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Hepatic Haematopoietic Stem Cells and the T Cell Development Potential of the Adult Human Liver.

A thesis submitted for the degree of Doctor of Philosophy

By

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Declaration.

I declare that this thesis is entirely my own work except where credited in the acknowledgements.

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Abbreviations.

Abbreviation	Description
°C	Degrees Centigrade
aa	Amino Acid
ABC	Avidin Biotin Complex
Abs	Absorbance
Ag	Antigen
AHL	Adult Human Liver
AO	Acridine Orange (3,6-bis[Dimethylamino]acridine)
APC	Antigen Presenting Cell
Apes	3-amino-propyl-triethoxy-saline
BCA	Bicinchoninic Acid
BCR	B Cell Receptor
BM	Bone Marrow
BSA	Bovine Serum Albumin
C	Constant
CD	Cluster of Differentiation
cDNA	complimentary Deoxyribonucleic Acid
cjTREC	coding joint TREC
CRP	C Reactive Protein
CSF	Colony Stimulating Factor
Cu	Copper
DAB	diaminobenzidine tetrahydrochloride
DEPC	Diethylpyrocorbonate
DMEM	Dulbeco's Modified Eagle's Medium
DN	Double Negative
DP	Double Positive
DSB	Double Strand Break
EB	Ethidium Bromide (2,7-Diamino-10-ethyl-9-phenyl-phenanthridinium bromide)
EDTA	Ethylenediaminetetra-Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EPO	Erythropoietin
FITC	Fluorescein Isothiocyanate
FTOC	Foetal Thymic Organ Culture
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
HAART	Highly Active Anti-Retroviral Therapy
HBSS	Hanks Balanced Salt Solution

HEV	High Endothelial Venule
HGF	Haematopoietic growth factor
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HMNCs	Hepatic Mononuclear Cells
HRP	Horse Radish Peroxidase
HSC	Haematopoietic Stem Cell
IEL	Intraepithelial Lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-(7)R	IL-(7) Receptor
IRF	Interferon Regulatory Factor
ISP	Immature Single Positive
KAR	Killer Activatory Receptors
Kb	Kilo Bases
KIR	Killer Inhibitory Receptors
L	Litre
LAK	Lymphokine Activated Killer
lin$^{+/-}$	Lineage positive or negative
LLC	Lewis Lung Carcinoma
LPS	Lipopolysaccharide
LSCM	Laser Scanning Confocal Microscopy
LT	Long Term
M	Molar
mAb	Monoclonal Antibody
MBP	Mannose Binding Protein
MFI	Median Fluorescent Intensity
mg/μg/ng/pg	milli-/micro-/nano-/pico-gram
$MgCl_2$	Magnesiun Chloride
MHC	Major Histocompatability
ml	Millilitre
Mø	Macrophage
mRNA	messenger Ribonucleic Acid
N	Normal
NaCl	Sodium Chloride
NaN_3	Sodium Azide
NaOH	Sodium Hydroxide
neg	Negative
NK	Natural Killer

nm	Nanometer
nt	Nucleotide
PBMC	Peripheral blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridin Chlorophyll Protein
PHSC	Pluripotent Haematopoietic Stem Cell
PKC	Protein Kinase C
PMN	Polymorphonuclear leukocytes
PMSF	PhenylMethylSulfonulFloride
pNPP	p-Nitrophenyl Phosphate
pos	Positive
QC-PCR	Quantitative Competitive PCR
RAG	Recombinase Activating Gene
RPMI	Roswell Park Memorial Institute
RSS	Recombination Signal Sequences
SAP	Serum Amyloid Protein
SCF	Stem Cell Factor
Sd	Standard deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
sjTREC	signal joint TREC
ST	Short Term
TAE	TRIS-Acetate-EDTA
TCR	T cell receptor
TGF-β	Transforming Growth Factor-beta
Th	T-helper cell
TN	Triple Negative
TNF-α	Tumour Necrosis Factor-α
TPO	Thrombopoietin
TRECS	T cell Receptor Excision Circles
TRIS base	TRIS[hydroxymethyl]aminomethane
Tween 20	Polyoxyethylene-sorbitan monolaurate
UTR	Untranslated Region
UV	Ultra Violet
V	Variable
V(D)J	Variable (Diversity) Joining
VCAM-1	Vascular Cell Adhesion Molecule-1

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There is a great number of people I would like to thank without whom this thesis would not have been started yet alone completed. I apologise in advance to anyone I may forget. The order of appearance of names is random and does not in anyway reflect the level of gratitude felt.

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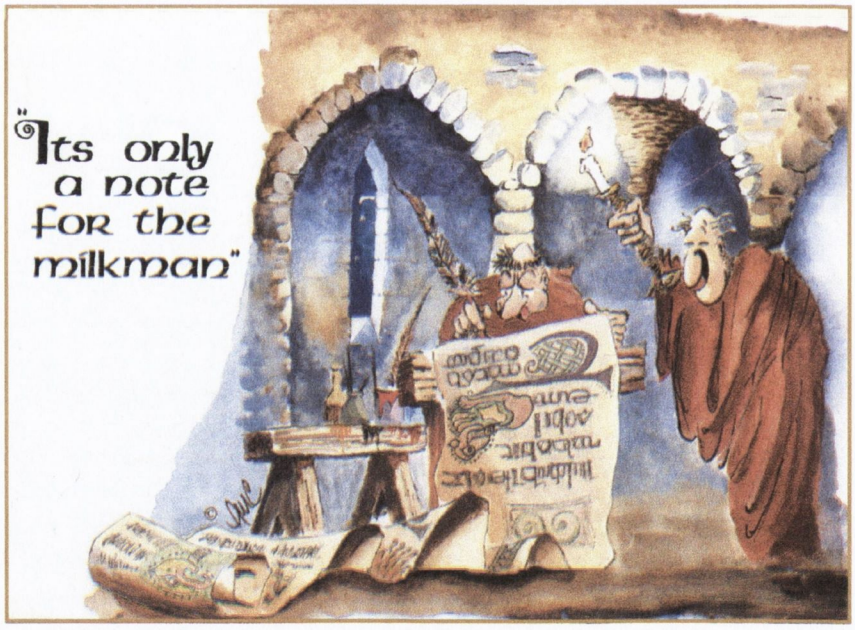
For Alex and my Parents



Summary.

Large populations of innate T cells are found in the normal adult human liver (AHL), some of which may differentiate locally. The AHL also contains populations of functional myeloid and erythroid progenitors. The co-expression of lineage-specific markers on hepatic haematopoietic progenitors has not been examined. In this study, the presence and phenotype of lineage-committed haematopoietic progenitors in the normal AHL was investigated and compared to the profiles of differentiating haematopoietic precursor populations detected in liver bearing metastases of colonic origin. Levels of haematopoietic stem cells (HSCs, $CD34^{+}CD45^{+}$) were increased six fold when compared to matched peripheral blood samples. In normal liver, less than 5% of HSCs expressed the myeloid associated antigen CD33 whereas considerable proportions expressed lymphoid associated markers (T cell 33.39%, B cell 17.39% and natural killer cell 37.17%). Significant increases were observed in the relative proportions of hepatic HSCs co-expressing CD33 (20.53%, $p = 0.001$), and the T cell associated antigen CD7 (58.13%, $p = 0.02$) in tumour-bearing liver compared to normal liver. HSCs with B-cell progenitor phenotype ($CD19^{+}$) were significantly decreased in tumour-bearing liver (0.06%, $p = 0.02$). Despite these differences, the activation status of haematopoiesis, as measured by the co-expression of the differentiation and activation markers (CD38 and CD45RA), did not differ significantly between normal and tumour-bearing liver. Using confocal microscopy, HSCs were localised to portal tract areas in normal hepatic tissue. These results indicate that the normal adult human liver harbours lineage committed haematopoietic progenitors and the vast majority of these progenitors express lymphoid associated antigens with changes occurring in both the myeloid and lymphoid compartments of the hepatic haematopoietic pathway on tumour challenge.

Recombinase activating gene(RAG)-1, RAG-2 and pre-TCR-α expression in the normal AHL, together with the presence of lymphoid-haematopoietic progenitors, is strong evidence that the AHL supports T cell maturation. Interleukin(IL)-7 and IL-15 have been shown to play an essential role in thymic-independent T-cell development. In this study, we investigated IL-7 and IL-15 mRNA and protein levels in order to determine whether AHL could provide a suitable microenvironment to support T-lymphocyte differentiation. Biopsies were snap frozen, powdered,

and RNA/protein was extracted. RT-PCR was used to detect IL-7 mRNA using primers that amplified 620 base pair (bp) fragments and other smaller transcripts. RT-PCR was also used to detect IL-15 transcripts that correspond to intracellular and secreted forms of this protein. A sandwich ELISA was developed to quantify IL-7 and IL-15 protein in tissue homogenates. IL-7 specific product (620 bp) was detected in 9/10 samples. Six samples were also positive for a smaller splice-variant (488 bp). Levels of the 620 bp product were 2.5 times greater than the 488 bp fragment. Hepatic tissue predominantly expressed the mRNA isoform associated with secreted IL-15. IL-7 protein was detected in all samples (mean 41.95 pg/100 mg tissue, range 18.47 - 76.93). Protein for IL-15 was detected in all samples tested (mean 116.34 pg/100 mg tissue, range 31.94 – 193.84). Immunohistochemistry demonstrated IL-7 protein in discrete cells of lymphoid-morphology, widely distributed throughout the parenchyma and within portal tracts.

The presence of lymphoid progenitors, IL-7 and IL-15 protein, taken together with RAG-1, RAG-2 and pre-TCR-α expression in the AHL provides evidence of local T cell development. We looked for evidence of ongoing $\alpha\beta$-TCR development in AHL by quantifying TRECS (T cell receptor excision circles produced during TCR gene rearrangement) in liver and matched blood. $CD3^+$ populations were isolated from the liver and blood of eight individuals. TRECs were detected in all PBMC samples at a mean level of 318 TRECS/1 x 10^5 naïve $\alpha\beta$ T cells, while only two hepatic mononuclear cell samples were positive at extremely low levels (48 and 5/1 x 10^5 naïve $\alpha\beta$ T cells). The absence of TRECS in liver provides evidence that the normal AHL is not a site for the development of conventional $\alpha\beta$ T cells. It is likely that hepatic lymphoid progenitors give rise to $\gamma\delta$ or unconventional $CD8^+$ $\alpha\beta$ T cell populations and that IL-7 and IL-15 produced locally play an important role in this pathway.

The demonstration of stem cells in the adult liver raises exciting possibilities for the future. Intervention in the normal development pathways of adult stem cells has the potential to provide us with a natural, safe tool to tailor the immune system to be more effective in the elimination of disease, induction of peripheral tolerance and stimulation of tissue regeneration.

CONTENTS.

Chapter 1: General Introduction.

1.1: Physiology of the adult human liver.

1.1.1: Macroanatomy.

The liver is the largest organ in the body weighing approximately 1.5 Kg (or 2% of body weight), and is positioned in the upper right side of the peritoneal cavity under the diaphragm. Classically, the liver is divided into a large right lobe and a smaller left lobe, however this bears no relationship to the true functional division of equal right and left lobes, which is based on blood supply (figure 1.1).

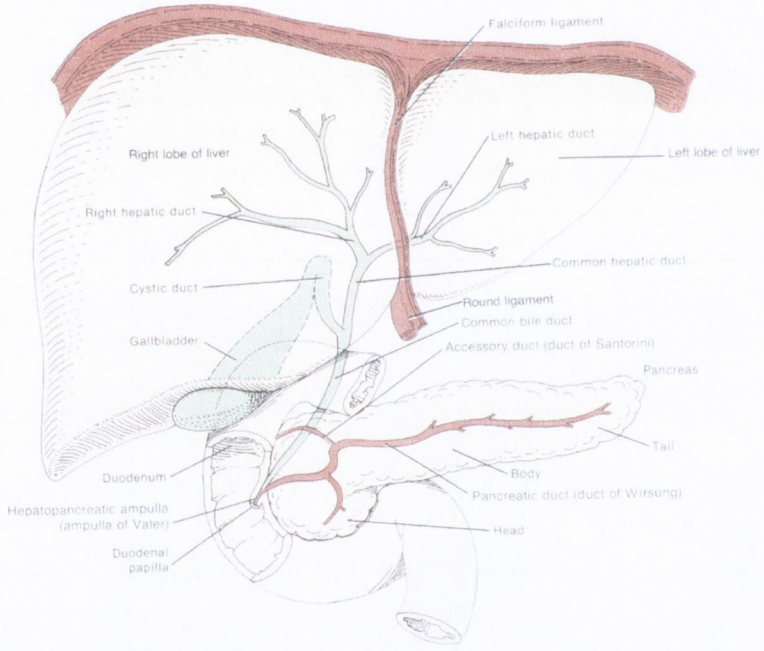


Figure 1.1: Macroanatomy of the liver (adapted from Tortora and Grabowski, 1990).

The liver receives a double blood supply from the hepatic artery, which is a branch of the coeliac axis, and from the portal vein, which is formed by the mesenteric and splenic veins. Total liver blood flow is 1,300 ml per minute, one quarter of the resting cardiac output. The hepatic artery supplies approximately 25% of total blood flow and 50% of oxygen. The portal vein, which drains the entire small intestine and most of the large bowel, provides the remaining liver blood flow and oxygen. Both hepatic artery and portal vein run to the liver hilum before dividing into major right and left branches. Venous drainage of the liver occurs via the hepatic veins, which drain directly into the inferior vena cava that runs posterior to the

liver between the functional right and left lobes. Lymph from the liver is drained directly into the thoracic duct. The biliary system collects bile, which it stores and concentrates in the gall bladder, and delivers to the duodenum when required. The gall bladder lies on the under-surface of the right lobe of the liver, and its cystic duct runs upwards to join the common bile duct. The main left and right hepatic ducts join in the hilum to form the common bile duct. The common bile duct runs with the hepatic artery and the portal vein behind the first part of the duodenum and pancreatic head to enter the second part of the duodenum through a shared channel with the pancreatic duct (ampulla of Vater) (Fleming, 1999; Guyton, 1986).

1.1.2: Microanatomy.

The basic structural functional unit of the liver is the liver lobule. The human liver contains 5 x 10^4 to 1 x 10^5 individual lobules. The liver lobule is constructed around a central vein, which is surrounded by radially oriented channels or sinusoids running between plates of parenchymal hepatocytes, and five or six portal tracts at the periphery. Each portal tract contacts three lobules and contains vessels of three main types: branches of the hepatic artery, portal vein and bile duct. Lymphatic vessels are also present, but since their walls are delicate and often collapsed, they are not readily seen. Each hepatic plate is one to two cells thick, and between the adjacent cells, lie small bile canaliculi that empty into bile ducts that originate in the fibrous septa that separates the adjacent liver lobules. In the septa are small portal venules that receive their blood from the portal veins. From these venules, blood flows into flat, branching hepatic sinusoids that lie between the hepatic plates and into the central vein. Hepatic arterioles are also present in the interlobular septa. These arterioles supply arterial blood to the septal tissues, and may also empty into the hepatic sinusoids. Endothelial cells lining the venous sinusoids contain large fenestrations of approximately 2 μm in diameter and do not have a basement membrane. Beneath this lining, lying between the endothelial cells and the hepatocytes, is a very narrow tissue space called the space of Disse. Plasma constituents can diffuse freely into the space of Disse where they are in direct contact with the sinusoidal surface of hepatocytes. Also present in the sinusoids are large tissue macrophages (Kupffer cells) capable of phagocytosing bacteria and other foreign matter in the portal blood. Kupffer cells are arranged in an apparently random fashion along sinusoids, resting on endothelial

cytoplasm or between the endothelial cells (figure 1.2). It is tempting to speculate that the distribution is not entirely random, as the density is greater in the periportal region (Fleming, 1999; Guyton, 1986).

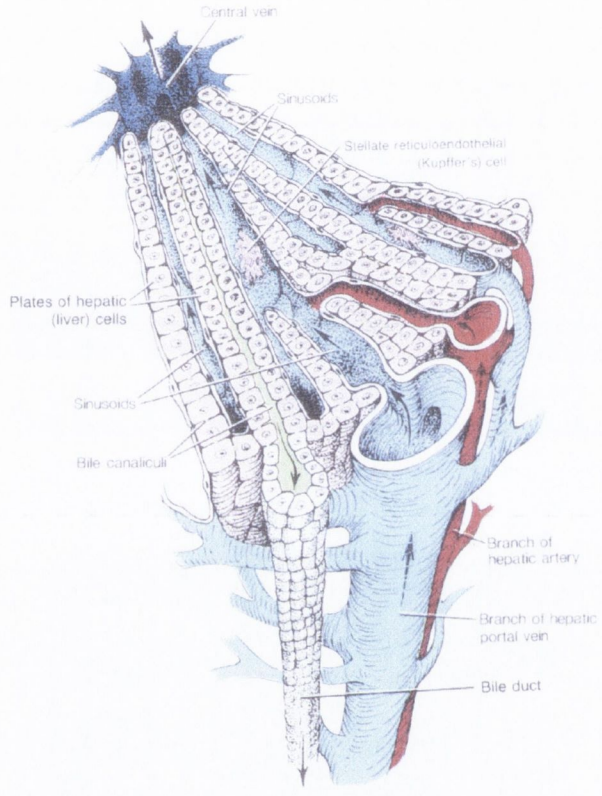


Figure 1.2: Diagrammatic representation of the microscopic appearance of the liver lobule (adapted from Tortora and Grabowski, 1990).

1.2: Functions of the adult human liver.

The liver is described as a secondary digestive organ, however this term is inadequate to describe the varied and multiple biochemical functions carried out by the liver.

1.2.1: Metabolic functions of the liver.

Over fifty separate functions have been described for this vital organ, many of which are concerned with screening and sorting of digested food. The liver plays a central role in the synthesis of proteins, carbohydrates and lipids and is involved in many anabolic and catabolic

reactions that regulate energy homeostasis in the post prandial and fasting states. The liver is especially important for maintaining a normal blood glucose concentration. The liver removes excess glucose from the blood, stores it, and then returns it to the blood when blood glucose concentration begins to fall. Gluconeogenesis occurs in the liver when blood glucose levels cannot be maintained by glycogen breakdown and, in the post absorptive state, is the only source of blood glucose. The liver is also the sole source of important plasma proteins including clotting factors and albumin. The liver is responsible for the biotransformation or detoxification and recycling of many endogenous and exogenous substances such as spent red blood cells, ammonia, steroid hormones, ethanol, drugs and toxins. Formation of urea by the liver removes toxic ammonia from body fluids, that if allowed to rise in concentration can rapidly result in hepatic coma. The liver therefore serves a biochemical regulatory or protective function for the whole organism (Guyton, 1986; Voet and Voet, 1990).

1.2.2: Immune functions of the liver.

The liver, located between gastrointestinal tract and cardiopulmonary system, receives a double blood supply. Total liver blood flow is approximately one and a half litres per minute. The hepatic artery supplies approximately 25% of total blood flow and the portal vein, which drains the entire small intestine and most of the large bowel, provides the rest (Emslie-Smith *et al.*, 1988; Guyton, 1986). As a result, the liver is continually exposed to pathogens, toxins, dietary antigens and metastatic cells that enter the circulation. The liver therefore needs to be armed with a range of protective mechanisms to deal with this large antigenic load. The hepatic immunological environment is required to mount aggressive reactions against infectious agents and malignant cells while at the same time generating tolerogenic responses to harmless dietary antigens. As well as innate defence mechanisms, the involvement of cells of the specific immune response would be required to enable the liver to carry out these diverse immune functions. However, the presence of lymphocytes in the liver has until recently been considered a pathological feature and the role of the normal liver in protective immunity has largely been ignored. As a site of early encounter of exogenous antigen, the liver plays a key role in innate immunity.

1.2.2.1: The role of the liver in innate immunity.

The role of innate immunity is to protect the host in the first minutes or hours after exposure to potentially harmful agents. The microorganisms that are encountered daily in the life of a normal healthy individual only occasionally cause perceptible disease, illustrating the effectiveness of physical defence barriers and innate immunity. It also makes a significant contribution to the systemic immune system by being the main source of components of the humoral innate response. A number of serum proteins of the complement system are produced by hepatocytes. In response to interleukin(IL)-1, IL-6 and Tumour Necrosis Factor(TNF)-α release by macrophages (Mø), changes occur in protein production and secretion by hepatocytes. This is termed the acute phase response and the proteins produced are fundamental in the initial response to microbial infection (Kushner and Rzewnicki, 1994; Steel and Whitehead, 1994). Acute phase proteins include serum amyloid protein (SAP), C-reactive protein (CRP), fibrinogen and mannose-binding protein (MBP), (figure1.3).

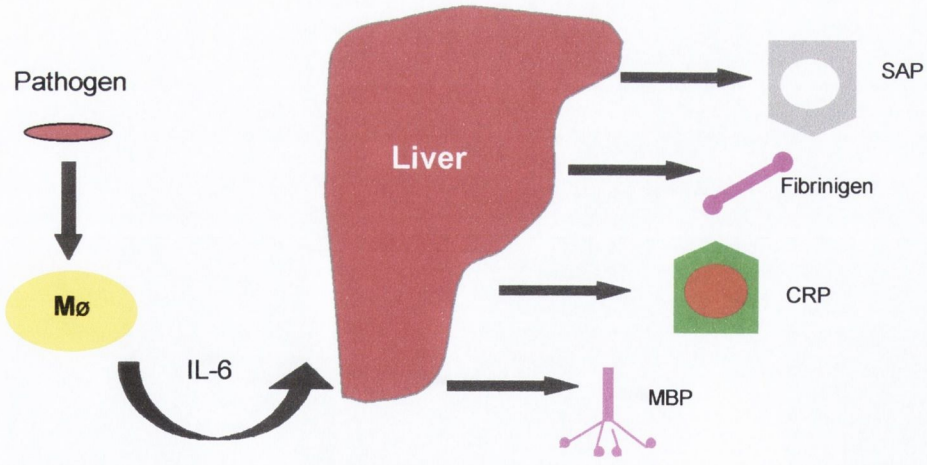


Figure 1.3: The acute phase proteins produced in response to IL-6.

CRP binds the phosphoryl choline portion of certain bacterial and fungal cell wall polysaccharides not found in mammalian cell membranes. When CRP binds to pathogens, not only is it able to opsonise, but it can also activate the complement cascade. MBP is found in normal serum at low levels and is increased during the acute phase response. MBP is a calcium-dependent sugar-binding lectin that interacts with mannose residues on microbes

and is structurally similar to the complement component C1q. Binding of MBP to pathogen surfaces activates complement (Kushner and Rzewnicki, 1994; Steel and Whitehead, 1994).

Complement is a is a powerful innate effector mechanism comprising a group of 25 plasma and cell membrane proteins that play a key role in host defence (Janeway and Travers, 1996). Activation of the complement system has three major consequences. Firstly, the lysis of bacteria and enveloped viruses is mediated by complement, which generates pores in plasma membranes. Secondly, enhancement of phagocytosis is achieved by opsonisation of pathogenic particles. This process involves the coating of the foreign particle with specific protein fragments that can be recognised by receptors on phagocytic cells. The third function of the complement system is the generation of peptide fragments that regulate the inflammatory response. These proteins cause vasodilation at inflammatory sites, induce the adherence of phagocytes to blood vessel endothelium, or act as chemoattractants in phagocyte recruitment (Frank, 1991). Components of the complement system can bind to bacterial surfaces, thus triggering a cascade of events that result in destruction of the pathogen. Host cells are protected from destruction by complement through expression of complement regulatory proteins (Liszewski *et al.*, 1996). Thus, within 24 – 48 hours, the liver, through the acute phase response, provides the host with molecules that can bind and activate clearance of a broad range of pathogens (figure 1.4). These proteins do not have structural diversity and are produced in response to any stimulus that triggers IL-1, IL-6 and TNF release. Thus, their synthesis by hepatocytes is not induced by or targeted to specific pathogens (Janeway and Travers, 1996).

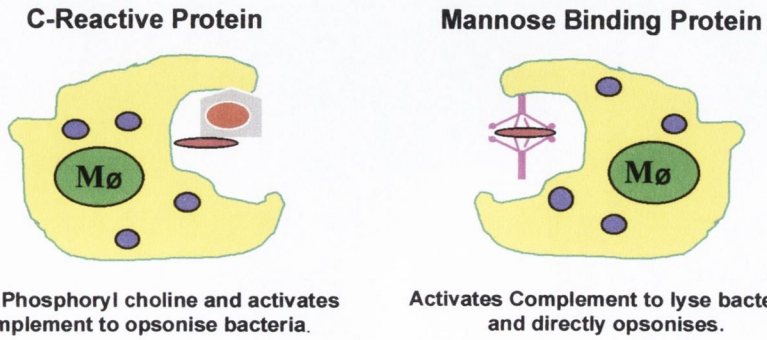


Figure 1.4: Action of acute phase proteins.

Cellular components of the innate immune system are also found in the liver. Central to innate immunity is the recognition of conserved molecular patterns shared by large numbers of pathogens. Nonclonal, germline-encoded host cellular defence receptors or molecules are responsible for this recognition (Medzhitov and Janeway, 1997b). Macrophages express many cell surface receptors that recognise microbial components. These receptors include the mannose receptor, Mac-1 (also known as CR3 or CD11b/CD18) and CD14 which binds lipopolysaccharide (LPS, a cell wall component of gram-negative bacteria). Trapping and engulfment of bacteria by macrophages at the site of entry may be sufficient to eliminate a pathogen (figure 1.5) (Janeway and Travers, 1996).

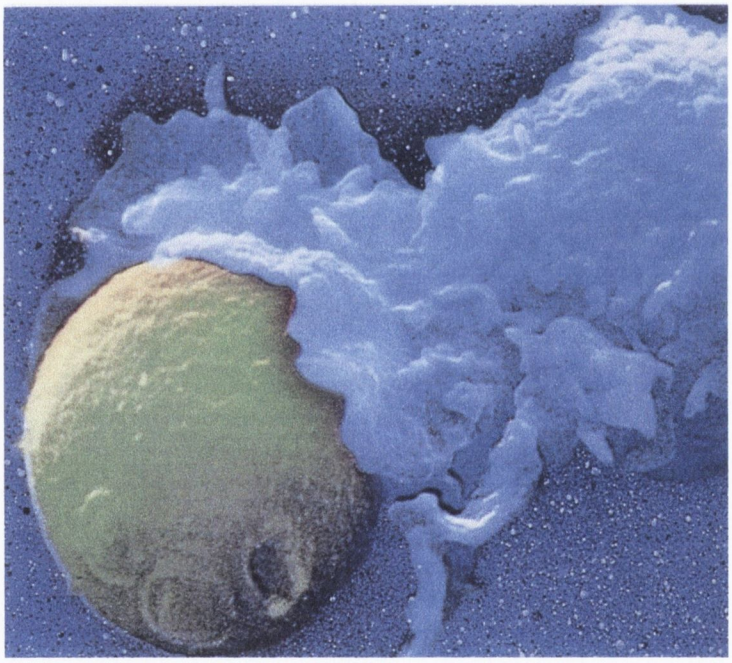


Figure 1.5: Macrophages engulf and destroy pathogens at the site of infection.

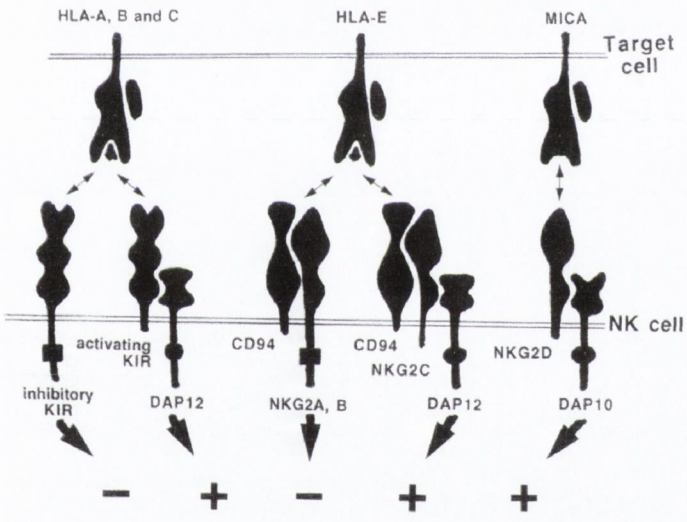
A macrophage (blue) swallowing an infectious yeast cell (green). Macrophages have been described as foot soldiers in the body's non-stop war against infection (reproduced from Krieger, 1998).

Hepatic macrophages, termed Kupffer cells, account for 15% of the total number of cells in the liver (Gates *et al.*, 1961). Kupffer cells express receptors for complement components (Jones and Altorfer, 1988). Removal of endotoxin from the portal circulation is a specialised function of Kupffer cells (Winwood and Arthur, 1993) and it has been demonstrated that they are actively involved in phagocytosis of macromolecules and colloidal particles from the

bloodstream (Pranning van Dalen *et al.*, 1981). Engagement of receptors on macrophages induces the expression of co-stimulatory molecules (B7) necessary for T cell activation. Macrophages also play an important role in antigen uptake and processing (Medzhitov and Janeway, 1997b). Kupffer cells have also been implicated as having an active role in the surveillance and prevention of hepatic metastases (Heuff *et al.*, 1995; Zhang *et al.*, 1993) and in mediating rejection (Sankary *et al.*, 1995). Kupffer cells further contribute to host defence mechanisms by secretion of cytokines such as IL-1, IL-6, IL-8 and TNF-α. These cytokines act as chemoattractants for phagocytes (IL-8), increase vascular permeability (IL-1, TNF-α), initiate systemic effects such as fever (IL-1, TNF-α, IL-6) and the acute phase response (IL-6) (Janeway and Travers, 1996). Secretion of IL-12 by tissue macrophages in response to LPS is crucial in the activation of natural killer (NK) cells and in diverting the differentiation of the subsequent CD4 T cell response towards production of pro-inflammatory mediators (Hall, 1995). Kupffer cells can also secrete IL-10 in response to LPS, which may influence the behaviour of other hepatic cells. IL-10 produced by Kupffer cells may be important in downregulating the production of IL-1 and IL-6 by sinusoidal endothelial cells (Knolle *et al.*, 1995) and diverting the differentiation of the CD4 T cell response towards production of humoral mediators.

In addition to Kupffer cells, the liver is a rich source of NK cells (Whiteside and Heberman, 1994; Norris *et al.*, 1998; Doherty *et al.,* 1999). NK cells are directly involved in the killing of virally infected cells and bacteria (Bancroft, 1993; Robertson and Ritz, 1990). Their killing mechanisms are activated on the detection of the absence of self-major histocompatability (MHC) class I molecules, which are highly polymorphic molecules expressed on the surface of all nucleated cells. Viral infection or transformation often results in decreased expression of MHC class I, therefore NK cells have a critical role in defence against infection and malignancy. Interferon (IFN)-γ production by activated NK cells augments the inflammatory response (Arase *et al.*, 1996; Kos and Engleman, 1996). An important role in the surveillance and the prevention of metastatic spread of cancers has been attributed to NK cells (van de Griend *et al.*, 1984; Whiteside and Heberman, 1995). Because of their potent direct cytotoxic ability, NK cells represent a potentially dangerous cell population, which if not strictly

regulated, could significantly harm the host. NK cells express on their cell surface a network of activatory receptors (Killer Activatory Receptors, KARs) which co-operate to induce optimal activation and killing of target cells (Lanier and Phillips, 1996). In addition, NK cells express distinct families of cell-surface receptors capable of antagonising the activatory signals (Killer Inhibitory Receptors, KIRs). KIRs are involved in the inhibition of cytotoxic responses against normal MHC class I expressing self-cells (Colonna *et al.*, 1997; Raulet *et al.*, 1997). NK cells are thus thought to be the principal cell population responsible for distinguishing self from non-self and ensuring the integrity of the host (Ljunggren and Karre, 1990). Regulation of NK cell activity appears to be provided by a complex, quantitative balance of inhibitory and activatory signals (Lanier, 1997; Rolstad and Seaman, 1998) (figure 1.6) .



The double-ended arrows represent the interactions between MHC class I or class I like molecules on a target cell (top) and their receptors on the natural killer (NK) cell (bottom).
The round or square symbols on the cytoplasmic portions of the molecules associated with the NK receptors represent ITAMS (immunoreceptor tyrosine-based activatory) and ITIMS (inhibitory motifs) respectively.
The activatory or inhibitory signals transduced to the NK cell are donated by +/-.

Figure 1.6: Natural killer cell receptors (adapted from Doherty and O'Farrelly, 2000).

1.2.2.2: The role of the liver in adaptive immunity.

The liver is not traditionally thought to play a primary role in the adaptive immune response. The presence of lymphocytes in the liver is commonly considered to be associated with disease pathogenesis. Lymphocyte infiltration is well documented in inflammatory conditions such as autoimmune and viral liver disease, and the term "liver-infiltrating lymphocytes" has been used to describe hepatic lymphocytes in these conditions (Hata *et al.*, 1990). The presence of lymphocytes in non-diseased liver has been documented in the setting of liver transplantation where they are described as "passenger lymphocytes", a term that implies a

trafficking event and does not convey the possibility that these lymphocytes may be resident in the donor liver (Schlitt *et al.*, 1993). However, recent studies have demonstrated that the normal adult human liver (AHL) contains significant populations of T and B cells, predominantly located in portal tracts, which appear to be tissue resident as they are not removed by extensive perfusion (Norris *et al.*, 1998) (figure 1.7). The origin and role of hepatic lymphocytes in normal liver remains controversial.

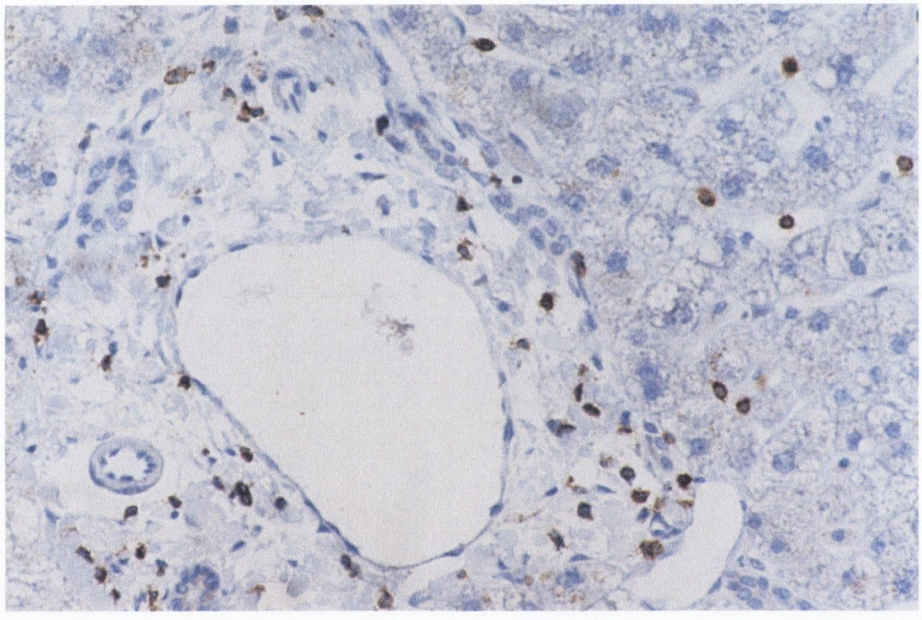


Figure 1.7: Staining for T lymphocytes ($CD3^+$, brown) in a normal liver section (x 200).

It has been proposed that the role of the liver in adaptive immunity is a scavenging one, analogous to its role in the clearance of spent red blood cells. The liver has been postulated to be a site for the elimination of activated cytotoxic $CD8^+$ T cells by apoptosis. The predominance of $CD8^+$ activated T cells in the normal liver supports the notion that the liver is a specific site for the deletion of peripheral activated cytotoxic T cells (Crispe and Huang, 1994; Huang *et al.*, 1994; Crispe and Mehal 1996, Mehal *et al.*, 2001). The high proportion of $CD8^+$ T cells in normal liver could be a reflection of this scavenging function, however, accumulation of activated $CD8^+$ T cells only occurs in circumstances where there is disruption of the apoptotic pathways and, due to rapid elimination, are not observed in normal liver.

Phenotypic and functional analysis of tissue-associated lymphocytes has demonstrated the presence of unusual T cells, rarely found in peripheral blood, in a number of peripheral tissues, whose primary function is not considered to be immunological (Abo *et al.*, 1999; Doherty *et al.*, 1999; Guy-Grand *et al.*, 1991; Guy-Grand *et al.*, 1994; Makino *et al.*, 1993; Mincheva-Nilsson *et al.*, 1997; Norris *et al.*, 1998; Norris *et al.*, 1999; Rocha *et al.*, 1995). The unique range of phenotypic and functional characteristics of gut and liver tissue-specific lymphocytes suggests a role specific for their location. The liver, *via* the portal circulation, is constantly exposed to a diverse antigenic load that includes harmless dietary antigens as well as toxins, pathogens and malignant cells. The liver immune system is therefore faced with the challenge of providing protection from harmful agents while remaining tolerant to harmless dietary antigen. Kupffer cells express cell-surface MHC class I and class II molecules and may be involved in antigen presentation to hepatic T cells *via* their clonogenic T cell receptors (TCRs), and are also more concentrated in the portal tract area (Fleming, 1999). Antigens in the liver are processed and presented in a poorly understood manner, leading to local and often systemic T cell tolerance, mediated by multiple overlapping mechanisms (Crispe, 1999; Sato *et al.*, 1996). Hepatic T cells are clearly implicated in tolerance induction, as portal vein tolerance is transferable to another animal through the intravenous adoptive transfer of liver lymphocytes (Koyamada *et al.*, 1993), however, the nature of the cells and the mechanisms involved are not understood.

The presence of lymphocytes in specialised tissues such as the gut and liver has been attributed to microenvironmental homing from the circulating pool (Butcher and Picker, 1996). Lymphocytes continuously circulate from peripheral blood to efferent lymph through secondary lymphoid tissues. This recirculation increases the likelihood that a naïve lymphocyte will encounter its particular antigen. Furthermore, migration is essential for recruitment of lymphocytes from sites of immune induction to effector sites (Butcher and Picker, 1996; Westermann and Pabst, 1996). Homing is controlled by the expression of selectin-type adhesion molecules, and rapidly increasing chemokine gradients, which actively recruit lymphocytes expressing the corresponding receptors (Baggiolini, 1998). Disparities between compartments may therefore be explained by vascular and lymphoid adhesion

molecules and local chemokine profiles. Compartmentalised expression of chemokines and their receptors in human liver, may account for selective recruitment of lymphocytes into the liver from the periphery (Shields *et al.*, 1999).

Selective lymphocyte homing may not be the only factor determining the unique nature of liver-specific T cell repertoires. It is also possible that the hepatic cytokine microenvironment influences the distribution of T cell subpopulations found in the liver. Cytokines such as IL-2, IL-12, IL-15 and IL-18 can induce the selective expansion of particular T cell subpopulations and may cause phenotypic changes to infiltrating cells. Thus, the hepatic cytokine milieu may be responsible for the selective accumulation of T cell subsets that are derived from circulating T cells. In pathological situations, increased production of any of these cytokines may result in expansion of local resident populations or increased infiltration of circulating cells (Dao *et al.*, 1998; Hashimoto *et al.*, 1995; Jin *et al.*, 1998; Leite-De-Moraes *et al.*, 1998; Satoh *et al.*, 1996).

Extrathymic T cell development is an additional factor which may contribute to the hepatic lymphocyte repertoire. Studies in mice have established the adult murine liver as an important lymphopoietic organ, mainly producing innate T cells (Abo *et al.*, 1999). Evidence supports the existence of a similar T cell development pathway in the AHL (Collins *et al.*, 1996; Norris *et al.*, 1998; Norris *et al.*, 1999). Recent studies have demonstrated that under non-pathological conditions, while there is consistent infiltration of peripheral T cells into the parenchyma of liver in normal mice, there is also *in situ* production of extrathymic T cells from resident stem cells. Locally derived hepatic T cells are not thought to migrate to the periphery under normal conditions, suggesting a site-specific role for these cells (Yamamoto *et al.*, 1999). The local production of T cell populations which complement and interact with circulating immune populations could provide a mechanism for fine-tuning organ specific immunity at regional sites.

In order to explore the immune mechanisms involved in hepatic immunity an understanding of some of the general principles involved in adaptive immunity is necessary.

1.2.2.3: Principles of adaptive immunity.

The hallmark of adaptive immunity is specific recognition of antigen and lasting protective immunity against the instigating pathogen in the form of immunological memory. Adaptive immune responses are mediated by B and T lymphocytes bearing clonogenic receptors which specifically recognise antigen. Effector functions that are triggered are therefore tailored to deal exclusively with the triggering antigen. The power of the adaptive immune system lies in its capacity to efficiently generate receptors with as many as 10^{11} different specificities. A highly regulated process of site-specific somatic recombination of a relatively small group of gene sequences gives rise to this enormous array of specificities (Davis and Bjorkman, 1988; Tonegawa, 1983). Immunological memory is established as a consequence of initial exposure to a pathogen, which leads to a clonally expanded pool of antigen-specific lymphocytes. After the original infection has been cleared, the majority of responding cells die off, some cells remain in the circulation in a resting state (memory B and T lymphocytes) and can be rapidly re-stimulated by a subsequent encounter with the same pathogen. Immunological memory allows the immune system to respond rapidly and more efficiently to pathogens that have previously been encountered by the host (Sprent, 1994).

a) B lymphocytes.

Many of the bacteria that are most important in human infectious disease multiply in the extracellular spaces of the body, and most intracellular pathogens must spread by moving from cell to cell through the extracellular fluids. The specific humoral immune response leads to the destruction of extracellular micro-organisms and prevents the spread of intracellular infections. This is achieved by antibodies secreted by B lymphocytes (Janeway and Travers, 1996).

(i) Classical (B-2) cells.

B cells originate from pluripotent haematopoietic stem cells (PHSCs) in the bone marrow. The B-lymphocyte clonotypic receptor for antigen recognition expressed on the cell surface is termed the B cell receptor (BCR). Secreted forms of the BCR found in circulation are responsible for the effector functions of the humoral response. Antibody genes are inherited

as gene segments which are randomly rearranged to form a unique functional BCR in individual B lymphocytes as they develop (Tonegawa, 1983). B cells encounter antigen trapped within the secondary lymphoid organs, such as the spleen or lymph nodes. The activation of B cells, and their differentiation into antibody-secreting cells, is directly triggered by antigen (Reth and Wienands, 1997) but usually requires helper T cells. Upon engagement of the BCR with its specific antigen, the BCR:antigen complex is internalised. The antigen is degraded intracellularly into peptides and returned to the cell surface bound to an MHC class II molecule. The antigen:MHC complex can then be recognised by antigen-specific $CD4^+$ T helper cells, that in turn cause B cell proliferation and terminal differentiation into antibody-producing plasma cells or memory B cells (Liu *et al.*, 1997). Antibodies mediate immunity in three main ways: neutralisation, opsonisation and activation of complement.

(ii) $CD5^+$ (B-1) cells.

A distinct population of B cells that display polyspecificity, with a preference for binding common bacterial polysaccharides, has been identified. These B cells are identified by the surface expression of CD5 in humans and Ly-1 in mice (Haughton *et al.*, 1993). Little is known about the function of $CD5^+$ B cells. They appear to make little contribution to the adaptive immune response and can produce antibody independently of T cell help. The antibodies produced by $CD5^+$ B cells can bind a wide range of ligands and express a far less diverse antigen-receptor repertoire than conventional B cells ($CD5^-$). Naturally occurring autoantibodies are thought to be products of $CD5^+$ B cells (Stein *et al.*, 1991). These aspects of their biology are strikingly similar to innate cells, thus $CD5^+$ B cells are considered to be more primitive than conventional B cells and thus to be part of the innate immune system. IL-10 production by $CD5^+$ B cells (O'Garra *et al.*, 1992) has a potential role in the regulation of the nature of the specific immune response.

b) T cells.

The T lymphocyte is considered to be the principal co-ordinator and regulator of the adaptive immune response, allowing the discrimination between self and non-self within the context of harmful and harmless antigen differentiation (Matzinger, 1994). Both the structure of the TCR

and the nature of antigen recognition by T cells are fundamental in allowing the T cell to carry out this role.

(i) Structure of the TCR.

The expression of an antigen receptor, of unique specificity, on each T cell clone provides the diversity required to recognise a universe of potentially hazardous antigens. T cell antigen receptors are heterodimers composed of two transmembrane glycoprotein chains, either $\alpha\beta$ or $\gamma\delta$. The $\alpha\beta$ heterodimers are expressed by the vast majority of peripheral T cells (> 90%) and have been well characterised. Both chains of the T cell receptor (TCR) have an amino-terminal variable (V) region which interacts with antigen, a constant (C) region, and a short hinge region with a cysteine residue that forms an interchain disulfide bond. Each chain spans the lipid bilayer by a hydrophobic transmembrane domain whose notable feature is the presence of positively charged amino acids. These charged residues play an important part in the interaction of the TCR chains with the invariant signal transducing CD3 γ, δ, and ε chains (Clevers *et al.*, 1988). The cytoplasmic domains of the CD3 γ, δ, and ε chains contain sequences called immunoreceptor tyrosine-based activation motifs (ITAMs) that allow them to associate with cytosolic protein tyrosine kinases following receptor stimulation. Another protein, known as ζ, also forms part of the CD3 complex as a disulfide-linked dimer. The ζ chain contains three sets of ITAM sequences that allow it to interact with cytosolic tyrosine kinases, and it is also a major target for tyrosine phosphorylation (Samelson and Klausner, 1992). The TCR is expressed on the cell surface in obligatory association with the CD3 complex of proteins, which are required for the cell surface expression of the TCR as well as signalling through the TCR. Many lines of evidence suggest that the TCR is expressed on the cell surface as a divalent structure (Male *et al.*, 1996) (figure 1.8). During antigen recognition by T cells, co-receptors associate with the TCR (CD4 or CD8). These co-receptors are involved in stabilising the antigen recognition complex and in amplifying the activation signal initiated by TCR recognition of antigen. CD4 and CD8 co-receptor expression delineates two functionally distinct subsets of T cells.

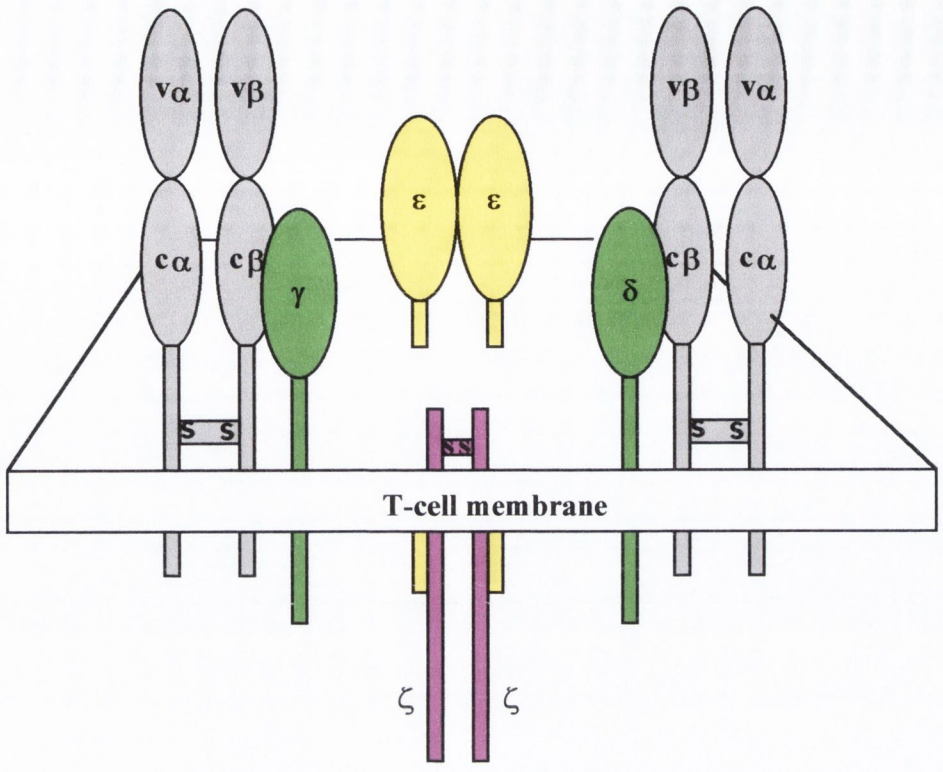


Figure 1.8: Representation of the αβ-TCR:CD3 complex *(adapted from Male et al. 1996).*

(ii) CD4 and CD8 subsets of T cells.

On the vast majority of mature T cells, the expression of CD4 or CD8 co-receptors is mutually exclusive. CD4 is a 55 kilo Dalton (kDa) transmembrane protein with an extracellular region containing four Ig-like domains which functions in cell-cell interaction (Wu *et al.*, 1997). Expression of CD4 defines a subset of T cells termed helper T cells (Th) which, upon activation, secrete cytokines that alter the behaviour and properties of other cells. CD4 cells are further divided into two subsets; Th1 and Th2, based on their cytokine secretion patterns which correlates with distinct functional properties (Abbas *et al.*, 1996; Mosmann and Coffman, 1989). Th1 cells mediate cellular immunity. Upon activation, they activate macrophages allowing them to destroy intracellular pathogens more efficiently. A Th1 response is characterised by the production of IFN-γ induced by IL-12 secreted by macrophages (Trinchieri and Scott, 1994). Th2 cells mediate humoral immunity. Upon activation, they in turn activate B cells to differentiate and secrete antibodies. A Th2 response is characterised by the production of IL-4, IL-5, IL-6, IL-10 and IL-13 and is induced by IL-4

release from innate T cells (Mak and Ferrick, 1998). A system of reciprocal regulation operates between Th1 and Th2 responses. IFN-γ, as well as promoting a Th1 response, down-regulates Th2 responses. IL-10 acts similarly to promote Th2 and down-regulate Th1 responses (Mosmann and Coffman, 1989).

More recently, additional Th-subtypes have been described (Th0 and Th3). Th0 cells are thought to be progenitors of the Th1 and Th2 subsets, or early regulators of the specific immune response, as they secrete cytokines associated with both cell types. Th0 cells are characterised by simultaneous production of IFN-γ and IL-4. In contrast, Th3 cells are a regulatory population involved in tolerogenic reactions, particularly *via* Th1 suppression and the production of TGF-β and IL-10 (Mosmann and Sad, 1996).

The CD8 molecule is a disulphide-linked heterodimer consisting of an α and β chain, each consisting of a single external Ig-like domain, involved in cell-cell interactions, and polypeptide linkers connecting the transmembrane and cytoplasmic domains (Zamoyska and Travers, 1995). Expression of CD8 defines a subset of T cells termed cytotoxic T lymphocytes (CTLs), which upon activation kill malignant or virally infected cells (figure 1.9.a). Evidence suggests that $CD8^+$ T cells may also have suppresser activity, perhaps mediated through cytokines such as TGF-β and IL-10 that inhibit Th1 subsets and preferentially induce Th2 subsets (Zamoyska, 1998) (figure 1.9.b). The majority of CTLs express the CD8-αβ heterodimer, however, the CD8 α-chain can form a homodimer (CD8αα) which can be expressed on the cell surface in the absence of the β-chain of the CD8 molecule. The functional significance of CD8αα expression is as yet unknown. Renard and colleagues (1996) compared the efficiency of the CD8αα and CD8αβ molecules in restoring the responsiveness of a T cell hybridoma. The CD8αβ heterodimers were found to be more efficient in promoting the response to antigen than the CD8αα homodimers (Renard *et al.*, 1996). The localisation of CD8αα populations to sites such as the gut and liver, and its reduced efficiency as a co-receptor suggests a possible role for this molecule in tolerance induction. Rare T cells (~3%) in the periphery co-express both CD4 and CD8 (double positive, DP). In the thymus, DP T cells ($CD4^+CD8\alpha\beta^+$) are an immature population which give rise to single positive ($CD4^+$/$CD8^+$)

cells, while the physiological role of peripheral DP T cells is unknown. Peripheral DP T cells express the CD8αα homodimer and not the CD8αβ heterodimer. They express activation markers and are thus thought to represent a primed memory cell population (Zuckermann, 1999). Occasional (~5%) peripheral T cells are negative for both the CD4 and the CD8 co-receptors (double negatives, DNs). It has been suggested that DN T cells are innate T cells that utilise their TCRs as pattern recognition receptors (Thomssen *et al.*, 1995), this hypothesis is supported by the observation that the majority of γδ T cells have a DN phenotype.

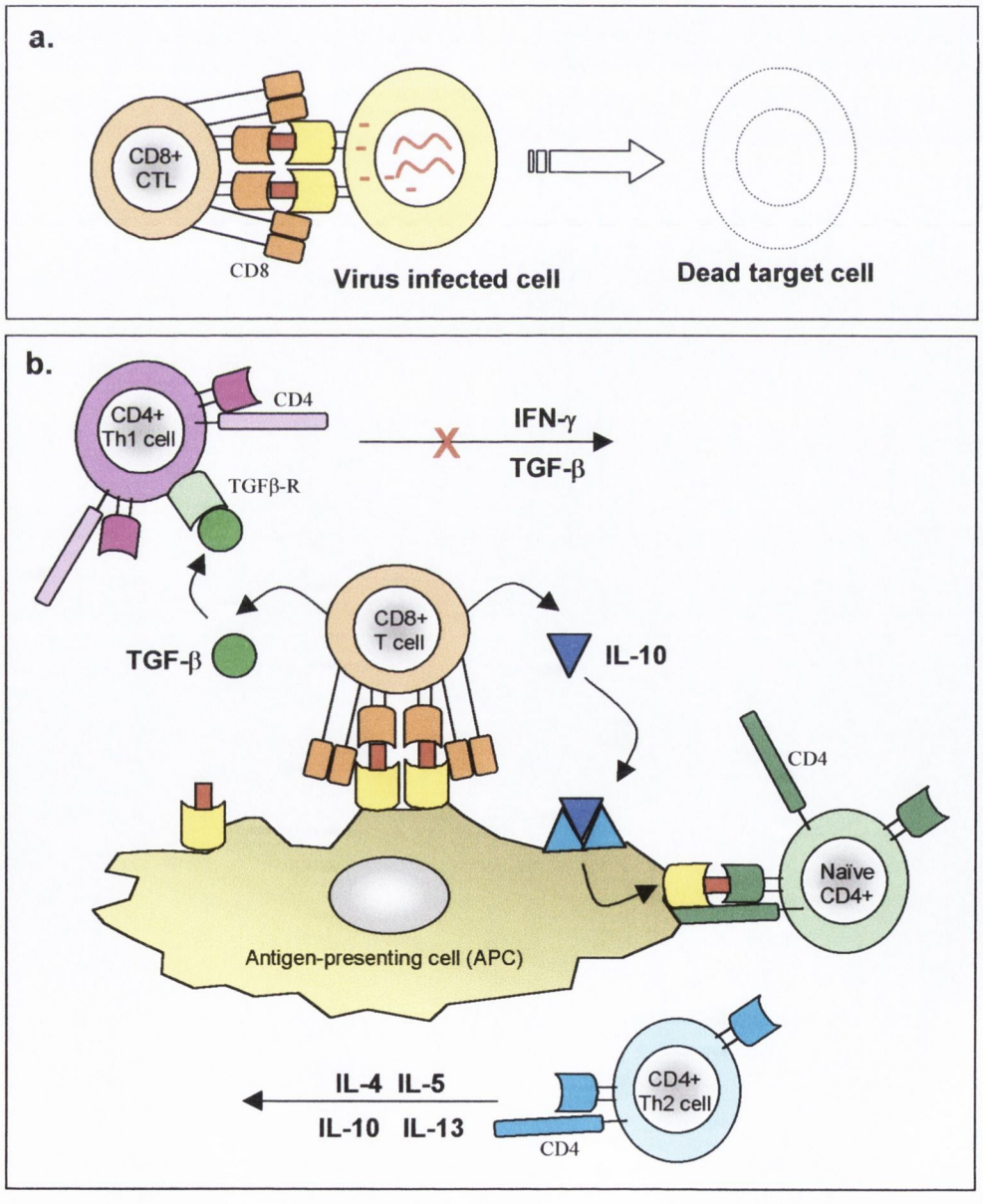


Figure 1.9: Effector actions of CD8⁺ T cells.

The localisation of DP and DN T cells to inflammatory sites such as the gut (Abuzakouk *et al.*, 1998) and the liver (Norris *et al.*, 1998) is suggestive of an immunoregulatory and/or immunosurveillance function (Zuckermann, 1999).

(iii) Antigen recognition by T cells and MHC-restriction.

Unlike B cells that recognise soluble antigen, classical T cells recognise processed antigen in the context of highly polymorphic self-MHC molecules expressed on the surface of antigen-presenting cells (APCs) (Accolla *et al.*, 1995). MHC polymorphism determines peptide-binding capacity and thus affects antigen recognition and ultimately the ability of the immune system to respond appropriately. Thus, the TCR exhibits dual specificities, one for the peptide and the other for the MHC molecule. The latter characteristic is referred to as self-MHC restriction (Zinkernagel and Doherty, 1974).

Two classes of MHC molecules, class I and class II (known as the classical MHC molecules), are involved in the presentation of antigen from different intracellular compartments to either $CD4^+$ or $CD8^+$ T cells. In humans, the MHC complex (termed the human leukocyte antigen [HLA] complex) extends over 4 x 10^6 base pairs on the short arm of chromosome 6, and contains more than 100 genes (Altmann and Trowsdale, 1989) (figure 1.10). The class I region codes for HLA-A, -B and -C which are involved in presentation of antigen to $CD8^+$ T cells. HLA-DP, -DQ and -DR are coded for by the class II region and are involved in antigen presentation to $CD4^+$ T cells. The class III region codes for many components of the complement system and TNF. The HLA codes for many other genes including non-classical MHC molecules, non-polymorphic class I-like (HLA-E, -F and -G, MICA and MICB) (Braud *et al.*, 1999) and class II-like molecules (HLA-DM and -DO), heat-shock proteins and cytokines (Altmann and Trowsdale, 1989). HLA class II molecules are composed of two transmembrane glycoprotein chains (α and β) each of which has two immunoglobulin-like domains. The HLA class I molecule consists of an α-chain (heavy chain) with three extracellular immunoglobulin-like domains. Cell-surface expression of the HLA class I molecule is stabilised by the β2-microglobulin (β2-m) chain which is coded for outside of the MHC locus. The peptide-binding site of the MHC molecule comprises a groove between the two anti-parallel α-helices of the

N-terminal domains of either the MHC class I (Bjorkman, 1997; Davis and Bjorkman, 1988), or the MHC class II molecule (Brown *et al.*, 1993).

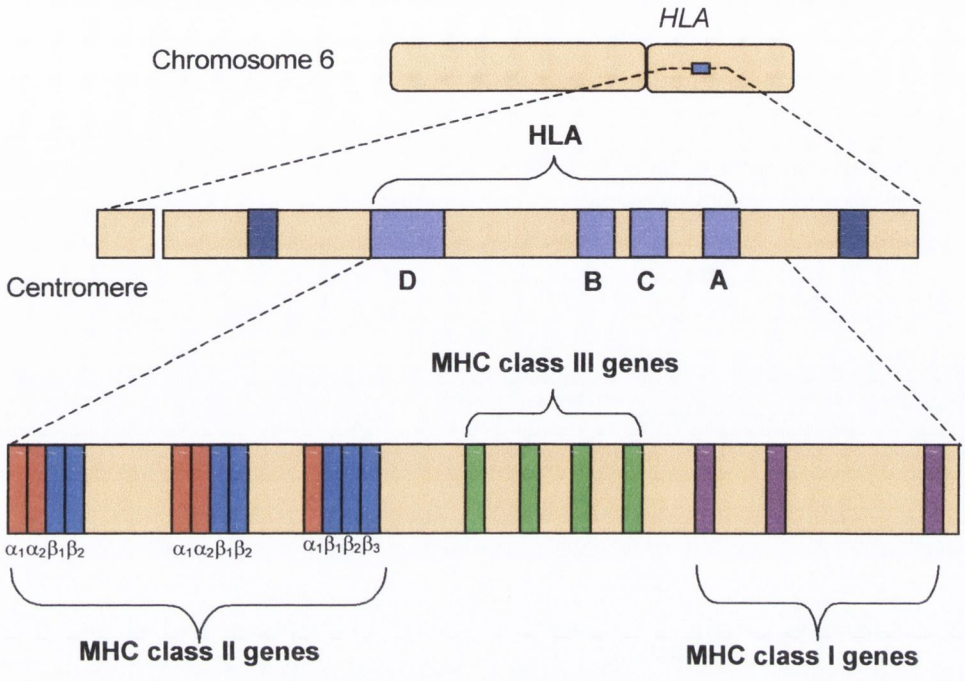


Figure 1.10: The human major histocompatability (MHC) locus.

MHC Class I molecules present peptides from pathogens degraded in the cytosol, such as viruses, to $CD8^+$ cytotoxic T cells (figure 1.11), which generally kill cells they specifically recognise.

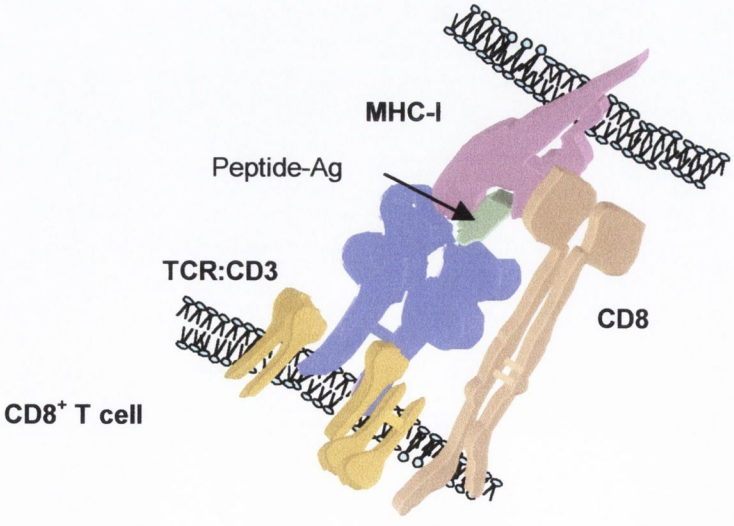


Figure 1.11: MHC class I molecules present processed antigen to $CD8^+$ T cells.

MHC Class II molecules, on the other hand, are expressed only on the surface of specialised APCs such as dendritic, monocyte/macrophage and B cells. These APCs endocytose and proteolytically process exogenous antigens into peptides, which are then bound to class II molecules and presented to $CD4^+$ T cells (figure 1.12). The main function of $CD4^+$ T cells is to activate other cells of the immune system, mainly through the production and secretion of cytokines (Engelhard, 1994).

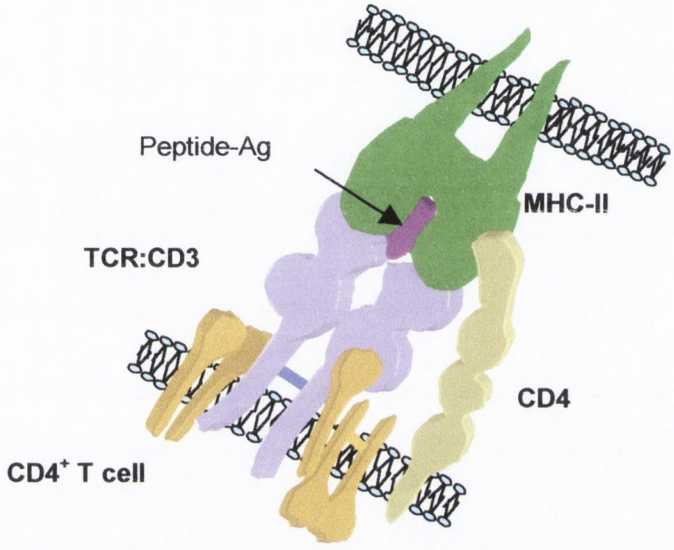


Figure 1.12: MHC class II molecules present processed antigen to $CD4^+$ T cells.

Until recently, only proteins encoded within the MHC locus were thought to be capable of antigen presentation to T cells. A separate lineage of antigen-presenting molecules (CD1), encoded outside the MHC locus, has now been shown to be capable of presenting antigen to T cells, both in mice and humans. Although CD1 is not polymorphic, multiple isotypes exist. Five CD1 genes have been identified in humans, CD1a, b, c, d and e. A protein product has been identified for the a, b, c and d isoforms. The CD1 molecule has a 45 kDa heavy chain which resembles the heavy chain of the MHC class I molecule in structure but does not display sequence homology (< 20%). Similar to MHC class I, the CD1 molecule associates with the β2-m chain, and can be expressed independently of MHC class I or class II molecules. Based on sequence homology, the CD1 proteins have been classified into two sub-groups: the group 1 proteins include the closely related CD1a, b and c, group 2 consists of the CD1d molecules. The mouse has only two related CD1d molecules and thus, lacks the

group 1 CD1 family (Wilson and Bjorkman, 1998). The most surprising finding of studies of the CD1 antigen presentation system is that antigens presented by CD1 are not peptides, but rather lipids or glycolipids (Beckman *et al.*, 1996; Beckman *et al.*, 1994; Sieling *et al.*, 1995). Antigen presentation by CD1 represents a third mechanism of T cell recognition complementary to the MHC class I and class II systems. The T cells recognising group 1 CD1:antigen complexes are double negative or $CD8^+$ and lyse targets via Fas:Fas-ligand interactions or through cytotoxic granule mechanisms respectively. They play an important role in anti-microbial and anti-metastatic immunity. A second type of CD1 recognition occurs in the absence of added foreign antigen. In both humans and mice, these directly CD1-reactive T cells include NK T cells, a heterogeneous population of T cells that co-express NK cell surface receptors. NK T cells produce large amounts of cytokines (especially IL-4) upon recognition of group 2 CD1 proteins, suggesting an immunoregulatory role for these cells. In humans, DN T cells producing IFN-γ or mediating cytolysis upon recognition of group 1 CD1 proteins have also been described (Porcelli *et al.*, 1998) (figure 1.13).

(iv) Gamma-delta ($\gamma\delta$) T cells

The clonogenic T cell receptor (TCR) has two forms $\alpha\beta$ and $\gamma\delta$. The $\alpha\beta$-TCR is found on more than 90% of peripheral lymphoid T cells, whereas, fewer than 10% express the $\gamma\delta$-TCR. However, significant populations of these cells are found at the primary sites of antigen exposure such as the gut, liver and skin. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells can directly recognise antigen in a non-MHC restricted manner by using the TCR as a pattern-recognition receptor to identify non-peptide conserved bacterial moieties (Boismenu and Havran, 1997; Bukowski *et al.*, 1999; Constant *et al.*, 1994). Prevention of anti-host reactivity appears to be achieved through recognition of self-MHC class I by a network of activatory and inhibitory receptors in a similar manner to NK cells (Battistini *et al.*, 1997; Bauer *et al.*, 1999; Carena *et al.*, 1997; Nakajima *et al.*, 1995). Intestinal $\gamma\delta$ T cells have been shown to recognise MHC class I related stress molecules MICA and MICB (Bauer *et al.*, 1999; Groh *et al.*, 1998), suggesting a role for $\gamma\delta$ T cells in the recognition of altered-self and a possible role in tumour surveillance. $\gamma\delta$ T cells secrete cytokines that can regulate both the innate and adaptive immune responses. Elaboration of chemokines upon activation of $\gamma\delta$ T cells facilitates the recruitment of

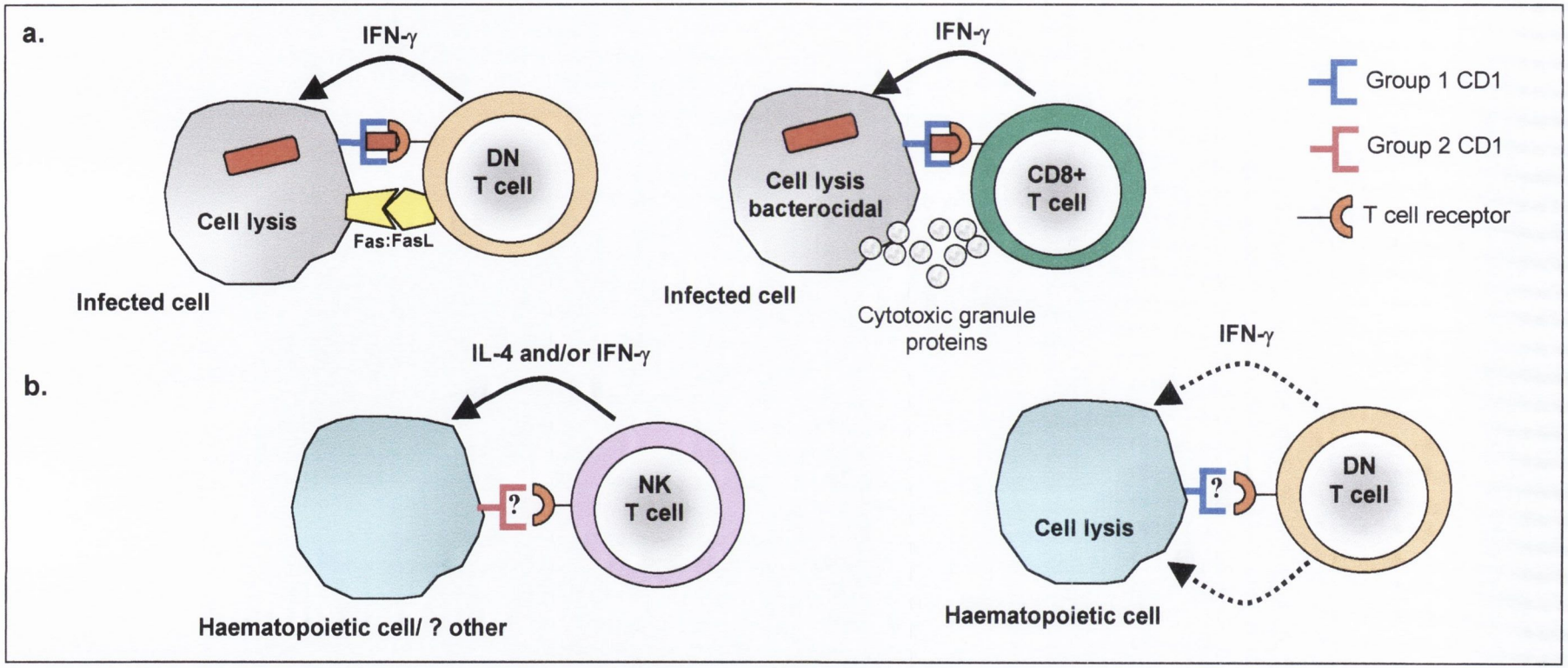


Figure 1.13: CD1-restricted T cells.

a) CD1 restricted foreign antigen-specific T-cell recognition has been described for cytotoxic $CD8^+$ T cells that lyse targets through cytotoxic granule mechanisms and for double negative (DN) T cells that use Fas:Fas ligand (FasL) interactions. These microbial antigen-reactive T cells produce interferon gamma (IFN-γ). **b)** A second type of CD1 recognition occurs in the absence of foreign antigen. In both humans and mice, these directly CD1 reactive T cells include NK T cells that produce large amounts of cytokines (IFN-γ/IL-4). In humans, DN T cells producing IFN-γ or mediating cytolysis upon recognition of group 1 CD1 proteins have also been described (adapted from Porcelli, 1998).

inflammatory cells to the site of insult (Boismenu *et al.*, 1996). Secretion of IFN-γ and IL-4 may play a role in innate regulation of specific immunity (Ferrick *et al.*, 1995). However, not all γδ T cells may be part of the innate system. Human γδ T cells can be stimulated by peptide antigens in the context of MHC class I or II (Guo *et al.*, 1995; Kozbor *et al.*, 1989), demonstrating that at least a subset of γδ T cells has a direct role in adaptive immune responses.

c) T cell development.

In the adult, the bone marrow is the major site of production of all mature circulating blood cells, with the exception of T cells, which require the specialised microenvironment of the thymus to complete their development (Hann *et al.*, 1983). T cell precursors traffic from the bone marrow to the thymus where they undergo a complex series of maturation steps, including somatic gene rearrangements at the TCR-locus, proliferation, rigorous positive and negative selection events and functional maturation. The objective of this thymic education process is to produce helper and cytotoxic subsets of self-MHC restricted T cells that can respond to foreign antigen but do not respond inappropriately to self-antigens.

(i) The TCR locus and somatic recombination.

The diversity of the antigen receptor repertoire is generated by random rearrangement of germline gene segments at the TCR loci. The genes that code for both classes of T cell antigen receptor, αβ or γδ, are not contiguous in the germline, but exist as a number of non-functional gene segments arranged in clusters at the TCR chain loci. Each unique antigen receptor specificity is generated by random rearrangement and juxtaposition of these gene segments (Bogue and Roth, 1996). These rearrangement events are dependent on the expression of the lymphoid specific recombinase activating gene 1 (RAG 1) and RAG 2 (Oettinger, 1992; Schatz *et al.*, 1989). TCR genes are assembled in developing lymphocytes from variable (V), joining (J) and in some cases diversity (D) gene segments, which are widely separated in the genome. These gene segments are brought together to form one functional continuous receptor chain gene, in a highly regulated manner by a somatic site-specific recombination reaction known as V(D)J recombination (figure 1.14).

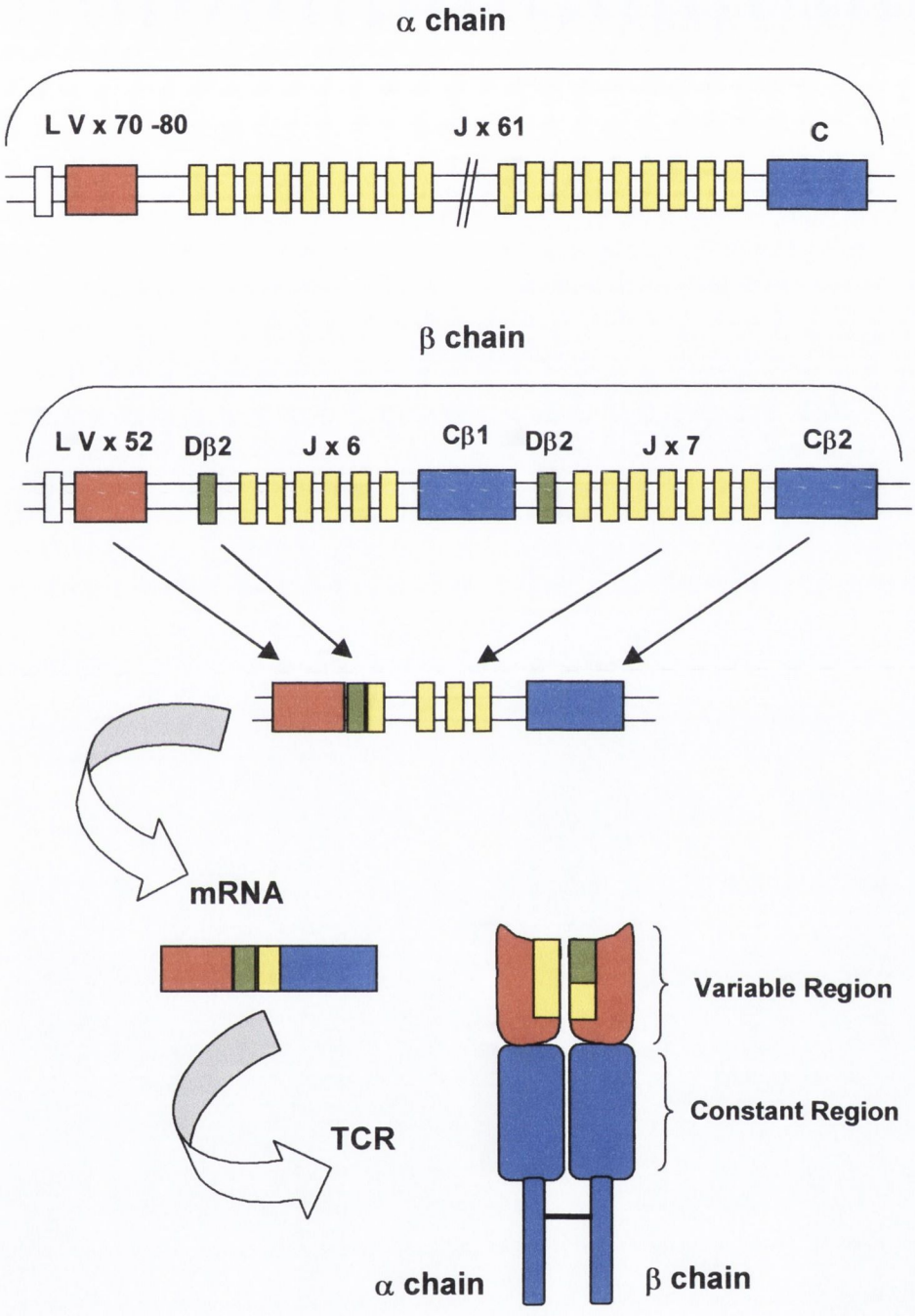


Figure 1.14: Germline configuration and rearrangement at the TCR loci.

The germline configuration for the TCR-α and -β loci is shown. Functional TCR genes are assembled in developing lymphocytes from variable (V), joining (J) and in some cases diversity (D) gene segments, which are widely separated in the genome. These gene segments are brought together to form one functional continuous receptor chain gene, in a highly regulated manner by a somatic site-specific recombination reaction known as V(D)J recombination (adapted from Janeway and Travers, 1996).

(ii) The thymus is the major site of T cell development.

The thymus lies above the heart and is made up of several lobules. On histological examination two distinct regions are observed, the outer cortex and inner medullary region. Both areas play unique roles in T cell development (figure 1.15). The architecture of the thymus dictates that early differentiation occurs in the outer cortical regions of the thymic lobe. Thymocytes then migrate towards the medulla while progressing through the discrete stages of their development. The progression of thymocytes through their differentiation can be monitored in terms of the changes in cell surface phenotype. Sequential loss of precursor cell markers such as CD34 is followed by the sequential acquisition of early (CD7, CD5, CD2, CD1a), and late (CD3, CD4, CD8) T cell markers.

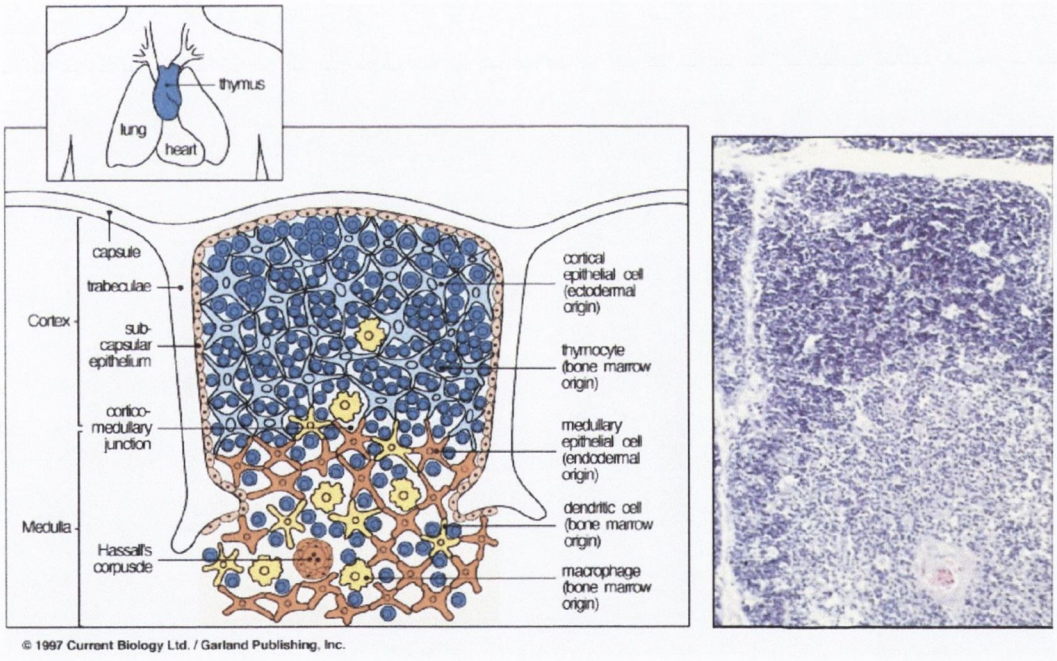


Figure 1.15:The cellular organization of the thymus.

The cortex consists of immature thymocytes (dark blue), branched cortical epithelial cells (pale blue), with which the immature cortical thymocytes are closely associated, and scattered macrophages (yellow) involved in clearing apoptotic thymocytes. The medulla consists of mature thymocytes (dark blue), and medullary epithelial cells (orange), along with macrophages (yellow) and dendritic cells (yellow) of bone marrow origin. Hassall's corpuscles found in the human thymus are probably also sites of cell destruction. The thymocytes in the outer cortical cell layer are proliferating immature cells, while the deeper cortical thymocytes are mainly cells undergoing thymic selection. The photograph shows the equivalent section of a human thymus, stained with hematoxylin and eosin (reproduced from Janeway and Travers, 1996).

(iii) T cell development stages.

Early T cell precursors entering the sub-capsular region of the thymic cortex from the bone marrow, do not express any of the cell surface markers associated with mature T cells; CD3, CD4 or CD8 and are therefore known as triple negatives (TNs). Thymic TNs are $CD34^+$ (stem cell marker), $CD44^+$ (putative thymic homing receptor) and express low levels of CD38 (stem cell differentiation marker) (Marquez *et al.*, 1995; Res *et al.*, 1996; Schmitt *et al.*, 1993). This population has multipotent differentiation capacity and depending on cytokine culture conditions, individual cells can develop into either NK cells or lymphoid dendritic cells (Res *et al.*, 1996). The majority of TNs appear to be uncommitted to T cell lineage as evidenced by the germline configuration of their TCR genes and their multipotency (Shortman and Wu, 1996). However, a small minority of these early thymic cells has initiated rearrangement at the TCR-δ locus and expresses low levels of RAG-1 and RAG-2, suggesting that they have already committed to the T cell lineage (Blom *et al.*, 1998). The signals that induce T cell commitment within the thymus are largely unknown. Cytokines such as IL-2 or IL-4, which are important growth factors for mature T cells, are not implicated in differentiation of T cell precursor cells. The only cytokine thus far that has been implicated as having an essential role in T cell development is IL-7 (Hofmeister *et al.*, 1999).

The expression of CD1a on $CD34^+$ TN thymocytes correlates with T cell commitment and concomitant initiation of rearrangements at the TCR-β and TCR-γ loci, downregulation of CD34 and upregulation of CD4 (Blom *et al.*, 1999). As immature thymocytes progress to the early double positive stage ($CD4^+CD8^+$), they begin to upregulate CD8 expression, with the expression of CD8α preceding that of CD8β. At this stage, rearrangement at the TCR-β locus is complete and the TCR-β chain is expressed on the cell surface in association with an invariant TCR-α (pre-TCR-α) chain and CD3. Signals through the pre-TCR complex initiate proliferation, upregulation of RAG and rearrangement at the TCR-α locus, allelic exclusion at the TCR-β locus (β-selection), as well as down-regulation of the pre-TCR complex. Completion of functional rearrangement at the α-locus results in the cell surface co-expression of CD4 and CD8 and the CD3:TCR-αβ complex. At this stage, the selection processes that will determine which specificities will be retained and which deleted in the TCR

repertoire begin (Blom *et al.*, 1999; Trigueros *et al.*, 1998). The vast majority of T cells (> 90%) which develop in the thymus express the αβ-TCR. A separate lineage of T cells bearing the γδ-TCR also arises from haematopoietic precursors in the thymus. The mechanism by which T cells become committed to one or other of these lineages is unknown. In contrast to αβ-TCR development stages, little is known about the stages involved in the intrathymic development of γδ T cells. As rearrangement at the TCR δ, γ and β chains occurs almost simultaneously in developing thymocytes, it is thought that competition between functional γ and δ chain rearrangement and association, and in-frame rearrangement at the β locus associated with the already transcribed invariant pre-TCR-α chain may be the determining factor in αβ/γδ T cell lineage commitment (Aifantis *et al.*, 1998; Livak *et al.*, 1995; Ramiro *et al.*, 1996).

(iv) Selection of the TCR repertoire.

On expression of a functional αβ-TCR, immature double positive (DP, CD3 ^{low} $CD4^+CD8^+$) thymocytes are subjected to rigorous selection processes. These selective events ensure that the TCR is self-MHC restricted (positive selection) and tolerant of self-peptides (negative selection), before they are allowed to develop into functional single positive (SP, $CD4^+$/$CD8^+$) thymocytes and released into the periphery (Robey and Fowlkes, 1994). Small DP thymocytes expressing low levels of the CD3:TCR complex interact with peptide:MHC complexes expressed on thymic stromal epithelial cells. Recognition of peptide:MHC complexes delivers a survival signal to developing thymocytes, whereas cells failing to recognise these complexes apoptose and die (Guidos, 1996). The second stage of T cell repertoire selection involves the deletion of potentially autoreactive T cell clones. The random nature of TCR gene rearrangement inevitably results in the generation of TCRs with self-reactivity. Thymocytes that react to self-peptides bound to MHC receive a death signal and are deleted from the repertoire. This process of negative selection is often termed clonal deletion and is the primary defence mechanism against autoimmune disease (von Boehmer, 1991). The selection of the mature T cell repertoire is a very tightly controlled process as illustrated by the fact that only approximately 2% of thymocytes trafficking through the thymus survive this rigorous screening regime (Surh and Sprent, 1994). Thymocytes that survive the

selection process begin to down-regulate one of the CD4 or CD8 co-receptors. Those thymocytes with TCR specific for MHC class I molecules always adopt a $CD8^+$ SP status, with thymocytes bearing receptors specific for MHC class II molecules adopting a $CD4^+$ SP status. The mechanism by which the receptor MHC specificity controls choice of co-receptor is not understood in detail.

(v) Extrathymic T cell development.

The thymus involutes with increasing age (figure 1.16), and is considered to lose its capacity to generate new T lymphocytes (George and Ritter, 1996).

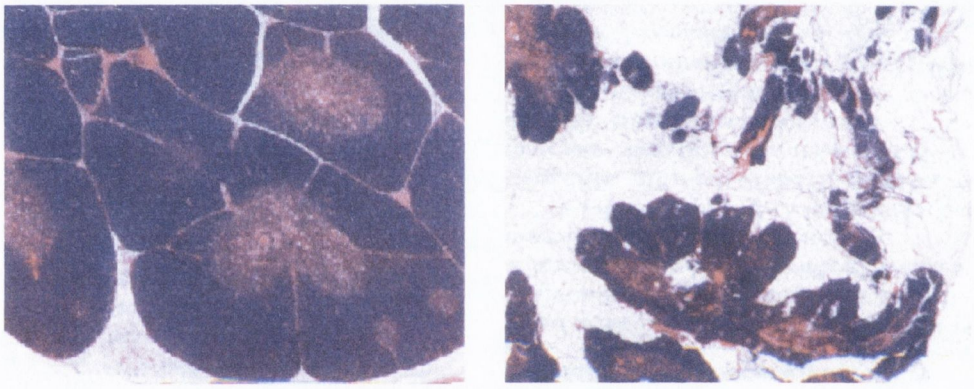


Figure 1.16: The thymus involutes with age.

Histological examples of thymus sections from a child (left) and an adult (right) indicate dense thymic tissue in the young thymus only. The adult thymus consists mostly of fat but still harbours islands of lymphoid tissue.

T cells leaving the thymus are short-lived, although stimulation by relevant antigen rescues them from death. The intense peripheral proliferation which ensues following stimulation is considered to be one of the factors which contributes to the maintenance of T cell homeostasis (Rocha *et al.*, 1989). However, the ability of T cells to respond to newly encountered antigens following thymic involution suggests that new antigen specificities are being continually generated (Miller, 1996). In addition, thymectomy, as a result of corrective heart surgery in infants (Brearley *et al.*, 1987) or as treatment for *Myasthenia gravis* (Melns *et al.*, 1993) does not result in T lymphocyte deficiency. HIV-1 infection results in progressive loss of peripheral $CD4^+$ T cells and leads to acquired immune deficiency syndrome (AIDS).

Highly active combination antiretroviral therapy (HAART) has been shown to result in immune reconstitution of naïve $CD4^+$ peripheral T cells in AIDS patients. Furthermore, thymectomy before HIV-1 infection did not preclude peripheral $CD4^+$ T cell rises after HAART (Haynes *et al.*, 1999). Therefore, questions have arisen as to whether alternative sites of T lymphopoeisis exist, or whether the adult thymus contributes to *de novo* T cell development throughout life.

T lymphocyte precursors traffic from the bone marrow (or the fetal liver) to the thymus (Hayes and Heinly, 1995). As this route of migration is *via* the peripheral circulation, there is a possibility that such precursors could seed other tissues with the capacity to support their further maturation into T lymphocytes. Thus, alternative sites of *de novo* T lymphopoiesis may exist that compensate for the diminution of thymic function with age (Franceschi *et al.*, 1995). T lymphopoiesis at different organ sites may contribute to immune homeostasis and may be important for local immune-regulation, allowing a tissue-specific T-cell repertoire to develop, appropriate for the specific immune needs of the organ (Abo *et al.*, 1994).

Persuasive evidence exists to support extrathymic pathways of T-cell generation. Although most of the data comes from murine studies, evidence is now accumulating to support the existence of extrathymic T-cell development sites in humans. T cells have been identified in athymic nude mice (Chen *et al.*, 1984; Rocha *et al.*, 1995) and in mice with genetic disruptions ($CD3\zeta^{-/-}$) that prevent major thymic T-cell development pathways (Malissen *et al.*, 1993). Local immune systems in organs such as the gut and liver are thought to predate the central, adaptive immune system, and the thymus may have evolved from primitive local immune systems as a specialised site for T-cell differentiation.

The most extensively studied extrathymic T cell maturation pathway is that occurring in the gastrointestinal tract. Intestinal intraepithelial lymphocytes (IELs) are believed to contain both thymus-derived and locally derived T cell populations bearing $\alpha\beta/\gamma\delta$ TCRs. TCR-$\alpha\beta$ cells with unconventional phenotypes, $CD8\alpha^+\beta^-$ and $CD4^+CD8^+$, are thought to be products of the gut T-cell differentiation pathway. These populations of TCR-$\alpha\beta$ IELs are refractory to activation stimuli and contain self-reactive clones (Rocha *et al.*, 1992). Similar unconventional T cell populations have been detected in the human IEL populations (Abuzakouk *et al.*, 1998; Lynch

et al., 1993). Differences between extrathymic and the classical pathway of T cell maturation are only currently being delineated. In mice, selection of intestinal CD8$\alpha\alpha^+$ T cells is induced most efficiently by high affinity ligands, whereas positive selection of conventional thymic T cells is induced by lower affinity ligands (Levelt *et al.*, 1999). It is thought that less stringent selection processes at extrathymic sites of T cell differentiation may account for the development of self-reactive clones, which are characteristic of thymic-independent T cell populations. Anatomical structures in the murine intestinal mucosa, termed cryptopatches, have been described that contain T-committed lymphocyte precursors expressing the receptors for stem cell factor (ckit, CD117) and IL-7 (IL-7Rα, CD127) (Kanamori *et al.*, 1996). Transplantation of cryptopatch cells from nude mice into severe combined immuno-deficient (SCID) recipients generates small numbers of TCR-$\gamma\delta$ IELs and TCR-$\alpha\beta$ cells in the IEL and mesenteric lymph node compartments (Saito *et al.*, 1998). IELs of the $\gamma\delta$ T cell lineage are also thought to differentiate extrathymically, due to their identification in nude mice and in athymic radiation chimeras (Bandeira *et al.*, 1991; Poussier and Julius, 1994). A recent study by Laky *et al.* (2000) has provided direct evidence for the development of $\gamma\delta$ IELs *in situ*. Further evidence to support the existence of an extrathymic T-cell development pathway within the IEL compartment includes: the expression of RAG transcripts in IELs derived from murine (Guy-Grand *et al.*, 1991) and human gut (Lundqvist *et al.*, 1995; Lynch *et al.*, 1995) and the expression of the pre-TCR-α gene in murine intestinal epithelium (Bruno *et al.*, 1995).

1.2.2.4: Extrathymic T cell development in the liver.

The concept that, in addition to infiltration of lymphocytes from peripheral blood into the liver, some hepatic T cells differentiate and mature locally is also gaining credibility (Yamamoto *et al.*, 1999). Studies of mice and bone marrow chimeric mouse models have produced strong evidence that some T cell sub-populations differentiate locally in the adult murine liver (Abo *et al.*, 1994; Abo *et al.*, 1999, Abo *et al.*, 2000; Ohteki and MacDonald, 1994; Sato *et al.*, 1995). In mice, extrathymically derived hepatic T cells can be identified by their unconventional or innate phenotype (Abo *et al.*, 1999). Approximately one third of T lymphocytes derived from the AHL have also been shown to display an innate phenotype, including CD8$\alpha^+\beta^-$, DN and $\gamma\delta$

T cells (Norris *et al.*, 1998; Norris *et al.*, 1999). The adult murine liver contains haematopoietic stem cell (HSC) populations (Taniguchi *et al.*, 1995; Watanabe *et al.*, 1996) and it has been suggested that extrathymic T cells in the liver might be generated *in situ* from pre-existing hepatic HSCs (Shimizu *et al.*, 1999). The normal AHL also contains HSC populations capable of differentiation into multiple erythroid and myeloid colonies *in vitro* (Crosbie *et al.*, 1999). The AHL may also harbour lymphocyte-committed progenitors with the ability to differentiate into T cells. However, the co-expression of lineage-specific markers on hepatic haematopoietic progenitors, or their ability to differentiate into T cells *in vitro,* has not previously been examined. Therefore, the presence of hepatic lymphoid progenitor populations has yet to be demonstrated. The detection of mRNA for RAG-1, RAG-2 and pre-TCR-α in human hepatic lymphocytes (Collins *et al.*, 1996), taken together with the unconventional phenotype of T cells normally found in the AHL (Norris *et al.*, 1998; Norris *et al.*, 1999), provides convincing evidence that the normal AHL is a site of extrathymic T cell development.

Because of thymic involution, scientists have assumed that the thymus is only functional early in life. However, residual thymic tissue is a possible source for new naïve T cells in adults. Recent studies using a novel technique which targets DNA intermediates (T cell receptor excision circles or TRECs) produced as a result of ongoing V(D)J recombination in $\alpha\beta$ T cells (figure 1.17), have provided direct proof that adult humans can produce T cells *de novo* (Douek *et al.*, 1998; Douek *et al.*, 2000; Poulin *et al.*, 1999). Douek and colleagues argue for a thymic rather than extrathymic origin for recently generated T cells entering the periphery. They found that the number of TRECs detected in the peripheral blood declined and correlated well with quantitative data on the lymphoid mass in the thymus relative to age (Douek *et al.*, 1998). Studies of adults recovering from autologous haematopoietic cell transplants, also showed that TREC counts correlated inversely with age but directly with counts of phenotypically naïve T cells (Douek *et al.*, 2000). The results of the study analysing TREC levels in the peripheral blood of normal adults, carried out by Poulin *et al.* (1999), confirm those of Douek *et al.* However, these studies do not provide direct evidence that recently generated $\alpha\beta$ T cells are produced by the thymus. Analysis of TRECs in

thymectomised patients receiving chemotherapy and haematopoietic cell transplantation could further define the origins of T cells generated *de novo*.

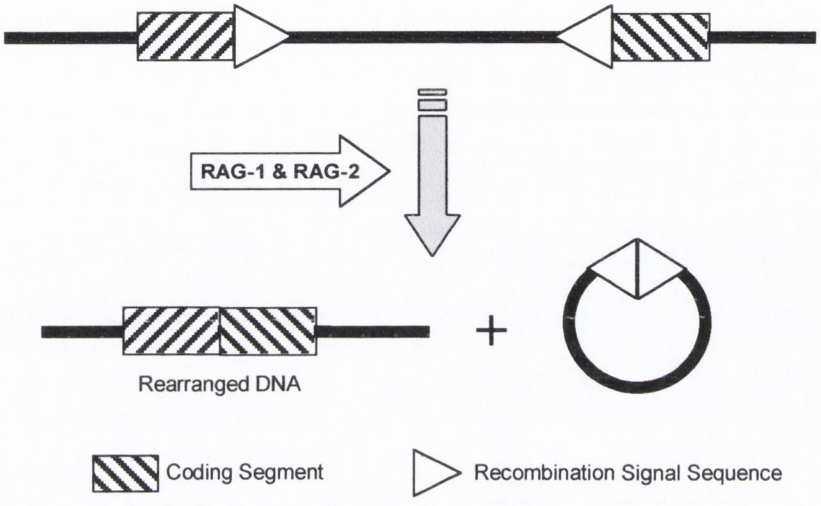


Figure 1.17: TCR rearrangement reaction intermediates.

During V(D)J recombination, cleavage occurs at the recombination signal sequences and the intervening DNA is spliced out and circularised to form a stable reaction intermediate, which is termed a T cell receptor excision circle or TREC.

1.2.2.5: The role of the liver at the interface of innate and adaptive immunity.

For many years, innate immunity has been considered as a separate entity from the adaptive immune response, and has been regarded to be of secondary importance in the hierarchy of immune functions (Medzhitov and Janeway, 1997a). It is now clear that the progression from an innate to a specific response is a continuum and that there is an important interplay between innate and adaptive immune systems. The initiation of an appropriate specific immune response is regulated by innate reactions and subsequent specific immune responses can in turn activate and harness innate immune functions (Fearon and Locksley, 1996). At the interface between innate and adaptive immune systems, operates what can almost be considered to be an intermediate system which co-ordinates and integrates innate and adaptive responses. This interface is localised at regional sites in the body, such as the

skin or gut, that are primary targets for entry of viruses and micro-organisms as well as malignant cells and innocuous environmental agents. These sites are characterised by cell populations that cannot be described exclusively as innate or adaptive as they display properties of both. An important requirement of the immune response at these regional sites is the ability to discriminate between harmful and harmless foreign antigen. The specific demands at these sites has led to the concept of organ-specific or regional immunity, where populations of cells with immunoregulatory or immunosuppressive properties form a functional bridge between the innate and adaptive immune systems (Mak and Ferrick, 1998).

a) Innate lymphocytes.

Innate lymphocytes are thought to play a primary role in the interplay between innate and adaptive immunity. They can respond immediately to immune challenge (without the need for a previous phase of expansion) because they express stereotyped receptors that recognise a similar exogenous or endogenous molecule. Their rapid activation and early secretion of key cytokines strongly supports the idea that they are pivotal regulators as well as effectors of the immune system (figure 1.18). They include natural killer (NK) cells (Bancroft, 1993), some subsets of γδ-TCR cells (Ferrick *et al.*, 1995), NK T cells (MacDonald, 1995) and $CD5^+$ B cells (Carroll and Prodeus, 1998). NK cells do not express antigen specific receptors and are thus thought to belong exclusively to the innate compartment of the immune system. γδ-TCR cells, NK T cells and $CD5^+$ B cells express clonotypic antigen receptors of limited diversity and are considered to be an intermediate population, sharing properties of both the innate and adaptive immune lymphocytes. Their antigen recognition mechanisms, cytokine profiles and responses to stimulation differ significantly from those of classical B and T cells and are almost innate in nature.

(i) Murine NK T cells.

The term NK T cells is used to describe a heterogeneous population of T cells that co-express NK cell surface receptors and a clonotypic TCR. In mice, such NK T cells comprise $CD4^+$ or DN T cells, can express low levels of the TCR (TCR^{int} cells) and the NK receptors, NK1.1, Ly49 and IL-2Rβ (Abo *et al.*, 1994; Abo *et al.*, 1999; Ohteki and MacDonald, 1994). $NK1.1^+$ T

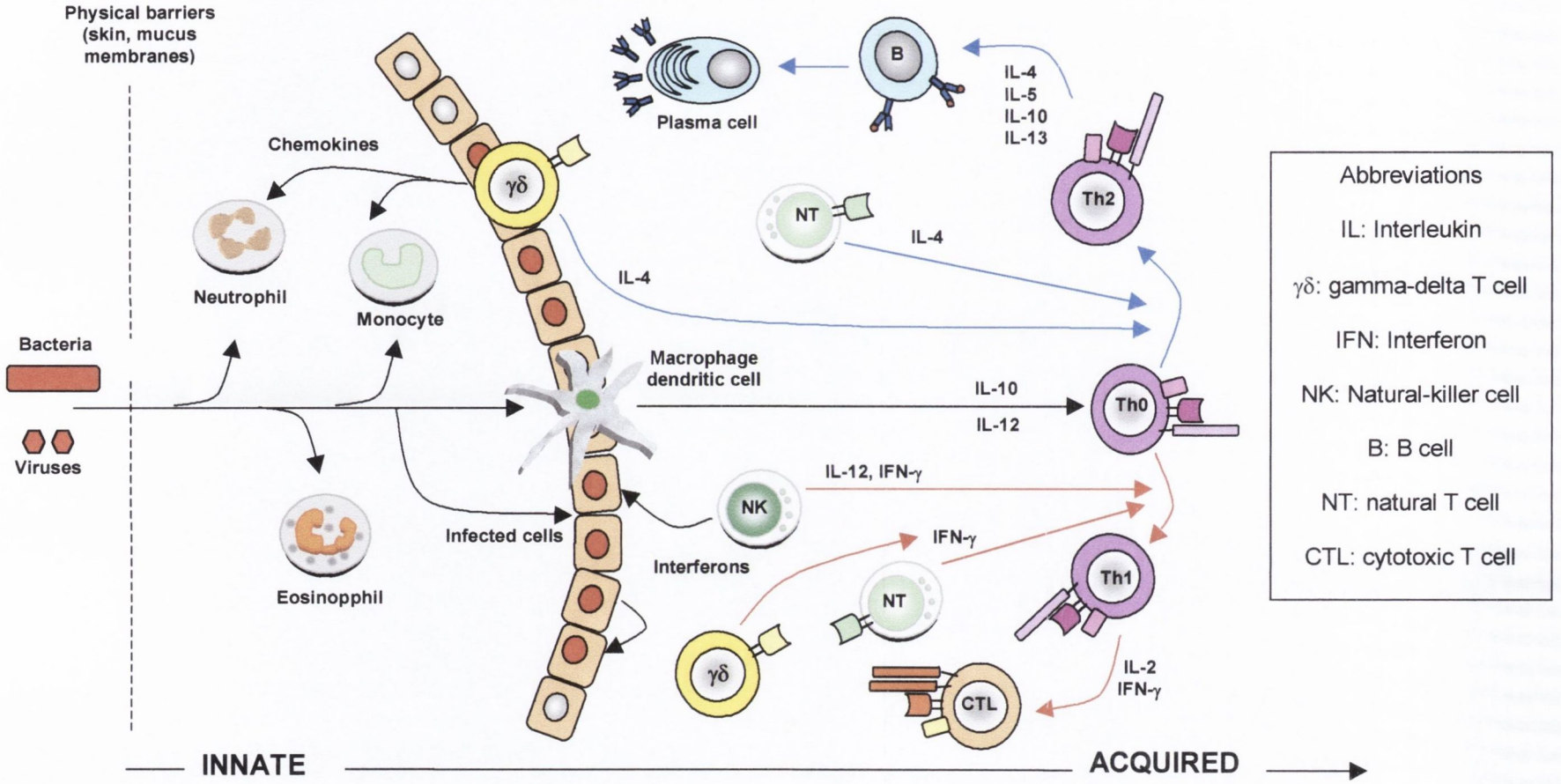


Figure 1.18: Innate and adaptive immunity are linked: innate lymphocytes play an important role in bridging the two systems.

As pathogens cross the physical barriers of the host and gain access to the body, both the innate and acquired immune systems are triggered. The innate response composed of neutrophils, monocytes, macrophages and interferons, is responsible for limiting the spread of infection. The acquired response, with its humoral and cell mediated arms, specifically recognises and dispatches pathogens and elicits memory cells. Recent findings suggest that innate lymphocytes, γδ T cells and natural T cells (T cells sharing functional and phenotypic characteristics of natural killer cells) may coordinate the interplay of acquired and innate immunity (adapted from Mak and Ferrick, 1998).

cells account for about 1-5% of T cells in the blood, spleen, thymus and lymph nodes, but they are present in remarkably high frequencies in the bone marrow and liver of mice, accounting for around 40% of all $CD3^+$ cells in these locations (MacDonald, 1995; Vicari and Zlotnik, 1996). As well as sharing phenotypic characteristics, NK T cells also share functional properties with NK cells. $NK1.1^+$ T cells are characterised by their potent effector function, as evidenced by their ability to lyse various tumour cells in the absence of prior antigenic stimulation and to rapidly produce high levels of cytokines upon activation through their TCR or NK1.1 molecules. These cells most notably produce IL-4 and are thought to have a role in the initiation and regulation of humoral immunity (Chen and Paul, 1997). They can also produce IFN-γ, IL-5 and IL-10, suggesting that they regulate both humoral and cell mediated responses (Arase *et al.*, 1996; Chen and Paul, 1997; Denkers *et al.*, 1996; Yoshimoto *et al.*, 1995). Murine $NK1.1^+$ $\alpha\beta$-T cells frequently express an invariant TCR α-chain encoded by Vα14 and Jα281, in association with a limited number of Vβ chains (Lantz and Bendelac, 1994). Vα14 cells can respond to non-peptide antigens in the context of a non-classical antigen-presenting molecule, CD1 (Bendelac *et al.*, 1995; Kawano *et al.*, 1997). The physiological role of murine NK T cells is thought to lie in the control of autoimmunity and in the surveillance and prevention of spread of malignant cells (Shin *et al.*, 2001, Cui *et al.*, 1997; Hashimoto *et al.*, 1995; Takeda and Dennert, 1993). The ability of these cells to carry out these two seemingly opposing roles is presumably mediated by the complex, quantitative balance of inhibitory and activatory signals provided by their KIR and KAR receptors, similar to those on NK cells (Lanier, 1997).

(ii) Human NK T cells.

Studies in humans have also identified NK T cell populations that co-express $\alpha\beta$ or $\gamma\delta$ TCRs and various NK receptors, including CD16, CD56, CD57, CD161, KIR and/or CD94 (Ferrini *et al.*, 1994; Mingari *et al.*, 1995; Ortaldo *et al.*, 1991; Phillips *et al.*, 1995), which account for about 2% of peripheral T cells (Doherty *et al.*, 1999; Ishihara *et al.*, 1999; Norris *et al.*, 1998; Norris *et al.*, 1999). These include a human Vα14Jα281 NK T cell homologue that expresses an invariant TCR chain, Vα24JαQ (Lantz and Bendelac, 1994). However, while Vα14Jα281

TCR-expressing NK1.1$^+$ T cells account for the majority of NK T cells in mice, the human Vα24Jα Q TCR is present on only a small percentage of NK T cells (Norris *et al.*, 1999). Thus, NK T cells in humans are a more heterogeneous population than in mice. However, their ability to mediate lysis, in both a T and NK cell manner, and their ability to produce cytokines that promote both humoral and cell mediated specific responses suggests that NKT cells in humans and mice share the same functional properties, and possibly the same physiological roles (Doherty *et al.*, 1999; Prussin and Foster, 1997; Wilson *et al.*, 1998).

b) Hepatic innate lymphocyte populations.

Phenotypic analysis of murine hepatic-derived T-lymphocytic populations has demonstrated that a considerable proportion (~ 40%) of these cells display an innate or primordial phenotype. Murine hepatic primordial T cells express the IL-2Rβ and express the CD3 complex and the TCR at a lower intensity than blood T cells or IELs, and have been termed TCR^{int} cells. T cells bearing the homodimeric form of the CD8 co-receptor (CD8-αα) and a high proportion of γδ T cells and self-reactive clones have also been detected in the adult murine liver (Ohtsuka *et al.*, 1994). Another (possibly overlapping) population of T cells in the murine liver co-expresses the NK cell surface antigen NK1.1. The abundant presence of TCR^{int} cells in the liver suggests an important role in organ specific immunity. Their function lies in recognition of abnormal self-cells, including rapidly dividing cells, cells infected with intracellular pathogens and malignant cells. They produce large amounts of cytokines upon activation and contribute to the regulation of specific immune responses. Over-activation or over-production of hepatic innate T cells may be responsible for the pathogenesis of autoimmune liver disease (Abo *et al.*, 1999).

Up to 65% of all lymphocytes present in the normal adult human liver consist of NK cells, T cells expressing γδ-TCR, and T cells co-expressing NK molecules, which are found in much lower proportions in the peripheral blood. Unlike their murine counterparts, human NK T cells do not constitutively express IL-2Rβ and have normal levels of TCR expression. Although hepatic NK T cells isolated from human liver express a number of NK markers, CD56 appears

to be the best marker of innate T cell phenotype and function, hence our group have defined human hepatic NK T cells as $CD3^+CD56^+$ T cells (figure 1.19).

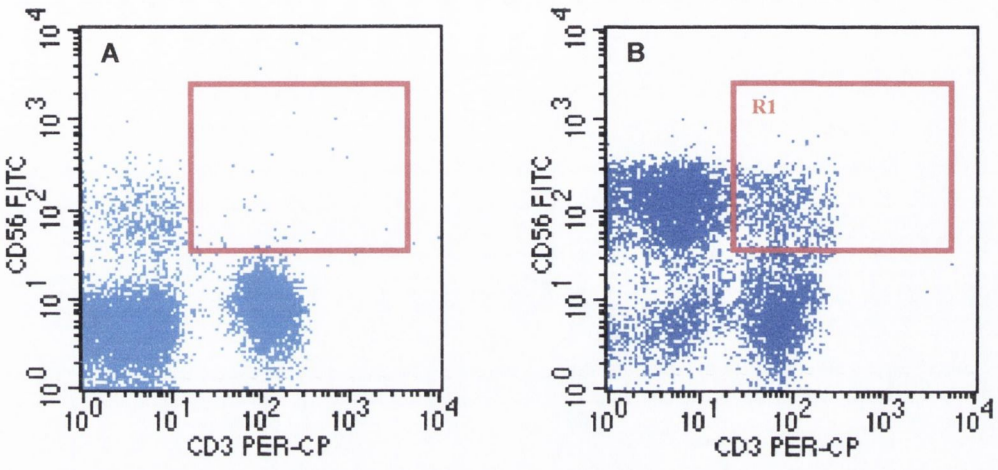


Figure 1.19: Human NK T cells ($CD3^+CD56^+$, R1) in peripheral blood (A) and matched liver mononuclear (B) cell populations.

There is evidence that there is a skewed TCR repertoire in the hepatic innate T cell population: as many as 4% may express the Vα24 chain of the TCR (Norris *et al.*, 1999). Functional studies have demonstrated that human hepatic $CD3^+CD56^+$ cells have lymphokine activated killing (LAK) as well as TCR-mediated cytotoxic activities and produce multiple cytokines upon activation *in vitro* (Doherty *et al.*, 1999; Norris *et al.*, 1999). These cytotoxic activities of human NK T cells are similar to those of murine $NK1.1^+$ T cells. LAK activity of $NK1.1^+$ T cells is strongly inducible by IL-2 and these cells are thought to be the major effectors of IL-12 mediated tumour rejection in mice (Cui *et al.*, 1997). Human cytotoxic $CD3^+CD56^+$ NK T cells are also selectively expanded by a combination of IL-2 and IL-12 (Jin *et al.*, 1998; Satoh *et al.*, 1996) suggesting a similar role for these cells *in vivo*. A major role for human hepatic NK T cells is the production of cytokines that regulate specific immunity. The vast majority of $CD3^+CD56^+$ hepatic cells produce Th1 cytokines with approximately 14% producing IL-4 (Th2). This is in contrast to murine NK T cells, which predominantly secrete IL-4, suggesting that there may be a functional distinction between murine and human NK T cells. However, murine NK T cells can be biased towards IFN-γ production by IL-12 (Leite-De-

Moraes *et al.*, 1998). Most human hepatic T cells that produce IL-4 also produce IFN-γ, indicating they are of the Th0 type, rather than conventional Th2 cells (Doherty *et al.*, 1999).

Approximately 6% of hepatic mononuclear cells are B cells ($CD19^+$). A small proportion of these co-express CD5 (B-1 cells), a marker of innate B cells thought to be the equivalent of γδ T cells. However levels of $CD5^+$ B cells are low in hepatic populations isolated from normal liver (8% of B cells, see appendix I) when compared to levels in normal peripheral blood (25.34%) (Curry *et al.*, 2000a). The physiological role of innate ($CD5^+$) B cells is unknown and thus the significance of low levels of $CD5^+$ B cells in liver is also unknown. However, it is interesting to note that while other sub-populations of innate lymphocytes (NK, NK T and γδ cells) are increased in liver relative to peripheral blood, B-1 cells are decreased.

The predominance of innate T cells in the liver suggests an important role for this organ in immune regulation at this regional site of primary antigen encounter.

1.2.3: The haematopoietic role of the liver.

1.2.3.1: Haematopoiesis.

Daily, there is a high demand for new blood cells: approximately 10^{12} cells/day. Thus, the haematopoietic system is in a state of constant dynamic flux to meet this demand in order to maintain haematological homeostasis. All mature circulating blood cells are derived from undifferentiated, self-renewing, pluripotent haematopoietic stem cells (PHSCs) thought to reside exclusively in bone marrow. The formation of blood cells represents a complex interaction between stem cells, cells making up the haematopoietic microenvironment and growth/differentiation regulatory proteins (Williams, 1995).

1.2.3.2: Hepatic haematopoiesis.

While the haematopoietic function of the liver in the human foetus is well documented (Hann *et al.*, 1983), the role of the AHL in haematopoiesis is thought to be relatively minor. However,

the haematopoietic potential of the liver is retained in the adult human and can become activated as demonstrated by the extramedullary erythropoiesis that occurs in liver or spleen at times of severe bone marrow dysfunction (Schlitt *et al.*, 1995). In addition, in adult humans with normal bone marrow function, extramedullary erythropoiesis in the liver and reconstitution of multilineage haematopoiesis by donor-derived cells has been reported following liver transplantation (Collins *et al.*, 1993). The normal AHL contains populations of functional myeloid and erythroid progenitors (figure 1.20) which may play a role in local blood cell homeostasis (Crosbie *et al.*, 1999).

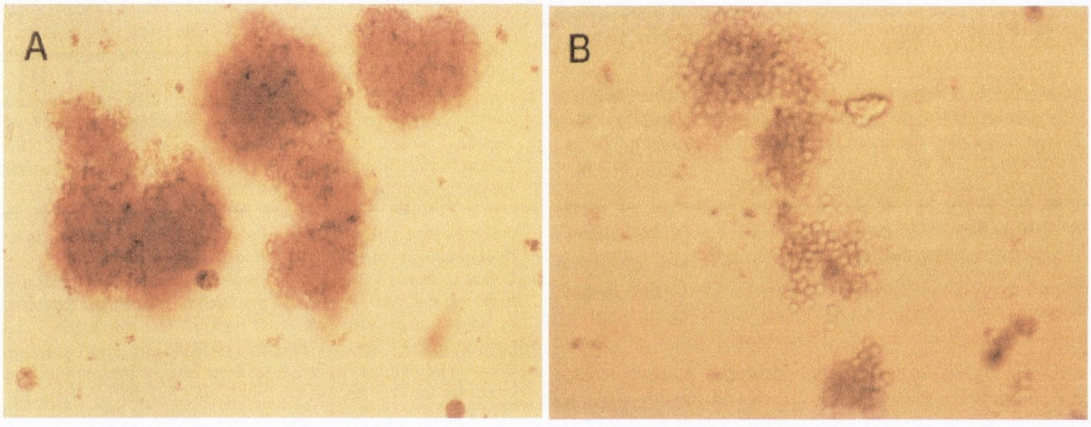


Figure 1.20: Haematopoietic stem cells derived from normal adult human liver tissue give rise to erythroid (A) and monocytic/granulocytic (B) colonies in methyl cellulose cultures.

1.2.3.3: Haematopoietic stem cells and their progeny.

PHSCs are generally described as clonogenic cells capable of self-renewal and multilineage differentiation (Till and McCulloch, 1961), and can be functionally described as cells with the capacity to restore long-term lymphohaematopoiesis in myeloablated transplant recipients (Lansdorp, 1995). At least two classes of PHSCs can be identified based on their self-renewal capacity; long-term and short-term PHSCs. The long term subset renews for the life of the host and gives rise to short term PHSCs which have a limited self-renewal capacity (Morrison and Weissman, 1994). Short term PHSCs give rise to oligolineage progenitors, more limited in their self renewal capacity (figure 1.21), which in turn give rise to progeny that are more restricted in their differentiating potential, and finally to functionally mature cells.

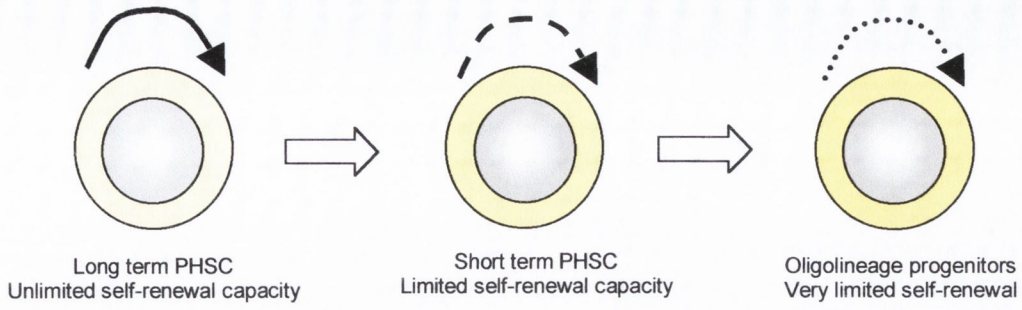


Figure 1.21: Long-term pluripotent haematopoietic stem cells (PHSCs) give rise to short term PHSCs.

Each stage of differentiation involves functionally irreversible maturation steps. There are two kinds of oligolineage-restricted progenitor cells. Common lymphocyte progenitors, which at a clonal level are restricted to give rise to NK-, T- and B-lymphocytes, and common myeloid progenitors, which are restricted to generation of myeloerythroid lineages (erythrocytes, platelets, monocytes, dendritic cells and granulocytes), (figure 1.22) (Akashi *et al.*, 2000). The haematopoietic system therefore has two arms: lymphoid (and the process of differentiation along this arm is termed lymphopoiesis) and myeloid (myelopoiesis). HSCs differentiating along either arm of the haematopoietic system can be identified by the differential expression of lineage-associated cell surface antigens, these will be discussed in chapter 2 which deals with the phenotypic characterisation of hepatic HSCs. The majority of mature blood cells lose their ability to proliferate once they have terminally differentiated. However B and T lymphocytes retain renewal capacity. This retention of renewal capacity is central to mounting an effective immune response. On antigenic stimulation these cells are activated to proliferate and produce a clone of identical offspring so that sufficient effector cells (specific for antigen) are available to mount a response.

1.2.3.4: The haematopoietic microenvironment.

Adult haematopoiesis takes place within a complex microenvironment in the medullary cavity of bone marrow. Adventitial reticular cells reside on the adluminal surface of venous endothelial cells, which branch through the medullary cavity. These cells provide a reticular

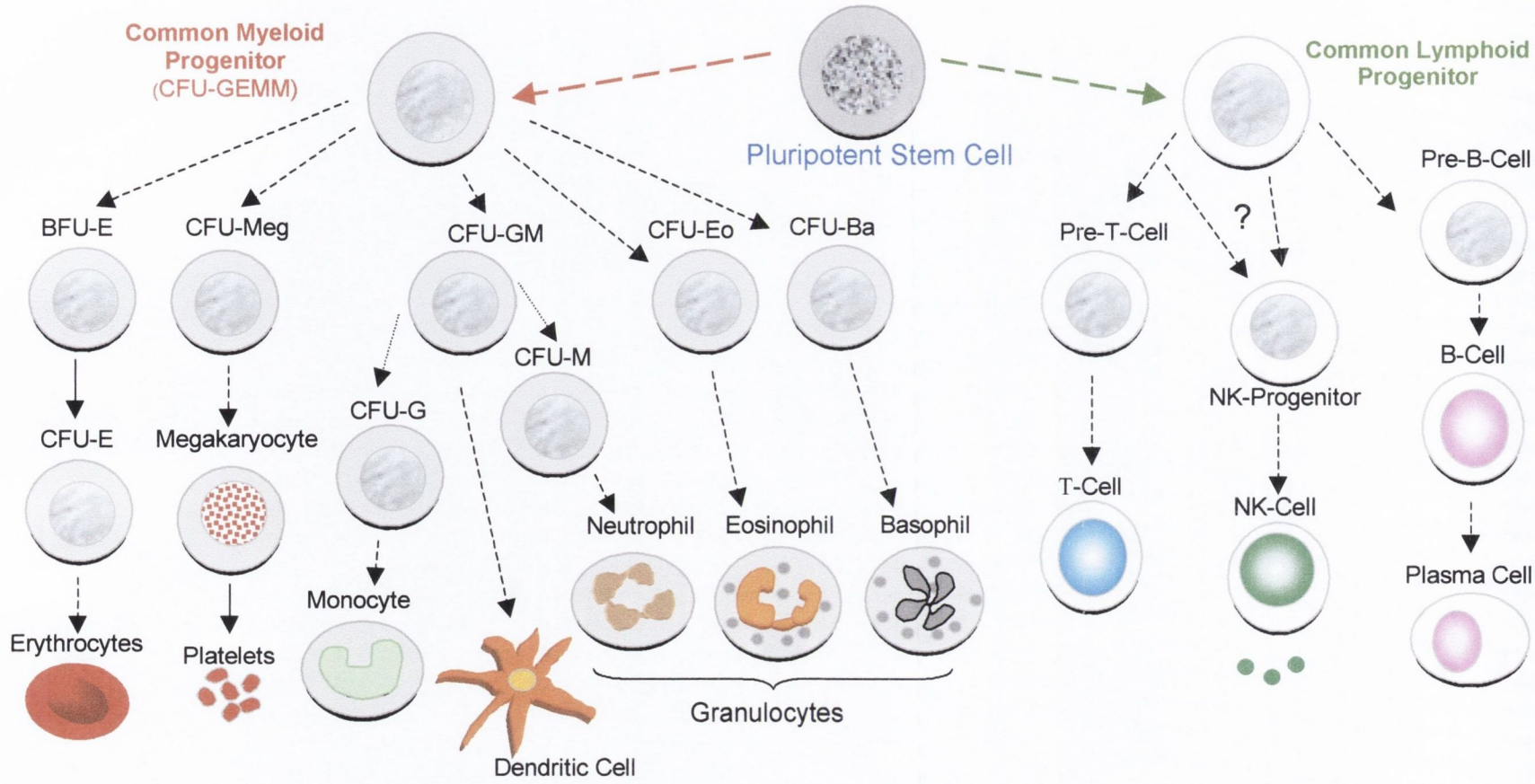


Figure 1.22: The Haematopoietic System.

There are two kinds of oligolineage-restricted progenitor cells derived from self-renewing pluripotent haematopoietic stem cells. Common lymphocyte progenitors, which at a clonal level are restricted to give rise to natural killer (NK)-, T- and B-lymphocytes, and common myeloid progenitors, which are restricted to generation of myeloerythroid lineages (erythrocytes, platelets, monocytes, dendritic cells and granulocytes). Abbreviations: CFU = colony forming unit; GEMM = granulocyte, erythrocyte, megakaryocyte, monocyte. BFU = burst forming unit. Meg = megakaryocyte. GM = granulocyte, monocyte. Eo = eosinophil. Ba = basophil. NK = natural killer.

network that supports developing blood cells, which develop in nests or cobblestone areas (figure 1.23).

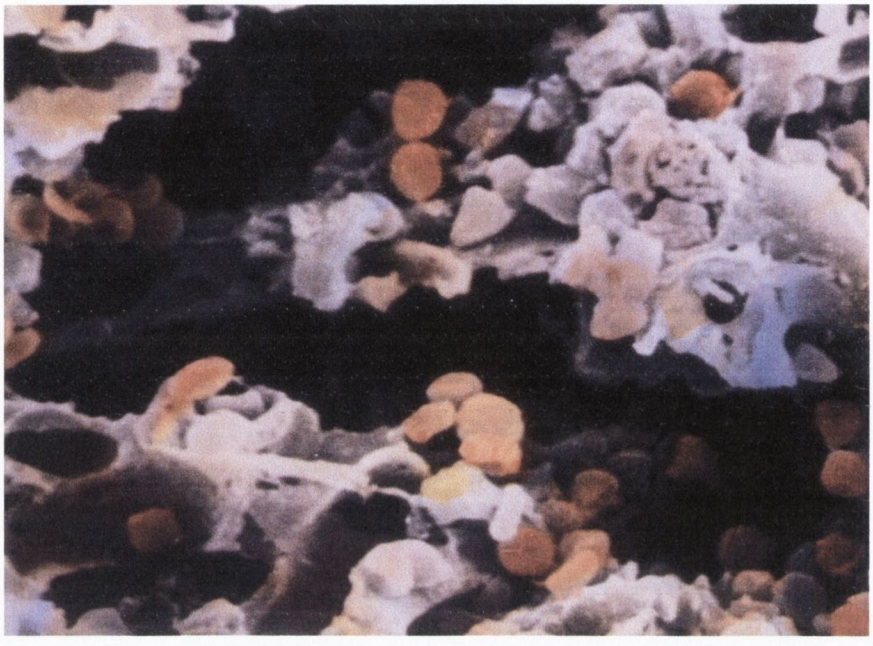


Figure 1.23: Bone marrow gives rise to both white and red blood cells.

The importance of this soft, fatty tissue occupying bone cavities was discovered only after the World War II bombings of Hiroshima and Nagasaki. Studies showed that nuclear radiation destroyed marrow, causing death by infection and internal bleeding. Fat cells provide a rich source of energy and essential fatty acids needed in cell metabolism during proliferation (reproduced from Krieger, 1998).

The haematopoietic microenvironment of the medullary cavity provides more than structural support for HSCs during proliferation and differentiation. Stromal cells are required both to produce and present haematopoietic growth factors (HGFs) to haematopoietic precursor cells. Many of the HGFs are synthesised in membrane and secreted forms and function as adhesion molecules by binding their respective receptors. Several investigators have suggested that stromal mesenchymal cells secrete HGFs at such low quantities that local presentation by stromal cells is necessary to support basal haematopoiesis (Bennaceur-Griscelli *et al.*, 1999; Williams, 1995). HGFs comprise a family of glycoproteins produced by macrophages, endothelial cells and lymphocytes and include colony stimulating factors (CSFs) or interleukins (ILs). Most growth factors act at several levels of haematopoietic proliferation and differentiation. Stem cell factor (SCF, also known as *c-kit* ligand or steel factor), Flt3 ligand, and IL-3 (also known as multi-CSF) primarily support the survival and

proliferation of long term and short term PHSCs (Lyman and Jacobsen, 1998; Sachs and Lotem, 1994). Both Flt3 ligand and *c-kit* ligand are produced in membrane-bound as well as secreted forms (Lyman and Jacobsen, 1998). Inflammatory mediators IL-6 and IL-1 synergise with the actions of Flt3 ligand, *c-kit* ligand and IL-3 on PHSCs, thus allowing for increased blood cell production in times of need such as blood loss or infection. The mediators responsible for stem cell differentiation are largely unknown. The CD34 molecule, which phenotypically defines HSCs (Krause *et al.*, 1996), is thought to play a role in stem cell differentiation. Two species of CD34 mRNA have been identified, which code for a full-length and truncated protein. The extracellular domain of both CD34 isoforms is identical and the truncated form lacks a potential target site for protein kinase C (PKC). There is evidence that the full-length CD34 molecule may impede terminal differentiation of HSCs (Fackler *et al.*, 1995). CD34 also functions as an adhesion molecule for interacting with bone marrow stroma (Rao *et al.*, 1996). Thus CD34 may function to retain HSCs in their niche and to inhibit their differentiation until the appropriate differentiation and homing signals are received. Although the factors responsible for differentiation of HSCs are poorly understood, several specific growth factors are known to be responsible for the subsequent proliferation and maturation of progenitor cells. Thrombopoietin (TPO) is the primary regulator of megakaryocyte and platelet production (Kaushansky, 1995). Granulocyte-monocyte colony stimulating factor (GM-CSF) stimulates the growth of granulocytic and monocytic cell lines, as well as the progenitors of megakaryocytes and erythrocytes. Granulocyte colony stimulating factor (G-CSF) promotes the growth of the granulocytic cell line and monocyte colony stimulating factor (M-CSF) fosters the monocytic lineage. Erythropoietin (EPO) promotes the growth of early erythroid precursors in conjunction with IL-3, and GM-CSF can act alone on later erythroid progenitors (Williams, 1995). IL-7 promotes the differentiation of lymphoid progenitors and B cells (Ryan *et al.*, 1997; Veiby *et al.*, 1996). IL-15 produced by bone marrow epithelial cells supports the differentiation of natural killer (NK) cells (Cavazzana-Calvo *et al.*, 1996; Liu *et al.*, 2000; Mrózek *et al.*, 1996). Negative regulatory cytokines such as transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α) antagonise the action of the positive cytokines on normal haematopoietic cells, (Herrmann *et al.*, 1988; Sachs and Lotem, 1994).

1.2.3.5: Mobilisation of HSCs from bone marrow.

HSCs differ from other stem cells found in the adult such as epithelial stem cells in that they do not adhere tightly to one another. Populations of HSCs and their progeny with more restricted potential are detectable in peripheral blood (Kessinger, 1995), spleen and liver (Fuchs and Segre, 2000). Large scale migration of adult HSCs from bone marrow into the cirrculation is observed in response to treatment with a wide variety of cytokines and cytoreductive drugs, but little is known about the mechanisms of either normal or drug-induced HSC migration (Morrison *et al.*, 1997). Down-regulation of integrins on HSCs which adhere to bone marrow stromal vascular cell adhesion molecule-1 (VCAM-1) may be an important regulator of HSC migration (Lasky, 1996; Papayannopoulou and Nakamoto, 1993). Down-regulation of or variation in the CD34 molecule may influence mobilisation events. Three major classes of CD34 have been defined based on their differential glycosylation, which may have important functional correlates and may influence the homing of $CD34^+$ cells to different tissues in both the foetus and adult (Lin *et al.*, 1995; Titley *et al.*, 1995). Differential glycosylation of CD34 may therefore influence the compartmentalisation of stem and progenitor cells (Krause *et al.*, 1996).

1.2.3.6: Ontogeny of haematopoiesis.

Development of the human haematopoietic system involves a series of co-ordinated transformations, which commence early in embryonic life. Formation and differentiation of the haematopoietic mesenchyme is initiated around the thirteenth day of gestation, with the formation of the yolk sac (Prindull, 1992). Haematopoiesis is established in the yolk sac by day sixteen and is most active at six weeks. With the establishment of blood vessels, yolk sac haematopoiesis transfers *via* the circulation to the liver (Hann *et al.*, 1983; Lansdorp, 1995). By five to six weeks gestation, the liver has begun to replace the yolk sac as the main site of haematopoiesis, with cells entering the circulation from the liver from approximately eight weeks onwards (Migliaccio *et al.*, 1986) (figure 1.24). At five to six weeks gestation, stem cells and early progenitor cells proliferate intensely in the liver undergoing little differentiation, leading to a rapid expansion of their numbers. The expansion phase is followed by maturation of this primitive HSC population, with erythropoiesis commencing at six weeks.

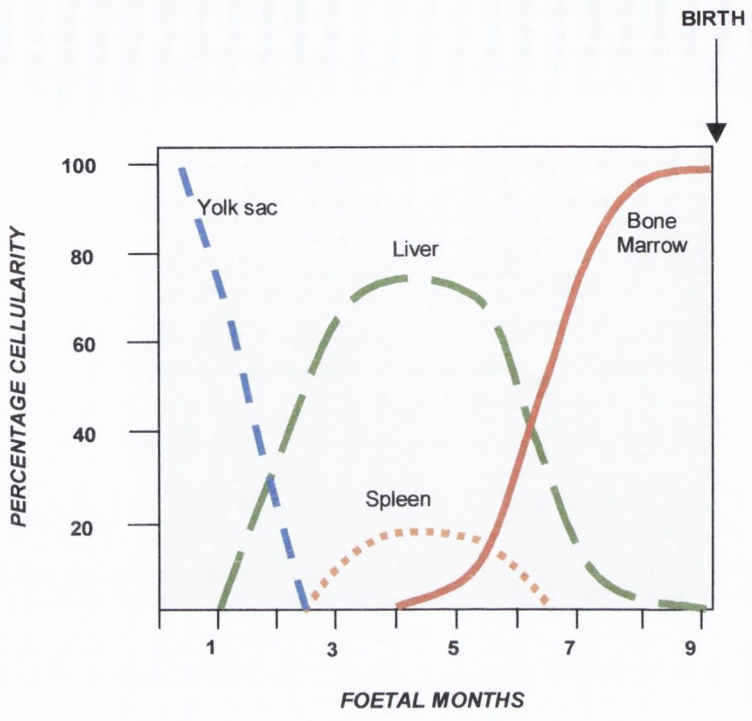


Figure 1.24: Contribution of various organs to haematopoiesis in the foetus.

Despite the preponderance of erythropoiesis in the foetal liver, myelopoiesis, B and T lymphopoiesis and megakaryocyte production can also be detected (Prindull, 1992). B cells are first detected in the foetal liver at eleven to fourteen weeks. These B cells subsequently colonise the bone marrow and then other sites such as the spleen and lymph nodes. T cell precursors are first detectable at seven weeks gestation, with thymic colonisation occurring at eight weeks (Haynes and Heinly, 1995). The liver undergoes a massive increase in size in the five to twenty week period of gestation and progenitors of both myeloid and lymphoid lineages can be identified in mid-trimester foetal liver. Stem cells are first observed in foetal bone marrow at fifteen to sixteen weeks gestation, subsequent to stromal cell development. The colonisation of bone marrow with stem cells from the liver occurs *via* circulating progenitors, which are detectable at high levels in foetal circulation from thirteen to twenty one weeks. The bone marrow gradually replaces the foetal liver as the primary haematopoietic site, and by the time of birth almost all haematopoiesis appears to take place in the bone marrow and it is currently understood that under normal circumstances, liver haematopoietic activity ceases shortly after birth. In the adult, the bone marrow is the major site of the production of all

mature circulating blood cells, with the exception of T cells, which require the specialised microenvironment of the thymus to complete their development (Hann *et al.*, 1983).

1.3: Rationale and overall objectives of thesis.

The haematopoietic role of the foetal liver (both in mice and humans) is well established. Although the bone marrow is thought to be the primary site of blood cell production in the adult, murine studies have demonstrated that the liver is also an important haematopoietic organ in adulthood. An important role of hepatic haematopoiesis in the adult mouse is the development of extrathymic T cells from liver derived HSCs (Abo *et al.*, 1999). Human case studies have demonstrated that the haematopoietic potential of the liver is retained in the adult and can become activated in times of stress such as bone marrow failure or following liver transplantation in patients with normal bone marrow function (Collins *et al.*, 1993; Schlitt *et al.*, 1995). The demonstration of resident HSCs with the functional capacity to differentiate into multiple erythroid and myeloid colonies *in vitro* in the normal AHL, suggests a possible haematopoietic role for the adult liver under normal circumstances (Crosbie *et al.*, 1999). The majority of hepatic T lymphocytes found in the normal AHL display the functional and phenotypic properties of innate T cells and are strikingly similar to their extrathymically derived murine counterparts, suggesting the possibility of local differentiation (Doherty *et al.*, 1999; Norris *et al.*, 1998; Norris *et al.*, 1999). Furthermore, RAG-1, RAG-2 and pre-TCR-α expression in $CD2^+/CD7^+$ lymphocytes isolated from normal AHL provides strong evidence that the AHL supports T cell development (Collins *et al.*, 1996). Taken together, these studies suggest that the normal AHL may be an important haematopoietic organ. Because of its location and function, the liver is continuously exposed to a large antigenic load that includes pathogens, toxins, tumour cells, and harmless dietary antigens. The liver must remain immunocompetent, while controlling inappropriate inflammatory responses to dietary and other harmless antigens encountered in the portal circulation. A specialised function of hepatic haematopoiesis in the adult human may be the generation of specialised T cell subsets to meet the demands of the diverse immunological challenges to which the liver is continuously exposed.

This thesis explores the hypothesis that human hepatic derived HSCs have a T-lymphopoietic function.

1.3.1: Specific objectives.

1. To phenotypically characterise hepatic HSCs with respect to expression of a range of differentiation, activation and lineage specific antigens. The cell surface phenotype of freshly isolated HSCs will be an indication of the role of these cells *in vivo* (chapter 2).

2. To examine the suitability of the hepatic microenvironment to support lymphopoiesis, by looking for the expression of IL-7 and IL-15. These two cytokines have been shown to play an essential role in thymic and extrathymic T-cell development (chapter 3).

3. To provide direct molecular evidence of gene rearrangements at the α-TCR locus, by quantifying TCR rearrangement-specific circular DNA reaction intermediates in hepatic lymphocyte populations (chapter 4).

Chapter 2 : Hepatic Haematopoietic Stem Cell (HSC) Characterisation.

2.1: Introduction.

2.1.1: Haematopoietic potential of the adult human liver.

While the haematopoietic function of the liver in the human foetus is well documented (Hann *et al.*, 1983; Williams, 1995) the role of the adult human liver (AHL) in haematopoiesis is thought to be relatively minor. However, the haematopoietic potential of the liver is retained in the adult human and can become activated, as demonstrated by the extramedullary erythropoiesis that occurs in liver or spleen at times of severe bone marrow dysfunction. In addition, in adult humans with normal bone marrow function, extramedullary erythropoiesis in the liver and reconstitution of multilineage haematopoiesis by donor-derived cells has been reported following liver transplantation (Collins *et al.*, 1993; Schlitt *et al.*, 1995). The normal AHL contains populations of functional haematopoietic stem cells (HSCs) with myeloid and erythroid differentiation capacity (Crosbie *et al.*, 1999). The co-expression of lineage-specific markers on hepatic haematopoietic progenitors has not previously been examined, therefore, the presence of hepatic lymphoid progenitor populations has yet to be demonstrated.

2.1.2: Lymphopoietic potential of the adult human liver.

Evidence suggests that the normal AHL may support extrathymic T cell development, as has been demonstrated in mice (Abo *et al.*, 1999). In humans, RNA transcripts specific for RAG-1 and RAG-2, the cell-specific components required for lymphoid development, have been detected in hepatic lymphocytes ($CD2^+/CD7^+$) isolated from normal liver tissue. In addition, pre-TCR-α, a T-cell specific chaperone expressed at an early stage of $\alpha\beta$ T cell development, has been demonstrated in the same cell populations (Collins *et al.*, 1996). These findings provide strong evidence that T cell development is ongoing in the normal AHL, and that at least a subset of cells is developing along the $\alpha\beta$ T cell pathway.

Further evidence for a lymphopoietic role for the normal AHL is provided by phenotypic and functional characterisation of lymphocytes derived from normal liver tissue (Doherty *et al.*, 1999; Hata *et al.*, 1990; Norris *et al.*, 1998; Norris *et al.*, 1999). These studies have

demonstrated that, while the hepatic lymphoid repertoire contains antigen-specific lymphocytes, innate immune cells dominate. Large numbers of γδ T cells, DN and CD8αα T cells, NK T lymphocytes ($CD3^+CD56^+$) and $V\alpha24^+$ cells are found in the liver. The phenotypic and functional properties of these innate hepatic cell populations resemble those of murine lymphocytes which are thought to be extrathymic in origin (Abo *et al.*, 1994; Laky *et al.*, 2000; Porter and Malek, 1999; Rocha *et al.*, 1995; Sato *et al.*, 1995). The origin of human hepatic lymphocyte populations is unknown. Hepatic lymphocyte populations appear to be tissue resident as they are not removed by extensive perfusion of the organ (Norris *et al.*, 1998). The location of the liver between the gastrointestinal tract and cardiopulmonary system results in constant exposure to the constituents of the major blood vessels of the body. As a result, there is continuous infiltration of peripheral blood cells into the liver. Selective homing and retention of peripheral lymphocytes and subsequent expansion and induction of cell surface molecules mediated by the hepatic cytokine milieu, may be responsible for the unique nature of hepatic lymphocyte populations. However, studies in mice have demonstrated that in addition to cells derived from the periphery, adult murine hepatic lymphocytes contain populations which are locally derived (Yamamoto *et al.*, 1999). Given, the presence of HSCs, the expression of RAG-1, RAG-2 and pTα, taken together with the "*extrathymic*" nature of hepatic lymphocytes, we have hypothesised that the normal AHL is a site for the extrathymic generation of liver resident T cell subsets. The demonstration of resident lymphocyte committed progenitor cell populations in the normal AHL would provide further evidence to support this hypothesis.

2.1.3: Characterisation of haematopoietic progenitor cell populations.

Flow cytometric analysis of individual cells provides a rapid, yet highly accurate method of assessing HSCs. The maturation of pluripotent HSCs (PHSCs) into non-lineage committed and subsequent lineage-committed progenitor cell populations is accompanied by changes in cell surface antigens. Using monoclonal antibodies (mAbs) directed against specific cell surface markers, it is possible to discern the differentiation status and potential of these cells.

Phenotypic characterisation of the HSCs derived from normal liver will give us some insight into the role of these cells *in vivo*.

2.1.3.1: Flow cytometry.

The analytical strength of flow cytometry lies in its ability to make quantitative multi-parameter measurements on statistically adequate numbers of cells to define the properties of a cell population or its component sub-populations. Flow cytometry provides for rapid measurements of several characteristics of single cells, confined in a stream of fluid, as they pass through an intense beam of light. The measurable characteristics derive from the scattering of light and the emission of fluorescence by particles confined in the fluid stream.

The cells subjected to flow cytometric analysis are prepared as a single cell suspension. The sample is forced from a container through sample tubing to enter a nozzle. Here the sample stream meets with the sheath fluid. The sheath fluid surrounds the sample stream emerging from the nozzle and keeps the sample stream stable and in focus. The stream passes at right angles to an argon laser (488nm). Transmitted and reflected light impinges on photodetectors *via* a series of strategically placed dichroic and band pass filters (figure 2.1.1). Photodetectors, or photomultiplier tubes (PMTs), amplify and convert the analogue signal to a digital format so that a computer can process it. One detector is placed directly in the path of the laser and detects low angle light scatter or forward angle scatter (FSC) and relates to cell size. A photodetector placed perpendicular to the laser detects 90° or side angle light scatter (SSC), and relates to the granularity of the cell. Using a combination of FSC and SSC allows us to distinguish cell populations based on their morphological properties and larger more granular cells such as macrophages are easily distinguished from smaller less granular cells such as lymphocytes (Pillemer, 1990).

Further information regarding the sample population can be obtained with the use of specific monoclonal antibodies (mAbs) conjugated to fluorescent molecules that emit light at various wavelengths after excitation in the laser beam. Cells positive for a particular marker against which the antibody is directed will emit a fluorescent signal of a particular wavelength,

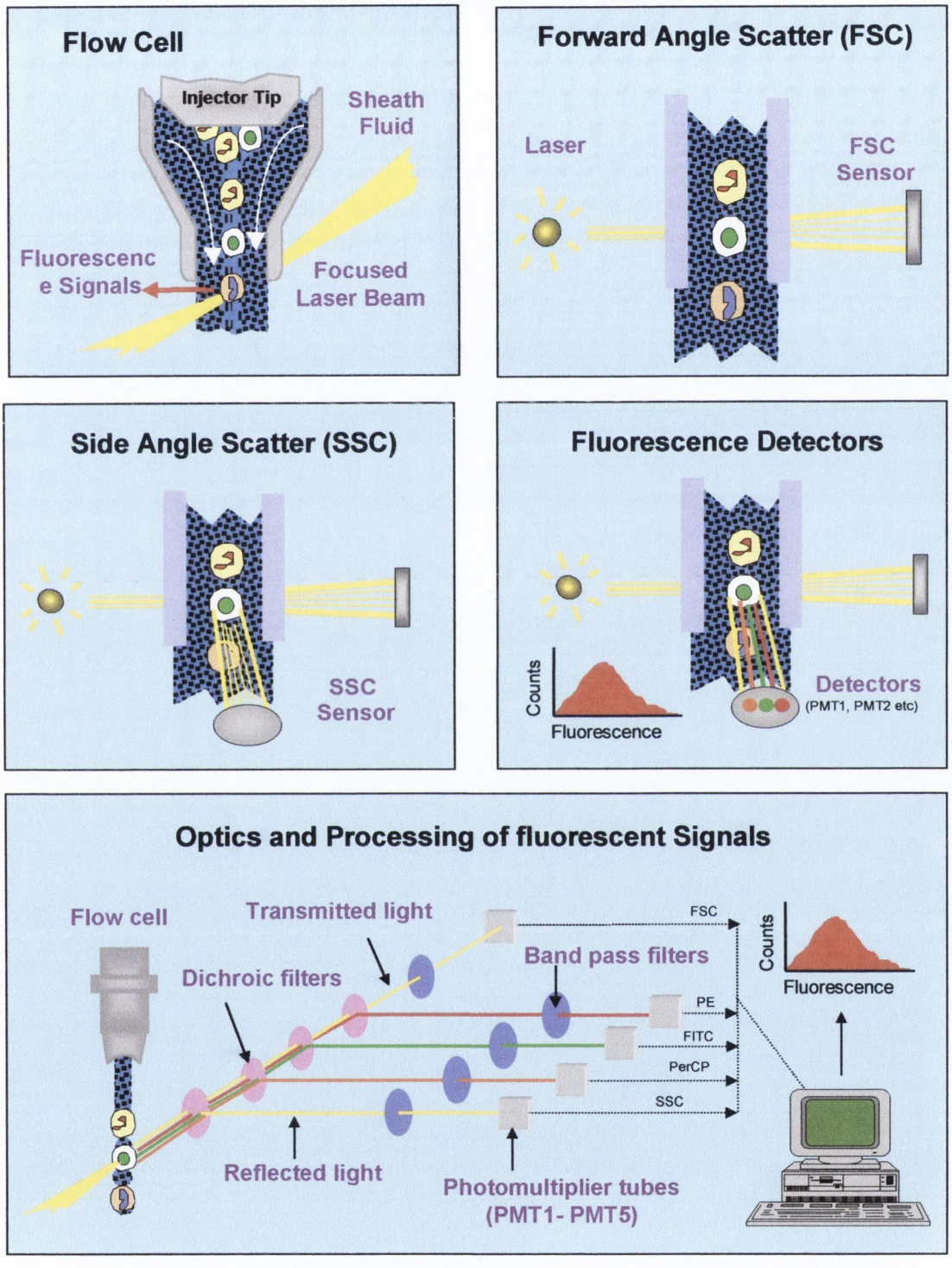


Figure 2.1.1: Principle of flow cytometry.

The measurable characteristics derive from the scattering of light and the emission of fluorescence by cells or particles confined in the fluid stream.

whereas a cell negative for the marker will not. The fluorescent signals of various wavelengths are picked up and processed by photomultiplier tubes. The intensity of the fluorescence signal relates to the number of molecules of interest detected on a single cell. The FacsScan™ flow cytometer (Becton Dickinson) allows the simultaneous measurement of three different fluorescent markers in addition to the FSC and SSC parameters. Thus this technology is ideal to for analysis of the cell surface phenotype of hepatic stem cell populations. The limitation of flow cytometric analysis is that it has to be applied to single cell suspensions and therefore we gain no information on the absolute numbers (as analysis is in terms of proportions), or the anatomic location of cells.

2.1.3.2: Phenotypic delineation of HSCs and their progeny.

All mature circulating blood cells are derived from self-renewing pluripotent HSCs characterised by the expression of CD34 (Krause *et al.*, 1996). The CD34 molecule is currently the only available phenotypic surface marker that selectively identifies all lineages of haematopoietic stem/progenitor cells (Fackler *et al.*, 1995). This glycoprotein is expressed on stem cells with *in vivo* reconstituting capacity, on virtually all colony forming cells and on early myeloid and lymphoid precursors, but not on mature blood cells (Krause *et al.*, 1996). During differentiation, CD34 is gradually down regulated and progenitors begin to express activation, differentiation and lineage-associated antigens as they proliferate and irreversibly commit to development along a particular haematopoietic lineage (Civin and Gore, 1993; Fritsch *et al.*, 1996). The relative levels of surface CD34 expression can distinguish two distinct populations of bone marrow $CD34^+$ cells by flow cytometric analysis. The $CD34^{bright}$ populations contains the majority of immature PHSCs, whereas the $CD34^{dim}$ populations contains more lineage committed progenitors (Andrews *et al.*, 1990; Herbein *et al.*, 1994). The $CD34^+$ population therefore comprises a heterogeneous population of cells at different stages of maturation that can be defined further by the presence or absence of differentiation and/or lineage markers ($lin^{+/-}$). As CD34 begins to be down regulated the expression of CD38 is concomitantly upregulated. $CD34^+CD38^{dim}$ cells are enriched for haematopoietic progenitors, a population which also expresses CD45RO (an isoform of the pan-leukocyte marker CD45), but does not express lineage-specific markers (Civin *et al.*, 1996; Civin and Gore, 1993). The expression of

CD38 is retained on progenitor cells, which commit to differentiation along both the myeloid and lymphoid lineages and thus can be considered a non-specific marker for HSC differentiation (Res *et al.*, 1996; Terstappen *et al.*, 1992; Terstappen *et al.*, 1991). The CD45RA isoform of the CD45 molecule is considered to be a marker of actively differentiating HSCs detectable on myeloid and lymphoid progenitors but not on erythroid precursors (Fritsch *et al.*, 1996). Thus, co-expression of CD38 and/or CD45RA identifies non-primitive $CD34^+$ progenitors that are actively differentiating, though not necessarily exclusively committed to differentiation along the myeloid or lymphoid lineages, but may contain populations of cells committed to either lineage. Committed progenitor populations can be identified by the co-expression of lineage specific markers. CD33 co-expression by $CD34^+$ cells identifies commitment to the myeloid lineage; CD19, B cell committed progenitors; CD7, T/NK cell committed progenitors; CD56, NK (and possibly NK T cell) committed progenitors (Civin and Gore, 1993; Fritsch *et al.*, 1996; Ship and Look, 1993) (figure 2.1.2).

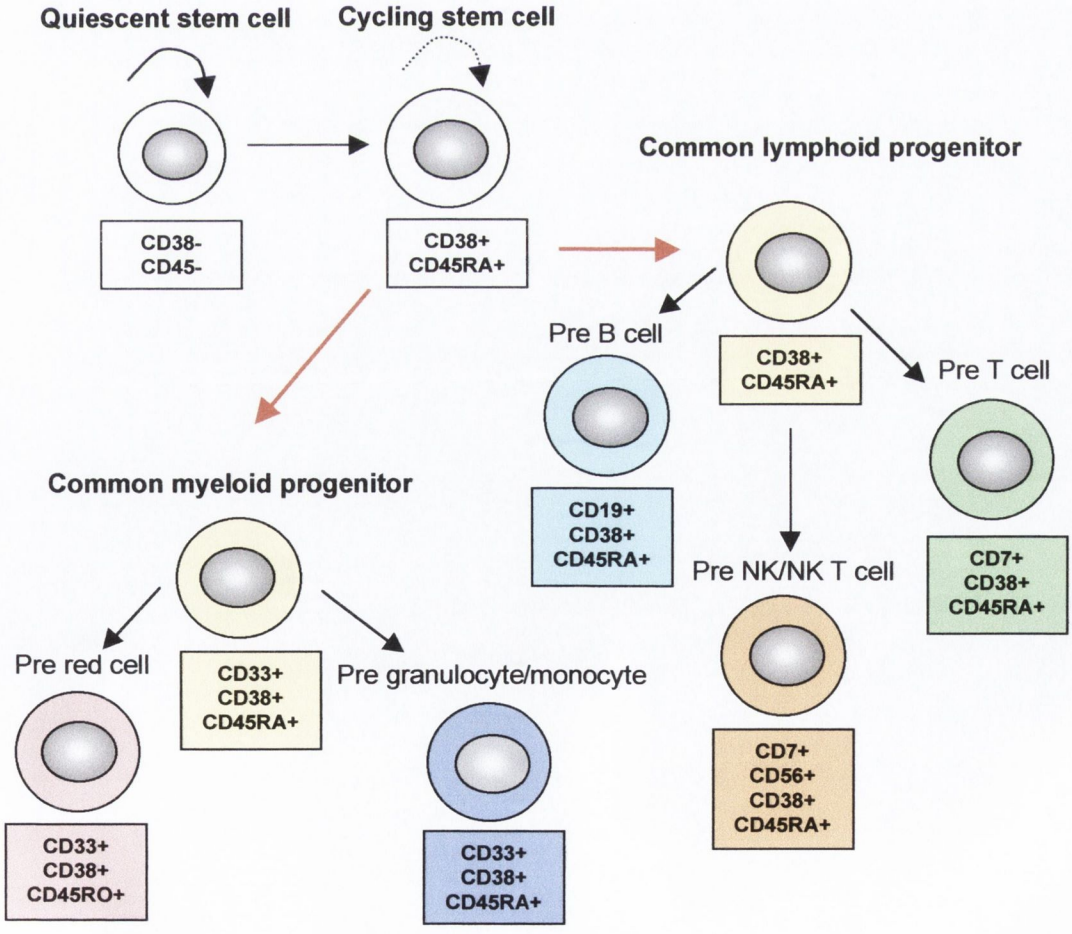


Figure 2.1.2: The $CD34^+$ haematopoietic stem/progenitor cell compartment.

While identifying all stem and progenitor cells, CD34 is not exclusively expressed on cells of the haematopoietic lineage. Vascular endothelial cells in human capillaries from most tissues and some large vessels express CD34 (Fina *et al.*, 1990). Recently, a new HSC marker, AC133 antigen, has been identified, which was thought to be specifically expressed on an immature subset of $CD34^{high}$ haematopoietic cells of bone marrow origin, but absent on more mature blood cells (de Wynter *et al.*, 1998; Miraglia *et al.*, 1997; Yin *et al.*, 1997). This glycoprotein has a predicted novel 5-transmembrane structure (figure 2.1.3).

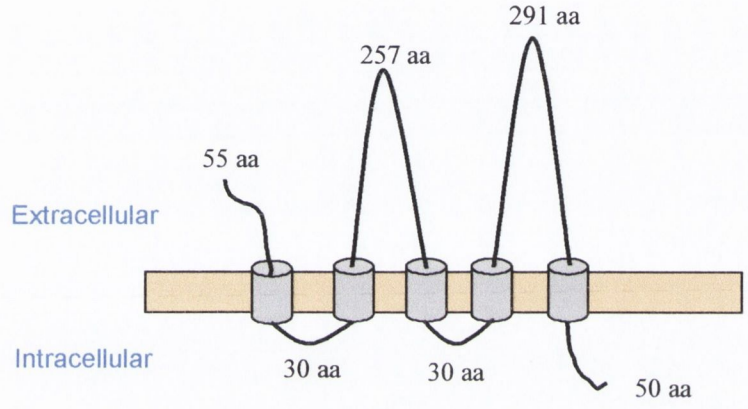


Fig. 2.1.3: Proposed structural model of the novel 5-transmembrane haematopoietic stem cell antigen AC133 (Miraglia *et al.* 1997).

As the $CD34^{+}AC133^{+}$ cell population contains all the $CD34^{+}CD38^{-}$ subset of HSCs, the AC133 antigen was thought to be a definitive marker of early pluripotent HSCs. However, a subsequent study has demonstrated the AC133 antigen to be expressed on undifferentiated Caco-2 cells (human epithelial cell line) and various human embryonic epithelia, including the neural tube, gut and kidney (Corbeil *et al.*, 2000). Thus, no single marker alone can define HSCs and the expression of stem cell antigens (CD34/AC133) must be looked at in conjunction with other markers. A combination of CD34 and CD45 allows the identification of all haematopoietic stem and progenitor cell subsets (see figure 2.1.2).

2.1.3.3: Localisation of HSCs within liver tissue.

To establish the anatomical location of HSC cell populations within normal hepatic tissue a technique that allows dual labelling of rare single cells (CD34 and CD45), within a much

larger population ($CD45^+$), on tissue sections is needed. Conventional, wide-field fluorescence microscopy only allows the observer to view a cell as a three-dimensional (3-D) structure. Fluorescent light emitted from points not within the focal plane can result in the apparent co-localisation of signals that are very close to each other but not necessarily derived from a single cell. Large numbers of $CD45^+$ cells are concentrated in the portal tract area of hepatic tissue sections in close proximity to vessel walls that may express CD34. This may lead to some ambiguity in the localisation of true CD45CD34 double positive HSCs in the peri-portal region. The addition of a confocal unit obstructs light above and below the focal plane, allowing generation of a 0.1 micron thick 2-D image. Thus, confocal fluorescence microscopy provides us with an ideal tool for the reliable visualisation of HSCs in hepatic tissue sections.

2.1.4: Physiological role of adult hepatic haematopoiesis.

The physiological significance of hepatic haematopoiesis in adult mice is illustrated by activation of this pathway by various stimuli including syngenic tumours (Abo *et al.*, 1992; Abo *et al.*, 1991; Ohmori *et al.*, 1993; Okuyama *et al.*, 1992). Mature cells of the haematopoietic system of both myeloid (monocytes and neutrophils) and lymphoid (T cells, natural killer and murine NK1.1 T cells) lineages are thought to play a role in the host response to tumour challenge. Anti-tumour effector cells can be broadly divided into two groups: early anti-metastatic, and those involved in the later stages of the elimination of established solid tissue metastases. Kupffer cells (Heuff *et al.*, 1995; Zhang *et al.*, 1993), neutrophils (Musiani *et al.*, 1996) and NK cells (Trinchieri, 1989) have all been implicated in the surveillance and prevention of hepatic metastases. NK1.1 T cells have been postulated to be the principal antimetastatic lymphocyte population in the murine liver (Hashimoto *et al.*, 1995), and play an important role in IL-12 mediated rejection of tumours (Cui *et al.*, 1997). NK cells facilitate the development of tumour specific cytotoxic T lymphocytes (CTLs) (Kurosawa *et al.*, 1995), which are thought to be the most important effectors in the rejection of established tumours (Xiang *et al.*, 1997). NK cells themselves may also participate directly in the elimination of established metastases (Whiteside and Heberman, 1995). Hepatic haematopoiesis (in adult mice) has been shown to be important in the generation of anti-tumour effector cells (Cui *et*

al., 1997; Hashimoto *et al.*, 1995; Kawamura *et al.*, 1996; Ohmori *et al.*, 1993). The normal AHL contains significant populations of functional myeloid and erythroid progenitors (Crosbie *et al.*, 1999), activation of which may contribute to the local pool of myeloid anti-tumour effector cells. Murine studies have demonstrated an increase in the extrathymic differentiation pathway of T cells in the livers of mice bearing syngenic tumours (Ohmori *et al.*, 1993). It has also been suggested that, in humans, as in mice, T cells of extrathymic origin may be involved in tumour immunity (Takii *et al.*, 1994). Strong evidence supports the existence of an extrathymic adult human T cell differentiation pathway in the liver (Collins *et al.*, 1996; Norris *et al.*, 1998). The AHL may contain lymphoid progenitors capable of contributing to the generation of lymphoid anti-tumour effector cells.

2.1.5: Overall objectives of this study.

The cell surface phenotype of hepatic stem cells, derived from normal adult human liver, has not previously been investigated. Characterisation of freshly isolated hepatic stem cells with respect to differentiation and lineage specific markers may give an indication as to the role of these cells *in vivo*. If a physiologically significant pathway of hepatic haematopoiesis operates in adult humans, one would expect to see upregulation of this pathway in a situation where there is an increased local need for immune cells, such as, in tumour challenge. The overall experimental strategy used to achieve these goals is illustrated in figure 2.1.4 and the specific objectives are outlined below.

2.1.5.1: Specific objectives.

1) To characterise the cell surface phenotype of isolated hepatic HSCs and thus verify the presence of lymphoid committed progenitor cells in the normal AHL.
2) To test the physiological significance of adult hepatic haematopoiesis by comparing the phenotype of hepatic stem cells in tumour-bearing hepatic tissue to those isolated from normal liver.
3) To localise hepatic HSCs within tissue sections from normal liver.

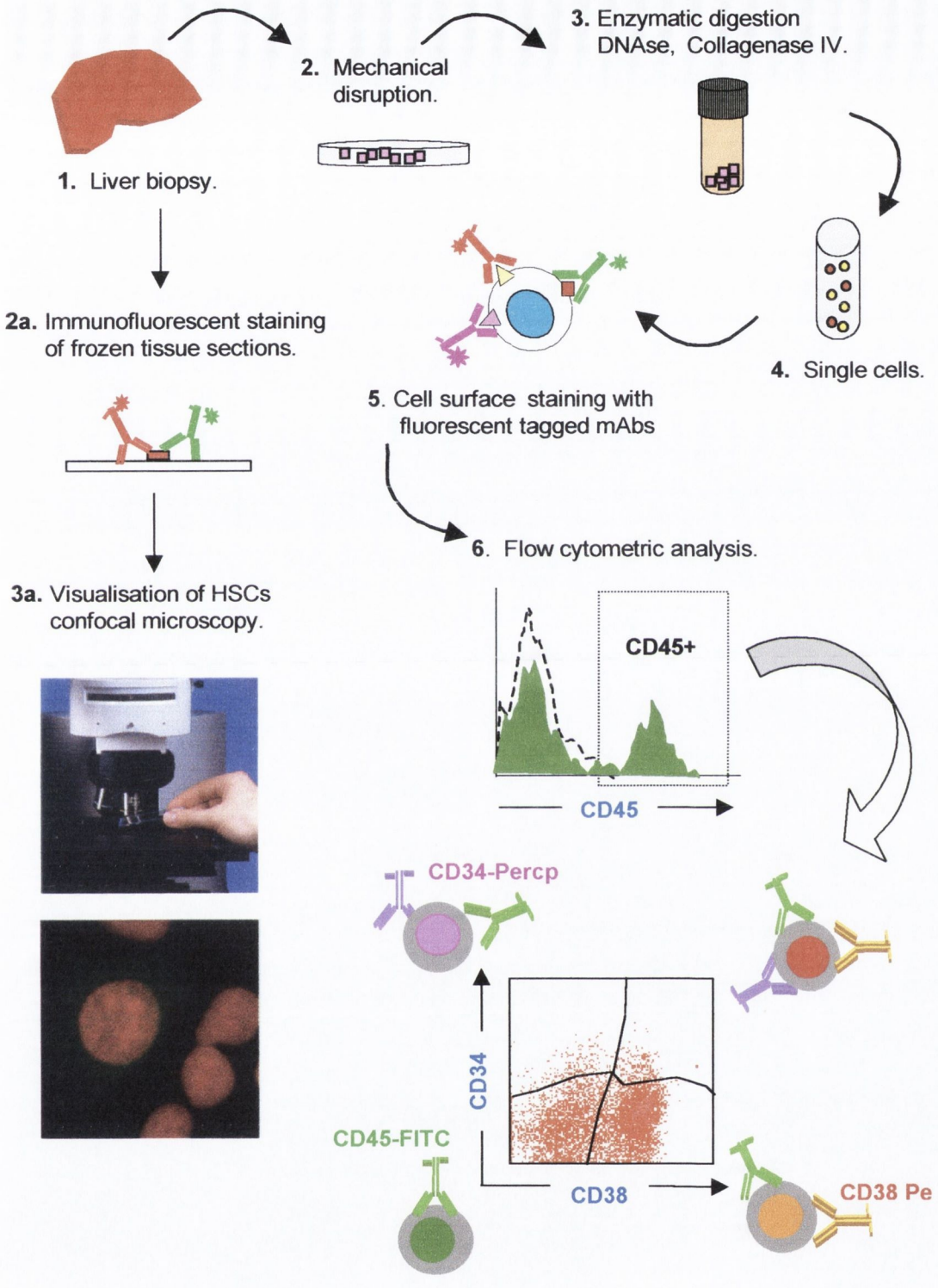


Figure 2.1.4: Experimental strategy for the characterisation and tissue localisation of hepatic haematopoietic stem cell (HSC) populations.

Hepatic mononuclear cells were prepared from fresh tissue biopsies using a combination of mechanical and enzymatic disruption. The single cell suspensions obtained from this process were stained with a panel of monoclonal antibodies directed against surface antigens associated with immature and differentiating HSCs. Expression of CD45 identified cells of haematopoietic origin. $CD45^{+}$ cells were analysed for the co-expression of CD34 (stem cell marker) and a panel of differentiation, activation and lineage specific markers (1 – 6). The anatomical location of hepatic HSCs was determined using fluorescent confocal microscopy (1 – 3a).

2.2: Materials and methods

2.2.1: The National liver transplant programme.

In the context of declining numbers of organ donations world wide, we in Ireland continue to have one of the highest per-capita organ donation rates in the world. The national liver transplant programme was established in 1993 in collaboration with the United Kingdom Transplant Service. Over the eight year period from 1993 to 2000, over 180 liver transplants have been performed at St. Vincent's University hospital (SVUH). Actuarial one-year survival is greater than 80%. The success rate at SVUH compares very favourably with the best survival figures obtained in liver transplant programmes throughout the world. The Liver Unit at SVUH has a firm commitment to training and research in all aspects of hepatology.

2.2.1.1: Ethical approval.

The Ethics committee at St. Vincent's University Hospital granted permission to carry out these studies.

2.2.2: Tissue specimens.

2.2.2.1: Normal liver.

Normal liver wedge biopsies (200-400mg) were obtained from donor organs (n = 20) at time of liver transplantation. Donor organs were extensively perfused with University of Wisconsin solution prior to obtaining the biopsy. Biochemical analysis was performed on all donors prior to hepatic surgery and liver biochemistry was normal in all cases. Part of the biopsy (approximately 5 mm^3) was placed in 10% formalin (Labscan, see Appendix II for alphabetical list of suppliers). Five micrometer sections of formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E, figure 2.2.1). The Pathology department, St. Vincent's Hospital, performed routine histological examination of tissue sections. For four of the normal liver samples a small section of tissue (approximately 5 mm^3) was mounted in cryopreservative embedding medium (OCT, Finetek Europe BV) before being transferred to liquid nitrogen.

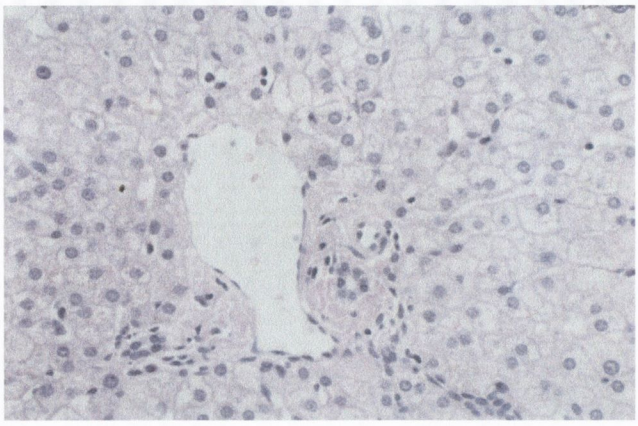


Figure 2.2.1: Photomicrograph of normal hepatic tissue stained with H&E.

2.2.2.2: Malignant liver.

Tumour-bearing tissue (n = 10) was obtained at the time of resection for isolated hepatic metastases of colonic origin. Wedge biopsies (200-400 mg) were taken 5-10 cm from tumour margin and appeared histologically normal at a gross level. Biopsies obtained at time of resection were washed three times in Hanks Balanced Salts Solution (HBSS, Gibco BRL) to remove residual blood. Immunohistochemical studies carried out in our laboratory have demonstrated that the washing procedure used for non-perfused samples is as effective as perfusion for the removal of intravascular non-resident leukocytes (Raghu Varadarajan, unpublished data, 1999). A comprehensive hematological and biochemical analysis was performed on all patients prior to hepatic surgery, and in none was myelodysplasia evident in peripheral blood smears. None of the patients had macrometastatic bone disease as assessed by radioisotope bone scan. Haemoglobin (Hb) levels and mean corpuscular volume (mcv) were normal for all but one of the patients. The majority of patients did not receive chemotherapy prior to hepatic surgery, while the interval between the primary colon surgery and hepatic surgery was 3 to 5 years. In three cases, the interval between primary colon surgery and hepatic surgery was approximately six months and two of these patients received 6 sessions of 5-fluorouracil (5-FU) + Leucovorin prior to hepatic surgery. The Pathology department, St. Vincent's Hospital, performed routine histological examination of sections of formalin fixed paraffin embedded liver tissue stained with H&E (figure 2.2.2).

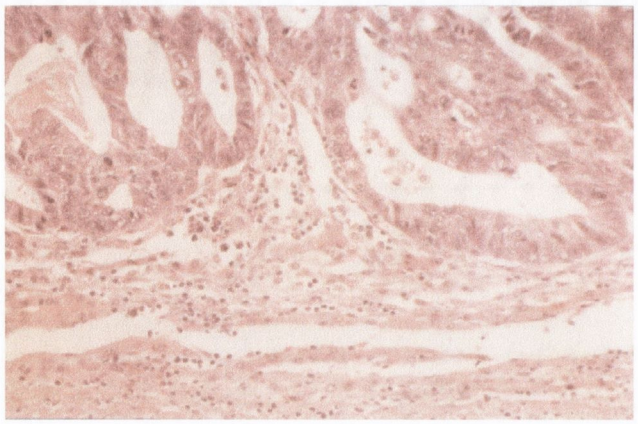


Figure 2.2.2: Photomicrograph of hepatic tissue with isolated metastatic carcinoma of colonic origin, showing tumour margin.

2.2.2.3: Peripheral blood.

In six cases (3 x normal, 3 x tumour-bearing), 10 ml of matched venous blood was collected in lithium-heparin tubes (Becton Dickinson [BD]).

2.2.2.4: Bone marrow.

Marrow aspirates (n = 3) were collected from patients undergoing routine examination by the Haematology department at St. Vincent's University Hospital. These samples were used as a positive control for haematopoietic stem cells (HSCs).

2.2.3: Preparation of single cell suspensions suitable for flow cytometric analysis.

2.2.3.1: Isolation of hepatic mononuclear cells (HMNCs).

A technique developed in our laboratory was used for the isolation of single cell suspensions, suitable for flow cytometric analysis, from hepatic tissue (Curry *et al.*, 2000b); a brief description of the method follows. Liver tissue was finely chopped on ice. HBSS (20 ml) containing 0.5 mg/ml type IV collagenase (Sigma-Aldrich), 0.02 mg/ml DNase I (Boehringer Mannheim), 2% foetal calf serum (FCS, Gibco BRL) and 0.6% bovine serum albumin (BSA, Sigma-Aldrich), was added to the dissected tissue. The tissue mix was then placed on a blood rotator and incubated at 37°C for 20 minutes. Following enzymatic digestion, the

solution was passed through a 30 μm nylon mesh (Caddisch Precision Mesh) to remove any undissociated tissue. The filtered suspension was washed twice in 20 ml HBSS with centrifugation at 300g for 8 minutes and the resulting pellet was resuspended in 10 ml HBSS and centrifuged at 30g for 1 minute to remove the hepatocyte rich matrix. The supernatant was centrifuged at 300g for 8 minutes and the pellet resuspended in 1 ml Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL) containing 10% FCS. Cell yields and viability were assessed by ethidium bromide acridine orange (EB:AO) staining and microscopic examination of cell suspension loaded on a haemocytometer (Hawskey, see Appendix III [APIII.1] for details). Hepatic mononuclear cell suspensions were adjusted to a concentration of 1 x 10^6 cells/ml in RPMI + 10% FCS.

2.2.3.2: Isolation of mononuclear cells from bone marrow and blood.

Bone marrow (BM) and peripheral blood mononuclear cells (PBMCs) were prepared by standard density gradient centrifugation over Lymphoprep™ (Nycomed, see Appendix III [APIII.2] for details). Cell yields and viability were determined by EB:AO staining as above and cell suspensions were adjusted to a concentration of 1 x 10^6 cells/ml in RPMI + 10% FCS.

2.2.4: Cell surface staining of cells for flow cytometric analysis.

2.2.4.1: General staining protocol.

Aliquots of 100 μl (1 x 10^5 cells) were labelled with monoclonal antibodies (mAbs) directed against cell surface markers classically associated with immature and differentiating haematopoietic progenitor cells. Prior to labelling, blocking of non-specific Fc receptor sites was performed by pre-incubation of cells with an equal volume of heat inactivated (63°C for ten minutes) normal human serum for 30 minutes at 4°C. Cells were then washed with 1ml of phosphate buffered saline (PBS, Sigma-Adrich) containing 3.33% BSA and 0.02% sodium azide (Sigma-Aldrich, PBS-BSA-Azide). The appropriate mAbs (0.3 μg/ml final concentration), were added to cell suspensions, and incubated in the dark at 4°C for 30 minutes. Samples were then incubated for 10 minutes at room temperature in 2 ml of 10% FACS lysis solution

(BD) to remove red blood cells, and washed twice with 1ml of PBS-BSA-Azide. Labelled cells were fixed in 0.5 ml 1% paraformaldehyde (Sigma-Aldrich).

2.2.5: Two colour and three colour flow cytometry.

In the first instance, two colour flow cytometry was used to detect the presence of $CD34^+CD45^+$ HSCs in mononuclear cell populations isolated from hepatic tissue. Three colour flow cytometry was used subsequently to further characterise hepatic HSC populations with respect to co-expression of activation, differentiation and lineage-specific markers.

2.2.5.1: Monoclonal antibodies used for two-colour staining.

Two colour staining of 15 liver samples was used to determine the presence and levels of haematopoietic stem cells (HSCs, $CD34^+CD45^+$, see table 2.2.1 for details). Two-colour flow-cytometric analysis also facilitated the determination of what proportion of $CD34^+$ cells were of haematopoietic origin (i.e. coexpressing CD45). Single cell suspensions prepared from hepatic tissue as described earlier (section 2.2.2.a) were stained with a combination of anti-CD34-PE (stem cell marker, phycoerythrin, BD, clone 8G12) and anti-CD45-FITC (pan-leukocyte marker, fluorescein isothiocyanate, BD, clone 2D1). Bone marrow aspirates, from three patients in whom haematological malignancy had been excluded, were also stained with the above antibodies. All samples were also stained with the appropriate fluorescent-labelled isotype-matched control antibodies.

Table 2.2.1: Demographics of hepatic tissue samples used for two-colour flow cytometric analysis of haematopoietic stem cells.

Status	n	Male:Female	Mean age (range)
Normal	10	5:5	49* (26 – 68 years)
Tumour-bearing	5	2:3	55 (35 – 67 years)

*Note: the lower age of donors is not statistically significant when compared to patients with malignancy.

2.2.5.2: Monoclonal antibodies used for three-colour staining.

Three colour staining was used to characterise the co-expression of cell surface markers on hepatic $CD34^{+}CD45^{+}$ cells from 26 liver samples (see table 2.2.2 for details). For the identification of cells of haematopoietic origin, FITC-labelled anti-CD45 mAb (BD, clone 2D1) was included in all tubes. Stem/progenitor cells were identified using Peridin Chlorophyll Protein (PerCP) labelled anti-CD34 mAb (BD, clone 8G12). In conjunction with anti-CD45-FITC and anti-CD34-PerCP, the following PE-labelled anti-differentiation/lineage marker mAbs were included: anti-CD33 (myeloid, clone P678.6), anti-CD38 (differentiation marker, clone HB-7), anti-CD56 (natural killer, clone MY 31) anti-CD19 (B-cell, clone 4G7, all from BD), anti-CD7 (T-cell, clone M-T701), anti-CD45RA (activation, naive T-cell marker, B-cells and monocytes, clone HI100, both from Pharmingen), anti-AC133 (early stem cell marker, clone AC133, Miltenyi Biotec).

Table 2.2.2: Demographics of hepatic tissue samples used for three-colour flow cytometric analysis of haematopoietic stem cells.

Status	n	Male:Female	Mean age (range)
Normal	16	8:8	42* (16 - 69 years)
Tumour-bearing	10	5:5	56 (33 - 68 years)

*Note: The age of donors is significantly lower than tumour-bearers ($p < 0.03$).

Appropriate fluorescent-labelled isotype-matched control antibodies were used to correct for any background staining. The antibodies used in this study to characterise hepatic HSC sub-populations are represented in diagrammatic form in figure 2.2.3. In six cases, matched blood had been obtained (3 x normal, 3 x malignant), and PBMCs prepared from these bloods were stained as above. Because of the low frequency of HSC populations in blood and liver mononuclear cell preparations, and, because liver mononuclear cell preparations have higher levels of auto-fluorescence than blood or bone marrow, a number of additional control stains were included to ensure a more accurate estimation of HSC levels and their co-expression of differentiation and/or lineage-specific markers. The additional controls included are described

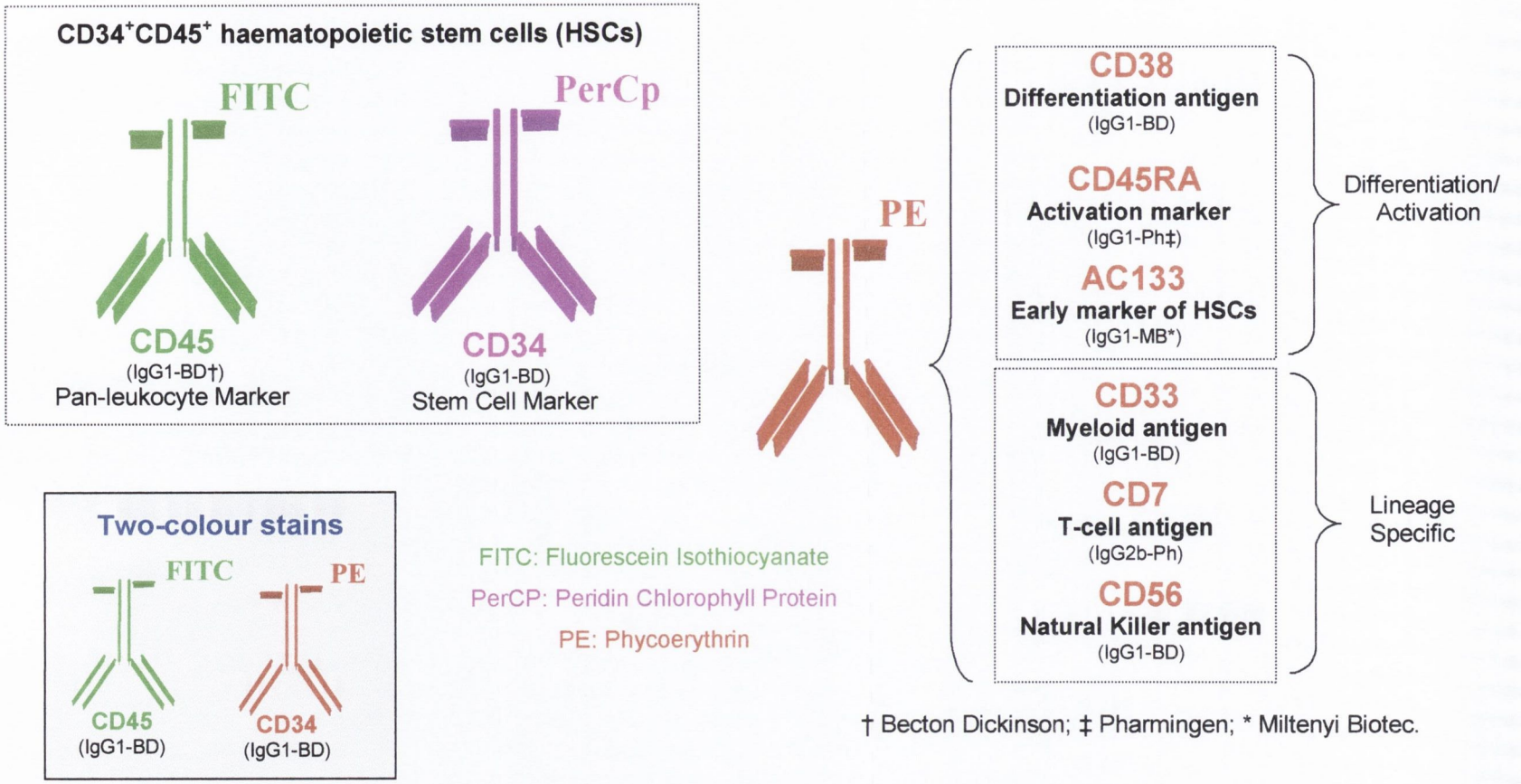


Figure 2.2.3: Antibodies used for cell surface staining of haematopoietic stem cells.

Three colour flow cytometric analysis was used to characterise hepatic haematopoietic stem cell (HSC) populations. For the identification of cells of haematopoietic origin, FITC-labelled anti-CD45 mAb (BD, clone 2D1) was included in all tubes. Stem/progenitor cells were identified using PerCP-labelled anti-CD34 mAb (BD, clone 8G12). In conjunction with anti-CD45 and anti-CD34, a number of PE-labelled anti-differentiation/activation/lineage-specific marker mAbs were included. Two-colour analysis was also used to determine hepatic HSC levels, the antibodies used are depicted in the shaded panel.

in table 2.2.3 and in figure 2.2.4 an example is shown of how the control stains consisting of each of the markers on their own were used to set the appropriate analysis regions used instead of standard quadrants.

Table 2.2.3: Additional control stains used for three-colour flow cytometric analysis of haematopoietic stem cells.

Control no	FITC	PE	PerCP
1	CD45-BD*	γ_1-BD	γ_1-BD
2	CD45-BD	γ_1-Ph‡	γ_1-BD
3	CD45-BD	γ_{2b}-Ph	γ_1-BD
4	CD45-BD	γ_1-BD	CD34-BD
5	CD45-BD	γ_1-Ph	CD34-BD
6	CD45-BD	γ_{2b}-Ph	CD34-BD
7	CD45-BD	CD38-BD	γ_1-BD
8	CD45-BD	CD33-BD	γ_1-BD
9	CD45-BD	CD56-BD	γ_1-BD
10	CD45-BD	AC133-Mi#	γ_1-BD
11	CD45-BD	CD45RA-Ph	γ_1-BD
12	CD45-BD	CD7-Ph	γ_1-BD

*BD = Becton Dickinson; ‡Ph = Pharmingen, #Miltenyi Biotec

2.2.6: Flow Cytometric Analysis.

2.2.6.1: Determination of acquisition/analysis gate.

To determine the optimal region for the detection and analysis of hepatic HSCs, HMNC preparations (n = 3) were stained with a combination of CD45-FITC, CD34-PerCP and IgG1-PE. Acquisition and analysis were carried out using a FACScan flow cytometer and Cell Quest software (BD). CD45 positive cells were gated using FL1 (FITC, $CD45^+$) and side-scatter (SSC, granularity) parameters (R1 in figure 2.2.5a). The forward-scatter (FSC, size) SSC morphology plot of $CD45^+$ cells was divided into two gates, a blast gate (low to medium granularity) and a granular gate (R2 and R3 respectively in figure 2.2.5). Cells were acquired twice, first with compensations and fluorescent detectors optimised for R2 and a second time

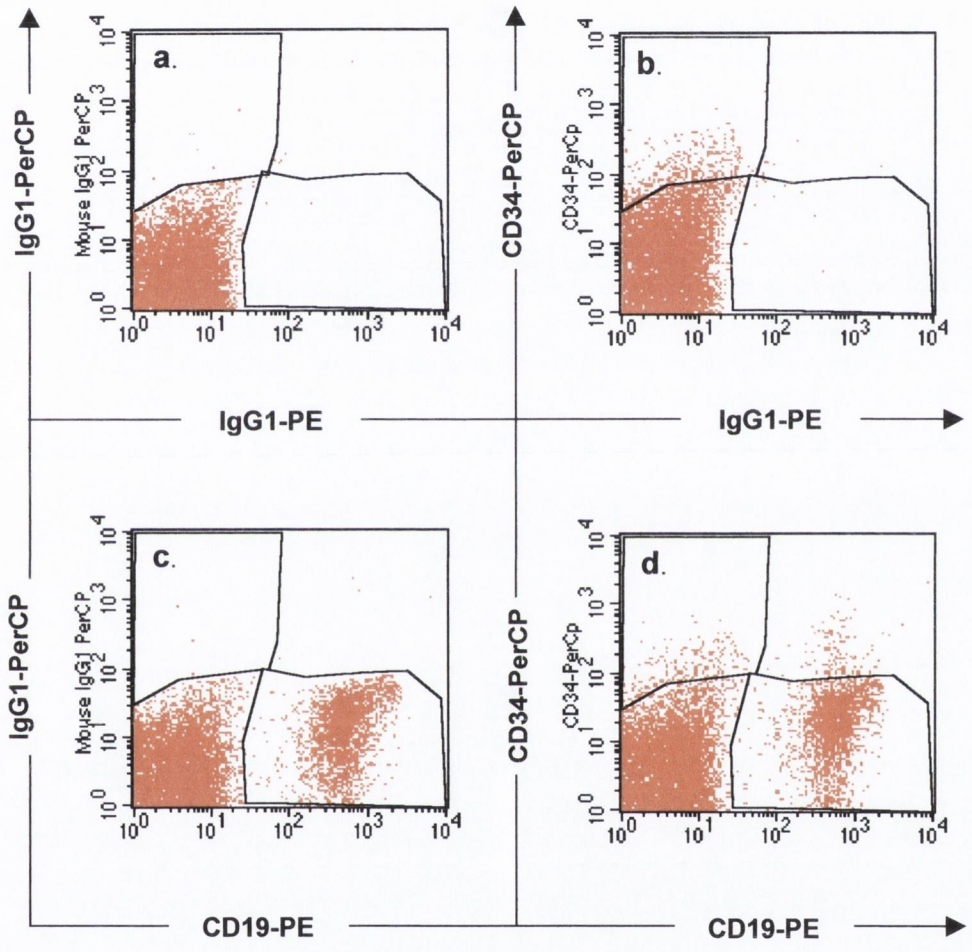


Figure 2.2.4: Controls used for the characterisation of hepatic haematopoietic stem cell sub-populations.

The dot plots show the PE and PerCP staining levels for CD45-positive hepatic mononuclear cells. **a.** The level of staining seen with isotype-matched control antibodies was used to set the fluorescent detectors to the appropriate levels. Staining with anti-CD34-PerCP and isotype-matched PE control antibody (**b**), and staining with PE-conjugated antibodies against each marker of interest (in the example shown CD19) with PerCP-labelled isotype-matched control antibodies (**c**) was used to set the compensations and to determine where the analysis regions were drawn. **d.** Estimation of the levels of CD19-positive (upper right region) and CD19-negative (upper left region) hepatic HSCs based on the control files.

with the acquisition parameters optimised for R3. Ten thousand $CD45^+$ events were acquired in each case. Analysis was performed on $CD45^+$ cells only and the level of staining for CD34-PerCP and IgG1-PE in R2 and R3 was determined. Levels of staining were calculated as levels above those detected with the appropriate isotype matched control antibodies.

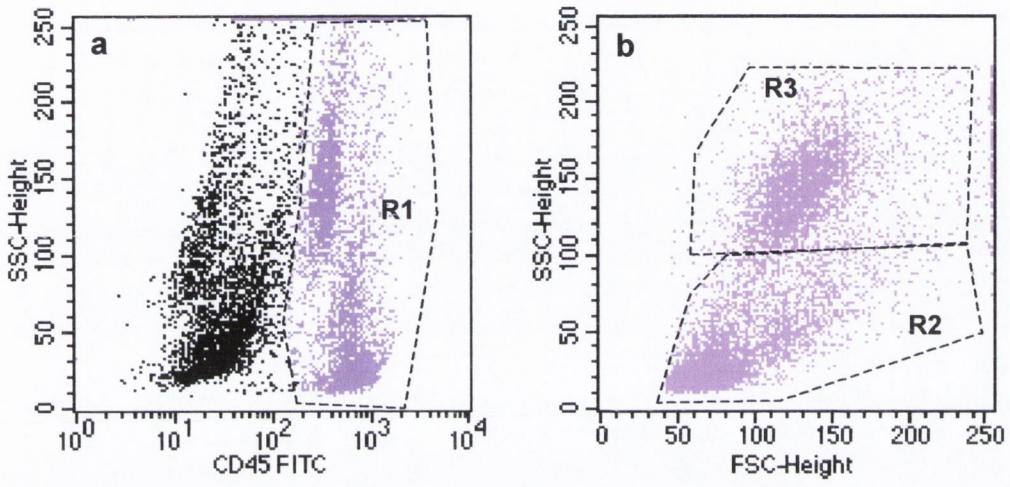


Figure 2.2.5: Gates used for the determination of the optimal region for the detection and characterisation of hepatic haematopoietic stem cell populations.

a. Forward scatter (FSC):FL1 (FITC) dot plot of hepatic mononuclear cells (HMNCs), CD45+ cells are shown in R1. **b.** FSC:side scatter (SSC) dot plot of total CD45+cells in hepatic mononuclear cell preparation (R1). The total CD45+ cells are divided into two gates based on granularity; R2 low to medium granularity (blast gate) and R3 high granularity (granular gate).

2.2.6.2: Two-colour flow cytometric analysis.

Acquisition and analysis were carried out using a FACScan flow cytometer and Cell Quest software (BD). PE (CD34) and FITC (CD45) staining levels, above those of appropriate isotype matched controls, were analysed for all cells falling in the blast gate (R2), once it had been established that >98% of $CD34^+CD45^+$ cells were detected in this region (see results section 2.3.2). Twenty thousand events were acquired for each sample. Analysis was carried out on all cells in the blast region to determine what proportion of the $CD34^+$ cells in HMNC preparation were of haematopoietic origin (co-expressed CD45). Results are expressed as a percentage of total $CD45^+$ cells or as a percentage of total $CD34^+$ cells where appropriate.

2.2.6.3: Three-colour flow-cytometric analysis.

Acquisition and analysis were carried out using a FACScan flow cytometer and Cell Quest software (BD). CD45 positive cells were gated using side-scatter (SSC) and FL1 (FITC) parameters. All further analysis was performed on $CD45^+$ cells only. Twenty thousand $CD45^+$ events were acquired in each case. PE (variable markers) and PerCP (CD34) staining levels above that of appropriate isotype matched controls were analysed for $CD45^+$ cells falling in the blast gate. Results are expressed as a percentage of total $CD45^+$ cells or as a percentage of $CD34^+CD45^+$ cells where appropriate.

2.2.6.4: Comparison of HSC levels detected using PE or PerCP labelled anti-CD34 mAb.

The assessment by flow cytometry of levels of $CD34^+$ haematopoietic stem cells varies greatly depending on the particular clone from which the antibodies used are derived (Titley *et al.*, 1995). The two anti-CD34 mAbs used in this study are derived from the same clone (8G12) but are conjugated to different fluorochromes. To assess if the particular fluorochrome used affects the detection sensitivity of the anti-CD34 mAb, levels of CD34 staining for twelve of the samples which were used for three-colour and two colour analysis were compared.

2.2.6.5: Effect of proteolytic enzymes on cell surface marker expression

The method used for the extraction of mononuclear cells from hepatic tissue involves the use of proteolytic enzymes which may disrupt cell surface marker expression (Abuzakouk *et al.*, 1996). To determine the effects of proteolytic enzymes (collagenase IV and Dnase I) on cell surface expression of haematopoiesis-associated antigens, bone marrow mononuclear cells (n = 2) were incubated in the enzyme mix used for the isolation of hepatic mononuclear cells for 20 minutes at 37°C. After incubation, cells were washed once to remove proteolytic enzymes. Aliquots of 1×10^5 cells were stained with a panel of monoclonal antibodies (mAb) which included anti-CD45-FITC, anti-CD34-PE, anti-CD34-PerCP and anti-AC133-PE. Cells were also stained for the same cell-surface markers prior to incubation. Flow cytometry was performed on cells to assess change in cell surface marker expression during incubation with the proteolytic enzyme solution used for the release of hepatic mononuclear cells. Histogram

analysis before and after incubation of the individual cell-surface antigens was used to monitor any changes in median fluorescent intensity (MFI) associated with treatment of cells with the enzyme mix.

2.2.6.6: Granular $CD45^+$ cell populations.

Granularity of hepatic mononuclear cells was assessed using forward and side scatter (FSC, SSC) morphology plots of total $CD45^+$ cells. Granular cells (identified by their characteristic high SSC properties) were gated and the proportion of the total $CD45^+$ cells falling within the granular gate was calculated.

2.2.7: Tissue distribution of hepatic HSCs.

Confocal fluorescence microscopy was used to determine the anatomic location of HSC populations in normal liver tissue (n = 4). Cryostat sections (5 μm) were acetone-fixed for 10 minutes and stored at - 70°C. Immediately before use, they were fixed in acetone for a further 10 minutes. Double immunostaining for CD45 (Southern Biotechnology Associates Inc, clone F10-89-4, IgG2a, 1/50) and CD34 (Novo-Castra, clone QBEnd/10, IgG1) was carried out on fixed cryostat sections. Colocalisation was visualised using the fluorescent conjugates anti-IgG1 Texas red and anti IgG2a fluorescein isothiocyanate (both from Southern Biotechnology Associates Inc, 1/50). The anti-CD45 and anti-CD34 monoclonal antibodies (diluted in PBS containing 10% FCS and 0.1% sodium azide) were applied to the tissue sections simultaneously. After 1 hour, the sections were washed in PBS (pH 7.4) for 30 minutes. A mixture of the fluorescent-conjugated antibodies against the IgG subclasses (1 and 2a) were added and incubated for 30 minutes. After a further wash in PBS (one hour, with constant agitation), the slides were mounted in 2.5% diazbicyclo-octane (Sigma Aldrich) to prevent fading. The histology was assessed under incident transmitted Fluorescence microscopy. The Negative control consisted of omission of the primary antibody. Anit-cytokeratin-19 (Dako, clone RCK108, IgG1), that specifically identifies biliary epithelial cells, served as a positive control for fluorescent staining.

2.2.8: Statistical analysis.

A two-tailed unpaired Student's t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant. Spearman Rank was used for correlation analysis.

2.3: Results.

2.3.1: Yields and viability of HMNC preparations.

Recovery of hepatic mononuclear cells (HMNCs) from normal liver biopsies was 1.45 – 8.67 x 10^6 cells/g tissue (wet weight), viability ranged from 60-95% (figure 2.3.1). There was no statistical difference between recovery and viability values for normal and tumour-bearing tissue (range 1.09 – 8.53 x 10^6 cells/g tissue, viability 50 – 95%).

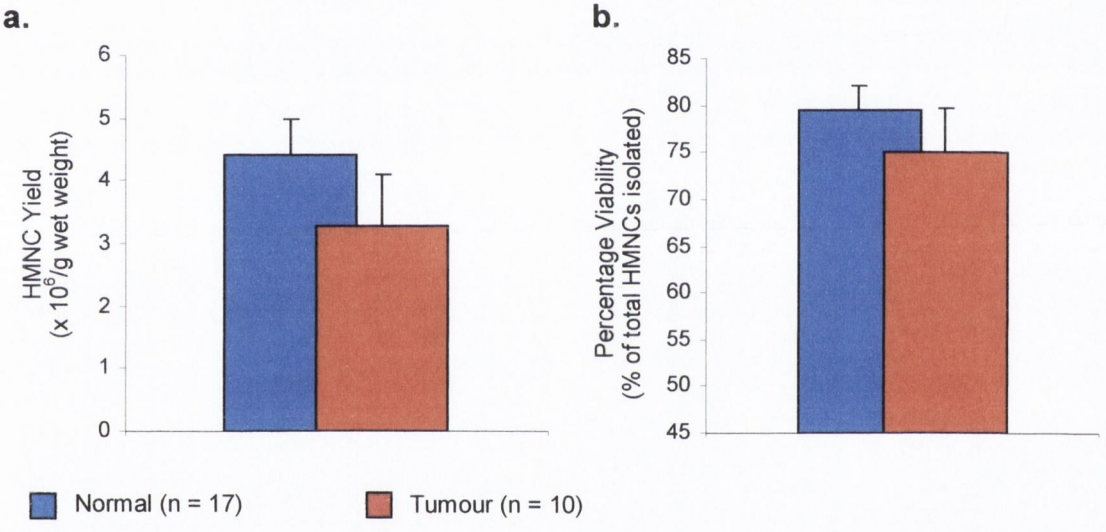


Figure 2.3.1: Yields and viability of mononuclear cells isolated from normal and tumour-bearing and hepatic tissue.

The columns represent the mean values for each tissue group and the error bars show the standard error of the mean (SEM). **a.** Cell yields are expressed as the number of hepatic mononuclear cells (HMNCs x 10^6) isolated from the equivalent of one gram of liver tissue (wet weight). **b.** Viability is expressed as a percentage of total mononuclear cells isolated.

2.3.2: Determination of the optimal acquisition/analysis region.

To determine the optimal region for the acquisition and analysis of hepatic haematopoietic stem cells (HSCs, $CD34^+CD45^+$), HMNC preparations from three liver biopsies (2 x normal, 1 x tumour-bearing), were acquired and analysed in two regions, a blast and a granular region (R2 and R3 in figure 2.2.1 respectively). The levels of HSCs detected in the granular region were in the range 0% – 0.14%. Table 2.3.1 details the levels of HSCs detected in the blast

and granular gates for the samples tested. The mean levels of HSCs detected in the blast gate was 1.37%, whereas mean levels detected in the granular region was 0.06% (or 4.38% of analysis gate levels, Figure 2.3.2). It was concluded that greater than 90% of hepatic HSCs lie within the blast region. The low-medium SSC blast gate was used for all subsequent acquisition and analysis of HSCs.

Table 2.3.1 : HSC levels detected in blast and granular regions.

Sample number	Sample status	CD34+CD45+ (% of total CD45+ cells) Blast gate(R2)	Granular gate (R3)
1	Normal	1.35	0.05
2	Normal	1.38	0
3	Tumour	1.37	0.14

2.3.3: Two-colour flow cytometric analysis.

2.3.3.1: Levels of hepatic HSCs.

Using the combination of CD45-FITC and CD34-PE, significant levels of haematopoietic stem cells (HSCs, $CD34^+CD45^+$) were detected in normal liver (mean 1.83%, n = 10, % of total $CD45^+$ cells), comparable to levels found in bone marrow (mean 2.21%, n = 3). Mean levels detected in tumour-bearing liver (1.40%, n = 5) did not differ significantly from normal levels (figure 2.3.3). However; the small sample numbers may be responsible for the lack of statistical significance.

2.3.3.2: Levels of CD34 expression on hepatic HSCs.

The median fluorescent intensity (MFI) for a particular cell surface marker is an index of the number of molecules expressed on the cell surface on a per-cell basis. The level of CD34 expression (MFI) on hepatic $CD45^+$ cells was lower than CD34 expression on bone marrow $CD45^+$ cells (mean MFI 58.91 [n = 15] and 343.61 [n = 3] respectively), suggesting that hepatic stem cells are more differentiated (Figure 2.3.4).

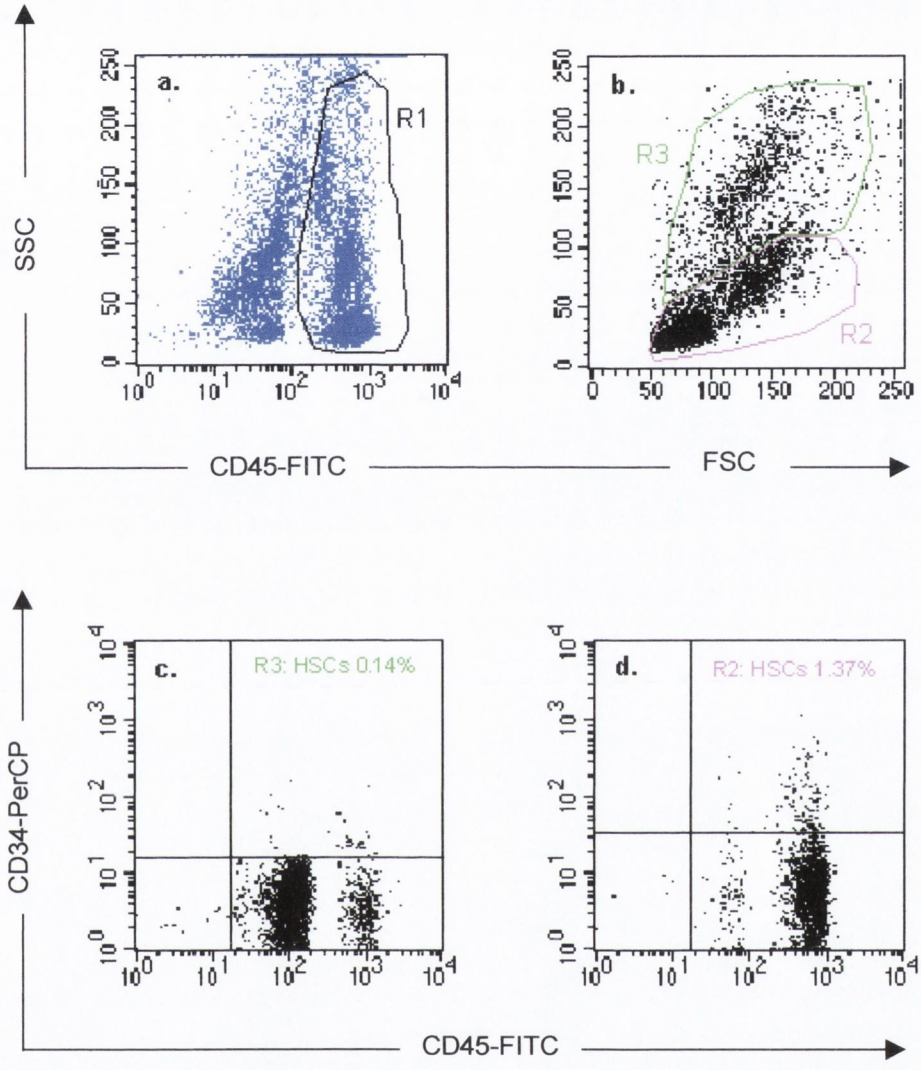


Figure 2.3.2: Levels of haematopoietic stem cells (HSCs) detected in hepatic mononuclear cell populations with high granularity and low-medium granularity.

Single-cell suspensions, prepared from hepatic tissue (n = 3), were stained with a combination of, anti-CD45-FITC (Fluorescein isothiocyanate, pan-leukocyte marker) and anti-CD34-PerCP (Peridin Chlorophyll Protein, stem cell marker). Samples were acquired and analysed on a FACScan flow cytometer using Cell Quest software (Becton Dickinson). **a.** $CD45^+$ cells were gated using SSC (side scatter, granularity) and FITC (CD45) parameters (R1). All further analysis was carried out on $CD45^+$ cells only. **b.** The granularity of $CD45^+$ cells was assessed using FSC (forward scatter, size) and SSC parameters. Granular cells were identified by their characteristic high SSC properties. $CD45^+$ cells were divided into two gates, R2 (blast gate) containing cells of low-medium granularity and R3 (granular gate) cells of high granularity. Each sample was acquired twice with the acquisition parameters optimised for R2 and R3. **c & d**. Haematopoietic stem cells (HSCs) were identified by the co-expression of CD34 and CD45. The level of HSCs was expressed as a percentage of the total $CD45^+$ population. To assess the optimal region for the acquisition and analysis of hepatic HSC populations, the levels of HSCs detected in R3 (**c**) and R2 (**d**) were compared. The example shown is sample 3 in table 2.3.1.

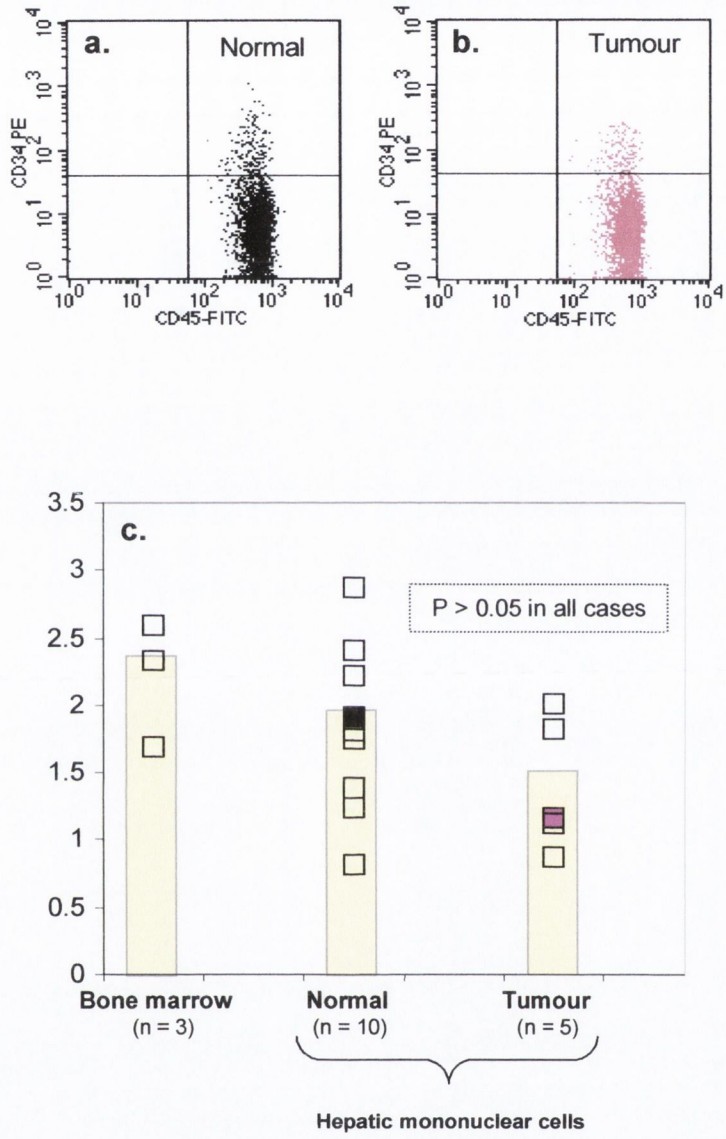


Figure 2.3.3: Levels of haematopoietic stem cells (HSCs) detected in bone marrow and liver samples using two-colour flow cytometric analysis.

Single-cell suspensions prepared from bone marrow aspirates or hepatic tissue, were stained with a combination of anti-CD45-FITC and anti-CD34-PE and analysed on a FACScan flow cytometer using Cell Quest software (Becton Dickinson). Haematopoietic stem cells (HSCs) were identified by the co-expression of CD34 and CD45. **a.** and **b.** Dot plots of $CD45^+$ hepatic mononuclear cells showing $CD34^+CD45^+$ HSCs in the upper right quadrant. **a.** In the example shown from normal liver, HSCs comprise 1.91% of total $CD45^+$ cells. **b.** HSCs in tumour-bearing liver comprise 1.12%. **c.** The level of HSCs detected in each sample was expressed as a percentage of the total CD45+ population detected in the sample. The levels of HSCs detected in BM (n = 3) were compared to the levels detected in normal liver (n = 10) and tumour-bearing liver (n = 5) tissues. The shaded bars show the mean values for each group and each point represents the level of HSCs detected in an individual sample. The filled points indicate the samples for which the dot plots are shown. A two-tailed unpaired Student's t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant.

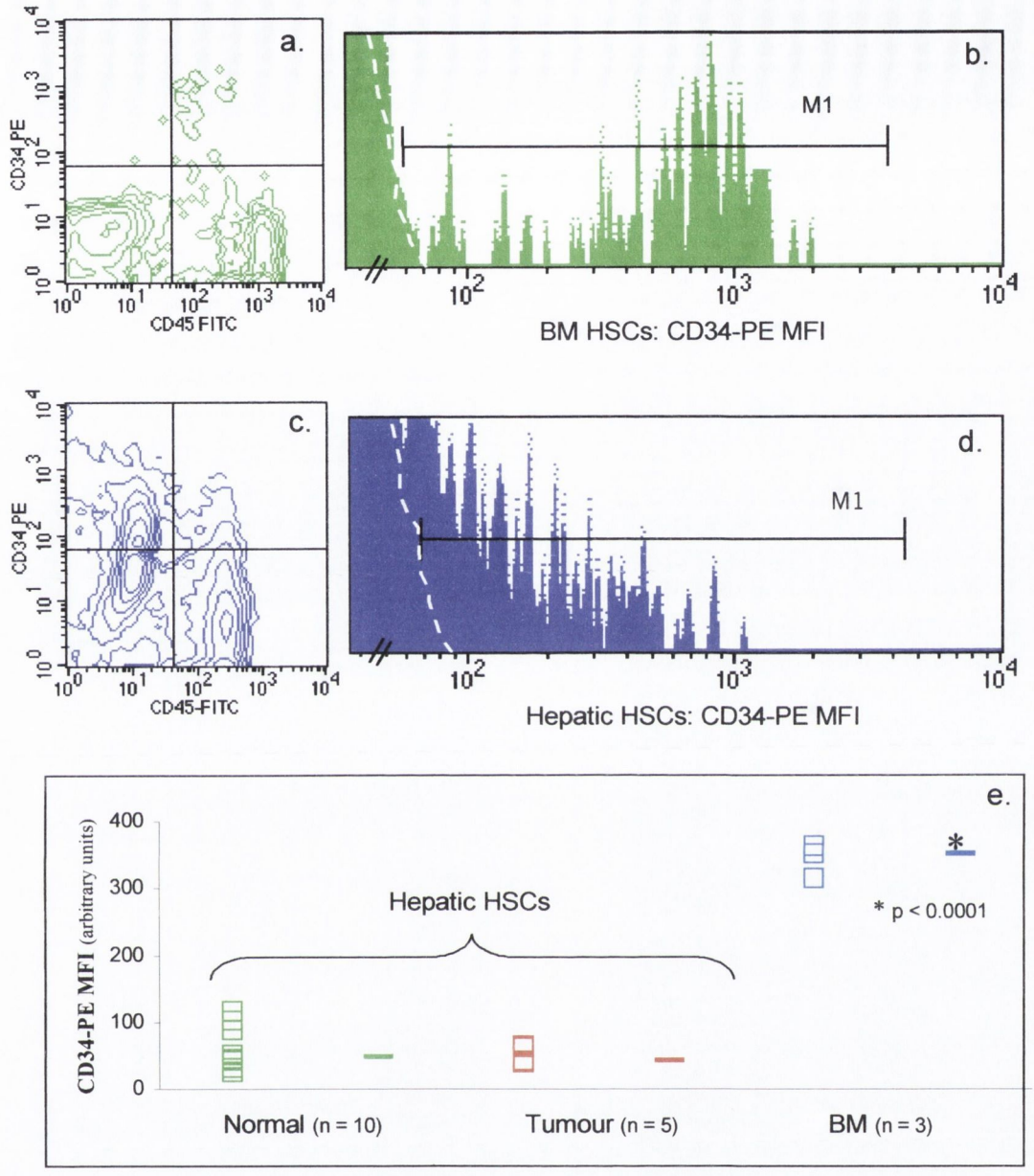


Figure 2.3.4: Level of expression of CD34 on haematopoietic stem cells (HSCs) from bone marrow (BM) and liver.

Bone marrow mononuclear cells were isolated by density centrifugation (1.077 g/ml, n = 3). A combination of mechanical and enzymatic disruption was used to isolate mononuclear cells from liver tissue (n = 15). Mononuclear cell suspensions were stained with a combination of anti-CD45-FITC and PE-labelled anti-CD34 mAbs. Samples were analysed on a FACScan flow cytometer using Cell Quest software (Becton Dickinson). Haematopoietic stem cells (HSCs) were identified by the co-expression of CD34 and CD45. The median fluorescent intensity (MFI, arbitrary units) for a particular fluorochrome is an index of the median number of molecules of the antigen (against which the attached antibody is directed) expressed on a per cell basis. **a.** Contour plot showing the typical $CD34^{high}$ profile of bone marrow HSCs (seen in the upper right quadrant). **b.** Histogram plot of the MFI of CD34 on bone marrow HSCs. **c.** Contour plot showing the typical $CD34^{low}$ profile of hepatic HSCs. **d.** Histogram plot of the MFI of CD34 on hepatic HSCs. The level of staining for the corresponding isotype matched control Pe-antibody is shown by the white dotted lines (b and d). **e.** Individual MFI values for hepatic and bone marrow samples are represented by the squares. The lines show the median. A two-tailed unpaired Student's t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant. There was no difference in the MFI for CD34 on hepatic HSCs isolated from normal and tumour-bearing liver tissue.

2.3.3.3: Hepatic CD34$^+$ cells of haematopoietic origin.

A variable proportion of the CD34$^+$ cells found in liver were of haematopoietic origin (co-expressed CD45, mean 41.28%, n = 15, range 19.33 – 78.57, Sd. 15.47), compared to > 95% in bone marrow (Figure 2.3.5). The variable proportion of CD34$^+$ cells of haematopoietic origin in HMNC preparations necessitated the use of three-colour flow cytometry to further characterise hepatic HSCs.

2.3.3.4: The effect of HMNC isolation enzyme mix on CD45-FITC and CD34-PE expression.

The technique to isolate HMNCs involves the use of proteolytic enzymes (collagenase IV and DNase I), which may disrupt cell-surface markers (Abuzakouk *et al.*, 1996). The relatively high levels of hepatic HSCs and/or the lower CD34 MFI, when compared to bone marrow, may be a consequence of the isolation technique used. To assess the effect of proteolytic enzymes on the levels of HSCs detected, bone marrow mononuclear cells (n = 2) were incubated in the liver enzyme mix under the same conditions as used for the release of hepatic mononuclear cells. Cells were stained before and after incubation with anti-CD45-FITC and anti-CD34-PE. The levels of CD45$^+$ cells (% of total cells) and CD34$^+$CD45$^+$ HSCs (% of total CD45$^+$ cells) were unaffected by incubation with the enzyme mix. The MFI values for both CD45 and CD34 were also unchanged by treatment with the proteolytic enzyme mix (table 2.3.2).

Table 2.3.2: Effect of Collagenase/DNase on CD34-PE and CD45-FITC expression.

a) CD34-PE.

Sample	Antibody (-label)	Before incubation (% of CD45$^+$ cells)	After incubation (% of CD45$^+$ cells)	MFI* Before	MFI After
1	CD34-PE	1.46	1.44	352.27	348.22
2	CD34-PE	1.57	1.55	316.23	318.55

b) CD45-FITC.

Sample	Antibody (-label)	Before incubation (% of total cells)	After incubation (% of total cells)	MFI Before	MFI After
1	CD45-FITC	98.05	97.98	523.30	528.62
2	CD45-FITC	93.7	94.13	562.12	556.93

*MFI = Median Fluorescent Intensity, an index of the number of molecules of a particular cell surface antigen on each individual cell in the sample (arbitrary units).

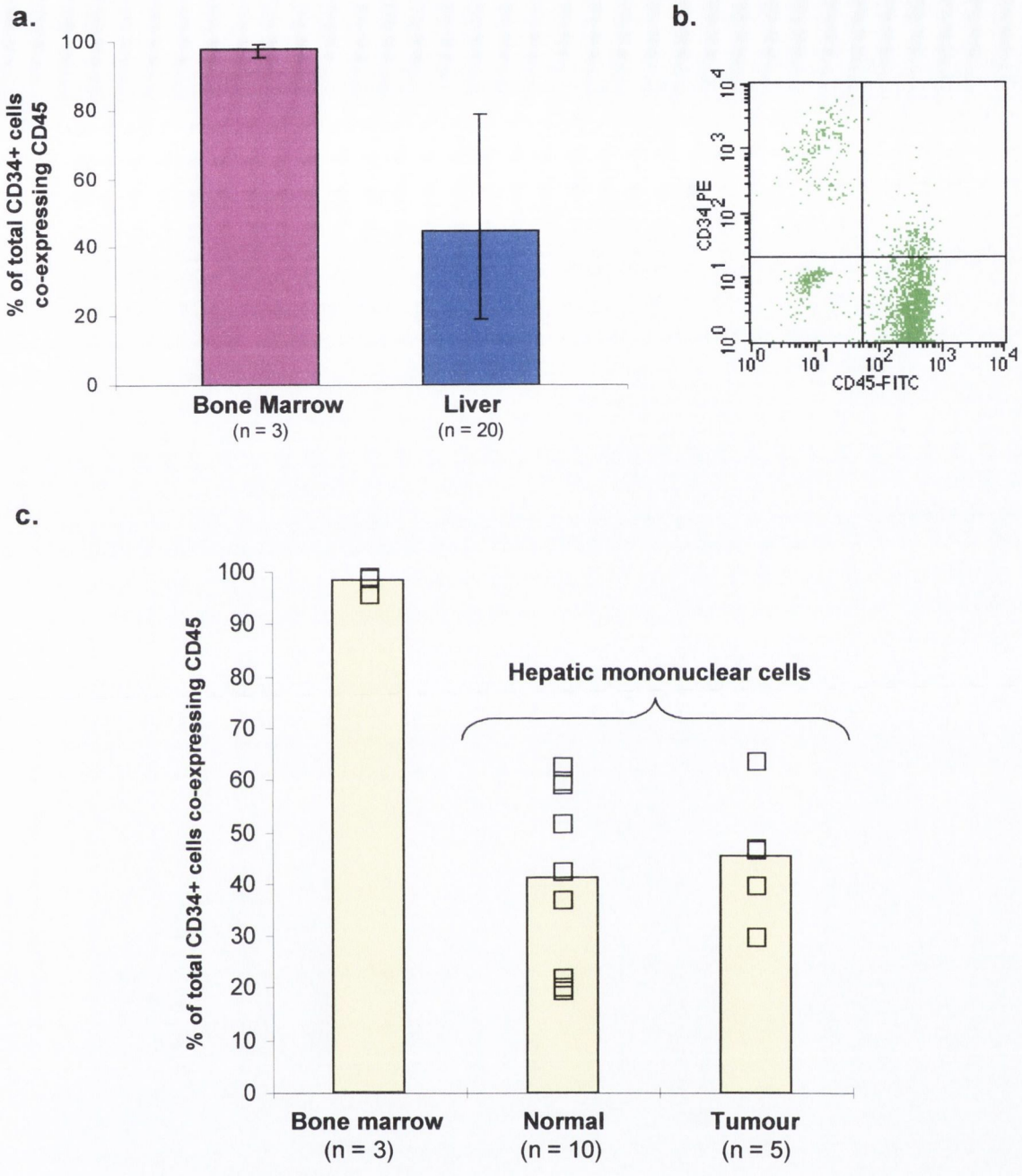


Figure 2.3.5: CD34-positive cells of haematopoietic origin in bone marrow and liver.

Mononuclear cell preparations isolated from bone marrow and hepatic tissue were stained with a combination of anti-CD45-FITC and anti-CD34-PE and analysed on a FACScan flow cytometer using Cell Quest software (Becton Dickinson). The proportion of CD34 positive cells coexpressing CD45 was expressed as a percentage of total $CD34^+$ cells. **a.** Greater than 95% of bone marrow $CD34^+$ cells are of haematopoietic origin. The proportion of $CD34^+$ cells of haematopoietic origin in hepatic mononuclear cell preparations varies greatly, on average less than 50% coexpress CD45. The columns represent the mean values for all livers and bone marrows stained for two-colour flow-cytometric analysis. The error bars show the range. **b.** Flow cytometry dot-plot showing CD34 and CD45 staining of hepatic mononuclear cells. The $CD34^+$ cells of haematopoietic origin (coexpress CD45) are shown in the upper right quadrant. $CD34^+$ cells, which are negative for CD45 expression, are shown in the upper left quadrant. **c.** The proportion of $CD34^+$ hepatic mononuclear cells of haematopoietic origin did not differ significantly between normal and tumour-bearing liver samples (the columns show the mean and each individual sample is represented by an open square).

2.3.4: Three-colour flow cytometric analysis.

2.3.4.1: The effect of fluorochrome on the detection of HSC populations.

The variability of the levels of HSCs detected using anti-CD34 mAbs derived from different clones is well documented (Titley *et al.*, 1995). The two anti-CD34 mAbs used in this study were derived from the same clone (8G12) but were conjugated with different fluorochromes (PE or PerCP). To assess if the particular fluorochrome used affects the detection sensitivity of the anti-CD34 mAb, levels of CD34 staining for ten of the samples which were used for three-colour and two-colour analysis were compared (figure 2.3.6). The PE conjugate was more sensitive than the PerCP. The significant difference in sensitivity observed prohibited direct comparison of levels between samples for which different fluorochrome conjugates were used. Although the CD34-PE antibody was more sensitive, the CD34-PerCP antibody was used in subsequent three colour flow cytometric analysis. This was due to the limited availability of PerCP-conjugated antibodies against other markers of interest.

2.3.4.2: Levels of hepatic HSCs using anti-CD34-PerCP.

A small but significant proportion of $CD45^+$ cells in the liver co-express CD34 (mean 1.23%, n = 16), on average, six times higher than the levels detected in matched peripheral blood samples (mean 0.18%, n = 3). Levels detected in Tumour-bearing liver were similar to normal liver (mean 1.29%, n = 10), greater than five times the levels detected in matched blood (mean 0.22, n = 3, figure 2.3.7).

2.3.4.3: AC133 antigen expression on hepatic and bone marrow HSCs.

Co-expression of the AC133 antigen on hepatic HSCs was tested in five HMNC preparations (2 x normal, 3 x tumour-bearing). Bone marrow mononuclear cell preparations (isolated by standard density centrifugation) were used as a positive control for AC133 staining (n = 3). The AC133 antigen was not detectable on hepatic HSC populations but was observed on approximately a quarter of the bone marrow HSC populations (mean 27.09%, figure 2.3.8, demonstrating that the antibody was working).

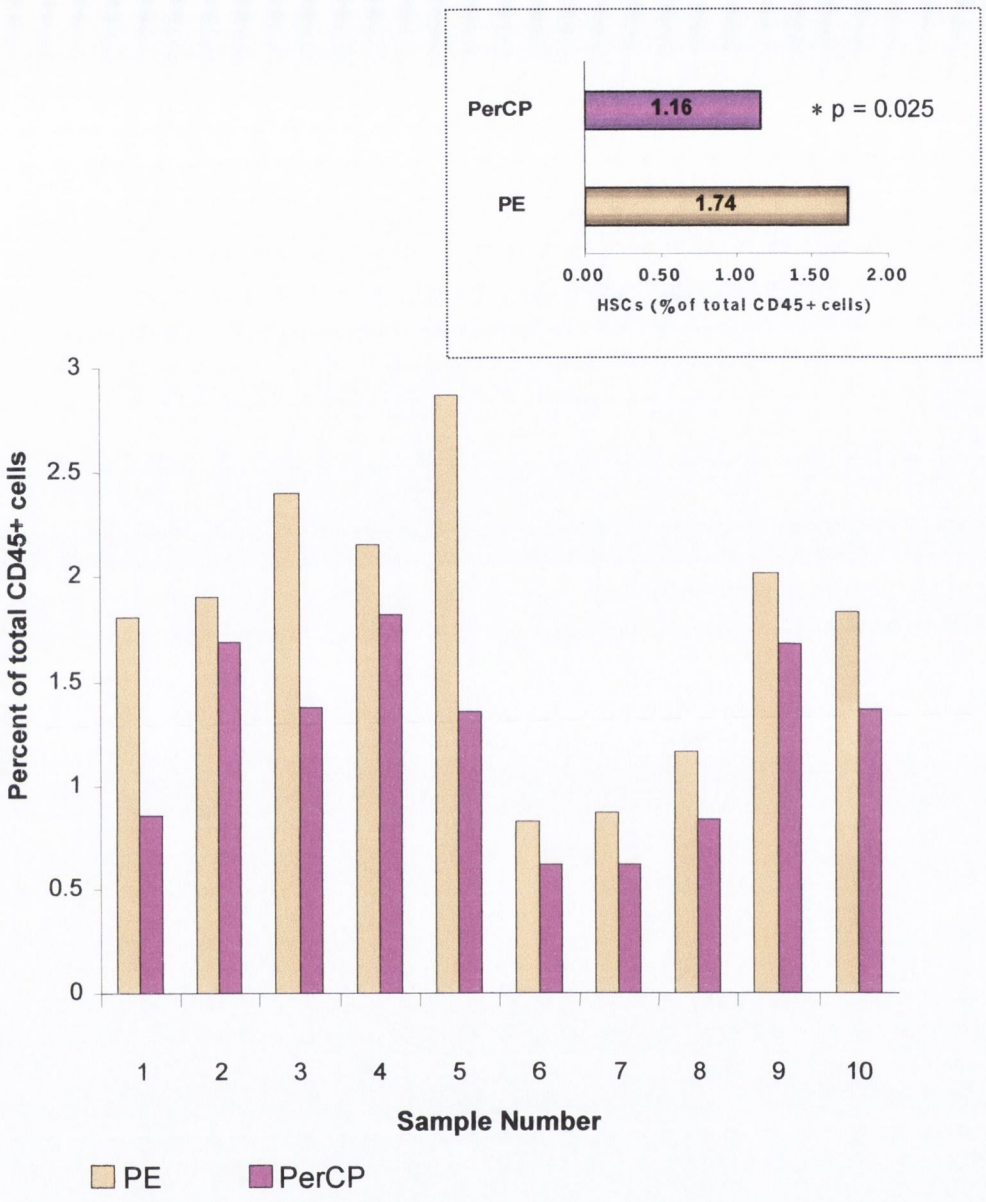


Figure 2.3.6: The levels of haematopoietic stem cells detected in hepatic mononuclear cell populations using anti-CD34 monoclonal antibodies of the same clone conjugated to different fluorochromes.

Hepatic mononuclear cells (HMNCs) were isolated from Normal (n = 6, samples 1 – 6) and tumour-bearing (n = 4, samples 7 – 10) liver tissue. HMNCs were stained with a combination of anti-CD45-FITC and anti-CD34 (clone 8G12, stem cell antigen) conjugated to either PE or PerCP. Haematopoietic stem cells (HSCs) were identified by the co-expression of CD34 and CD45 and the level of HSCs was expressed as a percentage of the total $CD45^+$ population detected in the sample. A two-tailed unpaired Student's t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant. The levels of HSCs detected with the PerCP-conjugated anti-CD34 antibody were significantly lower than the levels detected using PE-conjugated anti-CD34 antibody ($p = 0.03$). The inset shows the mean values for all samples.

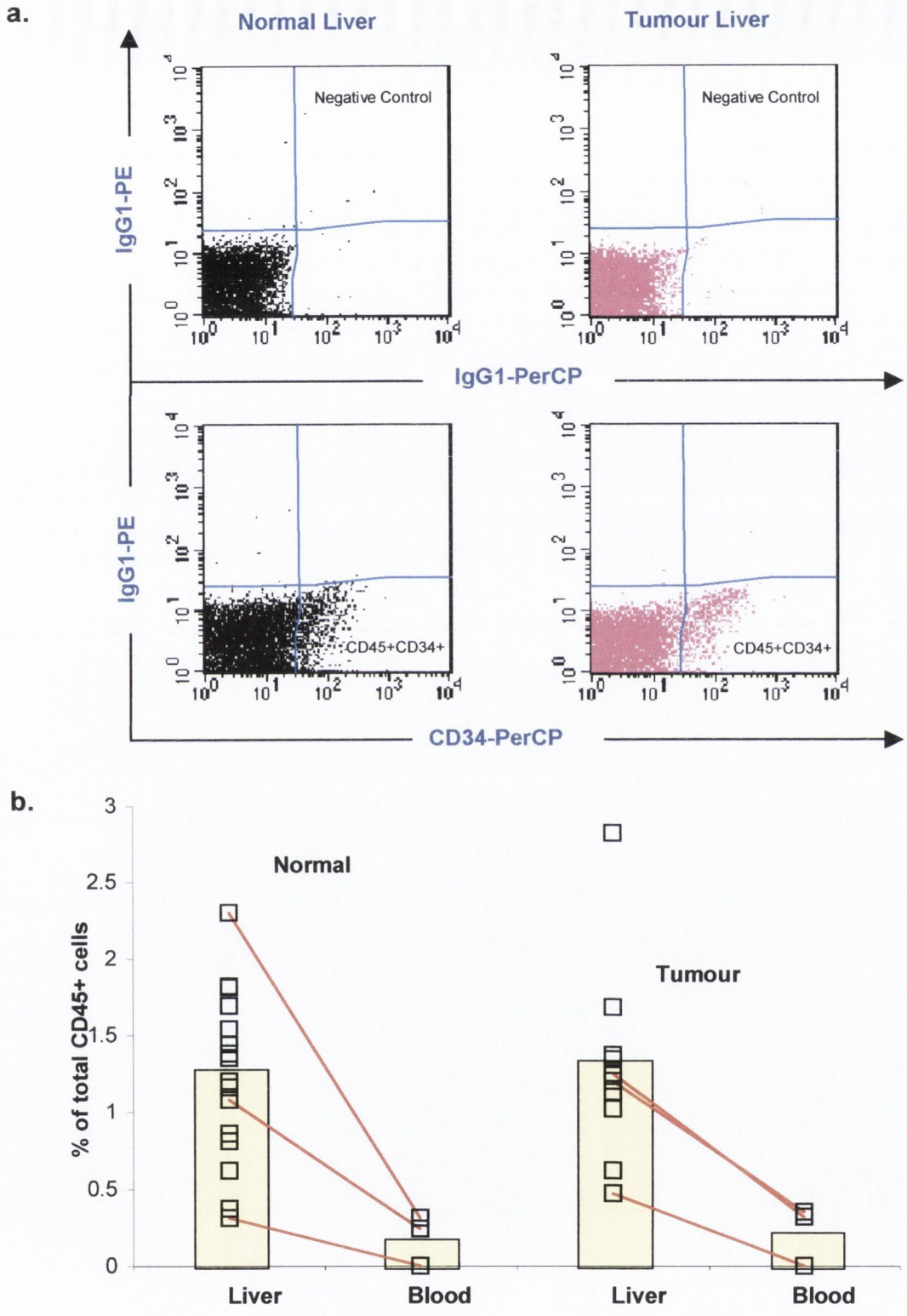


Figure 2.3.7: Levels of HSCs detected in normal and tumour-bearing liver and matched peripheral blood using three-colour flow cytometric analysis.

a) Flow cytometric dot plots showing the levels of CD34-PerCP staining in $CD45^+$ cell populations isolated from normal (black) and tumour-bearing (pink) liver. **b)** The level of HSCs detected in each sample was expressed as a percentage of the total $CD45^+$ cells. The shaded bars show the mean values and each point represents the level of HSCs detected in a single sample. In six cases, matched peripheral blood was available. The red lines show levels of HSCs detected in the matched samples.

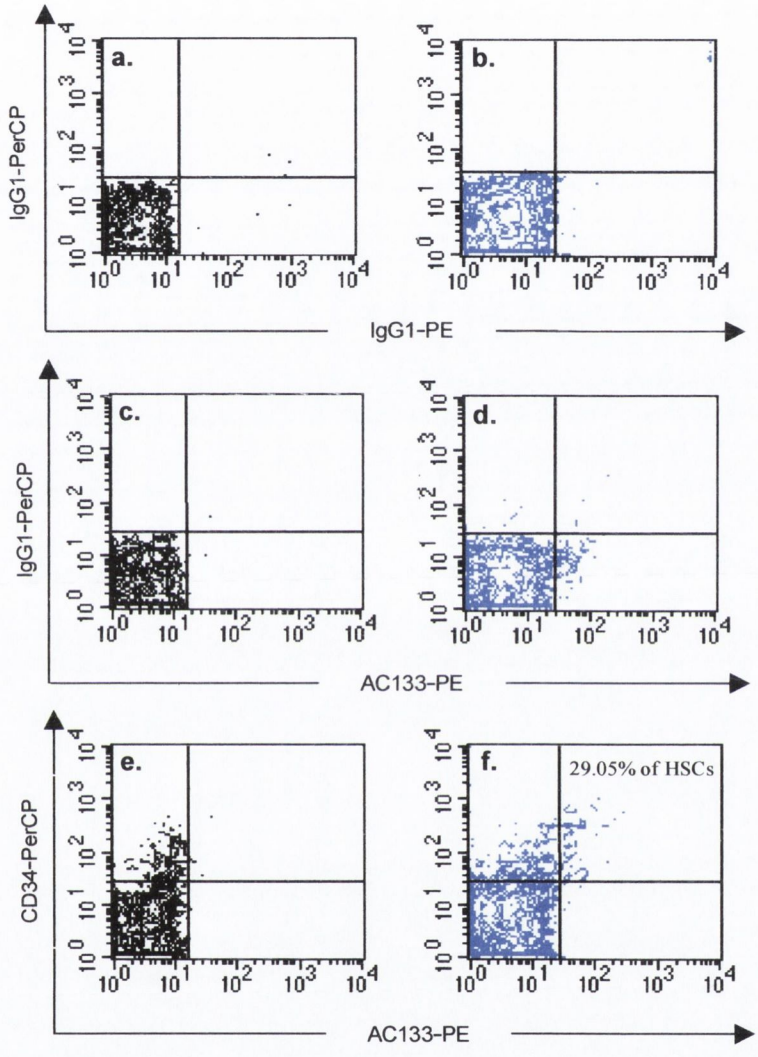


Figure 2.3.8: The novel stem cell antigen AC133 expressed on a subpopulation of bone marrow haematopoietic stem cells (HSCs) is not detectable on hepatic HSCs.

Density plots of the flow cytometric analysis of the expression of AC133 on $CD34^+CD45^+$ HSCs derived from liver tissue (n = 5) and bone marrow (n = 3). Mononuclear cells were isolated from hepatic tissue (2 x normal, 3 x tumour-bearing), using a combination of mechanical and enzymatic disruption. Bone marrow mononuclear cells were isolated using standard density centrifugation (1.077 g/ml). Single cell suspensions were stained with a combination of anti-CD45-FITC, anti-CD34-PerCP and anti-AC133-PE. CD45 positive cells were gated and CD34 and AC133 expression (above the levels detected with the appropriate isotype-matched controls) was analysed on the CD45 positive hepatic and bone marrow populations. Liver cells are shown in black and bone marrow in blue. **a & b**. Negative controls. **c & d.** AC133 staining on $CD45^+$ cells. **e**. AC133 expression was undectable on hepatic HSC ($CD45^+CD34^+$) populations but readily detected on bone marrow HSCs (**f**).

2.3.4.4: The effect of isolation enzyme mix on CD34-PerCp and AC133-PE expression.

The technique to isolate HMNCs involves the use of proteolytic enzymes (collagenase IV and DNase I), which may disrupt cell-surface markers (Abuzakouk *et al.*, 1996). The high levels of hepatic HSCs and/or the lack of AC133 expression on hepatic HSC populations may be a consequence of the isolation technique used. To assess the effect of proteolytic enzymes on the expression of CD34-PerCP and AC133-PE, bone marrow mononuclear cells (n = 2) were incubated in the liver enzyme mix under the same conditions as used for the release of hepatic mononuclear cells. Cells were stained before and after incubation with anti-CD45-FITC, anti-CD34-PerCP and anti-AC133-PE. The levels of expression of $CD34^+CD45^+$ (% of total $CD45^+$ cells) and AC133 (% of $CD34^+CD45^+$) were unaffected by incubation with the enzyme mix (table 2.3.3 and figure 2.3.9).

Table 2.3.3: Effect of Collagenase/DNase on CD34-PerCP and AC133-PE expression.

a) CD-34-PerCP.

Sample no	Antibody (-label)	Before incubation (% of $CD45^+$ cells)	After incubation (% of $CD45^+$ cells)
1	CD34-PerCp	1.23	1.21
2	CD34-PerCp	1.28	1.28

b) AC133-PE.

Sample no	Antibody (-label)	Before incubation (% of $CD34^+45^+$ cells)	After incubation (% of $CD34^+45^+$ cells)
1	AC133-PE	29.05	28.39
2	AC133-PE	25.0	26.28

2.3.4.5: Effect of age on hepatic HSC levels.

The age of the donors used for three-colour flow cytometric analysis was significantly lower than the tumour-bearing patients (see table 2.2.2). To assess if the age of the patients impacted on the levels of HSCs detected in liver, the levels of $CD34(PerCP)^+CD45^+$ hepatic stem cells (% of total $CD45^+$ cells) detected in all samples used for three-colour flow cytometric analysis (n = 26) were correlated with the age of the patients (years). There was

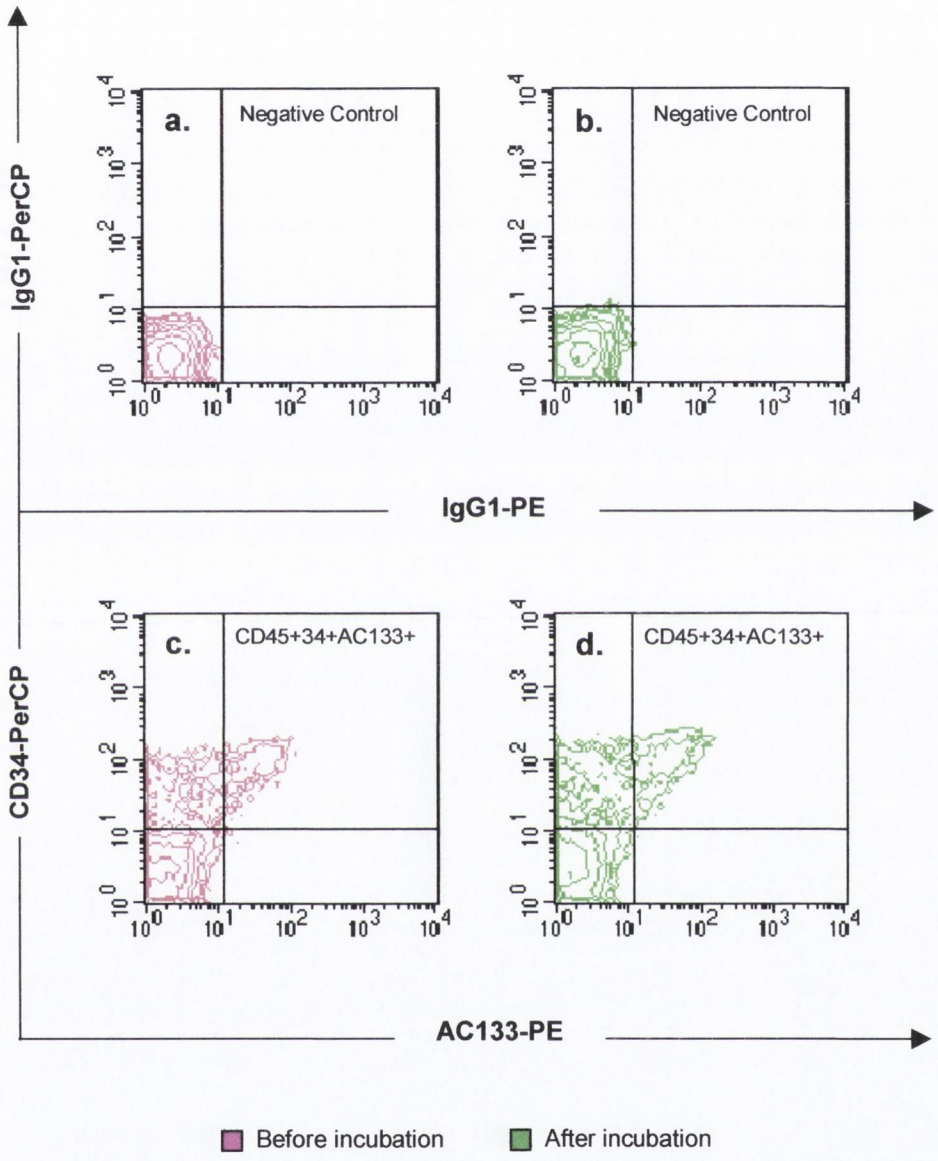


Figure 2.3.9: The effect of hepatic mononuclear cell isolation enzyme mix on CD34 and AC133 antigen expression.

To assess the effect of the proteolytic-enzyme mix used for the release of hepatic mononuclear cells (HMNCs) on the expression of CD34 and AC133, bone marrow mononuclear cells (n = 2) were incubated in the liver enzyme mix (collagenase IV and Dnase I) under the same conditions as used for the release of HMNCs from liver tissue (37°C for 20 minutes). Cells were stained before and after incubation with anti-CD45-FITC, anti-CD34-PerCP and anti-AC133-PE. **a.** and **b.** negative control stains before and after incubation in enzyme mix. **c.** and **d.** The flow-cytometric contour plots of $CD45^{+}$ bone marrow cells show that the levels of expression of CD34-PerCP and AC133-PE were unaffected by incubation in the enzyme cocktail. The MFI of CD34 and AC133 was also unaffected.

no association between age and the detectable levels of hepatic HSCs. When only normal samples were used, to negate the influence that the higher age and the detected HSC levels associated with tumour-bearing specimens would have on producing a trend, no significant correlation was observed between age and HSC levels (figure 2.3.10).

2.3.4.6: Effect of cell yield and viability on hepatic HSC levels.

There was a wide range in cell yield, viability and detected levels of HSCs. To assess if cell yield and/or viability impacted on the levels of HSCs, the levels of $CD34^{+}CD45^{+}$ hepatic stem cells (% of total $CD45^{+}$ cells) samples used for three-colour-flow-cytometric analysis (n = 26) were plotted against HMNC yield (x 10^{6}/g tissue wet weight) or HMNC viability (% of total HMNCs). Spearman rank was used for correlation analysis. There was no association between HMNC yield or percent viability of HMNCs and the levels of hepatic HSCs. Figure 2.3.11 a and b shows the correlation data for cell yield and percentage viability respectively.

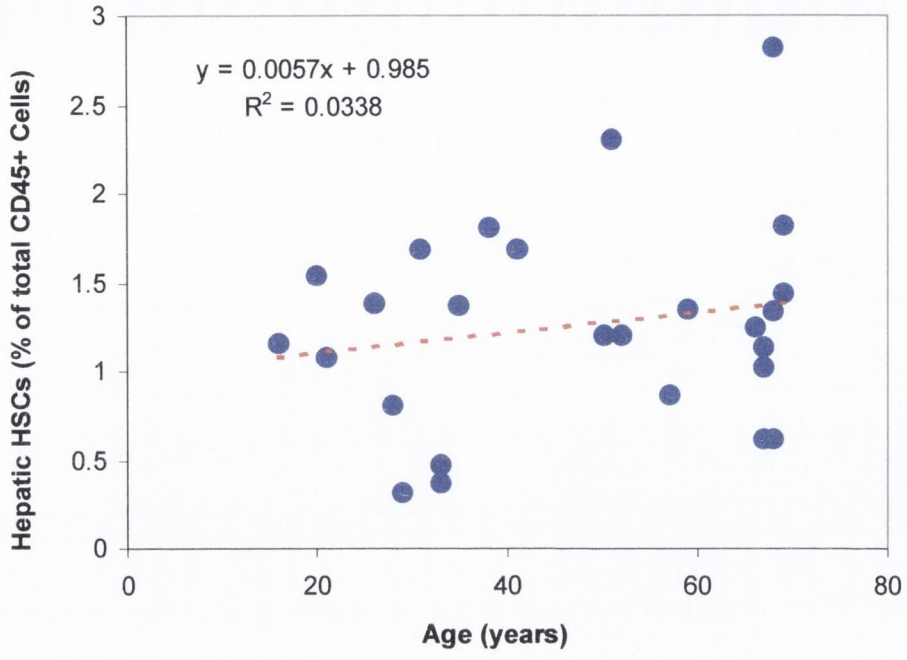
2.3.4.7: Co-expression of differentiation, activation and lineage markers on hepatic HSCs.

Three-colour flow cytometric analysis was used to characterise the co-expression of CD38 (the early differentiation marker) and CD45RA (activation marker) on hepatic HSCs isolated from eight normal and eight tumour-bearing liver specimens. Liver derived HSCs were also characterised with respect to the co-expression of a number of lineage specific markers: myeloid (CD33), NK and NK T cell (CD56), B cell (CD19) and T cell (CD7).

a) Normal hepatic HSCs.

For normal liver there was a wide variation in the proportions of HSCs in the individual samples which co-expressed differentiation and activation markers. Typical flow-cytometric profiles and the levels detected in individual samples can be seen in figure 2.3.12. There was also wide variation in the proportion of normal hepatic HSCs co-expressing lineage-specific markers. Approximately one third of HSCs co-expressed the natural killer cell marker (CD56) and the T cell marker (CD7), while a smaller proportion (13.23%) were positive for CD19 (B

a.



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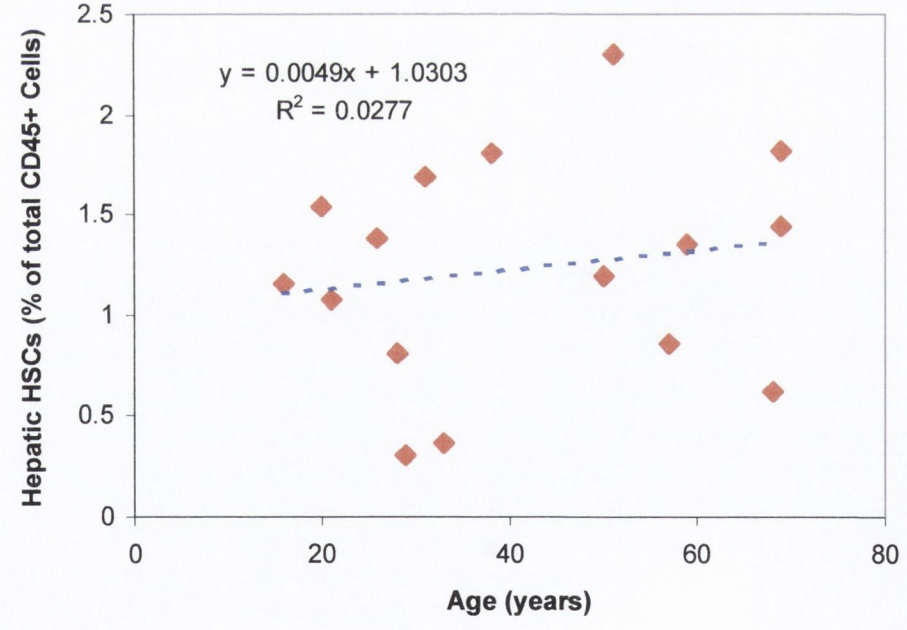
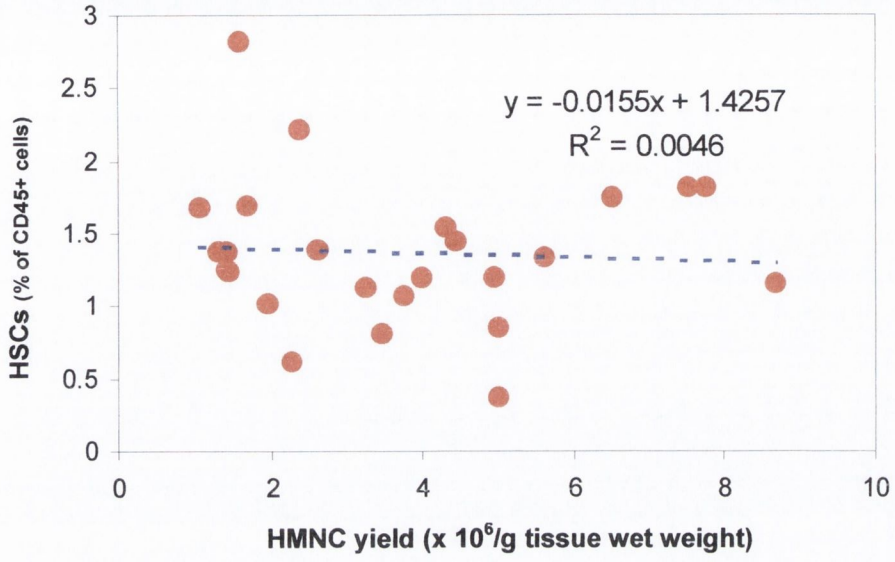


Figure 2.3.10: Association of age with hepatic haematopoietic stem cell (HSC) levels.

The age of the donors (mean 42 years, range 16 – 69, n = 16) used for three-colour flow cytometric analysis was significantly lower than the tumour-bearing (mean 54.5 years, range 29 – 67, $p < 0.02$, n = 10). To assess if the age of the donors/patients impacted on the levels of HSCs detected in liver, the levels of $CD34^+CD45^+$ hepatic stem cells (% of total $CD45^+$ cells) detected in all samples used for three-colour flow-cytometric analysis (n = 26) were plotted against the age of the donors/patients (years). Spearman rank was used for correlation analysis. There was no association between age and the levels of hepatic HSCs (**a**). This remained true when only normal samples were used (n = 16), negating the influence that the higher age and the detected HSC levels associated with tumour-bearing specimens would have on producing a trend (**b**).

a.



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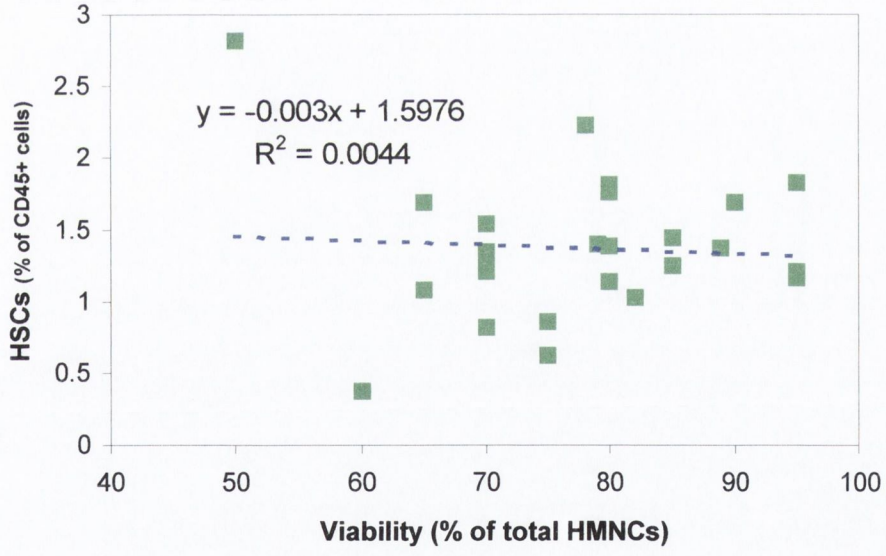


Figure 2.3.11: Association of hepatic mononuclear cell yield and viability with hepatic haematopoietic stem cell (HSC) levels.

To assess if cell yield impacted on the levels of HSCs, the levels of $CD34^{+}CD45^{+}$ hepatic stem cells (% of total $CD45^{+}$ cells) for all samples used for three-colour flow-cytometric analysis (n = 26) were plotted against HMNC yield (x 10^{6}/g tissue wet weight). To assess if HMNC viability impacted on the levels of HSCs, the levels of hepatic stem cells were plotted against HMNC viability (% of total HMNCs). Spearman rank was used for correlation analysis. There was no association between HSC levels and yield **(a)** or percent viability of HMNCs **(b)**.

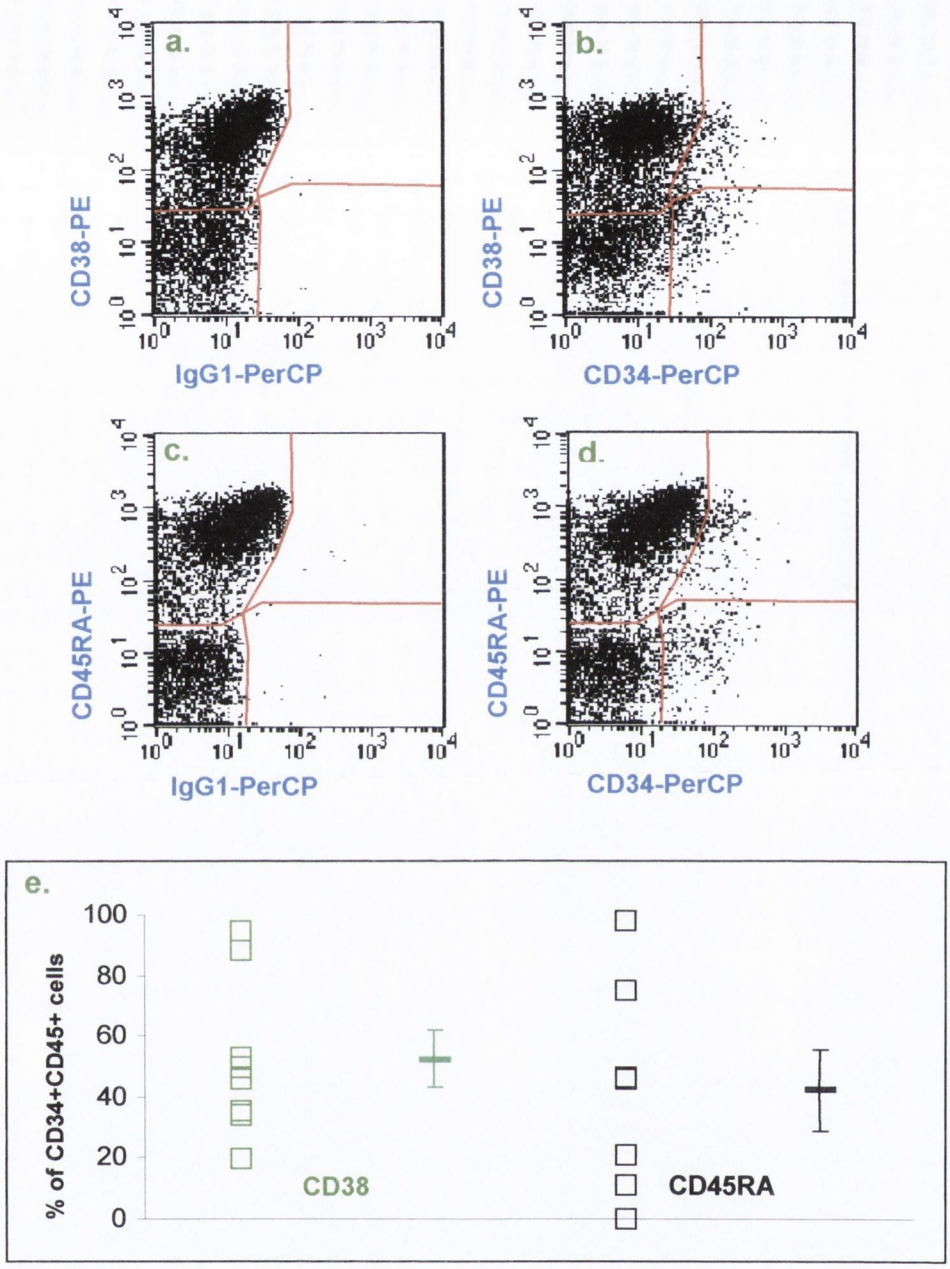


Figure 2.3.12: Co-expression of differentiation (CD38) and activation (CD45RA) markers on hepatic haematopoietic stem cells (HSCs) isolated from normal liver tissue.

Single-cell suspensions, prepared from normal hepatic tissue (n = 8), were stained with a combination of anti-CD45-FITC, PerCP-labelled anti-CD34 mAbs and PE-conjugated anti-CD38 or anti-CD45RA. Hepatic derived cell suspensions were also stained with appropriate isotype-matched control antibodies and each of the markers in conjunction with CD45, to correct for background staining and to ensure the analysis quadrants were set in the correct positions. $CD45^+$ cells were gated and the levels of PerCP and PE staining within the $CD45^+$ population were analysed. The dot plots shown are for $CD45^+$ cells only. **a.** Cells stained for CD38. **b.** Cells stained for CD38 and CD34, the $CD34^+CD38^+$ cells are seen the upper right quadrant, while $CD34^+CD38^-$ cells fall in the lower right quadrant. **c.** Cells stained for CD45RA. **d.** Cells stained for CD45RA and CD34, the $CD34^+CD45RA^+$ cells are seen the upper right quadrant, while $CD34^+CD45RA^-$ cells fall in the lower right quadrant. **e.** The level of co-expression of CD38/CD45RA on hepatic HSCs was expressed as a percentage of the total $CD34^+CD45^+$ cells, the open squares depict the values for individual samples, the error bars show the mean ± SEM.

cell marker). However, the myeloid marker (CD33) was detected on less than 5% of HSCs suggesting a bias towards lymphopoiesis in the normal liver. The levels detected in individual samples along with representative flow cytometry dot plots, can be seen in figure 2.3.13.

b) HSCs from tumour-infiltrated liver.

As for normal liver, there was a wide variation in the proportions of HSCs in the individual samples of tumour-bearing liver which co-expressed differentiation and activation markers. The levels detected in individual samples, along with representative flow cytometry dot plots, are depicted in figure 2.3.14. There was also wide variation in the proportion of hepatic HSCs co-expressing lineage-specific markers. Approximately one quarter co-expressed the natural killer cell marker CD56, the T cell marker CD7 was expressed on more than half, while a smaller proportion were positive the myeloid marker CD33, (approximately one fifth of the HSC populations isolated from tumour-bearing liver). However, the B cell marker was detected in only one of four samples tested. Typical flow-cytometric profiles and the levels detected in individual samples can be seen in figure 2.3.15.

2.3.4.8: Comparison of the cell-surface marker co-expression between normal and tumour-bearing liver derived HSC populations.

Significant increases were observed in the relative proportions of hepatic HSCs co-expressing the myeloid antigen (CD33, 20.53% of $CD34^+CD45^+$ HSCs vs 3.26%, $p = 0.001$) and the T-cell marker (CD7, 58.13% of $CD34^+CD45^+$ HSCs vs 33.39%, $p = 0.02$) in tumour-bearing liver, when compared to normal liver. HSCs with B-cell progenitor phenotype were significantly decreased in tumour-bearing liver (0.06% vs 13.23%, $p = 0.02$). Although there was a decrease in the co-expression of the natural killer cell marker (CD56, 26.94%) in tumour-bearing HSC populations compared with normal (37.17%), this decrease did not reach statistical significance. Despite these differences, the activation status of haematopoiesis, as measured by the co-expression of the differentiation and activation markers CD38 and CD45RA on hepatic HSC populations did not differ significantly between normal and tumour-bearing liver (figure 2.3.16).

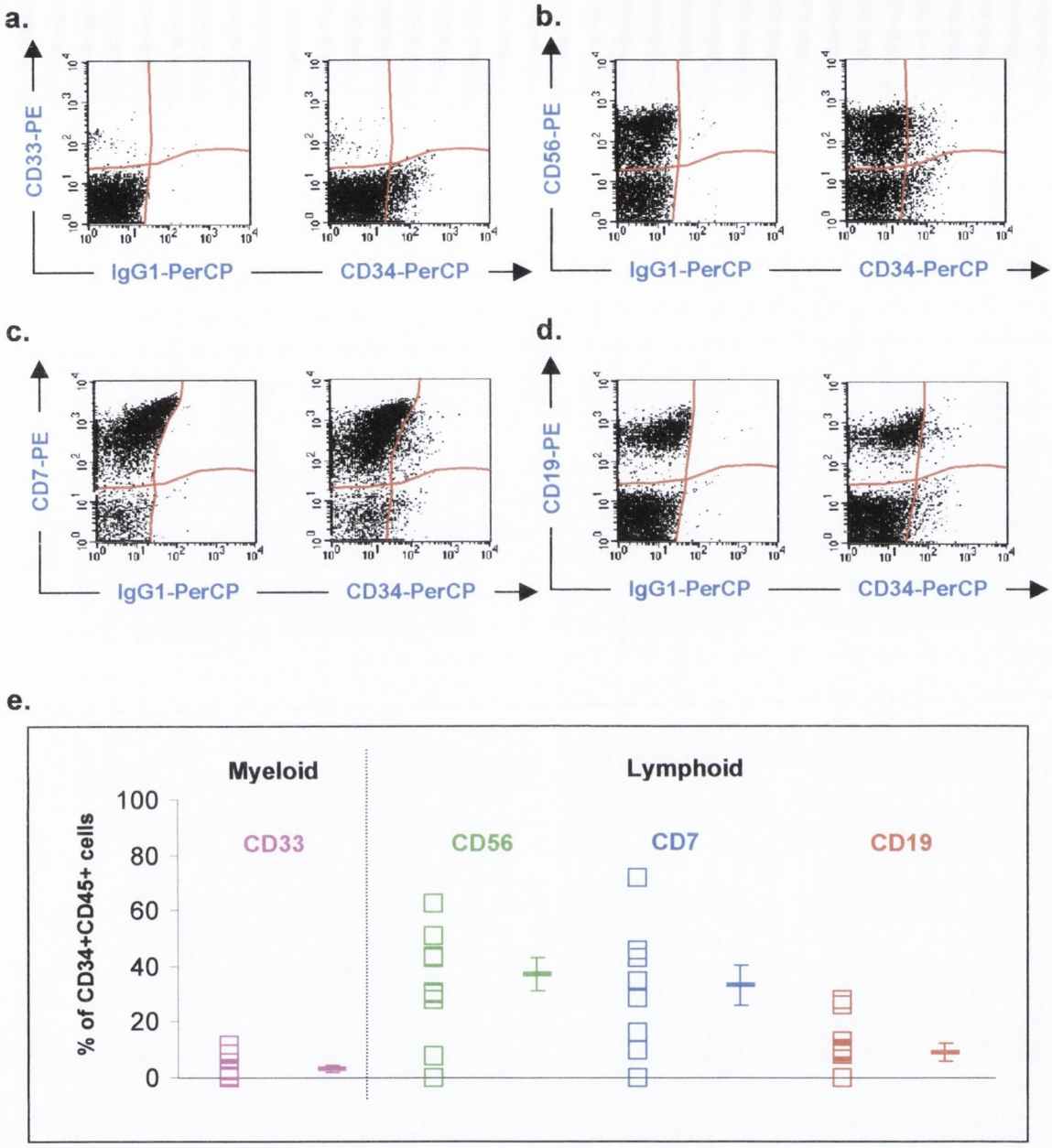


Figure 2.3.13: Co-expression of lineage-specific markers on hepatic haematopoietic stem cells (HSCs) isolated from normal liver tissue.

Single-cell suspensions, prepared from normal hepatic tissue (n = 8), were stained with a combination of anti-CD45-FITC, PerCP-labelled anti-CD34 mAbs and PE-conjugated anti-CD38 or anti-CD45RA. Hepatic derived cell suspensions were also stained with appropriate isotype-matched control antibodies and each of the markers in conjunction with CD45, to correct for background staining and to ensure the analysis quadrants were set in the correct positions. $CD45^+$ cells were gated and the levels of PerCP and PE staining with in the $CD45^+$ population were analysed. The dot plots shown are for $CD45^+$ cells only. **a.** CD45+ cells stained for CD33 (control) and, CD33 plus CD34. The CD34+CD33+ cells are seen the upper right quadrant (second plot), while $CD34^+CD33^-$ cells fall in the lower right quadrant. **b.** Cells stained for CD56 and for CD56 plus CD34. **c.** Cells stained for CD7 and, CD7 plus CD34. **d.** Cells stained for CD19 and for CD19 plus CD34. **e.** The level of co-expression of CD33/56/7/19 on hepatic HSCs was expressed as a percentage of the total $CD34^+CD45^+$ population, the open squares depict the values for individual samples, the error bars show the mean ± SEM.

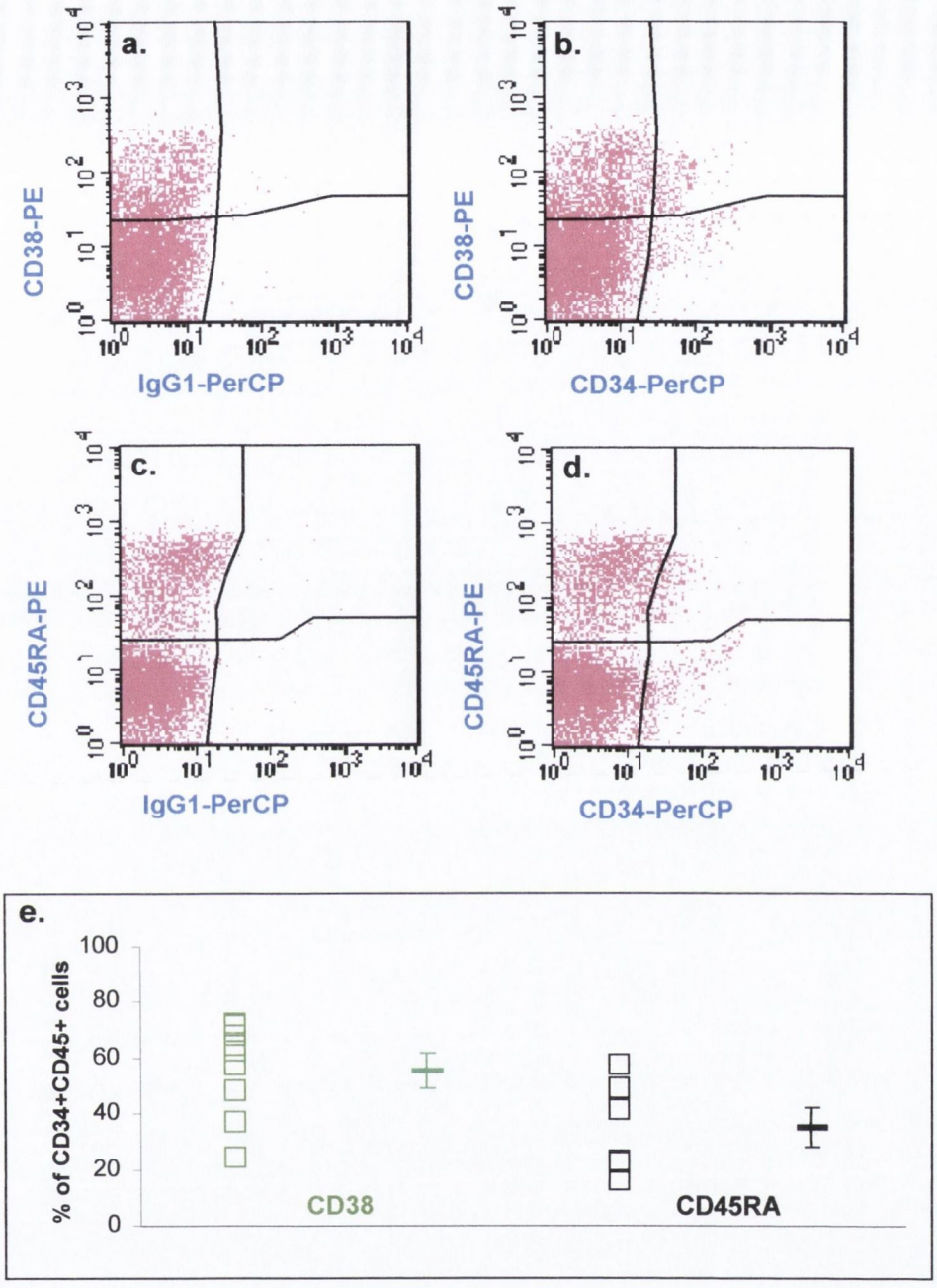


Figure 2.3.14: Co-expression of differentiation (CD38) and activation (CD45RA) on hepatic haematopoietic stem cells (HSCs) isolated from tumour-bearing liver tissue.

Single-cell suspensions, prepared from tumour-bearing hepatic tissue (n = 8), were stained with a combination of anti-CD45-FITC, PerCP-labelled anti-CD34 mAbs and PE-conjugated anti-CD38 or anti-CD45RA. Hepatic derived cell suspensions were also stained with appropriate isotype-matched control antibodies and each of the markers in conjunction with CD45, to correct for background staining and to ensure the analysis quadrants were set in the correct positions. $CD45^+$ cells were gated and the levels of PerCP and PE staining with in the $CD45^+$ population were analysed. The dot plots shown are for $CD45^+$ cells only. **a.** Cells stained for CD38. **b.** Cells stained for CD38 and CD34, the $CD34^+CD38^+$ cells are seen the upper right quadrant, while CD34+CD38- cells fall in the lower right quadrant. **c.** Cells stained for CD45RA. **d.** Cells stained for CD45RA and CD34, the $CD34^+CD45RA^+$ cells are seen the upper right quadrant, while $CD34^+CD45RA^-$ cells fall in the lower right quadrant. **e.** The level of co-expression of CD38/CD45RA on hepatic HSCs was expressed as a percentage of the total $CD34^+CD45^+$ cells, the open squares depict the values for individual samples, the error bars show the mean ± SEM.

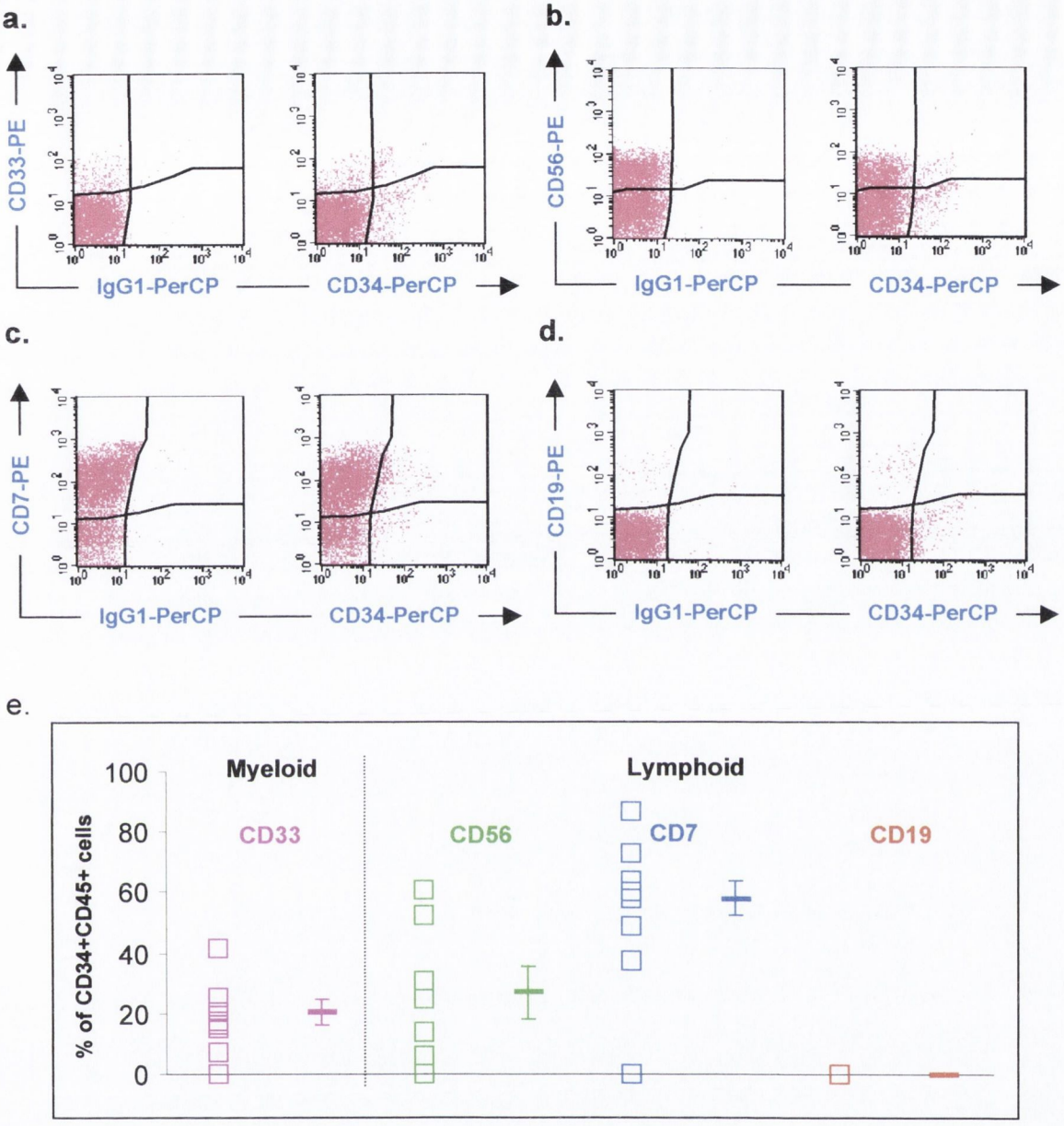


Figure 2.3.15: Co-expression of lineage-specific markers on hepatic haematopoietic stem cells (HSCs) isolated from tumour-bearing liver tissue.

Single-cell suspensions, prepared from tumour-bearing hepatic tissue (n = 8), were stained with a combination of anti-CD45-FITC, PerCP-labelled anti-CD34 mAbs and PE-conjugated anti-CD38 or anti-CD45RA. Hepatic derived cell suspensions were also stained with appropriate isotype-matched control antibodies and each of the markers in conjunction with CD45, to correct for background staining and to ensure the analysis quadrants were set in the correct positions. $CD45^+$ cells were gated and the levels of PerCP and PE staining with in the $CD45^+$ population were analysed. The dot plots shown are for $CD45^+$ cells only. **a.** $CD45^+$ cells stained for CD33 (control) and, CD33 plus CD34. The $CD34^+CD33^+$ cells are seen the upper right quadrant (second plot), while $CD34^+CD33^-$ cells fall in the lower right quadrant. **b.** Cells stained for CD56 and for CD56 plus CD34. **c.** Cells stained for CD7 and, CD7 plus CD34. **d.** Cells stained for CD19 and for CD19 plus CD34. **e.** The level of co-expression of CD33/56/7/19 on hepatic HSCs was expressed as a percentage of the total $CD34^+CD45^+$ population, the open squares depict the values for individual samples, the error bars show the mean ± SEM.

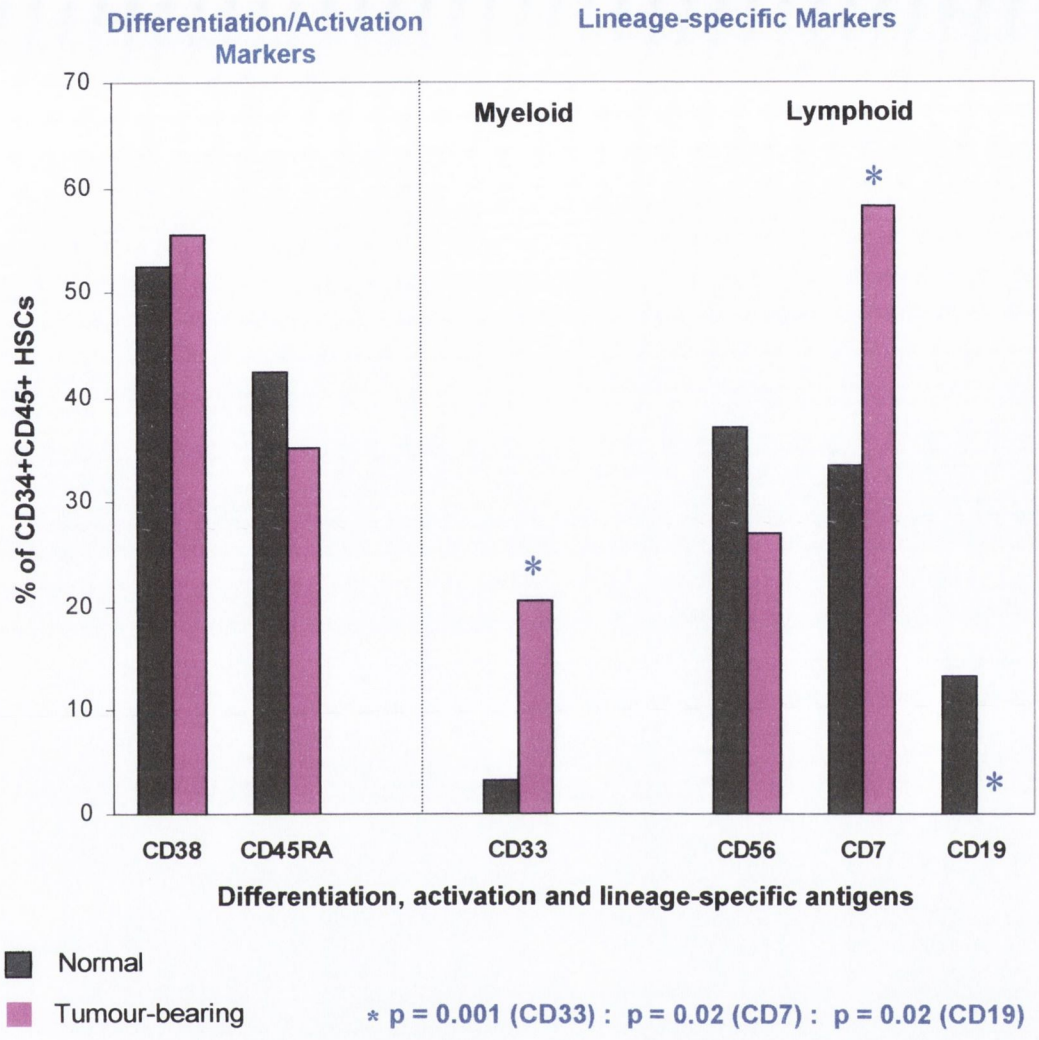


Figure 2.3.16: Comparison of the co-expression of differentiation, activation and lineage specific markers on hepatic haematopoietic stem cells ($CD34^+CD45^+$) isolated from normal and tumour-bearing liver.

Three-colour flow-cytometric analysis was used to characterise the co-expression of CD38 (the early differentiation marker) and CD45RA (activation marker) on hepatic haematopoietic stem cells (HSCs) isolated from eight normal and eight tumour-bearing liver specimens. Liver derived HSCs were also characterised with respect to the co-expression of a number of lineage specific markers: myeloid (CD33), natural killer cell (CD56), B cell (CD19) and T cell (CD7). The columns show the mean value obtained for all samples. A Students t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant. Significant increases were observed in the relative proportions of hepatic HSCs co-expressing the myeloid antigen and the T-cell marker in tumour-bearing liver, when compared to normal liver. HSCs with B-cell progenitor phenotype were significantly decreased in tumour-bearing liver. Although there was a decrease in the co-expression of the natural killer cell marker (CD56, 26.94%) in tumour-bearing HSC populations compared with normal (37.17%), this decrease did not reach statistical significance. Despite these differences, the activation status of haematopoiesis, as measured by the co-expression of the differentiation and activation markers CD38 and CD45RA, did not differ significantly between normal and tumour-bearing liver.

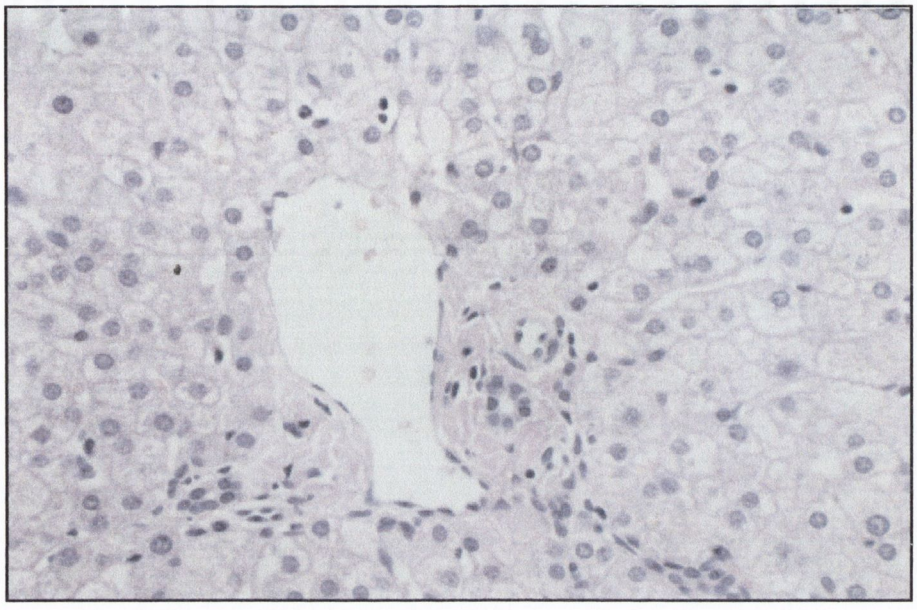
2.3.4.9: Granular CD45+ cell populations.

Microscopic examination of tumour-bearing liver (taken 5-10 cm from tumour margin), which on gross examination appeared to be histologically normal, revealed, in the majority of cases, mild portal tract inflammation and occasional to moderate numbers of granular polymorphonuclear (PMN) and lymphocytic leukocytes in the sinusoids. Inflammatory cells were observed in tumour-bearing tissue at levels above those seen in normal liver (figure 2.3.17). The presence of granular cell populations in tumour-bearing and normal liver was further examined using flow cytometry. The FSC:SSC morphology plots of $CD45^+$ hepatic mononuclear cells (HMNCs) isolated from distant tumour liver (5 – 10 cm from tumour margin) showed an increase in the proportion of large granular cells (figure 2.3.18.c and d, R3). The proportion of granular cells of haematopoietic origin (co-expressing CD45) in tumour-bearing liver was significantly higher than levels observed in normal liver ($p = 0.02$, figure 2.3.18.e), suggesting possible involvement of these granular populations in the tumour response. In view of the observed increase in HSCs co-expressing the myeloid antigen CD33 (figure 2.3.16), the levels of mature granular cell populations of haematopoietic origin ($CD45^+$, high FSC:SSC) were correlated with relative proportions of myeloid progenitors in the 15 liver biopsies, from normal and tumour-bearing organs, that had been analysed for stem cell myeloid progenitor populations. Immature $CD34^+CD45^+CD33^+$ (myeloid) HSCs showed a strong positive correlation with mature granular populations ($r = 0.784$, $p = 0.0005$, figure 2.3.19), suggesting a possible contribution of local differentiation to the increased mature granular cell populations.

2.3.5: Anatomic location of hepatic HSCs.

Confocal fluorescence microscopy was used to determine the location of HSC ($CD34^+CD45^+$) populations within normal liver tissue. Hepatic HSCs were localised to portal tract areas. Not all portal tracts stained positively for HSCs. On average clusters of two to three HSCs in close proximity to each other were seen per two portal tracts (figure 2.3.20). $CD34^+CD45^+$ cells were never observed in the parenchyma.

a.



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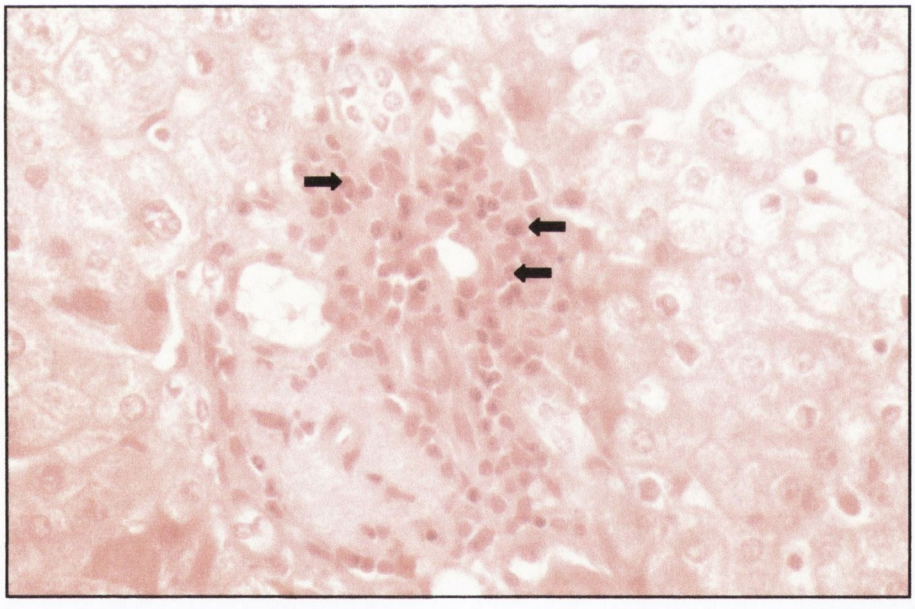
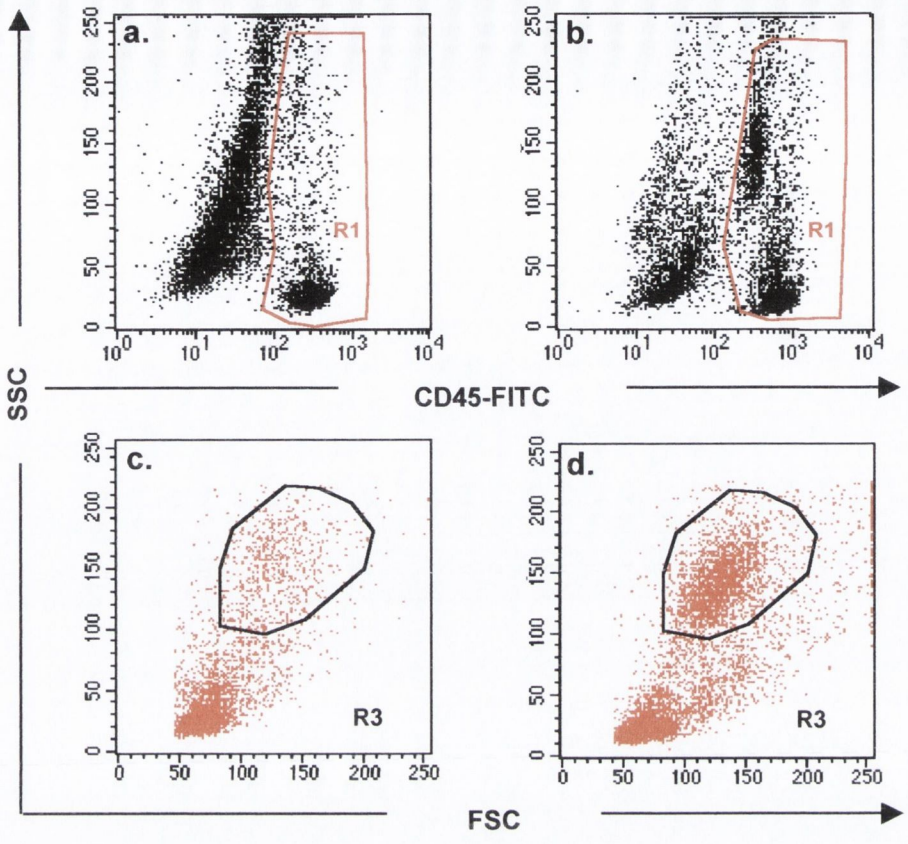


Figure 2.3.17: Portal tract and sinusoidal inflammatory cell populations are seen in distal tumor-bearing liver tissue.

Five micrometre sections of formalin-fixed paraffin-embedded tissue were stained with haematoxylin and eosin (H&E) and examined microscopically for evidence of inflammatory cell infiltrates. Magnification by 100 (objective). **a).** Normal liver section. **b).** An example is of a tumour-bearing hepatic tissue section in which portal tract inflammation was observed in most tracts. Granular polymorphonuclear and lymphocytic cells were also observed in sinusoids. Liver tissue was taken 5 – 10 cm away from the tumour margin. Arrows indicate some of the granular polymorphonuclear cells (PMNs).



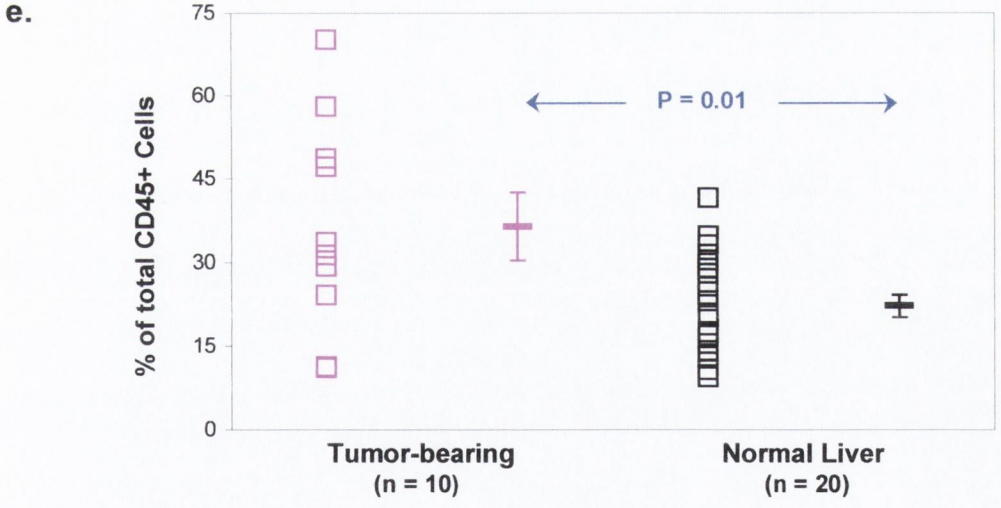


Figure 2.3.18: The proportion of cells of haematopoietic origin ($CD45^+$) with granular properties is significantly increased in tumour-bearing liver.

a) and **b**) Cells of hematopoietic origin ($CD45^+$) were gated (R1) using side scatter (SSC, granularity) and FITC fluorescence (CD45) parameters in single cell suspensions isolated from normal and tumour-bearing liver respectively. **c**) and **d**) Forward scatter (FSC, size), SSC morphology plots for $CD45^+$ cells only (R1), from normal and tumour-bearing liver respectively. Granular cells are identified by their high SSC property (R3). A higher proportion of the $CD45^+$ cells fall within R3 in cells isolated from tumour-bearing liver (**d**). The proportion of mature cells of haematopoietic origin ($CD45^+CD34^-$) isolated from liver tissue with granular properties (high SSC) was calculated as a percentage of total $CD45^+$ cells. Each symbol represents an individual sample. Error bars represent ± standard error of the mean (SEM). A two-tailed Students t-test was used to determine the significance of the difference between the groups, a p value of < 0.05 was taken as significant. The value for tumour-bearing tissue was significantly higher than that of normal tissue (**e**).

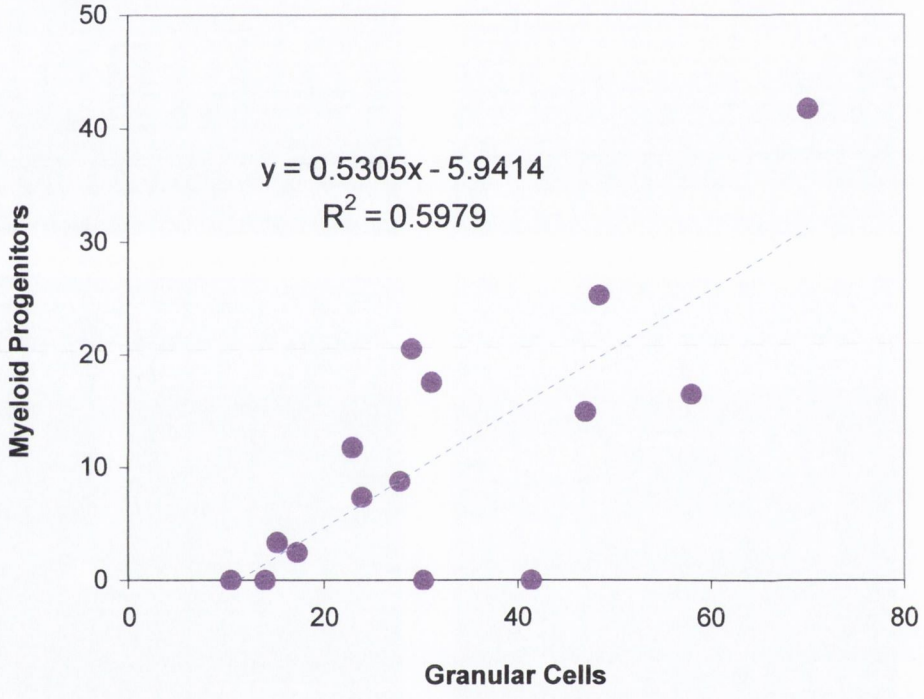


Figure 2.3.19: Correlation of mature granular cell levels observed in normal and tumour-bearing liver with myeloid progenitor levels.

Levels of mature granular cells of haematopoietic origin ($CD45^+CD34^-$, % of total CD45+ cells with high SSC) were correlated with relative proportions of myeloid progenitors ($CD45^+CD34^+CD33^+$, % of total $CD45^+CD34^+$ cells) for the 15 liver biopsies, from normal and tumour-bearing organs, that had been analysed for stem cell myeloid progenitor populations.

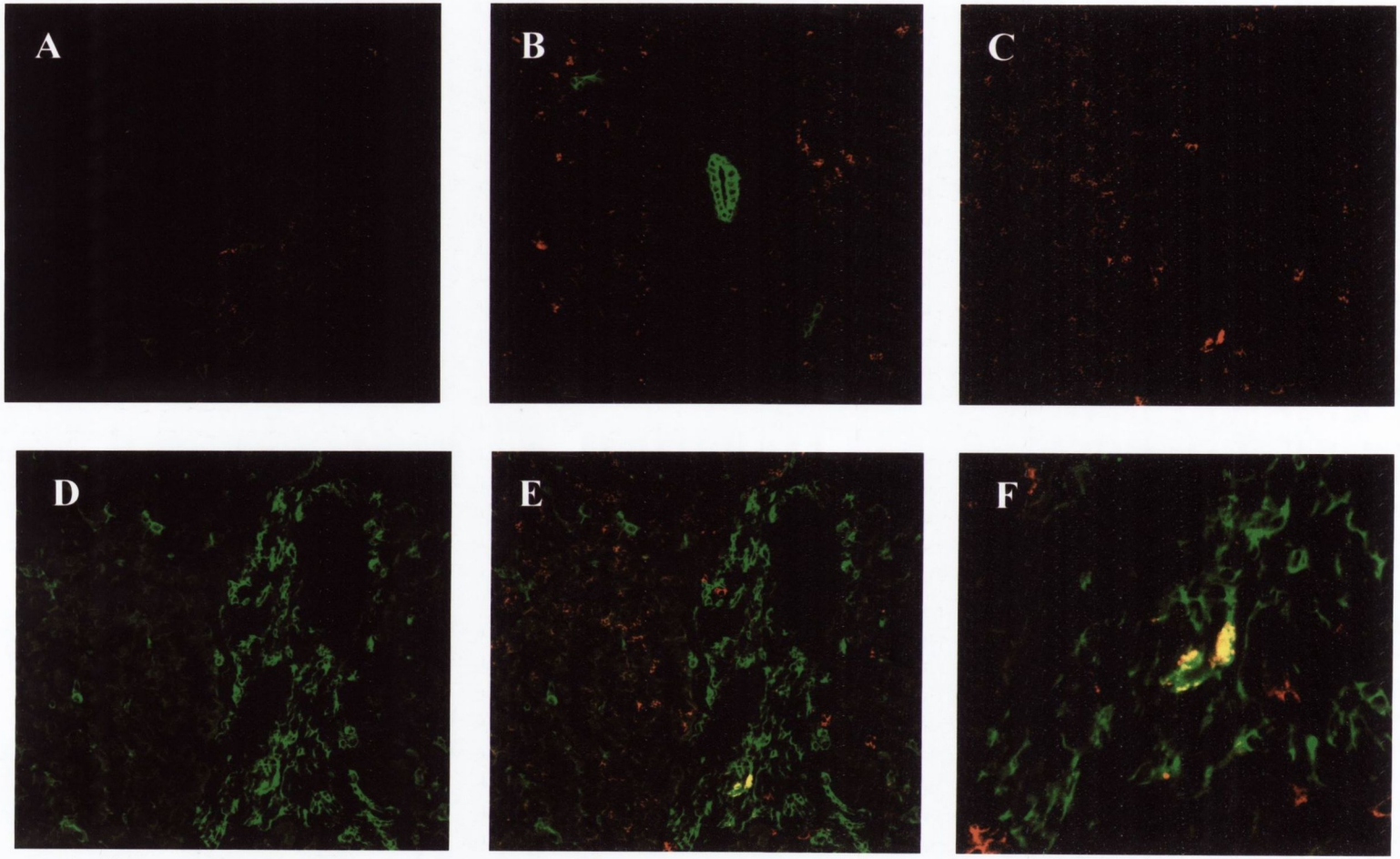


Figure 2.3.20: Localisation of hepatic HSC populations in liver tissue.
A. Negative control. **B.** Positive control. **C.** CD34 (Texas Red). **D.** CD45 (FITC). **E.** CD34 and CD45 showing double positive cells (yellow). **F.** Zoom of CD34+CD45+ HSCs (Original magnification x 250).

2.4: Discussion

The concept of extrathymic T-lymphocyte development predicts that T cell progenitors and all factors required for their maturation are present at extrathymic sites (Laky *et al.*, 2000). Strong evidence suggests the AHL is a T-lymphopoietic organ, in particular the expression of RAG-1, RAG-2 and pre-TCR-α (Collins *et al.*, 1996). The AHL contains functional HSCs which give rise to multiple erythroid, monocytic and granulocytic colonies *in vitro* (Crosbie *et al.*, 1999). However, the presence of lymphoid progenitor cells within this population has yet to be established. In the present study we set out to establish the presence of lymphoid committed progenitor cells in the AHL by characterising the cell surface phenotype of isolated hepatic mononuclear cell populations.

HSCs can be measured *in vitro* by their ability to form colonies in various tissue culture systems. However, no culture system supports the growth of all classes of HSCs. In our system, flow cytometry allows detection of simultaneous expression of up to three cell surface markers in conjunction with morphological properties such as size and granularity. Thus, it provides an ideal tool for phenotypic characterisation of the entire $CD34^+$ population, which includes all classes of HSCs.

Difficulties can arise with respect to non-specific uptake of antibodies, through high Fc receptor expression, when staining cells isolated from liver tissue (Barcena *et al.*, 1993). High background fluorescence levels can make interpretation of results difficult, particularly when analysing rare cell populations such as HSCs. A number of steps were taken to minimise non-specific signals and to ensure that the double- or triple-positive cells were true populations. Prior incubation with non-specific mouse antibody has been shown to block non-specific uptake of primary antibodies (Barcena *et al.*, 1993; Fritsch *et al.*, 1996). In this study we used heat-inactivated normal mouse serum as a source of irrelevant IgG to block Fc receptors. In addition, we used bone marrow as an assay control - as levels of HSCs in bone marrow are well documented, this would allow us to compare levels detected using our technique to known levels (Civin and Gore, 1993; Lansdorp *et al.*, 1992; Titley *et al.*, 1995). We detected

HSCs at a mean level of 2.21% of total leukocytes in bone marrow, well within the normal range (1% - 5%), indicating that our flow cytometry technique reflects true levels.

A previous study carried out in our laboratory had established the presence of $CD34^+$ cells in normal liver tissue. The presence of HSCs within this $CD34^+$ hepatic population was demonstrated by the ability to form erythroid, monocytic and granulocytic colonies in methyl-cellulose culture assays (Crosbie *et al.*, 1999). While all haematopoietic stem and progenitor cells express CD34 (Krause *et al.*, 1996), not all $CD34^+$ cells are HSCs, as CD34 is expressed on cells that are not of haematopoietic origin (Fina *et al.*, 1990). Two-colour staining for CD34 and CD45 was used initially to establish the presence of HSCs in hepatic samples. The anti-CD45 antibody used in this study recognises both the CD45RA and CD45RO isoforms of the CD45 molecule. CD45RA is expressed on all classes of haematopoietic stem and progenitor cells with the exception of rare, very early non-cycling stem cells and erythroid precursors. Red cell progenitors do not express CD45RA but do express the CD45RO molecule. (Civin *et al.*, 1996; Civin and Gore, 1993; Fritsch *et al.*, 1996). Using a combination of CD34 and CD45 we can therefore detect all cycling haematopoietic stem and progenitor cells.

Another problem associated with analysis of hepatic mononuclear cell populations is a high level of auto-fluorescence, arising from tissue debris in the sample preparation. This problem was overcome by gating first on the $CD45^+$ population, which allowed us to exclude debris and cells that were not of haematopoietic origin. Initial gating on $CD45^+$ cells eliminates virtually all background staining and small populations are clear. When dealing with rare cell populations, it is particularly important that compensation controls are correctly set so as to avoid false positives. We included additional controls, consisting of each of the specific antibodies on their own, to ensure that background fluorescence from any of the individual markers did not contribute to the double- or triple-positive populations.

Murine hepatic HSCs and bone marrow haematopoietic progenitor cells are contained within a low-medium FSC:SSC gate (Watanabe *et al.*, 1996). The present study confirms that human hepatic HSCs have similar morphological properties to their murine counterparts.

Using two-colour flow cytometry we detected levels of hepatic HSCs comparable to levels detected in bone marrow. Studies in mice have shown levels of adult hepatic HSCs to be approximately 50% those of bone marrow (Taniguchi *et al.*, 1996). The AHL is therefore a richer source of HSCs than murine liver. There was considerable variation in the levels of HSCs detected in the individual samples (range 0.82% - 2.87%), this may reflect variation in individual status with respect to infection, diet and genetic background.

Two-colour analysis, without initial gating on the $CD45^+$ population, allowed us to determine what proportion of the hepatic $CD34^+$ were of haematopoietic origin. Less than half (approximately 40%) the $CD34^+$ population co-expressed CD45. As our interest lay in haematopoietic progenitor cells, the $CD34^+CD45^-$ population was not analysed further. It is, however, interesting to speculate as to what these cells may be. It is unlikely that all non-haematopoietic $CD34^+$ cells are mature endothelial cells, as the gating technique used would have excluded the majority of endothelial cells on the basis of size. It is possible that this population consists of non-cycling HSCs that have yet to up-regulate CD45. In rodents the presence of hepatic oval, bipotential $CD34^+$ cells giving rise to hepatocytes and biliary epithelial cells, is well established (Coleman and Grisham, 1998; Dabeva *et al.*, 1993). While there is no direct proof of a similar cell population in humans, a number of recent studies provide persuasive evidence that a human oval cell equivalent is present in the liver (Baumann *et al.*, 1999; Crosby *et al.*, 1998; Haque *et al.*, 1996). The $CD34^+CD45^-$ hepatic cell population may therefore include small mature endothelial cells, quiescent HSCs and/or human oval cells.

The inclusion of bone marrow samples as an assay control highlighted the low level of expression of the CD34 molecule on hepatic HSC populations. As haematopoietic progenitor cells mature there is a gradual down-regulation of CD34 (Andrews *et al.*, 1990; Herbein *et al.*, 1994). The low level of CD34 expression seen on hepatic HSCs is consistent with a more differentiated phenotype. Interestingly, the $CD34^+CD45^-$ hepatic populations expressed higher levels of CD34 supporting the notion that quiescent HSCs may be contained in this fraction.

To further characterise $CD34^+CD45^+$ hepatic HSC populations with respect to co-expression of differentiation, activation and lineage-specific markers, three-colour flow cytometric analysis was used. The PE-conjugated anti-CD34 antibody was found to be a more sensitive marker for hepatic HSCs and would have been the antibody of choice for the three-colour study. However, the availability of antibodies dictated the use of the PerCP-conjugated antibody for three-colour staining. Because of the significant difference in sensitivity observed with the two fluorescent-conjugates, it is possible that we may be underestimating hepatic HSC levels in the three-colour study. Using the anti-CD34-PerCP small but consistent populations of $CD34^+CD45^+$ cells were detected in all hepatic samples tested.

The six fold higher levels of HSCs detected in hepatic mononuclear cell (HMNC) populations demonstrate that HMNCs are enriched for HSCs when compared to matched peripheral blood (PBMCs). The technique used for the isolation of HMNCs involves the use of proteolytic enzymes (Curry *et al.*, 2000b), which may result in the modulation of cell surface markers (Abuzakouk *et al.*, 1996). The levels of $CD34^+CD45^+$ cells detected in the hepatic cell samples was not due to upregulation of CD34 on $CD45^+$ cells, or CD45 on $CD34^+$ cells, as a result of the isolation technique, since neither of these markers were affected by incubation in the enzyme mix. Hepatic HSCs express low levels of CD34, indicating that they may be more differentiated than the majority of bone marrow HSCs. However, although all of the $CD34^+CD45^+$ cells had low levels of CD34, only half of these cells expressed differentiation (CD38) and activation (CD45RA) markers. In addition, the early bone marrow HSC marker AC133-antigen was undetectable on hepatic HSC populations. Lack of AC133-antigen expression could be evidence for a more differentiated phenotype as its down-regulation occurs earlier than CD34 (Yin *et al.*, 1997). As the AC133 antibody binds to sugar or sugar-dependent epitopes, it is possible that lack of reactivity with AC133 is simply due to a differentially glycosylated form of the molecule being expressed in liver. Alternatively, hepatic HSCs may not originate from bone marrow but have a local origin and thus a different phenotype from bone marrow HSCs. The high proportion of $CD45^+CD34^{low}$ HSCs which are negative for any of the activation, differentiation and lineage markers classically associated

with differentiating HSCs, and lack of AC133-antigen expression on hepatic HSCs is consistent with this notion.

In normal liver, co-expression of lineage specific markers on hepatic HSCs ($CD34^+CD45^+$) revealed that less than 5% of differentiating hepatic HSCs express the myeloid associated antigen (CD33) and that the majority express lymphoid-associated markers (CD56, CD7, CD19). This is in contrast to the phenotypic profiles observed in normal bone marrow aspirates, where the majority of $CD34^+$ cells express CD33, with $CD19^+$ B-cell precursors comprising approximately 25% and T cell progenitors ($CD7^+$) only 1% of HSCs (Civin and Gore, 1993; Lansdorp *et al.*, 1992). There is evidence that the normal AHL is capable of supporting T-cell development, as has been shown in mice (Abo *et al.*, 1999). RAG-1 and RAG-2, cell specific components required for lymphoid development, and pre-TCR-α, a T-cell specific chaperone expressed at an early stage of T cell development, have all been detected in hepatic lymphocytes isolated from AHL (Collins *et al.*, 1996), suggesting that the AHL may be an extrathymic site of T-cell development. The relatively high level of HSCs co-expressing CD7 detected in normal liver is consistent with this hypothesis. The co-expression of CD56 and CD19 on significant populations of hepatic HSCs suggests that the hepatic microenvironment may also support NK (or NK T) and B cell differentiation.

In adult mice, hepatic haematopoiesis plays an important role in the generation of innate T cells involved locally in the surveillance and rejection of tumours (Hashimoto *et al.*, 1995; Kawamura *et al.*, 1996; Ohmori *et al.*, 1993). It is as yet unknown if a similar mechanism of tumour immunity exists in humans. If there operates a physiologically significant haematopoietic pathway in the AHL, one would expect to see activation and/or alterations in this pathway in a situation where there is a local need for increased numbers of immune effector cells, such as in tumour challenge. In this study, using multi-parameter flow cytometry, we examined the levels and cell surface phenotype of differentiating HSCs in tumour-bearing liver and compared them to HSCs detected in the normal AHL.

The human hepatic haematopoietic pathway does not appear to undergo up-regulation in tumour-bearers. There is no increase in the overall levels of HSCs with similar yields of mononuclear cells isolated from normal and tumour-bearing liver, equal proportions of which co-expressed CD34. While age is a factor which influences the activation status of hepatic haematopoiesis in mice (Abo *et al.*, 1999), in this study the mean age of donors was lower than that of cancer patients and hepatic HSC levels showed no correlation with age. The majority of hepatic HSCs, from either source, had a differentiated phenotype with similar co-expression of differentiation (CD38) and activation (CD45RA) markers, suggesting a surprisingly similar activation status for haematopoiesis in both tissue types.

Although no overall up-regulation of haematopoiesis was observed in tumour-bearing liver, changes were observed in the nature of the hepatic haematopoietic pathway. A significant increase in the proportion of hepatic HSCs co-expressing the T cell marker (CD7) was observed in tumour-bearing livers. Murine studies have demonstrated an increase in the extrathymic differentiation pathway of T cells in the livers of mice bearing syngenic tumours (Ohmori *et al.*, 1993). It also has been suggested that, in humans as in mice, T cells of extrathymic origin may be involved in tumour immunity (Takii *et al.*, 1994). The significant increase in the levels of T cell precursors observed in tumour-bearing liver provides supportive evidence for the possible involvement of extrathymically derived T cells in the tumour response in humans. The upregulation of T cell precursors appears to be at the expense of other lymphoid cell types as, B cell precursor populations were virtually undetectable in tumour-bearing liver, and there was also a small decrease in progenitor cells with NK cell phenotype.

Although myeloid cells have been implicated in surveillance and prevention of hepatic metastases (Griffith *et al.*, 1999; Heuff *et al.*, 1995; Musiani *et al.*, 1996; Zhang *et al.*, 1993), it is unclear what role, if any, they play in the rejection of established tumours. Using flow cytometry, we found that a significantly higher proportion of cells of haematopoietic origin ($CD45^+$) isolated from tumour-bearing liver had a mature granular phenotype (high FSC:SSC, $CD34^-$). This observation was confirmed by microscopic examination of liver tissue which

indicated that an increase in granular cell populations is a feature of tumour-bearing liver. A six-fold increase was observed in HSCs with an immature myeloid phenotype ($CD33^+$) in tumour-bearing liver as compared with normal liver, which correlates strongly with the increase in mature granular cells. The increase in mature granular populations and CD33 positive HSCs observed in tumour-bearing liver may be a general feature of metastatic liver disease, resulting granular cell infiltration in response to tissue damage. The observed correlation is consistent with concomitant infiltration of immature myeloid cells along with mature granular cell populations. However, granulocytes as well as lymphocytes are generated in the hepatic parenchyma of adult mice (Narita *et al.*, 1998) and the presence of significant levels of hepatic HSCs committed to differentiation along the myeloid lineage in human tumour-bearing liver suggests the possibility of local promotion of granular populations.

The increases observed in myeloid and T cell precursor levels observed in tumour-bearing liver may be due to a number of factors other than tumour presence, such as pre-operative chemotherapy and/or chronic anaemia, which would influence extramedullary haematopoiesis. However, only one of the patients had evidence of anaemia prior to hepatic surgery, and thus, in this study, the hepatic HSC levels and the differences in phenotype observed cannot be attributed to the effects of anaemia. While the increase in myeloid precursors could reflect the bone marrow myelotoxic effects of drugs (MacDonald, 1999) only two of the patients in the study received preoperative chemotherapy, suggesting that these changes may result from the presence of tumour.

Generation of confocal images allowed us to visualise hepatic HSCs directly in tissue. As donor organs are extensively perfused prior to the biopsy been taken, it is unlikely that these cells represent residual circulating HSCs. These images provide direct evidence of the existence of resident HSCs in the normal AHL.

The results of this study show that the normal AHL contains significant populations of HSCs with a phenotype consistent with actively differentiating cells. The AHL harbours lineage

committed haematopoietic progenitors and the vast majority of these progenitors express lymphoid associated antigens suggesting a lymphoid bias in the hepatic haematopoietic pathway. The physiological significance of this pathway is evidenced by the alterations observed on tumour challenge. Changes occur in both the myeloid and lymphoid compartments of the hepatic haematopoietic pathway on tumour challenge. Tumour-bearing livers are enriched for intrahepatic myeloid precursors and T cell progenitor cells; further studies are required to establish the origin and *in situ* development potential of hepatic HSCs in the adult human and their role in tumour-immunity.

Chapter 3: The Hepatic Cytokine Microenvironment.

3.1: Introduction.

If lymphopoiesis is to progress successfully in adult human liver (AHL), the appropriate microenvironment will provide the necessary growth factors and cytokines.

3.1.1: Interleukin 7.

Interleukin-7 (IL-7) is a pleiotropic cytokine, whose primary physiological function is the promotion of lymphopoiesis (Goodwin *et al.*, 1989; Hofmeister *et al.*, 1999). Consistent with a role in lymphopoiesis, IL-7 is expressed by known haematopoietic tissues, bone marrow (Funk *et al.*, 1995) and thymus (Goodwin *et al.*, 1989). IL-7 exerts its influence *via* the IL-7 receptor (IL-7R) complex, which is composed of the IL-7-specific α-chain (Goodwin *et al.*, 1990) and the common γ-chain (γc) (Noguchi *et al.*, 1993), also shared by the receptors for cytokines IL-2, IL-4 (Kondo *et al.*, 1993), IL-9 (Kimura *et al.*, 1995) and IL-15 (Giri *et al.*, 1994). Lessons from *in vitro* studies, knockout mice and human mutations clearly point to IL-7 as a critical cytokine, controlling the survival, expansion and differentiation of pro-T cells during normal T cell development.

3.1.1.1: The role of IL-7 in murine thymic T cell development.

Studies in mice deficient in IL-7 (von Freeden-Jeffry *et al.*, 1995) or IL-7R (Peschon *et al.*, 1994) have established its role as a critical cytokine in normal T-cell development in mice. IL-7Rα deficient mice display a complete lack of γδ T cells, have normal numbers of NK cells and very low levels (less than 5% those of normal mice) of αβ T cells (He and Malek, 1996; Maki *et al.*, 1996b). These studies indicate an absolute requirement for IL-7 in γδ T cell development, and a critical role for IL-7 in the expansion and survival of early αβ T cells. It appears that IL-7 is required for TCR-γ chain rearrangement (Schlissel *et al.*, 2000) and/or transcription (Maki *et al.*, 1996a). In two, independent IL-7Rα$^{-/-}$ mouse strains, TCR-γ chain rearrangement was severely impaired, although the TCR-β and δ loci had normal rearrangement patterns. Since it is known that the same cells initiate rearrangement of TCR γ, β and δ and that a majority of αβ T cells have rearranged γ loci, it appears that the IL-7R signal is crucial for γ-chain rearrangement only (Maki *et al.*, 1996a). A role for IL-7 has also

been suggested during rearrangement of TCR-αβ genes (Candéias *et al.*, 1997; Muegge *et al.*, 1993), however this may represent survival effects as opposed to a distinct signalling function of IL-7/IL-7R. Muegge *et al.* (1993) reported that foetal thymocytes on embryonic day 14 underwent V-DJ recombination *in vitro* in the presence of IL-7. However, since these experiments were done *in vitro*, the successful detection of cells with rearranged TCR-β genes may simply be due to increased cell survival during V-DJ recombination in the presence of IL-7. Several studies provide compelling data that the IL-7R provides crucial survival signals. These studies demonstrated that transgenic expression of Bcl-2 in T-lymphoid cells of IL-7R deficient mice resulted in substantial restoration of thymic cellularity and function (Akashi *et al.*, 1997; Maraskovsky *et al.*, 1997). IL-7 has been shown to block apoptosis directly, to induce Bcl-2 expression and to promote cell-cycle progression of TN cells in normal mice (Kim *et al.*, 1998; Von Freeden-Jeffry *et al.*, 1997).

3.1.1.2: The role of IL-7 in human thymic T cell development.

Support for a pivotal role for IL-7 in normal T-cell development in humans comes from studies of patients that suffer from X-linked severe combined immuno-deficiency (X-SCID). Due to a mutation in the common γ–chain, patients that suffer from X-SCID have an arrest in T-cell development and thus lack mature T-cells. The T-cell phenotype of the common γ-chain-deficient patients has been attributed to defective signalling through the IL-7/IL-7R system (Lai *et al.*, 1997; Puel and Leonard, 2000) and suggests that IL-7 is essential for T-cell development in humans. A recently described missense mutation in the IL-7R α-chain (P132S) results in arrest of T cell development and presents clinically as a SCID phenotype (Roifman *et al.*, 2000). This substitution of proline with serine in the extracellular portion of the IL-7Rα does not affect protein expression but severely reduces IL-7 affinity, such that signalling is defective through the receptor complex. The severity of the phenotype resulting from this partial deficiency in IL-7Rα confirms the hypothesis that the γ-chain deficient phenotype is due to defective signalling through the IL-7R complex and that IL-7 is essential for normal T cell development in humans (figure 3.1.1). Further evidence for a central role for IL-7 in human T-cell development comes from studies carried out by Plum et al (1996), who

used a chimeric human-mouse foetal thymic organ culture (FTOC) system which supports differentiation to mature T-cells. In this system, the addition of monoclonal antibodies against IL-7 or IL-7Rα, resulted in a profound reduction in human thymic cellularity. The small number of cells able to differentiate in this system indicates a critical role for IL-7 in the expansion and maintenance of early T cell precursors, but not an absolute requirement for IL-7 in the differentiation of T cells (Plum *et al.*, 1994; Plum *et al.*, 1996). An absolute requirement for IL-7 in γ-chain rearrangement was not tested as Plum *et al.* (1996) based their observations on the cell surface expression of CD3 and did not look at αβ or γδ TCR expression, so these cells may well have expressed the αβ-TCR.

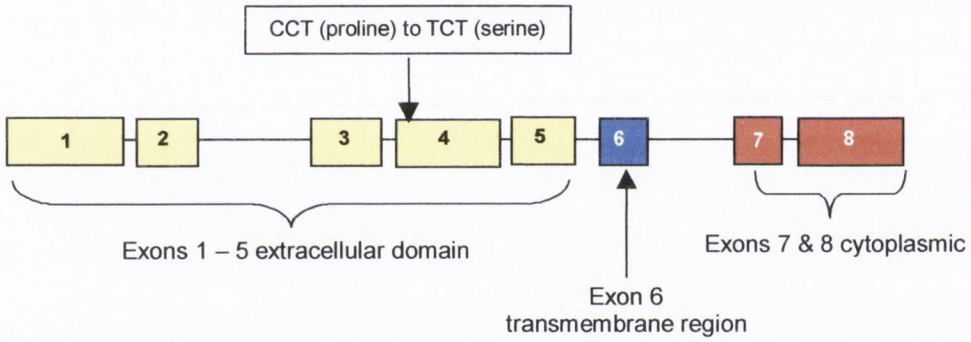


Figure 3.1.1: The IL-7Rα gene showing the position of the P132S missense mutation.

Cytosine to thymine transition at nucleotide 394 in exon 4 , leads to a proline to serine substitution in the extracellular domain of the IL-7Rα chain (adapted from Roifman *et al*, 2000).

3.1.2: Interleukin 15.

IL-15 was initially identified as a cytokine with similarities to IL-2, due to its ability to stimulate T cell proliferation and its utilisation of components of the IL-2R (Grabstein *et al.*, 1994). IL-15 is a 14-15 kDa glycoprotein whose mature form consists of 114 amino acids (aa). The IL-15R is composed of the IL-2Rβ chain (now referred to as the IL-2R/IL-15Rβ chain or CD122), the common γ-chain, and a unique IL-15Rα chain (Giri *et al.*, 1994). The IL-2 receptor has a similar trimeric structure comprising CD122, the common γ-chain and a specific IL-2Rα chain. Although the receptor arrangement suggests that IL-2 and IL-15 might have redundant roles,

IL-2 is critical for maintaining peripheral homeostasis of thymus derived T cells whereas IL-15 promotes extrathymic T and NK cell development. This response specificity is achieved through a combination of differential ligand production sites and regulation of receptor α-chain expression (DiSanto, 1997) (figure 3.1.2).

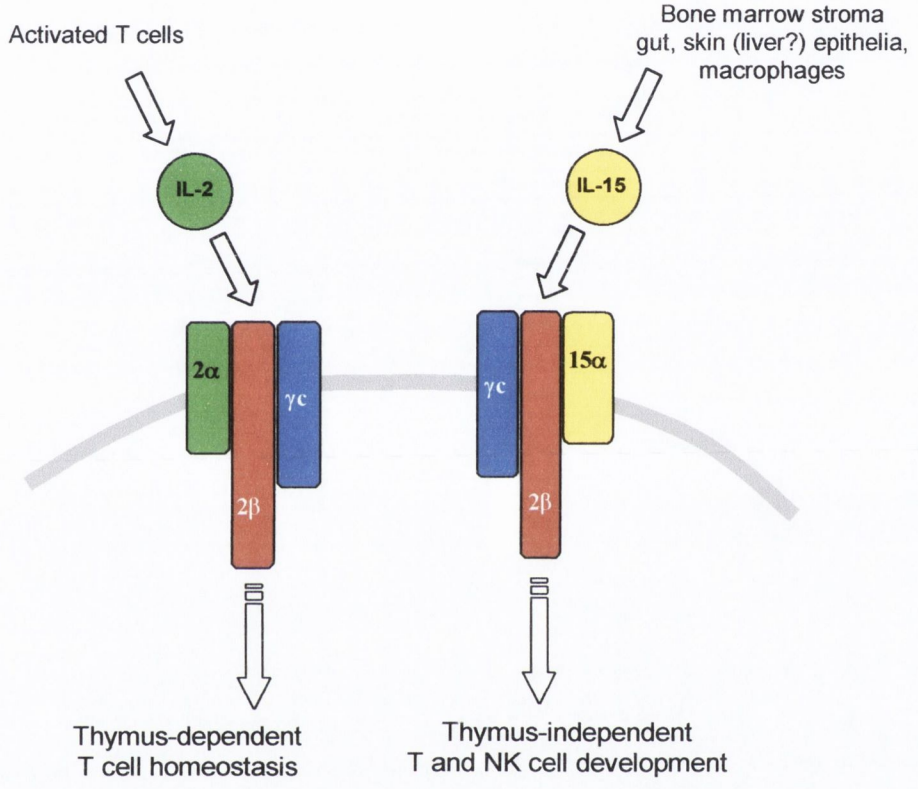


Figure 3.1.2: IL-15 and IL-2 share two out of three receptor components.

The human IL-15 gene has been mapped to chromosome 4q31 (Anderson *et al.*, 1995). The IL-15 gene consists of nine exons (1 – 8 and 4a) and 8 introns spanning at least 35 kb. One form of IL-15 mRNA contains a 5' untranslated region (UTR) of at least 352 nucleotides (nt), a coding sequence of 486 nt and a 3' UTR of at least 400 nt (Grabstein *et al.*, 1994). The alternatively spliced mRNA transcripts give rise to proteins with two alternative leader peptides, one composed of 48 amino acids (aa) and another of 21 aa (Grabstein *et al.*, 1994; Meazza *et al.*, 1996; Onu *et al.*, 1997; Tagaya *et al.*, 1997). The classical long (48 aa) signal peptide associated with all secreted IL-15 is present in a 1.6 kb mRNA encoded by exons 3, 4 and 5 (Anderson *et al.*, 1995). The short (21 aa) signal peptide is encoded by a 1.2 kb cDNA

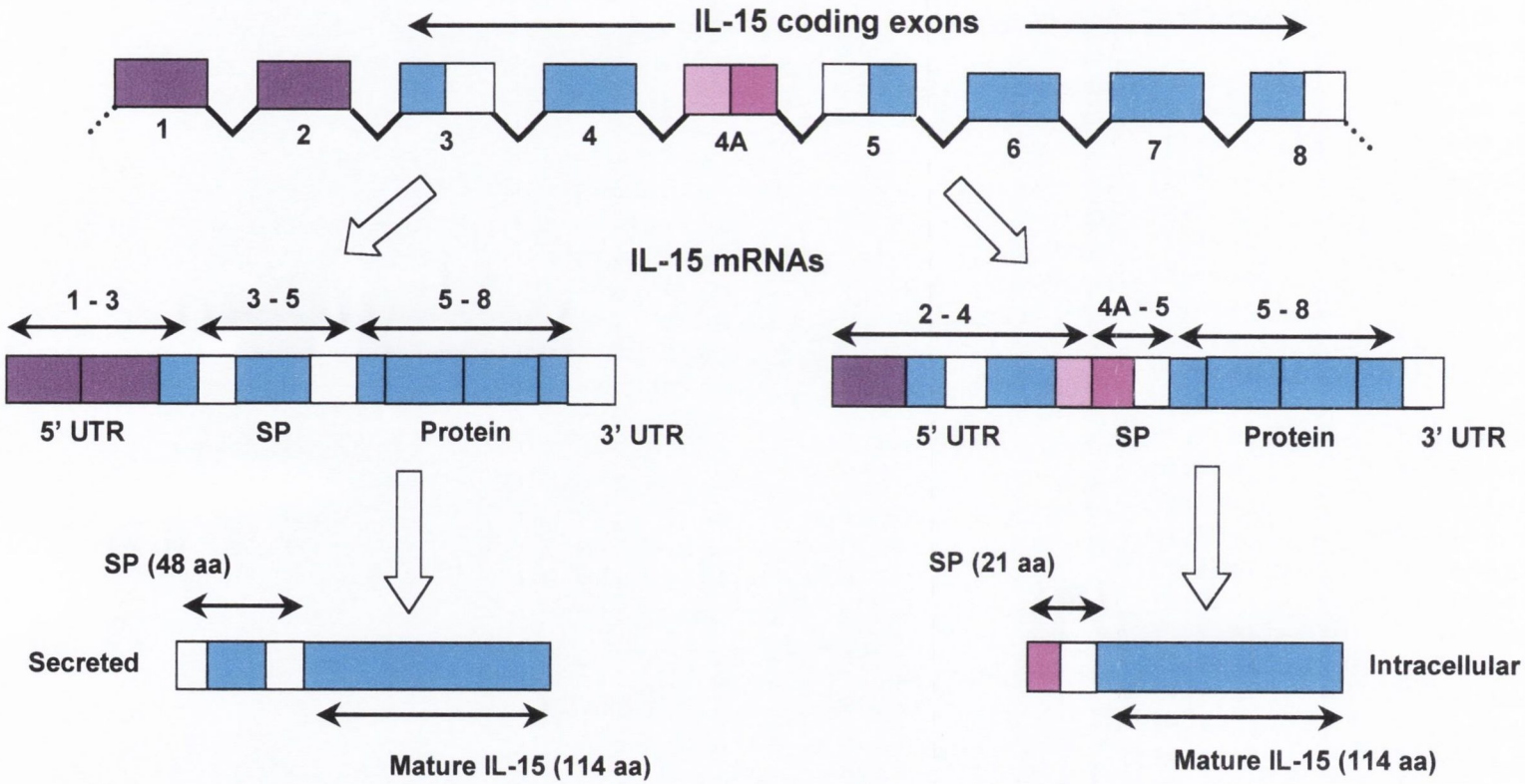


Figure 3.1.3: Alternative splicing of the IL-15 gene results in secreted and intracellular forms of the mature protein.
There are two isoforms of IL-15 that contain different signal peptides (SP), one with 48 amino acids (aa) and one with 21 aa. The 48 aa SP is encoded by exons 3, 4 and 5. The 21aa SP is encoded by exon 5 and an additional 119 nucleotide (nt) sequence inserted between exons 4 and 5 (exon 4A). The introduction of the 119-nt exon 4A disrupts the 48 aa SP by inserting a premature stop codon and then provides an alternative start codon. The 114 aa mature protein encoded by both transcripts is identical (adapted from Waldman and Tagaya, 1999).

that lacks the elements encoded by exon 1. This signal is encoded by exon 5 and by an additional 119 nt sequence inserted between exons 4 and 5 (exon 4a) (Meazza *et al.*, 1996; Onu *et al.*, 1997; Tagaya *et al.*, 1997). The 21 aa signal peptide is not secreted but stored intracellularly appearing in nuclear and cytoplasmic compartments (figure 3.1.3). The production of an intracellular lymphokine is not typical of other soluble interleukin systems suggesting a biological function for IL-15 as an intracellular molecule (Tagaya *et al.*, 1997). Alternatively, IL-15 mRNA may exist in translationally inactive pools leading to a very rapid induction of IL-15 on receipt of the appropriate signals (Waldmann and Tagaya, 1999).

3.1.2.1: The lymphopoietic role of IL-15.

The expression of IL-15 mRNA in thymic and bone marrow stromal and epithelial cells suggests that IL-15 may play a role in the development of NK and T cells (Waldmann and Tagaya, 1999). However, IL-15 mRNA is widely expressed constitutively (Grabstein *et al.*, 1994), thus, the expression of IL-15 mRNA in the thymus and bone borrow may be a reflection of a wider role for IL-15 in the immune system. Observations suggest that IL-15 may be essential for the development of NK cells in mice (Suzuki *et al.*, 1997) and in humans (Cavazzana-Calvo *et al.*, 1996; Mrózek *et al.*, 1996). NK cells are markedly deficient in both humans (Lai *et al.*, 1997) and mice (Cao *et al.*, 1995) that do not express the common γ-chain used by IL-2, IL-4, IL-7, IL-9 and IL-15. Mice deficient in IL-2 (Kundig *et al.*, 1993; Schorle *et al.*, 1991), IL-2Rα chain (Willerford *et al.*, 1995), IL-4 (Kuhn *et al.*, 1991; Sadlack *et al.*, 1994) and IL-7 (He and Malek, 1996) have no defect in NK cells. Although IL-9 deficient mice are not yet available, a mAb to IL-9 exerted no inhibitory effects on lymphoid development, suggesting that this cytokine does not play a role in NK cell development (He *et al.*, 1997). Recent reports of an IL-15 knockout mouse, which suffers a severe defect in NK cells, supports a role for this cytokine in NK cell development (Lodolce *et al.*, 1998). Treatment of mice with monoclonal antibody directed against IL-2/IL-15Rβ eliminates NK cells (Tanaka *et al.*, 1993). Mice made deficient in IL-2/IL-15Rβ by homologous recombination or through the use of Abs to this receptor subunit are markedly deficient in NK cells (Suzuki *et al.*, 1997). In

contrast, mice deficient in IL-2 or IL-2Rα have normal numbers of NK cells, suggesting that IL-15 may be required for NK cell development (Suzuki *et al.*, 1997).

Additional evidence supporting a role for IL-15 in NK cell development comes from mice deficient in the transcription factor Interferon-Regulatory-Factor-1 (IRF-1), which induces IL-15 gene expression. These mice completely lack NK cells. Bone marrow cells from IRF-1 deficient mice develop into NK cells when cultured with exogenous IL-15. This indicates that NK cell progenitors are present in these mice, but that the microenvironment cannot support NK cell maturation. The NK cell defect in these animals has been attributed solely to the lack of IL-15 (Ogasawara *et al.*, 1998; Ohteki *et al.*, 1998).

The role of cytokines in NK-cell development has been studied directly using stromal-independent cultures of haematopoietic precursors. Addition of IL-2 in the presence of other cytokines such as IL-7 or SCF leads to NK-cell differentiation of $CD34^+CD7^+$ precursor cells (Cavazzana-Calvo *et al.*, 1996; Puzanov *et al.*, 1997). However, IL-15 is even more effective in inducing bone marrow progenitor differentiation into NK cells. Furthermore, it is the one factor capable of inducing human $CD34^+CD7^-$ bone marrow, foetal liver or cord blood progenitor cells to undergo such differentiation (Cavazzana-Calvo *et al.*, 1996; Jaleco *et al.*, 1997; Mrózek *et al.*, 1996). A similar propagation of NK cells from their progenitors was observed in NK cell deficient IRF-$1^{-/-}$ mice in the presence of IL-15 (Ohteki *et al.*, 1998). This suggests that IL-15 is an important factor in the differentiation of NK cells from uncommitted precursors both in mice and humans. Signalling through the IL-15R during NK (or NK T) cell development is analogous to IL-7R signalling during T cell development (Baird *et al.*, 1999).

3.1.3: Lymphopoietic cytokines (IL-7 & IL-15) in extrathymic T cell development.

The role of cytokines in extrathymic T cell development has to date been studied only in mice. Because NK T cells share phenotypic and functional properties with T and NK cells, there may also be overlap in the developmental requirements of this subset of T cells. Whereas the majority of T cells depend upon the thymus and IL-7 for development, NK cells develop normally in athymic nude, SCID, IL-7Rα deficient and RAG-deficient mice and are dependent

on IL-15. Patients with partial deficiency in the IL-7Rα chain also have normal NK cell numbers and function (Roifman *et al.*, 2000), however, X-SCID patients suffer a severe defect in NK cells, suggesting that the cytokine requirements of developing T and NK cells are similar in mice and humans. Murine NK T cells can be either thymus derived or can develop at sites other than the thymus. NK T cells are readily detectable in the bone marrow, spleen and liver of athymic nude mice (Ohteki *et al.*, 1997). IL-7 produced by parenchymal hepatocytes plays a pivotal role in the generation of murine hepatic extrathymic T lymphocytes (Miyaji *et al.*, 1996). Normal profiles of conventional T cells are detected in the thymus and liver of IL-2Rβ deficient mice, but NK T cells are dramatically reduced (Ohteki *et al.*, 1997), suggesting a role for IL-15 in the development of hepatic derived NK T cells. The requirement for IL-15 defines a common portion of NK/NK T cell ontogeny (Ohteki *et al.*, 1997).

Another population of T cells, which can be of thymic or extrathymic origin, are TCR-$\gamma\delta^+$ or TCR-$\alpha\beta^+$ IEL sub-populations in the intestine. TCR-$\alpha\beta^+$ IELs of extrathymic origin can be distinguished phenothypically by the expression of the CD8αα homodimer (Rocha *et al.*, 1994). TCR-αβ IELs are found in near normal numbers in IL-2Rβ or IL-7Rα deficient mice (Maki *et al.*, 1996a), however, they are completely lacking in γc-deficient mice indicating an essential role for one or more γc-dependent cytokines in the development of all IEL subsets. The role for IL-7 in IEL development appears to be restricted to production of γδ-TCR$^+$ cells (He and Malek, 1996; Maki *et al.*, 1996a; Malek *et al.*, 1999). Although IL-7 and IL-7R deficient mice have low numbers of αβ-TCR$^+$ cells, these mice lack thymic and IEL γδ-TCR$^+$ populations (Malek *et al.*, 1999). It has recently been shown that extrathymic expression of IL-7 is sufficient to promote development of TCR-γδ IEL in thymectomised, irradiated, bone-marrow-reconstituted chimeric mice, whereas thymic expression of IL-7 was required for the development of thymic T cells (Laky *et al.*, 1998). Using the intestinal fatty acid binding protein (IFABP) promoter, IL-7 expression was reinstated exclusively to mature enterocytes of IL-7$^{-/-}$ mice. In these mice, γδ cells, cryptopatches and Peyer's patches were restored, while γδ T-cells remained absent from all other tissues (Laky *et al.*, 2000). These studies suggest that IL-7 acts in a paracrine fashion and is required at the T-cell development site, illustrating the

importance of local cytokine production in providing a microenvironment capable of supporting lymphopoiesis. Consistent with a role in the extrathymic development of T cells in humans, IL-7 is expressed in the normal intestine, a putative extrathymic T cell generation site (Watanabe *et al.*, 1995; Madrigal-Estebas *et al.*, 1997).

The dependence of certain subclasses of IELs on IL-2Rβ expression points to an essential role for IL-15 in the development of lymphocyte subsets of extrathymic origin (Suzuki *et al.*, 1997). Signals through the IL-2Rβ appear to be required for the production of the unique CD8αα subset of IELs as evidenced by the severe defect in this population observed in IL-2Rβ-deficient mice. This probably reflects a requirement for IL-15 in the development of CD8αα T cells, although a contributory role for IL-2 has not yet been excluded (Suzuki *et al.*, 1997). IL-15$^{-/-}$ mice (generated by gene targeting) reveal critical roles for IL-15 in the development of CD8αα IELs, which were also decreased in the spleen and liver of these mice. The specific defects associated with IL-15 deficiency were reversed by *in vivo* administration of exogenous IL-15. The observation that the NK T cells present in the IL-15$^{-/-}$ mice appear to have undergone a normal selection process suggests that IL-15 is not obligatory for NK T cell development but important for the expansion/survival of committed NK T cells (Kennedy *et al.*, 2000). The IL-15 knockout mouse supports the notion that IL-15 contributes to IEL development, further supported by the findings that similar defects were detected in the IL-15Rα deficient mouse (Lodolce *et al.*, 1998). Consistent with a role in extrathymic development of gut IELs, IL-15 mRNA and protein are expressed by human intestinal epithelial cells (Reinecker *et al.*, 1996). The studies outlined above point to a critical role for both IL-7 and IL-15 in normal T and NK cell development as well as in the development of T and NK T cells of extrathymic origin.

3.1.4. The haematopoietic microenvironment of the adult human liver.

The importance of the local microenvironment in determining the developmental fate of haematopoietic precursor cells is demonstrated by the ability of cells from the same clone to commit to different lineages under different environmental conditions. Kondo *et al.* (1997)

cultured single cells in methyl cellulose in the presence of SCF, IL-7 and flt-3 ligand to expand cell numbers, and a portion of the day 3 colonies were injected directly into the thymus. The remaining cells in methylcellulose formed B-lineage colonies, but the cells injected directly into the thymus differentiated into all stages of thymic lymphocytes, including mature T-cells. Thus, the commitment of common lymphoid progenitors (CLPs) to either the T- or B-cell lineage may possibly be determined simply by the microenvironment. CLPs that home successfully to the thymus, or to sites that support extrathymic T-cell development, differentiate mainly into T-cells whereas other CLPs differentiate into B-cells in the marrow (Kondo *et al.*, 1997).

The concept of extrathymic T-lymphocyte development predicts that T cell progenitors and all factors required for their maturation are present at extrathymic sites (Laky *et al.*, 2000). The adult murine liver harbours haematopoietic stem cells (HSCs) (Taniguchi *et al.*, 1996; Watanabe *et al.*, 1996) and IL-7 produced by parenchymal hepatocytes plays a pivitol role in the generation of murine hepatic extrathymic T lymphocytes (Miyaji *et al.*, 1996). Human liver also contains significant populations of functional HSCs (Crosbie *et al.*, 1999), the vast majority of which express lymphoid-associated lineage markers (Golden-Mason *et al.*, 2000). RAG-1 and RAG-2 (cell specific components required for lymphoid development), and pre-TCR-α (T-cell specific chaperone expressed at an early stage of T cell development), have all been detected in hepatic lymphocytes isolated from AHL (Collins *et al.*, 1996), suggesting that the AHL may be an extrathymic site of T-cell development. The relatively high level of HSCs co-expressing CD7 (Golden-Mason *et al.*, 2000), and the innate phenotype of mature T lymphocytes (in particular the high proportion of CD8$\alpha\alpha$, $\gamma\delta$ and NK T cells) detected in the normal AHL (Doherty and O'Farrelly, 2000) is consistent with this hypothesis (figure 3.1.4).

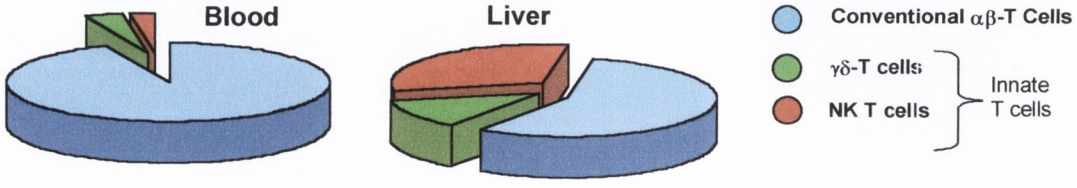


Figure 3.1.4: A significantly higher proportion of hepatic T cells display an innate phenotype when compared to matched peripheral blood (adapted from Doherty *et al.*, 2000).

Evidence suggests that IL-7 and IL-15, required for the promotion of local T-cell differentiation, may also be expressed in the normal AHL. Human IL-7 was first cloned from a hepatoma cell line (SK-HEP-1) (Goodwin *et al.*, 1989) and expression of IL-7 mRNA has been found in other human-derived hepatocyte cell lines (HepG2, HHH7 and HH25) (Rowell *et al.*, 1997). IL-15 mRNA has been detected in a human derived hepatoma cell line (HHH7) and in HH25 cells derived from normal liver, but was absent in another hepatoma cell line, HepG2 (Rowell *et al.*, 1997). IL-7 expression has been demonstrated in the adult murine liver (Miyaji *et al.*, 1996). A recent study has shown expression of IL-15 mRNA in leukocytes isolated from University of Wisconsin solution (UW) used to perfuse human liver donor organs prior to transplantation (Jonsson *et al.*, 2000). However, direct evidence for IL-7 or IL-15 expression in the normal AHL is presently lacking.

3.1.5: Overall objective of this study.

Many of the elements for extrathymic T cell development are present in the normal AHL, however, the status of the normal AHL with respect to expression of essential lymphopoietic cytokines, IL-7 and IL-15 is unknown. The aim of this study is to determine if the normal AHL has the potential to provide a suitable cytokine microenvironment to promote the differentiation of hepatic NK/T lymphoid progenitor cells. The experimental strategy used to achieve these goals is illustrated in figure 3.1.5 and the specific objectives are outlined below.

3.1.5.1: Specific objectives.

1) To detect the presence of IL-7 and IL-15 specific RNA transcripts and estimate their relative quantities in liver biopsies.
2) To quantify IL-7 and IL-15 protein levels in homogenised whole liver tissue.
3) To localise the anatomic distribution of IL-7 within hepatic tissue.
4) To determine if hepatic mononuclear cell and/or haematopoietic stem cell populations express IL-7/IL-15 receptors.

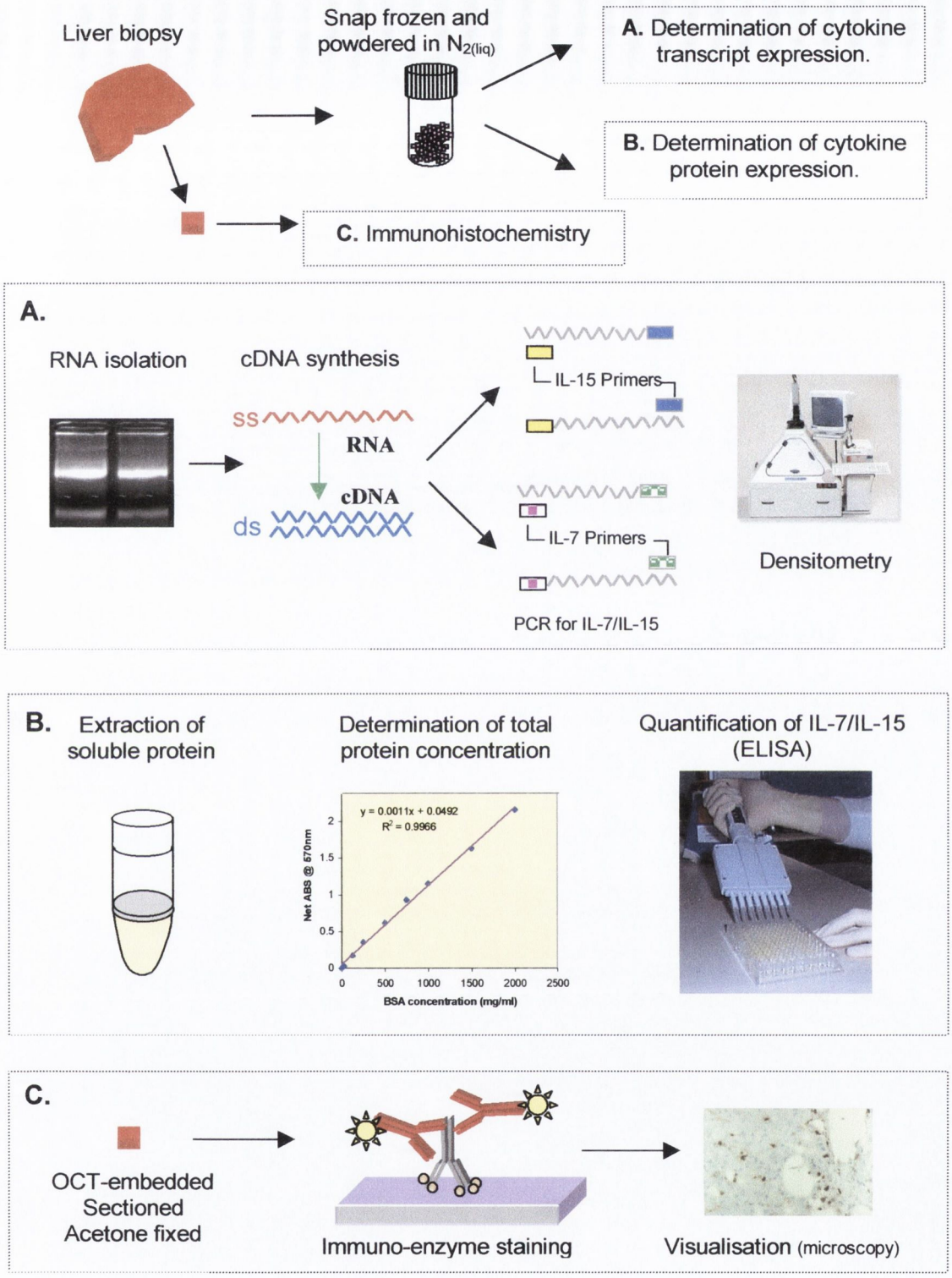


Figure 3.1.5: Experimental strategy for the determination of lymphopoietic cytokine expression in normal hepatic tissue.

A. Semi-quantitative RT-PCR was used to detect the presence and estimate the relative quantities of IL-7 and IL-15 specific RNA transcripts in liver biopsies. **B.** A sandwich Enzyme Linked Immunosorbent Assay (ELISA) was used to quantify IL-7 and IL-15 protein levels in whole homogenised tissue. **C.** The anatomic distribution of IL-7 within hepatic tissue was determined by immunohistochemical staining.

3.2: Materials and Methods.

3.2.1: Tissue specimens.

3.2.1.1: Normal liver (cytokine study).

Normal liver wedge biopsies (50-100 mg) were obtained from donor organs at time of liver transplantation (n = 16, mean age 45, range 19 – 65 years, 9 male and 7 female, see appendix V [table APV.1] for details). Donor organs were extensively perfused with University of Wisconsin solution prior to obtaining the biopsy. Liver samples were immediately snap-frozen in liquid nitrogen and powdered using the Braun Mikrodismembrator (see Appendix III [APIII.3]). The powder was stored at -80°C and used subsequently for RNA extraction and, (if sufficient powder remained) protein extraction. For two samples, a small section of tissue was dissected from the biopsy and mounted in cryopreservative embedding medium (OCT, Finetek Europe BV) before being transferred to liquid nitrogen.

3.2.1.2: Control cell lines (cytokine study).

The human liver adenocarcinoma cell line SK-HEP-1 (ATCC) was used as a positive control and the human Acute Lymphocytic Leukaemia (B-cell) line CRL-8286 (ATCC) served as a negative control for IL-7 mRNA production (figure 3.2.1).

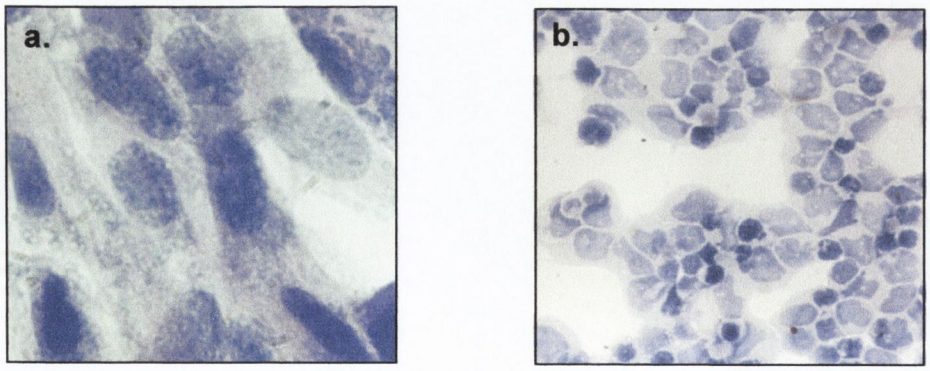


Figure 3.2.1: Cell lines used for the detection of IL-7 mRNA transcripts by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

a). The human liver adenocarcinoma cell line SK-HEP-1 (magnification x 1000). **b).** The Acute Lymphocyic Leukaemia (B-cell) line CRL-8286 (ATCC, magnification x 400).

SK-HEP-1 cells were grown to confluence in 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson) in Dulbecco's Modified Eagle's Medium (DMEM, supplemented with 25 mM Hepes, 1% non-essential amino acids, 4% heat inactivated foetal calf serum [FCS], 50 μg/ml streptomycin, 50 U/ml penicillin). SKHEP-1 cells were harvested by standard trypsinisation (see Appendix III [APIII.4]), washed twice in HBSS and pelleted, by centrifugation @ 300g for 5 minutes, for subsequent RNA extraction. CRL-8286 cells were maintained in RPMI1640 medium (supplemented with 2% heat inactivated FCS, 2 mM L-glutamine, 25 mM Hepes, 50 μg/ml streptomycin, 50 U/ml penicillin). CRL8286 cells are non-adherent and were harvested by centrifugation @ 500g for 5 minutes, washed twice and pelleted @ 300g for 5 minutes for subsequent RNA extraction. All of the tissue culture media used in this study were purchased from Gibco-BRL.

3.2.1.3: Normal liver (receptor status).

Normal liver biopsies were obtained from donor organs at time of liver transplantation (n = 13, mean age 44 years, range 18 – 65, 6 male and 7 female, see Appendix V [tables APV.5 and 6] for details). Hepatic mononuclear cells were prepared from normal liver wedge biopsies as described earlier (Chapter 2, Section 2.2.2.1).

3.2.2: Detection of IL-7 mRNA transcripts by RT-PCR.

3.2.2.1: RNA extraction.

Total RNA was extracted from tissue powders (approximately 50 - 100mg) or cell pellets using the RN*Ace* Total Pure™ RNA purification system (Bioline). All of the individual hepatic tissue powder samples were weighed immediately prior to isolation of RNA, which was carried out according to the manufacturer's instructions (see Appendix III [APIII.5] for details). The integrity of the RNA preparations was assessed by standard electrophoresis in a 2% agarose gel (Sigma-Aldrich). RNA yield was estimated spectrophotometrically (see Appendix III [APIII.6]).

3.2.2.2: Reverse Transcription.

One μg of total RNA was reverse transcribed to cDNA with 200 units Superscript II (Gibco-BRL), 1 x RT-buffer and 10 mM DTT (supplied with RT-enzyme), 2.0 mM dNTPs, 500 ng Oligo-dTs and 40 units of recombinant Rnasin Ribonuclease Inhibitor (all supplied by Promega). Nuclease free water (Sigma-Aldrich) was used to bring the total volume to 20μl. The mixture was incubated at 42°C for 40 minutes. The cDNA was stored at -20°C pending further use.

3.2.2.3: PCR amplification of IL-7 specific transcripts.

PCR amplification of IL-7 was carried out as described previously (Madrigal-Estebas *et al.*, 1997). A 25 μl reaction was performed containing 4 μl of cDNA, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton-X-100, 3 mM $MgCl_2$, 10 pM of each primer, 2.0 mM dNTPs and 0.625 U *Taq* DNA-Polymerase (all supplied by Promega). The IL-7 primers used:

5'-ATGAGAGTGTTCTAATGGTC 3' and 5' -GGAGCATTCAGTTTCCATTG- 3'

direct the amplification of a 620 bp cDNA fragment as well as other smaller transcripts (figure 3.2.2). PCR amplification of GAPDH served as a positive control for cDNA integrity and was performed under the same conditions as used for IL-7 (with 2 μl of cDNA) using the following primer sequences:

5' –GCC TCA AGA TCA TCA GCA A-3', and 5'-CCA GCG TCA AAG GTG GAG-3'.

Thermocycling conditions (MJ Research) consisted of; 3 minutes @ 94°C, followed by 16 cycles of (94°C for 30s, 56°C for 40s, 72°C for 30s) and a further 18 cycles of (94°C for 30s, 54°C for 40s, 72°C for 30s) and a final 10 minutes @ 72°C. Ten μl of IL-7 PCR product (5 μl for GAPDH) was visualised under UV light on a 2.0% agarose (Sigma Aldrich) gel containing ethidium bromide. Sizes of PCR transcripts amplified in each reaction tube were estimated against a 100 bp DNA ladder (Promega, range 100 bp – 1,200 bp) using RFLPSCAN™ software (Stratagene Cloning Systems). The optical density of the bands detected in the gel was determined using the EagleEye II™ still video system (Stratagene Cloning Systems) and expressed as arbitrary units.

..421	cctcccctga	tccttgttct	gttgccagta	gcatcatctg	attgtgatat	tgaaggtaaa
481	gatggcaaac	aatatgagag	tgttctaatg	gtcagcatcg	atcaattatt	ggacagcatg
541	aaagaaattg	gtagcaattg	cctgaataat	gaatttaact	tttttaaaag	acatatctgt
601	gatgctaata	aggaaggtat	gtttttattc	cgtgctgctc	gcaagttgag	gcaatttctt
661	aaaatgaata	gcactggtga	ttttgatctc	cacttattaa	aagtttcaga	aggcacaaca
721	atactgttga	actgcactgg	ccaggttaaa	ggaagaaaac	cagctgccct	gggtgaagcc
781	caaccaacaa	agagtttgga	agaaaataaa	tctttaaagg	aacagaaaaa	actgaatgac
841	ttgtgtttcc	taaagagact	attacaagag	ataaaaactt	gttggaataa	aattttgatg
901	ggcactaaag	aacactgaaa	aatatggagt	ggcaatatag	aaacacgaac	tttagctgca
961	tcctccaaga	atctatctgc	ttatgcagtt	tttcagagtg	gaatgcttcc	tagaagttac
1021	atggtcaaaa	cggattaggg	catttgagaa	atgcatattg	tattactaga	tgaatgcacc
1081..	agatgaatac	aaacaatgga	aactgaatgc	tccagtcaac	aaactatttc	ttatatatgt

Figure 3.2.2: Human interleukin-7 (IL-7) cDNA sequence [Goodwin, 1989 #259].

The primer target sequences (shown in blue) direct the amplification of a 620 base pair (bp) IL-7 specific product. The deleted portion of the splice variant resulting in the 488 bp product is shown in pink [Madrigal-Estebas, 1997 #478]. Accession no: J04156 EMBL/GenBank data base.

3.2.3: Detection of IL-15 mRNA transcripts by RT-PCR.

3.2.3.1: RNA extraction and Reverse Transcription.

RNA was extracted and cDNA synthesised as described for IL-7 RT-PCR.

3.2.3.2: PCR amplification of IL-15 specific transcripts.

PCR amplification of IL-15 was carried out as described previously (Meazza *et al.*, 1996). A 25 μl reaction was performed containing 2 μl of cDNA. The IL-15 primers used:

5'-GGATTTACCGTGGCTTTGATGTAATGAG 3' and 5' -GCCTTCATGGTATTGGGAAC- 3'

direct the amplification of a 524 bp cDNA fragment that corresponds to the secreted form of the IL-15 protein. The same primers amplify a larger (650 bp) transcript which corresponds to the intracellular form of IL-15 (figure 3.2.3). Thermocycling conditions (MJ Research), consisted of 35 cycles of (94°C for 60s, 55°C for 45s, 72°C for 90s). PCR products were visualised and the size of the amplified products was estimated as above.

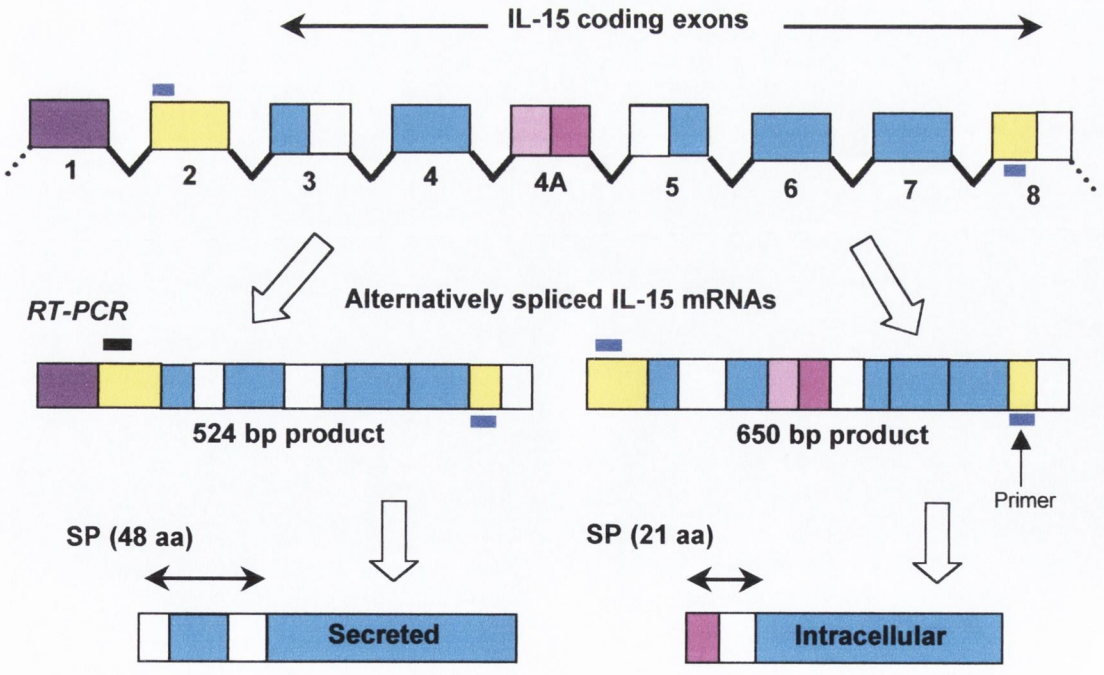


Figure 3.2.3: Alternative splicing of the Interleukin-15 gene results in secreted and intracellular forms of the mature protein.

Using primers directed against exons 2 and 8, two distinct PCR products can be detected, which represent the secreted and intracellular forms of IL-15 (adapted from Meazza *et al.*, 1996).

3.2.4: Quantification of IL-7 protein by ELISA.

An enzyme-linked immunosorbent assay (ELISA) technique was developed to measure cytokine levels in homogenised liver tissue.

3.2.4.1: Extraction of protein from tissue powders.

Protein was extracted from approximately 50 mg powdered tissue using 300 μl of lysis buffer (1% Igepal, 0.5% deoxycholic acid, 0.1% SDS in PBS) to which protease inhibitors (10 μg/ml of PMSF [PhenylMethylSulfonulFloride, disolved in isopropoanol] and a 1/33 dilution of Aprotinin) had been added. All reagents for the extraction of protein were supplied by Sigma-Aldrich. The tissue powder was homogenised by passaging several times through a 21-gauge needle (Beckton Dickinson). The homogenate was then incubated on ice for thirty minutes. The solution was centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was harvested and the soluble protein yield from hepatic tissue powders was assessed using the BCA Protein Assay Kit (Pierce, see Appendix III [APIII.7]). The protein concentration of the samples was adjusted to approximately 10 mg/ml in sample diluent (20 mM TRIS base [BDH], 150 mM NaCl, 0.1% BSA, 0.05% Tween 20® [Sigma-Aldrich]).

3.2.4.2: IL-7 Sandwich ELISA.

ELISA antibody (Ab) pairs for the development of an assay for the detection of IL-7 protein were obtained from R&D Systems. The general protocol as recommended by the manufacturers was followed (see appendix III [APIII.8]). The standard null control (exclusion of capture and detection Abs) was found to be an inadequate control for the specific problems associated with hepatic tissue. Isotype matched IgG control Ab (Dako) was used instead of capture Ab (2 μg/ml) to control for non-specific binding of sample to plates and the contribution of endogenous biotin or alkaline phosphatase to the signal generated. The readings obtained from control IgG were subtracted from the readings obtained from samples. The capture (monoclonal anti-human IL-7, clone 7417.111) and detection (biotinylated anti-human IL-7) Abs were used at concentrations of 2 μg/ml and 50 ng/ml respectively. The assay was performed in a 96-well Nunc-Immuno® plate (Nalgene Nunc International).

Extravidin® Alkaline Phosphatase conjugate (Sigma-Aldrich, 1/2,500) and 1 mg/ml p-Nitrophenyl Phosphate (pNPP in 10% diethanolamine buffer, pH 9.8, containing 0.2% sodium azide, Sigma-Aldrich) generated the colormetric reaction. Recombinant human IL-7 (R&D Systems) was used to set up a standard curve (range 0 - 1000 pg/ml, prepared with sample diluent). The optical density was determined using a microplate reader (Dynatech MR4000) set to 410 nm. Samples and standards were assayed in triplicate and an average was taken of the triplicate readings. The concentration of IL-7 protein in the unknown samples was calculated against the standard curve and expressed as pg IL-7/100 mg of hepatic tissue. The detection limit of the assay under these conditions was determined as 7.8 pg/ml.

3.2.5: Quantification of IL-15 protein by ELISA.

Protein was extracted and measured as described above. IL-15 was quantified using the same technique as for the quantitative measurement of IL-7. The capture (monoclonal anti-human IL-15, clone 34505.11, R&D Systems) and detection (biotinylated anti-human IL-15, clone 34593.11, R&D Systems) Abs were used at concentrations of 2 μg/ml and 200 ng/ml respectively. Recombinant human IL-15 (R&D Systems) was used to set up a standard curve (range 0 - 1000 pg/ml, prepared with sample diluent). The concentration of IL-15 protein in the unknown samples was calculated against the standard curve and expressed as pg IL-15/100 mg of hepatic tissue. The detection limit of the assay under these conditions was determined as 7.8 pg/ml.

3.2.6: Localisation of IL-7 protein expression in liver tissue by Immunohistochemistry.

Sections (7 μm) were cut on a microtome (Microm HM 505N) from frozen blocks of hepatic tissue embedded in OCT, placed on glass slides coated with 2% 3-amino-propyl-triethoxy-saline in acetone (APES, Sigma-Aldrich) and dried overnight at room temperature. Immunoperoxidase staining of sections was carried out using the Vectastain® Elite ABC Kit (Vector Laboratories) according to the manufacturer's guidelines. In brief: slides were fixed in acetone for 10 minutes and transferred to phosphate buffered saline (PBS). Endogenous peroxidase was quenced by incubation of the sections in 0.3% H_2O_2 (in 0.3% normal goat

serum) for five minutes and then washed in PBS. Blocking was performed for 90 minutes with dilute normal goat serum (150 μl/10 mls PBS). Sections were incubated for 15 minutes in avidin followed by 15 minutes in biotin (Vector Laboratories Avidin/Biotin blocking kit). The primary Ab (anti-IL-7, rabbit polyclonal IgG, Santa Cruz) was incubated overnight at room temperature (5 μg/ml in PBS). Polyclonal rabbit immunoglobulin (Dako, 5.0 μg/ml in PBS) was used in place of the primary Ab as a negative control or background staining reference stain. Secondary antibody (anti-rabbit, Vectastain) and Avidin-Biotin-Complex (ABC, Vectastain HRP [Horse Radish Peroxidase]-congugated) was applied to the sections for 30 minutes. The HRP-substrate (diaminobenzidine tetrahydrochloride, DAB, Sigma-Aldrich) reaction was allowed to proceed for 7 minutes and sections were counterstained for 30 seconds in Mayer's haemotoxylin (BDH). Further details of the ABC-staining technique can be found in Appendix III (APIII.9).

3.2.7: Expression of IL-7 and IL-15 receptors on hepatic mononuclear cells.

3.2.7.1: Staining of hepatic mononuclear cells.

Flow cytometric analysis was used to determine the IL-7 and IL-15 receptor status of hepatic mononuclear cell populations (n = 6). The general staining protocol described earlier in Chapter 2, section 2.2.3 was followed. Samples were stained with phycoerythrin-conjugated (PE) mAbs directed against the IL-7 receptor α-chain (IL-7R-α, Immunotech, CD127, clone R34.34). The same samples were also stained with PE mAbs directed against the IL-2 receptor β-chain (IL-2R-β, Becton Dickinson, CD122, clone TU27), which forms part of the IL-15 receptor and correlates with IL-15 responsiveness. Levels of staining above those of the appropriate isotype-matched IgG control Abs were taken as positive.

3.2.7.2: Staining of hepatic haematopoietic progenitor cells.

A further seven samples were stained for IL-7/IL-15 receptor status as above with the addition of mAbs directed against CD45 (FITC) and CD34 (PerCP) to distinguish haematopoietic progenitor cells ($CD34^+CD45^+$).

3.2.7.3: Flow cytometric analysis.

Forward scatter (size) and side scatter (granularity) parameters were used to select cells of low to medium granularity (R1, figure 3.2.4). Histogram analysis was used to assess the levels of staining for CD127 and CD122 on cells falling in R1 (figure 3.2.4). For assessment of the expression of CD122/CD127 on HSC populations, $CD34^{+}CD45^{-}$ (R2, figure 3.2.4) and $CD34^{+}CD45^{+}$ (R3 figure 3.2.4) cell populations were gated and the level of expression of CD122/CD127 was analysed.

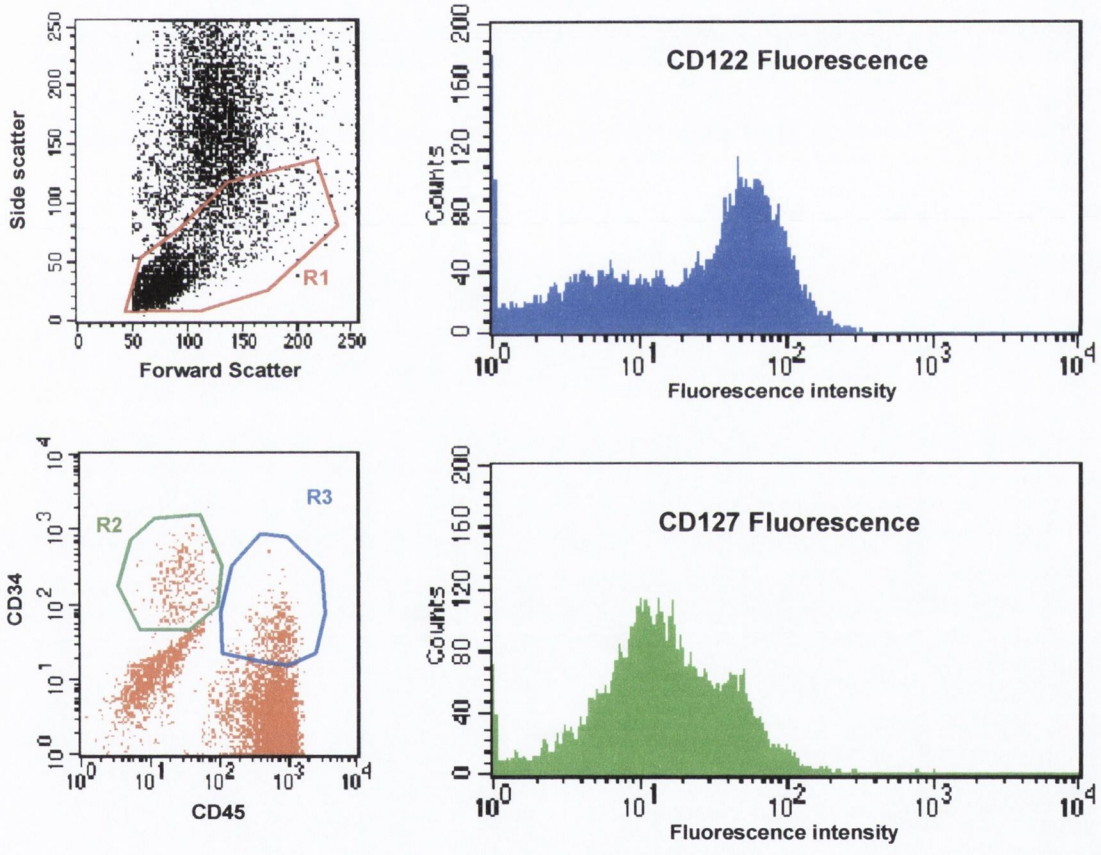


Figure 3.2.4: IL-7 and IL-15 receptor expression on hepatic mononuclear cell and stem cell populations.

Forward scatter (size) and side scatter (granularity) parameters were used to select cells of low to medium granularity (R1). Histogram analysis was used to assess the levels of staining for CD127 and CD122 on all cells falling in R1 which contains mature and immature lymphocyte populations. For assessment of the expression of CD122/CD127 on HSC populations $CD34^{+}CD45^{-}$ (R2) and $CD34^{+}CD45^{+}$ (R3) cell populations were gated and the level of expression of CD122/CD127 was analysed.

3.2.8: Statistical analysis.

A two-tailed unpaired Student's t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant. Spearman Rank was used for correlation analysis.

3.3 Results.

3.3.1: RNA extraction and cDNA integrity.

Total RNA was successfully extracted from hepatic tissue powders (n = 16) and control cell line pellets (n = 2) using the RN*Ace* Total Pure RNA purification system (Bioline). Mean yield of RNA from approximately 50 mg of hepatic tissue powder was 34.74 μg of total RNA (range 22.7 – 41.7, standard error of the mean [SEM] = 1.42, see appendix V for details on the individual samples). One μg of total RNA was successfully transcribed into cDNA in all cases as assessed by PCR amplification of the housekeeping gene GAPDH (figure 3.3.1).

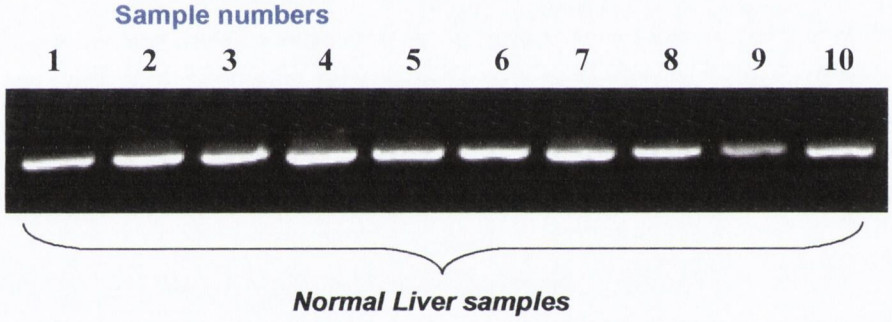


Figure 3.3.1: PCR amplification of the housekeeping gene GAPDH to assess the integrity of the cDNA generated from RNA isolated from normal liver tissue.

For normal liver samples (1 - 10) the GAPDH product is shown. The remaining 5 liver samples and control cell lines were also positive for GAPDH (not shown).

3.3.2: Detection of IL-7 mRNA transcripts.

Two distinct DNA fragments were obtained by PCR-amplification of reverse transcribed mRNA from the human liver adenocarcinoma cell line SK-HEP-1 which served as a positive control for IL-7 mRNA production. The detected transcripts included the predicted 620 bp fragment of IL-7, in addition to a smaller product, corresponding to a 488 bp fragment produced by alternative splicing of IL-7 mRNA (Goodwin *et al.*, 1989). No IL-7 products were detected in the Acute Lymphocytic Leukaemia cell line CRL8286, which served as a negative control in all PCR experiments. The 620 bp IL-7 specific product was detected in nine of the ten normal liver biopsy samples tested. Six of the nine samples that were positive for the 620

bp product were also positive for the 488 bp splice variant (figure 3.3.2). The sample in which no 620 bp fragment was detected was also negative for the smaller product (sample 8, table 3.3.1). The relative amount of the two distinct IL-7 products detected in each sample was estimated by comparison of the optical density (OD) of the bands in the gel (488/620 OD ratio). The 620 bp band was more intense in all but one of the samples. On average the lower molecular weight product was expressed at approximately 37% of the level of the 620 bp product. For the SK-HEP-1 cell line the 488 bp band was approximately one quarter of the intensity of the larger product (table 3.3.1).

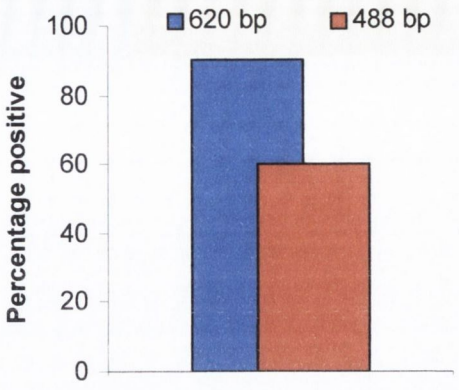


Figure 3.3.2: Percent of samples expressing IL-7 specific bands.

Table 3.3.1: The expression and relative levels of two distinct IL-7 transcripts detected in normal hepatic tissue and control cell lines.

Sample No.	Type	ID	620 bp PCR product	488 bp PCR product	OD* Ratio 488/620 (arbitrary units)
1	Liver	D160598	+	+	0.28
2		D080398	+	+	0.1
3		D180996	+	-	0
4		D120498	+	+	0.29
5		D130498	+	+	0.19
6		D210699	+	+	0.93
7		D161296	+	-	0
8		D150798	-	-	ND
9		D190796	+	+	1.01
10		D210497	+	-	0.52
0A	+ Control	SK-HEP-1	+	+	0.26
0B	- Control	CRL-8286	-	-	ND

*Abbreviations: OD: Optical Density. ND: not done.

To estimate the relative levels of IL-7 mRNA in normal liver samples, the intensity of IL-7 product (OD, arbitary units) band obtained with 10 μl of PCR product was normalised against the GAPDH-housekeeping gene product band obtained with 5 μl of PCR product (620/GAPDH or 488/GAPDH OD ratio, figure 3.3.3). Five μl of GAPDH product was used so that the OD of the IL-7 and GAPDH could be measured in the same gel using the same settings as 10 μl produced GAPDH bands that were too intense. A typical gel showing the GAPDH and IL-7 transcripts in control cell lines and two liver samples is shown in figure 3.3.4.

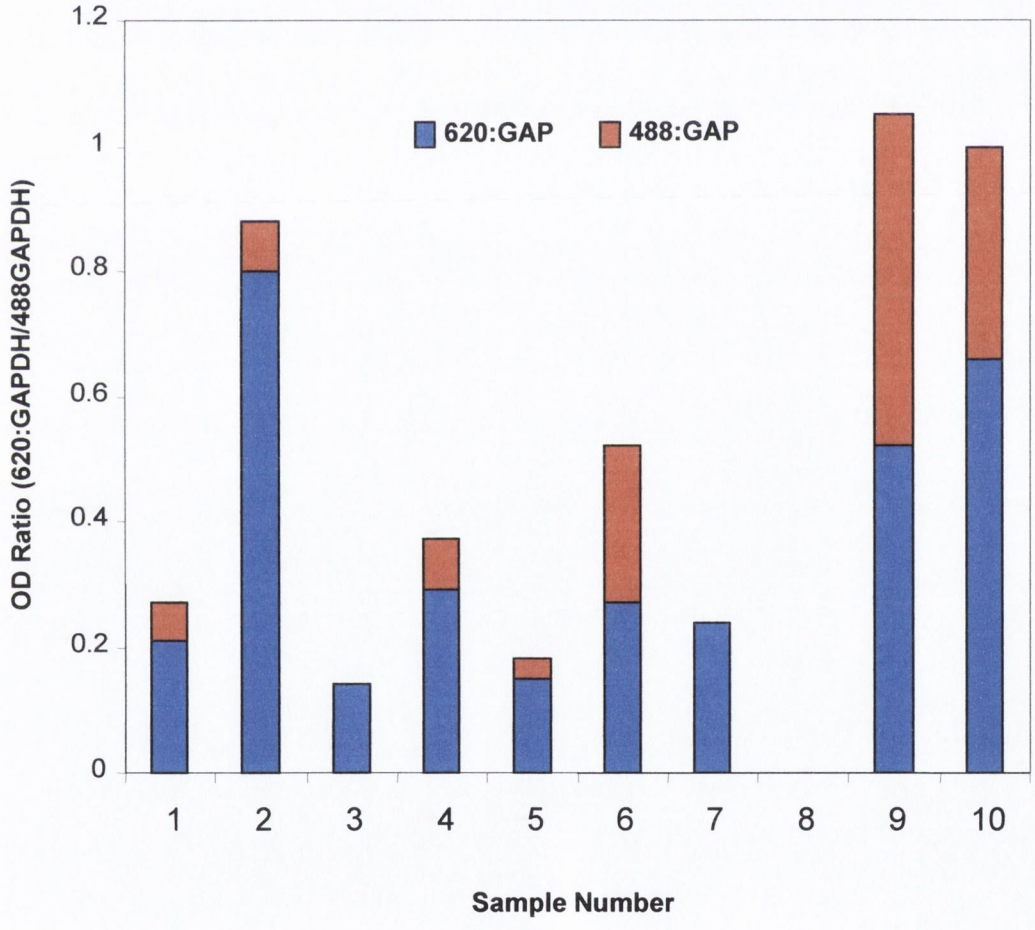


Figure 3.3.3: Relative levels of interleukin-7 (IL-7) transcripts in normal liver samples.

The blue columns represent the levels of the 620 bp product (which is known to give rise to the functional protein) and the red shows the level of the 488 bp product in the same sample. Nine of the ten samples were positive for the 620 bp product. Six of the nine were also positive for the 488 bp product (usually at a much lower intensity than the larger molecular weight product). Sample number 8 was negative for both bands.

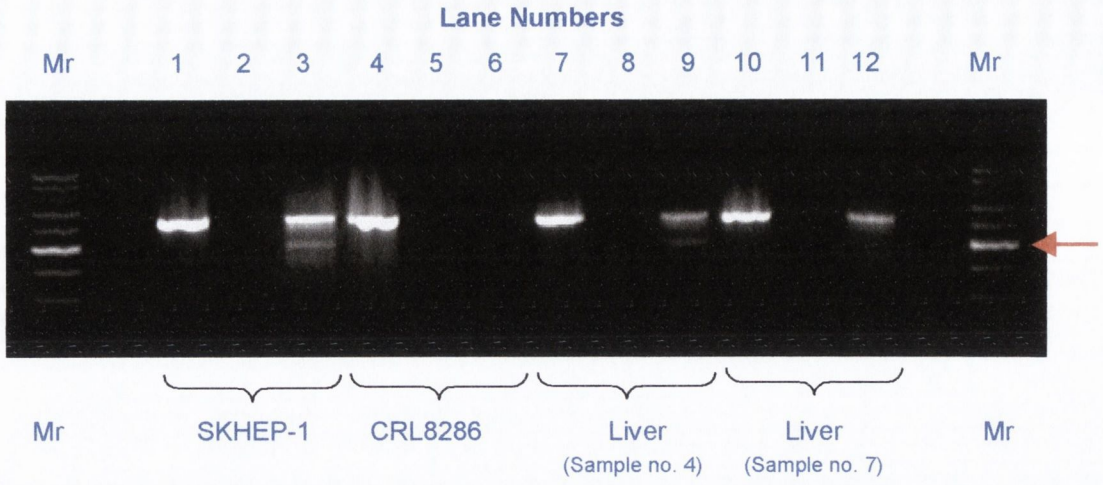


Figure 3.3.4: Representative gel of Interleukin-7 and GAPDH PCR products for control cell lines and two hepatic tissue samples.

PCR for IL-7 and GAPDH (housekeeping gene) was carried out as described in materials and methods. For each of the samples, the GAPDH product (lanes 1, 4, 7 and 10), the genomic control (negative, lanes 2, 5, 8 and 11) and the IL-7 products (620/488 bp) are shown. The genomic control consisted of products of the reverse transcription reaction to which water was added instead of the reverse transcriptase enzyme. The human liver adenocarcinoma cell line SK-HEP-1 (ATCC) was used as a positive control (lane 3) and the Acute Lymphocytic Leukaemia (B-cell) line CRL-8286 (ATCC) served as a negative control for IL-7 mRNA production (lane 6). The liver sample in lane 9 expressed two distinct IL-7 specific PCR products, 620 bp and 488 bp fragments, as did the positive control (lane 3). The second liver sample expressed only the 620bp fragment (lane 12). Mr = molecular weight (100 bp ladder standards). The red arrow indicates the 500 bp standard.

3.3.3: IL-15 mRNA transcripts detected in normal liver.

Nine normal liver samples were tested for the presence of IL-15 specific transcripts. The primers used amplify two distinct products (Meazza *et al.*, 1996). All of the samples tested (100%) were positive for the 524bp product, which corresponds to the secreted form of IL-15. Seven of the samples (78%) were also positive for the 650bp product, which corresponds to the intracellular form of IL-15 (figure 3.3.5). In all cases, the smaller 524bp product was expressed at a much higher intensity than the larger (650bp) product. The relative intensities of the two IL-15 product bands in each of the samples were estimated using densitometry (as described earlier for IL-7) and are shown in figure 3.3.6 (a

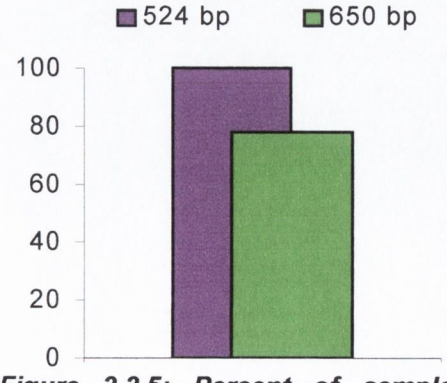
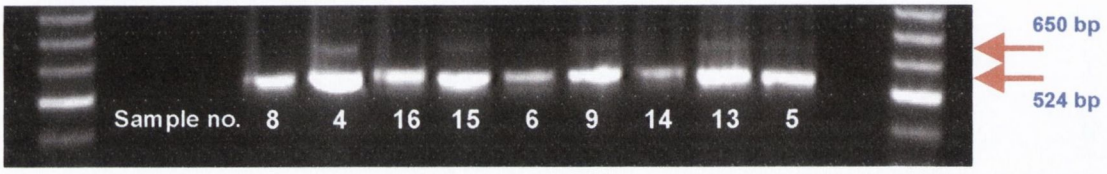


Figure 3.3.5: Percent of samples expressing IL-15 specific bands.

and b). On average the higher molecular weight product was expressed at approximately 18% of the level of the 524 bp product. The relative levels of IL-15 mRNA in individual samples was estimated by normalisation to the GAPDH-housekeeping gene (650/GAPDH or 524/GAPDH OD ratio, figure 3.3.6c).

a) Interleukin 15 PCR product bands electrophoresed on a 2% agarose gel.



b) Table showing the GAPDH and IL-15 PCR products in 10 normal liver samples.

Sample no.	GAPDH	524 bp (secreted)	650 bp (intracellular)	650/524 (OD ratio)
4	+	+	+	0.42
5	+	+	+	0.21
6	+	+	-	0
8	+	+	-	0
9	+	+	+	0.33
13	+	+	+	0.36
14	+	+	-	0
15	+	+	+	0.26
16	+	+	-	0

c) Relative levels of interleukin-15 transcripts in normal liver samples.

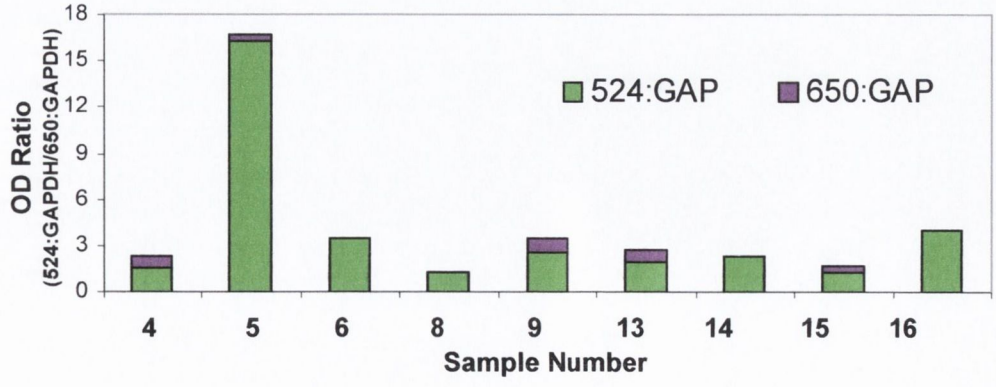


Figure 3.3.6: Normal liver expresses the secretory isoform of the Interleukin-15 gene.

All of the nine samples were positive for the 524 bp product. Five of the nine were also positive for the 650 bp product at a much lower intensity than the smaller molecular weight product.

Five of the samples, which were positive for IL-15 transcripts (numbers 4-6 and 8-9), had also been tested previously for the presence of IL-7 specific transcripts. Four of these five samples were also positive for IL-7 mRNA. Sample number 8 tested negative for IL-7 mRNA.

3.3.4: Protein extraction from hepatic tissue powders.

Soluble protein was extracted from seven normal liver samples and yield was estimated using the BCA protein assay. Mean protein yield per 100 mg of frozen tissue powder was 10.74 mg (range 5.87 – 14.60, Table 3.3.2).

Table 3.3.2: Protein extraction from normal hepatic tissue samples.

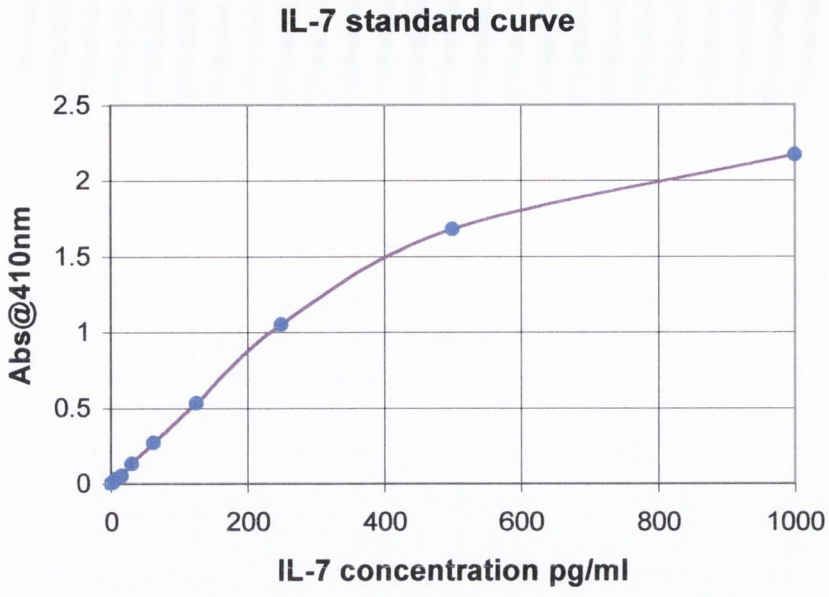
Sample No.	Type	ID	Protein [ ]* mg/ml	Protein [ ] mg/100 mg tissue
2	Liver	D080398	30.37	11.05
3		D180996	51.85	14.60
4		D120498	44.65	11.83
5		D130498	35.56	13.29
7		D161296	42.56	5.87
8		D150798	35.69	12.59
11		D060698	31.96	5.94

*[ ] = concentration.

3.3.5: Measurement of IL-7 protein in normal liver by ELISA.

A sandwich ELISA was used to quantify IL-7 protein levels in homogenised normal hepatic tissue (n = 7). The mean level of IL-7 cytokine protein detected in liver was 41.95 pg/100 mg tissue powder (range 18.47 – 76.93, figure 3.3.7). The intensity of the bands for IL-7 message (620 bp or 488 bp) detected in the PCR assay did not correlate with IL-7 protein levels (figure 3.3.8).

a.



b.

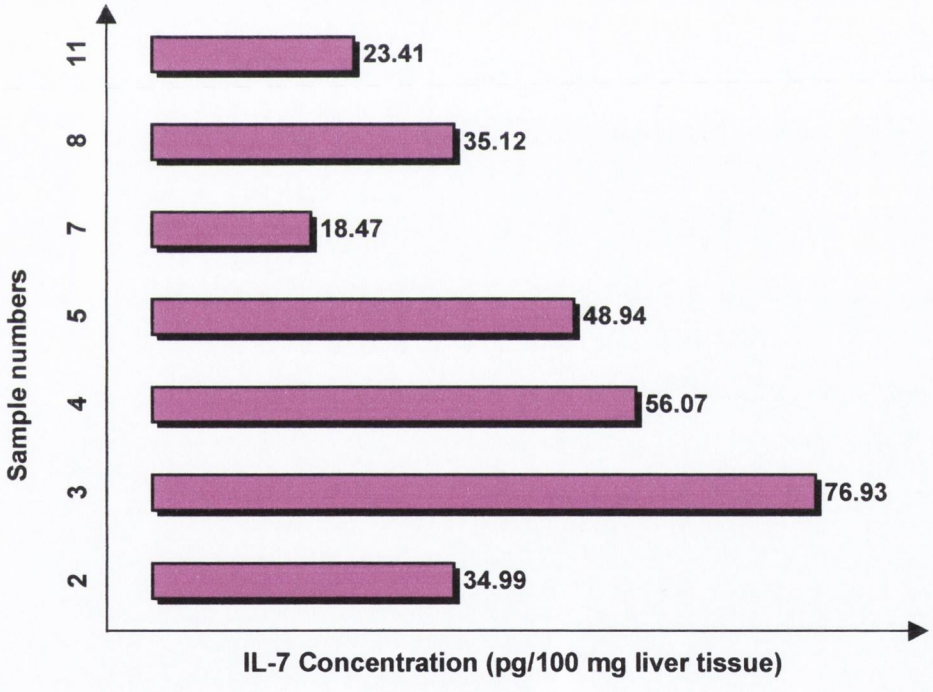
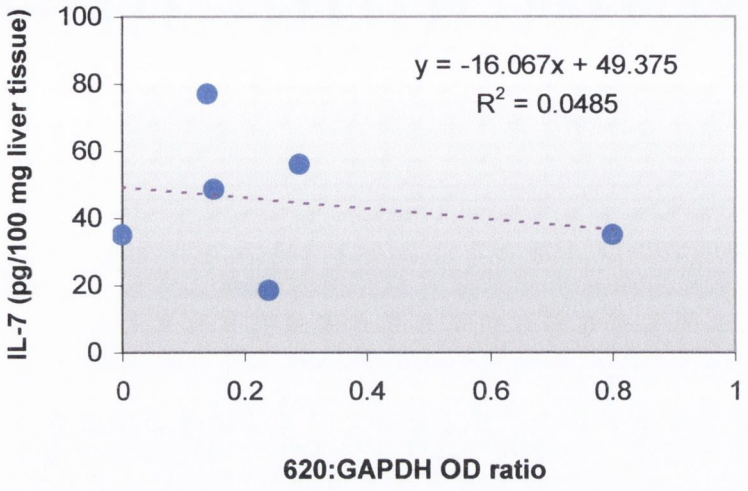


Figure 3.3.7: Levels of IL-7 protein detected in normal liver tissue by ELISA.

Normal liver tissue obtained from donor organs (n = 7) at the time of transplantation was snap frozen and powdered. Protein was extracted from the tissue powders and the amount of IL-7 protein in each of the liver samples was quantified using a sandwich ELISA system as described in materials and methods. Recombinant human IL-7 was used to construct a standard curve **(a).** The amount of IL-7 protein detected in the hepatic samples had a normal distribution (mean 41.95 pg/100 mg tissue powder, range 18.47 – 76.93) **(b)**.

a.



b.

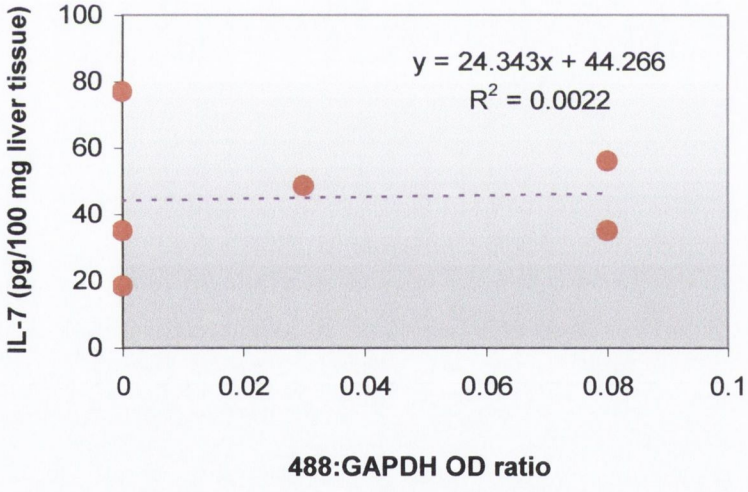


Figure 3.3.8: Relationship between IL-7 mRNA levels and protein levels.

Semi-quantitative RT-PCR was used to estimate the relative quantities of IL-7 specific RNA transcripts in normal liver biopsies. PCR amplification of IL-7 specific transcripts produced two distinct products, a 620 base pair (bp) fragment corresponding to the full length cDNA and a smaller 488 bp splice variant. The relative levels of IL-7 mRNA in the individual liver samples were estimated by comparing the optical density of the IL-7 product band (OD, arbitrary units) obtained with 10 μl of PCR product, normalised against the GAPDH-housekeeping gene product band obtained with 5 μl of PCR product (620/GAPDH or 488/GAPDH OD ratio). For 6 of the samples used in the PCR assay IL-7 cytokine levels were measured by ELISA (pg IL-7/100mg tissue). Comparison of the protein levels with the 620 bp **(a)** or the 488 bp **(b)** product levels showed no correlation existed between the levels of either PCR product and cytokine protein levels. Spearman rank was used for correlation analysis.

3.3.6: Measurement of IL-15 protein in normal liver by ELISA.

A sandwich ELISA was used to quantify IL-15 protein levels in homogenised normal hepatic tissue (n = 5). The mean level of IL-15 cytokine protein detected in liver was 116.34 pg/100 mg tissue powder (range 31.94 – 193.84, figure 3.3.9). For five normal liver samples there was sufficient tissue available to carry out ELISAs for both IL-7 and IL-15. On average 2-3 times more IL-15 was detected in hepatic tissue than IL-7 (figure 3.3.10).

3.3.7: Anatomic distribution of IL-7 protein within hepatic tissue.

Immunohistochemical staining of normal hepatic tissue showed that IL-7 protein was widely distributed throughout the parenchyma as well as being localised in portal tracts (figure 3.3.11). The cells staining positively for IL-7 were of lymphoid morphology. IL-7 secretion by hepatocytes was not detected under the conditions used for immuno-enzyme staining of frozen liver tissue.

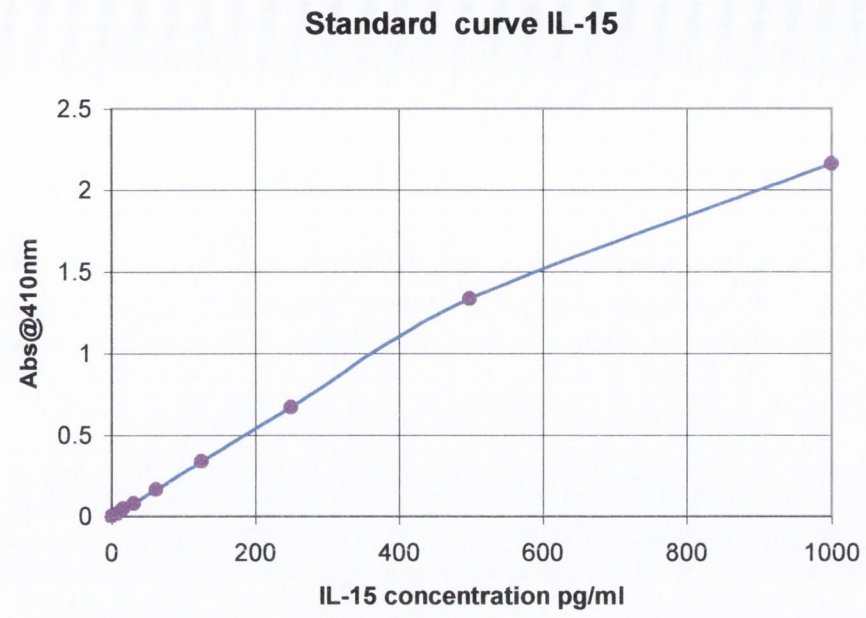
3.3.8: Expression of IL-7 and IL-15 receptors on hepatic mononuclear cells.

The expression of CD122 (IL-2/IL-15R-β) and CD127 (IL-7R-α) was analysed within a lymphogate which includes all mature lymphocyte populations as well as immature progenitor cells. CD122 was expressed by 35.88% (mean, n = 6, range 22.62 – 45.62) of cells falling within this gate. Fewer cells within the lymphogate expressed CD127 (mean 18.49%, n = 6, range 8.33 – 31.17, figure 3.3.12).

3.3.9: Expression of IL-7 and IL-15 receptors on hepatic stem/progenitor cells.

To determine if hepatic stem/progenitor cells expressed IL-15R-β or IL-7R-α, seven normal liver samples were stained with a combination of anti-CD45 and anti-CD34 to identify HSCs with anti-CD122 or anti-CD127. On average, 61% of $CD34^{+}CD45^{+}$ hepatic HSCs co-expressed CD122 (mean, range 40.8 – 78.82). Fewer $CD34^{+}CD45^{+}$ cells co-expressed CD127 (mean 46.66, range 25.37 – 61.46, figure 3.3.13). No expression of either CD122 or CD127 was detected on $CD34^{+}CD45^{-}$ hepatic cells.

a.



b.

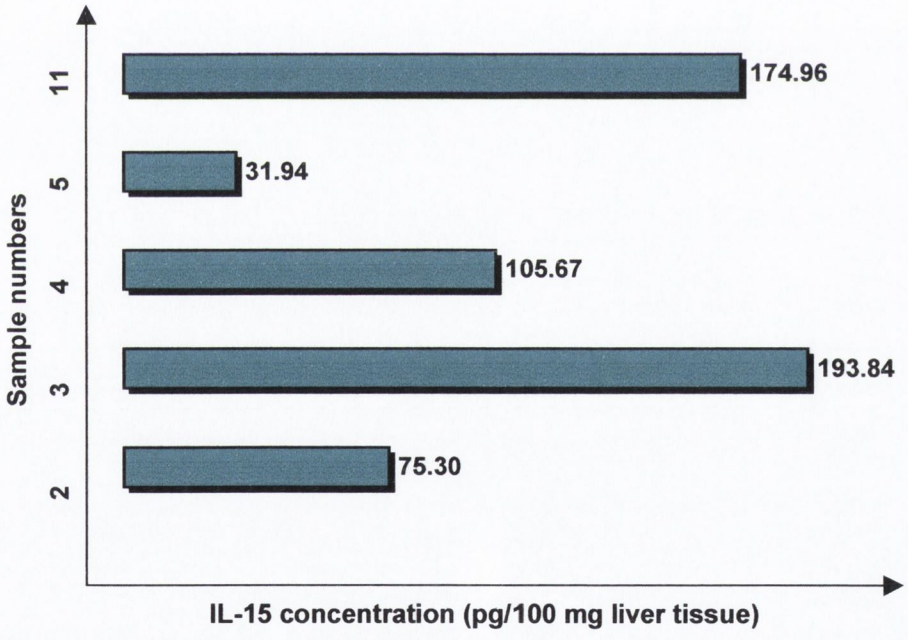


Figure 3.3.9: Levels of IL-15 protein detected in normal liver tissue by ELISA.

Normal liver tissue obtained from donor organs (n = 5) at the time of transplantation was snap frozen and powdered. Protein was extracted from the tissue powders and the amount of IL-7 protein in each of the liver samples was quantified using a sandwich ELISA system as described in materials and methods. Recombinant human IL-15 was used to construct a standard curve **(a).** The quantity of IL-15 protein detected in hepatic samples ranged between 31.94 and 193.84 pg/100 mg tissue (mean 116.34 pg) **(b)**.

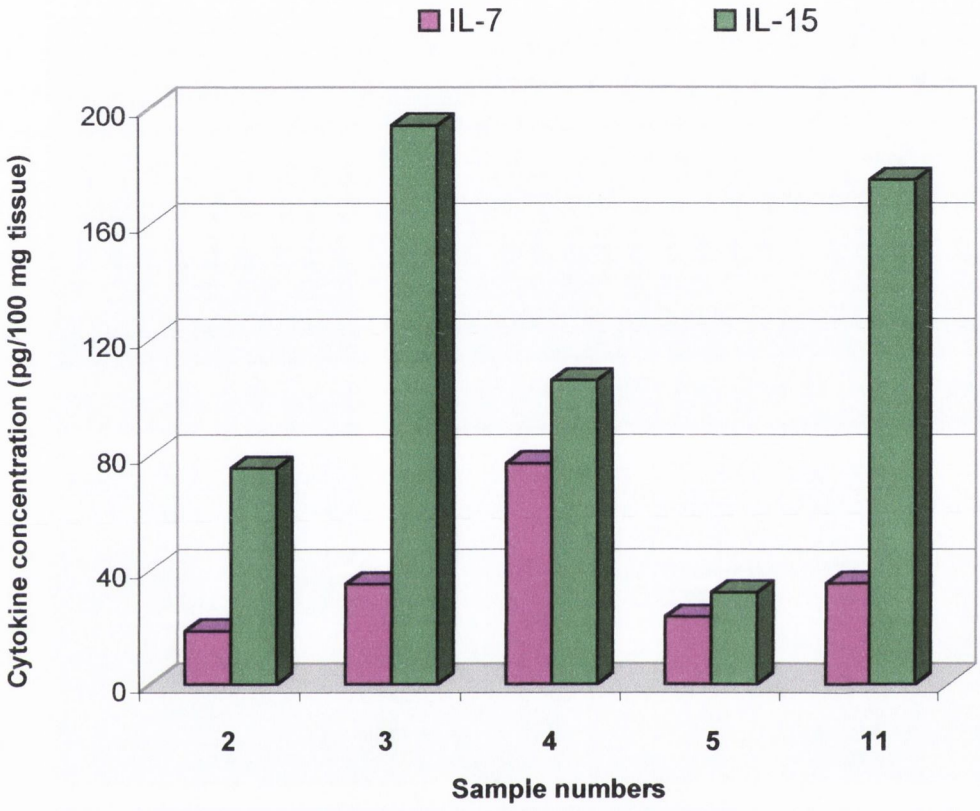


Figure 3.3.10: Comparison of IL-7 and IL-15 levels in normal liver samples tested for both lymphopoietic cytokines.

The five normal liver samples for which IL-15 levels had been measured had also been assessed for IL-7 levels. Seven normal liver samples had been assessed for IL-7 levels, but for two of these, insufficient sample remained to carry over to the IL-15 ELISA. All five of the samples tested for both IL-7 and IL-15 were positive for both cytokine proteins at varying levels. Comparison of the levels of IL-7 and IL-15 showed that on average IL-7 was expressed at approximately one third the levels of IL-15 (means 41.95 v 116.34 pg/100 mg tissue respectively). The level of each of the cytokines appear to be independent of each other in that a high level of IL-7 did not equate with a low or high level of IL-15.

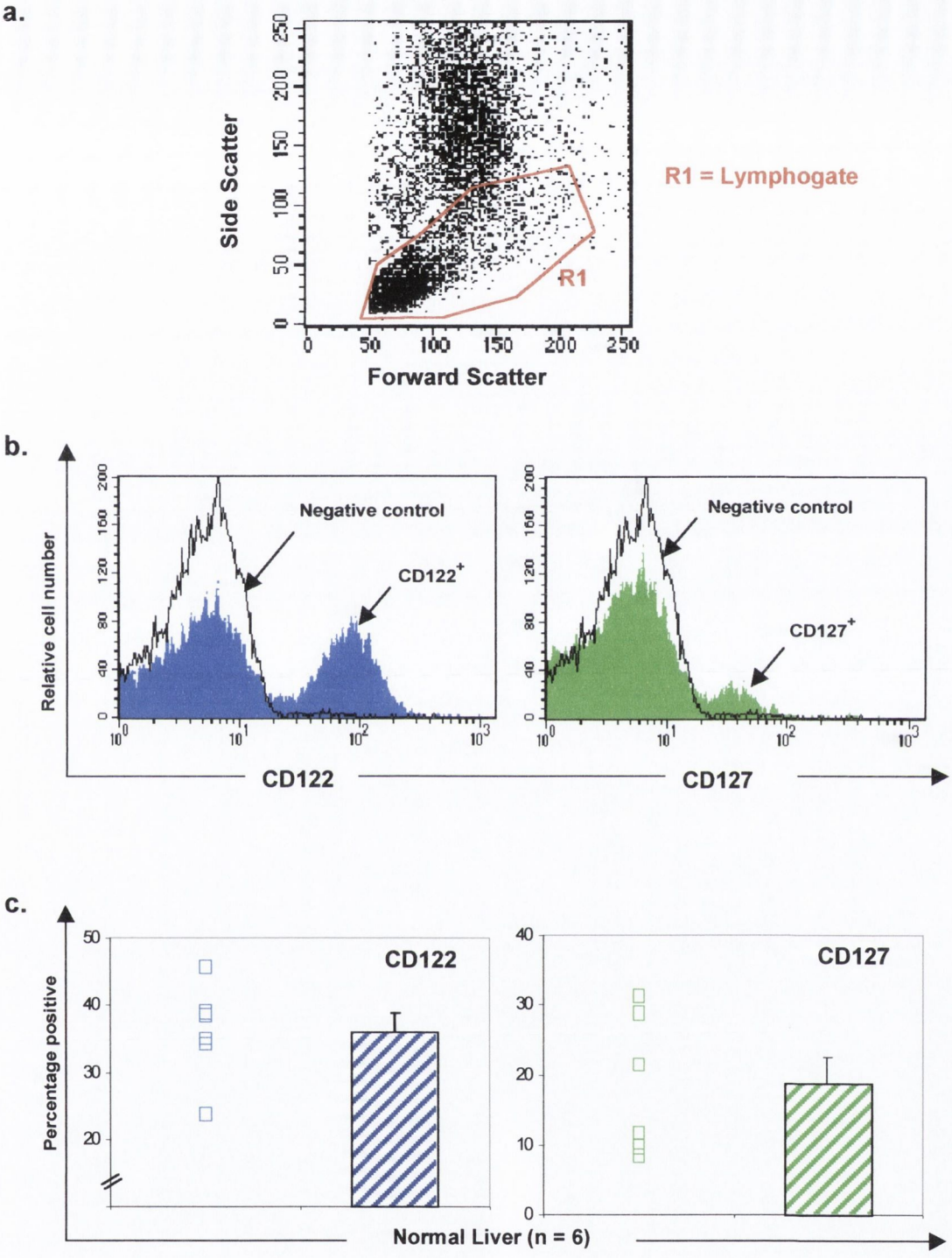


Figure 3.3.12: IL-2/IL-15 receptor-β (CD122) and IL-7 receptor-α (CD127) expression on hepatic mononuclear cells.

a) Forward:Side scatter morphology plot of an hepatic mononuclear cell preparation showing the lymphogate (R1) used for analysis. **b)** Histogram analysis of $CD122^+$ and $CD127^+$ cells within the lymphogate. **c)** Combination plots showing the percentage of cells positive in each of the individual samples for CD122 (blue) and CD127 (green) and the mean value for all six samples plus the standard error of the mean.

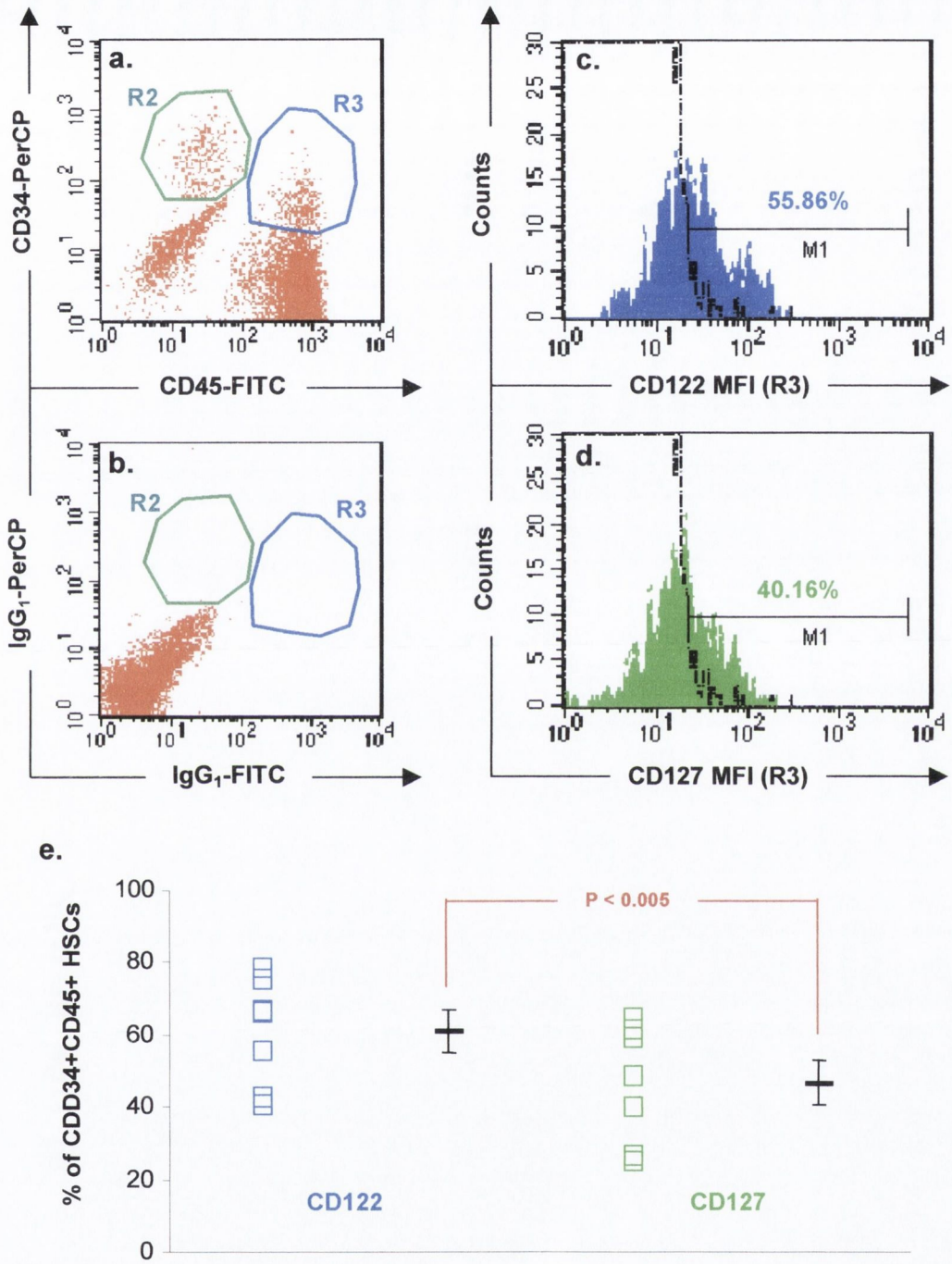


Figure 3.3.13: IL-2/IL15 receptor-β (CD122) and IL-7 receptor-α (CD127) expression on hepatic haematopoietic stem/progenitor ($CD34^+CD45^+$) cells.

a) Dotplot of CD45 (FITC) and CD34 (PerCP) staining of hepatic mononuclear cells showing $CD34^+CD45^-$ (R2) and $CD34^+CD45^+$ (R3) gated for analysis. **b)** Negative control. **c)** CD122 and **d)** CD127 levels on $CD34^+CD45^+$ hepatic cells. **e)** Percentage of $CD34^+CD45^+$ cells positive in each of the individual samples for CD122 (blue) and CD127 (green) and the mean value for all six samples plus the standard error of the mean. A significantly higher proportion of hepatic HSCs co-express CD122.

3.4 Discussion

In humans, IL-7 is a critical cytokine for normal T-cell development (Lai *et al.*, 1997; Plum *et al.*, 1996; Puel and Leonard, 2000) and IL-15 plays an essential role in the development of NK cells (Cavazzana-Calvo *et al.*, 1996; Mrózek *et al.*, 1996). Studies in mice suggest that the developmental needs of NK T cells include a dependence on IL-15 (Ohteki *et al.*, 1997). Thymic independent IEL subsets display a differential dependence on both these lymphopoietic cytokines, with IL-7 being essential for TCR-$\gamma\delta$ (Laky *et al.*, 2000) and IL-15 for TCR-$\alpha\beta$CD8$\alpha\alpha$ IEL development (Porter and Malek, 1999). The normal AHL contains non-conventional innate T-cell subsets, including large numbers of TCR-$\gamma\delta$, CD8$\alpha\alpha$ and NK T cells, some of which may differentiate locally from haematopoietic stem cells already described in the liver (Abo *et al.*, 1999; Collins *et al.*, 1996; Crosbie *et al.*, 1999; Golden-Mason *et al.*, 2000; Norris *et al.*, 1998). However the presence of both these critical lymphopoietic cytokines in normal adult human liver (AHL), a potential site extrathymic T-cell differentiation has not previously been demonstrated. In this study, whole hepatic tissue, isolated from perfused donor organs at the time of liver transplantation, was used to determine the expression of IL-7 and IL-15. Tissue was snap frozen and only manipulated in the presence of RNAse or protease inhibitors so as to avoid stimulation caused by cell isolation techniques in order to estimate *in vivo* expression as accurately as possible.

Several IL-7 transcripts have previously been described in human (and mouse) tissue. Human IL-7 activity has been attributed only to the protein translated from the complete mRNA (620 bp product). In the alternatively spliced transcript, the reading frame is maintained (488 bp product) (Goodwin *et al.*, 1989) although, it has not yet been confirmed that this smaller transcript is translated into a functional protein. In hepatic tissue, the smaller molecular weight IL-7 PCR product was less intense and only detected in 60% of liver samples. A similar expression pattern of these two IL-7 transcripts has been observed in the human intestine (Madrigal-Estebas *et al.*, 1997), a site believed to be important in the generation of local IEL subsets (Laky *et al.*, 2000). The biological significance of this finding is unknown. It has been suggested that splicing-derived IL-7 isoforms may play a role in modulating IL-7 mediated biological effects (Korte *et al.*, 1999). The mRNA species (620 bp

product), known to give rise to the functional protein, was detected in all but one of the liver samples, suggesting constitutive expression of IL-7 message in normal hepatic tissue. The negative result obtained for one of the liver samples may possibly be due to RNA degradation. RNA extraction from hepatic tissue is subject to some degree of degradation. Positive amplification of GAPDH in this sample indicates a relatively intact RNA preparation, however, the absence of IL-7 message may be a reflection of the relative lability of cytokine mRNA.

The control of IL-15 expression is complex, with regulation at the levels of transcription, translation and intracellular trafficking (Waldmann and Tagaya, 1999). Constitutive expression of IL-15 mRNA, as determined by Northern blot analysis, has been demonstrated in a variety of tissues such as placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells and monocytes. However, it has been difficult to demonstrate IL-15 in supernatants of many cells that express such mRNA (Bamford *et al.*, 1996; Grabstein *et al.*, 1994). This observation may be explained, in part, by the more recent discovery of two IL-15 mRNA splice variants (Meazza *et al.*, 1996), which give rise to identical mature cytokine proteins but differ in their signal sequences. The signal peptides of IL-15 isoforms direct intracellular trafficking of the protein. The isoform containing the long signal peptide (48aa) is directed to the secretory pathway while the short signal peptide (21aa) isoform is retained in the cytoplasm or the nucleus (Tagaya *et al.*, 1997). In this study we used primers which distinguish both species of the IL-15 mRNA (Meazza *et al.*, 1996). We found that the normal liver predominantly expresses the mRNA species associated with the long signal peptide suggesting that IL-15 is produced and secreted in the normal AHL. The alternative transcript of IL-15 associated with intracellular forms of the cytokine was also detected at a much lower intensity and in only 78% of samples. The physiological significance of the expression of an intracellular form of IL-15 in hepatic tissue is unknown, as a role for this form of the protein has not yet been elucidated.

Although detection of mRNA can give us valuable information regarding the expression of genes in tissue, cytokine genes are often subjected to translational regulation, thus,

physiologically significant data regarding the expression of cytokines is more likely to come from measurement of cytokine protein. Both IL-7 (Namen *et al.*, 1988) and IL-15 (Bamford *et al.*, 1996; Grabstein *et al.*, 1994) contain AUGs in their 5' untranslated regions which dramatically reduce the efficiency of mRNA translation into protein. Using an ELISA system, mean levels of 41.99 pg IL-7 per 100 mg hepatic tissue were detected in normal liver samples, which indicates that the cytokine protein is present in more than adequate amounts to exert its effect on responsive cells. The complex nature of the hepatic tissue homogenate used in the ELISA assay can generate false signals and thus obscure the true level of cytokine detectable in the sample. The specific problems associated with whole liver homogenate include adhesion to plastic and endogenous biotin and alkaline phosphatase activity. Null controls produce signals almost as strong as the signal generated using specific antibody as none of the binding sites on the plastic have been blocked by antibody. The endogenous biotin in the sample accounts for the signal generated in the absence of secondary antibody which is also contributed to by endogenous alkaline phosphatase activity (shown by the fact that a weak signal is produced when extravidin-alkaline phosphatase is omitted). The use of a null control would therefore lead to under-estimation of the level of cytokine. The binding of sample to plastic can be blocked using 5-10% BSA. However, this control may result in over-estimation of the levels of cytokines as it may be a more efficient block of non-specific sites than the primary antibody used in the assay. The use of an isotype matched IgG control antibody, at the same concentration as the primary anti-cytokine-specific antibody, controls for all the above problems and thus generates readings that reflect the true level of cytokine protein in liver homogenates. Interestingly, the sample which was negative in the IL-7 PCR assay, was positive for the protein, supporting the assumption that the lack of message was due to RNA degradation.

Local production of IL-7 appears to be required for successful T-cell differentiation, in particular for maturation of γδ-TCR bearing T cells. Lakay and colleagues (1998) established that extrathymic IL-7 expression was sufficient to promote development of the TCR-γδ subset of intestinal lymphocytes in thymectomised, irradiated, bone-marrow-reconstituted chimeric mice, whereas thymic expression of IL-7 was necessary to promote development of TCR-γδ

cells in the thymus (Laky *et al.*, 1998). In a further study, (using the intestinal fatty acid binding protein promoter to reinstate IL-7 expression in mature enterocytes of IL-$7^{-/-}$ mice), they demonstrated that IL-7 expression by enterocytes was sufficient for extrathymic development of TCR-$\gamma\delta$ cells *in situ* within the intestinal epithelium, but not at other sites (Laky *et al.*, 2000). Thus, IL-7 protein at the site of T-cell maturation is likely to be more important than systemic or thymic expression of IL-7 in promoting the generation of T cells from HSCs at extrathymic sites. Surprisingly high proportions of hepatic T lymphocytes are $\gamma\delta$-TCR^{+}, approximately 15% compared with 3.5% in peripheral blood (Norris *et al.*, 1998), a population likely to be of extrathymic origin.

A mean level of 116.34 pg of IL-15 per 100 mg hepatic tissue was detected in normal liver samples by ELISA. Whole tissue was used in this assay, which would result in detection of both secreted and intracellular forms of IL-15. However, the predominant expression of the mRNA isoform associated with secreted IL-15 suggests that the cytokine protein is secreted, and therefore available, in more than adequate amounts to exert its effect on responsive cells. The environmental conditions within which common lymphoid progenitors develop determines their fate (Kondo *et al.*, 1997). Addition of IL-15 to foetal thymic organ cultures (FTOCs) shifts differentiation away from conventional TCR-$\alpha\beta$ cell towards NK cell differentiation while differentiation towards TCR-$\gamma\delta$ cells is unaffected (Leclercq *et al.*, 1996). The predominance of NK, NK T and TCR-$\gamma\delta$ lymphocytes in liver (57% of lymphocytes) compared to peripheral blood (18%) (Doherty and O'Farrelly, 2000) may be due to the relatively high hepatic level of IL-15 acting in conjunction with IL-7 on lymphoid precursors (Le *et al.*, 2001).

Our results indicate that normal AHL has a constitutive supply of IL-7 and IL-15 protein, which may act on hepatic HSCs or other IL-7/IL-15 responsive cells. The amount of IL-7 protein detected in individual samples did not correlate with the intensity of the PCR-product bands. This may be indicative of the inherent instability of cytokine mRNA species or, of the inability of densitometry to pick up subtle differences, as protein concentrations did not vary widely between the individual samples. Alternatively, the lack of correlation could be suggestive of an indirect relationship between message and protein levels due to post-transcriptional

regulation as has previously been shown for IL-15 (Waldmann and Tagaya, 1999). This inconsistency demonstrates the importance of determining cytokine protein levels in addition to message.

Immunohistochemical staining of liver sections demonstrated IL-7 protein in discrete cells of lymphoid morphology, widely distributed throughout the parenchyma and within portal tracts, with some particularly strong staining of a number of cells in groups of two or three. The staining pattern observed was at first surprising as it was expected that the principle source of IL-7 would be hepatocytes, since IL-7 mRNA has been detected in human derived hepatocyte cell lines (Goodwin *et al.*, 1989; Rowell *et al.*, 1997). In the murine intestine, $\alpha\beta$-TCR$^+$ intra epithelial lymphocytes produce much higher levels of IL-7 mRNA than IELs which also secrete IL-7 (Fujihashi *et al.*, 1996). It is possible that hepatic T cells are the highest producers of IL-7 in the liver. Our staining did not detect IL-7 in hepatocytes, however, low-level production of IL-7 by hepatocytes may be below the detection limit of immunohistochemistry.

Despite reports of IL-15 mRNA production in a wide variety of tissues, only activated macrophages and epithelial cells are reported to be able to produce IL-15 protein (Yoshikai and Nishimura, 2000). In this study, despite the presence of higher amounts of IL-15 protein than IL-7 (as assessed by ELISA), we were unable to localise IL-15 expression in hepatic tissue. We tested two antibodies (polyclonal anti-human IL-15 (L-20), Santa Cruz Biotechnology, Inc. and anti-human IL-15, clone 34593.11, R&D Systems). Both the antibodies proved to be unsuitable for staining of hepatic tissue under the conditions used. IL-15 has successfully been detected by immunohistochemistry in frozen human intestinal sections using Immunex monoclonal antibodies (clones M110, M111 and M112), CD68 positive tissue macrophages scattered throughout the intestine were the highest producers of IL-15 protein (Maiuri *et al.*, 2000). The technique used by Maiuri *et al.* (2000) may be suitable for staining of hepatic tissue, however for the purposes of this study we did not pursue the localisation of IL-15 in hepatic tissue as we felt that the ELISA studies provided sufficient evidence as to the presence of IL-15 protein in normal liver tissue.

Studies of cytokine receptor expression have proven valuable in pinpointing where specific ligand-receptor pairs have biological activities. Cytokines, such as KL (c-Kit ligand) and FL (flt-3 ligand) are widely expressed in different tissues, in contrast to their receptors which have a more restricted expression pattern (Lyman and Jacobsen, 1998). Examination of the expression of CD122 (IL-15R-β) and CD127 (IL-7Rα) on whole hepatic mononuclear cell (HMNC) populations revealed that a large number of mature lymphocytes have the potential to respond to both IL-15 (approximately 36%) and IL-7 (approximately 18%). As immature hepatic HSC populations account for only 1 – 2 % of HMNCs, their contribution to CD122/CD127 expression within this population would be minimal. Significantly higher proportions of hepatic HSCs ($CD34^+CD45^+$) express receptors for IL-7 and/or IL-15 ($p < 0.002$). Hepatic IL-7 and /or IL-15 may act on these progenitor cells to promote their survival and differentiation.

The results of this study indicate that normal human liver tissue expresses IL-7 and IL-15 specific mRNA and more importantly, has an abundant supply of IL-7 and IL-15 protein. As both of these cytokines have an established lymphopoietic function (Hofmeister *et al.*, 1999; Ma *et al.*, 2000; Waldmann and Tagaya, 1999), it is likely that a major role of both hepatic IL-7 and IL-15 is to act on resident lymphoid (T/NK/NK T/B) progenitor cells, providing them with the appropriate signals to mature locally. In this study, we have also shown that the majority of hepatic HSC populations express receptors for these cytokines. However, as indicated by the high level of expression of receptors on mature HMNC populations, these cytokines have other functions in the normal liver. IL-7 has been shown to have diverse effects on mature T cells including induction of cytokine production (Armitage *et al.*, 1992) and enhancement of cytotoxic functions (Zoll *et al.*, 1998). Thus, hepatic IL-7 may also be involved in local T-cell activation and expansion. As predicted from their sharing of receptor subunits, IL-15 shares a number of redundant functions with IL-2 such as induction of T-cell proliferation and co-stimulation of immunoglobulin synthesis (Armitage *et al.*, 1995; Burton *et al.*, 1994; Wilkinson and Liew, 1995). Resting NK cells constitutively express IL-15 receptors (Carson *et al.*, 1997) and IL-15 acts as an important chemoattractant and activator of NK cells (Carson *et al.*, 1995). It is generally accepted that IL-15 plays an important role in the migration and

maintenance of memory-type $CD8^+$ T cells (Yoshikai and Nishimura, 2000). Hepatic NK cell populations constitute 30% of the HMNC pool and $CD8^+$ T cells outnumber $CD4^+$ T cells two to one in the liver (Norris *et al.*, 1998), IL-15 may thus be involved in the regulation of hepatic NK and $CD8^+$ T cell populations. IL-15 also has unique functions on non-lymphoid cells, including actions on intestinal epithelial cells (Reinecker *et al.*, 1996) and muscle (Quinn *et al.*, 1995) and may therefore be involved in hepatocyte regeneration.

In summary, the constitutive expression of IL-7 and IL-15 in the normal adult human liver suggests that the normal AHL provides a suitable cytokine microenvironment to support lymphocyte development. These cytokines may play a role in the generation and maintenance of the unique hepatic lymphoid repertoire.

Chapter 4: Hepatic T-cell Development.

4.1 Introduction

4.1.1: Is the normal adult human liver a T-lymphopoietic organ?

Studies in mice have established the adult murine liver as an important T lymphopoietic organ (Iiai *et al.*, 1992; Makino *et al.*, 1993; Abo *et al.*, 1994; Sato *et al.*, 1995: Emoto *et al.*, 1997; Narita *et al.*, 1998; Shimizu *et al.*, 1999; Abo *et al.*, 1999). Strong evidence supports the existence of similar T cell development activity in the normal adult human liver (AHL). Extramedullary erythropoiesis in the liver, and reconstitution of multilineage haematopoiesis by donor-derived cells, in patients with normal bone marrow function, has been reported following liver transplantation (Collins *et al.*, 1993). Human hepatic T lymphocytes differ from those in blood, a large number are NK T cells whose phenotypic and functional characteristics resemble murine hepatic-derived cells (Makino *et al.*, 1993; Norris *et al.*, 1998; Norris *et al.*, 1999). Recombinase activating gene 1 (RAG1) and RAG2, the molecular machinery required for T cell development, and pTα, a chaperone involved in $\alpha\beta$-T cell development, are expressed in lymphocyte populations derived from normal AHL (Collins *et al.*, 1996). The liver is also a rich source of interleukin 7 (IL-7) (Golden-Mason *et al.*, 2001) which has been shown to be critical for normal T cell development (Plum *et al.*, 1996; Porter *et al.*, 2001; Mackall *et al.*, 2001). Normal AHL contains functional haematopoietic stem cells (Crosbie *et al.*, 1999; Crosby *et al.*, 2000) the vast majority of which are lymphoid progenitors (Golden-Mason *et al.*, 2000). Taken together these studies argue for an active T lymphopoietic role for normal AHL. However, direct evidence of ongoing T cell development in the human liver is presently lacking.

4.1.2: V(D)J recombination.

The expression of an antigen receptor, of unique specificity, on each T cell provides the diversity required to recognise a universe of potentially hazardous antigens. T cell receptors are heterodimers composed of two transmembrane chains, either $\alpha\beta$ or $\gamma\delta$. The $\alpha\beta$ heterodimers are expressed by greater than 90% of peripheral T cells. Both chains of the T cell receptor have, a variable region which interacts with antigen, a constant region, a short hinge region with a cysteine residue that forms an interchain disulfide bond and a

hydrophobic transmembrane region (Male *et al.*, 1996). The genes which code for both classes of T cell receptor ($\alpha\beta$ or $\gamma\delta$) are not contiguous in the germline, but exist as a number non-functional variable (V) joining (J) and in some cases diversity (D) gene segments which code for the variable portion of the T cell receptor. Each unique antigen receptor is generated by random rearrangement or recombination of these gene segments, a process termed V(D)J recombination (Alt *et al.*, 1992; Gellert, 1992; Lewis and Wu, 1997; Ramsden *et al.*, 1997; Thompson, 1995).

4.1.2.1: Recombination signal sequences and the 12/23 rule.

The recombination machinery (or recombinase) consists of cell specific factors (the RAG1 and RAG2 proteins) as well as general factors involved in double strand break repair (Gellert, 1996; Hagmann, 1997, Sadofsky, 2001). The recombinase recognises conserved sequence elements flanking each of the gene segments termed recombination signal sequences (RSSs), which are positioned 3' of V regions, 5' of J regions and on both sides of D segments (Tonegawa, 1983). RSSs consist of highly conserved heptamer and nonamer elements that are separated by nonconserved 12 or 23 base pair (bp) spacers. Efficient recombination requires a pair of RSSs with different spacer lengths, the 12/23 rule (Bogue and Roth, 1996). The 12/23 rule ensures that recombination occurs only between two segments of different types (figure 4.1.1). The consensus RSS is defined by a heptamer (CACAGTG) adjacent to a coding region, followed by a spacer (12/23 bps) followed in turn by a nonamer (ACAAAAACC). The heptamer is the most conserved sequence element of the RSS, and the three highly conserved residues closest to the coding segment (CAC) are found to be the most important in maintaining efficient V(D)J recombination activity (Ramsden *et al.*, 1997).

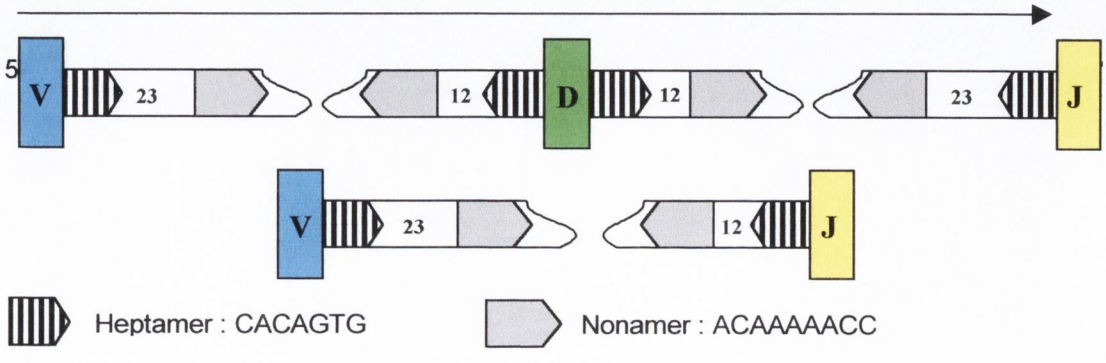


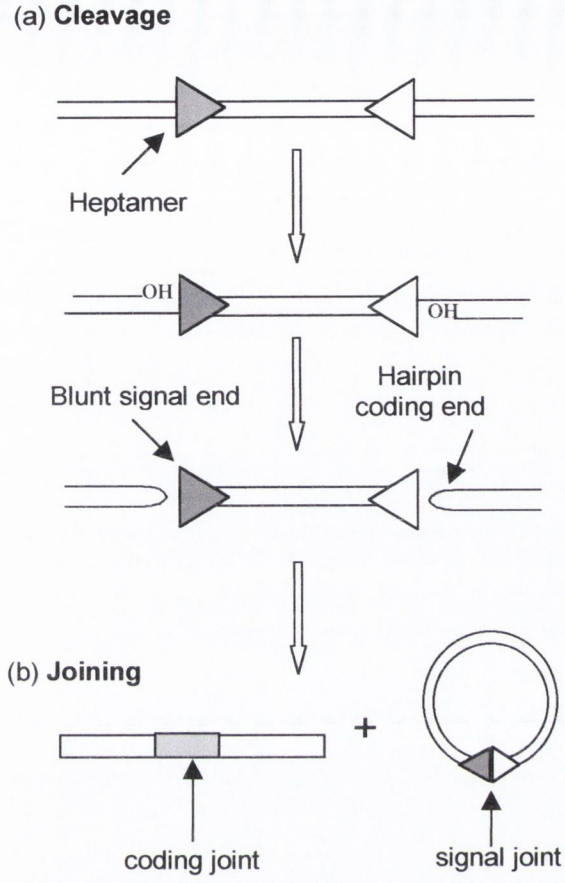
Figure 4.1.1: Recombination Signal Sequences.

4.1.2.2: Control of V(D)J recombination.

Strict regulation of V(D)J recombination is required to prevent the loss of functionally selected assembled antigen receptors, to avoid the generation of potentially auto-reactive receptor specificity in mature functionally competent lymphocytes and for the maintenance of the integrity of the genome during T-lymphocyte development. Control of recombination events is governed by locus accessibility (Sleckman *et al.*, 1996; Stanhope-Baker *et al.*, 1996), and by regulation of the expression of the RAG-1 and RAG-2 proteins (Lin and Desiderio, 1995) which are required for V(D)J recombination (Oettinger, 1992; Oettinger *et al.*, 1990; Schatz *et al.*, 1989; Schatz *et al.*, 1992). The RAG proteins are directly involved in the recognition of RSSs (Agrawal and Schatz, 1997; Difilippantonio *et al.*, 1996; Mo and Sadofsky, 1999; van Gent *et al.*, 1996).

4.1.2.3: Intermediates in the V(D)J recombination reaction.

Genes of the same transcriptional orientation rearrange by deletion to generate extra-chromosomal DNA by-products. The V(D)J recombination reaction can be divided into two steps, cleavage and joining. First double strand DNA breaks (DSBs) are introduced at the border of the RSS heptamer and the flanking coding region, followed by a second step in which the signal and coding ends are processed and linked into signal and coding joints (McBlane *et al.*, 1995) (figure 4.1.2). The signal ends are blunt 5'-phosphorylated and contain the complete RSS (Schlissel *et al.*, 1993). The coding ends however have a hairpin structure with the two strands being covalently linked. The coding ends are processed almost immediately, opened and incorporated into coding joints. The signal ends are processed much slower than the coding ends. RAG proteins remain attached to the coding ends and stabilise them (Agrawal and Schatz, 1997) until they are incorporated into signal joints, which form circular pieces of DNA containing two heptamer sequences back to back and the portion of germline DNA excised from the particular rearrangement (Ramsden and Gellert, 1995). The coding ends, but not signal ends, undergo modification by nucleotide insertion by terminal deoxynucleotidyltransferase (Knowles, 1990; Riley *et al.*, 1988) or nucleotide deletion, which alters the protein coding capacity of the original germ-line sequence (Tonegawa, 1983).



The 12-spacer signal is depicted as a closed triangle and the 23-spacer signal as an open triangle.

a) **Cleavage** at the RSSs by the RAG proteins takes place in two steps. First a nick is introduced at the boarder between the RSS and the coding elements. The second step results in hairpin formation.

b) **Joining** of the coding elements involves opening of the hairpins, nucleotide insertion/deletion. Joining of the signal sequences results in the formation of a circular DNA product containing a signal joint consisting of two heptamers back to back.

Figure 4.1.2 : The V(D)J recombination reaction can be divided into two steps, cleavage and joining (adapted from McBlane *et al.* 1995).

4.1.2.4: TCR-rearrangement circles (TRECs).

In humans there is no known way of phenotypically distinguishing long-lived naïve cells and recently generated T cells in the periphery. Episomal circles that are generated during excisional rearrangement of TCR genes have been found to correlate directly with thymic output of newly generated T cells (Kong *et al.*, 1998). These products termed TCR-rearrangement circles (TRECs) are stable, as demonstrated by their persistence in peripheral blood cells (Livak and Schatz, 1996), but diluted out at each cellular division as they are not replicated during mitosis (Douek *et al.*, 1998). The number of naïve T-cells remains constant from 20-60 years of age, whereas the number of TRECs continues to decline exponentially,

indicating that the presence of TRECs is not simply a surrogate for cells with a naïve phenotype. Thus the presence of these TRECs can be considered diagnostic of relatively recent TCR gene rearrangement. Because of the enormous diversity of TRECs produced, no single TREC can be considered as a reliable marker of recent TCR-rearrangement. However, because of the positioning of the δ-locus within the α-locus a common requirement for productive rearrangement at the α-locus is deletion of the encompassed δ-locus. Deletion of the δ-locus is facilitated by recombination between δRec sequences positioned within the δ-locus and the pseudo ΨJα region (figure 4.1.3).

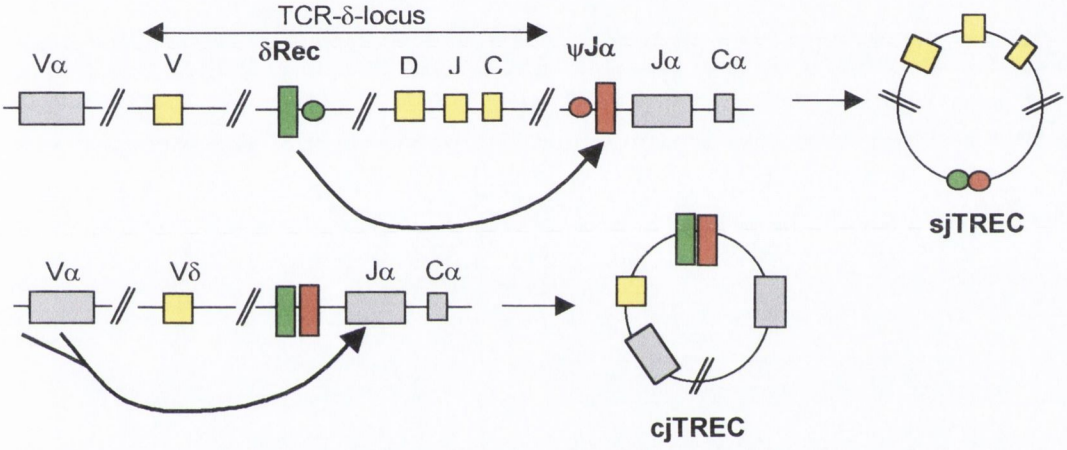


Figure 4.1.3 : Signal and coding joint TRECs generated by deletion of the TCR-δ-locus.

End to end ligation of the RSSs flanking the δRec and ψJα removes most of the TRC-δ-region, forming a single TREC containing a unique signal joint (sj) sequence. The recombined δRec to ψJα coding joint (cj) is retained on the germline DNA until TCR-αV to J recombination occurs, the coding joint then becomes part of a second TREC (adapted from Douek *et al.* 1998).

The two rearrangement events that occur during deletion of the δ-locus are identical in ~70% of αβ-T-cells, the first producing a signal joint TREC (sjTREC) and the second producing a coding joint TREC (cjTREC) (Verschuren *et al.*, 1997). Thus, the TRECs generated by deletion of the δ-locus are common to most conventional αβ-T-cells and therefore can act as an index of recent rearrangement at the TCR-α locus. A recently developed, quantitative competitive-PCR (QC-PCR), has been used to quantify sjTRECS generated by δRec to ΨJα recombination (Douek *et al.*, 1998). As TRECs do not replicate on cell division, they are found

only in naïve T cells, thus δ-deletion TRECS represent a direct index of *de novo* αβ-T cell generation.

4.1.3: Phentoypic markers of naïve and memory T cells.

The only currently available phenotypic marker of naïve T cells is differential expression of isoforms of CD45, a transmembrane tyrosine phosphatase expressed on all leukocytes. This molecule has classically been used to define the maturation status of T cells. The CD45 gene has three variable exons (A, B, and C) that encode part of its external domain. In naive T cells, the entire gene is expressed and the high molecular weight isoform (known as CD45RA) is found. In memory T cells, the variable exons are removed by alternative splicing of CD45 RNA, and low molecular weight isoforms known as CD45RO are expressed (figure 4.1.4). Several isoforms of CD45 can be expressed on mature T cells, thus, the expression of CD45RA does not by itself distinguish naïve T cell subsets, as recently activated T cells will be positive for both high and low molecular weight CD45 isoforms. The complete absence of CD45RO is therefore a more reliable marker of naïve T cells (Arlettaz *et al.*, 1999).

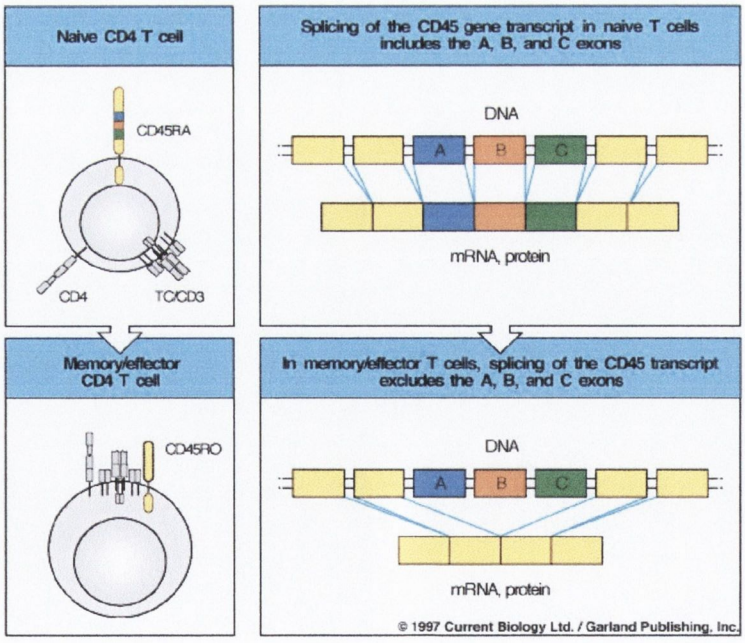


Figure 4.1.4: Memory T cells express altered CD45 isoforms.

In naive T cells, high molecular weight isoforms (CD45RA) are found that do not associate with either the T-cell receptor or co-receptors. In memory T cells, the variable exons are removed by alternative splicing of CD45 RNA, and this isoform, known as CD45RO, associates with both the T-cell receptor and the co-receptor. This assembled receptor appears to transduce signals more effectively than the receptor on naive T cells.

4.1.4: Skewed gene usage by putative extrathymic T cells.

Skewing of the TCR repertoire is thought to occur as a consequence of the development of T cells at sites other than the thymus (Emoto *et al.*, 1997, Abo *et al.*, 1999). The human intestine, a putative extrathymic T cell differentiation site (Lundqvist *et al.*, 1995; Lynch *et al.*, 1995, Madrigal-Estebas et al., 1997, Laky et al., 2000), has a skewed γδ T cell repertoire. Thymus derived γδ T cells found in normal blood predominantly express the Vδ2 gene segment whereas Vδ3 gene usage in the small intestine (Olive *et al.*, 1997) and Vδ1 gene usage in the colon (Deusch *et al.*, 1991) predominates. Murine hepatic αβ NK T cells, thought to be locally derived, frequently express an invariant TCR α-chain encoded by Vα14 and Jα281, in association with a limited number of Vβ chains (Vβ2, Vβ7 and Vβ8) (Lantz and Bendalac, 1994). αβ-TCR$^+$ NK T cells, isolated from human liver, display a TCR repertoire that is skewed towards Vα24JαQ usage (Norris *et al.*, 1999), a receptor that is almost identical to the murine Vα14Jα281 TCR chain (Prussin *et al.*, 1997). γδ T cells can account for up to 60% of human hepatic NKT cells (Norris et al., 1999). The high levels of γδ T cells in AHL is indicative of an organ specific role for this subpopulation of T cells (Doherty and O'Farrelly, 2000) however, it is not known if hepatic γδ T cell populations display a skewed TCR repertoire.

4.1.5: Overall objective of this study.

The aim of this study was to look for molecular evidence of ongoing conventional αβ T cell development in normal AHL (figure 4.1.5).

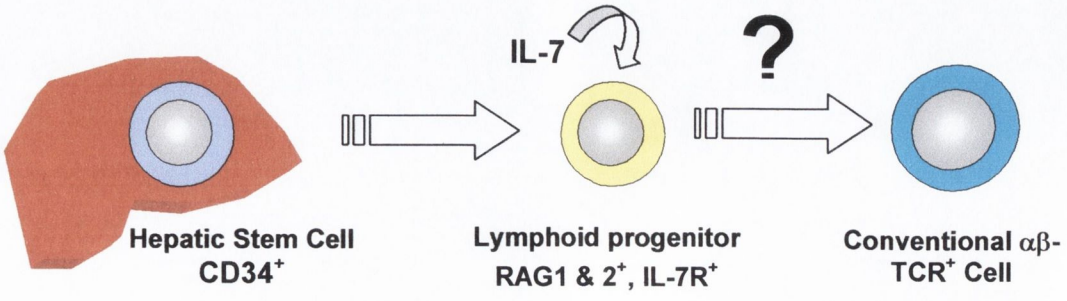


Figure 4.1.5: Do hepatic lymphoid progenitors give rise to conventional αβ T cells?

Using flow cytometry we examined the levels of naïve αβ T cells isolated from normal liver and matched peripheral blood samples. We quantified δ-deletion TRECs in naïve hepatic and blood T cell populations to provide molecular evidence of ongoing conventional αβ T cell development (figure 4.1.6).

4.1.4.1: Specific objectives.

1) To quantify δ-deletion TRECs in matched hepatic and peripheral blood T cell populations.
2) To provide evidence of differential gene-segment usage within hepatic γδ T cell populations.

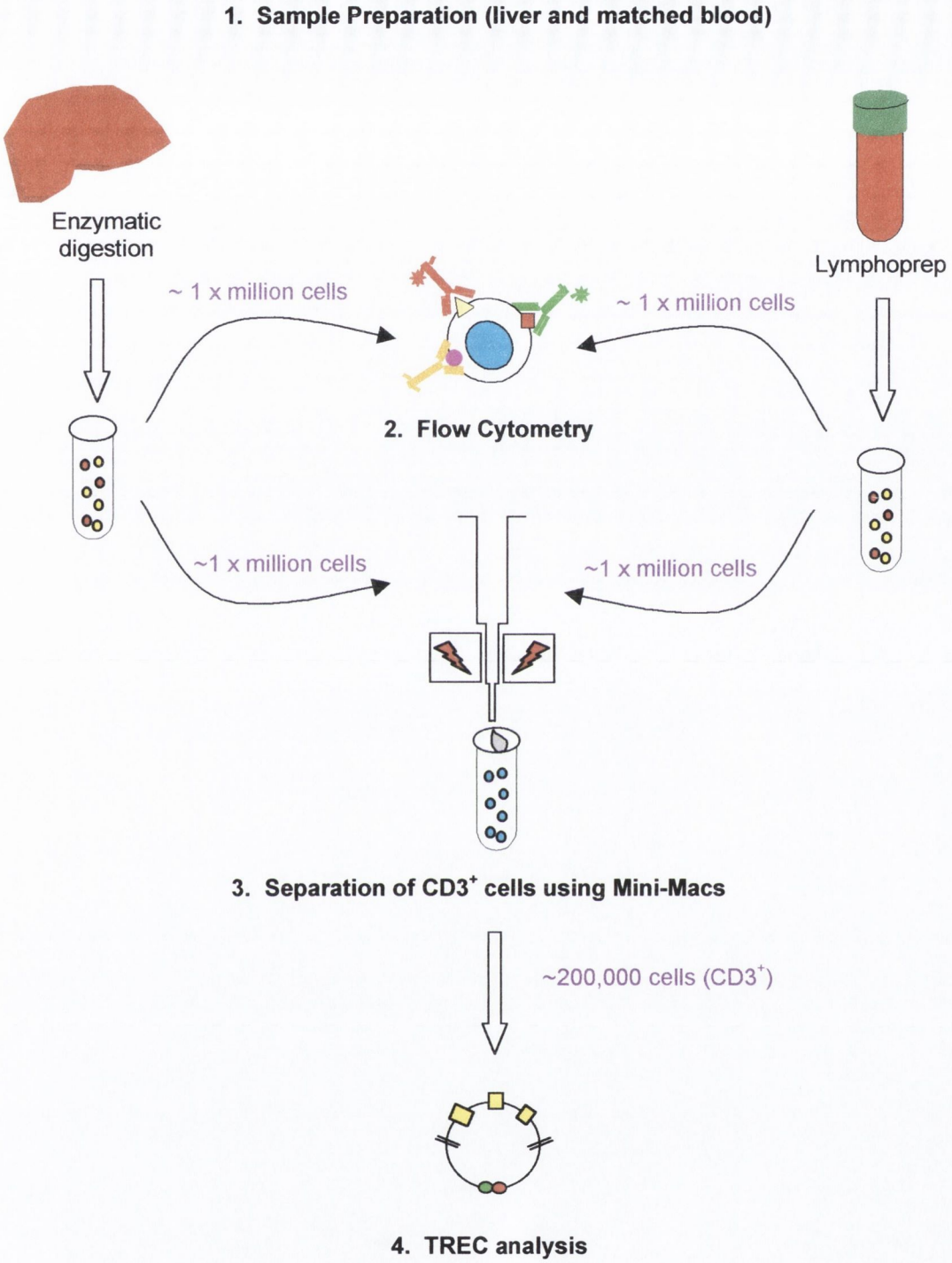


Figure 4.1.6: Experimental strategy for the detection of recent T cell receptor (TCR) gene rearrangements at the α-locus in hepatic lymphocyte populations.

Hepatic mononuclear cells were isolated from fresh liver biopsies using a combination of mechanical and enzymatic disruption. Peripheral blood mononuclear cells were prepared from 10 mls of matched blood by density centrifugation. The cells obtained were split into two equal fractions. Approximately 1 x 10^6 cells were used for flow cytometric analysis of TCR-gene usage ($\alpha\beta/\gamma\delta$), co-receptor expression (CD4/CD8) and memory status ($CD45RO^{+/-}$). $CD3^+$ cells were selected from the remaining fraction using the MiniMACS™ system. DNA was isolated from the $CD3^+$ cells and a quantitative PCR assay was used to quantify the number of δ-deletion TRECS in each of the samples.

4.2: Materials and methods

4.2.1: Tissue specimens.

4.2.1.1: Normal liver.

Normal liver wedge biopsies (n = 12, mean age 47 years, range 20 –75, 9 male and 3 female) were obtained from donor organs at the time of liver transplantation. Donor organs were extensively perfused with University of Wisconsin solution prior to obtaining the biopsy. Eight samples were used for the TREC study and a further 4 samples were used to characterise delta–gene usage (see appendix VI, tables APVI.1 and APVI.2 for demographic details).

4.2.1.2: Peripheral blood.

In all cases, 10 ml of matched venous blood was collected in lithium-heparin tubes (Becton Dickinson).

4.2.2: Preparation of hepatic and peripheral blood mononuclear cell suspensions.

4.2.2.1: Isolation of hepatic mononuclear cells.

Hepatic mononuclear cells (HMNCs) were prepared from liver biopsy samples as described in chapter 2 (section 2.2.2.1). Only samples with cell yields equal to or greater than 2×10^6 were used in the TREC study. Cell suspensions were diluted to 1×10^6 cells/ml in RPMI and divided into two equal fractions. One fraction was used for flow cytometric analysis and the remaining fraction was used for DNA isolation and subsequent TREC analysis. For the delta-gene usage study all cells were used for flow-cytometric analysis.

4.2.2.2: Isolation of mononuclear cells from peripheral blood.

Peripheral blood mononuclear cells (PBMCs) were prepared by standard density gradient centrifugation over Lymphoprep™ (Nycomed, see Appendix III [APIII.2] for details). Two

million cells were divided into two fractions (TREC study only) and used for analysis as described for HMNCs.

4.2.3: Flow cytometric analysis.

4.2.3.1: Monoclonal antibodies used for flow-cytometric analysis.

A panel of mAbs was used to characterise the naive or memory phenotype of hepatic and matched peripheral blood TCR-$\alpha\beta^+$ T lymphocytes (n = 8). Cell suspensions were stained with a range of fluorescent-labelled mAbs that included; anti-CD3-PerCP (clone SK7), anti-TCR-$\alpha\beta$-FITC (clone WT31), anti-TCR-$\gamma\delta$-PE (clone 11F2), anti-CD4 (PE/PerCP, clone SK3), anti-CD8-PerCP (clone SK1) and anti-CD45RO-PE (clone UCHL-1). All the above antibodies were supplied by Becton Dickinson. The stains carried out are described in table 4.2.1.

Table 4.2.1: Stains used to characterise the naive or memory phenotype of hepatic and matched peripheral blood TCR-$\alpha\beta^+$ T lymphocytes.

	Fluorescent label		
Stain no.	**FITC**	**PE**	**PerCP**
1	TCR-$\alpha\beta$	TCR-$\gamma\delta$	CD3
2	TCR-$\alpha\beta$	CD4	CD8
3	TCR-$\alpha\beta$	CD45RO	CD4
4	TCR-$\alpha\beta$	CD45RO	CD8
Control	IgG_1	IgG_1	IgG_1

A second panel of antibodies was used to establish the delta gene usage of hepatic and matched peripheral blood TCR-$\gamma\delta^+$ T lymphocytes (n = 4). Cell suspensions were stained with a range of fluorescent-labelled mAbs which included, as well as, anti-CD3, anti-TCR-$\alpha\beta$ and anti-TCR-$\gamma\delta$ (as above); anti-Vδ1 (unlabelled, clone R9.12, Immunotech), anti-Vδ2-FITC (clone Immu389, Immunotech) and anti-Vδ3-PE (clone P11.5B, Immunotech). The stains

carried out for this part of the phenotypic analysis are described in table 4.2.2. All samples were also stained with the appropriate isotype-matched control antibodies.

Table 4.2.2: Stains used to characterise the delta gene usage of hepatic and matched peripheral blood TCR-$\gamma\delta^+$ T lymphocytes.

Stain no.	Fluorescent label FITC	PE	PerCP
1	TCR-αβ	TCR-γδ	CD3
2	Vδ2	IgG_1	CD3
3	Vδ1*	IgG_1	CD3
4	IgG_1	Vδ3	CD3
Control (1)	IgG_1	IgG_1	CD3
Control (2)	IgG_1*	IgG_1	CD3

* Vδ1 and IgG1 were unlabeled antibodies the FITC label was added as part of the staining.

4.2.3.2: General staining protocol.

The general staining protocol as described in chapter 2 (section 2.2.4.1) was used to stain HMNC and PBMC preparations with directly labelled antibodies. One of the antibodies used in this study was unlabelled (anti-Vδ1, table 4.2.2) and a modified protocol for staining with unlabelled antibody (as described below) was used for this stain.

4.2.3.3: Staining protocol for use with unlabelled antibody.

Five μl (0.2 μg) of unlabelled antibody was placed in a tube to which an aliquot of 1 x 10^5 cells was added. Cells were incubated with the antibody for ten minutes at room temperature, then washed twice in 1 ml of PBS-BSA-Azide by centrifugation at 200 g for 7 minutes. After the second wash, cells were resuspended in 100 μl of PBS-BSA-Azide. Five μl of a 1/5 dilution of rabbit anti-mouse-FITC (Dako) was added to the cell suspension. The incubation and washing steps were repeated (as above). Fifty μl of a 1/25 dilution of filtered normal mouse serum (Vectastain) was added to the resuspended cells and the suspension was incubated at

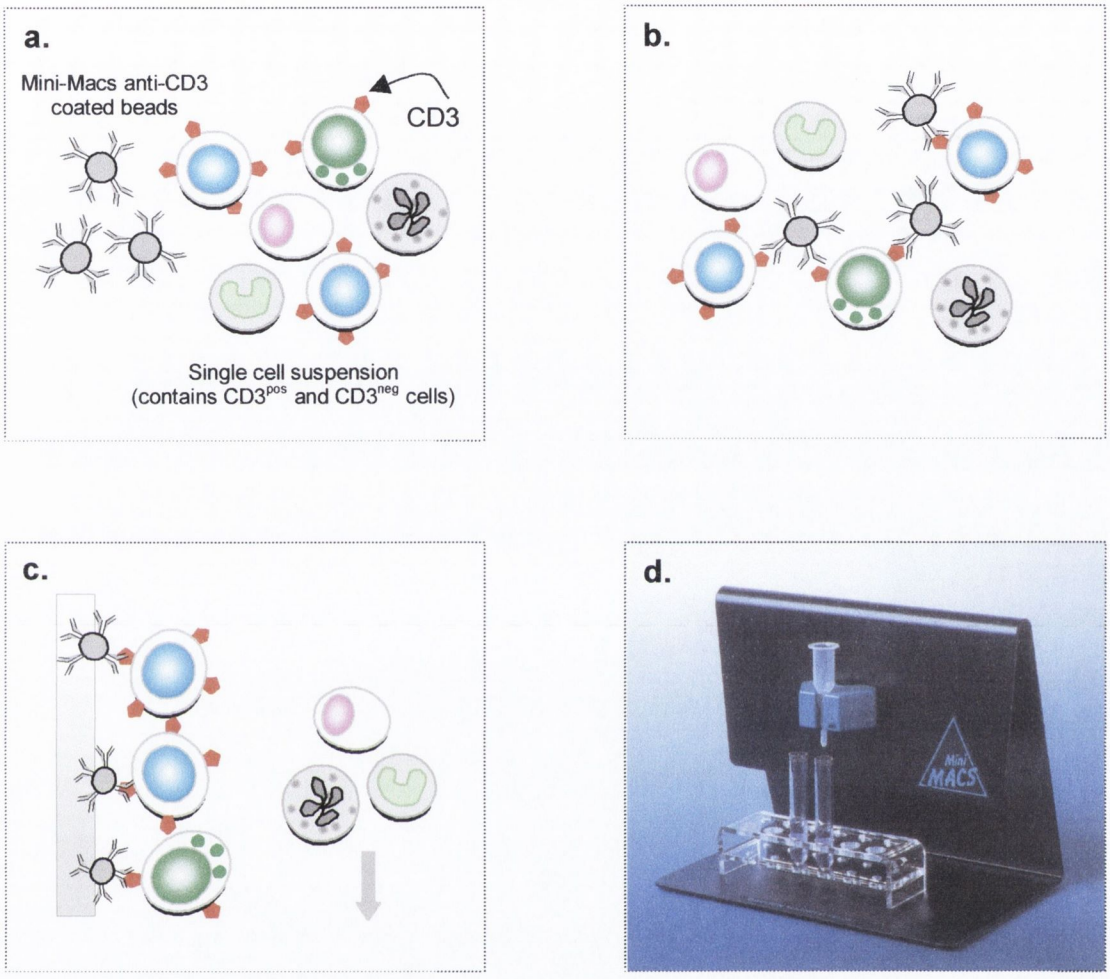


Figure 4.2.1: Separation of CD3 expressing cells using MiniMACS™ magnetic Micro-Beads.

MACS Micro-Beads are about 50nm in diameter. They react like magnetic antibodies and labelling is achieved within minutes. Their size and composition (iron oxide polysaccharide) make the Micro-Beads biodegradable. Cells are thought keep their biological function. Even positively selected cells can be used immediately after separation for flow cytometric analysis (as the beads do not affect the light-scattering properties of the attached cells) and functional assays. **a.** Single cell suspensions are prepared from the tissue of interest and CD3-Micro-Beads are added. **b.** The beads attach to $CD3^{positive}$ cells in the suspension. **c.** $CD3^{positive}$ cells are retained in the magnetic field while the follow-through contains all the $CD3^{negative}$ cells. Both positive and negative fractions can be used in subsequent assays. **d.** The MiniMACS™ apparatus.

room temperature for ten minutes. Cells were washed once in 1 ml and resuspended in 100 μl of PBS-BSA-Azide. Additional stains with labelled antibodies were carried out as described in the general staining protocol.

4.2.3.4: Flow cytometric analysis.

Acquisition and analysis were carried out using a FACScan flow cytometer and Cell Quest™ software (Becton Dickinson). CD3 or TCR-αβ positive cells were gated using FL3 (PerCP, $CD3^+$) or FL1 (FITC, TCR-$\alpha\beta^+$) and side-scatter (SSC, granularity) parameters. A minimum of ten thousand $CD3^+$/TCR-$\alpha\beta^+$ events were acquired for each tube. Due to the rarity of γδ T cells, twenty thousand events were acquired when examining the Vδ gene usage of peripheral blood cells. The levels of staining for the other two fluorescence markers (FITC and PE or PE and PerCP) above those observed with isotype-matched control antibodies were analysed within the $CD3^+$/TCR-$\alpha\beta^+$ populations.

4.2.4: TREC analysis of $CD3^+$ cells isolated from matched liver and peripheral blood.

4.2.4.1: Separation of $CD3^+$ cells using the Mini-MACS™ system.

Aliquots of 1 x 10^6 cells were placed in 2 ml Eppendorfs and pelleted by centrifugation at 10,000g for 5 minutes. Pelleted cells were resuspend in 90 μl of MiniMACS buffer (2 mM EDTA [BDH] in PBS [Sigma-Aldrich] plus 0.5% BSA [Sigma-Aldrich]). Twenty μl aliquots of CD3-MicroBeads (Miltenyi Biotec) were added to tubes and mixed. Samples were incubated for 15 minutes at 6ºC. Samples were then washed in 500 μl of MiniMACS buffer (10,000g for five minutes). The resulting pellet was resuspended in 500 μl of MiniMACS buffer. Positive separation columns (Miltenyi Biotec) were prepared with 500 μl of MiniMACS buffer. The cell sample was passed through column and washed through with 500 μl buffer (negative fraction). The column was removed from the magnet and the $CD3^+$ fraction was eluted with one ml MiniMACS buffer (figure 4.2.1). Cell yields ($CD3^+$) were assessed by EB:AO staining (see APIII.1). $CD3^+$ Cells were pelleted by centrifugation at 10,000g for 5 minutes for subsequent DNA extraction. Cells were stained before (whole fraction) and after separation

(positive and negative fractions) with anti-CD3-FITC and IgG-FITC (negative control) and the purity of the separated fractions was assessed by flow cytometric analysis.

4.2.4.2: Salting out of DNA.

Eighty μl of DNA-buffer (0.375 M NaCl [BDH], 1.2 M Na_2EDTA [BDH], pH 8.0), 30 μl of proteinase K (Sigma-Aldrich, 10 mg/ml in 0.2 M Na_2EDTA + 1% SDS [BDH]) and 260 μl of 1.5% SDS was added to the CD3-enriched cell pellet. The pellet was resuspended in the above mixture by vortexing vigorously. Suspensions were incubated for 30 minutes at 55°C. During the incubation period, the suspension was vortexed occasionally. One hundred μl of 6M NaCl was then added and the tubes were shaken for 15 seconds. Samples were centrifuged at 10,000g at 4°C for five minutes. The resulting supernatant was decanted into a clean tube and 1 ml of ice-cold 100% ethanol (Sigma-Aldrich) was added. The solution was then mixed gently, by inversion (the DNA was visible at this stage as a small white precipitate). Tubes were centrifuged at 10,000g at 4°C for two minutes. The supernatant was discarded and 1 ml of 70% ethanol (in sterile water) was added to the pellet. The sample was centrifuged again at 10,000g at 4°C for two minutes, the supernatant discarded and the DNA pellet was resuspended in 5 μl of sterile water. DNA samples were stored in liquid nitrogen and subsequently shipped to Dallas on dry ice, where the TREC assay was carried out (see below).

4.2.4.3: TREC assay.

A quantitative-competitive PCR assay developed by Douek et al (1998) was used to quantify the number of δ-deletion (signal joint) TRECS in the CD3-enriched cell populations isolated from HMNC and PBMC samples. This part of the study was carried out in collaboration with Danny Douek and Richard Koup at the University of Texas South Western Medical Centre at Dallas. Primers for the signal joint TRECS were based on previously published sequences (Verschuren *et al.*, 1997) and are located approximately 180 bp on either side of the recombination joints (5' –aaa gag ggc agc cct ctc caa ggc aaa- 3' and 5' agg ctg atc ttg tct gac att tgc tcc g- 3'). Each PCR reaction contained 1 U platinum™ *Taq* polymerase (Gibco), 1.8

mM $MgCl_2$, 0.2 mM dNTPs, 12.5 pM each primer and 1.7 nmol (5 μCi) ^{32}P-labelled dCTP in 50 μl platimun™ *Taq* buffer (and the DNA isolated from $CD3^+$ cells for each of the samples). To each PCR reaction 10^2, 10^3 or $10^{3.5}$ molecules of signal-joint internal standard was added. Internal standards have the same primer-binding sequences as the template for the target TREC but were modified to produce a PCR-product which is 60 bp shorter than the target sequence product (figure 4.2.2). PCR conditions were 95°C for 5 minutes, followed by 30 cycles of: 90°C, 60°C and 72°C, each for 30 seconds. PCR products were separated on 6% (non-denaturing) polyacrylamide gels. Bands were imaged and analysed using a Cyclone phosphorimager and Optiquant software (Packard). Because ^{32}P-labelled dCTP is incorporated in the reaction and there are fewer GC nucleotides in the internal standards (determined by sequencing), the intensity of the standard band was multiplied by a correction factor of 1.156. To quantify the number of TRECs in a PCR reaction, the reaction in which the equivqlance point was reached was selected and the target TREC-band was divided by the corrected standard-band intensity and multiplied by the number of standard molecules in the reaction. So that a direct comparison could be made between samples, the number of TRECs in each sample was expressed as TRECs/1 x 10^5 naïve TCR$\alpha\beta^+$ T cells. To calculate the number of TRECS/1 x 10^5 TCR-$\alpha\beta^+$ naïve cells: the total TREC number was normalised to TRECs/1 x 10^5 total cells, this figure was then adjusted to account for the purity of the sample and the proportion of $CD3^+$ cells which were TCR-$\alpha\beta^+$CD45RO$^-$.

4.2.5: Statistical analysis.

A two-tailed unpaired Student's t-test was used to determine the significance of differences between groups, a p value of < 0.05 was taken as significant. Spearman Rank was used for correlation analysis.

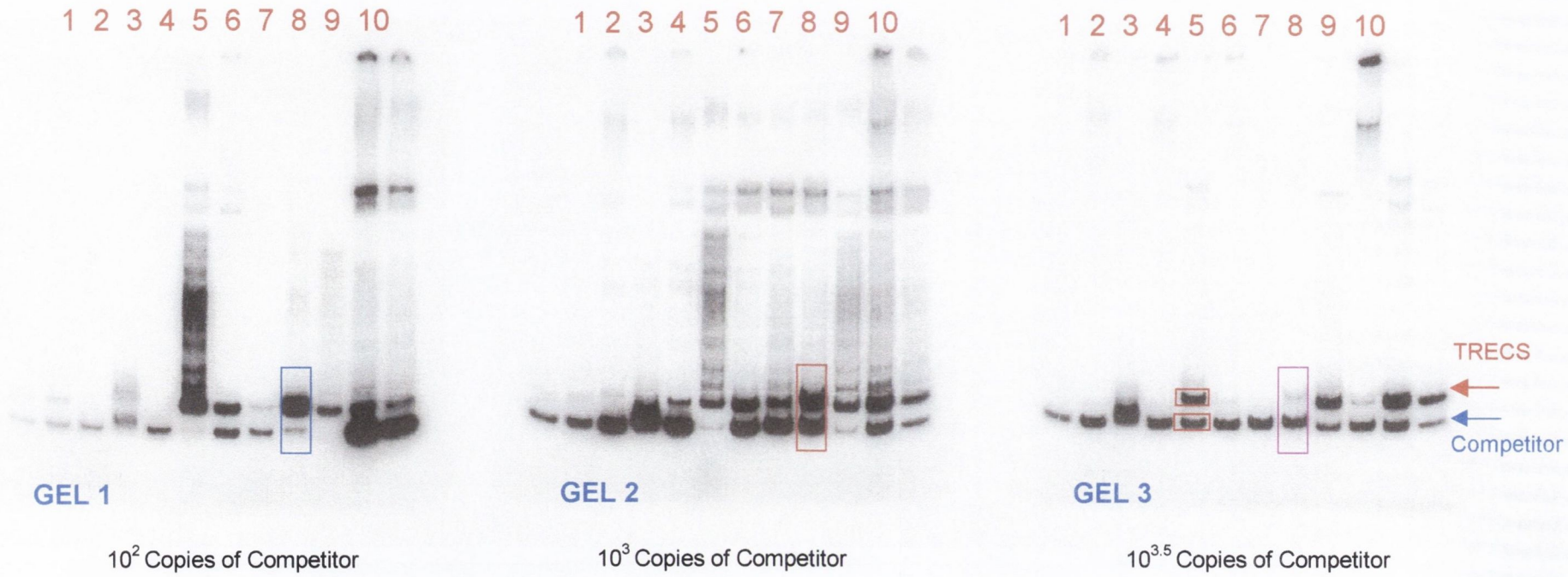


Figure 4.2.2: Quantitative PCR assay for the determination of δ-deletion signal-joint TREC levels.

A quantitative-competitive PCR assay developed by Douek et al (1998) was used to quantify the number of δ-deletion (signal joint) TRECS in the CD3-enriched cell populations isolated from HMNC and PBMC samples. To each PCR reaction 10^2, 10^3 or $10^{3.5}$ molecules of signal-joint internal standard was added. Internal standards have the same primer-binding sequences as the template for the target TREC but were genetically modified to produce a PCR-product which is 60 bp shorter than the target sequence product. PCR products were separated on 6% (non-denaturing) polyacrylamide gels. Bands were imaged and analysed using a Cyclone phosphorimager and Optiquant software™ (Packard). Because ^{32}P-labelled dCTP is incorporated in the reaction and there are fewer GC nucleotides in the internal standards (as determined by sequencing), the intensity of the standard band was multiplied by a correction factor of 1.156. To quantify the number of TRECs in a PCR reaction, the gel in which the equivalence point is reached was selected (for example GEL2 red box, lane 8). Too little competitor produces a falsely high result (GEL 1 blue box,lane 8), too much competitor produces a falsely low result (GEL 3 pink box, lane 8). The target TREC-band was divided by the corrected standard-band intensity and multiplied by the number of standard molecules in the reaction.

4.3 Results

4.3.1: Phenotypic analysis of hepatic and matched blood T cells.

Flow cytometric analysis was used to characterise T cells isolated from liver and matched blood samples with respect to T cell receptor (TCR) isoform expression. A significantly higher proportion of hepatic T cells express TCR-γδ than matched peripheral blood T cells (mean 11.12% v 5.64 %, n = 8, p = 0.02, figure 4.3.1).

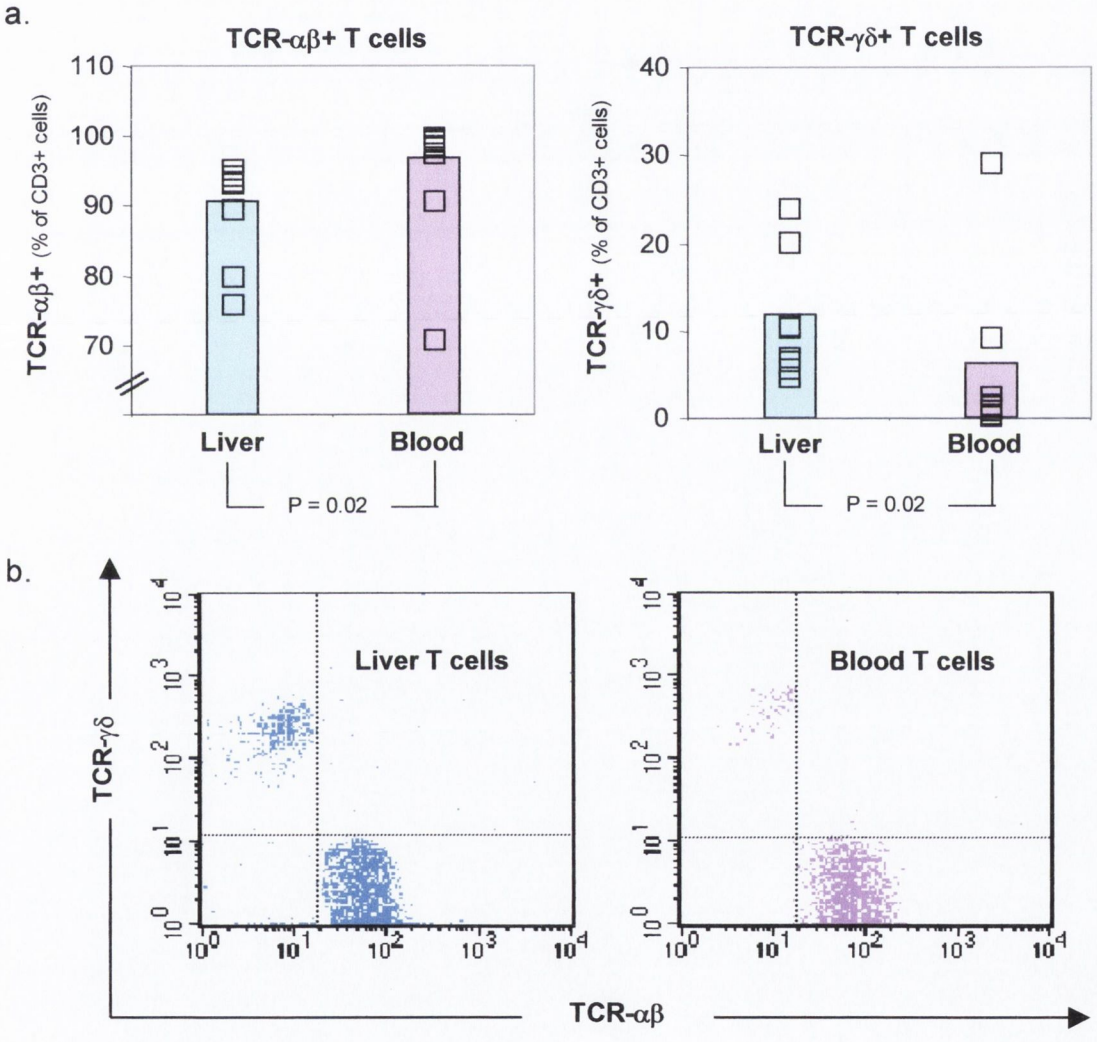


Figure 4.3.1: A higher proportion of hepatic T cells express the gamma-delta isoform of the T cell receptor (γδ-TCR) when compared to matched peripheral blood T cells.

a) The open squares represent the individual samples and the bars show the mean values. A significantly higher proportion of hepatic T cells express the γδ-isoform of the TCR. **b)** Representative flow cytometry dot plots of $CD3^+$ hepatic and matched peripheral blood T cells showing the higher frequency of TCR-$\gamma\delta^+$ T cells in hepatic populations.

The TCR-$\alpha\beta^+$ populations were further characterised with respect to CD8 and CD4 co-receptor expression and naïve or memory phenotype ($CD45RO^-$ or $CD45RO^+$ respectively). T cells with cytotoxic cell phenotype ($CD8^+$) occur more frequently in hepatic TCR-$\alpha\beta^+$ cell populations than in peripheral blood (mean 69.47% v 14.20%, n = 8, $p < 0.001$, figure 4.3.2). Double negative (cells which express neither CD8 nor CD4 co-receptors) were significantly increased in the liver-derived TCR-$\alpha\beta^+$ populations (mean 5.49% v 1.25%, n = 8, $p < 0.02$), while no statistically significant difference was observed in the double positive ($CD8^+CD4^+$) populations (figure 4.3.2).

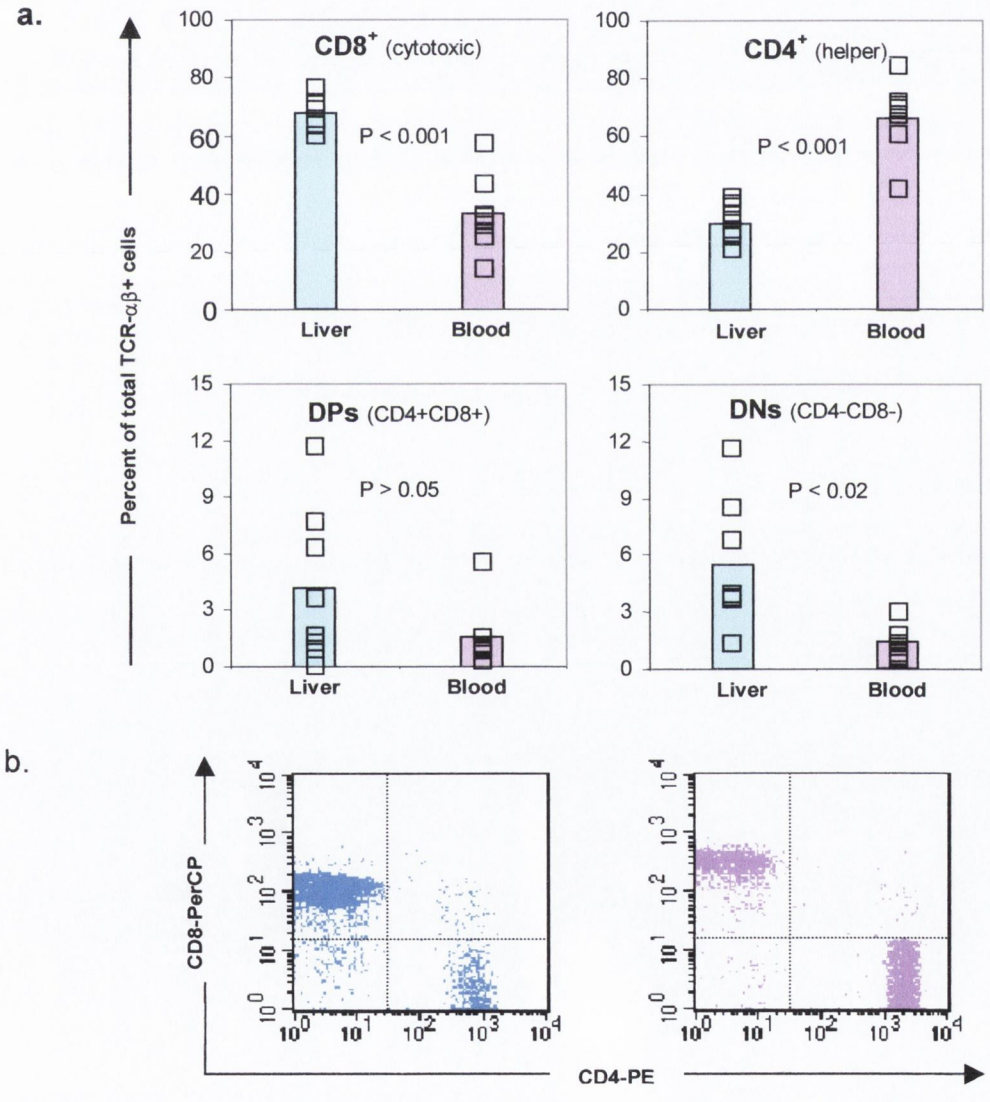


Figure 4.3.2: CD4 and CD8 co-receptor expression on TCR-$\alpha\beta^+$ hepatic and matched peripheral blood T cells.

a) A significantly higher proportion of hepatic αβ-T cells express the CD8 co-receptor or are double negative (DN, $CD8^-CD4^-$). **b)** Representative flow cytometry dot plots of TCR-$\alpha\beta^+$ hepatic and matched peripheral blood T cells showing the higher frequency of $CD8^+$ and DN T cells in hepatic populations.

The levels of naïve (CD45RO$^-$) TCR-$\alpha\beta^+$CD4$^+$ cells were significantly reduced in hepatic populations (mean 4.39% v 25.75%, n = 8, p <0.005, figure 4.3.3), while levels of naïve TCR-$\alpha\beta^+$CD8$^+$ cells were significantly increased in hepatic populations (mean 18.52% v 11.5%, n = 8, p < 0.05, figure 4.3.4).

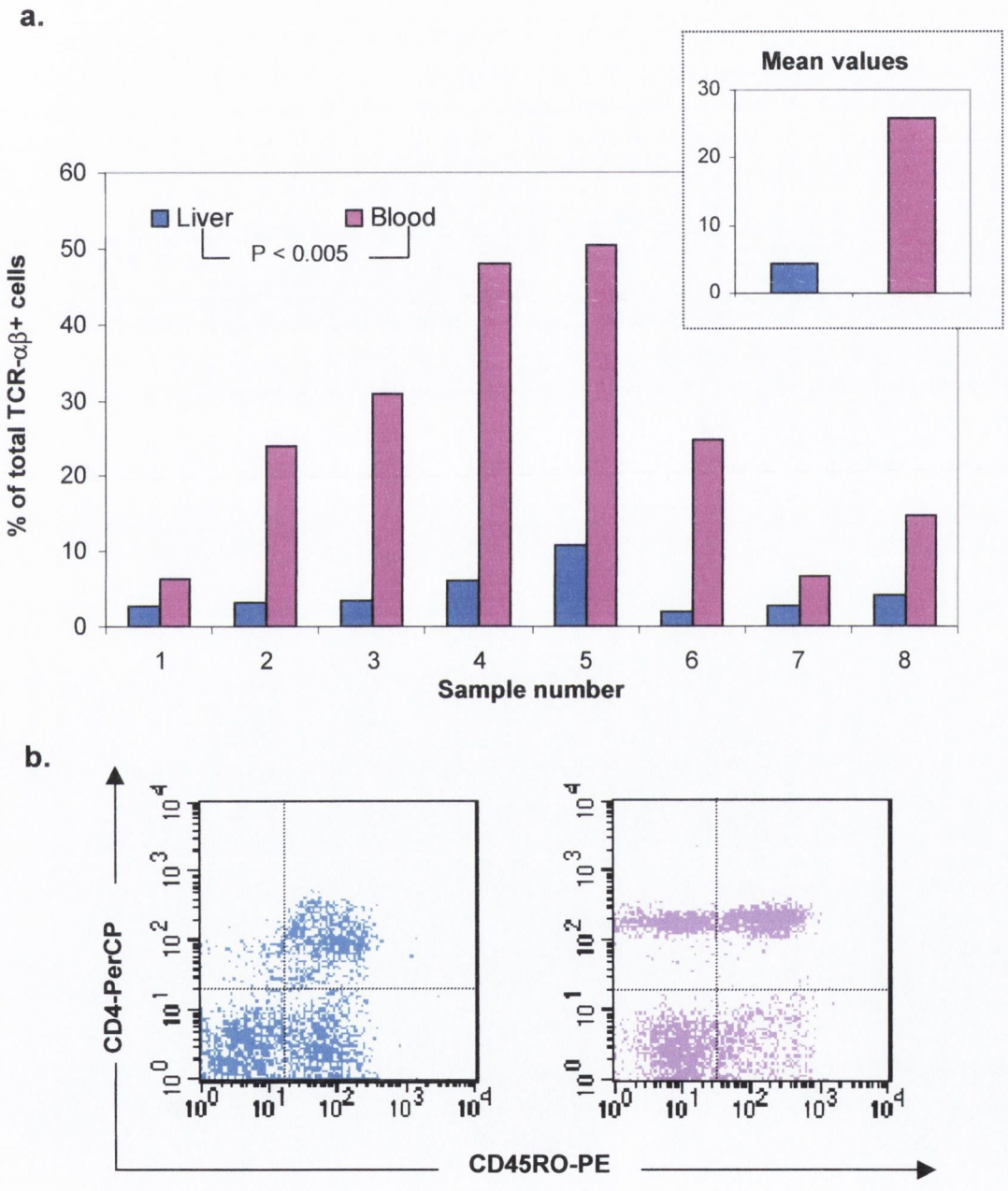
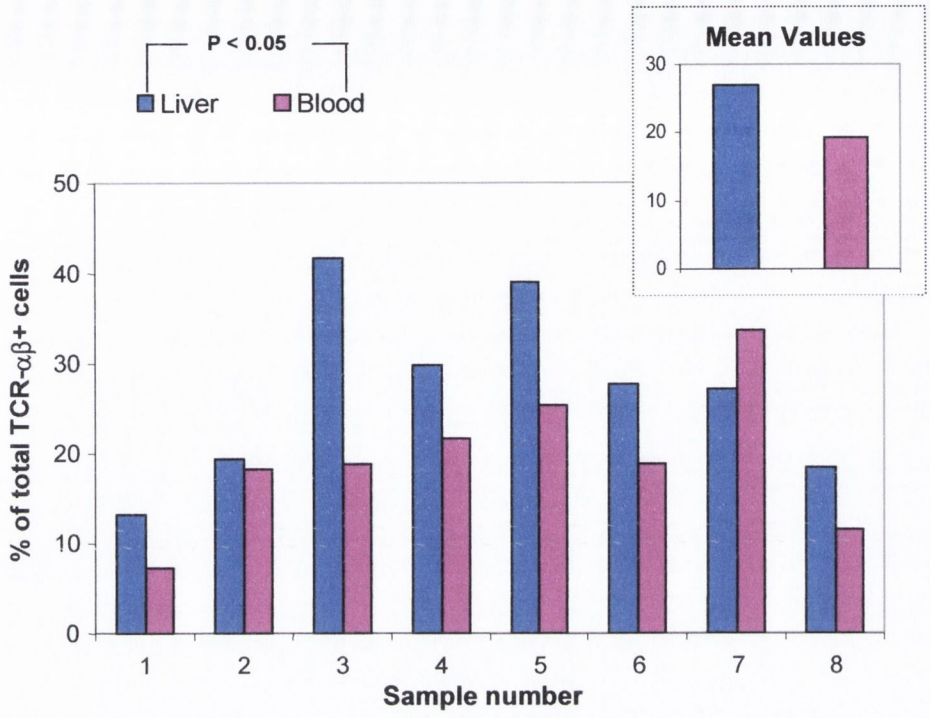


Figure 4.3.3: Naïve (CD45RO$^-$) TCR-$\alpha\beta^+$CD4$^+$ (helper) hepatic and peripheral blood T cells.

a) Bar chart showing the relative proportions of TCR-$\alpha\beta^+$CD4$^+$ cells with naïve phenotype in hepatic and matched blood samples. A significantly higher proportion of TCR-$\alpha\beta^+$CD4$^+$ PBMCs are of naïve phenotype. The inset shows the mean values. **b)** Representative flow cytometric dot plots of CD4 and CD45RO levels in liver and blood $\alpha\beta$-TCR$^+$ cell populations. The TCR-$\alpha\beta^+$CD4$^+$CD45RO$^-$ cells lie in the upper left quadrant.

a.



b.

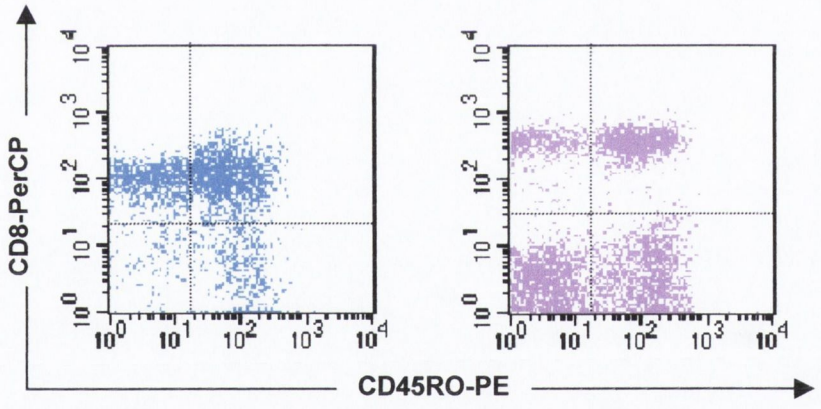


Figure 4.3.4: Naïve (CD45RO$^-$) TCR-αβ$^+$CD8$^+$ (cytotoxic) hepatic and peripheral blood T cells.

TCR-αβ+ cells were gated and the levels of PerCP and PE staining within the TCR-αβ$^+$ populations were analysed. **a)** Bar chart showing the relative proportions of TCR-αβ+CD8+ cells with naïve phenotype in hepatic and matched blood samples. The inset shows the mean values. **b)** Representative flow cytometric dot plots of CD8 and CD45RO levels in liver and blood αβ-TCR$^+$ cell populations. The TCR-αβ$^+$CD8$^+$CD45RO$^-$ cells lie in the upper left quadrant.

These differences were contributed to by the increased levels of TCR-αβ$^+$CD8$^+$ T cells observed in liver samples (figure 4.3.2). However, a significantly lower proportion of TCR-αβ$^+$CD8$^+$ hepatic cells displayed a naïve phenotype when compared to peripheral blood TCR-

$\alpha\beta^+CD8^+$ cells (40.59% v 62.27%, n = 8, % of TCR-$\alpha\beta^+CD8^+$ cells, $p < 0.02$). Similarly, a significantly lower proportion of TCR-$\alpha\beta^+CD4^+$ hepatic cells were of naïve phenotype (14.63% v 37.55%, n = 8, % of TCR-$\alpha\beta^+CD4^+$ cells, $p < 0.01$, figure 4.3.5).

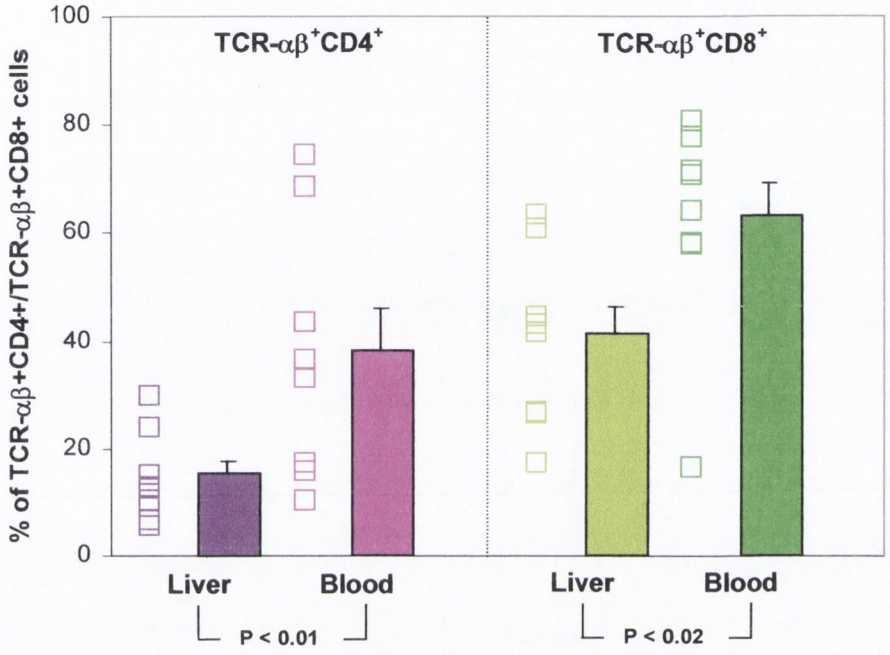


Figure 4.3.5: Naïve TCR-$\alpha\beta^+$ cells which co-express CD4/CD8 as a percentage of TCR-$\alpha\beta$+CD4+ or TCR-$\alpha\beta$+CD8+ cell.

The open squares represent the individual samples, colums show the mean values and the error bars represent the standard error of the mean (SEM). A significantly lower proportion of hepatic $\alpha\beta$-T cells that express the CD4/CD8 co-receptors have a naïve phenotype.

The total number of TCR-$\alpha\beta^+$ naïve cells in each of the samples was estimated by addition of the levels of naïve $CD8^+$ and $CD4^+$ (figure 4.3.6). Overall the levels of naïve TCR-$\alpha\beta^+$ cells was significantly higher in peripheral blood when compared to matched hepatic T lymphocyte populations (mean 45.19% v 31.46 %, % of total TCR-$\alpha\beta^+$ cells, n = 8, $p < 0.007$). Correlation of the levels of naïve TCR-$\alpha\beta^+$ lymphocytes in matched liver and blood samples, demonstrated that there was a direct relationship between the levels detected from either source (inset figure 4.3.6.b). A low level of naïve TCR-$\alpha\beta^+$ cells in the periphery was reflected in a low level in hepatic cells and the converse was true for high levels (samples 1 and 5 figure 4.3.6).

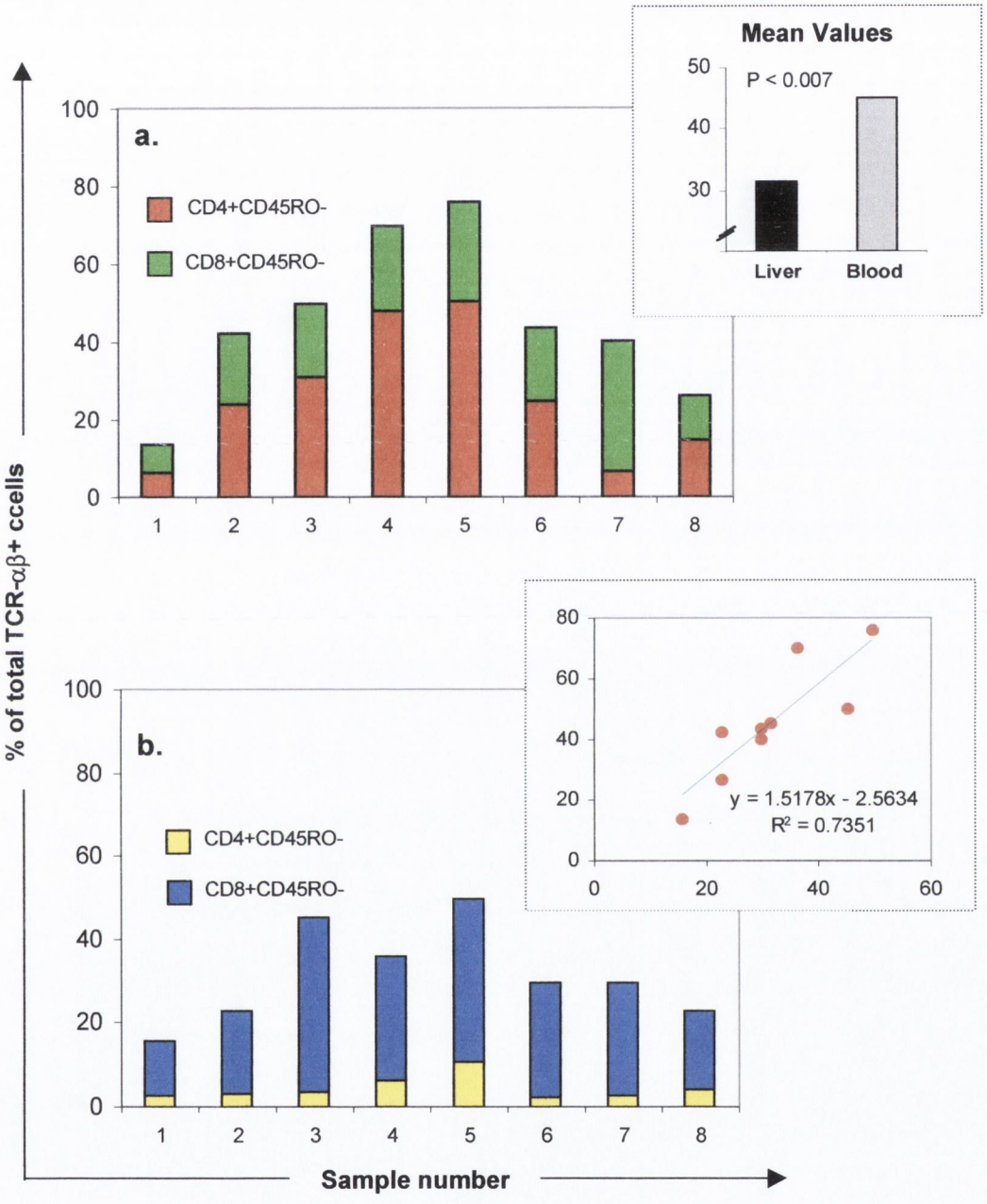


Figure 4.3.6: Total naïve TCR-$\alpha\beta^+$ cells in peripheral blood and hepatic T cell populations.

TCR-$\alpha\beta^+$ cells were gated and the levels of PerCP and PE staining within the TCR-$\alpha\beta^+$ populations were analysed. **a)** Bar chart showing total TCR-αβ cells with naïve phenotype ($CD45RO^{neg}$) in PBMC samples. **b)** Bar chart showing total TCR-αβ cells with naïve phenotype in matched HMNC samples. A significantly higher proportion of TCR-$\alpha\beta^+$ PBMCs are of naïve phenotype (**inset a**). There is a direct relationship between levels of naïve TCR-αβ cells in blood and liver (**inset b**).

4.3.2: Separation of $CD3^+$ cells.

4.3.2.1: $CD3^+$ cell yields.

Paramagnetic bead technology was used to separate $CD3^+$ cells from eight matched peripheral blood and hepatic mononuclear cell preparations. Mean yields were 256.25 and 531.25 thousand $CD3^+$ cells from a starting number of 1 million hepatic or blood mononuclear cells respectively. The lower yields from liver were due to tissue debris in the samples, which necessitated extra wash steps (figure 4.3.7).

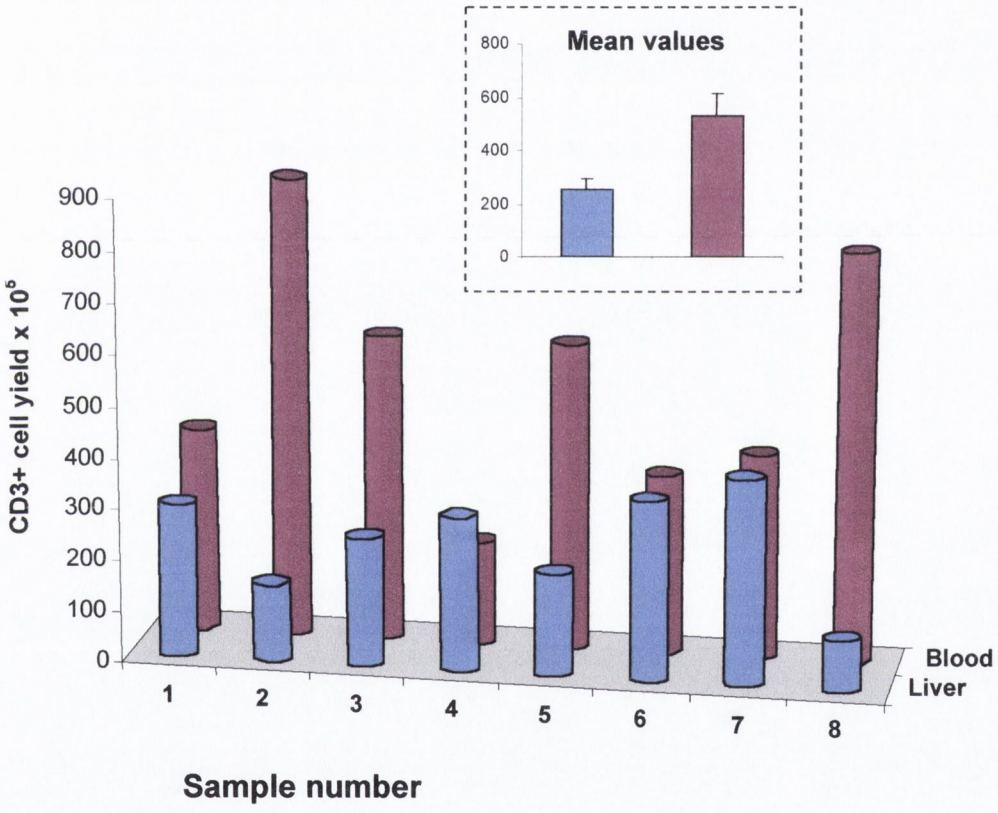


Figure 4.3.7: Yields of the $CD3^+$ cells isolated from hepatic and peripheral blood mononuclear cell preparations using magnetic beads.

4.3.2.2: $CD3^+$ cell purity.

The purity of $CD3^+$ cells separated from liver and matched blood was > 80% in all cases, as assessed by flow cytometry (figure 4.3.8). The purity of $CD3^+$ cells isolated from peripheral blood was significantly higher than that of $CD3^+$ cells isolated from matched HMNC samples

(figure 4.3.8 inset). Higher purities (approximately 90%) could be achieved from HMNC samples by passing the positive fraction through a second column. However this resulted in dramatically reduced yields. As the purity of the enriched fraction would be taken into consideration in the calculation of TREC levels it was decided that a purity of equal to or greater than 80% was acceptable. The histogram shown in figure 4.3.9 is an example of one hepatic sample before enrichment for CD3 (pink) and the positive and negative fractions after enrichment. DNA was successfully isolated from the CD3-enriched populations of the eight matched hepatic and blood samples. One μg of DNA from each of the samples was used in the QC-PCR assay to determine the number of TRECS in each of the samples.

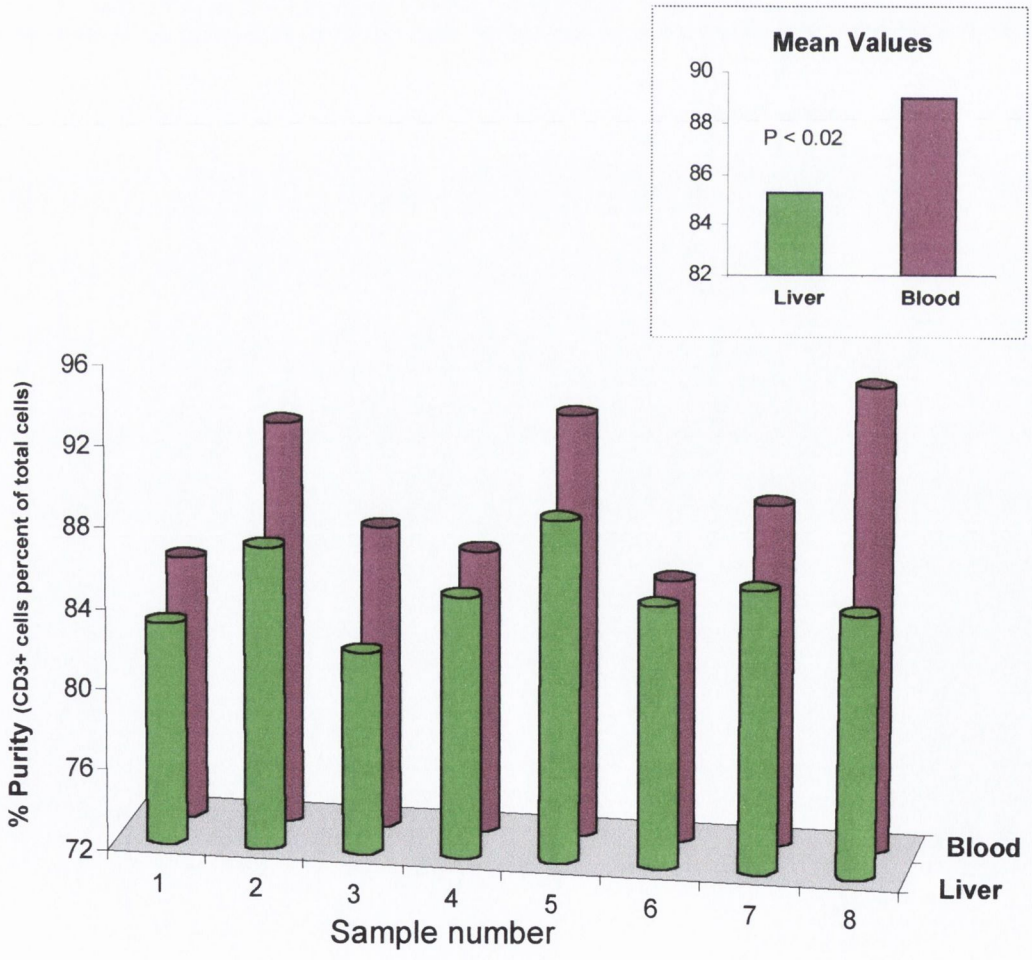


Figure 4.3.8: Purity of the CD3$^+$ fraction of cells isolated from hepatic and peripheral blood mononuclear cell preparations.

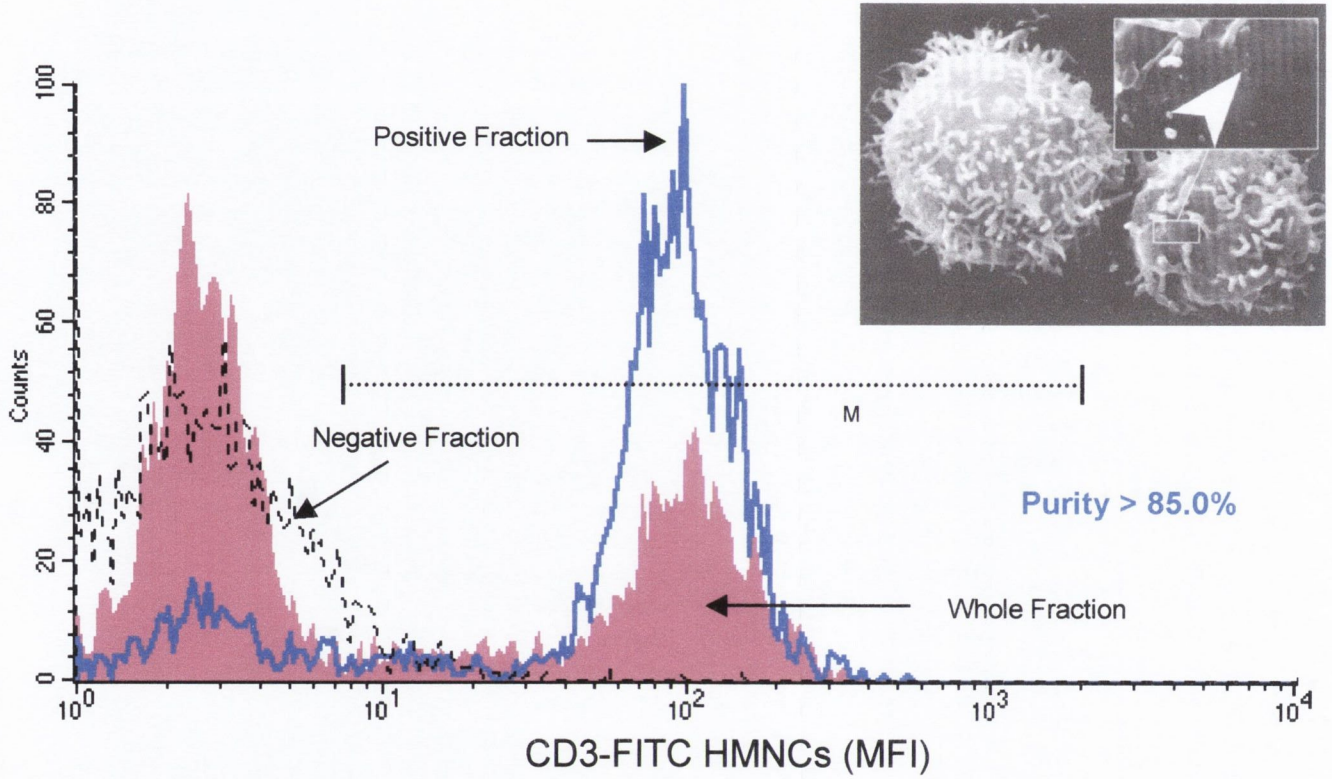


Figure 4.3.9: Separation of CD3$^+$ hepatic mononuclear cells using Mini-Macs CD3 microbeads

In the whole HMNC fraction 40.30% of cells are CD3$^+$, in the positive fraction the percentage CD3$^+$ cells is increased to 85.99, while 9.73% of the negative fraction express low levels of CD3. Inset CD3-microbeads bound to T-cells, beads are 1 million times smaller than the cell.

4.3.3: TREC levels.

The total number of TRECs detected in each of the samples was corrected for, yield, purity, the proportion of T cells expressing the αβ isoform of the TCR and, the proportion of αβ T cells of naïve phenotype ($CD45RO^-$). TRECs were detected in all blood samples (at a mean level of 318/100 thousand naïve αβ T cells), demonstrating that *de novo* αβ T cell development was ongoing in all individuals tested. Only two of the liver samples (samples 3 and 6) were positive at levels of 48 and 5 (figure 4.3.10).

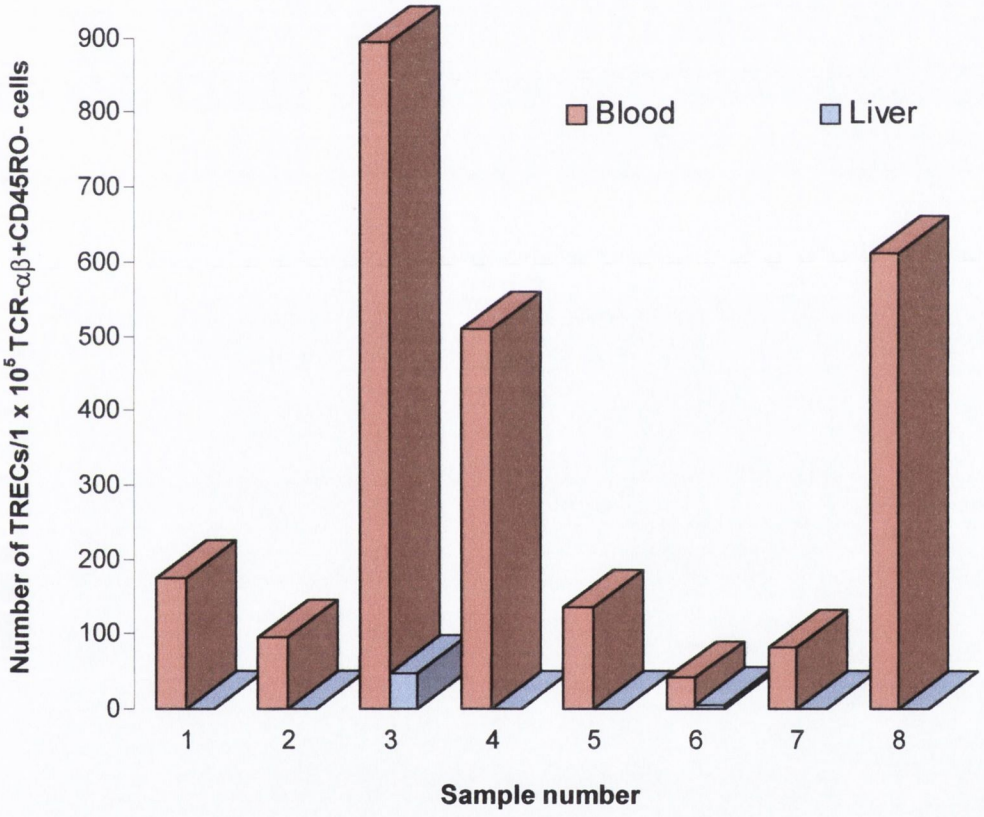


Figure 4.3.10: Levels of TRECS detected in peripheral blood and matched hepatic T cell ($CD3^+$) populations.

All of the blood samples tested were positive for δ-deletion TRECs while only two of the liver samples were positive at extremely low levels.

4.3.4: Delta-gene usage.

Using commercially available antibodies against Vδ1, Vδ2 and Vδ3, we looked for evidence of differential use of delta-gene V segments in hepatic T cell populations isolated from 4 liver biopsies. Vδ1 was expressed on a significantly lower proportion of hepatic γδ-T cells (mean

11.64% v 38.80%, $p < 0.04$). There was no difference in Vδ2 usage for hepatic and blood samples (mean 42.23% v 56.46% respectively). Vδ3 was expressed, on average, by 33.54% of hepatic γδ-T cells while matched blood from these patients were negative (figure 4.3.11). Flow cytometry plots of δ-gene V-segment usage on hepatic and matched blood γδ T cell populations are shown in figure 4.3.12.

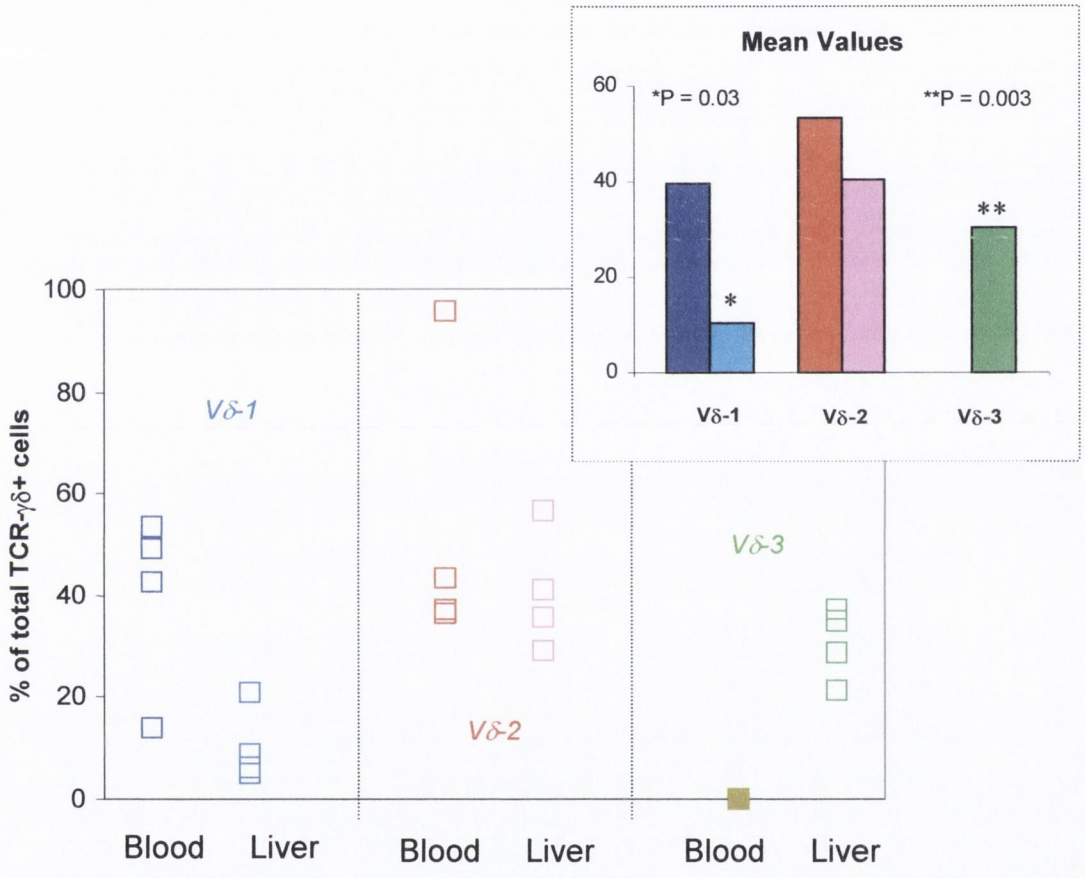


Figure 4.3.11: V-delta gene usage in hepatic and peripheral blood TCR-γδ⁺ T cells.

A Students paired T-test was used to test the significance of differences between groups ($p < 0.05$ was taken as significant). The inset shows the mean and p values for the Vδ gene usage for PBMC and HMNC TCR-$\gamma\delta^+$ populations respectively.

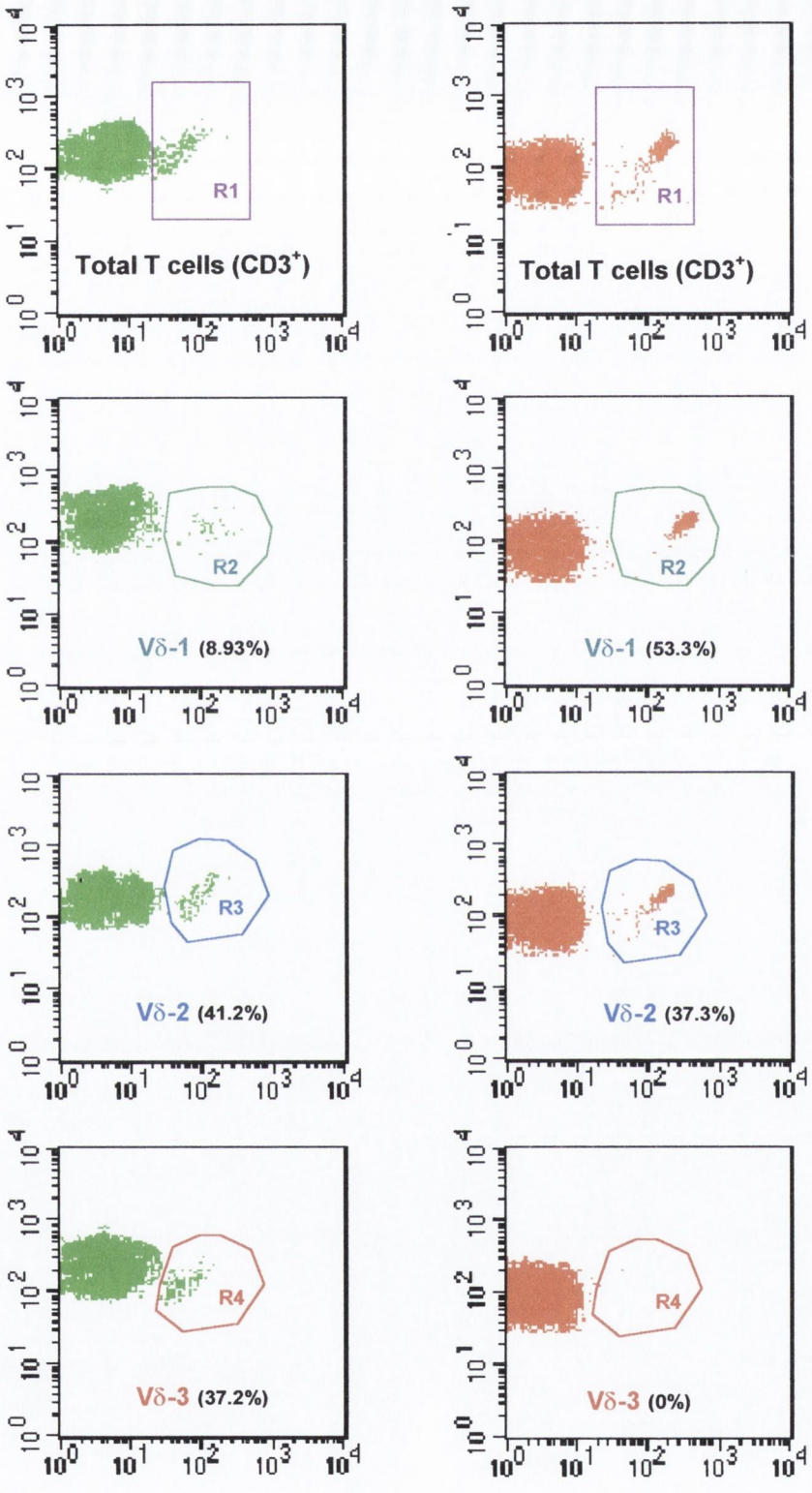


Figure 4.3.12: V-delta gene usage in hepatic and peripheral blood TCR-$\gamma\delta^+$ T cells.

T cells were gated and the proportion of T cells that expressed the γδ-TCR was estimated (R1). Vδ1 (R2), Vδ2 (R3) and Vδ3 (R4) expression was estimated in four matched hepatic and peripheral blood mononuclear cell preparations. Results are expressed as a percentage of the total γδ T cell population. One matched liver (green) and blood (red) sample is shown. Antibodies against the other five Vδ segments are not commercially available.

4.4: Discussion.

Phenotypic analysis of hepatic and matched blood mononuclear T cell populations demonstrated significant increases in γδ T cells, $CD8^+$ and DN ($CD4^-CD8^-$) αβ T cells in the liver. These findings confirm previous studies carried out in our laboratory which showed that lymphocyte populations isolated from normal AHL have phenotypic properties which distinguish them from their peripheral blood counterparts (Norris *et al.*, 1998; Norris *et al.*, 1999). Hepatic $CD8^+$ T cell populations contain a high proportion of cells bearing the CD8αα homodimer instead of the more conventional CD8αβ heterodimer (Norris *et al.*, 1998). Based on data generated from studies in mice, the T cell subsets occurring more frequently within hepatic populations, γδ, CD8αα and double negatives, may result from extrathymic T cell development pathways (Abo *et al.*, 1994; Laky *et al.*, 2000; Laky *et al.*, 1998; MacDonald, 1995; Porter and Malek, 1999).

Ongoing local T cell development would provide a constant supply of naïve T cells. Cell surface expression of CD45RA, a high molecular weight isoform of the pan leukocyte marker CD45, is classically used to define naive T cells, while memory or activated T cells have been defined by the expression of a low molecular weight isoform of CD45 (CD45RO). However several isoforms of CD45 can be expressed on mature T cells, thus, the expression of CD45RA does not by itself distinguish naïve T cell subsets. Recently, activated T cells have been shown to be positive for both high and low molecular weight CD45 isoforms. In addition, evidence suggests that memory T cells can re-express the CD45RA molecule but do not loose expression of CD45RO (Arlettaz *et al.*, 1999). The complete absence of CD45RO is therefore a more reliable marker of naïve T cells and was used in this study, to determine the naïve ($CD45RO^-$) or memory ($CD45RO^+$) status of TCR-$\alpha\beta^+$ sub-populations. Overall, the proportion of naïve TCR-$\alpha\beta^+$ cells was significantly higher in peripheral blood when compared to matched hepatic T lymphocyte populations. Naïve TCR-$\alpha\beta^+CD4^+$ cells were found in small proportions in hepatic preparations, suggesting that the majority of the $CD4^+$ TCR-αβ cells in the liver have previously encountered antigen. In contrast to blood, the majority of naïve αβ T cells isolated from liver samples were positive for the CD8 co-receptor. The liver has been postulated to be a site for the elimination of activated $CD8^+$ T cells by apoptosis following

activation in the periphery (Crispe and Huang, 1994; Huang *et al.*, 1994). The high proportion of CD8+ T cells in normal liver could be a reflection of this scavenging function. However, accumulation of activated $CD8^+$ T cells only occurs in circumstances where there is disruption of an apoptotic pathway and, due to rapid elimination, are not observed in normal liver. The high levels of $CD45RO^-$ cells within the $CD8^+$ hepatic T cell populations observed in this study argues for the existence of a local pool of naïve $CD8^+$ T cells.

Paramagnetic bead technology was used to separate $CD3^+$ cells from eight matched peripheral blood and hepatic MNC preparations. The cell yield and purity of the $CD3^+$ enriched fractions were lower for hepatic cells. The lower yields from liver were due to tissue debris in the samples, which necessitated two additional wash steps. The purity of $CD3^+$ cells isolated from HMNC samples could be improved by passing the positive fraction through a fresh column. However, this resulted in unacceptable cell loss, and as the purity of the enriched population would be taken into consideration in the calculation of TREC levels, it was considered that a higher purity was not necessary.

DNA was successfully isolated from the CD3-enriched populations of the eight matched hepatic and blood samples. TRECs were detected in all blood samples, demonstrating that *de novo* αβ T-cell development was ongoing in all individuals tested. Only two of the liver samples were positive at very low levels. The almost complete absence of TRECs specific for conventional αβ T cells in the liver provides evidence that the normal AHL is not a site for the development of conventional αβ T cells. The relatively high levels of TRECs detected in the matched blood samples argues for a thymic source of conventional αβ T cells (Douek *et al.*, 1998; Douek *et al.*, 2000; Poulin *et al.*, 1999), although it is also possible that they might arise at other putative extrathymic sites such as the gut or bone marrow. HIV-1 infection results in progressive loss of peripheral $CD4^+$ T cells and leads to severe immune deficiency and opportunistic infections (Pantaleo *et al.*, 1993). Adult HIV-infected patients treated with highly active anti-retroviral therapy (HAART) show increases in their number of naïve $CD4^+$ T cells (Autran *et al.*, 1997). Using the TREC assay, Douek *et al.* (1998) have shown that *de novo* T-cell development contributes to the expansion of naïve $CD4^+$ T cells observed in these

patients, and suggest that the thymus is the source of the newly generated T cells. However, Haynes *et al.* (1999) found that thymectomy before HIV-1 infection did not preclude rises peripheral naïve $CD4^+$ T cells or clinical response after retroviral therapy, suggesting an extrathymic source of naive $CD4^+$ T cells. The absence of TRECs in liver-derived T cell populations, despite their abundance in matched blood samples, further highlights that hepatic lymphocytes are a unique population differing from circulating blood lymphocytes.

Failure to detect δ-deletion TRECs in the liver could lead one to draw the conclusion that the AHL is not a site of extrathymic T cell synthesis. However, the presence of significant populations of lymphoid progenitor cells (Golden-Mason *et al.*, 2000) and the expression of RAG-1, RAG-2 and pTα (Collins *et al.*, 1996), suggests that T cell development is ongoing in normal AHL, as has been shown in mice (Abo *et al.*, 1999; Makino *et al.*, 1993). Furthermore, the expression of pTα in hepatic lymphocyte populations suggests that some of the T cells developing locally express the αβ-TCR. The assay used in this study detects TRECS in only 70% of αβ T cells and works well for conventional αβ T cells found in the adult circulation (Al-Harthi *et al.*, 2000; Douek *et al.*, 1998), which are dominated by Vδ2 segment usage mostly associated with Vγ9 (Casorati *et al.*, 1989; Constant *et al.*, 1994; Jaleco *et al.*, 1997). The TREC assay used is limited in that it depends on Vδ gene segment recombination that would leave intact δRec and ψJα sequences. Eight TCR Vδ subgroups (TRDV1-8) have been identified, all of which produce functional proteins. As can be seen in figure 4.4.1, the use of the majority of these Vδ segments would not leave the δRec sequence intact (Verschuren *et al.*, 1997). Differential Vδ gene segment usage in the liver may account for the absence of δ-deletion TRECs in immature hepatic lymphocyte populations. Using flow cytometry, we looked for evidence that γδ T cells in the liver differ in their Vδ segment usage from those in matched blood samples. As commercial antibodies suitable for flow cytometry against only three of these segments (Vδ1, Vδ2 and Vδ3) are available, we were not able to look at all Vδ segments. However, using this limited range of antibodies, a striking difference was observed in both Vδ1 and Vδ3 usage. Hepatic γδ T cells positive for the Vδ1 segment were significantly reduced while Vδ3 usage was confined solely to the liver. This finding provides some

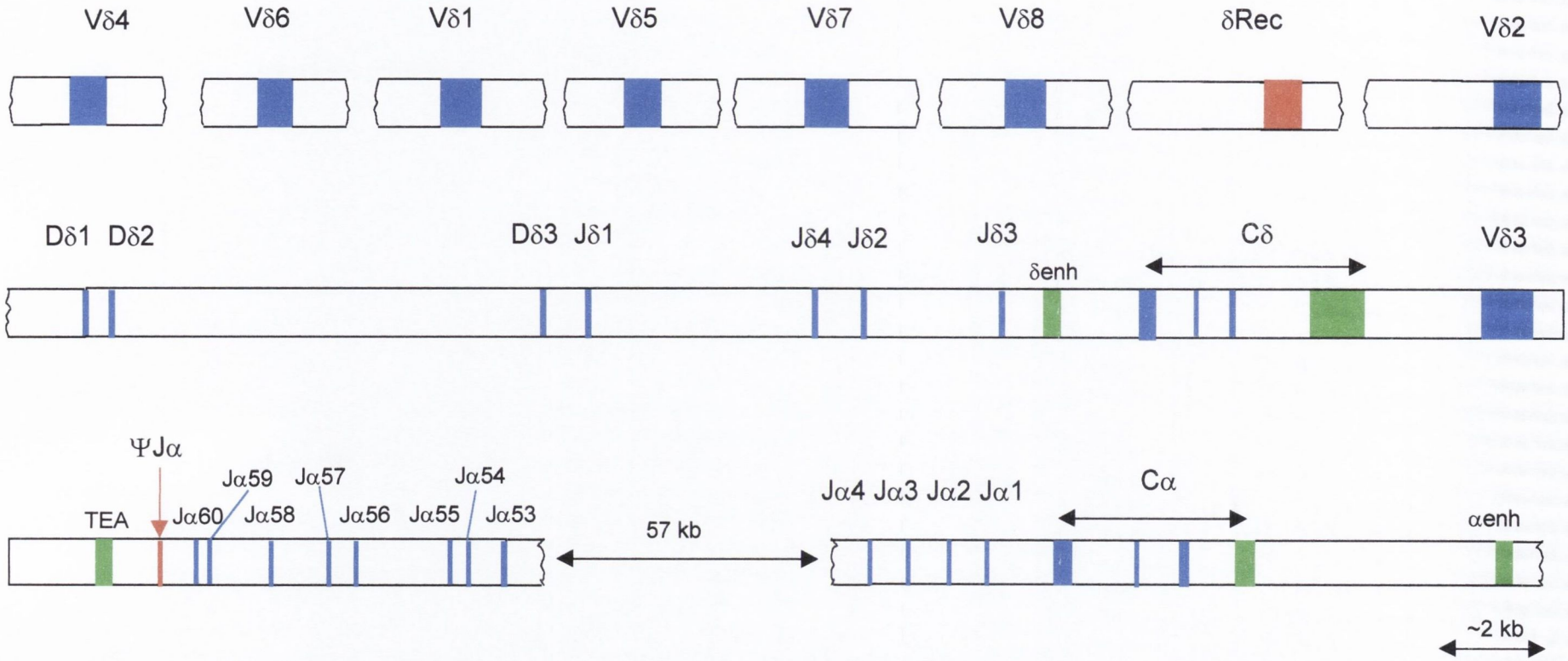


Figure 4.4.1: Schematic representation of the human TCR-δ gene and a part of the TCR-α gene.

For 70% of conventional αβ-TCR positive cells the primary event in the rearrangement of the alpha-chain is the deletion of the delta gene segment (which lies within the alpha gene locus), by bringing together the non-coding sequences δREC and ΨJα (shown in red). The TREC assay detects this particular excision product. As the majority of peripheral blood αβ-TCR cells use Vδ2 these sequences remain intact, however if for example Vδ4 was the main rearrangement then the sequence would no longer be intact. The V-delta usage in liver may account for the lack of δ-deletion TRECS. Exons are indicated as blue boxes in the bars; non-coding regions are shown in green, except for δRec and ΨJα which are shown in red. Abbreviations; enh = enhancer, kb= kilo bases, V = variable, D = diversity, J = joining (adapted from Verschuren *et al.*, 1997).

evidence for differential use of Vδ segments in hepatic γδ T cell populations. No significant difference was detected in Vδ2 segment usage. The antibodies used account for greater than 93% of peripheral blood but only 80% of hepatic γδ T cells, lending further weight to the hypothesis that γδ T cell populations in the liver differ from those in blood. The significant populations of hepatic γδ T cells bearing the Vδ2 segment is surprising in view of the virtual absence of δ-deletion TRECs. While the results of this part of the study do not explain the absence of δ-deletion TRECs, it can be concluded that Vδ2 positive γδ T cells do not develop locally in the normal liver.

Studies using parabiotic mice, which share the same blood supply, have shown that while there is continual infiltration of thymus derived conventional T cells into sites that support extrathymic T cell differentiation, such as the liver (Yamamoto *et al.*, 1999) and gut (Sugahara *et al.*, 1999), T cells that are generated *in situ* in these organs do not, under normal circumstances, enter the circulation (Narita *et al.*, 1998; Suzuki *et al.*, 1998). The high proportion of $V\delta2^+$ hepatic γδ T cells observed is likely to be derived from the circulation. Although we did not do so in this study, it would be interesting to look at CD45RO expression on this population to determine if they are memory cells. Differential recirculation of thymus-derived and thymus-independent T cells would predict that populations of T cells unique to the liver are the cells likely to be of local origin. In this respect $V\delta3^+$ cells are likely extrathymic candidates as they were abundant in hepatic tissue but absent from matched peripheral blood. Recombination involving the Vδ3 gene segment is complicated by the fact that this segment is located downstream of the Cδ gene segment and has been shown to also recombine to the Cα gene segment (Peyrat *et al.*, 1995). Recombination of Vδ3 to Cδ would leave intact δRec and ψJα sequences, and therefore under these circumstances δ-deletion TRECs would have been detected. However, if hepatic Vδ3 populations utilise the Cα gene segment, the ψJα sequence would be deleted and thus, δ-deletion TRECs would not be detected (see figure 4.4.1). We did not in this study include anti-TCR-αβ or anti-TCR-γδ antibodies in the same tubes as the individual anti-Vδ antibodies. The level of γδ T cells was estimated first then the levels of staining for the three individual Vδ gene segments tested

were expressed as a percentage of the total γδ T cell population. Thus, we have no way of knowing if the Vδ3$^+$ hepatic cell populations are true γδ T cells or hybrid αβ T cells. In retrospect, this series of stains was badly designed and would need to be repeated with the inclusion of anti-TCR-αβ and or anti-TCR-γδ together with the antibodies directed against the individual Vδ gene segments. Despite the inherent limitations of this part of the study due to availability of antibodies and poor design, we can conclude that the absence of TRECS in hepatic samples shows that Vδ2Cδ γδ T cells do not arise *de novo* in the liver. Due to the large proportion of Vδ3$^+$ cells in the liver and their absence from peripheral blood, Vδ3Cα T cells remain likely candidates, however their presence in liver remains to be determined. Further studies are required to determine which populations of T cells are developing locally in the normal AHL.

The evidence for the existence of an extrathymic T cell development site in the normal AHL is convincing even if classical αβ T cells do not differentiate in the liver. Furthermore, given the unusual nature of the hepatic lymphoid repertoire, the high proportion of γδ T cells, and, that up to 55% are NK T cells, which can use unusual α-gene segments such as Vα24 (Norris *et al.*, 1999), it is hardly surprising that we see no evidence of conventional αβ-T cell development in the liver, it is likely that a non-classical pathway of T cell differentiation exists. The virtual absence of TRECs specific for conventional αβ-T cells in liver suggests that hepatic lymphoid progenitors may give rise to specific γδ cell populations unique to the liver, or unconventional αβ T cells such as, Vδ3Cα^{pos} and Vα24JαQ^{pos} or other, as yet unidentified, invariant NK T cells.

Chapter 5: General Discussion and Future Directions.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS .

5.1: Hepatic haematopoietic stem cells (HSCs).

5.1.1: HSCs in the normal adult human liver (AHL).

This study has demonstrated the presence of significant populations of haematopoietic stem cells (HSCs, $CD34^+CD45^+$) in normal adult human liver (AHL), the majority of which express lymphoid associated antigens (CD56 NK, CD7 T cell and CD19 B cell). Approximately half of the hepatic HSC populations also express differentiation and activation antigens (CD45RA/CD38). These findings suggest that an active haematopoietic pathway operates in the normal AHL, and, that this pathway is biased towards lymphopoiesis. The expression of HLA-DR on almost all hepatic $CD34^+$ cells provides further evidence that this population of cells is actively differentiating (Crosbie *et al.*, 1999).

Evidence has accumulated to suggest that the physiological role of hepatic haematopoiesis, in the normal liver, is to give rise to organ specific immunoregulatory T or NK T lymphocytes (Doherty and O'Farrelly, 2000, Abo *et al.*, 1999; Doherty *et al.*, 1999; Norris *et al.*, 1999, Narita *et al.*, 1998; Norris *et al.*, 1998; Collins *et al.*, 1996; Makino *et al.*, 1993,). Stimulation of stem cell populations present in transplanted organs may be responsible for the phenomenon of chimerism and hence the development of tolerance following liver transplantation (Starzyl *et al.* 1992). Hepatic derived lymphocytes may play an important role in the pathogenesis and/or resolution of autoimmune liver disease (Abo *et al.* 1999), viral hepatitis (Nuti *et al.* 1998), and hepatic malignancy (Cui *et al.* 1997). Demonstration of a local hepatic pool of lymphocyte progenitor cells lends further weight to the hypothesis that the normal AHL is a primary T lymphoid organ. However, considerable proportions of hepatic HSCs co-express NK and B cell associated antigens. The expression of these antigens may reflect the presence of lymphoid progenitor cells as yet uncommitted to differentiation along the T cell development programme. Alternatively, they may reflect a wider role for hepatic lymphopoiesis. Hepatic HSC populations may give rise to other lymphoid cell types such as B cells and NK cells (Jaleco *et al.*, 1997). A major role for hepatic stem cells may be to provide a continual source of NK cells which account for almost one third of hepatic lymphoid cells (Doherty and O'Farrelly, 2000). Functional assays would be necessary to determine the full

developmental potential of hepatic HSCs. Modified hepatocyte co-culture systems (Appasamy, 1992; Appasamy *et al.*, 1993; Gunji *et al.*, 1991; Hata *et al.*, 1993; Nanno *et al.*, 1994), using cell lines derived from normal adult human liver would give the best indication of the role of normal hepatic lymphopoiesis *in vivo*.

Although the majority of $CD34^+$ hepatic cell populations co-expressed CD45, a small, but consistent population of $CD34^+CD45^-$ cells were detected in all samples. These cells may be a pool of quiescent stem cells that do not express activation or differentiation markers, which, when circumstances dictate, become activated and give rise to the differentiating haematopoietic progenitor cells described in chapter two of this thesis. During differentiation, CD34 is gradually down regulated and progenitors begin to express activation, differentiation and lineage-associated antigens as they proliferate and irreversibly commit to development along a particular haematopoietic lineage (Civin and Gore, 1993; Fritsch *et al.*, 1996). The high level of CD34 expression on these CD45 negative populations is consistent with this hypothesis (Krause *et al.*, 1996). CD34, while identifying all stem and progenitor cells, is not, however, exclusively expressed on cells of the haematopoietic lineage. Vascular endothelial cells in human capillaries from most tissues and some large vessels express CD34 (Fina *et al.*, 1990). Because of the gating technique used for flow cytometry, endothelial cells should be excluded on size criteria. It is, therefore, unlikely that $CD34^+CD45^-$ cell populations are composed of endothelial cells.

The absence of CD45 on some $CD34^+$ cells could be explained by the presence of epithelial stem cell populations that give rise to hepatocytes and/or biliary epithelium (Suzuki *et al.*, 2001). The capacity of the liver to regenerate has long been recognised (Bucher and Farmer, 1998; Michalopoulos and DeFrances, 1997). During massive hepatic necrosis in response to a variety of experimental regimens or toxin treatments, a population of epithelial progenitor or 'oval' cells arise which can give rise to either hepatocytes (Alison *et al.*, 1996; Dabeva *et al.*, 1993; Lazaro *et al.*, 1998) or biliary epithelium (Germain *et al.*, 1988; Lenzi *et al.*, 1992; Sirica *et al.*, 1990). By morphological or, immunohistochemical criteria, several groups have reported the presence of oval-like cells in human liver tissue in a number of different diseases

(Baumann *et al.*, 1999; Crosby *et al.*, 1998a; Crosby *et al.*, 1998b; Crosby *et al.*, 2000; De Vos and Desmet, 1992; Demetris *et al.*, 1996; Haque *et al.*, 1996; Hsia *et al.*, 1992; Roskams *et al.*, 1996; Ruck *et al.*, 1997). Hepatic $CD34^+$ cells can differentiate into erythroid and multiple myelomonocytic colonies *in vitro* (Crosbie *et al.*, 1999). *In vitro* studies have also shown that hepatic $CD34^+$ cells can also develop into epithelial cells (Crosby *et al.*, 2000), suggesting that there may be more than one stem cell compartment in the AHL (figure 5.1).

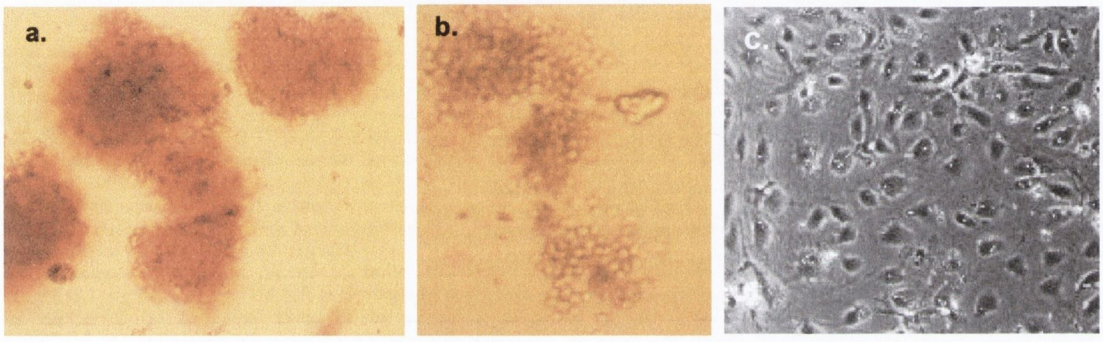


Figure 5.1: Hepatic $CD34^+$ cells differentiate into haematopoietic cells (a and b, Crosbie et al. 1999) and epithelial cells (c, Crosby et al. 2000) in vitro.

The publication of recent reports, which indicate that in rodents and in man, bone marrow-derived haematopoietic stem cells can differentiate into hepatic epithelium (Alison *et al.*, 2000; Lagasse *et al.*, 2000; Petersen *et al.*, 1999; Theise *et al.*, 2000a; Theise *et al.*, 2000b), has generated the intriguing possibility that hepatic cells of epithelial and haematopoietic lineages may arise from a common stem cell. However, the bone-marrow stem cell compartment comprises a heterogeneous population of cells at different stages of maturation (Civin and Gore, 1993; Krause *et al.*, 1996), which may contain separate haematopoietic, stromal and epithelial stem cell populations (Waller *et al.*, 1995). Defining whether a common stem cell gives rise to both epithelial and haematopoietic lineages has important implications for the design of therapeutic strategies which exploit the innate immunoregulatory and regenerative properties of the liver. Initially phenotypic studies using flow cytometry and/or three colour confocal microscopy techniques would give some indication of whether separate stem cell compartments for haematopoietic and epithelial cells are present in AHL. Segregated

expression of epithelial cell and haematopoietic markers on stem cell populations would provide evidence for two separate populations of stem cells. Conversely, co-expression of haematopoietic and epithelial antigens on hepatic stem cells would argue for a common stem cell giving rise to both lineages (figure 5.2).

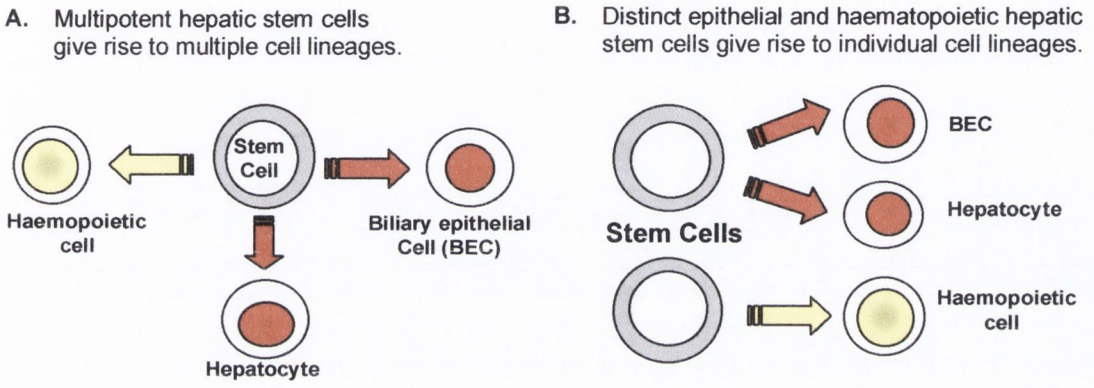


Figure 5.2: Does more than one stem cell compartment exist in the adult human liver?

5.1.2: HSCs in tumour-infiltrated AHL.

The significant changes observed in hepatic HSC populations on tumour challenge suggests a physiological role for hepatic haematopoiesis in immune mediated liver disease. The role of this pathway in immune and non-immune mediated liver disease should be examined further. Bone marrow derived cells with an immature myeloid phenotype have been implicated as suppressers of anti-tumour effector cells in a mouse model of Lewis lung carcinoma (LLC) metastases (Young and Wright, 1992), and granulocyte colony stimulating factor (G-CSF) production by melanoma cells has been linked with metastases of human melanoma (Safarians *et al.*, 1997). More recently, this myelopoiesis-associated suppression phenomenon has been linked to neck cancers in humans (Lathers *et al.*, 2000) It is conceivable that a similar immune suppression mechanism is in operation in liver bearing colonic metastases and the relatively high levels of myeloid progenitors detected in tumour liver suggests that these cells may be generated *in situ*. The high proportion of myeloid progenitors in tumour liver may be a reflection of the activation of myelopoiesis in liver in response to or as a prerequisite of metastases. Low dose combined IFN-γ plus TNF-α has

been found to diminish the presence of myeloid-associated suppressor cells and to reduce tumour recurrence and metastases in metastatic LLC bearing mice (Young and Wright, 1992). The immature myeloid immune-suppressor cells exert their effect, not on the numbers of anti-tumour effector cytotoxic T lymphocytes (CTLs), but by decreasing their sensitivity to activation by IL-12, and thus promoting tumour survival and growth. After treatment of LLC tumour beating mice with vitamin D3 to eliminate myeloid suppressor cells, IL-12 can induce select regional anti-tumour immune responses (Prechel *et al.*, 1996). The results of the present study indicate that myelopoiesis is activated in hepatic colorectal metastatic disease. A qualitative suppression mechanism, similar to that operating in murine LLC metastases may be in operation *in-situ* in human liver bearing secondary tumours of colonic origin. Despite local activation of T-lymphopoiesis, activation of hepatic myelopoiesis may be a tumour derived mechanism to overcome the effect of increased numbers of anti-tumour effector T-cells, facilitating progression of hepatic colorectal metastatic disease (figure 5.3). This hepatic haematopoietic pathway may serve as a potential therapeutic target for diminution of tumour metastases from colon and warrants further investigation.

5.2: The haematopoietic microenvironment of the liver.

The potential of the normal AHL to provide a suitable microenvironment to support local T/NK T cell development was explored in chapter 3. Constitutive expression of IL-7 and IL-15, and, the expression of receptors for these cytokines on the majority of hepatic HSCs, lends further weight to the hypothesis that the normal AHL can act as a primary lymphoid organ, as both of these cytokines have been demonstrated to be indispensable for thymic-independent T cell development (Laky *et al.*, 1998; Malek *et al.*, 1999; Ohteki *et al.*, 1997; Porter and Malek, 1999). However, both IL-7 and IL-15 act on a variety of mature leukocytes (Armitage *et al.*, 1995; Armitage *et al.*, 1992; Burton *et al.*, 1994; Ma *et al.*, 2000; Waldmann and Tagaya, 1999; Wilkinson and Liew, 1995; Yoshikai and Nishimura, 2000; Zoll *et al.*, 1998) and IL-15 also acts on cells that are not of haematopoietic lineage (Quinn *et al.*, 1995; Reinecker *et al.*, 1996). Thus, their expression in liver may be a reflection of a wider role for these cytokines in local immune responses (Li *et al.*, 2001, Mackall *et al*, 2001). Further studies are required to determine the presence in liver of other factors known to be necessary for T cell development,

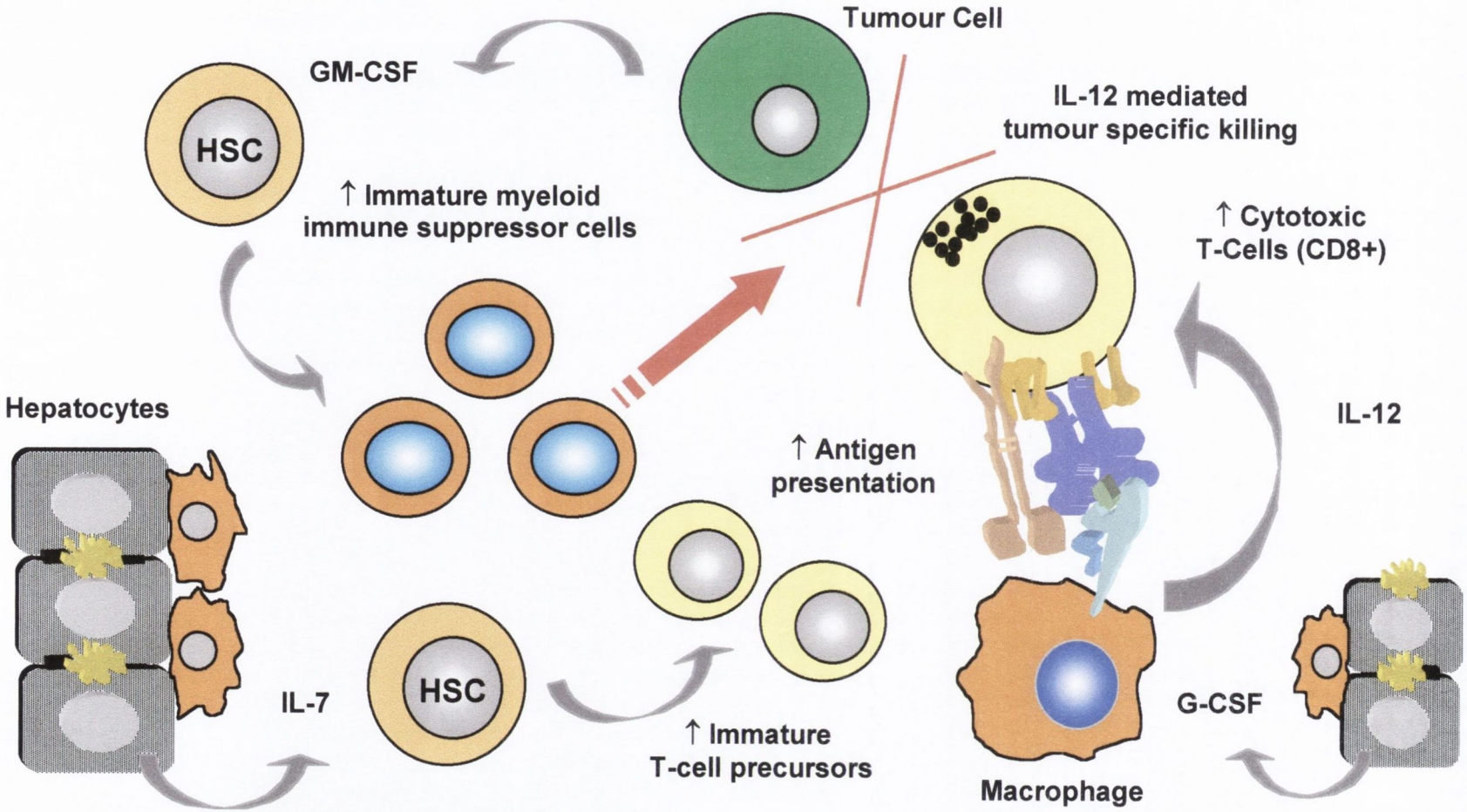


Figure 5.3. Myelopoiesis associated immune suppression (based on Young *et al.*, 1992).
Abbreviations: CSF = Colony stimulating Factor; G = Granulocyte; HSC = Haematopoietic Stem Cell; IL = Interleukin; M = Monocyte.

such as haematopoietic colony stimulating factors and antigen presentation molecules important in the selection of extrathymic T cells. A recent study on the differentiation of murine intestinal T cells indicated that CD8$\alpha\alpha^+$ lymphocyte maturation was induced most efficiently by high affinity ligands, a scenario that is in direct contrast to T cell selection in the thymus, emphasising the unique features of local T cell maturation (Levelt and al, 1999). T cells which mature locally in the liver may not be subjected to the same stringent selection processes which occur intrathymically, allowing auto-reactive clones to emerge which may contribute to the pathogenesis of auto-immune liver disease (Abo *et al.*, 1999). The same principle may facilitate differentiation of tolerant clones in the presence of donor MHC molecules following transplantation and may explain why donor organs do not need to be MHC matched and the tolerance of other organs co-transplanted with liver (Calne, 2000; Sfeir *et al.*, 2000). The importance of the hepatocyte-haematopoietic stem cell interaction has been highlighted by the discovery of a foetal hepatocyte cell clone that can support haematopoiesis *in vitro* (Hata *et al.*, 1993; Nanno *et al.*, 1994). Liver injury induced by carbon tetrachloride administration causes disruption of the hepatic T cell development pathway, suggesting that the intact microenvironment of the liver is important for supporting the differentiation of T cells *in situ* (Kawachi *et al.*, 1994). A better understanding of the selection processes and the molecules involved in hepatic T cell development will help to define the pathogenesis of auto-immune liver disease. It may then be possible to design specific treatment and prevention strategies for patients with auto-immune liver disease.

5.3: T-cell development in the liver.

Hepatic T lymphocytes consist of heterogeneous populations of lymphoid cells likely to have many functional characteristics in addition to classical T cell activity (Doherty and O'Farrelly, 2000). The possibility that some populations of hepatic T cells, which play an important role in organ-specific immunity, might mature and differentiate locally and be supplemented by infiltrating lymphocytes from the peripheral blood is an intriguing one that is now gaining credibility (Narita *et al.*, 1998; Suzuki *et al.*, 1998; Yamamoto *et al.*, 1999). The absence of δ-deletion TRECS in hepatic samples provides evidence that conventional $\alpha\beta$ T cells do not arise locally in the liver, however the evidence for local T cell development remains

convincing and the possibility that γδ or other unconventional T cells develop locally should be investigated. The presence of significant populations of $CD8^{pos}$ naïve ($CD45RO^{neg}$) T cells in all hepatic samples suggests that local contribution to the naïve T cell pool would be to this subset. The majority of hepatic NK T cells, a population likely to be extrathymic in origin (Makino *et al.*, 1993; Ohteki *et al.*, 1997), are $CD8^{+}$ (Norris *et al.*, 1998; Norris *et al.*, 1999) and, the majority of hepatic γδ T cells, another population likely to be extrathymic in origin (Laky *et al.*, 2000; Laky *et al.*, 1998), express $CD8^{+}$ or DN phenotypes (Norris *et al.*, 1999). NK T and γδ T cells are abundant in liver but occur relatively rarely in peripheral blood (Doherty and O'Farrelly, 2000). Studies in mice suggest that, while there is continued infiltration of peripheral blood T cells into the liver and gut, locally derived populations do not traffic into the blood under normal circumstances (Suzuki *et al.*, 1998). Thus, hepatic NK T and γδ T cells are likely candidates for locally derived populations (figure 5.4).

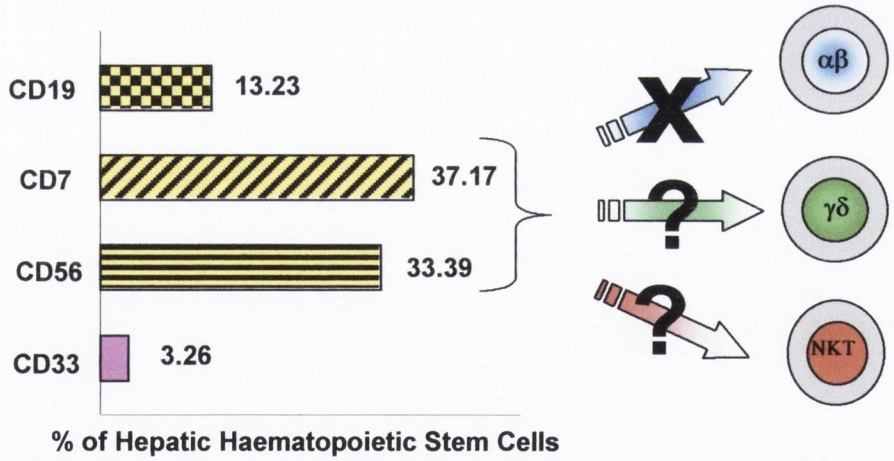


***Figure 5.4: Hepatic haematopoietic stem cells do not give rise to conventional αβ T cells* but may give rise to γδ *or unconventional αβ T cells such as Vα24Jα*Qs.**

Skewing of the TCR repertoire is thought to occur as a consequence of the development of T cells at sites other than the thymus (Abo *et al.*, 1999; Emoto *et al.*, 1997). NK T cells, isolated from human liver, display a TCR repertoire that is skewed towards Vα24JαQ usage (Norris *et al.*, 1999). Preliminary studies, (described in chapter 4 of this thesis), provide evidence that there is also skewing of the hepatic γδ TCR repertoire. Thirty percent of hepatic γδ T cells

express the Vδ3 gene segment whereas this segment was undetected in matched blood samples. The Vδ-gene usage of hepatic γδ T cells in the adult human has not been explored. As commercially available antibodies only recognise Vδ1, Vδ2 and Vδ3 regions, our ability to look at the full range of Vδ gene usage is limited using this approach. Genomic and cDNA sequences of the human δ-locus are now available facilitating the design and interpretation of PCR experiments. Real time quantitative PCR techniques would provide an excellent tool to look for further evidence of differential Vδ gene usage in liver. Comparison of profiles between γδ T cells in liver and blood may identify differences in Vδ gene segment usage (in addition to Vδ-3 usage) and hence demonstrate other hepatic skewed γδ TCR repertoires. TREC experiments designed to target deletion circles for Vα24s and Vδ genes used preferentially in the liver may help to delineate which populations are differentiating locally. A recent report suggests that γδ T cells may also be involved in tissue remodelling (Miller *et al.* 2000), thus, locally developing γδ T cells may play a role in liver regeneration.

5.4: Wider implications.

The demonstration of stem cells in the AHL raises exciting possibilities for the future. Intervention in the normal development pathways of adult stem cells has the potential to provide us with a natural, safe tool to tailor the immune system to be more effective in the elimination of disease, induction of peripheral tolerance and stimulation of tissue regeneration and repair. A basic understanding of the biology of adult hepatic stem cell is essential to being ultimately capable of manipulating these cells in therapeutic applications such as gene therapy. The liver is a fascinating complex organ composed of hepatic parenchymal cells and a variety of non-parenchymal cells including endothelial cells, Kupffer cells and several subsets of resident lymphocytes. Its role as the major site of energy conversion, detoxification and protein synthesis in the body render it susceptible to a host of pathogenic, pharmacological, metastatic and infectious challenges. Despite significant research initiatives, no progress has been made in the development of processes or products for reversing liver damage that results from these challenges. An exciting alternative to liver transplantation is to devise strategies for generating fresh liver tissue *in situ* by exploiting the liver's unique

capacity to regenerate. Regeneration is dependent on a continuous supply of stem cells and healthy human liver tissue has been shown to contain a significant population of dividing stem cells (Crosbie *et al.* 1999; Crosby *et al.* 2000, Golden-Mason *et al.*, 2000).

A major focus of the biomedical research community is to devise methods of growing replacement tissues for substitution of diseased damaged or aged tissues. The long-term aim is the development of the necessary technologies for the generation of new liver tissue from harvested stem cells. A more immediate aim is the generation of a safe, fully functional human cell model that can be substituted for xenogenic primary hepatocytes in bioartificial liver support devices that could also be used for evaluation of drug toxicity and pharmokinetics for the development of safe new therapeutic drugs. The ability to identify and exploit a human hepatic clonal stem cell could have important clinical implications for the treatment of metabolic disorders or fulminant hepatic failure. It is critical to know whether the same stem cells is capable of generating hepatocyte, epithelial cells as well as haematopoietic cells, to understand how these development pathways are regulated and how they interact. The studies described in this thesis have contributed to our understanding of this exciting area of research that is still at a very early stage but promises exciting possibilities for the future.

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Appendices.

Appendix I: $CD5^{+/-}$ B Cells ($CD19^{+}$) in Liver.

The levels of B cells ($CD19^{+}$) were estimated in 8 normal liver samples, results are expressed as a % of total leukocytes ($CD45^{+}$). The levels of B-1 ($CD19^{+}CD5^{+}$) cells were estimated in the same samples, results are expressed as a % of total B cells.

Table API.1. : Levels of B-2 ($CD19^{+}CD5^{-}$) and B-1 ($CD19^{+}CD5^{+}$) cells in normal liver.

Sample	B cells	B-1 cells
D030598	10.61	6.31
D060698	1.16	16.38
D160698	2.88	2.08
D150598	12.62	0.06
D180698	3.23	3.61
D270698	10.61	7.73
D300698	7.06	15.86
D070798	2.99	8.03
D150798	6.05	13.39
Mean	**6.36**	**8.16**

Appendix II (APII): List of Suppliers.

Suppliers of reagents and equipment are listed in alphabetical order.

Company	Address
ATCC	Rockville, MD, USA
BDH	Poole, England
Beckman Instruments	Fullerton, CA, USA
Becton Dickinson (BD)	Oxford, England
Bioline	London, England
Boehringer Manheim	Steinheim, Germany
Braun Apparate	Melsungen, Germany
Cadisch Precision Meshes	London, England
Dako	Cambridge, UK
Dynal (UK) Ltd	Wirral, England
Dynatech	Chantilly, VA, USA
Falcon (BD)	Oxford, England
Finetek Europe BV	Zoeterwoude, Holland
GIBCO BRL	Paisley, Scotland
Hawskey	Essex, England
Immunotech	Marseille, France
Labscan	Dublin, Ireland
Microm	Walldorf, Germany
Miltenyi Biotec	Gladbach, Germany
MJ Research Inc.	Cambridge, MA, USA
Nalgene Nunc International	Roskilde, Denmark
Nycomed (UK) Ltd	Birmingham, England
Packard	Meriden, CT, USA
Pharmingen	San Diego, CA, USA
Pierce	Rockford, IL, USA
Promega	Madison, WI, USA
R&D Systems (Europe)	Oxon, England
Sigma-Aldrich	Dublin, Ireland
Southern Biotechnology Associates Inc	Birmingham, AL, USA
Stratagene Cloning Systems	La Jolla, CA, USA
Vector Laboratories	Burlingame, CA, USA

Appendix III (APIII): Standard Protocols.

APIII Contents.

Figures included in Appendix III.

Tables included in Appendix III.

APIII.1: Assessment of cell viability and yields.

Introduction.

Ethidium bromide (EB) interchelates double-stranded DNA and fluoresces upon ultra-violet (UV) illumination. The viable cells appear green, non-viable cells take up the acridine orange (AO) counterstain and appear orange under UV light. The improved Neubauer haemocytometer is used to count the number of cells in a defined volume appearing in a grid which is etched onto the surface of the counting chamber and visible by microscopic examination.

Materials.

EB (stock solution: 4 mg/ml EB, Sigma-Aldrich* # E8751, stored at 4°C protected from light)

AO (stock solution: 1 mg/ml AO, Sigma-Aldrich # A6014, stored at 4°C protected from light).

Improved Neubauer haemocytometer (Hawskey, B.S. 748) *and coverslips.*

Glass test tubes.

RPMI (Gibco BRL #21875-034).

**Note: suppliers of reagents are listed alphabetically in Appendix II*

Protocol.

Cells isolated from all sources were resuspended in one ml of RPMI (+ 0.02 M Hepes buffer, Gibco BRL, # 15630-056) after the final wash. Twenty μl of cells was added to 380 μl of EB/AO working solution (made from 0.8 ml EB stock and 2.0 ml AO stock made up to 200 ml with 0.85% NaCl [BDH, # 102415K]) in a glass test tube and mixed well. Approximately 10 μl of the solution was loaded onto a haemocytometer, ensuring that the solution covered the entire surface of the counting chamber. Cells were visualised under UV-light. Total cells and live cells only, appearing in the four corners of the grid (the shaded areas in figure APIII.1), were counted.

Cell yield was calculated as follows:

(The sum of the counts from the four corners of the grid ÷ 2) x 10^5 = cell number /ml. The required concentration of cells/ml was achieved by dilution in the appropriate volume of RPMI (+ Hepes buffer).

Percentage viability was calculated as follows:

(Number of live cells/ml ÷ Number of total cells/ml) x 100 = percentage viability.

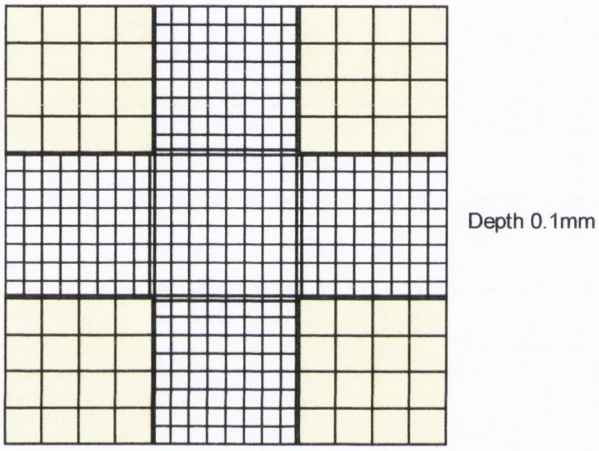


Figure APIII.1: Diagramatic representation of the grid of the Hawskey counting chamber.

APIII.2: Density gradient centrifugation.

Introduction.

Mononuclear cells (monocytes and lymphocytes) have a lower buoyant density than erythrocytes and polymorphonuclear leukocytes (PMNs). Mononuclear cells can therefore be isolated by centrifugation on an iso-osmotic medium with a density close to 1.077 g/ml, which allows the erythrocytes and granulocytes to sediment through the medium while retaining the mononuclear cells at the sample/medium interface. Lymphoprep™ (density 1.077 g/ml) developed by Bøyum (1983) is based on this principle.

Materials.

HBSS (Gibco BRL, # 24028-091).

Lymphoprep™ solution (Nycomed).

RPMI (Gibco BRL #21875-034).

Centrifuge (Jouan C312).

25 ml disposable containers (Sterilin).

Sterile plastic pasteur pipettes.

Protocol.

Ten ml of peripheral blood (or bone marrow aspirate), collected in a lithium-heparin container, was added to an equal volume of HBSS (+ 0.02 M Hepes buffer) and mixed gently. The diluted sample was then layered onto five ml of Lymphoprep™ in a 25 ml Sterilin and centrifuged at 300g for twenty five minutes (with the break off to ensure that the layers do not mix). Mononuclear cells are retained in the buffy-coat visible as a clouded band just above the clear lymphoprep™ solution (figure APIII.2). Mononuclear cells were removed with a Pasteur pipette and washed twice in twenty ml HBSS (+ Hepes), with centrifugation at 300g for 8 minutes. After the last wash, the pellet was resuspended in one ml of RPMI (+ Hepes) and cell yield and viability was calculated as described earlier (APIII.1).

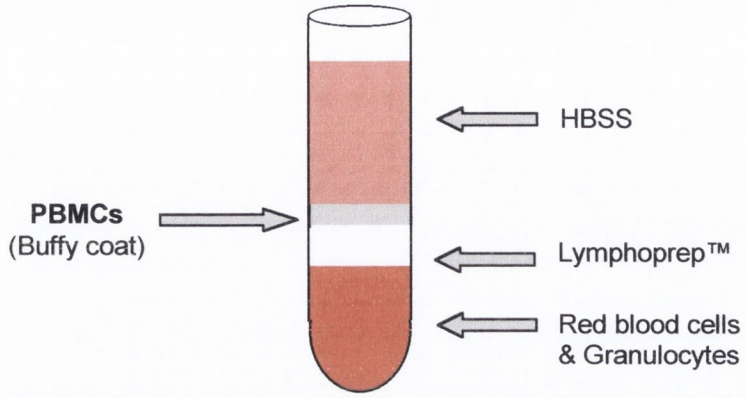


Figure APIII.2: Separation of mononuclear cells by density centrifugation.

APIII.3: Powdering of hepatic tissue samples.

Introduction.

The Braun Mikrodismembrator apparatus (Braun Apparate) consists of a Teflon chamber in which a ball bearing is vibrated at high speed (50 hz in a bilateral direction) for approximately 15 seconds, until the sample is converted to a fine powder (figure APIII.3). The production of a high quality powder (even consistency without degradation of RNA or protein) is dependent on the chamber, ball bearing and sample being kept frozen throughout the procedure.

Materials.

Braun Mikrodismembrator, Teflon vial and ball bearing.

Liquid Nitrogen.

DEPC-treated water (Diethylpyrocarbonate, Sigma-Aldrich #D-5758): One ml DEPC was added to 500 ml water, the solution was mixed well and left at room temperature for at least 2 hours and autoclaved before use).

Seventy percent Ethanol (in DEPC-treated water, Sigma-Aldrich # E-7023).

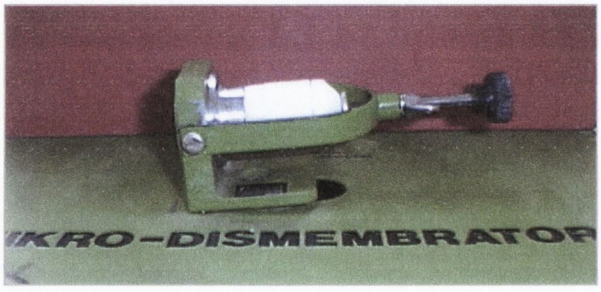
Petri dish, forceps, spatula, Sterilin containers, cryovial and scalpels.

Protocol.

Frozen liver tissue was cut into small pieces on a petri dish (snap-frozen in liquid nitrogen prior to addition of the sample). The sample was kept frozen by pouring liquid nitrogen into the dish periodically. The chopped tissue was placed in a Sterilin and returned to liquid nitrogen. The chamber and ball bearing were washed sequentially in 70% Ethanol and DEPC-treated water (to remove RNases) and frozen in liquid nitrogen. The chopped tissue sample was then poured into the chamber, the ball bearing added and the assembled chamber and sample were placed in liquid nitrogen for 20 seconds. The chamber was attached to the Mikrodismembrator and vibrated at 50 hz for 15 - 20 seconds. The chamber was removed and placed in liquid nitrogen for 10 seconds. The chamber was disassembled and the contents was checked to ensure the liver tissue had been completely and evenly powdered. If the sample was insufficiently powdered the vibration was repeated for another 15 seconds.

Tissue powder was then aliquoted into labelled cryovials and stored in liquid nitrogen until required.

a.



b.

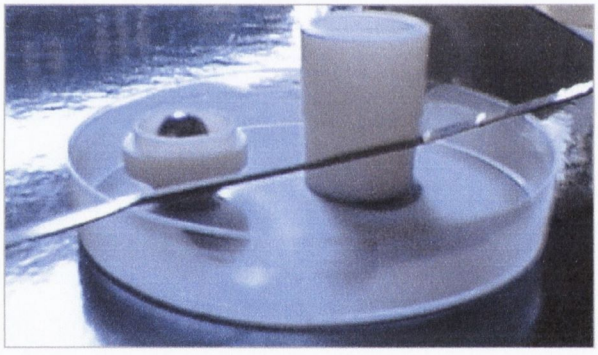


Figure APIII.3: Braun Mikrodismembrator, Teflon vial and ball bearing.

a. The assembled Teflon vial attached to the Braun Mikrodismembrator. **b.** Disassembled Teflon vial and ball bearing.

APIII.4: Trypsinisation of cultured adherent cells.

Introduction.

The growth of some cell lines is adherence dependent- they attach to the surface of plastic culture flasks and grow in tight colonies. Washing alone is insufficient to remove cells from the surface of the flask. Treatment with a protease such as trypsin is required to break the contact between cells and to remove them from the plastic surface of culture flasks (figure APIII.4).

Materials.

Trypsin 10 x liquid (25 g/L, Gibco BRL # 25090-010).

HBSS (Ca^{2+} and Mg^{2+} free, Gibco BRL # 14170-088).

Culture Medium (appropriate medium for the particular cell line).

25 ml disposable plastic Sterilin containers.

Centrifuge (Jouan C312).

Protocol.

The following procedure is described for cells grown in 75 cm^2 tissue culture flasks. The culture medium was decanted from the culture flask containing the cells. Twenty mls of Ca^{2+} and Mg^{2+} free HBSS was added to the flask and left for 5 minutes to remove FCS which is a strong inhibitor of trypsin. A working solution of 1 x trypsin was prepared (in Ca^{2+} and Mg^{2+} free HBSS) immediately before use. The HBSS was decanted and 10 mls of trypsin solution (1 x) was added to the cells and incubated for approximately seven minutes at 37°C. Cells remaining on the flask surface were removed by gently tapping the side of the flask for approximately 30 seconds. The trypsin solution containing the cells was decanted into a 25 ml disposable plastic Sterilin container and the solution was made up to 20 mls with the appropriate medium containing FCS. The suspension was centrifuged at 200g for 5 minutes and the cells resuspended in 1 ml of the appropriate growth medium. Cell yield and viability was assessed as described earlier (APIII.1). Cells were then either re-seeded into tissue culture flasks at a concentration of 0.5 x 10^6 cells/ml or pelleted for extraction of RNA/protein.

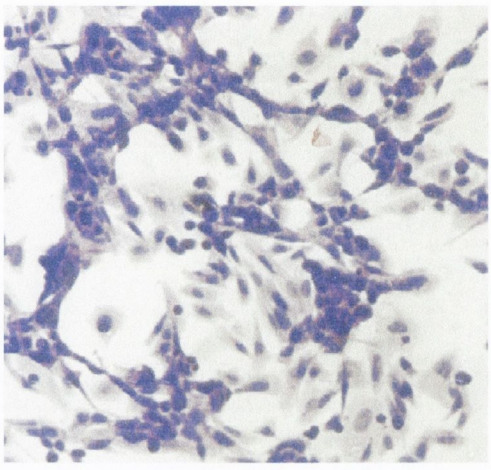


Figure APIII.4: Adherent cell line SK-HEP-1 stained with Haematoxylin and Eosin.

Cells were grown in Dulbeco's Modified Eagle's Medium (Gibco BRL # 2190-022). Treatment with trypsin is required to separate the cells and remove them from the surface of the flask.

APIII.5: RNA isolation (RNAce Total Pure purification system™).

Introduction.

The RN*Ace* system has been designed for the isolation of total RNA from different sources of biological material, such as cell culture or tissue samples (1 x 10^5 - >10^8 cells or 5 mg – 1 g tissue material). The system is based on a new procedure, which allows the isolation of cellular total RNA free of contamination with genomic DNA. The genomic DNA will bind with a carrier suspension after cell lysis and the resulting supernatant is pure total RNA. The procedure is based on the dual action of chaotrophic compounds that are responsible for cell lysis and the inactivation of endogenous RNases. The purified RNA is undegraded and of excellent quality and is suitable for a large number of RT/PCR reactions and can also be used for several RNA dot blots, Northerns, cDNA synthesis, mRNA isolation and RNase protection assays.

Materials.

RNAce Total Pure Kit™ (Bioline, components : Carrier suspension, Lysis solution [chaotropic salt], Buffer A and Isopropanol [ultra pure]).

DEPC-treated water (APIII.3 materials).

Water saturated phenol (BDH # 43672 2V).

Chloroform (BDH # 100774W)

70% Ethanol (in DEPC-treated water, Sigma-Aldrich # E-7023).

Refrigerated Epifuge (Micromax-RF, International Equipment Company [IEC])

Protocol.

Tissue powder (~ 50 mg) was placed in 2ml eppendorfs. Five hundred μl of Lysis solution was added and 10 minutes was allowed for lysis (the lysis suspension was pipetted up and down occasionally). When lysis was complete, 20-30μl of carrier suspension was added and the suspension was thoroughly vortexed. Tubes were then placed on ice for 5 minutes. Eppendorfs were centrifuged at 10,000g for 1 minute to sediment the carrier pellet, the supernatant was transferred into a new tube (taking care not to disturb or carry over the

pellet). Five hundred μl water saturated phenol, 100μl chloroform and 50μl Buffer A were added to the supernatant and vortexed vigorously for 10 seconds and then incubated on ice for 5 minutes. The organic and aqueous phases were separated by centrifugation at 10,000g for 10 minutes (figure APIII.5). The upper aqueous phase was carefully transferred to a new tube *(at least 2mm of the aqueous phase was left above the interface).* The volume of the aqueous phase transferred was estimated and an equal volume of isopropanol was added. Samples were mixed gently and incubated at -70°C for 30 minutes to precipitate the RNA. The RNA was harvested by centrifugation at 10,000g for 10 minutes (the supernatant was discarded without disturbing the RNA pellet). One ml ice-cold 70% ethanol (in DEPC-treated water) was added and the pellet washed by inversion of the tube several times and centrifugation at 10,000g for 5 minutes (the ethanol was discarded). The wash step was repeated twice more. After the last wash step, the residual ethanol was allowed to evaporate by placing the opened tube upside down on a tissue. The dried RNA pellet was resuspended in 50 μl of DEPC-treated water. RNA was isolated from pellets prepared from haematological samples or cultured cells using the same protocol.

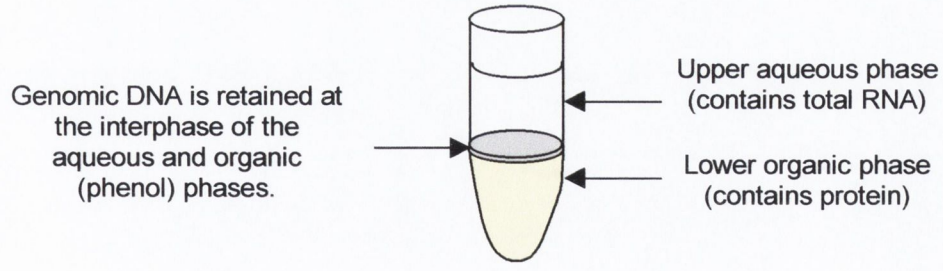


Figure APIII.5: Separation of aqueous and organic phases.

In this single step extraction, total RNA is separated from DNA and proteins. The upper aqueous phase contains total RNA, the DNA is retained at the interphase and proteins in the organic phase.

APIII.6: Assessment of the integrity and yield of RNA.

Introduction.

The quality of the RNA extracted was determined by agarose gel electrophoresis, ethidium bromide staining and UV transillumination. The 28S and 18S RNA bands should be apparent, with the 28S band being ideally twice the density of the 18S band (figure APIII.6). DNA contamination is visible in the well, or as a laddered band just before the 28S band. Degradation of the RNA will be visible as a long smear in the gel.

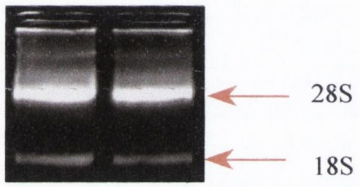


Figure APIII.6: Agarose gel electrophoresis of total RNA.

Materials.

50 x TRIS-Acetate-EDTA (TAE) Buffer (1 litre: 242g Tris Base [Sigma-Aldrich # T-6791], 57.1ml Glacial Acetic Acid [BDH # 27013], 100 ml 0.5M EDTA [pH8.0, BDH # 100935V]).

Ethidium Bromide (0.4mg/ml, APIII.1)

Blue/Orange Loading buffer (6 x, Promega # G190A, diluted in 1 x TAE).

Horizontal gel electrophoresis apparatus (Gibco BRL)

Agarose (Sigma-Aldrich # A-9539).

Protocol.

A) Preparation and running of gel.

Agarose (0.5g) was dissolved (boiled) in 25 mls 1 x TAE buffer (prepared from stock 50 x TAE with distilled water). The gel was allowed to cool to ~37°C before addition of 8-9μl of Ethidium Bromide. The gel was then poured immediately into a horizontal electrophoresis apparatus and an eight well comb was inserted. The gel was set for 20-30 minutes at room temperature and the comb removed. Ten μl was loaded in each lane (5μl of sample + 5μl of 2

x loading dye). The gel was run for approximately 15-30 minutes at 100V in 1 x TAE buffer (until samples had run three quarters way along the gel). The RNA bands were visualised using the Eagle Eye™ II still video system (Stratagene Cloning Systems).

B) Calculation of RNA yield.

Nucleic acids absorb UV light at 260 nm. An absorbance (Abs) of 1 at 260 nm = 40 μg/ml RNA. The absorbance at 280 nm is an indication of the extent of protein contamination in the sample; Abs260:Abs280 ratio should approximate 2 (indicates a clean RNA preparation). Five μl of total RNA was placed in a tube containing 45 μl of DEPC-treated water and used for spectrophotometric determination of total RNA yield. A micro cuvette fitting (50 μl volume) was used in a DU-64 Spectrophotometer (Beckman Instruments). Cuvettes were washed sequentially in ethanol and DEPC-water to ensure they were free of RNases. The light path was optimised and the machine calibrated using 50 μl of DEPC-water. Absorbance was read at 260 nm and 280 nm.

C) Calculation.

The total RNA yield was calculated as follows:

Abs @ 260 nm = 1 = 40 μg/ml

Abs @ 260 nm = x = x μg/ml

Multiply by dilution factor 10 (5μl sample + 45μl DEPC-water) = [RNA]/μl

Multiply by total volume (30-50μl) to estimate total yield.

One μg of total RNA was used for reverse transcription into cDNA.

D) Storage of RNA.

RNA was stored at -80°C in DEPC-treated water until required.

Note: Integrity and yield of DNA can be assessed in the same manner as RNA (a single band should be visible in the gel). The Abs @ 260 nm = 1 = 50 μg/ml dsDNA.

APIII.7: The BCA protein Assay™.

Introduction.

The Pierce BCA protein Assay™ is a detergent compatible formulation based on bicinchonic acid (BCA) for the colourimetric detection and quantitation of total protein. This method combines the well-known biuret reaction (reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium) with the highly sensitive and selective colourimetric detection of the cuprous cation (Cu^{1+}) using a unique reagent containing the BCA. The purple-coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex absorbs strongly at 570 nm, and absorbance remains linear with increasing protein concentration over a broad working range (20 μg/ml to 2,000 μg/ml).

Materials.

BCA Protein Assay Kit™ (Pierce, contents: reagent A [solution of sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in 0.2N sodium hydroxide], reagent B [solution of 4% cupric sulphate]).

Albumin Standard (2.0 mg/ml bovine serum Albumin [BSA] solution in 0.9% saline and 0.05% sodium azide, Pierce).

Microwell plate (96 well, Falcon).

ELISA plate reader (Dynatech MR4000).

Protocol.

Protein standards were prepared following the scheme set out in Table APIII.1. Unknown samples were diluted 1/40, 1/80 and 1/160 in PBS. The BCA working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part reagent B. Twenty five μl of each standard and unknown sample were pipetted into the appropriate wells (in triplicate), 25 μl of diluent was used for the blank wells. Two hundred μl of WR was added to each well and the plate was mixed on a shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. After incubation the plate was allowed to cool to room temperature and absorbance was read at 570 nm (Abs570) on an ELISA plate reader (Dynatech MR4000).

The average Abs570 reading for the blank wells was subtracted from the Abs570 reading for each BSA standard and unknown sample. A standard curve was constructed by plotting the average blank-corrected reading for each BSA standard against concentration in μg/ml. Using the standard curve, the protein concentration for each unknown sample was calculated.

Table APIII.1: Preparation of BSA standards.

Volume of BSA	Volume of diluent*	Final BSA concentration
300 μl of (stock)	0 μl	2,000 μg/ml
375 μl of (stock)	125 μl	1,500 μg/ml (A)
325 μl of (stock)	325 μl	1,000 μg/ml (B)
175 μl of (A)	175 μl	750 μg/ml (C)
325 μl of (B)	325 μl	5000 μg/ml (D)
325 μl of (D)	325 μl	250 μg/ml (E)
325 μl of (E)	325 μl	125 μg/ml (F)
100 μl of (F)	400 μl	25 μg/ml (G)

*Phosphate buffered saline (PBS, 0.1M, Sigma-Aldrich, # p-4417)

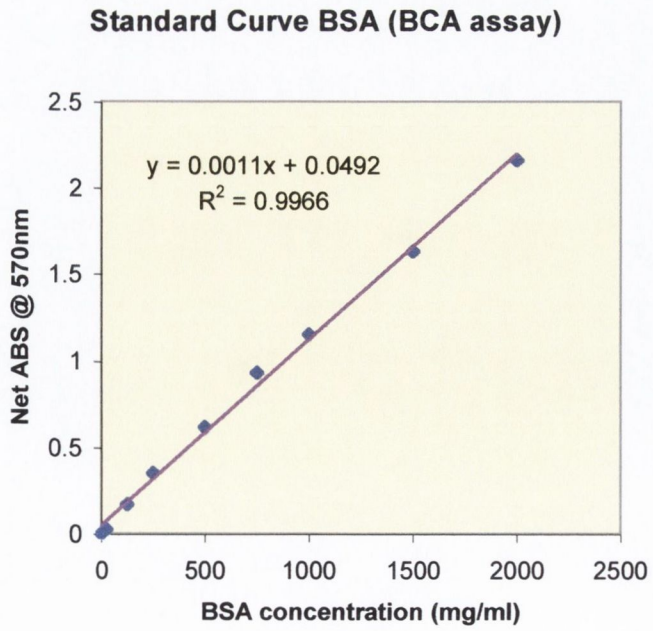


Figure APIII.7: Typical standard curve generated using the BCA protein assay.

APIII.8: Enzyme-Linked Immunosorbent Assay (ELISA).

Introduction.

Immunogenic methods of quantifying antigen concentration provide exquisite sensitivity and specificity and have become standard techniques for both research and clinical applications. When the indicator molecule is covalently coupled to an enzyme, it can be quantified by determining with a spectrophotometer the amount of clear substrate converted to a coloured product (ELISA). Several variations of ELISA are in common use, the one used in this study was the "Sandwich ELISA". A fixed quantity of one antibody (capture Ab), directed against the protein of interest, is attached to a solid support (96-well plate). Test solutions of unknown antigen concentration and a series of standards of known concentrations are allowed to bind. Unbound antigen is removed, and a second biotinylated-antibody (detection Ab, directed against the same antigen but a different epitope) is allowed to bind. Unbound antibody is removed and an enzyme-linked avidin complex is added which binds to the biotinylated-antibody. Excess avidin complex is removed and the addition of a substrate solution results in the formation of a coloured product which is directly proportional to the concentration of peroxidase, and thus, the antigen of interest in the sample or standard (figure APIII.8).

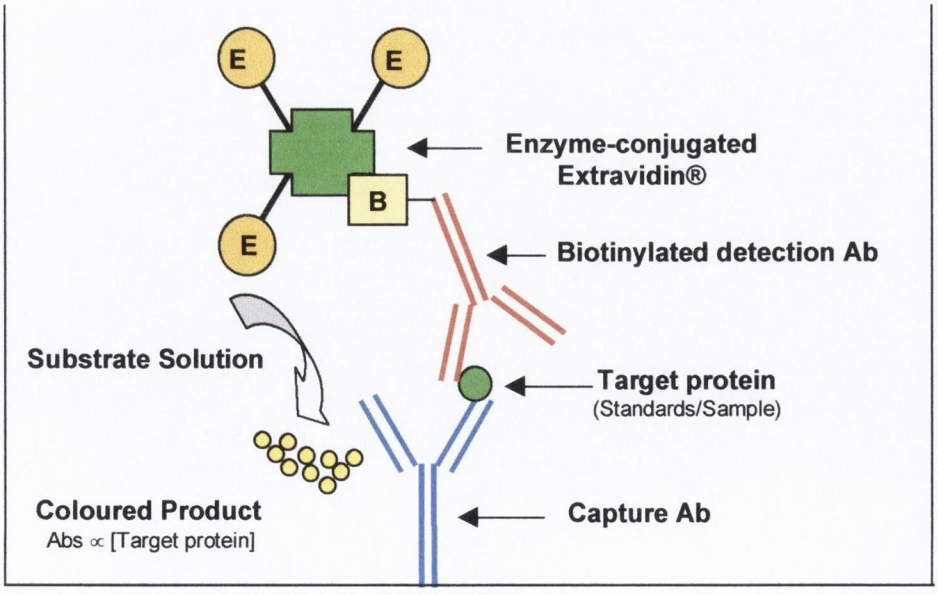


Figure APIII.8: Principle of the sandwich ELISA used to quantify protein.

Note: the following detailed protocol is given for the Interleukin-7 (IL-7) ELISA used for the measurement of IL-7 cytokine protein levels in normal hepatic tissue (Chapter 3). The same general protocol was used for the measurement of IL-15. Any differences between the IL-7 and IL-15 ELISAs (for example antibodys) are detailed at the end of the protocol.

Materials.

Capture mAb (anti-IL7, R&D Systems, MAB207, 2 μg/ml in sterile PBS).

Detection mAb (anti-IL7, R&D Systems, BAF207, 50 ng/ml in sterile TBS + 0.1% BSA [Sigma-Aldrich # A-1662]).

Recombinant human IL-7 (R-IL7, R&D Systems, 207-IL, stock solution 1 μg/ml in sterile PBS + 0.1% BSA).

Wash Buffer (0.5% Tween 20® [Sigma-Aldrich # P-1379] in PBS pH 7.4).

Blocking buffer (PBS + 3% BSA, 5% sucrose [Signa-Aldrich # S-7903], 0.05% NaN_3 [Sodium Azide, Sigma-Aldrich # S-8032]).

Sample Diluent (20mM TRIS base [Sigma-Aldrich # T-6791], 150mM NaCl [BDH # 10241], 0.1% BSA, 0.05% Tween 20®).

Extravidin® Alkaline Phosphatase conjugate: (Sigma-Aldrich, E-2636, 1/2,500 in PBS).

Diethanolamine Substrate buffer (97 ml Diethanolamine [BDH # 103934J], 1 ml 1% NaN_3, 100 mg $MgCl_2$ [Hexahydrate, Sigma-Aldrich # M-0250] , pH 9.8. made up to 1 litre with distilled water).

Substrate solution p-Nitrophenyl Phosphate (pNPP, Sigma-Aldrich, N-2765, 1mg/ml in Diethanolamine buffer).

Stop Solution (3M NaOH, [Sigma-Aldrich # S-5881, 12 g/100 ml).

Nunc-Immuno ® plate (Nalgene Nunc International).

ELISA plate reader (Dynatech MR4000).

Protocol.

Plate preparation.

The capture antibody was diluted to appropriate concentration in PBS immediately before use. Diluted capture antibody was transferred to an ELISA Plate (100 μl/well). One row of

wells did not receive capture Ab but instead isotype matched IgG control Ab (Dako, Cambridge, UK, 2 μg/ml in PBS, negative control). The plate was sealed and incubated overnight at room temperature. The following morning, each well was aspirated and washed with wash buffer (400 μl/well) for a total of three washes. Plates were blocked by the addition of 300 μl of blocking buffer to each well and incubation at room temperature for one hour. Aspiration and washing was repeated as above. During the blocking step, dilutions of samples and standards were prepared.

Preparation of solutions for standard curve (range 2-2000 pg/ml).

Dilution of standards and samples was carried out in polypropylene tubes using the sample diluent described above. Lysates prepared from powdered liver tissue were diluted 1/2 or 1/4. A series of nine doubling dilutions was carried out after an initial 1/500 dilution of the stock solution of R-IL-7. Diluent only was used as a blank. The final concentration of the IL-7 standards is detailed in table APIII.2.

Table APIII.2: Concentrations of IL-7 standards.

Standard no	Concentration pg/ml	Standard no	Concentration pg/ml
1	2000	**7**	31.25
2	1000	**8**	15.625
3	500	**9**	7.8125
4	250	**10**	3.906
5	125	**11**	1.953
6	62.5	**12**	0

Assay procedure.

One hundred μl of samples or standards was added to wells and the plate was mixed by gentle tapping of the plate frame for one minute. The plate was covered with an adhesive strip and incubated at room temperature for two hours. Aspiration and washing were repeated as

above. Detection antibody (100 μl to each well) diluted to 2 mg/ml in the appropriate diluent was added to the plate. The plate was covered with an adhesive strip and incubated at room temperature for two hours. Aspiration and washing were repeated as above. Extravidin (working solution, 100 μl) was added to each well, the plate was covered with a new adhesive strip and incubated at room temperature for 30 minutes. Aspiration and washing were repeated as above. Two hundred μl of substrate solution was to each well and incubated at room temperature for 30 minutes. Fifty μl of stop solution was added to each well and the plate frame was tapped gently to ensure thorough mixing. Absorbance was read at 410 nm (Abs410) on an ELISA plate reader (Dynatech MR4000). The average Abs410 reading for the negative wells was subtracted from the Abs410 reading for each IL-7 standard and unknown sample. A standard curve was constructed by plotting the average blank-corrected reading for each IL-7 standard against it's concentration in pg/ml. Using the standard curve, the IL-7 protein concentration for each unknown sample was calculated. Only readings above those of the negative control were taken as positive.

Reagents for IL-15 ELISA (used in place of IL-7 antibodies and standards).

Capture mAb (anti-IL15, R&D Systems, MAB647, clone 34505.11, 2 μg/ml in PBS).
Detection mAb (anti-IL15, R&D Systems, BAM247, clone 34593.11, 50μg/ml in TBS + 0.1% BSA).
Recombinant IL-15 (R-IL15, R&D Systems, 247-IL, 5 μg/ml in PBS + 0.1% BSA)

APIII.9: Immunohistochemistry (Vectastain® Elite® ABC Kit).

Introduction.

Antibodies (Abs) can be used to identify the anatomic distribution of an antigen within a tissue. The common principle behind these techniques is that a label is attached to the specific antibody and the position of the label in the tissue, determined with a microscope, is used to infer the position of the antigen. In an enzyme linked detection system (conversion of a colourless substrate to a coloured product), a conventional light microscope may be used to localise the antigen in a stained tissue. The signal can be enhanced using "Sandwich" or indirect techniques where amplification of the original signal is achieved with each additional layer. A technique developed by Hsu *et al.* (1981) uses the enzyme peroxidase conjugated to an avidin containing complex which binds a biotinylated secondary Ab directed against the primary antibody specific for the antigen of interest. This has been termed the ABC technique (figure APIII.9).

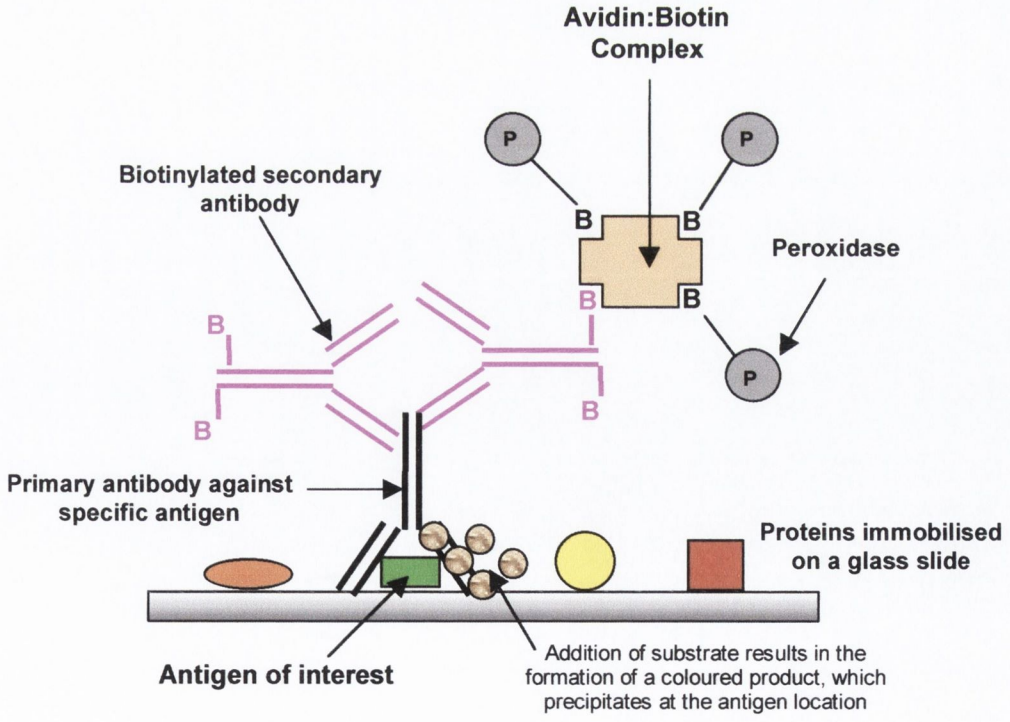


Figure APIII.9: Principle of the ABC Immunoperoxidase technique.

Note: the Vectastain® *Elite*® kit (Vector Laboratories) comes in two forms, one for use with a mouse monoclonal primary antibody (# PK-6102) and the other for use with a rabbit polyclonal primary Ab (# PK-6101). These kits differ in the nature of the secondary biotinylated Ab and the normal serum, which is prepared from the same species in which the secondary antibody is made. The preparation of these reagents and the general staining protocol are standard. The instructions detailed below are given in general terms and apply to both kits.

Materials.

Primary Ab directed against antigen of interest diluted to the appropriate concentration (~ 2.0 –5.0 μg/ml) in PBS.

Vectastain® *Elite*® kit (Vector Laboratories: Components; Normal serum, Secondary biotinylated Ab., Reagent A and Reagent B [Avidin-Biotin Complex]).

PBS buffer (10 mM sodium phosphate, pH 7.5, 0.9% sailne).

Chromagen: diaminobenzidine tetra hydrochloride (DAB, Sigma-Aldrich # D9015, x mg/ml in distilled water).

Meyers Hematoxylin (BDH # 350604T), clearing agents (graded alcohols) and mounting medium.

Protocol.

A) Preparation of Vectastain® working solutions.

Blocking serum: 150 μl of normal serum in 10 mls PBS.

Biotinylated secondary Ab: 50 μl Ab in 10 mls of blocking serum.

ABC: 100 μl of Reagent B added to 100 μl Reagent A in 5 mls of PBS (note: the ABC reagent needs to stand at room temperature for thirty minutes before use).

B) Staining protocol for frozen tissue sections.

Tissue sections (7 μm) were air dried. Immediately before staining sections were fixed in fresh acetone for ten minutes then transferred to PBS. Quenching of endogenous peroxidase was performed with 0.3% hydrogen peroxide (H_2O_2, in 0.3% normal serum in PBS) for 5

minutes (note: if necessary, due to antigen loss, H_2O_2 treatment may also be performed after the addition of secondary Ab). Slides were washed in PBS for 5 minutes. Blocking of non-specific sites was performed by incubation with blocking serum for 90 minutes. Excess serum was blotted from the sections. An endogenous avidin biotin blocking step was performed using the Avidin/Biotin Blocking kit (Vector Laboratories, # SP-2001) according to the manufacturers instructions (if endogenous biotin is not a problem in the tissue being stained this step can be omitted). The specific primary Ab (or isotype-matched control antibody) was applied to the sections and incubated overnight at room temperature in a moist chamber. Slides were washed (15 minutes in PBS) and incubated with secondary Ab for 30 minutes. Sections were washed, incubated with ABC reagent for 30 minutes and washed again. The peroxidase substrate (DAB, to which 10 μl of H_2O_2 was added immediately before use) was applied for 7 – 10 minutes. Sections were rinsed in tap water to stop the DAB reaction, counterstained for 15 seconds in Meyers Hematoxylin, cleared and mounted.

Appendix IV (APIV): HSC Characterisation Data Tables.

The data generated in the study to phenotypically characterise hepatic haematopoietic stem cells (HSCs, Chapter 2) has been presented in the text mainly as figures. The following tables detail the individual data used, the corresponding figure(s) is indicated for each table.

APIV Contents.

Table APIV.1: Demographics of all samples used for the characterisation of hepatic haematopoietic stem cells*.

ID	Status	Sex#	M:F~	Age†	Range	Mean
D160698	**Normal**	F		68		
D060698	**(n = 20)**	M		26		
D030598		M		57		
D050498		M		38		
D150598		F		31		
D250298		M		51		
D270698		M		20		
D300698		F		28		
D150798		F		59		
D180698		F		68		
D250898		M		16		
D090199		M		50		
D210199		M		33		
D250199		F		69		
161199.2		F		29		
D300100		F		21		
D181297		F		39		
D221197		M		58		
D200998		F		58		
D101098		M	10:10	29	16 - 69	42.4
R080698	**Tumour**	M		35		
R020298	**(n = 10)**	M		68		
R300398		M		68		
R240298		M		66		
R190698		M		67		
R290698		F		67		
R240798		F		41		
R180998		F		67		
R161199		F		33		
R210100		F	5:5	52	33 - 68	56.4

* Corresponding figure 2.3.10.

Tables 2.2.1 & 2.2.2 give the mean age for samples used for 2/3colour flow cytometry respectively.

M = Male, F = Female.

~ Male to female ratio.

† Age in years.

Table APIV.2: Yields and viability of mononuclear cells isolated from normal (donor) and tumour-bearing hepatic tissue*.

ID	Status	Yield#	Viability†	Range	Mean	SEM‡
D160698	**Normal**	ND~	ND			
D060698	**(n = 17)**	1.32	80			
D030598		5.0	75			
D050498		7.5	80			
D150598		1.7	90			
D250298		8.67	95			
D270698		4.31	70			
D300698		3.46	70			
D150798		ND	ND			
D180698		7.75	95			
D250898		8.67	95			
D090199		4.94	70			
D210199		5	60			
D250199		4.44	85			
161199.2		ND	ND			
D300100		3.75	65	Yield		
D181297		2.63	79	1.45 – 8.67	4.43	0.57
D221197		2.38	78			
D200998		1.45	85	Viability		
D101098		6.5	80	65 - 95	78.56	2.52
R080698	**Tumour**	1.43	89			
R020298	**(n = 10)**	8.53	82			
R300398		5.6	70			
R240298		1.43	70			
R190698		2.27	75			
R290698		3.25	80	Yield		
R240798		1.09	65	1.09 – 8.58	3.29	0.82
R180998		1.96	50			
R161199		ND	ND	Viability		
R210100		4.0	95	50 - 95	75.11	4.67

* Corresponding to figures 2.3.1 and 2.3.11.

Yields are expressed as cell no x 10^6/g of tissue wet weight.

† Viability is expressed as percentage of total hepatic mononuclear cells.

‡ SEM = Standard Error of the Mean.

~ ND = not done.

Table APIV.3: Levels of haematopoietic stem cells (HSCs) detected in liver samples using two-colour flow cytometric analysis (anti-CD34-PE)*.

ID	Status	Sex~	HSCs†	Range	Mean	SEM‡
D160698	**Normal**	M	2.40			
D060698	(n = 10)	M	1.91			
D030598		M	1.81			
D150598		F	1.91			
D150798		F	2.87			
D180698		F	0.82			
D181297		F	1.39			
D221197		M	2.22			
D200998		F	1.24			
D101098		M	1.75	0.82 – 2.87	1.83	0.18
R080698	**Tumour**	M	1.83			
R190698	(n = 5)	M	1.16			
R290698		F	0.87			
R240798		F	2.01			
R180998		F	1.12	0.87 – 2.01	1.40	0.22

* Corresponding to figure 2.3.3.

† CD34+CD45+ cells, percent of total CD45+ cells.

‡ SEM = Standard Error of the Mean.

~ M = Male, F = Female.

Table APIV.4: Levels of expression of CD34 on haematopoietic stem cells isolated from hepatic tissue*.

ID	Status	CD34 (MFI)†	Range	Mean	SEM‡
D160698	**Normal**	86.6			
D060698	**(n = 10)**	24.8			
D030598		115.48			
D150598		42.17			
D150798		86.6			
D180698		30.51			
D181297		103.22			
D221197		45.77			
D200998		52.33			
D101098		43.71	**24.8 – 115.48**	**63.12**	**10.12**
R080698	**Tumour**	64.94			
R190698	**(n = 5)**	42.71			
R290698		62.64			
R240798		39.24			
R180998		42.94	**39.24 – 64.94**	**50.69**	**5.37**

* Corresponding to figure 2.3.4.

† MFI = Median Fluorescent Intensity (arbitrary units)

‡ SEM = Standard error of the mean

Table APIV.5: CD34-positive cells of haematopoietic origin (co-express CD45) in hepatic mononuclear cell preparations*.

ID	Status	CD34+CD45+†	Range	Mean	SEM‡
D160698	**Normal** (n = 10)	58.97			
D060698		20.15			
D030598		36.94			
D150598		20.15			
D150798		59.7			
D180698		21.98			
D181297		51.53			
D221197		19.33			
D200998		42.34			
D101098		62.44	19.33 – 62.44	41.0	5.32
R080698	**Tumour** (n = 5)	39.52			
R190698		46.83			
R290698		46.32			
R240798		29.61			
R180998		63.38	29.61 – 63.38	45.13	5.52

* Corresponding to figures 2.3.5 and 2.3.6.

† Percent of total $CD45^+$ cells.

‡ SEM = Standard Error of the Mean.

Table APIV.6: Bone marrow samples used for two-colour flow cytometry*.

a). Levels of haematopoietic stem cells (HSCs, % of total $CD45^+$cells).

ID	Status	$CD34+CD45^{+\dagger}$	Range	Mean	SEM‡
BM1	**Normal** (n = 3)	2.34			
BM2		2.60			
BM3		1.69	1.69-2.60	2.21	0.27

b). Level of expression of CD34 on bone marrow HSCs.

ID	Status	MFI†	Range	Mean	SEM
BM1	**Normal** (n = 3)	352.27			
BM2		362.34			
BM3		316.23	316.23 – 362.34	343.61	13.99

c). $CD34^+$ cells of haematopoietic origin (co-express CD45, % of total $CD34^+$).

ID	Status	$CD45^+$	Range	Mean	SEM
BM1	**Normal** (n = 3)	95.47			
BM2		99.02			
BM3		98.59	95.47-99.02	97.69	1.12

* corresponds to figures 2.3.3 – 2.3.5.

‡ SEM = Standard error of the Mean.

† MFI = Median Fluorescent Intensity (arbitrary units).

Table APIV.7: Levels of haematopoietic stem cells detected in normal and tumour-bearing liver samples using three-colour flow cytometric analysis (CD34-PerCP)*.

ID	Status	Sex	HSCs†	Range	Mean	SEM‡
D160698	**Normal**	F	1.82			
D060698	**(n = 16)**	M	1.38			
D030598		M	0.86			
D050498		M	1.81			
D150598		F	1.69			
D250298		M	2.30			
D270698		M	1.54			
D300698		F	0.81			
D150798		F	1.35			
D180698		F	0.62			
D250898		M	1.16			
D090199		M	1.20			
D210199		M	0.37			
D250199		F	1.44			
161199.2		F	0.31			
D300100		F	1.08	031 – 2.30	1.23	0.14
R080698	**Tumour**	M	1.37			
R020298	**(n = 10)**	M	2.82			
R300398		M	1.34			
R240298		M	1.25			
R190698		M	0.62			
R290698		F	1.13			
R240798		F	1.68			
R180998		F	1.02			
R161199		F	0.47			
R210100		F	1.20	0.47 – 2.82	1.29	0.20

* Corresponding to figures 2.3.6 and 2.3.7.

† HSCs = haematopoietic stem cells ($CD34^+CD45^+$), % of total $CD45^+$ cells

‡ SEM = Standard Error of the Mean

Table APIV.8: Levels of co-expression of differentiation, activation and lineage-specifc markers on hepatic haematopoietic stem cell (HSC) populations*.

Sample	ID	CD38#	CD33	CD56	CD45RA	CD7	CD19
Normal	D030598	88.37	0	62.79	20.93	16.28	ND†
(n = 14)	D050498	33.70	3.31	43.09	97.79	28.73	ND
	D150598	94.67	2.37	43.79	46.15	34.91	ND
	D250298	45.65	8.69	51.30	45.65	45.65	ND
	D160698	35.16	0	7.69	10.99	9.89	13.33
	D060698	19.56	0	30.43	0	16.66	ND
	D150798	50.00	11.67	27.94	ND	72.17	27.83
	D250898	52.78	0	30.30	75.00	42.86	ND
	D270698	ND	ND	ND	ND	ND	10.78
	D300698	ND	ND	ND	ND	ND	8.69
	D250898	ND	ND	ND	ND	ND	26.32
	D090199	ND	ND	ND	ND	ND	0
	D210199	ND	ND	ND	ND	ND	10.00
	D250199	ND	ND	ND	ND	ND	8.91
Means		**52.49**	**3.26**	**37.17**	**42.36**	**33.39**	**13.23**
Tumour	R080698	48.90	7.30	13.87	16.06	72.99	ND
(n = 8)	R020298	57.80	ND	ND	22.70	57.80	ND
	R300398	24.63	16.42	5.97	ND	48.51	ND
	R240298	62.40	17.60	24.80	49.60	64.00	ND
	R190698	37.35	25.30	60.87	57.83	86.75	0.23
	R290698	68.49	41.67	0	41.67	37.50	0
	R240798	71.86	15.00	30.77	ND	60.00	0
	R180998	72.72	20.45	52.27	23.40	37.50	0
Means		**55.52**	**20.53**	**26.94**	**35.21**	**58.13**	**0.06**

* Corresponding to figures 2.3.12 – 2.3.16.

The results for each marker are expressed as a percentage of the total $CD34^+CD45^+$ population.

† ND = Not done.

Table APIV.9: The proportion of CD45$^+$ cells with granular properties in hepatic mononuclear cell preparations*.

ID	Status	Granular cells†	Range	Mean	SEM‡
D160698	**Normal**	30.29			
D060698	(n = 20)	41.47			
D030598		10.59			
D050498		15.32			
D150598		17.34			
D250298		27.86			
D270698		16.84			
D300698		20.86			
D150798		22.95			
D180698		30.29			
D250898		14.05			
D090199		20.39			
D210199		16.79			
D250199		14.05			
161199.2		33.04			
D300100		28.82			
D181297		9.33			
D221197		34.63			
D200998		25.59			
D101098		12.99	9.33 - 41.47	22.17	2.0
R080698	**Tumour**	24.0			
R020298	(n = 10)	11.02			
R300398		57.97			
R240298		31.2			
R190698		48.62			
R290698		70.08			
R240798		47.08			
R180998		29.2			
R161199		11.26			
R210100		33.47	11.02 - 70.08	36.39	6.13

*Corresponding to figures 2.3.18 and 2.3.19.

† percent of total CD45$^+$ cells.

‡ SEM = Standard Error of the Mean

Appendix V (APV): Hepatic Cytokine Data Tables.

The data generated in the study to characterise hepatic expression of lymphopoietic cytokines (IL-7 and IL-15, Chapter 3) has been presented in the text mainly as figures. The following tables detail the individual data used, the corresponding figure(s) is indicated for each table where appropriate.

APIIV Contents.

Table APV.1: Demographics of liver samples used for the detection of lymphopoietic cytokines (IL-7/IL-15) in hepatic tissue*.

No*.	ID	Sex#	M:F~	Age†	Range	Mean
1	D160598	F		65		
2	D080398	M		51		
3	D180996	M		44		
4	D120498	F		39		
5	D130498	M		43		
6	D210699	F		25		
7	D161296	M		32		
8	D150798	F		59		
9	D190796	M		22		
10	D210497	F		19		
11	D060698	F		48		
12	D270198	M		44		
13	D280596	F		58		
14	D210198	F		59		
15	D050696	M		58		
16	D230895	F	9:7	54	19 - 65	45

* The sample numbers assigned in this table are used throughout the cytokine study (chapter 3 and appendix V).

M = Male, F = Female.

~ Male to female ratio.

† Age in years.

Table APV.2: Absorbance readings and yields of total RNA extracted from normal hepatic tissue and control cell lines.

Sample (1/10)			Abs‡	Abs	OD ratio	RNA	Total
No.	Type	ID	260 nm	260 nm	260:280	µl/µg*	yield†
1	Liver	D160598	1.545	0.886	1.744	1.6	31.2
2		D080398	1.783	1.028	1.734	1.4	35.7
3		D180996	1.119	0.568	1.970	2.2	22.7
4		D120498	1.430	0.816	1.752	1.8	27.8
5		D130498	2.035	0.994	2.047	1.2	41.7
6		D210699	1.760	0.861	2.044	1.4	35.7
7		D161296	1.862	0.927	2.008	1.3	38.5
8		D150798	2.002	1.087	1.842	1.2	41.7
9		D190796	1.756	0.886	1.982	1.4	35.7
10		D210497	1.963	0.995	1.973	1.3	38.5
11		D060698	1.987	0.892	2.227	1.3	38.5
12		D270198	1.735	0.889	1.951	1.4	35.7
13		D280596	1.852	0.911	2.033	1.4	35.7
14		D210198	1.786	0.896	1.993	1.4	35.7
15		D050696	1.988	0.978	2.033	1.3	38.5
16		D230895	1.332	0.624	2.135	1.9	26.3
0A	+ Control	SK-HEP-1	1.302	0.653	1.994	1.9	26.3
0B	- Control	CRL-8286	1.208	0.609	1.984	2.1	23.8

‡ Abs = Absorbance

* Volume of RNA sample used for generation of cDNA (1 µg).

† Total volume = 50 µl (µg/50 µl).

Table APV.3: Levels of 620 bp and 488 bp (IL-7) transcripts detected in normal hepatic tissue samples normalised against GAPDH*.

Sample No.	Type	ID	IL-7 products 620 bp	488 bp
1	Liver	D160598	0.21	0.06
2		D080398	0.8	0.08
3		D180996	0.14	0
4		D120498	0.29	0.08
5		D130498	0.15	0.03
6		D210699	0.27	0.25
7		D161296	0.24	0
8		D150798	0	0
9		D190797	0.52	0.53
10		D210497	0.66	0.34

* Corresponding to figures 3.3.2, 3.3.3 and 3.3.8.

Table APV.4: Levels of 524 bp and 650 bp (IL-15) transcripts detected in normal hepatic tissue samples normalised against GAPDH*.

Sample No.	Type	ID	IL-15 products 524 bp	650 bp
4†	Liver	D120498	1.60	0.67
5		D130498	16.37	0.37
6		D210699	3.53	0
8		D150798	1.27	0
9		D190797	2.64	0.88
13		D280596	1.98	0.72
14		D210198	2.37	0
15		D050696	1.33	0.35
16		D230895	4.01	0

* Corresponding to figures 3.3.5 and 3.3.6.
† The five samples that were tested for both IL-7 and IL-15 transcripts are shown in purple.

Table APV.5: Demographics of liver samples used for the detection of cytokine receptors on hepatic mononuclear cells.

No.	ID	Sex[#]	M:F~	Age[†]	Range	Mean
17	D250200	M		21		
18	D010300	M		18		
19	D070200	F		42		
20	D150200	M		56		
21	D300100	F		21		
22	D230300	M	4:2	24	18 - 56	30.33

[#] M = Male, F = Female.

~ Male to female ratio.

[†] Age in years.

Table APV.6: Demographics of liver samples used for the detection of cytokine receptors on hepatic haematopoietic progenitor cells.

No.	ID	Sex[#]	M:F~	Age[†]	Range	Mean
23	D231000	M		55		
24	D191000	F		43		
24	D170800	F		63		
26	D201000	F		43		
27	D270900	M		65		
28	D191100	F		62		
29	D291000	F	2:5	63	43 - 65	56.28

[#] M = Male, F = Female.

~ Male to female ratio.

[†] Age in years.

Table APV.7: IL-7 receptor-α (CD127) and IL-2/IL-15 receptor-β (CD122) expression on hepatic mononuclear cells*.

No.	ID	CD127 (% of blast gate)	CD122 (% of blast gate)
13	D250200	8.33	33.96
14	D010300	11.64	45.62
15	D070200	9.8	38.10
16	D150200	21.25	23.62
17	D300100	28.77	38.98
18	D230300	31.17	34.79

* Corresponding to figure 3.3.12.

Table APV.8: IL-7 receptor-α (CD127) and IL-2/IL-15 receptor-β (CD122) expression on hepatic haematopoietic stem cells*.

No.	ID	CD127 (% CD34+CD45+ cells)	CD122 (% CD34+CD45+ cells)
19	D231000	26.88	40.8
20	D191000	48.4	66.71
21	D170800	40.16	55.86
22	D201000	25.37	43.02
23	D270900	64.86	78.82
24	D191100	59.47	66.33
25	D291000	61.46	75.49

* Corresponding to figure 3.3.13.

Appendix VI (APVI): Hepatic T-cell development Data Tables.

The data generated in the study to characterise hepatic T cell development (Chapter 4) has been presented in the text mainly as figures. The following tables detail the individual data used, the corresponding figure(s) is indicated for each table.

APVI Contents.

Table APVI.1: Demographics of samples used for T-cell receptor excision circle analysis.

Number	ID	Sex[#]	M:F~	Age[†]	Range	Mean
1	280599	M		57		
2	290799	M		36		
3	050899	F		25		
4	200499	F		20		
5	280699	M		53		
6	090899.1	M		69		
7	090899.2	M		75		
8	270999	F	5:3	42	20 - 75	47

[#] M = Male, F = Female.

~ Male to female ratio.

[†] Age in years.

Table APVI.2: Demographics of samples used for delta-gene use analysis.

Number	ID	Sex[#]	M:F~	Age[†]	Range	Mean
9	190400	M		40		
10	190500	M		62		
11	040600	M		62		
12	300600	M	4:0	26	26 - 62	47.5

[#] M = Male, F = Female.

~ Male to female ratio.

[†] Age in years.

Table APVI.3: T-cell receptor (TCR) usage on matched hepatic and peripheral blood CD3+ T cells*.

Sample No	TCR gene usage			
	TCR-$\alpha\beta^+$		TCR-$\gamma\delta^+$	
	Liver	Blood	Liver	Blood
1	94.31†	99.16	5.69	0.84
2	93.58	97.55	6.52	2.45
3	95.19	99.76	4.81	0.24
4	75.98	70.90	24.02	29.10
5	89.63	99.58	10.37	0.42
6	93.10	98.47	6.90	1.53
7	79.86	90.78	20.14	9.22
8	89.46	98.67	10.54	1.33
Mean	**88.89**	**94.39**	**11.12**	**6.64**

* Corresponding to figure 4.3.1.

† Results are expressed as a percentage of total $CD3^+$ T cells.

Table APVI.4: Characterisation of co-receptor (CD4/CD8) expression on TCR-$\alpha\beta^+$ T cells from liver and matched blood*.

Sample No	$CD4^+$		$CD8^+$		DPs (CD4+8+)		DNs (CD4-8-)	
	Liver	Blood	Liver	Blood	Liver	Blood	Liver	Blood
1	20.77	60.81	77.05	43.52	1.63	5.56	3.81	1.23
2	25.68	72.13	71.53	25.65	1.26	0.83	4.0	3.05
3	32.82	71.10	65.85	29.39	0.07	0.93	1.4	0.54
4	25.93	69.78	71.86	30.68	6.37	1.04	8.53	0.58
5	35.84	67.76	64.17	32.74	11.67	1.15	11.67	0.65
6	36.84	67.81	64.05	32.42	7.73	1.58	6.84	1.35
7	39.11	42.31	60.57	57.57	3.64	0.89	3.96	0.84
8	27.73	84.46	69.47	14.20	0.92	0.34	3.72	1.77
Mean	**30.59**	**67.02**	**68.07**	**33.27**	**4.16**	**1.54**	**5.49**	**1.25**

* Corresponding to figure 4.3.2.

† Results are expressed as a percentage of total TCR-$\alpha\beta^+$ cells.

Table APVI.5: Characterisation of memory phenotype ($CD45RO^+$) of $CD8^+/CD4^+$ TCR-$\alpha\beta^+$ T cells from liver and matched blood.

a) Expressed as a percentage of total TCR-$\alpha\beta^+$ lymphocytes*.

Sample No	$CD4^+CD45RO^-$		$CD8^+CD45RO^-$	
	Liver	Blood	Liver	Blood
1	2.58	6.34	13.26	7.28
2	3.29	23.92	19.36	18.36
3	3.37	30.96	41.73	18.86
4	6.24	47.99	29.85	21.75
5	10.74	50.53	39.02	25.44
6	2.05	24.84	27.72	18.77
7	2.64	6.70	27.12	33.59
8	4.22	14.70	18.52	11.50
Mean	4.39	25.75	27.07	19.44

* Corresponding to figures 4.3.3, 4.3.4 and 4.3.6.

b) Expressed as a percentage of TCR-$\alpha\beta^+CD4^+$/TCR-$\alpha\beta^+CD8^+$ lymphocytes*.

Sample No	$CD4^+CD45RO^-$		$CD8^+CD45RO^-$	
	Liver	Blood	Liver	Blood
1	12.42	10.43	17.21	16.73
2	12.81	33.162	27.05	71.58
3	10.27	43.61	63.37	64.17
4	24.07	68.77	41.54	70.89
5	29.97	74.57	60.81	77.70
6	5.56	36.63	43.28	57.90
7	6.75	15.84	44.78	58.16
8	15.22	17.40	26.66	80.99
Mean	14.63	37.55	40.59	62.27

* Corresponding to figure 4.3.5.

Table APVI.6: Purity and yield of $CD3^+$ cells isolated using MiniMACS™*.

Sample No	Purity~		Yield†	
	Liver	Blood	Liver	Blood
1	83	85	300	400
2	87	92	150	900
3	82	87	250	600
4	85	86	300	200
5	89	93	200	600
6	85	85	700	700
7	86	89	800	800
8	85	95	100	800
Mean	85.25	89.0	350	625

* Corresponding to figures 4.3.7, 4.3.8 and 4.3.9.

~ $CD3^+$ cells as a percentage of total cells.

† Total cell number in the $CD3^+$ fraction (1×10^6 cells were applied to the column in all cases)

Table APVI.7: TREC numbers detected in peripheral blood and matched hepatic CD3-enriched populations*.

Sample No	TRECs Total number~		TRECS/1 x 10^5 Naïve TCR-$\alpha\beta^+$ cells†	
	Blood	Liver	Blood	Liver
1	81	0	176.4	0
2	327	0	96.84	0
3	2316	99	892.7	48.29
4	434	0	510.31	0
5	572	0	135.49	0
6	107	9	41.86	5.44
7	218	0	83.71	0
8	1201	0	611.26	0

* Corresponding to figure 4.3.10.

~ DNA from the entire $CD3^+$ fraction was used in the TREC assay, starting cell number varied between $1 - 9 \times 10^5$ cells.

† To calculate the number of TRECS/1 x 10^5 TCR-$\alpha\beta^+$ naive cells: the total TREC number was normalised to TRECs/1 x 10^5 total cells, this figure was then adjusted to account for the purity of the sample and the proportion of $CD3^+$ cells which were TCR-$\alpha\beta^+CD45RO^-$.

Table APVI.8: V-delta gene usage of hepatic and peripheral blood T-cells*.

Sample No	Vδ-1†		Vδ-2		Vδ-3	
	Blood	Liver	Blood	Liver	Blood	Liver
9	14.0	4.94	95.7	56.4	0	28.5
10	49.1	21.0	36.4	29.0	0	34.9
11	53.3	8.93	37.3	41.2	0	37.2
12	42.7	6.18	43.3	35.7	0	21.2
Mean	39.78	10.26	53.18	40.58	0.00	30.45

* Corresponding to figure 4.3.11.

† % of total TCR-$\gamma\delta^+$ cell population

Appendix VII (APVII): Publications and presentations

Completed Manuscripts.

Expression of Interleukin 7 (IL-7) mRNA and Protein in the Normal Adult Human Liver: Implications for Extrathymic T-cell development. Lucy Golden-Mason, Anna M. Kelly, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty and Cliona O'Farrelly. **2001**. Cytokine **14,**143-151.

Differential Expression of Lymphoid and Myeloid Markers on Differentiating Hematopoietic Stem Cells in Normal and Tumor-bearing Adult Human Liver. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. **2000**. Hepatology **31**:1251-1256.

Isolation of Hepatic Mononuclear Cells from Normal Adult Human Liver Suitable for Phenotypic and Functional Characterisation. MP Curry, S Norris, L Golden-Mason, DG Doherty, C Collins, T Deignan, O Traynor, GP McEntee, JE Hegarty, C O'Farrelly. **2000**. J. Immunol. Meth. **242**:21-31.

Increased primordial B cells in the peripheral blood may be protective in chronic Hepatitis C virus infection. Michael P. Curry, Lucy Golden-Mason, Niamh Nolan, Nollaig A. Parfrey, John E. Hegarty, Cliona O'Farrelly. **1999**. J. Hepatology **32**:121-125

The normal adult human liver is not a site for the development of conventional $\alpha\beta$ TCR$^+$ cells. Lucy Golden-Mason, Danny Douek, Richard Koup, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. **2001.** J. Exp. Med. (Submitted)

Expansion of innate CD5$^+$ B cells expressing high levels of CD81 in HCV infected liver. MP Curry, L Golden-Mason, T Deignan, DG Doherty, S Norris, M Duffy, N Nolan, W Hall, JE Hegarty, C O'Farrelly. **2001**. Hepatology (Submitted).

Localisation of T and B lymphocytes in histologically normal adult human liver. Tina Deignan, Fiona Smith, Lucy Golden-Mason, Niamh Nolan, Oscar Traynor, Gerry McEntee, John Hegarty, Cliona O'Farrelly. **2001.** Eur. J. Gastro. & Hepatol. (Submitted).

Decrease in hepatic CD56$^+$ T cells and Vα24$^+$ natural T cells in chronic hepatitis C virus infection. T Deignan, MP Curry, DG Doherty, L Golden-Mason, Y Volkov, S Norris, N. Nolan, Oscar Traynor, Gerry McEntee, John E. Hegarty, Cliona O'Farrelly. J. Hepatology (Submitted).

Manuscripts in preparation.

Hepatic hematopoietic stem cells in end-stage alcoholic liver disease. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.

Hepatic Interleukin 15 expression: implications for NK/NK T cell maturation and homeostasis. Lucy Golden-Mason, Anna M. Kelly, Derek G. Doherty, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.

The role of Interleukin 12 and Natural T (NT) cells in hepatic malignancy. Anna M. Kelly, Lucy Golden-Mason, Oscar Traynor, Gerry McEntee, John E. Hegarty, Cliona O'Farrelly, Derek G. Doherty.

Characterisation of NK T cell populations in liquid and solid human bone marrow. J. Dean, L. Golden-Mason, M. Lawler, D. McCarthy, C. O'Farrelly.

Characterisation of $\gamma\delta$ T cells in normal and malignant adult human liver. Tony Kenna, Lucy Golden-Mason, Suzanne Norris, Oscar Traynor, Gerry McEntee, John E. Hegarty, Cliona O'Farrelly. Derek G. Doherty.

Abstracts published and presented.

Irish Society of Gastroenterology (ISG) Summer Meeting. June 11th – 12th 2001.

Expression of Ets transcription factors in normal and malignant human liver. L. Golden-Mason*, Y. Buggy*, B. Arniaz-Villaneuva, M. O'Brien, O. Traynor, G. McEntee, J.E. Hegarty C. O'Farrelly and T. Maguire. Ir. J. Med. Sc. (Supl. in press). *Joint first authors. (First prize poster presentation).

British Society for Immunology Annual Meeting. December 5th – 8th 2000.

The adult human liver is not a site for conventional $\alpha\beta$-TCR$^+$ T-cell Development. L. Golden-Mason, D.C. Douek, R.D. Koup, M.P. Curry, J. Kelly, O. Traynor, G. McEntee, J.E. Hegarty and C. O'Farrelly. Immunology 101(Supl.1):12.

American Association for the Study of Liver Disease (AASLD) Annual Meeting. October 27th – 31st 2000.

Interleukin-7 mRNA and protein levels in the Normal Adult Human Liver: Implications for hepatic T-cell Differentiation. L. Golden-Mason, A.M. Kelly, M.P. Curry, J. Kelly, O. Traynor, G. McEntee, J.E. Hegarty and C. O'Farrelly. Hepatology 32(2):202A.

Irish Society of Gastroenterology (ISG) G-2K Meeting. September 14th – 15th 2000.

Hepatic lymphoid progenitor cells do not give rise to conventional $\alpha\beta$-TCR$^+$ T-cells. L. Golden-Mason, D.C. Douek, R.D. Koup, M.P. Curry, J. Kelly, O. Traynor, G. McEntee, J.E. Hegarty and C. O'Farrelly. Ir.J.Med.Sc. 169(Suppl.1):17. (First prize poster presentation).

Lymphopoietic cytokines (IL-7 & IL-15): levels of expression in the normal adult human liver. Lucy Golden-Mason, Anna M. Kelly, Michael P. Curry, Jacinta Kelly, Oscar Traynor, Gerry McEntee, John E. Hegarty and Cliona O'Farrelly. Ir.J.Med.Sc. 169(Suppl.1):16.

British Society for Immunology Annual Meeting. November 30th - Decemer 3rd 1999.

Interleukin-7 expression in the normal adult human liver: Implications for extrathymic T-cell differentiation. Golden-Mason; L; Curry, M.P; Kelly, J; Traynor, O; McEntee, G; Hegarty, J.E. O'Farrelly, C. Immunology(Suppl.1):98:161.

American Association for the Study of Liver Disease (AASLD) 50th Annual Meeting. November 5th – 9th 1999.

Hepatic hematopoietic stem cells in end-stage alcoholic liver disease. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Hepatology 30(Suppl.):255A.

Differentiating hematopoietic stem cells in tumor-bearing adult human liver. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Hepatology 30(Suppl.):253A.

Irish Society of Gastroenterology (ISG) Summer Meeting. June 11th – 12th 1999.

Hepatic haematopoietic stem cells in alcoholic liver disease. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Ir.J.Med.Sc. 168(Suppl.5):3.

Irish Society of Gastroenterology (ISG) Winter Meeting. November 20th – 21st 1999.

Activation of myelopoiesis may contribute to progression of hepatic colorectal metastatic disease. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Ir.J.Med.Sc. 167(Suppl.11):7.

Differentiating haematopoietic stem cells in the normal adult human liver suggest a lymphoid bias. Lucy Golden-Mason, Michael P. Curry, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Ir.J.Med.Sc. 167(Suppl.11):25.

American Association for the Study of Liver Disease (AASLD) Annual Meeting. November 1998.

Differentiating hematopoietic stem cells in adult human liver suggest a lymphoid bias. Lucy Golden-Mason, Michael P. Curry, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Hepatology 28(Suppl.):521A.

British Society for Immunology Annual Meeting. Decemer 1st – 4th 1998.

Differentiating *stem cells in normal adult human liver may contribute to tumour immunity.* Lucy Golden-Mason, Michael P. Curry, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly. Immunology 95(Suppl.1):161.

Other presentations (not published as abstracts)

Irish Society for Immunlogy Ulster Immunology Group Joint Scientific Meeting. September 14th – 15th 2000.

Hepatic lymphoid progenitor cells do not give rise to conventional $\alpha\beta$-TCR$^+$ T-cells. L. Golden-Mason, D.C. Douek, R.D. Koup, M.P. Curry, J. Kelly, O. Traynor, G. McEntee, J.E. Hegarty and C. O'Farrelly.

St. Vincent's University Hospital 10th Anniversery Biomedical Symposium. November 23rd 2000.

Haematopoietic Stem Cells in the Adult Human Liver: Local T cell differentiation. Lucy Golden-Mason.

Irish Society for Immunlogy Annual Scientific Meeting. September 23rd – 24th 1999.

Hepatic expression of interleukin-7 mRNA and protein. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.

St. Vincent's University Hospital Annual Biomedical Symposium. November 26th 1999.

Lymphopoiesis in the adult human liver: Implications for tumour surveillance/progression. Lucy Golden-Mason, Michael P. Curry, Niamh Nolan, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.

Irish Society for Immunlogy Annual Scientific Meeting. October 8th – 9th 1998.

Differentiating stem cells in normal and tumour-bearing adult human liver. Lucy Golden-Mason, Michael P. Curry, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.

St. Vincent's University Hospital Annual Biomedical Symposium. November 27th 1998.

The hepatic microenvironment favours lymphopoiesis. Lucy Golden-Mason, Michael P. Curry, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.

Activation of myelopoiesis in tumour-bearing liver: Contribution to progression of hepatic metastases. Lucy Golden-Mason, Michael P. Curry, Oscar Traynor, Gerry McEntee, Jacinta Kelly, John E. Hegarty, Cliona O'Farrelly.