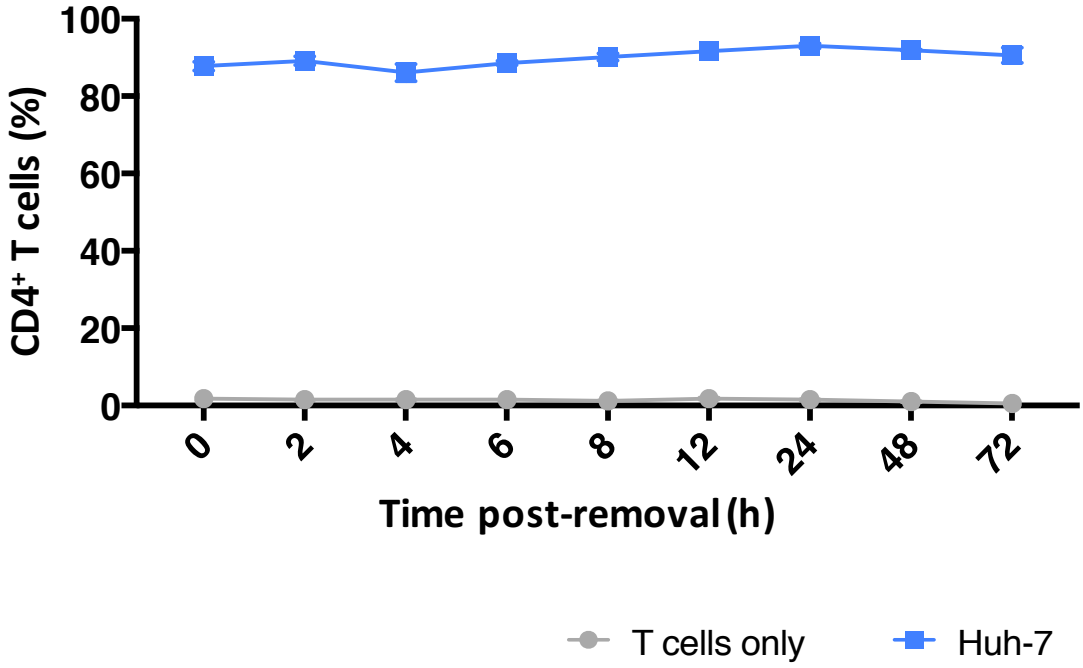


Figure 4.1.5 - CD69 expression does not diminish short term following removal. $CD4^+$ T cells were co-cultured with Huh-7 and then carefully harvested and placed into culture alone. Expression of CD69 was then monitored for up to 3 days. N=1 (2 technical replicates), median + range.

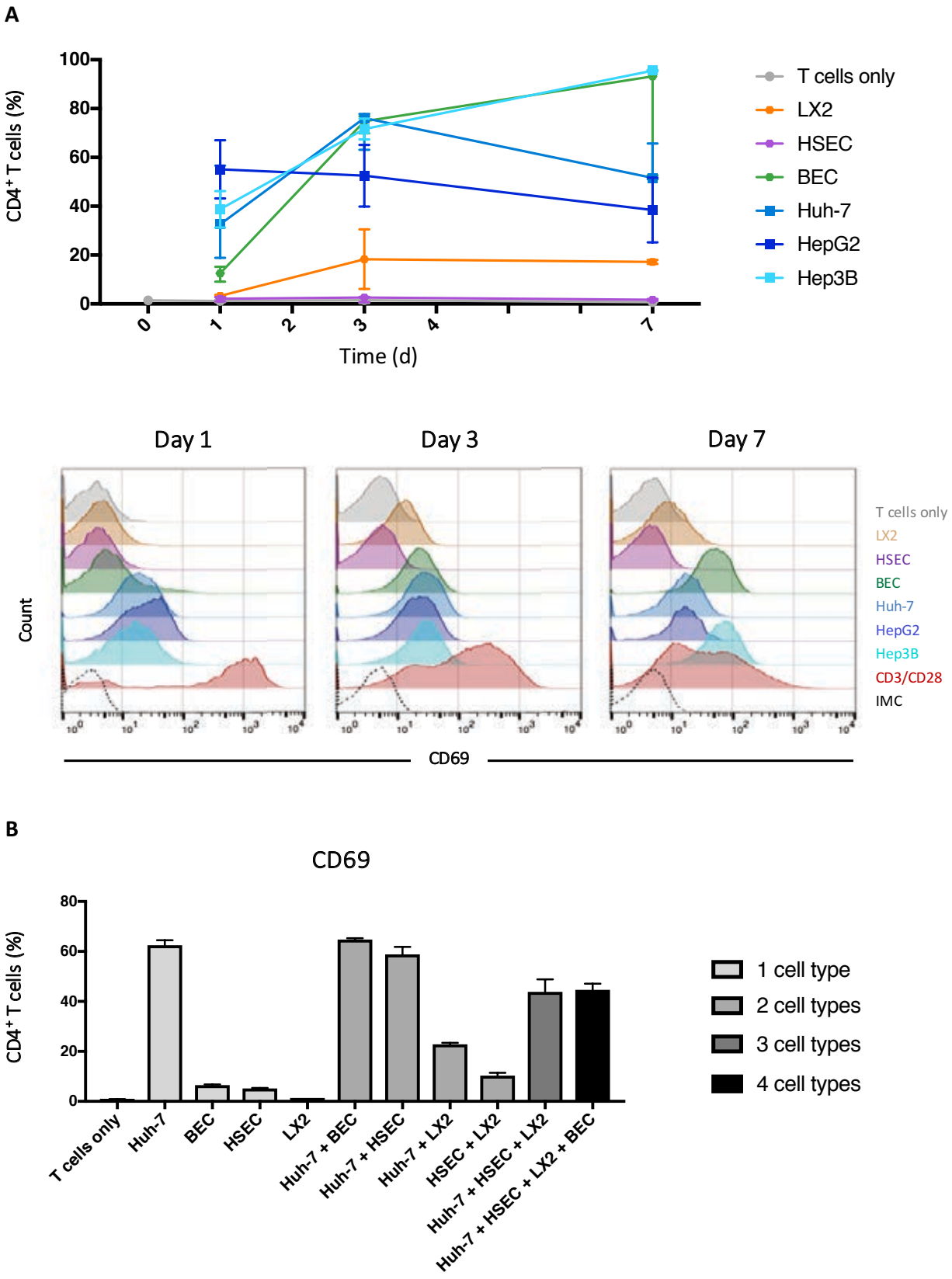


Figure 4.1.6 - CD69 upregulation can be induced by primary BEC as well as hepatoma cell lines. A - 7 day co-culture with different hepatoma cell lines (Huh-7, HepG2, Hep3B), hepatic stellate cell line LX2, primary HSEC, or primary BEC. Median (95% CI) expression shown (n=2-3). Below are representative flow cytometry histogram overlays showing the same data (plus included anti-CD3/CD28 stimulation, positive control; IMC, negative control). **B -** % CD69 expression following overnight co-culture with different liver cell lines in combinations shown. Darker shades of grey indicates more cell lines in culture together. N=1 (2 technical replicates), median + IQR.

would in migrating through the liver. We were only able to culture these cells overnight due to availability of donor material, but it appeared that BEC did not provide a synergistic effect on CD69 upregulation when combined with Huh-7; and neither did HSEC dampen the Huh-7 driven effect (Fig. 4.1.6B). However, LX2 cells did seem to attenuate CD69 upregulation when combined with Huh-7, but this attenuation was not as strong when HSEC and BEC were also present.

Together these data suggest the CD69 upregulation induced by Huh-7 cells does not reflect conventional T cell activation. This is due to intermediate expression levels, the failure to express other activation markers, and no discernible proliferation observed. This phenotype was stable following removal from the hepatocytes, and also detected in primary biliary epithelia, indicating that this is not a feature of neoplastic disease.

4.3 CD69 upregulation did not represent unconventional T_{REG} induction

With Huh-7-mediated CD69 upregulation unlikely to represent conventional T cell activation, we turned our attention to another documented function of CD69 on T cells – as an unconventional regulatory T cell marker. $CD69^+$ $CD4^+$ T cells have been shown to form an unconventional regulatory T cell subset that are associated with hepatic carcinomas in mice and men[620, 622, 623]. Furthermore, long-term CD69 expression has been associated with this cell type in preference to a more conventional transient upregulation[29, 625]. Therefore, we investigated whether the $CD69^+$ T cells generated by co-culture with Huh-7 cells possessed any regulatory capacity.

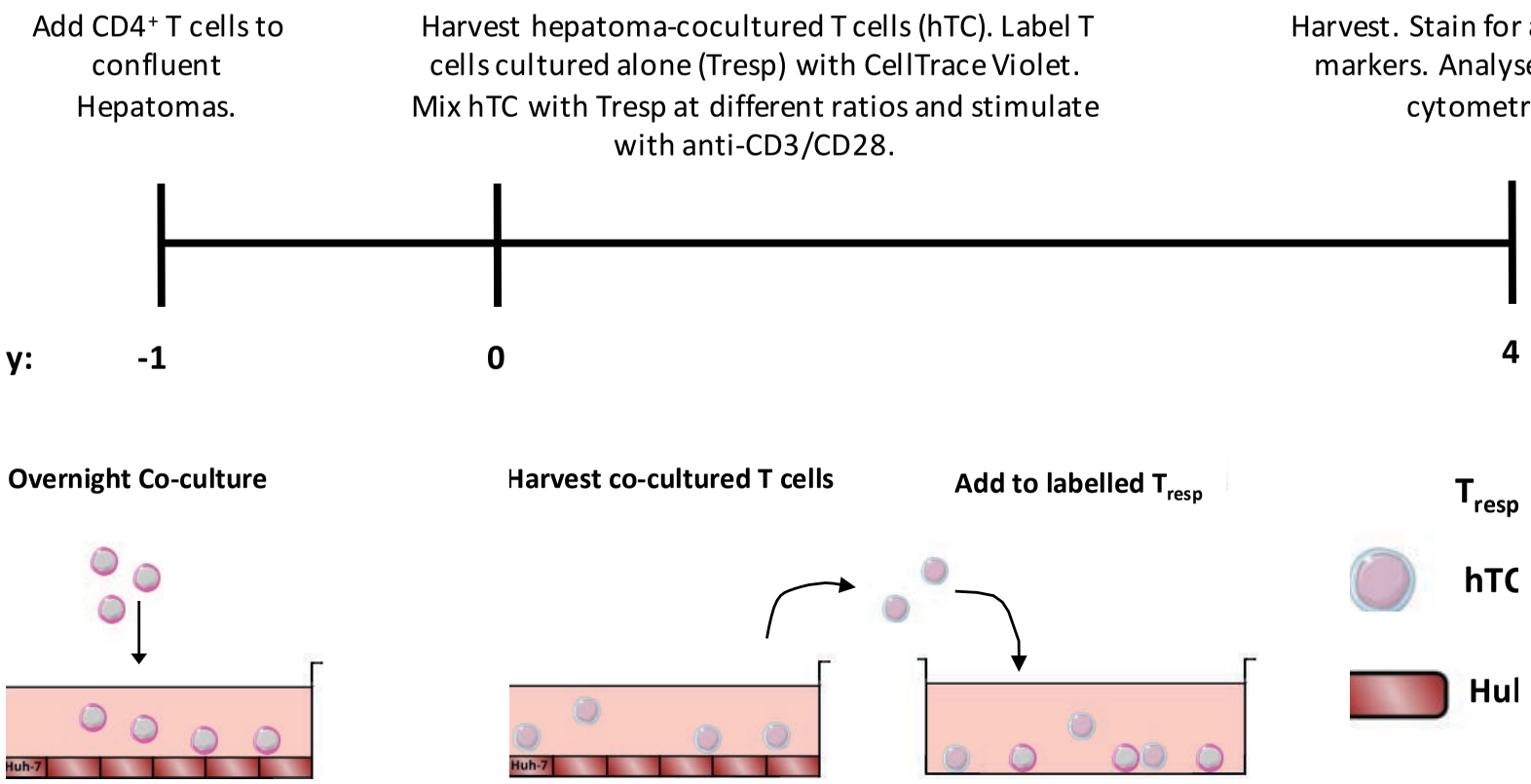
In order to test this, we devised suppression assay methodology based on the standardised work of others[540]. We generated $CD69^+$ T cells from overnight co-culture

with Huh-7 cells, before mixing the resulting T cells (hepatoma-cultured T cells - hTCs) with autologous untouched T cells (T responder cells - Tresp) that had been labelled with CellTraceViolet™ proliferation dye (Fig. 4.2.1). These two cell types were mixed at various ratios - 1 x 10^5 Tresp were present in all conditions, with numbers of hTCs varied to form simple doubling cell ratios. Anti-CD3/CD28 beads were added to the wells as the stimulant and TGF-β added to Tresp alone was used as a positive control for suppression. Cells were cultured together for 5 days, before harvesting, and the amount of proliferation determined by flow cytometry. From these proliferation rates, % suppression was calculated as follows:

$$\frac{\%\ division\ Tresp\ alone\ -\ \%\ division\ Tresp\ with\ hTC}{\%\ division\ Tresp\ alone} \times 100$$

This assay set up formed our standard suppression assay.

To our surprise, from 1:1 Tresp:hTC, the addition of more hTCs resulted in apparent suppression (Fig. 4.2.2A). This was statistically significant at 1:2 and 1:4, and reached a modest suppression of 34%. Furthermore, the inclusion of exogenous IL-2 in these cultures made no difference to the result, indicating failure of Tresp survival was not a critical factor (data not shown). Unfortunately, the positive control, TGF-β, yielded disappointing suppression results – 4.5% average suppression (data not shown). This led us to improve the controls for these experiments by other means. First, it is noteworthy that for these standard suppression assays we were keeping Tresp numbers constant across the hTC 'doses', therefore changing hTC numbers and thus overall cell number



4.2.1 - Suppression assay set up. Peripheral blood-derived CD4+
aneously, T cells were cultured alone, to be used as the responder population (Tresp). hTCs were then carefully harvested and added to CellTra
proliferation dye-labelled Tresps at different cell ratios in the presence of anti-CD3/CD28 beads. Proliferation, and phenotypic changes were analys
4 by flow cytometry.

between conditions (i.e 1:1 had $1x10^5$ Tresp + $1x10^5$ hTC; 1:4 had $1x10^5$ Tresp + $4x10^5$ hTC). Concerned that at higher hTC doses here we would just be seeing apparent suppression due to lack of space to proliferate, or lack of nutrient availability, instead of genuine regulatory cell-mediated proliferative inhibition, we devised a parallel experimental set up. For this second experimental iteration, we set the overall cell numbers/well at $1x10^5$, and varied both Tresp and hTC accordingly. This second set-up we termed constant suppression assays.

In the constant suppression assays, no suppression was observed at any dose (Fig. 4.2.2B). This seemingly confirmed our suspicion that the competition for space or nutrients in the 1:2 and 1:4 Tresp:hTC doses was behind what we initially interpreted as suppression. However, this interpretation was again questioned when we included our final control. Instead of using hTC as 'suppressors' we used T cells cultured alone (tTC) to directly compare the effect of hepatocyte imprinting on a T cell, as suggested by others[627]. As all T cells were autologous, using tTCs was effectively culturing T cells separately for a day before putting them back together – a scenario that should obviously not invoke any kind of suppressive ability. Indeed, this is exactly what we found – no clear suppression when tTCs were used as the suppressors (Fig. 4.2.2C). Whether tTC + Tresp numbers were kept constant (constant suppression assay), or varied with the ratios used (standard suppression assay), no suppression was seen. If competition for space/nutrients was the major controlling factor, we would expect tTC cells to show apparent suppression in the standard assays just like hTCs did. Therefore combined, although this data is not yet clear, it appears hTCs are not able to reliably act as a suppressive cell type due to their lack of ability to suppress in different experimental set-ups.

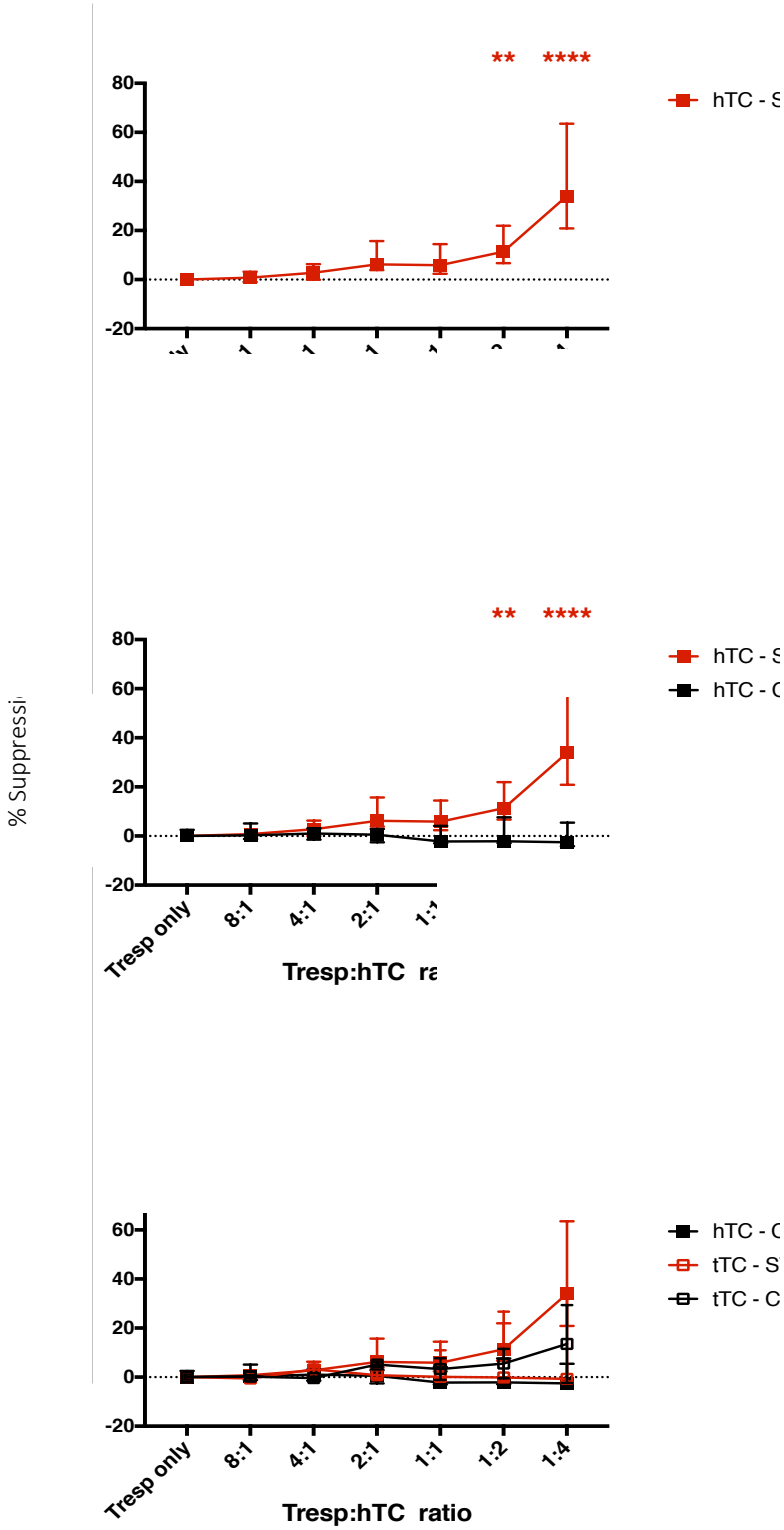


Figure 4.2.2 - hTCs are unlikely to be suppressive in vitro. hTCs and Tresps were mixed in different ratios, activated, and cultured for 4 days as described in Fig. 4.2.1. The three graphs shown represent the evolution of results as more internal assay controls are added in. In **A**, the standard suppression assay (STD) was carried out revealing statistically significant increases in apparent suppression. In **B**, these data were compared with the constant suppression assay (CON - where summed hTCs and Tresp numbers were constant across the different cell ratios - see text for more info.). Last, in **C**, suppression was assessed in Tresps cultured with tTCs (T cells cultured without Huh-7 cells) in both the STD and CON configurations. hTC STD - n=6; hTC CON, tTC STD - n=3; tTC CON - n=2. Median values +/- IQR shown in all graphs.

4.4 The CD69 upregulation mechanism involves direct cell-cell contact

An outstanding question in Huh-7-mediated CD69 upregulation lies in determining the mechanism. To investigate this, we first looked at the CD69 upregulation dynamics. We demonstrated that CD69 upregulation was evident after as early as 2h in co-culture (Fig. 4.3.1). This rapidity indicates that CD69 is likely pre-formed inside the T cell as others have suggested[589]. It is possible that the CD69-inducing pathway is likely already present on the hepatocyte, with no need for new transcription or translation following T cell contact. Isolation of memory and naïve T cell subsets allowed us to better determine the $CD4^+$ T cell type(s) that are capable of CD69 upregulation in response to hepatocyte contact. Although we saw CD69 upregulation in both naïve and memory $CD4^+$ T cells, this was more efficient in the latter subset (Fig. 4.3.2). Additionally, we reverted to transwell migration methodology in order to indirectly demonstrate CD69 upregulation was more efficient in the more activated cell types. By enabling migrated cells to land on a second hepatocyte monolayer, we showed an enhanced % of CD69 upregulation, when compared to $CD4^+$ T cells in static co-culture (Fig. 4.3.2B). As we already determined that migrated cells are more activated than their non-migrated counterparts (see section 3.3), it follows that the cells best able to upregulate CD69 are likely also a relatively activated contingent.

To see if CD69 upregulation required cell-cell contact with the hepatocyte, we included 0.4μm pore transwells in our co-culture set ups. T cells restrained above these did not upregulate CD69 and so we concluded cell surface contact was required, with soluble mediators unimportant (Fig. 4.3.3). Honing in on the mechanism further, we fixed Huh-7 cells before adding the $CD4^+$ T cells. Resulting CD69 upregulation was dramatically

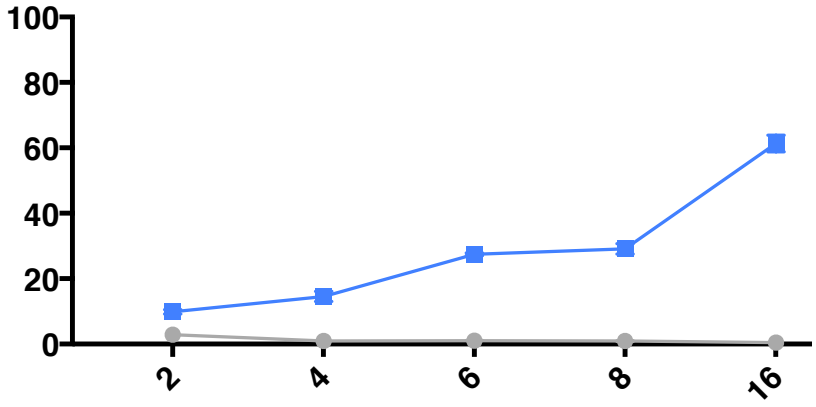


Figure 4.3.1 - Huh-7-induced CD69 upregulation happens very rapidly. % CD69 expression in $CD4^+$ T cells cultured alone (grey), or with Huh-7 cells (blue) at various early time points. N=1 (2 technical replicates - median + range shown).

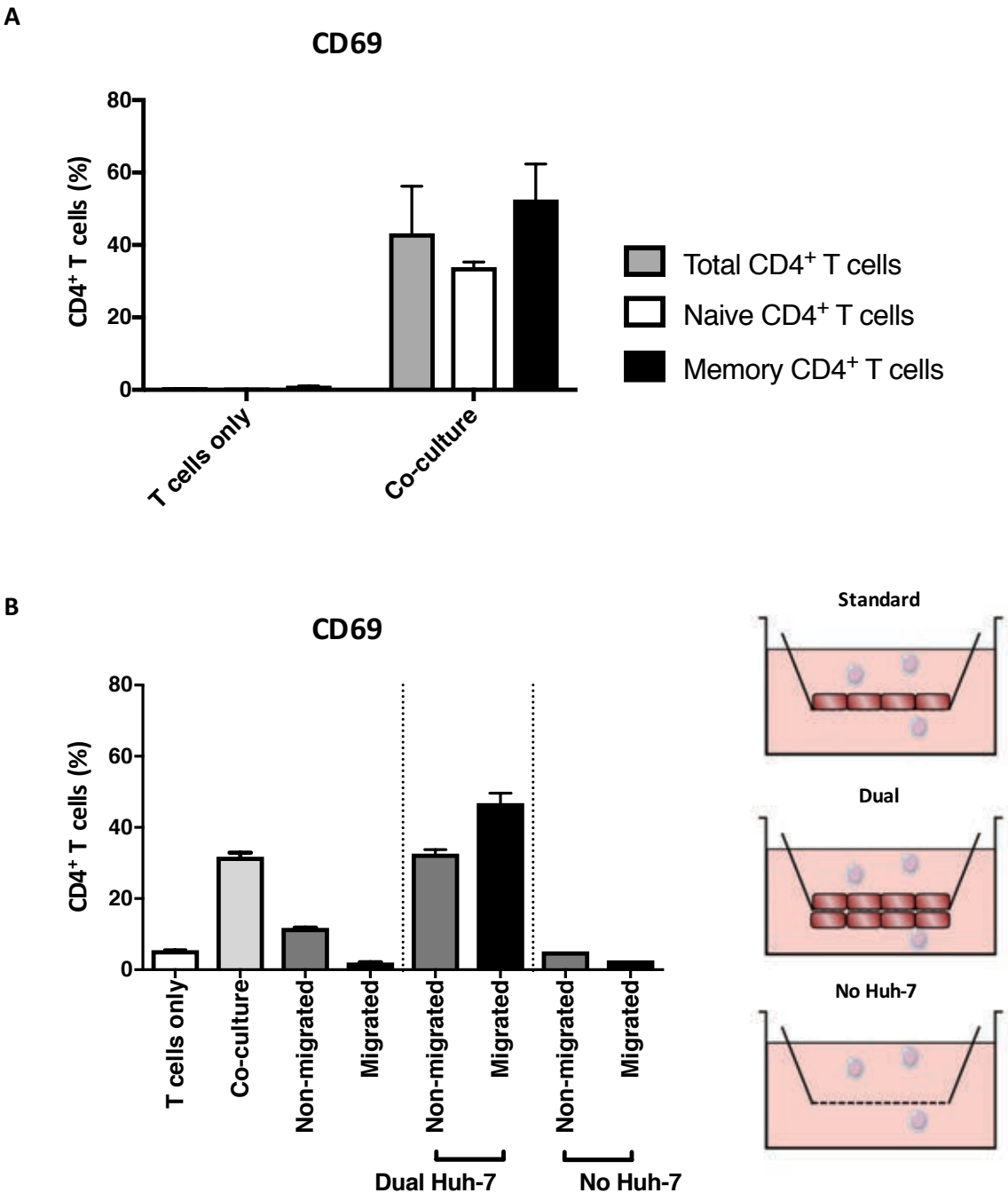


Figure 4.3.2 - CD69 upregulation efficiency is semi-dependent on memory and activation state. A - CD69 upregulation following overnight Huh-7 co-culture with purified isolated naïve (white), memory (black) or total (grey) $CD4^+$ T cells. N=1 (4 technical replicates, median + IQR shown). **B -** Transwell migration assay using Huh-7 cells. % of CD69-epressing $CD4^+$ T cells assessed in the following conditions: standard transwell conditions (T cells only, co-culture, non-migrated, migrated); dual Huh-7 layers (additional Huh-7 monolayer plated on bottom of culture well - non-migrated and migrated); and transwell with no-Huh7 (non-migrated and migrated). Diagram illustrates the three different transwell set ups described. N=1 (2 technical replicates, median + IQR shown).

reduced in cells fixed with three different fixatives (glutaraldehyde, formaldehyde, and methanol – Fig. 4.3.4). This result suggested that CD69 induction was an active process from the hepatocyte standpoint; the mere presence of surface ligand was not sufficient.

So to this point we learned that CD69 upregulation requires active cell-surface ligand binding. One such candidate molecule that fits these criteria is ICAM-1. ICAM-1 can be expressed on both human hepatocytes[628-631], and the Huh-7 cell line[632]; and can stimulate CD69 expression in human T cells[633, 634]. Initially we did see a reduction in CD69 upregulation when hepatocytes were pre-treated with blocking anti-ICAM-1 antibodies (in 3 of 4 donors), but this was not specific as isotype control antibodies produced the same effect (Fig. 4.3.5A). This was not due to a contamination of IMC, as the same result occurred in a new IMC batch from a different company (data not shown). Wondering whether the length of culture or pre-treatment time would affect the result, we included a short-term culture condition (4h), and did not wash off blocking antibodies before adding T cells to overnight (16h) co-cultures. None of these alterations affected the magnitude of the prevention of CD69 induction, and isotype controls for each produced the same effect as ICAM-1 blocking (Fig. 4.3.5B). Last, we targeted the T cell as well as the hepatocytes; blocking or stimulating components of LFA-1 (CD11a and CD18). We hypothesised that if ICAM-1 interactions were responsible for CD69 upregulation on $CD4^+$ T cells, then blocking or stimulating $CD4^+$ T cell LFA-1 components would dampen or enhance this process respectively. However, we saw no upregulation of CD69 on $CD4^+$ T cells when CD11a or CD18 were stimulated; nor did we see a reduction in CD69% expression following T cell CD18 blocking (Fig. 4.3.5C). Additionally, coating plates with recombinant ICAM-1 antibody did not lead to appreciable CD69 induction.

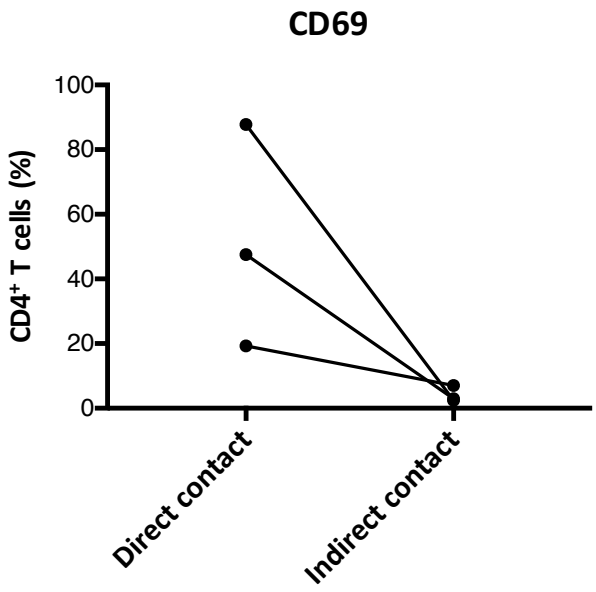


Figure 4.3.3 - CD69 upregulation requires direct hepatocyte contact. Huh-7 cells were cultured in 24-well plates before blood-derived $CD4^+$ T cells were added either directly on top (direct contact), or on top of 0.4µm pore transwell inserts (indirect contact) so that only Huh-7 derived soluble mediators can interact with the T cells. Shown are the % of CD69-expressing $CD4^+$ T cells following overnight co-culture in both conditions in 3 donors.

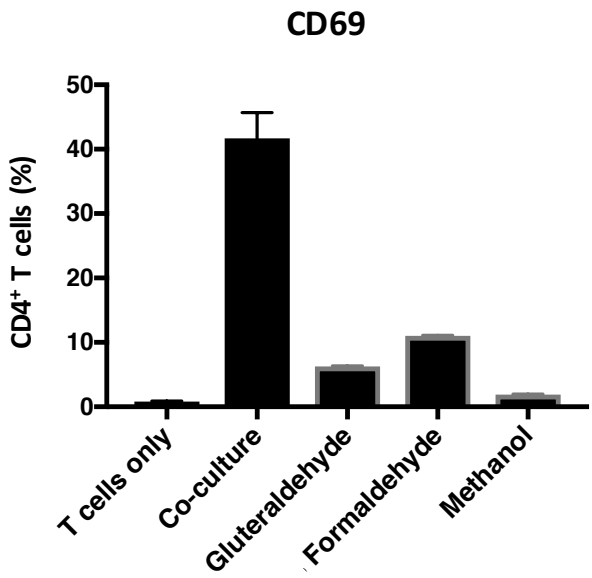


Figure 4.3.4 - CD69 upregulation depends on active hepatocyte ligand engagement. Blood-derived $CD4^+$ T cells were co-cultured overnight with live Huh-7 cells, or Huh-7 cells fixed by three different methods (Gluteraldehyde, Formaldehyde, or Methanol). Percentage CD69 expression shown (1 donor, 2 technical replicates, median + IQR shown).

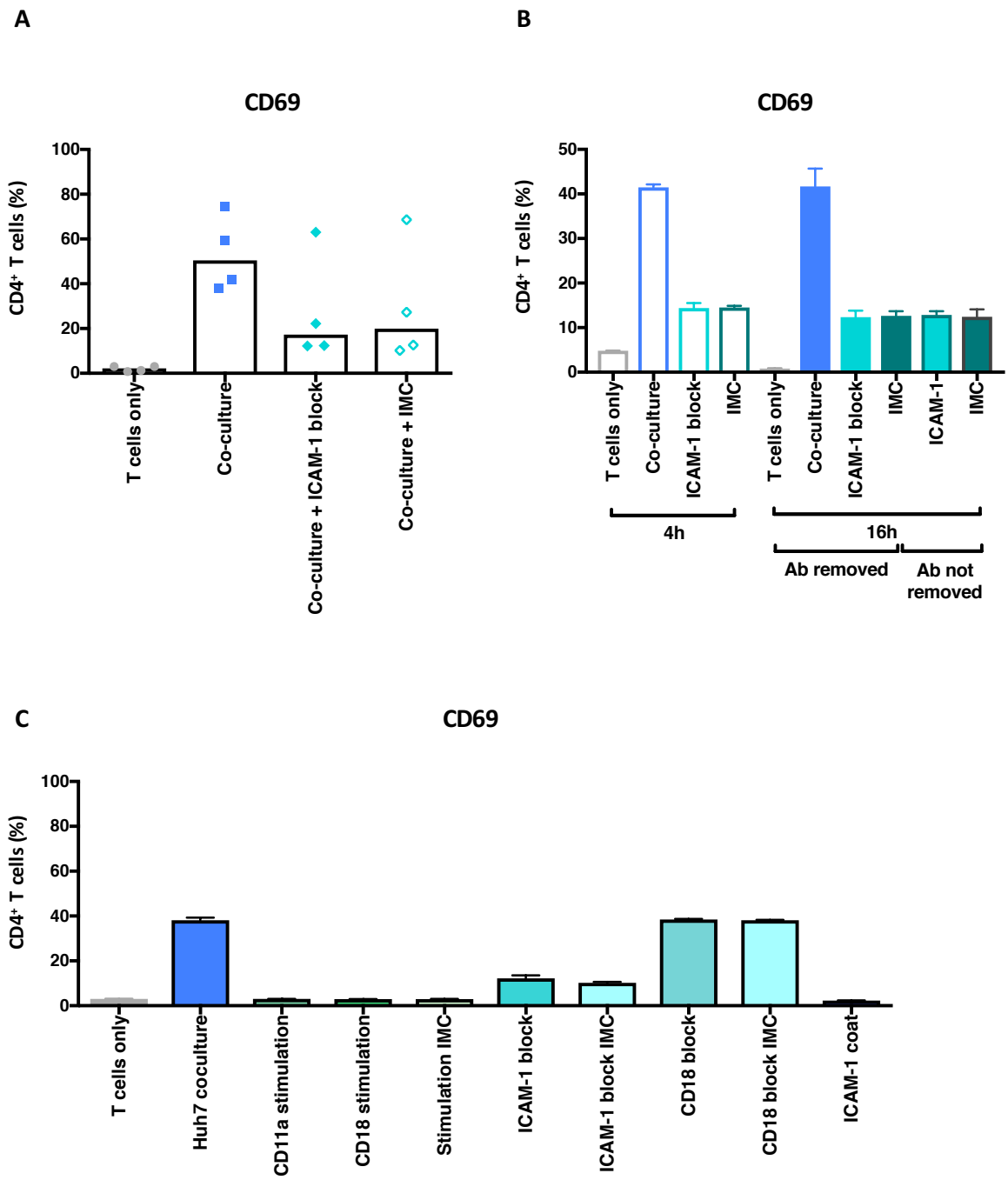


Figure 4.3.5 - ICAM-1 interactions may be involved in hepatocyte-induced CD69 upregulation. A - $CD4^+$ T cells from 4 donors were put into overnight co-culture assays with Huh-7 cells; that had been pre-treated with anti-ICAM-1 blocking antibodies (Ab), or IMC for 20 mins. **B -** This same experiment was carried out for different times - 4 hour co-culture, or overnight (16h co-culture). In one overnight iteration, the anti-ICAM-1 blocking antibody (or IMC) was not removed before $CD4^+$ T cells were added. **C -** T cells were pre-treated for 20mins with stimulatory anti-CD11a antibodies, stimulatory anti-CD18 antibodies, or blocking anti-CD18 antibodies plus their respective controls; or Huh-7 cells were pre-treated with anti-ICAM-1 blocking antibodies or IMC as before. Last, ICAM-1-coated culture plates (in the absence of Huh-7) were used. T cells and Huh-7 cells were co-cultured together overnight to produce the conditions shown. All experiments in this figure show % CD69 expression (median + IQR). Parts B and C - n=1 (2 technical replicates).

Together these data show that CD69 induction by Huh-7 cells happens rapidly, and most efficiently to activated, memory CD4$^+$ T cells. Direct cell-cell contact is key, and an active hepatocyte process is needed. We subsequently hypothesised that ICAM-1-LFA-1 interactions could be responsible for CD69 upregulation, but were unable to confirm this so far. Thus, this chapter has disproved conventional T cell activation, and argued against regulatory function explaining hepatocyte-induced CD69 upregulation in CD4$^+$ T cells, whilst contemporaneously beginning to unravel the mechanism behind this induction.

4.5 Discussion

In this chapter, we investigated the consequences of CD69 upregulation, as well as the mechanisms behind it. Our data first demonstrated that the process was independent of conventional T cell activation, before arguing against concomitant induction of regulatory T cell functions. Finally, we demonstrated that CD69 upregulation began very rapidly; was most efficient in activated memory CD4$^+$ T cells; and the process was active, cell surface contact-dependent, and may involve ICAM-1-LFA-1 interactions (Box 4.1).

Chapter 4 – Core Findings:

- Contact with hepatic and non-hepatic epithelia induced an upregulation of CD69 in peripheral blood CD4$^+$ T cells
- This upregulation was independent of activation as shown by intermediate expression levels, no correlation with other activation markers and no associated proliferation
- This phenomenon also did not represent acquisition of a regulatory phenotype
- The mechanism behind CD69 induction involved cell-surface contact with an actively expressed molecular determinant, potentially involving ICAM-1

Box 4 Box 4.1 – Core findings from chapter 4.

Six separate lines of evidence pointed to Huh-7-induced CD69 being independent of conventional T cell activation. First the interaction is not antigen-dependent as Huh-7 cells

do not express MHC class II (data not shown). The purity of $CD4^+$ T cells we were isolating averages at over 98%, so there was no possibility that the high percentage of CD69 expression seen (minimum 11.4%) was due to $CD8^+$ T cell contaminants recognising antigen-MHC class I complexes. Second, no other activation markers were upregulated across the 7-day co-culture period, in stark contrast to when conventional activation stimuli were used. CD25 and CD38 act as intermediate markers of activation, while HLA-DR is classified as late[25, 635]. Within the time period allowed, all markers should have sufficient time to upregulate following conventional activation, so it is telling that they were unaltered. Third, CD69 was only induced to an intermediate level, as opposed to the normal high level seen with conventional activation stimuli. We believe this is particularly significant not only in separating these cells from activated phenotypes, but in matching these cells with their intrahepatic $CD69^{INT}$ counterparts, discussed in chapter 5. Fourth, once induced by the Huh-7 cells, CD69 expression persisted for days, even following separation from the stimulus. Although we did not formerly demonstrate that removing the conventional activation stimuli would lead to a more transient CD69 expression profile, this well-studied aspect of CD69 biology can be strongly inferred[27]. Fifth, Huh-7-induced CD69 did not associate with any real increase in activation-induced proliferation. We are confident that the small number of lymphocytes seen in the divided gate were not truly divided as they were shifted far towards the negative end of the scale. Instead these cells, that were only present in Huh-7 co-cultures, could represent Huh-7 contamination in the lymphocyte gate, or other artefacts of the co-culture. Sixth and finally, apoptosis rates were no higher in the $CD69^+$ T cells than the $CD69^-$. This reaffirms the other data, showing activation-induced apoptosis was not occurring preferentially in either subset[565]. Therefore, together it is clear that although contact with Huh-7 cells induced CD69, this was not reflective of an activation event.

In future, it would be worth formerly proving that removal of activation stimulus causes a rapid decline in CD69 expression, while removal of Huh-7 cells does not. We also ran an interesting supplementary experiment where Huh-7 co-culture and different conventional activation stimuli were combined. Combining activation stimuli and hepatocytes together generally reduced the % upregulation of CD69, CD25 and CD38. It could be that some of the stimuli are also triggering phenotypic changes in the hepatocytes in our system, and these altered hepatocytes cannot induce CD69 as well, or do not permit upregulation of the other activation markers as much[636-639]. Alternatively, the result could be due to some form of sequestration of the activation stimuli by the hepatocytes, or due the additional competition for stimulus that the hepatocytes provide. The fact that T-cell specific anti-CD3/CD28 beads have the smallest diminishing effect when combined with Huh-7 supports these hypotheses, as hepatocytes cannot be competing for this stimulus. In our experiments, the stimuli were added into the wells already containing both hepatocytes and T cells. Therefore, although the stimuli are acting at the same concentration, they are now acting on a much larger cell number compared to with T cells alone. Future experiments could control for this by carrying out a matched stimulation per total cells (T cells + hepatocytes), or by pre-treating the T cells before adding them to hepatocytes. Interestingly, HLA-DR% expression synergistically increased when stimuli were combined. Perhaps therefore Huh-7 cells contain co-stimulatory abilities that are only important and additive once a T cell has been appropriately stimulated. Dose-dependent treatments with the conventional stimuli, and different sequential exposure of T cells to hepatocytes and the stimuli will help answer these questions.

CD69 upregulation was also caused by primary BEC. We had previously shown that CD69 upregulation occurred following contact with multiple epithelial cell lines from different tissues, but this finding showed for the first time that the induction was transferrable to primary hepatic epithelia. Additionally, the failure of primary HSEC to induce CD69 upregulation demonstrated the phenomenon does not happen following contact with any cell type. However, CD69 upregulation can occurr following co-culture with TNF-α-activated endothelial cells, as others have shown[568, 633]. BEC-induced CD69 induction was delayed by 1-2 days compared to that seen with hepatocyte cell lines, perhaps reflective of the different properties of primary cells compared to tumour cell lines. Last, LX2 showed a very discrete, but definite induction of CD69 upregulation compared to other cell types. We have not followed this up here, but it would be interesting in future to determine if this was consistent with primary liver HSCs, and if so to compare liver epithelial and non-epithelial cell-mediated CD69 upregulation mechanisms, dynamics, and associated phenotypic changes. Additionally, the combined liver cell co-cultures made an interesting initial inroad into uncovering the influence of multiple cell types on CD69 upregulation. This tentatively shows LX2 cells inhibit the process, but ultimately a much more detailed approach is needed. As $CD4^+$ T cells can contact all cells equally and in random orders in these simple mixed culture systems, liver chip systems (discussed in 3.6) that organise cell types into a more physiological 'liver-like' arrangement would be perfect successor assays, allowing $CD4^+$ T cells to interact with different liver cells in the order they would *in vivo*.

We next turned our attention to the possibility of our generated $CD69^+$ $CD4^+$ T cells possessing regulatory capabilities. This hypothesis was fuelled by previous data showing CD69 marks an unconventional T_{REG} subset with links to hepatic tumours[620-623]; as well

as our own data showing CD69 expression remains elevated long-term, an aspect linked to this regulatory subset[29, 625]. Therefore, we performed suppression assays to address this possibility. It would have been favourable to sort $CD69^+$ hTCs, instead of just using total hTCs as our suppressor population, but we felt total hTCs were sufficient for preliminary experimentation. Any evidence of suppression and we would have moved onto cell sorting. Additionally, the use of purified conventional T_{REG} cells as positive controls would be of interest, although technically challenging to achieve as a large amount of starting material was required. Instead we used potent inhibitory cytokine TGF-β as a positive control[640], with very limited success. Nevertheless, multiple experimental iterations failed to conclusively prove that hTCs were providing a suppressive effect. The standard assays where hTCs showed apparent suppression at ratios of 2 hTCs for every 1 Tresp and above, but even if this was real suppression and not an artefact of competition for physical space or nutrients, these ratios were not likely something that would ever be reached *in vivo*. Furthermore, it is commonly thought harder to prove a suppressive effect *in vivo* than *in vitro*[540]. Such assays are thought to be highly sensitive to many variables[627]. One such variable is TCR-stimulation strength – too strong and no suppression would be visible, too weak and insufficient proliferation will be seen to reliably calculate percentage suppression. Although, we tested soluble anti-CD3/CD28 antibodies, and anti-CD3/CD28 complexes, as well as anti-CD3/CD28 beads and found the latter to be optimum; this stimulation may have been slightly 'too strong'. Another caveat of our suppression assays is the lack of a live/dead marker. While we took great care with our live lymphocyte gating, dead cells could still form a part of those gates. Overall, the lack of proliferative inhibition, and no alterations in cytokine potential or activation marker phenotype provide fairly comprehensive evidence that hTCs are not

suppressive. Any future suppression assay experiments should include live/dead stains, and ideally sorted $CD69^+$ hTCs and positive control T_{REGS} to confirm this.

We attacked the question of the mechanism behind CD69 upregulation from two angles. The first investigated the $CD4^+$ T cell. We were able to show that CD69 upregulation was more efficient in memory T cells, and likely better in more activated T cells as shown through transwell methodology. This gives early indication of the type of T cell able to upregulate CD69. Future experiments could delve further, sorting specific memory subtypes, sorting different T helper subsets, or sorting on different activation marker-positive cells. As another example, KLRG-1-expressing and non-expressing T cells could be sorted and then added to Huh-7 co-cultures to see if CD69 is upregulated equally in both subsets. A bias towards $KLRG\text{-}1^-$ cells could indicate CD69 upregulation is related to a tissue-residence phenomenon[110]. Analyses of this sort may provide candidate molecules that would be involved in CD69 induction, with their partner molecules preferentially expressed on the more efficient CD69 upregulators. The second angle was from the hepatocyte side. We demonstrated the need for cell-cell contact with the hepatocyte, and the requirement of live, active hepatocytes for the interaction to be successful. Therefore, many cytokines that have been previously shown to upregulate CD69 in T cells such as type-1 IFNs, TNF-α and TGF-β can be ruled out[30, 171]. However, this does not preclude involvement of mTGF-β or IL-15 that can both function as cell surface ligands and are both linked to CD69 upregulation[148, 641]. Even so, the need for cell contact is interesting as we may have revealed a less recognised, or even novel upregulation mechanism here. This led us to target ICAM-1-LFA-1 interactions as the first candidate pathway as ICAM-1 has triggered CD69 upregulation in other settings[633]. Unfortunately, the IMC matched the downregulation observed by blocking ICAM-1, and

stimulating LFA-1 or culturing T cells on ICAM-1 coated plates did not produce a corresponding CD69 upregulation. In future, isotype matched antibodies against VCAM-1 (that is not expressed by Huh-7 cells [Z. Stamataki – personal communication]) could be used instead of the non-specific IMC to verify if ICAM-1 is involved, and plate bound ICAM-1 presence could be verified by anti-ICAM-1 staining and appropriate visualisation. If required to look for other candidate molecules, we could use transcription and translation inhibitors such as actinomycin D and cycloheximide to determine at which stage of protein processing is more important. Other candidate molecules to target could be include ICAM-3, another LFA-1 partner, that has also been held responsible for T cell CD69 upregulation by others[642]; and CD58 (LFA-3), that binds to the costimulatory receptor CD2 (LFA-2) on T cells, and, like ICAM-1, is known to be expressed on hepatocytes, especially during inflammation[643]. Alternatively, we could back-track and perform comparative transcriptomic analyses of $CD69^+$- and $CD69^-$ hTCs, allowing us to then target preferentially-expressed surface receptors that could be involved in CD69 induction.

While CD69 upregulation was influenced by primary and cancer cell hepatic epithelia alike, it is interesting to briefly consider the impact on anti-tumour responses. It has been proposed that all TILs are in fact a form of T_{RM}, and the generation of which is better associated with tumour eradication[23, 644, 645], substantiating their importance. We were unable to show induction of associated regulatory phenotypes through hepatoma contact like others have[623]; but we did show consistent CD69 upregulation amongst many different tumour cell lines, and propose this represents a form of tumour-residence[23, 645]. How the tumour cells could be altering the resident T cells in other ways is a potential avenue for future work.

In conclusion, we have proved CD69 induction by hepatomas likely represents neither activation, nor formation of a regulatory subset. If so what does this drastic change in CD69 represent for the T cell? Some have pointed to the 'dimmer switch hypothesis' using CD69 to explain a poised, intermediate activatory state[590]. While feasible, we prefer a different explanation that links these cells to tissue residence. Other arms of our investigation have now connected this $CD69^{+}$ phenotype to an analogous $CD69^{INT}$ phenotype seen in the liver. This finding will be discussed in depth in the next chapter. It is important to continue to elucidate the mechanism behind this change as it may be important in T_{RM} precursor formation, and/or short-term maintenance of $CD4^{+}$ T cells in the liver.

Chapter 5 - CD69 expression separates two distinct intrahepatic $CD4^+$ T cell lineages: $CD69^{HI}$ tissue resident cells, and a novel $CD69^{INT}$ subset

5.1 Introduction – Resident memory T cells and the Liver

Resident memory T cells have rapidly emerged as key constituents of systemic immunity. These cells embody scientific intelligent design – strategically placed at sites of reinfection, they respond rapidly to clear the pathogen directly, alert other arms of the immune system to its presence, and persist indefinitely[224, 238]. We already know that these cells provide superior protection to local infections versus circulating T cells, and contribute to a number of human inflammatory diseases[23, 84]. Given the intimate relationship of the liver and the immune system, and the potency of liver tolerance, it is of great importance that the field combine these aspects to investigate liver T_{RM} biology.

Until recently the best insights we had into liver T_{RM} biology came from the identification of liver-resident $CD8^+$ T cells in mouse models[123], and from resident human NK cells[395, 646-650]. In mice, $CD8^+$ T_{RM} that were generated in response to malaria sporozoite infection, were critical for clearance and depended on CXCR6 expression for their maintenance[123, 128, 393]. Atypically, they principally resided in the liver vasculature – unlike T_{RM} from any other organ[108, 128], and recent data showed a dependence on LFA-1 for their maintenance[651]. Three studies to date have characterised human liver T_{RM}[82, 148, 149]. All three have investigated $CD8^+$ T_{RM}, with Wong et al. also studying $CD4^+$ T_{RM}. Pallett et al. provides the most comprehensive study of this population, demonstrating tissue-resident $CD8^+$ T cells in the human liver with a distinct $CD69^+CD103^+CXCR6^+$ $CXCR3^+PD\text{-}1^+$ phenotype, and markedly elevated IL-2 production following stimulation. The authors were also able to induce a matching T_{RM} phenotype in circulating $CD8^+$ T cells in culture by the sequential addition of IL-15/anti-CD3 and then TGF-β. Interestingly, the order of signals provided appeared to be non-negotiable; with CD69 and homing receptor

expression imprinted by the first (IL-15/anti-CD3), and retention signals through CD103 imprinted by the second (TGF-β). This study also demonstrated a key role for liver T_{RM} in controlling HBV infection, the first direct evidence of T_{RM} involvement in human liver disease[148]. The study by Stelma et al. was published soon after, and supports the work of Pallett et al.. They too showed resident $CD8^+$ T cells in the liver largely express CD103 and CXCR6, and added low KLF-2 and S1PR1 gene expression to this phenotype[149]. The analysis in Wong et al. is part of a multi-organ high-dimensional mass spectrometry approach to analyse T cell compartments throughout the human body. As such it lacks liver-specific hypothesis-driven data, unlike the other studies. However, this work provided an excellent framework for the expression of homing receptors and activation/inhibitory markers in $CD8^+$ and $CD4^+$ T cells in the liver that express CD69, CD103, or both. According to this work, $CD69^+CD4^+$ T cells express more CXCR3, CXCR6, CCR5, CCR6, and PD-1 (amongst others) than $CD69^-$ cells, a trend that was also seen amongst the intrahepatic $CD8^+$ T cell pool, matching up well with the other two studies[82].

$CD4^+$ T_{RM} have been comparatively understudied next to $CD8^+$ T_{RM}. This is despite the key role $CD4^+$ T cells have in enhancing many aspects of the immune response, including B cell help, allowing $CD8^+$ T cell memory formation, and providing regulatory assistance to inflammatory environments[652-655]. All evidence to date points to T_{RM} acting as super-charged versions of their circulating counterparts, regardless of helper or killer designation, whether through providing superior cytokine production, or in some cases superior killing ability[23, 108, 186]. $CD4^+$ T_{RM} have already been shown to be important in the clearance of infections of the FRT, lungs, and skin of mice[99, 102, 112, 124, 199]; and outnumber $CD8^+$ T_{RM} in many body sites in mouse and man as revealed by recent multi-organ T cell

mapping studies[82, 87, 88, 90, 91]. In this chapter, we aimed to identify the hepatic CD4$^+$ T_{RM} cell type, and perform a thorough and detailed characterisation of this population's phenotype and function. We sought to place our cells in the ever-expanding human T_{RM} matrix, using other CD4$^+$ T_{RM} studies, and the liver CD8$^+$ T_{RM} studies as comparative axes. Furthermore, given our earlier evidence of a CD69^{INT} population generated through contact with hepatic epithelia (chapter 4), we asked whether an analogous population was present in liver, and if so, what their properties would be.

5.2 Identification of CD69^{HI} T_{RM} and CD69^{INT} CD4$^+$ T cells in human liver

First, we analysed CD69 expression amongst intrahepatic CD4$^+$ T cells in human livers. In this chapter, we utilised both healthy and chronic end-stage diseased livers from a variety of aetiologies (for patient donor information see table 2.2). Liver explant slices were diced and washed thoroughly to minimise blood cell contamination and digested mechanically. The LIMCs isolated from the resulting homogenate were then subjected to surface protein expression analysis by flow cytometry.

Combining all liver donors, we first gated on singlet lymphocytes. Any NK cells, NKT cells, and $\gamma\delta$-T cells were then excluded with CD56 and $\gamma\delta$-TCR staining; allowing downstream investigations to focus on the conventional $\alpha\beta$-TCR-expressing CD4$^+$ T cell population (Fig. 5.1.1A), that will henceforth simply be referred to as CD4$^+$ T cells. CD69 has been repeatedly described as the key discriminator of human T_{RM}[91, 186], and has been used for this purpose in multiple studies[82, 88, 90, 136]. Therefore, we first analysed CD69 expression in order to identify resident T cell subsets. Within the CD4$^+$ T cell compartment, three distinct populations were observed based on CD69 expression: CD69$^-$, CD69^{INT}, and

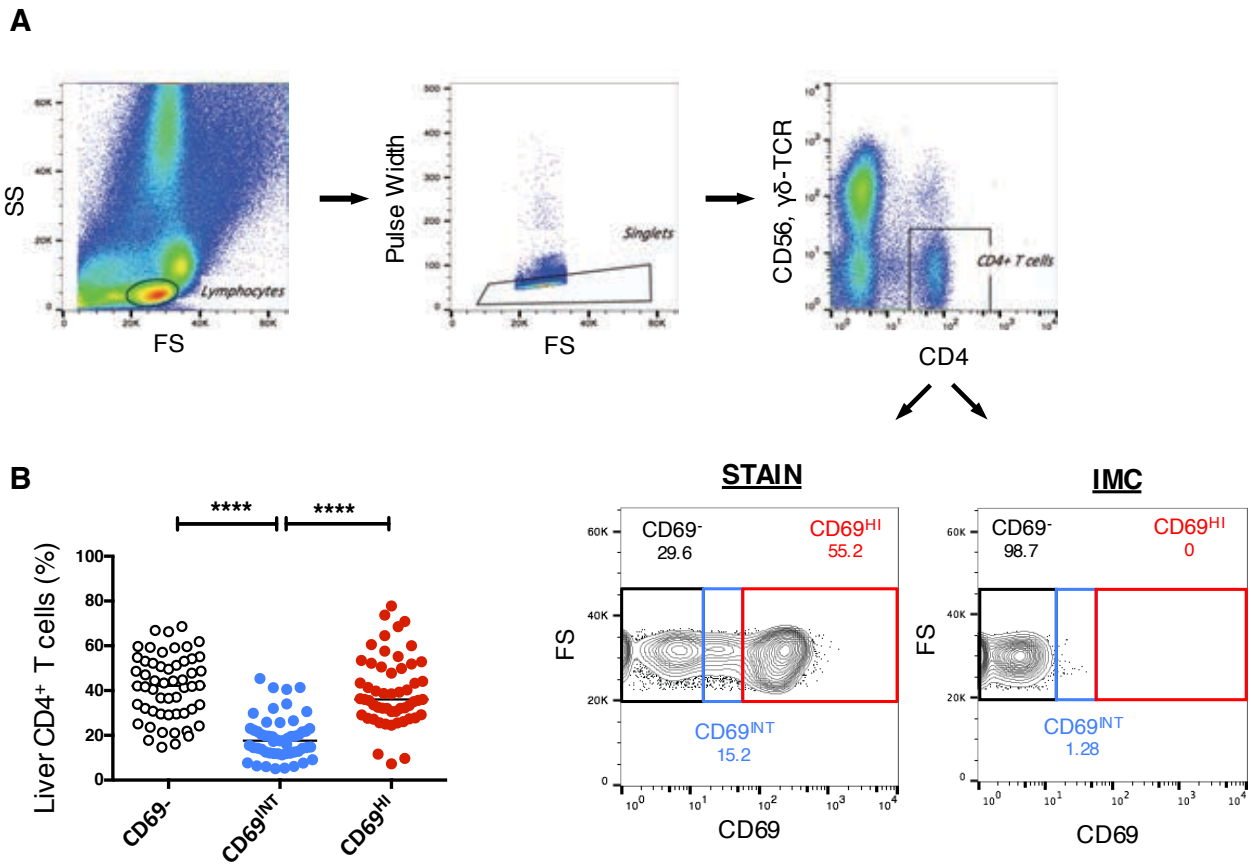


Figure 5.1.1 – Identification of CD69^{INT} and CD69^{HI} CD4^{+} T cells in the human liver. A – Gating strategy used. First live lymphocytes were gated on, then single cells, then CD4+ lymphocytes that did not express CD56 (excluding NK and NKT cells) or the γδ- T cell receptor (excluding γδ-T cells). The remaining CD4^{+} T cell population was split into three populations based on CD69 expression – CD69^{-}, CD69^{INT} and CD69^{HI} cells (left plot). Negative gates were set according to isotype matched control (right plot). **B** – Percentage of CD69^{-}, CD69^{INT} and CD69^{HI} intrahepatic CD4^{+} T cells in combined healthy and diseased livers (n=54). Lines show median values. Groups compared statistically with a Friedman test with Dunn's multiple comparisons test.

$CD69^{HI}$ (Fig. 5.1.1B). $CD69^{INT}$ cells were clearly elevated above IMC background, and we were confident we were not gating on the tail of another population, or seeing a fluorescence shift artefact as we have previously observed a similar intermediate CD69 level in T cells in culture with hepatic epithelia. Importantly, although the $CD69^{-}$ and $CD69^{HI}$ populations were consistently larger (medians of 42% & 39% respectively); $CD69^{INT}$ cells were ever-present.

Having found two CD69-expressing populations where other studies have only reported one, we delved deeper into tissue residence properties in order to address the question as to which population represented the true T_{RM} cell type. Using matched patient blood and liver samples we were able to ascertain that both $CD69^{INT}$ and $CD69^{HI}$ $CD4^{+}$ T cells were only appreciably present in liver tissue, not peripheral blood (Fig. 5.1.2A). Second, using classical memory and naïve T cell markers CCR7 and $CD45RA^{656}$, we noticed the first divergence between the two populations. While all populations from both diseased and healthy livers showed mostly a T_{EM} phenotype ($CD45RA^{-}CCR7^{-}$), $CD69^{HI}$ cells showed an especially strong, statistically significant preference for this subset – a phenotype shared with T_{RM}[71] (Fig. 5.1.2 B-D). When compared to the other two populations, $CD69^{HI}$ cells showed a corresponding underrepresentation of naïve, T_{CM}, and T_{EMRA} cells. In contrast, $CD69^{INT}$ cells were not different from the $CD69^{-}$ putatively non-resident $CD4^{+}$ T cells in any naïve or memory population, in either healthy or diseased livers. Third, we determined the expression of other tissue-residence associated markers across $CD69^{-}$, $CD69^{INT}$, and $CD69^{HI}$ $CD4^{+}$ T cells. Assessing data from the more numerous diseased livers first, percentage of CD49a positive cells was progressively increased with CD69 expression, although $CD49a^{+}$ MFI of $CD69^{HI}$ cells was no higher than in the $CD69^{INT}$ pool (Fig. 5.1.3). CD103 expression was low amongst intrahepatic

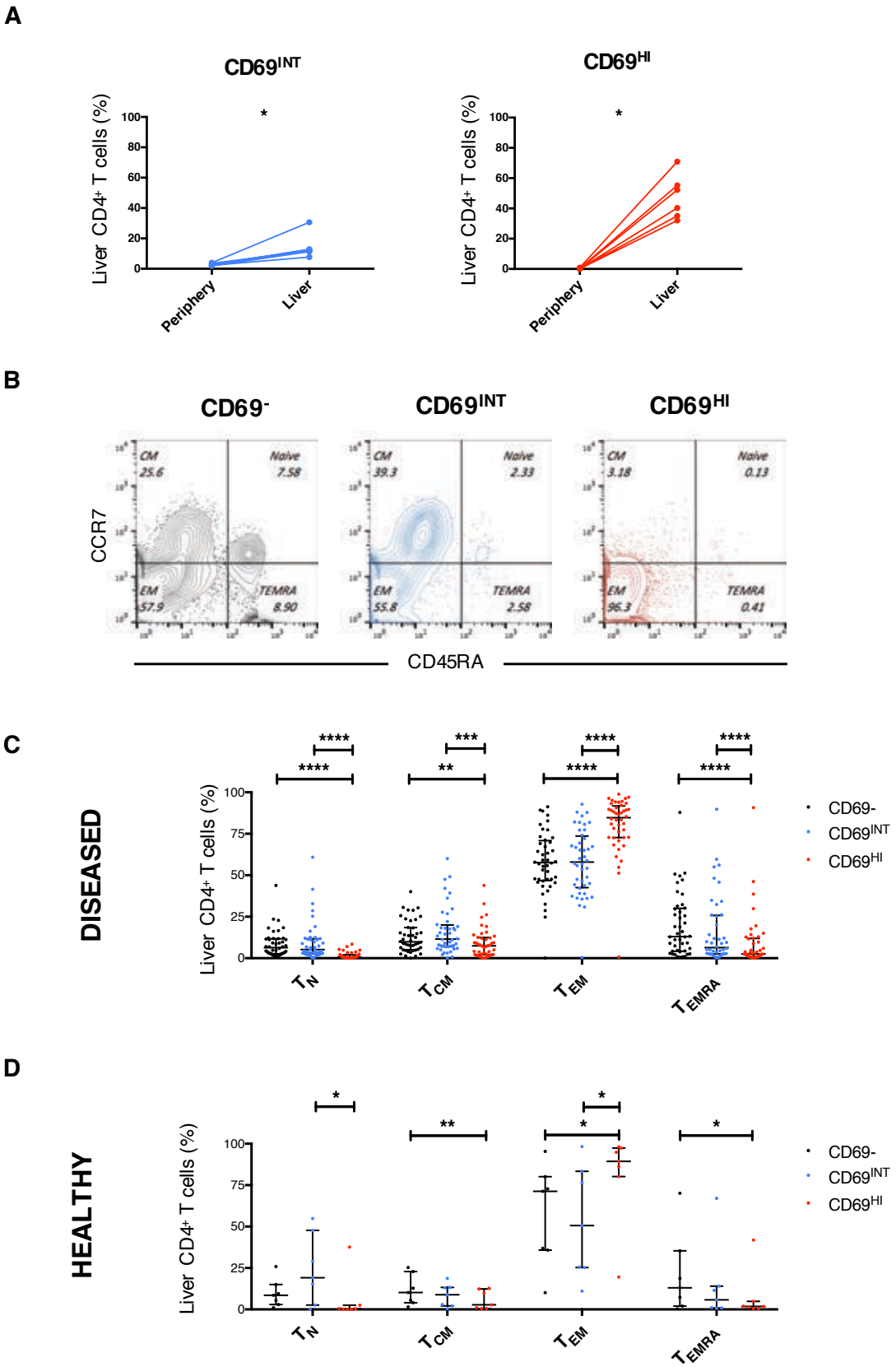


Figure 5.1.2 CD69^{HI} cells are not present in peripheral blood and display increased effector memory phenotype representation. A - CD69^{INT} and CD69^{HI} cells in liver compared to matched patient peripheral blood (n=6). **B-D** –Percentages of CD69^{-}, CD69^{INT} and CD69^{HI} cells that made up each naïve and memory subset (T_N – naïve; T_{CM} – central memory; T_{EM} – effector memory). **B –** Representative flow cytometry plots for each CD69-population. **C –** Combined data from all chronic liver disease donors (n=51). **D –** Combined data from all healthy liver donors (n=7). Median and IQR shown.

CD4+ T cells in general as previously described[23, 91, 186], but a modestly higher fraction of both CD69^{INT} and CD69^{HI} cells were positive for this marker. However, only CD69^{HI} cells had a higher expression level by MFI. We next assessed expression of tissue egress receptor S1PR1, which turned out to be a key factor in distinguishing CD69^{INT} and CD69^{HI} cells. A very small frequency of CD69^{HI} cells expressed S1PR1, and of those that did they only expressed low levels. By contrast, around a quarter of CD69^{INT} cells expressed S1PR1 on average (25.2%), comparable to CD69^{-} cells. Lastly, we established the expression of CXCR6 in the three populations as this chemokine receptor has been heavily associated with tissue retention in the liver[148, 180, 393, 395]. Compared to CD69^{-} cells, CD69^{INT} had slightly higher percentage of CXCR6+ cells, but the highest CXCR6+ proportion were found in CD69^{HI} cells. The healthy donor liver cells showed very similar CD49a, CD103, S1PR1, and CXCR6 differential expression patterns to the diseased donors, albeit with less observed statistical significance, likely due to smaller sample numbers (Fig. 5.1.4). Together these data point to CD69^{HI} cells representing the CD4+ T_{RM} population of the liver. While both populations were only found in liver tissue and not the periphery, the CD69^{HI} CD4+ T cell population expressed more T_{RM}-associated molecules, and crucially only CD69^{HI} cells did not express appreciable S1PR1, making these cells insensitive to tissue egress signals and promoting retention in the liver.

Next, we asked if we could pinpoint an even clearer T_{RM} phenotype using additional residence-associated markers. High expression of CD69 alone acted as our gold-standard control. We compared these cells to CD69^{HI}CXCR6+, CD69^{HI}CD103+, and CD69^{HI}CD49a+ cells in the context of CXCR6, CD103, and CD49a expression (using the other two proteins not already defined in the population – see Fig. 5.1.5A). To compare the new populations to CD69^{HI} control, the % expression of each protein in the three new

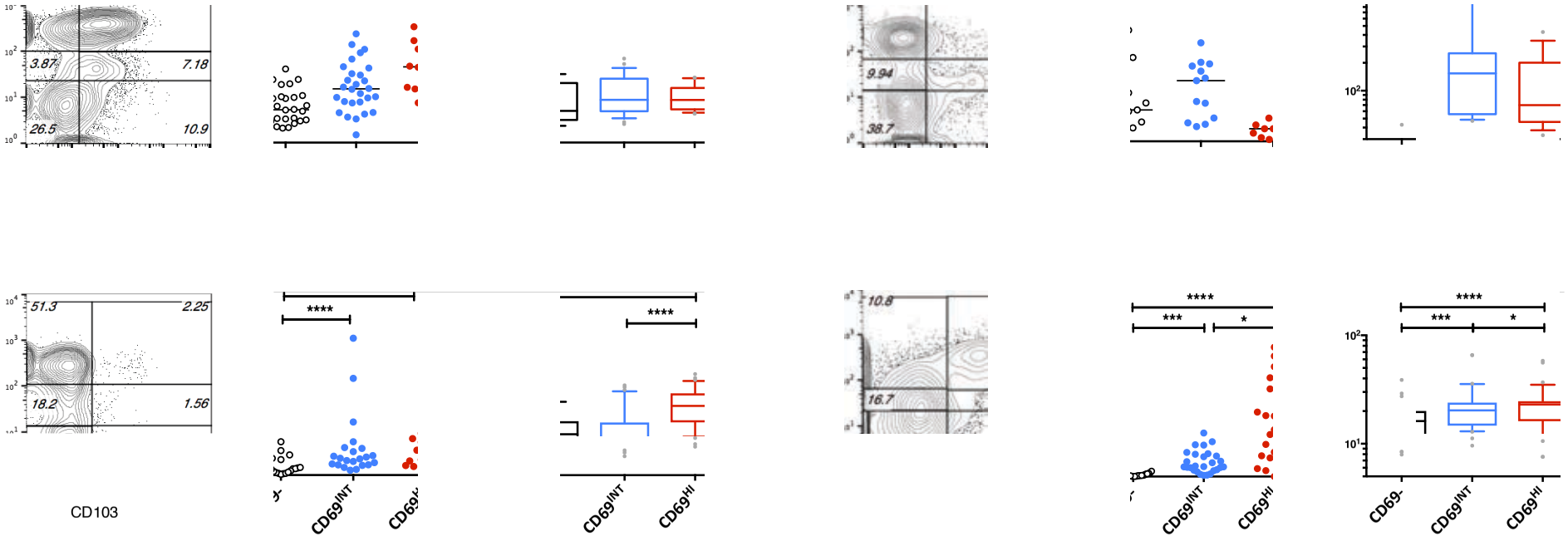


Figure 5.1.3 CD69HI cells represent the tissue resident CD4$^+$ T cells of the human liver. Expression of the T_{RM}-associated markers CD49a, CD103, S1PR and CXCR6 in each of the three liver populations (CD69$^-$, CD69^{INT}, CD69^{HI}). All chronic disease donors shown. From left to right of each triple: representative flo cytometry plots showing expression of each marker against CD69 (median values), percentage expression, and median fluorescence intensity (MFI) of the marke positive cells (median + 10-90% data range).

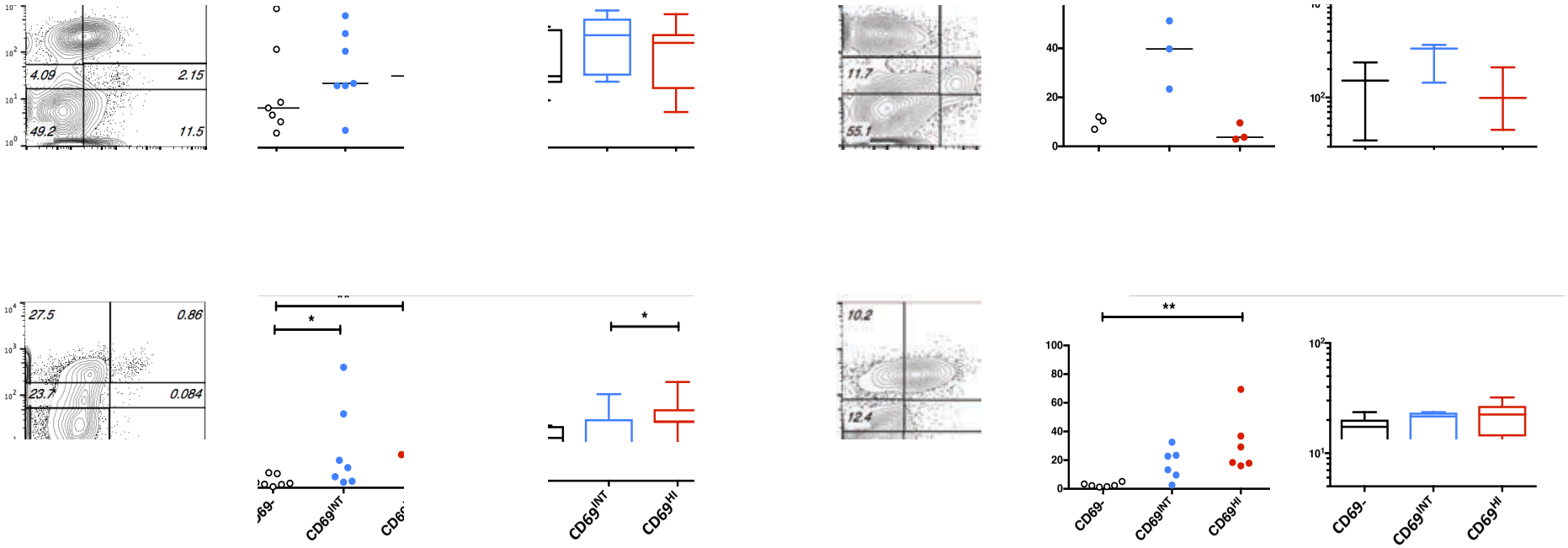


Figure 5.1.4 CD69HI cells appear to be T_{RM} in healthy livers. Layout as in Fig. 5.1.3, but using healthy donors. Expression of each T_{RM}-associated markers shown in each liver population; in representative flow plot, percentage expression, and MFI formats. Median values shown, box plots show 10-90% data range.

populations was normalised to expression in control $CD69^{HI}$ cells (i.e. % protein expression in new population/% protein expression in $CD69^{HI}$ control population). These normalised differences were then summed, and plotted to give a rank difference (Fig. 5.1.5B). This revealed each new population had a higher rank than $CD69^{HI}$ cells. With the highest rank, the population with most promise was $CD69^{HI}CXCR6^{+}$. Although CD103 showed a similar median difference this antigen expression was low on $CD69^{HI}$ cells and is not a good marker of $CD4^{+}$ T_{RM}; while $CD69^{HI}CD49a^{+}$ cells showed the smallest rank difference. To investigate the possibility of $CD69^{HI}CXCR6^{+}$ T cells representing the most likely liver T_{RM}, we compared $CD69^{HI}$ cells with and without CXCR6 expression for CD103, CD49a, S1PR1, and for a T_{EM} phenotype (Fig. 5.1.5C). Only CD103 expression was significantly elevated in this population, with none of the other phenotypes differentially expressed. Furthermore, back analysis on the rank difference data (in Fig. 5.1.4B) revealed that most of the differences between $CD69^{HI}CXCR6^{+}$ and $CD69^{HI}$ cells were due to this elevation in CD103 that was large in fold change, but small in absolute %. Therefore, based on this data combined, we decided that total $CD69^{HI}$ cells represent the likeliest liver T_{RM} cell phenotypically. While CXCR6 was a good additional qualifier, the real shifts in other T_{RM}-associated markers were not convincing enough to switch to this phenotype. Importantly, remaining with CD69 alone as our sole T_{RM} discriminator, allowed for more rigorous analysis as no trends could be missed that were only present within the CXCR6 negative gate.

Expression of the transcription factor Hobit is part of the core tissue residence signature in mice[174], but its involvement in human T cell tissue residence is thought to be non-essential[134, 135, 148]. We performed preliminary stains for Hobit expression in intrahepatic liver T cells. In 3 of 5 livers, no Hobit protein expression was observed above IMC

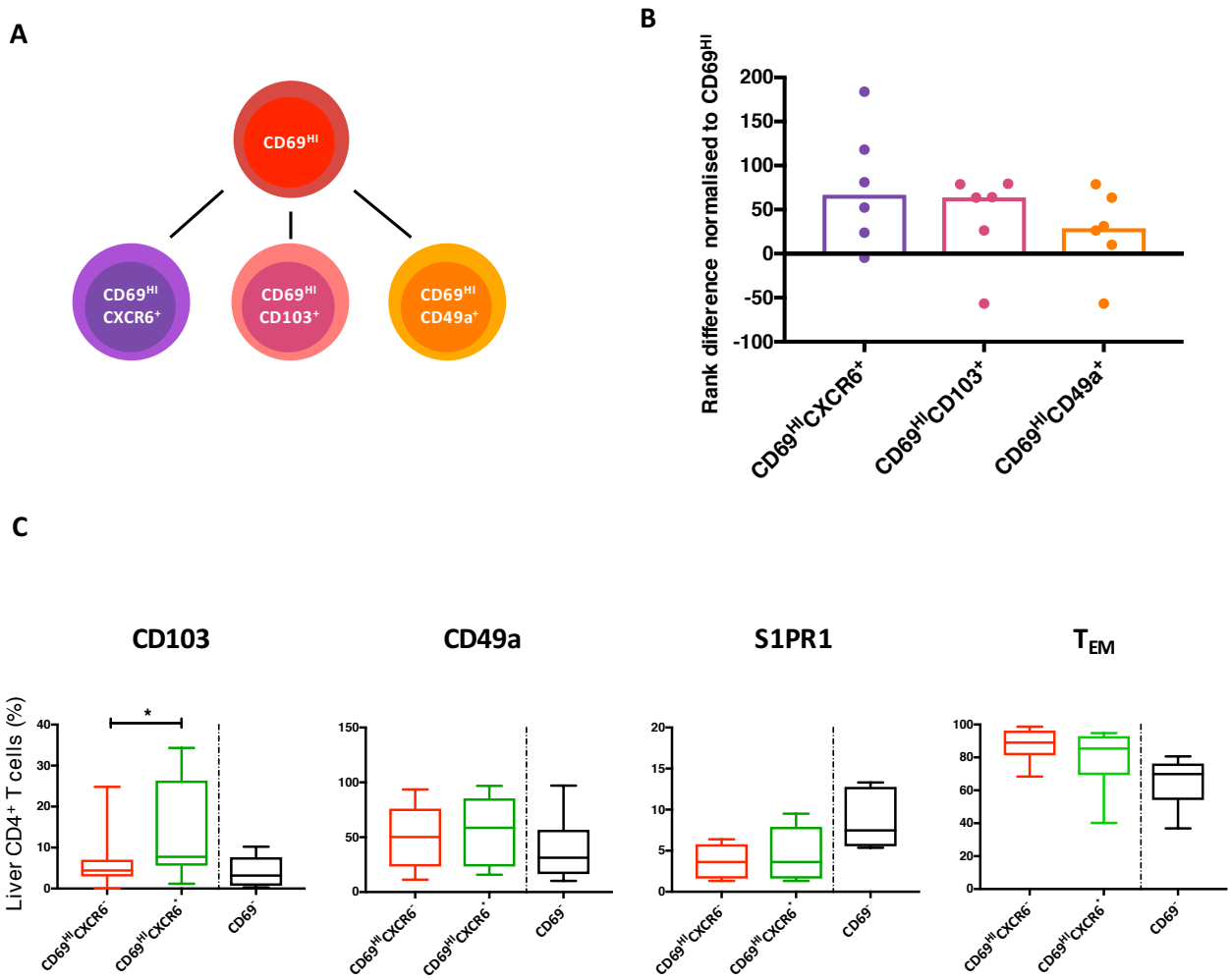


Figure 5.1.5 – Determining the best Tissue Resident population. A – The intrahepatic $CD4^+$ $CD69^{HI}$ T cells were compared with the $CD69^{HI}CXCR6^+$, $CD69^+CD103^+$, & $CD69^{HI}CD49a^+$ sub-populations in terms of expression of the other residence markers (CXCR6, CD103 and CD49a). **B –** 6 donor liver populations were analysed (from mixed aetiologies). For each donor the percentage expression of each residence marker was analysed (CXCR6, CD103 and CD49a) within each subpopulation. This expression was then normalised to that of the control $CD69^{HI}$ population. Finally, the differences found were summed within each population to allow a simple comparison to the control population, 0. Median average used. **C** – Comparison of residence factors, CD103, CD49a, S1PR1 and effector memory (T_{EM} – $CD45RA^-CCR7^-$) in $CD69^{HI}CXCR6^-$ cells (red) and $CD69^{HI}CXCR6^+$ cells (green). $CD69^-$ (black) populations are included as controls. Box and whisker plots show min. to max. (n=6-9).

background in $CD4^+$ or $CD4^-$ T cells (Fig. 5.1.6A). Naïve/memory cell compartmentalisation analysis dispelled any doubts that high IMC background was hiding true Hobit staining in $CD4^+$ T cells as naïve T cells showed the highest fluorescence in the channel occupied by Hobit staining (Fig. 5.1.6B). This is unlikely to be a true result as it is known that Hobit is specifically upregulated in effector T cell populations in humans[190]. However, the Hobit staining protocol did work, at least in part, as evidenced by positive Hobit expression in the remaining two livers (Fig. 5.1.6C). Interestingly, looking at $CD4^+$ T cells in the donor with the largest Hobit expression, there was a slight preference for Hobit expression in $CD69^{INT}$ cells, but no change in the more phenotypically resident $CD69^{HI}$ population (Fig. 5.1.6D). Thus, preliminary data pointed to no strong association of Hobit with $CD69^{HI}$ T_{RM}, with intrahepatic T cells from most livers seemingly lacking this transcription factor.

Next, we asked whether $CD69^{HI}$ T_{RM} cells, or the newly identified $CD69^{INT}$ $CD4^+$ T cell population were enriched in any specific liver disease aetiology. We saw no such enrichment in either population when comparing donors with dietary (ALD, NASH), or autoimmune end stage liver diseases (PBC, PSC) with healthy controls, although more donors may be needed to truly confirm this (Fig. 5.1.7). These results suggest neither $CD69^{HI}$ nor $CD69^{INT}$ frequencies are associated with non-viral liver disease, and instead are universally present, consistent components of human liver immunity.

In sum, these results identify two distinct intrahepatic $CD4^+$ T cell populations based on CD69 expression levels. $CD69^{HI}$ cells likely represent the true tissue resident cells of the liver due to their absence in the circulation, lack of S1PR1 expression, and the highest expression of other T_{RM} associated markers; while $CD69^{INT}$ present with a semi-resident

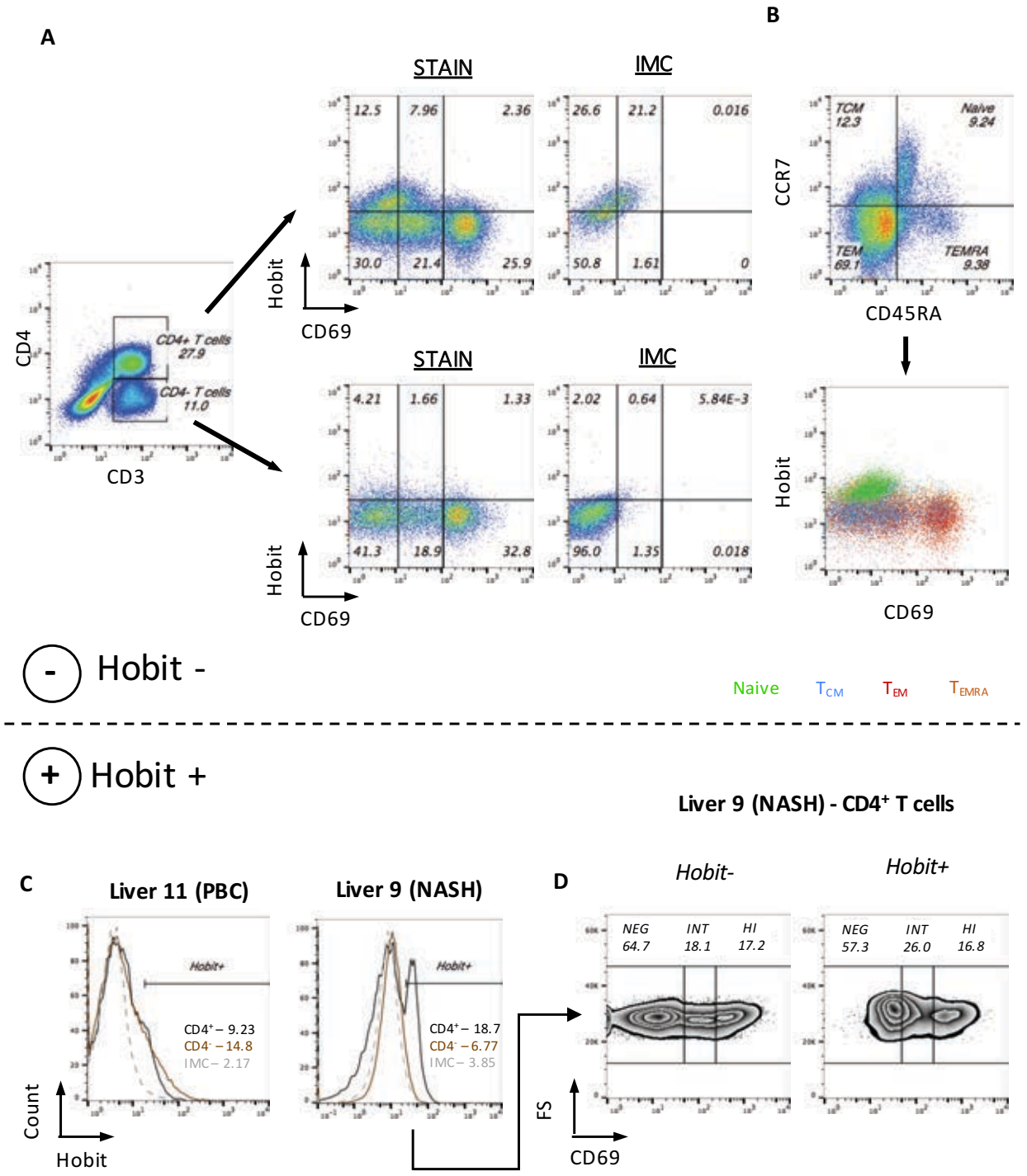


Figure 5.1.6 – Hobit expression in intrahepatic CD4$^+$ T cell populations. A,B – Hobit Negative liver T cells. **A –** In 3/5 livers no clear Hobit expression was detected (representative flow cytometry plots shown). CD4$^+$ and CD4- T cells were investigated. In each population the expression of Hobit was investigated in the context of CD69 allowing both Hobit$^+$ and Hobit$^-$ gating in conjuction with gating on CD69$^-$, CD69^{INT} and CD69^{HI} cells. **B -** Representative Hobit and CD69 co-expression in the four canonical naïve/memory subsets from the livers that showed no clear Hobit expression (naïve, green; central memory, blue; effector memory, red; & TEMRA, orange). **C,D –** Hobit positive liver T cells. **C –** Hobit expression in CD4$^+$ T cells (black) and CD4$^-$ T cells (brown) from the two livers where Hobit positivity was detected above isotype matched control (grey). Percentages of Hobit$^+$ cells are given in the legend. **D** – Percentage of cells in the CD69$^-$, CD69^{INT}, and CD69^{HI} CD4$^+$ T cell pools within both the Hobit$^-$, and Hobit$^+$ gates from liver 9 (where CD4$^+$ Hobit expression > CD8$^+$ Hobit expression).

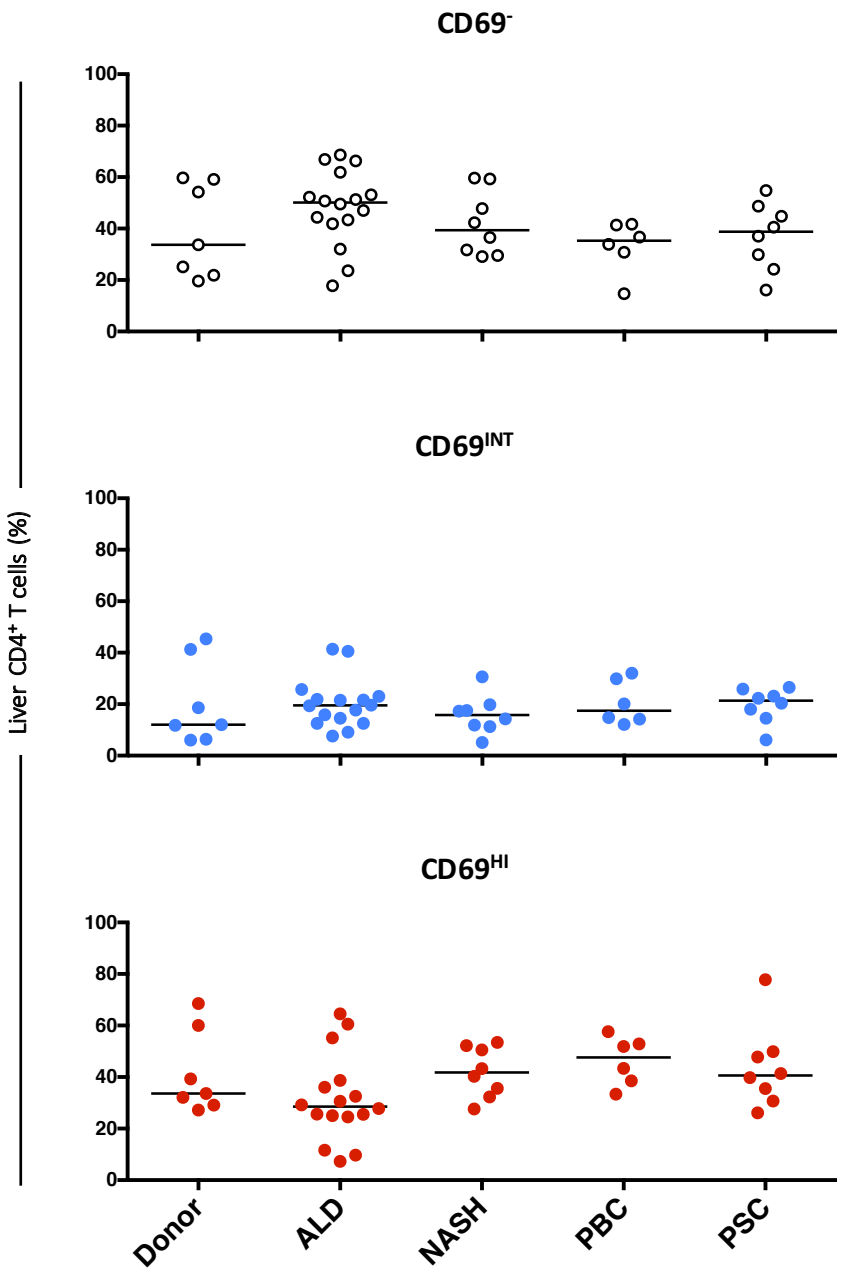


Figure 5.1.7 – CD69^{-}, CD69^{INT} or CD69^{HI} populations are not enriched in different liver diseases. Proportion of intrahepatic CD4^{+} T cells that are CD69^{-}, CD69^{INT} or CD69^{HI} from different chronic end-stage disease liver donors. Donor (n=7), ALD (n=16), NASH (n=8), PBC (n=6), PSC (n=8). Averages displayed are medians.

phenotype, also absent from the blood, and intermediary T_{RM} marker expression, but with clear S1PR1 expression. Neither population appeared to express Hobit, and neither were clearly associated with pathology. Future mentions of $CD69^{HI}$ and $CD69^{INT}$ cells here pertain to intrahepatic $CD4^+$ T cells.

5.3 $CD69^{HI}$ T_{RM} display a more restricted homing receptor profile than $CD69^{INT}$ cells

Next, we moved on to the analysis of further homing receptor expression patterns in order to ascertain migratory potential of the different populations *in vivo*. Chemokine receptors CCR5 and CXCR3 are involved in T cell recruitment to portal and sinusoidal endothelium[362], while CXCR6, CCR6, CCR10 and CX3CR1 are associated with bile duct infiltration[388, 389], and CXCR1 expression on a T cell allows migration towards inflamed hepatocytes[396]. In addition to maintenance in the liver, CXCR6 expression is important for a T cell to access the bile ducts, and liver parenchyma[391]. Of the three populations, $CD69^{HI}$ T cells only had the highest expression of CCR5, showed increased CCR6 from $CD69^-$ cells, and negligible expression of CX3CR1 (Fig 5.2.1). By stark contrast, $CD69^{INT}$ cells had the highest expression of CXCR1 and CXCR3, matched $CD69^{HI}$ expression of CCR6, retained consistent expression of CX3CR1, and had an intermediate expression of CCR5. CCR10 expression was not differentially expressed between the subsets. Furthermore, $CD69^{INT}$ cells expressed the most gut-homing chemokine receptor CCR9, and a significantly higher percentage of gut-homing integrin $\alpha4\beta7$ than $CD69^{HI}$ cells (Fig. 5.2.2). These data suggest that $CD69^{HI}$ T_{RM} cells have migration patterns largely restricted to the portal areas, bile ducts and parenchyma through elevated CCR5, CCR6 and CXCR6 expression. Contrastingly, $CD69^{INT}$ cells may have more of a free reign over the liver through their wider array of expressed chemokine receptors, and may be able to

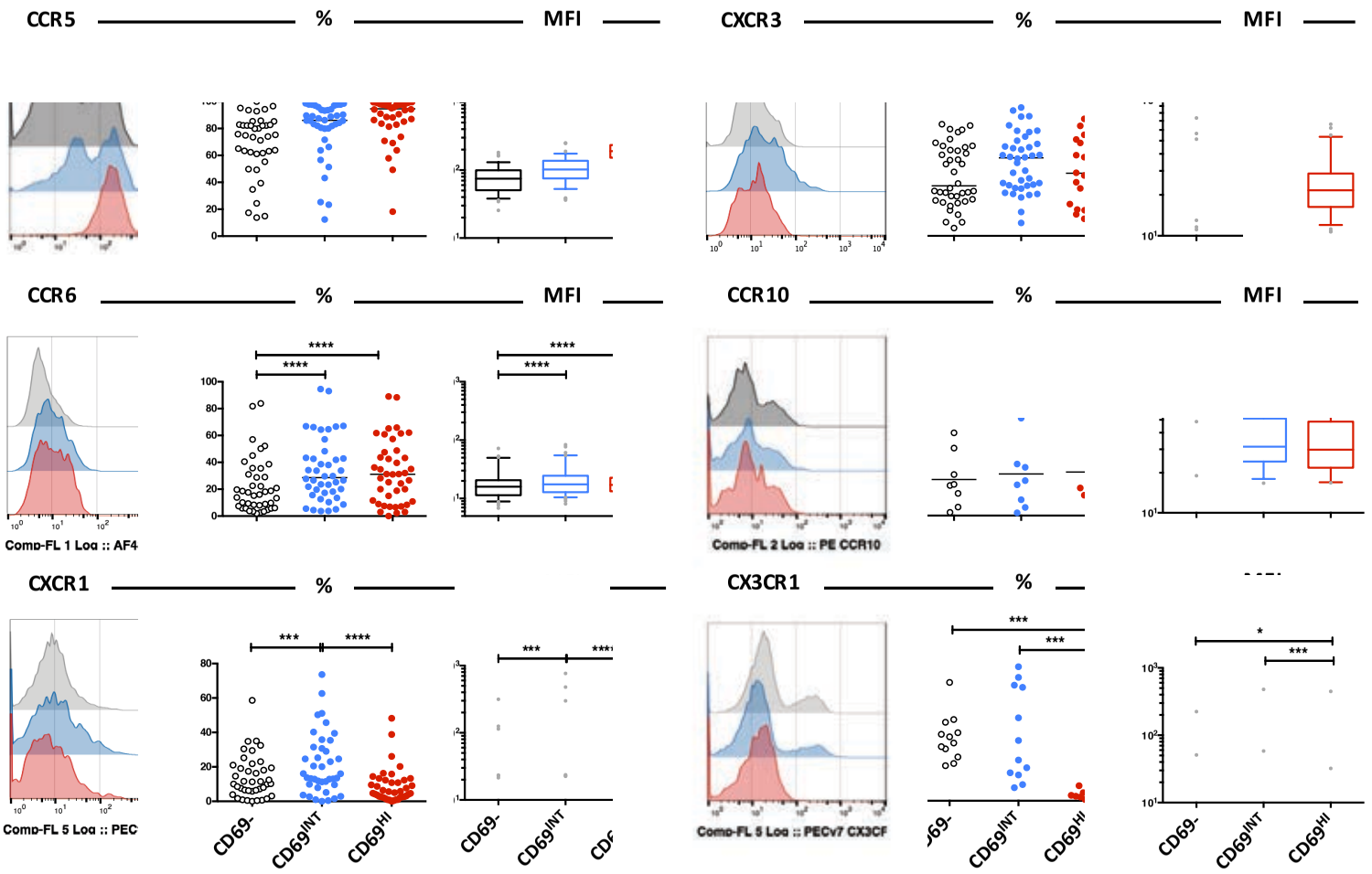


Figure 5.2.1 – CD69^{HI} cells show increased CCR5 and CCR6 expression, while CD69^{INT} cells show more varied chemokine expression patterns. Six different chemokine receptors are shown. As in 5.1.3, for each triple representative flow cytometry plots, combined percentage marker expression, and combined MFI of the marker-positive cells are shown. Combined healthy and disease donors shown. Lines represent median values, error bars show 10-90% data range

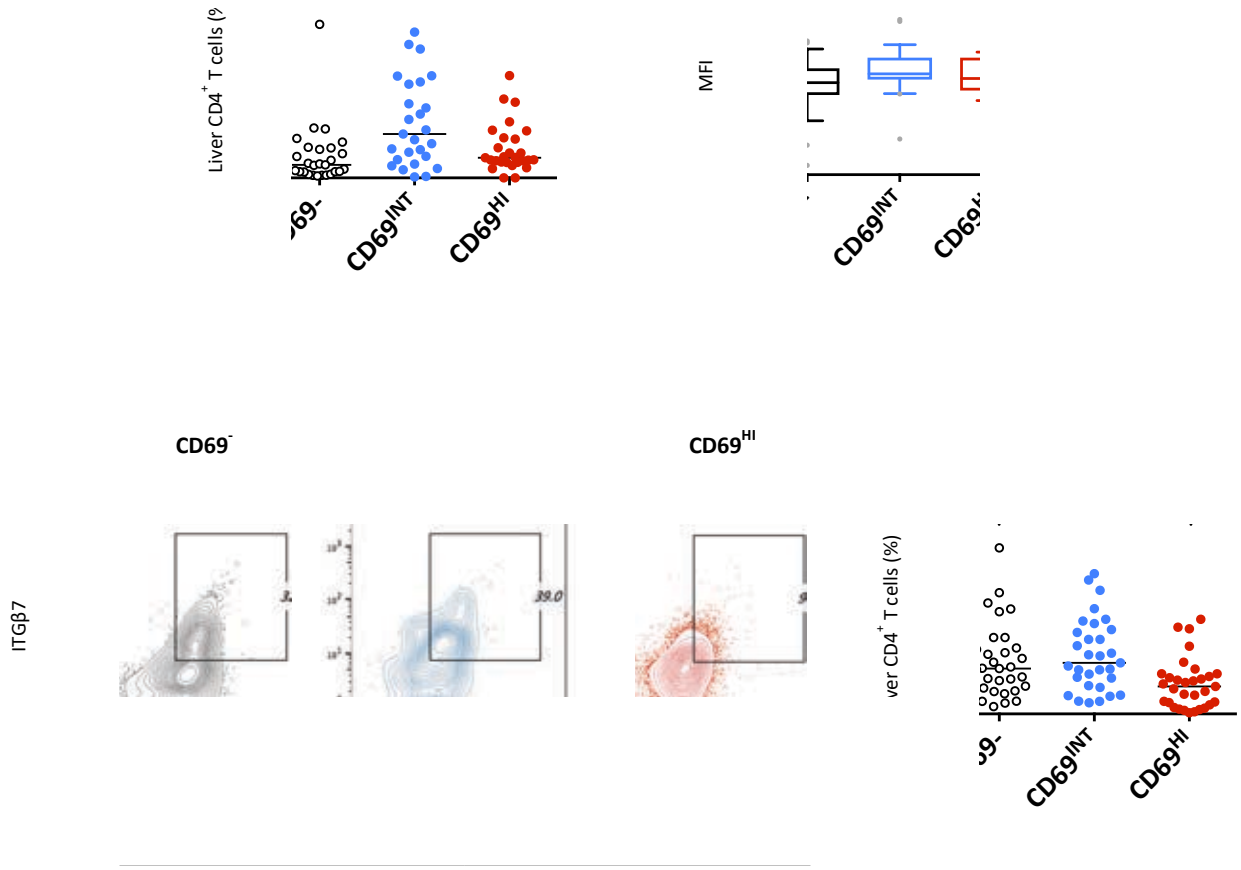


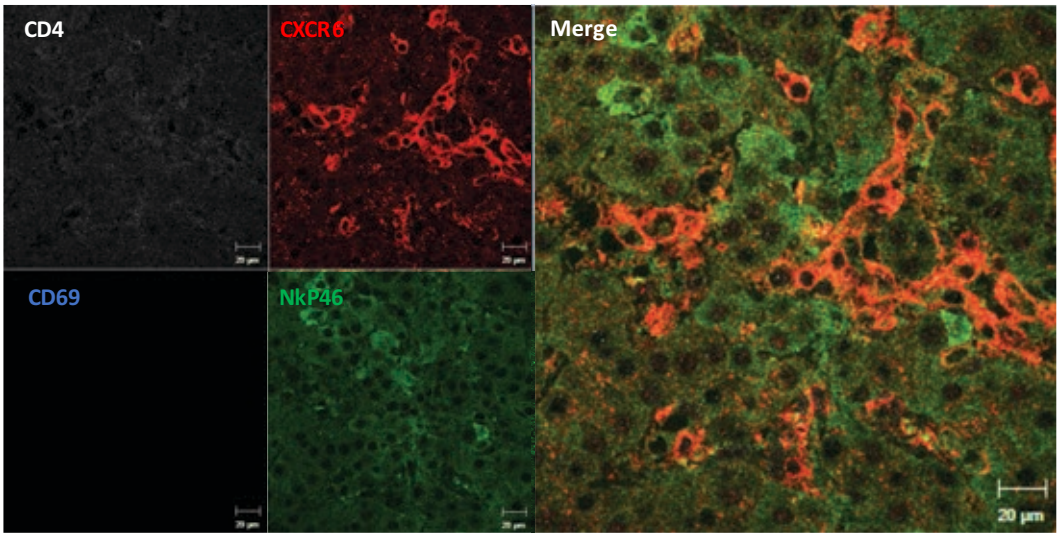
Figure 5.2.2 – CD69^{INT} cells have increased expression of the gut-homing markers CCR9 and Integrin α4β7. A – Combined expression of CCR9 (% and MFI) in the three liver CD4^{+} populations. **B –** Integrin α4 and Integrin β7 co-expression representative staining (left) and combined percentage expression data (right) in the three liver CD4^{+} populations. For both parts all healthy and diseased liver donors shown. Lines represent medians, error bars show 10-90% data range.

migrate to the gut through CCR9 and integrin $\alpha4\beta7$. These patterns are in keeping with our model of $CD69^{HI}$ cells acting as a tissue-resident population, restricted in tissue niches; while also suggesting $CD69^{INT}$ cells are less confined, arguing against their placement into a bona fide T_{RM} category.

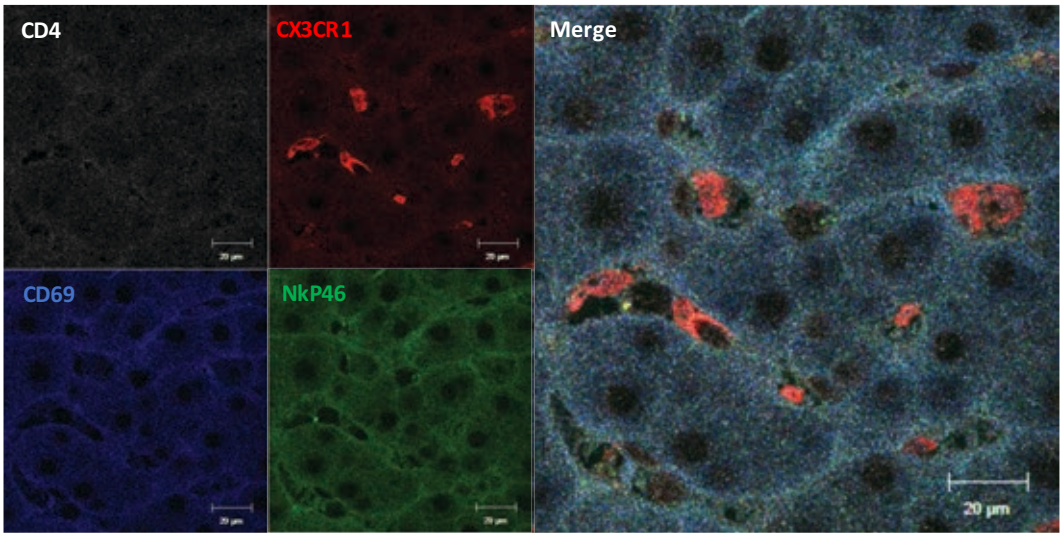
In an attempt to support these conclusions, we performed immunofluorescence staining of liver sections with the aim of identifying the positions of $CD69^{HI}$ and $CD69^{INT}$ cells in the liver. Unlike flow cytometry, this staining is not sensitive enough to differentiate cells expressing intermediate levels of CD69 from those expressing high levels. Instead, we stained for CXCR6 and CX3CR1 alongside CD69 to differentiate $CD69^{HI}$ and $CD69^{INT}$ cells respectively as we have already seen these receptors are largely population-specific (Fig. 5.1.3A; Fig 5.2.1). We combined these with stains for CD4 and NKp46 (in order to rule out contaminating NK or NKT cells, B cells and $CD8^+$ T cells). Unfortunately, we were forced to limit our conclusions as both CD4 and CD69 staining was unsuccessful in these experiments. However, CXCR6 and CX3CR1 staining worked well, allowing us to perform simple assessments of these chemokine receptor localisation patterns across the liver architecture. CXCR6 was detected heavily in the liver parenchyma and around bile ducts, but scarcely in the fibrotic tracts (Figs. 5.2.3; 5.2.4). CX3CR1 was detected in the parenchyma and in sinusoids, but not in fibrotic tracts or around bile ducts. So, although we could not detect specific lymphocyte subsets, these preliminary data largely agree with flow cytometry staining patterns – CXCR6-expressing cells may favour the parenchyma and bile ducts, whereas CX3CR1-expressing cells may additionally be found in sinusoids, but do not preferentially associate with biliary cells.

Parenchyma

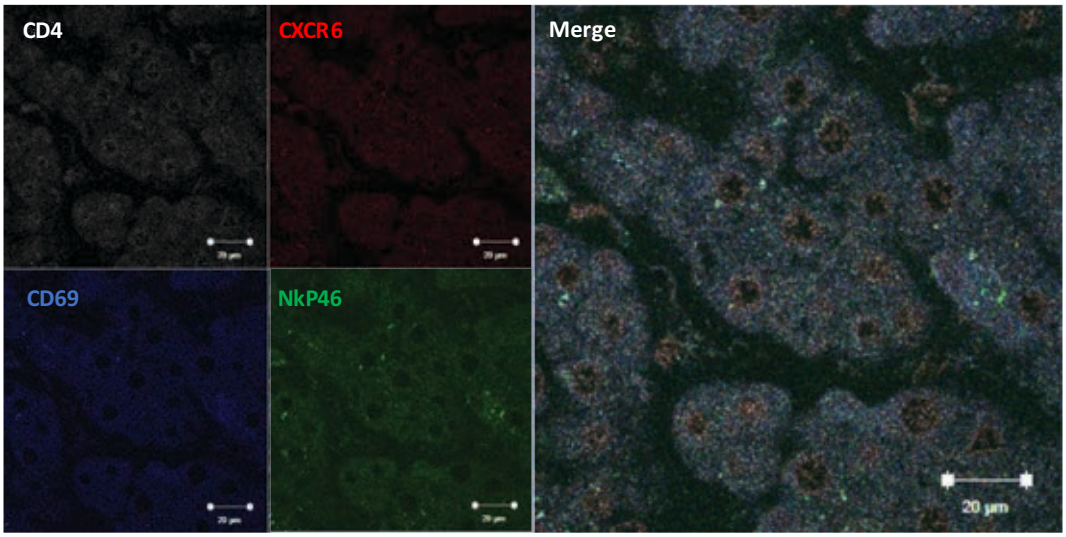
Panel 1 (CXCR6)



Panel 2 (CX3CR1)



IMC



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and merged immunofluorescence staining patterns for panel 1, panel 2 and IMC (top to bottom respectively). These data show the parenchymal regions. Representative staining from n=4 combined chronic liver disease donors. *(cont. from previous page)*

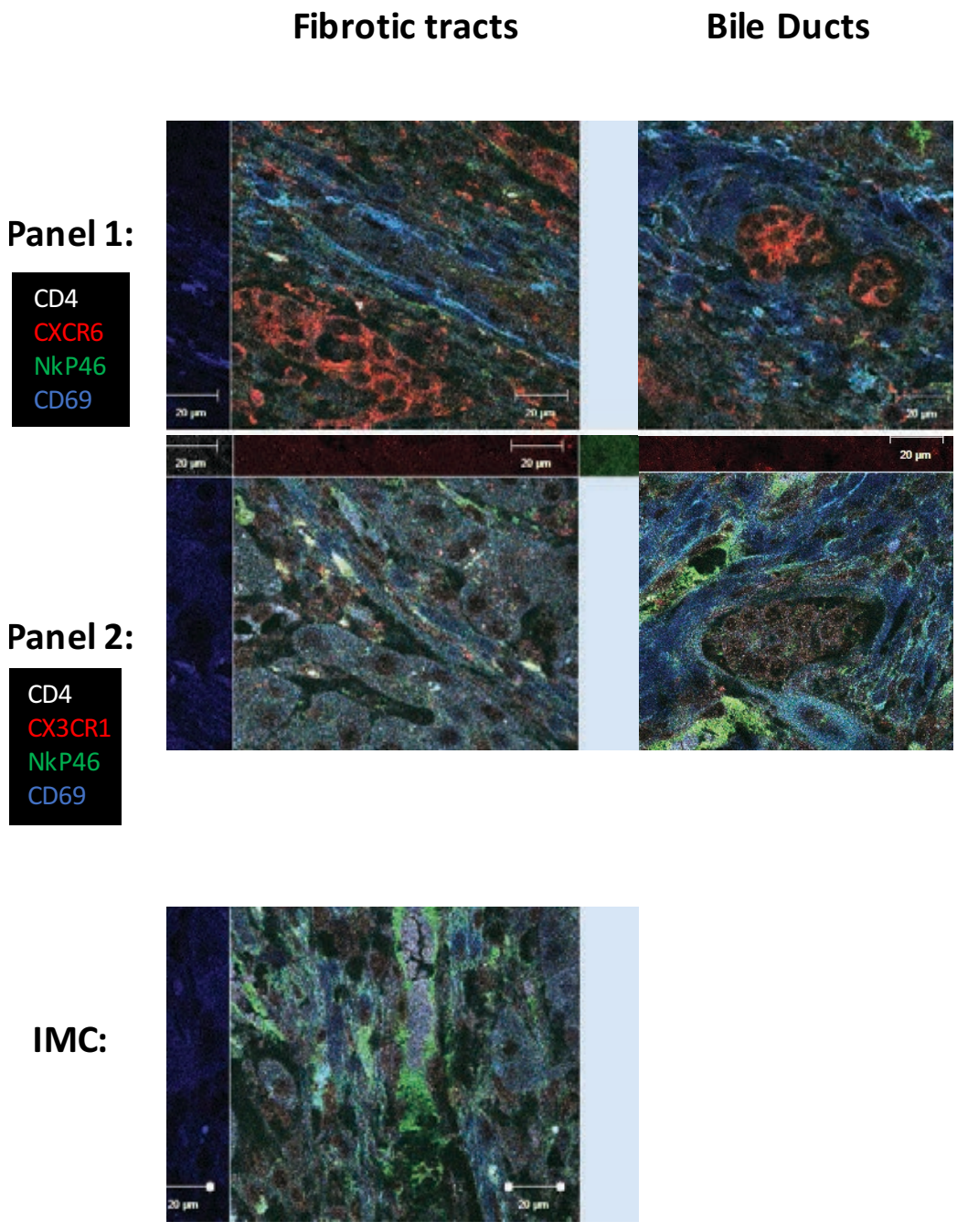

Figure 5.2.4 – CXCR6 and CX3CR1 staining in fibrotic tract and bile ducts of diseased human liver. Merged immunofluorescence images from both fibrotic tract areas and bile ducts for both panels and IMC. Representative images from n=4 combined chronic liver disease donors.

5.4 CD69^{HI} and CD69^{INT} cells possess distinct activation profiles and differentiation states

Continuing deeper into the characterisation of the three intrahepatic CD4^{+} T cell populations, we analysed activation marker expression patterns, beginning with commonly used activation markers CD25, CD38, and HLA-DR[24-26]. In combined end-stage disease livers, CD69^{HI} cells displayed the highest % of CD25 expression, but the expression level per cell was no higher than in the other two populations (Fig. 5.3.1A,B). CD38 expression was highest in CD69^{INT} cells, both at a population and per-cell level. Percentage HLA-DR expression was also highest in the CD69^{INT} cells, and CD69^{HI} cells had significantly lower expression levels compared to the other two subsets. In healthy donors, patterns were harder to interpret due to low donor numbers. However, it appeared that % CD25 expression correlated with CD69 expression, just like in the diseased donors (Fig 5.3.1C). CD38 and HLA-DR expression directional trends appeared to match those in the diseased donors as well. Additionally, analysis of proliferation marker Ki-67 demonstrated that CD69^{INT} cells were the most actively dividing, a statistically significant increase from CD69^{HI} cells (Fig. 5.3.2). Overall, CD69^{INT} appeared to be the most activated subset, displaying more CD38 and HLA-DR molecules, and contain a greater cycling fraction in the absence of artificial stimulation.

Human T_{RM} have been associated with elevated inhibitory receptors, such as PD-1 and CTLA-4[91, 134, 135, 148]. Similarly, in our study we noted a strong association of CD69^{HI} T_{RM} with PD-1 expression in both healthy and diseased livers, although no increases in CTLA-4 expression were seen compared to the CD69^{-} control cells (Fig. 5.3.3). The fraction of CD69^{INT} cells expressing PD-1 was midway between the other two subsets, and CTLA-

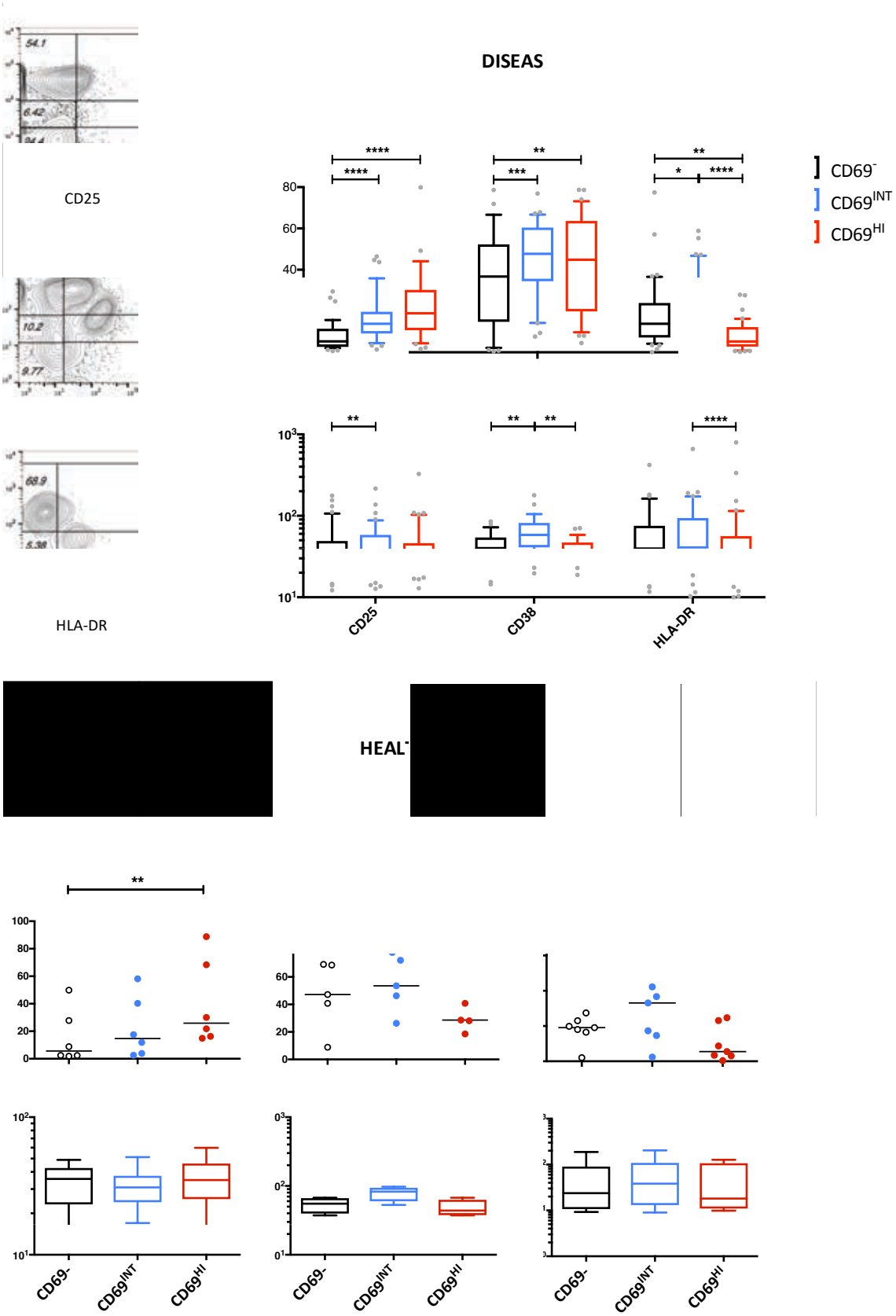

Figure 5.3.1 – CD69^{INT} represent the most activated intrahepatic CD4^{+} T cell population. A – Representative flow cytometry staining examples from diseased liver explants showing activation markers CD25, CD38 and HLA-DR in the context of CD69. **B –** Combined diseased liver explant data showing these activation markers as % of liver CD4^{+} T cells (top graph), and as MFI of marker-positive cells (bottom graph).

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C – Combined healthy liver explant data of these activation markers - % (top panel) and MFI of marker-positive cells (bottom panel). For all chart types median averages are used, error bars show 1—90% data range.

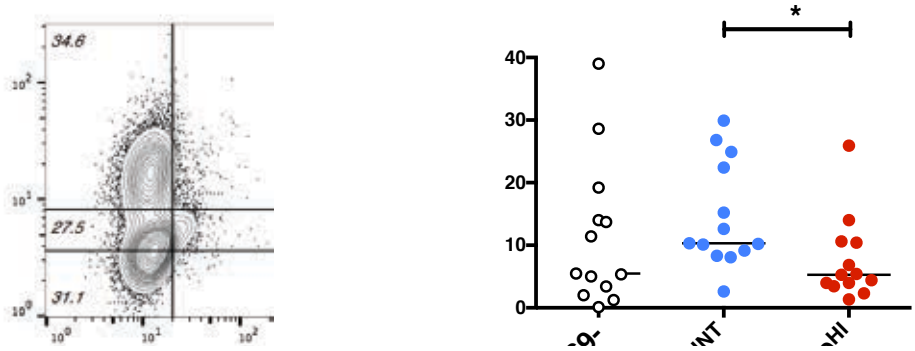

Figure 5.3.2 – Proliferation is highest among the CD69^{INT} CD4^{+} Liver T cell population. Representative flow cytometry staining (left) and combined percentage expression data from all healthy and diseased donors for proliferation marker Ki-67. All three liver CD69 populations shown. Ki-67 was detected by intracellular staining without stimulation directly following isolation from liver explants. Lines represent median values.

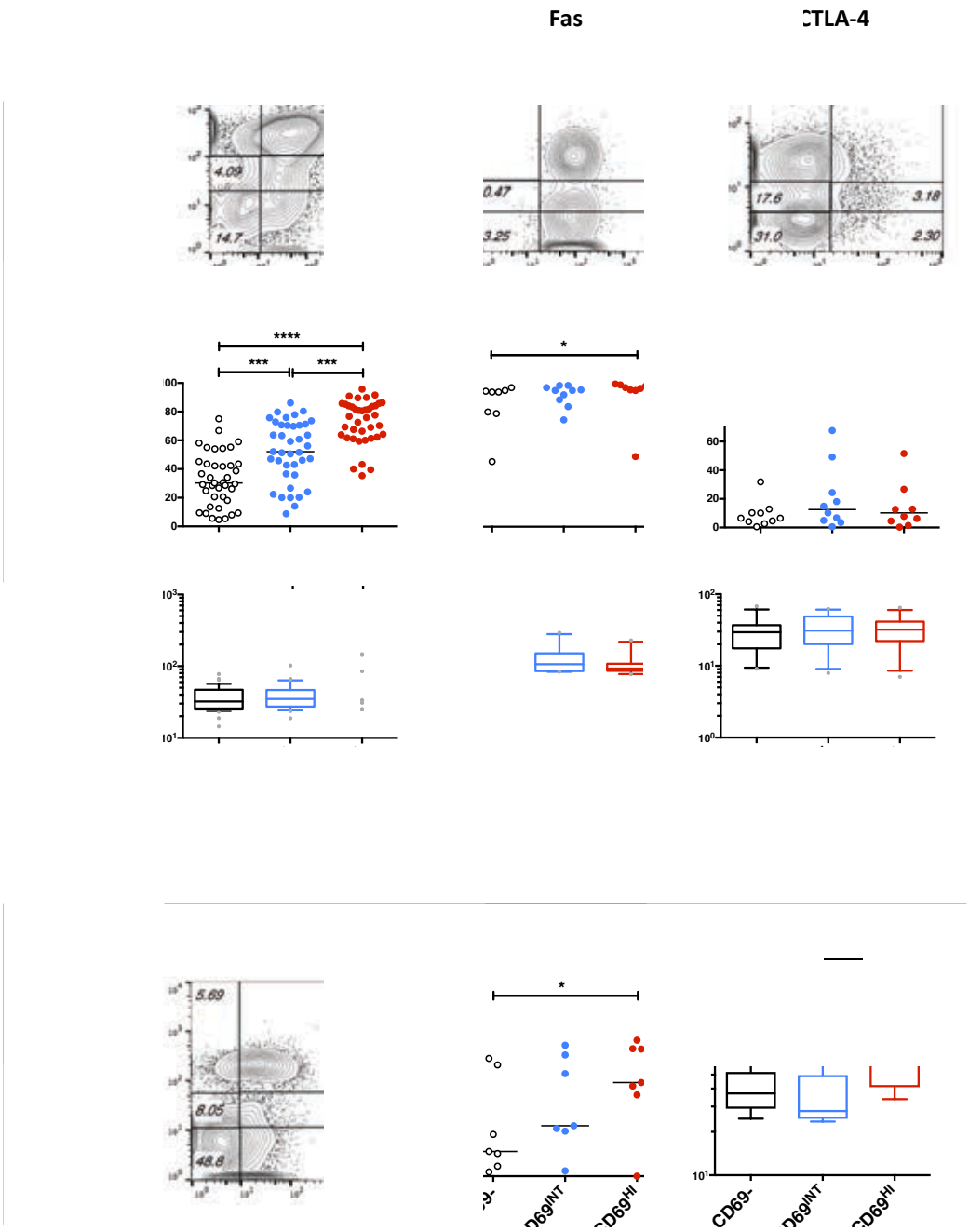

Figure 5.3.3 – Inhibitory marker expression is greatest amongst CD69^{HI} liver CD4^{+} T cells. A - Expression of inhibitory markers PD-1, Fas, and CTLA-4 in CD69^{-}, CD69^{INT} and CD69^{HI} cells from diseased liver donors. From top to bottom: representative staining in each CD69 population, expression (%) and expression (MFI + cells). **B –** As part A, but only PD-1 expression shown in healthy donor liver populations. Median used as average value, error bars show 10-90% data range.

4^+ cells made up a moderately higher fraction of the $CD69^{INT}$ subset than the $CD69^-$ cells. We also analysed expression of the death receptor Fas and saw that in most livers, nearly all intrahepatic $CD4^+$ T cells expressed this receptor, regardless of CD69 expression. Although there was a statistically significant difference between $CD69^-$ and $CD69^{HI}$ cells for % Fas expression ($p=0.042$), the absolute difference was minute and so unlikely physiologically relevant.

We next used three key proteins to determine the differentiation status of our three CD69- subsets: CD27, KLRG-1, and CD127. CD27 is a key costimulatory receptor, loss of which is a mark of a more differentiated memory cell type[19, 657-659]. Interestingly, the $CD69^{INT}$ population contained the greatest frequency and expression levels of CD27 in diseased livers; and at least the frequency data was matched in healthy livers (Fig. 5.3.4). KLRG-1 expression shows antigen experience, and advanced, late-stage differentiation[192, 193, 488, 660]. We noticed that for both diseased and healthy livers $CD69^{HI}$ T_{RM} cells had the lowest % expression of KLRG-1, while the other two subsets were similar. Correspondingly, CD127 expression, which marks a resting state[258], was at its highest level in both $CD69^{INT}$ and $CD69^{HI}$ cells; although in percentage terms, no statistical increase was seen in $CD69^{HI}$ cells from the $CD69^-$ subset ($p=0.061$), and these trends were not apparent in healthy liver donors.

The most information on differentiation status and antigen exposure history can be uncovered by using KLRG-1 and CD127 in combination. This strategy has been inspired by the work of Donna Farber's lab, who employed a very similar strategy using CD28 and CD127 to define four distinct stages of activation and homeostasis[90]. As KLRG-1 denotes antigen experience, and CD127 downregulation occurs both upon antigen recognition

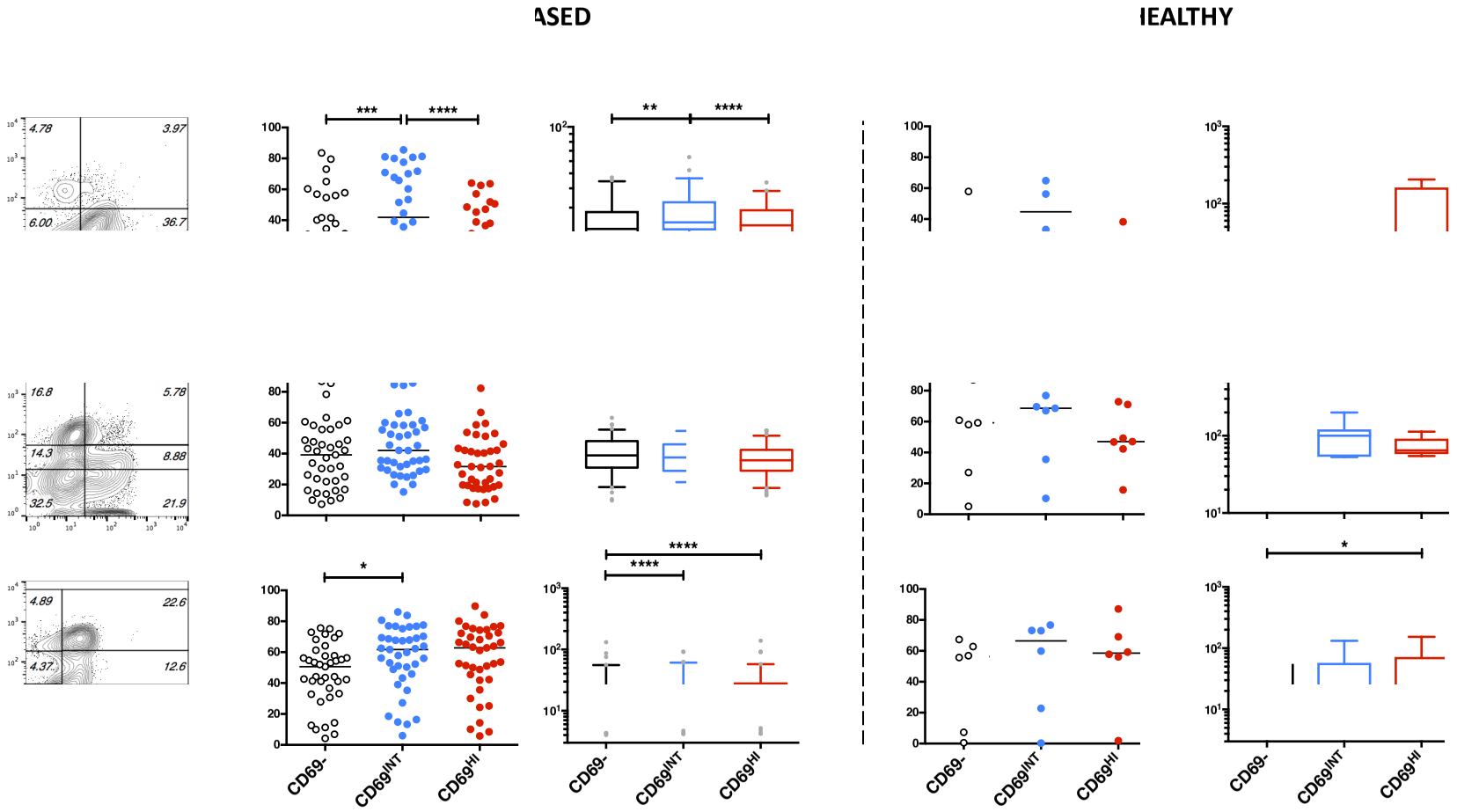

Figure 5.3.4 – Distinct differentiation profiles of CD69^{INT} and CD69^{HI} intrahepatic CD4^{+}T cells. Expression patterns of differentiation marke CD27, KLRG-1 and CD127 amongst the CD69^{-}, CD69^{INT}, and CD69^{HI} intrahepatic populations. From left to right: representative flow cytome staining, % of liver CD4^{+} T cell expression in diseased liver donors, MFI (of marker-positive cells) in diseased donors, % of CD4^{+}T cell expressi

and ligation with common-γ chain cytokines and IL-6[258], we defined these four stages as follows: KLRG-1^{-}CD127^{+} - resting/unstimulated, KLRG-1^{-}CD127^{-} - cytokine-stimulated, KLRG-1^{+}CD127^{-} - antigen-driven effector, KLRG-1^{+}CD127^{+} - previously responded to antigen. When comparing the three CD69-defined subsets in healthy and diseased livers, CD69^{HI} cells had a strong preference for resting (KLRG-1^{-}CD127^{+}) cells (Fig. 5.3.5). By contrast, the previously activated (KLRG-1^{+}CD127^{+}) phenotype was favoured by CD69^{INT} cells over both other subsets in disease, and seemingly over CD69^{HI} cells in healthy donors. Activated/effector (KLRG-1^{+}CD127^{-}) and cytokine-stimulated (KLRG-1^{-} CD127^{-}) cells were similar in frequency across the three subsets. Together, these results show a strong dichotomy of a non-terminally differentiated resting CD69^{HI} T_{RM} cell type, compared to a more differentiated previously activated CD69^{INT} cell population.

Finally, no CD69-subset contained a greater fraction of conventional regulatory cells (CD25^{HI}CD127^{LO}), and no subset strongly favoured FoxP3 expression (Fig. 5.3.6). We also performed a preliminary assessment of expression of components of the CD28 costimulatory pathway – CD28, CD80, and CD86. In the one donor tested (a PSC patient), we noted highest % expression of CD28 in the CD69^{HI} compartment (Fig. 5.3.7). This fits with previous data that argues CD69^{HI} cells are largely not terminally differentiated, as loss of CD28 correlates with late-stage differentiation of memory cells[659]. T cell expression of CD80 and CD86 is not well understood, but both could enhance survival and are likely involved in T cell - T cell antigen presentation[661, 662]. Here, CD80 was progressively and steadily increased with higher CD69 expression, but CD86 was markedly absent on CD69^{HI} T_{RM} cells.

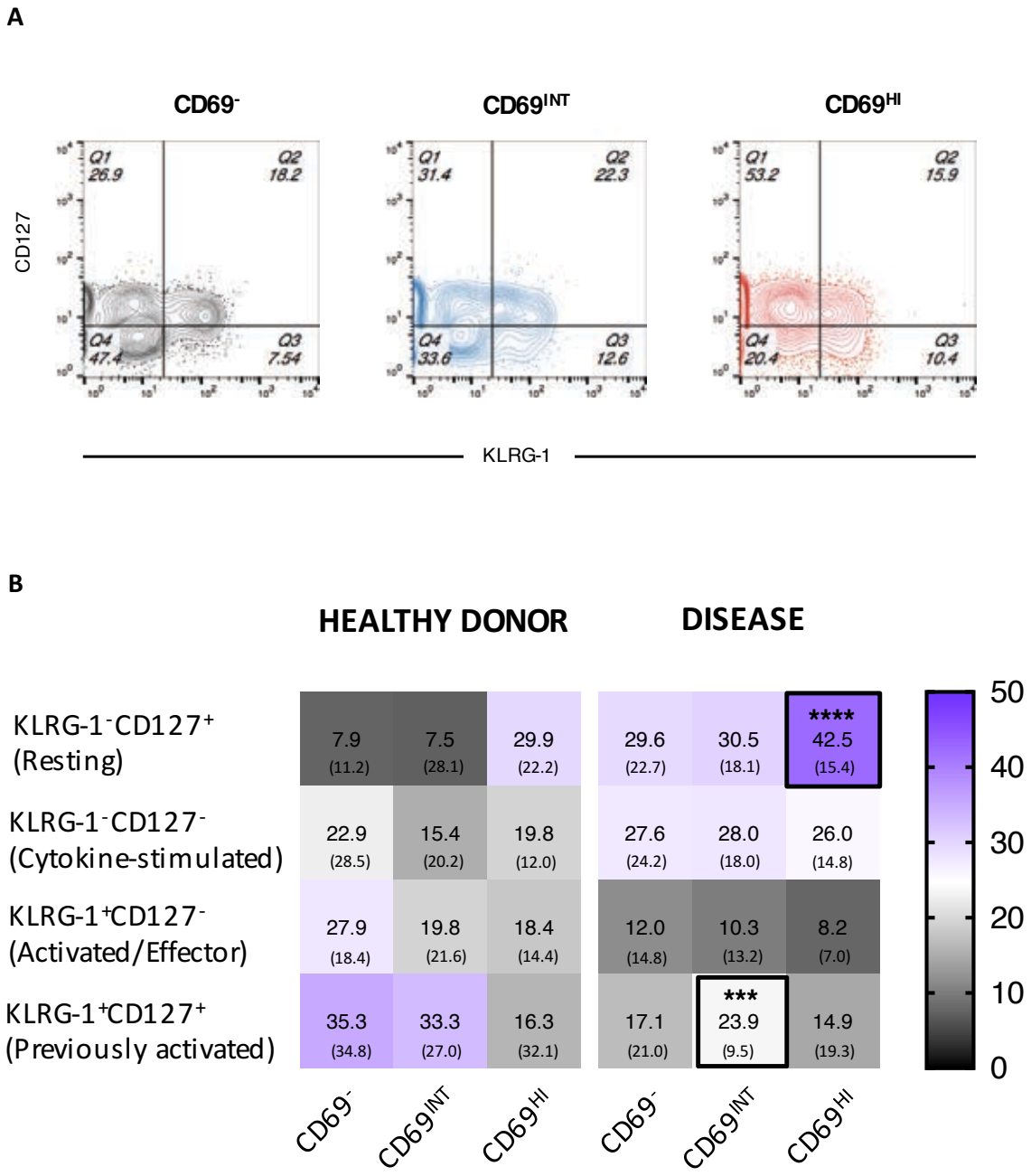

Figure 5.3.5 – KLRG-1 and CD127 co-expression patterns reveal CD69^{HI} and CD69^{INT} favour resting and previously activated phenotypes respectively. A – Representative flow cytometry staining in CD69^{-}, CD69^{INT}, and CD69^{HI} cells (from a diseased liver) showing co-expression patterns of differentiation and homeostasis markers KLRG-1 and CD127. Using these markers allows the divergence of populations into four subtypes as shown: resting (KLRG-1^{-}CD127^{+}), cytokine-stimulated (KLRG-1^{-}CD127^{-}), activated/effector (KLRG-1^{+}CD127^{-}), and previously activated (KLRG-1^{+}CD127^{+}). **B** - Heat-map showing combined % co-expression of the four aforementioned subsets. Central numbers in each cell represent median % expression, while values in parentheses represent the interquartile range. Asterisks in a cell denote the minimum statistically significant value when compared to both other values in the row (in each case highest p value used). Healthy donor, n=6; diseased donor, n=35.

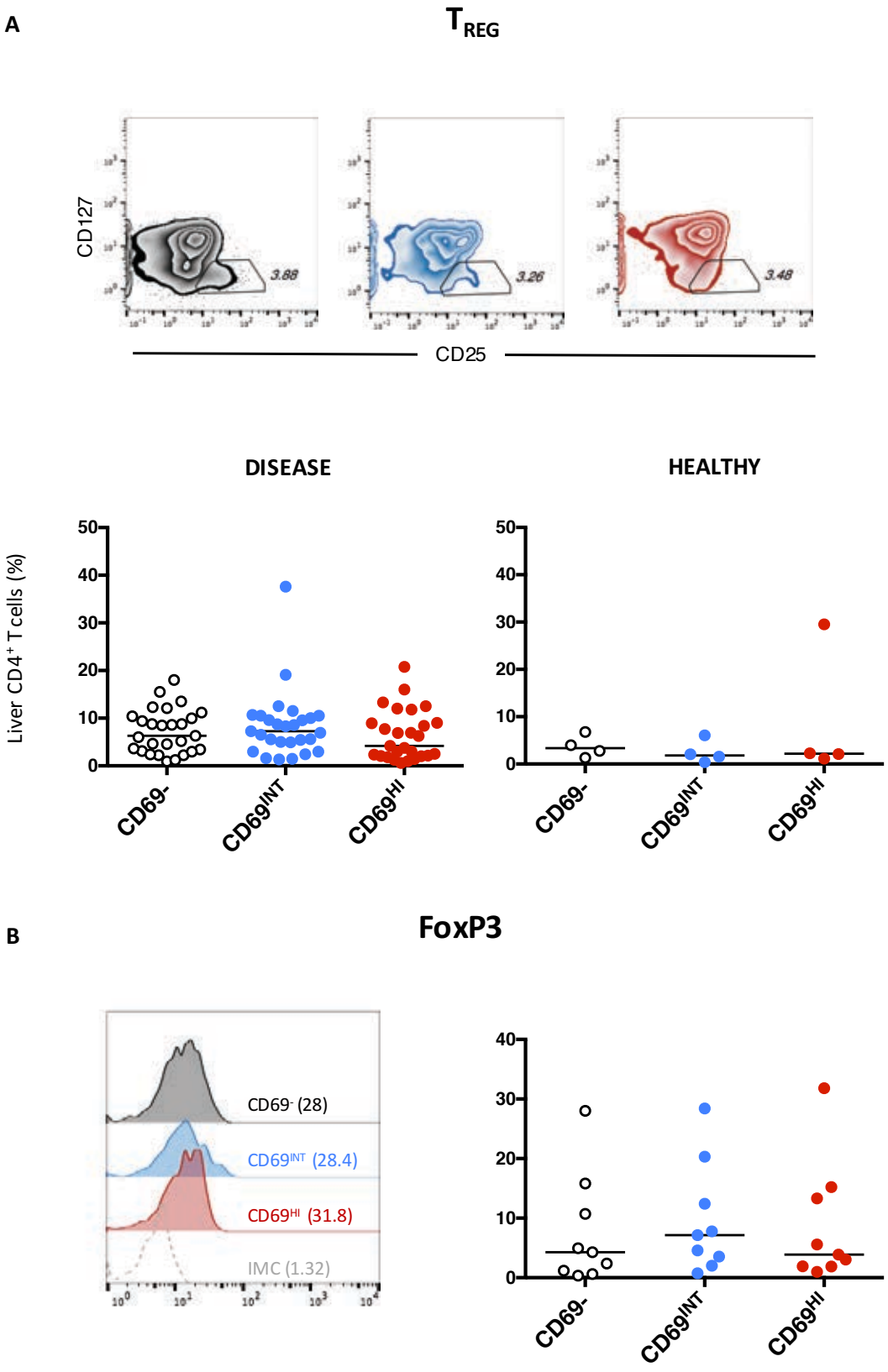

Figure 5.3.6 – Conventional regulatory cells are not markedly enriched across the different intrahepatic CD69-expressing populations. A – Percentage of conventional T_{REG} in $CD69^{-}$, $CD69^{INT}$, and $CD69^{HI}$ populations as representative staining (upper) and combined % in healthy and diseased donors (lower). **B –** Expression of FoxP3 in the three populations as representative staining and combined % expression for all diseased donors. Populations are indicated next to the histograms with the % expression for this example experiment listed in parentheses. For all scatter plots, lines represent median values.

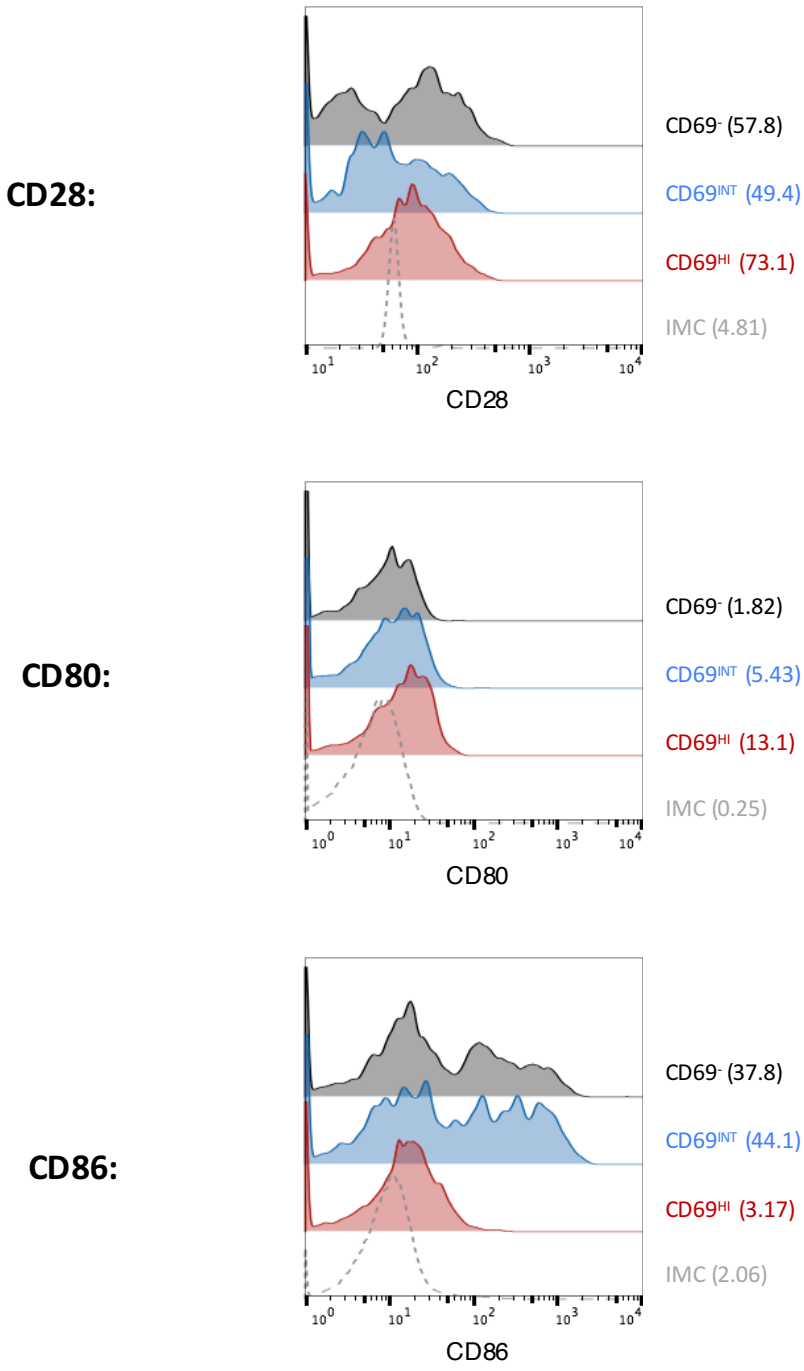

Figure 5.3.7 – Preliminary CD28, CD80, and CD86 staining in intrahepatic CD69 T cell subsets. Flow cytometry histograms of CD28, CD80, and CD86 expression in $CD69^-$ (dark grey), $CD69^{INT}$ (blue), and $CD69^{HI}$ (red) cells, as well as IMC controls (light grey – dashed). Percentage positive expression given in parentheses to the right of the plots. LIMCs isolated from a PSC liver explant. All histograms gated on $CD4^+$ T cells.

Collating all the phenotypic data so far, we have identified two distinct $CD4^+$ T cell populations in the liver. The first is a $CD69^{HI}$ T_{RM}-like cell that matched numerous residence characteristics including elevated residence-associated markers, elevated PD-1, and diminished KLRG-1. This cell type had a restricted chemokine receptor profile, and favoured a resting state. The second subset was a novel, more heterogeneous $CD69^{INT}$ cell type. This subset possessed a semi-resident phenotype, a wide array of homing receptors and contained a greater proportion of activated and previously activated cells. These differences are summarised in Fig. 5.3.8. Thus, we can conclude with some confidence that $CD69^{HI}$ cells are the $CD4^+$ T_{RM} of the human liver, whereas the role of $CD69^{INT}$ cells requires further investigation.

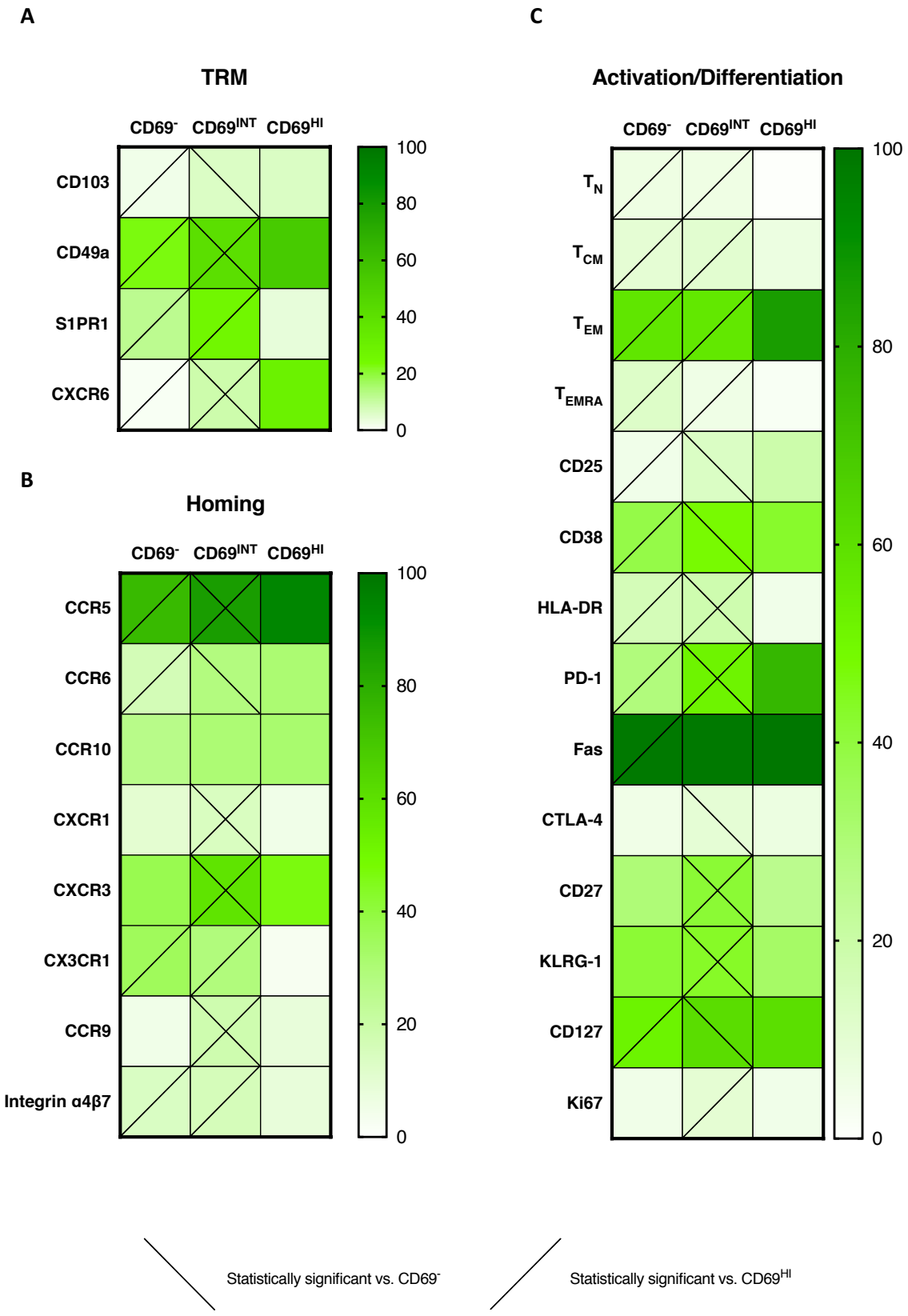

Figure 5.3.8 – In depth flow cytometric profiling of CD69^{-}, CD69^{INT} and CD69^{HI} CD4^{+} intrahepatic CD4^{+} T cells. Summary heat-maps showing median % expression of **(A)** TRM markers, **(B)** Homing receptors, and **(C)** Activation/differentiation markers across CD69^{-}, CD69^{INT} and CD69^{HI} cells. / in the box represents a statistically significant difference ($p<0.05$) in that population compared to CD69^{HI} cells, while \ demonstrates a statistically significant change to CD69^{-} cells. Total combined disease and healthy liver donors used for all plots.

5.5 Characterisation of CD69-expressing populations in blood and lymph nodes

Human studies on T cells have largely focussed on peripheral blood populations, and as such the roles and expression patterns of key T cell molecular determinants are mainly derived from these studies. We have used a plethora of surface antigens thus far to dissect the roles of our liver-infiltrating populations. To gain a better understanding of first what it means to be a T cell in the liver, and then to go deeper to examine how intrahepatic CD69-expressing subsets are distinct to blood T cells, we performed multi-parameter analyses on liver patient matched peripheral blood $CD4^+$ T cells. We have already shown that blood T cells did not appreciably express CD69 (Fig. 5.1.2A) and so all comparisons were made with blood $CD69^-$ $CD4^+$ T cells.

Blood $CD4^+$ T cells showed negligible expression of CD103 and CXCR6, and only a small fraction of $CD49a^+$ cells that was lower than all liver populations (Fig. 5.4.1A). S1PR1 expression was also extremely low, comparable to $CD69^{HI}$ cells in liver. As previously documented, we found blood contained much larger naïve and T_{CM} populations, and diminished T_{EM} cells compared to all liver populations (Fig. 5.4.1B)[314]. Although homing receptors CCR6 and integrin $\alpha4\beta7$ showed no change from the liver populations, % CCR5 and CXCR3 expression appeared lower in blood than the liver populations, CXCR1 was low – comparable to liver $CD69^{HI}$ cells, CCR9 was not expressed, and CX3CR1 was highly variable between donors (Fig. 5.4.1C). Activation and co-inhibitory markers CD25, CD38 and PD-1 were very similar in % expression to the $CD69^-$ liver cells, while $HLA\text{-}DR^+$ and $Ki\text{-}67^+$ fractions were low – more akin to the liver $CD69^{HI}$ population (Fig. 5.4.1D). There was a trend towards a higher T_{REG} representation in blood vs liver ($p=0.041$ vs. liver $CD69^{INT}$ cells), while CD27 appeared higher in blood populations ($p=0.022$ vs. liver $CD69^{HI}$ cells) (Fig. 5.4.1E). The most revealing differences on state of activation was seen

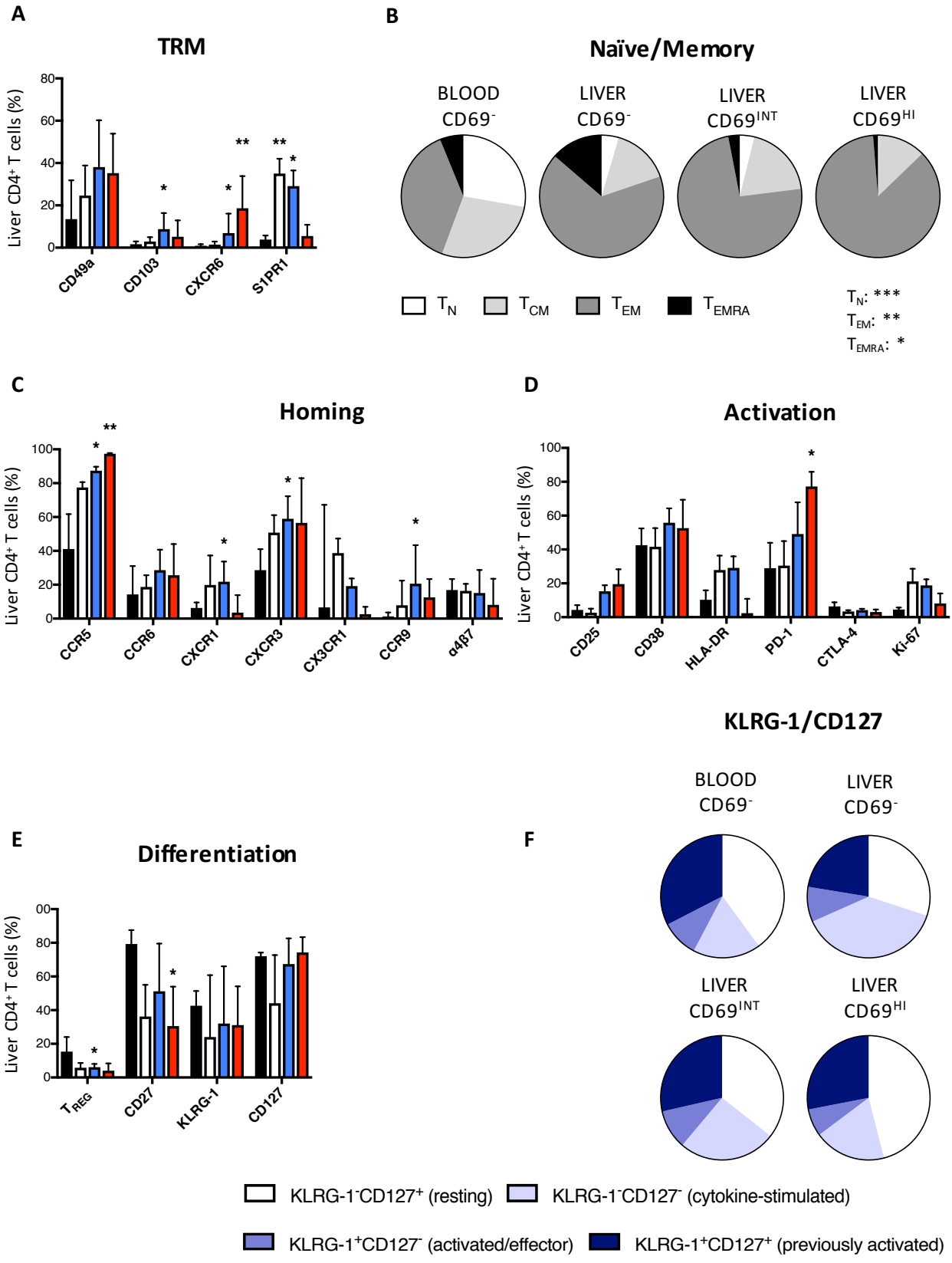

Figure 5.4.1 – Phenotype of matched blood and liver CD4$^+$ T cells. Bar charts show median (+IQR) % expression of different phenotypic markers in peripheral blood CD69$^-$ CD4$^+$ T cells (black); and liver CD69$^-$, CD69^{INT}, and CD69^{HI} CD4$^+$ T cells (white, blue, and red bars respectively. TRM (**A**), homing (**C**), activation (**D**) and differentiation (**E**) associated-markers are shown. **B, F –** Pie charts showing median proportion of each cell subset from liver and blood that fall into either different naïve and memory categories (**B**), or KLRG-1/CD127 delineations (**F**). *(Legend cont. on next page)*

For all bar charts, liver populations were all statistically compared to the blood $CD69^-$ population only. Asterisks above the bar indicate a statistical difference in that population to the control blood population. Similarly, for the pie charts liver populations were compared to the blood $CD69^-$ population only. Statistical differences in a population are listed below the corresponding pie chart. All data shown combination of 6 donors except naïve/memory, KLRG-1/CD127, S1PR1, CD25, CD127 (all n=5); T_{REG} (n=4), CX3CR1 (n=3); and CTLA-4, Ki-67 (both n=2).

using KLRG-1 and CD127 staining combinations, where interestingly blood CD69^{-} cells seemed to most closely resemble the liver CD69^{HI} subset, favouring a resting state for CD69^{HI} liver cells (Fig. 5.4.1E). Together, although still preliminary observations, these data allow us to put our findings in liver in the context of blood CD4^{+} T cells, and reveal that while blood CD69^{-} CD4^{+} T cells are most like their liver CD69^{-} counterparts in terms of low intrinsic activation, they are also akin to CD69^{HI} cells for resting homeostatic states.

Comparison with blood may have allowed us to examine how tissue T cells are distinct from their peripheral counterparts, but this falls short of demonstrating a liver-specific effect on T cell biology. To combat this, we were fortunate to gain access to limited human hepatic lymph node tissue. This not only provides another tissue environment to compare with blood, but also presents us with the opportunity to study lymphoid versus non-lymphoid organ tissue resident phenotypes. Unlike blood, but similar to liver, lymph nodes contained CD69^{HI} and CD69^{INT} CD4^{+} T cells, as well as CD69^{-} T cells (Fig. 5.4.2A). Although difficult to accurately assess due to low donor numbers and high patient-patient variation, it may be that CD69^{INT} cells were more frequent in hepatic lymph nodes than liver. Naïve/memory profiles were completely distinct in the two organs (Fig. 5.4.2B). Lymph node CD69^{-} and CD69^{INT} cells were similarly massively skewed towards naïve T cells, as anticipated due to the role of LNs in recirculation of naïve T cells. LN CD69^{HI} cells showed a much greater preference for T_{EM} cells, closer to their liver CD69^{HI} counterparts, but still retained a substantial T_{CM} and naïve component.

Focussing just on any differential expression between LN and liver populations with the same CD69 designation, CD49a % expression was consistently higher in Liver vs. LN (Fig. 5.4.2C). The same pattern was observed for CCR5, KLRG-1, HLA-DR, and PD-1;

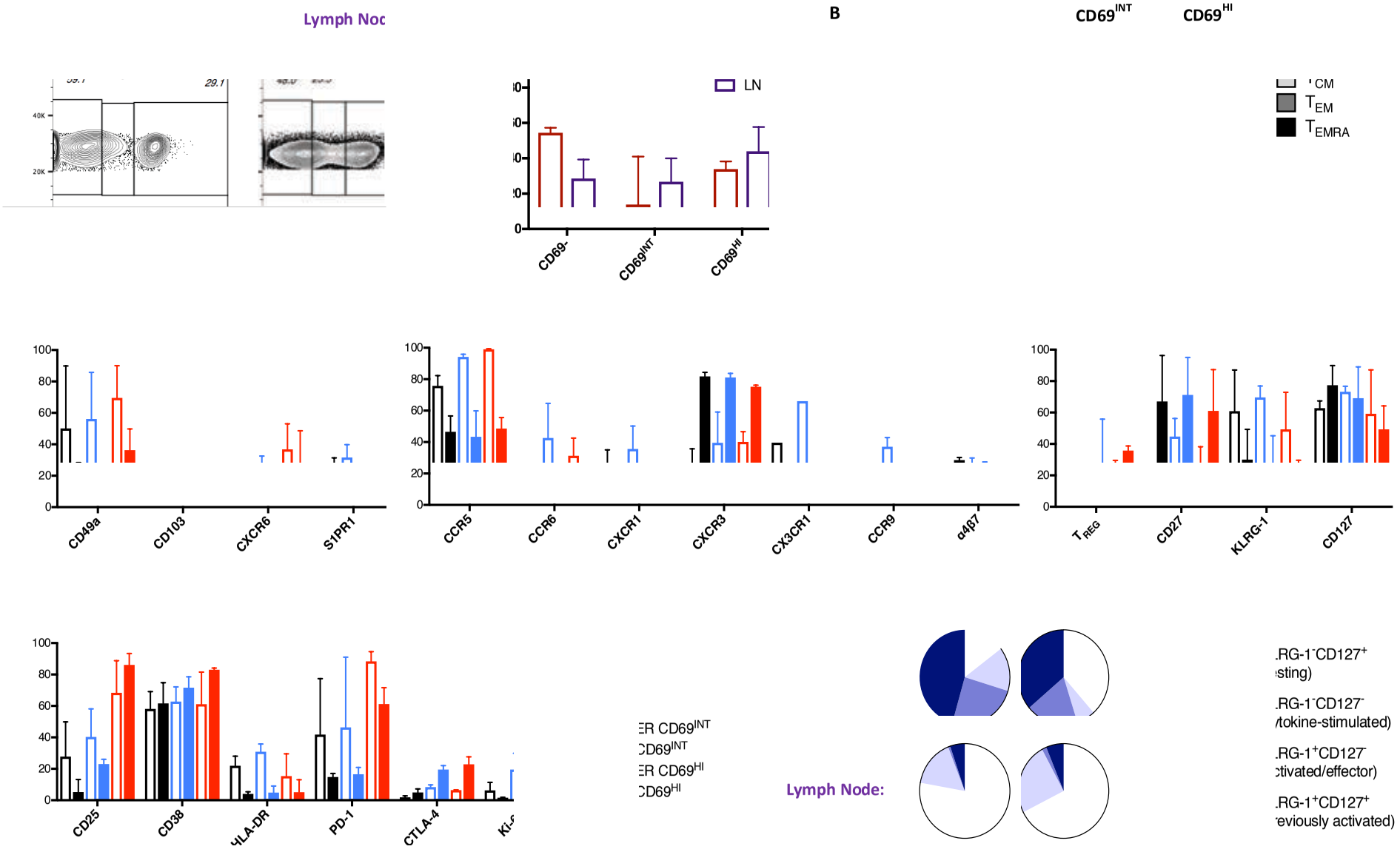

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Figure 5.4.2 – Phenotype of matched lymph node and liver $CD4^+$ T cells. A – Representative flow cytometry plots showing CD69 expression among $CD4^+$ T cells from liver and lymph node. Combined data (n=4) represented in a bar chart (median + IQR shown). **B –** Pie charts showing median composition of $CD4^+$ $CD69^-$, $CD69^{INT}$, and $CD69^{HI}$ T cells from liver and lymph node, in terms of their naïve and memory cell subsets. **C –** Series of TRM, homing, differentiation, and activation % marker expression in liver and lymph node populations: liver $CD69^-$ (black open bars), LN $CD69^-$ (black closed bars), liver $CD69^{INT}$ (blue open bars), LN $CD69^{INT}$ (blue closed bars), liver $CD69^{HI}$ (red open bars), LN $CD69^{HI}$ (red closed bars). **D –** Pie charts showing median composition of KLRG-1/CD127 populations amongst liver and lymph node $CD69^-$, $CD69^{INT}$, and $CD69^{HI}$ subsets. Unless otherwise stated, all data from this figure is a summary of 3 donors with the following exceptions: CD49a, CD103, and naïve/memory populations (all n=4); S1PR1, CCR9, integrin α4β7, CD38, CTLA-4, Ki-67, and CD27 (all n=2); and CX3CR1 (n=1).

while the inverse was seen for CXCR3, CD27, and potentially T_{REGS} for the CD69-expressing subsets. Like liver populations, as CD69 expression increased so did the expression of T_{RM} markers (↑CD49a, ↑CXCR6, ↓S1PR1); and certain activation/co-inhibitory markers (↑CD25, ↑PD-1). Just like for liver cells, the marked expression of S1PR1 and CX3CR1 in LN $CD69^{INT}$ cells was not observed in the LN $CD69^{HI}$ population. Also, analogous to liver patterns, CCR9 expression was highest in LN $CD69^{INT}$ cells. Finally, based on KLRG-1 and CD127 co-expression patterns, LN cells are strikingly more resting and less activated/previously activated (Fig. 5.4.2D). $CD69^{HI}$ LN cells were most like $CD69^{HI}$ liver cells for these markers, but contained a far greater cytokine-stimulated population, hinting at differential requirements for $CD69^{HI}$ $CD4^{+}$ T_{RM} maintenance across different organs. Overall, despite differences in proportional expression magnitudes between liver and LN, relative expression levels across the three CD69-defined subsets of the two organs matched remarkably well. We therefore propose that $CD69^{HI}$ cells also represent the tissue resident cells of the lymph node, and point out a liver-analogous $CD69^{INT}$ population in the LN that shares major characteristics with its putatively non-resident $CD69^{INT}$ liver counterpart.

5.6 Liver $CD69^{HI}$ and $CD69^{INT}$ cells favour T_H1 and T_H2 cytokine responses respectively

In order to determine the cytokine secretion potential of the three CD69-defined populations in the liver, isolated LIMCs were subjected to a pre-stain for CD69, before stimulation with PMA/Ionomycin, anti-CD3/CD28, or PHA for 2 or 5 hours (Fig. 5.5.1A). Intracellular stains for various cytokines were then carried out, and cells and analysed by flow cytometry. Preliminary work demonstrated that the intensity of CD69 staining was

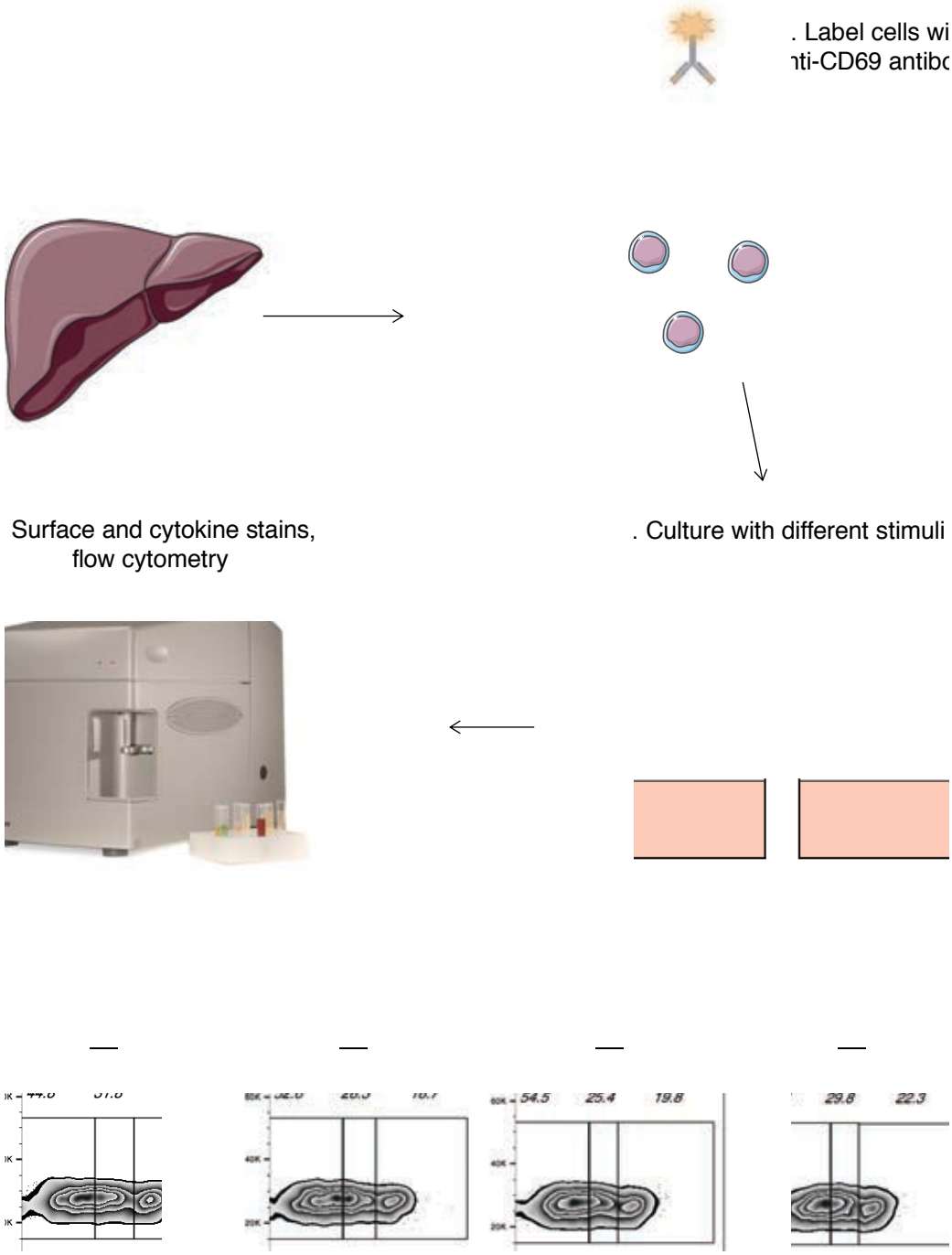
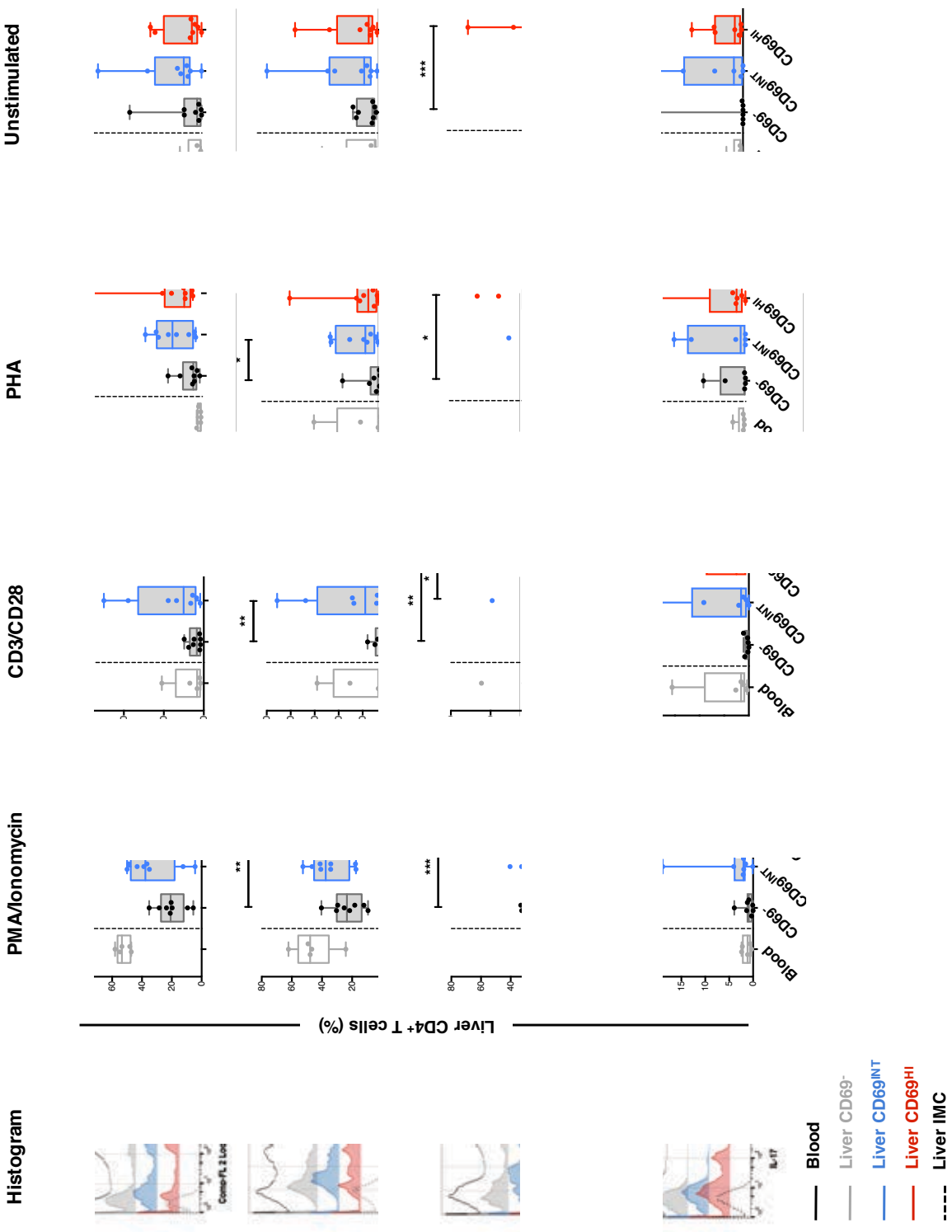


Figure 5.5.1 – LIMC cytokine staining protocol and CD69 expression confirmation experiment. A – Protocol used for investigating cytokine production in the different CD69-expressing liver $CD4^+$ T cell populations. Liver was first mechanically digested and the LIMCs harvested. LIMCs were pre-stained with an anti-CD69 antibody before being added into culture with various stimuli (PMA & Ionomycin, PMA/Iono; anti-CD3/CD28 beads; PHA; or untreated, UT) for either 2h or 5h in the presence of golgi stop Brefeldin A. Cells were then harvested; surface stained for CD4, CD56, and γδ-TCR; fixed and permeabilised; then stained for cytokines ready for flow cytometric analysis. **B –** Maintenance of CD69 expression over time in culture. LIMCs were stained for CD69 then left in culture for 0, 1, 2, or 3 hours and then CD69 expression analysed. Plots show CD69 expression on $CD4^+CD56^-$ γδ-TCR^- populations after indicated time points. N=1.

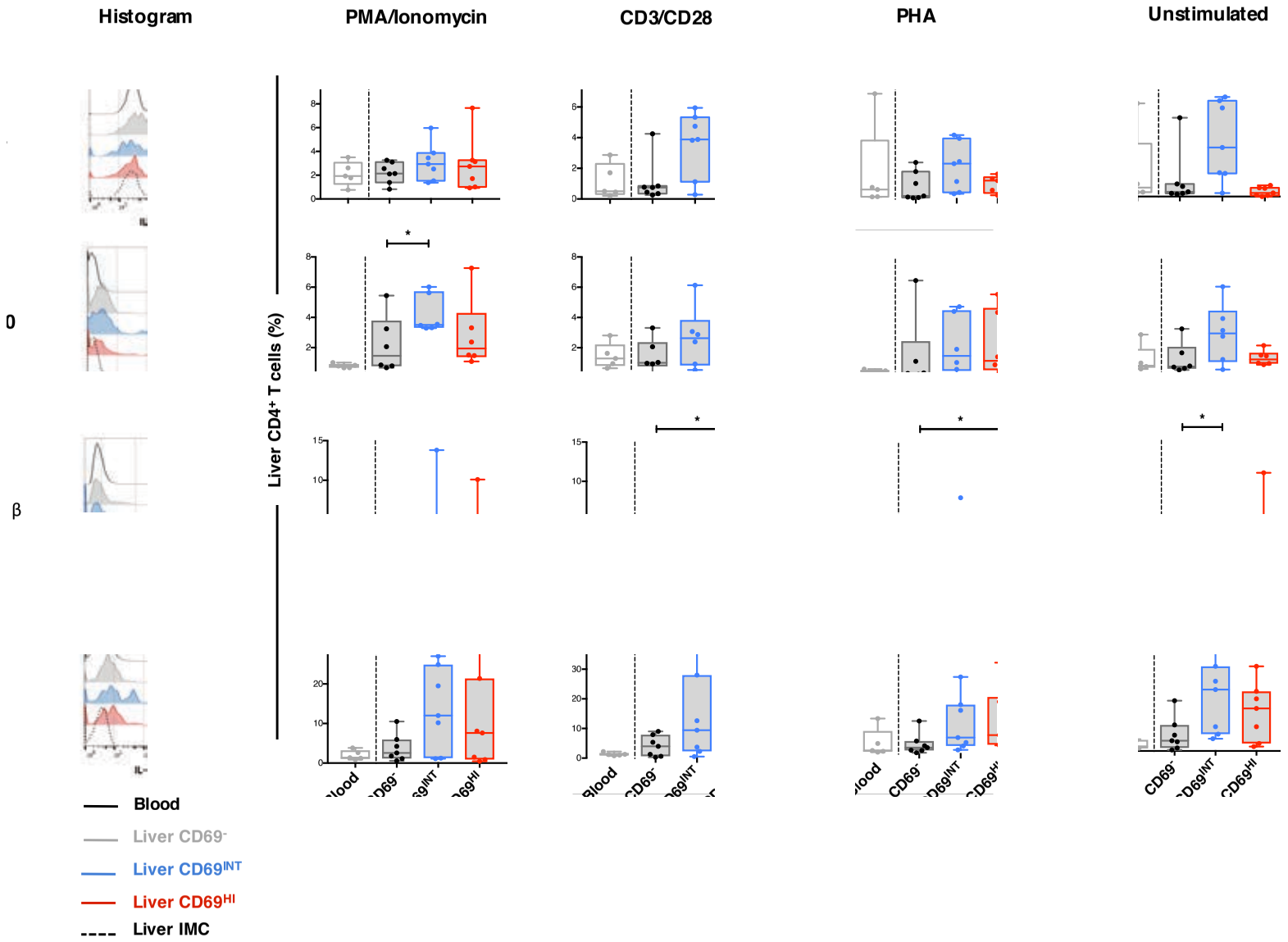
barely lost over time in culture, giving credence to our chosen methodology (Fig. 5.5.1B). We first Looked at the 5h time-point, where cells have had a greater chance to reveal their cytokine potential.

The first main observation from these experiments was that $CD69^{HI}$ cells favoured IL-2, TNF-α, IFN-γ, and IL-17A production (Fig. 5.5.2). This was most evident with the potent stimulants PMA and Ionomycin where consistent statistical increases were seen from $CD69^{-}$ cells. Statistical significance was not reached when comparing $CD69^{HI}$ and $CD69^{INT}$ cells, but a higher median percentage positivity was seen for every cytokine in the $CD69^{HI}$ subset. The other stimuli were less potent at inducing robust cytokine production in cells from both liver and blood, making interpretations harder. However, the proportion of IFN-γ^{+} cells was highest in $CD69^{HI}$ cells following anti-CD3/CD28, and PHA stimulation; and even in unstimulated controls. Interestingly, both CD3/CD28 and PHA stimulation appeared to reveal the highest median IL-2 and TNF-α % production was in the $CD69^{INT}$ population, although this was not statistically significant versus the $CD69^{HI}$ cells. Differences in IL-17A production were not apparent across the other stimuli.

$CD69^{INT}$ cells appeared to show better IL-4 and IL-10 responses (Fig. 5.5.3). For IL-4, this was clearly demonstrated following CD3/CD28, and PHA stimulation, as well as in unstimulated cells; though not in PMA/Ionomycin treated cells. For IL-10, the opposite effect was seen – $CD69^{INT}$ cells clearly contained the highest IL-10^{+} % following PMA/Ionomycin stimulation, but this pattern was less obvious with the other stimuli and unstimulated cells. Additionally, TGF-β and IL-6 were analysed. TGF-β production potential from the three subsets seemed to differ based on stimuli used. With CD3/CD28 and PHA stimulation, $CD69^{HI}$ cells possessed the highest percentage of TGF-β^{+} cells, a



Shown are cytokine production profiles (by intracellular cytokine capture) for IL-2, TNF-α, IFN-γ an IL-17 (top to bottom rows) in various stimulation conditions (PMA/Ionomycin, CD3/CD28, PHA, an unstimulated – left to right). All stimulations were for 5 hours. Box and whisker plots show a combined % expression data for blood CD69^{-} cells; and liver CD69^{-}, CD69^{INT}, and CD69^{HI} cell (error bars show min-max points). Example histogram overlays show the same populations plu


Histogram
PMA/Ionomycin
CD3/CD28
PHA
Unstimulated
Liver CD4+ T cells (%)
0
β
Blood
Liver CD69-
Liver CD69INT
Liver CD69HI
Liver IMC

trend that was not observed in the other two conditions. However, IL-6 % were highest on average in the $CD69^{INT}$ cells, the exception being following PHA stimulation where $CD69^{INT}$ and $CD69^{HI}$ cells were approximately equal.

Overall, we saw substantial functional differences between $CD69^{INT}$ and $CD69^{HI}$ subsets. Following the most efficient PMA/Ionomycin stimulation method, $CD69^{HI}$ cells favoured T_H1 and T_H17 production, and $CD69^{INT}$ cells were better at generating IL-10. Additional stimulations revealed a further preference for IL-4, and to a lesser degree IL-6 production in these $CD69^{INT}$ cells.

Using the additional 2-hour time-point allowed us to make preliminary observations about how cytokine production in these subsets changed over time. Only PMA/Ionomycin stimulation was studied in these experiments as it produced the greatest responses. $CD69^{HI}$ cells' production of IL-2, TNF-α, IFN-γ, and IL-17 was increased over time, whereas intriguingly the proportion of $CD69^{INT}$ cells making the same cytokines was the same at 2 hours as it was 5 hours post-stimulation (Fig. 5.5.4). IL-4-producing cells increased with stimulation time, regardless of the population studied. However, in the two CD69-expressing populations, TGF-β-secreting cells were actually at higher frequencies following 2 hours of stimulation, compared to 5 hours. Lastly, IL-6 production was independent of stimulation time in $CD69^{HI}$ cells, but $CD69^{INT}$ IL-6 production frequency was greatly enhanced at the later 5-hour time-point. Together, these data reveal intrinsic differences in the responsiveness of $CD69^{HI}$ and $CD69^{INT}$ cells to stimulation.

Multifunctional T_H1 cells are able to produce combinations of IL-2, TNF-α, and IFN-γ, and show superior immune protection compared to single cytokine-producing cells[241-243]. Like

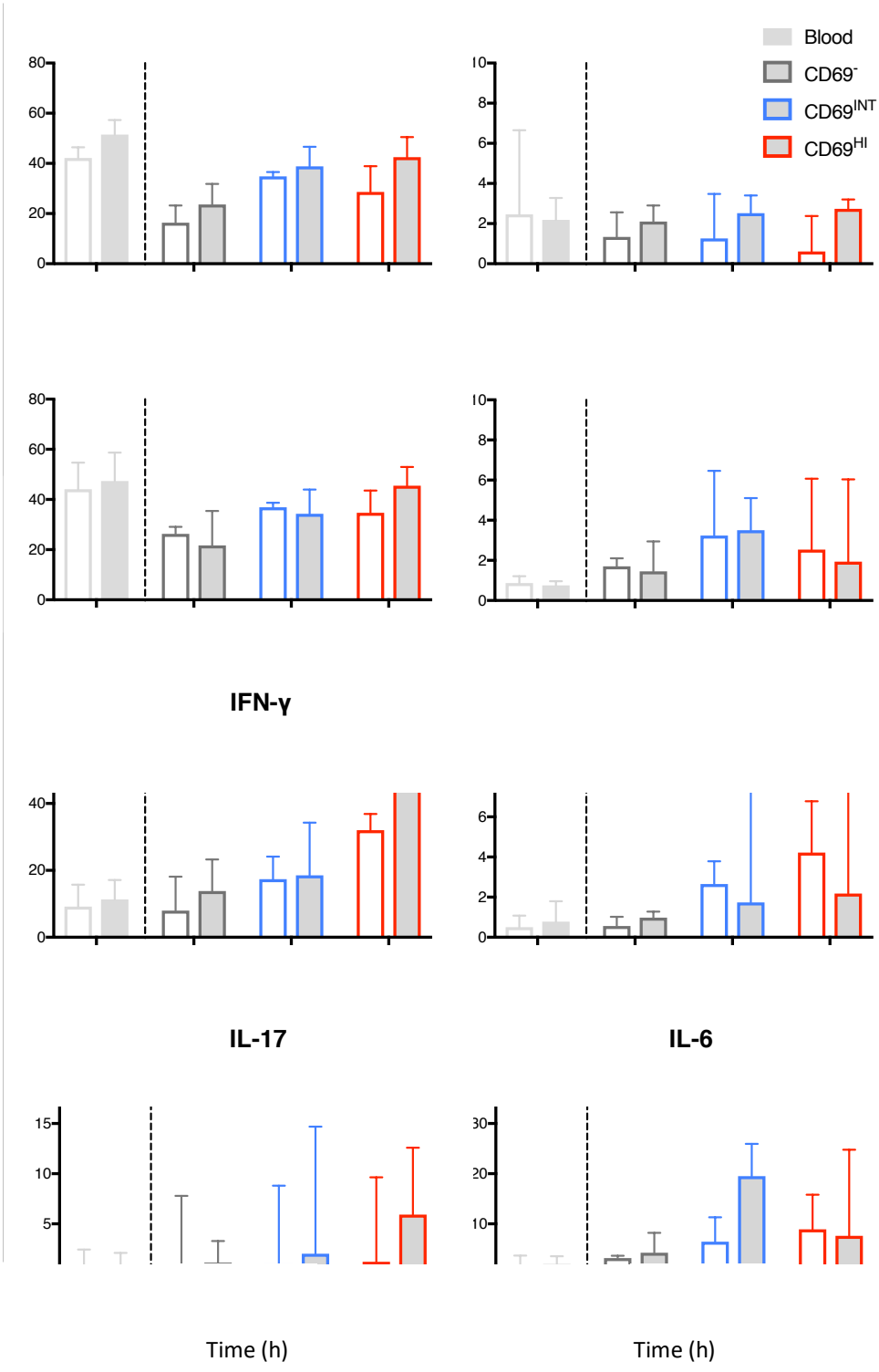


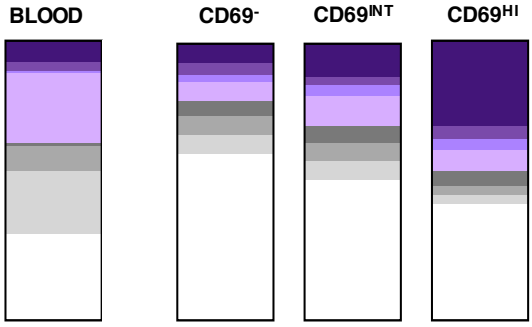
Figure 5.5.4 – Cytokine expression in liver CD69 populations over time. Bar charts show cytokine production in blood CD69^{-} cells (light grey), liver CD69^{-} cells (dark grey), liver CD69^{INT} cells (blue) and liver CD69^{HI} cells (red) post 2-, and 5-hour stimulations with PMA/Ionomycin. Bar heights show median and error bars IQR. N=5 for all liver data except for IL-10 and IL-17 (n=4). N=4 for all blood data except for IL-10 (n=3).

other similar studies on human tissue residence[82, 88, 131, 144], we calculated the proportions of liver CD69^{HI} T_{RM} cells that could make multiple cytokines, compared to liver CD69^{-} and CD69^{INT} cells, and blood CD4^{+} T cells following PMA/Ionomycin stimulation. CD69^{HI} cells contained the highest proportion of IL-2^{+}TNF-α^{+}IFN-γ^{+} cells, IL-2^{+}IFN-γ^{+} cells, and TNF-α^{+} cells (Fig. 5.5.5). All these differences were statistically different compared to CD69^{-} cells. CD69^{INT} cells on the other hand, fell in between the two liver populations, but were much closer aligned to CD69^{-} cells. Interestingly, blood CD4^{+} T cell responses were distinct from all liver CD4^{+} T cell populations, showing far greater fractions of T cells making IL-2 alone, and IL-2 with TNF-α. In these investigations, we also looked for IL-4 and IL-10 dual producers, but were unable to locate any double positives in any liver studied (data not shown).

These data demonstrate stark functional distinctions between CD69^{INT} and CD69^{HI} cells in terms of the type(s) of cytokines produced, and the speed of responses. CD69^{HI} favour T_H1 and T_H17 profiles, with the highest type-1 multifunctionality. Conversely, CD69^{INT} cells favoured IL-4 and IL-10, and generally reached their peak cytokine production faster than their CD69^{HI} counterparts.

5.7 Co-culture with hepatic cell lines imprints blood CD4^{+} T cells with a partial liver CD69^{INT} phenotype, while culture with intact liver slices may induce CD69^{HI} generation

We previously observed that co-culture of blood-derived CD4^{+} T cells with both hepatic and non-hepatic epithelia led to an upregulation of CD69 to an intermediate level (chapter 4). We showed this upregulation did not represent activation, nor did it indicate the



Population	Key	Significant?			IQR (%)		
		- vs. INT	- vs. HI	INT vs. HI	-	INT	HI
IL-2$^+$TNF-α$^+$IFN-γ$^+$		ns	***	ns	1.1	4.5	15
TNF-α$^+$IFN-γ$^+$		ns	ns	ns	1.4	1.7	3.3
IL-2$^+$IFN-γ$^+$		ns	*	ns	1.7	2.7	2.8
IL-2$^+$TNF-α$^+$		ns	ns	ns	4.4	8.5	4.6
IFN-γ$^+$		ns	ns	ns	4.3	2.6	3.9
TNF-α$^+$		ns	**	ns	2.7	3.6	1.7
IL-2$^+$		ns	ns	ns	4.2	4.7	2.3
IL-2$^-$TNF-α$^-$IFN-γ$^-$		ns	*	ns	15	26	16

Figure 5.5.5 – The CD69^{HI} liver population contain the most multifunctional IL-2$^+$TNF-α$^+$IFN-γ$^+$ cells. Stack charts display multifunctional responses in terms of single, dual, and triple IL-2, TNF-α, and IFN-γ responses to 5 hour PMA/Ionomycin stimulation. As in previous figures, blood CD69$^-$, liver CD69$^-$, liver CD69^{INT}, and liver CD69^{HI} CD4$^+$ T cell responses are shown. Stack chart heights represent median % of each cytokine expression pattern as shown. Accompanying table shows population key, statistical comparison between the three liver populations and interquartile range values (liver, n=7; blood n=5).

generation of a novel regulatory phenotype. Based on our discovery of a $CD69^{INT}$ $CD4^+$ T cell type in the liver, we hypothesised that the $CD69^{INT}$ phenotype we generated from blood $CD4^+$ T cells would phenotypically resemble their intrahepatic $CD69^{INT}$ counterparts.

To test this, we performed a comparative analysis of the $CD69^{INT}$ blood $CD4^+$ T cells that were the product of Huh-7 co-culture ($CD69^{INT}$ hTCs). We asked first if $CD69^{INT}$ hTCs are imprinted with a partial residence phenotype, like that displayed by liver $CD69^{INT}$ cells. $CD69^{INT}$ hTCs showed an upregulation of CD49a at day 1 following co-culture that remained consistent throughout the course of the 7-day co-culture period (Fig. 5.6.1). This difference was observed relative to the co-cultured $CD4^+$ T cells that did not upregulate CD69 ($CD69^-$ hTCs), but was also apparent when compared to T cells cultured alone (virtually all $CD69^-$) proving the effect a genuine upregulation. CD103 and CXCR6 were only expressed on very few $CD4^+$ T cells, but the $CD69^{INT}$ hTC cells contained a few more $CD103^+$ and $CXCR6^+$ cells than the $CD69^-$ hTC pool. S1PR1 was upregulated on all T cells following culture with Huh-7 cells, but this effect was greatest in magnitude, and longest lasting in $CD69^{INT}$ hTCs. Therefore, $CD69^{INT}$ hTCs seemed to recapitulate the partial residence phenotype of intrahepatic $CD69^{INT}$ cells, most notably through elevated S1PR1 expression.

Another key feature of liver $CD69^{INT}$ cells was relative increases in the percentages expressing KLRG-1 and CD127. KLRG-1 expression was higher among the $CD69^{INT}$ hTCs too, with $CD69^-$ hTCs more resembling T cell only controls (Fig. 5.6.2A). $CD69^{INT}$ hTCs also maintained CD127 expression well across the 7-day culture period, similar to the T cells alone. In stark contrast, $CD69^-$ hTCs drastically lost over 56% of their CD127

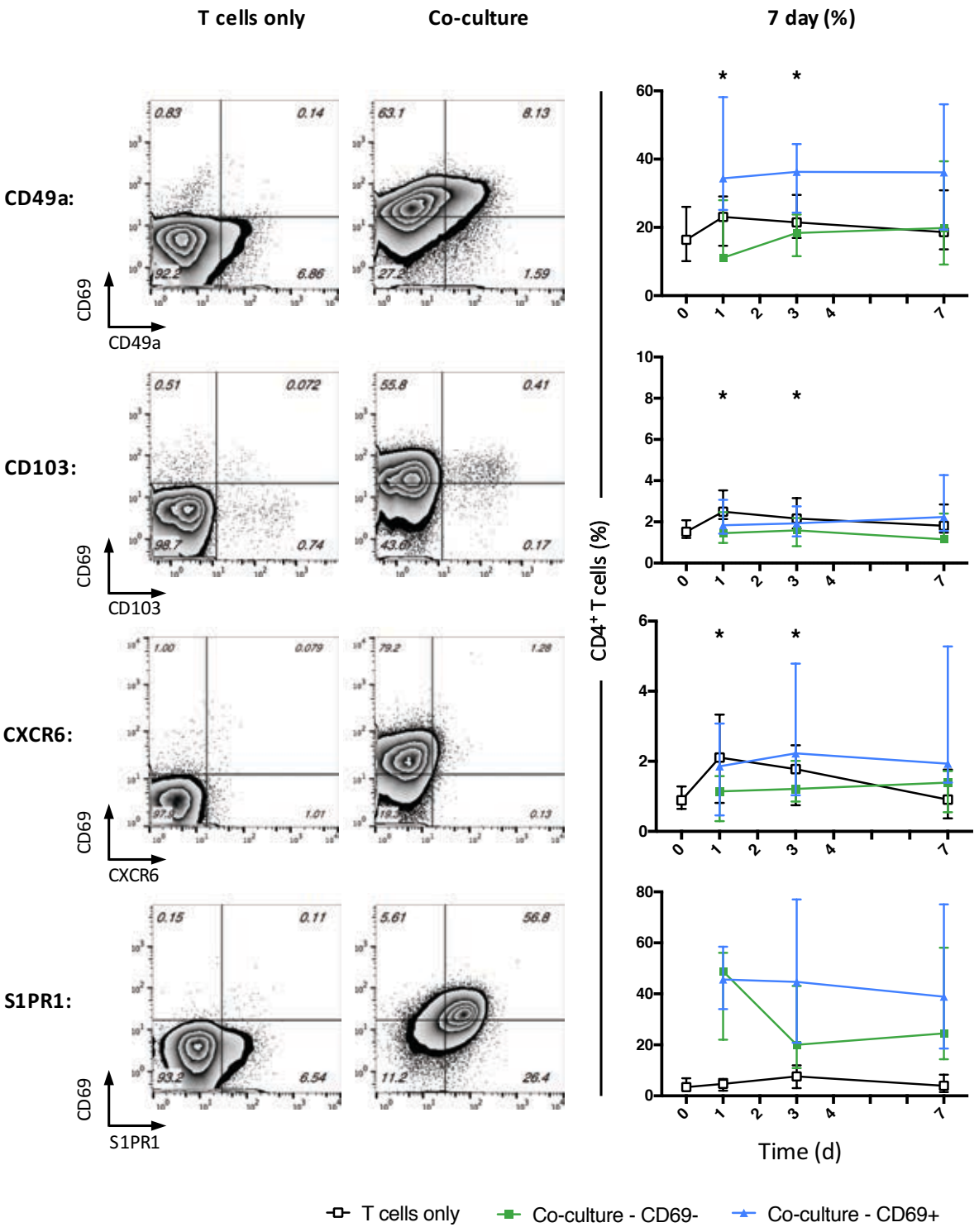


Figure 5.6.1 – Huh7 exposure induces blood-derived CD4$^+$ T cells to express a partial residence phenotype akin to liver CD69^{INT} cells. Shown are blood-derived CD4$^+$ T cell expression patterns of the tissue residence markers CD49a, CD103, CXCR6, and S1PR1, both with and without Huh-7 co-culture. Representative flow plots show expression of each marker against CD69 in both T cells only, and co-cultured cells after day 3 in culture. Graphs display change in % expression over time for T cells only (black), co-cultured CD69$^-$ cells (green), and co-cultured CD69$^+$ cells (blue). Points are medians, while error bars show IQR (all n=6 bar S1PR1 where n=5). Statistical analyses in this figure compare CD69$^-$ and CD69$^+$ co-cultured cells.

expression (Fig. 5.6.2B). Analysing combinatorial KLRG-1 and CD127 expression, T cell only controls were mostly resting, followed by smaller clusters of cytokine-stimulated, and previously activated cells, and very few activated/effector cells (Fig. 5.6.2C). In comparison, from day 3 onwards, many $CD69^{-}$ hTCs appeared to forgo their resting phenotype primarily becoming cytokine stimulated. These cells were much more susceptible to cytokine stimulation than $CD69^{INT}$ hTCs, which seemed to diverge into three main populations. First many more of these cells retained a resting phenotype. Second, some became cytokine stimulated, like their non CD69-expressing counterparts. Third, some of these cells presented with a previously activated phenotype. Of course, this last phenotype cannot be induced straight from resting cells, so instead it was inferred that the cells that upregulated CD69 through co-culture with hepatic cell lines were enriched for the existing previously activated population. This last feature was similar to liver $CD69^{INT}$ cells that were enriched for the previously activated phenotype.

We next sought to discover whether these findings held true in other hepatic cell lines, and primary liver epithelia. Preliminary data demonstrated HepG2 and Hep3B cells, as well as primary BEC cells were just as good as Huh-7 at inducing CD49a, CD103, CXCR6 and S1PR1 within the $CD69^{INT}$ gate when compared with the $CD69^{-}$ cells following co-culture (Fig. 5.6.3A). Additionally, KLRG-1 and CD127 upregulations among the different co-cultured $CD69^{INT}$ cells was fairly consistent (Fig. 5.6.3B). Following this, we looked at the KLRG-1 and CD127 co-staining patterns among the $CD69^{INT}$ cells in each co-culture condition (Fig. 5.6.3C). At day 1, we found that T cells co-cultured with all cell types showed similar division amongst the four activation and differentiation subsets. By day 3, those cultured with the hepatocyte cell lines remained similar, whereas BEC-cultured T cells showed a heavier naïve/resting bias by day 3. At day 7, with HepG2 there was also

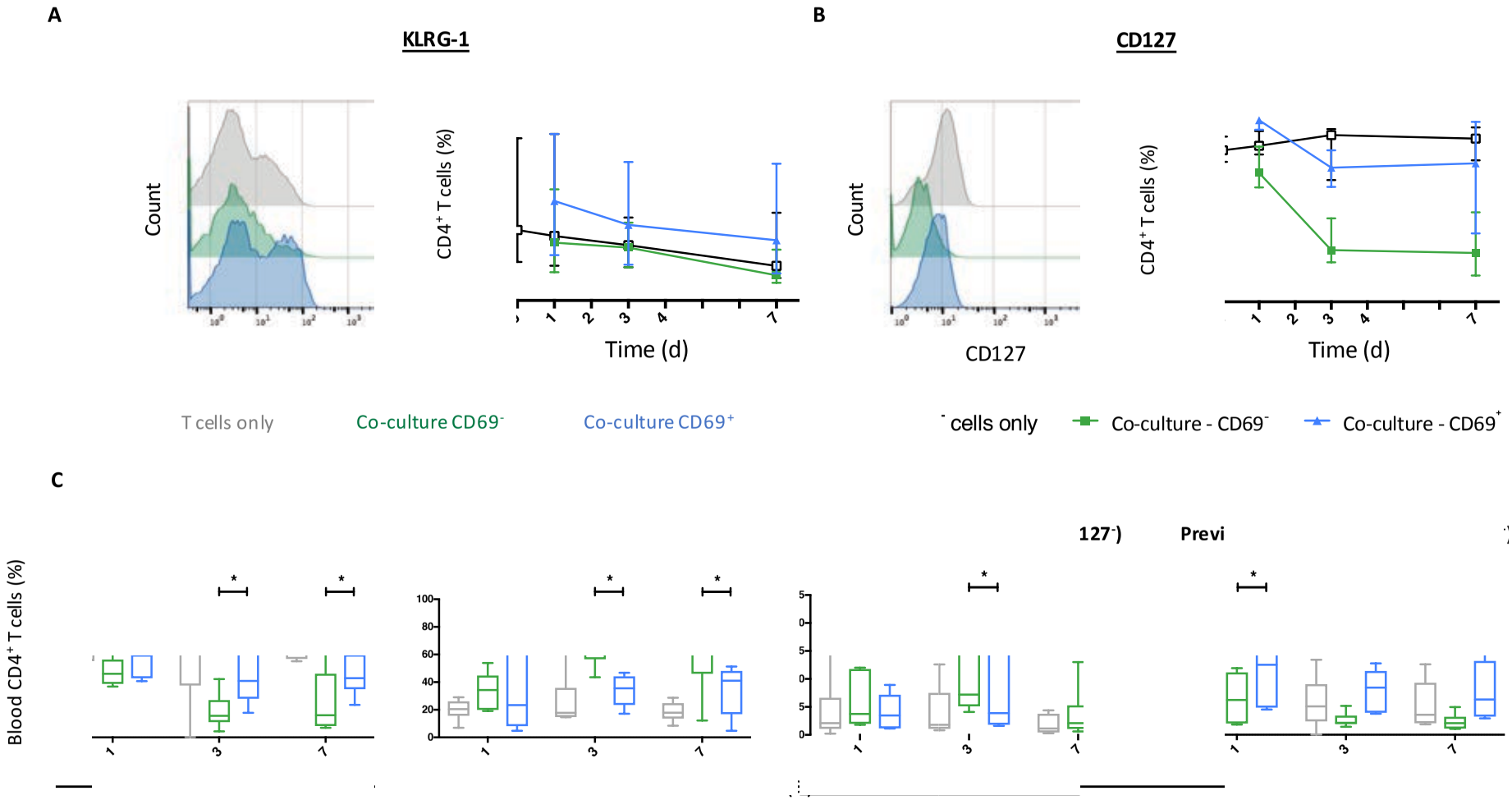
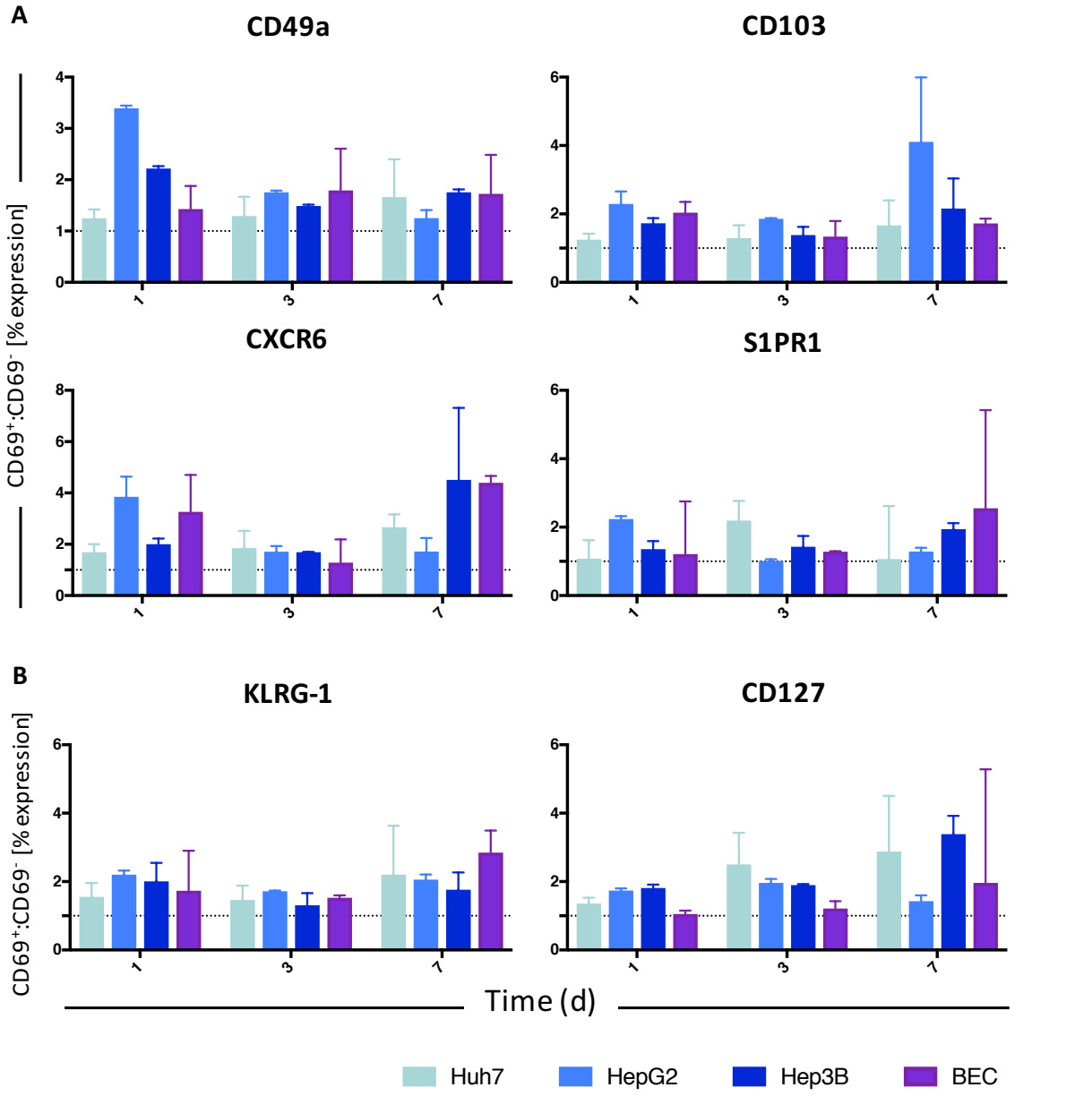


Figure 5.6.2 – hTCs are preferentially derived from KLRG-1+, and CD127+ populations; and display increased proportions of previously activated and resting phenotypes. Histogram overlay representative plots from day 7, and % expression over time for KLRG-1 (**A**), and CD127 (**B**). In both plot types gray/black represents T cells only, green the $CD69^{-}$ $CD4^{=}$ T cells following Huh-7 co-culture, and blue the $CD69^{+}$ $CD4^{=}$ T cells following culture with Huh-7 cells. Statistical tests compare only the two co-culture populations. **C** - Populations were divided into the four stages of activation and homeostasis based on expression of KLRG-1 and CD127 as previously (Fig.5.3.5, 5.4.1, 5.4.2). These four populations were analysed in $CD69^{+}$ and $CD69^{-}$ $CD4^{+}$ T cells at different times following Huh-7 co-culture (blue and green bars respectively), and compared with T cells only (grey bars). Box and whiskers span all data points (min to max). Statistical comparisons were only made between the two co-cultured populations. All data from this figure n=6.



C

	Day 1				Day 3				Day 7			
	Resting	Cytokine-stimulated	Activated/effector	Previously activated	Resting	Cytokine-stimulated	Activated/effector	Previously activated	Resting	Cytokine-stimulated	Activated/effector	Previously activated
Huh7	59	10	3	25	41	36	4	17	43	41	2	13
HepG2	69	14	2	15	54	29	3	14	65	5	1	16
Hep3B	62	21	4	13	56	31	3	10	52	36	2	9
BEC	48	14	4	33	76	7	4e-001	17	77	14	4e-001	10

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Figure 5.6.3 – The phenotype of CD69$^+$ cells induced by co-culture is similar regardless of inducing cell. A – Blood-derived CD4$^+$ T cells co-cultured with hepatocyte cell lines Huh-7, HepG2, or Hep3B; or primary biliary epithelial cells (BEC); for either 1, 3, or 7 days. Resulting difference in CD69$^+$ cell marker expression was shown as the median fold change compared to control CD69$^-$ cells. This was done for the TRM markers CD49a, CD103, CXCR6, and S1PR1. **B –** As part A, but for differentiation markers KLRG-1 and CD127. For both parts: light blue – Huh-7, mid-blue – HepG2, dark blue – Hep3B, purple – BEC; plots show median + IQR. **C –** KLRG-1/CD127 co-staining patterns (%) in the generated CD69$^+$ cells in each condition. Results shown as 3 heat-maps – 1 for each time point. Numbers in each cell denote median % expression in the CD69$^+$ cells. Resting – KLRG-1$^-$CD127$^+$, cytokine-stimulated – KLRG-1$^-$CD127$^-$, activated/effector - KLRG-1$^+$CD127$^-$, previously activated – KLRG-1$^+$CD127$^+$. For all parts of this figure, donor numbers as follows – Huh7 – 6, HepG2/Hep3B – 2, BEC – 3.

a higher resting cell proportion in expense of the cytokine-stimulated cells, distribution in BEC co-culture remained as at day 3, and Hep3B showed a constant population spread, similar to Huh-7 controls. Thus, the key partial T_{RM} and differentiation features seen following Huh-7 culture was largely consistent with different hepatic cell lines, and primary biliary epithelia.

Other key differences between $CD69^{INT}$ and $CD69^{-}$ hTCs included those amongst inhibitory and chemokine receptors. Both PD-1 and Fas expression was significantly more frequent in the $CD69^{INT}$ population at all time points (Fig. 5.6.4A). The proportion of CCR6-expressing cells fell sharply in the $CD69^{-}$ population, but this effect was delayed in the $CD69^{INT}$ (Fig. 5.6.4B). On the other hand, $CXCR3^{+}$ cells were clearly diminished in both co-cultured populations compared to T cells cultured alone, but $CD69^{INT}$ hTCs lost this chemokine receptor more effectively than $CD69^{-}$ hTCs. Last, after an initial delay, co-culture induced an CX3CR1 upregulation that was similar amongst CD69-expressing and non-expressing cells. These findings again show similarities to the $CD69^{INT}$ liver cells, with higher PD-1 and CCR6 than their $CD69^{-}$ counterparts; although unlike the liver cells, CXCR3 was lost, and CX3CR1 similarly upregulated in both populations *in vitro*.

To help determine whether the *in vitro*-generated $CD69^{INT}$ cells possessed the same cytokine biases as the earlier described liver $CD69^{INT}$ cells, we cultured blood-derived $CD4^{+}$ T cells with and without Huh-7 cells, before stimulation and intracellular cytokine staining. Only three donors were used for these experiments making definite conclusions difficult, but it appeared that a higher frequency of co-cultured T cells secreted IL-4 following PMA/Ionomycin, and especially CD3/CD28, stimulation (Fig. 5.6.5). The other cytokines were largely similar, but subtle distinctions may become significant with more

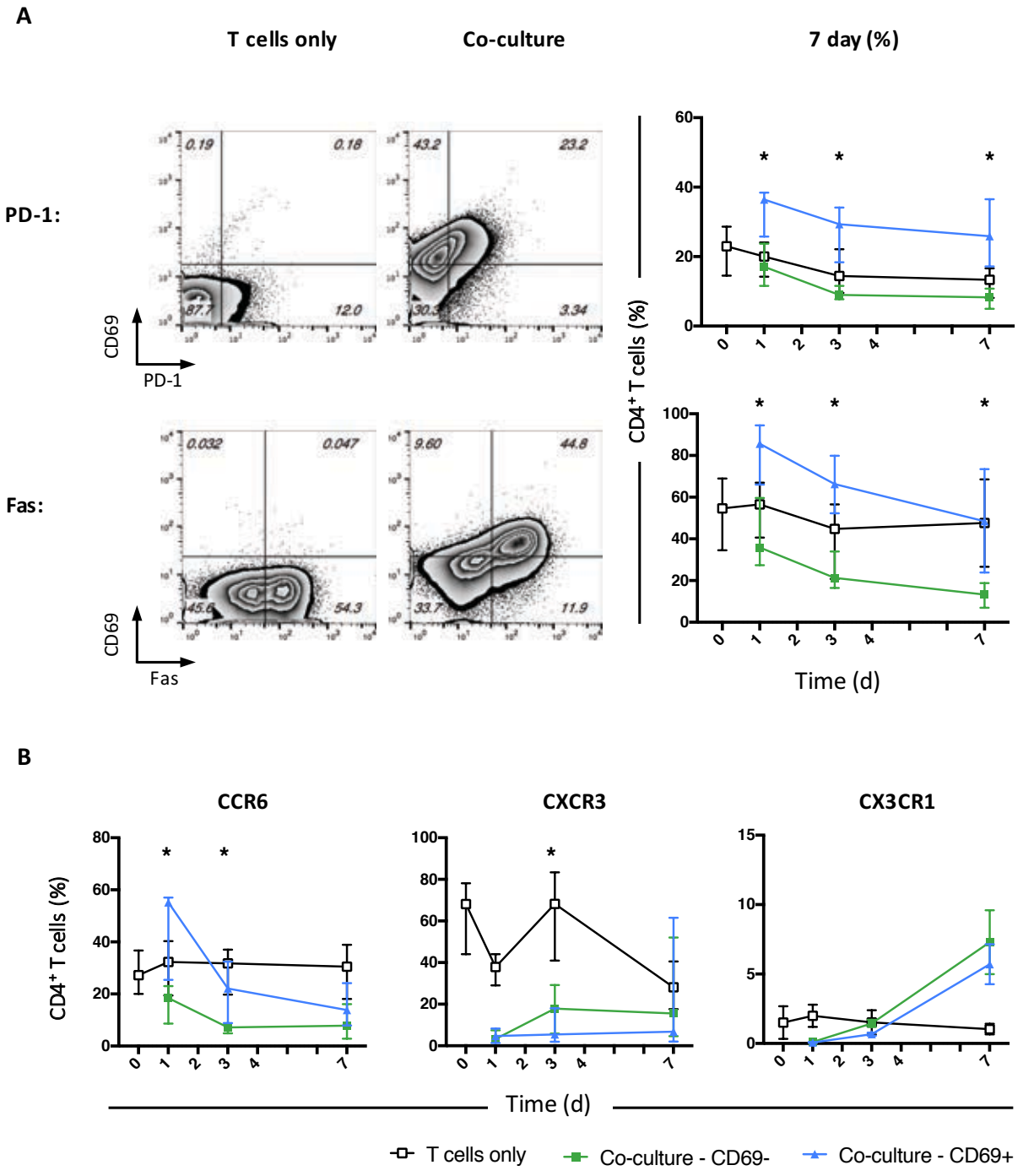

Figure 5.6.4 – Inhibitory receptor and chemokine receptor expression on hTCs. A – Representative flow cytometry plots showing PD-1, and Fas vs. CD69 expression in T cells only and T cells co-cultured with Huh-7 cells for 3 days. Percentage expression data from all donors in the different culture conditions (both n=6) is shown in the graph on the right. **B –** CCR6 (n=6), CXCR3 (n=6), and CX3CR1 (n=2) % expression over time following co-culture. For all graphs, T cells only, $CD69^-$ co-cultured cells, and $CD69^+$ co-cultured cells are shown in black, green, and blue respectively. Statistical comparisons tested the two co-culture populations only.

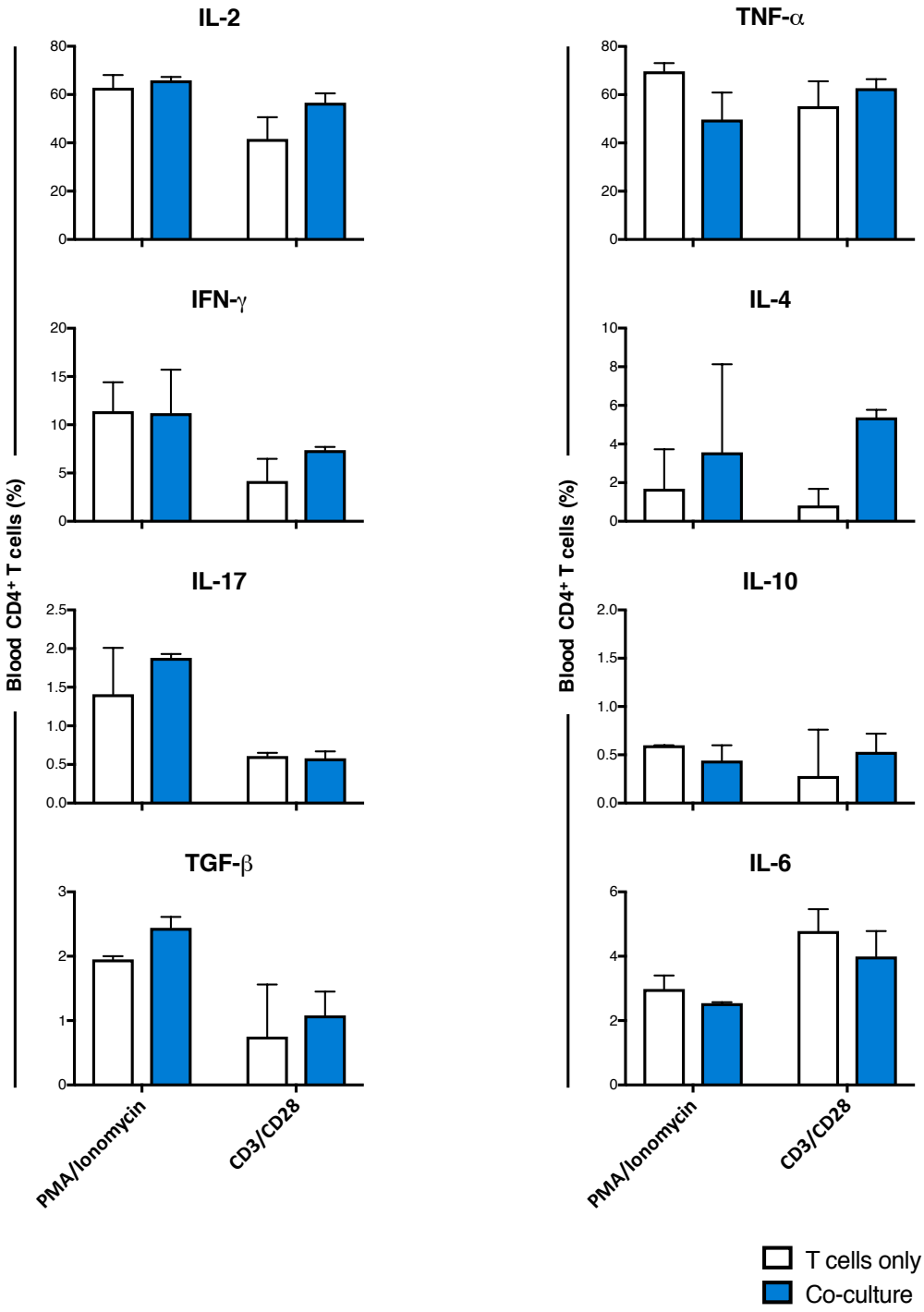

Figure 5.6.5 – CD69^{INT} cells generated through contact with Huh-7 cells show more pronounced IL-4 production than T cells cultured alone, similar to their liver CD69^{INT} counterparts. CD4^{+} T cells from peripheral blood were co-cultured with Huh-7 cells overnight, stimulated with PMA and Ionomycin, or anti-CD3/CD28 (both with golgi stop Brefeldin A), and stained for the same cytokines studied in liver CD4^{+} T cells (Figs 5.5). Bar charts show median (+IQR) percentage cytokine expression in T cells only (white bars) and co-culture (blue bars). N=3.

experimental repeats. Thus, similar to liver CD69^{INT} cells, hTCs upregulate IL-4 better than cells not exposed to Huh-7 cells in culture, but other cytokine distinctions seen in the liver counterparts were not seen in co-culture models.

Several key questions still remained in the generation of CD69^{INT} cells from blood. Would T cells adopt the same phenotype in coculture with primary hepatocytes, and if so what would be the time frame needed? How is the effect different within the complexities of the liver microenvironment and is this process different in diseased versus healthy patients? To begin to answer these questions, we cut 2mm slices of liver from patients with liver disease (n=2), and cultured them with autologous PBMCs for 5 hours. Although fairly crude experiments, determining whether intact liver slices induce a CD69^{INT} phenotype will show us a more physiological generation pathway, and allow us to unpick specific cell type(s) and cytokines responsible later down the line. Additionally, it will answer whether the CD69^{HI} T_{RM}-like phenotype can be induced under these conditions.

Like the healthy donor T cells, many blood CD4^{+} T cells from patients with liver disease also adopted the CD69^{INT} phenotype following Huh-7 contact, although curiously almost as many T cells alone gained this phenotype (Fig. 5.6.6A). This was observed in both donors, and CD69 positivity was low in T cells immediately following isolation. Importantly, a clear differentiation into CD69^{INT} cells was seen in the liver slice cultures. This was supplemented by a small, but distinct induction of CD69^{HI} cells.

First comparing CD69^{HI} cells from liver and slice culture, we noted many markers showed similar differential expression (Fig. 5.6.6B). Relative to their individual CD69^{-} controls, both populations showed similar changes in T_{RM} markers (↑CD49a, ↑CD103,

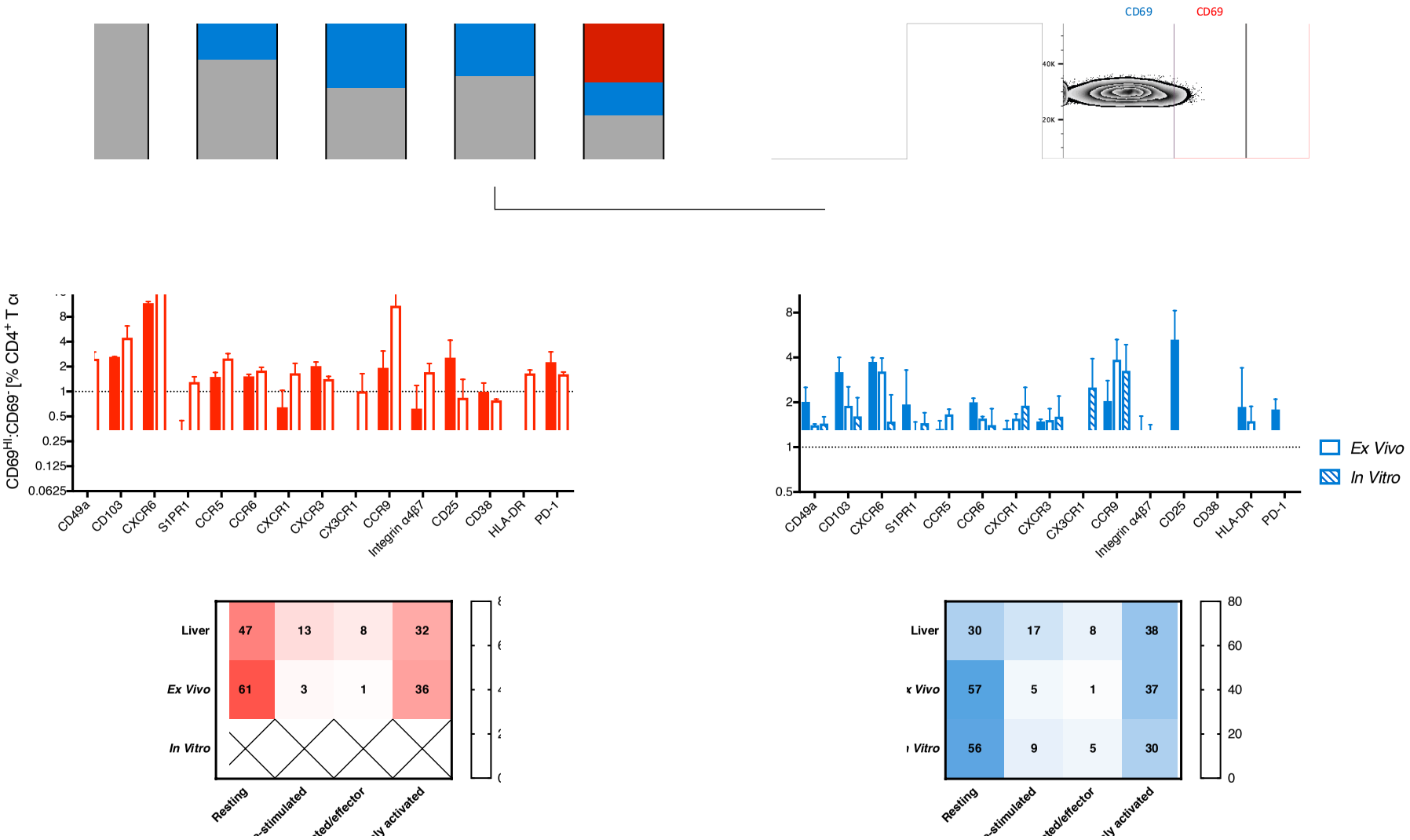

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Figure 5.6.6 – Contact with autologous liver slices may imprint CD69^{HI} and CD69^{INT} phenotypes analogous to that seen in human liver. Liver slices from diseased livers were cut to a thickness of 2mm and cultured with autologous PBMCs for 5 hours before harvesting and staining for flow cytometry. Live/Dead stains were included to eliminate dead cells. **A -** Stack charts showing median % representation of the three CD69 populations in blood, liver, and the three different culture conditions listed (T cells alone, T cells with Huh-7 cells – '*In Vitro*', and T cells with 2mm liver slices – '*Ex Vivo*'). For the *ex vivo* liver slice culture, a representative flow cytometry plot is shown. The remainder of the figure focusses on CD69^{HI} cells (**B**), and CD69^{INT} cells (**C**) where expression of multiple markers are compared in the liver, *ex vivo*, and *in vitro* Huh-7 co-culture conditions (T cells in the *in vitro*/Huh-7 co-culture are excluded from the CD69^{HI} analyses as no appreciable induction of CD69^{HI} cells was observed). Percentage marker expression displayed as a difference from CD69^{-} cells in each case (median + 95% CI). In the heat-maps below, median percentage expression in these three conditions was analysed in the four populations defined by KLRG-1 and CD127 coexpression patterns (as previously defined – see Fig. 5.3.5). All data in this figure derived from 2 donors (both NASH patients).

↑CXCR6), chemokine receptors (↑CCR5, ↑CCR6, ↑CXCR3 ↑CCR9), and activation/inhibitory markers (↑PD-1). However, while both tissue egress receptor S1PR1 and chemokine receptor CX3CR1 were strongly diminished amongst the liver $CD69^{HI}$ cells, they were unaffected in the liver slice culture $CD69^{HI}$ population. Additionally, in slice models, CCR9 expression was much higher and HLA-DR expression was not repressed, unlike in true liver $CD69^{HI}$ cells. Both $CD69^{HI}$ cell types favoured a resting ($KLRG\text{-}1^{-}CD127^{+}$), followed by previously activated ($KLRG\text{-}1^{+}CD127^{+}$), activated ($KLRG\text{-}1^{+}CD127^{-}$), and finally cytokine-stimulated ($KLRG\text{-}1^{-}CD127^{-}$).

$CD69^{INT}$ cells from the liver, liver slice cultures, and Huh-7 cultures were also mostly similar phenotypically. In all three, the four T_{RM} associated markers (CD49a, CD103, CXCR6, S1PR1) were increased in percentage from their $CD69^{-}$ counterparts; alongside increases in CCR6, CXCR1, CXCR3, and CCR9 (Fig. 5.6.6C). KLRG-1 and CD127 co-expression patterns were largely similar amongst the three $CD69^{INT}$ cell groups, although liver cells seemed to be more skewed towards cytokine-stimulated cells at the expense of resting cells.

Therefore, preliminary data shows it is possible to generate both $CD69^{INT}$ and $CD69^{HI}$ $CD4^{+}$ T cells from short-term liver slice culture, and these have similar, though not identical, expression profiles to their liver-isolated counterparts.

5.8 Discussion

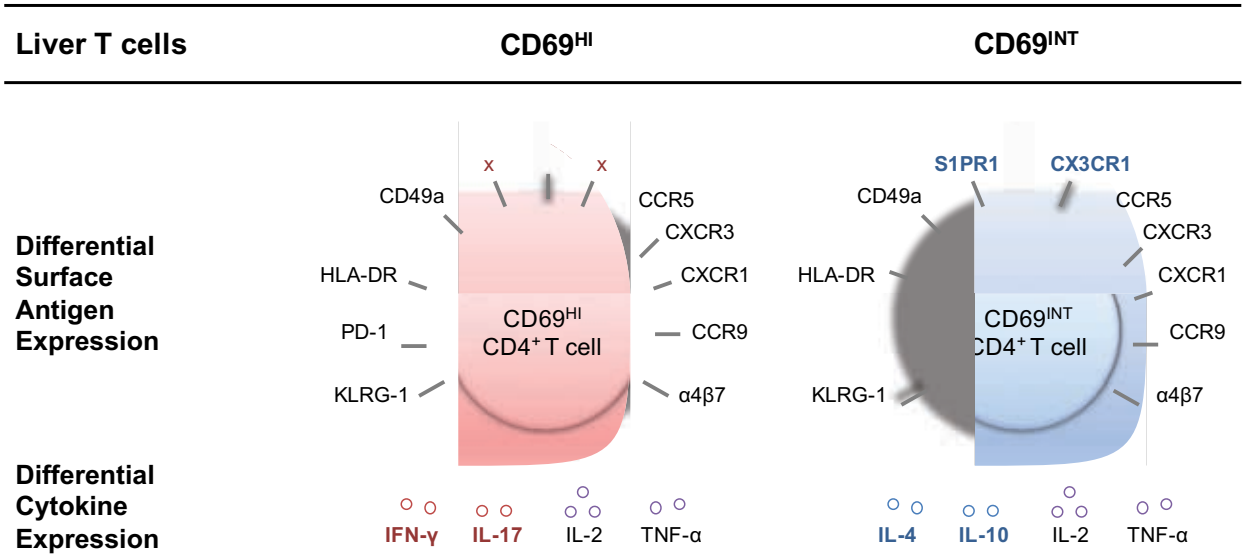
5.8.1 Summary of findings

The liver can be considered an immune organ in its own right, mediating direct activation of naive T cells and providing powerful immune tolerance mechanisms. As T_{RM} are key in immune homeostasis and memory immune responses, liver T_{RM} are expected to be of great importance in hepatic immunity. Although the $CD8^+$ T_{RM} compartment has been well described[148], $CD4^+$ T_{RM} are an unknown quantity. The aim of the research in this chapter was to address this gap in the literature and provide a thorough and detailed catalogue of their phenotype and function. On top of this, we asked whether a $CD69^{INT}$ population, analogous to that previously observed in hepatic co-culture systems could be identified in the liver, and if so what were its properties.

We demonstrated that both $CD69^{HI}$ and $CD69^{INT}$ $CD4^+$ T cells can be found in the human liver, appearing to be very distinct lineages (for summary see Fig. 5.7.1). $CD69^{HI}$ cells were true T_{RM} with restricted homing receptor expressions and preferences for resting phenotypes. Following stimulation these cells were the best producers of T_H1-cytokines. Conversely $CD69^{INT}$ did not appear fully resident phenotypically, expressed a wide range of homing receptors and mixed activation states, and were the highest IL-4 and IL-10 producers following stimulation. Phenotypically similar $CD69^{INT}$ cells were induced *in vitro* following hepatic cell co-culture, and it is possible we saw the development of $CD69^{HI}$-like cells following contact with intact liver slices (Box 5.1).

Chapter 5 – Core Findings:

- Intrahepatic $CD4^+$ T cells subdivided into three populations based on CD69 expression: $CD69^-$, $CD69^{INT}$ & $CD69^{HI}$
- $CD69^{HI}$:
  - Represented the $CD4^+$ T_{RM} of the liver through absence in blood and strong co-expression of tissue-residency markers
  - Expressed chemokine receptors predictive of restriction to portal and biliary areas
  - Favoured a predominantly resting ($KLRG\text{-}1^-CD127^+$) state and high co-inhibitory marker expression
  - Evoked robust multifunctional T_H1, and T_H17 response best following stimulation
- $CD69^{INT}$:
  - Displayed a semi-resident profile, with elevations of some residency markers, but also high expression of tissue egress receptor S1PR1
  - Were very heterogenous for homing receptors, suggestive of the ability to home to multiple liver niches as well as the gut
  - Were a more activated cell type than their $CD69^{HI}$ counterparts (based on CD38, HLA-DR, Ki-67, and CD127/KLRG-1 designations)
  - Were capable of producing IL-4 and IL-10 most efficiently
- Phenotypically similar $CD69^{INT}$ and $CD69^{HI}$ cells were generated from co-culture with hepatic epithelia and intact human liver slices respectively



$CD4^+$ liver T cells. Diagram shows first the significant differential expression of surface antigens between the two subsets ($p<0.05$). Two bars denotes a higher level of expression, while x means negligible expression of the corresponding marker. Markers highlighted in red or blue show subset-specific expression. Cytokines deemed differentially expressed following stimulation are shown below the cells.

5.8.2 Experimental justifications

When assaying phenotype and function, all $CD4^+$ T cell populations were pre-gated on $CD56^-\gamma\delta$-TCR^- singlet lymphocyte populations. This ensured that only conventional T cells were studied, not NK cells, NKT cells, or $\gamma\delta$ T cells that can be substantial hepatic populations[281]. This gating strategy also largely excludes MAIT cells that can make up between 20-50% of liver T cells, as MAIT cells from are predominantly $CD56^+$, and only a small proportion of MAIT cells express CD4[663, 664]. Next, although some panels contained a CD8 marker, there was not space in the 9-colour panels for this marker in all, so there is a possibility that some of our cells are $CD4^+CD8^+$ (double positive) T cells that have been described as a low frequency cell type in the human liver[275]. However, many of the livers we analysed did not contain appreciable amounts of these cells (data not shown). While we did not include a dead cell marker for the experiments where liver cells were immediately stained and fixed following their isolation (previous work in our lab has shown extremely low cell death in live lymphocytes in such experiments as LIMCs were obtained and stained immediately following density gradient centrifugation), live/dead dyes were always used to exclude dead cells in cytokine detection, and liver slice culture experiments that included prolonged culture periods and/or cell stimulation.

We used CD69 alone as the key discriminator of tissue-residence. The logic behind this was as follows. First, many other investigators of human tissue residence used this marker, enabling fair intra-study comparisons[82, 88, 90, 135, 136, 146]. Second, CD69 has shown repeatedly to be the best marker of human T_{RM}[91, 186]. CD69 expression completely segregates T_{RM} and non-T_{RM} phenotypically[91], has been demonstrated on every human T_{RM} type studied[186], and other T_{RM}-associated markers such as CD103 and CD49a are

only suitable for particular T cell subsets, tissues, or tissue niches[146, 152, 187, 188]. Furthermore, CD69 expression is strongly correlated with repression of the tissue egress phenotype through downregulation of S1PR1[153]. This process is allowed by KLF-2 downregulation that itself is downstream of other key T_{RM} inducers[84]. Thus, CD69 promotes tissue residence in a multitude of ways. Some murine studies have argued CD69 is an imperfect marker of tissue-residence, with some resident cells $CD69^{-}$ [87], expressing it while recirculating[130], or differentially modulating it as they travel through NLTs[213]. However, in humans the story is likely different as nicely illustrated by Beura et al. who showed 'dirty' pet shop mice possessed much greater CD69 expression in tissue-resident cells compared to specific pathogen free controls[214]. This provides a model closer to us 'dirty' humans[214]. Paradoxically, we argue both for and against CD69 as a true human T_{RM} discriminator. We believe that while binary CD69 expression is not enough to discriminate T_{RM} from non-T_{RM}, high expression of this molecule is an excellent marker of true T_{RM} cells.

An essential prerequisite to comparing and contrasting the $CD69^{INT}$ and $CD69^{HI}$ cells, is to prove they are truly distinct subsets. We have done this in a multitude of ways. First, both isotype-matched controls and our previous data on CD69 upregulation to an intermediate level in co-culture convinced us the $CD69^{INT}$ population was real and not just a fluorescence shift. Although staining in some livers clearly showed three distinct populations based on CD69 expression, some stains were less distinct, with the $CD69^{INT}$ cells closely positioned to the $CD69^{-}$ population. Thus, predictably, some have argued that $CD69^{INT}$ cells were simply a tail of the $CD69^{-}$ population. We can show this is not the case through our second, third, and fourth arguments – that $CD69^{INT}$ and $CD69^{HI}$ cells are phenotypically, and functionally distinct, and not all phenotypic markers show simple

linear correlations with CD69 expression. Multiple phenotypic distinctions separated the two subsets, including stark differences in CX3CR1, CXCR6, PD-1, and KLRG-1. These cells were also functionally distinct as only $CD69^{INT}$ cells were able to appreciably make IL-4 and IL-10. Lastly, and most importantly, many markers that were differentially modulated in one direction from $CD69^{-}$ to $CD69^{INT}$, were reversed in direction from $CD69^{INT}$ to $CD69^{HI}$. Examples include CXCR3, CD27, CCR9 and HLA-DR. This goes against what we would expect if the $CD69^{INT}$ were only a population tail where only linear changes with CD69 expression would be seen. Therefore, we have provided multiple lines of evidence that $CD69^{INT}$ cells are a true distinct population, and not an artefact of staining. It is interesting that we are the first to report this cell type when many others have investigated human tissue resident T cells. The $CD69^{INT}$ phenotype could of course be liver, or $CD4^{+}$ T cell specific, explaining away the vast majority of studies that look at $CD8^{+}$ T cells. Alternatively, this population may have been overlooked by researchers who were not aiming to find it. We ourselves may have missed the $CD69^{INT}$ cells we had not been specifically looking for an intermediate population thanks to our co-culture data, and also had the advantage of staining for CD69 with an antibody conjugated to the fluorochrome PE-Dazzle594, a very bright dye.

Finally, many of our literature comparisons have been made with $CD8^{+}$ T_{RM} as this reflects the availability of data in humans. Despite the clear differences between circulating $CD4^{+}$ and $CD8^{+}$ T cell biology, these differences may be largely diminished amongst T_{RM} subsets. This was meticulously demonstrated in the human lung recently[135]. Thus, it appears that tissue-specific factors are the most important regulators of T_{RM} identity, regardless of helper or killer designation.

5.8.3 Phenotypic and functional comparison of liver CD69-defined populations

We showed that high expression of CD69 marks the tissue-resident $CD4^+$ T cell population of the human liver. $CD69^{HI}$ cells were only present in liver not blood, were heavily skewed towards the T_{EM} phenotype, and showed the strongest association with T_{RM} markers (positive association with CD103, CD49a, CXCR6, PD-1; negative association with CCR7, S1PR1 and KLRG-1). These marker profiles were extremely similar to $CD8^+$ T_{RM} described in the liver[148, 149], skin[151], and lungs[131, 134]; as well as $CD4^+$ T_{RM} from the stomach, lungs, skin, and colon[82, 135, 136]. Comparing our results directly with the study by Wong et al. that also looked at liver $CD4^+$ T_{RM}, all of these markers (except for the lowly expressed CD103) showed the directional change in both studies (S1PR1 and KLRG-1 not studied by Wong et al.). Interestingly though, their study only found around 4% of liver $CD4^+$ T cells expressed CD69 compared to our average of 39%, and also reported no $CD69^{INT}$ cells. This could be partly due to methodology – we used digestion, whereas Wong et al. opted for portal flush[82].

$CD4^+$ T_{RM} were universally present in all livers, independent of disease aetiology, supporting a role for these cells in general immune homeostasis that supersedes any underlying inflammatory climates[23]. While we did not analyse differences in viral liver diseases here, intrahepatic $CD4^+$ T_{RM} are also thought to be unaltered in HCV infection[384], although $CD8^+$ T_{RM} are elevated in patients with chronic HBV[148]. In support of other human data, our cells did not appreciably express Hobit[134, 135, 148]. Although CD103 expression is generally not a good T_{RM} marker among $CD4^+$ T cells[84, 88, 665], CXCR6 expression in the $CD69^{HI}$ compartment was especially important. CXCR6 expression is thought to be vital for retention in the liver[180, 391, 393], and accordingly has been reported

on human liver-resident NK cells, as well as liver-resident T cells[148, 149, 384, 395, 646]. However, it should be noted that CXCR6 may be a more generic T_{RM} discriminator, as lung T_{RM} appear to rely on this receptor also[134, 135, 666].

Aside from CXCR6, other homing receptors with the strongest association with $CD69^{HI}$ cells were CCR5 and CCR6, suggestive of preferences for portal and bile duct areas. These chemokine receptor preferences not only exactly match previous findings on $CD8^+$, but also $CD4^+$ T_{RM} in the liver in lungs at both mRNA and protein levels[82, 134, 135, 384]. Our preliminary immunofluorescence data largely supported these conclusions showing CXCR6-expressing cells in the hepatic parenchyma and bile ducts. The two main limitations of these experiments were first we were not able to specifically stain $CD4^+$ T cells, and second a small proportion of $CD69^{INT}$ cells also expressed CXCR6. We reasoned the majority of CXCR6 cells observed that were also $CD4^+$ would be $CD69^{HI}$ cells as backed up by previous flow cytometry data, thus allowing the small discrepancy as acceptable error. Within the liver, CXCR6 is only expressed on lymphocyte populations (T cells, NK cells and NKT cells), except for in liver cancer where tumour cells can express this molecule[667]. As none of the livers used for staining had any information on file about liver cancer, again we deemed this latter scenario unlikely. Therefore, although we cannot pin down a specific lymphocyte subset from our immunofluorescence stains, we can argue that CXCR6 staining in these experiments at least limits the possibilities of where $CD4^+$ $CD69^{HI}$ cells can be.

Interestingly, we saw no association with sinusoidal homing receptor CXCR3 expression, and this was corroborated by little sinusoidal localisation patterns of $CXCR6^+$ cells by immunofluorescence. This was in obvious contrast to previous studies on $CD8^+$ and $CD4^+$

T_{RM} from the liver and lungs[82, 134, 135, 148]; but agreed with studies on $CD69^+$ intrahepatic $CD4^+$ T cells in HCV patients[384]. Perhaps these discrepancies stem from the other authors looking at only total CD69-expressing cells – our $CD69^{INT}$ did contain a greater $CXCR3^+$ population. As CXCR3 is used to enter the tissue through the sinusoids, it is possible that once a cell settles into a life of residency this receptor is no longer required and so downregulated. Perhaps this points to differences in the liver tissue niches of $CD8^+$ and $CD4^+$ T_{RM}; as the former have been suggested to reside in the sinusoids in mouse modes, and now too in human livers[128, 148], while our data supports the latter favour portal and biliary areas. This point brings us back to the low percentages of CD69-expressing intrahepatic $CD4^+$ T cells found by Wong et al., as perhaps only $CD8^+$ T_{RM} can be reliably isolated by portal flush methodology ($CD8^+$ T_{RM} frequencies in their study were comparatively far higher)[82].

As opposed to the $CD69^{HI}$ population, $CD69^{INT}$ cells are likely non-resident. Despite their negligible presence in blood, there was no T_{EM} phenotype enrichment within this subset compared to $CD69^-$ controls. $CD69^{INT}$ cells presented with a partial residence phenotype – expressing T_{RM}-associated markers CD49a, CD103, and CXCR6 more than $CD69^-$ cells, but not to the same degree as the truly resident $CD69^{HI}$ cells. $CD69^{INT}$ cells expressed S1PR1, and this was actually at a higher median percentage than S1PR1 expression in the $CD69^-$ cells. This was a key finding as possession of this receptor would allow $CD69^{INT}$ cells to leave the tissue. To our knowledge, our study is the first to report this association, with all others to date showing only negative associations between CD69 and S1PR1[153, 158, 668]. Our result indicates there could be a threshold for CD69 expression needed, before which S1PR1 is not appreciably out-competed and thus downregulation does not occur. As CD69 is thought to be upregulated early in the tissue residence

formation process[158], the S1PR1^{+} CD69^{INT} cells may be very recently formed tissue-residents that have not yet shut down the tissue-egress programme. Therefore, like others have suggested, it is conceivable that cells expressing CD69 could still return to the circulation before the T_{RM} programme is set, as exemplified in the skin where multiple resident and recirculating populations exist[152, 158, 213].

Homing molecule expression profiling provided additional evidence for the non-resident nature of the CD69^{INT} population. When compared to CD69^{-} controls, CD69^{INT} possessed greater CCR5, CCR6, CXCR1, CXCR3 and CX3CR1 percentage expression. This broad homing receptor profile suggested CD69^{INT} cells had not found a tissue niche within the liver in which to settle, in contrast to the CD69^{HI} cells which had a more restricted pattern. This is important as T_{RM} from both mouse and man have often been described as residing in particular tissue niches[133, 152, 218, 256, 261]. This was the first demonstration of the relative heterogeneity of CD69^{INT} cells versus CD69^{HI} cells in our study, an aspect of their biology we will return to later. This point is important when attempting to assign roles to this newly discovered subset. For homing receptor expressions, further research with the use of bioinformatic tools should investigate whether the fractions of the CD69^{INT} cells expressing different homing-receptors cluster together (i.e. are the CXCR3^{+} cells the same cells as those expressing CCR6 for example). Knowledge of these different receptors' roles in homing to different areas of the liver suggest CD69^{INT} cells would likely segregate, but it is important to test this hypothesis formally.

CX3CR1 was key in discriminating CD69^{INT} from CD69^{HI} cells. While the CD69^{HI} subset barely expressed this receptor, CX3CR1 expression in CD69^{INT} cells was clear. CX3CR1 is the receptor for fractalkine (CX3CL1), as well as CCL26 in humans[354, 669]. In the liver,

fractalkine can be expressed on inflamed bile ducts[398, 399], and perhaps may attract T_H1 cells on which CX3CR1 is traditionally expressed[353]. Data on CCL26 expression in the liver is scarce, but expression has been documented on human umbilical vein endothelial cells, raising the possibility of expression on liver endothelium[670]. Very little CX3CR1 expression in the $CD69^{HI}$ T_{RM} compartment fits well with the literature, as many have reported similar decreases in T_{RM}, and recently Kumar et al. even included its downregulation in the core human T_{RM} signature[91, 134, 135, 384]. Conversely, CX3CR1-expressing $CD69^{INT}$ cells could allow access to the bile ducts as part of the wide-ranging capabilities of this cell type. Further, CX3CR1 has been used to separate three distinct $CD8^+$ subsets in mice. High and low expressors represented T_{EM} and T_{CM} respectively, whilst intermediate expressors were more plastic, able to form both other populations, and were the predominant NLT-recirculating subset[671]. Perhaps this argues for the $CX3CR1^+$ $CD69^{INT}$ cells being a $CD69^{HI}$ T_{RM} precursor, as will be discussed in more detail in section 6.1.

We used CX3CR1 expression to identify the $CD69^{INT}$ cells by immunofluorescence. Unfortunately, we could not get around the same limitations posed by using CXCR6 to identify $CD69^{HI}$ cells for this subset. This was as CX3CR1 was comparably expressed by $CD69^-$ cells, and CX3CR1 expression is not limited to lymphocytes in the liver, but also BEC, vasculature, HSEC and the fibrotic septa[398, 399, 672]. Therefore, we limited our conclusions to state that CX3CR1 expression was seen in the parenchyma, sinusoids, and fibrotic septa, but not bile ducts in our hands. Future work will aim to successfully combine the stain for CX3CR1 with CD69 and CD4 to allow us to see exactly where our desired cell type is located.

CD69^{INT} cells also expressed the most gut-homing receptors CCR9 and integrin $\alpha4\beta7$. This is suggestive of the ability of CD69^{INT} cells to travel to and from the gut. This compliments the other homing receptor data as now we can suggest that not only are CD69^{INT} not restricted to one tissue niche within the liver, they are not restricted to the liver either. This is an interesting prospect, as it could be the recirculation of CD69^{INT} cells that contributes to sharing of immunological data between the gut and liver, either promoting tolerance, or immunity. The gut and liver have an intimate immune relationship – demonstrated best by antigen sharing via the portal vein, and the continuous nature of the intestinal and biliary epithelia[276, 673]. Consequently, all major liver autoimmune disorders show strong associations with bowel disease[414], the most apparent being between PSC and IBD[673], where CCR9^{+} gut-tropic lymphocytes can be greatly enriched in PSC patient livers[404]. Thus, discovering whether CCR9^{+} CD69^{INT} cells are similarly enriched in the liver or the gut of PSC patients, and if so discerning their roles in pathology, would be an interesting angle to take in the better understanding of these cells.

Some of the most important differences in CD69^{INT} and CD69^{HI} cells lay within their activation and differentiation states. Activation markers CD25, CD38 and HLA-DR were more frequently expressed in CD69^{INT} cells, and these cells proliferated the most of the three CD4^{+} T cell subsets. CD69^{HI} cells were comparatively less activated. While CD25 expression was still elevated in the population compared to CD69^{-} cells, there was no increase in proliferation. Additionally, HLA-DR and KLRG-1 were both decreased. A similar KLRG-1 decrease was seen in lung CD8^{+} T_{RM}[134], but in other liver studies, both CD4^{+} and CD8^{+} T_{RM} expressed higher HLA-DR levels[82, 148]. However, this was not consistent with other human NLTs, with HLA-DR levels low among T_{RM} isolated from these[91]. These differences could again be explained by the increased HLA-DR

expression among our distinguished $CD69^{INT}$ cells, and/or by differences in $CD8^+$ T_{RM} activation potential or the liver immune environment. T_{RM} cells in mice are thought to develop preferentially from KLRG-1^- precursors[110], a cell type also responsible for long-lived $CD8^+$ T_{CM}[193]. Our findings, combined with other human data, may hint at a similar mechanism in the human $CD4^+$ lineage[134]. Importantly, $CD69^{HI}$ T_{RM} were biased towards a resting (KLRG-1^-CD127^+) phenotype. Thome et al. used a similar marker set (CD28 and CD127) to demonstrate that lung and gut tissue $CD4^+$ T cells were also predominantly resting, whereas interestingly, $CD8^+$ T cells from these sites were mostly cytokine responsive[90]. Our $CD69^{INT}$ population by contrast was once again more heterogenous. However, the significant change from the other two subsets was in the previously activated (KLRG-1^+CD127^+) phenotype, in agreement with our earlier activation marker data. Following this logic, at least some of the intrahepatic $CD69^{INT}$ cells are not permanently resident, and/or perhaps too far differentiated to become resident through virtue of their KLRG-1 expression.

On the subject of differentiation status, CD27 and CD28 expression patterns were different among the two CD69-expressing subsets. CD27 and CD28 are both costimulatory receptors that are downregulated with advanced effector memory cell differentiation[659, 674]. In $CD4^+$ T cells, CD27 is usually lost first, followed by CD28 as differentiation progresses[488, 659]. Our data shows $CD69^{HI}$ cells have relatively low %CD27 expression, but high CD28, suggesting that T_{RM} occupy a specific intermediate-late differentiation stage. However, it is difficult to apply the same logic to $CD69^{INT}$ cells that have the highest CD27 expression, but lower CD28. As well as pointing out that CD28 stains were only performed on one donor to date, we suggest that assessing precise differentiation stages further would inevitably involve combining both these markers into

panels to compensate for cellular heterogeneity. Indeed, Larbi et al. state only five key markers (CD28, CD27, CD57, KLRG-1, PD-1) are necessary to encapsulate the entire spectrum of T cell differentiation, exhaustion, and senescence; four of which we have already studied[659]. Of interest, human lung $CD4^+$ T_{RM} contained the highest CD28 expression also[135], validating our results. Last, CD80 expression was marginally increased amongst $CD69^{HI}$ cells, but CD86 expression was markedly absent in this population (n=1). This could be a PSC-specific phenomenon, but other explanations are scarce due to the lack of knowledge of the implications for CD80/CD86 expression on T cells.

Analysis of the functional responsiveness of the three intrahepatic $CD4^+$ subsets revealed a greater propensity for $CD69^{HI}$ and $CD69^{INT}$ to produce T_H1 and T_H2 cytokines respectively. We decided to use multiple stimuli to test cytokine production. This provided a robust approach as no one stimulus was optimum for all cytokines (for example IFN-γ and IL-10 have been reported to be better stimulated by PMA/Ionomycin, and anti-CD3/CD28 respectively[675]); but also allowed better comparison with human T_{RM} studies who use both PMA/Ionomcyin[82, 88], and anti-CD3/CD28 stimulation[135, 136, 148], and a combination of the two[91, 131].

Our assay design involved a pre-stain of CD69 before stimulation and subsequent cytokine staining. This was similar to that employed by Wong et al. who stained for homing receptors prior to stimulation and cytokine staining[82]. In our case this pre-staining step ensured the process of stimulation did not mean we detected false CD69-positives. We also verified CD69 does not appreciably downregulate in culture for up to 3 hours, increasing confidence in our CD69 stain endurance.

Human $CD4^+$ T cells in the liver, like most other NLTs, have been show to favour T_H1 cytokine production the most[82, 88], and $CD4^+$ T_{RM} from the lungs also follow this pattern[135]. This lines up well with our findings – a consistent upregulation of IFN-γ with all stimulation methods, and IL-2 and TNF-α with PMA/Ionomycin in $CD69^{HI}$ T_{RM} cells. Also like T_{RM} from other studies[135, 136], the $CD69^{HI}$ cells in our study were the most type-1 multifunctional. It is well established that multifunctional cells are the most potent in multiple contexts[241, 243], and this finding also indicates that our cells are largely not functionally exhausted[564, 676]. Besides this, we found our T_{RM} were the best at producing IL-17 following PMA/Ionomycin stimulation, akin to $CD4^+$ T_{RM} in the stomach and lungs[91, 136]. These strong pro-inflammatory capabilities following stimulation suggest at a key role for the $CD69^{HI}$ T_{RM} in immune protection and pathology. There is also weaker evidence that $CD69^{HI}$ cells are the best TGF-β-producers, meaning these cells could also be self-regulating, and/or be contributing to fibrosis[677].

The word heterogenous must once again be used when describing the $CD69^{INT}$ cells. Although these cells appeared better than $CD69^-$ cells at making IL-2 and TNF-α (although not statistically significant) with PMA/Ionomycin stimulation, they did not make any more IFN-γ or IL-17, and neither are they any more type-1 multifunctional than the control $CD69^-$ cells. Instead, they make the highest IL-10 and IL-4, offering type-2 and regulatory responses that $CD69^{HI}$ cells cannot. Similar IL-4 and IL-10 findings were also observed with the other stimulation methods, but of course this is only the story at 5 hours or less. Once additional ways of extending the lives of LIMCs can be found, longer stimulations can be carried out. Interestingly though, $CD69^{INT}$ cells showed no clear enrichment of conventional regulatory T cells ($CD25^{HI}CD127^{LO}$), meaning if there is any

regulatory bias within the CD69^{INT} population, it must be within an unconventional subset. Lastly, IL-6^{+}-cells were most frequent within the CD69^{INT} gate. This could reflect a role of these cells in pro-inflammatory responses, maintenance of liver metabolic function, and/or hepatocyte proliferation depending on the levels and persistence of IL-6 secretion[678].

Increased expression of inhibitory markers such as PD-1 and CTLA-4 have been noted in many human T_{RM} studies[134, 135, 148], and PD-1 is now considered to be a T_{RM} core signature marker[91]. In agreement, we found PD-1 to be markedly upregulated in the CD69^{HI} population, but less so in CD69^{INT}. Contrastingly, CTLA-4 was not increased in CD69^{HI} cells, but was marginally elevated in the CD69^{INT} subset. Like others have put forward, we believe that the high inhibitory marker expression on T_{RM} cells serves to restrain this population's potent activatory capabilities[134, 135, 148]. The CD69^{HI} T_{RM} in this study likely behave similarly as they are mostly resting (KLRG-1^{-}CD127^{+}), and self-inhibitory (PD-1^{+}), but can potently produce type-1 and -17 cytokines upon stimulation, consistent with a key immunosurveillance role. It will be interesting to discern whether our CD69^{HI} cells have the highest levels of preformed cytokine mRNA, as is seen for resident CD8^{+} T cells in the lungs, although interestingly possession of pre-formed IFN-γ mRNA did not notably contribute to the rapidity of the response in some studies[134, 135]. CD69^{INT} cells by contrast are more activated as a population, and perhaps their smaller inhibition by lower surface PD-1 levels means they are less restrained than their CD69^{HI} counterparts. This hypothesis is supported by the time-course experiments that revealed that for many cytokines, CD69^{INT} had already reached their maximum % responsiveness at 2h post-stimulation; whereas CD69^{HI} cells generally produced more at the 5-hour time-point. Curiously, 5h anti-CD3/CD28 stimulation brought about the best IL-2 and TNF-α

responses in CD69^{INT} cells, whereas PMA/Ionomycin stimulation showed CD69^{HI} cells were the best at generating these cytokines. This again hints at potential differences responsiveness to stimulation signal types/strength, also supportive of a more restrained CD69^{HI} T_{RM} cell type.

5.8.4 Phenotypic comparison with blood and lymphoid organs

Many of the phenotypic markers studied in the liver were also analysed in blood and hepatic lymph nodes. Although the numbers of these samples obtained was much smaller as matched patient blood and lymph nodes were much more difficult to obtain, preliminary analyses into these populations allowed us to understand what is truly a liver-resident versus general tissue-resident phenotype. Many excellent human tissue atlas studies have been published in recent years, where multiple cell types across multiple human body tissues are studied[82, 88, 90, 91]. We used these studies as benchmarks wherever possible to verify our findings and to better understand the biology of both blood, and lymph node cells compared to the liver populations.

We found blood T cells contained many more naïve and T_{CM} cells, and as expected, displayed diminished expression of residency-associated markers compared to liver. This is in agreement with other studies[88, 90]. Levels of activation markers were often comparable to liver CD69^{-} cells, and displayed a predominantly resting (KLRG-1^{-}CD127^{+}) phenotype. Thome et al. also reported blood CD4^{+} T cells favour a resting (CD28^{+}CD127^{+}) phenotype, but in their study cytokine-stimulated cells comprised the second largest population, especially in the T_{EM} compartment. By contrast, in our cells, the previously activated cell type was the second largest fraction. These differences could

be down to disease status as all our donors were patients with chronic liver disease, whereas in the study by Thome et al., donors used were free of apparent immune-mediated disease[90].

Interestingly, human lymph nodes did not only contain $CD69^{HI}$ cells, but a substantial proportion of $CD69^{INT}$ cells as well. LN $CD69^{HI}$ cells appeared most phenotypically resident, with notable increases in CD49a, CXCR6, and PD-1; and decreases in S1PR1, and KLRG-1 expression from LN $CD69^{-}$ cells. CX3CR1 was also very lowly expressed in LN $CD69^{HI}$ cells. Therefore, Liver and LN $CD69^{HI}$ $CD4^{+}$ T cells were remarkably similar and likely both represent tissue-resident cells. Distinctions between the two exist however, and give us an idea about subtle differences in their biology. For example, LN $CD69^{HI}$ have much greater % CXCR3 expression, but less CCR5 and CD49a; indicating differing reliance for these molecules for their maintenance. Additionally, $CD69^{HI}$ LN cells have a much greater cytokine-stimulated component. This is largely similar to findings made by Thome et al. where $CD4^{+}$ T_{EM} from various lymph nodes trend towards a higher cytokine-stimulated fraction than T_{EM} equivalents in gut tissues[90]. This indicates that compared to liver, LN $CD4^{+}$ T_{RM} are much more reliant on common γ-chain cytokines for their survival.

As for $CD69^{INT}$ cells, intriguingly this population in LN may have even been a larger constituent than in liver. Like in liver, they display a partial residence phenotype, but retain S1PR1. $CD69^{INT}$ LN cells were also the best CCR9-expressors suggestive of gut-homing ability. A key difference however was that no LN population seemed to express CX3CR1 (n=1). This finding seems consistent with human lymphoid tissue[82]. KLRG-1/CD127 co-expression also revealed the majority of LN $CD69^{INT}$ are resting, compared to the much

larger expansion of previously activated cells in the liver. This highlights the ability of the liver to retain activated T cells[341], and suggests that while liver and LN $CD69^{HI}$ cells seem similar, the heterogeneous $CD69^{INT}$ populations differ substantially in activation state even if it turns out they fulfil similar roles.

5.8.5 Insights into the generation of liver $CD69^{INT}$ and $CD69^{HI}$ cells

Finally, we carried out preliminary investigations into the generation of liver $CD69^{INT}$ cells. Using blood-derived $CD4^{+}$ T cells cultured with hepatic epithelia, we managed to recreate a partial $CD69^{INT}$ phenotype. Alongside moderate increases in CD49a and CXCR6, S1PR1 was induced, indicating contact with the hepatic cell lines was not mimicking T_{RM} induction. This phenomenon was not solely due to tumour effects, as a similar S1PR1 upregulation was seen following contact with primary BEC cells. $CD69^{INT}$ hTC contained higher KLRG-1 proportions than $CD69^{-}$ hTCs, and maintained CD127 expression far better. Together, these markers suggested that generation of $CD69^{INT}$ cells from blood was slightly more efficient in both resting cells and previously activated cells than those currently activated or cytokine-stimulated. In particular, cytokine stimulated cells were far higher in the $CD69^{-}$ hTC cells, backing up earlier observations that soluble cytokines are less important in the generation of the $CD69^{INT}$ phenotype than direct surface cell contact (section 4.4). On top of this, co-culture revealed increases in % of inhibitory markers PD-1 and Fas in the $CD69^{INT}$ cells, the significance of which is unknown. However, depletions of CCR6 and CXCR3 expression in co-culture were seen, unlike the liver $CD69^{INT}$ cells. These could be artefacts of the tumour co-culture system[679], or novel signatures imprinted by hepatocytes that restrict migration to other areas of the liver.

The above observations were largely consistent regardless of hepatic cell line used, but interestingly also seen when using primary BEC, now allowing us to say that a $CD69^{INT}$ phenotype largely reminiscent of liver $CD69^{INT}$ cells can be induced with contact with primary liver epithelial cells. The only stark change in BEC-induced $CD69^{INT}$ cells from their liver counterparts, was a much greater preference for the resting ($KLRG\text{-}1^{-}CD127^{+}$) phenotype in the former. Surprisingly, co-culture with Huh-7 cells was enough to alter the cytokine production potential of these cells – IL-4 was preferably produced after co-culture, much the same as the liver $CD69^{INT}$ cells. However, total alignment was not seen as there were no other clear changes in cytokine potential.

So, in sum it appears that co-culture of blood $CD4^{+}$ T cells with a hepatic cell line is enough to induce a reasonably accurate replica of a liver $CD69^{INT}$ cell. This suggests that $CD69^{INT}$ cells in the liver form following direct contact with either hepatocytes or BEC. While not all markers matched, and not all cytokines were skewed the same way, this is to be expected as simple cell line systems cannot hope to recapitulate the full complexities of the liver microenvironment. However, as culture with BEC induced a similar phenotype, we gained confidence that most of the observations were not simply down to the use of a tumour cell line. Next steps would include the use of PHH alongside larger donor numbers, but even at this stage our data provide important clues to how these phenotypes might arise *in vivo*.

This data was supplemented by liver slice models which revealed that phenotypically similar $CD69^{INT}$ cells could be generated by contact with intact liver slices. Crucially, it appears that slices could also generate modest numbers of $CD69^{HI}$ cells too. These cells also showed well-matched phenotypes with liver-isolated $CD69^{HI}$ T cells. Although also

at an early stage, this data suggests that additional signals from other cell types in the liver, and/or exposure to the cytokine and antigenic milieu is enough to drive generation of resident T cells in the liver. As Pallett et al. show that $CD8^+$ T_{RM} can be similarly induced with IL-15 (or antigen) and TGF-β[148], perhaps similar signals in the context of the liver stroma are what is generating the $CD4^+$ $CD69^{HI}$ T_{RM} cells, a concept we are eager to put to the test.

Together these data suggest $CD69^{INT}$ cells develop from direct contact with hepatic epithelia in the liver, and additional signals may cause the development of $CD69^{HI}$ cells.

5.8.6 Conclusions & future work

In conclusion, we have found two phenotypically and functionally distinct liver $CD4^+$ populations based on differential CD69 expression. Our data strongly suggest that the $CD69^{HI}$ cells are the resident population in the liver. Profiling of residence-associated markers, chemokine receptors, differentiation markers, and coinhibitory molecules revealed significant alignments of our $CD69^{HI}$ cells with both $CD4^+$ T_{RM} from other organs[88, 90, 91, 135], and $CD8^+$ T_{RM} from the liver[148, 149], allowing us to place these cells in the matrix of human T_{RM} with confidence. The biggest key question that remains is what are the roles of the newly identified $CD69^{INT}$ cells, a question made more difficult to answer by the heterogeneous nature of this population. Based on our data, and excerpts from the data from others, we have proposed three linked hypotheses (Fig. 6.1). The first proposes that $CD69^{INT}$ cells are a transitional population, en route to becoming full-fledged $CD69^{HI}$ resident cells. This is supported by many residency-associated phenotypic markers being partially expressed on $CD69^{INT}$ cells, and their apparent need

for contact with hepatic epithelia in order to develop. Perhaps further signals are needed in order to drive high CD69 expression and the associated full residence phenotype, as supported by our preliminary liver slice findings. The second hypothesis postulates that $CD69^{INT}$ cells represent a short-term, or transiently resident population that remain in the liver longer than recirculating $CD69^{-}$ cells, but do not persist long-term like $CD69^{HI}$ cells. Partial residence marker expression would also argue for this, as well as some evidence from the literature of modulation of CD69 upon tissue egress[213], and recirculation of cells expressing intermediate CD69 levels in mice[153]. It follows that if this were the case, $CD69^{INT}$ cells would lose CD69 expression before leaving the liver. Of course, these hypotheses are not mutually exclusive. It is conceivable that a third possibility exists where $CD69^{INT}$ could be short-term resident cells that can undergo bidirectional differentiation to either a $CD69^{HI}$ T_{RM} cell, or a $CD69^{-}$ non-resident cell, in a signal-dependent manner. Just like in SLOs where CD69 is expressed for sufficient time to allow naive T cells chance to receive appropriate activation signals[31], $CD69^{INT}$ cells could remain in the liver for a limited time and if no appropriate signals (antigenic or otherwise) are received, the cells downregulate CD69 and return to the circulation. The expanded rationales behind all three hypotheses, and possible ways to test them will be discussed in greater detail in chapter 6.

Through this work we have delved deep into the biology of intrahepatic $CD4^{+}$ T cell populations in order to understand how these cells might work in inflammation and homeostasis. We have now built a platform on which to investigate both hepatic $CD4^{+}$ $CD69^{HI}$ T_{RM} and $CD69^{INT}$ cells, including their roles in specific diseases, and uncovering their preferred tissue niches and migration patterns. We can use this knowledge to further understand the generation requirements for both cell types, and in turn use this to uncover

the true roles of CD69^{INT} cells in the liver. Answering these questions will aid the field in understanding of immune tolerance, and hepatic immune memory; as well as ultimately helping us combat liver pathology.

Chapter 6 - Discussion

6.1 Summary of findings

6.1.1 Modelling CD4+ T cell transmigration into the liver parenchyma

The data presented in chapter 3 puts forward the idea that transwell migration through hepatic epithelia may be used as a tool for isolating CD4+ T cell populations. Migrated populations were able to better produce TNF-α, IFN-γ and IL-4; were enriched for T_{EM} and T_{EMRA} phenotypes, and presented with a distinct activated profile. Most significantly, CD4+ T cells from different chronic liver disease origins revealed intrinsic, disease-specific dispositions, that were only identifiable through the use of these migration assays. Whether we have accurately replicated transmigration into the parenchyma remains to be seen, but the strong possibility remains that migration assays reveal populations important in disease processes, and may also be able to act as a disease profiling mechanism.

While it is tempting to propose that transwell assays could be used for diagnostic purposes, only needing blood samples and with the ability to screen for multiple liver diseases at once, the reality is that there are more effective tools for this purpose. Liver biopsy is often no longer necessary for diagnosing PBC or HCV[680, 681], and the presence of specific auto-antibodies or anti-HCV antibodies in the serum is undoubtedly more accurate than monitoring directional cytokine changes in CD4+ T cells. Instead, we believe the greatest potential in this work lies in understanding disease mechanisms by studying the phenotypic and functional properties of CD4+ T cells likely best able to migrate into the heart of the diseased parenchyma.

Discovering the meaning behind the distinctions in cytokine profiles across different diseases will be of great importance. If the changes to the population we have shown following migration through hepatoma cell lines (the transmigration effect) is accurately recapitulated using primary hepatocytes, we can start to gather a wealth of information on what happens to a T cell after migration through HSEC, but before re-entry to the circulation, a topic that has been grossly understudied. We can also determine whether the differences seen in PBC and HCV are linked to disease pathology. Additionally, coupling this work to our other studies, we now know migration through hepatocytes is not enough to trigger induction of $CD69^{INT}$ or $CD69^{HI}$ T_{RM} phenotypes. Instead a period of contact is needed, although this may not necessarily be long – 2h was sufficient to see a modest upregulation of CD69 in culture (see section 4.4). Therefore, the most activated, migration-capable cells may not be the same precursors that form T_{RM}, as supported by $CD69^{HI}$ T_{RM} favouring resting, non-activated phenotypes (section 5.4). Instead, rapidly migrating cells may not have long enough contact time with hepatocytes for their phenotype to be largely altered. It will be fascinating in the future to determine the different roles of migratory recirculating cells, and T_{RM} in the liver in both immune homeostasis and disease.

6.1.2 Implications of CD69 upregulation on $CD4^+$ T cells with hepatocyte contact

Co-culture of peripheral blood-derived $CD4^+$ T cells with hepatocyte cell lines and primary BEC led to a robust upregulation of CD69. This phenotypic change was not only independent of activation (showing CD69 upregulation to an intermediate level, and no associated changes in other activation markers, or proliferative status compared to conventional activation stimuli), but also did not reflect a switch to a novel regulatory

phenotype as demonstrated through *in vitro* suppression assays. Instead, in chapter 5 we suggested that the CD69 upregulation is modelling differentiation into a phenotypically similar $CD69^{INT}$ phenotype seen in the liver. Furthermore, we began to investigate the mechanism behind the phenomenon, proving an active and rapid, contact-dependent process with potential, yet inconclusive, ICAM-1 involvement.

While we have not yet elucidated the key molecular determinants of CD69 induction, our current data prepares us well. First, we know that CD69 upregulation can happen to a wide variety of cells, but is more efficient in activated memory cell types. This tells us the molecules involved must be fairly T cell ubiquitous, making ICAM-1 and CD58 good candidate molecules as their receptors LFA-1 and CD2 are expressed on all T cells, and ICAM-1 increases in expression and changes to a more active confirmation on activated and memory T cells [refs [682-685] and my own unpublished data]. Second, we know the T cell requires cell contact with the hepatocyte, making these same target ligands possible. Third, we know the process is active from the hepatocyte standpoint, and begins rapidly following cell-contact. ICAM-1 expression on endothelial cells and hepatocyte cell lines could be induced rapidly (by 4 hours)[643, 686], supporting the possibility of its involvement. Fourth, we know that both ICAM-1 and CD58 are costimulatory ligands and therefore can increase activation in conjunction with TCR stimulation[683, 687], and for ICAM-1 at least, CD69 expression can be triggered in the absence of antigen recognition[633]. Therefore, ICAM-1 and CD58, known to be expressed on human hepatocytes[643], are good candidate molecules for us to continue our investigation in many different ways. An alternative approach would be to utilise RNA sequencing to assay the Huh-7 cells, $CD69^{+}$ hTCs, and $CD69^{-}$ hTCs. This would allow for the screening of differentially expressed receptors between the two T cell subsets, and allow alignment with any potential ligands expressed

on the Huh-7 cells. Expression of any candidate molecules could then be confirmed at the protein level, before they are blocked with antibodies or silenced genetically so CD69 upregulation in T cells can be monitored. Any candidates could then be tested in primary cells, first BEC, then PHH in the future.

Next, we must consider how determining the mechanism behind CD69 induction would benefit the field. We are currently hypothesising that liver $CD69^{INT}$ $CD4^+$ T cells represent either a short-term resident population and/or a transitionary precursor of $CD69^{HI}$ 'true' T_{RM} cells (see 6.1.3). Based on current evidence, the co-culture-induced $CD69^+$ $CD4^+$ T cells appear to mimic these $CD69^{INT}$ liver cells. Therefore, if translatable to primary cells, determining the mechanism behind CD69 induction would for the first time show how $CD4^+$ T cells begin to acquire residence properties in the liver. CD69 is key in initial residence formation in $CD8^+$ T cells[158], and important for multiple arms of the residence cascade, explaining why it is the central molecule in human T_{RM}-programmes[30, 91]. Strikingly, LFA-1 – ICAM-1 interactions were necessary for the maintenance of murine liver $CD8^+$ T_{RM} in the sinusoids[651]. Perhaps then a similar mechanism is at play for human hepatic $CD4^+$ T_{RM}. ICAM-1 and CD58 are both increased in expression on hepatocytes after stimulation or in pro-inflammatory environments[628, 630, 631]. Conceivably, interaction with these molecules in the liver, either alone or in combination with antigen, provides cues to differentiate into either $CD69^{INT}$ or $CD69^{HI}$ T_{RM} phenotypes. It may be, as Pallett et al. suggest, that following these cell-surface signals, cytokines or antigen are required to push T cells into the full $CD69^{HI}$ T_{RM} phenotype, a possibility we are excited to investigate[148]. If ICAM-1-LFA-1 interactions do turn out to be responsible, this represents a key step in understanding the settlement of these vital immune cells. In conclusion, we believe cell-surface ligands ICAM-1 and CD58 are two high potential molecules to

investigate in the CD69 upregulation mechanism, the revealing of which could have crucial implications for understanding the initiation of T cell retention in the liver, and ultimately the triggering of highly potent, disease relevant $CD69^{HI}$ T_{RM} cells.

6.1.3 *In vivo* $CD69^{INT}$ and $CD69^{HI}$ population relationships

Moving on to look at intrahepatic T cells, we isolated and defined three $CD4^+$ T cell populations based on CD69 expression – $CD69^-$, $CD69^{INT}$, and $CD69^{HI}$. $CD69^{HI}$ were deemed tissue-resident based on universal liver presence, and phenotypic and functional expression patterns allowing us to comfortably place them amongst other human tissue resident T cell types previously described. $CD69^{INT}$ were more difficult to define due to their previously undescribed nature, large heterogeneity, partial residence expression patterns and possession of a wide range of homing receptors. Moreover, a phenotype remarkably similar to liver $CD69^{INT}$ cells could be generated from blood $CD4^+$ T cells through simple co-culture with hepatic epithelia, hinting at similar generation pathways in the liver.

The next important question to answer is what are the roles of $CD69^{INT}$ cells in the human liver. Based on our data and from others, we devised three hypotheses to test (Fig. 6.1):

i. $CD69^{INT}$ are a transitional population, a precursor of $CD69^{HI}$ cells
ii. $CD69^{INT}$ are a short-term resident population, able to convert to $CD69^-$ cells upon liver exit

iii. Both the above are true. CD69^{INT} cells contain both short-term residents able to convert to CD69^{-} and exit liver, and differentiate into CD69^{HI} to become long-term residents.

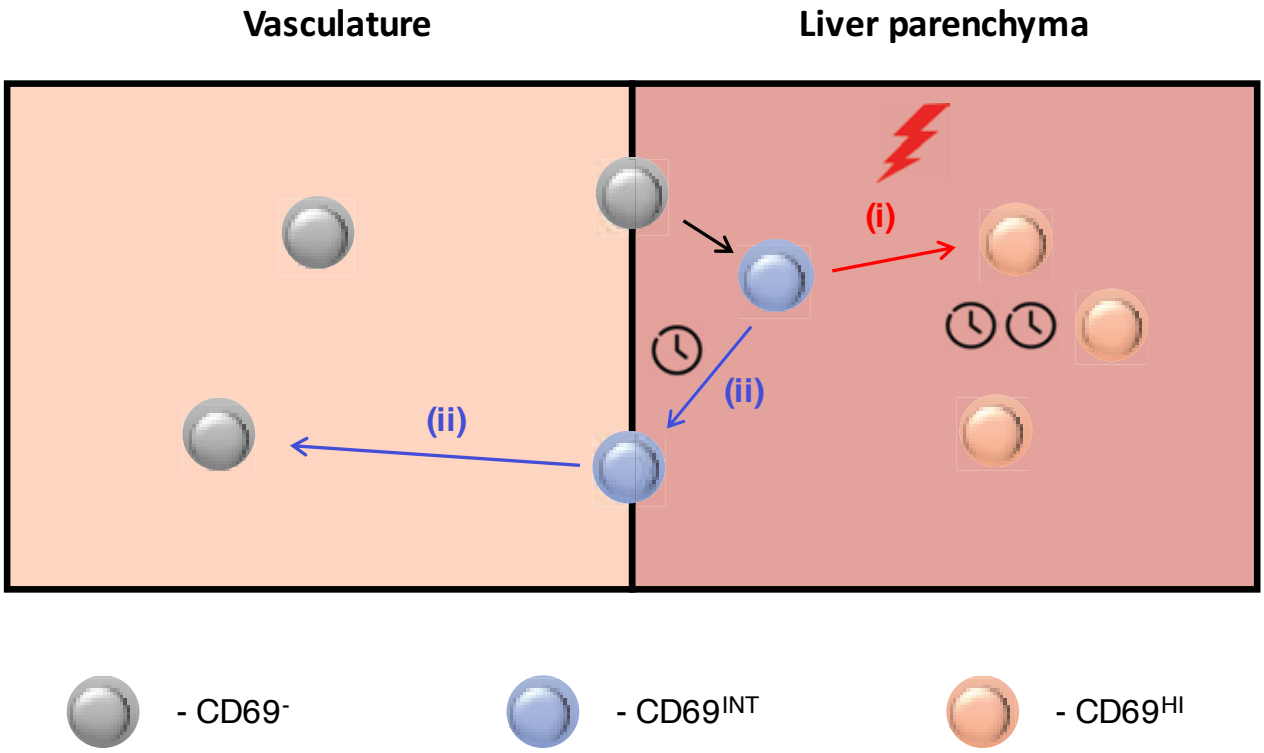


Figure 6.1 – Hypothesised roles of liver CD4^{+} CD69^{INT} cells. The diagram represents the liver vasculature and liver parenchyma as two separate compartments. We believe CD69^{-} CD4^{+} T cells differentiate into CD69^{INT} cells following contact with liver parenchymal cells (Hepatocytes, BEC), based on our hepatic cell co-culture and liver slice culture data. From here we believe one or both of two hypotheses is likely true. In the first situation (i – red arrow), CD69^{INT} cells can become long-lived CD69^{HI} T_{RM} cells upon receiving yet undefined signals, most likely cytokine and/or antigenic in nature (represented with red lightning bolt). In the second situation (ii – blue arrows), CD69^{INT} cells spend a short period of time in the liver, before downregulating CD69 expression and returning the circulation. Number of clock symbols represent relative dwell time: 1 - short-term, 2 - long term.

To our knowledge, ours is the first description of a distinct $CD69^{INT}$ phenotype in T cells in humans. Using parabiotic mice, Skon et al. described a rare population of $CD8^+$ cells that also expressed CD69 and KLF-2 at intermediate levels. These cells were recirculating, not resident, reaching NLTs of the parabiont mouse. The authors could not answer whether such cells were in the process of joining the T_{RM} population, or ready to re-join the circulation, lending equal support to all of our hypotheses[153]. Assumedly, partial KLF-2 downregulation would lead to incomplete S1PR1 reduction, as seen in our liver $CD69^{INT}$ cells. Thome et al. have reported the most activated human T cell populations re-enter the circulation, which matches the $CD69^{INT}$ population in our study[90]. Additionally, the 'migratory memory' (T_{MM}) cells in human skin grafts described by Watanabe et al. also share features with our liver $CD69^{INT}$ cells[152]. Although no data on CD69 expression levels per cell in this population was shown, the authors state that approximately a third of these cells expressed CD69, and some data indicated their slower migration through skin than T_{CM}. These cells made up the predominant skin-tropic population in the blood, and interestingly (like our liver $CD69^{INT}$) were good IL-4 producers following stimulation, although these comparisons were done using blood, not tissue populations. However, a major difference between these cells and ours was the T_{MM} cells were of the central memory lineage[152]. Nonetheless, the authors also suggest their T_{MM} cells may be in a transitional residency state, much like we have proposed for our $CD69^{INT}$ cells[152]. Gerlach et al. showed that $CD8^+$ T cells expressing CX3CR1 at an intermediate level were the major population entering NLTs of mice[671]. As our $CD69^{INT}$ cells were the highest CX3CR1-expressing cells amongst liver $CD4^+$ T cells, this study provides an argument for these cells being a T_{RM} precursor. Another study has demonstrated the up-, and down-regulation of CD69 on $CD4^+$ T cells upon respective entry and exit to the skin in mice[213], heavily supportive of a short-term resident population that could be analogous

to ours. Others have also reported the shorter tissue-dwell times of $CD4^+$ T cells compared to $CD8^+$ [218], and perhaps some of this difference is down to the $CD69^{INT}$ $CD4^+$ population that has not been found in $CD8^+$ T cells. Finally, Sakai et al. describe a $CX3CR1^+KLRG\text{-}1^{HI}$ lung-vasculature resident $CD4^+$ T cell population in mice that semi-parallels our $CD69^{INT}$ cells. Although this population did not express CD69 appreciably, they were able to lose their residence properties and migrate to multiple locations following adoptive transfer, unlike the $CD69^+$ lung-parenchymal resident cells that preferentially migrated back to the lungs[112]. This argues for a less committed resident role, that could support either a more short-term dwell time, as well as a T_{RM} precursor population.

Thus, using the literature alone cannot provide enough evidence to skew us towards either hypothesis, largely due to the lack of strongly applicable data. Lessons from our own data will help to shape our future research more. Expression of many of our phenotypic markers correlated directly with CD69 expression, supporting a transitional phenotype as cells transform from $CD69^-$ to resident $CD69^{HI}$. Also in support of our first hypothesis, culturing with liver slices allowed for the generation of $CD69^{HI}$ cells, building on the $CD69^{INT}$ generation seen with hepatic epithelia culture. This suggests that additional signals provided by the liver microenvironment may drive $CD69^{INT}$ cells towards a $CD69^{HI}$ phenotype. However, some of our data argues against this hypothesis as not all markers followed simple correlations with CD69. Lack of KLRG-1 appears important in the generation of T_{RM} cells[110], yet our $CD69^{INT}$ cells have much higher KLRG-1 expression than $CD69^{HI}$ cells. Functionally, the T_H2 cytokine-favouring $CD69^{INT}$ cells would also have to lose this cytokine production ability to become a more T_H1-skewed $CD69^{HI}$ population if the first hypothesis was correct, a switch that is possible, but perhaps

unlikely. On the other hand, given the heterogeneity of the CD69^{INT} pool, perhaps only some cells are able to convert, countering above arguments on non-linear surface antigen relationships, KLRG-1 preferences and cytokine switching.

In favour of the second hypothesis, our CD69^{INT} cells express a partial residence profile, but still retain S1PR1 expression. This latter feature means that they are unlikely to be resident by traditional definition (long-term), but none have yet described features of a transient/short-term population. Perhaps when researchers see discrepancies such as CD69^{-} resident cells[87], or modulations in CD69 expression upon entry and exit[213], this is actually reflective of short-term residence. It is very plausible that tissue-residence is a continuum, instead of a binary situation that many are treating it as. The liver CD69^{INT} cells we describe are the most activated subset, and it is the most activated cells that have been shown to be able to recirculate[90]. Additionally, they possess the widest homing receptor profiles, suggesting they have not settled in a liver niche, and can presumably migrate to the gut through CCR9 expression. In terms of naïve/memory profiles, CD69^{INT} were most similar phenotypically to CD69^{-} cells so perhaps interconversion with this phenotype was easier than with the more distinct CD69^{HI} cells (although this would be best tested with analysis of transcription factor profiles).

Of course, the third hypothesis (that CD69^{INT} cells contain both short-term resident cells and subsets able to differentiate into CD69^{HI} T_{RM}) could also be true. A lot of arguments from the literature suggest both possibilities. From our data, it could be that the CD69^{INT} pool contains both precursors able to convert to CD69^{HI} T_{RM}, and cells that can move back towards CD69^{-} cells after a short residency period. While we cannot favour one hypothesis at the current time, we can devise experiments to test them all. Extended

monitoring of CD69^{INT} hTCs would allow us to see whether additional signals such as cytokines or antigen stimulation would push these cells to a CD69^{HI} phenotype. Parallel experiments could dissect the components involved in the generation of CD69^{HI} in liver slice culture, perhaps by blocking certain cytokines or cellular interactions, before setting up simpler cultures with individual liver cell types. Further, we could culture liver-isolated CD69^{INT} cells under different stimulatory conditions, in the hope of generating CD69^{HI} cells, although feasibility would depend on the survival times of these cells in culture. Better determining where both CD4^{+} CD69^{INT} and CD4^{+} CD69^{HI} cells are located in the liver may give us additional clues about what liver parenchymal or stromal cells are involved in their maintenance and potential interconversion. Ascertaining whether analogous populations exist in mouse livers would allow tracking experiments, genetic manipulations and precise time-course experiments to monitor any differentiation and residency periods of CD69^{INT} cells. Lastly, full transcriptomic analyses of the different cell types would allow us to compare thousands of genes between CD69^{INT} and CD69^{HI}, answering whether they are genetically related, and thus more likely to possess a precursor-product relationship.

6.2 Limitations of studies

The major limitation of the transwell migration assays used in chapter 3 and the static co-culture assays used in chapters 4 and 5 were the use of tumour cell lines instead of primary cells. Cell lines are much more robust, and with careful culturing are in ample supply. The Huh-7 cells that we used for the majority of our experiments have also been particularly well studied[688]. While it is imperative we compare our findings to primary hepatocytes in the future, we believe that Huh-7 cell lines have given us a good platform to build on, allowing us to collect data reproducibly. Furthermore, analogous results with

BEC and liver slices in co-culture experiments increase our confidence that we were not observing a tumour-related effect in CD69 induction and associated phenotypic changes.

For the *ex vivo* liver characterisation work, the use of CD69 could represent another limitation. As discussed, some have argued that CD69 is not a perfect marker of T_{RM} cells[87, 213], although no evidence points to this in humans[91, 214]. Ideally, tissue-residence would be proved by demonstrating a clear lack of recirculation of these cells as done by using parabiotic mice, but these type experiments clearly cannot be performed in humans. The closest substitute others have used are antibodies to deplete circulating cells[150], human skin grafting into mice[152], or the study of repeated organ transplant recipients[646]. The feasibility of using the latter example to extend our studies will be discussed in the next section. Nonetheless, most, if not all, human T_{RM} studies have used CD69 as their primary residency marker allowing us easy intra-study comparisons. Thus, while CD69 may not be a completely perfect marker, we believe its use here can be more than justified.

Flow cytometry was the predominantly used end-result assay in these studies. Undoubtedly a powerful technique, there are obvious limitations with background fluorescence, fluorescence overlap, and undetectable antibody binding that we have done our utmost to minimise through the use of proper fluorescence minus one/isotype-matched controls, thorough compensation, and proper antibody screening and titration respectively. Panel size was occasionally an issue as with a 9-colour limit we were sometimes unable to look for all the marker associations we wanted to (pertinent for the *ex vivo* characterisation), or include important controls such as a live/dead marker (pertinent for suppression assays in chapter 4). This also relates to the use of flow

cytometry-linked bioinformatics that would have been extremely helpful in picking up marker associations in certain populations that we might have missed or were not realistically feasible to assess by repeated manual gating. Mass cytometry allows for over 30 parameters to be detected simultaneously, however it is laborious to analyse and unrealistic to perform for over fifty livers tested in this work within the given time frame. However, multi-parametric flow cytometry allowed the characterisation of 35 phenotypic markers and 8 intracellular cytokines in this study, the most comprehensive characterisation of human liver CD4+ T cells to date.

The poor survival of LIMCs in culture limited our study of their long-term responsiveness to stimuli. NLT immune cells are known to be particularly susceptible to death in culture[71, 170], presumably due to their removal from key tissue-rich survival factors they require. Until better culture methods are developed that allow LIMC survival without drastically altering their phenotype, this limitation will be difficult to overcome. More advanced culture systems such as liver microchips[573] may prove useful in this, while in themselves providing us better and more physiological models to study migration, allowing us to overcome the limitations of the relatively simple transwell migration assays.

Finally, as with all human studies, patient variability, sample size, and disease staging and management all play a role in shaping the results. In these studies, we have used mostly HFE patient blood as healthy controls. Our department has used this material with minimum resistance from the field for many years, as the immune cells are thought to be largely unaffected by liver pathology in this disease. However, this may not be a fool proof assessment, as some alterations in $CD8^+$ (though not $CD4^+$) T cell numbers and phenotypes have been reported[689, 690]. For liver samples, we have compared the

frequencies of $CD69^-$, $CD69^{INT}$, and $CD69^{HI}$ $CD4^+$ T cells in various end stage diseases, finding no proportional differences. This is not unexpected, given that end stage diseases share features of chronic inflammation that has led to fibrosis and cirrhosis. Antigen-driven autoimmune disorders such as AIH, PBC and PSC can resemble metabolic injuries such as high alcohol or high fat diet from an immunological perspective, as liver damage can result in neoantigen expression and local autoinflammatory injury. Of note, in the 8 livers with NASH tested, we did not observe a reduction in CD4+ T cell numbers compared to other diseases, as reported in one study[453]; and nor did we see large elevations in T_{REG} frequencies in HCV livers like others have reported, albeit only with n=2 here[518]. We have therefore grouped all chronic liver diseases together and so effects of different treatment regimens, disease stages, and patient demographics would have had a smaller impact than if investigating a disease-specific effect more susceptible to these compounding factors. This impact is decreased further by our relatively large sample sizes for the liver. Number of healthy donor livers is a bigger influence on our interpretations. Many of the donor livers used had been rejected for transplantation based on being too steatotic or due to surgical logistic reasons. Both of these factors could affect the cells' viability and phenotypes. Furthermore, donor tissue is much more infrequently available, yet ideally more 'healthy' donor data would be needed in our study to provide statistically significant data that back up the trends seen that match the diseased liver groups. Alternative sources of 'healthy' donor tissue have been suggested to come from tumour-free areas of liver cancer patients that undergo resection surgery, or patients with colorectal metastases in the liver; or from cyst-free areas of polycystic liver patients. None of these solutions are ideal however, as the impact of these diseases on T cell biology is largely unknown. Last, patient variability means that true differences are sometimes masked, and while with large sample numbers the true differences emerge through

statistical analysis, sufficient sample sizes have only been reached with most, not all of my data.

6.3 Future work

Our main future focusses in modelling transmigration of $CD4^+$ T cells into the liver are in expanding the diseases tested, determining disease relevance and expanding these data to more physiological systems. Determining the post-migrated cytokine potential of $CD4^+$ from other liver diseases such as HBV and PSC would allow for the expansion of our portfolio, in the hope of strengthening the transwell migration model. This would be most beneficial if disease relevance could be proved. We hypothesise that the more activated, more pro-inflammatory $CD4^+$ T cells separated out by migration are similar to the cells that would migrate into the hepatic parenchyma, and so by extension may participate in disease pathology and tissue damage. Downstream assays we could use to test these suspicions include antigen-presentation assays (with disease-specific antigens), further assessment of activation and exhaustion markers post-migration, or even transfer of these populations to humanised mouse models of liver disease. Lastly, the future use of more physiologically representative models of the human liver, such as the increasingly popular aforementioned liver chip system, will help translate these findings to human disease mechanisms.

The most pressing issue when studying CD69 upregulation *in vitro* is to uncover the mechanism behind this process, as discussed in depth above (sections 4.5, 6.1.2). This could either involve short term approaches of interfering with ICAM-1 - LFA-1, and/or CD58 – CD2 interactions; or more detailed whole transcriptome analyses to pick up likely candidate molecules from both hepatocyte and induced T cell perspectives. Another facet

of this overall project that might be worth studying is to address the issue of $CD69^+$ cell-associated suppression. We concluded that suppression was unlikely in these cells, but results were occasionally contradictory and confusing. What is likely needed here is robust comparisons with isolated conventional T_{REG} if possible, cell-sorted $CD69^+$ and $CD69^-$ hTC populations, upscaling of the plate well size so to remove competition for space as an issue, and the exclusion of dead cells with appropriate markers.

Key expansions in the hepatic $CD4^+$ T cells project would include first full transcriptomic analyses of the three intrahepatic $CD4^+$ T cell populations. This would involve the use of RNA sequencing or microarray technologies, similar to other human T_{RM} studies we have compared our data with[91, 134, 135]. Thousands of transcripts would be compared, hopefully identifying many more key differences between the populations. This would not only further inform us on how the liver $CD69^{HI}$ T_{RM} differ from described T_{RM} in different organs, but also how they differ from the liver $CD69^{INT}$ cells that could give more information on their specific roles. Further any shared transcriptional regulators between the populations would point to potential for subset interconversion. Thanks to other transcriptomic analyses in the literature, this need not be entirely a fishing exercise. Some have pointed to glucose deprivation and hypoxia signatures in lung T_{RM}[134, 135] that would be of interest to study in the liver. We could also look at chemokine production by liver T_{RM}, allowing their implication in auxiliary immune cell recruitment[134, 135]. Likewise, determining whether Notch-1 or any related pathway components are upregulated in liver $CD4^+$ T_{RM}, as seen in lung T_{RM}, would be key in helping to establish whether Notch-1 is a universal regulator of human T_{RM} maintenance[134, 135]. This would be a particularly exciting scenario, as many pharmaceutical Notch inhibitors have been developed, offering the possibility to specifically target T_{RM}[691].

We would also like to investigate the generation of both CD69-expressing subsets in *in vitro* systems more, as discussed in sections 5.8.5 and 6.1.3. It is inferred that in addition to undefined cell-surface signals that trigger development of the $CD69^{INT}$ phenotype, specific cytokine or antigenic signals are required to push further development into $CD69^{HI}$ T_{RM}-like cells. We would carry out parallel work to investigate both these steps, investigating both the initial CD69 upregulation mechanism that may be ICAM-1-, or CD58-dependent, before aiming to define the additional signals needed for $CD69^{HI}$ cell generation. For this latter part, we would start with sequential addition of IL-15, then TGF-β in a similar manner to Pallett et al.[148]. Transcriptomic analyses may also be of use in generation studies too, informing us of differentially expressed transcripts in $CD69^{INT}$ and $CD69^{HI}$ cells, isolated both *ex vivo* and generated *in vitro*, that would allow a targeted approach.

Differentially expressed transcripts would need confirming at protein level, and this could be done by more flow cytometry, or higher-parameter mass cytometry, as performed by Wong et al in their multi-organ approach[82]. The inclusion of more parameters would allow us to look at other populations in the liver, starting with conventional $CD8^+$ T cells. Although others have performed comprehensive studies on liver $CD8^+$ T_{RM}[148], none have specifically looked for $CD69^{INT}$ $CD8^+$ T cells, and there is great value in comparing populations from the same livers too.

I previously alluded to the benefits of incorporating bioinformatics into multi-parameter flow cytometry analyses. The example given was to see if the variety of homing receptors expressed by the $CD69^{INT}$ pool clustered together or formed separate subsets. Many

similar questions could be answered by clustering analyses such as t-distributed stochastic neighbour embedding (t-SNE), where all parameters are compared against all others and visualised in two dimensions, as many groups are performing now. For example, we could assess whether $CD69^{HI}$ and $CD69^{INT}$ cells with different cytokine preferences have different homing receptor preferences, or favoured a different KLRG-1/CD127 designation. The dangers of these are the loss of hypothesis-driven directions in the research, but on the plus side many unknown antigen co-localisation patterns could be uncovered. The heterogeneity of human cells compared to mouse, both within individuals and donor groups, strengthen the need for this type of research.

We, like many others, have used CD69 as the principal marker of tissue residence here. However, our data argue that $CD69^{INT}$ cells can recirculate, whereas $CD69^{HI}$ cells were the most likely resident type. We therefore discourage the use of CD69 as a binary marker of tissue residence and instead suggest the high expression of CD69 is an even better indicator. Despite the strength of this marker, it would of course be beneficial to formally prove our $CD69^{HI}$ $CD4^{+}$ T cells do not recirculate. The most feasible way to do this would be to mimic the approach of Cuff et al. who proved long-term residence in NK cells in patients with multiple liver transplants. By monitoring patients with HLA-mismatched transplants, the authors were able to prove the putative resident NK donor cells remained in the livers up to 13 years after transplant[646]. This would work just as well for our $CD69^{HI}$ T_{RM}, and short-term residence could be proved without the need to wait for a second transplant by taking blood both pre- and post-transplant and comparing it with liver perfusate. The presence of $CD69^{HI}$ cells in the perfusate, but their absence in post-transplant blood would indicate their true-resident status, although modulation of CD69 expression on liver egress could not be ruled out. Also, unless $CD4^{+}$ T_{RM} were similarly

present in the liver perfusate when compared to digested liver tissue, (like the NK cells described[646]), donor liver tissue biopsies would also be required.

Additionally, we would seek to extend our imaging of the different $CD4^+$ T cell subsets in the human liver. The preliminary data we have at present could be extended by finding reliable CD4 and CD69 immunofluorescence antibodies that fit in with CXCR6, CX3CR1 and NkP46 in antibody panels. This would allow us to verify the distribution of $CD69^{INT}$ and $CD69^{HI}$ cells in the liver.

Cementing our T_{RM} population in the human T_{RM} matrix further, it would be fascinating to study the metabolism of these cells. One candidate method to do this would be the use of Seahorse analysers (Agilent technologies, UK) that efficiently measure both mitochondrial respiration and glycolysis in real-time. Murine T_{RM} have recently been shown to adapt to consume free fatty acids that feed into oxidative phosphorylation pathways in order to survive for elongated periods in the skin[114, 692]. Determining whether liver $CD4^+$ T_{RM} are similar to this published study, and the contribution of glycolysis versus oxidative phosphorylation would be of great benefit to understanding the lives of these cells. These experiments would once again give us more ideas about the roles of $CD69^{INT}$ cells through their survival tactics.

Finally, ascertaining whether either CD69-expressing population is disease relevant will be important. We have looked at relative frequencies in autoimmune liver diseases and dietary liver injury, but would like to extend this to viral liver disease as others have done[148, 384]. Given the importance of T_{RM} cells in perpetuating, as well as controlling disease, we would expect $CD69^{HI}$ $CD4^+$ T_{RM} to be key in all liver pathologies. Therefore,

collecting more donors will allow for comparison of activation states and cytokine production ability across the different disease groups as part of an extended phenotypic and functional characterisation. These data would give researchers an idea of the $CD4^+$ T cell populations most important in the different diseases, along with indications on how to specifically target them therapeutically.

6.4 Conclusions

Chapter 6 – Core Findings and their Implications:

- Modelling $CD4^+$ T cell migration into the parenchyma revealed intrinsic chronic liver disease-specific differences in cytokine functionality, and may prove a useful way to study populations relevant to disease without the need to sample liver tissue
- Co-culture of blood-derived $CD4^+$ T cells with hepatic epithelia led to an activation-independent upregulation of CD69 that was also not linked to the acquisition of regulatory properties
- CD69 was also an important discriminator of intrahepatic $CD4^+$ T cells, allowing the divergence into $CD69^{HI}$ T_{RM} that acquiesce with the field in terms of a resting, tissue restricted, but multifunctional cytokine-capable cell type; and a novel $CD69^{INT}$ population, distinct through their semi-residency-associated, activated profile and ability to make T_H2 cytokines
- Generation of phenotypically similar $CD69^{INT}$ and $CD69^{HI}$ $CD4^+$ cells from blood through culture with hepatic epithelia, and intact liver slices respectively hints at untapped developmental pathways required for T_{RM} generation
- Together the data provide the first full characterisation of liver $CD4^+$ T_{RM} and identify $CD69^{INT}$ cells that are likely T_{RM} precursors and/or short-term resident liver cells; both likely important in immune homeostasis and disease pathogenesis

Box 6 Box 6.1 – Core findings from these studies combined and the implications for immunology.

Here we have explored multiple aspects of hepatic $CD4^+$ T cell biology, from the initial migration into the liver, to the short-term impact of hepatocytes on $CD4^+$ T cell phenotype and function, to the adaptations of long-term liver resident $CD4^+$ T cells (Box 6.1). Through modelling migration into the liver parenchyma, we found hepatocyte-selected migrated $CD4^+$ T cells displayed intrinsic and disease-specific enhanced cytokine

potential, that we postulated could be relevant to individual disease processes. The finding that simple co-culture with hepatocyte cell lines caused intermediate level CD69 upregulation in $CD4^+$ T cells that did not coincide with conventional T cell activation or acquired regulatory properties, gave us insights on how hepatocyte contact alters $CD4^+$ T cell phenotypes. This led to detailed characterisation of the entire intrahepatic $CD4^+$ T cell compartment and accompanied revelations that the pool contained two CD69-expressing subsets. The first was a putative T_{RM} cell type, high for CD69 expression, with the greatest association with residence-associated markers, restricted homing receptor profile, mostly resting profile, and a tendency towards multifunctional T_H1, and T_H17 cytokine production. The second, $CD69^{INT}$, subset seemed often diametrically opposed: thought non-resident with the potential for egress, with a wide-ranging predicted pattern of intra-and extra-hepatic movement, previously activated, and additionally able to produce T_H2 cytokines. Last, we demonstrated that modelling short-term hepatocyte contact was enough to mimic the generation of a phenotypically similar $CD69^{INT}$ cell type, while the more complex liver microenvironment additionally created $CD69^{HI}$ cells. Combined these findings document the powerful influence the liver microenvironment can have on the biology of a T cell and open multiple avenues to investigate how these alterations play roles in different liver disease processes. The properties of the novel $CD69^{INT}$ cells are of particular importance, and may represent a step in unravelling the generation and maintenance of human liver $CD4^+$ T_{RM}, or in understanding tissue residency as a dwell-time-dependent continuum.

References

1. Cooper, M.D. & Herrin, B.R. How did our complex immune system evolve? *Nat Rev Immunol* **10**, 2-3 (2010).

2. Flajnik, M.F. & Kasahara, M. Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nat Rev Genet* **11**, 47-59 (2010).

3. Cobey, S. Pathogen evolution and the immunological niche. *Ann N Y Acad Sci* **1320**, 1-15 (2014).

4. Shah, D.K. & Zuniga-Pflucker, J.C. An overview of the intrathymic intricacies of T cell development. *J Immunol* **192**, 4017-4023 (2014).

5. Rothenberg, E.V. & Taghon, T. Molecular genetics of T cell development. *Annu Rev Immunol* **23**, 601-649 (2005).

6. Germain, R.N. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* **2**, 309-322 (2002).

7. Takaba, H. & Takayanagi, H. The Mechanisms of T Cell Selection in the Thymus. *Trends Immunol* **38**, 805-816 (2017).

8. Klein, L., Kyewski, B., Allen, P.M. & Hogquist, K.A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* **14**, 377-391 (2014).

9. Chen, L. & Flies, D.B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* **13**, 227-242 (2013).

10. Weaver, C.T., Hatton, R.D., Mangan, P.R. & Harrington, L.E. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* **25**, 821-852 (2007).

11. Pennock, N.D., White, J.T., Cross, E.W., Cheney, E.E., Tamburini, B.A. & Kedl, R.M. T cell responses: naive to memory and everything in between. *Adv Physiol Educ* **37**, 273-283 (2013).

12. Murphy, K.M. & Reiner, S.L. The lineage decisions of helper T cells. *Nat Rev Immunol* **2**, 933-944 (2002).

13. Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K. IL-17 and Th17 Cells. *Annu Rev Immunol* **27**, 485-517 (2009).

14. Tripathi, S.K. & Lahesmaa, R. Transcriptional and epigenetic regulation of T-helper lineage specification. *Immunol Rev* **261**, 62-83 (2014).

15. DuPage, M. & Bluestone, J.A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol* **16**, 149-163 (2016).

16. Shahinian, A., Pfeffer, K., Lee, K.P., Kundig, T.M., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P.S., Thompson, C.B. & Mak, T.W. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609-612 (1993).

17. Howland, K.C., Ausubel, L.J., London, C.A. & Abbas, A.K. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J Immunol* **164**, 4465-4470 (2000).

18. Beyersdorf, N., Kerkau, T. & Hunig, T. CD28 co-stimulation in T-cell homeostasis: a recent perspective. *Immunotargets Ther* **4**, 111-122 (2015).

19. Taraban, V.Y., Rowley, T.F., Kerr, J.P., Willoughby, J.E., Johnson, P.M., Al-Shamkhani, A. & Buchan, S.L. CD27 costimulation contributes substantially to the expansion of functional memory CD8(+) T cells after peptide immunization. *Eur J Immunol* **43**, 3314-3323 (2013).

20. Schwartz, R.H. T cell anergy. *Annu Rev Immunol* **21**, 305-334 (2003).

21. Curtsinger, J.M. & Mescher, M.F. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* **22**, 333-340 (2010).

22. Tubo, N.J., Pagan, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S. & Jenkins, M.K. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell* **153**, 785-796 (2013).

23. Park, C.O. & Kupper, T.S. The emerging role of resident memory T cells in protective immunity and inflammatory disease. *Nat Med* **21**, 688-697 (2015).

24. Caruso, A., Licenziati, S., Corulli, M., Canaris, A.D., De Francesco, M.A., Fiorentini, S., Peroni, L., Fallacara, F., Dima, F., Balsari, A. & Turano, A. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry* **27**, 71-76 (1997).

25. Sandoval-Montes, C. & Santos-Argumedo, L. CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. *J Leukoc Biol* **77**, 513-521 (2005).

26. Reddy, M., Eirikis, E., Davis, C. & Davis, H.M. Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. *J Immunol Methods* **293**, 127-142 (2004).

27. Testi, R., Phillips, J.H. & Lanier, L.L. Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular [Ca++] and stimulation of protein kinase C. *J Immunol* **142**, 1854-1860 (1989).

28. Testi, R., D'Ambrosio, D., De Maria, R. & Santoni, A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol Today* **15**, 479-483 (1994).

29. Radulovic, K. & Niess, J.H. CD69 is the crucial regulator of intestinal inflammation: a new target molecule for IBD treatment? *J Immunol Res* **2015**, 497056 (2015).

30. Cibrian, D. & Sanchez-Madrid, F. CD69: from activation marker to metabolic gatekeeper. *Eur J Immunol* **47**, 946-953 (2017).

31. Masopust, D. & Schenkel, J.M. The integration of T cell migration, differentiation and function. *Nat Rev Immunol* **13**, 309-320 (2013).

32. Cyster, J.G. & Schwab, S.R. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu Rev Immunol* **30**, 69-94 (2012).

33. Lo, C.G., Xu, Y., Proia, R.L. & Cyster, J.G. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J Exp Med* **201**, 291-301 (2005).

34. Arnon, T.I., Xu, Y., Lo, C., Pham, T., An, J., Coughlin, S., Dorn, G.W. & Cyster, J.G. GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood. *Science* **333**, 1898-1903 (2011).

35. Zehn, D., Lee, S.Y. & Bevan, M.J. Complete but curtailed T-cell response to very low-affinity antigen. *Nature* **458**, 211-214 (2009).

36. Woerly, G., Brooks, N. & Ryffel, B. Effect of rapamycin on the expression of the IL-2 receptor (CD25). *Clin Exp Immunol* **103**, 322-327 (1996).

37. Minami, Y., Kono, T., Miyazaki, T. & Taniguchi, T. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol* **11**, 245-268 (1993).

38. Quarona, V., Zaccarello, G., Chillemi, A., Brunetti, E., Singh, V.K., Ferrero, E., Funaro, A., Horenstein, A.L. & Malavasi, F. CD38 and CD157: a long journey from activation markers to multifunctional molecules. *Cytometry B Clin Cytom* **84**, 207-217 (2013).

39. Pichler, W.J. & Wyss-Coray, T. T cells as antigen-presenting cells. *Immunol Today* **15**, 312-315 (1994).

40. Rivera Vargas, T., Humblin, E., Vegran, F., Ghiringhelli, F. & Apetoh, L. TH9 cells in anti-tumor immunity. *Semin Immunopathol* **39**, 39-46 (2017).

41. Purwar, R., Schlapbach, C., Xiao, S., Kang, H.S., Elyaman, W., Jiang, X., Jetten, A.M., Khoury, S.J., Fuhlbrigge, R.C., Kuchroo, V.K., Clark, R.A. & Kupper, T.S. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat Med* **18**, 1248-1253 (2012).

42. Eyerich, K. & Eyerich, S. Th22 cells in allergic disease. *Allergo J Int* **24**, 1-7 (2015).

43. Chatila, T.A. Role of regulatory T cells in human diseases. *J Allergy Clin Immunol* **116**, 949-959; quiz 960 (2005).

44. Zeng, H., Zhang, R., Jin, B. & Chen, L. Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance. *Cell Mol Immunol* **12**, 566-571 (2015).

45. Gol-Ara, M., Jadidi-Niaragh, F., Sadria, R., Azizi, G. & Mirshafiey, A. The role of different subsets of regulatory T cells in immunopathogenesis of rheumatoid arthritis. *Arthritis* **2012**, 805875 (2012).

46. Wawrzyniak, M., O'Mahony, L. & Akdis, M. Role of Regulatory Cells in Oral Tolerance. *Allergy Asthma Immunol Res* **9**, 107-115 (2017).

47. Palmer, M.T. & Weaver, C.T. Autoimmunity: increasing suspects in the CD4+ T cell lineup. *Nat Immunol* **11**, 36-40 (2010).

48. Jonuleit, H. & Schmitt, E. The Regulatory T Cell Family: Distinct Subsets and their Interrelations. *J Immunol* **171**, 6323-6327 (2003).

49. Bredo, G., Storie, J., Shrestha Palikhe, N., Davidson, C., Adams, A., Vliagoftis, H. & Cameron, L. Interleukin-25 initiates Th2 differentiation of human CD4(+) T cells and influences expression of its own receptor. *Immun Inflamm Dis* **3**, 455-468 (2015).

50. Vasanthakumar, A. & Kallies, A. IL-27 paves different roads to Tr1. *Eur J Immunol* **43**, 882-885 (2013).

51. Pot, C., Jin, H., Awasthi, A., Liu, S.M., Lai, C.Y., Madan, R., Sharpe, A.H., Karp, C.L., Miaw, S.C., Ho, I.C. & Kuchroo, V.K. Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. *J Immunol* **183**, 797-801 (2009).

52. Hooper, K.M., Kong, W. & Ganea, D. Prostaglandin E2 inhibits Tr1 cell differentiation through suppression of c-Maf. *PLOS ONE* **12**, e0179184 (2017).

53. Carrier, Y., Yuan, J., Kuchroo, V.K. & Weiner, H.L. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *J Immunol* **178**, 179-185 (2007).

54. Levings, M.K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal Malefyt, R. & Roncarolo, M.G. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* **166**, 5530-5539 (2001).

55. Vignali, D.A., Collison, L.W. & Workman, C.J. How regulatory T cells work. *Nat Rev Immunol* **8**, 523-532 (2008).

56. Rolf, J., Fairfax, K. & Turner, M. Signaling pathways in T follicular helper cells. *J Immunol* **184**, 6563-6568 (2010).

57. Weiner, H.L. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev* **182**, 207-214 (2001).

58. Pot, C., Apetoh, L. & Kuchroo, V.K. Type 1 regulatory T cells (Tr1) in autoimmunity. *Semin Immunol* **23**, 202-208 (2011).

59. Mittrucker, H.W., Visekruna, A. & Huber, M. Heterogeneity in the differentiation and function of CD8(+) T cells. *Arch Immunol Ther Exp (Warsz)* **62**, 449-458 (2014).

60. Murphy, K.M. & Stockinger, B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol* **11**, 674-680 (2010).

61. Koch, M.A., Tucker-Heard, G., Perdue, N.R., Killebrew, J.R., Urdahl, K.B. & Campbell, D.J. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* **10**, 595-602 (2009).

62. Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J.M., Chu, T.T., Corcoran, L., Treuting, P., Klein, U. & Rudensky, A.Y. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* **458**, 351-356 (2009).

63. Zhou, L., Chong, M.M. & Littman, D.R. Plasticity of CD4+ T cell lineage differentiation. *Immunity* **30**, 646-655 (2009).

64. Yang, X.O., Nurieva, R., Martinez, G.J., Kang, H.S., Chung, Y., Pappu, B.P., Shah, B., Chang, S.H., Schluns, K.S., Watowich, S.S., Feng, X.H., Jetten, A.M. & Dong, C. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* **29**, 44-56 (2008).

65. Wan, Y.Y. & Flavell, R.A. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* **445**, 766-770 (2007).

66. Tsuji, M., Komatsu, N., Kawamoto, S., Suzuki, K., Kanagawa, O., Honjo, T., Hori, S. & Fagarasan, S. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* **323**, 1488-1492 (2009).

67. Sakaguchi, S., Vignali, D.A., Rudensky, A.Y., Niec, R.E. & Waldmann, H. The plasticity and stability of regulatory T cells. *Nat Rev Immunol* **13**, 461-467 (2013).

68. Oldenhove, G., Bouladoux, N., Wohlfert, E.A., Hall, J.A., Chou, D., Dos Santos, L., O'Brien, S., Blank, R., Lamb, E., Natarajan, S., Kastenmayer, R., Hunter, C., Grigg, M.E. & Belkaid, Y. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* **31**, 772-786 (2009).

69. Gasper, D.J., Tejera, M.M. & Suresh, M. CD4 T-cell memory generation and maintenance. *Crit Rev Immunol* **34**, 121-146 (2014).

70. Mueller, S.N., Gebhardt, T., Carbone, F.R. & Heath, W.R. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol* **31**, 137-161 (2013).

71. Schenkel, J.M. & Masopust, D. Tissue-resident memory T cells. *Immunity* **41**, 886-897 (2014).

72. Kaech, S.M. & Cui, W. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol* **12**, 749-761 (2012).

73. Lees, J.R. & Farber, D.L. Generation, persistence and plasticity of CD4 T-cell memories. *Immunology* **130**, 463-470 (2010).

74. van Leeuwen, E.M., Sprent, J. & Surh, C.D. Generation and maintenance of memory CD4(+) T Cells. *Curr Opin Immunol* **21**, 167-172 (2009).

75. Kondrack, R.M., Harbertson, J., Tan, J.T., McBreen, M.E., Surh, C.D. & Bradley, L.M. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med* **198**, 1797-1806 (2003).

76. Li, J., Huston, G. & Swain, S.L. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J Exp Med* **198**, 1807-1815 (2003).

77. Schwendemann, J., Choi, C., Schirrmacher, V. & Beckhove, P. Dynamic differentiation of activated human peripheral blood CD8+ and CD4+ effector memory T cells. *J Immunol* **175**, 1433-1439 (2005).

78. Ahmadzadeh, M. & Farber, D.L. Functional plasticity of an antigen-specific memory CD4 T cell population. *Proc Natl Acad Sci U S A* **99**, 11802-11807 (2002).

79. Messi, M., Giacchetto, I., Nagata, K., Lanzavecchia, A., Natoli, G. & Sallusto, F. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat Immunol*, 78-86 (2002).

80. Pakpour, N., Zaph, C. & Scott, P. The central memory CD4+ T cell population generated during Leishmania major infection requires IL-12 to produce IFN-gamma. *J Immunol* **180**, 8299-8305 (2008).

81. Krawczyk, C.M., Shen, H. & Pearce, E.J. Functional Plasticity in Memory T Helper Cell Responses. *J Immunol* **178**, 4080-4088 (2007).

82. Wong, M.T., Ong, D.E., Lim, F.S., Teng, K.W., McGovern, N., Narayanan, S., Ho, W.Q., Cerny, D., Tan, H.K., Anicete, R., Tan, B.K., Lim, T.K., Chan, C.Y., Cheow, P.C., Lee, S.Y., Takano, A., Tan, E.H., Tam, J.K., Tan, E.Y., Chan, J.K., Fink, K., Bertoletti, A., Ginhoux, F., Curotto de Lafaille, M.A. & Newell, E.W. A High-Dimensional Atlas of Human T Cell Diversity Reveals Tissue-Specific Trafficking and Cytokine Signatures. *Immunity* **45**, 442-456 (2016).

83. Kumar, B.V., Connors, T.J. & Farber, D.L. Human T Cell Development, Localization, and Function throughout Life. *Immunity* **48**, 202-213 (2018).

84. Mackay, L.K. & Kallies, A. Transcriptional Regulation of Tissue-Resident Lymphocytes. *Trends Immunol* **38**, 94-103 (2017).

85. Wakim, L.M., Waithman, J., van Rooijen, N., Heath, W.R. & Carbone, F.R. Dendritic cell-induced memory T cell activation in nonlymphoid tissues. *Science* **319**, 198-202 (2008).

86. Clark, R.A. Resident memory T cells in human health and disease. *Sci Transl Med* **7**, 269rv261 (2015).

87. Steinert, E.M., Schenkel, J.M., Fraser, K.A., Beura, L.K., Manlove, L.S., Igyarto, B.Z., Southern, P.J. & Masopust, D. Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell* **161**, 737-749 (2015).

88. Sathaliyawala, T., Kubota, M., Yudanin, N., Turner, D., Camp, P., Thome, J.J., Bickham, K.L., Lerner, H., Goldstein, M., Sykes, M., Kato, T. & Farber, D.L. Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* **38**, 187-197 (2013).

89. Clark, R.A., Chong, B., Mirchandani, N., Brinster, N.K., Yamanaka, K., Dowgiert, R.K. & Kupper, T.S. The vast majority of CLA+ T cells are resident in normal skin. *J Immunol* **176**, 4431-4439 (2006).

90. Thome, J.J., Yudanin, N., Ohmura, Y., Kubota, M., Grinshpun, B., Sathaliyawala, T., Kato, T., Lerner, H., Shen, Y. & Farber, D.L. Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell* **159**, 814-828 (2014).

91. Kumar, B.V., Ma, W., Miron, M., Granot, T., Guyer, R.S., Carpenter, D.J., Senda, T., Sun, X., Ho, S.H., Lerner, H., Friedman, A.L., Shen, Y. & Farber, D.L. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. *Cell Rep* **20**, 2921-2934 (2017).

92. Hogan, R.J., Usherwood, E.J., Zhong, W., Roberts, A.A., Dutton, R.W., Harmsen, A.G. & Woodland, D.L. Activated antigen-specific CD8+ T cells persist in the lungs following recovery from respiratory virus infections. *J Immunol* **166**, 1813-1822 (2001).

93. Holtappels, R., Pahl-Seibert, M.F., Thomas, D. & Reddehase, M.J. Enrichment of Immediate-Early 1 (m123/pp89) Peptide-Specific CD8 T Cells in a Pulmonary CD62Llo Memory-Effector Cell Pool during Latent Murine Cytomegalovirus Infection of the Lungs. *J Virol* **74**, 11495-11503 (2000).

94. Podlech, J., Holtappels, R., Pahl-Seibert, M.F., Steffens, H.P. & Reddehase, M.J. Murine model of interstitial cytomegalovirus pneumonia in syngeneic bone marrow transplantation: persistence of protective pulmonary CD8-T-cell infiltrates after clearance of acute infection. *J Virol* **74**, 7496-7507 (2000).

95. Harris, N.L., Watt, V., Ronchese, F. & Le Gros, G. Differential T cell function and fate in lymph node and nonlymphoid tissues. *J Exp Med* **195**, 317-326 (2002).

96. Hawke, S., Stevenson, P.G., Freeman, S. & Bangham, C.R. Long-term persistence of activated cytotoxic T lymphocytes after viral infection of the central nervous system. *J Exp Med* **187**, 1575-1582 (1998).

97. Masopust, D., Vezys, V., Marzo, A.L. & Lefrancois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291**, 2413-2417 (2001).

98. Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R. & Carbone, F.R. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* **10**, 524-530 (2009).

99. Iijima, N. & Iwasaki, A. T cell memory. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* **346**, 93-98 (2014).

100. Jiang, X., Clark, R.A., Liu, L., Wagers, A.J., Fuhlbrigge, R.C. & Kupper, T.S. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483**, 227-231 (2012).

101. Schenkel, J.M., Fraser, K.A., Vezys, V. & Masopust, D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* **14**, 509-513 (2013).

102. Teijaro, J.R., Turner, D., Pham, Q., Wherry, E.J., Lefrancois, L. & Farber, D.L. Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection. *J Immunol* **187**, 5510-5514 (2011).

103. Boyman, O., Hefti, H.P., Conrad, C., Nickoloff, B.J., Suter, M. & Nestle, F.O. Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha. *J Exp Med* **199**, 731-736 (2004).

104. Clark, R.A., Chong, B.F., Mirchandani, N., Yamanaka, K., Murphy, G.F., Dowgiert, R.K. & Kupper, T.S. A novel method for the isolation of skin resident T cells from normal and diseased human skin. *J Invest Dermatol* **126**, 1059-1070 (2006).

105. Clark, R.A. & Kupper, T.S. IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. *Blood* **109**, 194-202 (2007).

106. Jungi, T.W. & Jungi, R. Immunological memory to Listeria monocytogenes in rodents. IV. Studies on origin and fate of tissue-positioned T memory cells. *Immunology* **44**, 789-798 (1981).

107. Tanchot, C. & Rocha, B. Peripheral selection of T cell repertoires: the role of continuous thymus output. *J Exp Med* **186**, 1099-1106 (1997).

108. Mueller, S.N. & Mackay, L.K. Tissue-resident memory T cells: local specialists in immune defence. *Nat Rev Immunol* **16**, 79-89 (2016).

109. Casey, K.A., Fraser, K.A., Schenkel, J.M., Moran, A., Abt, M.C., Beura, L.K., Lucas, P.J., Artis, D., Wherry, E.J., Hogquist, K., Vezys, V. & Masopust, D. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *J Immunol* **188**, 4866-4875 (2012).

110. Mackay, L.K., Rahimpour, A., Ma, J.Z., Collins, N., Stock, A.T., Hafon, M.L., Vega-Ramos, J., Lauzurica, P., Mueller, S.N., Stefanovic, T., Tscharke, D.C., Heath, W.R., Inouye, M., Carbone, F.R. & Gebhardt, T. The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat Immunol* **14**, 1294-1301 (2013).

111. Mackay, L.K., Wynne-Jones, E., Freestone, D., Pellicci, D.G., Mielke, L.A., Newman, D.M., Braun, A., Masson, F., Kallies, A., Belz, G.T. & Carbone, F.R. T-box Transcription Factors Combine with the Cytokines TGF-beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43**, 1101-1111 (2015).

112. Sakai, S., Kauffman, K.D., Schenkel, J.M., McBerry, C.C., Mayer-Barber, K.D., Masopust, D. & Barber, D.L. Cutting edge: control of Mycobacterium tuberculosis infection by a subset of lung parenchyma-homing CD4 T cells. *J Immunol* **192**, 2965-2969 (2014).

113. McMaster, S.R., Wilson, J.J., Wang, H. & Kohlmeier, J.E. Airway-Resident Memory CD8 T Cells Provide Antigen-Specific Protection against Respiratory Virus Challenge through Rapid IFN-gamma Production. *J Immunol* **195**, 203-209 (2015).

114. Pan, Y., Tian, T., Park, C.O., Lofftus, S.Y., Mei, S., Liu, X., Luo, C., O'Malley, J.T., Gehad, A., Teague, J.E., Divito, S.J., Fuhlbrigge, R., Puigserver, P., Krueger, J.G., Hotamisligil, G.S., Clark, R.A. & Kupper, T.S. Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nature* **543**, 252-256 (2017).

115. Wakim, L.M., Woodward-Davis, A. & Bevan, M.J. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc Natl Acad Sci U S A* **107**, 17872-17879 (2010).

116. Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S., Fraser, K.A., Webby, R.J., Brinkmann, V., Butcher, E.C., Newell, K.A. & Ahmed, R. Dynamic T cell migration program provides resident memory within intestinal epithelium. *J Exp Med* **207**, 553-564 (2010).

117. Hofmann, M. & Pircher, H. E-cadherin promotes accumulation of a unique memory CD8 T-cell population in murine salivary glands. *Proc Natl Acad Sci U S A* **108**, 16741-16746 (2011).

118. Shinoda, K., Tokoyoda, K., Hanazawa, A., Hayashizaki, K., Zehentmeier, S., Hosokawa, H., Iwamura, C., Koseki, H., Tumes, D.J., Radbruch, A. & Nakayama, T. Type II membrane protein CD69 regulates the formation of resting T-helper memory. *Proc Natl Acad Sci U S A* **109**, 7409-7414 (2012).

119. Anderson, K.G., Sung, H., Skon, C.N., Lefrancois, L., Deisinger, A., Vezys, V. & Masopust, D. Cutting edge: intravascular staining redefines lung CD8 T cell responses. *J Immunol* **189**, 2702-2706 (2012).

120. Cuburu, N., Graham, B.S., Buck, C.B., Kines, R.C., Pang, Y.Y., Day, P.M., Lowy, D.R. & Schiller, J.T. Intravaginal immunization with HPV vectors induces tissue-resident CD8+ T cell responses. *J Clin Invest* **122**, 4606-4620 (2012).

121. Mackay, L.K., Stock, A.T. & of the …, M.-J.Z. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proceedings of the National Academy of Sciences USA* **109**, 7037-7042 (2012).

122. Hofmann, M., Oschowitzer, A., Kurzhals, S.R., Kruger, C.C. & Pircher, H. Thymus-resident memory CD8+ T cells mediate local immunity. *Eur J Immunol* **43**, 2295-2304 (2013).

123. Tse, S.W., Cockburn, I.A., Zhang, H., Scott, A.L. & Zavala, F. Unique transcriptional profile of liver-resident memory CD8+ T cells induced by immunization with malaria sporozoites. *Genes Immun* **14**, 302-309 (2013).

124. Shin, H. & Iwasaki, A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* (2012).

125. Schenkel, J.M., Fraser, K.A. & Masopust, D. Cutting edge: resident memory CD8 T cells occupy frontline niches in secondary lymphoid organs. *J Immunol* **192**, 2961-2964 (2014).

126. Thom, J.T., Weber, T.C., Walton, S.M., Torti, N. & Oxenius, A. The salivary gland acts as a sink for tissue-resident memory CD8+ T cells, facilitating protection from local cytomegalovirus infection. *Cell Rep* (2015).

127. Smith, C.J., Caldeira-Dantas, S., Turula, H. & Snyder, C.M. Murine CMV Infection Induces the Continuous Production of Mucosal Resident T Cells. *Cell Rep* **13**, 1137-1148 (2015).

128. Fernandez-Ruiz, D., Ng, W.Y., Holz, L.E., Ma, J.Z., Zaid, A., Wong, Y.C., Lau, L.S., Mollard, V., Cozijnsen, A., Collins, N., Li, J., Davey, G.M., Kato, Y., Devi, S., Skandari, R., Pauley, M., Manton, J.H., Godfrey, D.I., Braun, A., Tay, S.S., Tan, P.S., Bowen, D.G., Koch-Nolte, F., Rissiek, B., Carbone, F.R., Crabb, B.S., Lahoud, M., Cockburn, I.A., Mueller, S.N., Bertolino, P., McFadden, G.I., Caminschi, I. & Heath, W.R. Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity* **45**, 889-902 (2016).

129. Steinbach, K., Vincenti, I., Kreutzfeldt, M., Page, N., Muschaweckh, A., Wagner, I., Drexler, I., Pinschewer, D., Korn, T. & Merkler, D. Brain-resident memory T cells represent an autonomous cytotoxic barrier to viral infection. *J Exp Med* **213**, 1571-1587 (2016).

130. Park, S.L., Mackay, L.K. & Gebhardt, T. Distinct recirculation potential of CD69(+)CD103(-) and CD103(+) thymic memory CD8(+) T cells. *Immunol Cell Biol* **94**, 975-980 (2016).

131. Purwar, R., Campbell, J., Murphy, G., Richards, W.G., Clark, R.A. & Kupper, T.S. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLOS ONE* **6**, e16245 (2011).

132. Piet, B., de Bree, G.J., Smids-Dierdorp, B.S., van der Loos, C.M., Remmerswaal, E.B., von der Thusen, J.H., van Haarst, J.M., Eerenberg, J.P., ten Brinke, A., van der Bij, W., Timens, W., van Lier, R.A. & Jonkers, R.E. CD8(+) T cells with an intraepithelial phenotype upregulate cytotoxic function upon influenza infection in human lung. *J Clin Invest* **121**, 2254-2263 (2011).

133. Turner, D.L., Bickham, K.L., Thome, J.J., Kim, C.Y., D'Ovidio, F., Wherry, E.J. & Farber, D.L. Lung niches for the generation and maintenance of tissue-resident memory T cells. *Mucosal Immunol* **7**, 501-510 (2014).

134. Hombrink, P., Helbig, C., Backer, R.A., Piet, B., Oja, A.E., Stark, R., Brasser, G., Jongejan, A., Jonkers, R.E., Nota, B., Basak, O., Clevers, H.C., Moerland, P.D., Amsen, D. & van Lier, R.A. Programs for the persistence, vigilance and control of human CD8(+) lung-resident memory T cells. *Nat Immunol* **17**, 1467-1478 (2016).

135. Oja, A.E., Piet, B., Helbig, C., Stark, R., van der Zwan, D., Blaauwgeers, H., Remmerswaal, E.B.M., Amsen, D., Jonkers, R.E., Moerland, P.D., Nolte, M.A., van Lier, R.A.W. & Hombrink, P. Trigger-happy resident memory CD4(+) T cells inhabit the human lungs. *Mucosal Immunol* **11**, 654-667 (2018).

136. Booth, J.S., Toapanta, F.R., Salerno-Goncalves, R., Patil, S., Kader, H.A., Safta, A.M., Czinn, S.J., Greenwald, B.D. & Sztein, M.B. Characterization and functional properties of gastric tissue-resident memory T cells from children, adults, and the elderly. *Front Immunol* **5**, 294 (2014).

137. Roberts, G.W., Baird, D., Gallagher, K., Jones, R.E., Pepper, C.J., Williams, J.D. & Topley, N. Functional effector memory T cells enrich the peritoneal cavity of patients treated with peritoneal dialysis. *J Am Soc Nephrol* **20**, 1895-1900 (2009).

138. Radenkovic, M., Uvebrant, K., Skog, O., Sarmiento, L., Avartsson, J., Storm, P., Vickman, P., Bertilsson, P.A., Fex, M., Korgsgren, O. & Cilio, C.M. Characterization of resident lymphocytes in human pancreatic islets. *Clin Exp Immunol* **187**, 418-427 (2017).

139. Kuric, E., Seiron, P., Krogvold, L., Edwin, B., Buanes, T., Hanssen, K.F., Skog, O., Dahl-Jorgensen, K. & Korsgren, O. Demonstration of Tissue Resident Memory CD8 T Cells in Insulitic Lesions in Adult Patients with Recent-Onset Type 1 Diabetes. *Am J Pathol* **187**, 581-588 (2017).

140. Damouche, A., Pourcher, G., Pourcher, V., Benoist, S., Busson, E., Lataillade, J.J., Le Van, M., Lazure, T., Adam, J., Favier, B., Vaslin, B., Muller-Trutwin, M., Lambotte, O. & Bourgeois, C. High proportion of PD-1-expressing CD4(+) T cells in adipose tissue constitutes an immunomodulatory microenvironment that may support HIV persistence. *Eur J Immunol* **47**, 2113-2123 (2017).

141. Zhu, J., Koelle, D.M., Cao, J., Vazquez, J., Huang, M.L., Hladik, F., Wald, A. & Corey, L. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *J Exp Med* **204**, 595-603 (2007).

142. Zhu, J., Hladik, F., Woodward, A., Klock, A., Peng, T., Johnston, C., Remington, M., Magaret, A., Koelle, D.M., Wald, A. & Corey, L. Persistence of HIV-1 receptor-positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. *Nat Med* **15**, 886-892 (2009).

143. Zhu, J., Peng, T., Johnston, C., Phasouk, K. & Kask, A.S. Immune surveillance by CD8 [agr][agr]+ skin-resident T [thinsp] cells in human herpes virus infection. *Nature* (2013).

144. McKinnon, L.R., Nyanga, B., Chege, D., Izulla, P., Kimani, M., Huibner, S., Gelmon, L., Block, K.E., Cicala, C., Anzala, A.O., Arthos, J., Kimani, J. & Kaul, R. Characterization of a human cervical CD4+ T cell subset coexpressing multiple markers of HIV susceptibility. *J Immunol* **187**, 6032-6042 (2011).

145. Trimble, C.L., Clark, R.A., Thoburn, C., Hanson, N.C., Tassello, J., Frosina, D., Kos, F., Teague, J., Jiang, Y., Barat, N.C. & Jungbluth, A.A. Human papillomavirus 16-associated cervical intraepithelial neoplasia in humans excludes CD8 T cells from dysplastic epithelium. *J Immunol* **185**, 7107-7114 (2010).

146. Bose, T., Lee, R., Hou, A., Tong, L. & Chandy, K.G. Tissue resident memory T cells in the human conjunctiva and immune signatures in human dry eye disease. *Sci Rep* **7**, 45312 (2017).

147. Okhrimenko, A., Grun, J.R., Westendorf, K., Fang, Z., Reinke, S., von Roth, P., Wassilew, G., Kuhl, A.A., Kudernatsch, R., Demski, S., Scheibenbogen, C., Tokoyoda, K., McGrath, M.A., Raftery, M.J., Schonrich, G., Serra, A., Chang, H.D., Radbruch, A. & Dong, J. Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc Natl Acad Sci U S A* **111**, 9229-9234 (2014).

148. Pallett, L.J., Davies, J., Colbeck, E.J., Robertson, F., Hansi, N., Easom, N.J.W., Burton, A.R., Stegmann, K.A., Schurich, A., Swadling, L., Gill, U.S., Male, V., Luong, T., Gander, A., Davidson, B.R., Kennedy, P.T.F. & Maini, M.K. IL-2(high) tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. *J Exp Med* **214**, 1567-1580 (2017).

149. Stelma, F., de Niet, A., Sinnige, M.J., van Dort, K.A., van Gisbergen, K., Verheij, J., van Leeuwen, E.M.M., Kootstra, N.A. & Reesink, H.W. Human intrahepatic CD69 + CD8+ T cells have a tissue resident memory T cell phenotype with reduced cytolytic capacity. *Sci Rep* **7**, 6172 (2017).

150. Clark, R.A., Watanabe, R., Teague, J.E., Schlapbach, C., Tawa, M.C., Adams, N., Dorosario, A.A., Chaney, K.S., Cutler, C.S. & LeBoeuf, N.R. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Sci Transl Med* **4** (2012).

151. Cheuk, S., Wiken, M., Blomqvist, L., Nylen, S., Talme, T., Stahle, M. & Eidsmo, L. Epidermal Th22 and Tc17 cells form a localized disease memory in clinically healed psoriasis. *J Immunol* **192**, 3111-3120 (2014).

152. Watanabe, R., Gehad, A., Yang, C., Scott, L.L., Teague, J.E., Schlapbach, C., Elco, C.P., Huang, V., Matos, T.R. & Kupper, T.S. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Sci Transl Med* **7** (2015).

153. Skon, C.N., Lee, J.Y., Anderson, K.G. & Masopust, D. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat Immunol* **14**, 1285-1293 (2013).

154. Ariotti, S., Haanen, J.B. & Schumacher, T.N. Behavior and function of tissue-resident memory T cells. *Adv Immunol* **114**, 203-216 (2012).

155. Masopust, D., Vezys, V., Wherry, E.J., Barber, D.L. & Ahmed, R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol* **176**, 2079-2083 (2006).

156. Lee, Y.T., Suarez-Ramirez, J.E., Wu, T., Redman, J.M., Bouchard, K., Hadley, G.A. & Cauley, L.S. Environmental and antigen receptor-derived signals support sustained surveillance of the lungs by pathogen-specific cytotoxic T lymphocytes. *J Virol* **85**, 4085-4094 (2011).

157. Sheridan, B.S., Pham, Q.M., Lee, Y.T., Cauley, L.S., Puddington, L. & Lefrancois, L. Oral infection drives a distinct population of intestinal resident memory CD8(+) T cells with enhanced protective function. *Immunity* **40**, 747-757 (2014).

158. Mackay, L.K., Braun, A., Macleod, B.L., Collins, N., Tebartz, C., Bedoui, S., Carbone, F.R. & Gebhardt, T. Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J Immunol* **194**, 2059-2063 (2015).

159. Ely, K.H., Cookenham, T., Roberts, A.D. & Woodland, D.L. Memory T cell populations in the lung airways are maintained by continual recruitment. *J Immunol* **176**, 537-543 (2006).

160. Ledgerwood, L.G., Lal, G., Zhang, N., Garin, A., Esses, S.J., Ginhoux, F., Merad, M., Peche, H., Lira, S.A., Ding, Y., Yang, Y., He, X., Schuchman, E.H., Allende, M.L., Ochando, J.C. & Bromberg, J.S. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat Immunol* **9**, 42-53 (2008).

161. Czeloth, N., Bernhardt, G., Hofmann, F., Genth, H. & Forster, R. Sphingosine-1-phosphate mediates migration of mature dendritic cells. *J Immunol* **175**, 2960-2967 (2005).

162. Lamana, A., Martin, P., de la Fuente, H., Martinez-Munoz, L., Cruz-Adalia, A., Ramirez-Huesca, M., Escribano, C., Gollmer, K., Mellado, M., Stein, J.V., Rodriguez-Fernandez, J.L., Sanchez-Madrid, F. & del Hoyo, G.M. CD69 modulates sphingosine-1-phosphate-induced migration of skin dendritic cells. *J Invest Dermatol* **131**, 1503-1512 (2011).

163. Maeda, Y., Matsuyuki, H., Shimano, K., Kataoka, H., Sugahara, K. & Chiba, K. Migration of CD4 T Cells and Dendritic Cells toward Sphingosine 1-Phosphate (S1P) Is Mediated by Different Receptor Subtypes: S1P Regulates the Functions of Murine Mature Dendritic Cells via S1P Receptor Type 3. *J Immunol* **178**, 3437-3446 (2007).

164. Pham, T.H., Baluk, P., Xu, Y., Grigorova, I., Bankovich, A.J., Pappu, R., Coughlin, S.R., McDonald, D.M., Schwab, S.R. & Cyster, J.G. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med* **207**, 17-27 (2010).

165. Schon, M.P., Arya, A., Murphy, E.A., Adams, C.M., Strauch, U.G., Agace, W.W., Marsal, J., Donohue, J.P., Her, H., Beier, D.R., Olson, S., Lefrancois, L., Brenner, M.B., Grusby, M.J. & Parker, C.M. Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol* **162**, 6641-6649 (1999).

166. Hadley, G.A. & Higgins, J.M. Integrin alphaEbeta7: molecular features and functional significance in the immune system. *Adv Exp Med Biol* **819**, 97-110 (2014).

167. El-Asady, R., Yuan, R., Liu, K., Wang, D., Gress, R.E., Lucas, P.J., Drachenberg, C.B. & Hadley, G.A. TGF-{beta}-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J Exp Med* **201**, 1647-1657 (2005).

168. Nestle, F.O., Di Meglio, P., Qin, J.Z. & Nickoloff, B.J. Skin immune sentinels in health and disease. *Nat Rev Immunol* **9**, 679-691 (2009).

169. Shimamura, K. & Takeichi, M. Local and transient expression of E-cadherin involved in mouse embryonic brain morphogenesis. *Development* **116**, 1011-1019 (1992).

170. Wakim, L.M., Woodward-Davis, A., Liu, R., Hu, Y., Villadangos, J., Smyth, G. & Bevan, M.J. The molecular signature of tissue resident memory CD8 T cells isolated from the brain. *J Immunol* **189**, 3462-3471 (2012).

171. Zhang, N. & Bevan, M.J. Transforming growth factor-beta signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. *Immunity* **39**, 687-696 (2013).

172. Haddadi, S., Thanthrige-Don, N., Afkhami, S., Khera, A., Jeyanathan, M. & Xing, Z. Expression and role of VLA-1 in resident memory CD8 T cell responses to respiratory mucosal viral-vectored immunization against tuberculosis. *Sci Rep* **7**, 9525 (2017).

173. Ray, S.J., Franki, S.N., Pierce, R.H., Dimitrova, S., Koteliansky, V., Sprague, A.G., Doherty, P.C., de Fougerolles, A.R. & Topham, D.J. The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* **20**, 167-179 (2004).

174. Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., Braun, A., Wynne-Jones, E., Behr, F.M., Stark, R., Pellicci, D.G., Godfrey, D.I., Belz, G.T., Pellegrini, M., Gebhardt, T., Busslinger, M., Shi, W., Carbone, F.R., van Lier, R.A., Kallies, A. & van Gisbergen, K.P. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science* **352**, 459-463 (2016).

175. Boddupalli, C.S., Nair, S., Gray, S.M., Nowyhed, H.N., Verma, R., Gibson, J.A., Abraham, C., Narayan, D., Vasquez, J., Hedrick, C.C., Flavell, R.A., Dhodapkar, K.M., Kaech, S.M. & Dhodapkar, M.V. ABC transporters and NR4A1 identify a quiescent subset of tissue-resident memory T cells. *J Clin Invest* **126**, 3905-3916 (2016).

176. Kohlmeier, J.E., Miller, S.C. & Woodland, D.L. Cutting edge: Antigen is not required for the activation and maintenance of virus-specific memory CD8+ T cells in the lung airways. *J Immunol* **178**, 4721-4725 (2007).

177. Zaid, A., Mackay, L.K., Rahimpour, A., Braun, A., Veldhoen, M., Carbone, F.R., Manton, J.H., Heath, W.R. & Mueller, S.N. Persistence of skin-resident memory T cells within an epidermal niche. *Proc Natl Acad Sci U S A* **111**, 5307-5312 (2014).

178. Laidlaw, B.J., Zhang, N., Marshall, H.D., Staron, M.M., Guan, T., Hu, Y., Cauley, L.S., Craft, J. & Kaech, S.M. CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. *Immunity* **41**, 633-645 (2014).

179. Slutter, B., Pewe, L.L., Kaech, S.M. & Harty, J.T. Lung airway-surveilling CXCR3(hi) memory CD8(+) T cells are critical for protection against influenza A virus. *Immunity* **39**, 939-948 (2013).

180. Heesch, K., Raczkowski, F., Schumacher, V., Hunemorder, S., Panzer, U. & Mittrucker, H.W. The function of the chemokine receptor CXCR6 in the T cell response of mice against Listeria monocytogenes. *PLOS ONE* **9**, e97701 (2014).

181. Zaid, A., Hor, J.L., Christo, S.N., Groom, J.R., Heath, W.R., Mackay, L.K. & Mueller, S.N. Chemokine Receptor-Dependent Control of Skin Tissue-Resident Memory T Cell Formation. *J Immunol* **199**, 2451-2459 (2017).

182. Kallies, A., Hawkins, E.D., Belz, G.T., Metcalf, D., Hommel, M., Corcoran, L.M., Hodgkin, P.D. & Nutt, S.L. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* **7**, 466-474 (2006).

183. Kallies, A., Xin, A., Belz, G.T. & Nutt, S.L. Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* **31**, 283-295 (2009).

184. Rutishauser, R.L., Martins, G.A., Kalachikov, S., Chandele, A., Parish, I.A., Meffre, E., Jacob, J., Calame, K. & Kaech, S.M. Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* **31**, 296-308 (2009).

185. Thome, J.J. & Farber, D.L. Emerging concepts in tissue-resident T cells: lessons from humans. *Trends Immunol* **36**, 428-435 (2015).

186. Turner, D.L. & Farber, D.L. Mucosal resident memory CD4 T cells in protection and immunopathology. *Front Immunol* **5**, 331 (2014).

187. Woon, H.G., Braun, A., Li, J., Smith, C., Edwards, J., Sierro, F., Feng, C.G., Khanna, R., Elliot, M., Bell, A., Hislop, A.D., Tangye, S.G., Rickinson, A.B., Gebhardt, T., Britton, W.J. & Palendira, U. Compartmentalization of Total and Virus-Specific Tissue-Resident Memory CD8+ T Cells in Human Lymphoid Organs. *PLoS Pathog* **12**, e1005799 (2016).

188. Cheuk, S., Schlums, H., Gallais Serezal, I., Martini, E., Chiang, S.C., Marquardt, N., Gibbs, A., Detlofsson, E., Introini, A., Forkel, M., Hoog, C., Tjernlund, A., Michaelsson, J., Folkersen, L., Mjosberg, J., Blomqvist, L., Ehrstrom, M., Stahle, M., Bryceson, Y.T. & Eidsmo, L. CD49a Expression Defines Tissue-Resident CD8(+) T Cells Poised for Cytotoxic Function in Human Skin. *Immunity* **46**, 287-300 (2017).

189. Vieira Braga, F.A., Hertoghs, K.M., Kragten, N.A., Doody, G.M., Barnes, N.A., Remmerswaal, E.B., Hsiao, C.C., Moerland, P.D., Wouters, D., Derks, I.A., van Stijn, A., Demkes, M., Hamann, J., Eldering, E., Nolte, M.A., Tooze, R.M., ten Berge, I.J., van Gisbergen, K.P. & van Lier, R.A. Blimp-1 homolog Hobit identifies effector-type lymphocytes in humans. *Eur J Immunol* **45**, 2945-2958 (2015).

190. Oja, A.E., Vieira Braga, F.A., Remmerswaal, E.B., Kragten, N.A., Hertoghs, K.M., Zuo, J., Moss, P.A., van Lier, R.A., van Gisbergen, K.P. & Hombrink, P. The Transcription Factor Hobit Identifies Human Cytotoxic CD4(+) T Cells. *Front Immunol* **8**, 325 (2017).

191. Hickey, W.F., Hsu, B.L. & Kimura, H. T-lymphocyte entry into the central nervous system. *J Neurosci Res* **28**, 254-260 (1991).

192. Bengsch, B., Spangenberg, H.C., Kersting, N., Neumann-Haefelin, C., Panther, E., von Weizsacker, F., Blum, H.E., Pircher, H. & Thimme, R. Analysis of CD127 and KLRG1 expression

on hepatitis C virus-specific CD8+ T cells reveals the existence of different memory T-cell subsets in the peripheral blood and liver. *J Virol* **81**, 945-953 (2007).

193. Kaech, S.M. & Wherry, E.J. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* **27**, 393-405 (2007).

194. Schwartzkopff, S., Woyciechowski, S., Aichele, U., Flecken, T., Zhang, N., Thimme, R. & Pircher, H. TGF-beta downregulates KLRG1 expression in mouse and human CD8(+) T cells. *Eur J Immunol* **45**, 2212-2217 (2015).

195. Grundemann, C., Bauer, M., Schweier, O., von Oppen, N., Lassing, U., Saudan, P., Becker, K.F., Karp, K., Hanke, T., Bachmann, M.F. & Pircher, H. Cutting Edge: Identification of E-Cadherin as a Ligand for the Murine Killer Cell Lectin-Like Receptor G1. *The Journal of Immunology* **176**, 1311-1315 (2006).

196. Gaide, O., Emerson, R.O., Jiang, X., Gulati, N., Nizza, S., Desmarais, C., Robins, H., Krueger, J.G., Clark, R.A. & Kupper, T.S. Common clonal origin of central and resident memory T cells following skin immunization. *Nat Med* **21**, 647-653 (2015).

197. Sowell, R.T., Rogozinska, M., Nelson, C.E., Vezys, V. & Marzo, A.L. Cutting edge: generation of effector cells that localize to mucosal tissues and form resident memory CD8 T cells is controlled by mTOR. *J Immunol* **193**, 2067-2071 (2014).

198. Nakanishi, Y., Lu, B., Gerard, C. & Iwasaki, A. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* **462**, 510-513 (2009).

199. Glennie, N.D., Yeramilli, V.A., Beiting, D.P., Volk, S.W., Weaver, C.T. & Scott, P. Skin-resident memory CD4+ T cells enhance protection against Leishmania major infection. *J Exp Med* **212**, 1405-1414 (2015).

200. Laidlaw, B.J., Craft, J.E. & Kaech, S.M. The multifaceted role of CD4(+) T cells in CD8(+) T cell memory. *Nat Rev Immunol* **16**, 102-111 (2016).

201. Pizzolla, A., Nguyen, T.H.O., Smith, J.M., Brooks, A.G., Kedzieska, K., Heath, W.R., Reading, P.C. & Wakim, L.M. Resident memory CD8(+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. *Sci Immunol* **2** (2017).

202. Davies, B., Prier, J.E., Jones, C.M., Gebhardt, T., Carbone, F.R. & Mackay, L.K. Cutting Edge: Tissue-Resident Memory T Cells Generated by Multiple Immunizations or Localized Deposition Provide Enhanced Immunity. *J Immunol* **198**, 2233-2237 (2017).

203. Khan, T.N., Mooster, J.L., Kilgore, A.M., Osborn, J.F. & Nolz, J.C. Local antigen in nonlymphoid tissue promotes resident memory CD8+ T cell formation during viral infection. *J Exp Med* **213**, 951-966 (2016).

204. Maru, S., Jin, G., Schell, T.D. & Lukacher, A.E. TCR stimulation strength is inversely associated with establishment of functional brain-resident memory CD8 T cells during persistent viral infection. *PLoS Pathog* **13**, e1006318 (2017).

205. Hu, Y., Lee, Y.T., Kaech, S.M., Garvy, B. & Cauley, L.S. Smad4 promotes differentiation of effector and circulating memory CD8 T cells but is dispensable for tissue-resident memory CD8 T cells. *J Immunol* **194**, 2407-2414 (2015).

206. Sowell, R.T., Goldufsky, J.W., Rogozinska, M., Quiles, Z., Cao, Y., Castillo, E.F., Finnegan, A. & Marzo, A.L. IL-15 Complexes Induce Migration of Resting Memory CD8 T Cells into Mucosal Tissues. *J Immunol* **199**, 2536-2546 (2017).

207. Xin, A., Masson, F., Liao, Y., Preston, S., Guan, T., Gloury, R., Olshansky, M., Lin, J.X., Li, P., Speed, T.P., Smyth, G.K., Ernst, M., Leonard, W.J., Pellegrini, M., Kaech, S.M., Nutt, S.L., Shi, W.,

Belz, G.T. & Kallies, A. A molecular threshold for effector CD8(+) T cell differentiation controlled by transcription factors Blimp-1 and T-bet. *Nat Immunol* **17**, 422-432 (2016).

208. Bergsbaken, T. & Bevan, M.J. Proinflammatory microenvironments within the intestine regulate the differentiation of tissue-resident CD8(+) T cells responding to infection. *Nat Immunol* **16**, 406-414 (2015).

209. Schenkel, J.M., Fraser, K.A., Casey, K.A., Beura, L.K., Pauken, K.E., Vezys, V. & Masopust, D. IL-15-Independent Maintenance of Tissue-Resident and Boosted Effector Memory CD8 T Cells. *J Immunol* **196**, 3920-3926 (2016).

210. Bergsbaken, T., Bevan, M.J. & Fink, P.J. Local Inflammatory Cues Regulate Differentiation and Persistence of CD8(+) Tissue-Resident Memory T Cells. *Cell Rep* **19**, 114-124 (2017).

211. Cipolletta, D., Feuerer, M., Li, A., Kamei, N., Lee, J., Shoelson, S.E., Benoist, C. & Mathis, D. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* **486**, 549-553 (2012).

212. Brestoff, J.R., Kim, B.S., Saenz, S.A., Stine, R.R., Monticelli, L.A., Sonnenberg, G.F., Thome, J.J., Farber, D.L., Lutfy, K., Seale, P. & Artis, D. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* **519**, 242-246 (2015).

213. Collins, N., Jiang, X., Zaid, A., Macleod, B.L., Li, J., Park, C.O., Haque, A., Bedoui, S., Heath, W.R., Mueller, S.N., Kupper, T.S., Gebhardt, T. & Carbone, F.R. Skin CD4(+) memory T cells exhibit combined cluster-mediated retention and equilibration with the circulation. *Nat Commun* **7**, 11514 (2016).

214. Beura, L.K., Hamilton, S.E., Bi, K., Schenkel, J.M., Odumade, O.A., Casey, K.A., Thompson, E.A., Fraser, K.A., Rosato, P.C., Filali-Mouhim, A., Sekaly, R.P., Jenkins, M.K., Vezys, V., Haining, W.N., Jameson, S.C. & Masopust, D. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature* **532**, 512-516 (2016).

215. Kaufman, D.R., Liu, J., Carville, A., Mansfield, K.G., Havenga, M.J., Goudsmit, J. & Barouch, D.H. Trafficking of antigen-specific CD8+ T lymphocytes to mucosal surfaces following intramuscular vaccination. *J Immunol* **181**, 4188-4198 (2008).

216. Neuber, K., Schmidt, S. & Mensch, A. Telomere length measurement and determination of immunosenescence-related markers (CD28, CD45RO, CD45RA, interferon-gamma and interleukin-4) in skin-homing T cells expressing the cutaneous lymphocyte antigen: indication of a non-ageing T-cell subset. *Immunology* **109**, 24-31 (2003).

217. Zammit, D.J., Turner, D.L., Klonowski, K.D., Lefrancois, L. & Cauley, L.S. Residual antigen presentation after influenza virus infection affects CD8 T cell activation and migration. *Immunity* **24**, 439-449 (2006).

218. Gebhardt, T., Whitney, P.G., Zaid, A., Mackay, L.K., Brooks, A.G., Heath, W.R., Carbone, F.R. & Mueller, S.N. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* **477**, 216-219 (2011).

219. Muschaweckh, A., Buchholz, V.R., Fellenzer, A., Hessel, C., Konig, P.A., Tao, S., Tao, R., Heikenwalder, M., Busch, D.H., Korn, T., Kastenmuller, W., Drexler, I. & Gasteiger, G. Antigen-dependent competition shapes the local repertoire of tissue-resident memory CD8+ T cells. *J Exp Med* **213**, 3075-3086 (2016).

220. Wu, T., Hu, Y., Lee, Y.T., Bouchard, K.R., Benechet, A., Khanna, K. & Cauley, L.S. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol* **95**, 215-224 (2014).

221. Zens, K.D., Chen, J.K. & Farber, D.L. Vaccine-generated lung tissue-resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight* **1** (2016).

222. Kinnear, E., Lambert, L., McDonald, J.U., Cheeseman, H.M., Caproni, L.J. & Tregoning, J.S. Airway T cells protect against RSV infection in the absence of antibody. *Mucosal Immunol* **11**, 249-256 (2018).

223. Smith, N.M., Wasserman, G.A., Coleman, F.T., Hilliard, K.L., Yamamoto, K., Lipsitz, E., Malley, R., Dooms, H., Jones, M.R., Quinton, L.J. & Mizgerd, J.P. Regionally compartmentalized resident memory T cells mediate naturally acquired protection against pneumococcal pneumonia. *Mucosal Immunol* **11**, 220-235 (2018).

224. Schenkel, J.M., Fraser, K.A., Beura, L.K., Pauken, K.E., Vezys, V. & Masopust, D. T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science* **346**, 98-101 (2014).

225. Stary, G., Olive, A., Radovic-Moreno, A.F., Gondek, D., Alvarez, D., Basto, P.A., Perro, M., Vrbanac, V.D., Tager, A.M., Shi, J., Yethon, J.A., Farokhzad, O.C., Langer, R., Starnbach, M.N. & von Andrian, U.H. VACCINES. A mucosal vaccine against Chlamydia trachomatis generates two waves of protective memory T cells. *Science* **348**, aaa8205 (2015).

226. Ariotti, S., Beltman, J.B., Chodaczek, G., Hoekstra, M.E., van Beek, A.E., Gomez-Eerland, R., Ritsma, L., van Rheenen, J., Maree, A.F., Zal, T., de Boer, R.J., Haanen, J.B. & Schumacher, T.N. Tissue-resident memory CD8+ T cells continuously patrol skin epithelia to quickly recognize local antigen. *Proc Natl Acad Sci U S A* **109**, 19739-19744 (2012).

227. Liu, L., Fuhlbrigge, R.C., Karibian, K., Tian, T. & Kupper, T.S. Dynamic programming of CD8+ T cell trafficking after live viral immunization. *Immunity* **25**, 511-520 (2006).

228. Liu, L., Zhong, Q., Tian, T., Dubin, K., Athale, S.K. & Kupper, T.S. Epidermal injury and infection during poxvirus immunization is crucial for the generation of highly protective T cell-mediated immunity. *Nat Med* **16**, 224-227 (2010).

229. Wilk, M.M., Misiak, A., McManus, R.M., Allen, A.C., Lynch, M.A. & Mills, K.H.G. Lung CD4 Tissue-Resident Memory T Cells Mediate Adaptive Immunity Induced by Previous Infection of Mice with Bordetella pertussis. *J Immunol* **199**, 233-243 (2017).

230. Jozwik, A., Habibi, M.S., Paras, A., Zhu, J., Guvenel, A., Dhariwal, J., Almond, M., Wong, E.H., Sykes, A., Maybeno, M., Del Rosario, J., Trujillo-Torralbo, M.B., Mallia, P., Sidney, J., Peters, B., Kon, O.M., Sette, A., Johnston, S.L., Openshaw, P.J. & Chiu, C. RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. *Nat Commun* **6**, 10224 (2015).

231. Rosato, P.C., Beura, L.K. & Masopust, D. Tissue resident memory T cells and viral immunity. *Curr Opin Virol* **22**, 44-50 (2017).

232. Iborra, S., Martinez-Lopez, M., Khouili, S.C., Enamorado, M., Cueto, F.J., Conde-Garrosa, R., Del Fresno, C. & Sancho, D. Optimal Generation of Tissue-Resident but Not Circulating Memory T Cells during Viral Infection Requires Crosspriming by DNGR-1(+) Dendritic Cells. *Immunity* **45**, 847-860 (2016).

233. Shin, H. & Iwasaki, A. Tissue-resident memory T cells. *Immunol Rev* **255**, 165-181 (2013).

234. Harris, T.H., Banigan, E.J., Christian, D.A., Konradt, C., Tait Wojno, E.D., Norose, K., Wilson, E.H., John, B., Weninger, W., Luster, A.D., Liu, A.J. & Hunter, C.A. Generalized Levy walks and the role of chemokines in migration of effector CD8+ T cells. *Nature* **486**, 545-548 (2012).

235. Schmidt, J.D., Ahlstrom, M.G., Johansen, J.D., Dyring-Andersen, B., Agerbeck, C., Nielsen, M.M., Poulsen, S.S., Woetmann, A., Odum, N., Thomsen, A.R., Geisler, C. & Bonefeld, C.M. Rapid allergen-induced interleukin-17 and interferon-gamma secretion by skin-resident memory CD8(+) T cells. *Contact Dermatitis* **76**, 218-227 (2017).

236. Landrith, T.A., Sureshchandra, S., Rivera, A., Jang, J.C., Rais, M., Nair, M.G., Messaoudi, I. & Wilson, E.H. CD103(+) CD8 T Cells in the Toxoplasma-Infected Brain Exhibit a Tissue-Resident Memory Transcriptional Profile. *Front Immunol* **8**, 335 (2017).

237. Steinfelder, S., Rausch, S., Michael, D., Kuhl, A.A. & Hartmann, S. Intestinal helminth infection induces highly functional resident memory CD4(+) T cells in mice. *Eur J Immunol* **47**, 353-363 (2017).

238. Ariotti, S., Hogenbirk, M.A., Dijkgraaf, F.E., Visser, L.L., Hoekstra, M.E., Song, J.Y., Jacobs, H., Haanen, J.B. & Schumacher, T.N. T cell memory. Skin-resident memory CD8(+) T cells trigger a state of tissue-wide pathogen alert. *Science* **346**, 101-105 (2014).

239. Glennie, N.D., Volk, S.W. & Scott, P. Skin-resident CD4+ T cells protect against Leishmania major by recruiting and activating inflammatory monocytes. *PLoS Pathog* **13**, e1006349 (2017).

240. Posavad, C.M., Zhao, L., Dong, L., Jin, L., Stevens, C.E., Magaret, A.S., Johnston, C., Wald, A., Zhu, J., Corey, L. & Koelle, D.M. Enrichment of herpes simplex virus type 2 (HSV-2) reactive mucosal T cells in the human female genital tract. *Mucosal Immunol* **10**, 1259-1269 (2017).

241. Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W., Davey, D.F., Flynn, B.J., Hoff, S.T., Andersen, P., Reed, S.G., Morris, S.L., Roederer, M. & Seder, R.A. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* **13**, 843-850 (2007).

242. Seder, R.A., Darrah, P.A. & Roederer, M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* **8**, 247-258 (2008).

243. Kannanganat, S., Ibegbu, C., Chennareddi, L., Robinson, H.L. & Amara, R.R. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J Virol* **81**, 8468-8476 (2007).

244. Clark, R.A. Skin-resident T cells: the ups and downs of on site immunity. *J Invest Dermatol* **130**, 362-370 (2010).

245. Ishizuka, A.S., Lyke, K.E., DeZure, A., Berry, A.A., Richie, T.L., Mendoza, F.H., Enama, M.E., Gordon, I.J., Chang, L.J., Sarwar, U.N., Zephir, K.L., Holman, L.A., James, E.R., Billingsley, P.F., Gunasekera, A., Chakravarty, S., Manoj, A., Li, M., Ruben, A.J., Li, T., Eappen, A.G., Stafford, R.E., K, C.N., Murshedkar, T., DeCederfelt, H., Plummer, S.H., Hendel, C.S., Novik, L., Costner, P.J., Saunders, J.G., Laurens, M.B., Plowe, C.V., Flynn, B., Whalen, W.R., Todd, J.P., Noor, J., Rao, S., Sierra-Davidson, K., Lynn, G.M., Epstein, J.E., Kemp, M.A., Fahle, G.A., Mikolajczak, S.A., Fishbaugher, M., Sack, B.K., Kappe, S.H., Davidson, S.A., Garver, L.S., Bjorkstrom, N.K., Nason, M.C., Graham, B.S., Roederer, M., Sim, B.K., Hoffman, S.L., Ledgerwood, J.E. & Seder, R.A. Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nat Med* **22**, 614-623 (2016).

246. Tan, H.X., Wheatley, A.K., Esterbauer, R., Jegaskanda, S., Glass, J.J., Masopust, D., De Rose, R. & Kent, S.J. Induction of vaginal-resident HIV-specific CD8 T cells with mucosal prime-boost immunization. *Mucosal Immunol* **11**, 994-1007 (2018).

247. Zaric, M., Becker, P.D., Hervouet, C., Kalcheva, P., Ibarzo Yus, B., Cocita, C., O'Neill, L.A., Kwon, S.Y. & Klavinskis, L.S. Long-lived tissue resident HIV-1 specific memory CD8(+) T cells are generated by skin immunization with live virus vectored microneedle arrays. *J Control Release* **268**, 166-175 (2017).

248. Shin, H. & Iwasaki, A. Generating protective immunity against genital herpes. *Trends Immunol* **34**, 487-494 (2013).

249. Ganusov, V.V. & De Boer, R.J. Do most lymphocytes in humans really reside in the gut? *Trends Immunol* **28**, 514-518 (2007).

250. Anderson, K.G., Mayer-Barber, K., Sung, H., Beura, L., James, B.R., Taylor, J.J., Qunaj, L., Griffith, T.S., Vezys, V., Barber, D.L. & Masopust, D. Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc* **9**, 209-222 (2014).

251. Marriott, C.L., Dutton, E.E., Tomura, M. & Withers, D.R. Retention of Ag-specific memory CD4(+) T cells in the draining lymph node indicates lymphoid tissue resident memory populations. *Eur J Immunol* **47**, 860-871 (2017).

252. Walrath, J., Zukowski, L., Krywiak, A. & Silver, R.F. Resident Th1-like effector memory cells in pulmonary recall responses to Mycobacterium tuberculosis. *Am J Respir Cell Mol Biol* **33**, 48-55 (2005).

253. Hegazy, A.N., West, N.R., Stubbington, M.J.T., Wendt, E., Suijker, K.I.M., Datsi, A., This, S., Danne, C., Campion, S., Duncan, S.H., Owens, B.M.J., Uhlig, H.H., McMichael, A., Oxford, I.B.D.C.I., Bergthaler, A., Teichmann, S.A., Keshav, S. & Powrie, F. Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. *Gastroenterology* **153**, 1320-1337 e1316 (2017).

254. Chapman, T.J. & Topham, D.J. Identification of a unique population of tissue-memory CD4+ T cells in the airways after influenza infection that is dependent on the integrin VLA-1. *J Immunol* **184**, 3841-3849 (2010).

255. Farber, D.L., Yudanin, N.A. & Restifo, N.P. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* **14**, 24-35 (2014).

256. Iijima, N. & Iwasaki, A. Tissue instruction for migration and retention of TRM cells. *Trends Immunol* **36**, 556-564 (2015).

257. Ma, C., Mishra, S., Demel, E.L., Liu, Y. & Zhang, N. TGF-beta Controls the Formation of Kidney-Resident T Cells via Promoting Effector T Cell Extravasation. *J Immunol* **198**, 749-756 (2017).

258. Huang, H.Y. & Luther, S.A. Expression and function of interleukin-7 in secondary and tertiary lymphoid organs. *Semin Immunol* **24**, 175-189 (2012).

259. Hondowicz, B.D., An, D., Schenkel, J.M., Kim, K.S., Steach, H.R., Krishnamurty, A.T., Keitany, G.J., Garza, E.N., Fraser, K.A., Moon, J.J., Altemeier, W.A., Masopust, D. & Pepper, M. Interleukin-2-Dependent Allergen-Specific Tissue-Resident Memory Cells Drive Asthma. *Immunity* **44**, 155-166 (2016).

260. Hondowicz, B.D., Kim, K.S., Ruterbusch, M.J., Keitany, G.J. & Pepper, M. IL-2 is required for the generation of viral-specific CD4(+) Th1 tissue-resident memory cells and B cells are essential for maintenance in the lung. *Eur J Immunol* **48**, 80-86 (2018).

261. Takamura, S., Yagi, H., Hakata, Y., Motozono, C., McMaster, S.R., Masumoto, T., Fujisawa, M., Chikaishi, T., Komeda, J., Itoh, J., Umemura, M., Kyusai, A., Tomura, M., Nakayama, T., Woodland, D.L., Kohlmeier, J.E. & Miyazawa, M. Specific niches for lung-resident memory CD8+ T cells at the site of tissue regeneration enable CD69-independent maintenance. *J Exp Med* **213**, 3057-3073 (2016).

262. Bromley, S.K., Yan, S., Tomura, M., Kanagawa, O. & Luster, A.D. Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern. *J Immunol* **190**, 970-976 (2013).

263. Wissinger, E.L., Stevens, W.W., Varga, S.M. & Braciale, T.J. Proliferative expansion and acquisition of effector activity by memory CD4+ T cells in the lungs following pulmonary virus infection. *J Immunol* **180**, 2957-2966 (2008).

264. Connor, L.M., Harvie, M.C., Rich, F.J., Quinn, K.M., Brinkmann, V., Le Gros, G. & Kirman, J.R. A key role for lung-resident memory lymphocytes in protective immune responses after BCG vaccination. *Eur J Immunol* **40**, 2482-2492 (2010).

265. Thawer, S.G., Horsnell, W.G., Darby, M., Hoving, J.C., Dewals, B., Cutler, A.J., Lang, D. & Brombacher, F. Lung-resident CD4(+) T cells are sufficient for IL-4Ralpha-dependent recall immunity to Nippostrongylus brasiliensis infection. *Mucosal Immunol* **7**, 239-248 (2014).

266. Marzo, A.L., Vezys, V., Williams, K., Tough, D.F. & Lefrancois, L. Tissue-level regulation of Th1 and Th2 primary and memory CD4 T cells in response to Listeria infection. *J Immunol* **168**, 4504-4510 (2002).

267. Iijima, N., Linehan, M.M., Zamora, M., Butkus, D., Dunn, R., Kehry, M.R., Laufer, T.M. & Iwasaki, A. Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. *J Exp Med* **205**, 3041-3052 (2008).

268. Takahara, M., Nemoto, Y., Oshima, S., Matsuzawa, Y., Kanai, T., Okamoto, R., Tsuchiya, K., Nakamura, T., Yamamoto, K. & Watanabe, M. IL-7 promotes long-term in vitro survival of unique long-lived memory subset generated from mucosal effector memory CD4+ T cells in chronic colitis mice. *Immunol Lett* **156**, 82-93 (2013).

269. Michalopoulos, G.K. Liver regeneration. *J Cell Physiol* **213**, 286-300 (2007).

270. Reshetnyak, V.I. Physiological and molecular biochemical mechanisms of bile formation. *World J Gastroenterol* **19**, 7341-7360 (2013).

271. Trefts, E., Gannon, M. & Wasserman, D.H. The liver. *Curr Biol* **27**, R1147-R1151 (2017).

272. Black, D.D. Protein Synthesis and Nutrient Metabolism, *The Gastrointestinal System*, Springer, 271-294 (2014).

273. Kuscuoglu, D., Janciauskiene, S., Hamesch, K., Haybaeck, J., Trautwein, C. & Strnad, P. Liver - master and servant of serum proteome. *J Hepatol* **69**, 512-524 (2018).

274. Anderson, E.R. & Shah, Y.M. Iron homeostasis in the liver. *Compr Physiol* **3**, 315-330 (2013).

275. Doherty, D.G. & O'Farrelly, C. Innate and adaptive lymphoid cells in the human liver. *Immunol Rev* **174**, 5-20 (2000).

276. Trivedi, P.J. & Adams, D.H. Gut-liver immunity. *J Hepatol* **64**, 1187-1189 (2016).

277. Crispe, I.N. The liver as a lymphoid organ. *Annu Rev Immunol* **27**, 147-163 (2009).

278. Sibulesky, L. Normal liver anatomy. *Clin Liver Dis* **2**, S1-S3 (2013).

279. Ishibashi, H., Nakamura, M., Komori, A., Migita, K. & Shimoda, S. Liver architecture, cell function, and disease. *Semin Immunopathol* **31**, 399-409 (2009).

280. Abdel-Misih, S.R. & Bloomston, M. Liver anatomy. *Surg Clin North Am* **90**, 643-653 (2010).

281. Racanelli, V. & Rehermann, B. The liver as an immunological organ. *Hepatology* **43**, S54-62 (2006).

282. Davies, S.P., Reynolds, G.M. & Stamataki, Z. Clearance of Apoptotic Cells by Tissue Epithelia: A Putative Role for Hepatocytes in Liver Efferocytosis. *Front Immunol* **9**, 44 (2018).

283. Gao, B., Jeong, W.I. & Tian, Z. Liver: An organ with predominant innate immunity. *Hepatology* **47**, 729-736 (2008).

284. Bogdanos, D.P., Gao, B. & Gershwin, M.E. Liver immunology. *Compr Physiol* **3**, 567-598 (2013).

285. Krishna, M. Microscopic anatomy of the liver. *Clin Liver Dis* **2**, S4-S7 (2013).

286. Lalor, P.F. & Adams, D.H. The liver: a model of organ-specific lymphocyte recruitment. *Expert Rev Mol Med* **4**, 1-16 (2002).

287. Nemeth, E., Baird, A.W. & O'Farrelly, C. Microanatomy of the liver immune system. *Semin Immunopathol* **31**, 333-343 (2009).

288. Bilzer, M., Roggel, F. & Gerbes, A.L. Role of Kupffer cells in host defense and liver disease. *Liver Int* **26**, 1175-1186 (2006).

289. Doherty, D.G. Immunity, tolerance and autoimmunity in the liver: A comprehensive review. *J Autoimmun* **66**, 60-75 (2016).

290. Rahman, A.H. & Aloman, C. Dendritic cells and liver fibrosis. *Biochim Biophys Acta* **1832**, 998-1004 (2013).

291. Crispe, I.N. Liver antigen-presenting cells. *J Hepatol* **54**, 357-365 (2011).

292. Norris, S., Collins, C., Doherty, D.G., Smith, F., McEntee, G., Traynor, O., Nolan, N., Hegarty, J. & O'Farrelly, C. Resident human hepatitis lymphocytes are phenotypically different from circulating lymphocytes. *J Hepatol* **28**, 84-90 (1998).

293. Warren, A., Le Couteur, D.G., Fraser, R., Bowen, D.G., McCaughan, G.W. & Bertolino, P. T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells. *Hepatology* **44**, 1182-1190 (2006).

294. Wong, Y.C., Tay, S.S., McCaughan, G.W., Bowen, D.G. & Bertolino, P. Immune outcomes in the liver: Is CD8 T cell fate determined by the environment? *J Hepatol* **63**, 1005-1014 (2015).

295. Crispe, I.N. Hepatic T cells and liver tolerance. *Nat Rev Immunol* **3**, 51-62 (2003).

296. Holz, L.E., Warren, A., Le Couteur, D.G., Bowen, D.G. & Bertolino, P. CD8+ T cell tolerance following antigen recognition on hepatocytes. *J Autoimmun* **34**, 15-22 (2010).

297. Lalor, P.F., Lai, W.K., Curbishley, S.M., Shetty, S. & Adams, D.H. Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo. *World J Gastroenterol* **12**, 5429-5439 (2006).

298. Gale, R.P. Development of the immune system in human fetal liver. *Thymus* **10**, 45-56 (1987).

299. Payushina, O.V. Hematopoietic Microenvironment in the Fetal Liver: Roles of Different Cell Populations. *ISRN Cell Biology* **2012**, 1-7 (2012).

300. Golden-Mason, L. & O'Farrelly, C. Having it all? Stem cells, haematopoiesis and lymphopoiesis in adult human liver. *Immunol Cell Biol* **80**, 45-51 (2002).

301. Bertolino, P., Bowen, D.G., McCaughan, G.W. & Fazekas de St. Groth, B. Antigen-Specific Primary Activation of CD8+ T Cells Within the Liver. *J Immunol* **166**, 5430-5438 (2001).

302. Tay, S.S., Wong, Y.C., McDonald, D.M., Wood, N.A., Roediger, B., Sierro, F., McGuffog, C., Alexander, I.E., Bishop, G.A., Gamble, J.R., Weninger, W., McCaughan, G.W., Bertolino, P. & Bowen, D.G. Antigen expression level threshold tunes the fate of CD8 T cells during primary hepatic immune responses. *Proc Natl Acad Sci U S A* **111**, E2540-2549 (2014).

303. Tay, S.S., Wong, Y.C., Roediger, B., Sierro, F., Lu, B., McDonald, D.M., McGuffog, C.M., Meyer, N.J., Alexander, I.E., Parish, I.A., Heath, W.R., Weninger, W., Bishop, G.A., Gamble, J.R., McCaughan, G.W., Bertolino, P. & Bowen, D.G. Intrahepatic activation of naive CD4+ T cells by liver-resident phagocytic cells. *J Immunol* **193**, 2087-2095 (2014).

304. Stein-Streilein, J. & Caspi, R.R. Immune privilege and the philosophy of immunology. *Front Immunol* **5**, 110 (2014).

305. Forrester, J.V. & Xu, H. Good news-bad news: the Yin and Yang of immune privilege in the eye. *Front Immunol* **3**, 338 (2012).

306. Tiegs, G. & Lohse, A.W. Immune tolerance: what is unique about the liver. *J Autoimmun* (2010).

307. Calne, R.Y., Sells, R.A., Pena, J.R., Davis, D.R., Millard, P.R., Herbertson, B.M., Binns, R.M. & Davies, D.A. Induction of immunological tolerance by porcine liver allografts. *Nature* **223**, 472-476 (1969).

308. Benseler, V., McCaughan, G.W., Schlitt, H.J., Bishop, G.A., Bowen, D.G. & Bertolino, P. The liver: a special case in transplantation tolerance. *Semin Liver Dis* **27**, 194-213 (2007).

309. Yang, R., Liu, Q., Grosfeld, J.L. & Pescovitz, M.D. Intestinal venous drainage through the liver is a prerequisite for oral tolerance induction. *J Pediatr Surg* **29**, 1145-1148 (1994).

310. Carambia, A. & Herkel, J. CD4 T cells in hepatic immune tolerance. *J Autoimmun* **34**, 23-28 (2010).

311. Crispe, I.N. Immune tolerance in liver disease. *Hepatology* **60**, 2109-2117 (2014).

312. Bamboat, Z.M., Stableford, J.A., Plitas, G., Burt, B.M., Nguyen, H.M., Welles, A.P., Gonen, M., Young, J.W. & DeMatteo, R.P. Human Liver Dendritic Cells Promote T Cell Hyporesponsiveness. *The J Immunol* **182**, 1901-1911 (2009).

313. Goddard, S., Youster, J., Morgan, E. & Adams, D.H. Interleukin-10 secretion differentiates dendritic cells from human liver and skin. *Am J Pathol* **164**, 511-519 (2004).

314. Klugewitz, K., Adams, D.H., Emoto, M., Eulenburg, K. & Hamann, A. The composition of intrahepatic lymphocytes: shaped by selective recruitment? *Trends Immunol* **25**, 590-594 (2004).

315. Limmer, A., Ohl, J., Kurts, C., Ljunggren, H.G., Reiss, Y., Groettrup, M., Momburg, F., Arnold, B. & Knolle, P.A. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med* **6**, 1348-1354 (2000).

316. Diehl, L., Schurich, A., Grochtmann, R., Hegenbarth, S., Chen, L. & Knolle, P.A. Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. *Hepatology* **47**, 296-305 (2008).

317. Knolle, P.A., Germann, T., Treichel, U., Uhrig, A., Schmitt, E., Hegenbarth, S., Lohse, A.W. & Gerken, G. Endotoxin down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells. *J Immunol* **162**, 1401-1407 (1999).

318. Jiang, G., Yang, H.R., Wang, L., Wildey, G.M., Fung, J., Qian, S. & Lu, L. Hepatic stellate cells preferentially expand allogeneic CD4+ CD25+ FoxP3+ regulatory T cells in an IL-2-dependent manner. *Transplantation* **86**, 1492-1502 (2008).

319. Dunham, R.M., Thapa, M., Velazquez, V.M., Elrod, E.J., Denning, T.L., Pulendran, B. & Grakoui, A. Hepatic stellate cells preferentially induce Foxp3+ regulatory T cells by production of retinoic acid. *J Immunol* **190**, 2009-2016 (2013).

320. Katz, S.C., Pillarisetty, V.G., Bleier, J.I., Shah, A.B. & DeMatteo, R.P. Liver sinusoidal endothelial cells are insufficient to activate T cells. *J Immunol* **173**, 230-235 (2004).

321. Leon, M.P., Bassendine, M.F., Wilson, J.L., Ali, S., Thick, M. & Kirby, J.A. Immunogenicity of biliary epithelium: investigation of antigen presentation to CD4+ T cells. *Hepatology* **24**, 561-567 (1996).

322. Burghardt, S., Erhardt, A., Claass, B., Huber, S., Adler, G., Jacobs, T., Chalaris, A., Schmidt-Arras, D., Rose-John, S., Karimi, K. & Tiegs, G. Hepatocytes contribute to immune regulation in the liver by activation of the Notch signaling pathway in T cells. *J Immunol* **191**, 5574-5582 (2013).

323. Pallett, L.J., Gill, U.S., Quaglia, A., Sinclair, L.V., Jover-Cobos, M., Schurich, A., Singh, K.P., Thomas, N., Das, A., Chen, A., Fusai, G., Bertoletti, A., Cantrell, D.A., Kennedy, P.T., Davies, N.A., Haniffa, M. & Maini, M.K. Metabolic regulation of hepatitis B immunopathology by myeloid-derived suppressor cells. *Nat Med* **21**, 591-600 (2015).

324. Chen, S., Akbar, S.M., Abe, M., Hiasa, Y. & Onji, M. Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus. *Clin Exp Immunol* **166**, 134-142 (2011).

325. Huang, L.R., Wohlleber, D., Reisinger, F., Jenne, C.N., Cheng, R.L., Abdullah, Z., Schildberg, F.A., Odenthal, M., Dienes, H.P., van Rooijen, N., Schmitt, E., Garbi, N., Croft, M., Kurts, C., Kubes, P., Protzer, U., Heikenwalder, M. & Knolle, P.A. Intrahepatic myeloid-cell aggregates enable local proliferation of CD8(+) T cells and successful immunotherapy against chronic viral liver infection. *Nat Immunol* **14**, 574-583 (2013).

326. Katz, S.C., Pillarisetty, V.G., Bleier, J.I., Kingham, T.P., Chaudhry, U.I., Shah, A.B. & DeMatteo, R.P. Conventional liver CD4 T cells are functionally distinct and suppressed by environmental factors. *Hepatology* **42**, 293-300 (2005).

327. Jinushi, M., Takehara, T., Tatsumi, T., Yamaguchi, S., Sakamori, R., Hiramatsu, N., Kanto, T., Ohkawa, K. & Hayashi, N. Natural killer cell and hepatic cell interaction via NKG2A leads to dendritic cell-mediated induction of CD4 CD25 T cells with PD-1-dependent regulatory activities. *Immunology* **120**, 73-82 (2007).

328. Peppa, D., Gill, U.S., Reynolds, G., Easom, N.J., Pallett, L.J., Schurich, A., Micco, L., Nebbia, G., Singh, H.D., Adams, D.H., Kennedy, P.T. & Maini, M.K. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. *J Exp Med* **210**, 99-114 (2013).

329. Crome, S.Q., Lang, P.A., Lang, K.S. & Ohashi, P.S. Natural killer cells regulate diverse T cell responses. *Trends Immunol* **34**, 342-349 (2013).

330. Sun, H., Sun, C., Tian, Z. & Xiao, W. NK cells in immunotolerant organs. *Cell Mol Immunol* **10**, 202-212 (2013).

331. Exley, M.A. & Koziel, M.J. To be or not to be NKT: natural killer T cells in the liver. *Hepatology* **40**, 1033-1040 (2004).

332. Herkel, J., Jagemann, B., Wiegard, C., Lazaro, J.F., Lueth, S., Kanzler, S., Blessing, M., Schmitt, E. & Lohse, A.W. MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocyutes. *Hepatology* **37**, 1079-1085 (2003).

333. Afford, S.C., Randhawa, S., Eliopoulos, A.G., Hubscher, S.G., Young, L.S. & Adams, D.H. CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. *J Exp Med* **189**, 441-446 (1999).

334. Lee, Y.C., Lu, L., Fu, F., Li, W., Thomson, A.W., Fung, J.J. & Qian, S. Hepatocytes and liver nonparenchymal cells induce apoptosis in activated T cells. *Transplant Proc* **31**, 784 (1999).

335. Bertolino, P., Trescol-Biemont, M.C. & Rabourdin-Combe, C. Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur J Immunol* **28**, 221-236 (1998).

336. Bertolino, P., Trescol-Biémont, M.-C., Thomas, J., de St Groth, B.F., Pihlgren, M., Marvel, J. & Rabourdin-Combe, C. Death by neglect as a deletional mechanism of peripheral tolerance. *International Immunol* **11**, 1225-1238 (1999).

337. Bowen, D.G., Zen, M., Holz, L., Davis, T., McCaughan, G.W. & Bertolino, P. The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *J Clin Invest* **114**, 701-712 (2004).

338. Benseler, V., Warren, A., Vo, M., Holz, L.E., Tay, S.S., Le Couteur, D.G., Breen, E., Allison, A.C., van Rooijen, N., McGuffog, C., Schlitt, H.J., Bowen, D.G., McCaughan, G.W. & Bertolino, P. Hepatocyte entry leads to degradation of autoreactive CD8 T cells. *Proc Natl Acad Sci U S A* **108**, 16735-16740 (2011).

339. Huang, L., Soldevila, G., Leeker, M., Flavell, R. & Crispe, I.N. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. *Immunity* **1**, 741-749 (1994).

340. Mehal, W.Z., Juedes, A.E. & Crispe, I.N. Selective retention of activated CD8+ T cells by the normal liver. *J Immunol* **163**, 3202-3210 (1999).

341. Crispe, I.N., Dao, T., Klugewitz, K., Mehal, Wajahat Z. & Metz, D.P. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunol Rev* **174**, 47-62 (2000).

342. Tu, Z., Bozorgzadeh, A., Crispe, I.N. & Orloff, M.S. The activation state of human intrahepatic lymphocytes. *Clin Exp Immunol* **149**, 186-193 (2007).

343. Polakos, N.K., Cornejo, J.C., Murray, D.A., Wright, K.O., Treanor, J.J., Crispe, I.N., Topham, D.J. & Pierce, R.H. Kupffer cell-dependent hepatitis occurs during influenza infection. *Am J Pathol* **168**, 1169-1178; quiz 1404-1165 (2006).

344. Wiegard, C., Wolint, P., Frenzel, C., Cheruti, U., Schmitt, E., Oxenius, A., Lohse, A.W. & Herkel, J. Defective T helper response of hepatocyte-stimulated CD4 T cells impairs antiviral CD8 response and viral clearance. *Gastroenterology* **133**, 2010-2018 (2007).

345. Luth, S., Huber, S., Schramm, C., Buch, T., Zander, S., Stadelmann, C., Bruck, W., Wraith, D.C., Herkel, J. & Lohse, A.W. Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. *J Clin Invest* **118**, 3403-3410 (2008).

346. Sana, G., Lombard, C., Vosters, O., Jazouli, N., Andre, F., Stephenne, X., Smets, F., Najimi, M. & Sokal, E.M. Adult human hepatocytes promote CD4(+) T-cell hyporesponsiveness via interleukin-10-producing allogeneic dendritic cells. *Cell Transplant* **23**, 1127-1142 (2014).

347. Muhlbauer, M., Fleck, M., Schutz, C., Weiss, T., Froh, M., Blank, C., Scholmerich, J. & Hellerbrand, C. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J Hepatol* **45**, 520-528 (2006).

348. Wiegard, C., Frenzel, C., Herkel, J., Kallen, K.J., Schmitt, E. & Lohse, A.W. Murine liver antigen presenting cells control suppressor activity of CD4+CD25+ regulatory T cells. *Hepatology* **42**, 193-199 (2005).

349. Lang, K.S., Georgiev, P., Recher, M., Navarini, A.A., Bergthaler, A., Heikenwalder, M., Harris, N.L., Junt, T., Odermatt, B., Clavien, P.A., Pircher, H., Akira, S., Hengartner, H. & Zinkernagel, R.M. Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling. *J Clin Invest* **116**, 2456-2463 (2006).

350. Derkow, K., Loddenkemper, C., Mintern, J., Kruse, N., Klugewitz, K., Berg, T., Wiedenmann, B., Ploegh, H.L. & Schott, E. Differential priming of CD8 and CD4 T-cells in animal models of autoimmune hepatitis and cholangitis. *Hepatology* **46**, 1155-1165 (2007).

351. Golden-Mason, L., Palmer, B., Klarquist, J., Mengshol, J.A., Castelblanco, N. & Rosen, H.R. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* **81**, 9249-9258 (2007).

352. Gardiner, D., Lalezari, J., Lawitz, E., DiMicco, M., Ghalib, R., Reddy, K.R., Chang, K.M., Sulkowski, M., Marro, S.O., Anderson, J., He, B., Kansra, V., McPhee, F., Wind-Rotolo, M., Grasela, D., Selby, M., Korman, A.J. & Lowy, I. A randomized, double-blind, placebo-controlled assessment of BMS-936558, a fully human monoclonal antibody to programmed death-1 (PD-1), in patients with chronic hepatitis C virus infection. *PLOS ONE* **8**, e63818 (2013).

353. Oo, Y.H., Shetty, S. & Adams, D.H. The role of chemokines in the recruitment of lymphocytes to the liver. *Dig Dis* **28**, 31-44 (2010).

354. Rot, A. & von Andrian, U.H. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* **22**, 891-928 (2004).

355. Ley, K., Laudanna, C., Cybulsky, M.I. & Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* **7**, 678-689 (2007).

356. Shetty, S., Lalor, P.F. & Adams, D.H. Lymphocyte recruitment to the liver: Molecular insights into the pathogenesis of liver injury and hepatitis. *Toxicology* **254**, 136-146 (2008).

357. Lee, W.Y. & Kubes, P. Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs. *J Hepatol* **48**, 504-512 (2008).

358. Steinhoff, G., Behrend, M., Schrader, B. & Pichlmayr, R. Intercellular immune adhesion molecules in human liver transplants: overview on expression patterns of leukocyte receptor and ligand molecules. *Hepatology* **18**, 440-453 (1993).

359. Adams, D.H., Hubscher, S.G., Fisher, N.C., Williams, A. & Robinson, M. Expression of E-selectin and E-selectin ligands in human liver inflammation. *Hepatology* **24**, 533-538 (1996).

360. Lalor, P.F., Shields, P., Grant, A. & Adams, D.H. Recruitment of lymphocytes to the human liver. *Immunol Cell Biol* **80**, 52-64 (2002).

361. Wong, J., Johnston, B., Lee, S.S., Bullard, D.C., Smith, C.W., Beaudet, A.L. & Kubes, P. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. *J Clin Invest* **99**, 2782-2790 (1997).

362. Adams, D.H., Ju, C., Ramaiah, S.K., Uetrecht, J. & Jaeschke, H. Mechanisms of immune-mediated liver injury. *Toxicol Sci* **115**, 307-321 (2010).

363. Liaskou, E. MAdCAM-1 Expression and Function in Human Liver. Doctor of Philosophy thesis, University of Birmingham, School of Immunology, Infection and Inflammation, 2010.

364. Lalor, P.F., Edwards, S., McNab, G., Salmi, M., Jalkanen, S. & Adams, D.H. Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. *J Immunol* **169**, 983-992 (2002).

365. Jung, M.-Y., Park, S.-Y. & Kim, I.-S. Stabilin-2 is involved in lymphocyte adhesion to the hepatic sinusoidal endothelium via the interaction with alphaMbeta2 integrin. *Journal of Leukocyte Biology* **82**, 1156-1165 (2007).

366. Ma, L., Cheung, K.C., Kishore, M., Nourshargh, S., Mauro, C. & Marelli-Berg, F.M. CD31 exhibits multiple roles in regulating T lymphocyte trafficking in vivo. *J Immunol* **189**, 4104-4111 (2012).

367. Trivedi, P.J. & Adams, D.H. Mucosal immunity in liver autoimmunity: a comprehensive review. *J Autoimmun* **46**, 97-111 (2013).

368. Salmi, M., Koskinen, K., Henttinen, T., Elima, K. & Jalkanen, S. CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood* **104**, 3849-3857 (2004).

369. Shetty, S., Weston, C.J., Oo, Y.H., Westerlund, N., Stamataki, Z., Youster, J., Hubscher, S.G., Salmi, M., Jalkanen, S., Lalor, P.F. & Adams, D.H. Common lymphatic endothelial and vascular

endothelial receptor-1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal endothelium. *J Immunol* **186**, 4147-4155 (2011).

370. McNab, G., Reeves, J.L., Salmi, M., Hubscher, S., Jalkanen, S. & Adams, D.H. Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium. *Gastroenterology* **110**, 522-528 (1996).

371. Yoong, K.F., McNab, G., Hubscher, S.G. & Adams, D.H. Vascular adhesion protein-1 and ICAM-1 support the adhesion of tumor-infiltrating lymphocytes to tumor endothelium in human hepatocellular carcinoma. *J Immunol* **160**, 3978-3988 (1998).

372. Bonder, C.S., Norman, M.U., Swain, M.G., Zbytnuik, L.D., Yamanouchi, J., Santamaria, P., Ajuebor, M., Salmi, M., Jalkanen, S. & Kubes, P. Rules of recruitment for Th1 and Th2 lymphocytes in inflamed liver: a role for alpha-4 integrin and vascular adhesion protein-1. *Immunity* **23**, 153-163 (2005).

373. Lalor, P.F., Sun, P.J., Weston, C.J., Martin-Santos, A., Wakelam, M.J. & Adams, D.H. Activation of vascular adhesion protein-1 on liver endothelium results in an NF-kappaB-dependent increase in lymphocyte adhesion. *Hepatology* **45**, 465-474 (2007).

374. Jalkanen, S., Karikoski, M., Mercier, N., Koskinen, K., Henttinen, T., Elima, K., Salmivirta, K. & Salmi, M. The oxidase activity of vascular adhesion protein-1 (VAP-1) induces endothelial E- and P-selectins and leukocyte binding. *Blood* **110**, 1864-1870 (2007).

375. Liaskou, E., Karikoski, M., Reynolds, G.M., Lalor, P.F., Weston, C.J., Pullen, N., Salmi, M., Jalkanen, S. & Adams, D.H. Regulation of mucosal addressin cell adhesion molecule 1 expression in human and mice by vascular adhesion protein 1 amine oxidase activity. *Hepatology* **53**, 661-672 (2011).

376. Trivedi, P.J., Tickle, J., Vesterhus, M.N., Eddowes, P.J., Bruns, T., Vainio, J., Parker, R., Smith, D., Liaskou, E., Thorbjornsen, L.W., Hirschfield, G.M., Auvinen, K., Hubscher, S.G., Salmi, M., Adams, D.H. & Weston, C.J. Vascular adhesion protein-1 is elevated in primary sclerosing cholangitis, is predictive of clinical outcome and facilitates recruitment of gut-tropic lymphocytes to liver in a substrate-dependent manner. *Gut* **67**, 1135-1145 (2018).

377. Kurkijarvi, R., Adams, D.H., Leino, R., Mottonen, T., Jalkanen, S. & Salmi, M. Circulating form of human vascular adhesion protein-1 (VAP-1): increased serum levels in inflammatory liver diseases. *J Immunol* **161**, 1549-1557 (1998).

378. Lalor, P.F., Tuncer, C., Weston, C., Martin-Santos, A., Smith, D.J. & Adams, D.H. Vascular adhesion protein-1 as a potential therapeutic target in liver disease. *Ann N Y Acad Sci* **1110**, 485-496 (2007).

379. Trivedi, P.J., Weston, C., Corbett, C., Liaskou, E. & Adams, D. PWE-124 Vap-1 Activity is Elevated in PSC and Modulates A4B7-Dependent Lymphocyte Adhesion to HSEC Under Flow. *Gut* **62**, A181.181-A181 (2013).

380. Claridge, L.C., Weston, C.J., Haughton, E.L., Westerlund, N., Reynolds, G.M., Lalor, P.F., Tomlinson, J.W., Smith, D.J., Day, C.P. & Adams, D.H. P87 Vascular Adhesion Protein-1 promotes increases in liver infiltrating CD4+ T cells and NKT cells and induction of fibrogenesis in steatohepatitis. *Gut* **60**, A40-A40 (2011).

381. Weston, C.J., Shepherd, E.L., Claridge, L.C., Rantakari, P., Curbishley, S.M., Tomlinson, J.W., Hubscher, S.G., Reynolds, G.M., Aalto, K., Anstee, Q.M., Jalkanen, S., Salmi, M., Smith, D.J., Day, C.P. & Adams, D.H. Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis. *J Clin Invest* **125**, 501-520 (2015).

382. Shields, P.L., Morland, C.M., Salmon, M., Qin, S., Hubscher, S.G. & Adams, D.H. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. *J Immunol*, 6236-6243 (1999).

383. Kunkel, E.J., Boisvert, J., Murphy, K., Vierra, M.A., Genovese, M.C., Wardlaw, A.J., Greenberg, H.B., Hodge, M.R., Wu, L., Butcher, E.C. & Campbell, J.J. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am J Pathol* **160**, 347-355 (2002).

384. Boisvert, J., Kunkel, E.J., Campbell, J.J., Keeffe, E.B., Butcher, E.C. & Greenberg, H.B. Liver-infiltrating lymphocytes in end-stage hepatitis C virus: subsets, activation status, and chemokine receptor phenotypes. *J Hepatol* **38**, 67-75 (2003).

385. Curbishley, S.M., Eksteen, B., Gladue, R.P., Lalor, P. & Adams, D.H. CXCR3 Activation Promotes Lymphocyte Transendothelial Migration across Human Hepatic Endothelium under Fluid Flow. *The Am J Pathol* **167**, 887-899 (2005).

386. Chuang, Y.H., Lian, Z.X., Cheng, C.M., Lan, R.Y., Yang, G.X., Moritoki, Y., Chiang, B.L., Ansari, A.A., Tsuneyama, K., Coppel, R.L. & Gershwin, M.E. Increased levels of chemokine receptor CXCR3 and chemokines IP-10 and MIG in patients with primary biliary cirrhosis and their first degree relatives. *J Autoimmun* **25**, 126-132 (2005).

387. Holt, A.P., Salmon, M., Buckley, C.D. & Adams, D.H. Immune interactions in hepatic fibrosis. *Clin Liver Dis* **12**, 861-882, x (2008).

388. Oo, Y.H., Banz, V., Kavanagh, D., Liaskou, E., Withers, D.R., Humphreys, E., Reynolds, G.M., Lee-Turner, L., Kalia, N., Hubscher, S.G., Klenerman, P., Eksteen, B. & Adams, D.H. CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J Hepatol* **57**, 1044-1051 (2012).

389. Eksteen, B., Miles, A., Curbishley, S.M., Tselepis, C., Grant, A.J., Walker, L.S.K. & Adams, D.H. Epithelial Inflammation Is Associated with CCL28 Production and the Recruitment of Regulatory T Cells Expressing CCR10. *J Immunol* **177**, 593-603 (2006).

390. Oo, Y.H., Weston, C.J., Lalor, P.F., Curbishley, S.M., Withers, D.R., Reynolds, G.M., Shetty, S., Harki, J., Shaw, J.C., Eksteen, B., Hubscher, S.G., Walker, L.S. & Adams, D.H. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. *J Immunol* **184**, 2886-2898 (2010).

391. Heydtmann, M., Lalor, P.F., Eksteen, J.A., Hubscher, S.G., Briskin, M. & Adams, D.H. CXC Chemokine Ligand 16 Promotes Integrin-Mediated Adhesion of Liver-Infiltrating Lymphocytes to Cholangiocytes and Hepatocytes within the Inflamed Human Liver. *J Immunol* **174**, 1055-1062 (2005).

392. Heydtmann, M., Hardie, D., Shields, P.L., Faint, J., Buckley, C.D., Campbell, J.J., Salmon, M. & Adams, D.H. Detailed analysis of intrahepatic CD8 T cells in the normal and hepatitis C-infected liver reveals differences in specific populations of memory cells with distinct homing phenotypes. *J Immunol* **177**, 729-738 (2006).

393. Tse, S.-W., Radtke, A.J., Espinosa, D.A., Cockburn, I.A. & Zavala, F. The chemokine receptor CXCR6 is required for the maintenance of liver memory CD8+ T cells specific for infectious pathogens. *J Infect Dis* **210**, 1508-1516 (2014).

394. Germanov, E., Veinotte, L., Cullen, R., Chamberlain, E., Butcher, E.C. & Johnston, B. Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. *J Immunol* **181**, 81-91 (2008).

395. Stegmann, K.A., Robertson, F., Hansi, N., Gill, U., Pallant, C., Christophides, T., Pallett, L.J., Peppa, D., Dunn, C., Fusai, G., Male, V., Davidson, B.R., Kennedy, P. & Maini, M.K. CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. *Sci Rep* **6**, 26157 (2016).

396. Clarke, C., Kuboki, S., Sakai, N., Kasten, K.R., Tevar, A.D., Schuster, R., Blanchard, J., Caldwell, C.C., Edwards, M.J. & Lentsch, A.B. CXC chemokine receptor-1 is expressed by hepatocytes and regulates liver recovery after hepatic ischemia/reperfusion injury. *Hepatology* **53**, 261-271 (2011).

397. Terada, R., Yamamoto, K., Hakoda, T., Shimada, N., Okano, N., Baba, N., Ninomiya, Y., Gershwin, M.E. & Shiratori, Y. Stromal cell-derived factor-1 from biliary epithelial cells recruits CXCR4-positive cells: implications for inflammatory liver diseases. *Lab Invest* **83**, 665-672 (2003).

398. Efsen, E., Grappone, C., DeFranco, R.M., Milani, S., Romanelli, R.G., Bonacchi, A., Caligiuri, A., Failli, P., Annunziato, F., Pagliai, G., Pinzani, M., Laffi, G., Gentilini, P. & Marra, F. Up-regulated expression of fractalkine and its receptor CX3CR1 during liver injury in humans. *J Hepatol* **37**, 39-47 (2002).

399. Isse, K., Harada, K., Zen, Y., Kamihira, T., Shimoda, S., Harada, M. & Nakanuma, Y. Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts. *Hepatology* **41**, 506-516 (2005).

400. Grant, A.J., Goddard, S., Ahmed-Choudhury, J., Reynolds, G., Jackson, D.G., Briskin, M., Wu, L., Hubscher, S.G. & Adams, D.H. Hepatic expression of secondary lymphoid chemokine (CCL21) promotes the development of portal-associated lymphoid tissue in chronic inflammatory liver disease. *Am J Pathol* **160**, 1445-1455 (2002).

401. Adams, D.H. & Eksteen, B. Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. *Nat Rev Immunol* **6**, 244-251 (2006).

402. Hillan, K.J., Hagler, K.E., MacSween, R.N., Ryan, A.M., Renz, M.E., Chiu, H.H., Ferrier, R.K., Bird, G.L., Dhillon, A.P., Ferrell, L.D. & Fong, S. Expression of the mucosal vascular addressin, MAdCAM-1, in inflammatory liver disease. *Liver* **19**, 509-518 (1999).

403. Grant, A.J., Lalor, P.F., Hubscher, S.G., Briskin, M. & Adams, D.H. MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). *Hepatology* **33**, 1065-1072 (2001).

404. Eksteen, B., Grant, A.J., Miles, A., Curbishley, S.M., Lalor, P.F., Hubscher, S.G., Briskin, M., Salmon, M. & Adams, D.H. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. *J Exp Med* **200**, 1511-1517 (2004).

405. Neumann, K., Kruse, N., Szilagyi, B., Erben, U., Rudolph, C., Flach, A., Zeitz, M., Hamann, A. & Klugewitz, K. Connecting liver and gut: murine liver sinusoidal endothelium induces gut tropism of CD4+ T cells via retinoic acid. *Hepatology* **55**, 1976-1984 (2012).

406. Guidotti, L.G., Inverso, D., Sironi, L., Di Lucia, P., Fioravanti, J., Ganzer, L., Fiocchi, A., Vacca, M., Aiolfi, R., Sammicheli, S., Mainetti, M., Cataudella, T., Raimondi, A., Gonzalez-Aseguinolaza, G., Protzer, U., Ruggeri, Z.M., Chisari, F.V., Isogawa, M., Sitia, G. & Iannacone, M. Immunosurveillance of the liver by intravascular effector CD8(+) T cells. *Cell* **161**, 486-500 (2015).

407. Inverso, D. & Iannacone, M. Spatiotemporal dynamics of effector CD8+ T cell responses within the liver. *J Leukoc Biol* **99**, 51-55 (2016).

408. Bertolino, P., Schrage, A., Bowen, D.G., Klugewitz, K., Ghani, S., Eulenburg, K., Holz, L., Hogg, N., McCaughan, G.W. & Hamann, A. Early intrahepatic antigen-specific retention of naive CD8+ T cells is predominantly ICAM-1/LFA-1 dependent in mice. *Hepatology* **42**, 1063-1071 (2005).

409. Pellicoro, A., Ramachandran, P., Iredale, J.P. & Fallowfield, J.A. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol* **14**, 181-194 (2014).

410. Lan, R.Y., Cheng, C., Lian, Z.X., Tsuneyama, K., Yang, G.X., Moritoki, Y., Chuang, Y.H., Nakamura, T., Saito, S., Shimoda, S., Tanaka, A., Bowlus, C.L., Takano, Y., Ansari, A.A., Coppel,

R.L. & Gershwin, M.E. Liver-targeted and peripheral blood alterations of regulatory T cells in primary biliary cirrhosis. *Hepatology* **43**, 729-737 (2006).

411. Wang, D., Zhang, H., Liang, J., Gu, Z., Zhou, Q., Fan, X., Hou, Y. & Sun, L. CD4+ CD25+ but not CD4+ Foxp3+ T cells as a regulatory subset in primary biliary cirrhosis. *Cell Mol Immunol* **7**, 485-490 (2010).

412. Lan, R.Y., Salunga, T.L., Tsuneyama, K., Lian, Z.X., Yang, G.X., Hsu, W., Moritoki, Y., Ansari, A.A., Kemper, C., Price, J., Atkinson, J.P., Coppel, R.L. & Gershwin, M.E. Hepatic IL-17 responses in human and murine primary biliary cirrhosis. *J Autoimmun* **32**, 43-51 (2009).

413. Zhao, L., Tang, Y., You, Z., Wang, Q., Liang, S., Han, X., Qiu, D., Wei, J., Liu, Y., Shen, L., Chen, X., Peng, Y., Li, Z. & Ma, X. Interleukin-17 contributes to the pathogenesis of autoimmune hepatitis through inducing hepatic interleukin-6 expression. *PLOS ONE* **6**, e18909 (2011).

414. Liaskou, E., Hirschfield, G.M. & Gershwin, M.E. Mechanisms of tissue injury in autoimmune liver diseases. *Semin Immunopathol* **36**, 553-568 (2014).

415. Muratori, L. & Longhi, M.S. The interplay between regulatory and effector T cells in autoimmune hepatitis: Implications for innovative treatment strategies. *J Autoimmun* **46**, 74-80 (2013).

416. Odin, J.A., Huebert, R.C., Casciola-Rosen, L., LaRusso, N.F. & Rosen, A. Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis. *J Clin Invest* **108**, 223-232 (2001).

417. Lleo, A., Selmi, C., Invernizzi, P., Podda, M., Coppel, R.L., Mackay, I.R., Gores, G.J., Ansari, A.A., Van de Water, J. & Gershwin, M.E. Apotopes and the biliary specificity of primary biliary cirrhosis. *Hepatology* **49**, 871-879 (2009).

418. Lleo, A., Bowlus, C.L., Yang, G.X., Invernizzi, P., Podda, M., Van de Water, J., Ansari, A.A., Coppel, R.L., Worman, H.J., Gores, G.J. & Gershwin, M.E. Biliary apotopes and anti-mitochondrial antibodies activate innate immune responses in primary biliary cirrhosis. *Hepatology* **52**, 987-998 (2010).

419. Bogdanos, D.P., Baum, H., Grasso, A., Okamoto, M., Butler, P., Ma, Y., Rigopoulou, E., Montalto, P., Davies, E.T., Burroughs, A.K. & Vergani, D. Microbial mimics are major targets of crossreactivity with human pyruvate dehydrogenase in primary biliary cirrhosis. *J Hepatol* **40**, 31-39 (2004).

420. Padgett, K.A., Selmi, C., Kenny, T.P., Leung, P.S., Balkwill, D.L., Ansari, A.A., Coppel, R.L. & Gershwin, M.E. Phylogenetic and immunological definition of four lipoylated proteins from Novosphingobium aromaticivorans, implications for primary biliary cirrhosis. *J Autoimmun* **24**, 209-219 (2005).

421. Rieger, R. & Gershwin, M.E. The X and why of xenobiotics in primary biliary cirrhosis. *J Autoimmun* **28**, 76-84 (2007).

422. Lohr, H.F., Schlaak, J.F., Lohse, A.W., Bocher, W.O., Arenz, M., Gerken, G. & Meyer Zum Buschenfelde, K.H. Autoreactive CD4+ LKM-specific and anticlonotypic T-cell responses in LKM-1 antibody-positive autoimmune hepatitis. *Hepatology* **24**, 1416-1421 (1996).

423. Martinez, O.M., Villanueva, J.C., Gershwin, M.E. & Krams, S.M. Cytokine patterns and cytotoxic mediators in primary biliary cirrhosis. *Hepatology* **21**, 113-119 (1995).

424. Yamashiki, M., Kosaka, Y., Nishimura, A., Watanabe, S., Nomoto, M. & Ichida, F. Analysis of serum cytokine levels in primary biliary cirrhosis patients and healthy adults. *J Clin Lab Anal* **12**, 77-82 (1998).

425. Nagano, T., Yamamoto, K., Matsumoto, S., Okamoto, R., Tagashira, M., Ibuki, N., Matsumura, S., Yabushita, K., Okano, N. & Tsuji, T. Cytokine profile in the liver of primary biliary cirrhosis. *J Clin Immunol* **19**, 422-427 (1999).

426. Shindo, M., Mullin, G.E., Braun-Elwert, L., Bergasa, N.V., Jones, E.A. & James, S.P. Cytokine mRNA expression in the liver of patients with primary biliary cirrhosis (PBC) and chronic hepatitis B (CHB). *Clin Exp Immunol* **105**, 254-259 (1996).

427. Harada, K., Van de Water, J., Leung, P.S., Coppel, R.L., Ansari, A., Nakanuma, Y. & Gershwin, M.E. In situ nucleic acid hybridization of cytokines in primary biliary cirrhosis: predominance of the Th1 subset. *Hepatology* **25**, 791-796 (1997).

428. Bo, X., Broome, U., Remberger, M. & Sumitran-Holgersson, S. Tumour necrosis factor alpha impairs function of liver derived T lymphocytes and natural killer cells in patients with primary sclerosing cholangitis. *Gut* **49**, 131-141 (2001).

429. Rong, G., Zhou, Y., Xiong, Y., Zhou, L., Geng, H., Jiang, T., Zhu, Y., Lu, H., Zhang, S., Wang, P., Zhang, B. & Zhong, R. Imbalance between T helper type 17 and T regulatory cells in patients with primary biliary cirrhosis: the serum cytokine profile and peripheral cell population. *Clin Exp Immunol* **156**, 217-225 (2009).

430. Janse, M., Lamberts, L.E., Franke, L., Raychaudhuri, S., Ellinghaus, E., Muri Boberg, K., Melum, E., Folseraas, T., Schrumpf, E., Bergquist, A., Bjornsson, E., Fu, J., Jan Westra, H., Groen, H.J., Fehrmann, R.S., Smolonska, J., van den Berg, L.H., Ophoff, R.A., Porte, R.J., Weismuller, T.J., Wedemeyer, J., Schramm, C., Sterneck, M., Gunther, R., Braun, F., Vermeire, S., Henckaerts, L., Wijmenga, C., Ponsioen, C.Y., Schreiber, S., Karlsen, T.H., Franke, A. & Weersma, R.K. Three ulcerative colitis susceptibility loci are associated with primary sclerosing cholangitis and indicate a role for IL2, REL, and CARD9. *Hepatology* **53**, 1977-1985 (2011).

431. Katt, J., Schwinge, D., Schoknecht, T., Quaas, A., Sobottka, I., Burandt, E., Becker, C., Neurath, M.F., Lohse, A.W., Herkel, J. & Schramm, C. Increased T helper type 17 response to pathogen stimulation in patients with primary sclerosing cholangitis. *Hepatology* **58**, 1084-1093 (2013).

432. Longhi, M.S., Ma, Y., Bogdanos, D.P., Cheeseman, P., Mieli-Vergani, G. & Vergani, D. Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease. *J Hepatol* **41**, 31-37 (2004).

433. Longhi, M.S., Ma, Y., Mitry, R.R., Bogdanos, D.P., Heneghan, M., Cheeseman, P., Mieli-Vergani, G. & Vergani, D. Effect of CD4+ CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. *J Autoimmun* **25**, 63-71 (2005).

434. Peiseler, M., Sebode, M., Franke, B., Wortmann, F., Schwinge, D., Quaas, A., Baron, U., Olek, S., Wiegard, C., Lohse, A.W., Weiler-Normann, C., Schramm, C. & Herkel, J. FOXP3+ regulatory T cells in autoimmune hepatitis are fully functional and not reduced in frequency. *J Hepatol* **57**, 125-132 (2012).

435. Taubert, R., Hardtke-Wolenski, M., Noyan, F., Wilms, A., Baumann, A.K., Schlue, J., Olek, S., Falk, C.S., Manns, M.P. & Jaeckel, E. Intrahepatic regulatory T cells in autoimmune hepatitis are associated with treatment response and depleted with current therapies. *J Hepatol* **61**, 1106-1114 (2014).

436. Sebode, M., Peiseler, M., Franke, B., Schwinge, D., Schoknecht, T., Wortmann, F., Quaas, A., Petersen, B.S., Ellinghaus, E., Baron, U., Olek, S., Wiegard, C., Weiler-Normann, C., Lohse, A.W., Herkel, J. & Schramm, C. Reduced FOXP3(+) regulatory T cells in patients with primary sclerosing cholangitis are associated with IL2RA gene polymorphisms. *J Hepatol* **60**, 1010-1016 (2014).

437. Liaskou, E., Jeffery, L.E., Trivedi, P.J., Reynolds, G.M., Suresh, S., Bruns, T., Adams, D.H., Sansom, D.M. & Hirschfield, G.M. Loss of CD28 expression by liver-infiltrating T cells contributes to pathogenesis of primary sclerosing cholangitis. *Gastroenterology* **147**, 221-232 e227 (2014).

438. Isse, K., Harada, K., Sato, Y. & Nakanuma, Y. Characterization of biliary intra-epithelial lymphocytes at different anatomical levels of intrahepatic bile ducts under normal and pathological conditions: numbers of CD4+CD28- intra-epithelial lymphocytes are increased in primary biliary cirrhosis. *Pathol Int* **56**, 17-24 (2006).

439. Tsuda, M., Ambrosini, Y.M., Zhang, W., Yang, G.X., Ando, Y., Rong, G., Tsuneyama, K., Sumida, K., Shimoda, S., Bowlus, C.L., Leung, P.S., He, X.S., Coppel, R.L., Ansari, A.A., Lian, Z.X. & Gershwin, M.E. Fine phenotypic and functional characterization of effector cluster of differentiation 8 positive T cells in human patients with primary biliary cirrhosis. *Hepatology* **54**, 1293-1302 (2011).

440. Beier, J.I. & McClain, C.J. Mechanisms and cell signaling in alcoholic liver disease. *Biol Chem* **391**, 1249-1264 (2010).

441. Albano, E. & Vidali, M. Immune mechanisms in alcoholic liver disease. *Genes Nutr* **5**, 141-147 (2010).

442. Albano, E. Role of adaptive immunity in alcoholic liver disease. *Int J Hepatol* **2012**, 893026 (2012).

443. Duwaerts, C.C. & Maher, J.J. Mechanisms of Liver Injury in Non-Alcoholic Steatohepatitis. *Curr Hepatol Rep* **13**, 119-129 (2014).

444. Narayanan, S., Surette, F.A. & Hahn, Y.S. The Immune Landscape in Nonalcoholic Steatohepatitis. *Immune Netw* **16**, 147-158 (2016).

445. Sutti, S., Jindal, A., Bruzzi, S., Locatelli, I., Bozzola, C. & Albano, E. Is there a role for adaptive immunity in nonalcoholic steatohepatitis? *World J Hepatol* **7**, 1725-1729 (2015).

446. Matos, L.C., Batista, P., Monteiro, N., Ribeiro, J., Cipriano, M.A., Henriques, P., Girao, F. & Carvalho, A. Lymphocyte subsets in alcoholic liver disease. *World J Hepatol* **5**, 46-55 (2013).

447. McClain, C.J., Shedlofsky, S., Barve, S. & Hill, D.B. Cytokines and alcoholic liver disease. *Alcohol Health Res World* **21**, 317-320 (1997).

448. Song, K., Coleman, R.A., Alber, C., Ballas, Z.K., Waldschmidt, T.J., Mortari, F., LaBrecque, D.R. & Cook, R.T. TH1 cytokine response of CD57+ T-cell subsets in healthy controls and patients with alcoholic liver disease. *Alcohol* **24**, 155-167 (2001).

449. Lemmers, A., Moreno, C., Gustot, T., Maréchal, R., Degré, D., Demetter, P., de Nadai, P., Geerts, A., Quertinmont, E., Vercruysse, V., Le Moine, O. & Devière, J. The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology* **49**, 646-657 (2009).

450. Sutti, S., Jindal, A., Locatelli, I., Vacchiano, M., Gigliotti, L., Bozzola, C. & Albano, E. Adaptive immune responses triggered by oxidative stress contribute to hepatic inflammation in NASH. *Hepatology* **59**, 886-897 (2014).

451. Chackelevicius, C.M., Gambaro, S.E., Tiribelli, C. & Rosso, N. Th17 involvement in nonalcoholic fatty liver disease progression to non-alcoholic steatohepatitis. *World J Gastroenterol* **22**, 9096-9103 (2016).

452. Wolf, M.J., Adili, A., Piotrowitz, K., Abdullah, Z., Boege, Y., Stemmer, K., Ringelhan, M., Simonavicius, N., Egger, M., Wohlleber, D., Lorentzen, A., Einer, C., Schulz, S., Clavel, T., Protzer, U., Thiele, C., Zischka, H., Moch, H., Tschop, M., Tumanov, A.V., Haller, D., Unger, K., Karin, M., Kopf, M., Knolle, P., Weber, A. & Heikenwalder, M. Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. *Cancer Cell* **26**, 549-564 (2014).

453. Ma, C., Kesarwala, A.H., Eggert, T., Medina-Echeverz, J., Kleiner, D.E., Jin, P., Stroncek, D.F., Terabe, M., Kapoor, V., ElGindi, M., Han, M., Thornton, A.M., Zhang, H., Egger, M., Luo, J., Felsher, D.W., McVicar, D.W., Weber, A., Heikenwalder, M. & Greten, T.F. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. *Nature* **531**, 253-257 (2016).

454. Inzaugarat, M.E., Ferreyra Solari, N.E., Billordo, L.A., Abecasis, R., Gadano, A.C. & Chernavsky, A.C. Altered phenotype and functionality of circulating immune cells characterize adult patients with nonalcoholic steatohepatitis. *J Clin Immunol* **31**, 1120-1130 (2011).

455. Rau, M., Schilling, A.K., Meertens, J., Hering, I., Weiss, J., Jurowich, C., Kudlich, T., Hermanns, H.M., Bantel, H., Beyersdorf, N. & Geier, A. Progression from Nonalcoholic Fatty Liver to Nonalcoholic Steatohepatitis Is Marked by a Higher Frequency of Th17 Cells in the Liver and an Increased Th17/Resting Regulatory T Cell Ratio in Peripheral Blood and in the Liver. *J Immunol* **196**, 97-105 (2016).

456. Boujedidi, H., Robert, O., Bignon, A., Cassard-Doulcier, A.M., Renoud, M.L., Gary-Gouy, H., Hemon, P., Tharinger, H., Prevot, S., Bachelerie, F., Naveau, S., Emilie, D., Balabanian, K. & Perlemuter, G. CXCR4 dysfunction in non-alcoholic steatohepatitis in mice and patients. *Clin Sci (Lond)* **128**, 257-267 (2015).

457. Tang, Y., Bian, Z., Zhao, L., Liu, Y., Liang, S., Wang, Q., Han, X., Peng, Y., Chen, X., Shen, L., Qiu, D., Li, Z. & Ma, X. Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease. *Clin Exp Immunol* **166**, 281-290 (2011).

458. Rolla, S., Alchera, E., Imarisio, C., Bardina, V., Valente, G., Cappello, P., Mombello, C., Follenzi, A., Novelli, F. & Carini, R. The balance between IL-17 and IL-22 produced by liver-infiltrating T-helper cells critically controls NASH development in mice. *Clin Sci (Lond)* **130**, 193-203 (2016).

459. Giles, D.A., Moreno-Fernandez, M.E., Stankiewicz, T.E., Cappelletti, M., Huppert, S.S., Iwakura, Y., Dong, C., Shanmukhappa, S.K. & Divanovic, S. Regulation of Inflammation by IL-17A and IL-17F Modulates Non-Alcoholic Fatty Liver Disease Pathogenesis. *PLOS ONE* **11**, e0149783 (2016).

460. Liu, Y., She, W., Wang, F., Li, J., Wang, J. & Jiang, W. 3, 3'-Diindolylmethane alleviates steatosis and the progression of NASH partly through shifting the imbalance of Treg/Th17 cells to Treg dominance. *Int Immunopharmacol* **23**, 489-498 (2014).

461. Ferreyra Solari, N.E., Inzaugarat, M.E., Baz, P., De Matteo, E., Lezama, C., Galoppo, M., Galoppo, C. & Chernavsky, A.C. The role of innate cells is coupled to a Th1-polarized immune response in pediatric nonalcoholic steatohepatitis. *J Clin Immunol* **32**, 611-621 (2012).

462. Vonghia, L., Magrone, T., Verrijken, A., Michielsen, P., Van Gaal, L., Jirillo, E. & Francque, S. Peripheral and Hepatic Vein Cytokine Levels in Correlation with Non-Alcoholic Fatty Liver Disease (NAFLD)-Related Metabolic, Histological, and Haemodynamic Features. *PLOS ONE* **10**, e0143380 (2015).

463. Ma, X., Hua, J., Mohamood, A.R., Hamad, A.R., Ravi, R. & Li, Z. A high-fat diet and regulatory T cells influence susceptibility to endotoxin-induced liver injury. *Hepatology* **46**, 1519-1529 (2007).

464. Shin, E.C., Sung, P.S. & Park, S.H. Immune responses and immunopathology in acute and chronic viral hepatitis. *Nat Rev Immunol* **16**, 509-523 (2016).

465. Schmidt, J., Blum, H.E. & Thimme, R. T-cell responses in hepatitis B and C virus infection: similarities and differences. *Emerg Microbes Infect* **2**, e15 (2013).

466. Swadling, L., Capone, S., Antrobus, R.D., Brown, A., Richardson, R., Newell, E.W., Halliday, J., Kelly, C., Bowen, D., Fergusson, J., Kurioka, A., Ammendola, V., Del Sorbo, M., Grazioli, F., Esposito, M.L., Siani, L., Traboni, C., Hill, A., Colloca, S., Davis, M., Nicosia, A., Cortese, R., Folgori, A., Klenerman, P. & Barnes, E. A human vaccine strategy based on chimpanzee adenoviral and MVA vectors that primes, boosts, and sustains functional HCV-specific T cell memory. *Sci Transl Med* **6**, 261ra153 (2014).

467. Ermis, F. & Senocak Tasci, E. New treatment strategies for hepatitis C infection. *World J Hepatol* **7**, 2100-2109 (2015).

468. Asabe, S., Wieland, S.F., Chattopadhyay, P.K., Roederer, M., Engle, R.E., Purcell, R.H. & Chisari, F.V. The size of the viral inoculum contributes to the outcome of hepatitis B virus infection. *J Virol* **83**, 9652-9662 (2009).

469. Grakoui, A., Shoukry, N.H., Woollard, D.J., Han, J.H., Hanson, H.L., Ghrayeb, J., Murthy, K.K., Rice, C.M. & Walker, C.M. HCV persistence and immune evasion in the absence of memory T cell help. *Science* **302**, 659-662 (2003).

470. Thimme, R., Wieland, S., Steiger, C., Ghrayeb, J., Reimann, K.A., Purcell, R.H. & Chisari, F.V. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* **77**, 68-76 (2003).

471. Guidotti, L.G. & Chisari, F.V. Immunobiology and pathogenesis of viral hepatitis. *Annu Rev Pathol* **1**, 23-61 (2006).

472. Raziorrouh, B., Ulsenheimer, A., Schraut, W., Heeg, M., Kurktschiev, P., Zachoval, R., Jung, M.C., Thimme, R., Neumann-Haefelin, C., Horster, S., Wachtler, M., Spannagl, M., Haas, J., Diepolder, H.M. & Gruner, N.H. Inhibitory molecules that regulate expansion and restoration of HCV-specific CD4+ T cells in patients with chronic infection. *Gastroenterology* **141**, 1422-1431, 1431 e1421-1426 (2011).

473. Fisicaro, P., Valdatta, C., Massari, M., Loggi, E., Biasini, E., Sacchelli, L., Cavallo, M.C., Silini, E.M., Andreone, P., Missale, G. & Ferrari, C. Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. *Gastroenterology* **138**, 682-693, 693 e681-684 (2010).

474. Golden-Mason, L., Palmer, B.E., Kassam, N., Townshend-Bulson, L., Livingston, S., McMahon, B.J., Castelblanco, N., Kuchroo, V., Gretch, D.R. & Rosen, H.R. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* **83**, 9122-9130 (2009).

475. Nebbia, G., Peppa, D., Schurich, A., Khanna, P., Singh, H.D., Cheng, Y., Rosenberg, W., Dusheiko, G., Gilson, R., ChinAleong, J., Kennedy, P. & Maini, M.K. Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. *PLOS ONE* **7**, e47648 (2012).

476. Semmo, N., Day, C.L., Ward, S.M., Lucas, M., Harcourt, G., Loughry, A. & Klenerman, P. Preferential loss of IL-2-secreting CD4+ T helper cells in chronic HCV infection. *Hepatology* **41**, 1019-1028 (2005).

477. Gerlach, J.T., Diepolder, H.M., Jung, M.C., Gruener, N.H., Schraut, W.W., Zachoval, R., Hoffmann, R., Schirren, C.A., Santantonio, T. & Pape, G.R. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology* **117**, 933-941 (1999).

478. Chang, K.M., Thimme, R., Melpolder, J.J., Oldach, D., Pemberton, J., Moorhead-Loudis, J., McHutchison, J.G., Alter, H.J. & Chisari, F.V. Differential CD4(+) and CD8(+) T-cell responsiveness in hepatitis C virus infection. *Hepatology* **33**, 267-276 (2001).

479. Ulsenheimer, A., Gerlach, J.T., Gruener, N.H., Jung, M.C., Schirren, C.A., Schraut, W., Zachoval, R., Pape, G.R. & Diepolder, H.M. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology* **37**, 1189-1198 (2003).

480. Day, C.L., Lauer, G.M., Robbins, G.K., McGovern, B., Wurcel, A.G., Gandhi, R.T., Chung, R.T. & Walker, B.D. Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection. *J Virol* **76**, 12584-12595 (2002).

481. Day, C.L., Seth, N.P., Lucas, M., Appel, H., Gauthier, L., Lauer, G.M., Robbins, G.K., Szczepiorkowski, Z.M., Casson, D.R., Chung, R.T., Bell, S., Harcourt, G., Walker, B.D., Klenerman, P. & Wucherpfennig, K.W. Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J Clin Invest* **112**, 831-842 (2003).

482. Urbani, S., Amadei, B., Fisicaro, P., Tola, D., Orlandini, A., Sacchelli, L., Mori, C., Missale, G. & Ferrari, C. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. *Hepatology* **44**, 126-139 (2006).

483. Lucas, M., Ulsenheimer, A., Pfafferot, K., Heeg, M.H., Gaudieri, S., Gruner, N., Rauch, A., Gerlach, J.T., Jung, M.C., Zachoval, R., Pape, G.R., Schraut, W., Santantonio, T., Nitschko, H., Obermeier, M., Phillips, R., Scriba, T.J., Semmo, N., Day, C., Weber, J.N., Fidler, S., Thimme, R., Haberstroh, A., Baumert, T.F., Klenerman, P. & Diepolder, H.M. Tracking virus-specific CD4+ T cells during and after acute hepatitis C virus infection. *PLOS ONE* **2**, e649 (2007).

484. Mueller, M., Spangenberg, H.C., Kersting, N., Altay, T., Blum, H.E., Klenerman, P., Thimme, R. & Semmo, N. Virus-specific CD4+ T cell responses in chronic HCV infection in blood and liver identified by antigen-specific upregulation of CD154. *J Hepatol* **52**, 800-811 (2010).

485. Raziorrouh, B., Heeg, M., Kurktschiev, P., Schraut, W., Zachoval, R., Wendtner, C., Wachtler, M., Spannagl, M., Denk, G., Ulsenheimer, A., Bengsch, B., Pircher, H., Diepolder, H.M., Gruner, N.H. & Jung, M.C. Inhibitory phenotype of HBV-specific CD4+ T-cells is characterized by high PD-1 expression but absent coregulation of multiple inhibitory molecules. *PLOS ONE* **9**, e105703 (2014).

486. Wedemeyer, H., He, X.S., Nascimbeni, M., Davis, A.R., Greenberg, H.B., Hoofnagle, J.H., Liang, T.J., Alter, H. & Rehermann, B. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. *J Immunol* **169**, 3447-3458 (2002).

487. Lauer, G.M., Barnes, E., Lucas, M., Timm, J., Ouchi, K., Kim, A.Y., Day, C.L., Robbins, G.K., Casson, D.R., Reiser, M., Dusheiko, G., Allen, T.M., Chung, R.T., Walker, B.D. & Klenerman, P. High resolution analysis of cellular immune responses in resolved and persistent hepatitis C virus infection. *Gastroenterology* **127**, 924-936 (2004).

488. Bengsch, B., Seigel, B., Ruhl, M., Timm, J., Kuntz, M., Blum, H.E., Pircher, H. & Thimme, R. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog* **6**, e1000947 (2010).

489. Kroy, D.C., Ciuffreda, D., Cooperrider, J.H., Tomlinson, M., Hauck, G.D., Aneja, J., Berger, C., Wolski, D., Carrington, M., Wherry, E.J., Chung, R.T., Tanabe, K.K., Elias, N., Freeman, G.J., de Kruyff, R.H., Misdraji, J., Kim, A.Y. & Lauer, G.M. Liver environment and HCV replication affect human T-cell phenotype and expression of inhibitory receptors. *Gastroenterology* **146**, 550-561 (2014).

490. Nakamoto, N., Kaplan, D.E., Coleclough, J., Li, Y., Valiga, M.E., Kaminski, M., Shaked, A., Olthoff, K., Gostick, E., Price, D.A., Freeman, G.J., Wherry, E.J. & Chang, K.M. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* **134**, 1927-1937, 1937 e1921-1922 (2008).

491. Ferrari, C., Penna, A., Bertoletti, A., Valli, A., Antoni, A.D., Giuberti, T., Cavalli, A., Petit, M.A. & Fiaccadori, F. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J Immunol* **145**, 3442-3449 (1990).

492. Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A. & Chisari, F.V. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. *J Exp Med* **181**, 1047-1058 (1995).

493. Larrubia, J.R., Benito-Martinez, S., Miquel, J., Calvino, M., Sanz-de-Villalobos, E., Gonzalez-Praetorius, A., Albertos, S., Garcia-Garzon, S., Lokhande, M. & Parra-Cid, T. Bim-mediated apoptosis and PD-1/PD-L1 pathway impair reactivity of PD1(+)/CD127(-) HCV-specific CD8(+) cells targeting the virus in chronic hepatitis C virus infection. *Cell Immunol* **269**, 104-114 (2011).

494. Radziewicz, H., Ibegbu, C.C., Fernandez, M.L., Workowski, K.A., Obideen, K., Wehbi, M., Hanson, H.L., Steinberg, J.P., Masopust, D., Wherry, E.J., Altman, J.D., Rouse, B.T., Freeman, G.J., Ahmed, R. & Grakoui, A. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection

display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J Virol* **81**, 2545-2553 (2007).

495. Boni, C., Fisicaro, P., Valdatta, C., Amadei, B., Di Vincenzo, P., Giuberti, T., Laccabue, D., Zerbini, A., Cavalli, A., Missale, G., Bertoletti, A. & Ferrari, C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* **81**, 4215-4225 (2007).

496. Maini, M.K., Boni, C., Lee, C.K., Larrubia, J.R., Reignat, S., Ogg, G.S., King, A.S., Herberg, J., Gilson, R., Alisa, A., Williams, R., Vergani, D., Naoumov, N.V., Ferrari, C. & Bertoletti, A. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* **191**, 1269-1280 (2000).

497. Webster, G.J., Reignat, S., Brown, D., Ogg, G.S., Jones, L., Seneviratne, S.L., Williams, R., Dusheiko, G. & Bertoletti, A. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. *J Virol* **78**, 5707-5719 (2004).

498. Schurich, A., Pallett, L.J., Jajbhay, D., Wijngaarden, J., Otano, I., Gill, U.S., Hansi, N., Kennedy, P.T., Nastouli, E., Gilson, R., Frezza, C., Henson, S.M. & Maini, M.K. Distinct Metabolic Requirements of Exhausted and Functional Virus-Specific CD8 T Cells in the Same Host. *Cell Rep* **16**, 1243-1252 (2016).

499. Bengsch, B., Martin, B. & Thimme, R. Restoration of HBV-specific CD8+ T cell function by PD-1 blockade in inactive carrier patients is linked to T cell differentiation. *J Hepatol* **61**, 1212-1219 (2014).

500. Raziorrouh, B., Schraut, W., Gerlach, T., Nowack, D., Gruner, N.H., Ulsenheimer, A., Zachoval, R., Wachtler, M., Spannagl, M., Haas, J., Diepolder, H.M. & Jung, M.C. The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology* **52**, 1934-1947 (2010).

501. Schurich, A., Khanna, P., Lopes, A.R., Han, K.J., Peppa, D., Micco, L., Nebbia, G., Kennedy, P.T., Geretti, A.M., Dusheiko, G. & Maini, M.K. Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-Prone CD8 T cells in persistent hepatitis B virus infection. *Hepatology* **53**, 1494-1503 (2011).

502. Nakamoto, N., Cho, H., Shaked, A., Olthoff, K., Valiga, M.E., Kaminski, M., Gostick, E., Price, D.A., Freeman, G.J., Wherry, E.J. & Chang, K.M. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog* **5**, e1000313 (2009).

503. McMahan, R.H., Golden-Mason, L., Nishimura, M.I., McMahon, B.J., Kemper, M., Allen, T.M., Gretch, D.R. & Rosen, H.R. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest* **120**, 4546-4557 (2010).

504. Kared, H., Fabre, T., Bedard, N., Bruneau, J. & Shoukry, N.H. Galectin-9 and IL-21 mediate cross-regulation between Th17 and Treg cells during acute hepatitis C. *PLoS Pathog* **9**, e1003422 (2013).

505. Owusu Sekyere, S., Suneetha, P.V., Kraft, A.R., Zhang, S., Dietz, J., Sarrazin, C., Manns, M.P., Schlaphoff, V., Cornberg, M. & Wedemeyer, H. A heterogeneous hierarchy of co-regulatory receptors regulates exhaustion of HCV-specific CD8 T cells in patients with chronic hepatitis C. *J Hepatol* **62**, 31-40 (2015).

506. Bertoletti, A. & Maini, M.K. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Curr Opin Immunol* **12**, 403-408 (2000).

507. Tsai, S.L., Liaw, Y.F., Chen, M.H., Huang, C.Y. & Kuo, G.C. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* **25**, 449-458 (1997).

508. Penna, A., Del Prete, G., Cavalli, A., Bertoletti, A., D'Elios, M.M., Sorrentino, R., D'Amato, M., Boni, C., Pilli, M., Fiaccadori, F. & Ferrari, C. Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapsid-specific T cells in acute self-limited hepatitis B. *Hepatology* **25**, 1022-1027 (1997).

509. Bertoletti, A., D'Elios, M.M., Boni, C., De Carli, M., Zignego, A.L., Durazzo, M., Missale, G., Penna, A., Fiaccadori, F., Del Prete, G. & Ferrari, C. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterology* **112**, 193-199 (1997).

510. Billerbeck, E., Kang, Y.H., Walker, L., Lockstone, H., Grafmueller, S., Fleming, V., Flint, J., Willberg, C.B., Bengsch, B., Seigel, B., Ramamurthy, N., Zitzmann, N., Barnes, E.J., Thevanayagam, J., Bhagwanani, A., Leslie, A., Oo, Y.H., Kollnberger, S., Bowness, P., Drognitz, O., Adams, D.H., Blum, H.E., Thimme, R. & Klenerman, P. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A* **107**, 3006-3011 (2010).

511. Grafmueller, S., Billerbeck, E., Blum, H.E., Neumann-Haefelin, C. & Thimme, R. Differential antigen specificity of hepatitis C virus-specific interleukin 17- and interferon gamma-producing CD8(+) T cells during chronic infection. *J Infect Dis* **205**, 1142-1146 (2012).

512. Seetharam, A.B., Borg, B.B., Subramanian, V., Chapman, W.C., Crippin, J.S. & Mohanakumar, T. Temporal association between increased virus-specific Th17 response and spontaneous recovery from recurrent hepatitis C in a liver transplant recipient. *Transplantation* **92**, 1364-1370 (2011).

513. Chang, Q., Wang, Y.K., Zhao, Q., Wang, C.Z., Hu, Y.Z. & Wu, B.Y. Th17 cells are increased with severity of liver inflammation in patients with chronic hepatitis C. *J Gastroenterol Hepatol* **27**, 273-278 (2012).

514. Sun, H.Q., Zhang, J.Y., Zhang, H., Zou, Z.S., Wang, F.S. & Jia, J.H. Increased Th17 cells contribute to disease progression in patients with HBV-associated liver cirrhosis. *J Viral Hepat* **19**, 396-403 (2012).

515. Zhao, J., Zhang, Z., Luan, Y., Zou, Z., Sun, Y., Li, Y., Jin, L., Zhou, C., Fu, J., Gao, B., Fu, Y. & Wang, F.S. Pathological functions of interleukin-22 in chronic liver inflammation and fibrosis with hepatitis B virus infection by promoting T helper 17 cell recruitment. *Hepatology* **59**, 1331-1342 (2014).

516. Raziorrouh, B., Sacher, K., Tawar, R.G., Emmerich, F., Neumann-Haefelin, C., Baumert, T.F., Thimme, R. & Boettler, T. Virus-Specific CD4+ T Cells Have Functional and Phenotypic Characteristics of Follicular T-Helper Cells in Patients With Acute and Chronic HCV Infections. *Gastroenterology* **150**, 696-706 e693 (2016).

517. Jung, M.K. & Shin, E.C. Regulatory T Cells in Hepatitis B and C Virus Infections. *Immune Netw* **16**, 330-336 (2016).

518. Ward, S.M., Fox, B.C., Brown, P.J., Worthington, J., Fox, S.B., Chapman, R.W., Fleming, K.A., Banham, A.H. & Klenerman, P. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatol* **47**, 316-324 (2007).

519. Boettler, T., Spangenberg, H.C., Neumann-Haefelin, C., Panther, E., Urbani, S., Ferrari, C., Blum, H.E., von Weizsacker, F. & Thimme, R. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. *J Virol* **79**, 7860-7867 (2005).

520. Rushbrook, S.M., Ward, S.M., Unitt, E., Vowler, S.L., Lucas, M., Klenerman, P. & Alexander, G.J. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. *J Virol* **79**, 7852-7859 (2005).

521. Cabrera, R., Tu, Z., Xu, Y., Firpi, R.J., Rosen, H.R., Liu, C. & Nelson, D.R. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology* **40**, 1062-1071 (2004).

522. Sugimoto, K., Ikeda, F., Stadanlick, J., Nunes, F.A., Alter, H.J. & Chang, K.M. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology* **38**, 1437-1448 (2003).

523. Stoop, J.N., van der Molen, R.G., Baan, C.C., van der Laan, L.J., Kuipers, E.J., Kusters, J.G. & Janssen, H.L. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* **41**, 771-778 (2005).

524. Yang, G., Liu, A., Xie, Q., Guo, T.B., Wan, B., Zhou, B. & Zhang, J.Z. Association of CD4+CD25+Foxp3+ regulatory T cells with chronic activity and viral clearance in patients with hepatitis B. *Int Immunol* **19**, 133-140 (2007).

525. Franzese, O., Kennedy, P.T., Gehring, A.J., Gotto, J., Williams, R., Maini, M.K. & Bertoletti, A. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. *J Virol* **79**, 3322-3328 (2005).

526. Stoop, J.N., Claassen, M.A., Woltman, A.M., Binda, R.S., Kuipers, E.J., Janssen, H.L., van der Molen, R.G. & Boonstra, A. Intrahepatic regulatory T cells are phenotypically distinct from their peripheral counterparts in chronic HBV patients. *Clin Immunol* **129**, 419-427 (2008).

527. Tacke, R.S., Lee, H.C., Goh, C., Courtney, J., Polyak, S.J., Rosen, H.R. & Hahn, Y.S. Myeloid suppressor cells induced by hepatitis C virus suppress T-cell responses through the production of reactive oxygen species. *Hepatology* **55**, 343-353 (2012).

528. Cnossen, W.R. & Drenth, J.P. Polycystic liver disease: an overview of pathogenesis, clinical manifestations and management. *Orphanet J Rare Dis* **9**, 69 (2014).

529. Schouten, J.N., Verheij, J. & Seijo, S. Idiopathic non-cirrhotic portal hypertension: a review. *Orphanet J Rare Dis* **10**, 67 (2015).

530. Strauss, E. & Valla, D. Non-cirrhotic portal hypertension--concept, diagnosis and clinical management. *Clin Res Hepatol Gastroenterol* **38**, 564-569 (2014).

531. Powell, L.W., Seckington, R.C. & Deugnier, Y. Haemochromatosis. *Lancet* **388**, 706-716 (2016).

532. Reimão, R., Porto, G. & de Sousa, M. Stability of CD4/CD8 ratios in man: new correlation between CD4/CD8 profiles and iron overload in idiopathic haemochromatosis patients. *Comptes rendus de l'Académie des sciences. Série III, Sciences de la vie* **313**, 481-487 (1991).

533. Porto, G., Reimɑo, R., Gonçalves, C., Vicente, C., Justiça, B. & Sousa, M. Haemochromatosis as a window into the study of the immunological system: A novel correlation between CD8+ lymphocytes and iron overload. *Eur J Haematol* **52**, 283-290 (2009).

534. Cruz, E., Melo, G., Lacerda, R., Almeida, S. & Porto, G. The CD8+ T-lymphocyte profile as a modifier of iron overload in HFE hemochromatosis: an update of clinical and immunological data from 70 C282Y homozygous subjects. *Blood Cells Mol Dis* **37**, 33-39 (2006).

535. Cardoso, E.M., Hagen, K., de Sousa, M. & Hultcrantz, R. Hepatic damage in C282Y homozygotes relates to low numbers of CD8+ cells in the liver lobuli. *Eur J Clin Invest* **31**, 45-53 (2001).

536. Fabio, G., Zarantonello, M., Mocellin, C., Bonara, P., Corengia, C., Fargion, S. & Fiorelli, G. Peripheral lymphocytes and intracellular cytokines in C282Y homozygous hemochromatosis patients. *J Hepatol* **37**, 753-761 (2002).

537. Joplin, R., Strain, A.J. & Neuberger, J.M. Immuno-isolation and culture of biliary epithelial cells from normal human liver. *In Vitro Cell Dev Biol* **25**, 1189-1192 (1989).

538. Wadkin, J.C.R., Patten, D.A., Kamarajah, S.K., Shepherd, E.L., Novitskaya, V., Berditchevski, F., Adams, D.H., Weston, C.J. & Shetty, S. CD151 supports VCAM-1-mediated lymphocyte adhesion

to liver endothelium and is upregulated in chronic liver disease and hepatocellular carcinoma. *Am J Physiol Gastrointest Liver Physiol* **313**, G138-G149 (2017).

539. Joplin, R. Isolation and culture of biliary epithelial cells. *Gut* **35**, 875-878 (1994).

540. Collison, L.W. & Vignali, D.A. In vitro Treg suppression assays. *Methods Mol Biol* **707**, 21-37 (2011).

541. Abbas, A.K., Murphy, K.M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793 (1996).

542. Islam, S.A. & Luster, A.D. T cell homing to epithelial barriers in allergic disease. *Nat Med* **18**, 705-715 (2012).

543. Sawant, D.V. & Vignali, D.A. Once a Treg, always a Treg? *Immunol Rev* **259**, 173-191 (2014).

544. Marrack, P., Scott-Browne, J. & MacLeod, M.K. Terminating the immune response. *Immunol Rev* **236**, 5-10 (2010).

545. Sugimoto, M.A., Sousa, L.P., Pinho, V., Perretti, M. & Teixeira, M.M. Resolution of Inflammation: What Controls Its Onset? *Front Immunol* **7**, 160 (2016).

546. Semmo, N. & Klenerman, P. CD4+ T cell responses in hepatitis C virus infection. *World J Gastroenterol* **13**, 4831-4838 (2007).

547. Holz, L. & Rehermann, B. T cell responses in hepatitis C virus infection: historical overview and goals for future research. *Antiviral Res* **114**, 96-105 (2015).

548. Ulsenheimer, A., Lucas, M., Seth, N.P., Tilman Gerlach, J., Gruener, N.H., Loughry, A., Pape, G.R., Wucherpfennig, K.W., Diepolder, H.M. & Klenerman, P. Transient immunological control during acute hepatitis C virus infection: ex vivo analysis of helper T-cell responses. *J Viral Hepat* **13**, 708-714 (2006).

549. Bes, M., Sauleda, S., Casamitjana, N., Piron, M., Campos-Varela, I., Quer, J., Cubero, M., Puig, L., Guardia, J. & Esteban, J.I. Reversal of nonstructural protein 3-specific CD4(+) T cell dysfunction in patients with persistent hepatitis C virus infection. *J Viral Hepat* **19**, 283-294 (2012).

550. Schulze Zur Wiesch, J., Ciuffreda, D., Lewis-Ximenez, L., Kasprowicz, V., Nolan, B.E., Streeck, H., Aneja, J., Reyor, L.L., Allen, T.M., Lohse, A.W., McGovern, B., Chung, R.T., Kwok, W.W., Kim, A.Y. & Lauer, G.M. Broadly directed virus-specific CD4+ T cell responses are primed during acute hepatitis C infection, but rapidly disappear from human blood with viral persistence. *J Exp Med* **209**, 61-75 (2012).

551. Kurktschiev, P.D., Raziorrouh, B., Schraut, W., Backmund, M., Wachtler, M., Wendtner, C.M., Bengsch, B., Thimme, R., Denk, G., Zachoval, R., Dick, A., Spannagl, M., Haas, J., Diepolder, H.M., Jung, M.C. & Gruener, N.H. Dysfunctional CD8+ T cells in hepatitis B and C are characterized by a lack of antigen-specific T-bet induction. *J Exp Med* **211**, 2047-2059 (2014).

552. Foster, R.G., Golden-Mason, L., Rutebemberwa, A. & Rosen, H.R. Interleukin (IL)-17/IL-22-producing T cells enriched within the liver of patients with chronic hepatitis C viral (HCV) infection. *Dig Dis Sci* **57**, 381-389 (2012).

553. Bjorkland, A., Festin, R., Mendel-Hartvig, I., Nyberg, A., Loof, L. & Totterman, T.H. Blood and liver-infiltrating lymphocytes in primary biliary cirrhosis: increase in activated T and natural killer cells and recruitment of primed memory T cells. *Hepatology* **13**, 1106-1111 (1991).

554. Wang, L., Sun, Y., Zhang, Z., Jia, Y., Zou, Z., Ding, J., Li, Y., Xu, X., Jin, L., Yang, T., Li, Z., Sun, Y., Zhang, J.Y., Lv, S., Chen, L., Li, B., Gershwin, M.E. & Wang, F.S. CXCR5+ CD4+ T follicular helper cells participate in the pathogenesis of primary biliary cirrhosis. *Hepatology* **61**, 627-638 (2015).

555. Lokhande, M.U., Thimme, R., Klenerman, P. & Semmo, N. Methodologies for the Analysis of HCV-Specific CD4(+) T Cells. *Front Immunol* **6**, 57 (2015).

556. He, X.S., Rehermann, B., Lopez-Labrador, F.X., Boisvert, J., Cheung, R., Mumm, J., Wedemeyer, H., Berenguer, M., Wright, T.L., Davis, M.M. & Greenberg, H.B. Quantitative analysis of hepatitis C virus-specific CD8+ T cells in peripheral blood and liver using peptide-MHC tetramers. *Proceedings of the National Academy of Sciences* **96**, 5692-5697 (1999).

557. Kita, H., Matsumura, S., He, X.S., Ansari, A.A., Lian, Z.X., Van de Water, J., Coppel, R.L., Kaplan, M.M. & Gershwin, M.E. Quantitative and functional analysis of PDC-E2-specific autoreactive cytotoxic T lymphocytes in primary biliary cirrhosis. *J Clin Invest* **109**, 1231-1240 (2002).

558. Sebode, M., Weiler-Normann, C., Liwinski, T. & Schramm, C. Autoantibodies in Autoimmune Liver Disease-Clinical and Diagnostic Relevance. *Front Immunol* **9**, 609 (2018).

559. Jeffery, H.C., Buckley, C.D., Moss, P. & Rainger, G.E. Analysis of the effects of stromal cells on the migration of lymphocytes into and through inflamed tissue using 3-D culture models. *Journal of Immunological Methods* **400**, 45-57 (2013).

560. Nuti, S., Rosa, D., Valiante, N.M., Saletti, G., Caratozzolo, M., Dellabona, P., Barnaba, V. & Abrignani, S. Dynamics of intra-hepatic lymphocytes in chronic hepatitis C: enrichment for Valpha24+ T cells and rapid elimination of effector cells by apoptosis. *Eur J Immunol* **28**, 3448-3455 (1998).

561. Rea, I.M., McNerlan, S.E. & Alexander, H.D. CD69, CD25, and HLA-DR activation antigen expression on CD3+ lymphocytes and relationship to serum TNF-alpha, IFN-gamma, and sIL-2R levels in aging. *Exp Gerontol* **34**, 79-93 (1999).

562. Depper, J.M., Leonard, W.J., Drogula, C., Kronke, M., Waldmann, T.A. & Greene, W.C. Interleukin 2 (IL-2) augments transcription of the IL-2 receptor gene. *Proc Natl Acad Sci U S A* **82**, 4230-4234 (1985).

563. Wyss-Coray, T., Gallati, H., Pracht, I., Limat, A., Mauri, D., Frutig, K. & Pichler, W.J. Antigen-presenting human T cells and antigen-presenting B cells induce a similar cytokine profile in specific T cell clones. *Eur J Immunol* **23**, 3350-3357 (1993).

564. Yi, J.S., Cox, M.A. & Zajac, A.J. T-cell exhaustion: characteristics, causes and conversion. *Immunology* **129**, 474-481 (2010).

565. Green, D.R., Droin, N. & Pinkoski, M. Activation-induced cell death in T cells. *Immunol Rev* **193**, 70-81 (2003).

566. Deterre, P., Berthelier, V., Bauvois, B., Dalloul, A., Schuber, F. & Lund, F. CD38 in T- and B-cell functions. *Chem Immunol* **75**, 146-168 (2000).

567. Bangs, S.C., Baban, D., Cattan, H.J., Li, C.K., McMichael, A.J. & Xu, X.N. Human CD4+ memory T cells are preferential targets for bystander activation and apoptosis. *J Immunol* **182**, 1962-1971 (2009).

568. Bruns, T., Zimmermann, H.W., Pachnio, A., Li, K.K., Trivedi, P.J., Reynolds, G., Hubscher, S., Stamataki, Z., Badenhorst, P.W., Weston, C.J., Moss, P.A. & Adams, D.H. CMV infection of human sinusoidal endothelium regulates hepatic T cell recruitment and activation. *J Hepatol* **63**, 38-49 (2015).

569. Friedl, P., Noble, P.B. & Zanker, K.S. Lymphocyte locomotion in three-dimensional collagen gels. Comparison of three quantitative methods for analysing cell trajectories. *J Immunol Methods* **165**, 157-165 (1993).

570. Molina-Jimenez, F., Benedicto, I., Dao Thi, V.L., Gondar, V., Lavillette, D., Marin, J.J., Briz, O., Moreno-Otero, R., Aldabe, R., Baumert, T.F., Cosset, F.L., Lopez-Cabrera, M. & Majano, P.L. Matrigel-embedded 3D culture of Huh-7 cells as a hepatocyte-like polarized system to study hepatitis C virus cycle. *Virology* **425**, 31-39 (2012).

571. Wang, Y.J., Liu, H.L., Guo, H.T., Wen, H.W. & Liu, J. Primary hepatocyte culture in collagen gel mixture and collagen sandwich. *World J Gastroenterol* **10**, 699-702 (2004).

572. Wiggins, B.G., Stamataki, Z. & Lalor, P.F. Using Ex Vivo Liver Organ Cultures to Measure Lymphocyte Trafficking. *Methods Mol Biol* **1591**, 177-194 (2017).

573. Beckwitt, C.H., Clark, A.M., Wheeler, S., Taylor, D.L., Stolz, D.B., Griffith, L. & Wells, A. Liver 'organ on a chip'. *Exp Cell Res* **363**, 15-25 (2018).

574. Du, Y., Li, N., Yang, H., Luo, C., Gong, Y., Tong, C., Gao, Y., Lu, S. & Long, M. Mimicking liver sinusoidal structures and functions using a 3D-configured microfluidic chip. *Lab Chip* **17**, 782-794 (2017).

575. Wiggins, B.G., Aliazis, K., Davies, S.P., Hirschfield, G., Lalor, P.F., Reynolds, G. & Stamataki, Z. In Vitro and Ex Vivo Models to Study T Cell Migration Through the Human Liver Parenchyma. *Methods Mol Biol* **1591**, 195-214 (2017).

576. Qian, S., Wang, Z., Lee, Y., Chiang, Y., Bonham, C., Fung, J. & Lu, L. Hepatocyte-induced apoptosis of activated T cells, a mechanism of liver transplant tolerance, is related to the expression of ICAM-1 and hepatic lectin. *Transplant Proc* **33**, 226 (2001).

577. Hall, C.H., Kassel, R., Tacke, R.S. & Hahn, Y.S. HCV+ hepatocytes induce human regulatory CD4+ T cells through the production of TGF-beta. *PLOS ONE* **5**, e12154 (2010).

578. Liu, Y., Meyer, C., Xu, C., Weng, H., Hellerbrand, C., ten Dijke, P. & Dooley, S. Animal models of chronic liver diseases. *Am J Physiol Gastrointest Liver Physiol* **304**, G449-468 (2013).

579. Gruener, N.H., Lechner, F., Jung, M.C., Diepolder, H., Gerlach, T., Lauer, G., Walker, B., Sullivan, J., Phillips, R., Pape, G.R. & Klenerman, P. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* **75**, 5550-5558 (2001).

580. Brosterhus, H., Brings, S., Leyendeckers, H., Manz, R.A., Miltenyi, S., Radbruch, A., Assenmacher, M. & Schmitz, J. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. *Eur J Immunol* **29**, 4053-4059 (1999).

581. Sancho, D., Gómez, M. & Sánchez-Madrid, F. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol* **26**, 136-140 (2005).

582. Labiano, S., Melendez-Rodriguez, F., Palazon, A., Teijeira, A., Garasa, S., Etxeberria, I., Aznar, M.A., Sanchez-Paulete, A.R., Azpilikueta, A., Bolanos, E., Molina, C., de la Fuente, H., Maiso, P., Sanchez-Madrid, F., de Landazuri, M.O., Aragones, J. & Melero, I. CD69 is a direct HIF-1alpha target gene in hypoxia as a mechanism enhancing expression on tumor-infiltrating T lymphocytes. *Oncoimmunology* **6**, e1283468 (2017).

583. Radulovic, K., Rossini, V., Manta, C., Holzmann, K., Kestler, H.A. & Niess, J.H. The early activation marker CD69 regulates the expression of chemokines and CD4 T cell accumulation in intestine. *PLOS ONE* **8**, e65413 (2013).

584. Martin-Gayo, E., Sierra-Filardi, E., Corbi, A.L. & Toribio, M.L. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* **115**, 5366-5375 (2010).

585. Radulovic, K., Manta, C., Rossini, V., Holzmann, K., Kestler, H.A., Wegenka, U.M., Nakayama, T. & Niess, J.H. CD69 regulates type I IFN-induced tolerogenic signals to mucosal CD4 T cells that attenuate their colitogenic potential. *J Immunol* **188**, 2001-2013 (2012).

586. Cortes, J.R., Sanchez-Diaz, R., Bovolenta, E.R., Barreiro, O., Lasarte, S., Matesanz-Marin, A., Toribio, M.L., Sanchez-Madrid, F. & Martin, P. Maintenance of immune tolerance by Foxp3+ regulatory T cells requires CD69 expression. *J Autoimmun* **55**, 51-62 (2014).

587. Zhu, J., Yamane, H. & Paul, W.E. Differentiation of effector CD4 T cell populations (*). *Ann Rev Immunol* **28**, 445-489 (2010).

588. Martin, P., Gomez, M., Lamana, A., Cruz-Adalia, A., Ramirez-Huesca, M., Ursa, M.A., Yanez-Mo, M. & Sanchez-Madrid, F. CD69 association with Jak3/Stat5 proteins regulates Th17 cell differentiation. *Mol Cell Biol* **30**, 4877-4889 (2010).

589. Risso, A., Smilovich, D., Capra, M.C., Baldissarro, I., Yan, G., Bargellesi, A. & Cosulich, M.E. CD69 in resting and activated T lymphocytes. Its association with a GTP binding protein and biochemical requirements for its expression. *J Immunol* **146**, 4105-4114 (1991).

590. Montufar-Solis, D., Garza, T. & Klein, J.R. T-cell activation in the intestinal mucosa. *Immunol Rev* **215**, 189-201 (2007).

591. Sancho, D., Santis, A.G., Alonso-Lebrero, J.L., Viedma, F., Tejedor, R. & Sanchez-Madrid, F. Functional analysis of ligand-binding and signal transduction domains of CD69 and CD23 C-type lectin leukocyte receptors. *J Immunol* **165**, 3868-3875 (2000).

592. Cebrian, M. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. *J Exp Med* **168**, 1621-1637 (1988).

593. Santis, A.G., Campanero, M.R., Alonso, J.L., Tugores, A., Alonso, M.A., Yague, E., Pivel, J.P. & Sanchez-Madrid, F. Tumor necrosis factor-alpha production induced in T lymphocytes through the AIM/CD69 activation pathway. *Eur J Immunol* **22**, 1253-1259 (1992).

594. Conde, M., Montano, R., Moreno-Aurioles, V.R., Ramirez, R., Sanchez-Mateos, P., Sanchez-Madrid, F. & Sobrino, F. Anti-CD69 antibodies enhance phorbol-dependent glucose metabolism and Ca2+ levels in human thymocytes. Antagonist effect of cyclosporin A. *J Leukoc Biol* **60**, 278-284 (1996).

595. Sancho, D., Gómez, M., Viedma, F., Esplugues, E., Gordón-Alonso, M., Angeles García-López, M., de la Fuente, H., Martínez-A, C., Lauzurica, P. & Sánchez-Madrid, F. CD69 downregulates autoimmune reactivity through active transforming growth factor-β production in collagen-induced arthritis. *J Clin Invest* **112**, 872-882 (2003).

596. Esplugues, E., Sancho, D., Vega-Ramos, J., Martinez, C., Syrbe, U., Hamann, A., Engel, P., Sanchez-Madrid, F. & Lauzurica, P. Enhanced antitumor immunity in mice deficient in CD69. *J Exp Med* **197**, 1093-1106 (2003).

597. McInnes, I.B., Leung, B.P., Sturrock, R.D., Field, M. & Liew, F.Y. Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. *Nat Med* **3**, 189-195 (1997).

598. Alari-Pahissa, E., Notario, L., Lorente, E., Vega-Ramos, J., Justel, A., Lopez, D., Villadangos, J.A. & Lauzurica, P. CD69 does not affect the extent of T cell priming. *PLOS ONE* **7**, e48593 (2012).

599. Feng, C., Woodside, K.J., Vance, B.A., El-Khoury, D., Canelles, M., Lee, J., Gress, R., Fowlkes, B.J., Shores, E.W. & Love, P.E. A potential role for CD69 in thymocyte emigration. *Int Immunol* **14**, 535-544 (2002).

600. Nakayama, T., Kasprowicz, D.J., Yamashita, M., Schubert, L.A., Gillard, G., Kimura, M., Didierlaurent, A., Koseki, H. & Ziegler, S.F. The generation of mature, single-positive thymocytes in vivo is dysregulated by CD69 blockade or overexpression. *J Immunol* **168**, 87-94 (2002).

601. Lauzurica, P., Sancho, D., Torres, M., Albella, B., Marazuela, M., Merino, T., Bueren, J.A., Martinez, A.C. & Sanchez-Madrid, F. Phenotypic and functional characteristics of hematopoietic cell lineages in CD69-deficient mice. *Blood* **95**, 2312-2320 (2000).

602. Martin, P., Gomez, M., Lamana, A., Matesanz Marin, A., Cortes, J.R., Ramirez-Huesca, M., Barreiro, O., Lopez-Romero, P., Gutierrez-Vazquez, C., de la Fuente, H., Cruz-Adalia, A. & Sanchez-Madrid, F. The leukocyte activation antigen CD69 limits allergic asthma and skin contact hypersensitivity. *J Allergy Clin Immunol* **126**, 355-365, 365 e351-353 (2010).

603. Cruz-Adalia, A., Jimenez-Borreguero, L.J., Ramirez-Huesca, M., Chico-Calero, I., Barreiro, O., Lopez-Conesa, E., Fresno, M., Sanchez-Madrid, F. & Martin, P. CD69 limits the severity of cardiomyopathy after autoimmune myocarditis. *Circulation* **122**, 1396-1404 (2010).

604. Vega-Ramos, J., Alari-Pahissa, E., Valle, J.D., Carrasco-Marin, E., Esplugues, E., Borras, M., Martinez, A.C. & Lauzurica, P. CD69 limits early inflammatory diseases associated with immune response to Listeria monocytogenes infection. *Immunol Cell Biol* **88**, 707-715 (2010).

605. Esplugues, E., Vega-Ramos, J., Cartoixa, D., Vazquez, B.N., Salaet, I., Engel, P. & Lauzurica, P. Induction of tumor NK-cell immunity by anti-CD69 antibody therapy. *Blood* **105**, 4399-4406 (2005).

606. Miki-Hosokawa, T., Hasegawa, A., Iwamura, C., Shinoda, K., Tofukuji, S., Watanabe, Y., Hosokawa, H., Motohashi, S., Hashimoto, K., Shirai, M., Yamashita, M. & Nakayama, T. CD69 controls the pathogenesis of allergic airway inflammation. *J Immunol* **183**, 8203-8215 (2009).

607. Murata, K., Inami, M., Hasegawa, A., Kubo, S., Kimura, M., Yamashita, M., Hosokawa, H., Nagao, T., Suzuki, K., Hashimoto, K., Shinkai, H., Koseki, H., Taniguchi, M., Ziegler, S.F. & Nakayama, T. CD69-null mice protected from arthritis induced with anti-type II collagen antibodies. *Int Immunol* **15**, 987-992 (2003).

608. Hasegawa, A., Iwamura, C., Kitajima, M., Hashimoto, K., Otsuyama, K., Ogino, H., Nakayama, T. & Shirai, M. Crucial role for CD69 in the pathogenesis of dextran sulphate sodium-induced colitis. *PLOS ONE* **8**, e65494 (2013).

609. Yu, X., Matsui, T., Otsuka, M., Sekine, T., Yamamoto, K., Nishioka, K. & Kato, T. Anti-CD69 autoantibodies cross-react with low density lipoprotein receptor-related protein 2 in systemic autoimmune diseases. *J Immunol* **166**, 1360-1369 (2001).

610. de la Fuente, H., Cruz-Adalia, A., Martinez Del Hoyo, G., Cibrian-Vera, D., Bonay, P., Perez-Hernandez, D., Vazquez, J., Navarro, P., Gutierrez-Gallego, R., Ramirez-Huesca, M., Martin, P. & Sanchez-Madrid, F. The leukocyte activation receptor CD69 controls T cell differentiation through its interaction with galectin-1. *Mol Cell Biol* **34**, 2479-2487 (2014).

611. Lin, C.R., Wei, T.Y., Tsai, H.Y., Wu, Y.T., Wu, P.Y. & Chen, S.T. Glycosylation-dependent interaction between CD69 and S100A8/S100A9 complex is required for regulatory T-cell differentiation. *FASEB J* **29**, 5006-5017 (2015).

612. Cedeno-Laurent, F., Opperman, M., Barthel, S.R., Kuchroo, V.K. & Dimitroff, C.J. Galectin-1 triggers an immunoregulatory signature in Th cells functionally defined by IL-10 expression. *J Immunol* **188**, 3127-3137 (2012).

613. Cibrian, D., Saiz, M.L., de la Fuente, H., Sanchez-Diaz, R., Moreno-Gonzalo, O., Jorge, I., Ferrarini, A., Vazquez, J., Punzon, C., Fresno, M., Vicente-Manzanares, M., Dauden, E., Fernandez-Salguero, P.M., Martin, P. & Sanchez-Madrid, F. CD69 controls the uptake of L-tryptophan through LAT1-CD98 and AhR-dependent secretion of IL-22 in psoriasis. *Nat Immunol* **17**, 985-996 (2016).

614. Shiow, L.R., Rosen, D.B., Brdickova, N., Xu, Y., An, J., Lanier, L.L., Cyster, J.G. & Matloubian, M. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* **440**, 540-544 (2006).

615. Dang, E.V., Barbi, J., Yang, H.Y., Jinasena, D., Yu, H., Zheng, Y., Bordman, Z., Fu, J., Kim, Y., Yen, H.R., Luo, W., Zeller, K., Shimoda, L., Topalian, S.L., Semenza, G.L., Dang, C.V., Pardoll, D.M. & Pan, F. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. *Cell* **146**, 772-784 (2011).

616. Zeng, H., Yang, K., Cloer, C., Neale, G., Vogel, P. & Chi, H. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* **499**, 485-490 (2013).

617. Mascanfroni, I.D., Takenaka, M.C., Yeste, A., Patel, B., Wu, Y., Kenison, J.E., Siddiqui, S., Basso, A.S., Otterbein, L.E., Pardoll, D.M., Pan, F., Priel, A., Clish, C.B., Robson, S.C. & Quintana, F.J. Metabolic control of type 1 regulatory T cell differentiation by AHR and HIF1-alpha. *Nat Med* **21**, 638-646 (2015).

618. Liu, G., Yang, K., Burns, S., Shrestha, S. & Chi, H. The S1P(1)-mTOR axis directs the reciprocal differentiation of T(H)1 and T(reg) cells. *Nat Immunol* **11**, 1047-1056 (2010).

619. Ishikawa, S., Akakura, S., Abe, M., Terashima, K., Chijiiwa, K., Nishimura, H., Hirose, S. & Shirai, T. A subset of CD4+ T cells expressing early activation antigen CD69 in murine lupus: possible abnormal regulatory role for cytokine imbalance. *J Immunol* **161**, 1267-1273 (1998).

620. Han, Y., Guo, Q., Zhang, M., Chen, Z. & Cao, X. CD69+ CD4+ CD25- T cells, a new subset of regulatory T cells, suppress T cell proliferation through membrane-bound TGF-beta 1. *J Immunol* **182**, 111-120 (2009).

621. Gandhi, R., Farez, M.F., Wang, Y., Kozoriz, D., Quintana, F.J. & Weiner, H.L. Cutting edge: human latency-associated peptide+ T cells: a novel regulatory T cell subset. *J Immunol* **184**, 4620-4624 (2010).

622. Zhu, J., Feng, A., Sun, J., Jiang, Z., Zhang, G., Wang, K., Hu, S. & Qu, X. Increased CD4(+) CD69(+) CD25(-) T cells in patients with hepatocellular carcinoma are associated with tumor progression. *J Gastroenterol Hepatol* **26**, 1519-1526 (2011).

623. Han, Y., Yang, Y., Chen, Z., Jiang, Z., Gu, Y. & Liu, Y. Human hepatocellular carcinoma-infiltrating CD4+ CD69+ Foxp3– regulatory T cell suppresses T cell response via membrane-bound TGF-β1. *J Mol Med* **92**, 539-550 (2014).

624. Lu, S.Y., Huang, X.J., Liu, K.Y., Liu, D.H. & Xu, L.P. High frequency of CD4+ CD25- CD69+ T cells is correlated with a low risk of acute graft-versus-host disease in allotransplants. *Clin Transplant* **26**, E158-167 (2012).

625. Saldanha-Araujo, F., Haddad, R., Farias, K.C., Souza Ade, P., Palma, P.V., Araujo, A.G., Orellana, M.D., Voltarelli, J.C., Covas, D.T., Zago, M.A. & Panepucci, R.A. Mesenchymal stem cells promote the sustained expression of CD69 on activated T lymphocytes: roles of canonical and non-canonical NF-kappaB signalling. *J Cell Mol Med* **16**, 1232-1244 (2012).

626. Kim, G., Jang, M.S., Son, Y.M., Seo, M.J., Ji, S.Y., Han, S.H., Jung, I.D., Park, Y.M., Jung, H.J. & Yun, C.H. Curcumin inhibits CD4(+) T cell activation, but augments CD69 expression and TGF-beta1-mediated generation of regulatory T cells at late phase. *PLOS ONE* **8**, e62300 (2013).

627. McMurchy, A.N. & Levings, M.K. Suppression assays with human T regulatory cells: a technical guide. *Eur J Immunol* **42**, 27-34 (2012).

628. Adams, D.H., Hubscher, S.G., Shaw, J., Rothlein, R. & Neuberger, J.M. Intercellular adhesion molecule 1 on liver allografts during rejection. *Lancet* **2**, 1122-1125 (1989).

629. Smith, M.E. & Thomas, J.A. Cellular expression of lymphocyte function associated antigens and the intercellular adhesion molecule-1 in normal tissue. *J Clin Pathol* **43**, 893-900 (1990).

630. Momosaki, S., Yano, H., Ogasawara, S., Higaki, K., Hisaka, T. & Kojiro, M. Expression of intercellular adhesion molecule 1 in human hepatocellular carcinoma. *Hepatology* **22**, 1708-1713 (1995).

631. Satoh, S., Nussler, A.K., Liu, Z.Z. & Thomson, A.W. Proinflammatory cytokines and endotoxin stimulate ICAM-1 gene expression and secretion by normal human hepatocytes. *Immunology* **82**, 571-576 (1994).

632. Liu, S., Li, N., Yu, X., Xiao, X., Cheng, K., Hu, J., Wang, J., Zhang, D., Cheng, S. & Liu, S. Expression of intercellular adhesion molecule 1 by hepatocellular carcinoma stem cells and circulating tumor cells. *Gastroenterology* **144**, 1031-1041 e1010 (2013).

633. Sancho, D., Yáñez-Mó, M., Tejedor, R. & Sánchez-Madrid, F. Activation of peripheral blood T cells by interaction and migration through endothelium: role of lymphocyte function antigen-1/intercellular adhesion molecule-1 and interleukin-15. *Blood* **93**, 886-896 (1999).

634. Nurmi, S.M., Autero, M., Raunio, A.K., Gahmberg, C.G. & Fagerholm, S.C. Phosphorylation of the LFA-1 integrin beta2-chain on Thr-758 leads to adhesion, Rac-1/Cdc42 activation, and stimulation of CD69 expression in human T cells. *J Biol Chem* **282**, 968-975 (2007).

635. Chadburn, A., Inghirami, G. & Knowles, D.M. The kinetics and temporal expression of T-cell activation-associated antigens CD15 (LeuM1), CD30 (Ki-1), EMA, and CD11c (LeuM5) by benign activated T cells. *Hematol Pathol* **6**, 193-202 (1992).

636. Mayati, A., Le Vee, M., Moreau, A., Jouan, E., Bucher, S., Stieger, B., Denizot, C., Parmentier, Y. & Fardel, O. Protein kinase C-dependent regulation of human hepatic drug transporter expression. *Biochem Pharmacol* **98**, 703-717 (2015).

637. Rojas, J., Teran-Angel, G., Barbosa, L., Peterson, D.L., Berrueta, L. & Salmen, S. Activation-dependent mitochondrial translocation of Foxp3 in human hepatocytes. *Exp Cell Res* **343**, 159-167 (2016).

638. Anwer, M.S. Mechanism of ionomycin-induced intracellular alkalinization of rat hepatocytes. *Hepatology* **18**, 433-439 (1993).

639. Kirschnek, S., Paris, F., Weller, M., Grassme, H., Ferlinz, K., Riehle, A., Fuks, Z., Kolesnick, R. & Gulbins, E. CD95-mediated apoptosis in vivo involves acid sphingomyelinase. *J Biol Chem* **275**, 27316-27323 (2000).

640. Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K. & Flavell, R.A. Transforming growth factor-beta regulation of immune responses. *Ann Rev Immunol* **24**, 99-146 (2006).

641. Jabri, B. & Abadie, V. IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. *Nat Rev Immunol* **15**, 771-783 (2015).

642. Berney, S.M., Schaan, T., Alexander, J.S., Peterman, G., Hoffman, P.A., Wolf, R.E., van der Heyde, H. & Atkinson, T.P. ICAM-3 (CD50) cross-linking augments signaling in CD3-activated peripheral human T lymphocytes. *J Leukoc Biol* **65**, 867-874 (1999).

643. Kvale, D. & Brandtzaeg, P. Immune modulation of adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58) in human hepatocytic cell lines. *J Hepatol* **17**, 347-352 (1993).

644. Rao, U.N., Lee, S.J., Luo, W., Mihm, M.C., Jr. & Kirkwood, J.M. Presence of tumor-infiltrating lymphocytes and a dominant nodule within primary melanoma are prognostic factors for relapse-free survival of patients with thick (t4) primary melanoma: pathologic analysis of the e1690 and e1694 intergroup trials. *Am J Clin Pathol* **133**, 646-653 (2010).

645. Amsen, D., van Gisbergen, K., Hombrink, P. & van Lier, R.A.W. Tissue-resident memory T cells at the center of immunity to solid tumors. *Nat Immunol* **19**, 538-546 (2018).

646. Cuff, A.O., Robertson, F.P., Stegmann, K.A., Pallett, L.J., Maini, M.K., Davidson, B.R. & Male, V. Eomeshi NK Cells in Human Liver Are Long-Lived and Do Not Recirculate but Can Be Replenished from the Circulation. *J Immunol* **197**, 4283-4291 (2016).

647. Marquardt, N., Beziat, V., Nystrom, S., Hengst, J., Ivarsson, M.A., Kekalainen, E., Johansson, H., Mjosberg, J., Westgren, M., Lankisch, T.O., Wedemeyer, H., Ellis, E.C., Ljunggren, H.G., Michaelsson, J. & Bjorkstrom, N.K. Cutting edge: identification and characterization of human intrahepatic CD49a+ NK cells. *J Immunol* **194**, 2467-2471 (2015).

648. Hudspeth, K., Donadon, M., Cimino, M. & Pontarini, E. Human liver-resident CD56 bright/CD16 neg NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways. *J Autoimmun* **66**, 40-50 (2016).

649. Harmon, C., Robinson, M.W., Fahey, R., Whelan, S., Houlihan, D.D., Geoghegan, J. & O'Farrelly, C. Tissue-resident Eomes(hi) T-bet(lo) CD56(bright) NK cells with reduced proinflammatory potential are enriched in the adult human liver. *Eur J Immunol* **46**, 2111-2120 (2016).

650. Aw Yeang, H.X., Piersma, S.J., Lin, Y., Yang, L., Malkova, O.N., Miner, C., Krupnick, A.S., Chapman, W.C. & Yokoyama, W.M. Cutting Edge: Human CD49e- NK Cells Are Tissue Resident in the Liver. *J Immunol* **198**, 1417-1422 (2017).

651. McNamara, H.A., Cai, Y., Wagle, M.V., Sontani, Y., Roots, C.M., Miosge, L.A., O'Connor, J.H., Sutton, H.J., Ganusov, V.V., Heath, W.R., Bertolino, P., Goodnow, C.G., Parish, I.A., Enders, A. & Cockburn, I.A. Up-regulation of LFA-1 allows liver-resident memory T cells to patrol and remain in the hepatic sinusoids. *Sci Immunol* **2** (2017).

652. Crotty, S. A brief history of T cell help to B cells. *Nat Rev Immunol* **15**, 185-189 (2015).

653. Novy, P., Quigley, M., Huang, X. & Yang, Y. CD4 T Cells Are Required for CD8 T Cell Survival during Both Primary and Memory Recall Responses. *J Immunol* **179**, 8243-8251 (2007).

654. Shedlock, D.J. & Shen, H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**, 337-339 (2003).

655. Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. & Yamaguchi, T. Regulatory T cells: how do they suppress immune responses? *Int Immunol* **21**, 1105-1111 (2009).

656. Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-712 (1999).

657. Hendriks, J., Gravestein, L.A., Tesselaar, K., van Lier, R.A., Schumacher, T.N. & Borst, J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol* **1**, 433-440 (2000).

658. Appay, V., van Lier, R.A., Sallusto, F. & Roederer, M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* **73**, 975-983 (2008).

659. Larbi, A. & Fulop, T. From "truly naive" to "exhausted senescent" T cells: when markers predict functionality. *Cytometry A* **85**, 25-35 (2014).

660. Henson, S.M. & Akbar, A.N. KLRG1--more than a marker for T cell senescence. *Age (Dordr)* **31**, 285-291 (2009).

661. Mukherjee, S., Maiti, P.K. & Nandi, D. Role of CD80, CD86, and CTLA4 on mouse CD4(+) T lymphocytes in enhancing cell-cycle progression and survival after activation with PMA and ionomycin. *J Leukoc Biol* **72**, 921-931 (2002).

662. Wyss-Coray, T., Mauri-Hellweg, D., Baumann, K., Bettens, F., Grunow, R. & Pichler, W.J. The B7 adhesion molecule is expressed on activated human T cells: functional involvement in T-T cell interactions. *Eur J Immunol* **23**, 2175-2180 (1993).

663. Kurioka, A., Walker, L.J., Klenerman, P. & Willberg, C.B. MAIT cells: new guardians of the liver. *Clin Transl Immunology* **5**, e98 (2016).

664. Kurioka, A., Jahun, A.S., Hannaway, R.F., Walker, L.J., Fergusson, J.R., Sverremark-Ekstrom, E., Corbett, A.J., Ussher, J.E., Willberg, C.B. & Klenerman, P. Shared and Distinct Phenotypes and Functions of Human CD161++ Valpha7.2+ T Cell Subsets. *Front Immunol* **8**, 1031 (2017).

665. Turner, D.L., Gordon, C.L. & Farber, D.L. Tissue-resident T cells, in situ immunity and transplantation. *Immunol Rev* **258**, 150-166 (2014).

666. Day, C., Patel, R., Guillen, C. & Wardlaw, A.J. The chemokine CXCL16 is highly and constitutively expressed by human bronchial epithelial cells. *Exp Lung Res* **35**, 272-283 (2009).

667. Wehr, A. & Tacke, F. The Roles of CXCL16 and CXCR6 in Liver Inflammation and Fibrosis. *Curr Pathobiol Rep* **3**, 283-290 (2015).

668. Bankovich, A.J., Shiow, L.R. & Cyster, J.G. CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *J Biol Chem* **285**, 22328-22337 (2010).

669. Nakayama, T., Watanabe, Y., Oiso, N., Higuchi, T., Shigeta, A., Mizuguchi, N., Katou, F., Hashimoto, K., Kawada, A. & Yoshie, O. Eotaxin-3/CC chemokine ligand 26 is a functional ligand for CX3CR1. *J Immunol* **185**, 6472-6479 (2010).

670. Shinkai, A., Yoshisue, H., Koike, M., Shoji, E., Nakagawa, S., Saito, A., Takeda, T., Imabeppu, S., Kato, Y., Hanai, N., Anazawa, H., Kuga, T. & Nishi, T. A novel human CC chemokine, eotaxin-3, which is expressed in IL-4-stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. *J Immunol* **163**, 1602-1610 (1999).

671. Gerlach, C., Moseman, E.A., Loughhead, S.M., Alvarez, D., Zwijnenburg, A.J., Waanders, L., Garg, R., de la Torre, J.C. & von Andrian, U.H. The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis. *Immunity* **45**, 1270-1284 (2016).

672. Wasmuth, H.E., Zaldivar, M.M., Berres, M.L., Werth, A., Scholten, D., Hillebrandt, S., Tacke, F., Schmitz, P., Dahl, E., Wiederholt, T., Hellerbrand, C., Berg, T., Weiskirchen, R., Trautwein, C. & Lammert, F. The fractalkine receptor CX3CR1 is involved in liver fibrosis due to chronic hepatitis C infection. *J Hepatol* **48**, 208-215 (2008).

673. Adams, D.H., Eksteen, B. & Curbishley, S.M. Immunology of the gut and liver: a love/hate relationship. *Gut* **57**, 838-848 (2008).

674. Hintzen, R.Q., de Jong, R., Lens, S.M., Brouwer, M., Baars, P. & van Lier, R.A. Regulation of CD27 expression on subsets of mature T-lymphocytes. *J Immunol* **151**, 2426-2435 (1993).

675. Olsen, I. & Sollid, L.M. Pitfalls in determining the cytokine profile of human T cells. *J Immunol Methods* **390**, 106-112 (2013).

676. Baitsch, L., Baumgaertner, P., Devevre, E., Raghav, S.K., Legat, A., Barba, L., Wieckowski, S., Bouzourene, H., Deplancke, B., Romero, P., Rufer, N. & Speiser, D.E. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *J Clin Invest* **121**, 2350-2360 (2011).

677. Friedman, S.L. Mechanisms of hepatic fibrogenesis. *Gastroenterology* **134**, 1655-1669 (2008).

678. Schmidt-Arras, D. & Rose-John, S. IL-6 pathway in the liver: From physiopathology to therapy. *J Hepatol* **64**, 1403-1415 (2016).

679. Liu, Y.Q., Poon, R.T., Hughes, J., Li, Q.Y., Yu, W.C. & Fan, S.T. Desensitization of T lymphocyte function by CXCR3 ligands in human hepatocellular carcinoma. *World J Gastroenterol* **11**, 164-170 (2005).

680. Bowlus, C.L. & Gershwin, M.E. The diagnosis of primary biliary cirrhosis. *Autoimmun Rev* **13**, 441-444 (2014).

681. Manns, M.P., Buti, M., Gane, E., Pawlotsky, J.M., Razavi, H., Terrault, N. & Younossi, Z. Hepatitis C virus infection. *Nat Rev Dis Primers* **3**, 17006 (2017).

682. Mak, T.W. & Saunders, M.E. The Immune Response. *Part I: Basic Immunology*, 373-401 (2006).

683. Pribila, J.T., Quale, A.C., Mueller, K.L. & Shimizu, Y. Integrins and T cell-mediated immunity. *Ann Rev Immunol* **22**, 157-180 (2004).

684. Kalinski, P., Lotze, M.T. & Kapsenberg, M.L. Dendritic Cells (Second Edition). *Part I: Origin and molecular biology of dendritic cells*, 51 (2001).

685. Naeim, F. Chapter 2: Principles of Immunophenotyping. *Hematopathology*, 27-55 (2008).

686. Maeno, N., Yoshiie, K., Matayoshi, S., Fujimura, T., Mao, S., Wahid, M.R. & Oda, H. A heat-stable component of Bartonella henselae upregulates intercellular adhesion molecule-1 expression on vascular endothelial cells. *Scand J Immunol* **55**, 366-372 (2002).

687. Wingren, A.G., Parra, E., Varga, M., Kalland, T., Sjogren, H.O., Hedlund, G. & Dohlsten, M. T cell activation pathways: B7, LFA-3, and ICAM-1 shape unique T cell profiles. *Crit Rev Immunol* **15**, 235-253 (1995).

688. Vecchi, C., Montosi, G. & Pietrangelo, A. Huh-7: a human "hemochromatotic" cell line. *Hepatology* **51**, 654-659 (2010).

689. Arosa, F.A., Oliveira, L., Porto, G., da Silva, B.M., Kruijer, W., Veltman, J. & de Sousa, M. Anomalies of the CD8+ T cell pool in haemochromatosis: HLA-A3-linked expansions of CD8+CD28- T cells. *Clin Exp Immunol* **107**, 548-554 (1997).

690. Costa, M., Cruz, E., Oliveira, S., Benes, V., Ivacevic, T., Silva, M.J., Vieira, I., Dias, F., Fonseca, S., Goncalves, M., Lima, M., Leitao, C., Muckenthaler, M.U., Pinto, J. & Porto, G. Lymphocyte gene expression signatures from patients and mouse models of hereditary hemochromatosis reveal a function of HFE as a negative regulator of CD8+ T-lymphocyte activation and differentiation in vivo. *PLOS ONE* **10**, e0124246 (2015).

691. Espinoza, I. & Miele, L. Notch inhibitors for cancer treatment. *Pharmacol Ther* **139**, 95-110 (2013).

692. Stolley, J.M. & Masopust, D. Tissue-resident memory T cells live off the fat of the land. *Cell Res* **27**, 847-848 (2017).