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"CD161+ Gamma-Delta T-cells in health and liver disease"

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DEDICATED TO ASHA AND AAKASH

Abstract

CD161 γδ T-cells have been implicated in the pathogenesis of Multiple Sclerosis however their role in health and chronic liver disease requires further exploration.

In health, the majority of γδ T-cells expressed CD161 – a C-type lectin, and predominantly expressed the Vδ2 chain. The CD161+ γδ T-cells demonstrated a Th1-like pattern, expressing IFN-γ, TNF-α and Granzymes/Perforin when compared to the CD161- subset. The CD161+ γδ T-cells also expressed CCR6 and IL-18R thus also displaying a Th17-like pattern. These cells were also found in the lamina propria in the gut and rapidly expanded in the 1st few weeks of life in the periphery.

On gene array analysis, there were 409 genes expressed on the CD161+ γδ T-cells when compared to their CD161-ve counterparts including those coding for β2 receptors, CCL20, Acetycholinesterase, CCR1 and IL-18R. A potential clinical correlation to cardiac diseases was found when the upregulated genes were analysed. When the CD161+ γδ and CD161+ αβ T-cell populations were compared via gene-array, an association with a risk variant for coeliac disease was found. Thus in health, CD161+ γδ T-cells are not only a distinct subset of T-cells (confirmed by a FACS approach and gene array methods), but also the expression of CD161 may be linked to common genetic signals downstream in cell processes and disease pathogenesis, irrespective of T-cell subset population.

In chronic liver disease there was a significant reduction in the periphery of CD161+ γδ T-cells in patients with chronic Hepatitis C (HCV) and an increase in patients with Primary Biliary Cirrhosis and Primary Sclerosing Cholangitis when compared with healthy individuals. The CD161+ γδ T-cells appeared to be of a different phenotype in HCV infection. There was no overall significant localisation into of CD161+ γδ T-cells patients with chronic liver disease or specifically in HCV infection. There was however a CD161+ γδ T-cell enrichment in the liver in patients with Non-Alcoholic Fatty Liver disease. The CD161+ γδ T-cells were also found in Hepatocellular Carcinoma tissue.

Overall it appears the CD161+ γδ T-cells are indeed a unique subset, playing a distinct role in health, as part of an early innate response, but also potentially involved in disease pathogenesis.

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Acknowledgements-1

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Publications during DPhil period (2009-2013)

"Genetic and pharmacodynamic factors determining activity and clinical effectiveness of interferons in managing Hepatitis C - new targets and new approaches: A review article"

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Published Abstracts from research (2009-2012)

"CD161+ Gamma-delta T-cells: defining their role in patients with and without chronic hepatitis C"

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Meetings (2009-2012)

CD161 γδ T-lymphocyte data from this thesis was presented in poster/oral form at the following conferences after a peer-review process for acceptance of abstracts:

1. 4th International γδ T-cell Conference, Kiel, Germany 2010- *Abstract awarded a full international travel scholarship.*

2. British Association for the Study of Liver (BASL), London, UK 2011

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ABBREVIATIONS-1

αβ	Alpha-beta
γδ	Gamma-delta
AB	Alpha-Beta
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cells / Allophycocyanin
AIDS	Acquired Immunodeficiency Syndrome
AIH	Autoimmune hepatitis
CD	Cluster of differentiation
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTLA	Cytotoxic T-Lymphocyte antigen
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EDTA	Ethylene diamine tetra-acetic acid
FACS	Fluorescence activated cell sorting
FES	Feature Extraction Software
FITC	Fluorescein
FMO	Fluorochrome minus one
GD	Gamma-Delta
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HMBPP	(e)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate

ABBREVIATIONS-2

HP	Helicobacter Pylori
IBD	Inflammatory bowel disease
ICS	Intracellular cytokine staining
IEL	Intraepithelial lymphocyte
IHL	Intrahepatic lymphocytes
IL	Interleukin
IPP	Isopentenyl pyrophosphate
LPMCs	Lamina propria mononuclear cells
MAIT	Mucosal-associated invariant T-cells
mcl	Microlitres
MHC	Major Histocompatibilty Complex
ml	Millilitres
mmol	Millimolar
MR1	MHC-Class 1 like molecule
MS	Multiple Sclerosis
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
NHS	National Health Service
PBC	Primary Biliary Cirrhosis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	R-Phycoerythrin
PercP	Peridinin chlorphyll protein
PGA	Polyglcolic acid
Phos-Ag	Pyrophosphomonoesters Antigens
PSC	Primary Sclerosing Cholangitis
PBS	Phosphate buffered Saline
PTX	Pentoxifylline

ABBREVIATIONS-3

SVR	Sustained virological response
T-cells	T-lymphocytes
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RORT	Retinoic acid-related orphan receptor γ-t
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RUNX	Runt-related transcription factor
TB	Tuberculosis
TCR	T-cell receptor
TLR	Toll-like receptor
UC	Ulcerative Colitis
µl	microlitre

CHAPTER 1: INTRODUCTION

1.1 Gamma Delta T-Lymphocytes

T-lymphocyte introduction

T-lymphocytes (T-cells) form part of the adaptive immune system. T-cells have a T-cell receptor (TCR) that interacts with molecules on antigen presenting cells (APCs) such as Dendritic cells (DCs). T-cells are broadly divided into two families depending on the composition of their TCR. "Conventional" T- cells recognize peptides presented via the Major Histocompatibility complex (MHC) class I or II molecules (for CD8 and CD4 T- lymphocytes respectively) and recognized by the TCR (Shawar *et al.* (1994)). They are composed of an alpha- and beta- chain (the αβ-TCR), which allows binding to MHC peptide complexes. Simultaneous binding of MHC molecules enhances signaling from the TCR complex by specific co-receptors. On T-helper cells this co-receptor is Cluster Differentiation (CD) 4 for MHC Class II molecules and on cytotoxic T-cells CD8 for MHC Class I. The mechanism whereby the TCR complex recognizes a specific Antigen (Ag) and thereafter triggers a specific response is a complex mechanism, not only involving TCR engagement with the peptide/MHC, but also involving other co-factors (such as CD45, Zap70, Integrin molecules) leading to T-cell activation. Together the TCR and co-molecules form the TCR complex (Figure 1).

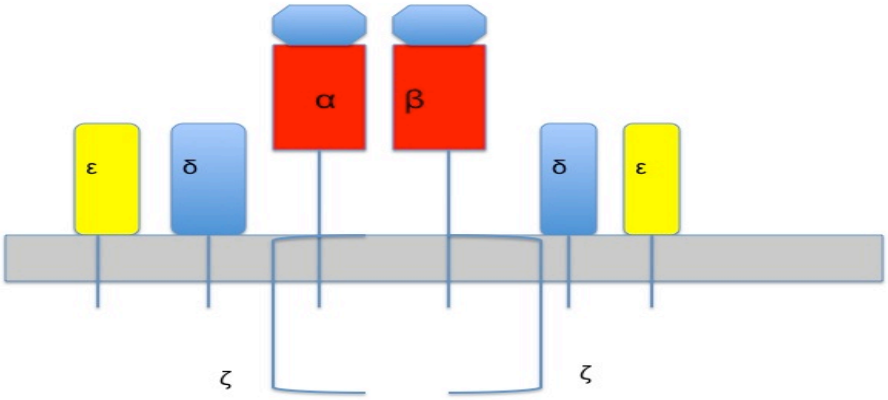


FIGURE 1.1: The T-cell receptor complex (TCR)

The TCR with TCR-α and TCR-β chains, CD3 and ζ-chain accessory molecules. Found on "conventional" αβ T- cells.

γδ T-cells – an introduction

Gamma-delta (γδ) T-cells were discovered after isolation of the TCR γ chain gene (Saito *et al.* (1984) and represent a subgroup of T-cells that have γ and δ glycoprotein chains linked by disulphide bonds (Figure 1.2) rather than the "conventional" αβ chain. Initially, γδ T-cells and αβ T-cells are thought to assemble in a similar fashion with their initial respective heterodimers binding to the invariant CD3δε heterodimer, then CD3γε heterodimer and thereafter to the CD247 (also called the TCRζ) homo-dimer. Large numbers of different receptors can be generated whereby different segments of genes: the Variable (V), Diverse (D) and Joining (J) segments combine at random to produce numerous gene sequences- VDJ recombination. This re-arrangement allows the generation of different receptors to a variety of different Ags

(Kabelitz, *et al*. (2000)). The TCR Gamma (γ) chain involves VJ recombination, and Delta (δ) chain V(D)J recombination; whilst in the αβ T-cells, the TCR alpha (α) chain is generated via VJ recombination whereas the beta (β) chain is generated by V(D)J recombination. γδ T- cells express CD3 – a protein complex forming part of the TCR, but are normally CD4/CD8 negative (double negative), although on occasion they may express variable CD4 and CD8 (Brenner, *et al.* (1986); McLean *et al.* (1986); Kabelitz *et al.* (1990)). Recent data (Munoz-Ruiz *et al.* (2013)) has shown that there are different structural constraints in the 2 (αβ and γδ) TCR isotypes, with regards to the incorporation of the CD3γε and CD3δε. The isotypes exhibit different discordant surface expression behavior when confronted with low levels of CD3γ chain but not the CD3δ chain. The γδ TCR expression was more dependent on available CD3γ than its αβ T-cell counterparts.

The γδ T- cells represent approximately 2-10% of all human T- cells in the peripheral blood (Chien and Bonneville (2006)) and are also found in other tissues within the body, most abundantly in gut epithelial tissue and others such as skin and bronchial epithelia. The strategic position of γδ T-cells at epithelial barriers, thus being constantly exposed to Ag and bacteria, may allow the cell population to have a key role is recognition of pathogens invading the body via ports of entry. Thus, the γδ T-cells are proposed as innate sentinels, guarding against Ag exposure or pathogen invasion.

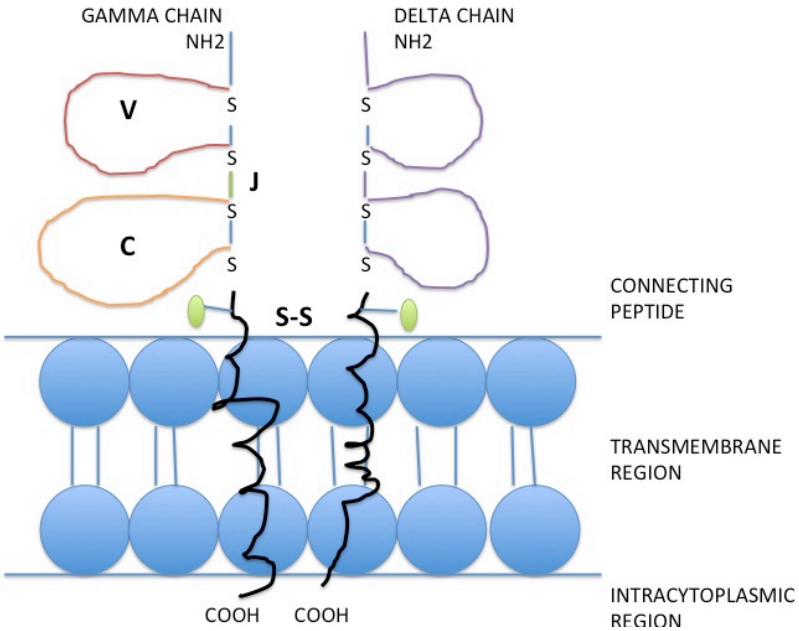


FIGURE 1.2: The γδ T-Cell Receptor (TCR)

The γδ TCR is composed of a γ-chain and δ-chain linked by a disulphide (S-S) bond. The Variable (V), Joining (J) and Diverse (D) regions seen.

Distribution of γδ T-cells

The subset of γδ T-cells, and their fluidity and predilection to certain anatomical locations is associated with their different Vδ-chain expressions. Two main subsets of γδ T-cells exist in humans determined by their Vδ chain re-arrangement. Vδ1 cells are predominantly found in the gut and other peripheral tissues (such as lung, kidney and spleen), whereas Vδ2 cells represent the major fraction of circulating γδ cells in blood (Triebel and Hercend (1989)). Although the main subsets in humans are Vδ1 and Vδ2, there are 6 expressed Vγ and Vδ genes and even more in mice. The Vδ2 subset (also known as Vδ2Vγ9) is unique to humans and primates making up to 50-95% of the total γδ population in the blood. The Vδ2 subset expands in acute infections such as: Tuberculosis (TB), Salmonella, Listeria, Brucella,

Malaria and Toxoplasmosis even before other lymphocyte populations expand (De Paoli *et al.* (1992); Eberl *et al*. (1992)). The Vδ1 T-cells can recognize MHC molecules, and this population is expanded in intracellular bacterial infections (Mycobacteria Tuberculosis and Listeria Monocytogenes), extracellular infections (Borrelia Burdofugeri) and have been shown to proliferate in response to stimulation with Ags from Cytomegalovirus (CMV) (Dechanet J, *et al.* 2010; Knight *et al.* (2010)) Vδ1γδ T-cells have also found to be expanded in the periphery in patients with Acquired Immunodeficiency Syndrome (AIDS) (Boullier *et al.* (1997)). Thus a small proportion of Vδ1 cells can also be expanded in the periphery in certain disease states.

Recent data has also show that although the main peripheral blood subset of γδ T-cells, the Vδ2 subset can also play an important role in local disease processes in barriers such as the skin. Laggner and colleagues (Laggner *et al*. (2011)) identified the Vδ2 as a pro-inflammatory subset likely involved in the pathogenesis of psoriatic lesions in the skin, with reduced circulating levels of these cells found in the periphery compared to healthy controls or patients with atopic dermatitis. Thus there remains a degree of fluidity between different subsets of γδ T-cells and the conditions/locations in which certain subsets expand/ are recruited to. Part of this fluidity and trafficking may indeed be determined by their CD expression, and importantly by CD161. Also there is a difference in these cells for the protective and deleterious roles in human physiology. Another subset, the Vδ3 T-cell population have been found to be enriched in the liver when compared to the periphery (Kenna T, *et al*. (2004)).

Recognition by γδ T-cells

γδ T-cells in contrast to αβ T-cells do not exclusively require MHC presentation of Ag. They in addition recognize small non-peptide Ag derived from infectious agents such as bacteria. The Vδ2 subset recognises small non-peptide pyrophosphomonoesters Ag, collectively known as Phos-Ag. The main Phos-Ag recognized is the microbial compound (e)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMB-PP): an essential metabolite in pathogenic bacteria but not in the commensal human microbiota. HMB-PP is an intermediate in the non-Mevalonate pathway leading to formation of Isopentenyl pyrophosphate (IPP), a compound needed for cell metabolic processes such as protein anchoring, N-glycosylation and cell membrane maintenance. The Vδ1 subset recognize stress-inducible MHC Class 1 chain-related Ags (MIC) A/B (Groh V *et al*. (1998)) or self-lipid presented Ags by CD1c (Spada FM *et al.* (2000)). The γδ T-cells can also be modulated by Toll-like receptor (TLR) ligands (Figure 1.3). TLR 1, 2, 3, 5 and 6 ligands can act directly *in combination* with TCR stimulation to enhance chemokine and cytokine production (Wesch D *et al.* (2011)). TLR ligands however on their own are not enough to fully trigger on γδ T-cells.

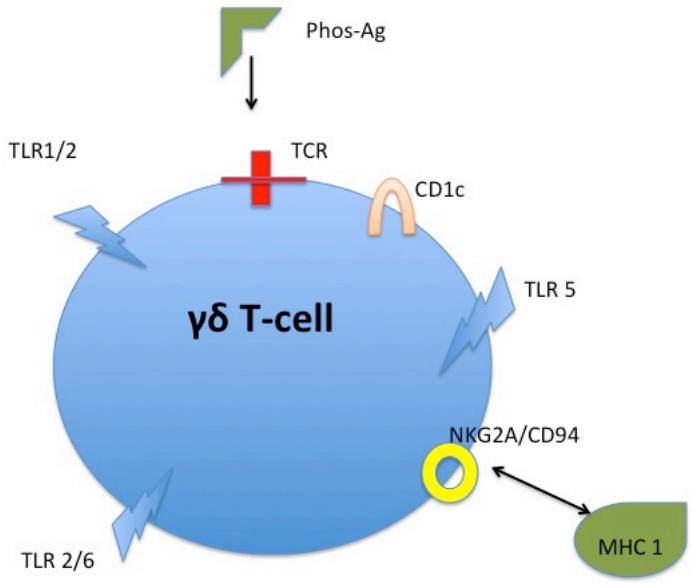


FIGURE 1.3: Recognition of Ags by γδ T-cell.

Recognition can occur via the TCR, via the TCR in combination with TLRs or via MHC Class 1 molecules.

Triggering of γδ T-cells

Bacteria synthesizing IPP via the classical Mevanolate pathway such as Streptococcus and Staphylococcus do not produce HMB-PP and thus do not recruit γδ T-cells, whereas other bacteria such as Listeria Monocytogenes and the parasite Toxoplasma Gondii which synthesise IPP (and intermediate HMB-PP) via the alternative non-Mevalonate pathway do indeed produce HMB-PP and thus recruit γδ T-cells. One drug group called the Aminobisphosphonates (used in clinical practice for bone strengthening in osteoporosis by the inhibition of bone digestion by osteoclasts) is comparable to IPP, and thus act as a Vδ2 agonists for potential therapeutic roles (Yuasa *et al.* (2009)). Other agonists of the Vδ2 cells include tumour cell lines and

alkylamines (used in the chemical industry). Although these agents/compounds stimulate γδ T-cell lines, few single non-peptide ligands have been shown to be both necessary and sufficient to stimulate γδ T-cells, and indeed T-cell activation by Phos-Ag requires cell-to-cell contact (Chien and Boneville. (2006)) suggesting the TCR alone may not be exclusively involved.

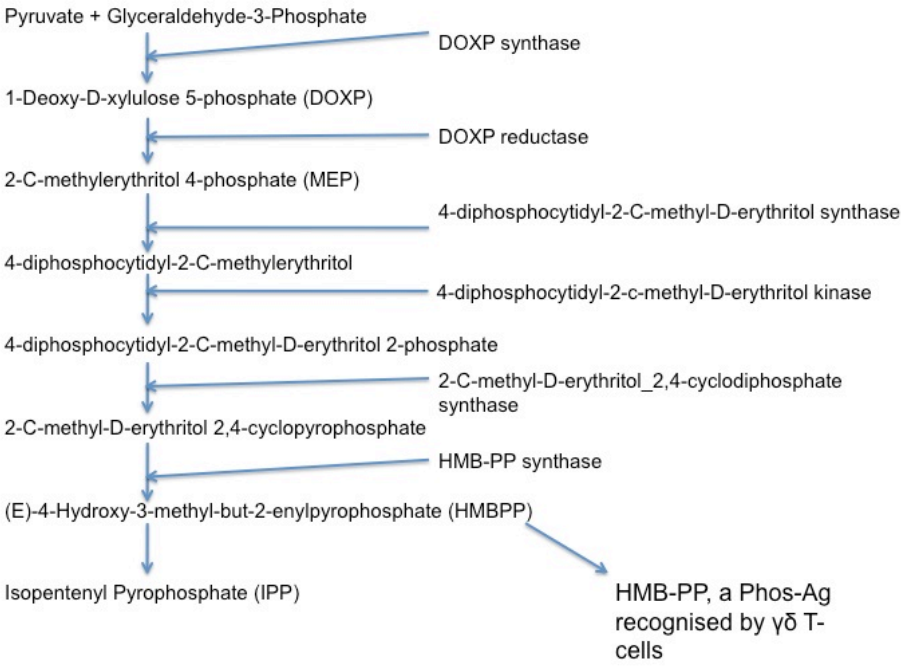


FIGURE 1.4: The non-mevalonate pathway

The non-mevanolate pathway of generating HMB-PP and subsequently IPP – which is recognized by γδ T-cells.

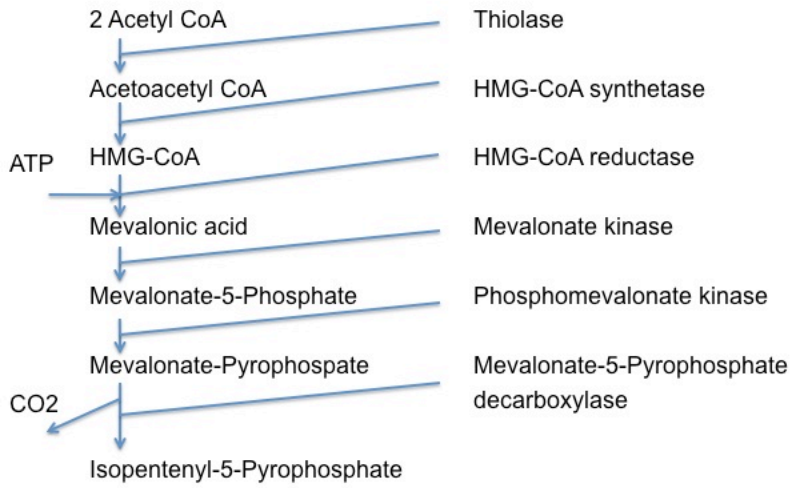


FIGURE 1.5 The "classical" Mevalonate pathway

Pathway whereby there is a generation of IPP with no generation of the intermediate HMB-PP, thus no recruitment of γδ T-cells.

Physiological role of γδ T-cells in mouse models

γδ T-cells are thought to serve as a "link" between the innate immune system and the adaptive immune system. Their role in the adaptive immune system stems from their ability to rearrange TCR genes to produce junctional diversity with memory phenotype, whereas an example of their role in the innate immune system is the Vδ2 subset responding within hours to a microbial infection, and the Vδ1 subset in the gastrointestinal tract responding within hours of an epithelial insult.

Mouse knockout models have confirmed the importance of γδ T cells in host defense with a lack of them conferring immunodeficiency (down-regulated CD8+ T cell responses against Encephalitozoon cuniculi infection (Moretto *et al.* (2001)). In contrast there are a number of human diseases where increased

numbers of γδ cells have been found including Graves disease, Multiple Sclerosis (MS) and Rheumatoid Arthritis (RA). This suggests a paradigm where the γδ T-cell population are protective against bacteria but also they may be implicated in disease pathogenesis too (Kjer-Nielsen *et al.* (2012)). This paradigm was illustrated in a mouse model of Staphylococcus aureus infection resulting in pneumonia (Cheng *et al* (2012)). On intranasal Staphylococcus aureus infection, the mice showed a rapid γδ T cell accumulation in the lung. In γδ T cell deficient mice there was an attenuated bacterial clearance and reduction of lung tissue damage along with impaired neutrophil recruitment and a reduction of cytokine production when compared with wild-type mice. The γδ T-cells produced Interleukin (IL)-17 (knockout mice produced less IL-17) and thus it was proposed in this model that γδ T cell accumulation in the lung was required for bacterial clearance, however that the accumulation may also contribute to parenchymal damage. The γδ T-cells were a major source in this setting for IL-17, which may thus influence the recruitment of neutrophils to the lung.

γδ T-cells in the human gut

γδ-T cells in the gut epithelium make up to 50% of the intra-epithelial lymphocyte population (Viney *et al* (1990)) a population that is expanded in certain disease states such as Coeliac Disease (Halstensen *et al.* (1989)). Helicobacter Pylori (HP) is a resident bacteria in the stomach and been found to be associated with peptic ulcers (Marshall (1985)). Recently, γδ-T cells have been shown to be activated by HP *in-vitro* (Romi *et al.* (2011)). Live HP activated CD3+ γδ-T cells with a non-specific increased expression of CD69 (activation marker), and also production of TNF-α and IFN-γ and chemokines

such as RANTES and MIP-1α. It was proposed HP bacteria may cause activation of γδ-T cells in the gut, resulting in a continuous chronic pro-inflammatory drive, and maybe future GI pathology such as ulceration. Treatment of HP with standard "triple therapy" (2 high dose antibiotics and a proton-pump inhibitor) may indeed eradicate HP, thus hypothetically reducing the stimulus for γδ-T cells activation and production of pro-inflammatory cytokines.

Physiological roles of γδ T-cells

Recently γδ T-cells have been found to be a source of IL-17 (Ness-Schwickerath *et al.* (2010)) promoting granulopoiesis and neutrophil recruitment to aid pathogen clearance. The production of IL-17 by γδ cells in the gut epithelium can lead to tight junction formation and mucin secretion in the face of insults to the gut epithelium. They have been found also to produce IL-17 in the face of TB infection in the lung (Peng *et al*. (2008)). Along with IL-17, γδ T-cells also produce IL-22, Granzyme B, Perforin, Tumour Necrosis Factor (TNF)-α and Interferon (IFN)-γ (Garcia *et al.* (1997); Bade *et al.* (1997); Wang *et al.* (2005)).

γδ T-cells and malignancy

γδ T-cells have been studied extensively in the oncology field. *Ex-vivo* expanded γδ T-cells from healthy volunteers have been shown to be cytotoxic to high-grade glioblastomas *in-vitro* and *in-vivo* (Bryant *et al.* (2009)). γδ T-cells have also been shown to mediate killing of other tumour cells and an important effector of the immune system with an anti-tumour peripheral surveillance role (Hayday (2009)). The Vδ2 T-cells are triggered by Phos-

Ags (which are indeed increased in malignancy) and produce cytokines typical of Th-1, Th-2 or Th-17 cells (Vermijlen *et al.* (2007); Caccamo *et al*. (2011; Wesch *et al.* (2001)), cross-talk with DCs (Mergavigalia *et al.* (2011)) and also have a direct cytotoxic effect via: perforin/granzyme, Fas/FasL, TNF /TNF-R and TRAIL-TRAIL-R pathways (Bonneville M *et al.* (2010)). The Vδ1 subset location in epithelia leads to surveillance of such malignancies as melanoma (Hayday (2009)). Recently Vδ1 and Vδ2 T-cell subsets have been found to be involved in killing of melanoma, showing both an effector phenotype but also terminally differentiated phenotype (Cordova *et al. (*2012)). The killing capacity of the Vδ2 T-cells was improved with pre-treatment Aminobisphosphonate of tumour target cells. The role and contribution of IL-17 producing γδ-T cells to the efficacy of anticancer chemotherapy was further studied by Ma and colleagues (Ma *et al.* (2011)). They demonstrated that chemotherapy led to a rapid and marked invasion of γδ-T cells into tumor bed, actually preceding accumulation of Tc-1 cytotoxic T-lymphocytes. The effect of chemotherapy on TCR-γ knockout mice was compromised, with no IL-17 production by the tumour infiltrating T-cells. Lack of a functional IL-17A-IL-17R pathway also reduced tumour specific T-cell responses and tumour cell death; however this was restored after adoptive transfer of γδ-T cells. The anticancer effect of the γδ-T cells was lost when lacking IL-17A or IL-1R1. Thus this data showed that γδ-T cells do indeed play a vital role in anticancer immune responses and are an important subset of T-cells in the immune system. Their role in the future of anticancer therapy may be either via adoptive transfer (Nakajima *et al.* (2010)) or *in-vivo* stimulation and recruitment through the Aminobisphosphoantes (Lucas C *et*

al. (2009)), and remains an ever developing area.

1.2 Gamma Delta T-cells in Hepatology

The epidemic and burden of chronic liver disease

The most common causes of chronic liver disease in the world include: Hepatitis C (HCV), Hepatitis B (HBV), alcoholic liver disease, Primary Sclerosing Cholangitis (PSC), Primary Biliary Cirrhosis (PBC) and Non-Alcoholic Fatty Liver disease (NAFLD)/ Non-Alcoholic Steatohepatitis (NASH). HCV infection is a worldwide epidemic that can be classified into 6 main genotypes based on sequence homology. The latest World Health Organisation figures estimate that more than 170 million people (3% of the world's population) are infected with HCV (World Health Organisation, 2004). Of those acutely infected, 50-80% of patients do not clear the virus and go on to develop persistent infection. Persistence of the virus in the long-term is associated with the development of liver fibrosis and eventually cirrhosis with its inherent risk of life threatening complications including oesophageal variceal bleeding, spontaneous bacterial peritonitis and hepatocellular carcinoma (HCC) (Lauer and Walker (2001)). The aim of therapy in the treatment of HCV is long-term viral eradication. The treatment regimens in chronic HCV have changed, with the previous standard of care treatment (a combination of Pegylated-Interferon and Ribavarin) being superseded in Genotype 1 patients with addition of the new Direct-Antiviral agents (Boceprivir and Telaprevir) to the previous standard of care (Zeuzem *et al.* (2008); Ghany *et al.* (2011). The treatment of chronic HCV is

constantly changing, with the aim soon of oral Interferon-free regimes coming of age such as Sofosbuvir (Lawitz and Gane. (2013)).

The liver and immune system: an introduction

The immune mediated mechanisms by which liver damage ensues in certain conditions/ infections and not in others remains a key question. Intra-hepatic T-cell populations has been well characterized in humans, with some intra-hepatic T-cells expressing CD4+, CD8+ and the αβ TCRs. However NK cells and γδ T-cells in the liver have also been identified with up to 15% of mouse and human IHLs being γδ T-cells (Mehal *et al.* (2001). With γδ T-cells playing a pivotal role in the immune system involved in viral and bacterial clearance, and their preponderance to localise preferentially in the liver compared to blood (Norris *et al.* (1999)), their contribution to liver disease remains of great interest. The porto-systemic circulation may provide a route for γδ T-cells to move from the gastrointestinal tract to the liver.

γδ T-cells in chronic hepatitis

The presence of γδ T-cells in chronic hepatitis biopsies was explored by Kasper and colleagues (Kasper et al. (2009)). In biopsies from 18 HBV and 25 HCV patients, they found the predominant portal tract infiltrate to be αβ T-cells. However the lobular infiltration frequencies between αβ and γδ T-cells were approximately equal. The authors concluded the γδ T-cell population was rarely found in patients with chronic hepatitis, and that the αβ T-cells were the key immunological cell population. Conflicting results however were described by Tseng and colleagues (Tseng *et al.* (2001)) who found, in T-cell lines generated from patient's liver biopsies with HCV/HBV *in vitro*,

significant numbers of γδ T-cells compared to expanded cells from the non-virally infected liver. These γδ T-cells had high levels of non-MHC-restricted cytotoxicity activity against primary hepatocytes and also produced high levels of IL-8, IFN-γ and TNF-α when activated by anti-CD3. Similar findings were described by Kanayama and colleagues (Kanayama *et al.* (1992)) finding increased γδ T-cells in immunohistochemical staining of patients with chronic liver disease.

The intra-hepatic γδ T-cell population was further described by Agrati and colleagues (Agrati *et al.* (2001)), analyzing 35 matched liver/blood samples from patients with chronic HCV. There was a specific compartmentalization of Vδ1 cells in preference to Vδ2 in the liver with the cells expressing a memory/effector phenotype (CD62L- CD45RO+ CD95+). Mitogenic stimulation of these cells revealed they produced IFN-γ and IL-4. A higher frequency of IFN-γ producing Vδ1 cells was associated with higher degree of necro-inflammation, suggesting that these cells may indeed contribute to the disease pathogenesis/ progression in HCV patients. Agrati et al (Agrati *et al.* (2011)) further analysed the antiviral functions of the Vδ2 T-cells on Huh7 hepatoma cells carrying the subgenomic HCV replicon. Activation of the Vδ2 cells was associated with a marked reduction of HCV RNA levels. The neutralization of IFN-γ by antibodies revealed the importance of this cytokine in inhibiting HCV replication. The inhibition of HCV was increased by the use of Aminobisphosphonates that activate γδ T-cells – thus pointing towards a potential future therapeutic strategy.

Another group (Par *et al.* (2002)) studied γδ T-cell responses in the blood in

patients who had HCV disease compared to healthy controls, and those patients who had cleared the virus with treatment. Patients with chronic HCV showed an impaired Natural Killer (NK) cytotoxicity, decreased percentage of Vδ2 cells, as well as reduced perforin-positive T lymphocytes compared to controls or to those who had cleared HCV infection. These data suggest that impaired function and number of γδ T-cells in the periphery may indeed contribute to the pathogenesis of HCV and that potentially the Vδ2 cells are protective, but Vδ1 not.

γδ T-cells in non-viral liver diseases

With γδ T-cells playing a key role in the immune system, their role has also been explored in the liver transplant setting and other liver diseases apart from viral hepatitis. Increased numbers of γδ T-cells have been identified in the circulation of patients with stable liver graft function (Malan-Borel *et al*. (2003); Kobisha *et al.* (2007); Martinez-Llordella *et al.* (2007)). The cell population has been characterized in murine models revealing that γδ T-cells are indeed involved in allogenic immune responses in the murine model of liver transplant, with numbers increasing in those mice with Cytotoxic T-Lymphocyte antigen (CTLA)-4/antiCD-25 induced rejection (Malone *et al*. (2009)). The role of γδ T-cells in the post-liver transplant setting has been studied (Puig-Pey *et al*. (2010)) with the finding of an increase in the total circulating peripheral γδ T-cell population in patients post-transplant when compared to healthy controls. The main circulating fraction of γδ T-cells was infact the Vδ1 T-cell population irrespective of type of transplant (single liver or combined liver/kidney) or type of immunosuppression. This population did not change over time either when tested in patients with stable graft function

over time. When correlated to viral infections in these patients, the physiological reversal of the normal peripheral blood Vδ2>Vδ1 was seen in patients with HCV and CMV positivity (rather than EBV or HSV positive patients). Thus it was proposed patients may have increased Vδ1 populations in the transplant setting as a consequence of certain chronic viral triggers. The Vδ1 cell population expressed CD4, CD8, NKG2D, Perforin and IL-17A.

The role of γδ T-cells in autoimmune liver disease has recently been studied in detail by Ferri and colleagues (Ferri *et al*. (2010)). This group explored these cells along with T-regulatory cells and Natural Killer-T (NKT)-cells in patients with autoimmune hepatitis (AIH). The γδ T-cell population frequency was comparable between the PBMCs of patients with AIH (irrespective of disease state- active vs. remission) and healthy control groups. Interestingly they observed a reversal of the physiological Vδ2:Vδ1 ratio with more Vδ1 cells found in the periphery ($p=0.001$). In patients with AIH, the γδ T-cell population expressed significantly more Granzyme B (with higher levels in patients with active versus remission of AIH). The number of Vδ1 cells producing IFN-γ was significantly higher in patients with AIH compared to healthy controls. However no differences were seen in disease activity states. Moreover, there was no significant difference in IFN-γ production by Vδ2 cells. Finally, γδ T-cells have also been described in patients with PBC/PSC with elevated numbers in the periphery compared to controls (Martins *et al.* (1996)).

1.3 CD161: an introduction, and MAIT cells

CD161: an introduction

CD161 (also known as Killer Lectin Receptor subfamily B member 1 (KLRB1)) is a C-type lectin membrane glycoprotein that is expressed on the majority of NK cells, and approximately 24% of peripheral T-cells (Lanier *et al.* (1994)). CD161 was originally identified as the human homologue of the NKRP1A glycoproteins expressed on rodent NK cells, demonstrating 46-47% homology with its rodent counterparts. It is composed of a disulfide-linked homodimer of 40kDa subunits, and has been shown to be expressed on both αβ and γδ T-cells (Maggi *et al.* (2010)). CD161 is present on CD4 and CD8 αβ T-cells with higher surface density on CD8 T-cells (Lanier *et al.* (1994)). CD8 cells expressing CD161 can be divided into further populations based on expression levels with a CD161+high and CD161+intermediate levels (also known as CD161 "bright" and CD161 "mid" populations) (Takahashi *et al* (2006)). CD161 T-cell expression has consistently been associated with memory phenotype in adult circulations in not only αβ T-cell populations (Takahashi *et al* (2006); Annunziato *et al.* (2007); Cosmi *et al*. (2008); Kleinschek *et al.* (2009) but in the γδ T-cell population too (Maggi *et al*. (2010)) with the CD8+ CD161+ predominantly an effector memory population. Less than 1% of CD161+(high) CD8+ CD45RO+ cells expressed a central memory phenotype (Takahashi *et al.* (2006)) suggesting CD161 expression may be acquired as a result of immune stimulation. CD161 itself has been shown not just to be a simple activation marker; as re-stimulation of influenza-specific cells in the presence of cytokines had no resultant expression of CD161 (Northfield *et al.* (2008)).

The timing of CD161 acquisition on T-cells has been studied, with a subset of T-cells in foetal liver expressing CD161 and in a minor population of CD3+ thymocytes (Lanier *et al* (1994); Martin *et al* (2009)). CD161positive CD4 T-cells have been found in umbilical cord blood (Cosmi *et al.* (2008)) whilst CD161++ CD8+ T-cells have also been found to be present in cord blood (Martin *et al.* (2009); Billerbeck *et al.* (2010); Walker *et al.* (2011)).

MAIT cells

CD161 positivity has been found in Mucosal-associated invariant T-cells (MAIT) cells (Dusseaux *et al.* (2011)) that are abundant in humans (comprising up to 45% of liver lymphocytes). High expression of CD161 is the main characterisctic of MAIT cells, and CD161expression is an early feature of MAIT cells (Walker *et al.* (2011)). Staining with anti-Vα7.2 mAb and either CD161 or IL-18R unequivocally identifies MAIT cells in the γδ-CD4- CD3+ compartment. Thus these cells are distinct from the γδ T-cell population. MAIT cells develop in the thymus, exiting as naïve cells, expanding rapidly after birth in humans (Dusseaux *et al*. (2011)), and express an invariant TCR Vα7.2-Jα33. Unlike conventional T-cells that use a diverse TCR repetroire to recognise a wide variety of Ags, MAIT cells have a more limited repetoire restricted by the MHC-Class 1 like molecule (MR1). After expansion these cells make up up to 10% of human PBMCs (Dusseaux *et al.* (2011)). Once in the periphery they encounter commensal flora, expand further, display an effector-memory phenotype (CD45RO(+)CD95(hi)CD62L(lo)), and their chemokine receptor expression

pattern (expressing high levels of CCR6 and CXCR6) indicating a preferential homing to tissues and particularly the intestine and the liver (Billerbeck *et al.* (2010); Northfield *et al.* (2008). The overlap between CD161+Tc17 T-cells and MAIT cells still needs further clarification. Both sets are CD161+ and produce IL-17/expressing Retinoic acid-related orphan receptor γ-t (RORγT), yet MAIT cells are unable to react to virus-infected APCs (Le Bourhis *et al.* (2010)) unlike the Tc17 cells (Billerbeck et al (2010)). A simple explanation is that in adults the MAIT cell population provides the vast majority of the CD161+ Tc17 populations, with a smaller fraction reserved for peptide specific responses (Walker et al. (2011)). These MAIT cells play an important role in host defense against organisms and bacteria reacting in a MR-1 dependant manner (Le Bouhris *et al.* (2010); Gold *et al.* (2010))– *(see Ligands for CD161+ T-cells).*

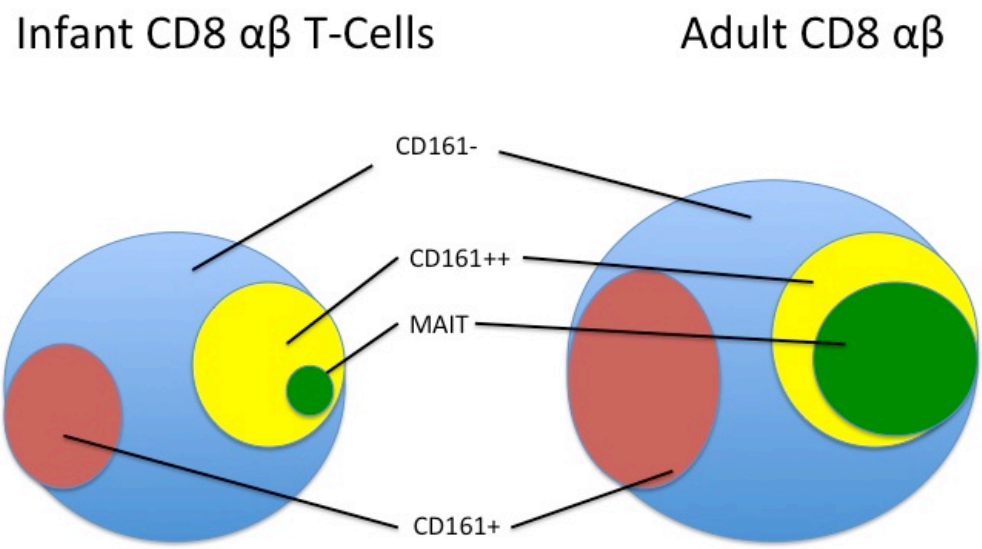


FIGURE 1.6: Expansion of CD161 and MAIT cells through life.

In infants there is a small CD161+, CD161++ and MAIT cell pool out of the total CD8 αβ T-cell pool. With time there is an expansion of the total CD161+ Tcell pool and CD161++ pool, however of this latter pool the MAIT cells grow and predominate.

Ligands for CD161+ T cells

The ligand for CD161 in humans is still an evolving field with to date 2 putative ligands identified. Lectin-like Transcript-1 (LLT1) is expressed by both activated APCs (Rosen et al. (2008)) and lymphocytes (Aldemir *et al.* (2005)). Ligation of CD161 to LLT in these cell subsets produced different effects, with an enhancement of IFN-γ production on lymphocytes, but inhibition of IFN-γ from APCs. Another ligand, Proliferation-induced lymphocyte-associated receptor (PILAR), related to LLT1 is expressed on lymphocytes being upregulated in a TCR-dependent fashion (Huarte *et al.*

(2008)). In the absence of CD28 co-stimulation, PILAR increased the proliferation of naïve T-cells, suggesting CD161 may be a co-stimulatory receptor enhancing proliferation via interaction with PILAR. It has been shown however that the PILAR gene encoded another ligand called keratinocyte-associated C-type Lectin (KACL) that interacted with a different distinct C-type lectin receptor called NKp65 (Spreu *et al.* (2010)) and not with CD161.

MR1 is a highly conserved MHC molecule and is a bacterial ligand that has been found to be involved in the presentation of exogenous conserved microbial molecules to MAIT cells. MR1 has a high sequence identity for the α1 and α2 domains, forming a putative ligand-binding groove (confirmed on mutational studies (Huang *et al.* (2005)). Overexpression of the invariant chain in APCs facilitates the trafficking of MR1 from the endoplasmic reticulum to endosomes (Huang *et al.* (2008)). This trafficking is required when microbes trigger MAIT cells via MR1, blocking endocytosis in APCs during infection and inhibiting MAIT cell activation (Le Bouhris *et al.* (2010)). MR1 also binds Vitamin B intermediates (Kjer-Nielsen *et al.* (2012)), thus providing another potential pathway for the triggering of MAIT cells via bacteria that have a functional riboflavin biosynthesis pathway. With the CD161 orthologs in mice existing and being both inhibitory and activating, there remains much work to be carried out on the CD161 ligands in humans. However the recent data on MR1 may explain at least the ligand for MAIT cells.

CD161 T-cells and viral Hepatitis

CD161 is expressed on a significant subset of HCV and HBV-specific T-cells, but to lower levels on CMV, Human Immunodeficiency Virus (HIV) or Influenza specific responding cells (Northfield *et al.* (2008)). Further exploration of the properties of CD161+ CD8+ T- cells in infected HCV showed an effector phenotype and the cells produced pro-inflammatory cytokines, such as IFN-γ and TNF-α, but express low levels of Granzyme B and Perforin, suggesting a reduced immediate ability to lyse virus-infected cells. CD161 was strongly correlated with expression of CXCR6, a chemokine receptor strongly implicated in liver homing. These CD8+CD161++ T cells have been studied (Billerbeck *et al.* (2010)) in patients with HCV (and in healthy controls) revealing a functionally distinct population of CD8+ cells expressing high levels of CD161 and a pattern of molecules consistent with Th-17. These CD8+CD161++ T-cells expressed numerous molecules including cytokines IL-17 and IL-22 (a cytokine promoting hepatocyte survival in the liver (Park *et al.* (2011))). They also expressed transcription factors RORγ-t and Runt-related transcription factor (RUNX)-2, cytokine receptors IL-23 receptor and IL-18 receptor, and chemokine receptors CCR6 and CCR2, along with CXCR6. CD161+CD8+ T cells were markedly enriched in liver tissue samples and co-expressed IL-17A with high levels of IFN-γ and/or IL-22. The levels of these poly-functional CD161+ T-cells in tissue were most marked in those with mild liver disease. The role of CD161+ in NK cells has further been studied in HCV infection. In the acute setting (Alter *et al.* (2011)) there were reduced frequencies of CD161 expressing NK cells in blood of patients who could clear acute HCV

compared to those going on to develop chronic HCV. This suggested at least on NK cells, a possible role of CD161+ NK cells in acute HCV infection.

1.4 CD161 & Gamma-Delta T-cells

CD161 and its link to γδ T-cells was first studied by Battistini et al (Battisitini *et al (*1997)). The expression of NK receptors on γδ T-cells was defined, with CD94 the most common NK receptor being found on γδ T-cells. γδ T-cells were found also to express CD161, but not on all cells. Hence, it was postulated that the CD161 phenotype could be acquired *in vitro* which was confirmed in culture experiments. All cells that were Vδ2 expressed CD161 in healthy controls. On cross-linking the γδ T-cells with CD161, the γδ T-cells expressed large amounts of IFN-γ. Experiments previously had shown that that CD161 was involved in trans-endothelial migration of CD4 T-cells (Poggi *et al.* (1997)), with CD161+CD4 T-cells migrating further across endothelial cell monolayers compared to CD161-ve CD4 T-cells. The migration was reduced by incubation with an anti-CD161 monoclonal antibody.

CD161+ γδ T-cells and the central nervous system

γδ T-cells have previously been shown to be cytotoxic towards oligodendrocytes (Freedman *et al.* (1991)) and increased frequencies of γδ T-cells in the peripheral blood and CSF of patients with MS has been observed (Stinissen *et al.* (1995)). The γδ T-cells involved in MS have also been shown to produce IFN-γ. Thus Poggi and colleagues (Poggi *et al.* (1998)) explored the role of CD161 in γδ T-cells in patients with MS. It was proposed that

CD161 was involved in the migration of γδ T-cells from the blood to the CSF and CNS. CD161+ γδ cells were increased in the peripheral blood of patients with MS (predominantly the Vδ2 subset) compared to healthy donors. CD161 expression was also found to be up-regulated on Vδ2 cells when cultured with IL-12 (known to up-regulate CD161 (Poggi *et al.* (1998)) irrespective of disease state. Transmigration was then studied by culturing γδ T-cells with IL-2 or IL-12, and on day 6 assessing transmigration through HUVEC monolayers using a double Transwell system. Vδ2 cell transmigration was higher and faster and enhanced by culture with IL-12. When blocking the cells with an anti-NKRP1A (191b8) monoclonal Ab, there was a significant reduction in transendothelial migration. It was postulated that the CD161+ γδ T-cells did indeed localize to sites of inflammation, and on exposure to IL-12 up-regulate expression of CD161.

Further experiments revealed that the Vδ1 subset rather than using CD161 used platelet endothelial cell adhesion molecule (PCAM1 or CD31: an adhesion molecule involved in lymphocyte extravasion) for trans-endothelial migration (Poggi *et al.* (2002)). There was no difference in expression of Vδ1 levels of CD161 between healthy donors and MS patient's peripheral blood. In the same model to assess trans-endothelial function as previously mentioned, CAMKII (a Calmodulin-dependant protein kinase) was found to be an important factor involved with the migration on Vδ2 cells as well as CD161. It was found that engagement of CD161 induced the activation of CAMKII on Vδ2 cells.

The migratory pattern on CD161 has been recently studied, with co-expression of CD161 and CCR6 in γδ T cells in CSF and peripheral blood studied in patients with MS (Schirmer *et al.* (2013)). The fraction of CD161high CCR6+ γδ T cells was significantly higher in the CSF of patients with MS in relapse than patients with systemic autoimmune disorders or even controls with non-inflammatory disease. The CD161high CCR6+ double-positive γδ T-cell population was enriched in the CSF in relation to blood in MS patients in relapse compared to other patients/controls .The CD161high CCR6+ γδ T-cell population was indeed characterized by its capacity to produce IL-17. Thus the CD161+CCR6+ γδ T-cell population may indeed be a subset in patients with MS correlating to poor disease outcome. The role of CD161+ T-cells in MS has been further studied via gene array techniques, (in the CD8 αβ T-cells (Annibali *et al.* (2011)) rather than γδ T-cells). Leukapheresis from disease-discordant monozygotic twins and gene expression profiling in CD4+ and CD8+ T-cell subsets was carried out. Disease-related differences emerged only in the CD8+ T-cell subset and not the CD4+ subset – γδ T-cells were not sub-analysed. The five differentially expressed genes identified included CD161, previously presented by the International Multiple Sclerosis Genetics Consortium as one of the non-MHC candidate loci (Hafler *et al.* (2007)). FACS on peripheral blood of healthy donors and patients with MS and RA confirmed an up-regulation of CD161 at the protein level, showing also a significant excess of CD161+(high)CD8+ T cells in MS. Thus irrespective of αβ T-cell or γδ T-cell, it appears the T-cells' CD161 status can play an important role in that cell's function that is relevant to disease pathology.

1.5 Aims of thesis

These aims follow on from published data (Northfield *et al.* (2008); Billerbeck *et al* (2010)) finding CD161 positivity to convey a distinct phenotypical and functional characteristic to CD161++ αβ T-cells, and their role in HCV infection; we applied this same premise to CD161+ γδ T-cells. If these T-cells were indeed different from their CD161- counterparts, we aimed to explore this cell subset not only in health but also in patients with chronic liver disease. Specifically we wanted to address the following questions throughout our experiments:

1. Does CD161 positivity identify a different subset of γδ T-cells with distinct phenotypic and functional characteristics in health?

2. Is CD161 positivity innate or acquired on γδ T-cells?

3. Do CD161+ γδ T-cells differ in number, phenotype or function in chronic HCV infection and other forms of chronic liver disease?

4. Are CD161+ γδ T-lymphocytes specifically linked to an intra-hepatic role?

2: Methods

2.1 Blood separation & PBMC storage/thawing

Blood separation/ PBMC isolation

Blood preparation was performed in a standard defined protocol used in the Peter Medawar Building for Pathogen Research laboratories (as defined below). All infectious blood (HBV and HCV in this project) or blood products isolated were stored and processed in a designated Category III laboratory. Venous blood was collected in heparinised tubes and then diluted 1:1 in R10 medium (Roswell Park Memorial Institute medium (RPMI) medium/ 10% fetal bovine serum/ 100 U/mL penicillin and 100 μg/mL streptavidin). Aliquots of diluted blood were slowly overlayed onto 10mls of Lymphoprep (Fresenius Kabi ™ Norge AS, Norway) at a 2:1 ratio in a 50ml Falcon tube. This tube was centrifuged for 25 minutes at 2200 revolutions per minute (rpm), 4 degrees with a slow start and stop (i.e. brake off). The lymphocyte layer was then removed with a pipette and re-suspended into a total of 50mls of R10 solution. The cells were then centrifuged at 1700rpm for 10 minutes at 20 degrees. The cells were then re-suspended into 10mls and counted with re-suspension into required volume for staining/experiment thereafter. All freshly isolated Peripheral Blood Mononuclear Cells (PBMCs) were used that day in experiments or rested overnight (i.e. none were frozen down).

PBMC thawing

Some pre-existing stored frozen PBMCs from patients with HBV, HCV, PSC/PBC and 10-week old infant samples were used for experiments. Thawing medium was prepared in the following dilutions:

-1:10 the 10X stock CTL-Wash (supplement medium solution in RPMI)
-1:100 L-glutamine
-30U/ml DNAse 1
-1:100 L-glutamine Penicillin Streptomycin

The thawing media and the R10 media were pre-warmed in 37^{o}C (+/- 1^{o}C) in the water bath. The required PBMC vial(s) were removed from liquid nitrogen and placed on dry ice until ready to thaw. The cells were thawed quickly with gentle agitation in 37^{o}C (+/- 1^{o}C) water bath. Using a sterile Pasteur pipette each thawed cell suspension was transferred to an appropriately labeled 15 ml tube. The vial(s) from which the PBMCs had been stored were rinsed with 1ml Thawing Media, and then this was added drop wise to the thawed cell suspension. Thawing Media was added to each tube drop wise in a 1-2 minutes of dispensing/mixing cycles diluting 5x the original volume. A further 4 ml Thawing media was added to each tube drop wise over 30 seconds of dispensing/mixing cycles adding 4mls of media. The total volume was made up per falcon to 10mls (per sample thawed) then centrifuged at room temperature (18-20 degree centigrade) at 16000 rpm for 10 minutes. The supernatant was aspirated and the cells re-suspended in R10 media and counted. The thawing protocol was adopted in The Peter Medawar Building for Pathogen research for the ongoing European multicenter Hepatitis C Vaccine trial (EudraCT Number: 2008-006127-32). Donors were used as healthy controls (either whole blood donations or PBMCs isolated thereafter).

2.2 FACS staining/analysis

FACS staining

All Fluorescence activated cell sorting (FACS) staining was carried out on whole blood or fresh or thawed PBMCs. Volumes of 200mcl were used for all staining. These volumes were stained with required antibodies (Abs) from panels established within our lab or from published data (Northfield *et al.* (2008); Billerbeck *et al* (2010). All Abs and manufacturers are listed per experiment in Chapters 3 and 5 where applicable. After the required Abs were added to blood/PBMCs, the samples were incubated at 4 degrees for 30 minutes. Thereafter 2mls of FACS lysing solution (BD – mixed with Saline) was added and the samples incubated in the dark for 10-30 min at room temperature, then centrifuged at 1600rpm for 7 minutes. The supernatant was removed and the cells were washed (Phosphate Buffered Saline (PBS) added in 1-2ml amount and a repeat centrifuge step at 1600rpm for 7 minutes to wash off excess Antibody). The cells were then fixed in 200mcl of PBS with 2% formaldehyde.

FACS analysis

When using the FACSCalibur ™ machine a 4-colour panel was used of antibodies conjugated with the following fluorochrome colours: PerCP, PE, FITC and APC. The LSR-II ™ machine was also used with more fluorochrome detection channels available. All cells were fixed with 2% Formaldehyde in PBS before being run on the FACS machines. When frozen cells were thawed and analsyed, or intra-hepatic lymphocytes analysed, a live/dead marker (Near Infra-red used at concentrations of 1 in 50) was added

to the panels to delineate dead cells, which were gated out on analysis. All data from the FACSCalibur ™ or LSR-II ™ machines were then analysed using FloJo ™ and statistical analysis carried out using PRISM ™.

2.3 *Intracellular cytokine staining*

Intracellular cytokine staining (ICS) was carried out from fresh PBMCS isolated from whole blood as per methods described. PBMCs were added to FACS tubes, and re-suspended to 1ml with R10. Leucocyte activation cocktail (Becton Dickinson™, Oxford UK) –a polyclonal cell activation mixture with phorbol ester PMA (Phorbol 12-Myristate 13-Acetate), a calcium ionophore (Ionomycin), and the protein transport inhibitor BD GolgiPlug™ (brefeldin A) was used to stimulate the cells. The cells were incubated at 37 degrees for 4 hours, then washed with PBS and centrifuged at 2000rpm for 5 minutes at 4 degrees. Required surface antibodies (Anti-human CD3, Anti-human CD161 and Anti-human γδ) were added to the pellet and 50 mcls of PBS, and incubated in the dark at 4ºC for 30 minutes. This was then washed with 1ml of PBS and then re-suspended into BD Perm. The cells were then washed and then permeabilisation buffers was added and cells rewashed. Thereafter, intracellular cytokine antibodies were added with 50ul PBS. After 30-minute incubation at room temperature, the cells were washed in PBS then re-suspended in 2% formaldehyde in 200 µl of PBS (FACS-Fix). There after the cells were processed on the FACS machine (FACSCalibur ™). All samples had corresponding un-stimulated controls whereby no leucocyte activation cocktail was added; however, the full staining was still carried out as above.

2.4 Gamma-Delta T-cell sorting

All samples on which γδ T-cell sorting were carried out on were from Leucocyte cones from the National Health Service (NHS) Blood and Transplant Services, John Radcliffe Hospital, Oxford in conjunction with the Bristol NHS Blood Transfusion Division. The leucocyte cone is a filter for leucocytes that is in place in the collection of blood (and then red blood cells) from people donating blood to the blood-bank services (Research product code NC24). No patient demographic data was available for any of the Leucocyte cones used, or data available on donor health status. However, all donors prior to giving blood were screened for blood-borne viruses per NHS Blood and Transplant guidelines (HBV, HCV, HIV and Human T-lymphotrophic virus).

Stage 1: Leucocyte cone preparation

With 70% ethanol, the hood, leucocyte cones, and the scissors were all cleaned. The cone tubing was cut off the bottom of the cone, as close to the cone as possible. The cone was placed into a 50 ml falcon and the top tubing was cut, again, as close to the cone as possible (Figure 2.1). The blood was allowed to slowly drain into a falcon tube. A needle was placed through the hole at the top of the cone and using the 50ml syringe, 40ml of sterile PBS was washed through the cone. 25 mls of the leucoctye fluid was thus layered over 15ml Ficoll (lymphoprep), and then the spun without accelerator or break at 22^{o}C, 2220 rpm, for 22 minutes. Thereafter, the PBMC layer was removed with a pasture pipette, washed with 50ml R10 and spun down again for 10 minutes at 1800 rpm. Repeat washing was carried out and re-

centrifuged for 5 minutes at 1500rpm. The pellet was broken and cells topped up with 50 ml with R10 and the cells were then counted, prior to being frozen for storage or used directly.

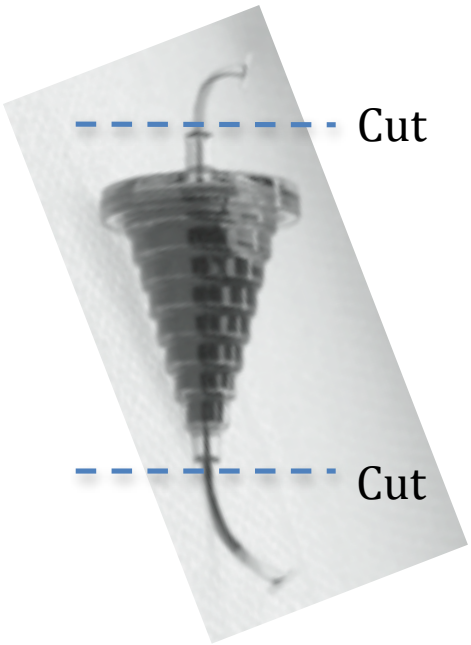


FIGURE 2.1: Leucocyte cone

Diagram of the leucocyte cones (approximately 6cm in longtitudinal axis) used to isolate large numbers of leucocytes. Areas shown where cuts are made for preparation of cells.

Stage 2: T-cell enrichment

The cells isolated from the leucocyte cone were enriched for T-cells prior to staining. The maximum total cells to be enriched by EasySep T-cell enrichment kit ™ (Stemcell, UK) was $1x10^8$ cells. Cell yields from the leukocyte cones were often near, or above, 10^9 total cells. Therefore, cells were reduced to the required quantity via separation into required volume (in media of PBS, 2% Foetal Calf Serum (FCS) and 5mmol Ethylenediaminetetra-acetic acid (EDTA)). The maximum number of cells per magnet separation was $5x10^7$ cells/ml, so cells reduced in 1-2mls of media with the total cell number not exceeding $5x10^7$ cells/ml. Using the T-

cell enrichment cocktail - 50mcl/1ml of cells were stained and incubated for 10 minutes. The Nanoparticle ™ solution was added at 50mcl/ml and incubated for 10 minutes. This solution bound to the non-T-cell population The total volume was made up to a total volume to 2.5 mls of media and placed in the magnet (purple Easysep "Big Easy"™ - Stemcell UK) for 5 minutes. The supernatant was collected (enriched T-cells – as the magnetically labeled non-T-cells remained stuck to sides of tube via the magnet), this was repeated 3 times to increase purity of T-cells.

Stage 3: Cell sorting/Staining

After the cell number was counted, each separated enrichment was pooled, spun (1800 rpm for 10 minutes), and the resulting pellet was re-suspended in 1ml of media. Staining panel was based of 200mcl staining protocol used in PBMC/whole blood staining experiments but quantities multiplied by 5 (as total 1ml instead of 200mcl). The panel (manufacturer and concentrations used detailed) was: Anti-CD3 (PercP – BD Biosciences, recommended dilution - 1 in 50), Anti-CD161 (FITC/APC – Miltenyi Biotech, recommended dilution 1in 20/ 1 in 50 respectively) and Anti-γδ (FITC: BD Biosciences – recommended dilution 1 in 20). Live/Dead marker Near Infra red, BD Biosciences, recommended dilution 1 in 50). The required antibody was added, and incubated at 4ºC for 30 minutes, then washed and re-suspended in PBS with FCS/EDTA. The cells were then sorted using the FACSAria ™ machine at The Translational Gastroenterology Unit at The John Radcliffe Hospital. Once the cells were sorted into CD161+ and CD161- γδ T-cells; they were centrifuged at high speed 1200rpm for 5 minutes, at

20ºC then the supernatants were aspirated off and then pellet snap frozen in 100% ethanol on dry ice. During stages 2 and 3 all cells were kept at 4ºC to minimize the chance of becoming activated.

2.5 Gene array analysis

Snap frozen sorted PBMCs (CD161+ and CD161- γδ T-cell populations) were sent to Miltenyi Biotech Genomic Services Department, Bergisch Gladbach, Germany for Ribonucleic acid (RNA) extraction and Gene array analysis. The RNA extraction was carried out there using RNeasy ® Mini Kit (Qiagen ™) with the quality being checked with an Agilent Bioanalyzer platform. RNA Integrity Numbers (RIN) were calculated to ensure the RNA's quality with RIN numbers > 6 of sufficient quality for gene expression profiling experiments (Fleige and Pfaffl 2006). A linear T7-based RNA amplification was carried out with 10ng of RNA used. To produce Cy2-labelled complement Deoxyribonucleic acid (cDNA), the RNA samples were amplified and labeled using Agilent Low Input Quick Amp Labelling kit (per manufacturer's protocol). Thereafter, a hybridization procedure was carried out according to an Agilent 60-mer oligo-microarray processing protocol using an Agilent gene Expression Hybridization kit (Agilent Technologies). The microarrays were run and fluorescent signals of the hybridized Agilent microarrays were detected using Agilent's Microarray Scanner system (Agilent technologies). Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files, determining feature intensities (for background subtraction), rejecting outliers and calculating statistical confidences. For determination of differential gene expression FES

derived output data files were further analysed using a Rosetta Resolver ® gene expression data analysis system (Rosetta Biosoftware). "Raw" data files were then retrieved for analysis (see Chapter 4 for methodology of data analysis).

2.6 Liver biopsies

(i) Study subjects & ethical approval

IHLs isolated for FACS analysis were obtained from patients having liver biopsies for clinical indications. The biopsies were performed by either a Consultant Radiologist or Hepatologist at 2 sites: The Churchill Hospital, Oxford, UK or at Freiburg Medical University Department, Freiburg, Germany. All patients in Oxford identified in a Hepatology clinic gave informed and written consent to have a 2nd pass liver biopsy performed after being given a Patient Information Sheet (See Appendix 1). Ethical approval for the local study and protocol (See Appendix 2) was obtained from the Thames Valley Research and Ethics committee (REC study 09/H0603/19, NIHR CSP 16343, R&D Ref: 5969). In the Department of Medicine II (Gastroenterology & Hepatology), University of Freiburg, Germany samples were acquired after obtaining written informed consent from the patients and approval by the ethics committee of the Albert-Ludwigs-University, Freiburg. All investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

(ii) Biopsy and processing

All biopsies were performed under aseptic technique using ultrasound guidance. No complications were encountered from the 2nd pass biopsies for research purposes. Once the samples were gained they were transported to the

laboratory in PBS. A paired blood sample from which PBMCs were isolated was taken from each patient (again with written consent). The liver samples were placed in a sieve and "mashed up" for 5 minutes with PBS filtered through the sieve. The fluid/cell mixture was then centrifuged at 1800rpm for 10 minutes at room temperature. The pellet gained was re-suspended in 200µl of PBS and cell staining with antibodies performed (with a live/dead marker) in concentrations equivalent to the PBMC staining. Once stained all cells were fixed in PBS/2% formaldehyde. The cells were analysed using an LSR-2 ™ or FACS-Canto ™ locally in Freiburg, Germany.

2.7 Gut biopsy processing

All colon samples were obtained from patients undergoing a resection of the colon for cancer. Healthy resection margins were used for this study to isolate Lamina propria mononuclear cells (LPMCs) – although it was not documented how far the resection margins were from the primary tumour. All patients signed consent forms and the use of tissue was approved by a local UK ethics committee (references: 11/YH/0022 for colon cancer resection patients and 09/H1204/30 for patients with Inflammatory Bowel Disease (IBD)). The following protocol (courtesy of the Powrie laboratory, Oxford) was used to isolate LPMCs from the human colon.

The surgical specimen was washed 2 times in PGA (5 ml Pen/Strep, 2ml G418 (Gentamicin, stock used was 10 mg/ml, stored in the fridge) and 50 µl Amphotericin B. Thereafter, the mucosa was detached and specimen weighed. The sample was placed in 1mM DTT solution (100 ml of PGA solution/1 ml DTT) and put on a shaker at room temperature for 15 minutes. The specimen

was then taken out of the bottle with sterile forceps, and any remaining submucosal (whitish) tissue cleaned off and the specimen was cut in pieces.

The pieces were placed into a in an Erlenmayer flask with 50 ml of EDTA solution and incubated at 37°C, with a stirrer in the incubator, for 30 minutes. Then the supernatant was removed and EDTA solution was added (repeated 3 times to release epithelial cells and intra-epithelial lymphocytes).

The sample was then washed with Polygylcolic acid (PGA) 3 times. The pieces were then placed in collagenase solution for 12 hours at 37°C, with a stirrer in the incubator.

Thereafter, the supernatant was removed, filtered and put it in a falcon tube and centrifuged for 10 minutes at 1500 rpm, followed by 3 washes with PGA. The cells were then placed in 2 mls 40% percoll and centrifuged 30 minutes at 1500 rpm at 4°C (with the break off). Thereafter, the lymphocytes and monocytes at the 40% - 60% interface were collected, washed twice with HBSS and counted. The LPMCs were then stained with required Abs. The base panel used was Anti-CD3 PercP-Cy 5.5 (1 in 50), Anti-CD161APC (1 in 50), Live/dead Fixable Violet Dead cell stain kit aFluoro450 (1 in 50), Anti-γδ FITC (1 in 20). Data was analysed on the LSR-II ™.

2.8 Statistical analysis

All statistical analysis was performed on PRISM ™ software packaging. Data was expressed as mean +/- Standard Deviation (SD) where applicable. When paired data was analysed a paired t-test was used. However, if sample were not paired, a Mann Whitney test was used (with non-assumption of normal

distribution). A p-value of < 0.05 was considered significant, except in the gene array study where a raw p-value of $p<0.01$ was used. For all raw data p-values in the gene array experiments a Bonferroni corrected p-value was calculated.

CHAPTER 3: CD161+ Gamma-Delta T-cells: defining their role & function in health

3.1 Introduction

It has been shown that CD161 positivity on CD8 αβ T-cell defines a distinct subset of T-cells with a distinct phenotype and function compared to CD161- T-cells (Billerbeck *et al*. (2010). The role/impact of CD161 positivity on γδ T-cells has not been fully established. Battistini et al (Battistini *et al.* (1997)) first detected the CD161 marker on γδ T-cells and postulated it could be acquired *in vitro*, which was confirmed in cell culture experiments. The timing of CD161 expression, however, on γδ T-cells are not known, unlike in MAIT cells where CD161 positivity occurs early in the lifespan of the cell in the thymus and in cord blood (Dusseaux *et al.* (2011)). CD161+ γδ cells have been studied in the setting of MS, and their role in transendothelial migration was studied by Poggi and colleagues (Poggi *et al.* (1999)). Transendothelial migration is a key event in the role of lymphocytes, especially γδ T-cells. Movement across an endothelial layer is key, and allows the γδ T-cells to migrate to an area of interest, aided by chemokine receptors such as CCR6. In the MS studies, CD161+ γδ cells were increased in the peripheral blood of patients with MS compared to healthy donors, thus potentially being implicated in disease pathogenesis in the MS setting. CD161 expression was also found to be up-regulated on Vδ2 cells when cultured with IL-12. Furthermore, Vδ2 cell transmigration was higher and faster and enhanced by culture with IL-12. When blocking the cells with an anti-NKRP1A (CD161) 191b8 monoclonal Ab, there was a significant reduction in trans-endothelial migration. Thus it was proposed that the CD161+ γδ T-cells did indeed localize to sites of inflammation (such as the CSF in patients with MS). To this end a further study (Schirmer *et al.* (2013)) explored the migratory pattern

of CD161+ γδ T-cells. Co-expression of CD161high/CCR6+ was found in γδ T cells in CSF and peripheral blood of patients with MS with increased expression in those with relapses of MS – and enrichment in the CSF compared to non-relapse patients, or controls. These CD161high /CCR6+ γδ T-cells also expressed IL-17. Thus the CD161+CCR6+ γδ T-cell population may indeed be a subset in patients with MS correlating to poor disease outcome. Whilst studied in the MS disease setting, the full biological and functional importance of CD161+ γδ T-cells as a distinct subset, has yet to be fully characterized.

3.2 Chapter aims

The aims of this chapter were:

-To fully characterize the CD161+ γδ T-cell population in humans and compare these to CD161- γδ T-cells.

-To define the phenotype and function differences between CD161+ and CD161- γδ T-cells.

-To examine if CD161 positivity was already present in naïve cord blood γδ T-cells.

3.3 Materials and methods

Whole blood lymphocyte and PBMC staining was carried out from healthy individuals, with subsequent FACS analysis to define the CD161+ and -ve populations of γδ T-cells. The base antibody panel used was Anti-CD3 (PercP – BD Biosciences, recommended dilution - 1 in 50), Anti-CD161 (FITC/APC

– Miltenyi Biotech, recommended dilution 1in 20/ 1 in 50 respectively) and Anti-γδ (FITC/PE: BD Biosciences – recommended dilution 1 in 20). Combinations were used on different fluorochromes (either PercP, FITC, APC or PE as 4 colour channel FACS caliber used). The following additional markers used: Anti-CD4 (FITC: BD Biosciences – recommended dilution 1 in 10), CD8 (PE- Beckmann Coulter, recommended dilution 1 in10), CD103 (FITC: BD Biosciences, recommended dilution 1 in 10), CD69 (FITC – eBiosciences, recommended dilution 1 in 10), CD85J (FITC: BD biosciences, recommended dilution 1 in 10), CD25 (FITC: BD Biosciences, recommended dilution 1 in10), CD27 (FITC: BD Biosciences, recommended dilution 1 in 10), CD28 (FITC: BD Biosciences, recommended dilution 1 in 10), CD38 (FITC –Milteny Biotech), CD45RA/RO (FITC: BD Biosciences, recommended dilution 1 in 10), CD69 (FITC – BD Biosciences and TGFR-II (PE: R+D, recommended dilution 1 in 20).

Other chemokine receptors included: CXCR3 (PE - R&D SYSTEMS), CXCR4 (PE – R+D System, recommended dilution 1 in 20), CXCR6 (PE: R+D systems, recommended dilution 1 in 20), and PD-1 and NKG2D (PE: R+D systems, recommended dilution 1 in 20) were stained for. Anti-IL-23R (PE: R+D SYSTEMS, recommended dilution 1 in 20), Vd1 (FITC – Thermo Scientific, recommended dilution 1 in 10) and Vd2 (PE: BD biosciences, recommended dilution 1 in 20) were also used. IL-18R was stained for (PE: E-biosciences, recommended dilution 1 in 20).

An ICS was carried out on fresh PBMCs to assess function of the CD161+ and CD161- γδ T-cell subsets, and permeabilisation with intracellular staining was carried out to assess the CD161 subset's killing capabilities (Chapter 2).

The following Abs (along with the base-panel) were used for the ICS: Anti-IL-17A (FITC/PE: Insight Biotechnology Ltd/ R+D systems, recommended dilution 1 in 10), IL-22 (PE: R&D Systems, recommended dilution 1 in 20), TNF-α (FITC: Biolegend, recommended dilution 1 in 10) and IFN-γ (FITC: Biolegend, recommended dilution 1 in 10). The following Ab for Granzymes were used: Granzyme A (Biolegend, recommended dilution 1 in 10), B (Biolegend, recommended dilution 1 in 10), K (Immunotools) and Perforin (FITC: Diaclonoe).

To explore the timeline of CD161 positivity/expression in γδ T-cells, antibody staining with subsequent FACS analysis on cells collected from cord blood was carried out – with full consent obtained from the mothers. The same staining was carried out on 6 frozen 10-week old infant samples PBMCs that were thawed. The panel used for this section was: Anti-CD3 Pacific Blue (1 in 50 eBiosciences, recommended dilution 1 in100), Anti-CD161 APC (Milteny Biotech, recommended dilution1 in 50), Live/Dead Near Infrared (Biotium, recommended dilution1 in 50) and Anti-γδ on PE (BD Biosciences, recommended dilution1 in 20). These samples were analysed on the LSR-II FACS machine. All data was analysed using FlowJo ™ and statistical analysis with PRISM ™

LPMCs and intra-epithelial lymphocytes were isolated from either colonic resection (of colorectal cancer) samples from the resection margins, or from endoscopic biopsies from patients with IBD. The cells were stained thereafter with an Ab panel (Chapter 2.7) to assess for CD161+ γδ populations.

3.4 Results

3.4.1 Defining CD161+ γδ T-Cells in fresh and frozen PBMC samples

The 1st experiment was carried out to explore what percentage (%) of human γδ T-cells were CD161+. To this end a FACS-based experiment was performed. Access was available to frozen PBMCs, fresh whole blood and freshly acquired PBMCs. In healthy adults (free from known chronic viral infections), *whole blood staining* of CD161+ γδ T-cells was carried out.

In healthy controls a distinct population of CD3+CD161+ γδ T-cells and then CD3+CD161- γδ T-cells was identified (Figure 3.1). In this healthy adult cohort, the mean CD3+ γδ % *of total lymphocytes* was 6.58%(+/-1.03). Of the γδ T-cells a mean of 82.1% (+/-12.8) expressed CD161 from fresh whole blood staining; thus the majority of circulating *in-vivo* γδ T-cells were indeed CD161+. A greater % of γδ T-cells were CD161+ when compared to the αβ population 21.7%(+/16.84)).

This gating strategy was repeated on *frozen* PBMCs (isolated from leucocyte cones). Of the total CD3+ T-cell population, 7.78%(+/-1.03) were CD3+γδ T-cells and of these 48.6% (+/-13.26) were CD161+. Correspondingly the mean CD3+ αβ population was 48.8%(+/-8.78) in the PBMCs thawed from frozen leucocyte cones. Thus, it appeared that in these samples there was, despite a small increase in the total CD3 T-cells being γδ, a marked reduction in the % being CD161+ (82.1% to 48.6% of CD3+ γδ T-cells).

The reduced frequency of the CD161+ population in the thawed PBMCs may

have been for a number of reasons including: disruption of cell populations during cone preparations, unknown disease processes in the leucocyte cone population or most likely due to the thawing process perhaps down-regulating the γδ CD161+ molecules (Chapter 3 discussion). Another possible mechanism was the CD161+ γδ T-cells being selectively stuck to the leucocyte cone.

To address this further, *freshly isolated PBMCs* from whole blood were stained (note data so far had focused on populations of whole blood and those from *frozen* PBMCs gained from frozen leucocyte cones). The results showed that in freshly isolated PBMCs taken *ex-vivo,* 4.75%(+/-2.78) of CD3+ T-cells were γδ T-cells, and of these 59.8%(+/- 5.28) were CD161+. Of the CD3+ T-cells, 94%(+/-3.35) were αβ and of these 20.5%(+/- 5.6) were CD161+.

In summary, the major decline in cell populations (irrespective of CD161 status – Table 3.1) seemed to be in the CD3+ αβ T-cells via thawing of frozen leucocyte cones (almost by half). However when focused on the γδ T-cells, the CD161+ cells were the major subset in fresh blood and freshly isolated PBMCs and, thus, likely are representative of *in-vivo* states. There did appear to be a drop off in CD161 positivity in the γδ T-cells on the thawing of frozen leucocyte cones despite comparable total γδ T-cell %s.

Cell Staining method	**T-cell subset**	**Mean % (+/-) of CD3+ T-cells**	**Mean % (+/- SD) of CD3+ cell type expressing CD161**
Whole Blood	**γδ**	6.58%(+/- 1.03)	82.1%(+/-12.8)
Whole Blood	αβ	92.6%(+/- 3.14)	21.7%(+/16.84)
PBMCs from fresh blood	**γδ**	4.75%(+/- 2.78)	59.8%(+/-5.28)
PBMCs from fresh blood	αβ	94%(+/- 3.35)	20.5%(+/-5.6)
PBMCs from thawed Leucocyte cone	**γδ**	7.78%(+/- 1.03)	48%(+/-13.6)
PBMCs from thawed Leucocyte cone	αβ	48.8%(+/- 8.78)	24.4%(+/-6.63)

TABLE 3.1: T-lymphocyte population summary per processing method

Summarises the T-cell populations and the mean %(+/-SD) of the subsets expressing CD161.

When gating on CD3+CD8+ γδ T-cells populations, there were typically two distinct separate CD161 positive and negative populations. This was different to published data (Billerbeck *et al* (2010)) on the CD3+CD8 αβ T-lymphocytes, where there were reliably 3 distinct cell sets expressing varying levels of CD161 (bright, mid and dim) – Figure 3.3. Thus, although the γδ T-cells are a smaller subset of the total lymphocyte population, there was a higher proportion of CD161+ T-cells when compared to the αβ T-cell population (whole blood, $p<0.0001$;PBMCs, $p =0.0099$).

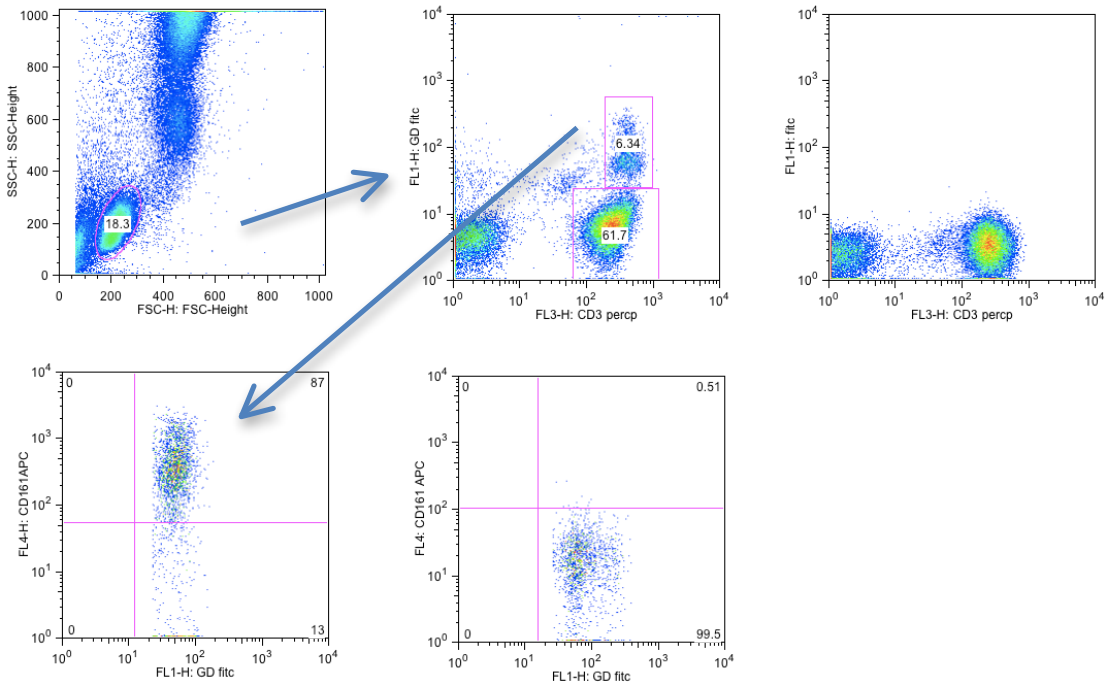


FIGURE 3.1: Gating strategy example for γδ T-cells from whole blood

A FACS based experiment was performed to assess the expression of CD161 on γδ T-cells. Cells were stained with Anti-CD3 PercP, CD161 APC and γδ FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data.

Top left panel: gating on the lymphocyte population.
Top middle panel: CD3 positive γδ T-cells (6.34% of total lymphocytes in this adult).
Top right panel: fluorochrome minus one (FMO) which the γδ T-cells gating strategy was based on. An isotype control was also performed to ensure correct gating strategy (not shown).
Bottom left panel: CD161+ (on APC) fraction of γδ T-cells (87% of γδ T-cells)
Bottom right panel: matching FMO stain for APC channel (an isotype control was used for verification – not shown).

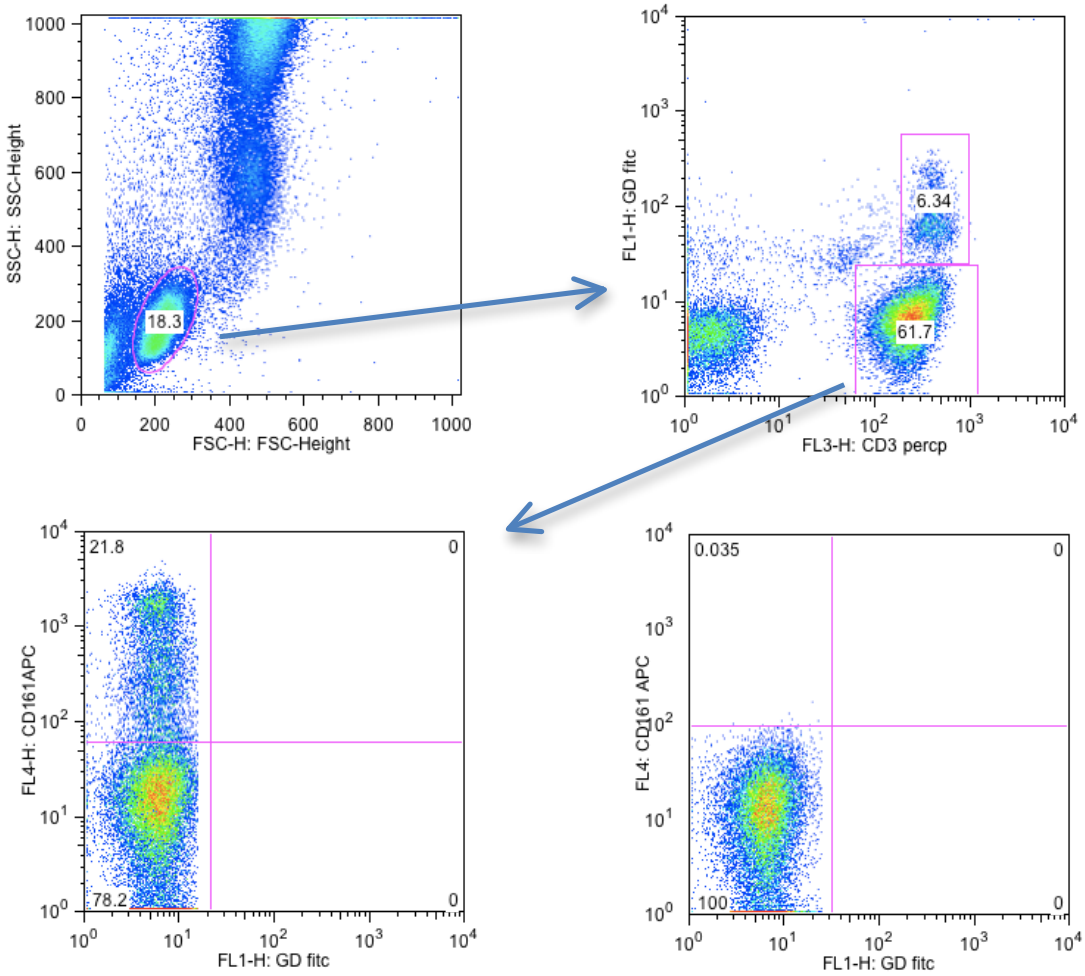


FIGURE 3.2: Gating strategy of CD161+ αβ T-cells from whole blood staining.

A FACS based experiment was performed to assess the expression of CD161 on αβ T-cells. Cells were stained with Anti-CD3 PercP, CD161 APC and γδ FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data.

Top left panel: gating on the lymphocyte population.
Top right panel: gating on the CD3+ αβ T-cells gated on.
Bottom left panel: CD3+CD161+ αβ T-cells
Bottom right panel: FMO to the CD161 αβ plot.
Gating based on published gating strategy (Northfield *et al.* (2008); Billerbeck *et al.* (2010))

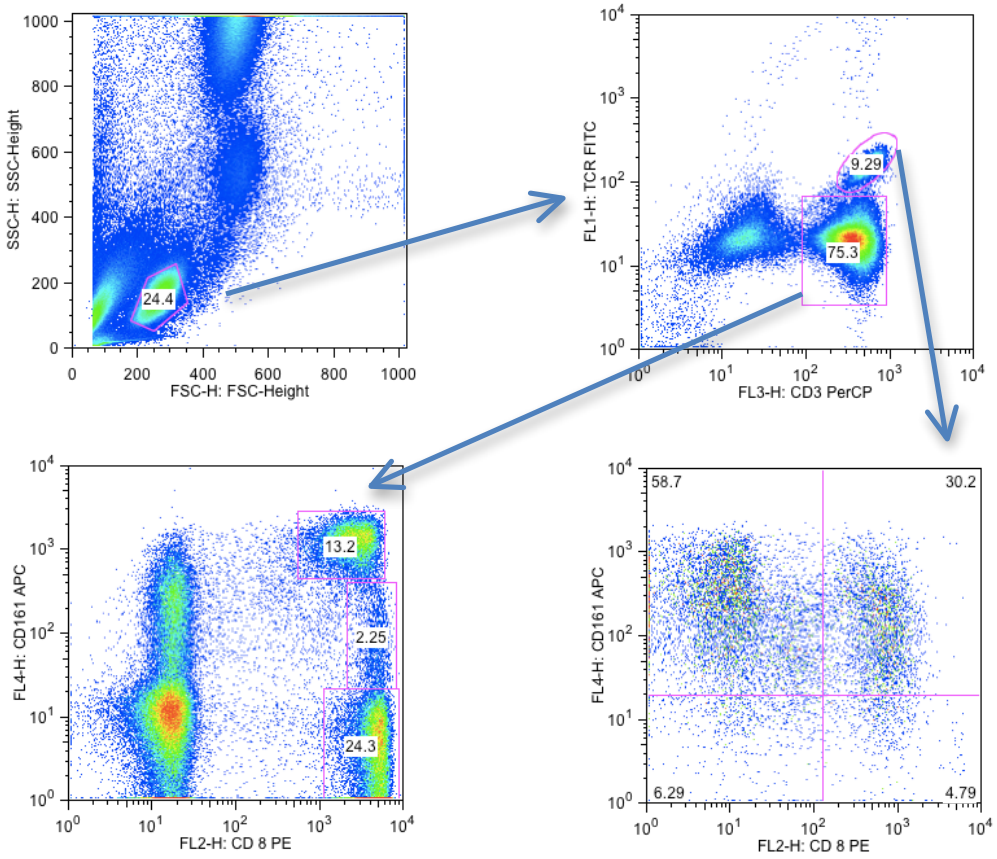


FIGURE 3.3: CD3+ CD8+ distinct CD161++ (high) and CD161+ (mid) αβ populations – not seen in CD161+ γδ T-cells

A FACS based experiment was performed to assess the expression of CD161 per CD8 subset of T-cells. Cells were stained with Anti-CD3 PercP, CD8 PE, CD161 APC and γδ FITC.FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data.

Top left panel: lymphocyte gating.
Top right panel: CD3+ γδ and αβ populations.
Bottom left panel: distinct CD161 bright (13.2%), dim (2.25%) and negative (24.3) CD8+αβ populations.
Bottom right panel: the distinct CD161 bright, mid and dim populations were not seen with the CD8+ γδ T-cell populations.
Gating based on published data from Billerbeck *et al.* (2010)

3.4.2 Phenotype characterization of CD161+ vs. CD161- γδ T-cells

To assess the CD161+ γδ T-cells subset further, whole blood staining was performed and analysed using a FACS approach. Similar methods to the previous experiments were used to phenotypically characterize the CD161+ γδ T-cells, and compare the difference between the CD161+ and CD161- subsets of γδ T-lymphocytes. The γδ T-cell whole populations expressed the following cell markers (data not shown): CCR7, CCR6, CD8, CD4, CD85J, CC27, CD28 and CD45 RA/RO (Table 2). This panel of markers was chosen based on prior data from CD161/CD8+ T-cell experiments in our lab (Billerbeck *et al*. (2010); Walker *et al*. (2011)).

When comparing CD161+ γδ T-cells vs. CD161- γδ T-cells in healthy adult populations, the former expressed significantly more CXCR3 and CCR6 ($p=0.03$ & 0.01 respectively- See Figures 3.4 and 3.5). CXCR3 is known to be involved in leucocyte trafficking, and is triggered by chemokines CXCL9 and IP10. CXCR3 is up-regulated on CD161+ CD4+ and CD161+ CD8+ αβ T cells compared to CD161- T-cells. CCR6 is expressed on Th17 cells and CD161++ (high) CD8 αβ T-cells have been shown to express CCR6 (Northfield *et al* (2008)). CCR6+ γδ T-cells have also been shown to be involved in the pathogenesis of MS ((Schirmer *et al.* (2013)).

Thus, in summary, in healthy controls γδ T-cells that are CD161 positive express significantly higher levels of CCR6 and CXCR3, suggesting they are primed to move between the liver/ gut and the blood i.e. in the porto-systemic circulation, or to inflamed sites.

MARKER	ROLE
CD4	Co-receptor assisting the TCR with MHC class II Ag presentation.
CD8	A transmembrane glycoprotein, expressed on cytolytic T-cells binding MHC class I molecules.
CCR6	Chemokine receptor regulating the migration and recruitment of T-cells during inflammatory responses.
CXCR3	Role in regulation of leucocyte trafficking. Ligation with its ligand attracts Th1 cells
CD27	TNF receptor, binding to its ligand (CD70) to activate NF-kB
CD28	Expressed on T-cells providing a co-stimulatory signal. Its ligands are CD80/86
CCR7	Controls the migration of T-cells to lymph nodes.
CD45 RA ISOFORM	Expressed on naïve/ non-activated (and revertant) T-cells
CD45 RO ISOFORM	Expressed on memory T-cells

TABLE 3.2: Phenotypical markers studied when comparing CD161+ γδ T-cells vs. CD161- γδ T-cell

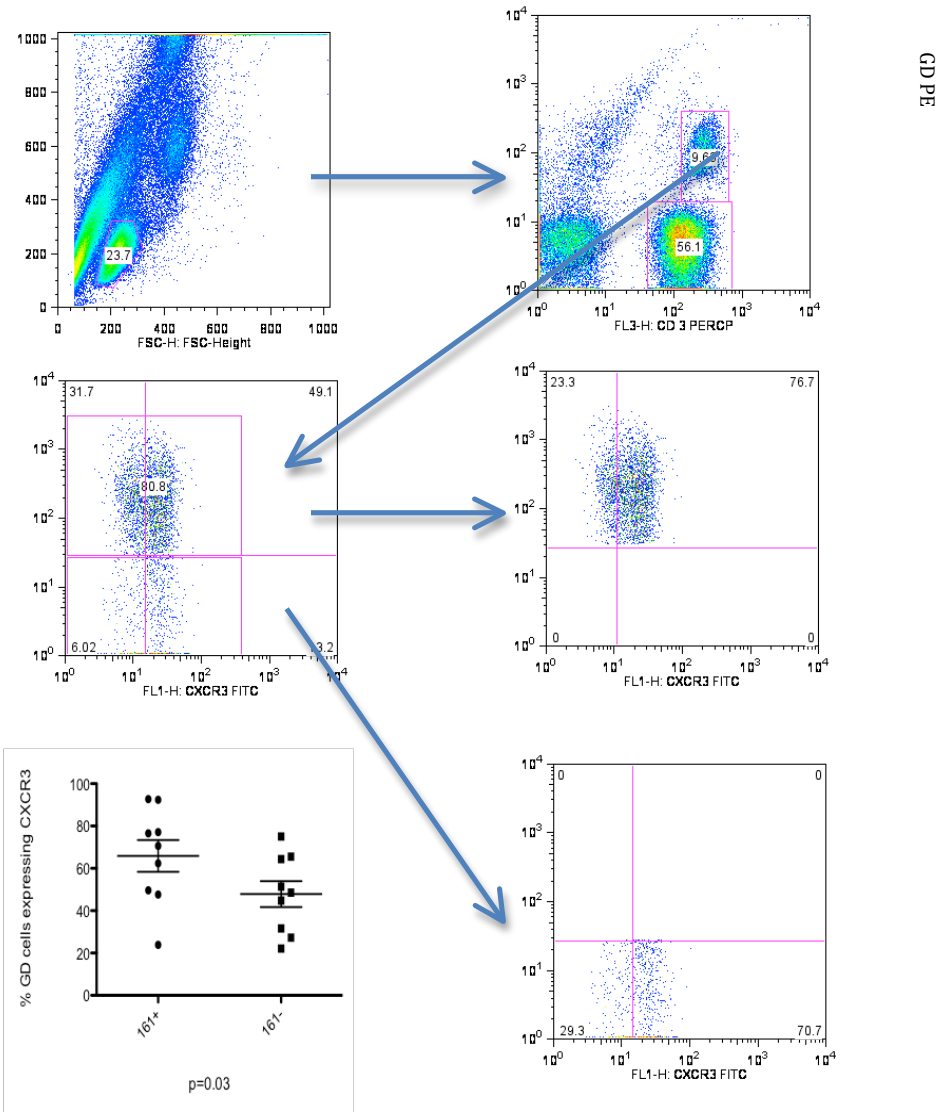


FIGURE 3.4: CXCR3 was significantly expressed in the CD161+ γδ T-lymphocytes compared to the negative subset.
A FACS based experiment was performed to assess the expression of CXCR3 by CD161+ compared to CD161- γδ T-lymphocytes. Nine samples were analysed with staining for Anti-CD3 PerCP, CD161APC and γδ PE and CXCR3 FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test. CXCR3 was significantly expressed in the CD161+ γδ T-cells compared to the negative subset.
Top left panel: lymphocyte gating.
Top right panel: CD3+γδ and αβ T-lymphocytes populations (y-axis denotes γδ PE).
Middle left panel: expression per CD161 status.
Middle right panel: CXCR3 expression in CD161+ γδ T-lymphocytes.
Bottom right panel: expression of CXCR3 in CD161- subset.
Bottom left panel: significant difference in expression of CXCR3 per CD161 status of γδ T-lymphocytes (p=0.03).

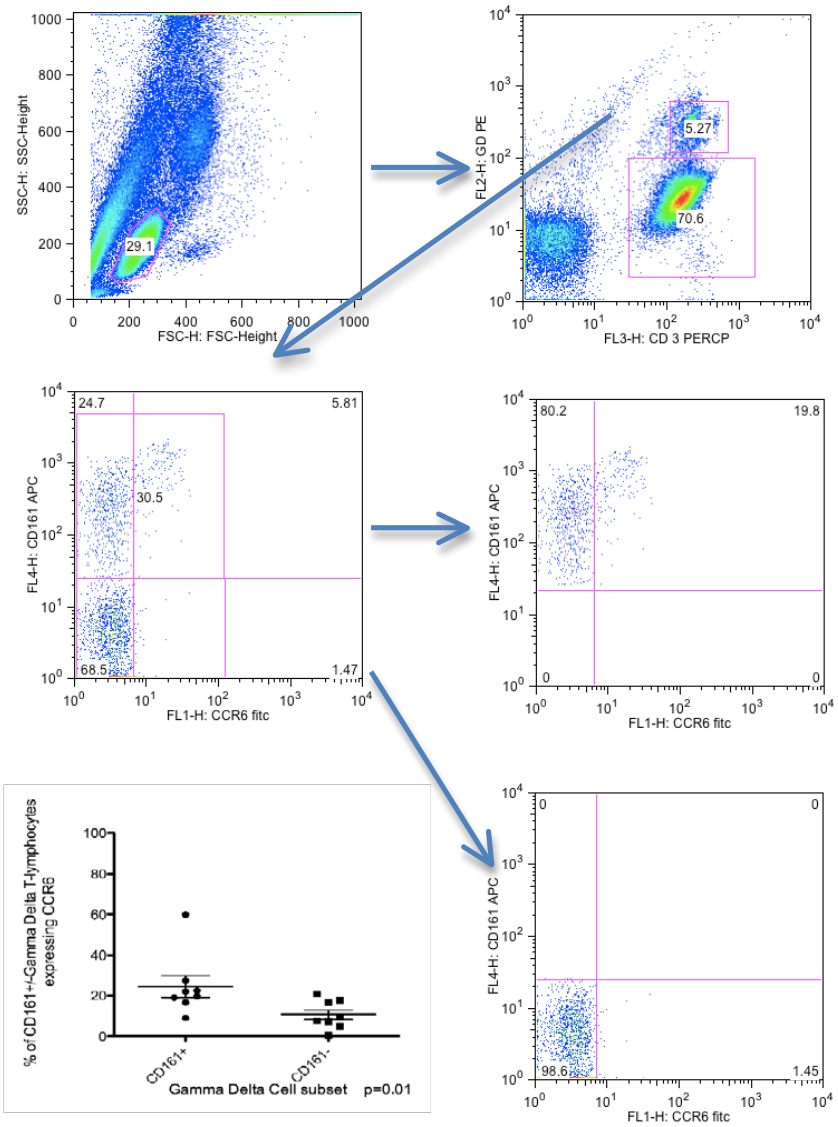


FIGURE 3.5: CCR6 was significantly expressed in the CD161+ γδ T-lymphocytes compared to the negative subset.

A FACS experiment was performed to assess the expression of CCR6 by CD161+ compared to CD161- γδ T-lymphocytes. Eight samples were analysed with staining for Anti-CD3 PerCP, anti-CD161APC and anti-γδ PE and anti-CCR6 FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Top left panel: shows lymphocyte gating.
Top right panel: shows CD3+γδ and αβ T-lymphocytes populations.
Middle left panel: shows expression per CD161 status.
Middle right panel: graph showing the CCR6 expression in CD161+ γδ T-lymphocytes
Bottom right panel: showing the expression of CCR6 in CD161- subset.
Bottom left panel: shows significant difference in expression of CCR6 per CD161 status of γδ T-lymphocytes (P=0.01)

3.4.3 Vδ chain expression of CD161+ γδ T-Cells

Following on from the phenotypical differences seen between the CD161+ and CD161- γδ T-cell subsets, the next experiment was performed to further characterize the CD161+ γδ T-cell subset, and specifically the Vδ chain expression. Due to the availability of commercial antibodies along with the main physiological subset, the 2 main Vδ chains expressed in humans were studied: Vδ1 and Vδ2. A similar panel of antibodies was used: Anti-human CD3 PercP, CD161 APC, γδ FITC/PE and Vδ1/Vδ2 on PE or FITC. Eight samples were analysed for Vδ2 and 11 samples for Vδ1.

In healthy controls, a mean of 2.70%(+/-1.85) of CD3+CD161+ γδ T-cells expressed Vδ1 and 84%(+/-16.9) expressed Vδ2. In comparison, 28.8%(+/-14.7) and 38.03%(+/-34.4) of CD161- γδ T-cells respectively expressed Vδ1 and Vδ2. Thus, there was significantly greater expression of Vδ2 on CD161+ γδ T-cells than Vδ1 ($p=0.005$) whereas there was no significant difference between expression of Vδ2 or Vδ1 on the CD161- subsets ($p=1.00$).

This data suggests that in healthy controls, not only are the γδ T-cells predominantly CD161+, but also that, in keeping with whole blood γδ T-cell populations, the predominant expression is of Vδ2.

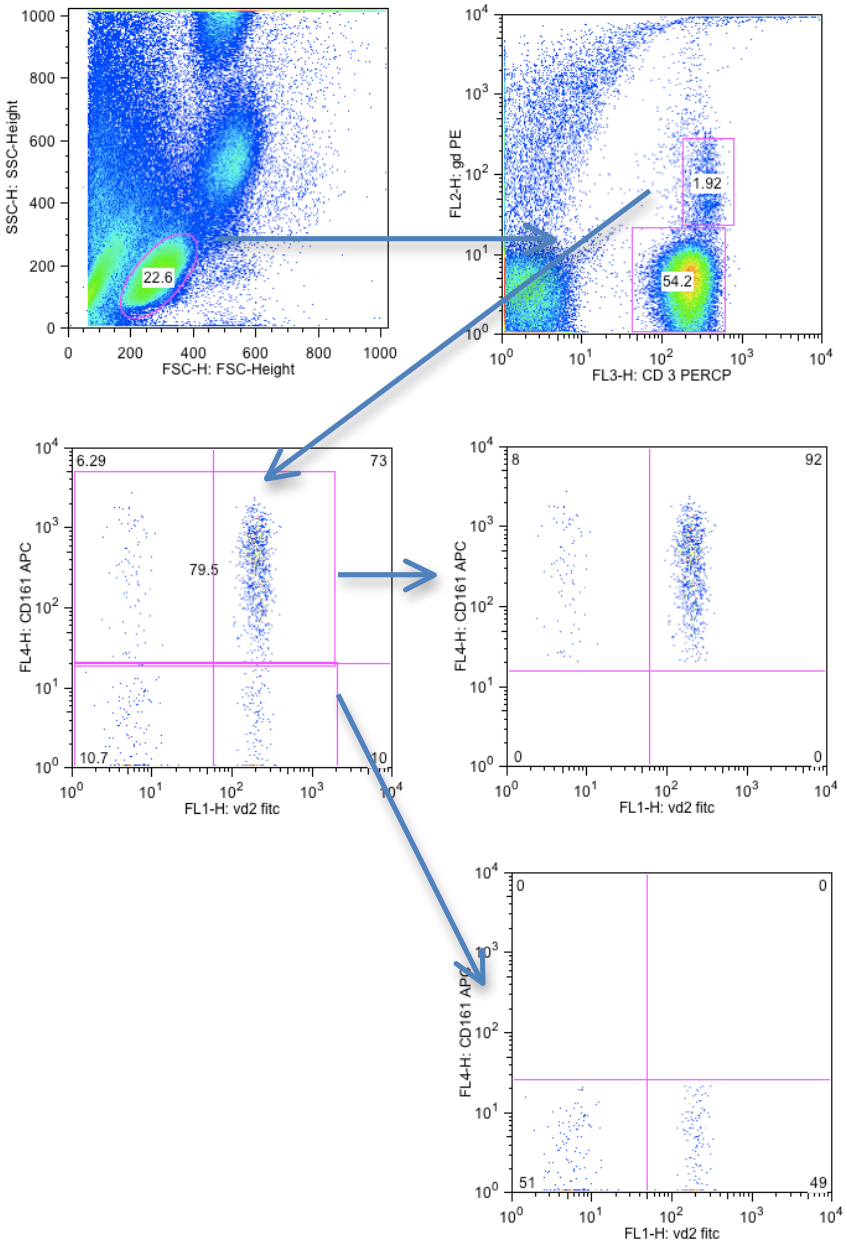


FIGURE 3.6: Vδ2 expression predominates in the CD161+ γδ T-lymphocytes
A FACS experiment was performed to assess the expression of the Vδ2 chain by CD161+ compared to CD161- γδ T-lymphocytes. Seven samples were analysed with staining for Anti-CD3 PerCP, CD161APC and γδ PE and Vδ2 FITC. A paired t-test was performed. Vδ2 was significantly expressed in the CD161+ γδ T-lymphocytes compared to the negative subset. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Top left panel: lymphocyte gating
Top right panel: CD3+ γδ population.
Middle left panel: gating of CD161+and CD161- subsets.
Middle right panel: expression of Vδ2 as a % of the CD161+ cells
Bottom right panel: expression of Vδ2 as a % of the CD161- cells

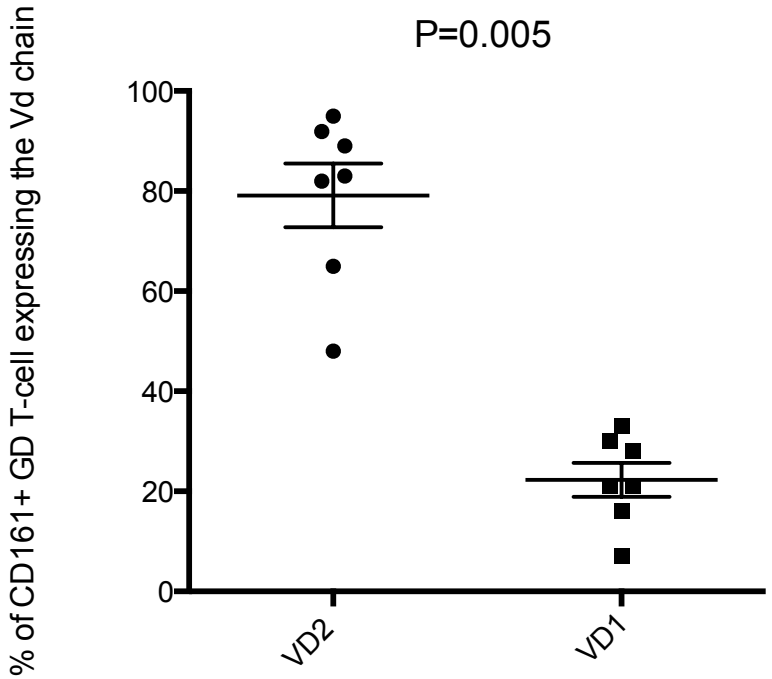


FIGURE 3.7: Significant expression of the Vδ2 chain on CD161+ γδ T-cells when compared with Vδ1 expression.
A FACS based experiment was performed (Figure 3.5) with 7 replicates used. A paired t-test was performed.

3.4.4 CD161+ γδ T-cells are functionally distinct subset of γδ T-cells

The next experiment's aim was to explore the function of the CD161+ γδ T-lymphocytes, by studying the production of cytokines via ICS after stimulation (Chapter 2: Methods). PBMCs were isolated from fresh blood and surface/intracellular staining carried out. All samples were from healthy control populations with the following Ab panel used: Anti-human CD3 PercP, Anti-human CD161 APC, Anti-human γδ PE/FITC, IF- γ FITC (eBiosciences, recommended dilution 1 in 10), TNF-α FITC (eBiosciences, recommended dilution 1 in 10) and IL-22/17A&F (BD Biosciences, recommended dilution 1 in 20) on PE.

Using the ICS protocol (Chapter 2.3), the whole γδ populations readily

expressed IFN- γ and TNF-α while only small amounts of IL-17A and IL-17F were expressed along with IL-22. When the CD161+ γδ T-cells were compared to the CD161- subsets, the CD161+ γδ T-cells expressed significantly higher amounts of TNF- α and IFN-γ ($p = 0.007$ and $p = 0.0059$ respectively). However, there was no difference between the subsets on their production of IL-17A/F or IL-22. The data suggested that in healthy controls, the CD161+ γδ T-cell population display a Th-1 profile as compared to their CD8+ CD161++ αβ counterparts, where a mixed Tc1/Tc17 phenotype/function predominated (Billerbeck *et al.* (2010)).

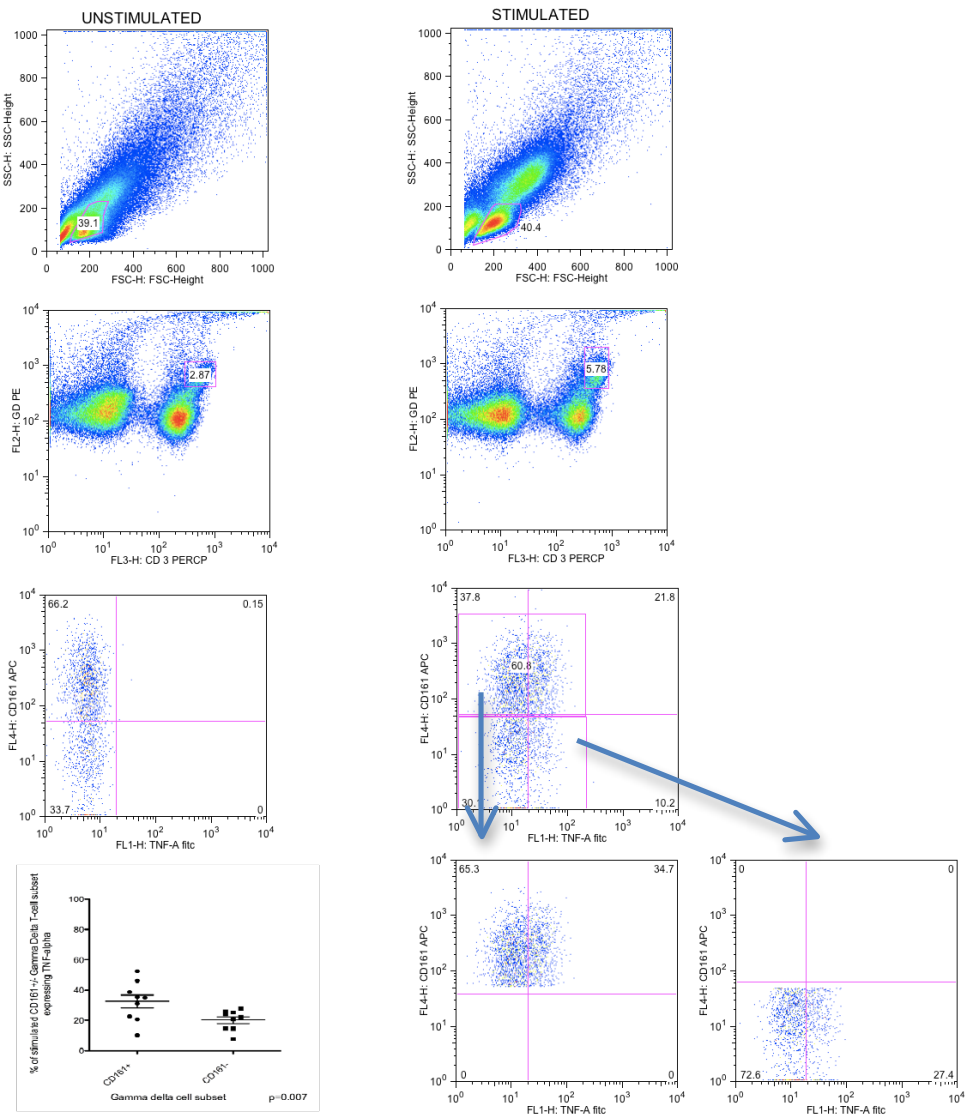


FIGURE 3.8: CD161+ γδ T-lymphocytes preferentially express TNF-α when stimulated compared to the CD161- subset.
An ICS was performed on 9 samples, with stimulation via PMA/Inomycin. Cell surface staining was performed with Anti-CD3 PercP, anti-CD161 APC, anti-γδ PE and cytokine staining with anti-TNF-α on FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Left column: shows un-stimulated control.
Right column: showing stimulated specimen.
Top panels: lymphocyte gating
2nd from top panels: CD3+γδ T-cell population,
2nd from bottom panels: production of TNF-α per CD161 status (below right).
Bottom middle panel: significant increase in production of TNF-α by CD161+ γδ T-lymphocytes (p=0.007)
Bottom right panel: CD161- subset production of TNF- α

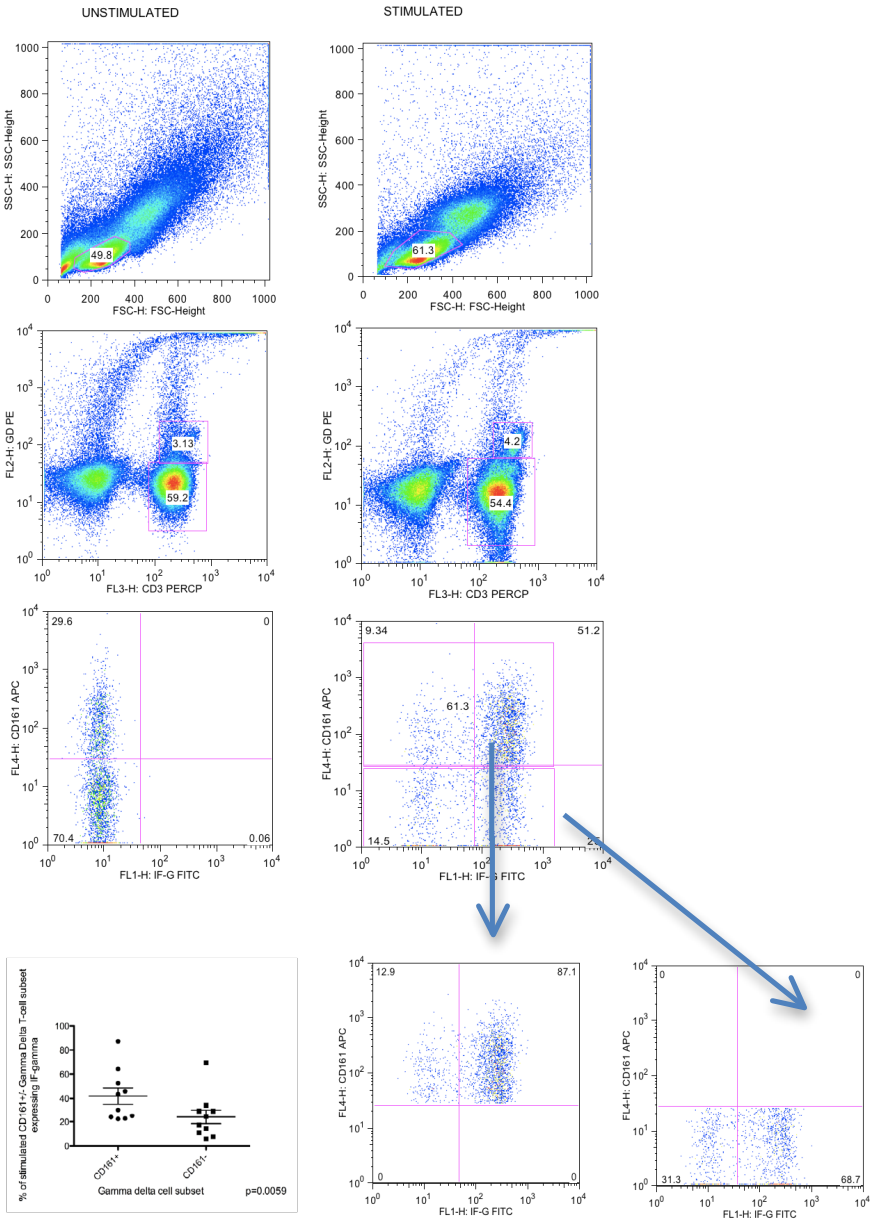


FIGURE 3.9: CD161+ γδ T-lymphocytes preferentially express IFN-γ when stimulated compared to the CD161- subset.
An ICS was performed on 10 samples with stimulation via PMA/Inomycin. Cell surface staining was performed with anti-CD3 PercP, anti-CD161 APC, anti-γδ PE and cytokine staining with anti-IFN-γ on FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Left column: shows un-stimulated control.
Right column: showing stimulated specimen.
Top panels: lymphocyte gating
2nd from top panels: CD3+γδ T-cell population,
2nd from bottom panels: production IFN-γ of per CD161 status (right).
Bottom middle panel: significant increase in production of IFN-γ by CD161+ γδ T-lymphocytes (p=0.0059)
Bottom right panel: CD161- subset production of TNF- α

3.4.5 The granule content of CD161+ γδ T-cells differs from the CD161- subset

γδ T-cells have been shown in previous studies to be potent cytolytic T-cells producing Granzyme B and Perforin (Fox and Meeusen. (1999)). This experiment was designed to explore if CD161+ γδ T-cells differed in their ability to produce mediators involved in killing. Whole blood staining was performed with a membrane permeabilisation stage and further intracellular staining for Granzyme B and Perforin. The following panel was used: Anti-human CD3 PercP, Anti-human CD161APC/ FITC, perforin FITC, Granzyme B APC/ Granzyme A Pacific Blue, Granzyme K PE and γδ on PE/FITC.

The CD161+ γδ T-cells expressed significantly higher levels of Perforin, Granzyme A, B and K than the CD161- subset ($p= 0.02, 0.002, 0.023$ and 0.01 respectively). This data supports previous evidence that γδ T-cells produce both Granzyme B and Perforin, however it appears that the CD161+ γδ T-cells express significantly higher levels of Perforin and Granzymes A, B and K compared to the CD161- subset. Thus this may indeed indicate the CD161+ γδ T-cells may be the predominant subset involved in killing however formal killing studies were not performed. The specific roles of Granzyme A, B and K are discussed in Chapter 3.4 Discussion.

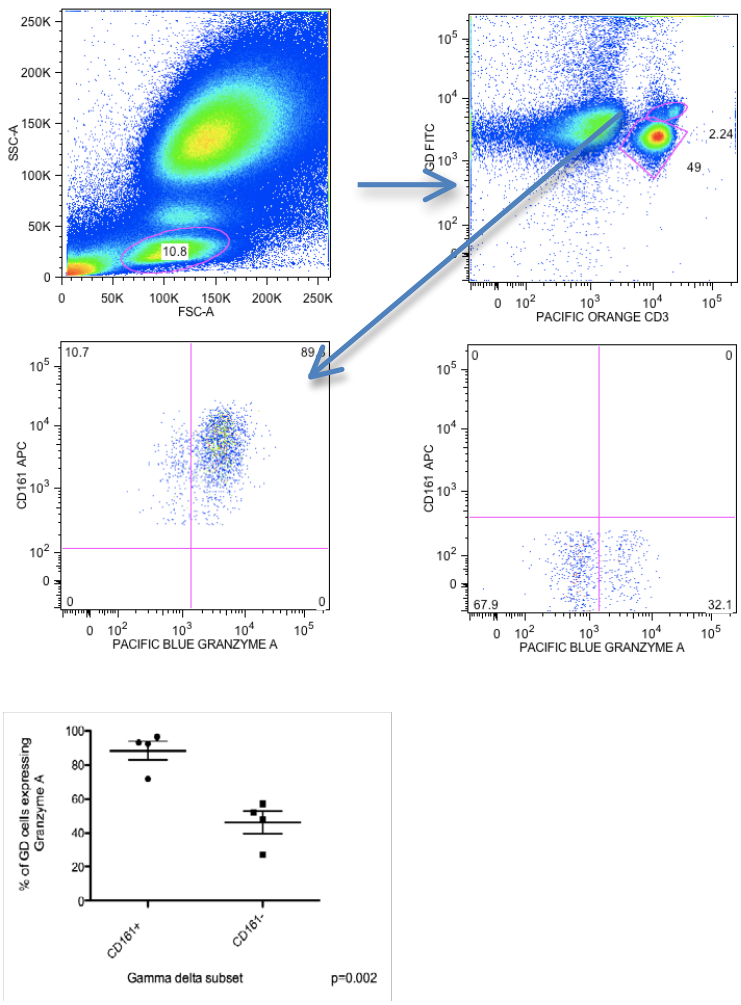


Figure 3.10: CD161+ γδ T-lymphocytes preferentially express Granzyme A when compared to the CD161- subset.
Cell surface staining was performed on 4 samples with a permeablisation stage followed by internal staining of the Granzyme. Cell surface staining was performed with anti-CD3 Pacific Orange, anti-CD161 APC, anti-γδ FITC and anti-Granzyme A ion Pacific Blue. FACS analysis was performed on the LSR-II with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Top left panel: lymphocyte gating
Top right panel: CD3 γδ gating.
Middle left panel: CD161+ γδ expression of Granzyme A,
Middle right panel: CD161- γδ expression of Granzyme A
Bottom left panel: significant expression of Granzyme A by CD161+ subset of γδ T-lymphocytes (p=0.002)

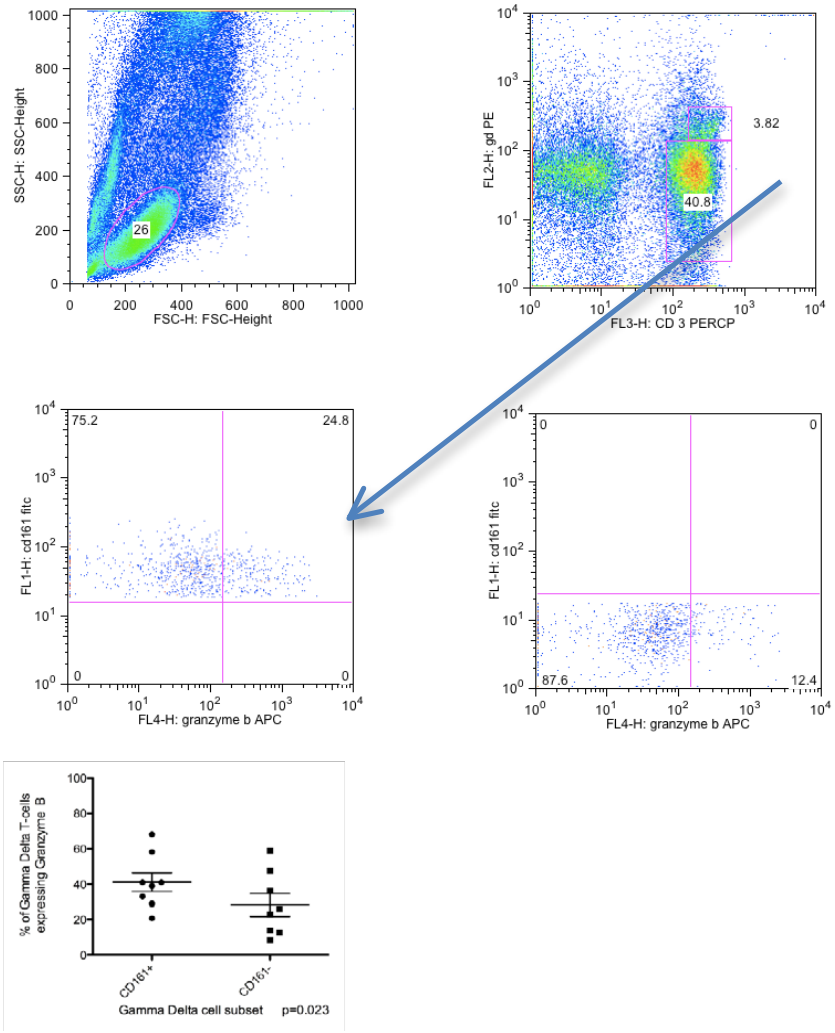


Figure 3.11: CD161+ γδ T-lymphocytes preferentially express Granzyme B when compared to the CD161- subset.
Cell surface staining was performed on 8 samples with a permeablisation stage followed by internal staining of the Granzyme. Cell surface staining was performed with anti-CD3 PercP, anti-CD161 APC, anti-γδ PE and anti-Granzyme B on APC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Top left panel: lymphocyte gating
Top right panel: CD3 γδ gating.
Middle left panel: shows CD161+ γδ expression of Granzyme B.
Middle right panel: CD161- γδ expression of Granzyme B.
Bottom left panel: significant expression of Granzyme B by CD161+ subset of γδ T-lymphocytes (p=0.023)

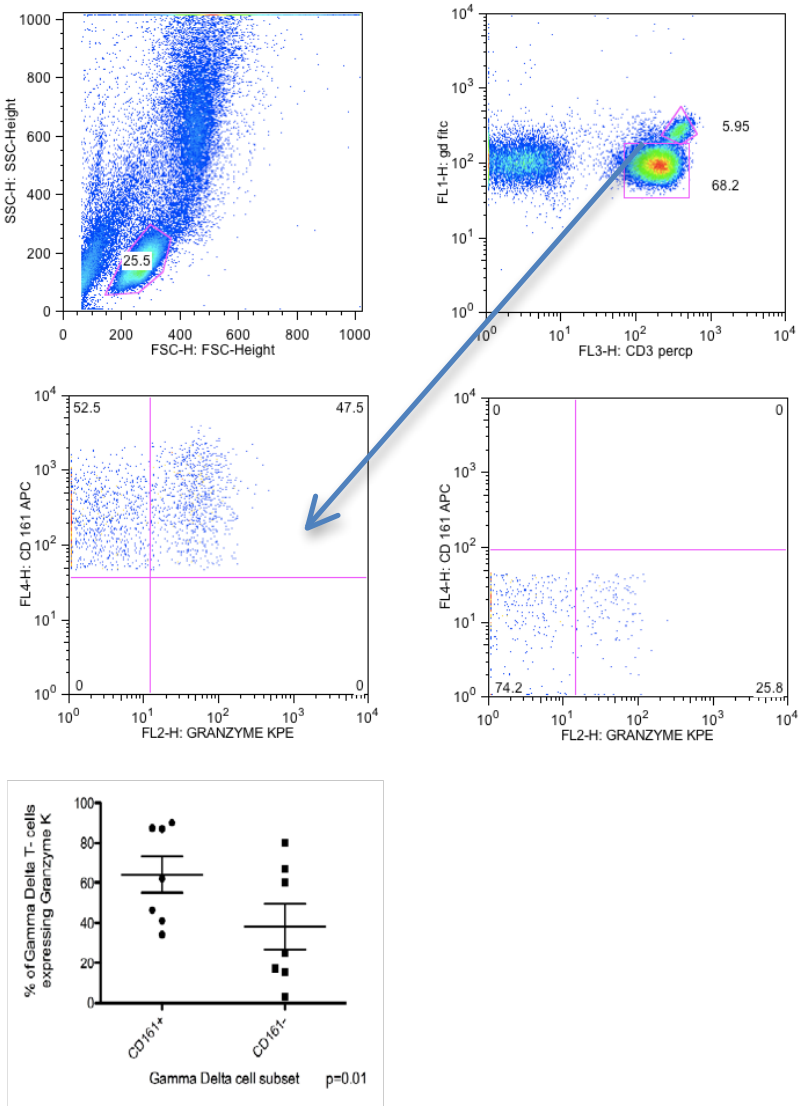


Figure 3.12: CD161+ γδ T-lymphocytes preferentially express Granzyme K when compared to the CD161- subset.
Cell surface staining was performed on 7 samples with a permeablisation stage followed by internal staining of the Granzyme. Cell surface staining was performed with anti-CD3 PercP, anti-CD161 APC, anti-γδ FITC and anti-Granzyme K on PE. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Top left panel: lymphocyte gating
Top right panel: CD3 γδ gating.
Middle left panel: CD161+ γδ expression of Granzyme K
Middle right panel: CD161- γδ expression of Granzyme K
Bottom left panel: significant expression of Granzyme K by CD161+ subset of γδ T-lymphocytes (p=0.01)

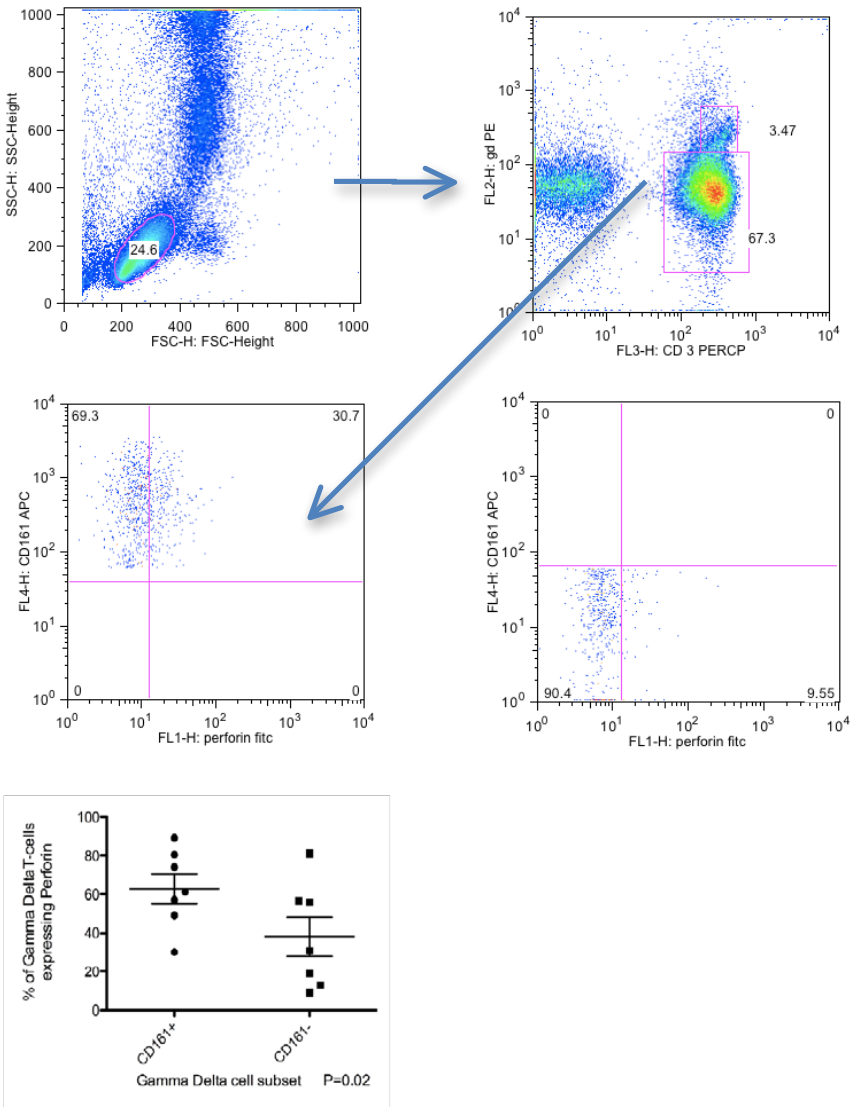


Figure 3.13: CD161+ γδ T-lymphocytes preferentially express Perforin when compared to the CD161- subset.
Cell surface staining was performed on 7 samples with a permeablisation stage followed by internal staining of the Perforin. Cell surface staining was performed with anti-CD3 PercP, anti-CD161 APC, anti-γδ PE and anti-Perforin on FITC. FACS analysis was performed on the LSR-II with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test.

Top left panel: lymphocyte gating
Top right panel: CD3 γδ gating.
Middle left panel: CD161+ expression of Perforin
Middle right panel: CD161- of Perforin.
Bottom left panel: significant expression of Perforin by CD161+ subset of γδ T-lymphocytes (p=0.02)

3.4.6 Fas expression

To explore the mechanism of being killed (rather than killing) and predisposition to apoptosis, the expression of the Fas receptor (CD95) – a key receptor for the induction of apoptosis was explored on CD161+ γδ T-cells. CD95 engagement by its physiological ligand (Fas-L) mediates cell death following TCR engagement in activated T-lymphocytes (Nagata and Goldstein. (1995)). γδ T-cells have been shown to be sensitive to CD95-mediated apoptosis when cultured in-vitro (Rovere *et al.* (1996)). Activation induced cell death of γδ T-lymphocytes has been implicated in the resolution phases of microbial infections when often peripheral γδ expanded frequencies reduce (Janssen *et al.* (1991)). Manfredi and colleagues (Manfredi *et al.* (1998)) showed that Mycobacterial Ags induced expression of the CD95 ligand by chronically activated CD95+/CD95L- γδ T-lymphocytes leading to apoptosis.

Thus, we examined the surface expression of CD95 (Fas receptor) on CD161+ γδ T-lymphocytes. Surface staining was carried out with Anti-human CD3 PercP, CD161 APC, γδ PE and CD95 FITC (BD Biosciences, recommended dilution 1 in 10). There was significantly higher expression of CD95 by CD161+ γδ T-lymphocytes compared to the CD161- subset ($p=0.005$ – Figure 3.13), which was echoed in the αβ populations too ($p=0.001$).

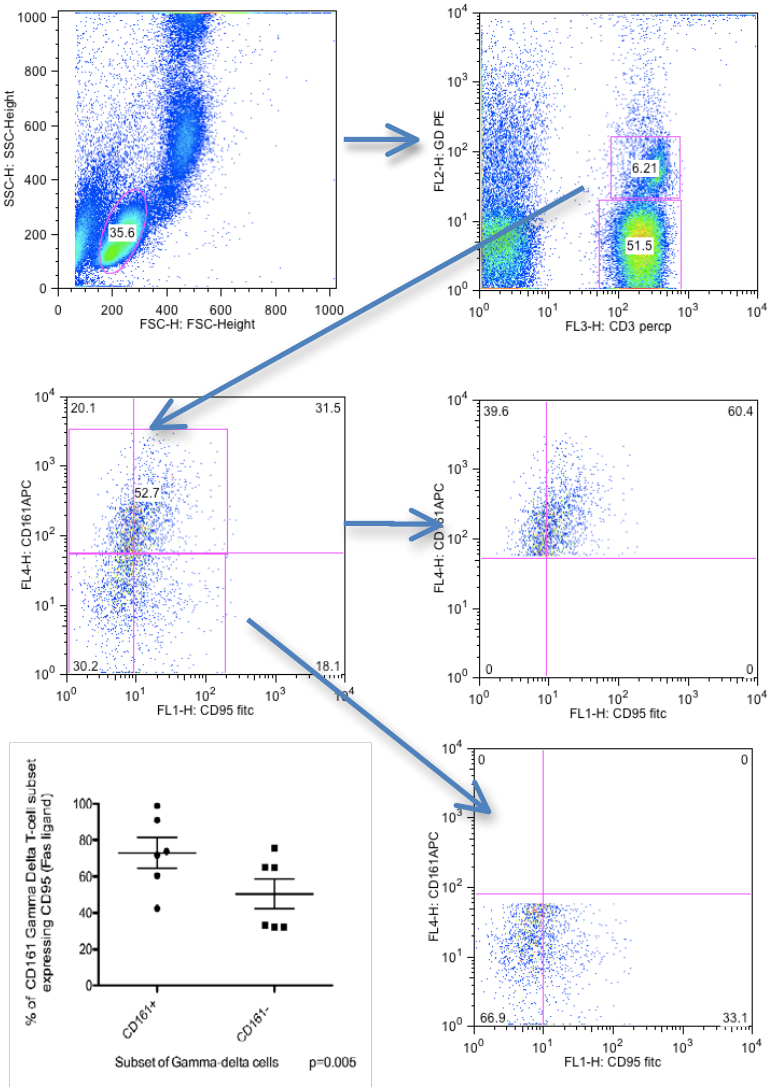


FIGURE 3.14: CD161+ γδ T-lymphocytes expressed higher levels of CD95 compared to CD161- subsets.
A FACS experiment was performed to assess the expression of CD95 by CD161+ compared to CD161- γδ T-lymphocytes. Six samples were analysed with staining for anti-CD3 PerCP, anti-CD161APC and anti-γδ PE and anti-CD95 FITC. FACS analysis was performed on the FACSCalibur with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test. CD95 was significantly expressed in the CD161+ γδ T-cells compared to the negative subset.
Top left panel: lymphocyte gating strategy
Top right panel: CD3+ γδ and αβ T-lymphocyte populations.
Middle left panel: CD161+ gating
Middle right panel: expression of CD95 on the CD161+ γδ T-lymphocytes, and
Bottom right panel: CD161- subset expression of CD95.
Below left panel: significantly more expression of CD95 by CD161+ γδ T-lymphocytes (p=0.005), echoed in CD161+αβ population (p=0.001 – data not shown).

3.4.7 CD161+ γδ T-cells are present at birth but expand quickly in early life compared to CD161- γδ counterparts

With the CD161 status of γδ T-lymphocytes isolated from blood indicating different phenotypical and functional properties, experiments to answer the question of whether CD161 positivity was an innate or acquired marker of γδ T-cells were next performed.

PBMCs were initially isolated from cord blood obtained from 7 neonates (blood donated via the John Radcliffe hospital, with all mothers fully consented). The PBMCs were stained with the following panel: Anti-human CD3 PercP, Anti-human CD161 APC, Anti-human γδ FITC. All samples were analysed as before. Six frozen PBMC samples from a South African cohort of 10-week old infants (TB Vaccination initiative field site, Worcester, South Africa) were thawed to study the frequency of CD3+CD161+ γδ T-cells. Adulthood PBMCs were gained from 8 frozen leucocyte cone samples. In the frozen samples a Near Infrared live/dead marker was also used.

The neonates total CD3+ γδ T-cell population was 1.29% (+/-0.64), which expanded to 2.86% (+/-1.57) in 10-week old infants to 10.03% (+/-1.61) in adults (all total % of CD3+ T-cells) – i.e. an incremental rise in the total γδ T-cell population with age. This expansion was a significant expansion of total CD3+ γδ T-cells in the 1st 10 weeks of life ($p=0.019$) and then again from 10 weeks to adulthood ($p<0.0001$).

An expansion was not seen however in the 1st 10 weeks of life in the CD3+ αβ population (non-significant difference of total CD3+ αβ T-lymphocytes

(p=0.7)), and when comparing 10-week old infant with adult total αβ populations there was a significant reduction ($p<0.0001$).

When the specific CD161+ and CD161- subsets were analysed, the neonates had a significantly lower proportion of CD161+ γδ T-lymphocytes compared to both 10-week old infants and adults ($p<0.0001$ and 0.005 respectively). This suggests that in the 1st 10 weeks of life, not only does the total γδ population increase, but also it is the CD161+ γδ T-lymphocyte subset that expands preferentially.

Thereafter, there was a significant reduction in CD161+ γδ T-cells from infanthood to adulthood ($p=0.002$) despite an overall significant increase in γδ T-cells ($p<0.0001$). Consequently, the CD161 γδ T-cells may either re-distribute to other sites from the periphery, or the CD161+ T-cells were reducing with the CD161- subset increasing (less likely). One factor in this may be that frozen leucocyte samples were used rather than fresh PBMCs (Chapter 3.4.1); and that in-vivo the drop off in CD161+ γδ T-cells may not actually be that marked significant between childhood and adults (if the mean values of the fresh PBMCs were used instead). Also from Chapter 3.3.1, it was clearly show in adulthood that the main subset of γδ T-cells were CD161+.

It follows that in the 1st few weeks of life, the % of total γδ T-cells and CD161+ increase likely due to exposure to Ags. This increase was not, however, so significant in the total (or CD161+) αβ populations ($p=0.06$ in CD161+ increase in 1st 10 weeks), although there was a significant increase in the CD161+ αβ T-cell population thereafter, ($p=0.01$ between 10 week old infants and adult populations).

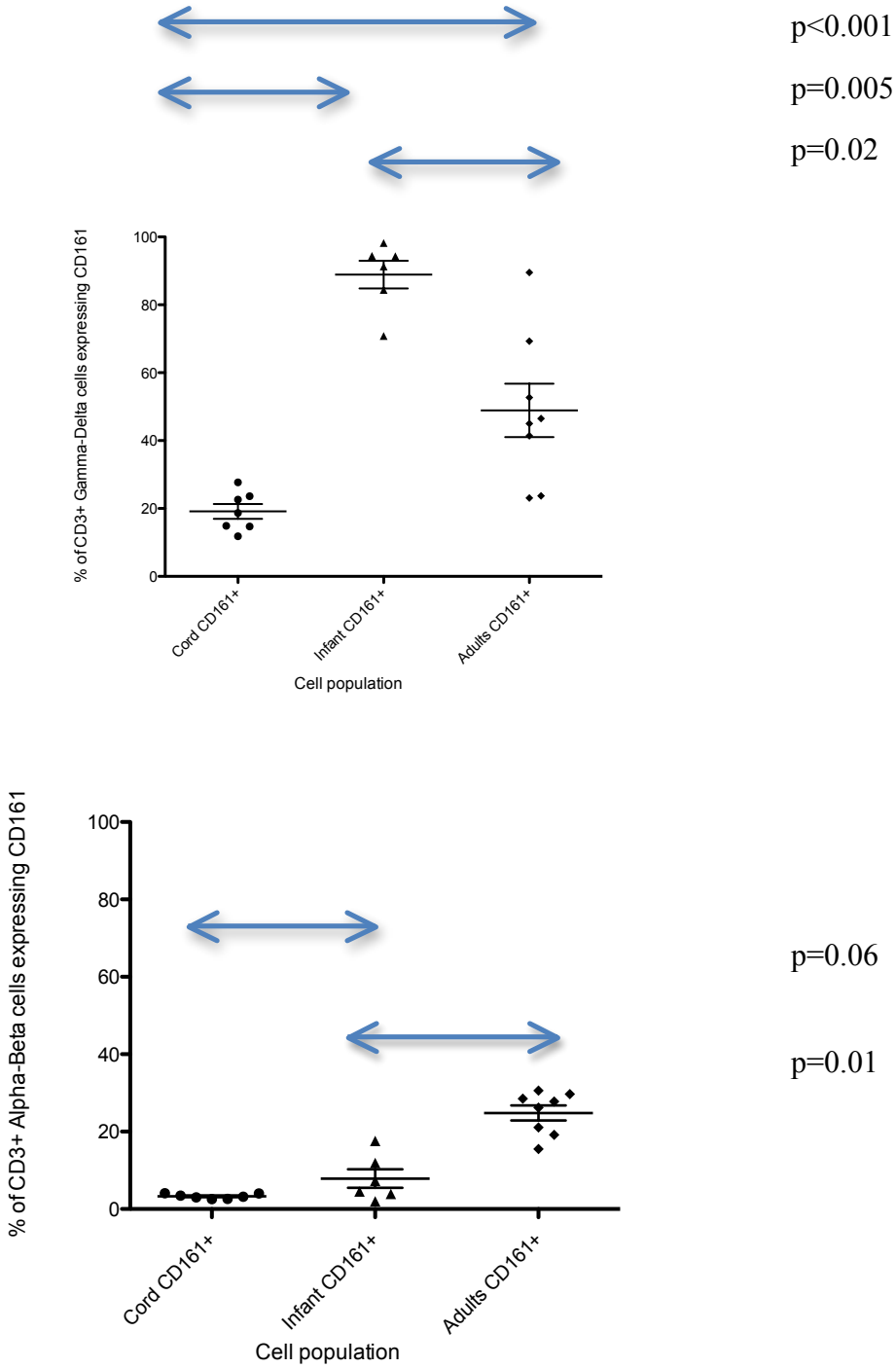


Figure 3.15: A signifincant expansion of the CD161+ γδ T-cells within the 1st weeks of life.
A FACS based experiment was carried out with staining of PBMCs isolated from cord blood, 10 week old infant samples (frozen) and frozen leucocyte cones (8, 6 and 8 samples resepectively). Staining was performed with anti-CD3 PercP, anti-CD161 APC, anti-γδ T-cells FITC and a live/dead marker Near Infrared. Once staining was performed all samples were analysed on the LSR-II and analysed using FloJo. Mann-whitney statistical testing was performed.

Thus, it appears the initial expansion of CD161+ T-cells in the 1st 10 weeks is in life is predominantly in the γδ T-cell population. Also, there appears to be a small fraction of γδ cells in neonates that are CD161+, however, the acquisition or expansion thereafter contributes to the later dominance.

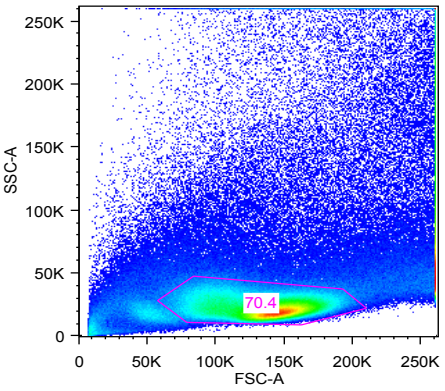
3.4.8: CD161+ γδ T-cell populations in the human colon

Following on from the data established that the CD161+ γδ T-cells are the main subset in the periphery, the next experiment was to ascertain if indeed this was the case in the Gastrointestinal tract. In the intestine, γδ T-cells are a major component of the lymphocyte population comprising of 30-40% of intra-epithelial lymphoctyes with the majority expressing Vδ1 (Deusch *et al* (1991)). With the phenotypical analysis showing CD161+ γδ T-cells expressing significantly more CCR6 and CXCR3 – involved in gut homing, we isolated γδ T-cells from the lamina propria of gut tissue (intestinal tissue from tumour resection margins) and stained the cells for anti-CD3 (PE/PE-Cy7), Live-dead marker (Pacific Blue), anti-CD161 (APC) and anti- γδ (FITC/Alexa-Fluro 788) in an attempt to confirm/refute the hypothesis that CD161+ γδ T-cells may indeed be the main subset of γδ T-cells in the gut.

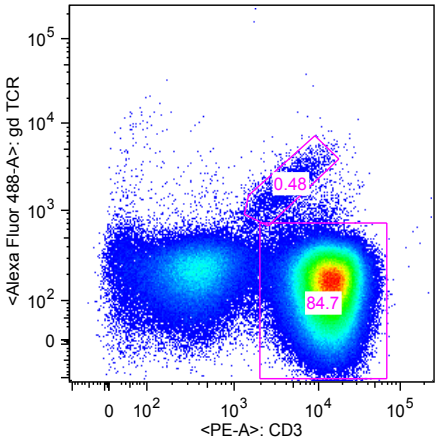
Samples from gut were studied and from patients with IBD for a comparison in view of the proposed hypothesis that CD161+ may be a marker for T-cell subsets to migrate to sites of inflammation. Only five patients were recruited (3 healthy controls and 2 patients with Ulcerative Colitis). In the healthy patients the samples were taken from the tumour margin resections (distance from tumour to margin unknown). With the potential recruitment of γδ T-cells

to tumour areas, our focus was on % of the γδ T-cells being CD161+ (as per previous experiments) rather than absolute numbers of γδ T-cells. It was hypothesisied that there may be an enrichment of the γδ T-cells to areas however around the tumour cells.

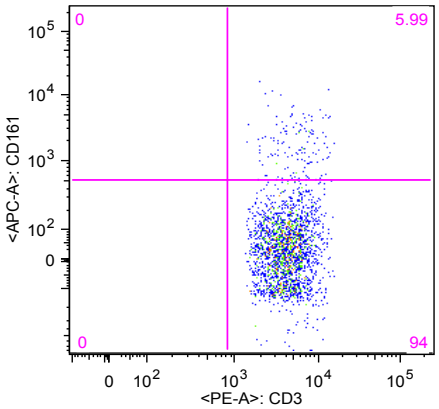
In view of the small numbers of patients, all staining is shown (Figures 3.16-3.20).



Lymphocyte gating



CD3 GAMMA DELTA GATING



CD161 SUBSET

FIGURE 3.16: FACS analysis of CD161+ γδ T-cell populations isolated from lamina propria.

Predominant subset in healthy control 1 colon was CD161- subset. See methods 2.7 for gut tissue processing. FACS staining was performed with anti-CD3 (PE/PE-Cy7), Live-dead marker (Pacific Blue), anti-CD161 (APC) and anti- γδ (FITC/Alexa-Fluro 788). All samples were processed on the LSR-II and anaysed using FloJo.

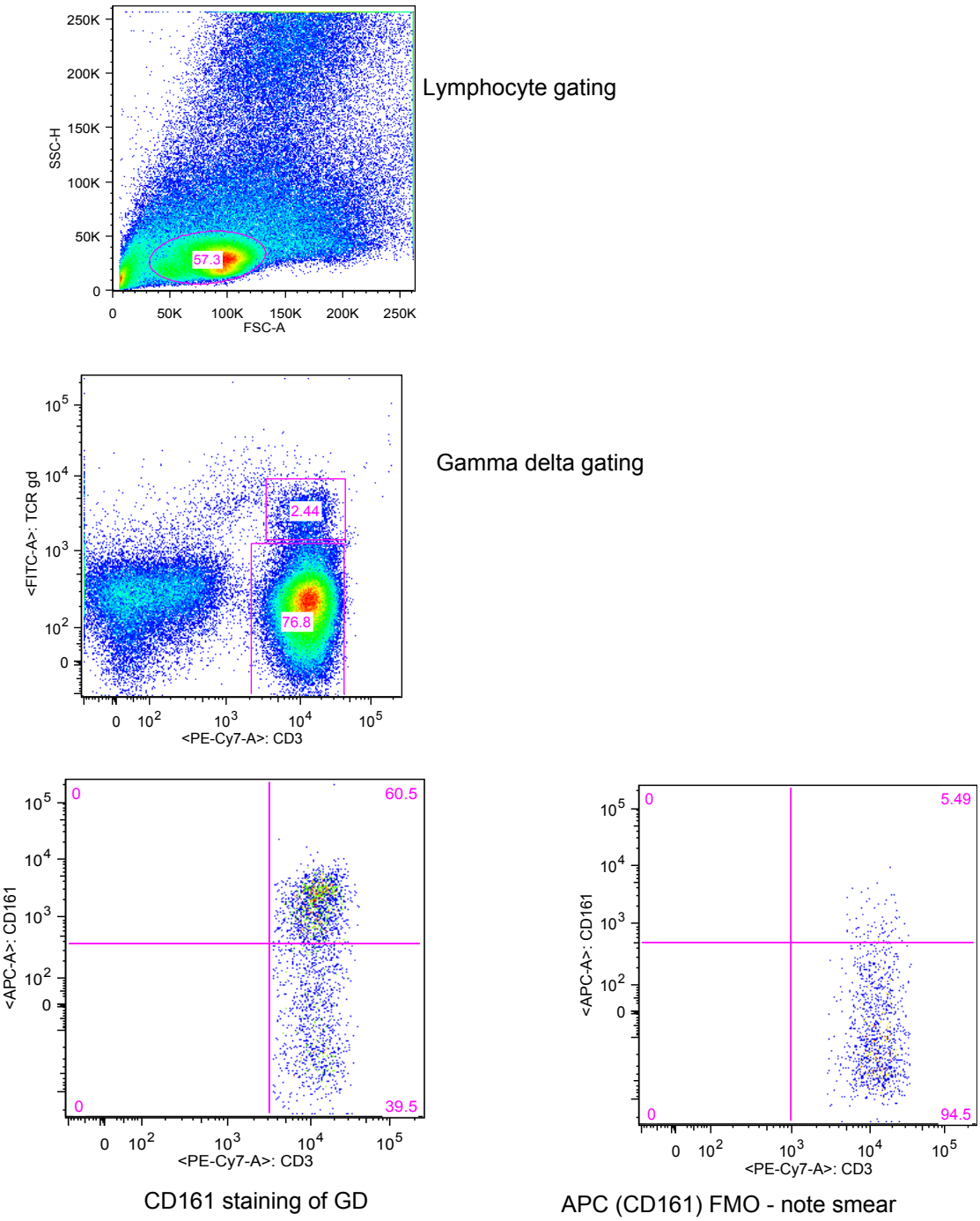
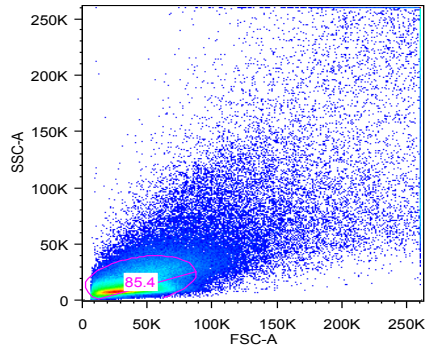
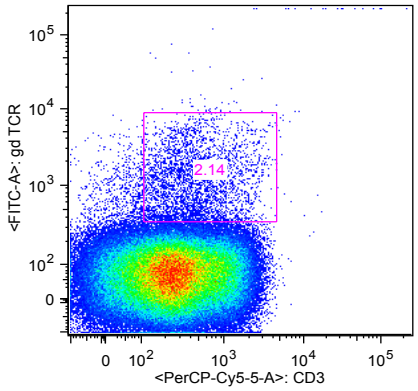


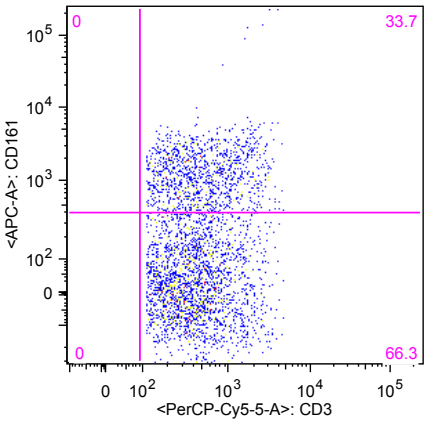
FIGURE 3.17: FACS analysis of CD161+ γδ T-cell populations isolated from lamina propria . Predominant subset in healthy control 2 colon was CD161+ subset –note gating with smear on APC channel (bottom right panel). See Methods 2.7 for gut tissue processing. FACS staining was performed with anti-CD3 (PE/PE-Cy7), Live-dead marker (Pacific Blue), anti-CD161 (APC) and anti- γδ (FITC/Alexa-Fluro 788). All samples were processed on the LSR-II and anaysed using FloJo.



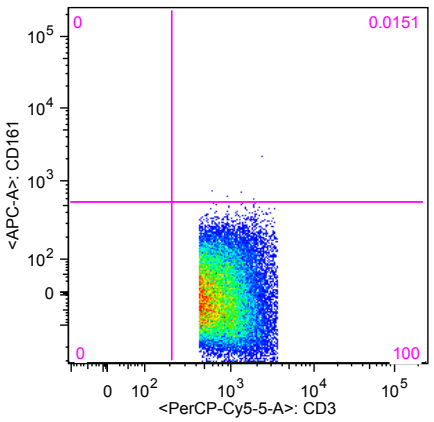
lymphocyte gating



CD3 GAMMA DELTA GATING
(BASED ON A FITC FMO)

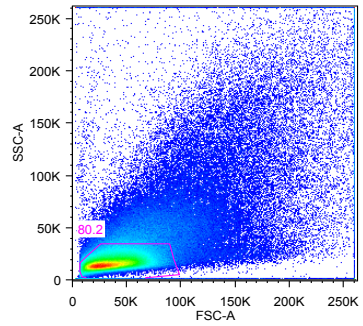


CD161 GAMMA DELTA
STAINING

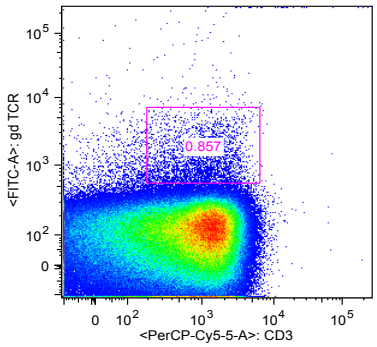


APC FMO

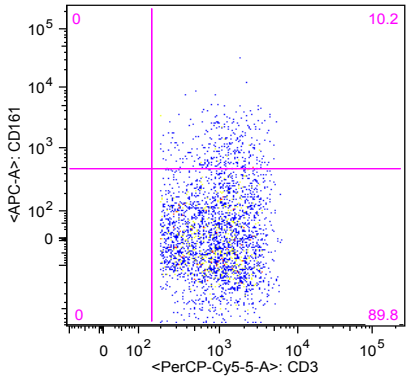
FIGURE 3.18: FACS analysis of CD161+ γδ T-cell populations isolated from lamina propria. Predominant subset in healthy control 3 colon was CD161- subset. See Methods 2.7 for gut tissue processing. FACS staining was performed with anti-CD3 (PE/PE-Cy7), Live-dead marker (Pacific Blue), anti-CD161 (APC) and anti- γδ (FITC/Alexa-Fluro 788). All samples were processed on the LSR-II and anaysed using FloJo.



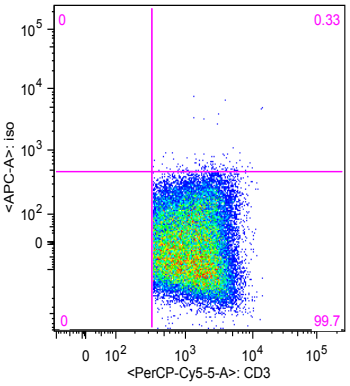
Lymphocyte gating



Gamma delta T-cells

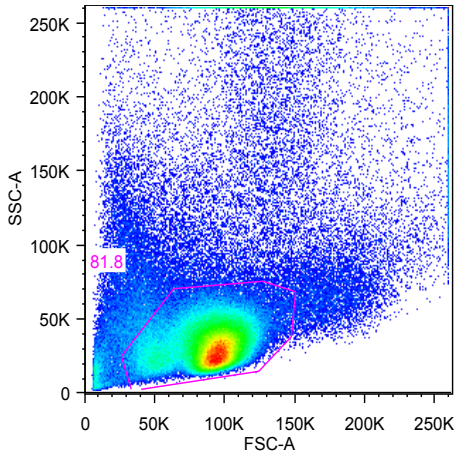


CD161 gating of Gamma delta T-cells

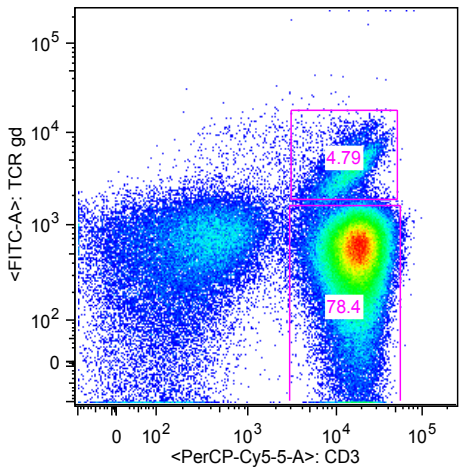


APC FMO

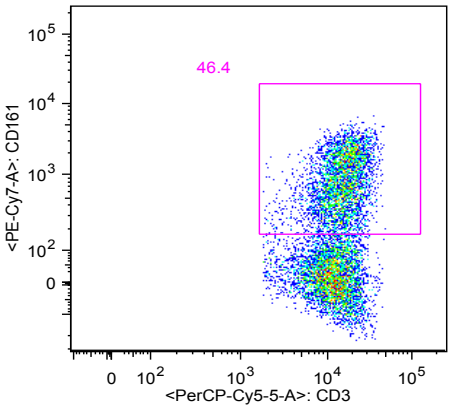
FIGURE 3.19: FACS analysis of CD161+ γδ T-cell populations isolated from lamina propria. CD161- γδ T-cells are the main subset in this patient with Ulcerative Colitis. See Methods 2.7 for gut tissue processing. FACS staining was performed with anti-CD3 (PE/PE-Cy7), Live-dead marker (Pacific Blue), anti-CD161 (APC) and anti- γδ (FITC/Alexa-Fluro 788). All samples were processed on the LSR-II and anaysed using FloJo.



Lymphocyte gating



CD3+ Gamma delta population



CD161 staining

FIGURE 3.20: FACS analysis of CD161+ γδ T-cell populations isolated from lamina propria. CD161+ subset predominant population in patient with UC. See Methods 2.7 for gut tissue processing. FACS staining was performed with anti-CD3 (PE/PE-Cy7), Live-dead marker (Pacific Blue), anti-CD161 (APC) and anti- γδ (FITC/Alexa-Fluro 788). All samples were processed on the LSR-II and anaysed using FloJo.

Thus out of the 3 healthy controls, 2 patients had the majority of γδ T-cells are CD161-ve whilst in 1 patient the majority were CD161+. In the 2 patients with Ulcerative Colitis, in 1 patient only 10% of γδ T-cells and in a second patient 46% expressed CD161. The staining of the LPL for the γδ T-cells was not optimal as seen in Figures 3.18 and 3.19 thus it was difficult to drawn firm conclusions from these experiments.

3.5 Discussion

CD161 γδ T-cells are the main subset in the periphery in humans

The data described in Chapter 3 has shown that CD161+ γδ T-cells are a distinct subset compared to CD161- γδ T-cells. On whole blood staining and freshly isolated PBMC staining, more CD3+ γδ T-cells express CD161 compared to their αβ counterparts. When gated on CD3+CD8+ populations, unlike the CD161 αβ populations which express 2 distinct subsets of CD161 (bright and mid populations) (Billerbeck *et al.* (2010)), it appears there are only reliably 2 distinct CD161+ or CD161- γδ T-cells - not varying expression/intensity of CD161 on the γδ T-cells using these stains (i.e. no "bright or mid" populations).

CD161 downregulation on γδ T-cells

An interesting finding was made upon exploration of the CD161+ γδ T-cells on PBMCs that had been isolated from Leucocyte Cones, frozen down then thawed and stained for CD3/CD161+ αβ and γδ T-cell populations (Table 1). It was expected that there would be similar proportions of CD161+ γδ T-cells

in lymphocytes from fresh whole blood, freshly isolated PBMCs and from frozen leucocyte cones (with maybe a small drop off in the total % s of γδ cells from the frozen samples due to some cell death in thawing processes). The findings showed preserved similar *total* CD3+ γδ T-cell populations (despite a large drop off in the total αβ population isolated from thawing methods). The CD161+ subset was the main subset of γδ T-cells in the *freshly isolated populations*, likely reflecting *in-vivo* populations, however the CD161+/- split in the γδ populations from *thawing of frozen leucocyte cones* was more 50/50 split (despite comparable CD161+/- split in the αβ populations). One explanation for this could be that the freezing process and thawing process had somehow affected the CD161+ γδ T-cell lineage specifically with their potential preponderance to being killed as seen by their CD95 expression. This however is less likely and instead we hypothesise that there might have been a down-regulation potentially of the CD161 molecule via the thawing process. One potential other explanation however explaining this may be that the true proportions of CD161+ γδ T-cells may indeed be less in the population who donate blood for the Leucocyte cones. Unfortunately, due to patient anonymity there was no data available pertaining to the Leucocyte cones regarding demographic or diseases states. These were anonymous patients attending the out-patient Blood transfusion service at the John Radcliffe Hospital undergoing venesection. All donors, prior to donation of blood are screened for common viral infections, although not specifically for other potential disease states that may reduce peripheral γδ T-cells, and specifically the CD161+ fraction. Another possibility may have been the adherence of the CD161+ γδ T-cells to the actual leucocyte cone plastic.

CD161+ γδ T-cells are phenotypically different to their CD161- counterparts

When comparing CD161+ γδ T-cells to the CD161- γδ T-cells in healthy adult populations, the former expressed significantly more CXCR3 and CCR6 (p=0.03 & 0.01 respectively) – CXCR3 is known to be involved in leucocyte trafficking, and present in tissues that are inflamed (Garcia-Lopez *et al.* (2001)) CXCR3 is up-regulated on CD161+ CD4+ and CD8+ αβ T-cells compared to CD161- cells. Thus there remains a close link between CD161 positivity and CXCR3.
Poggi and colleagues (Poggi *et al.* (2007)) studied phenotypical and transendothelial migratory capacities of γδ T-cells from healthy donors and patients with relapsing-remitting MS finding not only (in MS patients) Vδ2 subset expressing CD161, but also that this subset was expanded and capable of transendothelial migration. Moreover, the Vδ2 subset were CXCR3 (bright) CXCR4 (dull), while Vδ1 subset were mostly CXCR4 (+). CXCR3 is capable of inducing CAMKII activation - which is involved in the migration of the Vδ2 subset. However we would postulate that the CD161+ (predominantly Vδ2) may use CXCR3 in migration from the periphery to other sites.

CCR6 is expressed on Th17 cells, while CD161 + CD8 T-cells have been shown to express CCR6 (Northfield *et al.* (2008)). Martin and colleagues (Martin *et al*. (2009)) found that IL-17-producing γδ-T cells share characteristic features with Th17 cells, such as expression of CCR6, RORγT, aryl hydrocarbon receptor (AhR), and IL-23R . CCR6+ IL-17-producing γδ T cells, but not other γδ T cells expressed Toll-like receptors TLR1 and TLR2. Thus, CCR6 and CD161 are not only closely linked in αβ T-cells but on γδ T-cells sharing similar surface phenotypical markers. Co-expression

of CD161 and CCR6 in γδ T cells in CSF and peripheral blood has recently been studied in patients with MS (Schirmer *et al.* (2013)). A higher fraction of CD161high CCR6+ γδ T cells was observed in the CSF of MS patients in relapse than patients with systemic autoimmune disorders and controls with non-inflammatory disease. These cells produced IL-17 as seen in our experiments in the CD161+ CD8+ T cell population. We did not co-stain our healthy populations for CCR6 and CD161 to assess cytokine production, yet one could postulate that this specialized subset may be indeed involved in disease pathogenesis producing pro-inflammatory cytokines.

The Vδ split holds firm for CD161+ γδ T-cells only

With the 2 main γδ T-cell subsets being the Vδ1 and Vδ2 subsets, we explored to see the preferential expression of these subsets on CD161+ γδ T-cells. Previously the link between CD161 and Vδ2 had been established (Poggi *et al.* (2007)) and our data confirmed that the predominant expression in the CD161+ subset was of the Vδ2 chain- with this subset classically based in the periphery (rather than the sentinels at epithelial layers –the Vδ1 subset). Interestingly there was no significant difference between expression of Vδ2 or Vδ1 on the CD161- subsets (p=1), suggesting the CD161- subset may well be a less heterogeneous population or even less developed, or perhaps are waiting priming to become CD161+.

When comparing Vδ1 expression between CD161+/- subsets, there was significantly greater expression in the CD161- subset (p=0.02) and when comparing the Vδ2 expression between CD161+/- subsets, there was significantly greater expression in the CD161+ subset (p=0.03). Therefore, the CD161- subset may possibly already be primed to epithelial residency as they

express the Vδ1 chain. An interesting experiment not performed would be to ascertain the Vδ split in cord blood, infants and then compare to the adult population. Also to look at the Vδ split in the intra-epithelial γδ T-cell according to CD161 subsets.

CD161 γδ T-cell functionally differ from their CD161- counterparts.

On functional assessment the CD161+ γδ T-cells expressed significant levels (compared to the CD161- subset) of not only Granzymes A, B, K and Perforin, but also, upon stimulation, of TNF-α and IFN-γ. This suggests that the CD161+ subset of γδ T-cells are the key cells in inflammation as well as the main subset in killing foreign Ags. Granzymes are serine proteases contained in granules that are released when a T-cell recognizes a target cell to be killed. Granzyme A and K are tryptases, while Granzyme B activates a caspase-dependant pathway of apoptosis. Granzymes A and B are the most abundant granymes in mice and humans (Lieberman *et al.* (2003)) and along with granzyme K and Perforin (a membrane-perturbing pore forming protein) were described by Bade and colleagues (Bade *et al.* (2005)) to be produced by γδ T-cells. Further work is required to actually demonstrate killing, although this will depend on the targets used and the specific Ag.

These cells rather than their CD161+ (CD8+) αβ counterparts appear to display a more Th1 phenotype compared to their CD161 αβ counterparts displaying a mixed Tc1/Tc17 phenotype (Billerbeck *et al.* (2010)). The CD161+ subset also expressed significant levels of CD95 (Fas-ligand) compared to the CD161- γδ counterparts suggesting that it may well be this main subset that is more susceptible to being killed via apoptotic mechanisms.

This may be relevant to homeostasis during inflammation.

The CD161+ γδ T-cells upon stimulation expressed significant levels of TNF-α, an endogenous pyrogen, which can induce: fever, apoptosis, sepsis, cachexia, or inflammation. TNF-α can also inhibit viral replication. The CD161+ γδ population expressed significant levels of IFN-γ compared to the CD161- subset. IFN-γ, or type II interferon, is a cytokine that is critical in the innate and adaptive immune systems, active against viral and intracellular bacterial infections and for tumor surveillance- all facets of the immune system in which γδ T-cells have been shown play a role (Carding and Egan 2002). Aberrant IFN-γ expression, however, can be associated with a number of autoimmune diseases and again some papers have postulated that in autoimmune diseases such as in PBC/PSC/Auto-Immune liver disease that the γδ T-cells may actually be contributing to disease pathogenesis (Martins *et al.* (1996)). The importance of IFN-γ in the immune system stems, in part, from its ability to inhibit viral replication directly, which is critical in combating chronic HCV infection. Thus it may be that the CD161+ γδ T-cells are a key player in the generation of IFN-γ in chronic HCV infection and other viral infections (see Chapter 5).

The ontology of the CD161+ γδ T-cell subset

To finally explore the chronological expression of CD161+, the subsets were studied in cord blood, a 10-week old infant population and healthy adults. A rapid expansion in total γδ T-cells was seen with the CD161+ γδ expanding essentially in the 1st 10 weeks (unlike their αβ CD161+ counterparts). The brisk expansion of CD161 positivity in the γδ T-cell subset compared to the αβ

T-cells suggests that although an innate feature, a major, specific expansion of CD161 or induction of cells in the 1st few weeks of life occurs. This is likely due to Ag exposure and the CD161+ γδ T-cell subset in fact may be the key mediators in response to early childhood infection while the αβ population slowly develop and expand, and are more critical later in life.

CD161+ γδ T-cells and the gut

On exploration of the CD161+γδ T-cell subset in the human gut the % of γδ T-cells T-cells expressing CD161 did indeed seem to vary significantly. However in this pilot study, due to lack of specimens and thus numbers it was difficult to draw firm conclusions. This area requires further investigation, with a well-matched case series of normal human colon tissue, Ulcerative Colitis and Crohn's colon tissue. It would be ideal to gain matched PBMCs from such cohorts of patients. Only then would the potential role of γδ T-cells in relation to CD161 status be addressed. The pilot data to date do not indicate a marked enrichment of these cells in this tissue either in health or disease; however there was significant variability and matched peripheral blood samples would be needed to interpret these data.

3.6 Chapter summary

From this series of experiments in health:

-CD161 γδ T-cells appear to be a distinct subset of the peripheral γδ T-cell populations.

-Directly ex-vivo, the predominant population of γδ T-cells in blood are indeed CD161+, whereas in PBMCs isolated from frozen PBMCs the CD161

expression can be more variable.

-CD161+ express significantly higher levels of chemokine and IL receptors linked to CD161+ expression in αβ T-cells compared to their CD161- subset.

-CD161+ express more TNF-α and IF-γ after stimulation suggesting these cells are more key inflammatory mediators compared to the CD161- subset.

-CD161+ γδ T-cells also express significantly more Granzymes involved in cell killing compared to the CD161- subset.

-CD161 γδ T-cells express the Vδ2 chain preferentially suggesting in fact the CD161+ γδ T-cell's role is to reside in the periphery ready for recruitment to areas of infection/inflammation.

- In early life there is a fraction of CD161+ cells present at birth in the γδ T-cell population, however this subset increases dramatically within the 1st 10 weeks of life.

- In the human gut the expression of CD161 seems variable, and needs further exploration in normal colons and inflamed tissue.

CHAPTER 4: Gene expression analysis of CD161+ γδ T-lymphocytes by microarray

4.1 Introduction

Microarray technology

Gene array (microarray) technology was developed to scan different cell types looking for up-regulated or down-regulated gene sets. By identifying large numbers of complementary DNAs (cDNAs) from humans, a rapid overview of different classes of genes in a messenger RNA (mRNA) population can be identified for different patient cohorts, cell subsets or diseases (Wei *et al.* (2004)). Using gene array hybridization techniques, DNA in the form of oligonucleotides is placed onto a slide in an array format, which is probed by hybridization to a pool made from mRNAs from an organism/cell population (specifically γδ T-cells in this chapter's experiments); the hybridization is performed by first reverse transcribing mRNA into single stranded cDNA with a labeled nucleotide (Leung and Cavalieri. (2003)).

With current whole genome gene arrays consisting of thousands upon thousands of potentially expressed genes, the oligonucleotides are chosen from Expressed sequence Tag (EST) collections – with known and also predicted genes included (Agilent gene ™ array was used in following experiments). Down-stream, gene array technology allows molecular level differences to be identified in diseases allowing potential review of disease classifications, sub-types and prognostication, or aberrant functional pathways to be identified allowing for potential development of novel therapies to treat diseases.

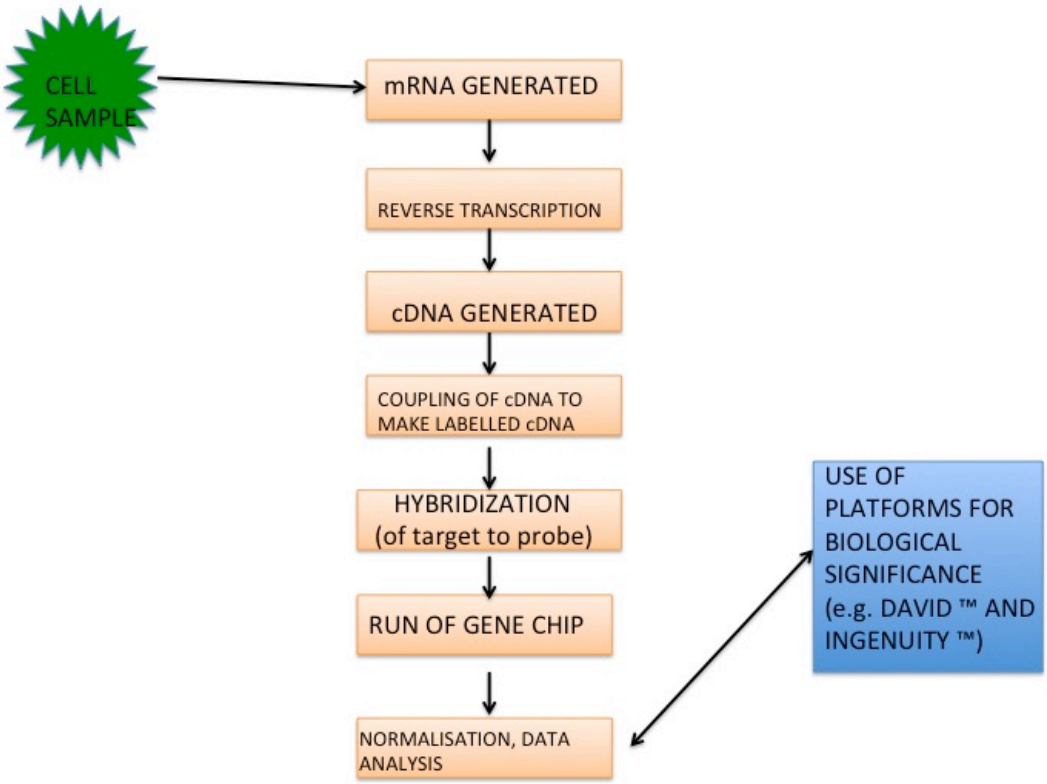


FIGURE 4.1: Summary of gene-array technology.

The "upstream" steps in generation of data from the gene array include:

1. Obtaining the cell sample (including cell sorting and purification as needed)
2. mRNA generation and thereafter, using reverse transcription, the generation of cDNA.
3. The cDNA is then labeled and hybridisation occurs.

Gene-arrays and γδ T-cells

Gene array technology has been used in research into γδ T-cell populations, with the IEL population being profiled by Faher and colleagues (Faher *et al.* (2001)) in a mouse model of Yersinia infection, discovering transcriptional

profiles suggesting that γδ IELs were constitutively activated whereas αβ T-cells required activation. Other findings included the observation that γδ IELS were involved with lipid metabolism and cholesterol homeostasis. Hedges and colleagues (Hedges *et al.* (2003)) studied the GD3.5+/- subsets (GD3.5 a lineage specific γδ T-cell Ag (Jones *et al.* (1996)). The GD3.5+ subsets were CD8- and the GD3.5-ve subset were CD8+. The CD3.5+/CD8- γδ T-cell subset expressed genes involved in proliferation, activation, transcription and translation, compared to a more quiescent/anti-proliferative GD3.5-CD8+ subset, which were also more apoptotic. More recently Kabilitz's group from Kiel, Germany used an Affimetrix ™ gene array to explore γδ T-cells in humans. They found different genes regulated between TCR or TCR/TLR3 stimulation on γδ T-cells (Pietschmann *et al.* (2009)). To our knowledge, γδ T-cell populations have not been separated *per* CD161 status and analysed using gene array technology to date.

4.2 Aims

The aim of this Chapter were:

1. To explore at a molecular level if there were distinct gene-expression differences between CD161+ and CD161- γδ T-cells.
2. To explore any functional / biological significance of the gene signatures up-/ down-regulated in CD161+ γδ T-cells.
3. To explore if there were any specific gene signatures associated with CD161+ status of T-cells consistent across the T-cell lineages – i.e. similar signatures between both the CD161+γδ and CD161+ (mid) αβ T-cell populations.

4.3 Materials and Methods

CD161+ and CD161- subsets of γδ T-cells were isolated (using protocols in Chapter 2.4). Cells were gained from leucocyte cones, enriched for T-cells, with the CD161+ and CD161- subsets then isolated using FACS sorting based on surface antibody staining (Figure 4.2). Cell sorting was carried out using a FACSAria ™ cell sorter (in the Translational Gastroenterology Unit, John Radcliffe Hospital), where the discrete γδ/ αβ T-cell populations could be visualized and gated upon/out (see Figure 4.2), followed by CD161 gating of the γδ T-cell populations. The cells were then transferred in PBS with 2% FCS media to the laboratory where they were spun 1500g for 3 minutes into a small pellet with the supernatant discarded. The cells were then snap frozen in their pellet using 70% ethanol and dry ice and sent for RNA extraction.

To be able to isolate ample amounts of RNA, 100,000 cells were required. Initially, cells isolated from leucocyte cones of 4 donors were sent for RNA extraction and subsequent gene array analysis. Unfortunately all the RNA had RIN values (see Chapter 2.5) between 2.1 and 2.9 – i.e. deemed as poor quality RNA and thus unable to run on the Agilent ™ gene array chip. The experiment was thus repeated and replacement samples from another 4 donors were sent. This time RIN values between 6.6-9.1 (good quality RNA suitable for gene chip) were obtained. The RNA was isolated from the cells, and the cells run on an Agilent gene array chip ™.

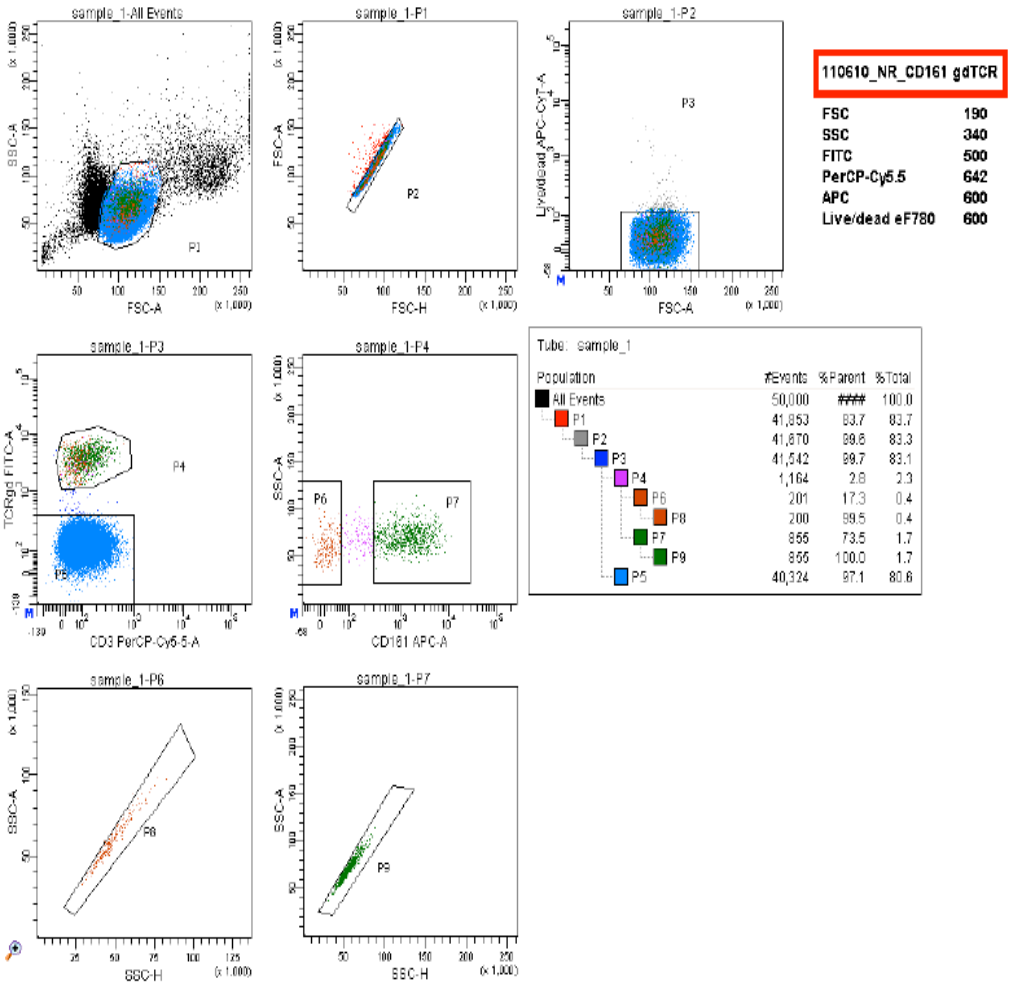


FIGURE 4.2: FACSAria gating strategy of CD3+CD161+ γδ T-cells.

An example of gating strategy and cell sorting from T-cells enriched from a leucocyte cone on FACSAria.
Top left panel: Lymphocyte gating ("All events")
Top right panel: live/dead gating (P2).
*Middle left panel: (*sample 1-P3) γδ T-cell population (in green) and αβ T-cells in blue.
*Middle panel:(*sample 1-P4) delineated CD161+ (green) and CD161- (brown) subsets of γδ T-cells used.
Lower panels: CD161+ γδ T-cells (green) and CD161- γδ T-cells (brown)

Data was sent back from Miltenyi from the Agilent ™ gene array as a raw data files and as processed data sets with genes up-regulated (+ Fold change (FC)), or down-regulated (- FC). The data was reanalyzed in Oxford by

collaboration with the Oxford Centre for Human Genomics. All data was also expressed according to p-value. Initially, a p value of <0.01 was taken as significant (with a FC>2). This threshold was reduced from an original cut-off from 0.05 in view of the potential number of significant genes evaluable. Corrected p-values thereafter were also plotted using a Bonferroni correction method. This method reduced the amount of genes reaching statistically significant differential expression.

One issue identified from the Agilent ™ data generated was that in the gene array data generation there were some probes listed had a signal classed as "undetected". As a result a filter was used to remove any probe not detected in at least 50% (> or equal to 4 out of the 8 samples) to form a new list. This meant that low intensity signals were thus filtered out that may have confounded results; giving a more precise gene list with only signatures that may have had a potential physiological and mechanistic role downstream included. As a result, the gene list was reduced (and thus referred to as "*filtered lists*" in Chapter 4) making the analysis more comprehensive and reliable (Appendix 2).

Heat maps were generated using R-software package. The "filtered lists" were used to compare the CD161 subsets (γδ v αβ) for matching gene signatures, and the "filtered lists" were used for functional mapping via DAVID. All data analysis was done in conjunction with The Wellcome Centre for Human Genomics, Oxford.

Validation FACS staining was carried out (as per Chapter 2 and 3 methods) on either whole blood samples from healthy volunteers or from PBMCs isolated

from frozen leucocyte cones. Antibodies used included: Anti-CD3 PerCP (BD Biosciences, recommended dilution 1 in 50) / PE-Cy7 (eBioscience, recommended dilution 1 in 100), Anti-CD161APC (as before)/ Pacific Blue (eBioscience, recommended dilution1 in 100), Live dead (Near InfraRed – as before), Anti-TCR γδ APC (Biolegend, recommended dilution 1 in 4)/ FITC (as before)/PE (as before), Anti-CCR6 FITC (as before), IL-18Ra PE (BD Biosciences, recommended dilution1 in 20), Anti-CCR1 Alx Fluoro 647 (R&D, recommended dilution 1 in 25).

As a comparison of CD161 T-cell types, data from the Klenerman group in Oxford (Fergusson, Fleming, Rajoriya *et al* 2014) of gene array analysis from CD161+ (mid (or "dim") αβ cells (with naïve cells excluded by a positive selection process) was surveyed. These cells had been compared on the same Agilent gene chip ™ to the CD8+ CD161- αβ T-cell subsets in a similar experiment (data unpublished). For this subset in previous experiments, PBMCs were isolated from leucocytes cones as per Chapter 2.4. CD8+ cells were purified (using a Stem cell kit ™) and then sorted by flow cytometry using the MoFlo ™ (Dunn School, Oxford), excluding the naive population. CD161 dim and CD161 negative populations were thereafter sorted, RNA extracted and a gene array performed in a manner analogous to that described.

The list of genes that was gained was analysed using the Database for Annotation, Visualization and Integrated Discovery (DAVID™) bioinformatics software, allowing biological meaning to be gained from large lists of genes. DAVID was used according to its published protocol (Huang *et al.* (2009)).

4.4 Results

4.4.1 CD161+ γδ T-lymphocytes have a distinct genetic signature

From the Agilent ™ gene chip experiment, there were 702 significant genes ($p<0.01$ and Fold changes (FC) > 2) from the raw data that was gained from CD161+ γδ T-cells when compared to the CD161- γδ T-cell subset (242 genes up-regulated and 460 down-regulated) – (Appendix 3).

The filter (removing undetectable signatures – those in less than 50%) was placed on to this original list, reducing it to the CD161+ γδ "filtered list" of 409 genes found (97 up-regulated on CD161+ and 312 down-regulated). From this list a Heat Map was generated (Figure 4.3) to depict the up-regulated (red) and down-regulated genes (green). The full list of 409 genes is included in Appendix 2. The genes were ranked by p-value (rather than FC - to account for any variability in the data – in that if FC used alone but only a single gene in a single subset showed a major FC, this could have skewed the data). The top gene hits of the filtered list are showed in Figure 4.4.

From this filtered list, a Bonferroni (BF) corrected p-value list was made, reducing the statistically significant list ($p<0.05$) to 53 (6 up-regulated and 47 down regulated). This list is shown in Figure 4.5. The top hit of up-regulated genes on the CD161+ γδ T-cells by FC, raw p-value or BF corrected p-value was KLRB1 – the gene coding for CD161. The functions of the genes on this gene list were derived from the gene-card database (www.genecards.org) with the function of each protein encoded by the genes listed in Tables 4.1 and 4.2 depending on expression (up-regulated and down-regulated respectively).

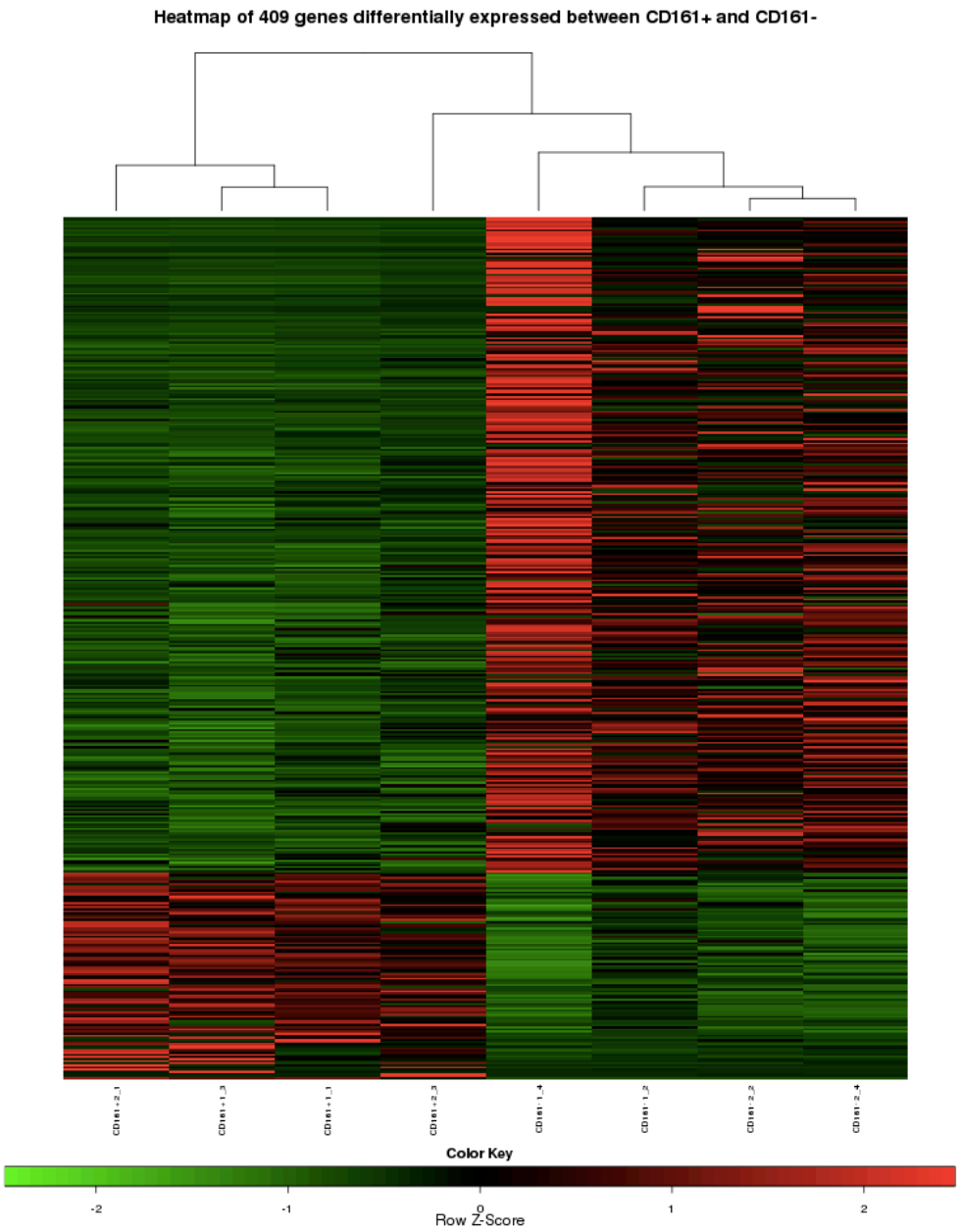


FIGURE 4.3: Heat map representation of the 409 genes from the "filtered list"

Left four columns depict CD161+ γδ T-cells groups (and 4 on right CD161-). Green depicts up-regulated genes whilst red depicts down-regulated. The figure conveys marked differences in genetic signatures between the CD161+ and CD161- γδ T-cells.

(Note: The lines from the top depict the Euclidean clustering programme used for the drawing rather than samples themselves or any related phylogenetic tree).

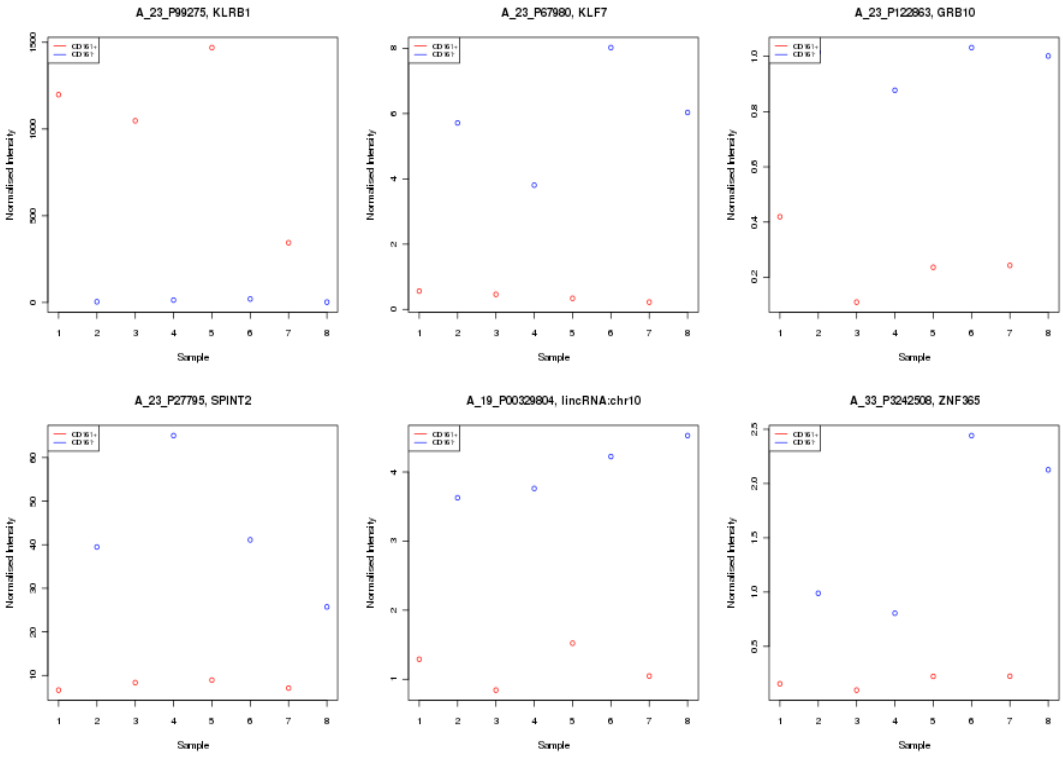


FIGURE 4.4: Top 6 gene hits from the "filtered list" on CD161+ γδ T-cells

Figure shows the top gene hits displayed – arranged by Fold Change (i.e. prior to Bonferroni correction of p-values). The figure includes up-regulated and down-regulated genes.
The red circles are CD161+ γδ samples and blue are CD161-, with sample number referring to the numbers of samples analysed (2 samples per donor – i.e. CD161+ and –ve subsets of γδ T-cells).
The y-axis represents normalized intensity and the data shown are from (top row) KLRB1, KLF7, GRB10. On the bottom row SPINT2, LinncRNA.chr10 and ZNF365.

Gene name	Fold Change	p-value	BF corrected p-value
KLRB1	64.32418	7.24E-36	4.34E-31
KLF7	-14.51668	3.28E-22	1.97E-17
GRB10	-5.29576	2.46E-20	1.48E-15
SPINT2	-4.97917	2.46E-20	1.48E-15
lincRNA:chr10:6623494-6637944_R	-3.45988	5.12E-20	3.07E-15
ZNF365	-10.07362	1.78E-18	1.07E-13
AK5	-8.8468	1.87E-17	1.12E-12
AIF1	-6.41572	1.12E-16	6.72E-12
WNT7A	-9.39575	2.92E-16	1.75E-11
PECAM1	-3.54409	1.65E-15	9.90E-11
PECAM1	-3.05985	2.90E-15	1.74E-10
TCEA3	-6.53897	2.95E-15	1.77E-10
KCTD12	-16.63801	5.28E-15	3.17E-10
VIPR1	-3.29631	5.70E-15	3.42E-10
ADRB2	2.66527	2.72E-14	1.63E-09
INF2	-5.7716	6.89E-14	4.13E-09
NOG	-22.15534	8.46E-14	5.08E-09
KLHL29	-2.90399	1.22E-13	7.32E-09
CACNA1I	-11.89334	3.13E-13	1.88E-08
ZNF365	-7.70655	2.92E-12	1.75E-07
MAL	-14.09853	7.05E-12	4.23E-07
PRKAR2B	-2.45258	9.45E-12	5.67E-07
BEX1	-7.337	9.13E-11	5.48E-06
CDC42BPB	-2.6155	1.09E-10	6.54E-06
BEND5	-15.82532	2.17E-10	1.30E-05
KANK1	-4.48669	2.39E-10	1.43E-05
NGFRAP1	-9.99433	8.74E-10	5.24E-05
FAM164A	-7.12951	1.15E-09	6.90E-05
NBEA	-5.71821	2.49E-09	1.49E-04
PVT1	-2.15998	2.78E-09	1.67E-04
INF2	-4.37732	4.44E-09	2.66E-04
FLJ45983	-6.12813	5.34E-09	3.20E-04
PASK	-8.96053	5.55E-09	3.33E-04
FBLN2	-8.43219	8.82E-09	5.29E-04
KANK1	-11.96443	1.37E-08	8.22E-04
lincRNA:chr18:68297754-68318093_R	-14.12603	1.63E-08	9.78E-04
PCSK5	-3.7059	2.75E-08	1.65E-03
CPAMD8	-4.97185	5.70E-08	3.42E-03
PECAM1	-2.79547	7.13E-08	4.28E-03
LRRC8B	-2.22473	7.50E-08	4.50E-03
CCL20	9.56603	7.88E-08	4.73E-03
CYTH3	2.37096	1.15E-07	6.90E-03
LRRC8B	-2.0662	1.92E-07	1.15E-02
ACVR2A	-2.72423	2.30E-07	1.38E-02
SESN3	-3.78098	3.18E-07	1.91E-02
MDS2	-6.4935	3.56E-07	2.14E-02
MYB	-5.34642	4.58E-07	2.75E-02
COLQ	3.56566	5.10E-07	3.06E-02
CR2	-14.866	5.59E-07	3.35E-02
MPP3	3.12469	5.92E-07	3.55E-02
lincRNA:chr7:79099906-79100510_F	-8.97082	6.20E-07	3.72E-02
KCNQ1	-5.44691	6.35E-07	3.81E-02
ZP1	-6.35382	7.39E-07	4.43E-02

FIGURE 4.5: Significantly up-/down-regulated genes on CD161+ vs CD161- γδ T-cells after BF correction from the Agilent ™ gene array.

Up-regulated Gene name	Function
KLRB1	Gene encoding for CD161
ADRB2	Gene encoding for B2-adrenergic receptor – a clinically important receptor (e.g. in "fight or flight" situations)
CCL20	Gene encoding for chemokine recruiting macrophages/neutrophils
CYTH3	Gene encoding for protein involved in Golgi structure/ regulation of ADP-ribosylation factor protein
COLQ	Gene encoding for Acetylcholinesterase (Ach-e) collagenic tail peptide. Mutations of gene related to end-plate Ach-e deficiency
MPP3	Gene encodes for membrane-associated proteins termed MAGUKs (membrane-associated guanylate kinase homologs which regulate cell proliferation, signaling pathways, and intracellular junctions.

TABLE 4.1: Up-regulated genes (after BF correction) on CD161+ γδ T-cells and their function.

Information available on www.genecard.org

Down-regulated Gene name	**Function**
KLF7	Transcriptional activator
GRB10	Encodes for an adaptor protein that interacts with tyrosine kinase receptors. Overexpression related to growth suppression.
SPINT2	Encodes for a Kunitz family of serine protease inhibitor protein. The protein inhibits a HGF activator and is thought to be involved in the regulation of the proteolytic activation of HGF in injured tissues
lincRNA:chr10:6623494-6637944_R	Nil documented
ZNF365	Encodes for Zinc-Finger protein 365. Mutations in the gene linked to uric acid nephrolithiasis
AK5	Encodes a member of the adenylate kinase family.
AIF1	This gene is induced by cytokines and interferon. Protein to be involved in negative regulation of growth of vascular smooth muscle cells, which contributes to the anti-inflammatory response to vessel wall trauma. Enhances lymphocyte migration.
WNT7A	Encoded proteins have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis
TCEA3	Necessary for efficient RNA polymerase II transcription elongation.
KCTD12	Potassium channel tetramerisation domain containing 12 – involved in G-protein receptor signalling
VIPR1	Encodes a receptor for vasoactive intestinal peptide. Vasoactive intestinal peptide is involved in smooth muscle relaxation and water/ion flux in the bronchial and intestinal epithelium
NOG	Encodes for a secreted polypeptide, that binds and inactivates members of the transforming growth factor-beta (TGF-β)
KLHL29	Kelch-like 29.

CACNA1I	Encodes for calcium channels
MAL	Encodes for T-cell differentiation protein
PRKAR2B	Encodes for protein kinase A
BEX1	Plays a role in cell cycle progression and neuronal differentiation. Inhibits neuronal differentiation in response to nerve growth factor.
CDC42BPB	Encodes for a member of the serine/threonine protein kinase family
BEND5	Ben-domain-containing 5
NGFRAP1	Nerve growth factor receptor (TNFRSF16) associated protein Plays a role in zinc-triggered neuronal death
FAM164A	Nil documented
NBEA	Neurobeachin: encodes for an A-kinase anchor proteins. Mutations in this gene may be associated with forms of Autism.
PVT1	Identified as a regulator of Gemcitabine sensitivity in human pancreatic cancer cells.
INF2	Gene represents a member of the formin family of proteins, with mutations linked to Focal sclerosing glomerulonephritis
FLJ45983	hypothetical LOC399717
PASK	The PAS domains regulates the function of many intracellular signaling pathways.
FBLN2	Encodes an extracellular matrix protein, which binds various extracellular ligands and calcium. The protein may play a role during organ development.
KANK1	Encodes for a protein with the suggested role in tumor genesis of renal cell carcinoma
lincRNA:chr18:68297754-68318093_R	Nil found
PCSK5	Encodes for protein mediates post-translational endoproteolytic processing for several integrin alpha subunits including HIV-1 glycoprotein gp160
CPAMD8	Encodes for complement protein.
PECAM1	The protein encoded by this gene is found on platelets, monocytes, neutrophils, and some types of T-cells. Protein involved in leucocyte migration, angiogenesis, and integrin activation.

LRRC8B	Leucine rich repeat containing 8 family member B
ACVR2A	Encodes activin A type II receptor (activins belong to TGF-β superfamily).
SESN3	Sestrin 3
MDS2	myelodysplastic syndrome 2 translocation associated gene. ? role in myelodysplastic syndrome
MYB	Encodes for MYB protein involved in transcriptional repression. This protein plays an essential role in the regulation of hematopoiesis
CR2	Encodes for a membrane protein, which functions as a receptor for EBV binding on B- and T-Lymphocytes
lincRNA:chr7:79099906-79100510_F	Nil found
KCNQ1	Encodes for potassium voltage-gated channel KQT subfamily member 1 – involved in cardiac repolarization.
ZP1	Encodes for glycoproteinZP1 ensures the structural integrity of the zona pellucida, which mediates species-specific sperm binding.

TABLE 4.2: Down-regulated genes (after BF correction) on CD161+ γδ T-cells and their function.

Where no information available on www.genecard.org, or from other databases "Nil documented" stated in table.

From the Agilent ™ data, the majority of statistically significant genes on the CD161+γδ T-cells (when compared to the CD161- subset) were down-regulated genes. This was the case on the original (unfiltered) gene list, the corrected "filtered" list and also then when a BF correction implemented.

From the gene array data the gene most highly up-regulated gene was KLRB1 encoding for CD161, and also of note were genes encoding for β2-receptors (ADRB2) and for CCL20 – the ligand for CCR6. Li and colleagues (Li *et al.*

(2011)) previously have shown that a CCL20-dependent influx of CCR6+ IL-17+ IL-22+ γδ T cells occurs in the setting of corneal abrasions, and is involved in the inflammatory response and epithelial healing. The COLQ gene also was highly up-regulated, a gene involved potentially in neurological conditions (see Chapter 4.5: Discussion). Also a significant "hit" was the gene encoding for IL-18R on the filtered 409 list – however this was lost after BF correction (see Chapter 4.4.3).

4.4.2 Functional and biological pathways of gene signatures found on CD161+ γδ T-lymphocytes

DAVID Functional significance analysis

Using the data generated from the Agilent ™ gene array, the filtered data (409 gene) was placed into the DAVID bioinformatics resource site (http://david.abcc.ncifcrf.gov/) to explore any functionally significant pathways from the dataset up-regulated/down-regulated genes of CD161+ γδ T-cells. The filtered data was used instead of the BF corrected data in view of the marked reduction (409 to 53 genes) of genes using this correction method for this stage – whereby a gene may not have reached statistical significance with the very stringent BF correction, however it may have played a key functional role in the CD161+ γδ subset. A published protocol by Huang and colleagues (Huang *et al.* (2009)) was used to aid data analysis.

When the filtered list of 409 genes was analysed in DAVID software, initially the Functional Annotation chart analysis was performed. This was performed to examine the significance of *gene-term* enrichment, using a modified Fisher's exact test: a cut-

off of $p<0.05$ was taken as significant. The clusters of genes from DAVID hit significance are outlined in Table 4.3.

From the functional annotation, important gene groups (especially pertinent to CD161+ γδ T-cells) included:

Response to wound healing *Inflammatory response*

Cell and biological adhesion *Regulation of chemotaxis*

Defense responses *Regulation of leucocyte migration*

Regulation of locomotion

Term	Count of genes	p-Value	Fold Enrichment
Signal	92	3.32E-12	2.046986698
signal peptide	92	3.75E-12	2.041678955
Glycoprotein	106	1.97E-10	1.775144786
glycosylation site:N-linked (GlcNAc...)	98	1.14E-08	1.711843975
GO:0009611~response to wounding	27	5.48E-08	3.480617496
GO:0005886~plasma membrane	95	1.19E-07	1.599482071
disulfide bond	74	1.52E-07	1.830058217
GO:0044459~plasma membrane part	65	2.12E-07	1.876297134
GO:0006954~inflammatory response	19	1.27E-06	3.994281274
disulfide bond	69	1.83E-06	1.765375116
topological domain:Extracellular	66	4.54E-06	1.750724115
GO:0007389~pattern specification process	16	8.49E-06	4.094276094
topological domain:Cytoplasmic	76	9.63E-06	1.624618895
GO:0007155~cell adhesion	27	1.03E-05	2.635324675
GO:0022610~biological adhesion	27	1.06E-05	2.631565296
GO:0005887~integral to plasma membrane	39	1.50E-05	2.087617468
GO:0040012~regulation of locomotion	13	2.44E-05	4.626052189
GO:0031226~intrinsic to plasma membrane	39	2.48E-05	2.041225969
Membrane	119	2.67E-05	1.375500572
GO:0051051~negative regulation of transport	11	2.77E-05	5.567078189
GO:0044421~extracellular region part	33	3.58E-05	2.185976368
GO:0050921~positive regulation of chemotaxis	6	5.56E-05	14.13584117
GO:0050920~regulation of chemotaxis	6	7.77E-05	13.22385142
GO:0040017~positive regulation of locomotion	9	9.01E-05	6.27458256
GO:0031012~extracellular matrix	17	1.05E-04	3.133520802
GO:0032101~regulation of response to external stimulus	11	1.11E-04	4.7267645
Transmembrane	97	1.12E-04	1.410469921
GO:0051270~regulation of cell motion	12	1.23E-04	4.248076621

GO:0048520~positive regulation of behavior	6	1.23E-04	12.057041
transmembrane region	96	1.26E-04	1.409886931
extracellular matrix	13	1.36E-04	3.900648925
IPR001849:Pleckstrin homology	14	1.75E-04	3.537693778
GO:0002685~regulation of leukocyte migration	5	1.76E-04	17.08080808
GO:0006955~immune response	24	1.78E-04	2.376460255
GO:0030334~regulation of cell migration	11	1.84E-04	4.447074293
GO:0005083~small GTPase regulator activity	14	2.32E-04	3.419407028
GO:0051240~positive regulation of multicellular organismal process	13	2.40E-04	3.640172214
GO:0006952~defense response	22	2.50E-04	2.444083107
GO:0046578~regulation of Ras protein signal transduction	12	2.54E-04	3.904184704
IPR013032:EGF-like region, conserved site	14	3.02E-04	3.344509135
GO:0051056~regulation of small GTPase mediated signal transduction	13	3.22E-04	3.524611191
GO:0002690~positive regulation of leukocyte chemotaxis	4	3.38E-04	27.32929293
GO:0043009~chordate embryonic development	15	3.55E-04	3.096218987
GO:0070161~anchoring junction	11	3.76E-04	4.066932778
GO:0010324~membrane invagination	12	3.81E-04	3.726721763
GO:0006897~endocytosis	12	3.81E-04	3.726721763
GO:0009792~embryonic development ending in birth or egg hatching	15	3.91E-04	3.068408637
GO:0002688~regulation of leukocyte chemotaxis	4	4.60E-04	24.84481175
GO:0016044~membrane organization	16	4.66E-04	2.869217105
IPR006209:EGF	9	4.67E-04	4.960332164
domain:PH	12	4.76E-04	3.651874851
GO:0051960~regulation of nervous system development	11	5.10E-04	3.914351852
GO:0005578~proteinaceous extracellular matrix	15	5.16E-04	2.980876866
GO:0050795~regulation of behavior	6	5.25E-04	8.911725955
domain:EGF-like 3	7	5.95E-04	6.731622642
GO:0003002~regionalization	11	6.26E-04	3.81500282
IPR006210:EGF-like	11	6.27E-04	3.830615828
GO:0051241~negative regulation of multicellular organismal process	10	6.67E-04	4.166050751
GO:0050767~regulation of neurogenesis	10	7.28E-04	4.115857369
GO:0005912~adherens junction	10	7.39E-04	4.102712245
GO:0051094~positive regulation of developmental process	13	7.79E-04	3.194971296
GO:0003012~muscle system process	10	7.92E-04	4.066859067
IPR013320:Concanavalin A-like lectin/glucanase, subgroup	7	7.95E-04	6.363254393
GO:0019899~enzyme binding	19	8.05E-04	2.431225483
GO:0030029~actin filament-based process	12	8.14E-04	3.401986672
GO:0060284~regulation of cell development	11	8.47E-04	3.666124661
GO:0048762~mesenchymal cell differentiation	6	8.49E-04	8.038027332
GO:0014031~mesenchymal cell development	6	8.49E-04	8.038027332
GO:0008016~regulation of heart contraction	7	8.88E-04	6.211202938

sequence variant	191	9.24E-04	1.148747907
GO:0060485~mesenchyme development	6	9.28E-04	7.883449883
GO:0019955~cytokine binding	8	0.001195449	4.911756361
GO:0002687~positive regulation of leukocyte migration	4	0.001216199	18.21952862
egf-like domain	11	0.001375695	3.458401438
domain:Ig-like C2-type 2	10	0.001382704	3.776153314
GO:0007610~behavior	17	0.001399909	2.476535074
transmembrane protein	20	0.001523185	2.252711217
SM00233:PH	14	0.001584492	2.764255578
GO:0031091~platelet alpha granule	6	0.001784157	6.813432836
GO:0006936~muscle contraction	9	0.001795951	4.019013666
domain:Laminin EGF-like 5; first part	3	0.001849164	43.27471698
domain:Laminin EGF-like 5; second part	3	0.001849164	43.27471698
GO:0030335~positive regulation of cell migration	7	0.001885643	5.373737374
IPR000742:EGF-like, type 3	10	0.001888372	3.608030841
GO:0044057~regulation of system process	13	0.001913458	2.874440195
GO:0005085~guanyl-nucleotide exchange factor activity	9	0.001954895	3.96252713
Polymorphism	183	0.0021038	1.145723074
GO:0045765~regulation of angiogenesis	6	0.002207477	6.506974507
Immunoglobulin domain	16	0.002307946	2.46168613
GO:0006935~chemotaxis	9	0.002366573	3.843181818
GO:0042330~taxis	9	0.002366573	3.843181818
GO:0032103~positive regulation of response to external stimulus	6	0.002366723	6.40530303
GO:0048584~positive regulation of response to stimulus	11	0.002431483	3.184557439
IPR018097:EGF-like calcium-binding, conserved site	7	0.002485567	5.103860294
IPR001881:EGF-like calcium-binding	7	0.002485567	5.103860294
GO:0030695~GTPase regulator activity	15	0.002875006	2.484752986
IPR012680:Laminin G, subdomain 2	5	0.002925627	8.332833133
GO:0051272~positive regulation of cell motion	7	0.003067496	4.88023088
GO:0035023~regulation of Rho protein signal transduction	7	0.003226675	4.830935619
Secreted	38	0.003264817	1.626913643
GO:0009986~cell surface	14	0.003293199	2.558300452
GO:0045664~regulation of neuron differentiation	8	0.003317693	4.10966811
GO:0001569~patterning of blood vessels	4	0.003333802	13.01394901
GO:0048585~negative regulation of response to stimulus	7	0.003391936	4.782626263
GO:0010648~negative regulation of cell communication	11	0.003455109	3.03046595
GO:0060589~nucleoside-triphosphatase regulator activity	15	0.003502276	2.430605826
IPR013091:EGF calcium-binding	6	0.003604467	5.832983193
SM00181:EGF	11	0.003607423	2.993136726
IPR001791:Laminin G	5	0.003767358	7.777310924
hsa04512:ECM-receptor interaction	7	0.003898828	4.556451613
domain:Ig-like C2-type 4	6	0.004027864	5.694041708
GO:0005576~extracellular region	47	0.004229514	1.486978045
hsa04640:Hematopoietic cell lineage	7	0.004381433	4.450487622

GO:0000302~response to reactive oxygen species	6	0.004712305	5.465858586
GO:0035295~tube development	10	0.004968149	3.105601469
domain:Ig-like C2-type 1	9	0.004985363	3.416425025
GO:0009968~negative regulation of signal transduction	10	0.00511085	3.091548974
GO:0005925~focal adhesion	7	0.005287966	4.364159594
IPR000034:Laminin B type IV	3	0.005333263	26.24842437
IPR007110:Immunoglobulin-like	16	0.005446412	2.235394757
GO:0045669~positive regulation of osteoblast differentiation	4	0.005524316	10.93171717
GO:0030278~regulation of ossification	6	0.005566188	5.255633256
IPR013783:Immunoglobulin-like fold	17	0.005798346	2.151769569
GO:0030036~actin cytoskeleton organization	10	0.005898867	3.023151873
GO:0016202~regulation of striated muscle tissue development	5	0.005956655	6.832323232
IPR003961:Fibronectin, type III	9	0.005967855	3.315590447
GO:0031252~cell leading edge	8	0.005977283	3.686495061
GO:0005924~cell-substrate adherens junction	7	0.006365211	4.199474326
GO:0048634~regulation of muscle development	5	0.00639134	6.69835611
domain:Laminin EGF-like 11	3	0.006418131	24.04150943
domain:EGF-like 1	7	0.006466255	4.207264151
IPR013548:Plexin cytoplasmic region	3	0.006793003	23.33193277
GO:0003044~regulation of systemic arterial blood pressure mediated by a chemical signal	4	0.006877456	10.12196034
wnt signaling pathway	7	0.006911998	4.149050906
GO:0007626~locomotory behavior	11	0.006920543	2.742903487
domain:Ig-like C2-type 5	5	0.006988483	6.5567753
GO:0050801~ion homeostasis	14	0.006990067	2.338692549
domain:Ig-like C2-type 3	7	0.006995252	4.138292607
GO:0001501~skeletal system development	12	0.007020848	2.57015294
hsa04060:Cytokine-cytokine receptor interaction	12	0.00719906	2.504309283
GO:0005604~basement membrane	6	0.00748419	4.891695369
domain:EGF-like 2; calcium-binding	5	0.007922815	6.326713009
SM00179:EGF_CA	7	0.007938471	3.988014558
GO:0048878~chemical homeostasis	16	0.007993545	2.13510101
GO:0002684~positive regulation of immune system process	10	0.008174502	2.870724047
GO:0030055~cell-substrate junction	7	0.008268576	3.974502488
GO:0043588~skin development	4	0.008410758	9.423894114
GO:0016021~integral to membrane	101	0.008424278	1.212534646
hsa04916:Melanogenesis	7	0.008661193	3.866080156
SM00282:LamG	5	0.008781277	6.076974565
domain:Sema	4	0.00880228	9.306390749
GO:0032403~protein complex binding	9	0.009006357	3.072980223
IPR003598:Immunoglobulin subtype 2	9	0.009265758	3.072986268
domain:Fibronectin type-III 2	7	0.009427364	3.883628447
IPR002165:Plexin	4	0.009527202	9.031715912
IPR001627:Semaphorin/CD100 antigen	4	0.009527202	9.031715912
domain:Laminin EGF-like 10	3	0.009628554	19.6703259

domain:Laminin EGF-like 8	3	0.009628554	19.6703259
domain:IPT/TIG 1	3	0.009628554	19.6703259
GO:0001568~blood vessel development	10	0.00974264	2.78870336
GO:0006928~cell motion	15	0.00976124	2.157575758
domain:Fibronectin type-III 1	7	0.009768897	3.853982428
GO:0005088~Ras guanyl-nucleotide exchange factor activity	6	0.009986195	4.562910028
GO:0031092~platelet alpha granule membrane	3	0.010092968	19.07761194
GO:0016192~vesicle-mediated transport	17	0.010120875	2.016484287
Endocytosis	6	0.010571068	4.51950188
GO:0035239~tube morphogenesis	7	0.010672748	3.765847451
GO:0007166~cell surface receptor linked signal transduction	40	0.010810586	1.472483455
GO:0005615~extracellular space	20	0.011054171	1.856701892
GO:0009953~dorsal/ventral pattern formation	5	0.011274181	5.693602694
GO:0001944~vasculature development	10	0.011325626	2.722041128
GO:0005178~integrin binding	5	0.011402947	5.671413594
domain:Laminin EGF-like 7	3	0.011449731	18.03113208
GO:0007167~enzyme linked receptor protein signaling pathway	12	0.011584963	2.397306397
GO:0048514~blood vessel morphogenesis	9	0.012171359	2.914261094
GO:0031224~intrinsic to membrane	103	0.012298867	1.194162279
IPR001331:Guanine-nucleotide dissociation stimulator, CDC24, conserved site	5	0.012355754	5.555222089
IPR000152:EGF-type aspartate/asparagine hydroxylation conserved site	6	0.012491687	4.329637009
GO:0043583~ear development	6	0.012543915	4.315151515
GO:0045909~positive regulation of vasodilation	3	0.012650556	17.08080808
GO:0016055~Wnt receptor signaling pathway	7	0.01318657	3.595959596
GO:0051271~negative regulation of cell motion	5	0.013317567	5.422478756
domain:IPT/TIG 3	3	0.013409219	16.64412192
domain:Laminin EGF-like 6	3	0.013409219	16.64412192
domain:IPT/TIG 2	3	0.013409219	16.64412192
GO:0042592~homeostatic process	20	0.013469798	1.819526826
domain:DH	5	0.01381895	5.382427485
IPR013098:Immunoglobulin I-set	7	0.014124274	3.550511509
GO:0003073~regulation of systemic arterial blood pressure	4	0.014136815	7.808369408
IPR004075:Interleukin-1 receptor, type I/Toll precursor	3	0.014177482	16.15287654
GO:0002526~acute inflammatory response	6	0.014201739	4.18305504
domain:Ig-like C2-type 6	4	0.014322249	7.797246303
cell adhesion	13	0.014533664	2.22762178
GO:0051674~localization of cell	11	0.014564962	2.448063699
GO:0048870~cell motility	11	0.014564962	2.448063699
GO:0048598~embryonic morphogenesis	11	0.014564962	2.448063699
GO:0048754~branching morphogenesis of a tube	5	0.014802605	5.255633256
GO:0008034~lipoprotein binding	4	0.014929063	7.648306333
GO:0007242~intracellular signaling cascade	29	0.015103804	1.577526861

GO:0000902~cell morphogenesis	12	0.015249754	2.303030303
GO:0030193~regulation of blood coagulation	4	0.01525977	7.591470258
GO:0008217~regulation of blood pressure	6	0.015385181	4.099393939
GO:0043394~proteoglycan binding	3	0.015396625	15.44369548
GO:0019722~calcium-mediated signaling	4	0.016431684	7.386295386
IPR008957:Fibronectin, type III-like fold	8	0.0165219	3.043295579
IPR000219:Dbl homology (DH) domain	5	0.016793488	5.072159299
GO:0051130~positive regulation of cellular component organization	8	0.01679824	3.019811373
hsa05217:Basal cell carcinoma	5	0.016950121	4.970674487
GO:0045823~positive regulation of heart contraction	3	0.017112843	14.64069264
Fertilization	3	0.01764092	14.46240602
GO:0006874~cellular calcium ion homeostasis	8	0.017739596	2.98680797
GO:0005794~Golgi apparatus	23	0.017917058	1.677312976
GO:0010720~positive regulation of cell development	5	0.018076804	4.950958864
SM00630:Sema	4	0.018250116	7.057131753
GO:0008092~cytoskeletal protein binding	15	0.018375913	1.991746441
GO:0000267~cell fraction	27	0.018561221	1.585397114
GO:0009725~response to hormone stimulus	12	0.018637352	2.234002147
IPR008085:Thrombospondin, type 1 repeat, subgroup	3	0.018732476	13.99915966
GO:0019725~cellular homeostasis	14	0.018972791	2.052629297
GO:0045597~positive regulation of cell differentiation	9	0.019044253	2.685192537
GO:0003013~circulatory system process	8	0.019213953	2.938633648
GO:0008015~blood circulation	8	0.019213953	2.938633648
GO:0016477~cell migration	10	0.019859905	2.475479432
IPR001478:PDZ/DHR/GLGF	7	0.019869037	3.28839321
IPR003599:Immunoglobulin subtype	11	0.020055565	2.333193277
domain:LDL-receptor class A 4	3	0.020076592	13.52334906
domain:SEC7	3	0.020076592	13.52334906
GO:0019838~growth factor binding	6	0.020147522	3.824153166
GO:0055074~calcium ion homeostasis	8	0.020237159	2.907371588
IPR002049:EGF-like, laminin	4	0.020371761	6.828858373
GO:0042127~regulation of cell proliferation	20	0.020964057	1.736295612
GO:0006873~cellular ion homeostasis	12	0.02119438	2.192189272
IPR000904:SEC7-like	3	0.021210215	13.12421218
GO:0001525~angiogenesis	7	0.02122754	3.231504232
actin-binding	9	0.021484022	2.634851298
GO:0019932~second-messenger-mediated signaling	9	0.021594035	2.616634429
GO:0050818~regulation of coagulation	4	0.021612152	6.665681202
inflammatory response	5	0.021762141	4.695586369
GO:0042060~wound healing	8	0.021850959	2.861706066
GO:0048041~focal adhesion formation	3	0.022140994	12.81060606
GO:0001655~urogenital system development	6	0.022291307	3.726721763
GO:0048545~response to steroid hormone stimulus	8	0.022408824	2.846801347
SM00060:FN3	9	0.022468827	2.590710209
GO:0001763~morphogenesis of a branching structure	5	0.02275822	4.616434616

GO:0007368~determination of left/right symmetry	4	0.023030922	6.506974507
GO:0009617~response to bacterium	8	0.023068569	2.832051081
GO:0030141~secretory granule	8	0.023330861	2.82631288
GO:0055082~cellular chemical homeostasis	12	0.02347276	2.157575758
mutagenesis site	40	0.023519797	1.41074872
GO:0055066~di-, tri-valent inorganic cation homeostasis	9	0.023870938	2.572841385
GO:0005089~Rho guanyl-nucleotide exchange factor activity	5	0.024306741	4.521802731
GO:0051347~positive regulation of transferase activity	9	0.024349558	2.562121212
GO:0055080~cation homeostasis	10	0.024398701	2.388924207
GO:0009855~determination of bilateral symmetry	4	0.024499201	6.355649518
GO:0045667~regulation of osteoblast differentiation	4	0.024499201	6.355649518
GO:0009799~determination of symmetry	4	0.024499201	6.355649518
GO:0051098~regulation of binding	7	0.024515167	3.125899518
GO:0006875~cellular metal ion homeostasis	8	0.024737294	2.78870336
GO:0005086~ARF guanyl-nucleotide exchange factor activity	3	0.025822767	11.80988478
GO:0048706~embryonic skeletal system development	5	0.025889876	4.436573527
GO:0046700~heterocycle catabolic process	5	0.025889876	4.436573527
GO:0007010~cytoskeleton organization	13	0.02598875	2.037160597
IPR013151:Immunoglobulin	8	0.026003322	2.772110825
GO:0032012~regulation of ARF protein signal transduction	4	0.02601695	6.211202938
IPR003659:Plexin/semaphorin/integrin	4	0.026023285	6.221848739
guanine-nucleotide releasing factor	6	0.026147555	3.5857205
sh3 domain	8	0.02653151	2.754744003
Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci	3	0.026653926	11.40062112
GO:0030501~positive regulation of bone mineralization	3	0.027698896	11.38720539
GO:0042803~protein homodimerization activity	11	0.027787435	2.204040373
GO:0006959~humoral immune response	5	0.02811458	4.32425521
h_nktPathway:Selective expression of chemokine receptors during T-cell polarization	4	0.028132453	5.748
IPR013300:Wnt-7 protein	2	0.028251536	69.99579832
GO:0005605~basal lamina	3	0.028393742	11.22212467
GO:0030111~regulation of Wnt receptor signaling pathway	4	0.029200535	5.941150637
IPR011993:Pleckstrin homology-type	10	0.02978769	2.310092354
GO:0010035~response to inorganic substance	8	0.0305556	2.666272481
GO:0055065~metal ion homeostasis	8	0.0305556	2.666272481
GO:0070169~positive regulation of biomineral formation	3	0.030665658	10.78787879
GO:0050927~positive regulation of positive chemotaxis	3	0.030665658	10.78787879
GO:0050926~regulation of positive chemotaxis	3	0.030665658	10.78787879
GO:0032989~cellular component morphogenesis	12	0.031045997	2.065185864
GO:0032940~secretion by cell	8	0.032045926	2.640511394
GO:0005509~calcium ion binding	22	0.032305468	1.602066343
IPR001438:EGF-like, type 2	3	0.03235919	10.49936975
GO:0006979~response to oxidative stress	7	0.032914772	2.916235526

GO:0030054~cell junction	15	0.033096158	1.841468334
SM00408:IGc2	9	0.033293543	2.401146048
SM00222:Sec7	3	0.033338526	10.25489458
IPR001452:Src homology-3 domain	8	0.033346815	2.628950172
Phosphoprotein	116	0.033496049	1.154921587
domain:LDL-receptor class A 3	3	0.033585585	10.30350404
short sequence motif:Cell attachment site	5	0.033668956	4.097984563
GO:0001990~regulation of systemic arterial blood pressure by hormone	3	0.033752036	10.24848485
GO:0042312~regulation of vasodilation	3	0.033752036	10.24848485
GO:0007163~establishment or maintenance of cell polarity	4	0.034344108	5.57740672
cleavage on pair of basic residues	9	0.034555691	2.401506534
GO:0009719~response to endogenous stimulus	12	0.035012134	2.024392069
GO:0015026~coreceptor activity	3	0.035044685	10.03840206
GO:0004620~phospholipase activity	5	0.035065729	4.031486772
compositionally biased region:Ser-rich	12	0.035076206	2.03645727
domain:SH3	7	0.036345931	2.852382475
SM00325:RhoGEF	5	0.036527086	3.963244281
domain:Laminin EGF-like 3	3	0.036613767	9.83516295
GO:0044420~extracellular matrix part	6	0.036651297	3.261130246
IPR000483:Cysteine-rich flanking region, C-terminal	5	0.036851666	3.977033995
repeat:LRR 12	5	0.037391751	3.96288617
SM00180:EGF_Lam	4	0.037968223	5.335880106
GO:0007507~heart development	8	0.03822262	2.542259807
GO:0030169~low-density lipoprotein binding	3	0.038361886	9.560382916
Newly identified genetic risk variants for celiac disease related to the immune response	3	0.038442878	9.388746803
IPR003129:Laminin G, thrombospondin-type, N-terminal	3	0.038621164	9.544881589
GO:0008285~negative regulation of cell proliferation	11	0.038778675	2.081871345
IPR003591:Leucine-rich repeat, typical subtype	6	0.039350334	3.205914427
domain:TSP N-terminal	3	0.039742611	9.40754717
GO:0030246~carbohydrate binding	11	0.039769547	2.079518318
GO:0048568~embryonic organ development	7	0.040050965	2.780596664
GO:0042325~regulation of phosphorylation	13	0.040178734	1.906012919
GO:0002027~regulation of heart rate	3	0.040267433	9.316804408
GO:0016323~basolateral plasma membrane	8	0.040545307	2.506090238
Short QT syndrome	2	0.040766198	48.20802005
domain:Laminin IV type A 1	2	0.040870146	48.08301887
glycosylation site:O-linked (Fuc...)	2	0.040870146	48.08301887
domain:Laminin IV type A 2	2	0.040870146	48.08301887
region of interest:Endothelin-like	2	0.040870146	48.08301887
GO:0001932~regulation of protein amino acid phosphorylation	7	0.04103189	2.764523851
GO:0044431~Golgi apparatus part	10	0.041753593	2.162994551
IPR000157:Toll-Interleukin receptor	3	0.041910796	9.129886737
IPR001928:Endothelin-like toxin	2	0.042077818	46.66386555
IPR019764:Endothelin-like toxin, conserved site	2	0.042077818	46.66386555

IPR006931:Calcipressin	2	0.042077818	46.66386555
domain:TIR	3	0.042968781	9.015566038
GO:0002507~tolerance induction	2	0.043057202	45.54882155
GO:0048643~positive regulation of skeletal muscle tissue development	2	0.043057202	45.54882155
GO:0016525~negative regulation of angiogenesis	3	0.043688578	8.911725955
GO:0007044~cell-substrate junction assembly	3	0.043688578	8.911725955
GO:0050886~endocrine process	3	0.043688578	8.911725955
GO:0031705~bombesin receptor binding	2	0.043940442	44.61512027
GO:0031708~endothelin B receptor binding	2	0.043940442	44.61512027
PIRSF001632:endothelin	2	0.044751434	43.63421829
GO:0045860~positive regulation of protein kinase activity	8	0.04481009	2.451057662
GO:0048562~embryonic organ morphogenesis	6	0.045000207	3.082251082
GO:0051048~negative regulation of secretion	4	0.045935065	4.968962351
domain:EGF-like 4	4	0.046118571	4.9741054
GO:0051338~regulation of transferase activity	11	0.046182971	2.020310633
GO:0002252~immune effector process	6	0.046215619	3.059249209
GO:0042391~regulation of membrane potential	6	0.046215619	3.059249209
site:Not glycosylated	3	0.046289015	8.654943396
Receptor	31	0.046470043	1.416091555
GO:0017016~Ras GTPase binding	5	0.046612554	3.677070352
IPR016186:C-type lectin-like	5	0.046652548	3.683989385
GO:0045778~positive regulation of ossification	3	0.047213585	8.54040404
GO:0048732~gland development	6	0.047450469	3.036588103
SM00423:PSI	4	0.047978761	4.861579652
hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	5	0.04799131	3.597198642
domain:IQ	4	0.048110515	4.889798529
GO:0048729~tissue morphogenesis	7	0.048138293	2.65701459
GO:0002253~activation of immune response	5	0.048359479	3.634214485
GO:0030005~cellular di-, tri-valent inorganic cation homeostasis	8	0.048574266	2.407867218
IPR013767:PAS fold	3	0.048789391	8.399495798
Deafness	5	0.049541993	3.615601504
GO:0005624~membrane fraction	20	0.049823455	1.572114705
GO:0030155~regulation of cell adhesion	6	0.049978576	2.99225835

TABLE 4.3: Significant gene term enrichment when list of 409 genes entered into DAVID.

The same list was put into the *functional clustering* section of DAVID to rank overall importance of gene groups in the gene list recovered from the CD161+ v CD161- γδ gene array experiment. A higher enrichment score was equivalent to the gene members in that group having a potentially more important role in disease processes. An enrichment score > 1.3 was taken to be significant (equivalent to non-log scale < 0.05) and values can be seen in Table 4.4.

Processes that had highest enrichment scores included: responses to wound healing, inflammatory/immune and defense response. Cellular processes such as glycosylation, peptide signaling, cell adhesion and endocytosis had high enrichment values, as did regulation of chemotaxis and leucocyte migration – in keeping with the previously established role of CD161 γδ T-cells (Chapter 1).

Enrichment Score: 8.851852669218447

Term	Count	p-Value	Fold Enrichment
Signal	92	3.32E-12	2.046986698
signal peptide	92	3.75E-12	2.041678955
Glycoprotein	106	1.97E-10	1.775144786
glycosylation site:N-linked (GlcNAc...)	98	1.14E-08	1.711843975
disulfide bond	74	1.52E-07	1.830058217
disulfide bond	69	1.83E-06	1.765375116

Enrichment Score: 5.36182136650751

Term	Count	p-Value	Fold Enrichment
GO:0044459~plasma membrane part	65	2.12E-07	1.876297134
topological domain:Extracellular	66	4.54E-06	1.750724115
GO:0005887~integral to plasma membrane	39	1.50E-05	2.087617468
GO:0031226~intrinsic to plasma membrane	39	2.48E-05	2.041225969

Enrichment Score: 5.126963621397969

Term	Count	p-Value	Fold Enrichment
GO:0009611~response to wounding	27	5.48E-08	3.480617496
GO:0006954~inflammatory response	19	1.27E-06	3.994281274
GO:0006955~immune response	24	1.78E-04	2.376460255
GO:0006952~defense response	22	2.50E-04	2.444083107

Enrichment Score: 4.935183242574214

Term	Count	p-Value	Fold Enrichment
Glycoprotein	106	1.97E-10	1.775144786
glycosylation site:N-linked (GlcNAc...)	98	1.14E-08	1.711843975
topological domain:Extracellular	66	4.54E-06	1.750724115
topological domain:Cytoplasmic	76	9.63E-06	1.624618895
Membrane	119	2.67E-05	1.375500572
Transmembrane	97	1.12E-04	1.410469921
transmembrane region	96	1.26E-04	1.409886931
GO:0016021~integral to membrane	101	0.008424278	1.212534646
GO:0031224~intrinsic to membrane	103	0.012298867	1.194162279

Enrichment Score: 3.9327017994621367

Term	Count	p-Value	Fold Enrichment
GO:0007155~cell adhesion	27	1.03E-05	2.635324675
GO:0022610~biological adhesion	27	1.06E-05	2.631565296
cell adhesion	13	0.014533664	2.22762178

Enrichment Score: 2.9390948360401654

Term	Count	p-Value	Fold Enrichment
GO:0031012~extracellular matrix	17	1.05E-04	3.133520802
extracellular matrix	13	1.36E-04	3.900648925
GO:0005578~proteinaceous extracellular matrix	15	5.16E-04	2.980876866
GO:0005604~basement membrane	6	0.00748419	4.891695369
GO:0044420~extracellular matrix part	6	0.036651297	3.261130246

Enrichment Score: 2.8279788001880735

Term	Count	p-Value	Fold Enrichment
GO:0010324~membrane invagination	12	3.81E-04	3.726721763
GO:0006897~endocytosis	12	3.81E-04	3.726721763
GO:0016044~membrane organization	16	4.66E-04	2.869217105
GO:0016192~vesicle-mediated transport	17	0.010120875	2.016484287
Endocytosis	6	0.010571068	4.51950188

Enrichment Score: 2.8264666590999146

Term	Count	p-Value	Fold Enrichment
GO:0040012~regulation of locomotion	13	2.44E-05	4.626052189
GO:0050921~positive regulation of chemotaxis	6	5.56E-05	14.13584117
GO:0050920~regulation of chemotaxis	6	7.77E-05	13.22385142
GO:0040017~positive regulation of locomotion	9	9.01E-05	6.27458256
GO:0032101~regulation of response to external stimulus	11	1.11E-04	4.7267645
GO:0051270~regulation of cell motion	12	1.23E-04	4.248076621
GO:0048520~positive regulation of behavior	6	1.23E-04	12.057041
GO:0002685~regulation of leukocyte migration	5	1.76E-04	17.08080808
GO:0030334~regulation of cell migration	11	1.84E-04	4.447074293
GO:0002690~positive regulation of leukocyte chemotaxis	4	3.38E-04	27.32929293
GO:0002688~regulation of leukocyte chemotaxis	4	4.60E-04	24.84481175
GO:0050795~regulation of behavior	6	5.25E-04	8.911725955
GO:0002687~positive regulation of leukocyte migration	4	0.001216199	18.21952862
GO:0030335~positive regulation of cell migration	7	0.001885643	5.373737374
GO:0042330~taxis	9	0.002366573	3.843181818
GO:0006935~chemotaxis	9	0.002366573	3.843181818
GO:0032103~positive regulation of response to external stimulus	6	0.002366723	6.40530303
GO:0048584~positive regulation of response to stimulus	11	0.002431483	3.184557439
GO:0051272~positive regulation of cell motion	7	0.003067496	4.88023088
GO:0006928~cell motion	15	0.00976124	2.157575758
GO:0051674~localization of cell	11	0.014564962	2.448063699
GO:0048870~cell motility	11	0.014564962	2.448063699
GO:0030193~regulation of blood coagulation	4	0.01525977	7.591470258

GO:0016477~cell migration	10	0.019859905	2.475479432
GO:0050818~regulation of coagulation	4	0.021612152	6.665681202

Enrichment Score: 2.8161520743916886

Term	Count	p-Value	Fold Enrichment
GO:0007389~pattern specification process	16	8.49E-06	4.094276094
GO:0043009~chordate embryonic development	15	3.55E-04	3.096218987
GO:0009792~embryonic development ending in birth or egg hatching	15	3.91E-04	3.068408637
GO:0003002~regionalization	11	6.26E-04	3.81500282
GO:0048598~embryonic morphogenesis	11	0.014564962	2.448063699
GO:0048568~embryonic organ development	7	0.040050965	2.780596664
GO:0048562~embryonic organ morphogenesis	6	0.045000207	3.082251082

Enrichment Score: 2.815711262011046

Term	Count	p-Value	Fold Enrichment
GO:0044421~extracellular region part	33	3.58E-05	2.185976368
Secreted	38	0.003264817	1.626913643
GO:0005576~extracellular region	47	0.004229514	1.486978045
GO:0005615~extracellular space	20	0.011054171	1.856701892

Enrichment Score: 2.3997545276461416

Term	Count	p-Value	Fold Enrichment
IPR001849:Pleckstrin homology	14	1.75E-04	3.537693778
GO:0005083~small GTPase regulator activity	14	2.32E-04	3.419407028
GO:0046578~regulation of Ras protein signal transduction	12	2.54E-04	3.904184704
GO:0051056~regulation of small GTPase mediated signal transduction	13	3.22E-04	3.524611191
domain:PH	12	4.76E-04	3.651874851
SM00233:PH	14	0.001584492	2.764255578
GO:0005085~guanyl-nucleotide exchange factor activity	9	0.001954895	3.96252713
GO:0030695~GTPase regulator activity	15	0.002875006	2.484752986
GO:0035023~regulation of Rho protein signal transduction	7	0.003226675	4.830935619
GO:0060589~nucleoside-triphosphatase regulator activity	15	0.003502276	2.430605826
GO:0005088~Ras guanyl-nucleotide exchange factor activity	6	0.009986195	4.562910028
IPR001331:Guanine-nucleotide dissociation stimulator, CDC24, conserved site	5	0.012355754	5.555222089
domain:DH	5	0.01381895	5.382427485
IPR000219:Dbl homology (DH) domain	5	0.016793488	5.072159299
GO:0005089~Rho guanyl-nucleotide exchange factor activity	5	0.024306741	4.521802731
guanine-nucleotide releasing factor	6	0.026147555	3.5857205
IPR011993:Pleckstrin homology-type	10	0.02978769	2.310092354
SM00325:RhoGEF	5	0.036527086	3.963244281

GO:0008624~induction of apoptosis by extracellular signals	5	0.080905988	3.0501443

Enrichment Score: 2.346017458229793

Term	Count	p-Value	Fold Enrichment
GO:0051960~regulation of nervous system development	11	5.10E-04	3.914351852
GO:0050767~regulation of neurogenesis	10	7.28E-04	4.115857369
GO:0051094~positive regulation of developmental process	13	7.79E-04	3.194971296
GO:0060284~regulation of cell development	11	8.47E-04	3.666124661
GO:0045669~positive regulation of osteoblast differentiation	4	0.005524316	10.93171717
GO:0030278~regulation of ossification	6	0.005566188	5.255633256
GO:0010720~positive regulation of cell development	5	0.018076804	4.950958864
GO:0045597~positive regulation of cell differentiation	9	0.019044253	2.685192537
GO:0045667~regulation of osteoblast differentiation	4	0.024499201	6.355649518

Enrichment Score: 2.3013516993068928

Term	Count	p-Value	Fold Enrichment
GO:0030029~actin filament-based process	12	8.14E-04	3.401986672
GO:0030036~actin cytoskeleton organization	10	0.005898867	3.023151873
GO:0007010~cytoskeleton organization	13	0.02598875	2.037160597

Enrichment Score: 2.283475252848667

Term	Count	p-Value	Fold Enrichment
GO:0070161~anchoring junction	11	3.76E-04	4.066932778
GO:0005912~adherens junction	10	7.39E-04	4.102712245
GO:0005925~focal adhesion	7	0.005287966	4.364159594
GO:0005924~cell-substrate adherens junction	7	0.006365211	4.199474326
GO:0030055~cell-substrate junction	7	0.008268576	3.974502488
GO:0030054~cell junction	15	0.033096158	1.841468334
GO:0016323~basolateral plasma membrane	8	0.040545307	2.506090238

Enrichment Score: 2.173724290687737

Term	Count	p-Value	Fold Enrichment
GO:0007610~behavior	17	0.001399909	2.476535074
GO:0042330~taxis	9	0.002366573	3.843181818
GO:0006935~chemotaxis	9	0.002366573	3.843181818
GO:0007626~locomotory behavior	11	0.006920543	2.742903487

Enrichment Score: 2.129423234158086

Term	Count	p-Value	Fold Enrichment
IPR013032:EGF-like region, conserved site	14	3.02E-04	3.344509135
IPR006209:EGF	9	4.67E-04	4.960332164

domain:EGF-like 3	7	5.95E-04	6.731622642
IPR006210:EGF-like	11	6.27E-04	3.830615828
egf-like domain	11	0.001375695	3.458401438
IPR000742:EGF-like, type 3	10	0.001888372	3.608030841
IPR001881:EGF-like calcium-binding	7	0.002485567	5.103860294
IPR018097:EGF-like calcium-binding, conserved site	7	0.002485567	5.103860294
IPR013091:EGF calcium-binding	6	0.003604467	5.832983193
SM00181:EGF	11	0.003607423	2.993136726
domain:EGF-like 1	7	0.006466255	4.207264151
domain:EGF-like 2; calcium-binding	5	0.007922815	6.326713009
SM00179:EGF_CA	7	0.007938471	3.988014558
IPR000152:EGF-type aspartate/asparagine hydroxylation conserved site	6	0.012491687	4.329637009
IPR001438:EGF-like, type 2	3	0.03235919	10.49936975
domain:EGF-like 4	4	0.046118571	4.9741054

Enrichment Score: 2.0141293128569644

Term	Count	p-Value	Fold Enrichment
IPR013320:Concanavalin A-like lectin/glucanase, subgroup	7	7.95E-04	6.363254393
IPR012680:Laminin G, subdomain 2	5	0.002925627	8.332833133
IPR001791:Laminin G	5	0.003767358	7.777310924
GO:0005604~basement membrane	6	0.00748419	4.891695369
SM00282:LamG	5	0.008781277	6.076974565
GO:0044420~extracellular matrix part	6	0.036651297	3.261130246

Enrichment Score: 1.9744594647503417

Term	Count	p-Value	Fold Enrichment
GO:0001569~patterning of blood vessels	4	0.003333802	13.01394901
GO:0035295~tube development	10	0.004968149	3.105601469
GO:0001568~blood vessel development	10	0.00974264	2.78870336
GO:0035239~tube morphogenesis	7	0.010672748	3.765847451
GO:0001944~vasculature development	10	0.011325626	2.722041128
GO:0048514~blood vessel morphogenesis	9	0.012171359	2.914261094
GO:0048754~branching morphogenesis of a tube	5	0.014802605	5.255633256
GO:0001525~angiogenesis	7	0.02122754	3.231504232
GO:0001763~morphogenesis of a branching structure	5	0.02275822	4.616434616

Enrichment Score: 1.9386769493534968

Term	Count	p-Value	Fold Enrichment
GO:0030029~actin filament-based process	12	8.14E-04	3.401986672
GO:0008092~cytoskeletal protein binding	15	0.018375913	1.991746441
actin-binding	9	0.021484022	2.634851298

Enrichment Score: 1.9019258392389724

Term	Count	p-Value	Fold Enrichment

GO:0051960~regulation of nervous system development	11	5.10E-04	3.914351852
GO:0050767~regulation of neurogenesis	10	7.28E-04	4.115857369
GO:0060284~regulation of cell development	11	8.47E-04	3.666124661
GO:0045664~regulation of neuron differentiation	8	0.003317693	4.10966811

Enrichment Score: 1.85055815540084

Term	Count	p-Value	Fold Enrichment
domain:Laminin EGF-like 5; second part	3	0.001849164	43.27471698
domain:Laminin EGF-like 5; first part	3	0.001849164	43.27471698
hsa04512:ECM-receptor interaction	7	0.003898828	4.556451613
IPR000034:Laminin B type IV	3	0.005333263	26.24842437
domain:Laminin EGF-like 11	3	0.006418131	24.04150943
GO:0005604~basement membrane	6	0.00748419	4.891695369
domain:Laminin EGF-like 8	3	0.009628554	19.6703259
domain:Laminin EGF-like 10	3	0.009628554	19.6703259
domain:Laminin EGF-like 7	3	0.011449731	18.03113208
domain:Laminin EGF-like 6	3	0.013409219	16.64412192
IPR002049:EGF-like, laminin	4	0.020371761	6.828858373
GO:0005605~basal lamina	3	0.028393742	11.22212467
domain:Laminin EGF-like 3	3	0.036613767	9.83516295
GO:0044420~extracellular matrix part	6	0.036651297	3.261130246
SM00180:EGF_Lam	4	0.037968223	5.335880106

Enrichment Score: 1.81323005685353

Term	Count	p-Value	Fold Enrichment
domain:Ig-like C2-type 2	10	0.001382704	3.776153314
Immunoglobulin domain	16	0.002307946	2.46168613
domain:Ig-like C2-type 4	6	0.004027864	5.694041708
domain:Ig-like C2-type 1	9	0.004985363	3.416425025
IPR007110:Immunoglobulin-like	16	0.005446412	2.235394757
IPR013783:Immunoglobulin-like fold	17	0.005798346	2.151769569
IPR003961:Fibronectin, type III	9	0.005967855	3.315590447
domain:Ig-like C2-type 5	5	0.006988483	6.5567753
domain:Ig-like C2-type 3	7	0.006995252	4.138292607
IPR003598:Immunoglobulin subtype 2	9	0.009265758	3.072986268
domain:Fibronectin type-III 2	7	0.009427364	3.883628447
domain:Fibronectin type-III 1	7	0.009768897	3.853982428
IPR013098:Immunoglobulin I-set	7	0.014124274	3.550511509
domain:Ig-like C2-type 6	4	0.014322249	7.797246303
IPR008957:Fibronectin, type III-like fold	8	0.0165219	3.043295579
IPR003599:Immunoglobulin subtype	11	0.020055565	2.333193277
SM00060:FN3	9	0.022468827	2.590710209
IPR013151:Immunoglobulin	8	0.026003322	2.772110825
SM00408:IGc2	9	0.033293543	2.401146048

Enrichment Score: 1.7842511270801795

Term	Count	p-Value	Fold Enrichment
IPR013320:Concanavalin A-like lectin/glucanase, subgroup	7	7.95E-04	6.363254393
IPR003129:Laminin G, thrombospondin-type, N-terminal	3	0.038621164	9.544881589
domain:TSP N-terminal	3	0.039742611	9.40754717

Enrichment Score: 1.7181082407898811

Term	Count	p-Value	Fold Enrichment
GO:0003002~regionalization	11	6.26E-04	3.81500282
GO:0014031~mesenchymal cell development	6	8.49E-04	8.038027332
GO:0048762~mesenchymal cell differentiation	6	8.49E-04	8.038027332
GO:0060485~mesenchyme development	6	9.28E-04	7.883449883
GO:0030278~regulation of ossification	6	0.005566188	5.255633256
GO:0016202~regulation of striated muscle tissue development	5	0.005956655	6.832323232
GO:0048634~regulation of muscle development	5	0.00639134	6.69835611
GO:0043588~skin development	4	0.008410758	9.423894114
GO:0009953~dorsal/ventral pattern formation	5	0.011274181	5.693602694
GO:0048598~embryonic morphogenesis	11	0.014564962	2.448063699
hsa05217:Basal cell carcinoma	5	0.016950121	4.970674487
GO:0001655~urogenital system development	6	0.022291307	3.726721763
GO:0048732~gland development	6	0.047450469	3.036588103
GO:0048729~tissue morphogenesis	7	0.048138293	2.65701459

Enrichment Score: 1.6197949360741022

Term	Count	p-Value	Fold Enrichment
GO:0007368~determination of left/right symmetry	4	0.023030922	6.506974507
GO:0009799~determination of symmetry	4	0.024499201	6.355649518
GO:0009855~determination of bilateral symmetry	4	0.024499201	6.355649518

Enrichment Score: 1.616633934012377

Term	Count	p-Value	Fold Enrichment
IPR013548:Plexin cytoplasmic region	3	0.006793003	23.33193277
domain:Sema	4	0.00880228	9.306390749
IPR001627:Semaphorin/CD100 antigen	4	0.009527202	9.031715912
IPR002165:Plexin	4	0.009527202	9.031715912
domain:IPT/TIG 1	3	0.009628554	19.6703259
domain:IPT/TIG 3	3	0.013409219	16.64412192
domain:IPT/TIG 2	3	0.013409219	16.64412192
SM00630:Sema	4	0.018250116	7.057131753
IPR003659:Plexin/semaphorin/integrin	4	0.026023285	6.221848739
SM00423:PSI	4	0.047978761	4.861579652

Enrichment Score: 1.604111877837217

Term	Count	p-Value	Fold Enrichment

domain:SEC7	3	0.020076592	13.52334906
IPR000904:SEC7-like	3	0.021210215	13.12421218
GO:0005086~ARF guanyl-nucleotide exchange factor activity	3	0.025822767	11.80988478
GO:0032012~regulation of ARF protein signal transduction	4	0.02601695	6.211202938
SM00222:Sec7	3	0.033338526	10.25489458

Enrichment Score: 1.5998985496537386

Term	Count	p-Value	Fold Enrichment
wnt signaling pathway	7	0.006911998	4.149050906
hsa04916:Melanogenesis	7	0.008661193	3.866080156
GO:0016055~Wnt receptor signaling pathway	7	0.01318657	3.595959596
hsa05217:Basal cell carcinoma	5	0.016950121	4.970674487

Enrichment Score: 1.4846557212793356

Term	Count	p-Value	Fold Enrichment
GO:0030278~regulation of ossification	6	0.005566188	5.255633256
GO:0030501~positive regulation of bone mineralization	3	0.027698896	11.38720539
GO:0070169~positive regulation of biomineral formation	3	0.030665658	10.78787879
GO:0045778~positive regulation of ossification	3	0.047213585	8.54040404

Enrichment Score: 1.4735845372267304

Term	Count	p-Value	Fold Enrichment
GO:0051051~negative regulation of transport	11	2.77E-05	5.567078189
GO:0003012~muscle system process	10	7.92E-04	4.066859067
GO:0008016~regulation of heart contraction	7	8.88E-04	6.211202938
GO:0006936~muscle contraction	9	0.001795951	4.019013666
GO:0044057~regulation of system process	13	0.001913458	2.874440195
GO:0003044~regulation of systemic arterial blood pressure mediated by a chemical signal	4	0.006877456	10.12196034
GO:0045909~positive regulation of vasodilation	3	0.012650556	17.08080808
GO:0003073~regulation of systemic arterial blood pressure	4	0.014136815	7.808369408
GO:0008217~regulation of blood pressure	6	0.015385181	4.099393939
GO:0045823~positive regulation of heart contraction	3	0.017112843	14.64069264
GO:0008015~blood circulation	8	0.019213953	2.938633648
GO:0003013~circulatory system process	8	0.019213953	2.938633648
GO:0042312~regulation of vasodilation	3	0.033752036	10.24848485
GO:0001990~regulation of systemic arterial blood pressure by hormone	3	0.033752036	10.24848485
GO:0050886~endocrine process	3	0.043688578	8.911725955
GO:0051048~negative regulation of secretion	4	0.045935065	4.968962351

Enrichment Score: 1.4163134899322325

Term	Count	p-Value	Fold Enrichment
GO:0002685~regulation of leukocyte migration	5	1.76E-04	17.08080808

GO:0001818~negative regulation of cytokine production	3	0.105536091	5.393939394
GO:0001817~regulation of cytokine production	5	0.270760594	1.887382108
GO:0009967~positive regulation of signal transduction	6	0.429714973	1.389625064

Enrichment Score: 1.4143707023002026

Term	Count	p-Value	Fold Enrichment
IPR004075:Interleukin-1 receptor, type I/Toll precursor	3	0.014177482	16.15287654
IPR000157:Toll-Interleukin receptor	3	0.041910796	9.129886737
domain:TIR	3	0.042968781	9.015566038

Enrichment Score: 1.4033707786137661

Term	Count	p-Value	Fold Enrichment
GO:0000267~cell fraction	27	0.018561221	1.585397114
GO:0005624~membrane fraction	20	0.049823455	1.572114705

Enrichment Score: 1.3823873087992529

Term	Count	p-Value	Fold Enrichment
IPR001478:PDZ/DHR/GLGF	7	0.019869037	3.28839321

Enrichment Score: 1.380715968216337

Term	Count	p-Value	Fold Enrichment
sh3 domain	8	0.02653151	2.754744003
IPR001452:Src homology-3 domain	8	0.033346815	2.628950172
domain:SH3	7	0.036345931	2.852382475

Enrichment Score: 1.35261096593115

Term	Count	p-Value	Fold Enrichment
GO:0009725~response to hormone stimulus	12	0.018637352	2.234002147
GO:0048545~response to steroid hormone stimulus	8	0.022408824	2.846801347
GO:0009719~response to endogenous stimulus	12	0.035012134	2.024392069

Enrichment Score: 1.3142716932764074

Term	Count	p-Value	Fold Enrichment
Endocytosis	6	0.010571068	4.51950188

TABLE 4.4: DAVID functional clustering of CD161+ γδ 409 genes with enrichment scores >1.3 being significant.

When the 409 genes were entered into DAVID's *disease* functional classification, to assess if the gene signatures clustered together are involved in potential disease processes downstream, the results in Table 4.5 were gained.

Term	P-Value	Genes
Coronary atherosclerosis	**0.01955573**	**IL18R1, IL18RAP, HMOX1, PECAM1, EDN1, TLR2, EPHX2, MYB, IFNGR2**
Periodontitis	**0.02911927**	**EDN1, TLR2, HSPG2, CTLA4, IL6R, TIMP2**
Cerebral malaria	0.06494346	HMOX1, PECAM1
Brugada syndrome	0.06494346	KCNH2, KCNQ1
Asthma	0.06972782	ADRB2, CYSLTR1, EDN1, MS4A1, TLR2, EPHX2, CTLA4, ALOX5, IFNGR2
Graft-versus-host disease	0.08010247	PECAM1, CTLA4, ABCB1, IFNGR2
idiopathic dilated Cardiomyopathy	0.08565381	EDN1, CTLA4

TABLE 4.5: DAVID disease classification output when 409 genes entered.

(Significant associations in bold)

Thus using DAVID, it did appear that the CD161+ γδ T-cells did indeed have a distinct genetic signature, and may have therefore distinct and important roles is key cellular and physiological processes within the body, as well as potentially in disease. The disease pathogenesis are discussed in Chapter 4.5.

FACS confirmation

In order to confirm the gene signature hits (where possible), FACS analysis was performed with commercially available labeled antibodies. One gene identified in the filtered gene list was that encoding for CCR1. CCR1, also known as CD191, is a G-coupled receptor expressed by T-cells, monocytes and dendritic cells. Its ligands include macrophage inflammatory protein 1-α (MIP-1α) and RANTES. The ligands are known to be involved in the progression of autoimmune diseases (Snowden *et al*. (1994); Karpus *et al.* (1995); Rayachaudhuri *et al.* (1999)). CCR1 is therefore a

receptor that may indeed mediate pathogenesis of such diseases. Thus, there remains interest in the development of CCR1 antagonists in clinical trials (Cheng and Jack (2008)) for treatment or prevention of many disease processes. CCR1 expression has been studied on γδ T-cells with expression confirmed (Glatzel *et al.* (2002)), but not strictly defined, in relation to CD161+/- γδ T-cell subsets.

On antibody staining and FACS analysis of 9 leucocyte cones, there were significantly higher levels of expression of CCR1 on the CD161+ γδ T-cell subset compared to the CD161- subset ($p=0.0078$). Although the differences identified using this staining approach were small they confirm the findings from the Agilent ™ array (Figure 4.5).

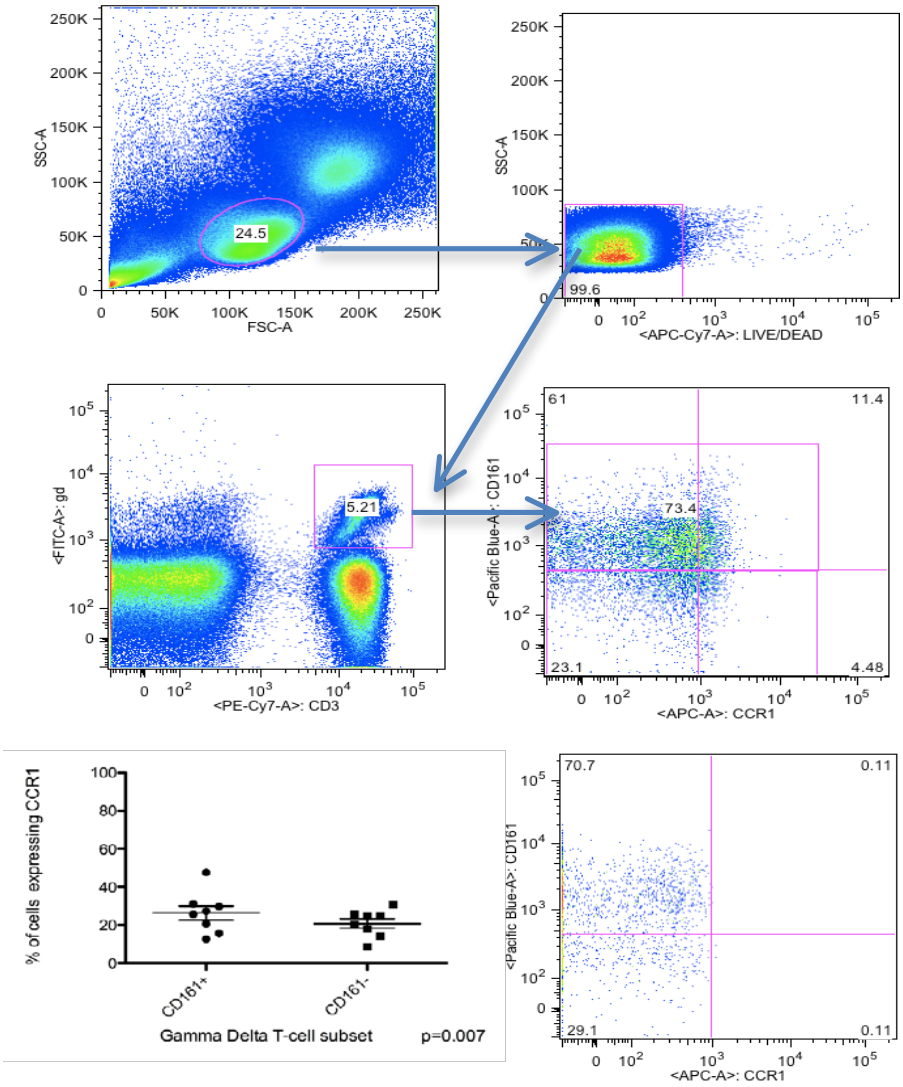


Figure 4.5: CCR1 expression on γδ T-lymphocytes. per CD161 status confirming gene array findings and also signifincantly more expression on CD161+ subset.

A FACS based experiment was performed to assess the expression of CCR1 by CD161+ compared to CD161- γδ T-lymphocytes. Eight samples were analysed with staining for Anti-CD3 PE-Cy7, Anti-CD161Pacific Blue and Anti-γδ FITC and Anti-CCR1 APC. FACS analysis was performed on the LSR-II with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test. CCR1 was significantly expressed in the CD161+ γδ T-cells compared to the negative subset.

Top left panel: lymphocyte gating
Top right panel: live/dead gating
Middle left panel: gating of γδ T-cells
Middle right panel: Expression of CCR1 per CD161 status of the γδ T-cells.
Bottom right panel: FMO for CCR1.

4.4.3 IL-18R linkage to CD161+ γδ T-cells

IL-18R is an IL-receptor and been shown by Billerbeck and colleagues (Billerbeck *et al.* (2010)) to be tightly linked to CD161 expression, with the CD8+CD161++ (bright) T-cells expressing significantly more IL-18R than the CD8+CD161 mid/dim subsets. Although the genes encoding for IL-18R chains were both removed on application of a BF corrected p-value in Chapter 4.4.1, in view of the previous tight linkage to CD161, its analysis by FACS was performed as the gene came up on the filtered list. PBMC staining was carried out, revealing that the CD3+CD161+ γδ T-cells expressed significantly greater IL-18R expression than the CD161- subset (p=0.0019 and 0.0059 respectively between whole blood and PBMCs isolated from Leucocyte cones – (Figure 4.6). This echoes the findings of CD8 αβ T-lymphocytes, and confirms the gene array analysis data by FACS methodology.

An interesting incidental feature was the pattern of FACS plot of IL-18R expression against CD161. This pattern/profile was very similar to the published data from the CD8+CD161+ αβ analysis by Billerbeck and colleagues (Billerbeck *et al.* (2010)). The data from several plots analyzing expression of CD161 on γδ T-cells against that of IL-18R is shown in Figure 4.7, with a corresponding plot from the published IL-18R staining of the CD161+ αβ populations. The αβ data suggested 2 distinct populations of IL-18R+CD161+ subsets. On comparing the γδ T-cells data in such a way, it did indeed appear there are 2 subsets of CD161+ populations based on IL-18R expression, a CD161++ (bright) and CD161+ (mid) subset of IL-18R expressing γδ T-cells – equivalent to their αβ counterparts.

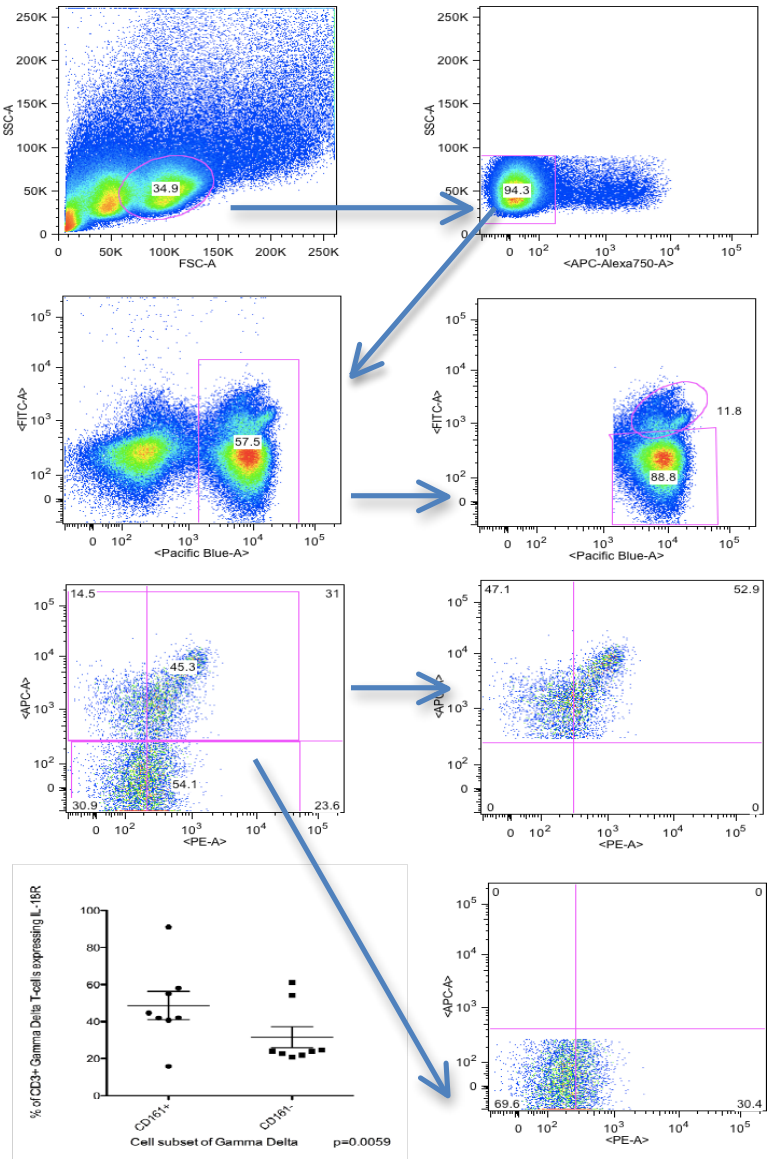


FIGURE 4.6: CD161+ γδ T-cells express IL-18R with a pattern suggestive of a bright and mid subset based on co-expression of CD161/IL-18R

A FACS based experiment was performed to assess the expression of IL-18R by CD161+ compared to CD161- γδ T-lymphocytes. Eight samples were analysed with staining for Anti-CD3 Pacific Blue, Anti-CD161APC and Anti-γδ FITC and Anti-IL18R PE. FACS analysis was performed on the LSR-II with FloJo ™ used to analyse data. Paired data was analysed with a paired t-test. IL-18R was significantly expressed in the CD161+ γδ T-cells compared to the negative subset.
Top left *panel*: lymphocyte gating
Top right *panel*: live/dead cell gate (APC-Alx 750).
2nd row left *panel*: gating on CD3 Pac Blue population
2nd row right *panel*: γδ-FITC T-cells as a % of the total CD3 population.
3rd row left *panel*: gating on CD161 APC
3rd row right *panel*: shows CD161+ γδ T-cells
Right bottom *panel*: CD161- subset of γδ T-cells expressing IL18R PE.
Left bottom *panel*: significant expression of IL-18R on the CD161+ subset

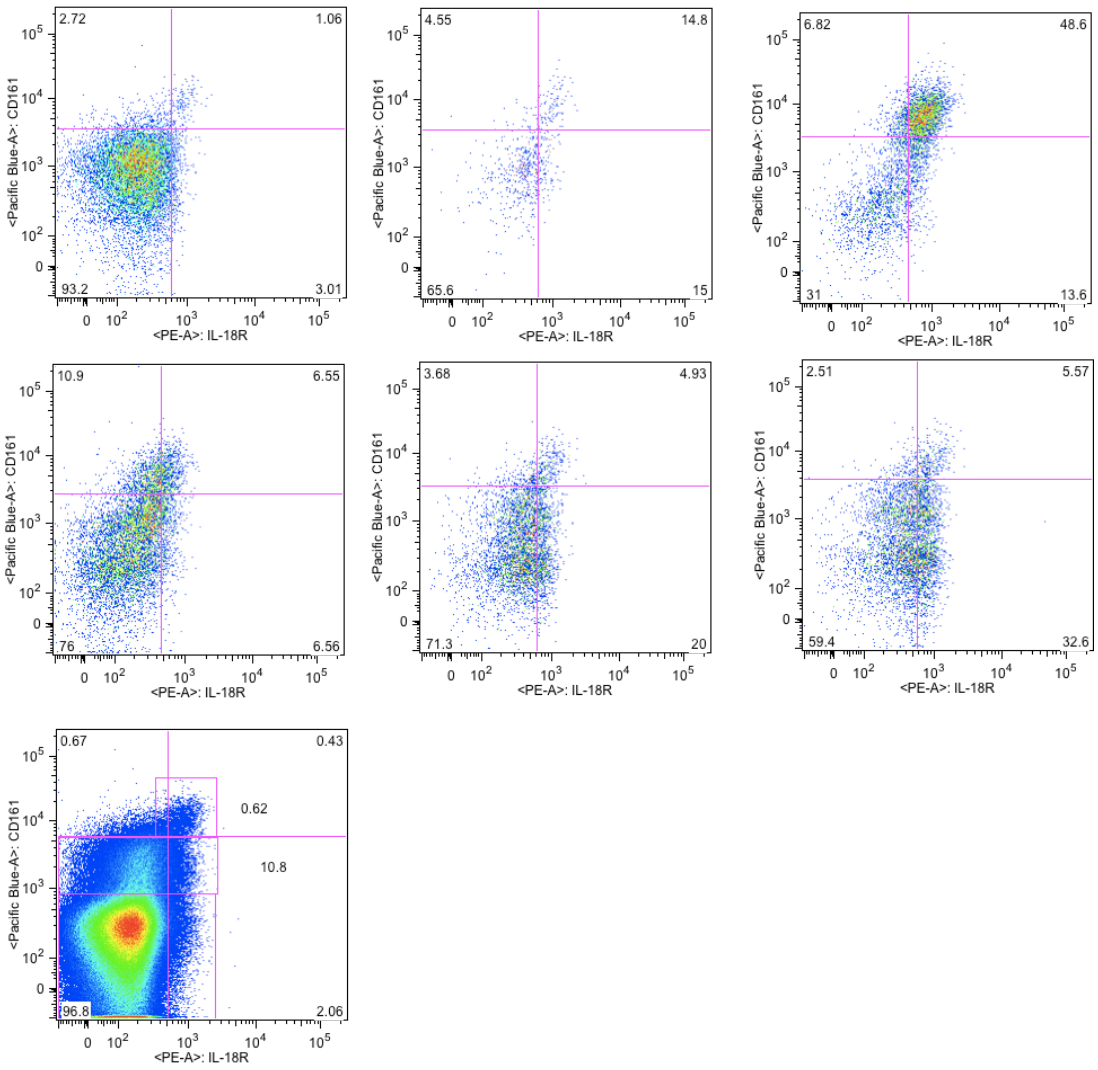


Figure 4.7: FACS plots of IL-18R expression in the CD161+ γδ T-cells in 6 different samples showing the CD161+/IL18R+ dual populations.

Top 2 rows: Suggestion of 2 distinct CD161+ subsets of γδ based on IL-18R expression.
Bottom left *panel*: shows comparative IL-18R expression per CD161 status of the αβ subset (Billerbeck, Kang *et al.* 2001).

This CD161++ vs. CD161+ staining profile was indeed suggested from the

CCR6 fresh blood PBMC stains in Chapter 3.3.2 – however was not

reproducible from staining of PBMCs isolated from frozen leucocyte cones

(data not shown). Once again the freezing/thawing process may have altered

the expression of CCR6 with a down-regulation as for the CD161 expression. Nevertheless, it remains there may indeed be subsets of CD161++ and CD161+ γδ T-cells when based on co-expression of Il-18R and CCR6. One would postulate that these CD161++/CCR6++ γδ T-cells might play a more vital role in pathogenesis aided by their ability to transmigrate. Further studies into these subsets would be warranted in case of distinct functions, as suggested by their CD161++/+ αβ counterparts.

4.4.4 Triggering of CD161+ γδ T-cells through the IL-12/IL-18 pathway

It has recently been shown from the Willberg/Klenerman group that $CD161^{++}CD8^{+}$ T cells, including the MAIT cell subset, are specifically activated by IL-12 & IL-18 in a TCR-independent manner (Ussher, Bilton, et al, 2013). The group hypothesized that given the high expression of the IL-18 receptor observed on the CD161++$CD8^{+}$T cell population (Billerbeck *et al.* (2010)), the CD161++$CD8^{+}$ T cells, including the MAIT cell subset, could be activated by cytokine stimulation with IL-12 and IL-18 – as has been reported with NK cells (Gorski *et al.* (2006)). The CD161++$CD8^{+}$ T-cell population responded to the co-stimulation (mean approx. 50%), whilst both CD16+CD8+T cell and CD161+CD4+T cell populations made limited responses (mean response ≤10%). The $CD161^{-}CD8^{+}$ and $^{-}CD4^{+}$ T-cell populations did not respond significantly. Also stimulation with either IL-12 or IL-18 alone did not induce IFN-γ expression from any T-cell subset in their studies. Also CD161 expression was also found to be up-regulated on Vδ2 cells when cultured with IL-12 (known to up-regulate CD161 (Poggi *et al.* (1998)).

To this end it was hypothesized that with the expression of IL-18R on the CD161+ γδ T-cell subset, stimulation with IL-12/18 may indeed activate them too. An ICS was performed as previously in PBMCs from leucocyte cones from healthy volunteers. The PBMCs were stained for IFN-γ secretion after co-culture with IL-12 and IL-18 as in the Ussher/Willberg study (PBMCs were stimulated for 24 hours with IL-12 (Miltenyi Biotech) and IL-18 (R&D Systems Europe) at 50 ng/ml).

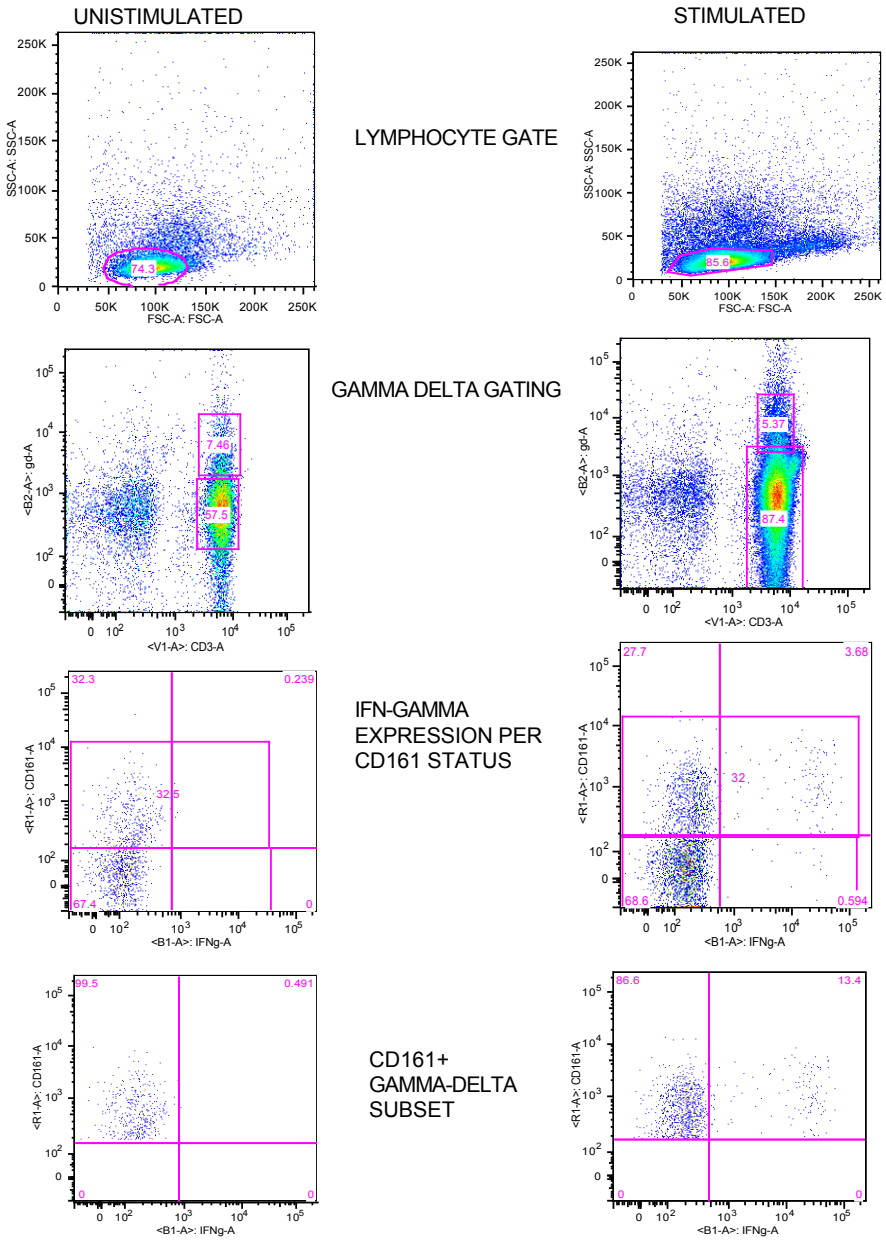


FIGURE 4.8: CD161+ γδ T-cells when co-stimulated by IL-12 and IL-18 produce IFN-γ
Left column: unstimulated cells
Right column: stimulated cells with I-12/18.

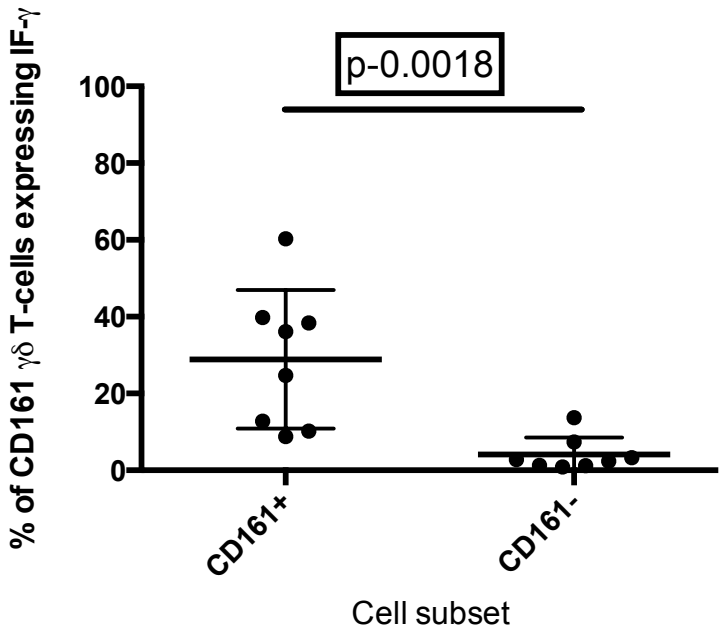


FIGURE 4.9: CD161 γδ T-cells express significantly more IFN-γ than the CD161- subset when stimulated via IL-18/12 co culture

This experiment suggests that the CD161 subset (like the CD161++ αβ T-cells and MAIT cells) are able to respond directly to the pro-inflammatory cytokines IL-12 and IL-18 to produce IFN-γ (Figure 4.8), and may be a way in which the CD161+ only subset are able to function in areas of viral, as well as bacterial infection. Also this may be a way in which the cell subset contributes to inflammation in inflammatory sites in autoimmune conditions.

4.4.5 CD161 positivity confers some similar genetic signatures to different T-lymphocyte subsets

Together with the data obtained from the CD161+ γδ T-cell subset, a similar data set was available from Fergusson J *et al* (unpublished data, 2014) whereby RNA from CD161+ vs CD161- αβ-T-cells had been run on the Agilent ™ gene array. A direct comparison of data thus was carried out

looking at the up-/down-regulated genes shared between the CD161+ γδ T-cells and the CD161+(mid) αβ T-cell subset to explore any common signatures conferred by CD161 positivity irrespective of the T-cell subtype. A similar filter method was used on the CD161+ αβ v CD161- gene list that had been generated from the Agilent ™ data earlier in Chapter 4.

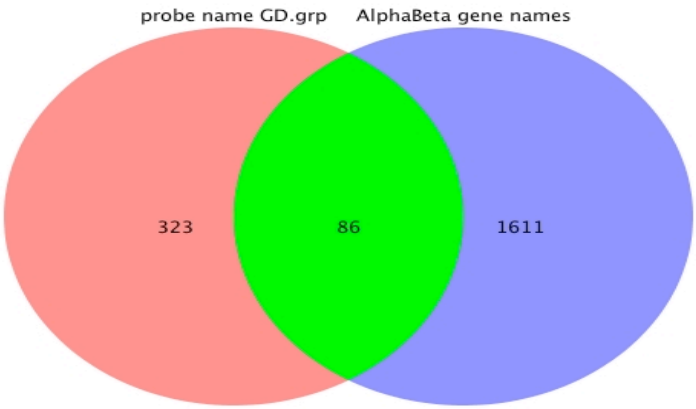


FIGURE 4.10: Venn diagram comparing common CD161+ gene signatures irrespective of γδ or αβ T-cell type

On comparison of the filtered CD161+ γδ gene list v the filtered CD161+ (mid) αβ list there were 86 shared genes (409 γδ v 1697 αβ genes analysed) with 19 up-regulated in both CD161 αβ and γδ subsets, and 67 down-regulated in both subsets. Of interest in the up-regulated genes were those encoding for CCR1 and IL-18R in both subsets. Also COLQ was a common gene – encoding for Acetylcholinesterase collagenic tail peptide.

Gene name	Fold Change γδ	p-value γδ	Fold Change αβ	p-value αβ
KLRB1	64.32418	7.24E-36	24.21392	1.27E-35
COLQ	3.56566	5.10E-07	3.10718	4.43E-09
IL18RAP	3.63652	1.00E-05	4.65393	3.16E-12
IL18RAP	3.42817	5.00E-05	3.32945	1.51E-19
BC037972	6.41639	7.00E-05	7.18212	1.00E-14
COL5A1	2.28729	8.00E-05	2.57466	4.00E-04
TLE1	2.45245	9.00E-05	2.86719	1.08E-09
ABCB1	2.40902	0.00032	2.247	0.00741
LTK	3.2452	0.00053	4.65134	4.81E-16
BLK	3.052	0.0015	4.02465	0.00553
IL18R1	2.61051	0.00195	3.13596	5.06E-22
B3GALT2	11.08968	0.00263	4.3441	0.00061
PLCB1	2.70977	0.00341	2.18196	0.00823
lincRNA:chr1:852362-859037_R	2.51008	0.00384	4.26722	0.00104
CCR1	3.87797	0.00419	3.44304	0.00025
LOC100128668	2.73951	0.00551	2.98372	0.00324
LOC619207	2.99192	0.00578	3.53604	1.00E-05
IL18R1	2.39579	0.0061	2.25129	5.00E-05
C8orf83	2.91678	0.00851	4.03277	0.00741

TABLE 4.6: The common genes up-regulated in CD161+ γδ and CD161+ (mid) αβ populations.

Gene names	Fold change γδ	p-value γδ	Fold change αβ	p-value αβ
KLF7	-14.51668	3.28E-22	-8.43959	1.71E-11
GRB10	-5.29576	2.46E-20	-2.82221	0.00053
TCEA3	-6.53897	2.95E-15	-2.65327	3.74E-08
KCTD12	-16.63801	5.28E-15	-5.97062	4.90E-08
INF2	-5.7716	6.89E-14	-2.08675	7.00E-05
NOG	-22.15534	8.46E-14	-13.36458	1.58E-09
MAL	-14.09853	7.05E-12	-2.23009	0.00028
BEND5	-15.82532	2.17E-10	-4.67489	3.12E-06
KANK1	-4.48669	2.39E-10	-3.53988	0.00216
NGFRAP1	-9.99433	8.74E-10	-3.18824	6.49E-10
FLJ45983	-6.12813	5.34E-09	-4.13991	0.00422
FBLN2	-8.43219	8.82E-09	-4.43046	0.00065
KANK1	-11.96443	1.37E-08	-4.31412	0.00049
lincRNA:chr18:68297754-68318093_R	-14.12603	1.63E-08	-6.86953	5.10E-09
SESN3	-3.78098	3.18E-07	-2.38159	0.00476
MDS2	-6.4935	3.56E-07	-3.29245	0.00907
MYB	-5.34642	4.58E-07	-4.73501	3.00E-05
lincRNA:chr14:96555696-96556712_F	-17.28899	9.54E-07	-2.82391	0.00839
FHIT	-7.01578	1.58E-06	-3.6986	0.00175
CDCA7L	-4.77938	1.98E-06	-2.59196	0.00354
DENND5A	-2.02524	3.15E-06	-2.74153	3.91E-13
EPHX2	-2.67111	3.44E-06	-2.07558	0.00145
ENST00000512129	-9.7622	3.46E-06	-4.08288	0.00271
PHGDH	-3.74009	5.87E-06	-7.98159	2.13E-11
ZWINT	-2.59923	6.17E-06	-5.01339	0.00034
HTR7P1	-2.09118	6.26E-06	-2.83546	0.00415
ACTN1	-9.01087	2.00E-05	-4.20495	5.92E-10
SGPP2	-7.34943	3.00E-05	-2.85925	0.00582
KCNH2	-4.75084	5.00E-05	-2.817	0.00098
KCTD12	-9.45734	6.00E-05	-11.22348	1.56E-23
lincRNA:chr2:74193717-74210392_R	-3.90015	6.00E-05	-2.85918	0.00253
LOC100131662	-12.52592	9.00E-05	-7.3586	1.03E-08
SLC22A23	-2.34588	0.00021	-4.22516	0.00953
LIMS2	-4.71416	0.00023	-2.83723	0.00722
ADAMTS4	-5.26849	0.00026	-2.96586	0.00576
SEMA5A	-3.6247	3.00E-04	-2.51082	0.00763
MAP3K1	-2.22641	0.00032	-2.08924	4.94E-07
ARHGEF4	-2.46205	0.00037	-2.59257	0.00025
NELL2	-3.32387	0.00039	-2.52096	0.00018
LY96	-5.2306	0.00051	-3.70914	3.00E-05

PPARGC1B	-2.01099	6.00E-04	-2.02013	0.00897
HLA-DOA	-2.27838	0.00071	-2.84276	0.00426
TMEM45B	-10.18063	0.00077	-4.08131	0.00135
ENST00000510551	-3.80026	0.00078	-3.01108	0.00056
RFPL2	-3.91816	0.00094	-5.63206	8.52E-06
TIAM1	-3.08198	0.00095	-3.09862	1.86E-06
PTPRK	-4.4411	0.00096	-2.62953	0.00175
LEF1	-4.62937	0.00103	-3.43907	2.60E-22
C2orf89	-3.33068	0.00126	-2.30036	0.00461
ENST00000419160	-3.50313	0.00169	-3.27212	0.00109
C5orf13	-5.41453	0.0019	-8.15249	3.00E-08
AEBP1	-4.21814	0.00193	-7.48813	3.33E-15
CES4A	-2.11958	0.00239	-2.20252	0.0015
LOC729680	-3.14467	0.00251	-2.5692	0.00311
ZDHHC23	-2.00819	0.00283	-2.53914	0.002
MGC15705	-2.81363	0.00302	-3.10064	0.00635
MRC2	-2.76152	0.00316	-2.13544	0.00264
PRKCA	-2.27724	0.0034	-2.10755	0.00076
lincRNA:chr11:110066285-110066727_R	-4.08899	0.00457	-2.42727	0.0052
GYPE	-3.53269	0.00478	-2.47354	0.00745
MYO1E	-6.23001	0.00533	-3.23663	0.00579
SPIN3	-3.95963	0.0057	-3.08552	0.00135
C2orf40	-2.42955	0.00701	-2.86465	0.00323
PKIA	-2.58484	0.00725	-2.23731	7.00E-05
AM408129	-4.4369	0.00754	-2.55957	0.0024
HCRP1	-2.41426	0.00769	-2.45046	0.00307
LAT2	-2.79712	0.00807	-3.45755	2.00E-05

TABLE 4.7: The common genes down-regulated in CD161+ γδ and CD161+ (mid) αβ populations.

The common up-/down- regulated genes in both lists were analysed using DAVID ™. The gene list was inserted into DAVID ™ and the functional annotation clustering used (on highest stringency setting for a tighter, cleaner analysis/clustering). Enrichment scores >1.3 (equivalent to non-log scale 0.05 or less) were taken to be significant.

When the upregulated genes were compared in DAVID, 4 common pathways on clustering had an enrichment score of 1.76 (> 1.3 so statistically significant) – with the top association being a strong association to risk

variants for coeliac disease and the immune response involved in it (Table 4.8).

Term	*p-Value*	*Genes*	*Fold Enrichment*
Newly identified genetic risk variants for celiac disease related to the immune response	3.00E-04	IL18R1, IL18RAP, CCR1	92.546218
hsa04060:Cytokine-cytokine receptor interaction	0.0345667	IL18R1, IL18RAP, CCR1	8.3178844
GO:0006952~defense response	0.0864875	IL18R1, IL18RAP, CCR1	5.4991869
GO:0006955~immune response	0.1053341	IL18R1, IL18RAP, CCR1	4.9014492

TABLE 4.8: Common genetic signatures in the upregulated genes common in the upregulated genes from the CD161+ γδ and αβ T-cells.

When the down-regulated common genes from the CD161+ γδ and αβ T-cells were compared, more clusterings were found than the upregulated genes as seen in Table 4.9.

The genes seem to be mainly focused on cell survival. It might be speculated that the survival programme of the cells is altered, although this does not indicate on its own if they have improved survival or shortened.

Enrichment Score: 2.212556027420177

Term	p-Value	Genes	Fold Enrichment
GO:0043065~positive regulation of apoptosis	0.00597401	PRKCA, ARHGEF4, TIAM1, MAP3K1, NGFRAP1, MAL	4.9674418
GO:0043068~positive regulation of programmed cell death	0.00614983	PRKCA, ARHGEF4, TIAM1, MAP3K1, NGFRAP1, MAL	4.9330254
GO:0010942~positive regulation of cell death	0.00626906	PRKCA, ARHGEF4, TIAM1, MAP3K1, NGFRAP1, MAL	4.9103448

Enrichment Score: 1.940324479607347

Term	p-Value	Genes	Fold Enrichment
GO:0000904~cell morphogenesis involved in differentiation	0.00427415	PRKCA, SEMA5A, KLF7, NOG, LEF1	7.2950819
GO:0000902~cell morphogenesis	0.01571181	PRKCA, SEMA5A, KLF7, NOG, LEF1	5.00
GO:0032989~cellular component morphogenesis	0.02248794	PRKCA, SEMA5A, KLF7, NOG, LEF1	4.4836272

Enrichment Score: 1.668485161196371

Term	p-Value	Genes	Fold Enrichment
GO:0042981~regulation of apoptosis	0.02072604	PRKCA, ARHGEF4, TIAM1, MAP3K1, ACTN1, NGFRAP1, MAL	3.0995024
GO:0043067~regulation of programmed cell death	0.02165157	PRKCA, ARHGEF4, TIAM1, MAP3K1, ACTN1, NGFRAP1, MAL	3.0689655
GO:0010941~regulation of cell death	0.02200587	PRKCA, ARHGEF4, TIAM1, MAP3K1, ACTN1, NGFRAP1, MAL	3.0576687

TABLE 4.9: Common genetic signatures in the down-regulated genes common in the up-regulated genes from the CD161+ γδ and αβ T-cells.

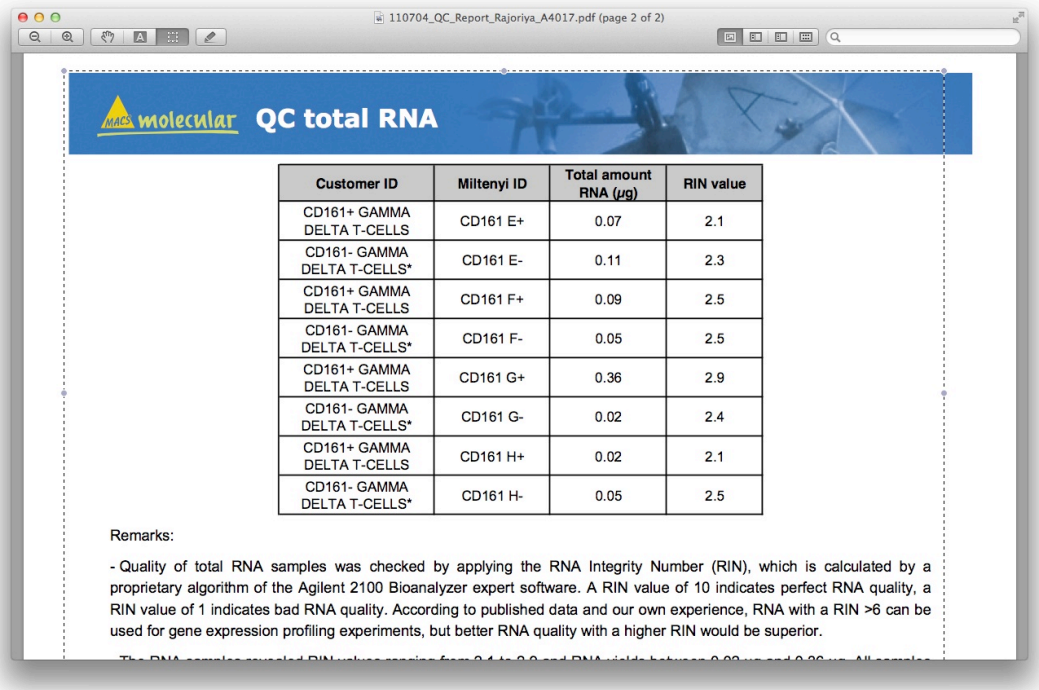
Thus it was concluded that CD161 positivity, irrespective of cell subtype, may indeed confer distinct genetic signatures to it's cell type, and these cells may be involved in disease processes such as coeliac disease, or in host defence.

4.5 Discussion

Experimental challenges

Further to the findings in Chapter 3, the aim of this chapter was to use gene array analysis to provide additional evidence for CD161+ γδ T-cells being a distinct cell subset, using different techniques from Chapter 3. After isolating CD161+ and CD161- γδ T-cells from leucocyte cones, RNA extraction was performed with the Agilent ™ gene array platform generating data for 4 chronic viral disease free- individuals. The data provided gene signatures that were up-regulated or down-regulated on the CD161+ γδ T-cells v their CD161- counterparts (i.e. a gene array comparison of ex-vivo CD161+ v CD161- γδ T-cells in health).

Initially the progress of the experiment was thwarted by the poor quality of RNA isolated after a long and complex extraction process. This was cited due to “degradation” of the RNA isolated.



110704_QC_Report_Rajoriya_A4017.pdf (page 2 of 2)

molecular **QC total RNA**

Customer ID	Miltenyi ID	Total amount RNA (µg)	RIN value
CD161+ GAMMA DELTA T-CELLS	CD161 E+	0.07	2.1
CD161- GAMMA DELTA T-CELLS*	CD161 E-	0.11	2.3
CD161+ GAMMA DELTA T-CELLS	CD161 F+	0.09	2.5
CD161- GAMMA DELTA T-CELLS*	CD161 F-	0.05	2.5
CD161+ GAMMA DELTA T-CELLS	CD161 G+	0.36	2.9
CD161- GAMMA DELTA T-CELLS*	CD161 G-	0.02	2.4
CD161+ GAMMA DELTA T-CELLS	CD161 H+	0.02	2.1
CD161- GAMMA DELTA T-CELLS*	CD161 H-	0.05	2.5

Remarks:

- Quality of total RNA samples was checked by applying the RNA Integrity Number (RIN), which is calculated by a proprietary algorithm of the Agilent 2100 Bioanalyzer expert software. A RIN value of 10 indicates perfect RNA quality, a RIN value of 1 indicates bad RNA quality. According to published data and our own experience, RNA with a RIN >6 can be used for gene expression profiling experiments, but better RNA quality with a higher RIN would be superior.

FIGURE 4.11: INITIAL REPORT OF RNA QUALITY GAINED

Thus the experiment was repeated from scratch after 6 months of optimizing.

The "optimization" stages included a few problems. Prior to gaining RNA from 4 leucocyte cones, there were problems encountered with the experiment in isolating the CD161+ subset of the γδ T-cells. A problem initially encountered was gaining sufficient γδ T-cells from samples to go on to extract the RNA. Initially this was attempted from fresh blood samples and then PBMCs from donors (data not included). However it quickly became apparent that not enough γδ T-cells would be gained to then isolate both the CD161+ and –ve subsets to allow extraction of RNA (recommended required value was 1x10 (5) cells after discussion with gene array collaborators).

Another issue was the extraction/ separation techniques of the γδ and the CD161 subsets from the total lymphocyte pool. Initially this was attempted via

the Automacs ™ separation machine to no avail (data not included), so an alternative separation machine was decided on (The FACSAria ™) – this proved fruitful. To ensure enough γδ T-cells were gained, fresh (not thawed – in view of the potential down regulation of CD161 in Chapter 3) leucocyte cones were used instead of fresh PBMCs. This provided a larger starting population of T-cells (often 1x10(9) cells). Thereafter to improve the yield, a T-cell enrichment kit was used which could process 1 x 10 (8) maximum cells in one sitting. With γδ T-cells representing 3-5% of the maximum T-cell population, we aimed to thus gain at least 1 million γδ T-cells, and, hence, (with the predominant fraction CD161+) at least 1x 10(5) CD161 cells. Thus enough cells were gained for the RNA extraction process.

Once sufficient cells were gained, RNA extraction was performed with good quality RNA gained (RIN > 6). The gene array analysis confirmed that CD161+ γδ T-cell do indeed have a distinct genetic profile compared to the CD161- subset. From the gene array analysis, 409 statistically significant genes (Appendix 2) were initially found after a filter was put in place due to errors in "undetected probes" from data generated from the Agilent ™ gene array from Miltenyi Biotech.

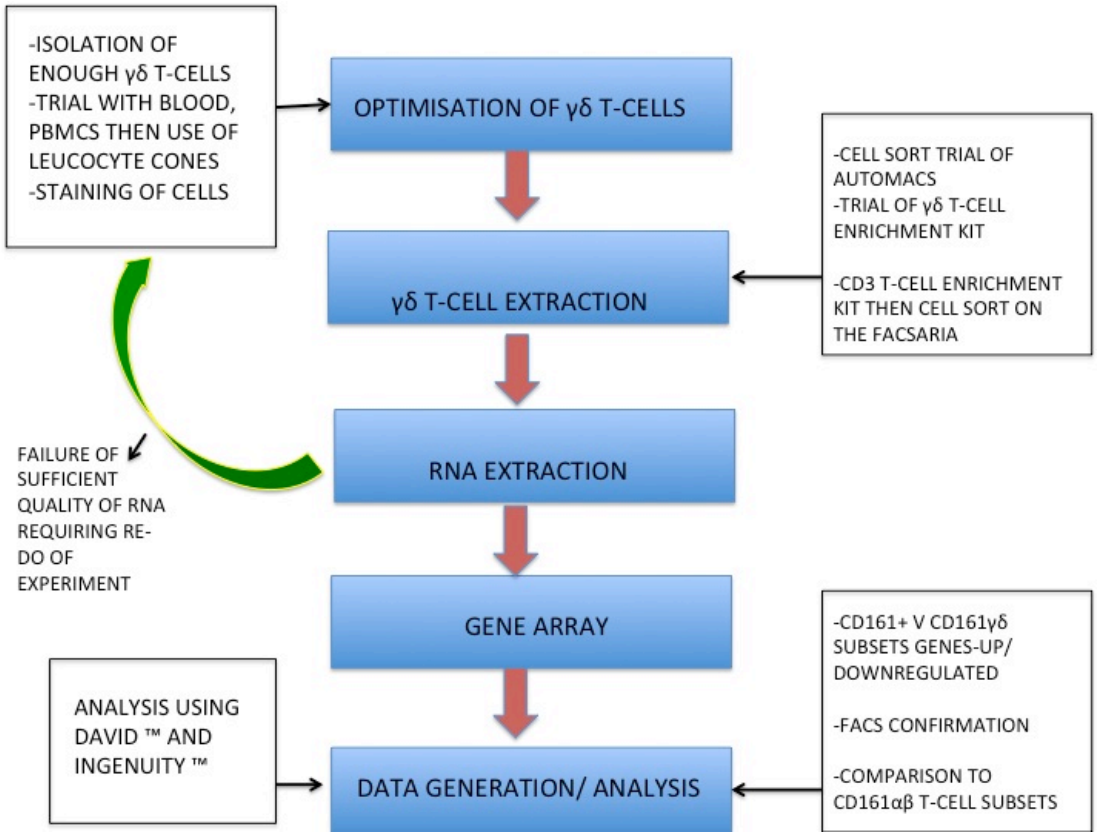


FIGURE 4.12: Summary of the experiment design, difficulties and progress

CD161+ γδ T-cells have a distinct genetic signature

Of these 409 genes gained from the experiment, 76% were down-regulated. When a Bonferroni correction was applied, the list dropped substantially to genes 53, with 89% of these being down-regulated genes on the CD161+ γδ subset (compared to CD161- γδ subset). Of note, relevant hits included CCL20 - the ligand of CCR6 (known to be preferentially expressed on CD161+ γδ T-cell subset – see Chapter 3), ADRB2- encoding for clinically important β2 receptors in the human body and KLRB1 – the gene encoding for CD161. Other up-regulated genes included: CYTH3, COLQ and MPP3. With the ever increasing interest clinically in CCR1 antagonists as an

approach for reducing neutrophil/ leucocyte migration to tissues (Gladue *et al* (2010)). CCR1 expression was confirmed by FACS. The CD161+ γδ T-cells preferentially expressed CCR1, the ligand postulated to play a role in autoimmune diseases. This may lead to a mechanism whereby γδ T-cells, specifically CD161+ subsets may be involved in autoimmunity. Thus in disease processes where γδ T-cells are involved (and maybe specifically CD161+ subsets – such as MS), CCR1 antagonism may hypothetically be an interesting approach to abrogating the effects of the CD161+ γδ T-cells.

CD161 γδ T-cell gene signatures and disease/cellular processes

The filtered gene list of 409 genes was entered into the DAVID analysis programme with some interesting findings. Looking down-stream at potential disease processes, the clustering of genes were found to be significantly linked to cardiac conditions such as atherosclerosis, and potentially also to conduction defects (Brugada syndrome) and cardiomyopathy. It has been shown that γδ T-cells are involved in the genesis of atherosclerotic lesions (Kleindienst R, *et al.* (1993)), and it follows that the CD161+ subset may be responsible given the predilection to produce pro-inflammatory cytokines (Chapter 3). Other diseases that were found to be potentially related to the clustering of genes included asthma and cerebral malaria – both inflammatory conditions with the involvement of T-cells and leucocytes, and periodontitis.

CD161 positivity confers some similar genetic signatures to different T-cell subsets

To review if CD161 positivity conferred a mechanistic signature across T-cell subtypes as suggested by the data in chapter 3, the CD161+ γδ T-cells were

compared directly to the CD161+ (mid) αβ CD8+ T-cells – both on Agilent ™ gene array platforms. There were 87 common genes – with 77% commonly down-regulated. Of the up-regulated genes, CCR1 and IL-18R genes both were featured, again expressing a close linkage of IL-18R and CCR1 with CD161. CCL20 however was not a commonly expressed up-regulated gene (being absent on the CD161αβ significant filtered list). Thus CCL20 may only be pertinent to the migration of the CD161 γδ T-cells as opposed to the CD161+αβ T-cells. When the upregulated genes were assessed using DAVID, a link to coeliac disease was found and a thus potential role for CD161 T-cells from both subsets. Again the pro-inflammatory effects may be a mechanism whereby the CD161+ T-cells (especially γδ T-cells cause villous atrophy).

The IL-18R gene was removed after the final BF correction was applied to the filtered 409 CD161+ γδ T-cell gene list. However it was studied by FACS analysis in view of the previously published tight linkage between CD161 and IL-18R (Billerbeck, Kang *et al*. 2010). Moreover, it was present in the 409 "filtered" gene list, and was a commonly expressed gene in both CD161 γδ and αβ gene lists. FACS analysis from whole blood confirmed CD161+ γδ T-cells preferentially expressed IL-18R compared to the CD161- subset. An interesting feature was the graph-plot of IL-18R expression against CD161. The pattern of expression on the IL-18R was very similar to that published from our group on the CD161+ αβ subset: with 3 specific γδ T-cell subsets based on their co-expression of CD161 and IL-18R. This may indeed suggest different functional subtypes of the γδ T-cell based on their co-expression.

Given the high expression of IL-18R by the CD161+ γδ T-cells, their effect after co-stimulation by IL-12/-18 was studied. This was following on from

data from Ussher et al (2013) as part of the Willberg/Klenerman group. They found co-stimulation of the CD161+ + (bright) αβ T-cells by IL-12/18 led to their activation; yet this was not the case following stimulation with either IL-12 or IL-18 alone. Thus, the same experiment was done with co-culture of IL-12 and IL-18 of mixed cell populations, then an ICS for IFN-γ, gating on the γδ T-cell subset. The CD161+ subset clearly preferentially expressed IFN-γ compared to the CD161- subset. Therefore, it seems likely that it is the CD161+ subsets (of both ab and γδ cells) which are the T cell populations involved in reaction to pro-inflammatory cytokines, producing IFN-γ in response to triggering with IL-12 and IL-18 in a manner analogous to NK cell triggering. This implicates this cell population potentially in a range of infectious and non-infectious inflammatory diseases. *It also remains possible that there may be subsets of CD161++ and CD161+ γδ T-cells when based on expression of CD161 and co-expression of IL-18R and CCR6.* One would postulate that these CD161+ γδ T-cells may play a more vital role in pathogenesis aided by their ability to transmigrate. Further studies into these subsets would be warranted in case of varying function as suggested by their CD161++/+ αβ counterparts.

Another interesting area was the common finding of the upregulation of the COLQ gene in both CD161+ αβ and γδ T-cells. This gene encodes for Acetylcholinesterase collagenic tail peptide. Mutations in this gene have been associated with end-plate acetylcholine esterase deficiency at the neuromuscular junction (Ishigaki *et al.* (2003)), with enzyme deficiency implicated in Myasthenia Gravis (MG). CD161+ γδ T-cells may play a role in neurological conditions such as MS (see Chapter 1.4). However, directly

extrapolating to a link between CD161+ γδ T-cells and Myaesthenia Gravis in view of the up-regulation of COLQ (based on mRNA expression) is difficult at this juncture. We propose that further investigation would focus initially on whether such a receptor was expressed at the protein level on T cells using FACS or western blot.

The data in this chapter has indeed confirmed CD161+ γδ T-cells to be a unique subset – with a distinct response to innate cytokine triggering - and provided more of an insight into the potential roles they may have in health and disease.

4.6 Chapter summary

From the gene array analysis of CD161+ γδ T-cells the following observations were made:

- CD161+ γδ have a distinct genetic profile compared to CD161- subsets.

-The majority of genes on CD161+ γδ T-cells were down-regulated ones compared to the CD161- subset.

-KLRB1, IL18R, CCR1, CCL20 and ADRB2 were important genes with potential biological effect up-regulated on CD161+ γδ T-cells

-When using DAVID some important cellular pathways highlighted from the genes found in the CD161 γδ included: cellular adhesion, chemotaxis, cellular defence. When the same gene list was compared to disease processes, cardiac disease (e.g. atherosclerosis) was an interesting area, seen to be common to the genes up/down-regulated.

- When comparing the CD161+ γδ T-cell and αβ T-cell commonly expressed genes, there were 87 shared genes, with 77% down-regulated common genes. Of the up-regulated genes on both subsets, IL-18R, CCR1 (and KLRB1) were important genes.

- CD161+γδ expressed on FACS analysis significantly more IL-18R than CD161- subsets.

-CD161+ γδ T-cells are significantly activated by IL-12/18 compared to the CD161- subset, suggesting a potential pathway for their activation in diseases.

-The profile of expression of IL18R on CD161+ γδ T-cells, suggested two distinct populations (CD161++ (bright) and CD161+ (mid) γδ T-cell when gating used as per published CD161+αβ expression of IL-18R.

- There were some commonalities found when the gene expression shared between CD161+ αβ and γδ T-cells were explored using DAVID. These included a potential link of both in coeliac disease and immune defence.

CHAPTER 5: The impact of chronic liver disease on CD161+ γδ T-cells

5.1 Introduction

Chronic liver disease can occur for a number of reasons, with common causes including viral hepatitis, alcohol, fatty liver disease (NAFLD/NASH) and autoimmune disease/PSC/PBC. The spectrum of liver diseases resulting from these conditions can vary from acute insults such as an acute alcoholic/ viral / autoimmune hepatitis or an acute drug induced liver injury, to chronic illnesses such as chronic hepatitis B/C or PSC/PBC. When a chronic insult continues, scarring (fibrosis) of the liver occurs and eventually cirrhosis. Severe fibrosis and cirrhosis can lead to the development of portal hypertension (although the development of cirrhosis is not a pre-requisite), which may result in portosystemic collaterals in the form of varices, ascites formation and hepatic encephalopathy.

The liver is strategically positioned within the body. It receives blood supply from the GI tract - through the portal venous system. It is thus in a position to scan for microbial invasion from the gut – a prime site of γδ T-cells (predominantly the Vδ1 population). Primary or secondary bacterial infections are common in cirrhotic patients (Fernandez *et al.* (2002); Borzio *et al.* (2001)) due to bacterial translocation into the portal system from impaired mucosal integrity and also impaired immune function. Antibiotics have been found to reduce bacterial infections, reduce recurrent variceal bleeding and improve mortality in patients bleeding from oesophageal varices (Soares-Weiser *et al. (*2003); (Hou MC *et al.* 2004) (Garcio-Tsao *et al.* 2007).

The normal intrahepatic lymphocyte populations include: αβ T-cells, NK cells,

NKT cells and γδ T-cells. The role of γδ T-cells has been studied in the liver (Chapter 1.2), with up to 15% of intrahepatic T-cells in the liver being γδ T-cells (Mehal *et al.* (2001)). It is worth noting that based on published data it is apparent that γδ T-cells, depending on the disease process, have different roles to play in chronic liver disease conditions – not only within the liver but also in the periphery (Figure 5.1). With respect to γδ T-cells in disease pathogenesis, Tseng and colleagues (Tseng *et al. (*2001) concluded that the γδ T-cells expanded from liver biopsies (from patients with chronic HCV or HBV) may indeed be pathogenic, producing high levels of IFN-γ and TNF-α. Production of these cytokines preferentially by the CD161+ γδ T-cells have been described in Chapter 3 in the peripheral blood setting. Agrati and colleagues (Agrati *et al.* (2001)) noted that the main population in chronic HCV infection were indeed the Vδ1 γδ T-cell subset, expressing a Th1 phenotype and may well have contributed to necro-inflammation. This finding was echoed by Yonekura and colleagues (Yonekura *et al.* (2000)) who found the γδ T-cells correlated with higher necro-inflammatory scores.

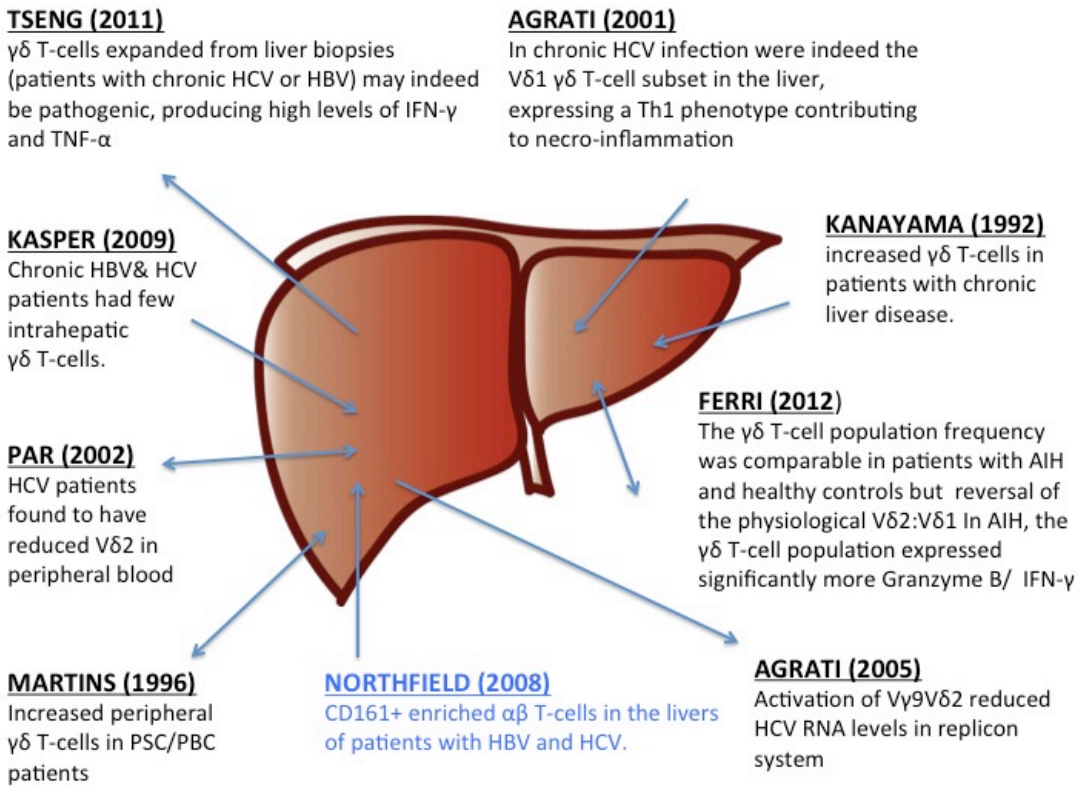


FIGURE 5.1: Summary of published data of the role of γδ T-cells and the liver.

In blue the study from Northfield et al (2008) highlighting the link between CD161+ cells and the liver, serving a platform for this chapter's work.

The subset of γδ T-cells in liver disease however may be important. In chronic HCV, Par and colleagues (Par *et al.* (2002)) found there was a decreased percentage of Vδ2 cells compared to controls or to those who had cleared HCV infection (with treatment). Agrati and colleagues (Agrati *et al*. (2006)) analysed the antiviral functions of the Vδ2 T-cells on Huh7 hepatoma cells with the subgenomic HCV replicon. Activation of the Vδ2 cells was associated with a marked reduction of HCV RNA levels. The neutralization of IFN-γ by antibodies led to clear loss of inhibition of HCV replication. The inhibition of HCV was furthered by the use of Aminobisphosphonates that

activate γδ T-cells – thus providing a potential future therapeutic strategy, although one requiring further studies.

Ferri and colleagues (Ferri *et al.* (2010)) explored γδ T-cell populations in patients with AIH and observed that, despite comparable numbers of γδ T-cells between the PBMCs of patients with AIH (irrespective of disease state-active vs. remission) and healthy control groups, there was a reversal of the physiological Vδ2:Vδ1 ratio with more Vδ1 cells found in the periphery ($p=0.001$). In patients with AIH, the γδ T-cell population expressed significantly more Granzyme B (with higher levels in patients with active vs. remission of AIH). The number of Vδ1 cells producing IFN-γ was significantly higher in patients with AIH compared to healthy controls. Moreover there were no significant differences in IFN-γ production by Vδ2 cells when AIH was compared to healthy controls. Thus one could propose the normal sentinel epithelial Vδ1 population may indeed be the subset more involved with liver disease pathogenesis, perhaprs via migration in the portal circulation.

To our knowledge there has been no assessment specifically of CD161+ γδ T-cells in chronic liver diseases after Northfield and colleagues' (Northfield *et al.* (2008)) findings of CD161+ enriched αβ T-cells in the livers of patients with HBV and HCV.

5.2 Aims

Following on from the findings in Chapters 3 and 4 that the CD161+ population of γδ T-cells are a distinct subset, the aims of this chapter were:

1. To explore the impact of chronic liver disease on the CD161+ subset of γδ T-cells.
2. To identify any differences in frequency or phenotype of the CD161+ between viral liver disease and other causes.
3. To explore if the CD161+ γδ subset, as a distinct population within the liver were enriched in chronic viral liver diseases like their αβ counterparts.

5.3: Materials and Methods

A FACS-based approach was used as in Chapter 3, with blood, PBMCs (fresh and frozen) and intra-hepatic T-lymphocytes used to study the γδ populations in different chronic liver disease states. A well-characterized cohort of patients from the John Radcliffe Hepatology/ Viral Hepatitis clinics were used, that had consented for their samples to undergo immune study. These samples were from chronically infected patients prior to treatment (to correct for confounders of potential treatment). Frozen PBMCs from patients with PSC/PBC were also studied with a thawing protocol adopted as in Chapter 2. The treatment histories of the PSC/PBC patients were not included in these analyses; however it was not envisaged that standard of care treatment in these patients would specifically affect the γδ populations. No "overlap" PBC/AIH patients were used incase of the confounder of steroid therapy. The intrahepatic lymphocytes from patients having liver biopsies for clinical

indications were isolated (detailed in Chapter 2) and used. This study was set-up as part of this DPhil (REC 09/HO306/19). All samples were stained with required Ab panels, live/dead markers – if appropriate, and processed in the FACSCalibur ™ or the LSR-II™ FACS machines. A similar antibody panel used per Chapter 3 and 4 were used apart from the additional antibody of Anti CD3 Pacific Blue (Biolegend) and a Live/Dead marker on APC-Cy7 (BD Biosciences).

5.4 Results

5.4.1 Chronic liver disease/ infection impacts upon *total* γδ T-cells in frequency.

Frozen PBMCs were thawed from patients with chronic HCV (n=8), chronic HBV (n=9), PSC (n=6) and PBC (n= 5). Healthy control data (n=8) was taken from Chapter 3. A gating strategy was used as per Figure 3.1, Chapter 3 (with live dead marker and γδ/αβ populations taken as % of total CD3 population). Using this gating strategy, the % of CD161+ γδ T-cells was measured from each of the disease states (as was the % of CD161 αβ T-cells for comparison). As comparing the % populations of CD161 T-cells was one of the aims of this Chapter, initially a full assessment of *total* CD3 T-cell populations was made in the different disease states. All the samples for this experiment thus were reflecting the peripheral cell T-populations.

There was a significant reduction in the total CD3+ T-cell γδ populations in HCV and HBV when compared to healthy controls (p=0.0006/0.0002 respectively). There was no significant difference between healthy controls and PSC (p=0.16). There was however a significant reduction in PBC

compared to healthy controls (p=0.002) – Figure 5.2.

Disease	CD3+ γδ (% of total T-cell population)	CD3+αβ (% of total T-cell population)
Healthy controls	4.75(+/-2.78)	94(+/3.35)
PSC	5.1(+/-7.97)	93.14(+/-8.30)
PBC	0.81(+/-0.68)	96.1(+/-3.16)
HCV	1.58(+/-0.89)	93.65(+/-3.18)
HBV	0.80(+/-0.33)	95.85(+/-1.09)

TABLE 5.1: Summary of αβ/γδ populations in chronic liver disease patients studied with the CD161 γδ column highlighted.

All data expressed as mean% (+/-SD).

* as a % of T-lymphocytes

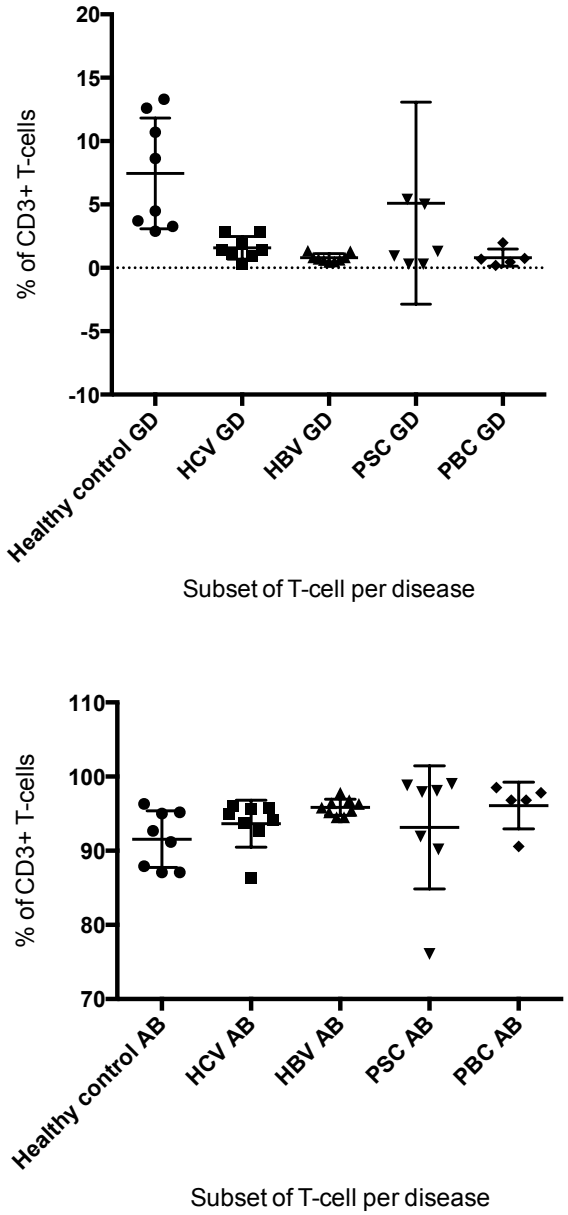


FIGURE 5.2: Breakdown of total peripheral CD3+ T-cell populations per disease.

A FACS based experiment with staining for the different T-cell subsets was performed of PBMCs. Cells were stained for CD3 and γδ, and thus the proportion αβ and γδ T-cell populations could be calculated. Cells were also stained with a live/dead marker (as from frozen specimens). The disease specimens included samples from patients with HCV (n=8), HBV (n=9), PSC (n=7) and PBC (n=5) along with healthy controls (n=8). Statistical analysis is shown in Table 5.2 and 5.3. Graphs show % of CD3+ T-cell subsets in the periphery of PBMCs isloted from blood. Populations were not divided into CD161 subsets in this experiment.

When comparing the 2 viral aetiologies there was a significant reduction of the total γδ populations in HBV compared to HCV (p=0.02). There were no significant reductions when comparing the 2 viral hepatitis conditions with PSC or PBC (See Table 5.2).

Comparison	Condition where *reduced* γδ T-cell populations	p-value
Healthy v HCV	HCV	0.0006
Healthy v HBV	HBV	0.0002
Healthy v PSC	Healthy	0.164
Healthy v PBC	PBC	0.002
HBV v HCV	HBV	0.02
HCV v PSC	HCV	0.9
HCV v PBC	PBC	0.12
HBV v PSC	HBV	0.3
HBV v PBC	Equivalent	0.7

TABLE 5.2: Comparison of the CD3+ γδ populations per disease.

p-values expressed after Mann-Whitney tests. All samples taken from frozen PBMCs.

Comparison	Condition where *reduced* αβ T-cell populations	p value
Healthy v HCV	Healthy	0.37
Healthy v HBV	Healthy	0.001
Healthy v PSC	Equivalent	0.22
Healthy v PBC	Healthy	0.03
HBV v HCV	HCV	0.054
HCV v PSC	Equivalent	0.53
HCV v PBC	HCV	0.07
HBV v PSC	Equivalent	0.68
HBV v PBC	Equivalent	0.20

TABLE 5.3: Comparison of the CD3+ αβ populations per disease.

p-values expressed after Mann-Whitney tests. All samples taken from frozen PBMCs.

Thus from this initial data, it would appear that chronic viral illnesses leads to a specific reduction in total CD3 γδ T-cells; however this appeared not to be the case in the αβ T-cell population. Also in patients with PBC there was a reduction of the CD3 γδ T-cells in the periphery, contrary to the published data to date (Martins *et al*. (1996)).

5.4.2 Chronic liver disease/ infection impacts upon the frequency of circulating *CD161+ γδ T-cells.*

Using the same experiment and data as Chapter 5.3.1, the CD161+ T-cell subsets were specifically analysed thereafter to assess the impact of liver diseases on the CD161 subsets of γδ T-cells.

Disease	**CD3+ γδ***	**CD3+ αβ***	***CD161+ γδ****	**CD161+ αβ*****
Healthy controls	4.75(+/-2.78)	94(+/-3.35)	***59.8 (+/-5.28)***	20.5 (+/-5.6)
PSC	5.1(+/-7.97)	93.14(+/-8.30)	***81.48 (+/-10.46)***	21.7 (+/-12.53)
PBC	0.81(+/-0.68)	96.1(+/-3.16)	***73.5 (+/-14.6)***	16.92 (+/-6.16)
HCV	1.58(+/-0.89)	93.65(+/-3.18)	***22.02 (+/-13.11)***	11.02 (+/-6.27)
HBV	0.80(+/-0.33)	95.85(+/-1.09)	***39.21 (+/-29.51)***	16.44 (+/-5.66)

TABLE 5.4: Summary of αβ/γδ populations in chronic liver disease patients.

CD161 γδ T-cell column highlighted. All data expressed as mean (+/-SD).

* as a % of T-lymphocytes

** as a % of CD3+ γδ T-cells

*** as a % of CD3% αβ T-cells

On analysis of the CD161+ subsets (See Figure 5.2), compared to the healthy control leucocyte population, there was a significant increase in the CD161+ γδ T-cell subsets in patients with PSC and PBC. This was despite an overall reduction in total γδ T-cell populations in patients with PBC, and equivalent γδ T-cell population when healthy controls compared to PSC (Chapter 5.3.1) Thus as a fraction of the total γδ T cell population, the CD161+ subset appeared to be enriched in the periphery in patients with PSC/PBC.

There was also a significant reduction (P=0.02) in patients CD161+ γδ T-cells with chronic hepatitis C (in keeping with an overall γδ T-cell reduction – however the CD161+ subset taken as a fraction/% of the total of the γδ T-cells). There was however no significant reduction of the CD161+ γδ subset in chronic HBV (p=0.61).

The CD161+ αβ T-cell subsets were analyzed in a similar fashion for comparison. There was a significant reduction in the CD161+ αβ T-cells as a % of the CD3+ αβ T-cells in patients with chronic HCV *and HBV* compared to healthy controls (p=0.002 and 0.02 respectively). In PSC/PBC there was no significant variations between the CD161 αβ subsets compared to the viral hepatitides or healthy controls (Table 5.6).

This data suggests that in the periphery, chronic HCV and HBV infection not only impacts on the total CD3+ γδ T-cell populations, but also as a % of the γδ T-cells the CD161+ γδ T-cell subsets reduce specifically in chronic HCV - however not in HBV. Also in PBC there was on overall reduction in the γδ T-cell frequencies, however an increase in the CD161+ γδ T-cell subset. In PSC there was an increase in CD161+ subset of γδ T-cells, but no statistically

significant reduction in the total CD3 γδ T-cell populations.

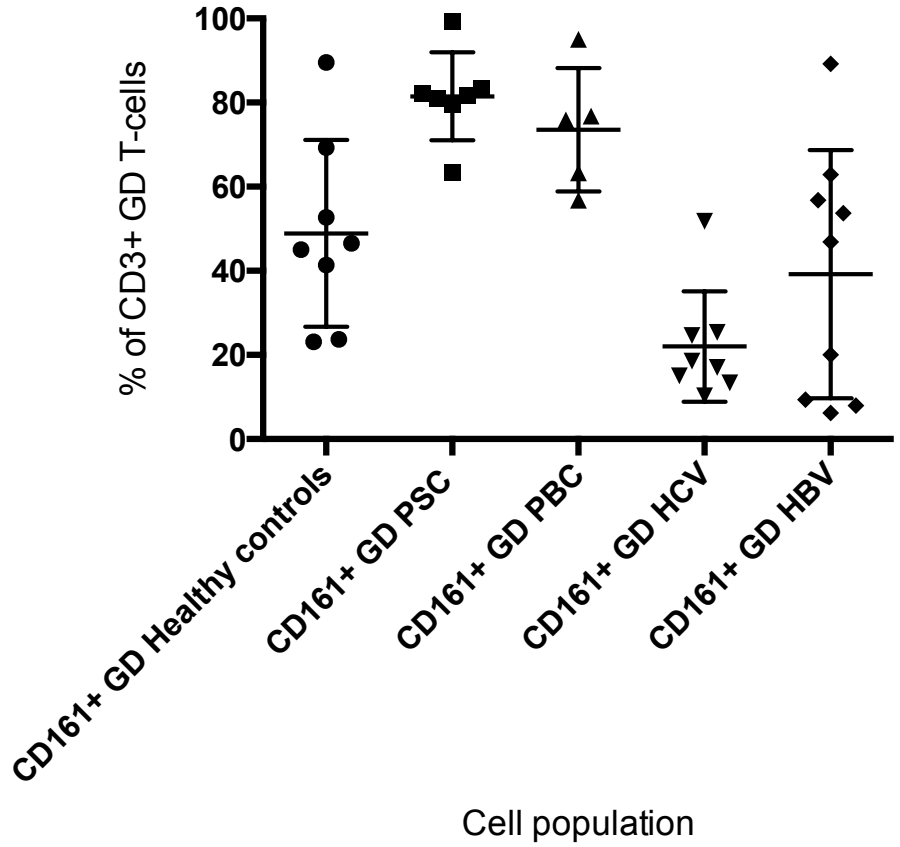


Figure 5.3: Breakdown of CD161 γδ T-cell subsets per chronic liver disease aetiology.

Using a FACS based experiment, the populations of CD161+ γδ T-cells were explored in the periphery of patients with chronic liver disease. Compared to healthy controls there was a significant reduction in the HCV group ($p=0.02$ but not in HBV ($p=0.61$). In PSC and PBS there was a significant increase in the CD161+ subset (0.04 and 0.01 respectively) compared to healthy controls. All statistical tests comparing populations in this experiment were using Mann-Whitney test.

Comparison	Condition where ***increased*** CD161+ γδ T-cell populations	p value
Healthy v HCV	**Healthy**	**0.02**
Healthy v HBV	Equivalent	0.61
Healthy v PSC	**PSC**	**0.04**
Healthy v PBC	**PBC**	**0.01**
HBV v HCV	HBV	0.48
HCV v PSC	**PSC**	**0.003**
HCV v PBC	**PBC**	**0.002**
HBV v PSC	**PSC**	**0.005**
HBV v PBC	**PBC**	**0.03**

Table 5.5: Comparison of CD161 γδ subsets per disease state.

Data shows significant reduction in chronic HCV when compared to healthy controls. Also significant increase in CD161+ γδ T-cells in PSC/PBC when compared to healthy controls or the viral hepatitides. All p-values expressed after Mann-Whitney tests.

Comparison	Condition where ***increased*** CD161+ αβ T-cell populations	p value
Healthy v HCV	**Healthy**	**0.002**
Healthy v HBV	**Healthy**	**0.02**
Healthy v PSC	Equivalent	0.69
Healthy v PBC	Healthy	0.06
HBV v HCV	HBV	0.14
HCV v PSC	PSC	0.12
HCV v PBC	PBC	0.22
HBV v PSC	Equivalent	0.61
HBV v PBC	Equivalent	0.61

TABLE 5.6: Breakdown of differences in CD161+ αβ T-cells by chronic liver disease state.

Data shows significant reduction in chronic HCV and HBV when compared to healthy controls (p=0.002 and 0.02 respectively). All p-values expressed after Mann-Whitney tests.

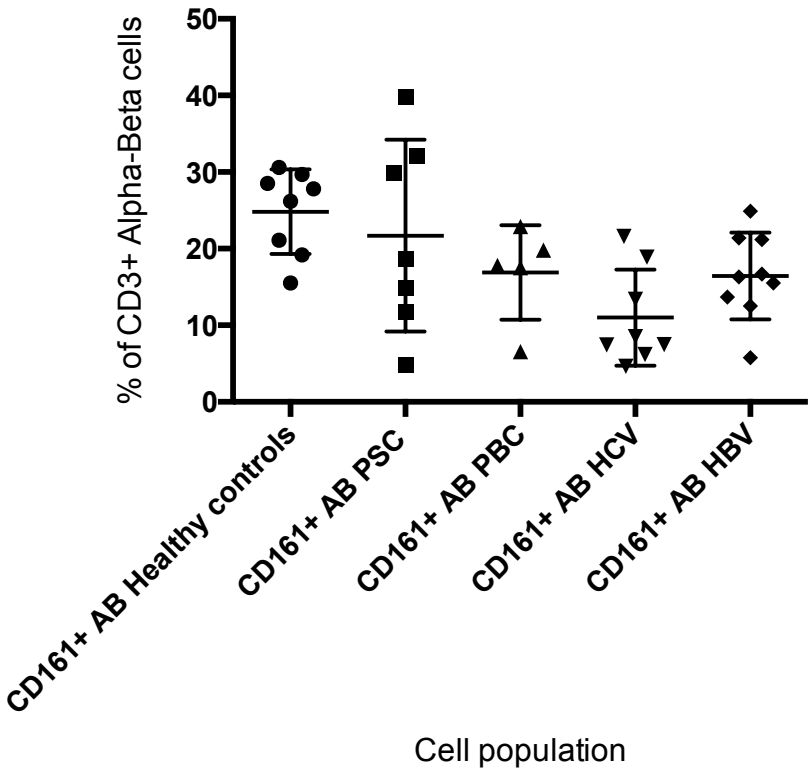


FIGURE 5.4: Breakdown of differences in CD161+ αβ T-cells per chronic liver disease state.

Using a FACS based experiment, the populations of CD161+ αβ T-cells were explored in the periphery of patients with chronic liver disease Data shows significant reduction in chronic HCV and HBV when compared to healthy controls (p=0.002 and 0.02 respectively). A Mann whitney test was used for statistical analysis.

Thus in summary this experiment showed that in chronic HCV infection the CD161+ γδ T-cells were the main subset altered / attenuated potentially by HCV infection. This led to the hypothesis that they may be enriched in viral HCV in the liver. This was not the case however for chronic HBV infection.

In patients with PSC and PBC it appeared the CD161+γδ T-cells were the

subset that were enriched/expanded in the periphery in these disease states (as a % of the total γδ population).

5.4.3 Phenotypic analysis of CD161+ γδ T-cells in chronic HCV infection

In view of the marked reduction in the CD161+ γδ T-cell subset (as a% of the total γδ T-cell population) in chronic HCV infection (p=0.02), yet preserved % in HBV (compared to healthy controls – p=0.61), a set of whole blood phenotyping experiments were set up (as per Chapter 3.4.2) to examine for any major phenotypic differences between the CD161+ subset in healthy controls and chronic HCV infection. The following markers/chemokine receptors were studied: CD4, CD8, CD38, CD69, CD28, CD45RA/RO, CD85j, CD103, CCR6, CXCR3, CXCR4 and CXCR6.

Total CD3+γδ T-cells expressed significantly more CXCR6, CD38 and CD69 in HCV infection (p=0.01 respectively) compared to the γδ T-cell populations in healthy controls. This finding suggested these cells are more activated in HCV and in the face of infection develop the ability to express a key chemokine involved in liver homing (CXCR6). The cells did not express CXCR4 or CD103.

On CD161 subset examination (Figure 5.4), HCV infection led to a significant up-regulation of NKG2D expression on the CD161+ γδ subset (p=0.015) when compared to the CD161- γδ subset. The expression of NKG2D in the CD161+ subset was not significantly different from the CD161- subset when this analysis was performed in HBV (p=0.183). There was also a significant

reduction in the expression of CXCR6 on the CD161+ subset (compared to the CD161-) in HCV infection (p=0.003). This staining was repeated in chronic HBV however we were unable to gain a good staining pattern. There was also a significant reduction in the expression of CD45RA (p=0.02) in HCV. There was a non-significant increase in the expression of CD8 and CD28 in the CD161- subset (p=0.08, 0.19 respectively) in HCV.

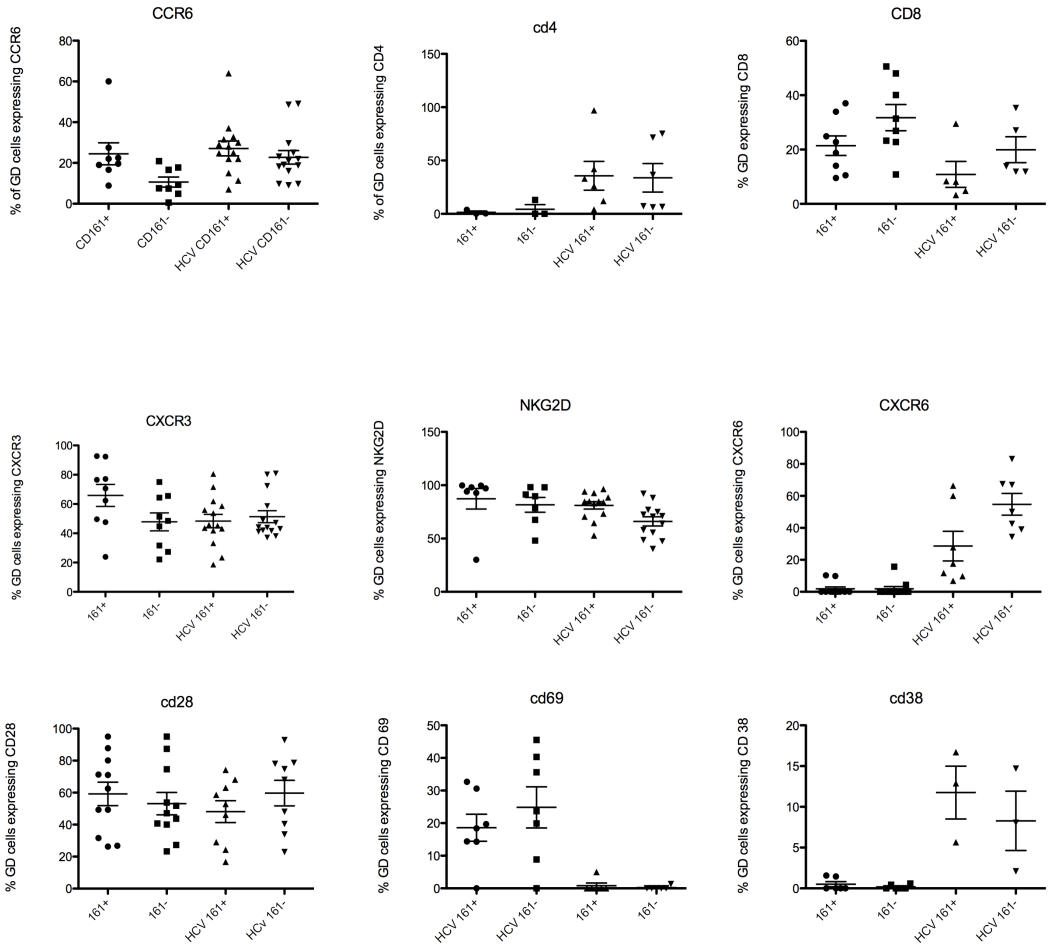


FIGURE 5.5: CD161+ γδ expression comparing healthy controls and chronic HCV infection

There was increased expression of CXCR6, CD38 and CD69 in HCV infection (p=0.01 respectively). A paired t-test was performed.

5.4.4 CD161+ expression on Vδ1 and Vδ2 γδ T-cells differs in chronic liver disease

With the established data suggesting that in chronic HCV the Vδ2 cell fraction of γδ T-cells drops compared to controls or to those who have cleared HCV infection (with treatment) (Par *et al.* (2002)), and that Vδ1 T-cells are the major γδ T-cell subset in the livers of HCV infected patients (Agrati *et al.* (2001)), we sought to explore if there were any differences in the CD161 expression per Vδ subsets of γδ T-cells and if this potentially differed per chronic liver disease. In Chapter 3, in healthy controls there was significantly greater expression of Vδ2 on CD161+ γδ T-cells than Vδ1 ($p=0.0035$), whereas there was no significant difference between expression of Vδ2 or Vδ1 on the CD161- subsets ($p=0.45$)(Figure 5.6).

Healthy controls compared to Hepatitis C

In Healthy controls (HC) there is significantly more expression of the Vδ2 chain compared to Vδ1 ($p=0.0035$) in CD161+ cells γδ T-cells. Using similar whole blood staining methods, in *chronic HCV* CD161+ γδ T-cells indeed still expressed significantly more Vδ2 than Vδ1 ($p=0.0012$). The data from these experiments are shown in Figures 5.6.

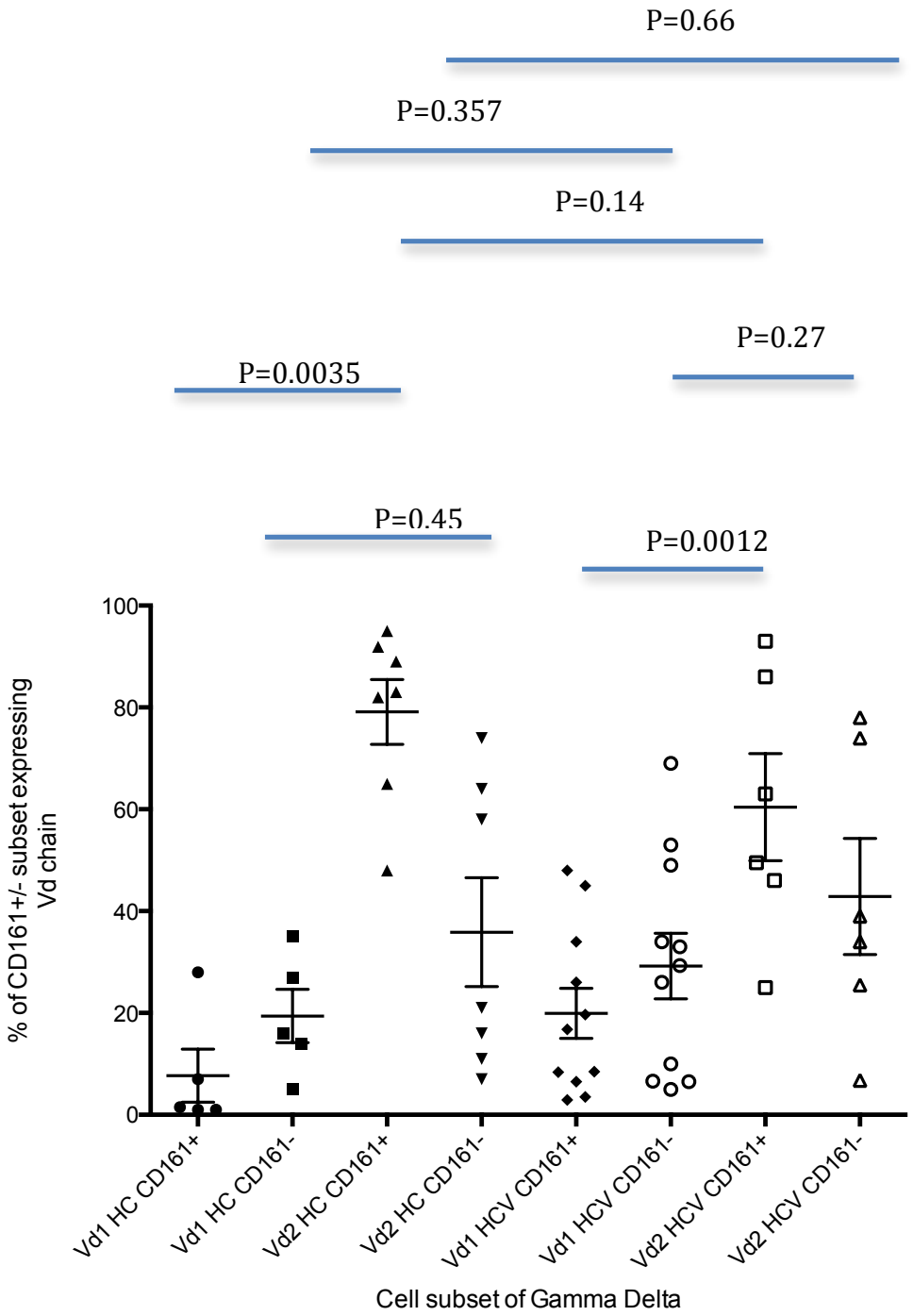


FIGURE 5.6: Expression of the Vδ1/2 chains in health and chronic HCV in blood.

In Healthy controls (HC) there is significantly more expression of the Vδ2 chain compared to Vδ1 (p=0.0035) in CD161+ γδ T-cells. In CD161- population the expression of the Vδ1/2 chains is not significantly different (p=0.45). In chronic HCV infection, the Vδ2 expression is still the predominant in the CD161+ subset (p=0.0012) with no difference in expression of the Vδ1 and Vδ2 on the CD161- subset (p=0.27).

In HCV there was an increase in the expression of Vδ1 on CD161+ γδ T-cells (p=0.04). This was not the case in the CD161- subset expressions of the Vδ1 between healthy controls (HC) and chronic HCV (p=0.357). There was a non-significant reduction of the Vδ2 expression in the CD161+ subset. A unpaired t-test (Mann Whitney was performed)

When comparing the healthy controls to HCV, in HCV there was a significant rise in the expression of Vδ1 in the CD161+ γδ T-cells (p=0.04), however this was not the case in the CD161- γδ T-cell subset (p=0.357). Thus in patients with chronic HCV infection, it is the CD161+ γδ T-cells that display a shift in the expression of their Vδ chain expression, with an enrichment of Vδ1 γδ T-cells making up the CD161 subset – a finding not occurring in the CD161- subset in chronic HCV infection. This can be described as a "physiological reversal".

CD161 expression in Vδ1 subset per disease

The expression of CD161 per actual Vδ subsets was analysed per disease (and compared to healthy controls (HC)). Firstly the expression of CD161 on the Vδ1 subset was studied.

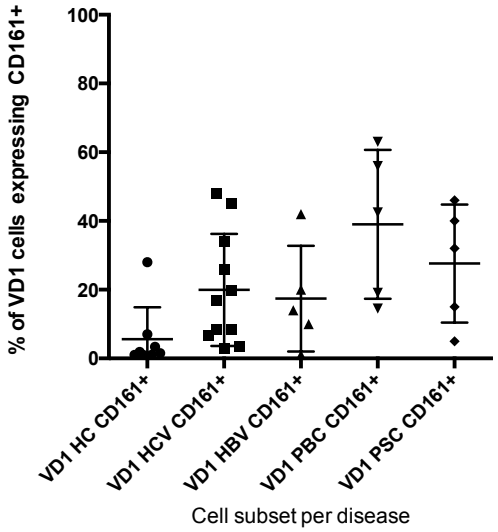


FIGURE 5.7: of the Vδ1 T-cells expressing CD161 per disease.

p-values per diseases seen in Table 5.7

When comparing the expression of CD161 between the Vδ1 subsets of γδ T-cells, there were some differences per disease. There was – as indicated above - an increase in expression of CD161 in HCV (p=0.04), PSC (p=0.01) and PBC (p=0.002) when compared to healthy controls.

Disease comparison	**Disease where more CD161 expressing Vδ1 T-cells**	**p-value**
Healthy control v HCV	HCV	0.04
Healthy control v HBV	No difference	0.109
Healthy control v PSC	PSC	0.01
Healthy control v PBC	PBC	0.002
HBV v HCV	No difference	0.77
HBV v PSC	No difference	0.35
HBV v PBC	No difference	0.106
HCV v PSC	No difference	0.4
HCV v PBC	PBC (ns)	0.07
PSC v PBC	No difference	0.38

TABLE 5.7: Analysis of CD161+ expression per Vδ1 subset per diseases.

In the Vδ1 subsets, *CD161-* γδ T-cells were significantly reduced in HCV when compared to all disease conditions apart from healthy controls. Also the *CD161-* subsets were significantly increased in HBV, PSC and PBC when compared to healthy controls ($p<0.001$, 0.0005 and 0.0005 respectively).

CD161 expression in Vδ2 subset per disease

The expression of CD161 on the Vδ2 subsets per disease were studied in a similar fashion.

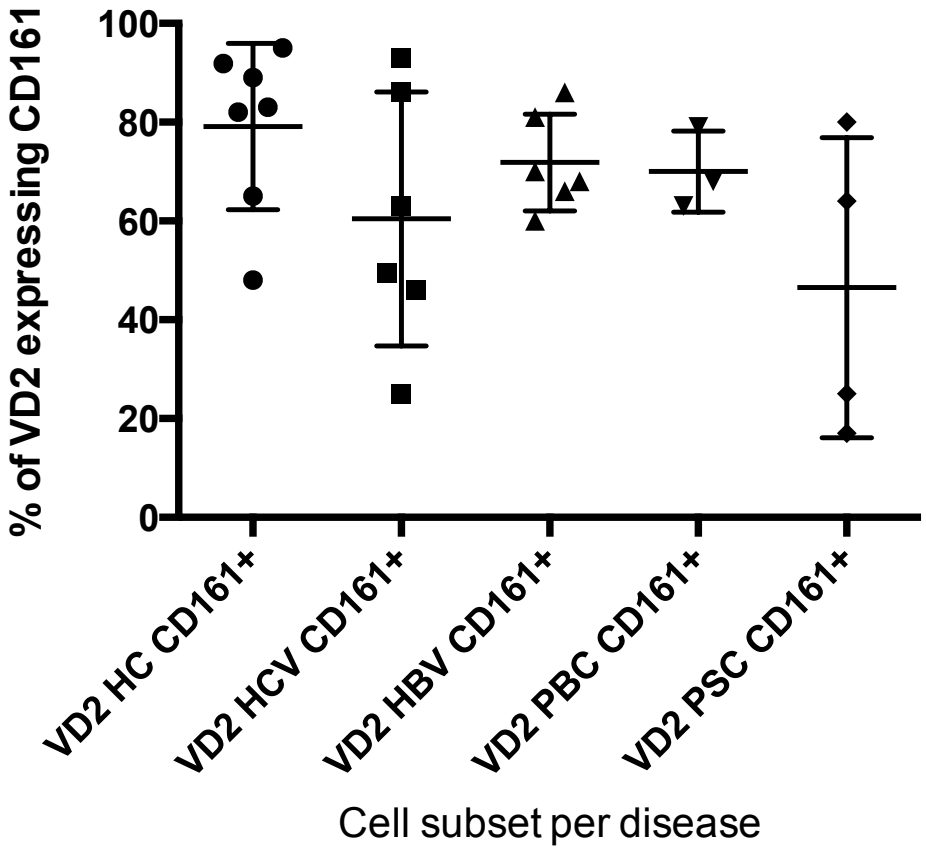


FIGURE 5.8: % of the Vδ2 T-cells expressing CD161 per disease.

p-values in Table 5.8

When comparing the expression of CD161 in the Vδ2 subsets, the only significant finding was the reduction in expression in PSC patients when compared to healthy controls (p=0.04)

Disease comparison	**Disease where more CD161 expressing Vδ2 T-cells**	**p-value**
Healthy control v HCV	No difference	0.14
Healthy control v HBV	No difference	0.37
Healthy control v PSC	Healthy controls	0.04
Healthy control v PBC	No difference	0.41
HBV v HCV	No difference	0.33
HBV v PSC	No difference	0.08
HBV v PBC	No difference	0.78
HCV v PSC	No difference	0.45
HCV v PBC	No difference	0.56
PSC v PBC	No difference	0.26

TABLE 5.8: Comparison of Vδ2 T-cells that were CD161+ per disease

The CD161- T-cells were studied in a similar fashion There was no significant differences in the Vδ2 γδ T-cells CD161- T cell subsets per disease.

Thus in summary, there were differences seen in the expression of the Vδ chains on the CD161 subset, and differences in the expression of CD161 per Vδ subsets depending on chronic liver disease aetiology (see Chapter 5 Discussion). The change in expression of CD161 seemed to be more applicable in the face of chronic liver disease to the Vδ1 subset. Overall an increase in CD161 expression on Vδ1 cells was noted in both infectious and

hepatitis and cholestatic liver diseases in blood.

5.4.5 Intrahepatic analysis of CD161 γδ T-cells in chronic liver disease

The aims of this experiment was to assess if CD161+ γδ T-cells were the predominant population of γδ T-cells localizing to the liver from the periphery, and to explore if there were any differences in the population sizes dependent on aetiology of liver disease. Paired liver biopsy and blood samples were gained from patients undergoing a liver biopsy for clinical indications (via collaboration with Professor Thimme's group, Freiburg, Germany and via a local study set up in Oxford as part of this DPhil). PBMCs were freshly isolated from the blood of the patients, and then IHLs were isolated from the liver biopsy samples gained (see Chapter 2 for methodology).

Paired biopsies from a total of 24 patients were gained from a sabbatical in Freiburg. Fifteen patients entered the local study in Oxford, having a liver biopsy and blood taken for PBMC isolation, however only paired data could be gathered for 5 patients (i.e. 33.3% success rate). This was due to either problems with isolation of the cells, staining, FACS processing, and in 1 patient kidney was biopsied rather than liver! This was reported as an adverse event by the clinical team dealing with the patient as occurred on both passes on the liver needle rather than only on the 2nd pass for the research tissue.

Patient (disease)	% of CD3 cells are γδ	% of CD3 cells are αβ	% of CD3+ γδ cells are CD161+	% of CD3+ αβ are CD161+
1 (iatrogenic)	62	38	56	57
2 (HBV)	5.34	94.1	74.6	57.4
3 (HCV)	10	90	50.2	42
4 (HCV)	9	91	55	46
5 (HCV)	9.4	90	84	66
6 (HCV)	0.27	.35	57	20
7 (NAFLD)	3.95	96	50	39
8 (HBV)	8.89	91.1	63	58.9
9 (HBV)	14.7	85	36	21.2
10 (HBV)	10.7	89.3	62.3	47.7
11 (NAFLD)	4.7	94.8	61.3	47
12 (Wilsons)	21.1	76.7	87	49
13 (NAFLD)	14.6	85.2	33.6	41.6
14 (NAFLD)	25	73	80	51
15 (NAFLD)	2.2	97.8	50.8	97.6
16 (GVHD)	5.45	94.5	19.9	20.4
17 (HCV)	16.1	83.9	34.1	66.3
18 (HCV)	12.5	87.2	67.8	48.8
19 (HCV)	18.3	81.5	82.4	51.5
20 (HCV)	6.69	93.1	43	41.6
21 (HCV)	9.16	86.7	90	65
22 (HCV)	8.34	91.6	55.6	48.1
23 (HCV)	7.39	91	46	54.5
24 (HCV)	6.6	93.2	42.2	39.6
25 (ALD)	3.52	94.6	67	22
26 (NAFLD)	11.8	87.6	23.9	15
27 (NAFLD)	7.07	91.1	61	34.5
28 (NAFLD)	3.87	85.5	40	21
29 (HCV)	2.3	89	42	34.1

Table 5.9: Cell populations gained from Liver biopsy specimens

Patient (disease)	% of CD3 cells are γδ	% of CD3 cells are αβ	% of CD3+ γδ cells are CD161+	% of CD3+ αβ are CD161+
1 (iatrogenic)	4.1	95.9	76	23
2 (HBV)	2.4	97	77	27
3 (HCV)	5.65	94	55	21
4 (HCV)	1.9	98	41	18.4
5 (HCV)	4	96	65	19.6
6 (HCV)	1	99	40	18
7 (NAFLD)	0.6	99	45	17
8 (HBV)	2.62	97.4	68	26
9 (HBV)	4.41	95.4	19.3	10.8
10 (HBV)	8.4	91.6	42.4	23.6
11 (NAFLD)	3.12	96.8	47.6	16.2
12 (Wilsons)	7.3	92.7	7.57	17.3
13 (NAFLD)	3.2	96.8	27.5	17
14 (NAFLD)	8.8	91.2	73	23
15 (NAFLD)	0.46	99.5	14.7	13.1
16 (GVHD)	0.55	99.1	14.7	13.1
17 (HCV)	11.2	88.8	70.4	26.6
18 (HCV)	2.5	97.3	40.7	15
19 (HCV)	6.98	93.1	85.7	21
20 (HCV)	3	97	85.7	21
21 (HCV) KH	3.7	96.3	54	14
22 (HCV)	3	97	45.1	23.7
23 (HCV)	2.06	97.6	51.2	20.4
24 (HCV)	5.5	94.5	54.4	21.9
25 (ALD)	5.52	93.5	68.6	46.2
26 (NAFLD)	7.17	92.5	5.41	14.5
27(NAFLD)	0.35	98.5	61	20
28 (NAFLD)	1.31	98.6	30.5	14.4
29 (HCV)	1.86	96	39	32

Table 5.10: Paired T-cell populations for PBMCs isolated from blood of patients having liver biopsy.

On analysis, there was enrichment of the CD3+ γδ T-cells in the liver compared to the periphery (p=0.001), most marked in viral conditions (HBV and HCV p<0.0001). However there was a small but significant reduction in the liver of CD3+αβ T-cells compared to the periphery (Figure 5.9) (p=0.037). There was a significant enrichment in the liver compared to the periphery of T-cells in patients with NASH/NAFLD too (p=0.01).

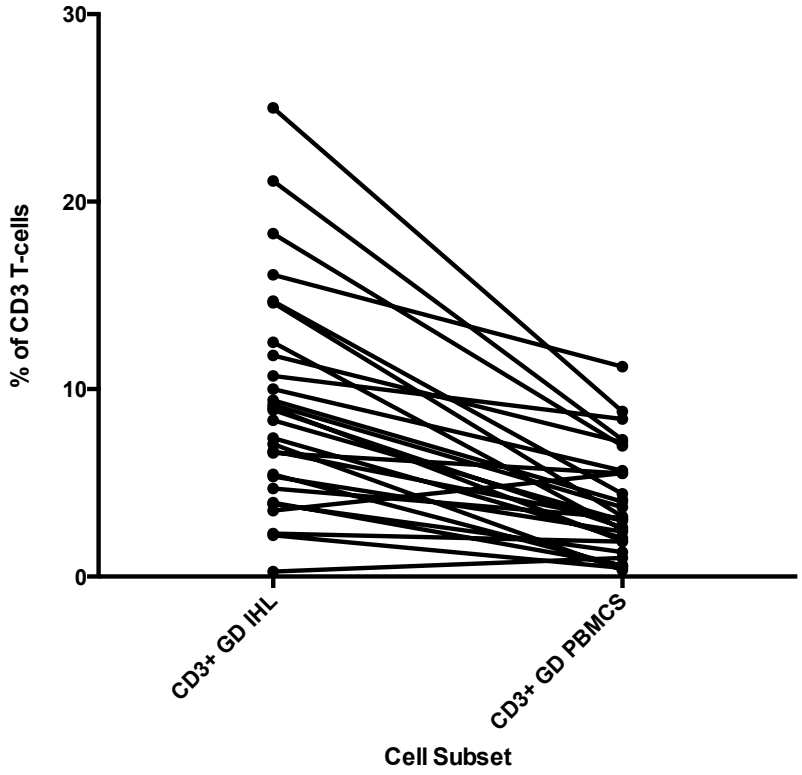


FIGURE 5.9: Enrichment of γδ T-cells into the liver from the periphery (p=0.001) in patients with chronic liver disease.

No such enrichment was seen in the αβ T-cell population (which reduced in frequency (p=0.037)). The patient with iatrogenic liver injury (drug-induced (patient 1 from Table 5.9/5.10) although kept in analysis, removed from above graph as out-with the set margins.

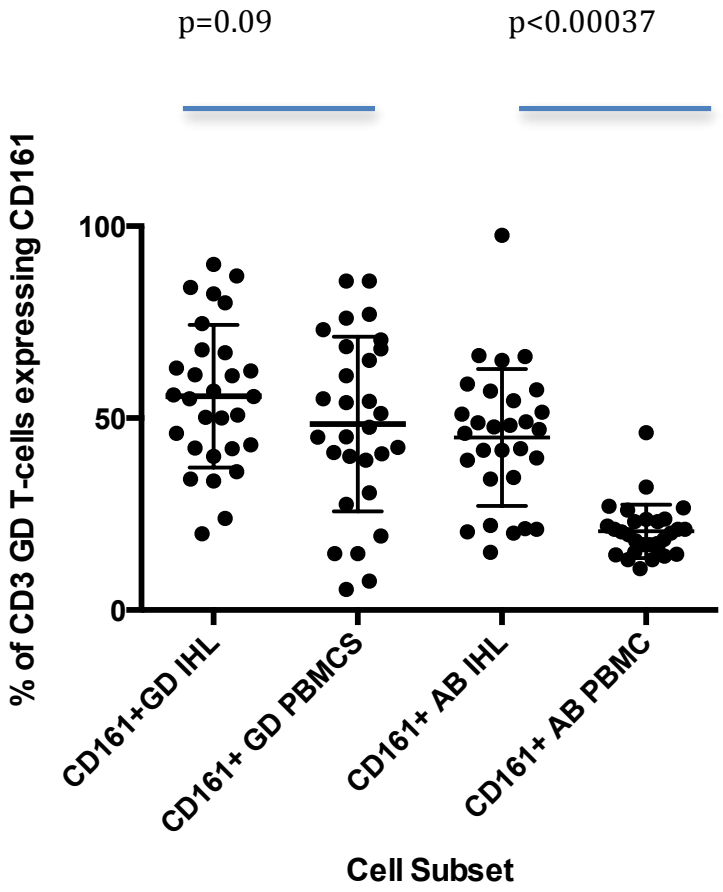


FIGURE 5.10: Enrichment of γδ T-cells into the liver was not specific to CD161+γδ T-cells from the periphery (p=0.09) in patients with chronic liver disease compared to an enrichment of the CD161+ αβ T-cells (p<0.00037)

The intrahepatic enrichment of γδ T-cells was not however confined to the CD161+ γδ T-cell subset (p=0.09); there was enrichment in the CD161αβ T-cell population (p<0.00037) – Figure 5.10.

To explore if this lack of CD161+ γδ T-cell enrichment was disease specific,

we subdivided the whole group of CD161+ γδ subsets from the liver and periphery by type of chronic liver disease. The sub-divisions were HBV, HCV, NAFLD/NASH. In view of the small numbers and heterogeneity of the other conditions (ranging from alcoholic liver disease, Graft versus host disease (GVHD) to Wilson's disease), this group were not grouped together and analysed.

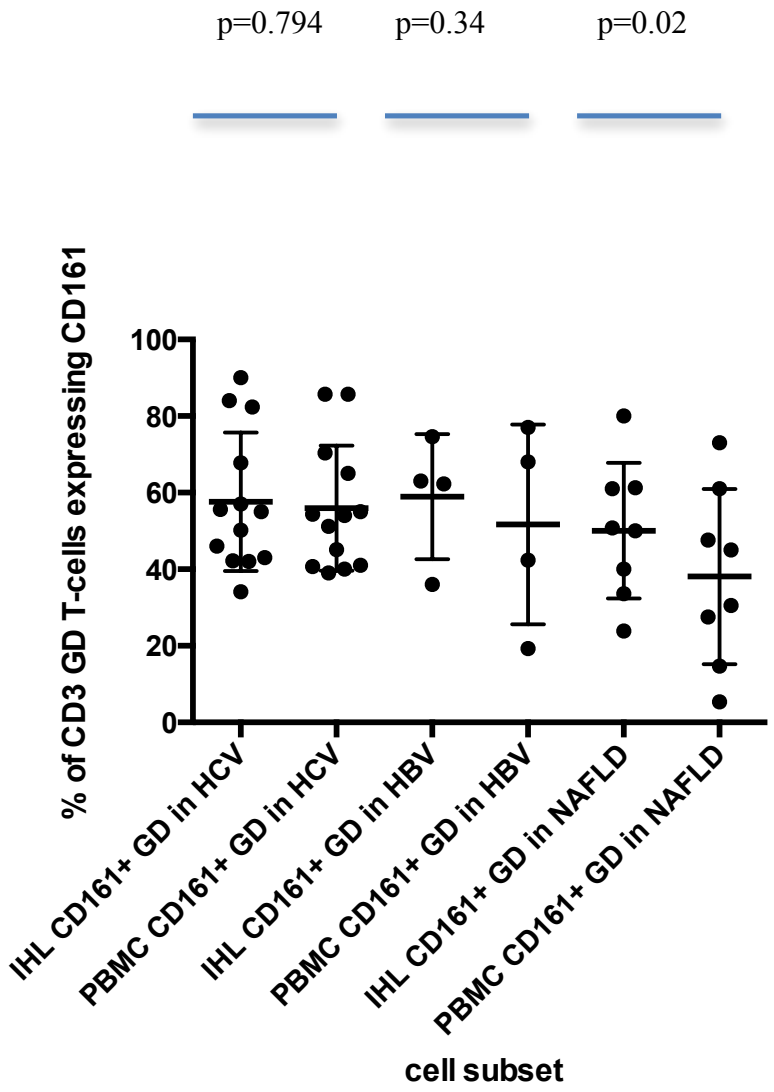


FIGURE 5.11: CD161γδ T-cells were only enriched from the periphery into the liver significantly in patients with NAFLD (p=0.02)

In HCV there was no significant enrichment of the CD161+γδ T-cells from

periphery to liver (p=0.794). In HBV this was true also (p=0.34). However in patients with NAFLD/NASH there was an enrichment of the CD161+ γδ subset into the liver from the periphery (p=0.02). In view of this finding, we checked the CD161 αβ populations too.

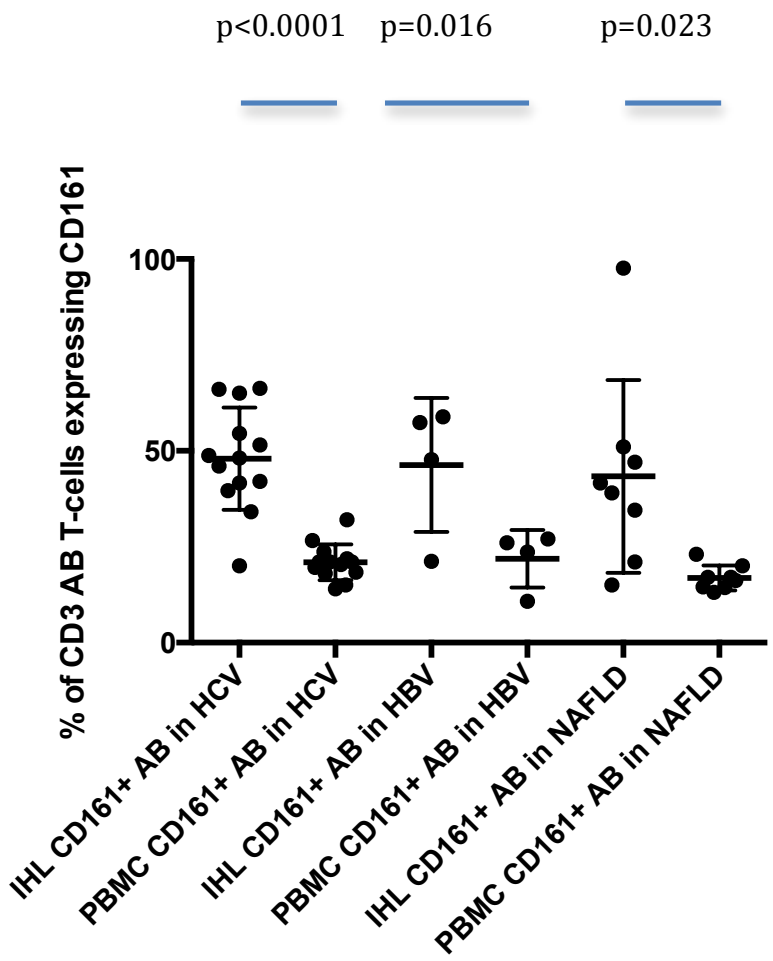


FIGURE 5.12: CD161αβ populations showing enrichment in all disease into the liver

There was a definite intrahepatic enrichment in the CD161 αβ population as described before in HCV, but also in the chronic HBV (p=0.016) and in the patients with NAFLD (p=0.023).

Thus in summary, there was an enrichment of γδ T-cells (unlike the αβ T-

cells) in patients with chronic liver disease - marked in viral conditions (HBV and HCV $p<0.0001$). This enrichment however was not confined to the CD161 subset however. When observing for any enrichment in the CD161+γδ T-cell subsets per disease, the only enrichment was seen in patients with NAFLD/NASH.

5.4.6 Expression of CD161+ γδ T-cells in Hepatocellular carcinoma

Via our collaboration with Professor Thimme's group in Freiburg, Germany, tissue from HCC (biopsied or resected tissue that had IHL isolated from, frozen and stored) was gained with corresponding "neighboring" tissue from a cirrhotic liver. The aetiologies of the cirrhosis were unknown (however only n=3). Three patients paired samples were gained with FACS analysis carried out. With the established role of γδ T-cells in tumour surveillance (Chapter 1), we hypothesed that γδ T-cells may preferentially locate in the HCC, compared to the external cirrhotic liver – preferentially the CD161 subset.

Patient 1

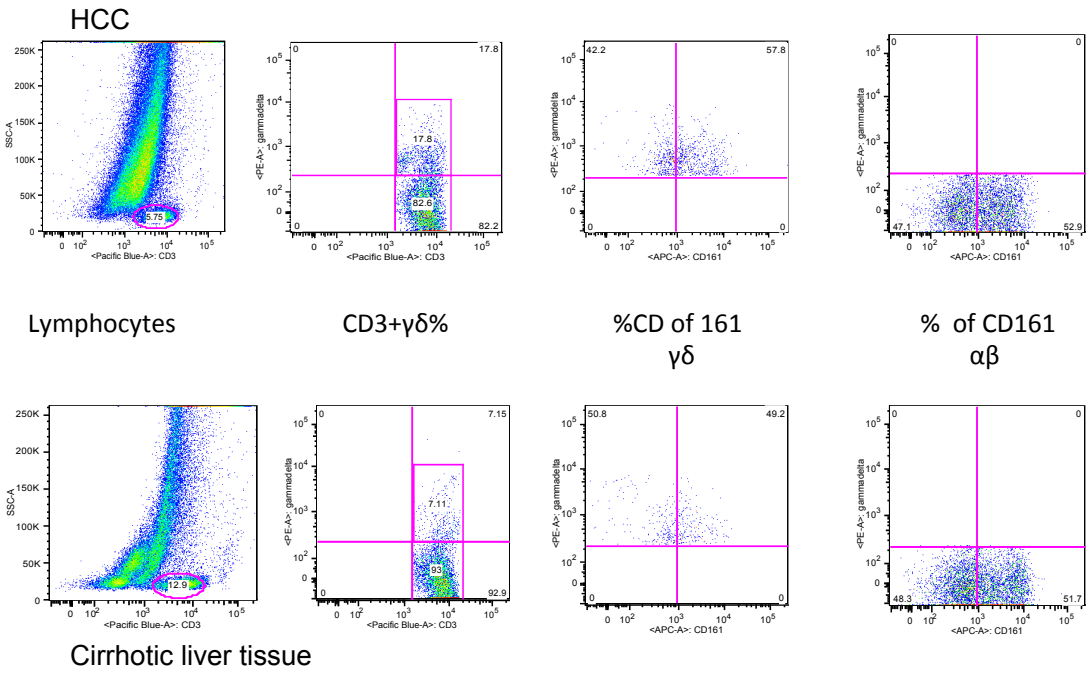


Figure 5.13: FACS plots isolated from HCC tissue and corresponding cirrhotic tissue.

Patient 2

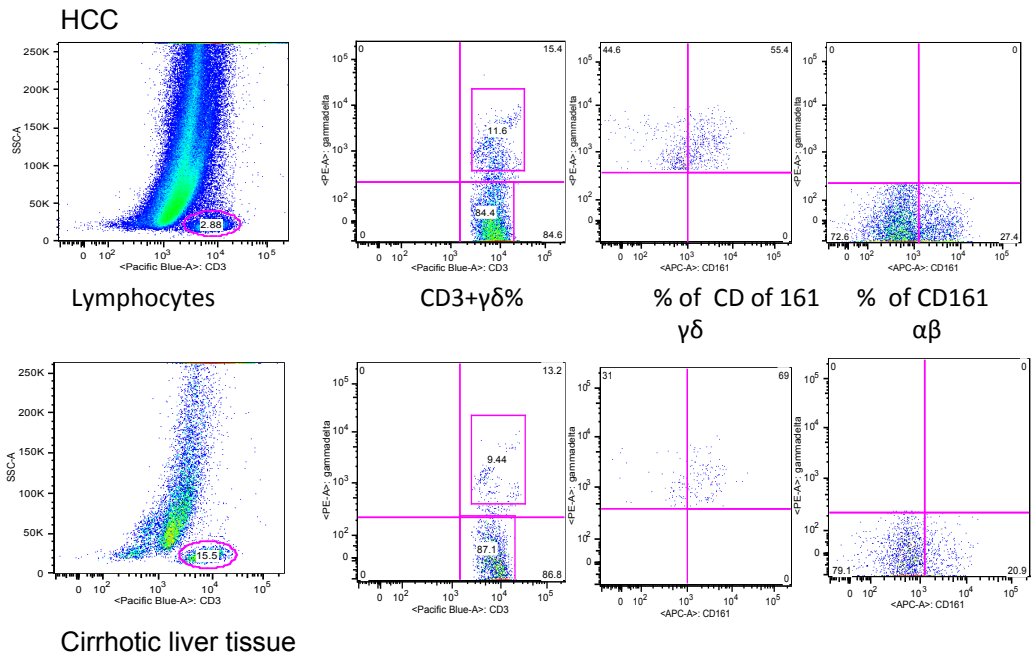


Figure 5.14: FACS plots isolated from HCC tissue and corresponding cirrhotic tissue

Patient 3

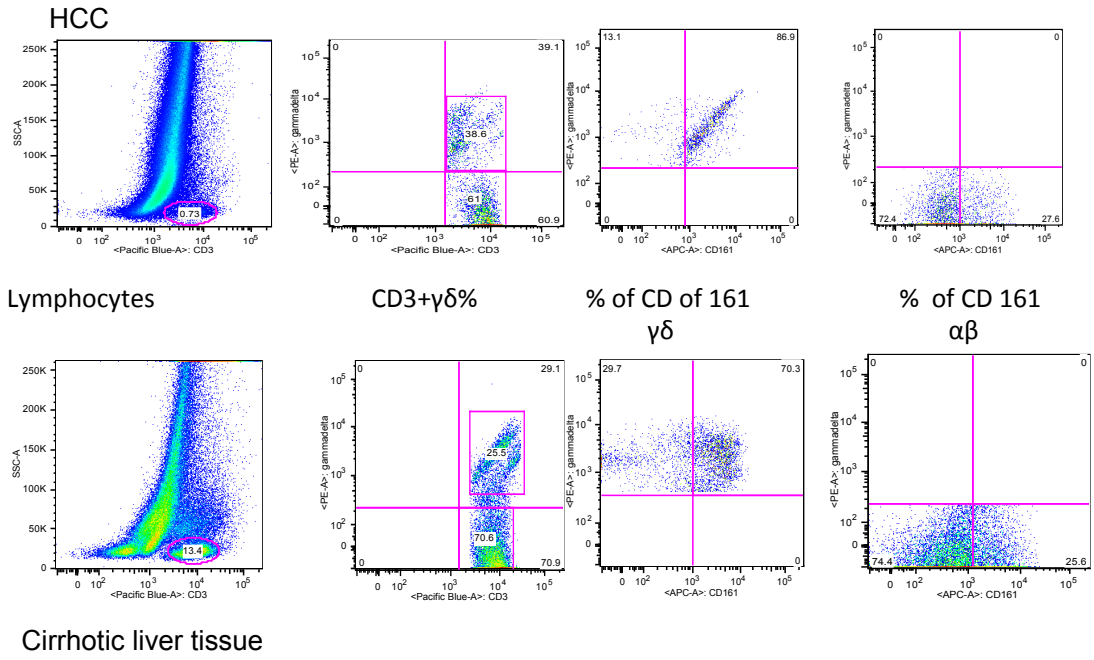


Figure 5.15: FACS plots isolated from HCC tissue and corresponding cirrhotic tissue.

In the cirrhotic tissue the % of CD3 γδ T-cells were 7.15%, 9.44% and 25.5%. The % of these being CD161+ were 49.2%, 69% and 70.3% respectively. In the HCC tissue, the % of CD3 γδ T-cells were 17.8%, 15.4% and 39.1% and of these 57.8%, 55.4% and 85.9% were CD161+ respectively. Thus there was indeed a tendency for the localization of γδ T-cells from cirrhotic liver to the HCC tissue (p=0.06). Due to the small numbers of available tissue there was no significant difference in the CD161 subset of γδ T-cells between HCC and surrounding liver (p=0.33) however this is an area that does warrant further exploration, however may be clinically difficult to gain tissue/ samples from out-with the UK transplant explanted livers in patients transplanted for HCC/ cirrhosis.

5.5 Discussion

In this chapter the role of CD161+γδ T-cells in chronic liver disease has been explored in more detail. With the heterogeneity of the role of γδ T-cells in chronic liver disease as described in Figure 5.1, and the established role of CD161+ αβ T-cells in chronic HBV and HCV (Northfield *et al.* (2008)), this chapter's focus was to explore the role and place of CD161+ γδ T-cells via FACS based experiments using available frozen PBMCs from patients with chronic HBV, HCV infection and also PBC/PSC. In addition, a new clinical study was set up, with Ethical/Research and Development approval to gain intra-hepatic lymphoctyes via a 2nd pass liver biopsy. These samples were aggregated to those gained via a collaborative visit to Professor Thimme's laboratory in Freiburg, Germany in 2010.

Chronic liver disease impacts upon total γδ T-cell populations and also the CD161 subsets

Initially using the samples from the disease states available the total CD3+γδ T-cell populations were studied in the periphery. In chronic viral aetiologies (HBV and HCV) there was a significant reduction in the total γδ T-cell populations (p=0.0002 and 0.0006 respectively. There was no change in PSC, however there was also a significant reduction in the periphery in patients with PBC (p=0.002).

On analysis of the CD161+ subsets there was a significant increase in the CD161+ γδ T-cell subsets in patients with PSC and PBC (in the face of an overall reduction in total γδ T-cell populations in patients with PBC and equivalent γδ T-cell population when healthy controls compared to PSC).

Thus it seemed that in these disease processes, in the periphery, the CD161+ fraction, which from Chapter 3 produced more pro-inflammatory cytokines, were relatively enriched. Unfortunately due to a lack of liver tissue from patients with PSC and PBC we could not explore if there was an enrichment of the CD161+ subset in the liver, maybe contributing to disease pathogenesis via the preferential expression of TNF-α. We also did not have samples (peripheral or intrahepatic) for patients with true autoimmune hepatitis.

Following on from this potential "pathological" effect of γδ T-cells in liver disease, in patients with NAFLD there was an enrichment of the CD161+γδ subset into the liver from the periphery ($p=0.02$). In patients with NAFLD and more so in NASH (whereby a pro-inflammatory drive and pathological changes within the liver of inflammatory cells, oxidative stress and pro-inflammatory cytokines that can eventually lead to fibrosis and cirrhosis) the enrichment of CD161+ γδ T-cells may propose a potential target thus for anti-inflammatory agents or antifibrotic agents. Unfortunately we did not have enough samples to look at phenotypical or functional studies in the γδ T-cells in patients with NASH, however this may be an interesting area for future studies. The NAFLD/NASH finding was indeed interesting following on from the association seen in Chapter 4 with ischaemic heart disease and atherosclerosis.

Further validation is also required for the homing of γδ T-cells to HCC tissue from the peripheral cirrhotic tissue that was seen as a trend. Although too small numbers to assess for specific CD161+ γδ T-cell enrichment, this concept is promising, again potentially allowing recruitment to tissue via immunomodulatory therapies such as aminobisphosphonates in patients with

HCC.

CD161 γδ T-cells and HCV

In HCV the CD161+ subset was reduced when compared to healthy controls ($p=0.02$) in the periphery; however this was not the case in chronic HBV. From this it was postulated that perhaps with the reduction of the CD161+ subset in chronic HCV they would be enriched in the liver like the published CD161αβ T-cell subset (Northfield *et al.* (2008)).

On studying the γδ T-cells in more detail in HCV, CD161+ γδ T-cells expressed significantly more Vδ2 than Vδ1 still ($p=0.0012$) whilst there was no difference between expression of the Vδ chains on the CD161- subset of γδ T-cells. Following on from the postulated theory that the reduction of peripherally circulating CD161+ γδ T-cells in chronic HCV home to the liver (with the preferential expression of CXCR6 of γδ T-cells in HCV). Disappointingly there was no statistically significant enrichment of the CD161+ γδ T-cells in the liver when compared to the periphery, despite there being an enrichment of the CD161+ αβ T-cells. Thus it is not clear why this subset (the CD161+) is reduced in the periphery in patients with HCV. However it may be that as there is no enrichment within the liver, that these cell subsets may be targeted by the virus (e.g. via myeloid dendritic cells (Zhao *et al.* (2013)) or that this subset is redirected to other (e.g. to the gut). Alternatively they may undergo activation induced cell death, as is postulated for CD8+ CD161++ T cells in chronic viral infection (Cosgrove *et al.* (2012)). The data from Agrati and colleagues was compelling (Agrati *et al.* (2011)) whereby inhibition of HCV was furthered by the use of aminobisphosphonates

that activated γδ T-cells – thus providing a potential future therapeutic strategy requiring studies. One could postulate that the Bisphosphonate therapy may indeed restore the number, and potentially function, of the CD161+ subset of γδ T-cells. In HCV infection when comparing the expression of CD161 between the Vδ1 subsets of γδ T-cells, there was an increase in expression of CD161 in HCV when compared to healthy controls ($p=0.04$). This was not the case for the CD161- T-cells. There was however no difference in the CD161 expression in the Vδ2 subset when comparing HCV and healthy controls. The overall significance of the Vδ1/Vδ2 shifts in chronic liver disease are not established however data does point towards the Vδ1 subset as a more deleterious subset in HCV. In HCV the clearest impacts are of a reduction in the % of γδ cells and the % of these that are CD161+; there may be relative sparing within this of the Vδ1 cells although such impacts are not as dramatic.

5.6 Chapter summary

From these sets of experiments in liver disease:

-CD161+ γδ T-cells were enriched in the periphery in patients with PSC and PBC.

-CD161+ γδ T-cells were specifically reduced in the periphery in patients with chronic HCV infection but not in chronic HBV.

-Chronic HCV infection led to a significant up-regulation of NKG2D expression on the CD161+ γδ subset and a significant reduction in the expression of CXCR6.

- In HCV there was a significant rise in the expression of Vδ1 in the CD161+ γδ T-cells.

- On exploration of the CD161+ expression on the Vδ2 T-cells per disease, the only significant change was a reduction in expression in patients with PSC compared to healthy controls.

-In Vδ1 γδ T-cells there was an increase in expression of CD161 in HCV, PSC and PBC when compared to healthy controls.

- There was no overall enrichment of CD161+ γδ T-cells from the periphery in patients with HCV however there was in patients with NAFLD/NASH cirrhosis. Further experiments to define the shifts in gd T cell populations in the periphery in NASH would be of interest in future and links in to the potential pro-inflammatory pathological effect seen in cardiac disease in Chapter 4.

-There was an enrichment of γδ T-cells in HCC tissue when compared to surrounding cirrhotic tissue.

CHAPTER 6: CD161 γδ T-cells in health and liver disease - The Discussion

6.1 CD161 γδ T-cells in health

Background to thesis revisited

This thesis aimed initially to explore in healthy humans the role of CD161 γδ T-cells in more detail. Experiments from our laboratory had previously shown CD161 positivity to confer a unique phenotype and function to αβ T-cells (Billerbeck *et al.* (2010)) in health. CD161 is expressed on both αβ and γδ T-cells (Maggi *et al.* (2010)) with its expression consistently associated with memory phenotype in adult γδ T-cell populations. The timing of CD161 acquisition on T-cells had been studied on αβ T-cells (Martin *et al.* (2009); Cosmi *et al.* (2008); Billerbeck *et al.* (2010); Walker et al. (2011)), however to our knowledge was unknown in the γδ T-cell populations. CD161 positivity had been found in MAIT cells (Dusseax et al (2010)) with a strong link to IL-18R expression too. In the periphery these cells expanded to express high levels of CCR6 and CXCR6 indicating a preferential gut and liver tissue homing. CD161 γδ T-cells have been implicated in MS with the CD161 positivity linked to migration of the cells from periphery to CNS (Poggi *et al.* (1998)) and recently the co-expression of CCR6/CD161 in γδ T cells in CSF and peripheral blood studied in patients with MS (Schirmer *et al.* (2013)). This dual IL-17-producing population was linked to patients with relapsing MS. The potential roles and published data (to date) of CD161 + γδ T cells is summarised in Figure 6.1.

Thus Chapter 3 and 4 of this thesis intended to explore further the role, biology, function and phenotype of CD161+ γδ T cells.

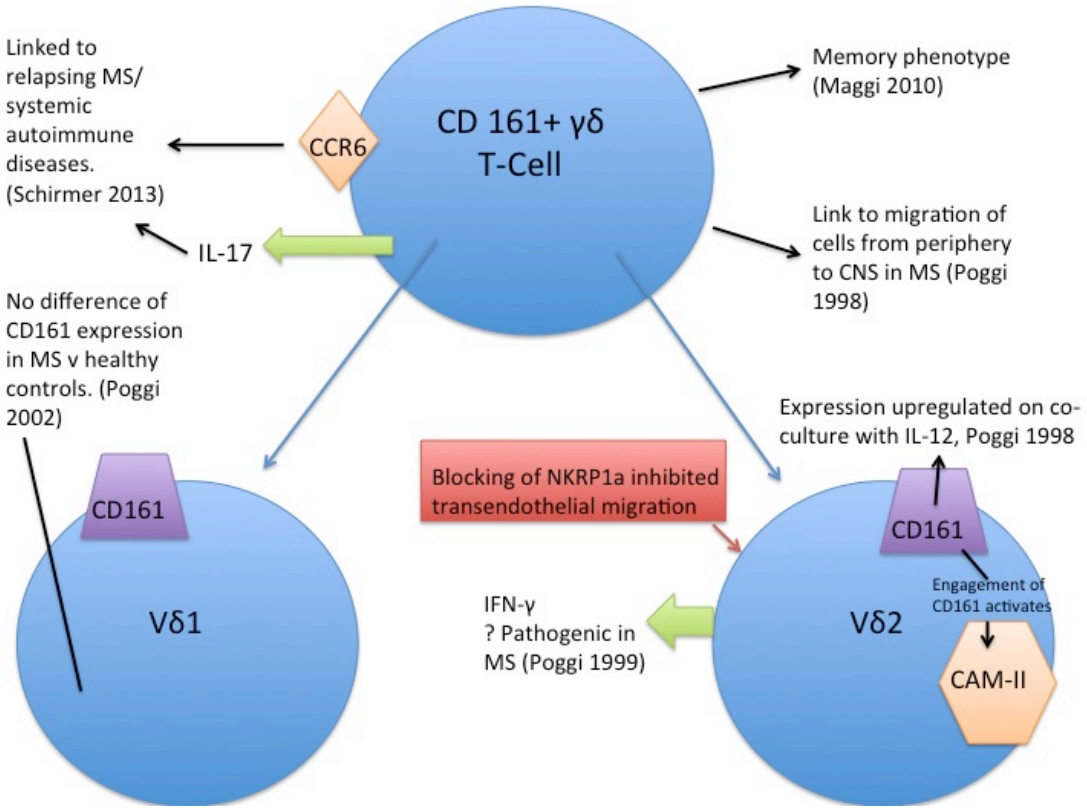


FIGURE 6.1: Summary of CD161 γδ T cell studies to date in health and disease

CD161 γδ T-cells are a distinct subset in health

Initially it was found that the CD161+ subset was the main circulating population in health. This was reassuring, and somewhat justified their exploration in further detail. Using a FACS based approach in Chapter 3, the CD161+ γδ T-cells were found to be phenotypically different from CD161- γδ T-cells. Some important findings included: the preferential expression of CXCR3 (the chemokine receptor involved in leucocyte trafficking, recruitment of inflammatory cells and implicated in wound healing). This preferential expression compared to the CD161- subset suggested the CD161+ γδ T-cells may indeed be the main subset involved in not only disease processes such as MS, but in physiological responses to inflammation and infection. The potential

dichotomy of disease pathogenesis versus host pathogen clearance/defence was once again introduced here, and something continually evident throughout the thesis.

The CD161+ γδ T-cells also expressed CCR6, expressed on Th17 cells and CD161++ (high) CD8 T-cells (Northfield *et al.* (2008)). CCR6 has been implicated in T-cell migration in disease states such as Crohns disease, thus again suggesting this subset may be involved in other potential disease processes – however this was not proven until colonic tissue was stained in a Crohns patient showing CD161+ γδ T-cells. The expression of CCR6 tied in with the established data in MAIT cells, and their link to liver homing. Thus a link between the CD161 γδ and αβ T-cells was established early on in this thesis.

On functional analysis by ICS the CD161+ γδ subset, after stimulation with PMA/Ionomycin, was found to express significantly more pro-inflammatory cytokine TNF-α, which is involved in conditions such as inflammation within the liver (e.g. alcoholic hepatitis and NASH) and gastrointestinal tract (e.g. Crohns disease and Ulcerative Colitis). In these latter conditions anti-TNF-α therapies (such as Infliximab and Adulimumab) have become an everyday part of the armamentarium. The CD161+ γδ T-cells also (when compared to their CD161- counterparts) expressed significantly more IFN-γ. This cytokine is important for T-cells to control viral and bacterial infections; however aberrant production can be associated with autoimmune conditions, and γδ T-cells have been linked to these (Ferri *et al.* (2010)). Thus one could postulate that it was again the CD161+ subset that is key in clearance of viral and bacterial pathogens (Chapter 1). On the other hand, potentially if increased in numbers or even activity they may contribute to disease pathogenesis – again the γδ

T-cell dichotomy. The CD161+ γδ T-cell subset produced significantly greater Granzymes and Perforin. Thus this specific cell subset seems to be the key mediator in killing compared to their CD161- counterparts. All this data suggested that the CD161+ subset compared to the CD161- subset may be the more "primed" and ready subset to combat disease, displaying a Th1-type of profile. The CD161+ γδ T-cells also preferentially expressed the commoner Vδ2 chain compared to the Vδ1, in keeping with them being the main circulating subset.

To explore when γδ T-cells gain this hallmark (CD161 positivity) a set of experiments were performed to assess for CD161 expression in cord blood and also in infants. There was some CD161 expression in cord blood. However, it was during the 1st few weeks of life that the total γδ population increased, and the CD161+ γδ T-lymphocyte subset expanded preferentially. This suggests that the naïve γδ T-cell expands upon Ag exposure and may acquire CD161 positivity, whilst the already circulating CD161+ γδ T-cells from birth fight pathogens in those initial critical weeks of life until till the adaptive immune system has developed.

The CD161 γδ T-cells, with their established phenotype in Chapter 3, were then assessed in human colons. Unfortunately the rate-limiting step in this experiment was a lack of samples and also a lack of paired PBMCS. It was difficult to draw major conclusions from this experiment. Nevertheless, it did *"whet the appetite"* for study of the CD161+ γδ T-cell subset in more detail, ideally gaining sufficient numbers of tissues samples from healthy colons, Ulcerative Colitis and Crohns, and even from GI tumour tissue, to characterise the cell subsets in more detail. The response to treatment too would be interesting, to see if changes in phenotype and function

potentially. This could be done also for the small bowel, examining disease states such as Coeliac disease (see later in discussion).

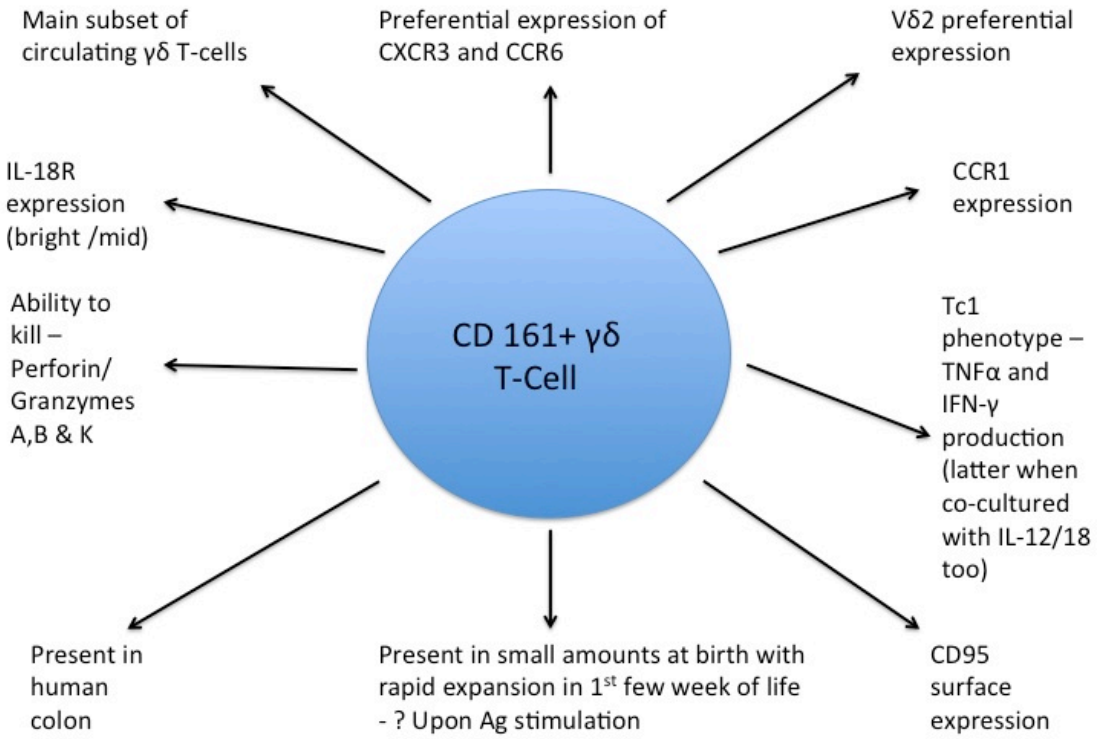


FIGURE 6.2: Summary of thesis FACS based findings of CD161+ γδ T-cells in health

The next Chapter focused on a totally different approach, using gene array technology to assess for fundamental differences between CD161+ and - γδ T-cells. Often gene array experiments are described as a "fishing experiments". However this technology allowed a method to consolidate on the findings from Chapter 3 using a different technique. Despite the numerous challenges detailed in Chapter 4 with the experiment, data was gained. Four hundred and nine genes were significantly associated with the CD161+ γδ T-cells when compared to the CD161- subset- with the majority (76%) down-regulated. Of the up-regulated genes, some genes of interest included ADRB2 – a gene encoding for B2-adrenergic receptor, CCL20 – a gene encoding for cytokine

recruiting macrophages/neutrophils, and COLQ- a gene encoding for Acetylcholinesterase collagenic tail peptide. Again these suggested novel, potential physiological roles for CD161+ γδ T-cells, although much more work would be required to define a functional role for some of these unexpected molecules on T-cells.

The genes were then inputted into the DAVID ™ Bioinfomatic software tool to look for any significant signatures/pattern profiles of the aforementioned 409 genes. Patterns/mechanisms/pathways that came out for the CD161+ γδ T-cells included: wound healing, defence and inflammatory responses, cell adhesion pathways, regulation of chemotaxis, regulation of leucocyte migration, and regulation of locomotion. This once again provided a key insight into the distinct role of the CD161+ γδ T-cells. The genes were then entered into the DAVID ™ *disease* functional classification to explore if the significant genes were associated with biological pathways/ diseases. There was association with cardiac disease including: coronary atherosclerosis, Brugada syndrome, and idiopathic dilated cardiomyopathy. The link with cardiac diseases – in all aspects is interesting and begs the question if this is related to pro-inflammatory mediators (in conduction tissue, arteries or in cardiac muscle) potentially from the CD161 γδ T-cells. Other diseases linked to the CD161+ γδ T-cells included periodontitis, cerebral malaria, asthma and GVHD - all pro-inflammatory conditions whereby the CD161 γδ T-cells may play a pathogenic rather than protective role, via the aforementioned released of pro-inflammatory cytokines. Thus with different specific mechanisms of action cited via input into DAVID ™, and also the genes having biological significance, this added weight to the fact that CD161 γδ T-cells are indeed a unique subset of T-cells, and shed light into their potential role in novel disease areas.

The next finding was to compare the γδ and αβ CD161+ subsets via 2 different gene array analyses. Using 2 separate lists of up- and down-regulated genes per subset, these were again analysed in DAVID ™ to explore for any common pathways or diseases that CD161 T-cells, irrespective of αβ or γδ TCR, may be involved with. Eighty-six common genes were found -19 upregulated and 67 down-regulated. Of note, the genes coding for CCR1 and IL-18R were upregulated. Both CCR1 expression and IL-18R expression were confirmed by FACS analysis, however the key role of IL-18R to both αβ and γδ CD161 is interesting. IL-18R staining has been a key to the identification of MAIT cells. IL-18 itself has also been shown by Li and colleagues (Li *et al.* (2010)) to expand the γδ T-cells when co-stimulated with IL-2 and a bisphosphonate. The cells that were expended were effector memory cells and expressed NKG2D and perforin. Thus one could hypothesis that these cells were indeed the CD161+ γδ T-cell subsets. When the γδ T-cells were incubated with IL-18 they produced GM-CSF, IFN-γ, and TNF-α at much higher levels than those incubated without IL-18. The cells also showed strong cytotoxicity against tumor cells including mesothelioma cells. Therefore, it is postulated that the CD161+ γδ T-cells are this key subset, expressing IL-18R, and as shown in Chapter 3 having a greater potential to kill cells and produce cytokines. The other interesting finding was a potential dual population of IL18R/CD161+ γδ T-cells found when stained. This may be a novel area worth pursuing regarding different functions and phenotype.

The CD161+ γδ T-cells also expressed CCR1. CCR1 and the antagonism of its pathway has been proposed in reducing liver inflammation (Zimmerman and Tacke. (2011)) and the prevention of collagen deposition. CCR1 antagonists are also in

clinical development for diseases such as Rheumatoid Arthritis and IBD. Blockage of CCR1 may be a potential therapeutic strategy in conditions whereby CD161 γδ T-cells play a pathogenic role however this has yet to be established.

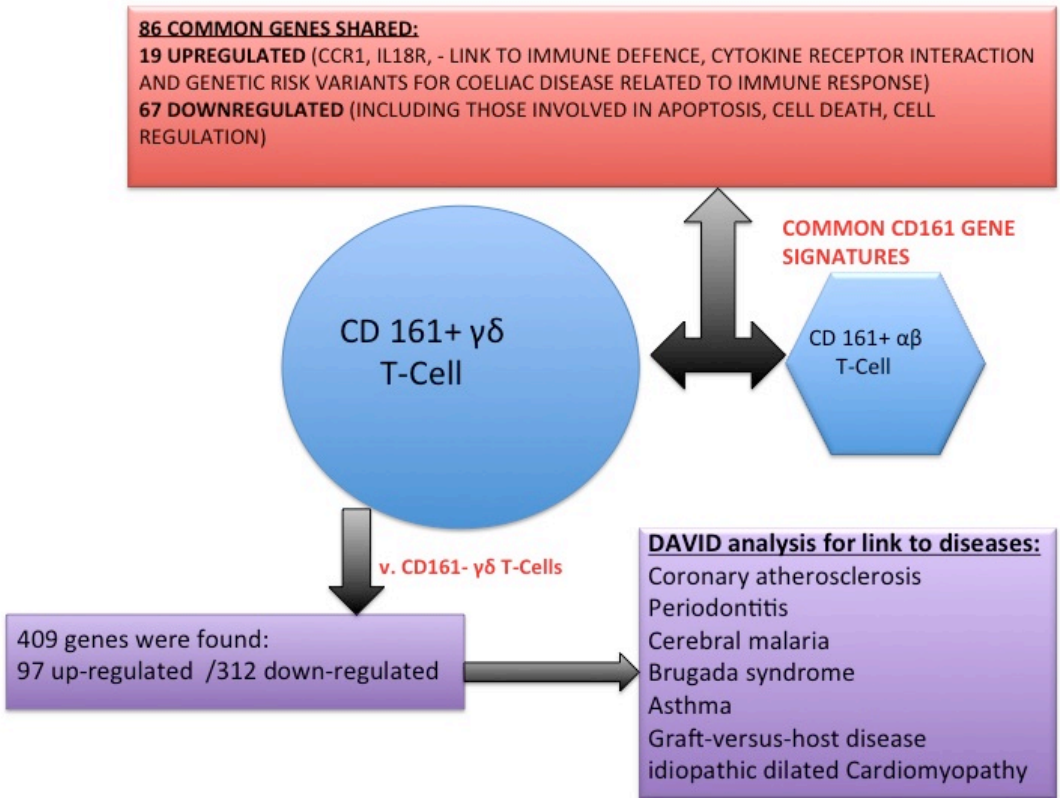


FIGURE 6.3: Overview of gene-array findings of CD161+ γδ T-cells in health and the link between CD161 γδ and αβ T-cells

The data from Chapters 3 and 4 is supportive of the concept that CD161+ γδ T-cells are a functionally distinct subset of γδ T-cells, sharing some properties of their CD161αβ counterparts, but in many respects unique. We propose that they play an important role in not only disease prevention but also in disease pathogenesis.

6.2 CD161 γδ T-cells in liver disease

Background to thesis revisited

Following on from defining the CD161+ γδ T-cells in health, their role in chronic liver disease was then studied. One of the main drivers for this thesis was the finding that CD161 was expressed on a significant subset of HCV and HBV-specific T-cells (Northfield *et al.* (2008)) with the exploration of the properties of CD161+ CD8+ αβ T- cells in infected HCV persons. These cells displayed an effector phenotype and produced pro-inflammatory cytokines (IFN-γ and TNF-α), but expressed low levels of granzyme B and perforin, suggesting a reduced immediate ability to lyse virus-infected cells.

From Chapter 3 it was shown that CD161+γδ T-cells were the main subset producing these pro-inflammatory cytokines, although higher levels of granzymes and perforin (compared to the CD161- subset). The CD161+ CD8+ αβ T- cells were strongly associated with CXCR6 – thus implicated in liver homing. CD8+CD161++ T cells had also been found to express a pattern of molecules in keeping with a Th-17 differentiation (Billerbeck *et al.* (2010)) in patients with HCV (and in healthy controls), revealing a functionally distinct population of CD8+ cells expressing high levels of CD161 and a pattern of molecules consistent with Th17. The role of CD161+ (in NK cells) had been studied in acute HCV (Alter *et al.* (2011)) with reduced frequencies of NK cells expressing CD161 in blood in patients clearing HCV. The role of γδ T-cells to date in liver disease is summarised once again in Figure 6.4 (as per Figure 5.1 in Chapter 5).

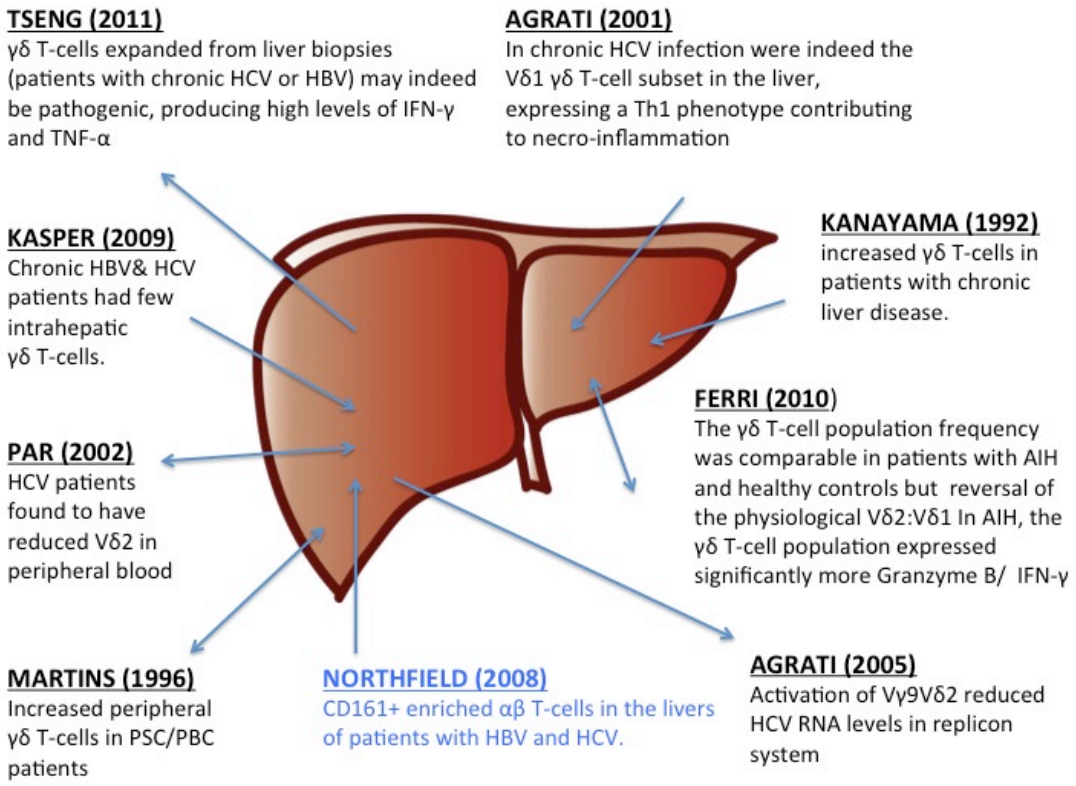


FIGURE 6.4: Summary of published data of the role of γδ T-cells and the liver.

CD161 γδ T-cells are a unique subset playing a role, and being attenuated in chronic liver disease

In Chapter 5 the role of CD161+ γδ T-cells was explored in the setting of chronic liver disease. As part of this chapter a new study was set up in Oxford, allowing 2nd pass biopsy samples to be studied for intrahepatic T-cell responses. This study has not only generated data for this thesis but is on-going allowing sample usage for subsequent T-cell studies in the Klenerman laboratory. Using frozen PBMCs from patient cohorts (including those with chronic HCV, HBV, PSC and PBC) the CD161+ γδ T-cell populations were analysed. As part of this Chapter, HCC tissue was also analysed via a collaboration with Professor Thimme's group in Freiburg, Germany.

The main findings of this chapter were that CD161+ γδ T-cells are altered in liver disease conditions. When exploring the frequencies of the CD161+ subset (as a % of the total γδ T-cell pool in the periphery) in patients with HCV, the CD161+ subset was reduced. This was not the case however in patients with chronic HBV. This led to the hypothesis that in HCV the CD161+ γδ T-cells may be (like the previously published CD161 αβ T-cells) enriched in the liver. However it was surprising that this was not the case in chronic HBV. In this cohort the patients were not on antiviral therapies such as Tenofovir/Entecavir for HBV and the % of γδ T-cells that were CD161+ were similar to that in healthy controls. Thus the question of why HCV was affecting the CD161+ γδ T-cells specifically was interesting. The main potential answer to this was enrichment within the liver, however this was not found to be the case.

On analysis of intrahepatic T-cells, there was an overall enrichment of γδ T-cells, however not of the CD161+ γδ T-cells in HCV (or overall in chronic liver diseases that there was biopsy access to). The only enrichment specifically (of the conditions that statistical analysis could be performed in) was observed in NASH/NAFLD. No phenotypical or functional studies were performed in the NASH cohort due to a late finding in the course of this thesis – however correlation with histology is needed and comparison of populations in NALFD v NASH would be required. Nevertheless this is an interesting finding of this thesis (discussed in Chapter 6.3). The lack of enrichment in the liver in HCV could be explained by either homing to another source (e.g. the gut) or attenuation of the circulating CD161+ γδ T-cells by the virus.

With the availability of HCV samples, and also the unanswered question as to why

there was a specific HCV reduction, phenotyping studies were carried out in the HCV cohort. Vδ chain expression was assessed, with the predominant expression of Vδ2 on the CD161 γδ cells; however when compared to healthy controls, on the Vδ1 T-cells, there was an increased expression of CD161. Agrati and colleagues (Agrati *et al.* 2001) found in chronic HCV the intrahepatic subset of γδ T-cells to be the Vδ1 type associated with pro-inflammatory capacity and linked to higher necro-inflammation. One could hypothesise thus that HCV potentially affects the CD161+ γδ T-cells, reducing their frequency, and maybe indeed it is the Vδ2 subset that (although predominating) are reduced preferentially (with the Vδ1 set homing to the liver in chronic HCV for a pathogenic effect). An increase in CD161 expression by the Vδ1 subset may tie in with an increase in Th-1 role, and a pro-inflammatory drive and follow on from them being related to persistence of virus. The Vδ1 T-cells have been shown also to be expanded in the periphery in other chronic viral illnesses like in HIV with AIDS complicating it. There was also (in the CD161+ subset) a significant over expression of NKG2D. HCV is known to be able to evade NKG2D-dependant NK cell responses via a NS5A mediated imbalance of inflammatory cytokines (Sene *et al*. (2010)), and one could propose this over-expression of NKG2D on the CD161+ γδ T-cells could be part of the virus's ability to evade the immune system. However, on NK cells the expression of NKG2D was actually reduced and as a result the NK cell cytolytic and IFN-γ producing functions were impaired. Certainly this area requires further studies.

In chronic HCV the expression of CXCR6 was also significantly reduced in CD161+ γδ T-cells. CXCR6 is strongly associated with attraction of cells to liver inflammation. Thus, one potential scenario is that by down-regulating its expression, HCV may

prevent CD161+ γδ T-cells from migrating to the liver to combat the virus.

The increase in CD161+ γδ T-cells was seen in the periphery in patients with PSC and PBC. Also in PSC there was a significant over expression of the CD161+ on Vδ1 T-cells compared to healthy controls. Unfortunately due to sample selection, no conclusions about the enrichment of the CD161+ γδ T-cells could be made for PSC/PBC. We postulate there would be an enrichment following on from Ferri and colleagues data (Ferri et al. (2010)), with the Vδ1 T-cells being the more predominant subset (echoed by a reduction in CD161+ expression in the periphery in PSC patients compared to controls in Chapter 5's experiments).

The other area explored was the enrichment of γδ T-cells into HCC tissue. Unfortunately due to inadequate sample number, conclusions regarding the enrichment of the CD161 subset could not be readily made. Nevertheless, γδ T-cells can have an anti-tumerogenic property, enhanced and potentiated in clinical trials with aminobisphosphonates. Therefore, it is not out-with the realms of possibility to propose that potentiation on the CD161+ subset may be a potential novel therapeutic strategy in patients with HCC – even as a bridge to downstage HCC into transplant criteria. This could be a potentially exciting area.

In summary, it is indeed possible that CD161+ γδ T-cells play different roles in different chronic liver diseases – dependent on their Vδ chain expression. Also their number, frequency and function within the liver (migrating from the periphery) or their attenuation in the blood by viruses, may be a novel mechanism by which HCV evades the adaptive and innate immune system. This requires further clarification.

The findings of Chapter 5 are summarized in Figure 6.5.

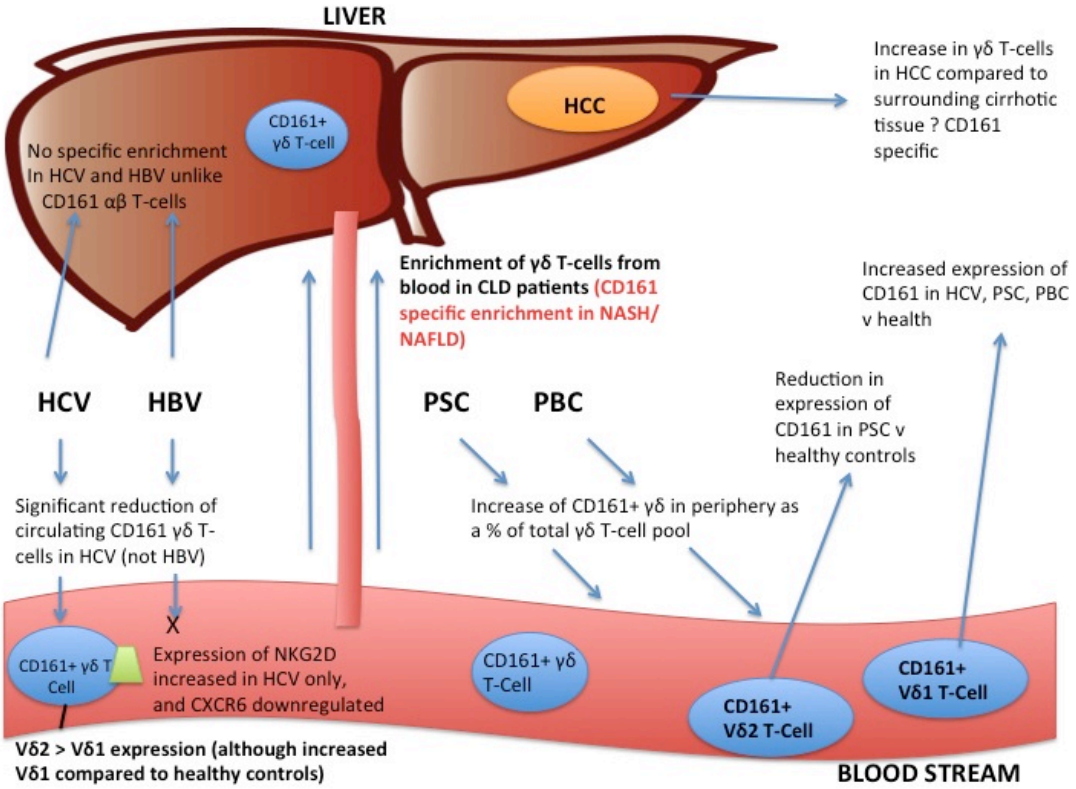


FIGURE 6.5: Summary of thesis findings of the role of CD161+ γδ T-cells in chronic liver disease.

6.3 The future of CD161+ γδ T-cells?

The future of the CD161γδ T-cells is bright not dim.

This thesis has highlighted the important role of CD161+ γδ T-cells in health and shed light into how they may be involved in some disease processes. There are some unanswered key clinical questions from this thesis that have come to light warranting exploration. These include:

1. *Are the CD161+ γδ T-cells functionally altered in chronic liver disease?*

To answer this 2 different approaches could be made. One could be, as per Chapter 3, a FACS based approach to cells from chronic liver disease. Phenotyping studies were

attempted in the PBMCs isolated from frozen samples however not much success was gained except in staining for NKG2D. Furthermore, the ideal would be to gain samples from healthy controls and HCV, HBV, PSC, PBC and NASH/NAFLD patients, and to perform functional assessments such as ICS looking at the production of IL-17A/F, IL-22, TNF-α and IFN-α. Patients with classical Autoimmune hepatitis too would be an interesting cohort, whereby the γδ T-cells may indeed be pathogenic. The killing ability of the CD161+ γδ T-cells between each disease warrants study. Looking at these experiments not only in the periphery but moreso in the liver and the intra-hepatic CD161+ γδ T-cell population would be key.

Unfortunately our protocol did have problems with isolation of the cells from the liver and this would need to be strengthened. Also potentially hepatic tissue samples could be gained from resections (partial hepatectomies) in metastatic colorectal cancer patients – however this may require ethical approval to use such tissue. The other option for studying intrahepatic γδ T-cells may be via gene array technology as per Chapter 4, to gain an insight into which genes are switched on and off in such disease conditions – however this may be difficult, say, in HCV infection due to the vast different quasispecies, let alone genotypes of virus, and constant attenuation of gene responses by the virus.

2. *With an enrichment of the CD161+ γδ T-cells to the liver in patients with NASH/NAFLD, are they playing a potential pathogenic role in inflammatory stimulus in these patients?*

CD161 γδ T-cells have been shown to produce IFN-γ, and have a pathological role in patients with MS. The co-expression of CCR6 and production of IL-17 has also been postulated to play a role in not only MS but also systemic autoimmune diseases. In

Chapter 5, the CD161 subset was enriched in the periphery in patients with PSC/PBC. Targeting of pro-inflammatory pathways has been critical in patients with liver disease. Steroids can be used in alcoholic hepatitis (Forrest *et al* (2007)) and established in the treatment of autoimmune liver disease. The pro-inflammatory pathways in NAFLD/NASH are key, and targets of these pathways have been studied. Pentoxyphyline (PTX), a phosphodiesterase inhibitor has been studied (reducing TNF-α) levels in not only Alcoholic Hepatitis (Akriviadis *et al* (2000)) but also in patients with NASH (Zein *et al* (2011)). In mouse models, PTX has been shown to have protective effects on hepatic and arterial function, partially through inhibition of advanced glycation end products (AGEs) and their receptor expression (Wu *et al.* (2010)). With the gene array studies in Chapter 4 also linking CD161 positivity to atherosclerosis in humans, it would be interesting to explore further CD161+ γδ T-cells in NASH (peripherally and intrahepatically), explore their frequency and function, and then potentially after treatment of PTX along with histological regression of fibrosis. With the proven pro-inflammatory role of CD161 γδ T-cells, it is not unreasonable to hypothesise they may be involved in the inflammatory drive in NASH. Unfortunately, a clear homologous marker for CD161 on mouse T cells has not been firmly established, despite analysis of the closest genetic relation (NKRP1D) (O'Hara *et al*, manuscript in preparation). Such a marker would allow definition of the role of CD161+ cells in disease models in vivo.

3. Do CD161+ γδ T-cells play a role in gastrointestinal conditions?

The role of CD161 γδ T-cells does require exploration in the GI tract. The main Vδ1 subset in the GI tract, although possibly expressing less CD161, may be a key subset in GI pathologies. Unfortunately due to a lack of samples in Chapter 3, no definitive conclusions could be made about the localisation of CD161+γδ T-cells in the colon, in

health or in IBD. However it stands to reason, with the expression of CD161+ T-cells of CCR6, CXCR3 and CXCR6 that these cells are indeed gut homing cells. Also, the common genetic signatures found on DAVID ™ analysis of the CD161+ αβ and γδ T-cells in Coeliac disease serves as evidence of a potential role. With the marked intra-epithelial lymphocytosis observed in Coeliac disease, it would be exciting to explore the intra-epithelial CD161+ γδ T-cells population in more detail – pre- and post-Gluten free diet. Also in patients with refractory Coeliac who display the TCR gene re-arrangement, the role, function and presence of CD161+ γδ T-cells is a potential area for exploration. Patients with refractory Coeliac disease can be treated with steroids or even Anti-TNFα therapies (Rajoriya *et al.* (2006)) and thus if there is an inflammatory drive/component from CD161γδ T-cells, then further studies could provide an insight into this condition, if the CD161+ γδ or even αβ T-cells are present. By the same token, their proposed potential pathogenic role in IBD does require further studies and clarification especially with the link to CCR1 found.

4. *Can CD161+ be used or attenuated by drugs such as Bisphosphonates in chronic liver disease and related HCC?*

This is an important question in the advent of immunotherapy in tumours. With γδ T-cells being used in other oncological fields, immunotherapy with CD161+ γδ T-cells does require further clinical studies.

6.4 Conclusion

In conclusion, CD161+ γδ T-cells are a unique subset of γδ T-cell, not only in health but also in chronic liver diseases. They are the dominant subset of γδ T-cells in adults, with different phenotype and function. They have a distinct and different inherent genetic signature to CD161- γδ T-cells. Their role in chronic liver disease has been

initially explored in this thesis and areas of further study have been highlighted. In Chapter 1.5 the primary aims were stated, following on from published data (Northfield *et al.* (2008); Billerbeck *et al.* (2010)) finding that CD161 positivity conveyed a distinct phenotypical and functional characteristic to CD161++ αβ T-cells. These specific aims have been answered, showing CD161+ γδ T-cells are a distinct subset with distinct phenotype and function. The CD161 positivity is something that is indeed innate however there is a rapid expansion early in life, maybe upon antigenic exposure in the 1st few weeks of life, when the γδ T-cell's role compared to the αβ may be key.

In liver diseases, the γδ T-cells do change compared to in health, changing not only in phenotype, but also in distribution and also in number. It is yet to be established if the roles are different in HCV compared to the cholestatic liver diseases (PBC/PSC). However it seems that in the latter the cells may play a role in disease pathogenesis whereas in HCV, following on from published data, their role may be potentially protective. Studies with the bisphosphonates in the HCV/HCC arena are warranted. Although there was an enrichment of γδ T-cells in liver disease, this was not clearly evident in HCV (and HBV) infection as is the case for the CD161+ αβ T-cells. There was however enrichment in patients with NASH/NAFLD and this warrants further investigation. Importantly, this work has highlighted a fluid and dichotomous role of CD161+ γδ T-cells potentially between disease pathogenesis and disease prevention.

In conclusion, CD161+ γδ T-cells are a key T-cell subset, different in health and in chronic liver diseases to their CD161- counterparts. Their role in disease pathogenesis requires further studies, however they appear to be a key

subset that can be identified/targeted for attenuation or even expansion in certain key disease areas in the future.

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Appendices

APPENDIX 1: POSTERS GENERATED FOR CONFERENCES

The impact of chronic liver disease on CD161+ γδ T-cells

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BACKGROUND:

Gamma-delta (γδ) T-cells fall at the interface between innate and adaptive immunity. Their role in liver inflammation is not clear but they have been identified in the livers of patients infected with chronic hepatitis C (HCV) (Agrati et al 2001).

CD161+ is a C-type lectin found on the surface of Natural Killer and T-cells in humans. HCV specific T-cells have been found to express CD161 with enrichment in the liver (Northfield et al 2008) and is associated with liver-homing T-cell populations. However the specific functions of CD161+ γδ T-cells and their role in chronic liver disease (CLD) has not been established.

AIM:

To explore the phenotype and function of CD161+ γδ T-cells in humans – initially in healthy individuals, and then assess the impact of chronic liver disease on these cells. CD161+ γδ T-cells were initially characterised in healthy controls and then studied in patients with CLD.

METHODS:

Whole blood & Peripheral Blood Mononuclear cell (PBMC) staining was carried out on healthy controls. patients with CLD (Hepatitis B & C (HBV/ HCV), Primary Biliary Cirrhosis (PBC) and Primary Sclerosing cholangitis (PSC); with subsequent Fluorescence-activated cell sorting (FACS) performed on all samples. All samples were analysed via a FACScalibur ™ or LSRII ™ Flow cytometers. PBMC samples and intra-hepatic lymphoctye (IHL) samples were all gained after local/international ethical approval. All patients donating IHLs to the study had a liver biopsy for clinical indications at The Churchill Hospital, Oxford or University of Freiburg, Germany with no complications encountered from the 2nd pass liver biopsy sample for research purposes.

RESULTS:

EXPERIMENT 1: TO DEFINE THE ROLE/FUNCTION OF CD161+ IN HEALTHY CONTROLS

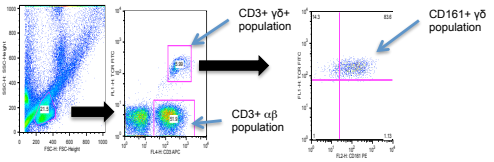

Figure 1: Whole blood staining showing gating strategy for CD3+/γδ cells per CD161 status. A mean of 82.1% (+/-12.8) of CD3+ γδ T-cells were CD161+ (a significantly higher % of CD161+ compared to the predominant CD3+ Alpha-Beta (αβ) population (p=0.01)

In healthy controls, when compared to CD161- subset:
-The CD161+ γδ subset expressed significantly more CXCR3, CCR6, TNF-α & IFN-γ (p=0.03, 0.01, 0.01 & 0.004 respectively).
-The CD161+ γδ subset also expressed significant levels of IL-18R (p=0.03) which has been show to be closely linked to CD161 expression on αβ T-cells (Billerbeck/Kang et al 2010).
-The CD161+ γδ subset expressed significant greater levels of Granzymes A, B & K along with Perforin (p=0.01,0.023,0.01 & 0.01 respectively) suggesting the CD161+ subsets are the major ones involved in cell killing. The CD161+ subset also preferentially expressed significant the FAS receptor (p=0.005).
-The CD161+ subset expressed significantly more Vδ2 when compared to the CD161- subset(p=0.01) with equivalent Vδ1/Vδ2 expression in the CD161- subsets (p=1).

Gene array analysis confirmed the CD161+ subset as a distinct subset. There were 409 genes identified on CD161+ subset (97 up-regulated on CD161+ and 312 down-regulated). After Bonoferroni correction, the list was reduced to 53 (6 up-regulated and 47 down regulated) .

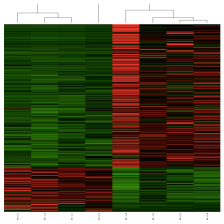

Figure 2: Heat map representation of the 409 genes post-filter of CD161+ v CD161- γδ T-cells.

Up-regulated Gene name	Function
KLRB1	Gene encoding for CD161
ADRB2	Gene encoding for B2-adrenergic receptor - a clinically important receptor in the body (ie "fight or flight" situations)
CCL20	Gene encoding for cytokine recruiting macrophages/neutrophils
CYTH1	Gene encoding for protein involved in Golgi structure/ regulation of ADP-ribosylation factor protein
COCQ	Gene encoding for Acetylcholinesterase (Ach-e) collagenic tail peptide. Mutations of gene related to end-plate Ach-e deficiency
MPP3	Gene encodes for membrane-associated proteins termed MAGUKs (membrane-associated guanylate kinase homologs which regulate cell proliferation, signaling pathways, and intracellular junctions.

Table 1: Up-regulated genes on CD161+ - γδ T-cells.

This data suggests a distinct phenotype of the CD161+ γδ T cells – the major subset of γδ T-cells in healthy donor's whole blood when compared to the CD161- subset . Furthermore, the CD161+ γδ T cells display a TH1-type profile compared to the TH17 profile of their CD161++ αβ counterparts (Billerbeck et al 2010). Gene array analysis confirmed the CD161+ γδ T cells had different gene signatures expressed compared to the CD161- subset. There were also some common gene signatures expressed when the CD161+ γδ T cells were compared to a CD161+(mid) αβ T-cell subset (data not shown).

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EXPERMIENT 2 : TO EXPLORE THE IMPACT OF CLD ON CD161+ γδ T-CELLS

Disease	CD3+ γδ	CD3+ αβ	CD161+ γδ	CD161+αβ
Healthy controls (leucocyte cone)	7.78(+/-1.03)	48.8(+/-8.78)	**48(+/-13.6)**	24.4(+/-6.63)
PSC	5.1(+/-7.97)	93.14(+/-8.30)	**81.48(+/-10.46)**	21.7%(+/-12.53)
PBC	0.81(+/-0.68)	96.1(+/-3.16)	**73.5(+/-14.6)**	16.92%(+/-6.16)
HCV	1.58(+/-0.89)	93.65(+/-3.18)	**22.02(+/-13.11)**	11.02(+/-6.27)
HBV	0.80(+/-0.33)	95.85(+/-1.09)	**39.21(+/-29.51)**	16.44(+/-5.66)

Table 2: *Breakdown of CD3+ γδ/αβ subsets per disease (and CD161+ subsets)*

There was a significant reduction in the total CD3+ γδ populations in HCV and HBV when compared to healthy controls (p=0.0006/0.0002 respectively). There was no significant difference between healthy controls and PSC (p=0.16) but a significant reduction in PBC (p=0.002)

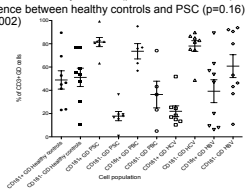

Figure 3: Breakdown of CD161+/- γδ T-cells per disease

On analysing the CD161+ subsets, compared to the healthy control leucocyte population, there was a significant increase in the CD161+ γδ T-cell subsets in patients with PSC and PBC. This was despite an overall reduction in total γδ T-cell populations in patients with PBC.
There was also a significant reduction (p=0.02) in patients CD161+ γδ T-cells with chronic hepatitis C (in keeping with an overall γδ T-cell reduction – however the CD161+ subset taken as a fraction of the γδ T-cells). There was however no significant reduction of the CD161+ γδ subset in chronic HBV (p=0.61).

This data suggests that in the periphery, chronic HCV and HBV infection not only impacts on the total CD3+ γδ T-cell populations, but as a % of the γδ T-cells the CD161+ γδ T-cell subsets reduce specifically. Also in PBC there was on overall reduction in the γδ T-cell frequencies, however an increase in the CD161+ γδ T-cell subset. Also in PSC there was an increase in CD161+ subset of γδ T-cells but no statistically significant reduction in the total CD3 γδ T-cell populations.

In HCV infections (compared to healthy controls):
-γδ cells were significantly more activated (CD38 & CD69)
-Expressed significantly more CXCR6 (p=0.0087 respectively).
-There was an increased expression of NKG2D in CD161+ subsets compared to CD161-in chronic HCV (p=0.0016).
-CD161+ γδ T-cells expressed significantly more Vδ1 (p=0.02), with a corresponding reduction in Vδ2. This was, however, not the case in the CD161- subsets. This was despite total reduction of CD161+ γδ T-cells in HCV (p=0.06).

To further explore the effects of HCV on CD161+ γδ, we isolated IHL from patients having liver biopsies, and paired PBMCs (n=21 – 9 HCV, 3 HBV, 7 NAFLD & 2 other).

From this data, we found an overall enrichment of γδ T-cells in the liver from the blood in all patients with CLD (p=0.01). However there was no specific enrichment in the liver of CD161+ γδ from the periphery when all CLD grouped together (p=0.13), compared to the CD161+ αβ (p<0.0001). Furthermore there was no enrichment from the periphery to the liver in HCV or HBV of the CD161+ γδ (p=0.783 & 0.3104 respectively). There was an enrichment of the CD161+γδ subset in other (non-viral)forms of CLD (p=0.0502).

CONCLUSIONS:
-We have shown that CD 161+ γδ T-cells are a distinct population of γδ T-cells that have a different function/phenotype when compared to their CD161- counterparts in healthy individuals. This CD161+ status is present in small numbers at birth but gained rapidly in early life (data not shown).
-In HCV infection the CD161+ γδ T-cells are reduced and alter in phenotype (with reduction of the physiological CD161+ Vδ 2 phenotype). In PSC/PBC the CD161+ subset are enriched in the periphery (despite an overall reduction of total CD3+ γδ T-cells in PBC).
-Our IHL data shows despite an increased enrichment of total γδ T-cells to the liver in all aetiologies of CLD, this is not specific to CD161+ subsets of γδ T-cells in HCV i.e. the reduction of this subset in HCV is not due to liver enrichment.
- In HCV infection, the virus reduces the total γδ population but preferentially affects the CD161+ subset of cells and may alter/attenuates their function. This needs explored in other CLD too with further studies ongoing

EASL IMMUNE MEDIATED LIVER INJURY, STRATFORD UPON AVON 2012

CD161+ γδ T-cells: defining their role in humans with or without Chronic Hepatitis C

Rajoriya N*, Willberg CB*, Seigel B**, Kang YH*, Philips-Hughes J***, Collier J****,
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**Department of Medicine, University of Freiburg, Germany
***Department of Radiology, Churchill Hospital, Oxford, UK
****Department of Gastroenterology, John Radcliffe Hospital, Oxford, UK

BACKGROUND:
Gamma-delta (γδ) T-cells fall at the interface between innate and adaptive immunity. Their role in liver inflammation is not clear but they have been identified in the livers of patients infected with chronic hepatitis C (HCV) (Agrati et al 2001) and enriched in the periphery in patients with PSC/PBC/AI hepatitis (Martins et al 1996).
CD161+ is a C-type lectin found on the surface of Natural Killer and T-cells in humans. HCV specific T-cells have been found to express CD161 with enrichment in the liver (Northfield et al 2008) and is associated with liver-homing T cell populations. However the specific functions of CD161+ γδ T-cells and their role in chronic hepatitis have not been established.

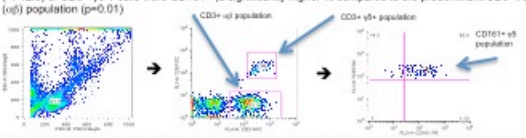
AIM:
To define the phenotype and function of CD161+ γδ T-cells compared to CD161- cells in humans without chronic liver disease (CLD) and to also assess the impact on these cells by chronic HCV infection.

METHODS:
The research was Fluorescence-activated cell sorting (FACS) -based with staining of: Whole blood/ Peripheral Blood Mononuclear cell (PBMC), cord blood samples, infant PBMCs and intra-hepatic lymphocytes (IHLs). All patients donating samples (or their guardians) consented, with local or national ethical approval for all recruitment.

RESULTS:

AIM 1: TO DEFINE THE ROLE/FUNCTION OF CD161+ v CD161- T-CELLS IN HEALTHY CONTROLS

FIGURE 1: Whole blood staining showing gating strategy for CD3+γδ cells and their CD161 status. A mean of 82.1% (+/-12.8) of CD3+ γδ T-cells were CD161+ (a significantly higher % compared to the predominant CD3+ Alpha-Beta (αβ) population (p=0.01)



On phenotypical/functional (via Intracellular Cytokine Staining) analysis, compared to the CD161- subset, the CD161+ γδ T-cells expressed significantly more CXCR3 (p=0.03) and CCR6 (p=0.01). The CD161 subset also expressed significantly more IL-18R (p=0.03) and Granzymes A, B, K and Perforin (p= 0.02, 0.02, 0.01,0.02 respectively) suggesting this cell subset in the major one involved in cell killing. On studying Vδ chain expression, the CD161+ subset expressed significantly more Vδ2(p=0.03) with greater Vδ1 expression in the CD161- subset(p=0.02).

This data suggests a distinct surface & intracellular phenotype of the CD161+ γδ T cells – the major subset of γδ T-cells in healthy donor's whole blood. CD161+ γδ T cells also appear to have a distinct function compared to the CD161- subset and display a TH1-type profile compared to the TH17 profile of their CD161+ αβ counterparts (Billerbeck et al 2010).

AIM 2: TO EXAMINE IF CD161 POSITIVITY IN γδ T-CELLS IS AN ACQUIRED OR INNATE PHENOTYPE
Using PBMCs isolated from cord blood, a 10-wk old cohort and adult healthy controls we explored differing CD161+/- status of γδ T-cells and their αβ counterparts.

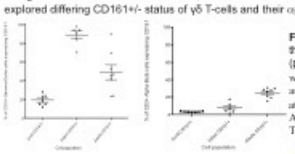
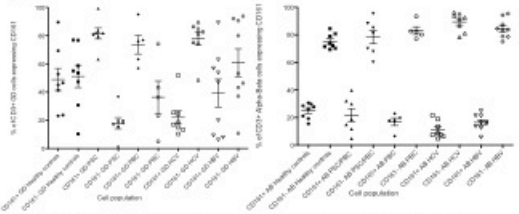


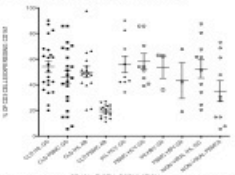
FIGURE 2: A rapid expansion of the CD161+ γδ T cells in the 10 weeks of life (p=0.005), with a reduction then in adult life (p=0.02). This however was not the case for the αβ CD161+ T-cells where the expansion was later in life (p<0.01 between 10 week olds and adults).This data suggests the γδ CD161+ phenotype is present at birth, but there is a massive expansion in the [illegible] Ag exposure, and that these cells predominate over the CD161 αβ T-cells in playing an early role in the immune system.

AIM 3: TO EXPLORE THE IMPACT OF CHRONIC LIVER DISEASE ON CD161+ γδ T-CELLS ISOLATED FROM PBMCS
In keeping with previous published data, we found reduced circulating levels of total γδ T-cell HCV and HBV infections, however a significant increase in patients with PSC/PBC.
FIGURE 3: On CD161+ subset analysis, only in chronic HCV was the CD161+γδ subset significantly reduced compared to healthy controls (p=0.01) but not in HBV (p=0.6). In PSC and PBC the CD161+ population was significantly increased compared to healthy controls/HBV/HCV. These findings were generally echoed in the αβ population apart from a reversal in the PSC/PBC cohort.



AIM 4: TO ASSESS THE IMPACT OF HCV ON CD161+ γδ T-CELLS IN BLOOD AND LIVER
When whole blood γδ populations in HCV were compared to healthy controls, the γδ cells were significantly more activated (CD38 & CD69) and expressed significantly more CXCR6 (p=0.0087). There was an increased expression of NKG2D in CD161+ subsets compared to CD161-in chronic HCV (p=0.002). When assessing for changes in expression of Vδ chains, in HCV there was expansion of Vδ1 expression in the CD161+ subset (p=0.02) with corresponding reduction in Vδ2. This was however not the case in CD161- subsets.

On paired IHL/PBMC date, there was an enrichment of γδ T-cells in the liver from the blood in all patients with CLD (p=0.01) but not specifically of CD161+ (p=0.13) γδ T-cells compared to the CD161+ αβ (p<0.0001). Furthermore there was no enrichment from the periphery to the liver in HBV/HCV but an enrichment of the CD161+ γδ subset in other (non-viral)forms of CLD (p=0.0502).



CONCLUSIONS:
We have shown that CD 161+ γδ T-cells are a distinct population of γδ T-cells that have a different phenotype compared to their CD161- counterparts. CD161+ status is present in small numbers at birth but is gained rapidly in early life. When compared to their CD161+αβ counterparts, CD161+ γδ T-cells display a more Th1-type profile, and are specifically reduced in HCV infection and not in other CLD. Also in HCV infection the phenotype of CD161+ γδ T-cell alters with a more activated profile than in healthy controls. Our IHL data shows an enrichment of γδ T-cells in the liver in all aetiologies of CLD, but this is not specific only to CD161+ subsets of γδ T-cells. Overall we observe in HCV infection, the virus reduces the total γδ population but preferentially affects the CD161+ subset of cells and alters their function, compared to other liver pathologies.

For enquiries, questions, collaborations please email: neil.rajoriya@gmail.com

[illegible]

AMERICAN ASSOCIATION FOR THE STUDY OF LIVER DISEASE (AASLD), SAN FRANCISCO 2011

CD161+ Gamma-Delta T-cells: defining their role in humans with or without Chronic Hepatitis C

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****Department of Medicine, University of Freiburg, Germany**
*****Department of Radiology, Churchill Hospital, Oxford, UK**
******Department of Gastroenterology, John Radcliffe Hospital, Oxford, UK**

UNIVERSITY OF OXFORD

BACKGROUND:

Gamma-delta (γδ) T-cells fall at the interface between innate and adaptive immunity. Their role in liver inflammation is not clear but they have been identified in the livers of patients infected with chronic hepatitis C (HCV) (Agrati et al 2001). CD161+ is a C-type lectin found on the surface of Natural Killer and T-cells in humans. HCV specific T-cells have been found to express CD161 with enrichment in the liver (Northfield et al 2008) and is associated with liver-homing T cell populations. However the specific functions of CD161+ γδ T-cells and their role in chronic hepatitis have not been established.

AIMS:

To define the phenotype and function of CD161+ γδ T-cells compared to CD161- cells in humans, and the assess the impact of HCV on these cells of HCV infection. Specific questions included:
Q1: Does CD161 positivity reflect a different subset of γδ T-cells with distinct phenotypic and functional characteristics?
Q2: Is CD161 positivity of γδ T-cells innate or acquired?
Q3: What type of γδ T-cell is found in the liver during chronic liver disease?
Q4: Is there an impact of HCV infection on the γδ T-cell populations specifically the CD161+ subset?

METHODS:

Whole blood/ Peripheral Blood Mononuclear cell (PBMC) staining was carried out on healthy controls, patients with HCV infection and other liver diseases; with subsequent Fluorescence-activated cell sorting (FACS) performed on all samples. Function of cells was assessed by Intracellular Cytokine staining (ICS). All samples were analysed via a FACScalibre ™ or LSRII Flow cytometers.
Cord blood samples, childhood PBMC samples and intra-hepatic lymphocyte (IHL) samples were all gained after local/international ethical approval. All patients donating IHLs to the study had a liver biopsy for clinical indications at The Churchill Hospital, Oxford or University of Freiburg, Germany with no complications encountered.

RESULTS:

AIM 1: TO DEFINE THE ROLE/FUNCTION OF CD161+ v CD161- T-CELLS.

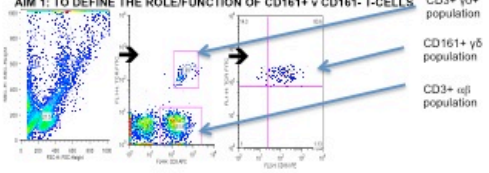


Figure 1: Whole blood staining showing gating strategy for CD3+/γδ cells and CD161 status. A mean of 82.1% (+/-12.8) of CD3+ γδ T-cells were CD161+ (a significantly higher % compared to the predominant CD3+ Alpha-Beta (αβ) population (p=0.01)

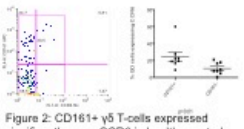


Figure 2: CD161+ γδ T-cells expressed significantly more CCR6 in healthy controls compared to their CD161- subset (p= 0.01)

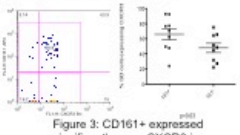


Figure 3: CD161+ expressed significantly more CXCR3 in controls v. CD161- subset (p=0.03)

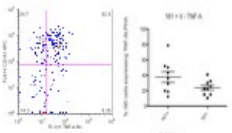


Figure 4: CD161 γδ T-cells expressed significantly more TNFα than CD161- subset (p=0.001)

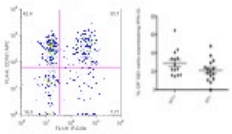


Figure 5: CD161 γδ T-cells expressed significantly more IF-γ than CD161- subset (p=0.004)

When compared to CD161- subset:
-The CD161+ subset also expressed significant levels of IL-18R (p=0.03) which has been show to be closely linked to CD161 expression on αβ T-cells (Billerbeck et al 2010).
-The CD161+ γδ expressed significant levels of Granzyme A, B and K along with Perforin suggesting these subsets are the major ones involved in cell killing.

When comparing expression of TCR Vd1 or Vd2 chains, of the CD161+ γδ T-cells majority of these expressed Vδ2 chain (75%) and overall the CD161+ subset expressed significantly more Vδ2 when compared to the CD161- subset.

This data suggests a distinct surface & intracellular phenotype of the CD161+ γδ T cells – the major subset of γδ T-cells in healthy donor's whole blood. CD161+ γδ T cells also appear to have a distinct function compared to the CD161- subset and display a TH1-type profile compared to the TH17 profile of their CD161+ αβ counterparts (Billerbeck et al 2010).

ACKNOWLEDGEMENTS:
-THE WELLCOME TRUST
-NIHR BIOMEDICAL RESEARCH PROGRAMME
-MRC UK
-JAMES MARTIN SCHOOL FOR THE 21ST CENTURY

AIM 2: TO EXAMINE IF CD161 POSITIVITY IN γδ T-CELLS IS AN AQCUIRED OR INNATE PHENOTYPE

Using PBMCs isolated from cord blood, a South African childhood cohort and adult healthy controls we explored differing CD161+/- status of γδ T-cells and their αβ counterparts.

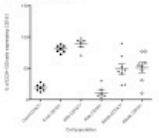


Figure 6: Shows CD161 on γδ T cells as age increases

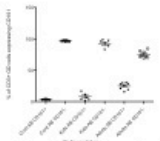


Figure 7: CD161 on αβ cells T-cells as comparison (as age increases)

These data suggest that although a small % of γδ express CD161+ from birth, positivity is acquired very quickly in the 1st few years of life till adulthood. This is compared to the CD161+ in αβ where there is not as marked expansion of the CD161+ subset and this appears to develop later.

AIM 3: TO EXPLORE THE IMPACT OF CHRONIC LIVER DISEASE ON CD161+ γδ T-CELLS ISOLATED FROM PBMCS

In keeping with previous published data, we found reduced circulating levels of total γδ T-cell populations in patients with viral hepatitides.

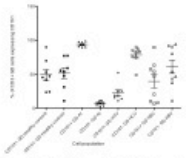


Figure 8: CD161 subset of γδ T-cells per Chronic liver disease (CLD) state

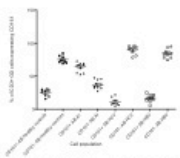


Figure 9: CD161 subset of αβ T-cells per per CLD state

On subset analysis, only in chronic HCV was the CD161+γδ subset significantly reduced compared to healthy controls. In Autoimmune (AI) liver disease the CD161+ population was significantly increased compared to healthy controls & other liver diseases. These findings are echoed in the αβ populations in liver diseases.

AIM 4: TO ASSESS THE IMPACT OF HCV ON CD161+ γδ T-CELLS IN BLOOD AND LIVER

When whole blood γδ populations in HCV were compared to healthy controls, the γδ cells were significantly more activated (CD38 & CD69) and expressed significantly more CXCR6 (p=0.0087). There was an increased expression of NKG2D in CD161+ subsets compared to CD161-in chronic HCV.

When assessing for changes in expression of Vδ chains, in HCV there was expansion of Vδ1 expression specifically so in the CD161+ subset (p=0.02) with corresponding reduction in Vδ2. This was however not the case in CD161- subsets.

We isolated IHL from patients having liver biopsies, and paired PBMCs (n=17).

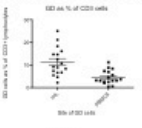


Figure 10: Enrichment of γδ T-cells from periphery to liver in CLD (p=0.01)

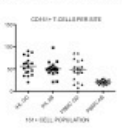


Figure 11: No specific enrichment of CD161+ γδ from blood to liver

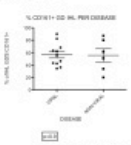


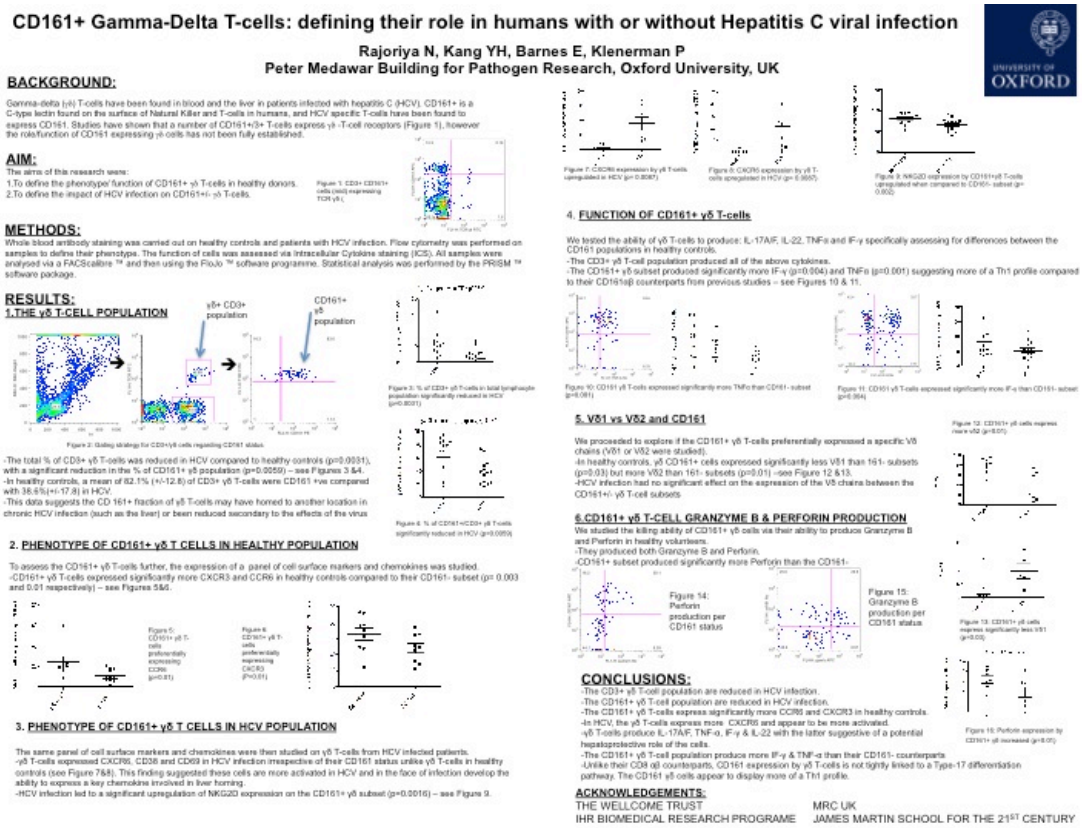
Figure 12: No difference in CD161+ γδ IHLs per disease state

Overall, we found an enrichment of γδ T-cells in the liver from the blood in patients with CLD. However there was no specific enrichment per CD161 status, or per viral v non-viral CLD. As previously conventional T cells show marked enrichment of CD161.

CONCLUSIONS:

We have shown that CD 161+ γδ T-cells are a distinct population of γδ T-cells that have a different phenotype compared to their CD161- counterparts. CD161+ status is present in small numbers at birth but is gained rapidly in early life. When compared to their CD161+αβ counterparts, CD161+ γδ T-cells display a more Th1-type profile, and are specifically reduced in HCV infection and not in other CLD. Also in HCV infection the phenotype of CD161+ γδ T-cell alters with a more activated profile than in healthy controls. Our IHL data shows an enrichment of γδ T-cells in the liver in all aetiologies of CLD, but this is not specific only to CD161+ subsets of γδ T-cells. Overall we observe in HCV infection, the virus reduces the total γδ population but preferentially affects the CD161+ subset of cells and alters their function, compared to other liver pathologies..

BRITISH ASSOCIATION FOR THE STUDY OF LIVER DISEASE (BASL), LONDON 2011



WORLD INTERNATIONAL GAMMA-DELTA CONFERENCE, KIEL, GERMANY 2010

APPENDIX 2: LIST OF 409 GENES FROM "FILTERED" LIST GAINED FROM EXTRACTION OF RNA COMPARING CD161+ γδ T-CELLS & CD161- γδ T-CELLS.

Gene name	Fold Change	p-value	BF p-value
KLRB1f	64.32418	7.24E-36	4.34E-31
KLF7	-14.51668	3.28E-22	1.97E-17
GRB10	-5.29576	2.46E-20	1.48E-15
SPINT2	-4.97917	2.46E-20	1.48E-15
lincRNA:chr10:6623494-6637944_R	-3.45988	5.12E-20	3.07E-15
ZNF365	-10.07362	1.78E-18	1.07E-13
AK5	-8.8468	1.87E-17	1.12E-12
AIF1	-6.41572	1.12E-16	6.72E-12
WNT7A	-9.39575	2.92E-16	1.75E-11
PECAM1	-3.54409	1.65E-15	9.90E-11
PECAM1	-3.05985	2.90E-15	1.74E-10
TCEA3	-6.53897	2.95E-15	1.77E-10
KCTD12	-16.63801	5.28E-15	3.17E-10
VIPR1	-3.29631	5.70E-15	3.42E-10
ADRB2	2.66527	2.72E-14	1.63E-09
INF2	-5.7716	6.89E-14	4.13E-09
NOG	-22.15534	8.46E-14	5.08E-09
KLHL29	-2.90399	1.22E-13	7.32E-09
CACNA1I	-11.89334	3.13E-13	1.88E-08
ZNF365	-7.70655	2.92E-12	1.75E-07
MAL	-14.09853	7.05E-12	4.23E-07
PRKAR2B	-2.45258	9.45E-12	5.67E-07
BEX1	-7.337	9.13E-11	5.48E-06
CDC42BPB	-2.6155	1.09E-10	6.54E-06
BEND5	-15.82532	2.17E-10	1.30E-05
KANK1	-4.48669	2.39E-10	1.43E-05
NGFRAP1	-9.99433	8.74E-10	5.24E-05
FAM164A	-7.12951	1.15E-09	6.90E-05
NBEA	-5.71821	2.49E-09	1.49E-04
PVT1	-2.15998	2.78E-09	1.67E-04
INF2	-4.37732	4.44E-09	2.66E-04
FLJ45983	-6.12813	5.34E-09	3.20E-04
PASK	-8.96053	5.55E-09	3.33E-04
FBLN2	-8.43219	8.82E-09	5.29E-04
KANK1	-11.96443	1.37E-08	8.22E-04
lincRNA:chr18:68297754-68318093_R	-14.12603	1.63E-08	9.78E-04

PCSK5	-3.7059	2.75E-08	1.65E-03
CPAMD8	-4.97185	5.70E-08	3.42E-03
PECAM1	-2.79547	7.13E-08	4.28E-03
LRRC8B	-2.22473	7.50E-08	4.50E-03
CCL20	9.56603	7.88E-08	4.73E-03
CYTH3	2.37096	1.15E-07	6.90E-03
LRRC8B	-2.0662	1.92E-07	1.15E-02
ACVR2A	-2.72423	2.30E-07	1.38E-02
SESN3	-3.78098	3.18E-07	1.91E-02
MDS2	-6.4935	3.56E-07	2.14E-02
MYB	-5.34642	4.58E-07	2.75E-02
COLQ	3.56566	5.10E-07	3.06E-02
CR2	-14.866	5.59E-07	3.35E-02
MPP3	3.12469	5.92E-07	3.55E-02
lincRNA:chr7:79099906-79100510_F	-8.97082	6.20E-07	3.72E-02
KCNQ1	-5.44691	6.35E-07	3.81E-02
ZP1	-6.35382	7.39E-07	4.43E-02
CDR2	-2.18459	8.38E-07	5.03E-02
lincRNA:chr14:96555696-96556712_F	-17.28899	9.54E-07	5.72E-02
MB21D2	-5.70448	1.02E-06	6.12E-02
FAAH2	-3.57845	1.50E-06	9.00E-02
FHIT	-7.01578	1.58E-06	9.48E-02
TMIE	-4.78042	1.80E-06	1.08E-01
LOC100128252	-14.53508	1.81E-06	1.09E-01
CDCA7L	-4.77938	1.98E-06	1.19E-01
IQSEC2	2.02977	2.45E-06	1.47E-01
CRB3	-2.37513	3.03E-06	1.82E-01
DENND5A	-2.02524	3.15E-06	1.89E-01
EPHX2	-2.67111	3.44E-06	2.06E-01
ENST00000512129	-9.7622	3.46E-06	2.08E-01
CFP	-2.85913	3.53E-06	2.12E-01
GPC2	-2.23719	3.84E-06	2.30E-01
RAB34	-4.5616	4.61E-06	2.77E-01
ACCN2	-7.25086	5.15E-06	3.09E-01
LOC100130865	3.0009	5.20E-06	3.12E-01
HSPG2	-4.30086	5.27E-06	3.16E-01
PLXNA4	-4.66571	5.38E-06	3.23E-01
PHGDH	-3.74009	5.87E-06	3.52E-01
TSPAN33	-2.33934	6.13E-06	3.68E-01
ZWINT	-2.59923	6.17E-06	3.70E-01
HTR7P1	-2.09118	6.26E-06	3.76E-01
DIRC3	-8.63851	6.31E-06	3.79E-01
LEPREL2	2.00505	6.50E-06	3.90E-01
B3GNT8	2.56255	6.60E-06	3.96E-01
CXCL2	-8.69003	6.66E-06	4.00E-01

C16orf75	-7.69534	7.50E-06	4.50E-01
DIP2B	-2.1251	8.20E-06	4.92E-01
lincRNA:chr13:30510667-30524625_F	-3.9294	8.26E-06	4.96E-01
GP1BB	-9.16597	8.41E-06	5.05E-01
CR2	-8.64149	8.76E-06	5.26E-01
RCAN3	-2.40415	9.14E-06	5.48E-01
ZC3H12D	-2.56822	9.86E-06	5.92E-01
CD8B	-7.10319	1.00E-05	6.00E-01
ARRDC4	-6.62767	1.00E-05	6.00E-01
IL6R	-6.0671	1.00E-05	6.00E-01
IL18RAP	3.63652	1.00E-05	6.00E-01
LOC641518	-12.75298	2.00E-05	1.20E+00
ANK1	-9.70869	2.00E-05	1.20E+00
ACTN1	-9.01087	2.00E-05	1.20E+00
ZNF80	-5.9793	2.00E-05	1.20E+00
ENST00000536986	-4.47364	2.00E-05	1.20E+00
TXNRD3	-3.87342	2.00E-05	1.20E+00
CD248	-3.81965	2.00E-05	1.20E+00
AGMAT	-2.40834	2.00E-05	1.20E+00
A_33_P3216621	4.32409	2.00E-05	1.20E+00
SGPP2	-7.34943	3.00E-05	1.80E+00
CYSLTR1	-3.79875	3.00E-05	1.80E+00
X58330	-3.22625	3.00E-05	1.80E+00
SERINC5	-2.79921	3.00E-05	1.80E+00
A_24_P230057	-2.71628	4.00E-05	2.40E+00
ENST00000541447	-4.83858	5.00E-05	3.00E+00
KCNH2	-4.75084	5.00E-05	3.00E+00
ARHGEF10	-3.24126	5.00E-05	3.00E+00
PLCL1	-3.07477	5.00E-05	3.00E+00
TRIM58	-2.31874	5.00E-05	3.00E+00
IL18RAP	3.42817	5.00E-05	3.00E+00
KCTD12	-9.45734	6.00E-05	3.60E+00
KRT1	-6.48379	6.00E-05	3.60E+00
lincRNA:chr2:74193717-74210392_R	-3.90015	6.00E-05	3.60E+00
lincRNA:chr11:82789751-82790485_R	2.06434	6.00E-05	3.60E+00
BC037972	6.41639	7.00E-05	4.20E+00
FAM19A2	-14.02457	8.00E-05	4.80E+00
WNT7B	-6.66545	8.00E-05	4.80E+00
ENST00000450721	-3.61674	8.00E-05	4.80E+00
COL5A1	2.28729	8.00E-05	4.80E+00
lincRNA:chr11:104771490-104780740_R	2.29719	8.00E-05	4.80E+00
LOC100131662	-12.52592	9.00E-05	5.40E+00
CLC	-6.8611	9.00E-05	5.40E+00
USP6NL	-5.52878	9.00E-05	5.40E+00

TLE1	2.45245	9.00E-05	5.40E+00
TRIP10	2.48743	9.00E-05	5.40E+00
LAYN	-9.80777	1.00E-04	6.00E+00
RBM20	-7.44797	1.00E-04	6.00E+00
lincRNA:chr8:32853730-32902348_F	-3.87106	1.00E-04	6.00E+00
TNFRSF10D	-5.03448	0.00011	6.60E+00
EPHB4	-4.19257	0.00011	6.60E+00
THBS1	2.81117	0.00011	6.60E+00
LAMP3	-2.45717	0.00012	7.20E+00
CLEC11A	-6.65056	0.00013	7.80E+00
DKK3	-2.20097	0.00013	7.80E+00
LAMA2	3.91962	0.00013	7.80E+00
lincRNA:chr8:129108772-129113316_F	-2.04415	0.00014	8.40E+00
SORBS2	-6.20762	0.00015	9.00E+00
LARGE	-2.8971	0.00015	9.00E+00
ANKRD57	-4.11979	0.00016	9.60E+00
LMO7	-3.26442	0.00018	1.08E+01
lincRNA:chr8:129015583-129108805_F	-2.04515	0.00018	1.08E+01
ZC3H12D	-4.02378	0.00019	1.14E+01
IKZF2	-3.69881	0.00019	1.14E+01
KRT18	-2.94157	0.00019	1.14E+01
FAM7A1	2.61014	0.00019	1.14E+01
ANK1	-9.26477	0.00021	1.26E+01
APBA2	-4.19371	0.00021	1.26E+01
SLC22A23	-2.34588	0.00021	1.26E+01
SYTL2	2.51507	0.00021	1.26E+01
BCL2	-2.00476	0.00022	1.32E+01
LIMS2	-4.71416	0.00023	1.38E+01
FOXJ1	-4.62477	0.00023	1.38E+01
KRT72	-7.67685	0.00024	1.44E+01
PLXNB2	-8.08336	0.00025	1.50E+01
GRB10	-5.31776	0.00025	1.50E+01
CTLA4	-2.6444	0.00025	1.50E+01
ADAMTS4	-5.26849	0.00026	1.56E+01
TMEM171	3.36485	0.00027	1.62E+01
HEPH	-2.84546	0.00029	1.74E+01
FAM172BP	5.23987	0.00029	1.74E+01
SEMA5A	-3.6247	3.00E-04	1.80E+01
A_33_P3416196	2.78092	3.00E-04	1.80E+01
LOC439949	-2.36403	0.00032	1.92E+01
MAP3K1	-2.22641	0.00032	1.92E+01
ABCB1	2.0693	0.00032	1.92E+01
ABCB1	2.40902	0.00032	1.92E+01
EDN1	-2.18373	0.00035	2.10E+01

ARHGEF4	-2.46205	0.00037	2.22E+01
lincRNA:chr8:32785498-32902078_F	-3.66396	0.00039	2.34E+01
NELL2	-3.32387	0.00039	2.34E+01
C2orf89	-3.12851	0.00039	2.34E+01
MS4A1	2.06562	0.00039	2.34E+01
MME	-6.02433	4.00E-04	2.40E+01
E2F5	-3.33095	4.00E-04	2.40E+01
lincRNA:chr11:17370980-17371527_F	-2.35141	4.00E-04	2.40E+01
lincRNA:chr21:44751672-44819297_R	2.0703	0.00043	2.58E+01
TIMP2	-4.47009	0.00044	2.64E+01
TNS4	2.49658	0.00044	2.64E+01
CBX2	-5.49179	0.00045	2.70E+01
SPATA6	-3.46528	0.00045	2.70E+01
lincRNA:chr7:105525814-105538089_F	-2.08321	0.00047	2.82E+01
ENST00000426318	-4.09836	0.00048	2.88E+01
FZD6	-4.94768	5.00E-04	3.00E+01
NCRNA00173	-3.70556	5.00E-04	3.00E+01
LY96	-5.2306	0.00051	3.06E+01
LTK	3.2452	0.00053	3.18E+01
GCNT4	-3.08122	0.00054	3.24E+01
lincRNA:chr21:29817053-30047175_R	3.02618	0.00055	3.30E+01
CYP4F22	4.76812	0.00055	3.30E+01
INMT	-2.919	0.00056	3.36E+01
PLXND1	2.34094	0.00056	3.36E+01
NT5E	-3.07926	0.00059	3.54E+01
LOC100131541	-3.70345	6.00E-04	3.60E+01
PPARGC1B	-2.01099	6.00E-04	3.60E+01
ENST00000493544	-2.56549	0.00062	3.72E+01
CCR4	-5.31817	0.00064	3.84E+01
SEP-10	-5.58362	0.00069	4.14E+01
LRRN3	-9.36969	7.00E-04	4.20E+01
HLA-DOA	-2.27838	0.00071	4.26E+01
LOC100128252	-8.29684	0.00073	4.38E+01
SLC9A7	-2.41376	0.00074	4.44E+01
TMEM45B	-10.18063	0.00077	4.62E+01
TSHZ2	-8.06025	0.00078	4.68E+01
ENST00000510551	-3.80026	0.00078	4.68E+01
TOX	-2.25405	0.00078	4.68E+01
CACNA2D2	2.13052	8.00E-04	4.80E+01
DSEL	-2.40477	0.00083	4.98E+01
ACVR1	-2.25162	0.00089	5.34E+01
KLF5	-3.1611	9.00E-04	5.40E+01

RFPL2	-3.91816	0.00094	5.64E+01
TIAM1	-3.08198	0.00095	5.70E+01
PTPRK	-4.4411	0.00096	5.76E+01
CDCA7L	-3.90445	0.00097	5.82E+01
ENST00000390357	-4.69774	0.00099	5.94E+01
DNAH11	-3.81405	0.00099	5.94E+01
RASGRP3	-2.19581	0.00099	5.94E+01
ZNF415	-8.21277	0.00102	6.12E+01
LEF1	-4.62937	0.00103	6.18E+01
GPR55	-4.68537	0.00105	6.30E+01
DLL3	-4.42168	0.00105	6.30E+01
CR1L	-3.95503	0.00105	6.30E+01
AFF3	-2.45852	0.00107	6.42E+01
ITPKA	2.47005	0.00107	6.42E+01
lincRNA:chr4:56803193-56814239_R	-2.23601	0.00109	6.54E+01
TLR2	-3.23359	0.0011	6.60E+01
AUTS2	2.07791	0.0011	6.60E+01
A_33_P3339696	-2.85275	0.00112	6.72E+01
LOC641510	-2.79211	0.00116	6.96E+01
PCSK5	-2.60882	0.00116	6.96E+01
KATNAL2	-2.17452	0.00119	7.14E+01
PHACTR2	2.03592	0.00119	7.14E+01
AGPAT4	2.05183	0.00119	7.14E+01
DCHS1	-3.00924	0.0012	7.20E+01
SLC6A10P	5.53656	0.00122	7.32E+01
C2orf89	-3.33068	0.00126	7.56E+01
PACSIN1	2.84102	0.00127	7.62E+01
ZNF532	3.483	0.00129	7.74E+01
lincRNA:chr9:123606154-123611279_R	-3.0705	0.00135	8.10E+01
lincRNA:chr15:67339521-67346496_R	2.79702	0.00135	8.10E+01
IKZF2	-3.58926	0.00147	8.82E+01
BLK	3.052	0.0015	9.00E+01
P2RY14	3.91504	0.00151	9.06E+01
MOXD1	-7.44463	0.00159	9.54E+01
ARHGEF11	-5.57934	0.00159	9.54E+01
DLL1	-3.94184	0.00163	9.78E+01
VLDLR	3.65034	0.00165	9.90E+01
ENST00000419160	-3.50313	0.00169	1.01E+02
HMOX1	-2.65525	0.0017	1.02E+02
lincRNA:chr6:3637801-3719951_R	-2.15597	0.00177	1.06E+02
MALL	-11.6019	0.0018	1.08E+02
LOC100509011	-2.65428	0.00185	1.11E+02
A_33_P3324680	7.5169	0.00186	1.12E+02
A_33_P3361217	4.4742	0.00187	1.12E+02

C5orf13	-5.41453	0.0019	1.14E+02
AEBP1	-4.21814	0.00193	1.16E+02
A_33_P3282988	-2.12745	0.00195	1.17E+02
IL18R1	2.61051	0.00195	1.17E+02
DKK3	-2.87759	0.00198	1.19E+02
MYOM2	-3.20536	0.00199	1.19E+02
PERP	2.36978	0.00199	1.19E+02
ZWINT	-3.12969	0.00201	1.21E+02
ARHGAP32	-3.05334	0.00209	1.25E+02
RBM11	-2.70464	0.00209	1.25E+02
HAPLN3	-2.8409	0.00212	1.27E+02
HHEX	-4.56684	0.00226	1.36E+02
PLEKHH2	-4.31806	0.00226	1.36E+02
lincRNA:chr9:132260617-132265944_F	3.84924	0.00233	1.40E+02
lincRNA:chr3:42056396-42105521_R	-2.76202	0.00234	1.40E+02
CHST2	-5.55443	0.00236	1.42E+02
SNTG2	-4.5603	0.00236	1.42E+02
RCAN2	-4.71112	0.00237	1.42E+02
CES4A	-2.11958	0.00239	1.43E+02
C9orf53	2.47181	0.0025	1.50E+02
LOC729680	-3.14467	0.00251	1.51E+02
PTCH1	2.18179	0.00251	1.51E+02
ENST00000444775	2.09435	0.0026	1.56E+02
lincRNA:chr2:196188105-196512905_F	-2.58276	0.00262	1.57E+02
B3GALT2	11.08968	0.00263	1.58E+02
lincRNA:chr14:66896649-66896875_R	-4.34115	0.00266	1.60E+02
PTCH1	2.10859	0.00273	1.64E+02
CPNE5	2.21248	0.00279	1.67E+02
ZDHHC23	-2.00819	0.00283	1.70E+02
RNF157	-3.23231	0.00284	1.70E+02
HOXA11-AS1	5.23368	0.00293	1.76E+02
RORA	2.00842	0.00295	1.77E+02
lincRNA:chr8:43518693-43531493_R	-5.20466	0.00297	1.78E+02
MGC15705	-2.81363	0.00302	1.81E+02
TYRO3	2.50261	0.00302	1.81E+02
DDX25	-10.55716	0.00308	1.85E+02
MRC2	-2.76152	0.00316	1.90E+02
PLEKHG3	2.142	0.00316	1.90E+02
PRKAR2B	-3.20834	0.00317	1.90E+02
PDZD7	9.80099	0.00323	1.94E+02
X13953	-2.53542	0.00331	1.99E+02
DSCAML1	-4.01435	0.00332	1.99E+02
SDK2	-3.27799	0.00332	1.99E+02

GP5	-6.37488	0.00338	2.03E+02
PTPRK	-3.01633	0.0034	2.04E+02
PRKCA	-2.27724	0.0034	2.04E+02
PLCB1	2.70977	0.00341	2.05E+02
NCRNA00299	2.71123	0.00347	2.08E+02
ZNF618	12.80783	0.0035	2.10E+02
BACH2	-2.3204	0.00353	2.12E+02
SVOPL	-3.42048	0.00364	2.18E+02
FAM83E	-2.29319	0.00367	2.20E+02
lincRNA:chr2:203179905-203194480_F	-4.05529	0.0037	2.22E+02
lincRNA:chr10:4944575-4977950_F	-7.8166	0.00378	2.27E+02
INF2	-2.5875	0.0038	2.28E+02
lincRNA:chr19:56806434-56816195_F	-9.83277	0.00381	2.29E+02
AB529250	-7.96879	0.00383	2.30E+02
ENST00000528029	3.97521	0.00383	2.30E+02
ALOX5	-3.43447	0.00384	2.30E+02
lincRNA:chr1:852362-859037_R	2.51008	0.00384	2.30E+02
SERPINF1	-2.02595	0.00388	2.33E+02
ENST00000404098	2.2111	0.00399	2.39E+02
MAOA	-3.84862	0.00409	2.45E+02
ENST00000425777	-2.66692	0.00413	2.48E+02
MSL3	-3.35738	0.00415	2.49E+02
lincRNA:chr7:19951575-20160200_F	2.20924	0.00415	2.49E+02
CCR1	3.87797	0.00419	2.51E+02
RNF157	-2.15972	0.00421	2.53E+02
MEGF6	-2.00007	0.00422	2.53E+02
ZNF532	2.93992	0.00423	2.54E+02
ENST00000422143	-3.773	0.00425	2.55E+02
ENST00000390394	-2.04495	0.00427	2.56E+02
EDN3	-2.72018	0.00431	2.59E+02
TSPAN15	3.72847	0.00441	2.65E+02
CDH2	2.24548	0.00451	2.71E+02
CD8B	-4.03043	0.00453	2.72E+02
lincRNA:chr4:47840518-47846712_F	5.10335	0.00455	2.73E+02
lincRNA:chr11:110066285-110066727_R	-4.08899	0.00457	2.74E+02
IFNGR2	-4.02803	0.00469	2.81E+02
GYPE	-3.53269	0.00478	2.87E+02
AURKC	-2.48565	0.00482	2.89E+02
FLJ35390	-3.006	0.00486	2.92E+02
lincRNA:chr2:213703255-213723530_F	9.53894	0.00512	3.07E+02
PLEKHG3	2.1082	0.00513	3.08E+02
lincRNA:chr15:74779247-74805647_R	7.68175	0.00514	3.08E+02

A_33_P3231319	2.6641	0.00518	3.11E+02
A_24_P256063	-2.3156	0.00519	3.11E+02
EDA	-2.50061	0.00521	3.13E+02
lincRNA:chr8:138331518-138433693_F	7.78379	0.00525	3.15E+02
PHC1	-7.76505	0.0053	3.18E+02
MYO1E	-6.23001	0.00533	3.20E+02
lincRNA:chr10:119344760-119356235_R	2.80347	0.00535	3.21E+02
PLD1	4.13159	0.00545	3.27E+02
lincRNA:chr6:111858507-111866738_R	3.80206	0.0055	3.30E+02
LOC100128668	2.73951	0.00551	3.31E+02
ENST00000508741	-2.11933	0.00556	3.34E+02
GAS6	-4.42179	0.00557	3.34E+02
SPIN3	-3.95963	0.0057	3.42E+02
A_33_P3356413	3.75144	0.0057	3.42E+02
LOC619207	2.99192	0.00578	3.47E+02
IL18R1	2.39579	0.0061	3.66E+02
CNN3	-5.11674	0.00632	3.79E+02
C14orf132	-8.793	0.0064	3.84E+02
lincRNA:chr8:129008804-129108894_F	-3.41439	0.00646	3.88E+02
MYH7	5.78991	0.00651	3.91E+02
lincRNA:chr14:96507172-96661947_F	-6.72447	0.00656	3.94E+02
lincRNA:chr5:38025798-38184036_R	2.01418	0.00658	3.95E+02
RTP1	2.05281	0.00665	3.99E+02
FLJ22536	-3.86504	0.00666	4.00E+02
NPAS2	-2.21943	0.00672	4.03E+02
ZNF662	-3.94323	0.00674	4.04E+02
A_33_P3274710	4.30746	0.00674	4.04E+02
RIMKLB	-3.09093	0.00701	4.21E+02
C2orf40	-2.42955	0.00701	4.21E+02
lincRNA:chr13:24717800-24728575_R	-2.86429	0.00707	4.24E+02
lincRNA:chr20:31438639-31447464_R	-2.37912	0.00716	4.30E+02
PKIA	-2.58484	0.00725	4.35E+02
KIAA1958	-2.19715	0.00725	4.35E+02
ZAR1L	-2.95071	0.00734	4.40E+02
AP1S3	2.4257	0.00735	4.41E+02
AK123797	-4.22919	0.00748	4.49E+02
LRRC32	-2.9625	0.00751	4.51E+02
KIAA0408	-2.139	0.00751	4.51E+02
AM408129	-4.4369	0.00754	4.52E+02
PLXNA4	-3.05035	0.00756	4.54E+02

PXDN	-4.98696	0.00758	4.55E+02
HCRP1	-2.41426	0.00769	4.61E+02
THNSL2	-2.8902	0.0077	4.62E+02
IQSEC3	-2.94734	0.00792	4.75E+02
PPARGC1B	-2.02612	0.00798	4.79E+02
LAT2	-2.79712	0.00807	4.84E+02
lincRNA:chr3:149102101-149103587_R	2.27363	0.0081	4.86E+02
MFGE8	-2.02291	0.0083	4.98E+02
NT5E	-3.46588	0.00837	5.02E+02
AK128421	-3.00087	0.00844	5.06E+02
C8orf83	2.91678	0.00851	5.11E+02
lincRNA:chr8:128807043-128820943_F	-4.06848	0.00865	5.19E+02
lincRNA:chr12:79972444-79982744_R	-2.04414	0.00878	5.27E+02
A_33_P3262028	-2.83083	0.00899	5.39E+02
AGAP1	2.10888	0.00899	5.39E+02
EDA	-3.54402	0.00905	5.43E+02
VSIG1	-3.74082	0.00924	5.54E+02
CDC42EP3	2.15738	0.00924	5.54E+02
LRP12	3.28293	0.00927	5.56E+02
SNORA65	19.81467	0.00934	5.60E+02
lincRNA:chr22:25423157-25423764_F	-8.35917	0.00955	5.73E+02
LAMC3	-2.86666	0.0096	5.76E+02
CD8B	-3.28531	0.00969	5.81E+02
ZP3	-2.15681	0.00978	5.87E+02
lincRNA:chr5:115631476-115644551_F	-2.03853	0.00978	5.87E+02
ENST00000390434	-3.18719	0.0098	5.88E+02
STXBP1	-2.97479	0.00982	5.89E+02
F2RL1	-4.91431	0.0099	5.94E+02
lincRNA:chr1:90090112-90097487_R	-2.39235	0.00994	5.96E+02

APPENDIX 3: FULL LIST OF GENES GAINED FROM EXTRACTION OF RNA COMPARING CD161+ γδ T-CELLS & CD161- γδ T-CELLS.

Name	Log(Ratio)	Fold Change	P-value	BF p-value
KLRB1f	1.80837	64.32418	7.24E-36	4.34E-31
SLC22A23	-1.12955	-13.47573	1.00E-34	6.00E-30
DSC1	-0.99906	-9.97848	2.52E-32	1.51E-27
CR1	-0.92376	-8.38987	1.13E-24	6.76E-20
KLF7	-1.16187	-14.51668	3.28E-22	1.97E-17
GRB10	-0.72393	-5.29576	2.46E-20	1.48E-15
SPINT2	-0.69716	-4.97917	2.46E-20	1.48E-15
UNKNOWN	-0.53906	-3.45988	5.12E-20	3.07E-15
ZNF365	-1.00319	-10.07362	1.78E-18	1.07E-13
AK5	-0.94679	-8.8468	1.87E-17	1.12E-12
AIF1	-0.80725	-6.41572	1.12E-16	6.73E-12
WNT7A	-0.97293	-9.39575	2.92E-16	1.75E-11
PECAM1	-0.5495	-3.54409	1.65E-15	9.92E-11
PECAM1	-0.4857	-3.05985	2.90E-15	1.74E-10
TCEA3	-0.81551	-6.53897	2.95E-15	1.77E-10
KCTD12	-1.2211	-16.63801	5.28E-15	3.17E-10
VIPR1	-0.51803	-3.29631	5.70E-15	3.42E-10
ADRB2	0.42574	2.66527	2.72E-14	1.63E-09
INF2	-0.7613	-5.7716	6.89E-14	4.13E-09
NOG	-1.34548	-22.15534	8.46E-14	5.08E-09
KLHL29	-0.46299	-2.90399	1.22E-13	7.33E-09
SULT1B1	-0.93471	-8.60418	2.60E-13	1.56E-08
CACNA1I	-1.0753	-11.89334	3.13E-13	1.88E-08
ZNF365	-0.88686	-7.70655	2.92E-12	1.75E-07
MAL	-1.14917	-14.09853	7.05E-12	4.23E-07
PRKAR2B	-0.38962	-2.45258	9.45E-12	5.67E-07
C6orf105	-1.02145	-10.50625	3.26E-11	1.95E-06
BEX1	-0.86552	-7.337	9.13E-11	5.48E-06
CDC42BPB	-0.41755	-2.6155	1.09E-10	6.52E-06
BEND5	-1.19935	-15.82532	2.17E-10	1.30E-05
KANK1	-0.65193	-4.48669	2.39E-10	1.43E-05
KIAA0125	-0.92432	-8.40077	5.68E-10	3.41E-05
NGFRAP1	-0.99975	-9.99433	8.74E-10	5.24E-05
SLC40A1	-1.03824	-10.92048	1.15E-09	6.88E-05
FAM164A	-0.85306	-7.12951	1.15E-09	6.92E-05
PLXDC2	0.8047	6.37829	1.77E-09	1.06E-04

NBEA	-0.75726	-5.71821	2.49E-09	1.49E-04
PVT1	-0.33445	-2.15998	2.78E-09	1.67E-04
INF2	-0.64121	-4.37732	4.44E-09	2.66E-04
FLJ45983	-0.78733	-6.12813	5.34E-09	3.20E-04
PASK	-0.95233	-8.96053	5.55E-09	3.33E-04
FBLN2	-0.92594	-8.43219	8.82E-09	5.29E-04
KANK1	-1.07789	-11.96443	1.37E-08	8.20E-04
UNKNOWN	-1.15002	-14.12603	1.63E-08	9.80E-04
PCSK5	-0.56889	-3.7059	2.75E-08	1.65E-03
VSIG4	-1.05267	-11.28941	5.06E-08	3.03E-03
UNKNOWN	-1.14964	-14.1138	5.53E-08	3.32E-03
CPAMD8	-0.69652	-4.97185	5.70E-08	3.42E-03
SDK2	-1.02019	-10.47585	5.82E-08	3.49E-03
UNKNOWN	-0.93364	-8.58297	6.90E-08	4.14E-03
PECAM1	-0.44645	-2.79547	7.13E-08	4.28E-03
LRRC8B	-0.34728	-2.22473	7.50E-08	4.50E-03
CCL20	0.98073	9.56603	7.88E-08	4.73E-03
CYTH3	0.37492	2.37096	1.15E-07	6.90E-03
CA6	-0.45066	-2.82265	1.32E-07	7.93E-03
SLC16A10	-0.95941	-9.1078	1.55E-07	9.30E-03
LRRC8B	-0.31517	-2.0662	1.92E-07	1.15E-02
ACVR2A	-0.43524	-2.72423	2.30E-07	1.38E-02
SESN3	-0.5776	-3.78098	3.18E-07	1.91E-02
MDS2	-0.81248	-6.4935	3.56E-07	2.13E-02
MYB	-0.72806	-5.34642	4.58E-07	2.75E-02
COLQ	0.55214	3.56566	5.10E-07	3.06E-02
CR2	-1.17219	-14.866	5.59E-07	3.36E-02
MPP3	0.49481	3.12469	5.92E-07	3.55E-02
UNKNOWN	-0.95283	-8.97082	6.20E-07	3.72E-02
KCNQ1	-0.73615	-5.44691	6.35E-07	3.81E-02
ZP1	-0.80303	-6.35382	7.39E-07	4.44E-02
CDR2	-0.33937	-2.18459	8.38E-07	5.03E-02
UNKNOWN	-1.23777	-17.28899	9.54E-07	5.73E-02
MB21D2	-0.75622	-5.70448	1.02E-06	6.10E-02
FAAH2	-0.55369	-3.57845	1.50E-06	8.97E-02
TUBB6	1.13696	13.70764	1.53E-06	9.16E-02
FHIT	-0.84608	-7.01578	1.58E-06	9.46E-02
TMIE	-0.67947	-4.78042	1.80E-06	1.08E-01
LOC100128252	-1.16242	-14.53508	1.81E-06	1.09E-01
CDCA7L	-0.67937	-4.77938	1.98E-06	1.19E-01
CD200	-0.63276	-4.29299	2.12E-06	1.27E-01
IQSEC2	0.30745	2.02977	2.45E-06	1.47E-01
CRB3	-0.37569	-2.37513	3.03E-06	1.82E-01
DENND5A	-0.30648	-2.02524	3.15E-06	1.89E-01
EPHX2	-0.42669	-2.67111	3.44E-06	2.06E-01
UNKNOWN	-0.98955	-9.7622	3.46E-06	2.07E-01

CFP	-0.45623	-2.85913	3.53E-06	2.12E-01
GPC2	-0.3497	-2.23719	3.84E-06	2.31E-01
RAB34	-0.65912	-4.5616	4.61E-06	2.77E-01
ACCN2	-0.86039	-7.25086	5.15E-06	3.09E-01
LOC100130865	0.47725	3.0009	5.20E-06	3.12E-01
ZNF285	-0.86169	-7.27265	5.20E-06	3.12E-01
HSPG2	-0.63356	-4.30086	5.27E-06	3.16E-01
PLXNA4	-0.66892	-4.66571	5.38E-06	3.23E-01
PHGDH	-0.57288	-3.74009	5.87E-06	3.52E-01
TSPAN33	-0.36909	-2.33934	6.13E-06	3.68E-01
ZWINT	-0.41485	-2.59923	6.17E-06	3.70E-01
HTR7P1	-0.32039	-2.09118	6.26E-06	3.76E-01
DIRC3	-0.93644	-8.63851	6.31E-06	3.79E-01
LEPREL2	0.30213	2.00505	6.50E-06	3.90E-01
B3GNT8	0.40867	2.56255	6.60E-06	3.96E-01
CXCL2	-0.93902	-8.69003	6.66E-06	4.00E-01
C16orf75	-0.88623	-7.69534	7.50E-06	4.50E-01
HLX	1.10359	12.6937	8.04E-06	4.82E-01
DIP2B	-0.32738	-2.1251	8.20E-06	4.92E-01
UNKNOWN	-0.59433	-3.9294	8.26E-06	4.96E-01
GP1BB	-0.96218	-9.16597	8.41E-06	5.05E-01
CR2	-0.93659	-8.64149	8.76E-06	5.26E-01
RCAN3	-0.38096	-2.40415	9.14E-06	5.49E-01
UNKNOWN	-1.14503	-13.96458	9.25E-06	5.55E-01
ZC3H12D	-0.40963	-2.56822	9.86E-06	5.92E-01
FST	-1.19919	-15.81954	0.00001	6.00E-01
CD8B	-0.85145	-7.10319	0.00001	6.00E-01
ARRDC4	-0.82136	-6.62767	0.00001	6.00E-01
IL6R	-0.78298	-6.0671	0.00001	6.00E-01
GTF2IRD1	-0.43837	-2.74393	0.00001	6.00E-01
IL18RAP	0.56069	3.63652	0.00001	6.00E-01
LOC641518	-1.10561	-12.75298	0.00002	1.20E+00
ANK1	-0.98716	-9.70869	0.00002	1.20E+00
ACTN1	-0.95477	-9.01087	0.00002	1.20E+00
UNKNOWN	-0.86842	-7.38622	0.00002	1.20E+00
ZNF80	-0.77665	-5.9793	0.00002	1.20E+00
DACT1	-0.73739	-5.46246	0.00002	1.20E+00
CD9	-0.67843	-4.76903	0.00002	1.20E+00
UNKNOWN	-0.65066	-4.47364	0.00002	1.20E+00
TXNRD3	-0.58809	-3.87342	0.00002	1.20E+00
CD248	-0.58202	-3.81965	0.00002	1.20E+00
AGMAT	-0.38172	-2.40834	0.00002	1.20E+00
UNKNOWN	0.63589	4.32409	0.00002	1.20E+00
UNKNOWN	-0.97259	-9.38847	0.00003	1.80E+00
SGPP2	-0.86625	-7.34943	0.00003	1.80E+00
UNKNOWN	-0.70415	-5.06	0.00003	1.80E+00

CYSLTR1	-0.57964	-3.79875	0.00003	1.80E+00
UNKNOWN	-0.5087	-3.22625	0.00003	1.80E+00
SERINC5	-0.44703	-2.79921	0.00003	1.80E+00
MPPED2	0.80009	6.3109	0.00003	1.80E+00
KRT2	-0.67477	-4.72899	0.00004	2.40E+00
UNKNOWN	-0.49914	-3.15604	0.00004	2.40E+00
UNKNOWN	-0.43397	-2.71628	0.00004	2.40E+00
TMEM163	0.73282	5.40527	0.00004	2.40E+00
DNAJC5G	0.77337	5.93433	0.00004	2.40E+00
VNN3	1.10047	12.60279	0.00004	2.40E+00
CYP2J2	-0.76208	-5.78202	0.00005	3.00E+00
UNKNOWN	-0.68472	-4.83858	0.00005	3.00E+00
KCNH2	-0.67677	-4.75084	0.00005	3.00E+00
ARHGEF10	-0.51071	-3.24126	0.00005	3.00E+00
NRCAM	-0.50379	-3.18999	0.00005	3.00E+00
PLCL1	-0.48781	-3.07477	0.00005	3.00E+00
TRIM58	-0.36525	-2.31874	0.00005	3.00E+00
IL18RAP	0.53506	3.42817	0.00005	3.00E+00
UNKNOWN	-1.12844	-13.44121	0.00006	3.60E+00
KCTD12	-0.97577	-9.45734	0.00006	3.60E+00
KRT1	-0.81183	-6.48379	0.00006	3.60E+00
UNKNOWN	-0.59108	-3.90015	0.00006	3.60E+00
UNKNOWN	0.31478	2.06434	0.00006	3.60E+00
WDFY4	-0.53488	-3.42674	0.00007	4.20E+00
	0.80729	6.41639	0.00007	4.20E+00
FAM19A2	-1.14689	-14.02457	0.00008	4.80E+00
WNT7B	-0.82383	-6.66545	0.00008	4.80E+00
UNKNOWN	-0.55832	-3.61674	0.00008	4.80E+00
COL5A1	0.35932	2.28729	0.00008	4.80E+00
UNKNOWN	0.3612	2.29719	0.00008	4.80E+00
TLCD2	0.47468	2.98322	0.00008	4.80E+00
LOC100131662	-1.09781	-12.52592	0.00009	5.40E+00
CLC	-0.83639	-6.8611	0.00009	5.40E+00
USP6NL	-0.74263	-5.52878	0.00009	5.40E+00
TLE1	0.3896	2.45245	0.00009	5.40E+00
TRIP10	0.39575	2.48743	0.00009	5.40E+00
LAYN	-0.99157	-9.80777	0.0001	6.00E+00
RBM20	-0.87204	-7.44797	0.0001	6.00E+00
CDH1	-0.76988	-5.88675	0.0001	6.00E+00
UNKNOWN	-0.58783	-3.87106	0.0001	6.00E+00
UNKNOWN	1.11467	13.02184	0.0001	6.00E+00
MYH4	-1.24481	-17.57171	0.00011	6.60E+00
FCER1A	-0.86073	-7.25648	0.00011	6.60E+00
TNFRSF10D	-0.70195	-5.03448	0.00011	6.60E+00
EPHB4	-0.62248	-4.19257	0.00011	6.60E+00
THBS1	0.44889	2.81117	0.00011	6.60E+00

CNTN5	1.03474	10.83287	0.00011	6.60E+00
LAMP3	-0.39044	-2.45717	0.00012	7.20E+00
CYP24A1	-0.93394	-8.58899	0.00013	7.80E+00
KATNAL2	-0.91641	-8.24913	0.00013	7.80E+00
CLEC11A	-0.82286	-6.65056	0.00013	7.80E+00
DKK3	-0.34261	-2.20097	0.00013	7.80E+00
LAMA2	0.59324	3.91962	0.00013	7.80E+00
LY86-AS1	1.0496	11.20991	0.00013	7.80E+00
UNKNOWN	-1.19397	-15.63054	0.00014	8.40E+00
GATM	-0.8345	-6.83118	0.00014	8.40E+00
UNKNOWN	-0.31051	-2.04415	0.00014	8.40E+00
PRSS35	1.11254	12.95793	0.00014	8.40E+00
FAM69C	-1.20363	-15.98209	0.00015	9.00E+00
IL1R1	-0.98817	-9.7313	0.00015	9.00E+00
SORBS2	-0.79292	-6.20762	0.00015	9.00E+00
LARGE	-0.46196	-2.8971	0.00015	9.00E+00
CCR8	-0.83972	-6.91379	0.00016	9.60E+00
ANKRD57	-0.61488	-4.11979	0.00016	9.60E+00
GUCY2E	-1.22366	-16.7364	0.00017	1.02E+01
UNKNOWN	-1.35559	-22.67728	0.00018	1.08E+01
FLJ20444	-1.16864	-14.74484	0.00018	1.08E+01
UNKNOWN	-0.95879	-9.0948	0.00018	1.08E+01
LMO7	-0.51381	-3.26442	0.00018	1.08E+01
UNKNOWN	-0.31072	-2.04515	0.00018	1.08E+01
LOC285441	-1.37371	-23.64318	0.00019	1.14E+01
ZC3H12D	-0.60463	-4.02378	0.00019	1.14E+01
IKZF2	-0.56806	-3.69881	0.00019	1.14E+01
KRT18	-0.46858	-2.94157	0.00019	1.14E+01
FAM7A1	0.41666	2.61014	0.00019	1.14E+01
UNKNOWN	0.96327	9.189	0.00019	1.14E+01
ANK1	-0.96683	-9.26477	0.00021	1.26E+01
APBA2	-0.6226	-4.19371	0.00021	1.26E+01
SLC22A23	-0.37031	-2.34588	0.00021	1.26E+01
SYTL2	0.40055	2.51507	0.00021	1.26E+01
FLJ40536	1.24985	17.77651	0.00021	1.26E+01
UNKNOWN	-1.36147	-22.98624	0.00022	1.32E+01
BCL2	-0.30206	-2.00476	0.00022	1.32E+01
LIMS2	-0.6734	-4.71416	0.00023	1.38E+01
FOXJ1	-0.66509	-4.62477	0.00023	1.38E+01
SERPINB11	1.13389	13.61087	0.00023	1.38E+01
KRT72	-0.88518	-7.67685	0.00024	1.44E+01
SLC7A8	-0.94922	-8.89659	0.00025	1.50E+01
PLXNB2	-0.90759	-8.08336	0.00025	1.50E+01
GRB10	-0.72573	-5.31776	0.00025	1.50E+01
CTLA4	-0.42233	-2.6444	0.00025	1.50E+01
CCR2	0.70891	5.11571	0.00025	1.50E+01

MS4A12	1.20744	16.1228	0.00025	1.50E+01
ADAMTS4	-0.72169	-5.26849	0.00026	1.56E+01
UNKNOWN	-0.57559	-3.76349	0.00027	1.62E+01
TMEM171	0.52697	3.36485	0.00027	1.62E+01
TPBG	0.90829	8.09639	0.00028	1.68E+01
HEPH	-0.45415	-2.84546	0.00029	1.74E+01
FAM172BP	0.71932	5.23987	0.00029	1.74E+01
CTRB1	0.99595	9.90712	0.00029	1.74E+01
UNKNOWN	-1.12238	-13.25515	0.0003	1.80E+01
SEMA5A	-0.55927	-3.6247	0.0003	1.80E+01
	0.44419	2.78092	0.0003	1.80E+01
FANK1	-0.58436	-3.84023	0.00032	1.92E+01
LOC439949	-0.37365	-2.36403	0.00032	1.92E+01
MAP3K1	-0.34761	-2.22641	0.00032	1.92E+01
ABCB1	0.31582	2.0693	0.00032	1.92E+01
ABCB1	0.38184	2.40902	0.00032	1.92E+01
UNKNOWN	1.14136	13.84698	0.00032	1.92E+01
NANOS2	-1.21694	-16.47937	0.00034	2.04E+01
GOT1L1	1.32955	21.35734	0.00034	2.04E+01
EDN1	-0.3392	-2.18373	0.00035	2.10E+01
UNKNOWN	1.07533	11.89393	0.00036	2.16E+01
GPR44	-0.62938	-4.25971	0.00037	2.22E+01
ARHGEF4	-0.3913	-2.46205	0.00037	2.22E+01
PRDM13	0.69259	4.92703	0.00037	2.22E+01
UNKNOWN	-1.00094	-10.02164	0.00039	2.34E+01
UNKNOWN	-0.56395	-3.66396	0.00039	2.34E+01
NELL2	-0.52164	-3.32387	0.00039	2.34E+01
C2orf89	-0.49534	-3.12851	0.00039	2.34E+01
MS4A1	0.31505	2.06562	0.00039	2.34E+01
MME	-0.77991	-6.02433	0.0004	2.40E+01
E2F5	-0.52257	-3.33095	0.0004	2.40E+01
UNKNOWN	-0.37133	-2.35141	0.0004	2.40E+01
UNKNOWN	1.16745	14.70435	0.0004	2.40E+01
UNKNOWN	1.34319	22.03907	0.0004	2.40E+01
UNKNOWN	-1.05919	-11.46008	0.00042	2.52E+01
UNKNOWN	1.31244	20.53248	0.00042	2.52E+01
UNKNOWN	0.31603	2.0703	0.00043	2.58E+01
LOC284395	1.18036	15.14825	0.00043	2.58E+01
UNKNOWN	1.28273	19.17477	0.00043	2.58E+01
UNKNOWN	-0.87258	-7.45734	0.00044	2.64E+01
TIMP2	-0.65032	-4.47009	0.00044	2.64E+01
TNS4	0.39735	2.49658	0.00044	2.64E+01
UNKNOWN	1.08677	12.21144	0.00044	2.64E+01
UNKNOWN	-1.34055	-21.90537	0.00045	2.70E+01
UNKNOWN	-0.99798	-9.95349	0.00045	2.70E+01
CBX2	-0.73971	-5.49179	0.00045	2.70E+01

HPSE2	-0.6807	-4.79405	0.00045	2.70E+01
SPATA6	-0.53974	-3.46528	0.00045	2.70E+01
UNKNOWN	1.08097	12.04945	0.00045	2.70E+01
ZNF385B	1.17692	15.02858	0.00045	2.70E+01
ZNF135	-1.11657	-13.07896	0.00047	2.82E+01
	-0.31873	-2.08321	0.00047	2.82E+01
	-0.61261	-4.09836	0.00048	2.88E+01
GEM	-1.05643	-11.38753	0.0005	3.00E+01
FZD6	-0.6944	-4.94768	0.0005	3.00E+01
NCRNA00173	-0.56885	-3.70556	0.0005	3.00E+01
LY96	-0.71855	-5.2306	0.00051	3.06E+01
	-1.14688	-14.02434	0.00052	3.12E+01
	-1.02856	-10.67964	0.00052	3.12E+01
	-0.86252	-7.28654	0.00052	3.12E+01
LTK	0.51124	3.2452	0.00053	3.18E+01
DEFB108B	1.14274	13.89135	0.00053	3.18E+01
GCNT4	-0.48872	-3.08122	0.00054	3.24E+01
	1.14113	13.83987	0.00054	3.24E+01
	0.48089	3.02618	0.00055	3.30E+01
CYP4F22	0.67835	4.76812	0.00055	3.30E+01
	-1.0639	-11.58501	0.00056	3.36E+01
INMT	-0.46523	-2.919	0.00056	3.36E+01
PLXND1	0.36939	2.34094	0.00056	3.36E+01
PVRL2	0.87871	7.56332	0.00056	3.36E+01
	1.14355	13.91701	0.00056	3.36E+01
E2F8	-1.06379	-11.58219	0.00058	3.48E+01
	-1.05991	-11.47928	0.00059	3.54E+01
NT5E	-0.48845	-3.07926	0.00059	3.54E+01
LOC100131541	-0.56861	-3.70345	0.0006	3.60E+01
PPARGC1B	-0.30341	-2.01099	0.0006	3.60E+01
	1.1914	15.53816	0.00061	3.66E+01
	-0.40917	-2.56549	0.00062	3.72E+01
	1.25909	18.1588	0.00062	3.72E+01
	1.29619	19.77842	0.00062	3.72E+01
CCR4	-0.72576	-5.31817	0.00064	3.84E+01
LYPD6B	1.26423	18.37529	0.00064	3.84E+01
Sep-10	-0.74692	-5.58362	0.00069	4.14E+01
LRRN3	-0.97173	-9.36969	0.0007	4.20E+01
LOC440925	1.32709	21.23677	0.0007	4.20E+01
HLA-DOA	-0.35763	-2.27838	0.00071	4.26E+01
CLMN	1.18635	15.35862	0.00071	4.26E+01
ZYG11A	-1.00226	-10.05217	0.00072	4.32E+01
LCE2C	1.08335	12.11568	0.00072	4.32E+01
LOC100128252	-0.91891	-8.29684	0.00073	4.38E+01
SLC9A7	-0.38269	-2.41376	0.00074	4.44E+01
OR51A2	1.1709	14.82184	0.00075	4.50E+01

TMEM45B	-1.00777	-10.18063	0.00077	4.62E+01
TSHZ2	-0.90635	-8.06025	0.00078	4.68E+01
	-0.57981	-3.80026	0.00078	4.68E+01
TOX	-0.35296	-2.25405	0.00078	4.68E+01
CACNA2D2	0.32849	2.13052	0.0008	4.80E+01
LOC389332	-0.85032	-7.08462	0.00081	4.86E+01
DSEL	-0.38107	-2.40477	0.00083	4.98E+01
SH3RF3	-0.97431	-9.42565	0.00084	5.04E+01
	-0.92562	-8.4259	0.00084	5.04E+01
LOC100130071	0.8442	6.98552	0.00084	5.04E+01
	1.03165	10.75596	0.00084	5.04E+01
GS85	1.03304	10.79057	0.00085	5.10E+01
LOC388780	-0.70978	-5.126	0.00086	5.16E+01
	-1.48337	-30.43481	0.00089	5.34E+01
ACVR1	-0.3525	-2.25162	0.00089	5.34E+01
UBE2E2	-0.80619	-6.40018	0.0009	5.40E+01
KLF5	-0.49984	-3.1611	0.0009	5.40E+01
	-1.02148	-10.50704	0.00093	5.58E+01
RFPL2	-0.59308	-3.91816	0.00094	5.64E+01
FLJ22763	1.37796	23.87591	0.00094	5.64E+01
TIAM1	-0.48883	-3.08198	0.00095	5.70E+01
PTPRK	-0.64749	-4.4411	0.00096	5.76E+01
LOC100133654	1.16884	14.75147	0.00096	5.76E+01
WFDC10B	1.18329	15.25085	0.00096	5.76E+01
CDCA7L	-0.59156	-3.90445	0.00097	5.82E+01
	-0.67189	-4.69774	0.00099	5.94E+01
DNAH11	-0.58139	-3.81405	0.00099	5.94E+01
RASGRP3	-0.34159	-2.19581	0.00099	5.94E+01
	1.14519	13.96973	0.00099	5.94E+01
ZNF415	-0.91449	-8.21277	0.00102	6.12E+01
	1.11809	13.12473	0.00102	6.12E+01
	-1.04607	-11.11908	0.00103	6.18E+01
LEF1	-0.66552	-4.62937	0.00103	6.18E+01
LOC286189	1.42248	26.45324	0.00103	6.18E+01
	0.93447	8.59943	0.00104	6.24E+01
GPR55	-0.67074	-4.68537	0.00105	6.30E+01
DLL3	-0.64559	-4.42168	0.00105	6.30E+01
CR1L	-0.59715	-3.95503	0.00105	6.30E+01
AFF3	-0.39067	-2.45852	0.00107	6.42E+01
ITPKA	0.39271	2.47005	0.00107	6.42E+01
	-0.66396	-4.61276	0.00108	6.48E+01
PARVA	0.40814	2.55943	0.00108	6.48E+01
	-0.34947	-2.23601	0.00109	6.54E+01
NWD1	1.14793	14.0583	0.00109	6.54E+01
TLR2	-0.50969	-3.23359	0.0011	6.60E+01
AUTS2	0.31763	2.07791	0.0011	6.60E+01

FAM55B	-0.93948	-8.69921	0.00112	6.72E+01
	-0.45526	-2.85275	0.00112	6.72E+01
LOC641510	-0.44593	-2.79211	0.00116	6.96E+01
PCSK5	-0.41644	-2.60882	0.00116	6.96E+01
	-0.93968	-8.70331	0.00117	7.02E+01
FNDC4	0.92809	8.47398	0.00117	7.02E+01
KATNAL2	-0.33736	-2.17452	0.00119	7.14E+01
PHACTR2	0.30876	2.03592	0.00119	7.14E+01
AGPAT4	0.31214	2.05183	0.00119	7.14E+01
DCHS1	-0.47846	-3.00924	0.0012	7.20E+01
MGAT3	-0.72333	-5.28852	0.00122	7.32E+01
SLC6A10P	0.74324	5.53656	0.00122	7.32E+01
VN1R2	1.10999	12.88216	0.00123	7.38E+01
	1.41475	25.98642	0.00125	7.50E+01
C2orf89	-0.52253	-3.33068	0.00126	7.56E+01
PACSIN1	0.45347	2.84102	0.00127	7.62E+01
ZNF334	-0.85347	-7.13632	0.00129	7.74E+01
ZNF532	0.54195	3.483	0.00129	7.74E+01
SERPINE2	-0.37142	-2.3519	0.00133	7.98E+01
GALNTL2	1.1798	15.1288	0.00133	7.98E+01
FAM135B	1.45997	28.83853	0.00133	7.98E+01
	-0.48721	-3.0705	0.00135	8.10E+01
	0.4467	2.79702	0.00135	8.10E+01
FAM20A	1.45515	28.52014	0.00139	8.34E+01
TANC1	0.81761	6.57069	0.00143	8.58E+01
RTN4RL1	-0.66025	-4.5735	0.00145	8.70E+01
METTL11B	-0.59246	-3.91256	0.00147	8.82E+01
IKZF2	-0.55501	-3.58926	0.00147	8.82E+01
BLK	0.48458	3.052	0.0015	9.00E+01
P2RY14	0.59274	3.91504	0.00151	9.06E+01
OTOL1	-0.97569	-9.45564	0.00154	9.24E+01
IL3	0.77254	5.92292	0.00155	9.30E+01
LOC732455	1.08056	12.0382	0.00155	9.30E+01
MOXD1	-0.87184	-7.44463	0.00159	9.54E+01
ARHGEF11	-0.74658	-5.57934	0.00159	9.54E+01
ADD2	-0.6529	-4.49676	0.00161	9.66E+01
OR52L1	1.1192	13.1584	0.00162	9.72E+01
DLL1	-0.5957	-3.94184	0.00163	9.78E+01
VLDLR	0.56233	3.65034	0.00165	9.90E+01
BTBD3	-0.73862	-5.47803	0.00167	1.00E+02
	-0.97889	-9.52545	0.00168	1.01E+02
	-0.54446	-3.50313	0.00169	1.01E+02
HMOX1	-0.42411	-2.65525	0.0017	1.02E+02
LOC647020	-0.91633	-8.24772	0.00171	1.03E+02
	-0.33364	-2.15597	0.00177	1.06E+02
	-0.7	-5.01191	0.00178	1.07E+02

CYP24A1	-0.8792	-7.57187	0.00179	1.07E+02
MALL	-1.06453	-11.6019	0.0018	1.08E+02
	-0.95992	-9.11851	0.0018	1.08E+02
	-0.49193	-3.10406	0.0018	1.08E+02
	0.82361	6.66212	0.0018	1.08E+02
LOC100509011	-0.42395	-2.65428	0.00185	1.11E+02
	0.87604	7.5169	0.00186	1.12E+02
	0.65072	4.4742	0.00187	1.12E+02
TXNRD3NB	1.08275	12.09903	0.00188	1.13E+02
C5orf13	-0.73356	-5.41453	0.0019	1.14E+02
ST6GALNAC3	-0.9615	-9.15162	0.00193	1.16E+02
AEBP1	-0.62512	-4.21814	0.00193	1.16E+02
	1.41632	26.08064	0.00194	1.16E+02
	-0.32786	-2.12745	0.00195	1.17E+02
IL18R1	0.41672	2.61051	0.00195	1.17E+02
LOC400622	-0.86216	-7.28044	0.00196	1.18E+02
	-0.86839	-7.38575	0.00198	1.19E+02
DKK3	-0.45903	-2.87759	0.00198	1.19E+02
MYOM2	-0.50588	-3.20536	0.00199	1.19E+02
PERP	0.37471	2.36978	0.00199	1.19E+02
LOC440910	-0.76952	-5.882	0.002	1.20E+02
ZWINT	-0.4955	-3.12969	0.00201	1.21E+02
LOC730668	1.22492	16.78481	0.00201	1.21E+02
ARHGAP32	-0.48478	-3.05334	0.00209	1.25E+02
RBM11	-0.43211	-2.70464	0.00209	1.25E+02
LOC100132344	-0.44598	-2.79241	0.00211	1.27E+02
HAPLN3	-0.45346	-2.8409	0.00212	1.27E+02
GJB6	-0.67433	-4.72426	0.00215	1.29E+02
	-0.98738	-9.7136	0.00217	1.30E+02
	0.87376	7.47749	0.00222	1.33E+02
HHEX	-0.65962	-4.56684	0.00226	1.36E+02
PLEKHH2	-0.63529	-4.31806	0.00226	1.36E+02
	1.20317	15.96488	0.00227	1.36E+02
REG4	-0.51104	-3.24367	0.00232	1.39E+02
	0.58538	3.84924	0.00233	1.40E+02
	-0.44123	-2.76202	0.00234	1.40E+02
CHST2	-0.74464	-5.55443	0.00236	1.42E+02
SNTG2	-0.65899	-4.5603	0.00236	1.42E+02
RCAN2	-0.67312	-4.71112	0.00237	1.42E+02
LRRN1	-0.84598	-7.01417	0.00239	1.43E+02
CES4A	-0.32625	-2.11958	0.00239	1.43E+02
FTCD	-0.55804	-3.61447	0.00247	1.48E+02
	1.36843	23.35782	0.00248	1.49E+02
C9orf53	0.39301	2.47181	0.0025	1.50E+02
LOC729680	-0.49757	-3.14467	0.00251	1.51E+02
PTCH1	0.33881	2.18179	0.00251	1.51E+02

PPBP	-0.61022	-4.07587	0.00254	1.52E+02
IL23R	0.76003	5.75476	0.00255	1.53E+02
GAGE7	1.34863	22.31681	0.00256	1.54E+02
NOX3	-0.83322	-6.81115	0.00258	1.55E+02
	0.32105	2.09435	0.0026	1.56E+02
	-0.6119	-4.09163	0.00262	1.57E+02
	-0.41208	-2.58276	0.00262	1.57E+02
	-0.90162	-7.97299	0.00263	1.58E+02
B3GALT2	1.04492	11.08968	0.00263	1.58E+02
	-0.6376	-4.34115	0.00266	1.60E+02
PTCH1	0.32399	2.10859	0.00273	1.64E+02
CPNE5	0.34488	2.21248	0.00279	1.67E+02
	1.26427	18.3768	0.00279	1.67E+02
	0.9636	9.19599	0.0028	1.68E+02
ZDHHC23	-0.3028	-2.00819	0.00283	1.70E+02
RNF157	-0.50951	-3.23231	0.00284	1.70E+02
LIMCH1	-0.92138	-8.34411	0.00285	1.71E+02
PSMB11	1.13301	13.58353	0.00286	1.72E+02
IGKV1D-13	0.89745	7.89686	0.00287	1.72E+02
CDH20	0.58821	3.87449	0.0029	1.74E+02
HOXA11-AS1	0.71881	5.23368	0.00293	1.76E+02
	0.88776	7.7226	0.00293	1.76E+02
RORA	0.30285	2.00842	0.00295	1.77E+02
	-0.71639	-5.20466	0.00297	1.78E+02
	-0.91917	-8.3018	0.00301	1.81E+02
C17orf99	-0.62482	-4.21522	0.00301	1.81E+02
MGC15705	-0.44927	-2.81363	0.00302	1.81E+02
TYRO3	0.39839	2.50261	0.00302	1.81E+02
	1.29589	19.76462	0.00303	1.82E+02
TSHZ3	0.73109	5.38376	0.00306	1.84E+02
	0.90867	8.10344	0.00306	1.84E+02
DDX25	-1.02355	-10.55716	0.00308	1.85E+02
BCL11A	0.94385	8.78726	0.0031	1.86E+02
POTED	0.69111	4.91035	0.00311	1.87E+02
PER4	-0.91061	-8.13973	0.00316	1.90E+02
MRC2	-0.44115	-2.76152	0.00316	1.90E+02
PLEKHG3	0.33082	2.142	0.00316	1.90E+02
SYCE1	0.87588	7.51412	0.00316	1.90E+02
PRKAR2B	-0.50628	-3.20834	0.00317	1.90E+02
LOC284798	-0.78677	-6.1202	0.00318	1.91E+02
	0.8887	7.73923	0.00321	1.93E+02
	-0.74584	-5.56978	0.00323	1.94E+02
PDZD7	0.99127	9.80099	0.00323	1.94E+02
C1orf227	0.99722	9.93618	0.00329	1.97E+02
	-0.40405	-2.53542	0.00331	1.99E+02
DSCAML1	-0.60362	-4.01435	0.00332	1.99E+02

SDK2	-0.51561	-3.27799	0.00332	1.99E+02
	0.99309	9.8422	0.00336	2.02E+02
	0.8699	7.41137	0.00337	2.02E+02
GP5	-0.80447	-6.37488	0.00338	2.03E+02
PTPRK	-0.47948	-3.01633	0.0034	2.04E+02
PRKCA	-0.35741	-2.27724	0.0034	2.04E+02
PLCB1	0.43293	2.70977	0.00341	2.05E+02
NCRNA00299	0.43317	2.71123	0.00347	2.08E+02
ZNF114	-0.79913	-6.29696	0.00349	2.09E+02
ROR2	0.51991	3.31066	0.00349	2.09E+02
ZNF618	1.10748	12.80783	0.0035	2.10E+02
KIF24	0.43157	2.70125	0.00351	2.11E+02
BACH2	-0.36556	-2.3204	0.00353	2.12E+02
LOC100131053	0.80557	6.39107	0.00355	2.13E+02
	0.84125	6.93821	0.00355	2.13E+02
	0.73799	5.47001	0.00358	2.15E+02
	-0.87278	-7.46062	0.00359	2.15E+02
	-0.65369	-4.50494	0.00359	2.15E+02
C9orf84	-0.88886	-7.74217	0.00363	2.18E+02
SVOPL	-0.53409	-3.42048	0.00364	2.18E+02
FAM83E	-0.36044	-2.29319	0.00367	2.20E+02
	0.86195	7.27696	0.00367	2.20E+02
	-0.60802	-4.05529	0.0037	2.22E+02
	-0.89302	-7.8166	0.00378	2.27E+02
INF2	-0.41288	-2.5875	0.0038	2.28E+02
	-0.99268	-9.83277	0.00381	2.29E+02
HGC6.3	-0.52615	-3.35854	0.00382	2.29E+02
	-0.90139	-7.96879	0.00383	2.30E+02
	0.59936	3.97521	0.00383	2.30E+02
ALOX5	-0.53586	-3.43447	0.00384	2.30E+02
	0.39969	2.51008	0.00384	2.30E+02
PRDM5	0.694	4.94311	0.00384	2.30E+02
SEC14L3	-0.90025	-7.94784	0.00386	2.32E+02
SERPINF1	-0.30663	-2.02595	0.00388	2.33E+02
LRP4	0.92189	8.35388	0.00389	2.33E+02
	0.80163	6.33323	0.0039	2.34E+02
	-0.89778	-7.90282	0.00392	2.35E+02
	0.34461	2.2111	0.00399	2.39E+02
ANK2	0.91417	8.20675	0.004	2.40E+02
MAOA	-0.5853	-3.84862	0.00409	2.45E+02
CSN1S2BP	-0.49649	-3.13682	0.0041	2.46E+02
DEFB126	0.89422	7.83832	0.0041	2.46E+02
	-0.42601	-2.66692	0.00413	2.48E+02
MSL3	-0.526	-3.35738	0.00415	2.49E+02
	0.34424	2.20924	0.00415	2.49E+02
HSP90AB6P	0.87949	7.57693	0.00416	2.50E+02

	-0.88271	-7.63328	0.00417	2.50E+02
	0.96035	9.12737	0.00418	2.51E+02
CCR1	0.5886	3.87797	0.00419	2.51E+02
	-0.88204	-7.62146	0.00421	2.53E+02
RNF157	-0.3344	-2.15972	0.00421	2.53E+02
MEGF6	-0.30104	-2.00007	0.00422	2.53E+02
AIFM2	0.86296	7.29395	0.00422	2.53E+02
ZNF532	0.46834	2.93992	0.00423	2.54E+02
	-0.57669	-3.773	0.00425	2.55E+02
	-0.31068	-2.04495	0.00427	2.56E+02
EDN3	-0.4346	-2.72018	0.00431	2.59E+02
PDE7B	-0.80365	-6.3629	0.00439	2.63E+02
RBFOX1	0.40456	2.53838	0.00441	2.65E+02
TSPAN15	0.57153	3.72847	0.00441	2.65E+02
NCRNA00244	0.73257	5.40218	0.00443	2.66E+02
CDH2	0.35131	2.24548	0.00451	2.71E+02
CD8B	-0.60535	-4.03043	0.00453	2.72E+02
	0.70786	5.10335	0.00455	2.73E+02
GSG1L	-1.05414	-11.32778	0.00456	2.74E+02
	-0.61162	-4.08899	0.00457	2.74E+02
NPCDR1	-0.57475	-3.75621	0.00463	2.78E+02
NCRNA00311	0.66794	4.65517	0.00464	2.78E+02
IFNGR2	-0.60509	-4.02803	0.00469	2.81E+02
	0.56192	3.64685	0.0047	2.82E+02
GYPE	-0.5481	-3.53269	0.00478	2.87E+02
AURKC	-0.39544	-2.48565	0.00482	2.89E+02
KRTAP4-8	0.80732	6.41683	0.00482	2.89E+02
	0.82503	6.68388	0.00482	2.89E+02
FLJ35390	-0.47799	-3.006	0.00486	2.92E+02
GALR2	-0.84816	-7.04959	0.00491	2.95E+02
PTK2	-0.5698	-3.7136	0.00497	2.98E+02
	0.73844	5.47573	0.00509	3.05E+02
	0.9795	9.53894	0.00512	3.07E+02
PLEKHG3	0.32391	2.1082	0.00513	3.08E+02
	0.88546	7.68175	0.00514	3.08E+02
	0.42555	2.6641	0.00518	3.11E+02
	-0.36466	-2.3156	0.00519	3.11E+02
EDA	-0.39805	-2.50061	0.00521	3.13E+02
	0.89119	7.78379	0.00525	3.15E+02
BEND4	-0.66042	-4.57535	0.00529	3.17E+02
PHC1	-0.89014	-7.76505	0.0053	3.18E+02
MYO1E	-0.79449	-6.23001	0.00533	3.20E+02
	0.4477	2.80347	0.00535	3.21E+02
	-1.28835	-19.42451	0.00542	3.25E+02
PLD1	0.61612	4.13159	0.00545	3.27E+02
	0.58002	3.80206	0.0055	3.30E+02

LOC100128668	0.43767	2.73951	0.00551	3.31E+02
	0.58819	3.87428	0.00551	3.31E+02
MSH4	-0.87835	-7.55692	0.00552	3.31E+02
	0.87312	7.46655	0.00553	3.32E+02
	-0.3262	-2.11933	0.00556	3.34E+02
NCRNA00282	-0.7317	-5.39133	0.00557	3.34E+02
GAS6	-0.6456	-4.42179	0.00557	3.34E+02
RNF144B	-0.6443	-4.40857	0.00568	3.41E+02
SPIN3	-0.59766	-3.95963	0.0057	3.42E+02
	0.5742	3.75144	0.0057	3.42E+02
LOC619207	0.47595	2.99192	0.00578	3.47E+02
IQCA1	-0.57161	-3.72919	0.00585	3.51E+02
TSHR	0.89305	7.81718	0.0059	3.54E+02
TUSC3	-0.91244	-8.17412	0.00594	3.56E+02
CCDC164	0.87792	7.5495	0.00601	3.61E+02
IL18R1	0.37945	2.39579	0.0061	3.66E+02
	-0.85195	-7.11131	0.00616	3.70E+02
TCF7L1	-0.34058	-2.19067	0.00624	3.74E+02
CNN3	-0.70899	-5.11674	0.00632	3.79E+02
C14orf132	-0.94414	-8.793	0.0064	3.84E+02
SEMA5B	-0.56187	-3.64644	0.00641	3.85E+02
	-0.53331	-3.41439	0.00646	3.88E+02
MYH7	0.76267	5.78991	0.00651	3.91E+02
	-0.82766	-6.72447	0.00656	3.94E+02
PWRN1	0.86976	7.40903	0.00657	3.94E+02
	0.3041	2.01418	0.00658	3.95E+02
S100A9	0.39305	2.47201	0.0066	3.96E+02
C6orf58	0.80635	6.40253	0.00663	3.98E+02
RTP1	0.31235	2.05281	0.00665	3.99E+02
FLJ22536	-0.58715	-3.86504	0.00666	4.00E+02
NPAS2	-0.34624	-2.21943	0.00672	4.03E+02
ZNF662	-0.59585	-3.94323	0.00674	4.04E+02
	0.63422	4.30746	0.00674	4.04E+02
SOX5	-0.69944	-5.00538	0.00683	4.10E+02
	0.52778	3.37113	0.00693	4.16E+02
RIMKLB	-0.49009	-3.09093	0.00701	4.21E+02
C2orf40	-0.38553	-2.42955	0.00701	4.21E+02
	-0.45702	-2.86429	0.00707	4.24E+02
	-0.79532	-6.24195	0.00711	4.27E+02
	-0.37642	-2.37912	0.00716	4.30E+02
	0.56214	3.64874	0.00723	4.34E+02
PKIA	-0.41243	-2.58484	0.00725	4.35E+02
KIAA1958	-0.34186	-2.19715	0.00725	4.35E+02
ME1	0.64192	4.38454	0.00731	4.39E+02
CREG2	-0.30421	-2.0147	0.00733	4.40E+02
ZAR1L	-0.46993	-2.95071	0.00734	4.40E+02

AP1S3	0.38484	2.4257	0.00735	4.41E+02
LOC100128651	0.86028	7.249	0.00738	4.43E+02
	-0.62626	-4.22919	0.00748	4.49E+02
LRRC32	-0.47166	-2.9625	0.00751	4.51E+02
KIAA0408	-0.33021	-2.139	0.00751	4.51E+02
	-0.64708	-4.4369	0.00754	4.52E+02
PLXNA4	-0.48435	-3.05035	0.00756	4.54E+02
	-0.72942	-5.36313	0.00758	4.55E+02
PXDN	-0.69784	-4.98696	0.00758	4.55E+02
QPCT	0.72631	5.32493	0.00764	4.58E+02
SSX1	0.85708	7.19578	0.00768	4.61E+02
HCRP1	-0.38278	-2.41426	0.00769	4.61E+02
THNSL2	-0.46093	-2.8902	0.0077	4.62E+02
ENTPD1	-0.60508	-4.02796	0.00772	4.63E+02
TRPM3	1.0865	12.20394	0.00775	4.65E+02
MAPK15	0.39619	2.48996	0.00785	4.71E+02
IQSEC3	-0.46943	-2.94734	0.00792	4.75E+02
LOC284412	0.89925	7.92964	0.00792	4.75E+02
PPARGC1B	-0.30667	-2.02612	0.00798	4.79E+02
PAGE3	0.5991	3.97281	0.00798	4.79E+02
SPINK5	-0.8407	-6.92944	0.00801	4.81E+02
LAT2	-0.44671	-2.79712	0.00807	4.84E+02
	-0.57172	-3.7301	0.00808	4.85E+02
	0.35672	2.27363	0.0081	4.86E+02
HCN1	-0.78617	-6.11179	0.00821	4.93E+02
SCN5A	0.99562	9.89959	0.00822	4.93E+02
SIGLEC11	0.61858	4.15509	0.00825	4.95E+02
LMO7	-0.52813	-3.37391	0.00826	4.96E+02
MFGE8	-0.30598	-2.02291	0.0083	4.98E+02
NT5E	-0.53981	-3.46588	0.00837	5.02E+02
P2RY2	-0.78112	-6.04118	0.00842	5.05E+02
	-0.47725	-3.00087	0.00844	5.06E+02
SLC16A8	0.66806	4.65645	0.00845	5.07E+02
C8orf83	0.4649	2.91678	0.00851	5.11E+02
	-0.58353	-3.83295	0.00854	5.12E+02
	0.58764	3.86936	0.00859	5.15E+02
GALR1	1.02522	10.59778	0.00861	5.17E+02
TBX3	0.50331	3.18647	0.00862	5.17E+02
	-0.60943	-4.06848	0.00865	5.19E+02
SLC22A4	0.53726	3.44555	0.00868	5.21E+02
C22orf41	0.79033	6.17065	0.00874	5.24E+02
	-0.31051	-2.04414	0.00878	5.27E+02
SERPINA10	-0.79303	-6.20913	0.00896	5.38E+02
	-0.45191	-2.83083	0.00899	5.39E+02
AGAP1	0.32405	2.10888	0.00899	5.39E+02
C9orf44	0.56847	3.7023	0.009	5.40E+02

EDA	-0.5495	-3.54402	0.00905	5.43E+02
VSIG1	-0.57297	-3.74082	0.00924	5.54E+02
CDC42EP3	0.33393	2.15738	0.00924	5.54E+02
	0.78427	6.08511	0.00924	5.54E+02
LRP12	0.51626	3.28293	0.00927	5.56E+02
SULT1E1	1.02183	10.51547	0.00933	5.60E+02
SNORA65	1.29699	19.81467	0.00934	5.60E+02
GRIK1-AS1	-0.59998	-3.98084	0.00938	5.63E+02
	0.82143	6.62865	0.00951	5.71E+02
	-0.92216	-8.35917	0.00955	5.73E+02
LAMC3	-0.45738	-2.86666	0.0096	5.76E+02
HHIPL1	0.81678	6.55812	0.00963	5.78E+02
CD8B	-0.51658	-3.28531	0.00969	5.81E+02
	0.87398	7.48133	0.00977	5.86E+02
ZP3	-0.33381	-2.15681	0.00978	5.87E+02
	-0.30932	-2.03853	0.00978	5.87E+02
	-0.50341	-3.18719	0.0098	5.88E+02
	0.7711	5.90337	0.00981	5.89E+02
STXBP1	-0.47346	-2.97479	0.00982	5.89E+02
F2RL1	-0.69146	-4.91431	0.0099	5.94E+02
ACSBG2	0.52898	3.38047	0.00992	5.95E+02
	-0.37882	-2.39235	0.00994	5.96E+02

APPENDIX 4:

LIVER BIOPSY STUDY (REC:09/HO603/19) – includes Protocol, Patient information sheet, Consent form and Consent to contact form.

PROTOCOL

TITLE: "ANALYSIS OF HEPATIC T-CELL RESPONSES FROM LIVER BIOPSIES"

REC No: 09/HO603/19
Chief Investigator: Dr Neil Rajoriya
Date: 20th June 2009
Version 1

Investigator: Dr Eleanor Barnes, Dr Jane Collier, Dr Jane Philips-Hughes, Dr Katie Jeffries and Professor Paul Klenerman

Sponsor: Oxford University

Trial site: Gastroenterology Unit, Level 5, John Radcliffe Hospital, Headly Way, Headington, Oxford OX39DU Tel 01865281232 and The Churchill Hospital, Oxford

Funder: Wellcome Trust, London
Clinical Laboratories: Peter Medawar Building for Pathogen Research, South Parks Road, Oxford, OX13SY

Confidentiality statement:
This document contains confidential information that must not be disclosed to anyone other than Sponsor, the investigator team, host NHS Trust and members of the research Ethics committee. This information cannot be used for any other purpose other that the evaluation or conduct of the clinical investigation without prior consent of:

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AMENDMENT HISTORY

AMENDMENT NO	PROTOCOL VERSION NO	DATE ISSUED	AUTHOR OF CHANGE	DETAILS OF CHANGES MADE

ABBREVIATIONS

AE	Adverse event
ALD	Alcoholic liver disease
AR	Adverse reaction
CI	Chief investigator
CT	Clinical trial
CTA	Clinical trials Authorisation
CTRG	Clinical Trials, and Research Governance, University of Oxford
EC	Ethic committee
eCRF	electronic Case report form
GCP	good clinical practice
GP	General Practitioner
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
ICF	Informed consent form
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NHS	National Health Service
NRES	National Research Ethics Service (previously known as COREC)
PI	Principal investigator
REC	Research Ethics Committee
SAE	Serious Adverse event
SAR	Serious Adverse reaction

BACKGROUND AND RATIONALE

Hepatitis C (HCV) is an increasing worldwide problem, currently affecting more than 170 million people worldwide. Complications include: the development of liver cirrhosis and liver cancer, each having a high mortality and can have major worldwide cost implications. Thus understanding the disease and how HCV affects the liver is vital. The body's defence system to HCV varies and the immune response mounted to initial infection varies from patient to patient. The strength and breadth of the immune response generated to HCV plays a role in determining clinical outcome. Following acute infection, HCV is cleared in 20% of patients, but the majority become persistently infected. T-cells play an important role in the early control of infection as determined by associations with HLA, functional studies of acute disease with diverse outcomes, and depletion experiments in chimpanzee models. However, the key features of a liver-specific antiviral CD8+ T-cell response have yet to be defined, both in terms of phenotype and function.

OBJECTIVES/ AIMS

The aim of this prospective study is to evaluate and explore the function and biology of T cells in HCV infection (specifically in the liver) with diverse clinical outcomes. By studying the phenotype and function of HCV specific T-cells within the liver, comparing them to T-cells in blood, and also to the cells in other liver diseases; we aim to achieve an understanding into the possible reasons why HCV persists in the liver; and thus different potential pathways for therapeutic interventions.

STUDY DESIGN

The study is a pilot, longitudinal study in patients with chronic HCV. Our control arm will be patients with liver disease of other aetiologies than HCV (such as HBV, NAFLD, Alcoholic liver disease, Autoimmune liver disease and NASH). We aim to recruite 40 patients from each group. The study will last 2 years, or until the numbers needed are recruited.

STUDY PROCEDURE

Listed are the steps that will be carried out in this trial.

1. Patients will be reviewed at hepatology clinics (general liver clinic or viral hepatitis clinic). At review there, if they are deemed to require a liver biopsy for diagnostic or therapeutic management/interventions by a Consultant or Specialist Registrar, they will be offered a chance to take part in the study by the clinical team responsible for the care of the patient.

2. The study team will be present at clinic to discuss the study with the patient should they wish. The patient will be given the patient information sheet (PIS) away with them (which includes a reply slip to be sent back to the research team if they are keen to take part). Consent will be taken by the clinical team for the research team to contact the patient a week post-clinic by phone to discuss whether they would be keen to take part or not, and answer any questions.

3. After a week post-clinic, the research team will contact the patient by phone (if they consented at clinic) to see if they are agreeable to participate. If so they will be offered an appointment to attend the hospital to see the research team.

4. At this appointment (which the research team will pay any expenses incurred by participants), the patients will have a chance to meet the research team and

discuss the research. At this appointment, they will have the research blood test carried out and they will be consented. Potential subjects will be given sufficient time to study the implications of participation in the study, to seek further information from the study staff and to discuss the possibility of involvement with relatives, friends or other healthcare professionals. Subjects who comply with the entry criteria and have been informed about the study will provide and signed and dated consent form. The original will be stored in the hospital notes, one photocopy will be given to the patient and another stored in the trial master file.

5. The patient will attend the John Radcliffe Hospital or Churchill Hospital for liver biopsy. The patient will have an ultrasound guided liver biopsy done with a 2nd pass for research sample. The patient will then be observed for a 6 hours and discharged (and exit the research protocol). If the patient did not attend the appointment (step 4) then the research blood sample will be taken at this point.

6. Post liver biopsy and blood sample the patient will exit the study (however will be followed up by the liver clinical team).

Potential study subjects will be identified via the hepatology clinic at the John Radcliffe hospital (either via the viral or general hepatology clinics). Any patient deemed to require a liver biopsy (except where malignancy suspected) by a hepatologist will be invited to participate in the study. The indication for biopsy will be made (on clinical grounds, diagnostic conundrums or for therapeutic management considerations) by the consulting hepatologist. At clinic, the potential study subject if interested will be given information on the study and given sufficient time to make a decision whether to participate or not in the study.

CLINICAL AND RESEARCH VARIABLES AND SAMPLES RECORDED OR STORED

Clinical: Demographic information, cause of liver disease, past medical history, onset of HCV infection and route of transmission, clinical course, severity, treatment details, alcohol consumption, and current clinical status including body mass index and signs of cirrhosis will all be stored. These are routine "parameters" recorded in case-notes.

Diagnostic: Liver function tests, coagulation profile, full blood count, viral genotype, liver ultrasound report, liver biopsy result and endoscopic imaging results if indicated.

Laboratory: Histological immunocytochemistry or other analysis of tissue samples.

Blood: 80mls of blood will be taken to store peripheral blood mononuclear cells.

Liver tissue: Liver tissue will be stored to obtain liver T-lymphocytes. The liver tissue gained will be immediately placed in a saline container and taken to the secure location at The Peter Medawar Building by the research team; where it

will be processed.

STUDY TREATMENT/ INTERVENTION

The study involves assessing the immune responses generated in the livers of patients (specifically to assess the immune responses in HCV infection). To do this we wish to gain liver tissue from liver biopsies for research purposes. The samples will be from patients already having a liver biopsy taken for clinical reasons/ indications. We wish to compare and contrast the immune responses in these liver samples with those in the patient's blood. We also aim to study the differences in the liver between HCV and other diseases affecting the liver. To do this we also wish to take an extra blood sample from patients.
The main interventions pertaining to this application include obtaining the following samples from patients: 1. Liver biopsy 2. Blood sample

1. Liver biopsies.
Liver biopsy is the biopsy (removal of a small sample of tissue) from the liver. It is a medical test that is done to aid diagnosis of liver disease, to assess the severity of known liver disease, and to monitor the progress of treatment. A liver biopsy involves patients being admitted as a day case to hospital. Prior to the biopsy being carried out, patients have a blood test carried out 5-7 days pre-liver biopsy. This blood test checks the patients "Coagulation profile" and "Full blood count": with components being checked to assess the patients ability to form clot incase of bleeding, and also how thin the patients blood is (incase of bleeding).

Patients are then admitted on the day of procedure (with consent forms already having been signed at the out-patient clinic). The patient attends the outpatient x-ray department where a trained interventional radiologist (a doctor trained specialising in the field of procedures done under x-ray guidance) will perform the procedure. The patient may be given a mild sedative/painkiller pre-procedure. Using an ultrasound machine, the liver is located. The skin overlying this area is cleaned/sterilised, and a small needle inserted to inject local anaesthetic. After a few minutes, a larger needle is inserted deeper (under ultrasound imaging - to ensure no other organ punctured) into the liver itself and a piece of tissue obtained. This insertion of the needle is called a "pass". Normally only one pass is made, unless clinically indicated, or if the liver team feel that the tissue from only one sample may not be enough to clinch a diagnosis.

Thereafter, the patient is observed for 6 hours (normal protocol) to observe for any complications post-liver biopsy (see later). The patient is thereafter discharged from hospital unless they live a long distance from the hospital, or have multiple health problems that may necessitate extra observation period (may entail an overnight stay).

2.Blood sample
An extra blood sample will be taken either at the time of liver biopsy, or at a meeting beforehand between the research team and the patient. The patient will

be having blood normally taken pre-biopsy for assessment of their clotting, so an extra sample will be taken at this point in time. We feel there is no major ethical issue in these patients having an extra 80mls of blood taken for research reasons (note: the Oxford Hepatitis C group do already have ethical approval on liver disease patients to take samples for research purposes).

DURATION OF STUDY

Once patients have the liver biopsy, they will exit our study. The actual study will take 3 years (or until 40 patients in each arm recruited – whichever time point comes first). Study participants themselves will only be in the study for 4-6 weeks.

6 SELECTION AND WITHDRAWEL OF RESEARCH PATIENTS/SUBJECTS

6.1 Inclusion/ Exclusion criteria

INCLUSION
1. Male and female patients
2. Aged between 18-75 years of age
3. Patients with chronic liver disease having a liver biopsy for other reasons than purely for research.
4. Patients who have had an ultrasound of liver within 6 months of biopsy.

EXCLUSION

1. Patients who are coagulopathic (INR > 1.3)/ thrombocytopenic (platelets < 150) (i.e. at increased risk of bleeding from a liver biopsy).
2. Patients unable to consent.
3. Patients with a clinical suspicion or ultrasound evidence of ascites
4. Patients with known malignancy.
5. Patients with documented evidence (radiologically or serologically or from previous biopsy) of cirrhosis.
6. Pregnancy

7. TREATMENT/ INTERVENTION

7.1 Description of Treatment/ Interventions

Treatment is given by the primary care or specialist clinic looking after the patient. No treatments will be given to participants directly as a result of this research.

Participants will be invited to have a second “pass” liver biopsy sample taken by an experienced interventional radiologist for research purposes. Meeting the strict inclusion and avoiding the exclusion criteria will minimize risks (as detailed before). The risk is also minimized using Ultrasound guidance.

8. ASSESSMENTS

Patients will only be assessed in this trial pre-biopsy and immediately post-biopsy by the operator (as per normal procedure, being ob). Participants will be followed up at clinic by the specialist team for review and biopsy results

9. SAFETY.

Clinically significant events will be collected as part of the general information on the patient's condition. Strict inclusion and exclusion criteria will be in place to minimize adverse events related to a 2nd pass of liver biopsy.

10 SAMPLE PROCESSING/ ANALYSIS/ STATISTICS

Samples gained (liver biopsy and blood) will be taken from the John Radcliffe / Churchill Hospitals to the Peter Medawar Building. Here the samples will be given a unique code to provide anonymity. The samples will then have a series of procedures carried out on them to isolate the T-cell lymphocyte population. The samples then will be studied in further detail including experiments on the biology of the T-cells using FACS analysis and gene expression profiling. Thereafter the results will be analysed using FLOJO - a computer programme to analyse FACS plot data, and PRISM – a statistical analysis package.

This is a pilot study into t-cell responses in HCV. Thus no formal statistical calculation has been performed. On the basis on previous studies published by our group, we intend to recruit 40 patients from each group.

40 HCV
40 HBV
40 NAFLD
40 NASH
40 ALD
40 Autoimmune liver disease
TOTAL: 240

11. ETHICS

The ethical concern in our opinion is the 2nd pass for liver biopsy, the taking of blood and maintaining patient confidentiality.

1. Liver biopsies.
Liver biopsy is the biopsy (removal of a small sample of tissue) from the liver. It is a medical test that is done to aid diagnosis of liver disease, to assess the severity of known liver disease, and to monitor the progress of treatment. A liver biopsy involves patients being admitted as a day case to hospital. Prior to the biopsy being carried out, patients have a blood test carried out 5-7 days pre-liver biopsy. This blood test checks the patients "Coagulation profile" and "Full blood

count": with components being checked to assess the patients ability to form clot incase of bleeding, and also how thin the patients blood is (incase of bleeding).

Patients are then admitted on the day of procedure (with consent forms already having been signed at the out-patient clinic). The patient attends the outpatient x-ray department where a trained interventional radiologist (a doctor trained specialising in the field of procedures done under x-ray guidance) will perform the procedure. The patient may be given a mild sedative/painkiller pre-procedure. Using an ultrasound machine, the liver is located. The skin overlying this area is cleaned/sterilised, and a small needle inserted to inject local anaesthetic. After a few minutes, a larger needle is inserted deeper (under ultrasound imaging - to ensure no other organ punctured) into the liver itself and a piece of tissue obtained. This insertion of the needle is called a "pass". Normally only one pass is made, unless clinically indicated, or if the liver team feel that the tissue from only one sample may not be enough to clinch a diagnosis.

Thereafter, the patient is observed for 6 hours (normal protocol) to observe for any complications post-liver biopsy (see later). The patient is thereafter discharged from hospital unless they live a long distance from the hospital, or have multiple health problems that may necessitate extra observation period (may entail an overnight stay).

The main ethical issues will be performing an extra pass (i.e. 2 biopsies instead of 1) at time of liver biopsy (i.e. taking two samples - one for clinical indication and an additional one for research purposes, instead of the standard one for clinical reasons). All patients will have the "2 pass"-liver biopsy performed by an experienced interventional radiologist under ultrasound guidance (thus eradicating the previously recognised complication of biopsy of the wrong organ).

Obtaining liver tissue via liver biopsy has recognised complications including:

1. Pain: the most common complication, with up to 84%* of patients experiencing mild discomfort. This pain is normally managed with small amounts of analgesia including paracetamol, ibuprofen or codeine. If a patient were to experience severe pain, they would be continually monitored in the hospital (incase of bleeding or puncture of other organs - very unlikely). If pain was severe, a repeat ultrasound or even CT scan may be performed to assess for any major complications. We will be monitoring patients (as part of the John Radcliffe standard protocol for post-liver biopsies) for 6 hours.

2. Bleeding: The risk of severe bleeding (defined as a clinical change in patients condition with a change in vital signs with radiological evidence of intraperitoneal bleed, and requiring transfusion) is quoted in recent AASLD (American Association of the study of liver disease guidelines*) as between 1 in 2500 to 1 in 10,000 biopsies. Less severe bleeding (defined as that sufficient to cause pain, lowering of blood pressure and a tachycardia (raised heart rate

above 100 beats per minute), but not requiring radiological intervention or transfusion) is quoted as occurring in approximately 1 in 500 biopsies.
Risk factors associated with an increased risk of bleeding include: less experienced operators, patients with bleeding diathesis, patients with ascites (fluid in the abdomen), active malignancy and in some patients who have an increased number of passes (however this risk appears in studies to be of statistical significance when > 3 biopsies carried out).

To minimise the risks of bleeding we will ensure the following:
1. All patients have an up to date (within 1 week) blood sample checked to assess the patients clotting and platelet count.
2. Avoid biopsy in patients who have platelet counts less than 150,000 (U/mmc) or whose INR > 1.3. If patients do have the aforementioned parameters, they will be excluded from the study.
3. All patients with malignancy (which confers an increased risk of bleeding) are excluded.
4. An experienced operator carries out procedure (again inexperience linked in studies to increased risk of bleeding).
5. To minimise bleeding further, patients who have ascites (fluid in the intra-abdominal compartment) will be excluded. This will be done on the basis of clinical examination and also by the presence/absence being confirmed on Ultrasound within 6 months prior to liver biopsy.

The current UK recommendations (from the British Society of Gastroenterology in 1999) state: "Usually one pass of the biopsy needle retrieves enough hepatic tissue for diagnostic purposes; however, if there may be a sampling error (such as may occur in
macronodular cirrhosis) which will result in an inappropriate diagnosis, then two passes may be made without significantly affecting the complication rate (Grade B recommendation)" (Grant & Neuberger GUT 1999;45;iv1-iv11).

2.BLOOD SAMPLE:
An extra blood sample will be taken either at the time of liver biopsy, or at a meeting beforehand between the research team and the patient. The patient will be having blood normally taken pre-biopsy for assessment of their clotting, so an extra sample will be taken at this point in time. We feel there is no major ethical issue in these patients having an extra 80mls of blood taken for research reasons (note: the Oxford Hepatitis C group do already have ethical approval on liver disease patients to take samples for research purposes).

3.PATIENT CONFIDENTIALITY
Once gained the liver biopsy samples will be anonymised (also their matching blood samples will be) and experiments can be done on them.
The main ethical issues in our opinion will be taking liver samples from patients.

11.1 Informed consent

The patient information sheet (P.I.S) describes the objectives of the research, the procedure involved and any risks/discomfort of the liver biopsy. All patients are given copies of information leaflets and have sufficient time to read these, ask any questions arising from the written information or accompanying verbal information and to discuss the possibility of taking part in the research with family members or health professionals (such as GP). The information leaflet confirms that patients or subjects are free to withdraw from the study at any time and for any reason without any effects on future medical care and without being obliged to give a reason for their withdrawal.

11.2 Patient/Subject confidentiality
Confidentiality and patient data protection are vital elements in our research lab. The investigators take responsibility for this under the oversight of the independent steering committee. All participating patients give informed consent for their data to be stored on the research database. The methods for achieving security of this electronic database are described below (section 12). All paper documents recording patient information will be stored securely in compliance with the data protection act.

12 DATA HANDLING AND REDCORD KEEPING

Security of data collection, handling, transfer, storage and use is a key component of research performed in our Laboratory; and central to the continuing integrity and viability of research projects in our area.

Data will be collected in hospital clinics, wards and radiology departments. Medical/ research staff trained in high quality handling and secure procedures will collect all data. All other information and data will be stored electronically.

The security of the dataset is maintained by 3 key principles:

1. Access to clinical and personal data in the database is limited to the named investigators and named research staff (typically research fellow and research nurse). Access is allowed only from site-specific computers with personal usernames and passwords; the latter being changed regularly. The research individuals will sign confidentiality agreements specific for the database and will be trained in electronic security.

2. All patient identifiable data (name, hospital number, dates of birth, address) will be stored in one database and all clinical and research data are stored in another separate one. When data is downloaded for research purposes patient identifiable data cannot be included and individuals are only identified by their encryption code, although age (but not date of birth) and sex can be downloaded.

3. The database system is maintained in a secure (Category III) laboratory, with intruder detection and encryption.

The number of staff who have access to all fields of the disease cohort database is limited, with them having to sign a confidentiality agreement specific for the database and are trained in electronic security. It is also permitted for auditors, and/or inspectors from regulatory bodies and ethics committees to have direct access to the source documents and data.

13. FINANCE AND INSURANCE/INDEMNITY

The costs of the study are funded by the Wellcome Trust as part of the CI's fellowship award. Indemnity and/or compensation for negligent harm arising specifically from an accidental injury for which the University is legally liable as the Research Sponsor may be covered by the University of Oxford.

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The Peter Medawar Building for Pathogen Research
South Parks Road, Oxford OX1 3SY, Tel: 01865281230

Oxford Radcliffe Hospitals NHS
NHS Trust

PATIENT INFORMED CONSENT SHEET

STUDY TITLE:
"ANALYSIS OF HEPATIC T-CELL RESPONSES FROM LIVER BIOPSY SPECIMENS"
DATE AND VERSION: 28th June 2010 Version 3
REC NO: **09/H0603/19**

PLEASE INITIAL EACH BOX

1. I confirm that I have read and understood the information sheet dated 28th June 2010, for the above project and have had the opportunity to ask questions and have had these added satisfactorily. ☐

2. I agree to take part in the above research project, and agree to have a 2nd pass liver biopsy carried out to gain another sample for research purposes. I also consent to a sample of blood to be given for research purposes. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason.

4. I understand that my blood and liver samples and information about me will be anonymised before analysis. I give permission for members of the Oxford research team to have access to my anonymised information and samples.

5. I wish my GP to be informed that I am taking part in this study.

6. I agree to be contacted regarding future studies that are ethically approved.

7. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Oxford University, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records

PLEASE INITIAL EACH BOX

8. I agree to gift blood/liver samples taken for the purpose of the research study to the University of Oxford. If a commercial product were developed as a result of this study, I will not profit financially from such a study.

9. I understand that samples may be stored for use in possible research on liver disease. I give permission for my samples to be used for any such research that has been approved by a research ethics committee.

Name of participant (or legal representative)	Date	Signature

Name of person taking Consent (if different from Lead researcher) | Date | Signature

(TO BE SIGNED AND DATED IN PRESENCE OF THE PARTICIPANT)

Lead Researcher | Date | Signature

(TO BE SIGNED AND DATED IN PRESENCE OF THE PARTICIPANT)

Copies:
Once all parties have signed this, the participant should receive a copy of the signed and dated participant consent form, the letter/pre-written script/ information sheet and any other written information provided to the participants. A copy for the signed and dated consent form should be placed in the project's main record (e.g. a site file), which must be kept in a secure location.

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The Peter Medawar Building for Pathogen Research
South Parks Road, Oxford OX1 3SY, Tel: 0186528123

PATIENT INFORMED CONSENT TO BE CONTACTED BY RESEARCH TEAM

STUDY TITLE:
"ANALYSIS OF HEPATIC T-CELL RESPONSES FROM LIVER BIOPSY SPECIMENS "

DATE AND VERSION: 4th August 2009 version 2
REC NO: 09/H0603/19

Please initial box

I hereby consent to the research team contacting me by telephone 7 days after my clinic appointment to see if I am interested in taking part in the research.
My daytime telephone number I wish to be contacted on is:

Name of participant (or legal representative)	Date	Signature

Name of person taking Consent (if different from Lead researcher)	Date	Signature

(TO BE SIGNED AND DATED IN PRESENCE OF THE PARTICIPANT)

Lead Researcher	Date	Signature

Copies:
Once all parties have signed this, the participant should receive a copy of the signed and dated participant consent form, the letter/pre-written script/ information sheet and any other written information provided to the participants. A copy for the signed and dated consent form should be placed in the project's main record (e.g. a site file), which must be kept in a secure location.

University of Oxford
The Peter Medawar Building for Pathogen Research
South Parks Road, Oxford OX1 3SY,
Tel: 01865281230

PATIENT INFORMATION SHEET

STUDY TITLE: "ANALYSIS OF HEPATIC T-CELL RESPONSES FROM LIVER BIOPSY SPECIMENS"

RESEARCH PROJECT

DATE & VERSION NO: 4th August 2009 VERSION 2
REC NO: 09/HO603/19

INVITATION

You are being invited to take part in a research study, which involves potentially taking a sample of your liver and a blood test to be used for research purposes. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully, and discuss it with others if you wish.

1. WHAT IS THE PURPOSE OF THIS STUDY

The purpose of this study is to assess the body's immune response to conditions affecting the liver. The liver is an organ in the body that can be affected by various diseases, but has the capacity to regenerate after damage. However often, chronic damage can lead to permanent irreversible damage to the liver and to liver cirrhosis.

Our aim is to explore how the body reacts to various injuries to the liver specifically Hepatitis C, and how the body fights against this disease affecting the liver. We also wish to study the livers responses to other diseases affecting it, and how this response differs from Hepatitis C. To do this we wish to look at samples of liver biopsies taken from people who require liver biopsies for medical reasons (whether it be to diagnose conditions, to accordingly alter treatment or aid treatment decisions) and also look at blood samples. We will not be asking patients to have a liver biopsy for research reasons unless they actually require one for clinical reasons.

2. WHY HAVE I BEEN CHOSEN?

You have been chosen as you are having a liver biopsy carried out on the basis of clinical decisions made by the liver doctors looking after you.

4. DO I HAVE TO TAKE PART?

No. It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep, and offered an opportunity to meet the research team at your initial clinic appointment.

You will be given a reply slip to post to the research team, offered to be contacted by the research team and also be offered in time another appointment to meet the research team. At this appointment you will have a chance to speak to the research team, ask any questions and be asked to sign a consent form to confirm that you understand what is involved when taking part in this study.

If you decide to withdraw from the study we will still keep the records or data relating to any samples if these have already been taken, unless you object to this. A decision to withdraw at any time, or a decision not to take part, will not affect the quality of care you receive.

5. WHAT WOULD HAPPEN IF I TAKE PART?

You will be given this information sheet to take home with you. You will be asked at clinic whether it would be ok for the research team to contact you at home 7 days after your clinic appointment to see if you wish to take part. If you wish to take part, you will be offered another appointment before your liver biopsy to

meet with the research team to discuss the study, answer any of your questions, and to have a blood sample taken. If you do not wish to or cannot attend the appointment, then a blood sample form will be sent to you to have a blood test carried out 5-7 days prior to liver biopsy, and then another sample taken on the day of the liver biopsy.
If you decide to take part, you will have a liver biopsy carried carried out as normal except 2 samples of liver tissue would be taken instead of one. This means the needle that takes the biopsy sample will be entered twice into the liver instead of once. You will be given local anaesthetic prior to this in the area of the liver to numb any sensation – thus decreasing any pain felt. Prior to the liver biopsy (at your initial clinic appointment) you will be given an explanation sheet of liver biopsies. After your liver biopsy, you will be observed for 6 hours in the hospital before being allowed home. After having the liver biopsy taken, you will no longer be part of the study, and will be followed up by the liver team for results in due course. Often after 5 years, patients are invited to have a liver biopsy 5 years after their initial biopsy, however this will be a decision made after 5 years by the hospital team, and not involve the research. However if you have had a liver biopsy 5 years previous to now, and require another one; you will be invited to participate in the study.

6. WHAT WOULD HAPPEN TO ANY SAMPLES THAT I GIVE?

Blood samples would be used to do tests (in the hospital) usual for your condition and would be processed in the University laboratory to prepare them for analysis of the immune system. The same would happen for the liver biopsy sample sent for research purposes. The other sample of liver tissue would be sent to the pathology lab for processing to aid with your medical condition. This result would be conveyed to you within 4 weeks.
Samples collected for this research would not have your name on them but would be identified by a unique research number. If the samples reveal anything relevant to your care, your study doctor would be informed.
<u>The majority of the samples gained (blood and liver biopsy) will be used in experiments shortly after you have given them. So blood and liver cells may be stored in a high security laboratory freezer to be used only during the duration of the study.</u>

7. PREGNANCY

If you were pregnant you would not be asked to take part in this study.

8. WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS?

Obtaining liver tissue via liver biopsy is not without risk and complications. These include:

1. Pain. Pain is the most common complication with up to 84% of people experiencing mild discomfort. This pain is normally managed with small amounts of painkillers including paracetamol, ibuprofen or codeine. If you were to experience severe pain, you would be continually monitored in the hospital. If pain was severe, a repeat ultrasound jelly scan or even C.A.T scan may be performed to assess for any major complications.

2. Bleeding. The risk of severe bleeding requiring a hospital stay and blood transfusion is between 1 in 2500 to 1 in 10,000 biopsies. Less severe bleeding (but not requiring transfusion) occurs in approximately 1 in 500 biopsies.

The risk of increased bleeding will be minimized by the following ways:
-Less than 3 biopsies being done (as >3 biopsies associated with an increased risk of significant bleeding).
-An experienced doctor carrying out the procedure under Ultrasound guidance.
-If you take part, you will have an up to date (within 1 week) blood sample checked to assess the thinness (clotting) of your blood. If any problems are found in the blood clotting you will not be entered into the study, but may still have a biopsy performed after discussions with the doctors looking after you.
-All patients with a known cancer (which can increase the risk of bleeding) will be excluded.
-To minimise bleeding further, all patients who have ascites (fluid in the abdominal compartment normally requiring diuretic tablets) will be excluded. This will be done on the basis of clinical examination and also by the presence/absence being confirmed on Ultrasound within 6 months prior to liver biopsy.
-If you are pregnant, you will not be entered into the study.
The blood samples that you give during this study involve minimal risk but may be associated with minor discomfort, redness or bruising.

9. WHAT ARE THE POSSIBLE BENEFITS?

By carrying out research of the immune system in the liver itself, it will improve our understanding of why certain disease affect the liver, why the liver reacts in certain ways to certain stimuli, and why some diseases persist in the liver causing chronic conditions whereas some conditions resolve quickly and spontaneously.

10. WHAT WILL HAPPEN IF I DON'T WANT TO CARRY ON

You are free to decide if you do not want to carry on with this research at any time, without giving a reason and without any affect on your future care or treatment.

11. WHAT IF THERE IS A PROBLEM?

Compensation for harm arising from an accidental injury and occurring as a consequence of your participation in the study will be covered by the University of Oxford.
If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against the University of Oxford (in respect of any harm arising out of the participation in the Clinical Study) or the NHS (in respect of any harm which has resulted from the clinical procedure being undertaken).
If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you should contact the <*name of investigator*> or you may contact the University of Oxford Clinical

Trials and Research Governance (CTRG) office on 01865 743005. or the head of CTRG, email heather.house@admin.ox.ac.uk

12. WOULD MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

Every effort is made to keep records of personal information confidential and secure but an absolute guarantee of this can never be given. The information that you give will be stored with code rather than your name, on a secure computer system, which will only be accessible by the few individuals that are specifically named in this study.

Complete confidentiality is paramount to us, the research team. Personal information will be kept on a computer to which access is limited. Everyone who works on a computer that contains names of participants will be trained on the importance of confidentiality and how to protect this. They will sign confidentiality agreements agreeing to the arrangements and confirming that they understand how important it is. You would also be allocated a unique cohort number that would be used for all of your tests done on your samples and for most of the research on the information collected at clinic.

Nearly all the information necessary for the research will be used through your unique disease cohort research number. The researchers using this information are aware of the importance of security and confidentiality even though they do not have access to personal information such as your name or address. The small numbers of researchers that do have the key to personal details all sign agreements confirming that they understand the need for confidentiality have been trained in the ways that information is kept secure and agree to everything within their power to look after and secure your information and samples. Responsible members of the University of Oxford or the Oxford Radcliffe Hospitals NHS Trust may be given access to anonymised data to audit of the study to ensure we are complying with regulations.

13. WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

The results of this research study may be presented at local, national and international medical and scientific meetings or in publications. You will not be identified in any way in publications or reports.

14. WHO IS ORGANISING AND FUNDING THE RESEARCH?

The research is funded by the Wellcome Trust, and being arranged by the group of Professor Paul Klenerman. The study is sponsored by the University of Oxford.

15. WHO HAS REVIEWED THIS STUDY?

The research study has been reviewed by an NHS research ethics committee in accordance with local and national regulations.

16. CONTACT FOR FURTHER INFORMATION

YOUR DOCTORS:
Dr Neil Rajoriya – Tel. Number 01865281880 (**MAIN CONTACT)**

Dr Jane Collier – Tel. Number 01865228757
Dr Paul Klenerman – Tel. Number 01865281885
Dr Ellie Barnes – Tel. Number 01865271290
YOUR RESEARCH/SPECIALIST NURSE
(NAME TO BE INSERTED ONCE POST FILLED) Tel. Number 01865220757

If you choose to participate in this study – thank you. You will be given a copy of the patient information sheet and consent form to take home with you and keep for future reference.