Circulating Natural Killer Cells and Natural Killer Receptor Positive T Cells In Patients with Hepatitis C Virus Infection

By

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A thesis sumitted for the degree of Master of Science

October 2003

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NUI MAYNOOTH

Ollscoil na hÉireann Má Nuad

To my husband and my son

Mohamed and Abdul Elfkhkhri

Abstract

Over 170 million people worldwide are infected with hepatitis C virus (HCV) The immune system is effective at clearing the virus in a small proportion of infected individuals while most people become persistently infected with the virus and develop chronic liver disease. Natural killer (NK) cells are thought to mediate immunity against viruses. They can lyse virus infected cells and release anti-viral cytokines. The roles of NK cells and natural killer receptor positive (NKR^+) T cells in immunity against HCV is largely unknown. The aim of the present study was to investigate the hypothesis that NK cells and NKR^+ T cells numbers and/or functions are depleted in patients with chronic HCV infection. Flow cytometry was used to evaluate the numbers and phenotypes of NK cell and NKR^+ T cells in peripheral blood of 33 patients with chronic HCV infection, 22 patients who spontaneously resolved HCV infection and 26 healthy controls. Natural cytotoxicity and interleukin-2-activated killing of K562 and Daudi cells by peripheral blood mononuclear cells (PBMCs) were assessed using ^{51}chromium release assays. NK cells and NKR^+ T cells were depleted in patients with chronic HCV compared to the healthy controls (3.4% vs 13.7% for NK cells; $p<0.001$ and 1.3% vs 3.7% for $CD56^+$ T cells; $p=0.0001$). NK cells and $CD56^+$ T cells were also depleted in HCV resolvers compared to controls. The frequencies of expression of CD16, CD56, CD161, KIR2DL1 and KIR3DL1/S1 by T and non-T cells were also altered in chronic HCV patients compared to controls. Natural cytotoxicity by PBMCs against K562 cells was impaired in patients with chronic HCV infection compared with controls (mean specific lysis at effector/target ratios of 50:1 were 1.8% vs 10.2%; $p<0.001$) and HCV resolvers (7.4%, $p=0.0012$). IL-2 induced cytotoxicity against K562 and Daudi targets was also impaired in patients with chronic HCV disease compared to

HCV resolvers and controls (2.4% vs 7.6% and 12.6% respectively, $p<0.001$ for K562 and 0% vs 2.5% and 8.1%; $p<0.001$ for Daudi). Spearman analysis indicated that the impaired cytotoxic activity and LAK activity in the patients is not solely due to decreased NK or NKR^+ T cell numbers. This study suggests that altered NK levels and activities are associated with HCV infection and may be responsible, at least in part, for the chronicity of the infection

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ACKNOWLEDGEMENTS

I am indebted to my supervisor Dr. Derek Doherty for his helpful comments and suggestions and for his encouragement.

I am also grateful to Lucy Golden-Mason for doing the phenotypic analysis of patients samples and for providing us with patients blood samples.

I would like to thank Laura Madrigal Estebas and I appreciate her help.

I thank my colleagues in the lab, Tony and Jane. And I thank all the volunteers, who provided blood for this study.

This project was supported by the People's Bureau of the Great Socialist People's Libyan Arab Jamahiriya. London.

DECLARATION

This thesis has not been submitted in whole or in part, to this, or any other University for any degree, and is, except where stated the original work of the auther.

Signed:.....

Hameda Asrafel

ABBREVIATIONS

Abbreviation	Description
°C	Degrees Centigrade
2D	Two Domains
3D	Three Domains
α-Galcer	Alpha-Galactosylceramide
ADCC	Antibody Dependent Cellular Cytotoxicity
AO	Acridine Orange
APC	Antigen Presenting Cells
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CD1d	MHC class I like presenting molecules expressed by APC and other cells
Cpm	Count Per Minute
^{51}Cr	^{51}Chromium
CTL	Cytotoxicity T Lymphocyte
DC	Dendritic Cells
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
E/T	Effector/ Target
EB	Ethidium Bromide
EDTA	Ethylenediaminetetra Acetic acid
FACS	Fluorescence Activated Cell Sorting

FC	Fragment Crystallisable
FCS	Foetal Calf Serum
FH	Fulminant Hepatitis
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GPI	Glycophosphatidylinositol
HAI	Histological Activity Index
HVR1	Hypervariable region1
HAV	Hepatitis A Virus
HBSS	Hank's Balanced Salt Solution
HBV	Hepatitis B Virus
HCC	HepatoCellular Carcinoma
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSP	Heat Shock Protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin Like Transcrpt
ITAM	Immunoreceptor Tyrosine Based Activation Motifs
ITIM	Immunoreceptor Tyrosine Based Inhibitory Motifs
IVDA	Intra Venous Drug Abuse
KARAP	Killer cell Activating Receptor Associated Protein
KIR	Killer cell Ig-like Receptors

L	Long
L	Litre
LAIR	Leukocyte Associated Inhibitory Receptor
LAK	Lymphokıne Actıvated Killer
LGLs	Large Granular Lymphocytes
LIR	Leukocyte Inhibitory Receptor
LILR	Leukocyte Immunoglobulin Like Receptor
LPS	LipoPolySaccharide
LRC	Leukocyte Receptors Complex
M	Molar
MΦ	Macrophages
MAbs	Monoclonal Antibodies
mg/μg	milli/ Microgram
MHC	Major Histocompatibility Complex
mL	milliliter
NCR	Natural Cytotoxicity Receptors
NK cells	Natural Killer cells
NKRs	NK Receptors
NKR^+ T cells	Natural Killer Receptors Positive T cells
NKT cells	Natural Killer T cells
PBA	PBS/BSA/Azıde Buffer
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Salıne
PE	Phycoerythrin

PerCP	Peridin Chlorophyll Protein
RNA	Ribonucleic Acid
S	Short
SE	Standard Error
SEM	Standard Error of the Mean
SSC	Side Scatter
TCR	T Cell Receptors
TGF	Transforming Growth Factor
Th	T helper
TLR	Toll- Like Receptors
TNF	Tumour Necrosis Factor
Tr	T regulatory

Chapter 1: Introduction

1.1 Brief introduction to the immune system

There are many different defence mechanisms that protect an individual against micro-organisms and potentially harmful material These include physical barriers like the skin, phagocytic cells, chemicals and enzymes, which are present prior to any exposure to the foreign material. These innate or natural immune mechanisms are not enhanced by previous exposure to, nor do they discriminate between, most foreign substances. Other defence mechanisms, collectively known as acquired or adaptive immunity, have components that are able to recognise structures present on foreign material.

The immune system consists of a number of organs and several different cell types. The cells of the immune system, which include tissue cells and white blood cells known as leukocytes, develop from pluripotent stem cells The production of leukocytes takes place through two main pathways of differentiation The lymphoid lineage produces T-lymphocytes, B-lymphocytes and natural killer (NK) cells while the myeloid pathway gives rise to monocytes and polymorphonuclear leukocytes, neutrophils and granulocytes as well as platelets (Weir & Stewart 1997)

1.1.1 The innate immune system

The innate immune system, which evolved with the appearance of multicellular organisms, can use a variety of effector mechanisms to recognise the pathogen and clear an infection These mechanisms rely on cells with killing potential, such as monocytes, macrophages, neutrophils, dendritic cells (DC) and NK lymphocytes, and hard-wired detection systems involving cell surface molecules

that detect common structures found on infections agents or changes in host cells that signal danger (Fig.1). Such "pattern-recognition receptors" include the Toll-like receptors that recognise common components of microorganisms (lipopolysaccharides, lipoproteins, glycolipids, flagellin, and bacterial DNA) as well as endogenous ligands (heat shock proteins released by damaged or necrotic host cells) (Aderem *et al.* 2000, Akira *et al* 2001). Engagement of these molecules initiates the activation of monocytes, macrophages, neutrophils and/or DC. The result is the targeted destruction of the activating organism, infected cell, or tumour cell by phagocytosis or the release of cytotoxic agents. A second type of detection system in the innate immune system is a variety of activating receptors on NK cells that recognise changes to host cells that signify danger such as infection or tumour transformation. Ligation of these natural cytotoxicity receptors results in immediate killing of the infected or tumour cell by the NK cell (Moretta *et al* 2001).

Inflammation is a general term given to the mobilisation and effector activity of the innate immune system in response to signals of danger. It is initiated by the release of a variety of polypeptide chemical messengers from activated cells of the innate immune system and from pathogen-infected and tumour cells These chemical messengers include chemokines and cytokines, which diffuse rapidly through the tissues and into the circulation. A key function of this activity is the recruitment of additional inflammatory cells from other sites of the body.

Innate immune strategies are activated within seconds of detection of danger. It is likely that such innate defence functions are regular events in the healthy individual, occurring throughout the body, but perhaps, more frequently at sites of

high cell turnover (where there is likely to be a higher incidence of mutation) and increased exposure to foreign antigens (such as the gastrointestinal tract, liver, lungs and uterus) (O'Farrelly and Doherty 2003).

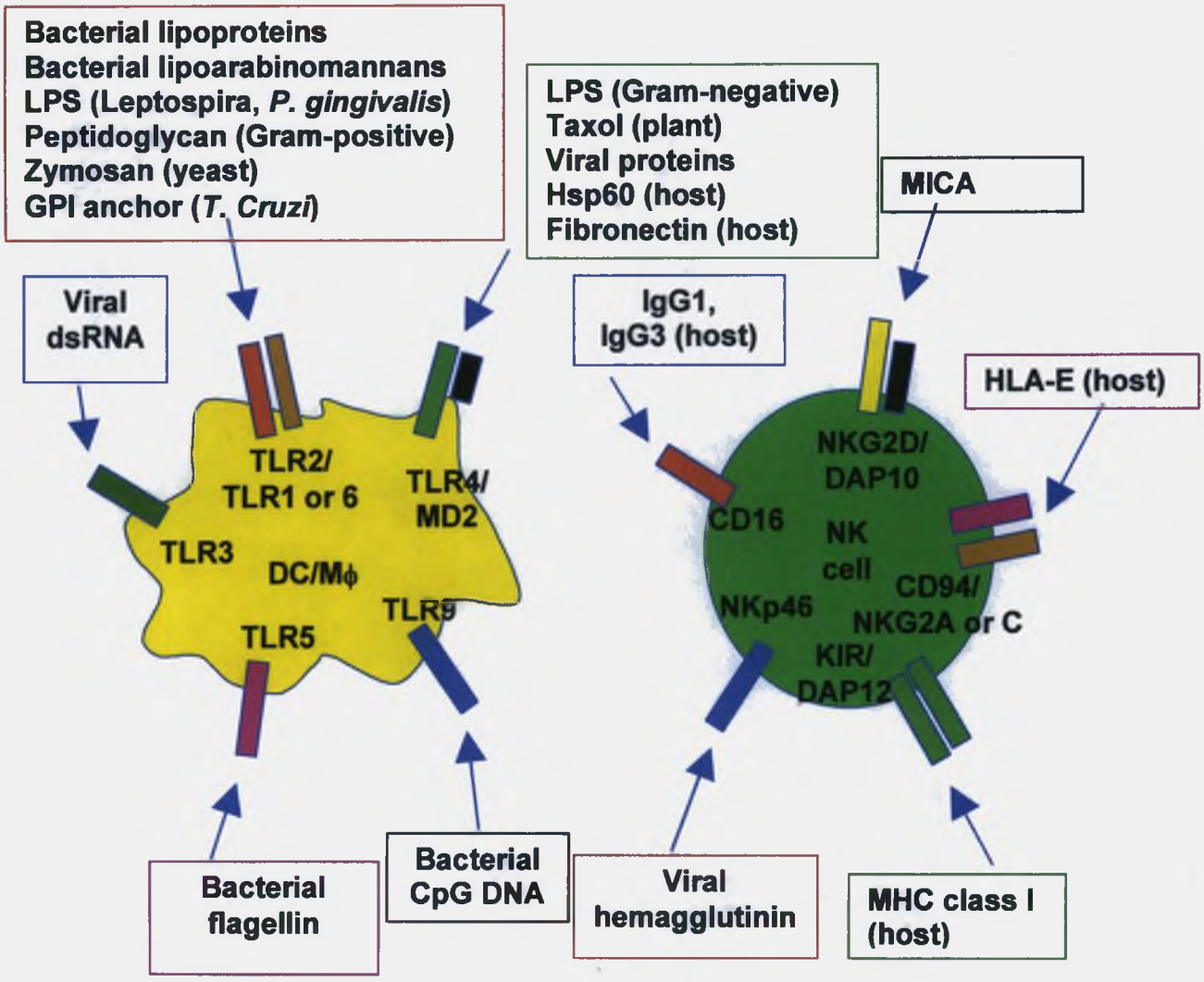

Figure 1: Recognition of danger by the innate immune system. Conserved pathogen-associated molecules and host cell-surface changes that signify danger are recognised by dendritic cells (DC), macrophages (MΦ) and natural killer (NK) cells. Toll-like receptors (TLR) on DC and MΦ recognise viral and bacterial products and stress-inducible molecules released by host cells. Natural cytotoxicity receptors on NK cells recognise viral products, changes in major histocompatibility complex (MHC) class I expression that signify danger, Fc portions of IgG1 and IgG3 antibodies, and the stress-inducible molecule, MICA. LPS, lipopolysaccharide; GPI, glycophosphatidylinositol; KIR, killer immunoglobulin-like receptor; hsp, heat shock protein. (Reproduced from O'Farrelly and Doherty 2003).

1.1.2 Adaptive immunity

If a micro-organism or tumour is able to evade the innate defence mechanisms and succeeds in expressing a threshold level of antigen, inflammation is not resolved and the adaptive immune system is initiated. The first and crucial step is the activation of T lymphocytes by antigen presenting cells (APC), which are capable of capturing, processing, and displaying antigen on their cell surface in association with MHC molecules. T cells can recognise specific peptide-MHC complexes by highly specific clonotypic T cell receptors (TCR) (O'Farrelly and Doherty 2003).

The adaptive immune system uses two different pathways to eliminate intracellular and extracellular antigens. Peptides derived from endogenously synthesised antigens, such as self-peptides or viral peptides (in infected cells), are loaded onto MHC class I molecules in the endoplasmic reticulum and presented on the cell surface to $CD8^+$ T cells. $CD8^+$ T cells typically kill the infected or tumour cells by either Fas-mediated or perforin/granzyme-mediated induction of apoptosis and release of interferon-γ (IFN-γ), which disrupts viral replication. Peptides derived from exogenous antigens are processed in the endocytic pathway, loaded onto MHC class II molecules and brought to the cell surface for presentation to $CD4^+$ T cells, which, in turn, activate other cells of the adaptive immune system. MHC class I molecules are expressed on almost all cells of the body and recognition of antigenic peptides presented by MHC class I renders a cell susceptible to lysis by $CD8^+$ T cells. In contrast, MHC class II molecule expression is restricted to "professional" APC that can activate $CD4^+$ T cells.

Three cell types include DC, macrophages and B lymphocytes.

DCs are the most effective of the APCs for naïve T cells. These cells express a high level of class II MHC molecules and costimulatory activity. The function of DC is to present antigen to T cells, and the mature DC found in lymphoid tissues are by far the most potent stimulators of naïve T cells. DC arise from myeloid progenitors within the bone marrow, and emerge from the bone marrow to migrate in the blood to peripheral tissues. In these tissues, they have an immature phenotype that is associated with low levels of MHC proteins and lack of co-stimulatory B7 molecules. They are not yet equipped to stimulate naïve T cells. However, they share with their close relatives the macrophages the ability to recognise and ingest pathogens through receptors that recognise features common to microbial components (Toll-like receptors), and they are very active in taking up antigens by phagocytosis. Upon ingestion of antigen, DC migrate to the lymph nodes, where they acquire mature DC phenotypes. The DC in lymphoid tissue are no longer able to engulf antigens by phagocytosis. However, they now express very high levels of long-lived MHC class I and MHC class II molecules; this enables them to stably present peptides from proteins acquired from the infecting pathogens. They also express very high levels of adhesion molecules, including DC-SIGN and high levels of B7 molecules, that make these cells able to potently activate naïve T cells (Janeway *et al* 2001).

Macrophages must be activated by antigens before they express class II MHC molecules or the co-stimulatory B7 membrane molecule (Goldsby *et al.* 2000). Macrophages that have bound and ingested microorganisms contribute to the adaptive immune response by acting as APC.

Macrophages, like tissue DC, have a variety of receptors that recognise microbial components, including the mannose receptor, the scavenger receptor, complement receptors, and several Toll-like receptors. Toll-like receptors bind conserved structures present on pathogens and mediate DC or macrophage activation. This is associated with the upregulation of MHC and costimulatory receptor expression and the release of cytokines such as interleukin-10 (IL-10) and IL-12 that induce the activation of cells of the adaptive immune system.

B cells are uniquely adapted to bind specific soluble molecules through their cell-surface immunoglobulin. B cells internalise the antigens bound by their surface immunoglobulin receptors and then display peptide fragments of antigen as peptide: MHC class II complexes. Because this mechanism of antigen uptake is highly efficient and B cells constitutively express high levels of MHC class II molecules, high levels of specific peptide· self MHC class II complexes are generated (Janeway *et al* 2001). Antibodies are the secreted from of the B cell receptor. The antibody molecule has two separate binding regions. The variable or V region recognises and specifically binds to molecules present in the pathogen that elicits the immune responses The constant (C) region binds to receptors on other cells that can destroy the pathogen. Antibody molecules are Y-shaped molecules consisting of three equal sized portions and form 2 heavy and 2 light polypeptide chains. The heavy and light chains contain the antigen binding site, and these are termed the Fab fragments, for fragment antigen binding. The Fab fragments correspond to the arms of the antibody molecule, which contain the complete light chains paired with the V_H and C_{H1} domains of the heavy chains. The other fragment contains no antigen-binding activity but were originally

observed to crystallise readily, and for this reason was named the Fc fragment (for fragment crystallisable). This fragment corresponds to the paired C_{H2} and C_{H3} domains and is the part of the antibody molecule that interacts with effector molecules and cells (Janeway *et al* 2001).

Release of IL-12 and IL-18 by macrophages and DC promotes the development of $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ T helper 1 (Th1) cells. Release of IL-4 and IL-6 promotes the development of $CD4^+$ Th2 cells. Th1 cells are generally induced by viruses and intracellular bacteria, whereas Th2 cells are induced by allergens and helminth pathogens. Th1 cells secrete IFN-γ and tumour necrosis factor-β (TNF-β) and activate macrophages. They also provide helper function for B cells production of complement-fixing and virus-neutralising antibodies of the IgG2a isotype in mice. Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and are considered to be the true helper cells, activating differentiation and class switching of B cells to secrete IgE, IgA and IgG1 (Abbas *et al* 1996, O'Garra and Murphy 1994). A third population of $CD4^+$ T cells with regulatory function, termed Th3 or T regulatory 1 (Tr1) cells, produce IL-10 and transforming growth factor-β (TGF-β) They suppress Th1 responses and have been implicated in the maintenance of immunological tolerance at mucosal surfaces (Maloy and Powrie 2001).

An important consequence of the adaptive immune response is protective immunity against reinfection and the establishment of a state of immunological memory. Immunological memory is the ability of the immune system to respond more rapidly and effectively to pathogens that have been encountered previously,

and reflects the preexistence of clonally expanded populations of antigen specific lymphocytes.

1.1.3 Hepatic lymphocyte repertoires

The liver contains a diversity of lymphocytes that include conventional αβ T cells, B cells, and NK cells that are similar to those found in blood, as well as large numbers of unconventional lymphoid cells that are rarely present in blood. Many of these cells appear to have roles in innate immunity, as evidenced by their ability to recognize common, conserved antigenic structures, their ability to mediate immediate killing of target cells, and/or their capacity to release cytokines that activate and regulate subsequent adaptive immune responses (Doherty and O'Farrelly 2000). These include large numbers of NK cells, B-1 cells, γδ T cells, natural killer T (NKT) cells, other T cells that express NK receptors (NKRs), and lymphoid DCs (Norris *et al* 1998, Norris *et al* 1999, Ishihara *et al* 1999, Hata *et al* 1990, Winnock *et al* 1991, Ohteki and MacDonald 1994, Tsukahara *et al* 1997, Wen *et al* 1999). Figure 2 shows the relative proportions of the major lymphocyte subsets that are present in the healthy adult human liver compared with those found in blood.

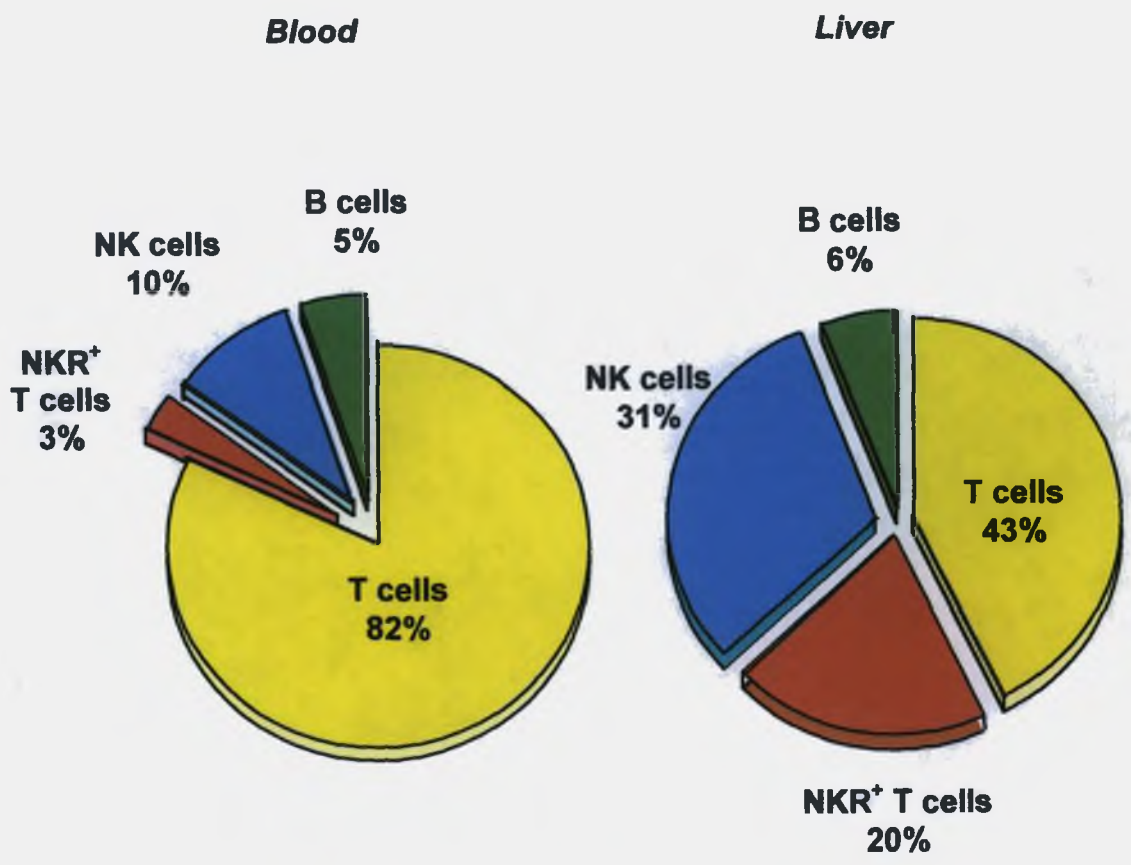

Fig. 2: Distribution of lymphocyte subpopulations in healthy human blood and liver (Reproduced from Doherty and O'Farrelly 2003).

1.2 Natural killer cells

Natural killer (NK) cells comprise approximately 10% of the mononuclear cell fraction in normal peripheral blood (Miller 2001). They contribute to immune defence against cancer and viruses. The term "natural killer" cells was originally assigned on a functional basis to define lymphoid cells capable of lysing certain tumours in the absence of prior stimulation (Moretta *et al.* 2002). NK cells are found in several lymphoid and nonlymphoid compartments, such as the spleen, liver, blood and lungs but they are relatively sparsely represented in the lymph nodes under normal conditions (Ven den *et al.* 1991). NK cells are predominantly large granular lymphocytes (LGLs) whose proliferation is induced by IL-15 (Fig.

3). They can mediate cytolysis in the absence of MHC class I or II antigen expression on target cells (Brittenden *et al* 1996).

In humans, CD56, an isoform of the human neural cell adhesion molecule serves as a tag to distinguish NK cells from other non-T lymphocytes (Rolstad and Seaman 1998). Whereas NK cells express CD16 and CD56, they lack the expression of CD3 and CD4 (Middleton and Curran 2002). Approximately half of human NK cells express CD8 but in contrast to most T cells, which express CD8αβ hetrodimers, NK cells express CD8αα homodimers (Seaman 2000).

1.2.1 Role of NK cells in immune responses

NK cells lyse target cells that have lost (or express insufficient amounts of) MHC class I molecules, as frequently occurs in tumours and in cells infected by certain viruses (Moretta *et al* 2002). NK cells can also directly identify and destroy normal MHC-mismatched hematopoietic or lymphoid cells, thus relating to the capacity of NK cells *in vivo* to reject transplants of bone marrow cells or lymphoid cells (Bennett 1987).

NK cells also play an important role in natural surveillance against viral infection and neoplasic diseases, and they can play a role in the induction of specific CTL during viral infections (Kos and Engleman 1996, Huang *et al* 1993). The role of NK cells in early antiviral immune defence mechanisms seems to be crucial. Defects in NK cells function can leave the host vulnerable to several viral infections and is correlated with a history of recurrent infectious diseases (Pawelec *et al* 1998).

NK cells produce higher levels of IFN-γ in response to IL-12, which is released from macrophages and from DC early during viral infections (Orange *et al* 1995). IFN-γ has antiviral effects both directly and indirectly It disrupts viral replication, induces activation of macrophages, increases antigen presentation by upregulating MHC class I expression in target cells (thus making them more susceptible to attack by cytotoxic T cells) and diverts the T-cell response toward a Th1 response (Rolstad and Seaman 1998)

Although NK cells that can kill sensitive targets can be isolated from uninfected individuals, this activity is increased by between 20-100 fold when NK cells are exposed to IFN-α and IFN-β, the NK cell-activating factor IL-12 and the T and NK cell growth factors IL-2 and IL-15. These cytokines are typically produced in virus infections (Fig.3) (Janeway *et al* 2001). NK cells also exert cell cytotoxicity by recognising and inducing lysis of antibody-coated target cells (antibody-dependent cell cytotoxicity) through antibody binding to the receptor CD16 (Fc γ receptor III) (Middleton and Curran 2002). NK cell functions are regulated by a number of surface receptors that bind specific ligands expressed by target cells (Moretta *et al* 2001). Binding of an NK cell to a susceptible target cell is followed by an increase in phosphoinositide metabolism with a resulting increase in cellular calcium concentration, the magnitude of which is proportional to the sensitivity of the cell to subsequent lysis (Trincheri 1989).

1.2.2 Mechanisms of NK cytotoxicity

NK cell-mediated cytotoxicity of target cells takes place by apoptosis induction, mediated by effectors molecules, which include perforin, granzymes and Fas/Fas

ligand. NK and lymphokine-activated killer (LAK) cell cytolysis can be considered to occur in four stages: 1- target cell binding (adhesion), 2-effector cell activation (recognition/signal transduction), 3-delivery of the lethal signal to the target cell and 4- effector cell detachment and recycling (Biron *et al* 1999, Bennett 1987, Graldo and Hiserodt 1989, Orange *et al* 1995, Young and Cohn 1987).

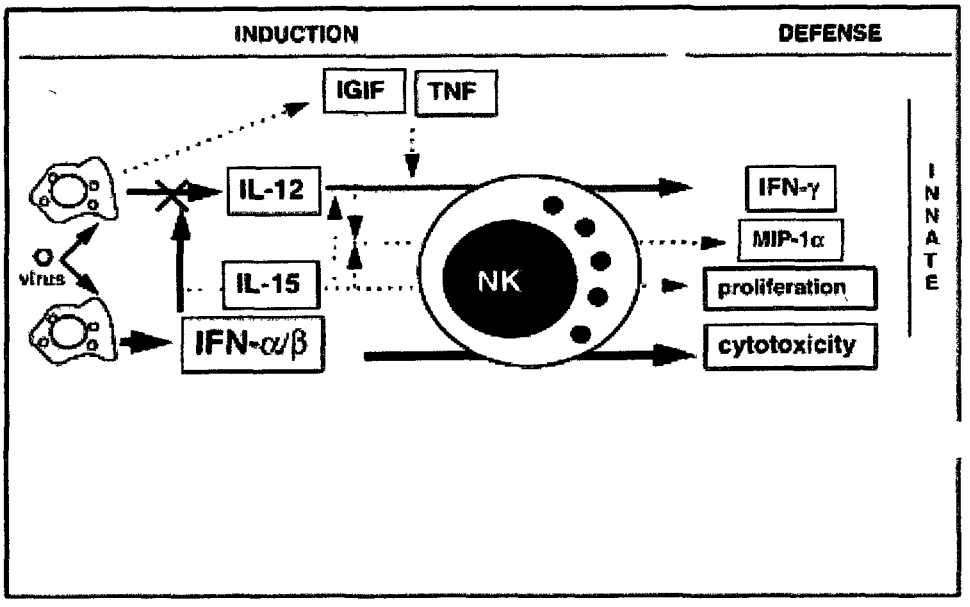

Figure 3: Cytokine-mediated pathways and responses in NK cells. Many viral infections induce production of IFN-α/β and these cytokines are good inducers of NK cell cytotoxicity. IL-15 induces proliferation of NK cells. IL-12 is induced in *some, but not all, viral infections* and stimulates NK cell IFN-γ responses to viruses. Dark solid arrows represent in vivo pathways or responses. Broken arrows represent proposed pathways. The X represents inhibition (Biron *et al.* 1999).

1.2.2.1 Perforin/granzyme granules

NK cells eliminate target cells by exocytosis of lytic granules, the contents of which kill target cells in a Ca^{++} -dependent manner. Binding of NK cell to a susceptible target cell infected with intracellular pathogens or to a tumour cell is

followed by an increase in phosphoinositide metabolism, with a resulting increase in intracellular calcium. The magnitude of this increase is proportional to the sensitivity of the cell to subsequent lysis (Trincheri 1989). The next stage involves polarisation of preformed lytic granules towards the part of the NK cell which is in intimate contact with the target cell. The contents of the lytic granules, which include perforins and granzymes (proteinases and chondroitin sulphate proteoglycans) are then released by a process of exocytosis (Trincheri 1989, Robertson and Ritz 1990). Perforins are pore-forming enzymes, which cause the appearance of channels on the target cell membrane and subsequent lysis by the release of soluble factors, mainly cytokines. Granzyme, in contrast, is an effector molecule of activated NK cells. It mainly mediates cell apoptosis.

1.2.2.2 Fas/Fas L

Apoptosis can be induced by the interaction of Fas ligand on NK cells with Fas on target cells. Fas ligand is a member of the TNF family of membrane-associated cytokines, whereas Fas is a member of the TNF receptor family. Both Fas and its ligand are normally induced during the course of an adaptive immune response. TNF and its receptor TNFR-1 can act in a similar way to Fas ligand and Fas but their actions are far less significant.

1.2.3 NK cell receptors

Structurally, there are two families of NK cell receptors, the immunoglobulin superfamily and the C-type lectin receptors. There are numerous members of the immunoglobulin superfamily, such as human killer cell Ig-like receptors (KIR), leucocyte immunoglobulin-like receptor (LILR), leucocyte inhibitory receptor

(LIR), leucocyte-associated inhibitory receptor (LAIR) and the activating NK receptor NKp46 (Fig. 4). The C-type lectin-like receptors, includes NKG2 and CD94.

NK cells can be phenotypically defined in humans by their surface expression of a number of receptors, that are involved in recognition of target molecules and activation/inhibition. These include activating receptors (CD16, NKG2D, natural cytotoxicity receptors (NCRs)), which recognise structures expressed on the target cells and transmit intracellular signals that initiate cytotoxicity. They also include costimulatory receptors (CD161) and inhibitory receptors (LIR, LAIR) that, likewise, recognise cell-surface structures but generate counter- activating signals that block the induction of cytotoxicity. Finally, NK cells express receptors that can be either be activating or inhibitory, such as KIRs and CD94/NKG2 (Fig.4).

In the mouse, the Ly49 family of the C-type lectin-like superfamily is expressed on NK cells and also on a subset of cytotoxic T cells. The repertoire of Ly49 expressed by an individual NK cell includes both activating and inhibitory types (Natarajan *et al* 2002). Another group of molecules in the mouse, the NKR-P1 family that includes the NK1.1 antigen, has been implicated in activation rather than inhibition. Cross-linking of NK1.1 on NK cells results in cytotoxicity and production of INF-γ. However, some of the NKR-P1 molecules are inhibitory (Long 1999).

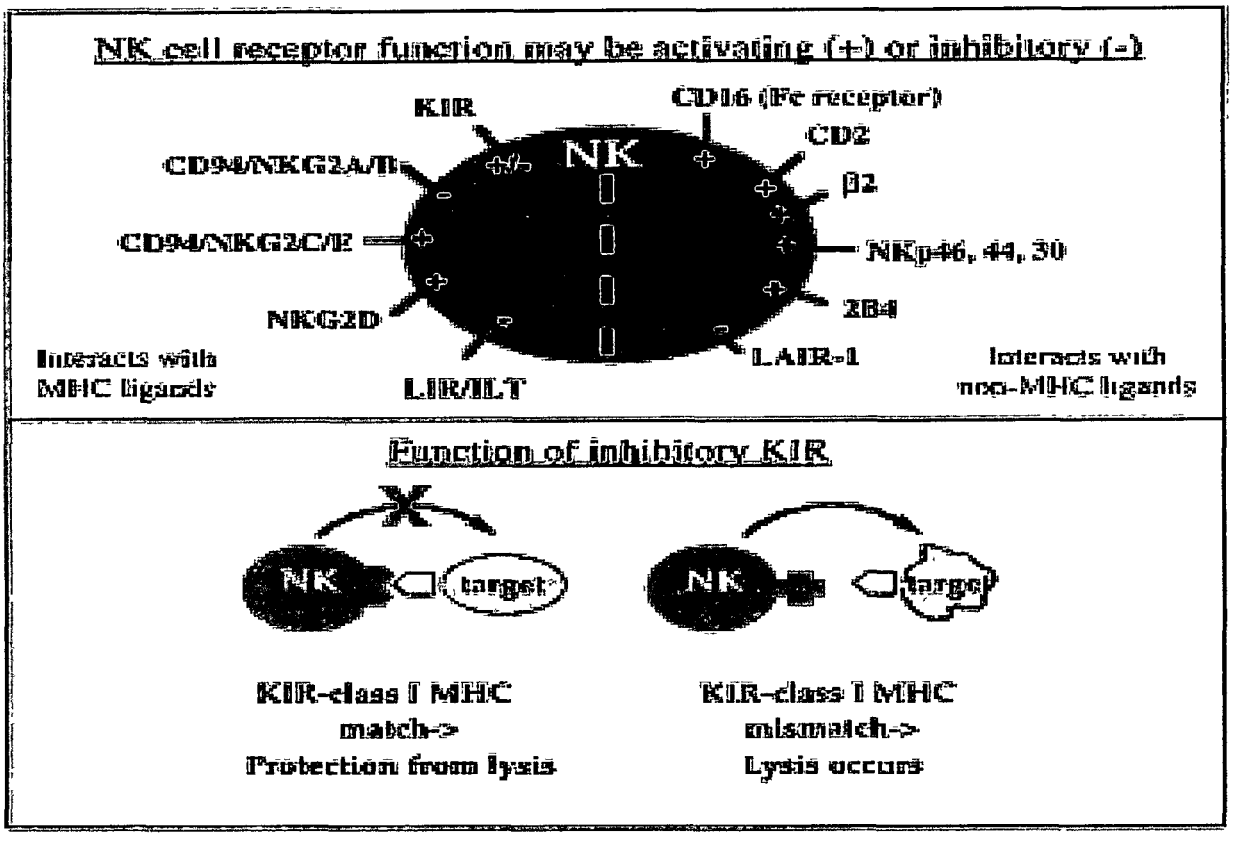

Figure 4: NK cells function through a repertoire of activating and inhibitory receptors. Activating receptor ligation triggers cytotoxicity while inhibitory receptors protect from NK cell-mediated lysis. Multiple receptors are involved in the interactions between NK cells and their targets, and inhibitory receptor signalling is dominant over stimulating receptor signalling. Both classical and nonclassical MHC class I [left side, upper panel] and non-MHC ligands [right side, upper panel] are known for many NK cell receptors. In contrast to T cells, which require self class I MHC to kill their targets, inhibitory KIR can have the opposite effect [lower panel] (Miller 2001).

1.2.3.1 Human activating receptors

Several membrane receptors have been implicated in NK cell activation, and the ligands for some of these receptors are known as shown in Table 1.

Table 1: NK cell activating receptors and their ligands

Activating receptors	Ligand	References
2B4	CD48	Brown *et al.* 1998
CD16	IgG	Perussia *et al* 1983, Lanier *et al.* 1983
NKG2D		Bauer *et al.* 1999
CD94/NKG2C	HLA-E	Brand *et al* 1998
LY6		Karlhofer and yokoyama 1991
CD44		Galandrini *et al* 1994, Sconcocchoia *et al* 1994.
CD69		Moretta *et al* 1991
NKR-P1 (Rat, mice only)		Chambers *et al* 1989
DNAM-1		Shibuya *et al* 1996
NKP44		Vitale *et al* 1998
NKP46		Sivori *et al* 1997
NKP30		Pende *et al* 1999
CD2	CD58	Dustin *et al* 1987

The cytoplasmic tail of activating receptors contains a charged residue (lysine) (Biassoni *et al* 2000). This residue is involved in the association of activating receptors with signalling molecules containing immunoreceptor tyrosine-based activation motifs (ITAM). These signalling molecules include DAP12 (also known as killer cell activating receptor-associated protein or KARAP), which is involved in the transduction of activating signals (Olcese *et al.* 1996, Lanier 1998).

CD16 is the human Fc receptor for IgG1 and IgG3. It permits NK cells to mediate antibody-dependent cell mediated cytotoxicity (ADCC) (Timonen *et al* 1981) by binding the Fc portions of these antibodies, while the Fab antigen-binding portions would be bound to a specific cellular antigen. Thus, the cell is targeted for lysis by the NK cell. CD16 is most frequently present on NK cells expressing low levels of

CD56, and this phenotype identifies mature NK cells (Miller 2001). CD16 is able to transduce activating signals through its association with the γ subunit of the high affinity IgE receptor (FcεRIγ) and the CD3ζ subunit (Lanier 1998, Orloffet 1990, Wirthmueller *et al* 1992). The signal transduction pathway occurs via activation of Src-family tyrosine kinases and phosphorylation of ITAM (Biron 1999)

Recently, three novel immunoglobulin-like type-1 membrane protein NKP46, NKP44 and NKP30, whose expression is confined to NK cells have been identified, and called natural cytoxicity receptors (NCR) (Vitale *et al.* 1998, Sivori *et al* 1997, Pende *et al* 1999). NKP46 and NKP30 are constitutively expressed on NK cells, and NKP44 on activated NK cells only (Middleton and Curran 2002). The different NCR cooperate in target cell recognition and killing. In most instances, blocking of individual NCR results in partial inhibition of cytotoxicity (Moretta *et al.* 2001). All three molecules have been implicated in NK cell mediated recognition of tumour cells because monoclonal antibodies (mAbs) with reactivity towards them have the ability to both induce NK cytotoxicity in a redirected CTL assay and to mask NK cell killing of tumour cells (Soloski 2001). MAb-mediated cross-linking of NKP46 results in increases in cytotoxicity, Ca^{++} mobilisation, and cytokine production (Moretta *et al* 2001).

NKP46 is implicated in the NK mediated lysis of human tumours, including lung, liver, breast carcinoma, melanoma and EBV–infected cell lines (Biassoni *et al* 2000). NKP46 is able to recognise viral ligands, such as haemagglutinin of influenza virus or haemagglutinin-neuraminidase of parainfluenza virus

(Mandelboim *et al.* 2001). The ligands for NKP44 and NKP30 are currently unknown.

1.2.3.2 Costimulatory receptors

Human NK activity is also influenced by costimulatory receptors, including CD161, CD2, CD11a/CD18, CD69 and CD49d/CD29. CD161 is involved in triggering cytotoxicity. IL-2 and IL-12 differentially modulate CD161 expression, suggesting a mechanism whereby these cytokines modulate NK cell activity (Azzoni *et al.* 1998)

1.2.3.3 Inhibitory receptors

The LIR/ Immunoglobulin like transcrpt (ILT) family was discovered by identifying receptors that bind the class I homologous cytomegalovirus UL18 protein (Cosman *et al* 1997). At least eight LIR genes have been identified (Colonna *et al* 1997). LIRs are encoded in a gene complex centromeric to the KIR complex (Wagtmann *et al* 1997), and LIR proteins contain various numbers of extracellular Ig-like domains. LIR-1 (ILT-2), a member with 4 Ig-like domains, is found on NK cells, monocytes, DC, and B cells (Miller 2001). It binds several different human leukocyte antigen (HLA) class I molecules, including HLA-G. It inhibits CD16-mediated NK cells activation. LIR-1 also inhibits T cell cytotoxicity induced by the TCR, and intracellular Ca^{++} mobilisation in B cells triggered by the B cell antigen receptor (BCR) (Colonna *et al* 1997) ILT3 has two Ig domains and is expressed in monocytes and DC (Cella *et al* 1997). ILT4 is less widely expressed than ILT2 and binds HLA-A and HLA-B allotypes (Colonna *et al.* 1998).

Leukocyte associated immunoglobulin-like receptor -1 (LAIR-1) has a similar cell distribution to LIR-1 and contains immune receptor tyrosine-based inhibitory motifs (ITIMs), but LAIR-1 does not appear to bind class I molecules (Meyaard *et al.* 1997). LAIR -1 shares its size (40KDa) and other properties with an inhibitory receptor called p40 that is expressed on hematopoietic cells and inhibits NK and T cell responses (Poggi *et al.* 1995, 1997).

1.2.3.4 Stimulatory/inhibitory receptors

The stimulatory/inhibitory receptors specific for HLA class I consist of two structurally distinct families of molecules, the killer cell Ig-like receptors (KIR) and the killer cell lectin like receptors (CD94/NKG2) The main function of HLA class I specific inhibitory receptors is to avoid damage to normal tissue. The lack of expression, or simply the expression of inadequate amounts, of MHC class I molecules can make cells susceptible to NK mediated killing (Moretta *et al* 2001).

KIR specifically recognise different groups of HLA class alleles (Colonna and Samaridis 1995, Dohring *et al* 1996, Wagtmann 1995). All mature human NK cells express at least one inhibitory receptor specific for self HLA class I (Moretta *et al* 2002). This allows the whole NK cell pool of a given individual to sense the loss of even single class I alleles on self cells (Moretta *et al* 1996). The HLA class I specific inhibitory receptors block NK mediated cytotoxicity and cytokine secretion (Biassoni *et al.* 2000).

The genes coding for KIRs have been mapped to chromosome 19q13, 42 in a

region called leukocyte receptors complex (LRC) (Barten *et al* 2001, Wende *et al.* 1998).

KIRs can signal either inhibition or activation of the NK cell when class I MHC is recognised (Fig.5). Inhibitory KIR isoforms have long cytoplasmic tails that contain ITIMs that recruit cytoplasmic tyrosine phosphatases which signal inactivation of NK cell function. In contrast, stimulatory KIRs have short, truncated cytoplasmic tails lacking such inhibitory motifs, and these molecules associate with DAP12, a protein with an ITAM that enhances cell activation. At least 14 KIR family members have been identified and these include 8 inhibitory and 6 noninhibitory receptors (Valiante *et al* 1997).

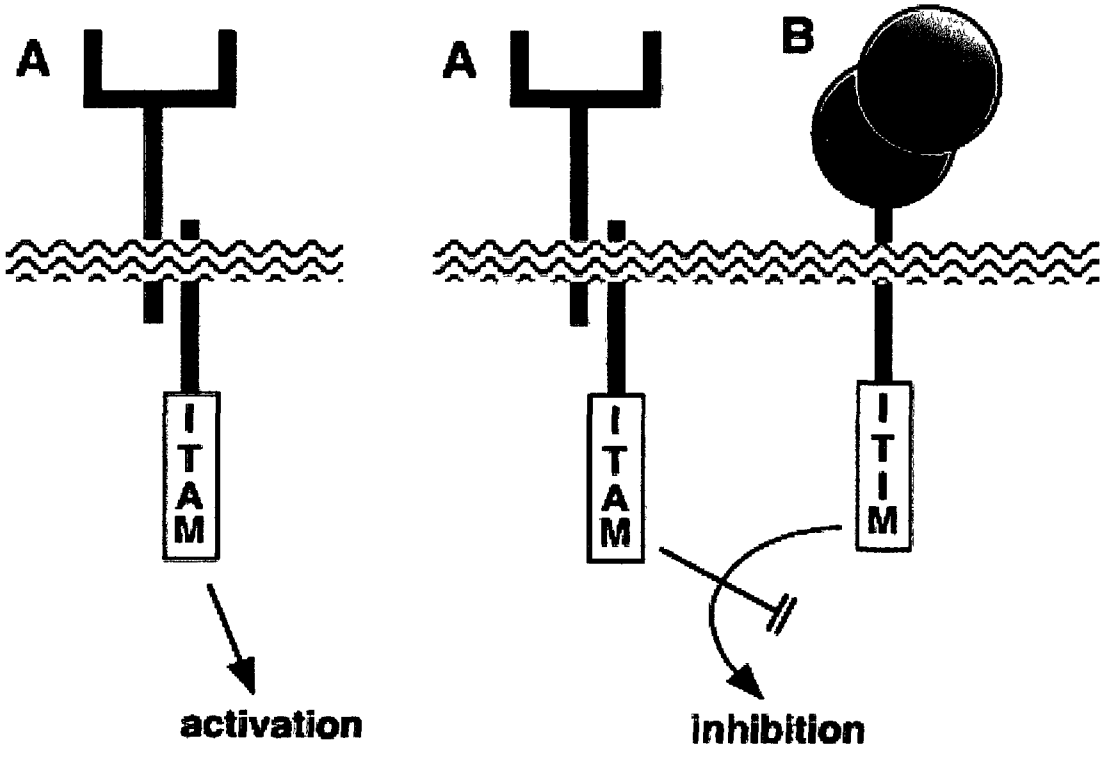

Figure 5: NK cell stimulatory and inhibitory receptors. The stimulatory receptor A with immune receptor tyrosine-based activation motif (ITAM) is inhibited by inhibitory receptor B with immune receptor tyrosine-based inhibitory motif (ITIM) (Long 1999).

KIRs are named according to whether they have two domains (2D) or three domains (3D) in their extracellular portion and to whether they possess a short (S) or long (L) cytoplasmic tail (Fig 6) (Middleton and Curran 2002). They have also been named according to the CD nomenclature system as CD158a, CD158b, etc., based on an approximate centromeric-telomeric order of the genes on chromosome 19.

Unfortunately, the CD nomenclature does not reflect structure, function, expression or localisation. This system also presents the possibility of confusion when mAbs against the CD158a and CD158b are used, since each binds to the extracellular domins of several different KIR molecules (Carrington and Norman 2003).

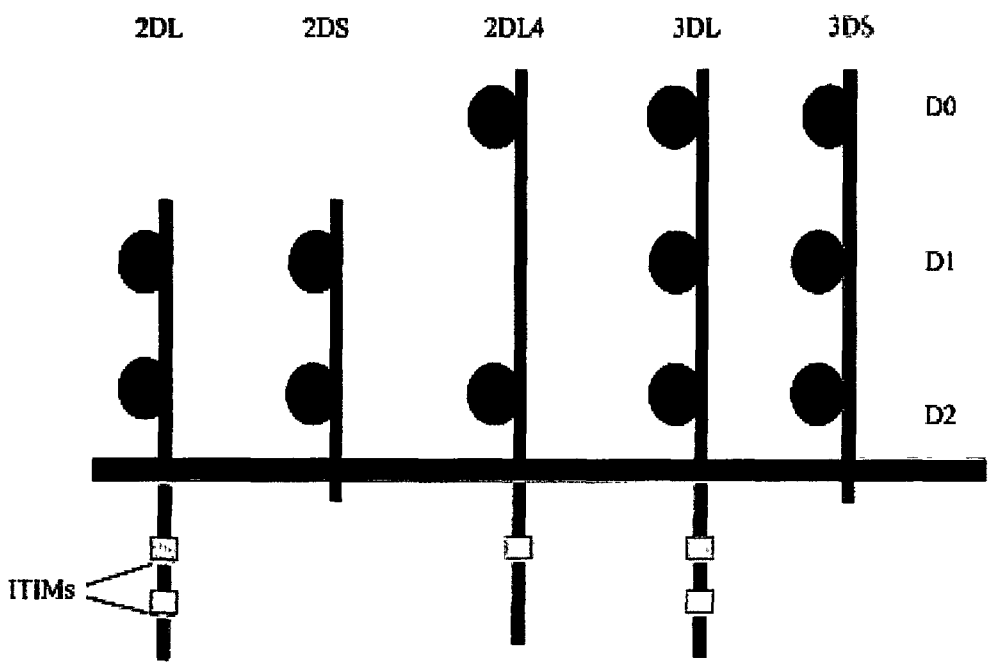

Figure 6: KIR receptors and their domains (Middleton and Curran 2002).

The shared structure and sequence similarity indicates that KIRs evolved from a common ancestor (Middleton and Curran 2002). The topology of the domains and their relative orientation resemble those found in haematopoietic receptors such as human growth hormone receptor (De Vos *et al.* 1992), erythropoietin receptor (Livnah *et al* 1999) and prolactin receptor (Somers *et al* 1994)

KIR2DL1 and KIR2DL2 bind different HLA-C allotypes, and this discrimination of binding resides in the first Ig domain of these KIR molecules (Winter *et al* 1998, Biassoni *et al.* 1997, Boyington *et al* 2000, Miller 2001). The KIR with three Ig domains (KIR3DL1) recognizes HLA-B allotypes that belong to the Bw4 serologic specificity (Long 1999). The ligand for KIR2DL4 is thought to be HLA-G, and that for KIR3DL2 is HLA-A3 (Selvakumar *et al* 1996, Litwin *et al* 1994). Ligands for the other receptors, including the recently found KIR2DL5, are unknown (Vilches *et al* 2000).

CD94 and NKG2 are lectin-like, disulfide-linked heterodimers expressed on human and rodent NK cells and a subpopulation of T cells (Natarajan *et al* 2002) CD94/NKG2 heterodimers recognise non-classical class I HLA-E molecules bound with peptide leader sequences from HLA -A, B, C and G (Leibson 1998, Brand *et al* 1998, Borrego *et al* 1998). The CD94/NKG2 receptor is designed to gauge the overall level of HLA class I synthesis in cells (Long 1999). CD94 essentially lacks a cytoplasmic domain, thus lacking signal transduction capacity. It dimerises with NKG2A (containing ITIM) or with NKG2C/DAP12 (containing ITAM), and can these function as an inhibitory or stimulatory receptors.

NKG2D is a member of the C-type lectin- like family of NK receptors. It is also expressed by γδ T cells, some $CD8^+$ T cells, and NKT cells. NK cell triggering via NKG2D has been shown to over ride the negative signalling generated by the engagement of HLA class-I-specific inhibitory receptors (Bauer *et al.* 1999). The NKG2D gene is located adjacent to NKG2A, B and C within the NK complex. Its encoded protein shows little homology to other NKG2s and does not pair with CD94. (Natarajan *et al* 2002). Various families of cell surface ligands have been identified, including the MICA/B and ULBP proteins expressed predominantly (but not exclusively) by cells of epithelial origin (Bauer *et al* 1999). Some of these ligands are upregulated on transformed, infected or distressed cells, and ligand expression by a tumour cell leads to activation of NK cells and $CD8^+$ T cells and induction of tumour immunity (Diefenbach *et al.* 2002). A key issue with respect to NKG2D function is whether it provides a stimulatory or costimulatory signal to responding cells. Its engagement in NK cells and macrophages results in direct cellular activation; in contrast, engagement of NKG2D on T cells costimulates effector T cell function (Jamieson 2002). This versatility is achieved by the selective expression of DAP10 and DAP12 by T cells and NK cells on different cells and by their distinct association with alternative isoforms of NKG2D (Diefenbach *et al.* 2002).

1.3 Natural killer receptor positive T cells (NKR^+ T cells)

A proportion of T cells can express NK cell stimulatory, costimulatory and inhibitory receptors (NKRs). Human NKR^+ T cells can be $CD8^+$, $CD4^+$ or $CD4^-$ $CD8^-$, they express αβ or γδ TCRs and most express activated/memory T cell

phenotypes They possess a variety of NKRs, including CD16, CD56, CD161, NKG2D/CD94, and KIR receptors for MHC class I molecules, such as KIR2DL1, KIR2DL2, KIR2DL3 and KIR3DL1 (Mingari *et al* 1995, Doherty and O'Farrelly 2000, Lanier *et al.* 1986, Lanier *et al.* 1987, Ortaldo *et al.* 1991, Mingari *et al* 1996, Norris *et al* 1999)

NKR^+ T cells have multiple effector activities. They can be rapidly activated without the need for prior antigen priming. They are capable of MHC-unrestricted cytotoxicity and the release of multiple cytokines (Ishihara *et al* 1999, Dunne *et al* 2001, Doherty *et al.* 1999). $CD56^+$ T cells and $CD161^+$ T cells can be induced by IL-2, IL-12, or IL-15 to directly kill a variety of tumour cell types and thus they display properties of LAK cells (Doherty *et al* 1999, Satoh *et al* 1996, Dunne *et al* 2001). They are also capable of TCR-mediated cytotoxicity. Their potent cytotoxic activities appears to be kept under regulatory control via inhibitory signals through KIRs and CD94. The majority of $CD56^+$ and $CD161^+$ T cells produce IFN-γ, TNF-α, and IL-2 within hours of activation, indicating that they can function in inflammatory or Th1 immune responses (Doherty *et al.* 1999, Nuti *et al* 1998). A significant proportion (~15 %) of NKR^+ T cells produce the Th2 cytokine IL-4. Since NKR^+ T cells can be rapidly activated without the need for prior antigen priming to produce large amount of Th1 or Th2 cytokines, it is thought that they represent a bridge between the innate and adaptive immune systems by influencing Th1/Th2 cell differentation (Fig.7) (Doherty and O'Farrelly 2003).

Although NKR^+ T cells display properties of cells of the innate immune system, they express memory T cell phenotypes (Norris *et al.* 1999) and homing

chemokine receptors (Campbell *et al.* 2001), suggesting that they may be antigen-specific memory T cells. NKR expression may therefore identify a subset of memory T cells that have acquired the less specific and more rapid and potent effector functions characteristic of NKR^+ T cells (Doherty and O'Farrelly 2003).

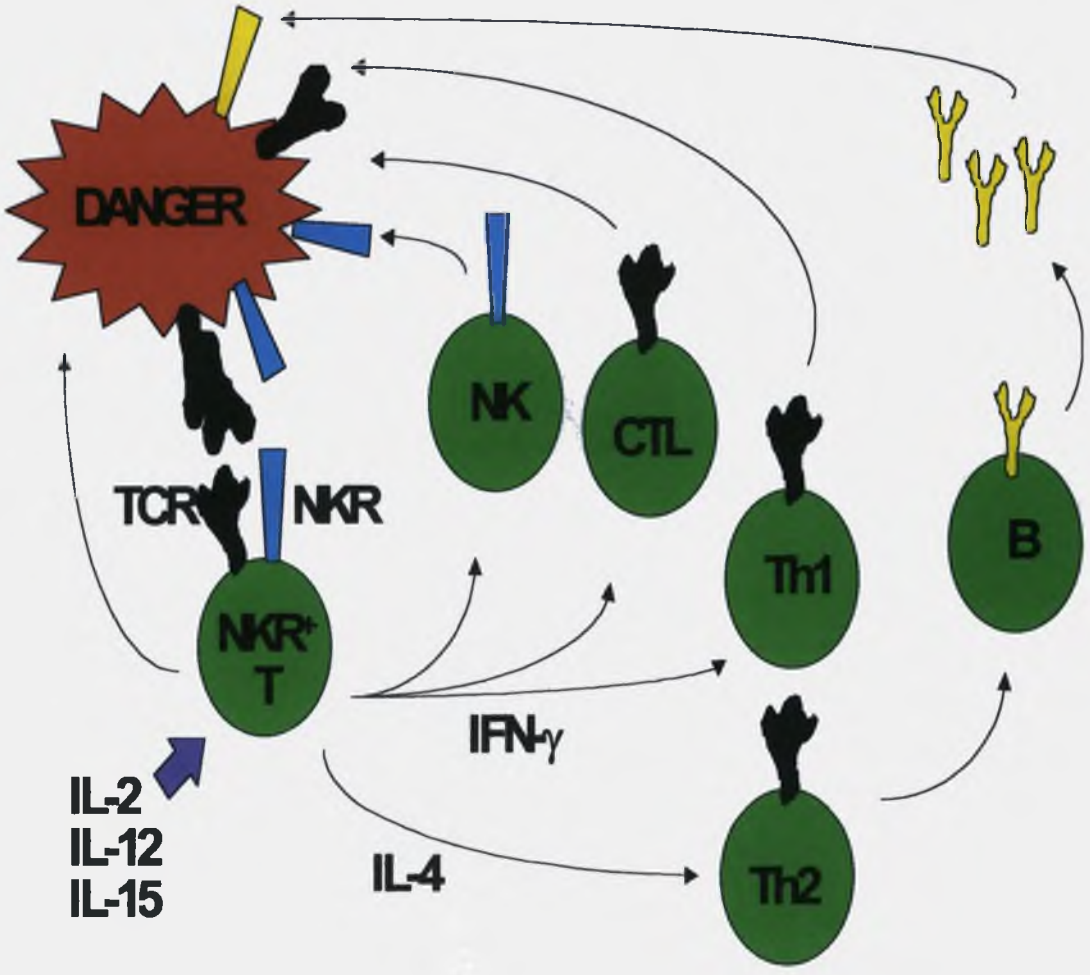

Figure 7: Natural killer receptor-positive (NKR^+) T cells can initiate and regulate innate and adaptive immune responses in humans (Doherty and O'Farrelly 2003).

1.4 Natural killer T cells (NKT cells)

NKT cells are a population of T cells that share some characteristics of NK cells and have immunoregulatory functions (Bendelac 1995, Bendelac *et al* 1997, MacDonald 1995). These cells are thought to be antitumour effectors (Bendelac *et al* 1997). The TCRs of NKT cells are specific for glycolipid antigens bound by the MHC class I-like protein CD1d (Wilson *et al* 2002). CD1d molecules are encoded outside of the MHC gene locus, and their expression is largely restricted to thymocytes, B cells, macrophages, DC and hepatocytes. The physiological ligands that are recognised by NKT cells in the context of CD1d remain unknown (Wilson *et al.* 2002). Most NKT cells recognise CD1d in conjunction with hydrophobic ligands (glycolipids) (Benlagha *et al* 2000, Burdin and Kronenberg 1999). One population of NKT cells expresses rearranged TCRs that are limited in heterogeneity using an invariant TCR α-chain (Vα24Jα18 in human, Vα14Jα18 in mouse) paired with one of a limited number of β-chains (Vβ11 in humans, Vβ8 in mouse) (Soloski 2001). These invariant TCR-expressing NKT cells account for about 5% of peripheral blood T cells in mice but <0.1 in human (Kenna *et al* 2003). NKT cells are more abundant in the thymus, liver, and bone marrow but not in lymph nodes (Fig. 8) (Wilson *et al* 2002).

A glycolipid extracted from sponges, alpha- galactosylceramide (α–GalCer), and its synthetic equivalent KRN7000, bind CD1d molecules and stimulate NKT cells (Brossay *et al* 1998, Spada *et al* 1998).Studies on the interaction of the NKT TCR and CD1d with α-GalCer have demonstrated that the acylchain of α-GalCer interacts with CD1d and that its carbohydrate portion is recognized by the invariant TCR (Joyce 2001). α-GalCer stimulates antitumour responses in mice by

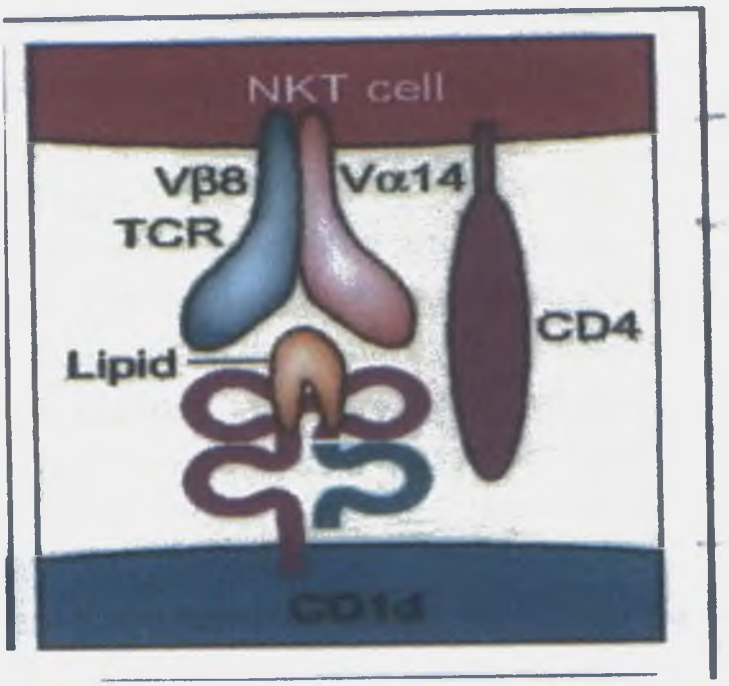

Figure 8: CDld molecules present lipid antigens to natural killer T (NKT) cells. NKT cells recognise CD1d using invariant Vα14/Vβ8 T cell receptors in mouse and Vα24/Vβ11 in humans (Wilson *et al.* 2002).

activating NKT cells (Soloski 2001). Murine models, NKT cells mediate cytotoxicity against tumour cell lines *in vitro* (Bendelac *et al.* 1997, Smyth *et al.* 2000), suggesting an important role for these cells in tumour rejection (Godfrey *et al.* 2000). They constitutively express Fas-L and can kill Fas-expressing target cells, and they can also kill tumour target in a perforin dependent manner (Smyth *et al.* 2000). Recent preclinical studies in mice have revealed significant efficacy of α-GalCer for treatment of metastatic cancers and infections, and for prevention of autoimmune diseases. These findings suggest that appropriate stimulation of NKT cells could be exploited for prevention or treatment of human diseases (Wilson *et al.* 2002). The potential usefulness of α-GalCer in immunotherapy resides on its ability to activate NKT cells, which secrete IFN-γ which in turn activate cytotoxic NK cells. α-GalCer is not a physiological ligand of NKT cells because mammals are unable to synthesize such α-anomeric glycosphingolipids

(Wilson *et al* 2002). NKT cells appear to participate in immune responses to a range of different infectious agents, including mycobacteria, the protozoan parasites *Leishmania* (Ishikawa *et al* 2000), and *Tyrpanosoma cruzi* (Duthine *et al* 2002), the spirochete *Birelia burgdorferi* (Kumar *et al*. 2000), the fungal pathogen *Cryptococcus neoformans* (Kawakami *et al* 2001), diabetogenic encephalomyocarditis virus (Exley *et al* 2001), and respiratory syncytial virus (Johnson *et al* 2002).

One striking property of NKT cells is their capacity to rapidly produce a spectrum of cytokines, including IL-4, IL-10, IL-13, INF-γ and TNF-α, upon TCR engagement (Godfrey *et al* 2000, Joyce 2001, Bendelac *et al* 1997). This makes NKT cells important potential regulatory cells of the adaptive immune system. As explained above, naïve $CD4^+$ T cells that are activated in the presence of IL-12 (from APC) or IFN-γ become committed to differentiate into Th1 cells, whereas $CD4^+$ T cells activated in the presence of IL-4 become committed to differentiate into Th2 cells. Because NKT cells can secrete both IFN-γ and IL-4, these cells can promote Th1 responses under certain conditions and Th2 responses in others (MacDonald 1995). A significant amount of evidence indicates that NKT cells play a critical role in the regulation of autoimmmune responses (Godfrey *et al* 2000, Joyce 2001, Bendelac *et al* 1997). Although the precise mechanism by which NKT cells regulate autoimmunity remains to be established, most studies have provided evidence for a crucial role of the cytokines IL-4, IL-10 and IL-13. Defects in NKT cell numbers and function have been observed in patients with systemic sclerosis, type 1 diabetes, rheumatoid arthritis and lupus (Wilson *et al* 2002).

1.5 Hepatitis C

1.5.1 Hepatitis C virus

The hepatitis C virus (HCV) is a positive stranded RNA virus with a genetic structure similar to that of flaviviruses. The single stranded RNA genome of HCV is approximately 10000 nucleotides in length. The virus is highly variable with nucleotide sequence varying considerably from one isolate to another, forming the basis for at least six known genotypes and these genotypes may also be subdivided into subtypes. In addition, nucleotide variability may be present in viruses circulating within an individual. These are referred to as quasispecies and may reflect the consequences of ongoing immune surveillance and viral mutation. Some genotypes such as 1a, 1b and 4 seem to be less responsive to interferon therapy and infection with genotype 1b is more likely to lead to cirrhosis or hepatocellular carcinoma (HCC) (Bisceglie 1998)

HCV protein is translated as one large polyprotein composed of 3010 amino acids. Viral protein translation is initiated by the ribosome binding to the internal ribosomal entry site in the 5' non-translated region (Fig. 9). The viral polyprotein is subject to processing to make up ten polypeptides. The virus structural proteins can be cleaved by the host signal peptidase and it includes a nucleocapsid core protein and two envelope proteins, designated E1 and E2, which coat the virus Extensive variation is present in the envelope glycoproteins of HCV, particularly within the amino-terminal region of E2 known as hypervariable region 1 (HVR1) (Forns *et al* 1999). HVR1 is composed of about 30 amino acids located at the N-terminus of E2. This region appears to operate as an antibody epitope (Kohara

2000). The non-structural proteins seem to be critical for viral replication and include a viral protease (Fig. 9) (Bisceglie 1998).

HCV infection may occur through binding of the envelope protein E2 to the cellular receptor CD81 (Kohara 2000). CD81 is a widely expressed cell surface protein, a tetraspanin which is conserved among different mammalian species (Meola *et al* 2000). Like all tetraspanins, CD81 is organised in four highly hydrophobic transmembrane domains, which force the protein to traverse the membrane four times (Petracca *et al* 2000) and forms a small extracellular loop and a large extracellular loop on the cell surface (Masciopinto *et al.* 2001). The large extracellular loop of CD81 is sufficient to bind HCV via interaction with the major virus envelope protein E2 (Pileri *et al.* 1998).

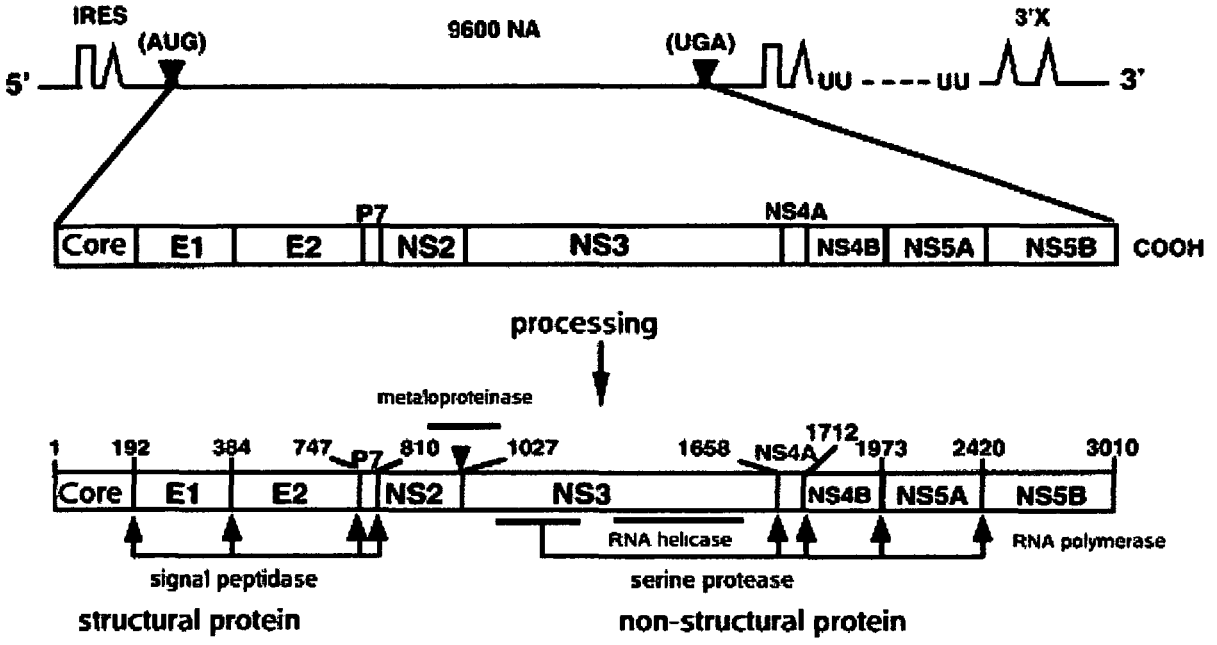

Figure 9: Organisation of the HCV genome. The 9600 nucleotide genome is translated as a single polyprotein composed of 3010 amino acids. The viral polyprotein is processed to make 10 proteins, which include a nucleocapsid core protein, two envelope peptides and non-structural peptides. IRES, internal ribosomal entry site.

1.5.2 HCV disease

HCV infection is endemic in most parts of the world, with an estimated overall prevalence of 3% (Wasley and Alter 2000). It is estimated that over 170 million people are infected (Klenerman *et al* 2002). Significantly higher rates of infection have been found in parts of Eastern Europe and Africa (Alter 1995) while Egypt seems to have one of the highest prevalence rates of all, approaching 15% of the general population. Nearly 4 million people are infected with HCV in the USA (Bisceglie 1998). Most infections are found among persons 30-44 years old, indicating that the risk for HCV infection was greatest in the relatively recent past (10-30 years ago) and primarily affected young adults (Wasley and Alter 2000).

HCV is most frequently transmitted by contact with contaminated blood among populations of intravenous drug users, and it is acquired rapidly so that within 6 to 12 months up to 80% of users are infected (Klenerman *et al* 2002). Invasive medical procedures, particularly those performed in non-medical settings or involving blood transfusion, may also transmit the hepatitis C virus (Gabriel *et al* 1996). Health care workers are at slightly increased risk of HCV infection compared with the general population, and the rate of infection following needle stick injury is 5 to 10%. HCV may also be transmitted from mother to infant (Bisceglie 1998). While it has been difficult to demonstrate that HCV is a sexually transmitted disease, individuals with multiple sexual partners are clearly at greater risk of having HCV (Alter *et al* 1990).

Although HCV infection is very common, it rarely presents acutely, as the disease is usually not accompanied by overt jaundice and patients rarely seek medical

attention at early stages of the disease (Lechner *et al* 2000)

HCV is able to set up chronic infection in about 80% of those infected (Gruener *et al* 2001), the remaining 20% appear to be able to clear the viruses and recover. The clinical course of chronic hepatitis C is highly variable. In about 70% of patients with chronic HCV infection the disease is mild and stable over several decades, whereas in the remaining 30% it is more rapidly progressive (Farci *et al* 2000). Very rarely HCV causes fulminant hepatitis (FH), the most severe form of hepatitis. In an estimated 15% to 20% of persons infected with HCV, the infection progresses to cirrhosis. Alcohol intake is an important factor in this progression (Herrine 2002). The initial appearance of hepatic fibrosis seems to be an important predictor of the development of cirrhosis (Yano *et al* 1996). HCC may develop in 1.2 to 4% of patients infected with HCV (Bisceglie 1997). Liver failure and HCC appear mainly in individuals with cirrhosis, and because cirrhosis may take many years to develop, HCV infection may be silent for decades (Seeff *et al* 1992). In addition to causing liver disease, HCV infection is frequently associated with the development of autoimmune diseases such as sjogren syndrome and systemic lupus erythematosus (Trejo *et al.* 2003).

In 1977, a cohort of Irish women was infected with HCV genotype 1b via contaminated anti-D immunoglobulin. (Barrett *et al.* 2001). The source of the infection was identified as an acutely infected female. As part of a voluntary serological screening programme, 704 individuals were identified as seropositive for exposure to the HCV. Of these, 55 4% were found to be positive for the viral genome 17 years after exposure. Of these women, 98% had evidence of inflammation, but surprisingly, 49% showed no evidence of fibrosis (Fanning

2002).

1.5.3 Diagnosis of hepatitis C

Two types of tests are available for the laboratory diagnosis of HCV infection: the detection of antibody to various HCV antigens, and molecular methods to detect and quantitate the nucleic acid of the virus (American Academy of Pediatrics 1998). There are several sensitive enzyme-linked immunoassays available for the detection of anti-HCV antibodies based on the use of recombinant viral proteins in a microtitre plate or bead format (Silva *et al.* 1994). Molecular testing for HCV is used to confirm a positive result on antibody testing and to provide prognostic information for treatment (Herrine 2002).

Liver biopsy is a very important tool in the assessment of patients with chronic HCV because it permits determination of the degree of inflammation (grade of hepatitis) and the amount of fibrosis present (stage of hepatitis) (Bisceglie 1998). Most specialists prefer to include an examination of liver histology in the management of patients with chronic HCV infection to aid prognostic and treatment decisions (Herrine 2002).

1.5.4 Treatment of hepatitis C

Interferon alpha (IFN-α) with ribavirin are the only products licensed for the treatment of chronic HCV infection in adults, and up to 66% of patients treated have a sustained response (Balistreri 1994, Poynard 1998, McHutchison 1998). IFN-α appears to augment host immune responses enabling the elimination of HCV. The use of IFN-α is associated with a decrease in serum aminotransferase

activity and a decline in HCV RNA to undetectable levels in some but not all individuals (Hoofnagle *et al* 1986). No controlled trials of IFN-α therapy in children with chronic hepatitis C have been conducted, and IFN-α is not approved by the food and drug administration of the USA for use in individuals aged less than 18 years (American Academy of Pediatrics 1998). The optimal response is defined by persistently normal serum amino transferase concentrations and absence of HCV RNA from serum at the end of therapy and for at least 6 months thereafter (Bisceglie 1998). Factors associated with a good chance of responding to interferon include the absence of cirrhosis, lower pretreatment serum HCV RNA levels, and infection with HCV genotypes other than type 1 (Bennett *et al* 1997). Several forms of interferon are available, but most seem to have similar effects with only small differences in response rates.

Other adjuncts to IFN-α have also been tested and these include non-steroidal anti-inflammatory agents, nacetyl cysteine, ursodeoxycholic acid, and iron reduction therapy. Side effects of IFN-α therapy include myalgias, fever, nausea, irritability and depression (Herrine 2002).

The current standard of pharmacologic treatment of chronic HCV is weekly subcutaneous IFN-α in combination with daily oral ribavirin. Ribavirin is a guanosine analogue, and is active against various RNA and DNA viruses (Herrine 2002, Manns *etal* 2001, Fried *et al* 2002). When ribavirin is used together with IFN-α, there is a significantly higher rate of sustained response than with IFN-α alone (Brillanti *et al* 1994, Reichard *et al* 1988).

For patients with advanced liver disease, liver transplantation is sometimes the

only therapeutic option. Hepatitis C is now the most frequent indication for liver transplantation among adults in many western countries (Bisceglie 1998).

No vaccine exists for the prevention of HCV infection.

1.5.5 Host immune response to HCV

Antiviral immune responses comprise a large array of cell types, effector functions, and signalling molecules as summarised in Fig. 10 (Klenerman *et al.* 2002).

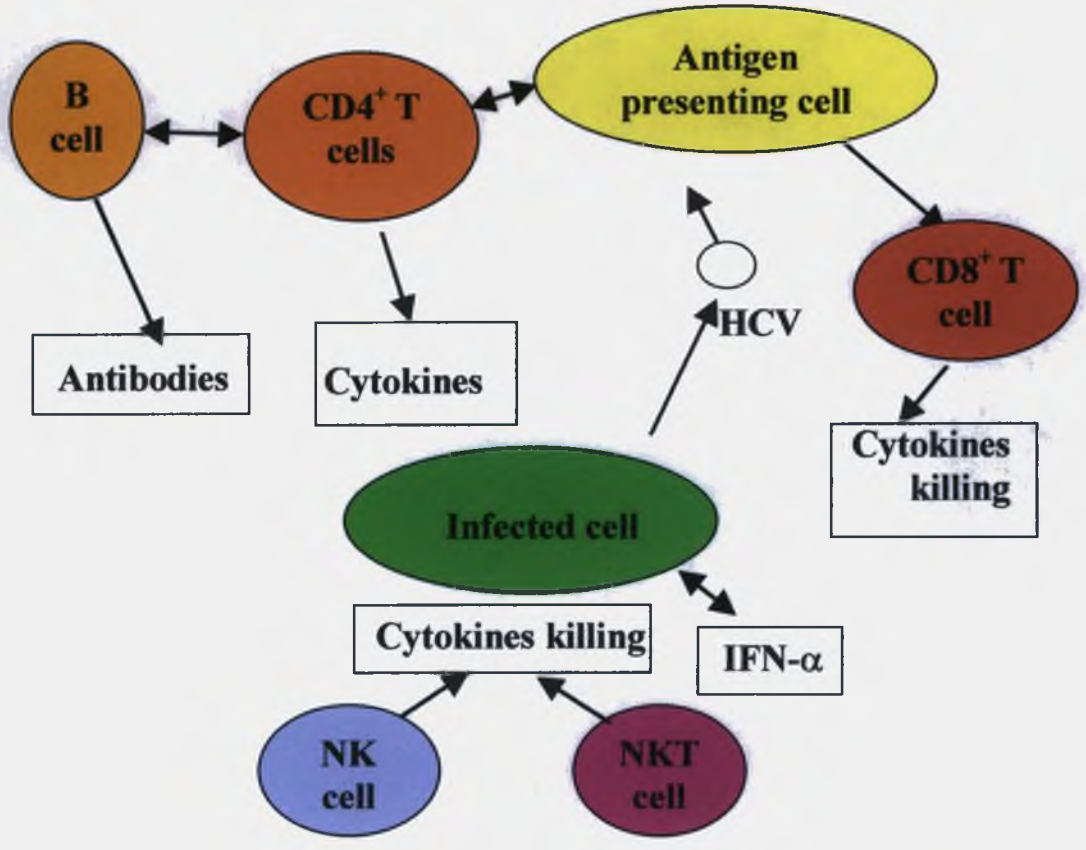

Figure 10: Players in the immune response against HCV and their roles (Klenerman *et al.* 2002).

The innate and adaptive immune responses are believed to play a crucial role in the host response to viruses and other pathogens. Although HCV infection induces

strong cellular and humoral immune responses (Alter 1995, Ferrari *et al* 1994), they are generally insufficient to eradicate the virus or to prevent reinfection (Farci *et al.* 1992, Prince *et al* 1992). The mechanisms responsible for the high rate of viral persistence and for the variable clinical course of hepatitis C are unknown, but are thought to represent a complex interplay between viral diversity and host immunity.

Multiple potential mechanisms have been postulated to explain why people infected with HCV fail to resolve this virus, despite generating both cellular and humoral immune responses. One possibility is the mutability of the RNA of this virus that leads to quasispecies that evade immune recognition (Weiner *et al.* 1991, Kumar *et al* 1994). Another possibility is that HCV inhibits crucial immune functions. HCV-encoded proteins such as envelope protein E2, the nonstructural protein and core protein have been repoted to have immunosuppressive potentials (Gale *et al* 1997, Taylor *et al* 1999, Large *et al* 1999). HCV is able to replicate in immune cells, such as mononuclear cells and B cells (Sung *et al* 2003, Hamaia *et al.* 2001).

Replication of HCV in immune cells may serve to transport HCV to lymphoid tissue where it can inhibit immune responses or to sequester virions from such recognition (Fig. 11) (Hahn 2003).

1.5.6 Quasispecies

A feature of hepatitis C is the simultaneous presence of different but closely related viral variants, commonly defined as quasispecies. This genetic heterogeneity is a result of the accumulation of mutations during viral replication.

This high mutation rate, which is characteristic of RNA viruses, can be attributed to an error-prone RNA-dependent RNA polymerase that lacks proofreading activity (Rice 1996). The existence of extensive HCV quasispecies has been suggested as a potential mechanism for viral persistence, mainly by allowing the virus to escape the host immune response (Manzin *et al.* 1998, Ray *et al.* 1999).

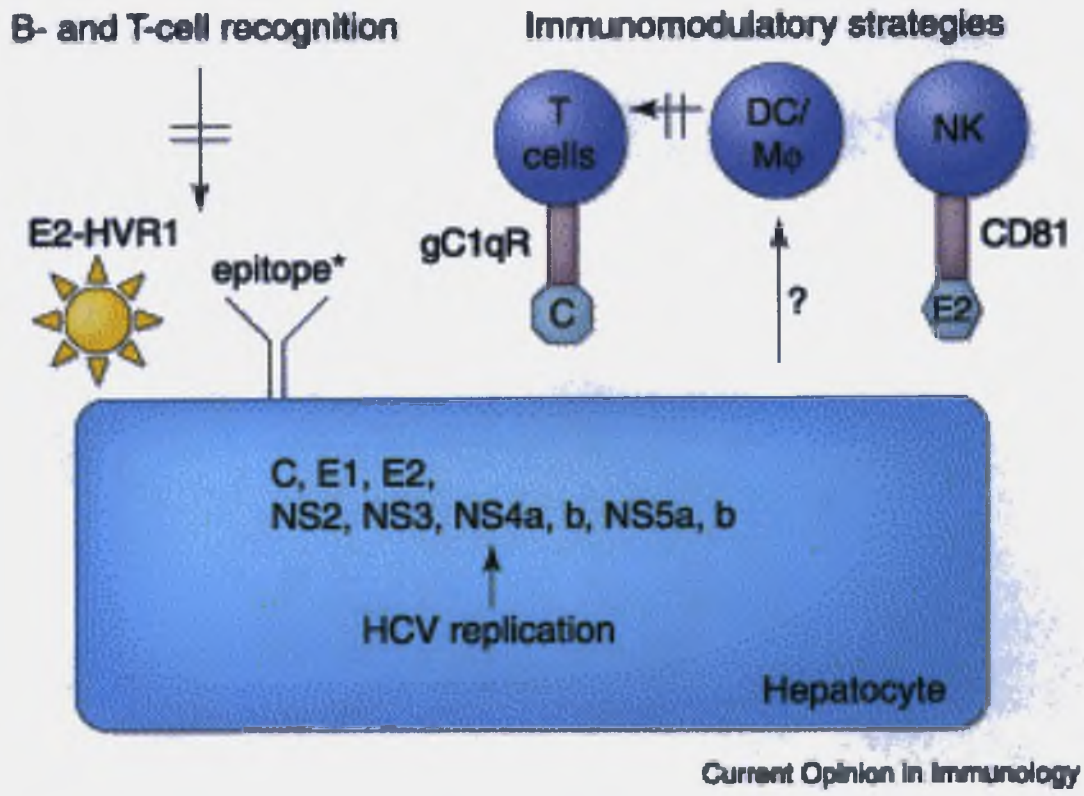

Fig. 11: HCV evolves strategies to evade and modulate immune responses. Multiple potential mechanisms have been postulated to account for the high rate of persistent HCV infection. For example, mutations in E2 HVR1 are associated with viral escape from neutralising anti-E2 antibody. Another strategy is the modulation of immune cell (e.g. T cell and NK cell) function by HCV gene products; the binding of HCV core protein (C) to the complement receptor gC1qR inhibits T cell function, whereas insufficient cytokine production by DCs or MΦs may be also involved in T cell dysfunction. In addition, the E2-CD81 intraction is capable of impairing NK cell activation (Hahn 2003).

Extensive variation in the envelope glycoproteins of HCV has been demonstrated (Hijikata *et al.*1991, Weiner *et al.* 1991), particularly within HVR1. Indeed,

sequence analysis of HVR1 has been used extensively to characterise the quasispecies distribution of HCV in infected patients (Forns *et al* 1999). Such hypervariability within the E2 protein may be under selective pressure from protective B cell or T cell responses and be able to escape immune recognition by rapid mutation at this antigenic site. Diversity of viral HVR1 quasispecies is increased in the acute phase of HCV infection in patients who develop chronic hepatitis, compared with acute resolving cases, suggesting that, in patients who develop a chronic HCV infection, the immune system is not capable of controlling the infection because of the emergence of multiple escape mutants (Forns *et al* 1999). Antibodies against the E2 protein of HCV can be detected in infected individuals. In a study of the acute phase of HCV infection, CTL activity against a particular epitope within HVR1 was significantly stronger in patients who cleared HCV infection than in those who developed a chronic infection (Tsai *et al.*1998), suggesting that T cell responses against HVR1 of the E2 protein are critical in determining the outcome of HCV infection.

1.5.7 Role of antibodies in immunity against HCV

Antibodies have a role in the containment of HCV infection (Hadlock *et al* 2000). While antibodies are produced in HCV infection, and probably mediate selective pressure on viral sequences (Farci *et al* 2000), few studies have demonstrated an important role for the humoral immune response in the control or clearance of infection. HCV persistence and hepatitis progression are observed even when circulating antibodies to HCV are present (Hahn 2003). Moreover, at least 70% of HCV infected individuals have circulating autoreactive antibodies (Cacoub *et al* 2000). This may be due to an effect of HCV infection directly on autoreactive B

cells (Wack *et al* 2001)

HCV infection is associated with disturbances of B lymphocyte activation and function. It has been proposed that these abnormalities reflect either chronic antigenic stimulation or aberrant signalling through the B cell coreceptor; the latter which may be mediated by binding of the HCV E2 glycoprotein to CD81 (Ni *et al* 2003). On B cells, CD81 associates with CD19 and CD21, forming a complex which is known to lower the threshold of activation by B cell receptor-mediated stimuli (Fearon and Carter 1995).

1.5.8 Role of T cells in immunity against HCV

T cells are thought to be the main players in antiviral defence. Studies of patients and chimpanzees (the only animal model of HCV infection) who spontaneously cleared acute HCV infection indicate that successful control of the infection requires a broad response of helper and cytolytic T cells (Lechner *et al* 2000, Cooper *et al* 1999, Takaki *et al* 2000). Recent studies have provided compelling evidence that resolution or persistence of HCV infection depends on the strengths of inflammatory T cell (Th1) and CTL responses that are generated in the liver soon after infection. Patients who clear the virus exhibit vigorous $CD4^+$ Th1 cell and $CD8^+$ CTL responses against multiple regions of the HCV polyprotein within weeks of infection. In contrast, a lack of an optimal T cell response to HCV is associated with the development of cirrhosis during chronic HCV infection (Sreenarasimhaiah *et al* 2003, Cooper *et al* 1999, Cerny and Chisari 1994).

The $CD8^+$ CTL immune response is an important mechanism for clearance of

most viruses. However, in the case of chronic HCV infection, the virus persists despite the presence of a detectable $CD8^+$ CTL response (Chang *et al.* 2001) Cooper *et al* (1999) showed that CTL responses in chimpanzees are essential for clearance of HCV They monitored antibody and intrahepatic CTL responses during acute infection in chimpanzees Two animals who terminated infection made strong CTL but poor antibody responses. In both resolvers, CTL targeted at least six viral regions. In contrast, animals developing chronic HCV generated weaker acute CTL responses against fewer viral epitopes. Extensive analysis of the fine specificity of the CTLs in one resolver revealed nine peptide epitopes and restriction by all six MHC class I allotypes of the chimpanzee. Every CTL specificity seen during acute HCV persisted in normal liver tissue more than 1 yr after resolution. These results suggest that $CD8^+$ CTL are better correlated with protection against HCV infection than antibodies.

The role of CTLs for controlling early HCV infection has been established for a small number of patients (Thimme *et al* 2001). However, several studies revealed that the effector function of HCV-specific $CD8^+$ T cells is significantly impaired during HCV infection (Wedemeyer *et al.* 2002, Urbani *et al* 2002, Gruener *et al.* 2001). The numbers of intrahepatic $CD8^+$ T cells are lower in patients with chronic HCV disease compared with healthy livers (Deignan *et al* 2002) When compared to the hepatitis B virus (HBV)-specific $CD8^+$ T cells, HCV-specific $CD8^+$ T cells express significantly less perforin and show evidence of diminished effector function (Hahn 2003) In some situations, $CD8^+$ T cells responses show a reduced capacity to secrete antiviral cytokines early in the infection (Klenerman *et al.* 2002). One study demonstrated that individuals with a

histology of fibrosis/cirrhosis showed a significantly lower $CD8^+$ CTL responses to five epitopes derived from the core, NS3, NS4 and NS5 proteins as compared with patients with histology of mild inflammation (Sreenarasimhaiah *et al.* 2003).

Several studies have shown that $CD4^+$ T cells also play an important role in the immune response against HCV infection (Eckels *et al* 2000). A $CD4^+$ T lymphocyte response directed against all of the putative HCV proteins occurs in chronically infected patients despite their failure to clear the virus (Cerny and Chisari 1994). The HCV-specific $CD4^+$ T cell response in infections with subsequent viral clearance has been shown to be more vigorous than in chronic HCV infections (Nakamura and Imawari 2000) $CD4^+$ T cell responses of Th1-type are important determinants of HCV resolution, since Th1 cells produce IFN-γ and IL-2 and promote CTL activity (Diepolder *et al.* 1995, Missale *et al* 1996). It has been shown that chronic HCV infection may be associated with a predominant $CD4^+$ Th2 response, that induces production of IL-4, IL-5 and IL-10 and promotion of antibody responses (Tsai *et al* 1997). The HCV core and NS4 proteins appear to be most immunogenic for peripheral blood lymphocytes, and NS4-specific $CD4^+$ T lymphocytes are preferentially compartmentalised to the liver (Spengler *et al* 1996). MacDonald *et al* (2002) examined the $CD4^+$ T cell responses to the HCV core protein in a cohort of women infected with a single genotype of HCV. $CD4^+$ T cells from HCV infected patients secreted IFN-γ in response to peptides from 4 immunodominant regions of the core protein, and these responses were stronger in persistently infected women. IL-10 was also produced by $CD4^+$ T cells from HCV infected subjects in response to the same core peptides. Furthermore, HCV core-specific $CD4^+$ T cell clones secreted either

IFN-γ or IL-10 but not IL-4. These findings demonstrate that T helper type 1 and regulatory T cells are induced against the same epitopes on the core protein during HCV infection

Other T cells population that reside in the liver are likely to play a key role in determining whether host immune response to HCV infection results in viral clearance or immune pathology and chronic hepatitis (Cerny and Chisari 1999). The proportions of $CD56^+$ T cells and $V\alpha24^+$ NKT cells are decreased in the livers of patients with chronic HCV infection compared with histologically normal donor livers (Deignan *et al* 2002). Hepatic γδ T cells have also been implicated in immunity against HCV The percentages of Vδ2/Vγ9 TCR^+ PBMCs are decreased in patients with chronic HCV (Par *et al.* 2002). It is not yet known if this decrease is reflected in the livers of these individuals.

1.5.9 Role of the DCs in immunity against HCV

DCs are highly specialised APCs that can activate resting T cells and NK cells (Yu *et al* 2001, Nishioka *et al* 2001, Gerosa *et al.* 2002). MICA/B are induced on DCs by IFN-α stimulation and are capable of activating NK cells However, in chronic HCV patients, IFN-α cannot induce DC-mediated NK cell activation (Jinushi *et al* 2003). This impairment might contribute to be the persistence of this viral infection, and/or an insufficient responses to IFN-α therapy (Jinushi *et al* 2003). There is some evidence that DC can be infected by HCV. Furthermore, some expressed HCV genes may inhibit DC function, leading to reduction in IL-12 production. Changes in cytokine production by HCV infected DCs and macrophages lead to impaired effector function of T cell activation (Bain *et al*

2001, Lee *et al* 2001, Sarobe *et al* 2002).

1.5.10 Role of NK cell in immunity against HCV

NK cells provide a critical first line of defense against viral infections both through their rapid and potent cytotoxic activity and the production of inflammatory cytokines. The importance of NK cells in host defense to various viruses has been extensively studied using animal models where NK cell depletion or deficiency results in a significant increase in the sensitivity of the animal to a number of viruses (Biron *et al.* 1999). However, the role of NK cells in HCV-related chronic liver disease is largely unknown. In the liver of patients with chronic HCV infection, NK cells numbers were slightly reduced (Deignan *et al* 2002). The circulating number of $CD3^{-}CD8^{+}$ (NK cell subtype) have been shown to be decreased in peripheral blood of chronic HCV patients (Par *et al.* 2002). The same study also found that NK cell cytotoxicity against K562 targets is impaired in chronic HCV infection. Although NK cell activity is enhanced by IFN-α, Par *et al.* (2002) found that NK cytotoxicity does not differ between responders and non-responders to IFN-α treatment This suggests that NK activity does not help to predict the outcome of IFN-α therapy. Increased NK activity was sustained in subjects who were successfully treated with IFN-α and remained HCV negative, indicating that the HCV infection itself may be responsible for the altered NK cell function and clearance of the virus may result in normalisation of NK activity.

Corado *et al.* (1997) found that spontaneous NK cytotoxicity in blood of HCV patients was four-fold lower than in normal donors by using ^{51}Cr-labeled K562 as target cells. They did not find any differences in cytotoxic activity between

patients with HCV infection and healthy controls when they used U937 cells as target cells. In another study, Lirussi *et al* (2002) studied peripheral NK cell cytotoxicity using an ELISA based assay to assess of apoptosis of target K562 cells. They found that NK cell cytotoxic activity was impaired in HCV patients. In contrast, Duesberg *et al.* (2001) investigated spontaneous and cytokine-induced (IL-2, IFN-γ) cytotoxicity and ADCC in patients suffering from chronic HCV and healthy controls using a flow cytometric assay As target cells they used the colorectal tumour cell line HT29 and the lymphoma cell line Raji. They found no significant differences in spontaneous cytotoxicity and ADCC between patients and normal controls, even if isolated NK cells were studied. Preincubation and stimulation of effector cells with cytokines increased both natural cytotoxicity and ADCC by 20-30% in all study groups. However, natural cytotoxicity and ADCC after stimulation did not differ between the patients with HCV and healthy controls.

Recently, the major HCV envelope protein E2 has been shown to inhibit NK cell production of IFN-γ and cytotoxic activity by cross-linking CD81 (Tseng and Klimpel 2002). The functional inhibition can be explained by the fact that CD81 engagement blocks tyrosine phosphorylation of important upstream and downstream signalling moieties induced by cross-linking CD16. The direct inhibition of NK cells via binding of HCV-E2 to CD81 provides an "off" signal for NK cell activation and efficiently blocks cytokine production, activation marker expression, cytotoxic granule release and proliferation (Crotta *et al.* 2002). This mechanism, which specifically down-regulates the NK cell response, may provide the virus with an efficient immune escape strategy capable of limiting the

antiviral activities of NK cells early in the infections process (Crotta *et al.* 2002). In contrast, this interaction elicits an opposing effect in T cells, such that CD81 is co-stimulatory and enhances proliferation. Alterations in NK cell function could set up a situation where the virus might have an initial growth advantage that could then lead to the development of a chronic and persistent infection that could not be completely cleared by T cell and B cell responses.

1.6 The aims of the project

The role of NK cells in chronic HCV is controversial and poorly understood. Some observations suggest that NK cells might be impaired in chronic HCV while others do not support this. To address this controversy, we determined the numbers, phenotypes and natural and LAK cytotoxic activities of circulating NK cells in large, well defined cohorts of patients with chronic HCV infection, patients who spontaneously resolved HCV infection and healthy donors.

Chapter 2: Materials and methods

2.1 Subjects

Thirty-three patients with chronic HCV infection (twelve males, twenty-one females, median age 46, range 21 to 69 years) were studied. All patients were anti HCV antibody positive and HCV RNA positive. Of these patients, nineteen were infected with HCV genotype 3, thirteen were infected with genotype 1 and virological data was not available for one patient. Fifteen patients became infected with HCV as a result of intravenous drug abuse (IVDA), twelve patients as a result of receiving anti-D immunoglobulin, four patients as a result of blood transfusion, one patient as a result of sexual transmission and history was not available for the remaining patient. Thirteen patients had no liver cirrhosis as determined by a histological stage of less than four using the modified histological activity index (HAI) scoring system (Ishak *et al* 1995). Three patients had HCV cirrhosis and histological data was not available for seventeen patients. None of the HCV patients had been treated at the time of study Twenty-two subjects (sixteen females, six males, median age 51, range 22 to 63 years) who spontaneously resolved HCV infection were also studied. All of these were consistently HCV RNA negative but HCV antibody positive. Of these, three individuals became infected with HCV as a result of IVDA, eight individuals as a result of receiving anti-D immunoglobulin, seven individuals as a result of blood transfusion and one patient as a result of sexual transmission History was not available for three of these subjects.

All patients were recruited at St.Vincent s University Hospital (SVUH), Dublin. Twenty-six (fourteen females, twelve males) healthy volunteers served as controls. For ethical reason none of the controls were tested for HCV but these

individual had no history of risk factors for HCV infection. Ethical approval for this study was obtained from the Research and Ethics committee, SVUH and the Ethics Committee of NUI Maynooth. Informed consent was obtained from all subjects.

2.2 Materials

2.2.1 General reagents

Bovine serum albumin (BSA), foetal calf serum (FCS), paraformaldehyde, phosphate buffered saline (PBS) and sodium azide were purchased from Sigma Chemie, Steinheim, Germany. Fungizone, Hank's balanced salts solution (HBSS), Hepes buffer, L-glutamine, penicillin-streptomycin and RPMI were purchased from Gibco-BRL, Paisley, UK. Acridine orange (AO), ethidium bromide (EB) and recombinant human IL-2 were purchased from Sigma Chemical Co. Ltd, Poole, UK Virkon was purchased from Antec International Limited, Suffolk, UK. Decon 90 was purchased from Decon laboratories limited, Sussex, England. Optiphase cocktail was purchased from Wallac, Turku, Finland. Dimethylsulphoxide (DMSO) was purchased from BDH Laboratory Supplies, Poole, England. Ethylenediaminetetraacetic acid (EDTA) was purchased from Scharlau Chemie. Barcelona, Spain. Lymphoprep was purchased from Nycomed, Oslo, Norway. ^{51}Chromium was purchased from Radiochemicals Division, Irvine, California.

2.2.2 Antibodies

The monoclonal antibodies (mAbs), Anti-CD158a-congugated with fluorescein isothiocyanate (FITC), Anti-CD161-FITC, Anti-CD16-FITC, Anti-CD4-FITC,

Anti-NKB1-FITC, Anti-CD56-congugated with phycoerythrin (PE), Anti-CD8-PE, Anti-CD3- congugated with peridin chlorophyll protein (PerCp) and Isotype control IgG1 were purchased from BD Pharmingen, Oxford, UK.

2.2.3 Cell lines

K562 and Daudi were obtained from Derek Doherty, NUI Maynooth. Ireland

2.2.4 Plastic ware

96-well plates and cryovials were purchased from Nunc, Roskilde, Denmark. Tissue culture flasks, Microfuge tubes, Transfer pipettes and Tips were purchased from Sarstedt, Numbrecht, Germany Wallac 96-well Isoplates were purchased from Wallac, Turku, Finland. Adhesive sheets was purchased from Sigma Chemie, Steinheim, Germany. Plate cassette was purchased from Perkin Elmer, USA.

2.3 Preparation of peripheral blood mononuclear cells (PBMCs)

Venous blood was taken into 0.5 % EDTA (1/10 dilution of 5% EDTA stock) as anticoagulant, and diluted 1:1 with HBSS medium containing 1% FCS which was heat-inactivated for 30 min at 56°C. The diluted blood was carefully layered onto 7.5 ml Lymphoprep in a 30 ml Universal tube and centrifuged for 25 min at 400 g (in a Centra CL3 centrifuge) with the brake off (minimum deceleration). The top (plasma) layer was removed by aspiration and discarded. The buffy coat (cloudy layer that sits on top of the Lymphoprep) plus the Lymphoprep layer were transferred to a clean Universal tube, ensuring that none of the red cell pellet was

taken. Buffy coat layers were resuspended in 20 ml HBSS containing 1% FCS, mixed and centrifuged for 5 min at 800 g with the brake on.

The supernatant was discarded by tipping off once, and resuspending the pellet in a small volume (~1 ml or the amount of liquid remaining) then vortexing. This was again resuspended in HBSS containing 1% FCS, mixed and centrifuged for 10 min at 400 g with the brake on. The supernatant was again discarded and the pellet was resuspended in a small amount of complete RPMI medium (RPMI 1640 containing 25mM HEPES, 2mM L-gluamine, 50 μg/ml streptomycin, 50U/ml penicillin and 10% heat-inactivated FCS).

2.4 Enumeration of PBMCs

A haemocytometer was used to enumerate PBMCs in suspension. The haemocytometer is a glass chamber, which holds a thin suspension of blood cells that can be examined with a microscope. The improved Neubauer haemocytometer has a chamber that is 0.1 mm deep and is divided into a grid precisely 3mm x 3 mm, and subdivided into squares of 0.25 mm x 0.25 mm and 0.05 mm x 0.05 mm.

Ten μl of cell suspension were added to 190 μl of a solution containing 16 μg/ml EB and 10 μg/ml AO in an Eppendorf tube and mixed well. Cells were counted on Neubauer haemocytometer slide, ensuring that the solution covered the entire surface of the counting chamber. Live cells only appearing in the two corners of the grid 2 x (1.0 mm x 1.0 mm) were counted. Taking into account the 1/20 dilution in EB/AO, the counts from two corners of the grid x 10^5 gives cell number/ml The total number of PBMCs was calculated by multiplying the

concentration in cell/ml by the number of ml of RPMI used to resuspend the cells

2.5 Cryopreservation of cells

For long-term storage, PBMCs were pelleted by centrifugation for 10 min at 800g, the supernatant was discarded, and the cells were resuspended in freezing mixture (90 % FCS containing 10 % DMSO, made up freshly, but in advance to allow it to cool as FCS and DMSO produce heat when mixed). Immediately cells were transferred to previously labelled cryovials and they were frozen at -80°C overnight. Next day, cryovials were transferred to liquid nitrogen.

Cryopreserved cells were recovered by thawing using warm water for few seconds. Cells were transfered immediately to universal tubes and RPMI was added dropwise. The cells were centrifuged at 400 g. The supernated was discarded and the cells were resuspended in complete RPMI.

2.6 Phenotypic analysis by flow cytometry

2.6.1 Brief background to flow cytometry

Flow cytometry employs instrumentation that scans single cells flowing past excitation sources in a liquid medium. The technology is unique in its ability to provide rapid, quantitative, multiparameter analyses on single living (or dead) cells. Measurement of visible and fluorescent light emission allows quantitation of antigenic, biochemical, and biophysical characteristics of individual cells. Flow cytometry can also separate distinct subpopulations of cells on the basis of these measured characteristics, usually by electrostatic deflection. This separation technology is called electronic cell sorting. A graphical representation of up to six

analytical measurements made on individual cells and can be used to quantity subpopulation of cells.

Performing flow cytometry experiments generally involves three distinct, interdependent phases. First is the pre-flow cytometry phase, which involves reagent preparation, cell preparation, and staining of cells with fluorescent reagents. Second is the flow cytometry phase, which involves processing the stained cells using flow cytometry instrumentation and collecting data for one, or more measurements (parameters) made on each individual cell Finally, the analysis phase involves analysing the collected data (Colican *et al.* 2002).

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 keeping it straight and in focus. The stream passes at right angles to an argon laser (488 nm). Transmitted and reflected light impinges on photodetetors via a series of strategically placed dichroic and band pass filters. Photodetectors and photomultiplier tubes amplify and convert the analogue signal to a digital format so that a computer can process the information. 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). Photodetectors that

measure fluorescence at four different wavelengths can then distinguish phenotypic variants of cells stained with mAb conjugated with fluorochromes. In the present study phenotypic analysis of PBMCs was performed by three color mAb staining and flow cytometry using a FACSCalibur (Becton Dickinson) and analysis-using cell Quest software (Becton Dickinson).

2.6.2 Monoclonal antibody (mAb) staining of PBMCs

1.2 x 10^6 cells were resuspended in 600 µl PBA buffer which is composed of 600 ml of PBS containing 1 % BSA and 0.02 % sodium azide. 100 µl of cells (0.2 x 10^5 cells) in PBA buffer were transferred to each of 6 labelled FACS tubes. 5 µl of each fluorochrome-conjugated mAb were added to the cells. The mAb and appropriate fluorescence-labelled isotype-matched control antibodies, used to correct for any background staining, are shown in Table 2. All mAbs were directly conjugated with fluorochromes. Cells were then vortexed and incubated for 15 min at room temperature in the dark (covered in tin foil) Two millilitres of PBA buffer were added, vortexed and centrifuged for 5 min at 800 g force. Supernatants were discarded and pellet were resuspended in ~0.5 ml PBA buffer.

Table 2. Monoclonal antibodies used for staining of PBMCs.

Tubes	PerCP-labelled mAb	PE- labelled mAb	FITC-labelled mAb
CONTROL	MOUSE IgG	MOUSE IgG	MOUSE IgG
2	CD3	CD56	CD16
3	CD3	CD56	CD161
4	CD3	CD56	KIR2DL1
5	CD3	CD56	KIR3DL1/S1
6	CD3	CD8	CD4

2.6.3 Flow cytometric acquisition and analysis of data

Freshly isolated PBMCs were stained with mAbs as described in section 2.7.2. With the flow cytometer in 'setup' mode, cells were run through the cytometer and the detectors and parameters were set so that lymphocytes could be distingushed by virtue of their FSC and SSC properties, as shown in Figure 12A. The detectors and parameters were then set so that gated lymphocytes that were stained with isotype-matched control mAbs located to the bottom left-hand quadrant of dot plots of fluorescence-1 (FL1) vs FL2, FL1 vs FL3, and FL2- FL3 (Fig. 12B). Cells stained with mAbs specific for CD3, CD4 and CD8 (see Table 2) were then run through the cytometer and compensations were set to eliminate fluorescence overlap of two fluorochromes (Fig. 12C). The cytometer was then set to 'run' and data for all tubes were acquired and saved to disk.

Analysis of data involved the enumeration of monocytes, lymphocytes, T cells defined by the expression of CD3, and NK cells defined by $CD3^{-}CD56^{+}$ or $CD3^{-}CD161^{+}$ phenotypes. The expression of other NK receptors (NKRs) by non-T ($CD3^{-}$) and T ($CD3^{+}$) lymphocytes was further quantified.

2.7 Cell culture

Experimentation with human PBMCs poses safety problems associated with the risk of infection with human disease agents For this reason, all procedures were performed according to the guidelines set out for working in the Lymphocyte Biology Laboratory at NUI Maynooth These include wiping with 70% alcohol before and after the work, use of laboratory coats and safety gloves, hand washing

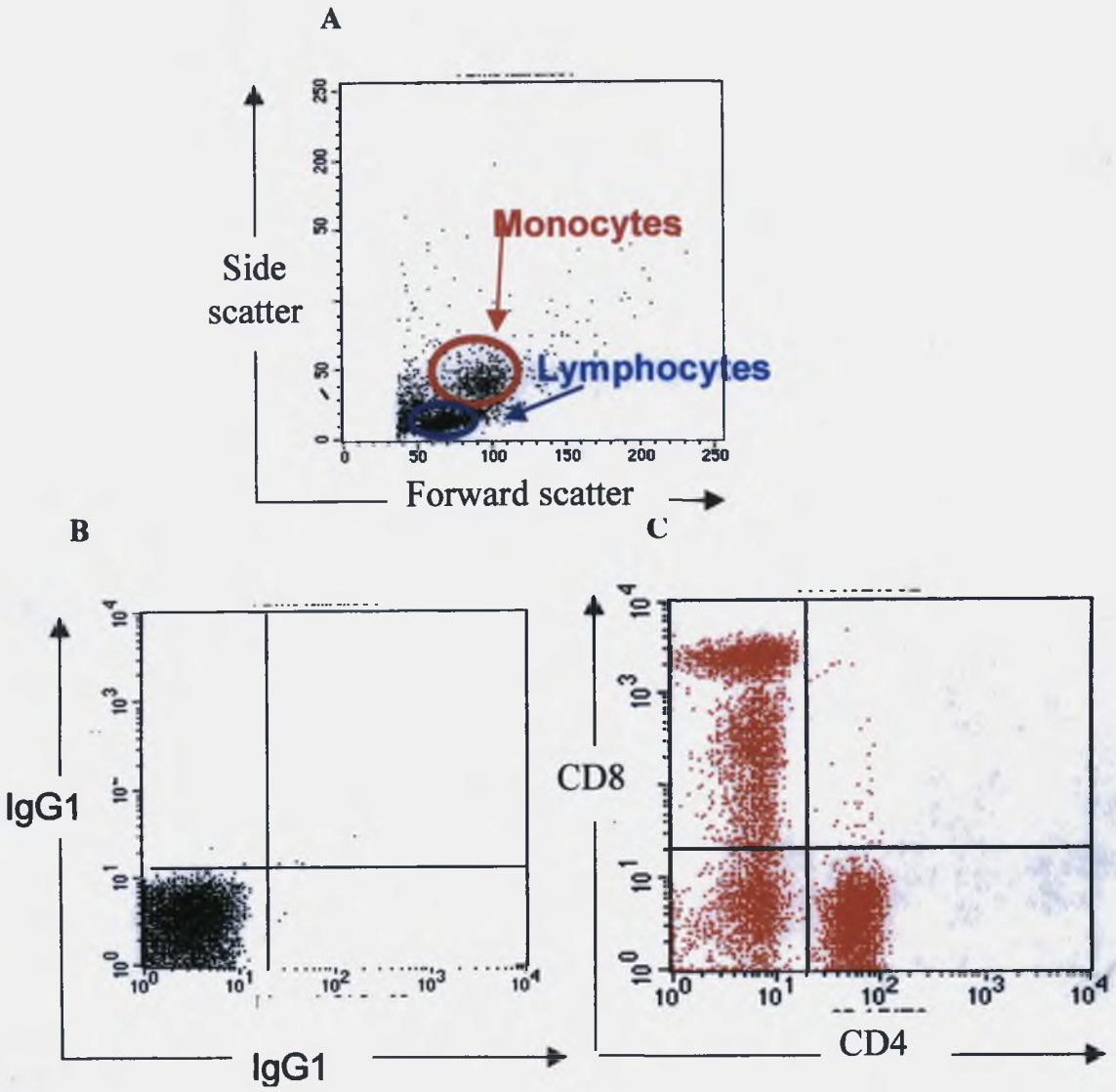

Fig. 12: Setting up the flow cytometer for 3-colour analysis of lymphocyte. (A) Flow cytometric dot plot showing forward scatter and side scatter properties of human peripheral blood monocytes and lymphocytes. (B) The level of staining seen with isotype matched control antibodies was used to set the fluoroscent detectors to the appropriate levels. (C) Cells stained with mAbs specific for CD3, CD4 and CD8 to set the compensations.

and daily decontamination of work surfaces. Cells were processed in a laminar

airflow cabinet. All the liquid wastes were discarded in Virkon at a concentration of 10mg/L of water. Solid waste (tips, transfer pipettes, gloves, 96 well plates, tubes and flasks) were autoclaved before discarding.

PBMCs were cultured for 1-2 days in complete RPMI medium in 75 cm^2 tissue culture flasks with loose lids in a Thermoforma CO_2 incubator at 37 °C and 5% CO_2. In some cultures recombinant human IL-2 was added at a concentration of 25 ng/ml.

K562 and Daudi cells were kept in continuous culture at 37 °C and 5% CO_2 in complete RPMI medium. Culture were diluted with medium every 3-4 days to maintain cells at densities of $<0.3 \times 10^6$ cells/ml.

2.8 Analysis of cytotoxicity

The human chronic myeloid leukemia derived cell line K562 is often used as a system to address scientific problems of hematological relevance. In the present study K562 cells were chosen because they are sensitive cytotoxicity by NK cells. Daudi cells, a human lymphoblastoid line, was also used as a target for cytotoxicity assays because these cells are resistant to natural cytotoxicity by NK cells, but NK cells can be induced to kill Daudi cells by activation with cytokines.

It is important that the target cells are viable and healthy on the day of cytotoxicity assays. This can be encouraged by feeding them with RPMI medium the previous day. All effector cells were cryopreserved before use so that they were treated under similar situation. An internal control containing frozen PBMCs from the same healthy individual was used in all experments to monitor day-to-day

variation in cytotoxic activity.

To avoid the risks associated with the use of radioactive materials, many safety procedures were taken in radioactive suite. Laboratory coat, lead jacket, radioactive dosimeter and two pairs of disposable gloves were worn at all the times. All work was carried out behind the lead-acrylic screen designated for ^{51}Cr use. All containers of radioactive materials were clearly labelled and all contaminated glassware were soaked in appropriate detergent (Decon 90%) and washed in running water. Contamination checks were carried out with the Mini-monitor and by taking wipes for counting in the liquid scintillation counter. Wipes were taken with a cotton bud wetted with 2% Decon, and placed in a liquid scintillation vial with scintillation fluid and counted. Any areas, which recorded above background, were cleaned thoroughly with detergent and water. Use and disposal forms were filled with details of amounts of waste generated. In addition, the suite user's logbook was filled in every time the radiation suite is used.
All solid waste was bagged with a tag including name, isotope and date. This was stored in large lead box in the radiation suite until no radiation could be detected using the Mini-monitor. It could then be disposed of with the ordinary waste. Liquid waste was collected in labelled Winchester waste bottles containing radioactive tape and stored underneath the fume hood until the activity is undetectable.

Target cells were labelled with 50 μCi ^{51}chromium (per ~0 $5x10^6$ cells). They were resuspended in 1 ml complete RPMI medium then incubated at 37 °C in a hybridisation oven in the radioactive suite for 2 hours in a lead box. The cells

were then pelleted by centrifugation at 110 g for 5 min. with the brake off The supernatant was removed using a wide-gauge pastette and discarding in the liquid waste container. To avoid cell damage that might lead to non-specific leakage of ^{51}Cr, cell pellets were not resuspended. 15 ml complete RPMI were added to and cells which were pelleted as above. This wash step was repeated three more times. Only after the final wash, cells were resuspended, this time in 1 ml complete RPMI

The densities of labelled cells were counted in a counting chamber designated for radioactive use only. Complete RPMI was added to bring the cell density to 13,333 or 26,666 cells/ml and 75µl of cells (1000 or 2000) were added to each well of a sufficient number of 96-well plates. The actual number of targets used per well is not critical but it is important that the same number of targets is placed in each well. Triplicate samples were analysed in all cases.

Effector cells were prepared by culturing PBMCs in the absence or presence of IL-2 as described in section 2 8. The cells were then washed and an aliquot stained in EB/AO for a viable cell count. Complete RPMI was added to bring the cell density so that 75µl can be added to each well to get effector/target (E/T) ratios of 5/1, 50/1 and 100/1 In some case insufficient cells were available so E/T ratios of 5/1, 10/1, 50/1 were used. 75µl effectors were aliquoted per well onto the ^{51}Cr-labelled targets. As controls for each target cell, triplicate samples containing equal numbers of target cells were included. In the first triplicate sample 75µl of complete RPMI was added instead of effector cells to determine spontaneous ^{51}Cr release. In the second, 75µl of 0.1% triton X-100 was added to determine maximum release.

Cells were concentrated at the bottoms of the wells by centrifugation of the plates at 110 g with the brake off and allowing them to cruise back to zero Cells were then incubated for 2 hours at 37 °C in lead box in a hybridisation oven to allow for target cell lysis to occur. After 2 hours, cells were again pelleted in the wells by centrifugation of the 96-well plates at 110 g for 5 min. with the brake off. 150 µl Optiphase supercocktail and 25 µl cell supernatant were added to each well in a wallac 96-well isoplate. Isoplates were covered with adhesive sheets and gently mixed for 15 minutes in hybridisation oven. The Isoplates were then attached to a Perkin Elmer plate cassette and placed in the Wallac Microbeta plate counter, which automatically counts the amounts of ^{51}Cr in the supernatents of each well .

Specific lysis was calculated as (cpm of sample- cpm of spontaneous release)/(cpm of maximum release- cpm of spontaneous release) x100 %.

2.9 Statistical analysis

All data on the patients and controls were entered into a computerised data base using Microsoft Excel. Minitab was used to analyse data. Cell numbers were expressed as median percentages ± standard errors. Cytolytic activities were expressed as means of triplicate samples and as means of mean specific activities within subject populations. The Mann Whitny U test for non-parametric data was used to compare median cell numbers and mean cytolytic activities. P values of <0.05 were taken as significant. Spearman Rank analysis was used for correlation studies.

Chapter 3: Results

3.1 A variety of NKRs are expressed by human T cells and non-T lymphocytes

PBMCs from 33 patients with chronic HCV disease, 22 patients who spontaneously cleared HCV infection (HCV resolvers) and 26 healthy control individuals were stained with mAbs specific for CD3 and either CD16, CD56, CD161, KIR2DL1 or KIR3DL1/S1 and analysed by flow cytometry. Representative dot plots obtained are shown in Figure 13. The results indicate that different NKRs are differentially expressed by T cells and non-T cells and that multiple NK cells subsets ($CD3^-$ NKR^+) and NKR^+ T cell subsets ($CD3^+$ NKR^+) can be defined phenotypically.

3.2 Circulating NK cell numbers are depleted in patients with chronic HCV disease

The percentages of lymphocytes expressing $CD3^-CD56^+$ phenotypes were significantly reduced in patients with chronic HCV disease compared to controls (median= 3.4 ± 0.8 vs 13.7 ± 1.3, $P<0.001$, Fig 14A). NK cells defined by the $CD3^-CD161^+$ phenotype were also decreased in chronic HCV group compared to controls (median=6.6 vs 15.5; $p<0.001$, Fig. 14B). The percentages of lymphocytes bearing $CD3^-CD56^+$ and $CD3^-CD161^+$ phenotypes were also lower in HCV resolvers group compared to controls (median=6.1 vs 13.7; p=0.0002 for CD56, 6.7 vs 15.5; p=0.0001 for CD161), but no significant differences were seen in the frequencies of expression of $CD3^-CD56^+$ and $CD3^-CD161^+$ phenotypes between chronic HCV and HCV resolver groups.

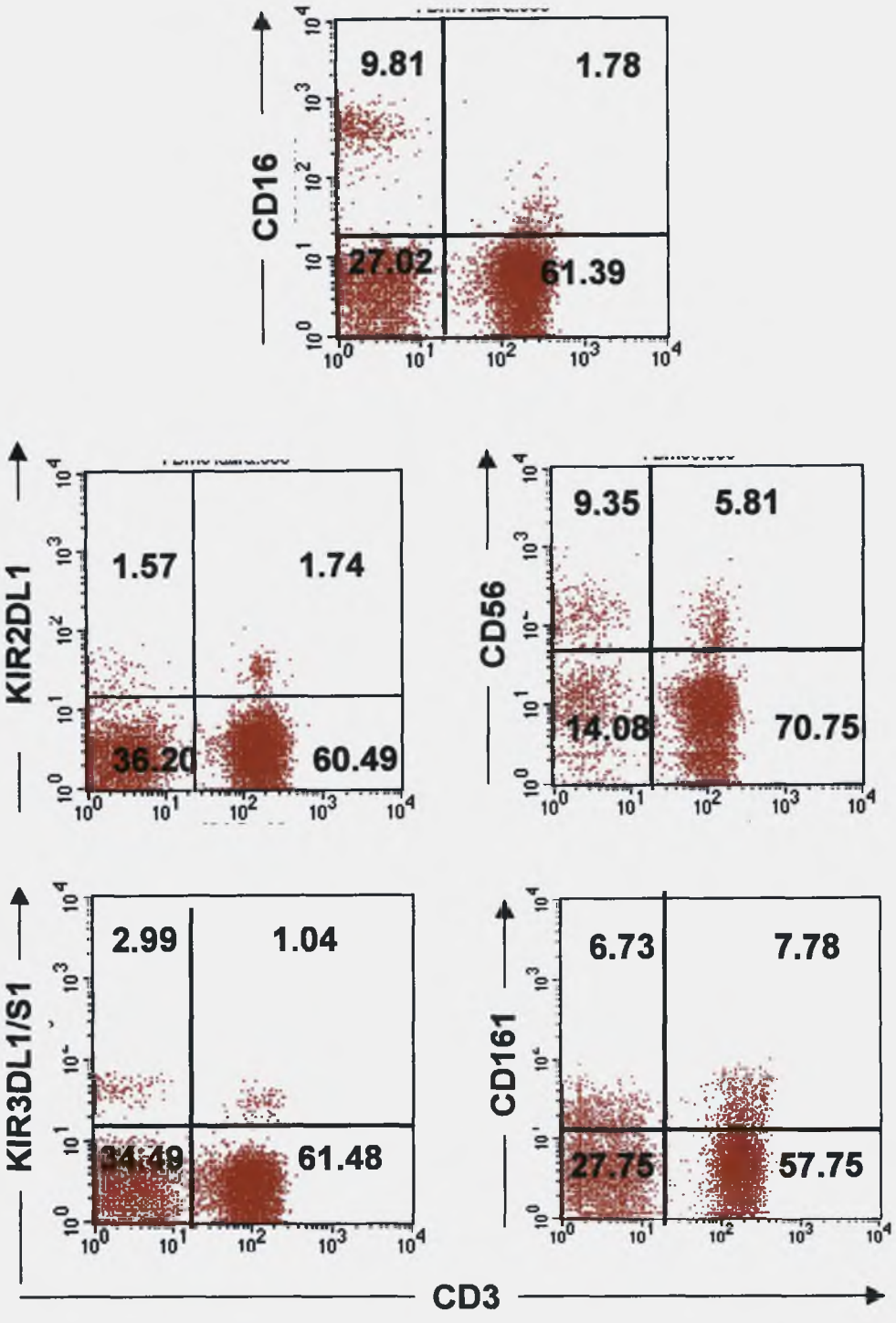

Fig.13: Flow cytometric analysis of CD16, CD56, CD161, KIR2DL1 and KIR3DL1/S1 by T cells ($CD3^+$) and non-T lymphocytes ($CD3^-$) in the preipheral blood of a healthy individual after gating on lymphocytes.
The number in the quadrants indicate the percentages of lymphocytes that are negative (bottom left), single positive (upper left and lower right) and double positive (upper right) for each pair of receptors.

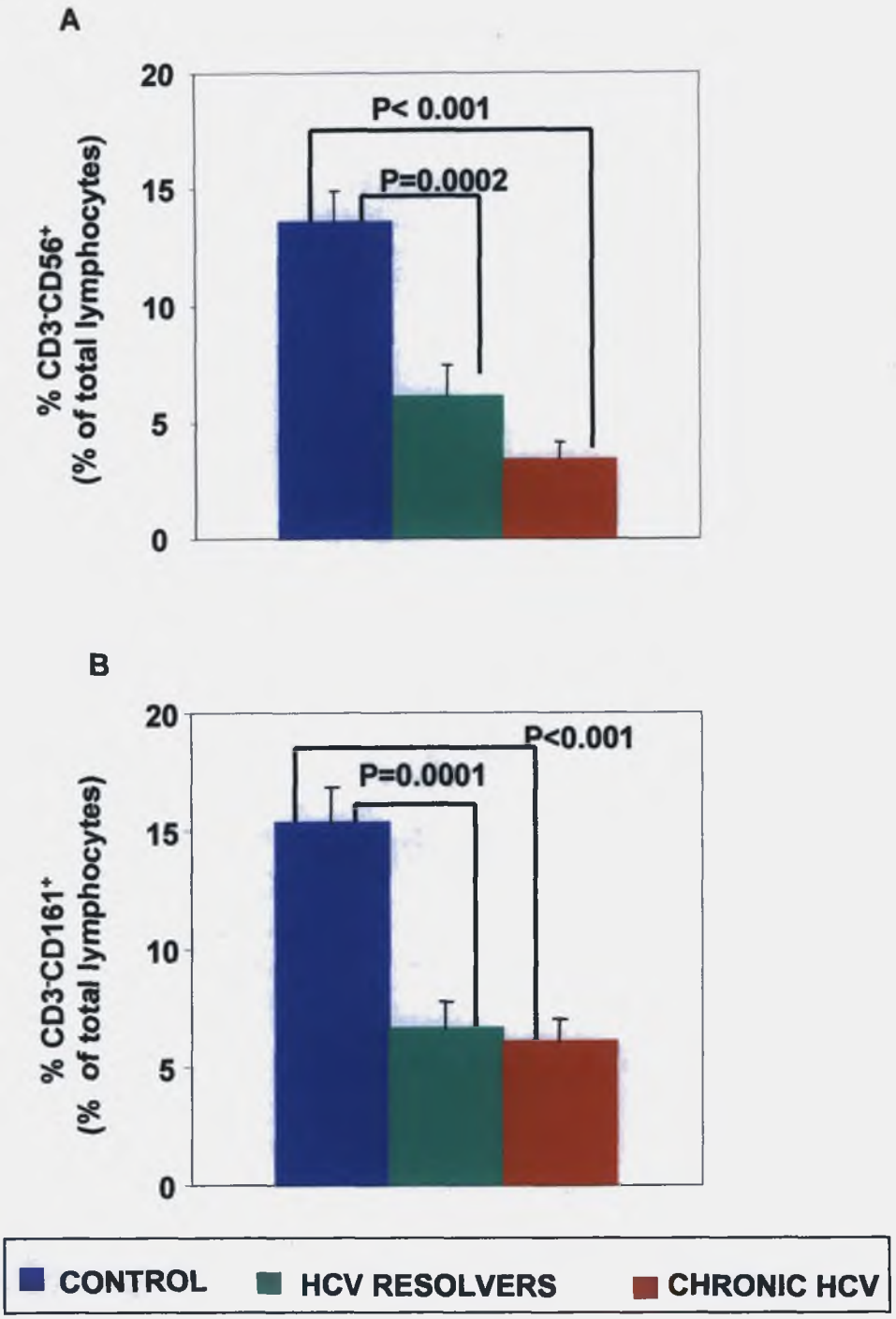

Fig. 14: Circulating NK cell numbers are depleted in patients with chronic HCV disease. Median percentage of total lymphocytes expressing $CD3^-CD56^+$ (A) and $CD3^-CD161^+$ (B) phenotypes in 33 patients with chronic HCV disease, 22 HCV resolvers and 26 healthy controls. Error bars show stander errors.

3.3 Circulating NKR$^+$ T cell numbers are depleted in patients with chronic HCV disease

The proportion of lymphocytes that coexpress CD3 and either CD56 or CD161 were also found to be depleted in patients with chronic HCV disease. Figure 15A shows that $CD3^+CD56^+$ cells accounted for median of 1.3% of lymphocytes in patients with chronic HCV disease, compared with 3.7% in healthy controls (P=0.0001) and 1.5% of HCV resolvers (P not significant when compared with chronic HCV, but there was a significantly decreased when compared to healthy control; p<0.001). Similarly $CD3^+CD161^+$ lymphocytes were depleted in patients with chronic HCV disease (5.6%) compared with controls (11.5%, P<0.001; Fig. 15B). Numbers of $CD3^+CD161^+$ lymphocytes were also decreased in HCV resolvers (5.3%) compared to controls (p<0.001), but these was not significantly different when comparing to the chronic HCV group.

3.4 NK cell phenotypes in patients and controls

Since different NKRs are differentially expressed by NK cells (Fig. 13), the expression of CD56, CD161, CD16, KIR2DL1 and KIR3DL1/S1 by circulating $CD3^-$ negative lymphocytes in chronically HCV-infected patients (n=33), HCV resolvers (n=22) and healthy controls (n=26) were compared. Figure 16 shows that the frequency of expression of CD161 and CD16 by non-T cells is reduced in patients with chronic HCV infection compared with controls (medians = 17.3% vs 32.7 for CD161; p=0.0004 and 13.9% vs 27.1% for CD16; p=0.038; Fig 16). Expression of CD161 in HCV resolvers was significantly lower when compared to healthy controls (medians=21.1 vs 32.7, p=0.0036), but there were not any

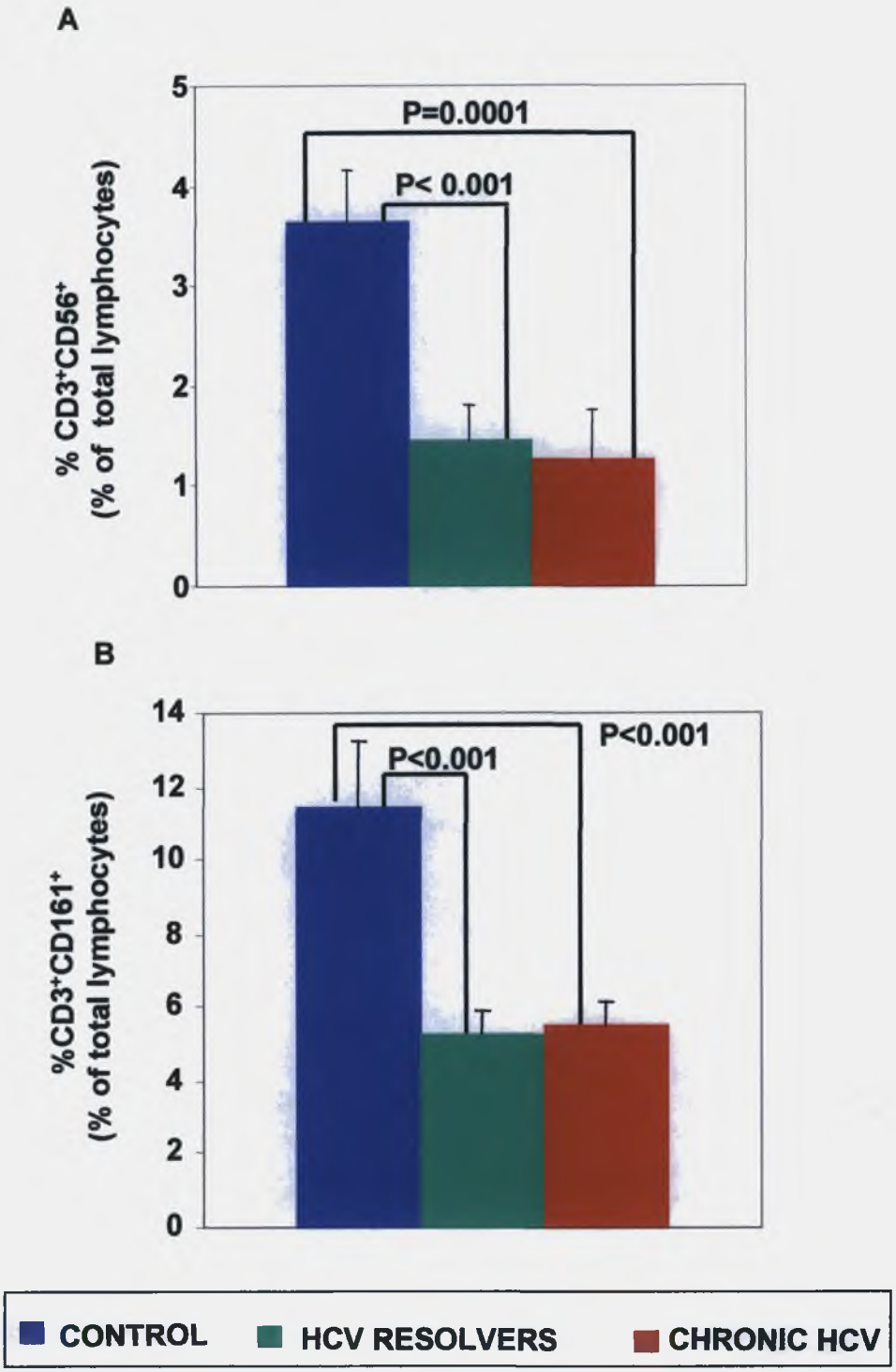

Fig.15: Circulating NKR$^+$ T cells are depleted in chronic HCV disease. Median percentage of total lymphocytes expressing CD3$^+$CD56$^+$ (A) and CD3$^+$CD161$^+$ (B) phenotypes in 33 patients with chronic HCV disease, 22 HCV resolvers and 26 healthy controls. Erorr bars show standard errors.

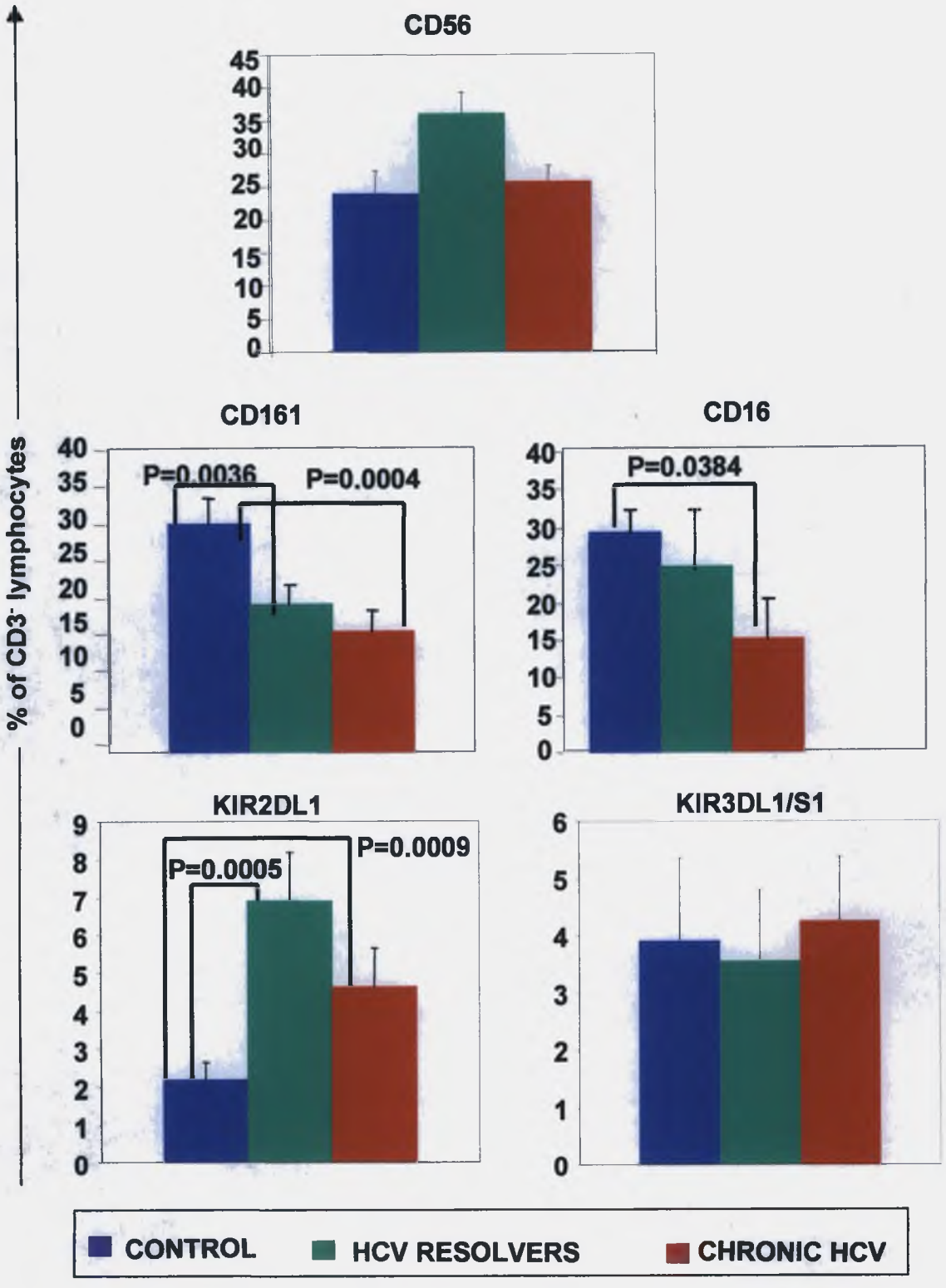

Fig. 16: Expression of CD56, CD16, CD161, KIR2DL1 and KIR3DL1/S1 by circulating $CD3^-$ lymphocytes in healthy controls (n=26), patients with chronic HCV disease (n=33) and HCV resolvers (n=22). Data show median percentages and standard errors.

differences in expression of CD16 from both groups Expression of CD16 by NK cells was slightly lower in chronic HCV compared to HCV resolvers, but this was not significant. KIR2DL1 was more frequently expressed by NK cells in patients with chronic HCV infection (4.6%) and in HCV resolvers (6.9%) compared to controls (2.2%, p=0.0009 and 0.0005 respectively; Fig 16). The frequencies of non-T cells expressing CD56 and KIR3DL1/S1 were similar in all subject groups (Fig. 16). These results indicate that, while overall NK cell numbers are depleted in patients with chromc HCV disease, changes m the distributions of NK phenotypic variants of NK cells are also seen.

3.5 NKR$^+$ T cell phenotypes in patients and controls

The expression of CD56, CD161, CD16, KIR2DL1, and KIR3DL1/S1 by T lymphocytes in chronically infected patients, HCV resolvers and healthy controls groups was also compared. Figure 17 shows that the frequencies of T cells that express CD56 and CD161 were decreased in patients with chronic HCV disease compared to controls (3.8% vs 6.7% for CD56; p=0.0007; 9.6% vs 13.1% for CD161; p=0.0047). They were also decreased in HCV resolvers group compared to controls (3.8 vs 6.7; p=0.0076 for CD56, 8.3 vs 13.1; p=0.0015 for CD161), but were similar in the HCV resolvers and chronic HCV groups.

The frequences of T cells expressing KIR2DL1 were increased in patients with chronic HCV disease compared to healthy controls (0.43% vs 0.21%; p=0.0009, Fig 17). The frequences of T cells expressing KIR2DL1 were similar in HCV resolvers (0.33) and chronic HCV. CD16 and KIR3DL1/S1 were found on similar proportion of T cells in all subject groups (Fig. 17).

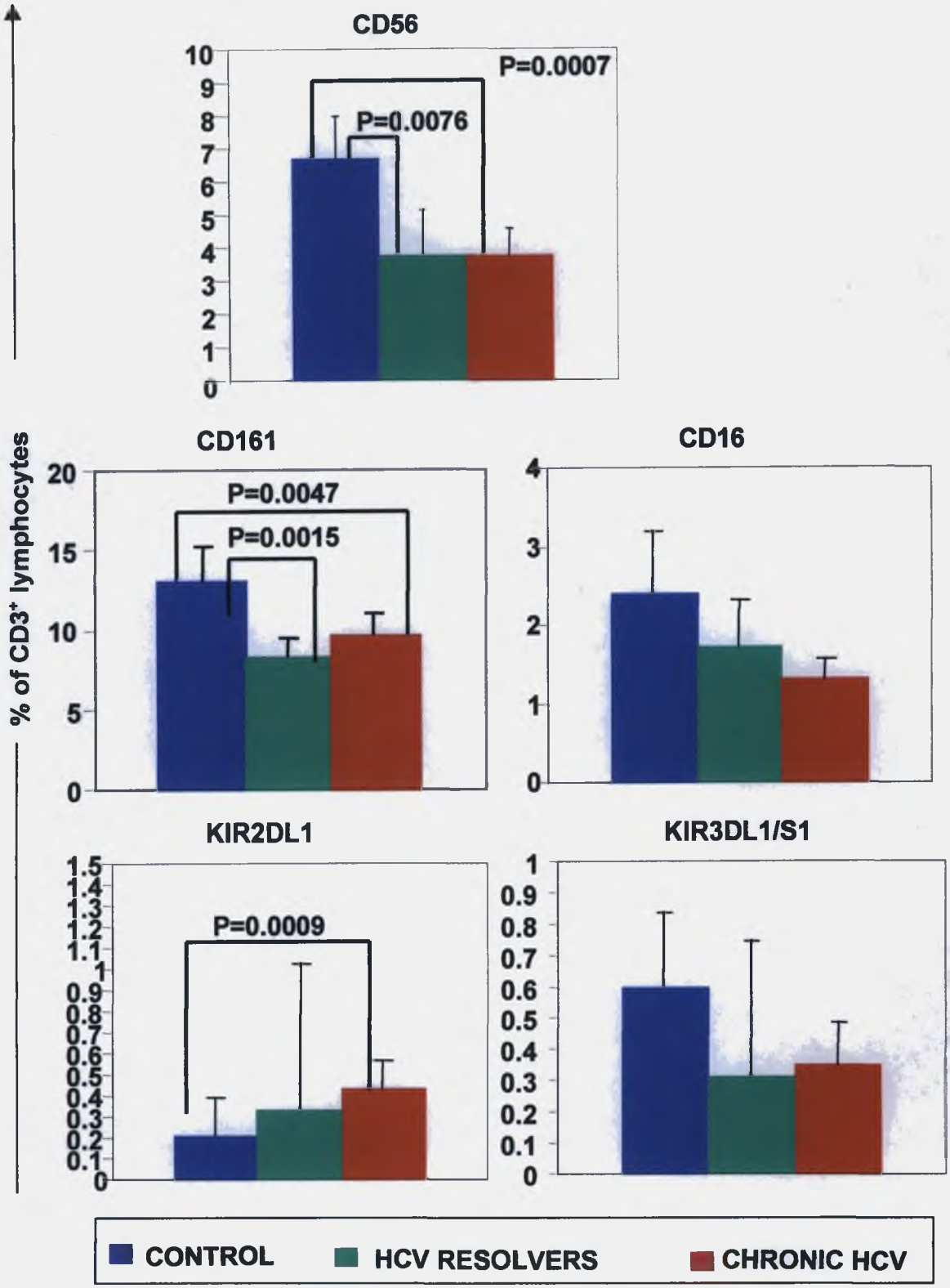

Fig. 17: Expression of CD56, CD16, CD161, KIR2DL1 and KIR3DL1/S1 by circulating $CD3^+$ lymphocytes in healthy controls (n=26), patients with chronic HCV disease (n=33) and HCV resolvers (n=22). Data show median percentages and standard errors.

3.6 Natural cytotoxicity by PBMCs is impaired in patients with chronic HCV infection

NK cells are capable of killing a range of tumour target cells lines. We compared the natural cytotoxic activities of PBMCs from 33 patients with chronic HCV disease, 22 HCV resolvers and 26 healthy controls against K562 (Fig. 18) and Daudi (Fig. 19) targets. In these experiments, an internal PBMCs standard, which had been cryopreserved in a similar fashion to the test effector PBMCs was included to monitor for day-to-day variations in background ^{51}chromium release. Figure 18A shows that the percent specific lysis of K562 cells was higher at E/T ratios of 50/1 compared with 5/1, but was consistently lower at E/T ratios of 100/1, suggesting the presence of an inhibitor that is active at E/T ratios of 100/1. Comparsion of the specific lysis obtained with PBMCs from the three subject groups at E/T ratios of 50:1 (Fig. 18B) showed significantly reduced natural cytotoxicity in the chronically infected patients (1.8%) compared to HCV resolvers (7.4%; $p=0.0012$) and controls (10.2%; $p<0.001$). There was no significant difference in comparing HCV resolvers group to the controls. When Daudi was used as the target cell line in similar experiments, no natural cytotoxicity was observed at all E/T ratios tested in the HCV resolvers and chronic HCV groups. However, natural cytotoxicity against Daudi cells was detected in the PBMCs of 8 out of 26 healthy volunteers giving a mean specific activity of 3.3 at an E/T ratio of 50:1 (Fig. 19). No correlation was seen between NK cell phenotype and natural cytotoxicity against Daudi cells as shown for CD16 in figure 20.

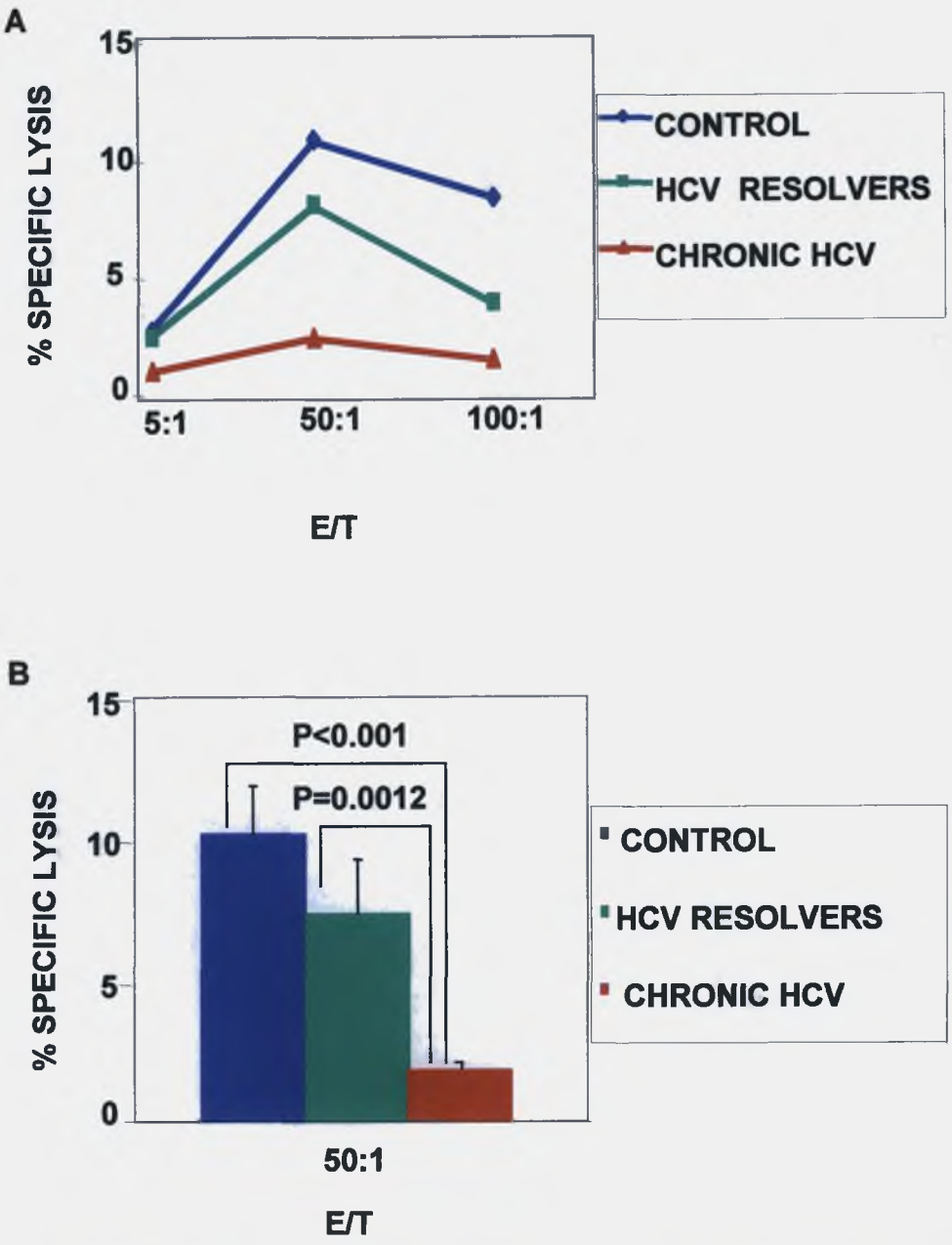

Fig. 18: Natural cytotoxicity against K562 targets is impaired in chronic HCV infection. A, mean cytolytic activities of total PBMCs from 33 patients with chronic HCV disease, 22 HCV resolvers and 26 controls against K562 targets at effector/target (E/T) ratios of 5:1, 50:1 and 100:1. B, comparison of mean specific lysis at E/T ratios of 50:1 between subject groups.

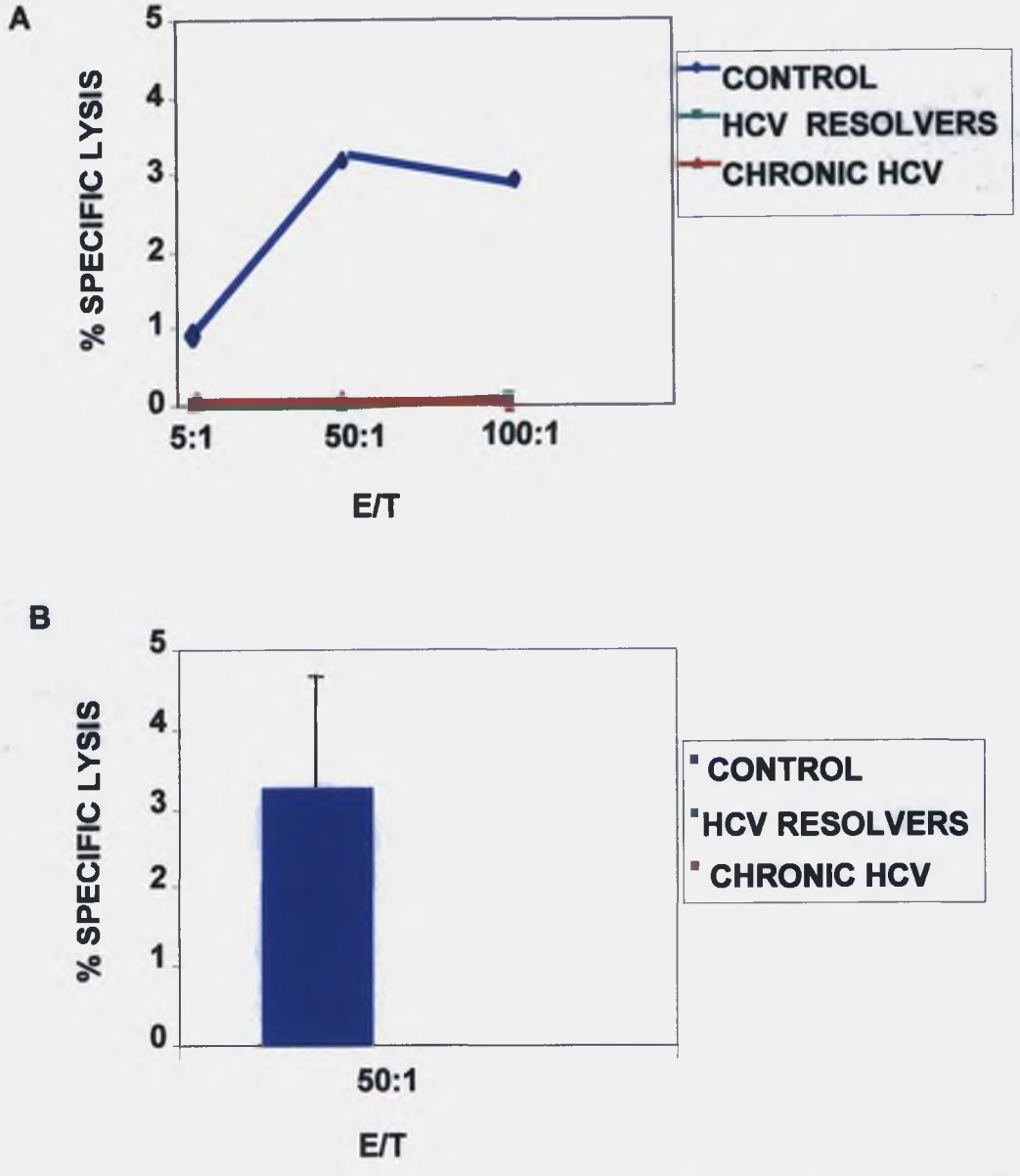

Fig. 19: Natural cytotoxicity against Daudi targets is impaired in chronic HCV infection. A,mean cytolytic activities of total PBMCs from 33 patients with chronic HCV disease,22 HCV resolvers and 26 controls against Daudi targets at effector/target (E/T) ratios of 5:1, 50:1 and 100:1. B, comparison of mean specific lysis at E/T ratios of 50:1 between subject groups.

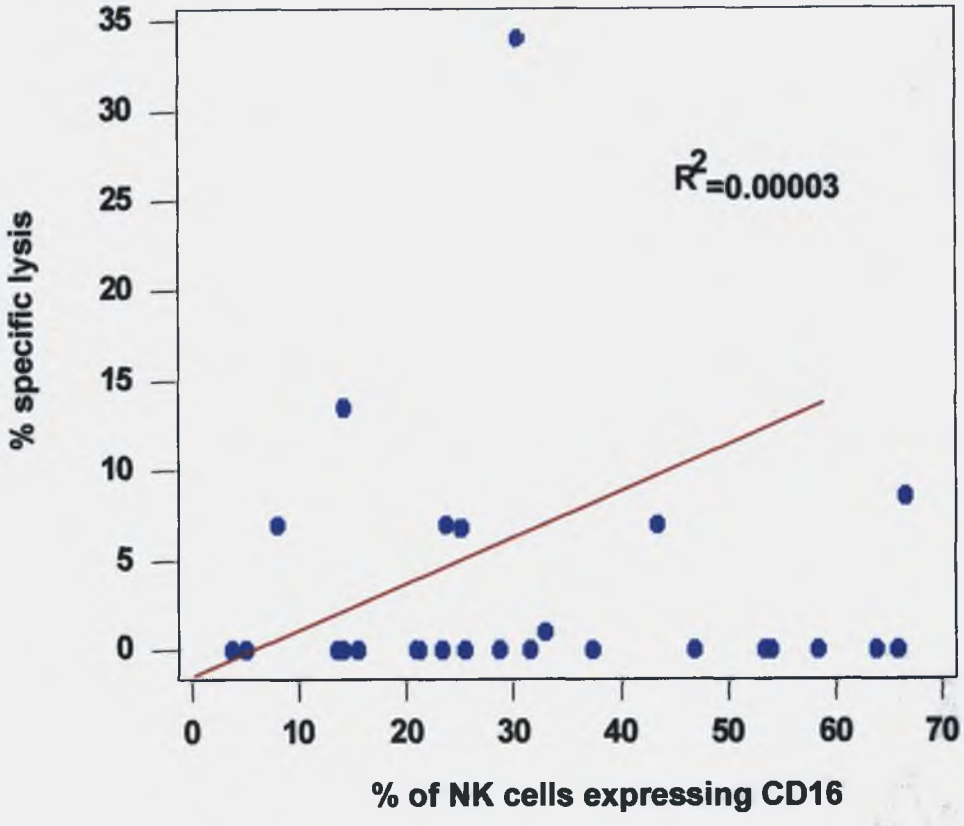

Fig. 20: Natural cytotoxicity of Daudi targets does not correlate with NK cell phenotype. The expression of CD16 by NK cells in 26 healthy donors is plotted against % specific lysis of Daudi cells.

3.7 Lymphokine-activated killing (LAK) by PBMCs is impaired in patients with chronic HCV infection

Cytotoxic activity of PBMCs from chronically HCV-infected patients, HCV resolvers and healthy controls against K562 and Daudi targets was also tested after incubating the PBMCs effectors for 1-2 days with 25 ng/ml IL-2. IL-2 activation led to increased cytolysis against both targets. Figure 21A and 22A show the LAK activity of PBMCs against K562 and Daudi at E/T ratios of 5/1, 50/1 and 100/1. Comparing groups of subjects at E/T ratios of 50:1, LAK activity against K562 was significantly reduced in chronic HCV disease (2.5%) compared with HCV resolvers (7.55%; $p=0.0001$) and controls (12.6%; $p<0.001$, Fig. 21B). There was no significant difference in comparing HCV resolvers group with the healthy controls. LAK activity against Daudi targets was similarly reduced in chronically-infected patients (0%) compared with HCV resolvers (2.5%, $p<0.001$) and controls (8.1%; $p<0.001$; Fig.22 B). A significant difference was also seen when HCV resolvers and controls were compared ($p=0.0074$).

3.8 Impaired cytotoxic activity of PBMCs in chronic HCV infection is not solely due to decreased NK cell numbers or NKR^+ T cells numbers

The present study has demonstrated that NK and NKR^+ T cell numbers are depleted in chronic HCV disease and that natural cytotoxicity and LAK activity are also impaired. To determine if impairment in cytotoxicity is merely due to low NK numbers we performed Rank Spearman analysis of NK cell numbers and % specific lysis at E/T of 50/1 (Fig. 23). The results indicate that impaired cytotoxicity in chronic HCV infection is not solely due to the decrease in NK cell

numbers.This impairment in cytotoxic function is also not solely due to decreased numbers of total lymphocytes (both NK cells and T cells) expressing CD56 (Fig. 24).

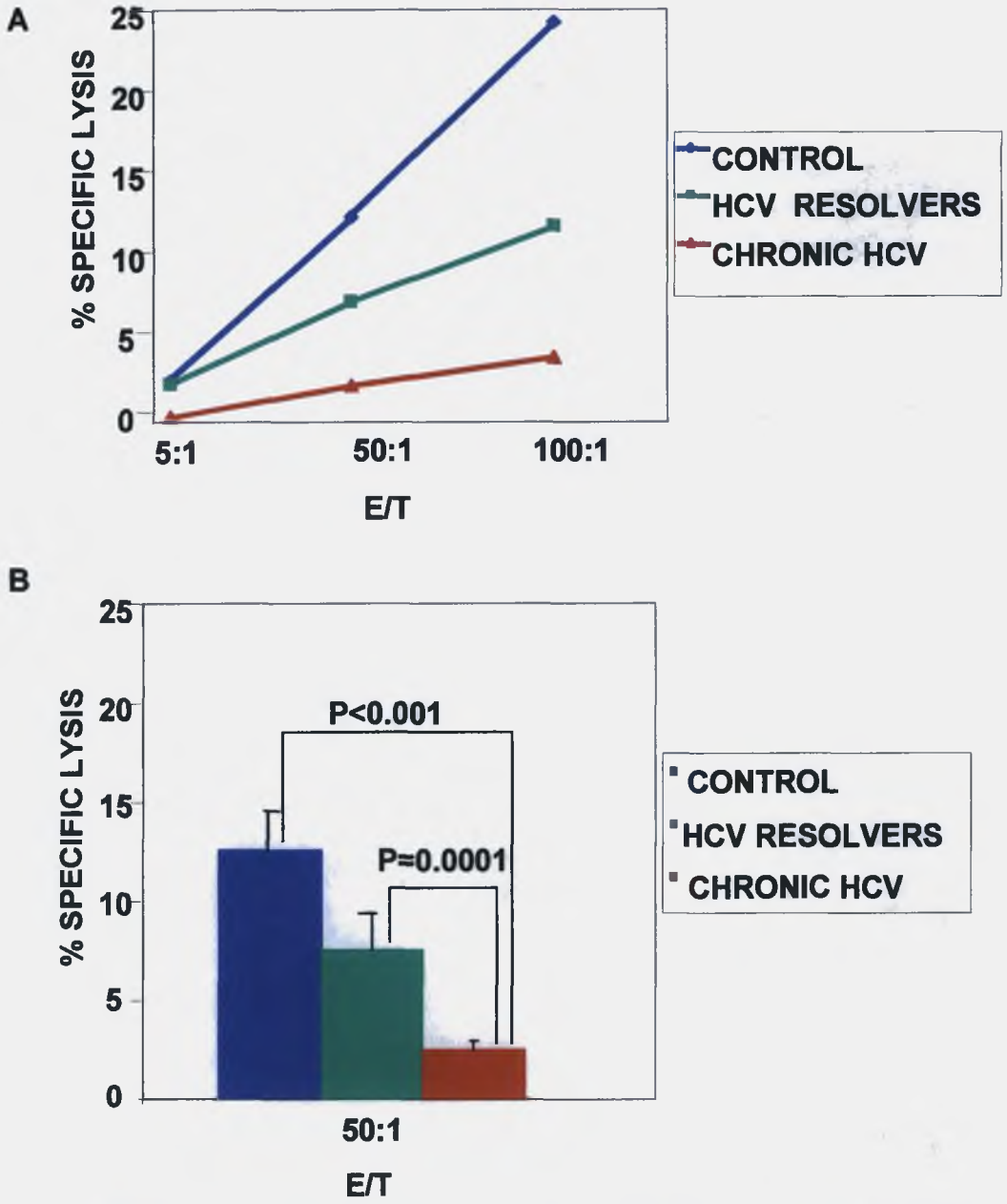

Fig. 21: Lymphokine activated killing of K562 target is impaired in chronic HCV disease. A, mean cytolytic activities of total PBMCs from 33 patients with chronic HCV disease, 22 HCV resolvers and 26 controls against K562 targets at effector/target (E/T) ratios of 5:1, 50:1 and 100:1. B, comparison of mean specific lysis at E/T ratios of 50:1 between subject groups.

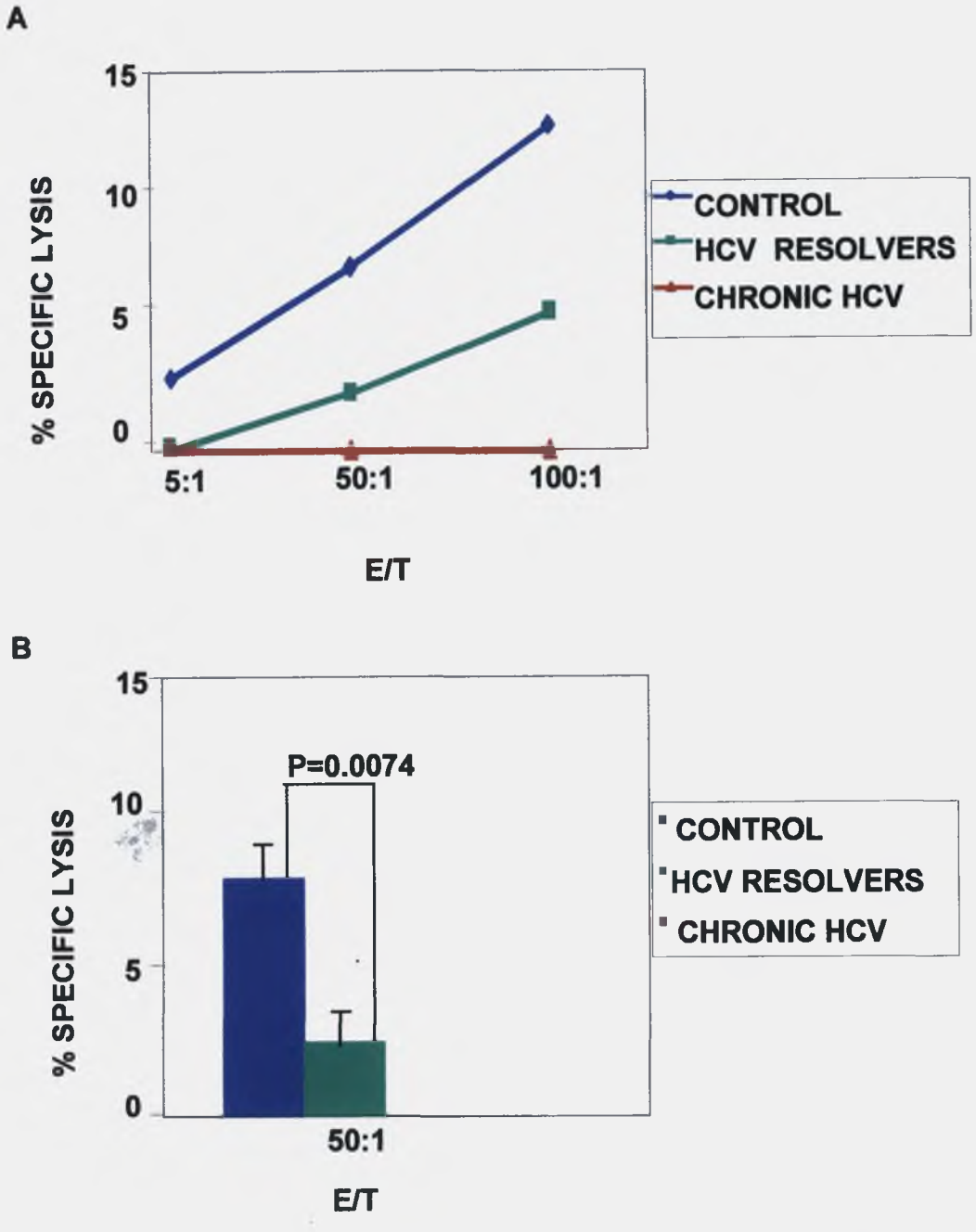

Fig. 22: Lymphokine activated killing of Daudi target is impaired in chronic HCV disease. A, mean cytolytic activities of total PBMCs from 33 patients with chronic HCV disease, 22 HCV resolvers and 26 controls against Daudi targets at effector/target (E/T) ratios of 5:1, 50:1 and 100:1. B, comparison of mean specific lysis at E/T ratios of 50:1 between subject groups.

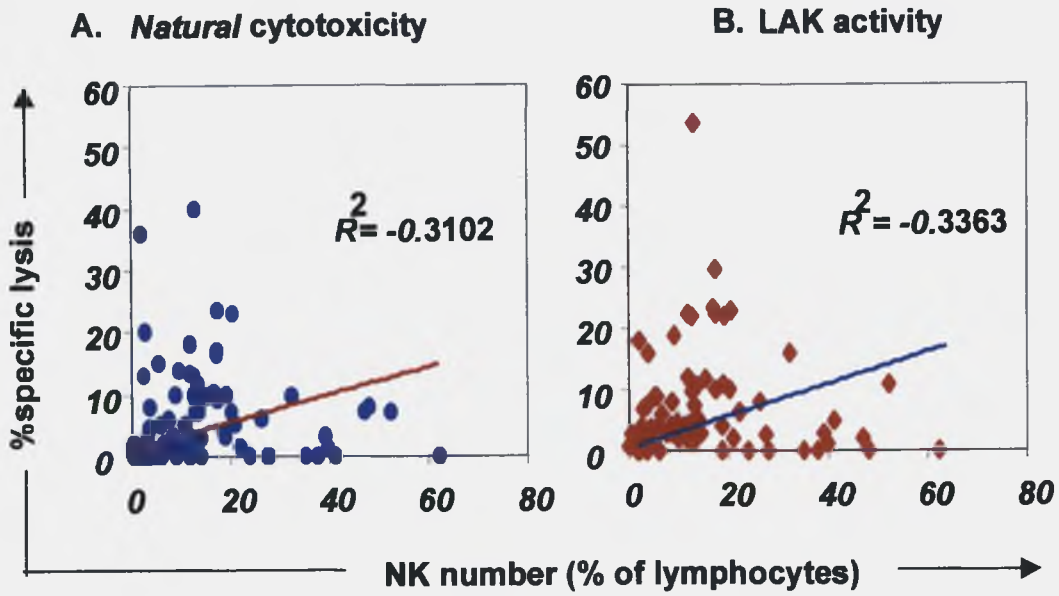

Fig. 23: Reduced natural cytotoxicity and LAK activity in chronic HCV disease is not solely due to depletion of NK cell numbers. Plot of median NK number (% of lymphocytes) against mean natural cytotoxicity (A) and LAK activity (B) at E/T ratios of 50/1 for all individuals.

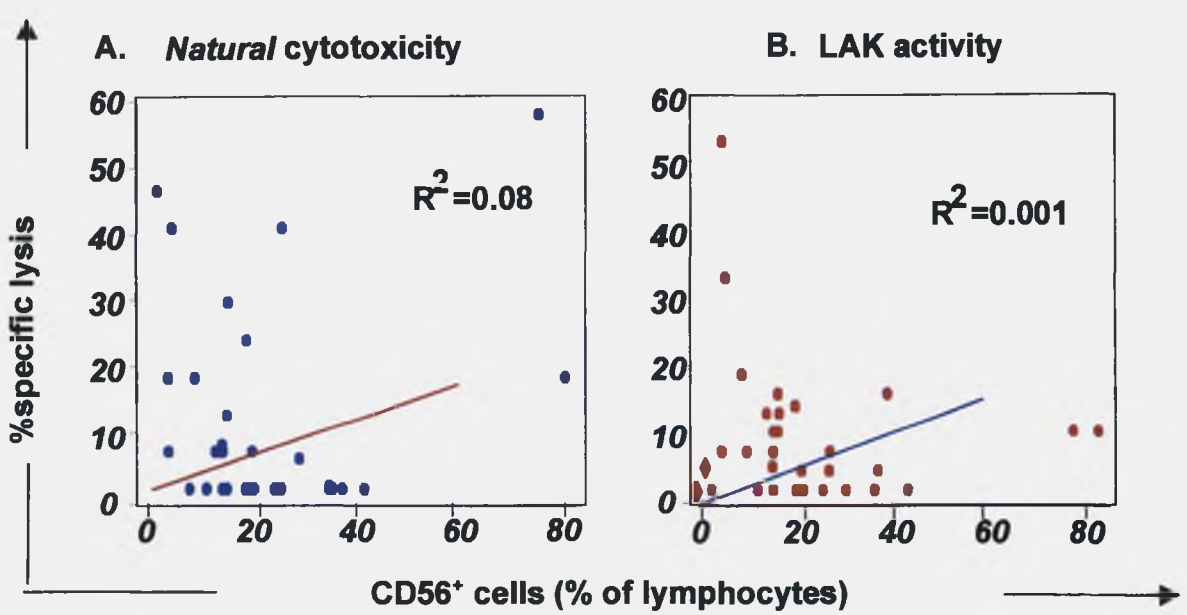

Fig. 24: Reduced natural cytotoxicity and LAK activity in chronic HCV disease is not solely due to depletions of total NK and NKR⁺ T cell numbers. Plot of median CD56⁺ cell (NK and T cell) number (% of lymphocytes) against mean natural cytotoxicity (A) and LAK activity (B) at E/T ratios of 50/1 for all individuals.

Chapter4: Discussion

NK cells are thought to play an important role in immunity against viruses. They can recognise virus infected cells and release IFN-γ, which not only inhibits virus replication but also can activate other cells involved in cell-mediated immunity (Biron *et al* 1999, Trinchieri 1995). NK cells also mediate cytolysis, which plays an important role in host antiviral defence mechanisms (see Chapter 1).

NK cell deficiency states are associated with a range of diseases and susceptibilities to infections. Current evidence suggests that a decreased NK cell number or cytotoxic activity is often associated with the development and/or progression of cancer, acute or chronic viral infection, autoimmune diseases, immune deficiency syndromes and psychiatric illness (Whiteside and Herberman 1994, Shibatomi *et al* 2001, Wilson *et al* 1994, Jovic *et al* 2001). Low NK cell function has been linked to increased susceptibility to Epstein-Barr virus, human cytomegalovirus, and human immune deficiency virus (HIV) (Ching and Lopez 1979, Merino *et al* 1986, Biron *et al* 1999). However, the role of NK cells in immunity against HCV has not been fully explored. NK cell function has been shown to be significantly lower in patients with chronic HCV infection, compared with healthy controls (Lirussi *et al* 2002, Corado *et al* 1997, Par *et al* 2002), however this observation has not been confirmed in another study (Duesberg *et al* 2001). In the present study, we evaluated the NK cell numbers, phenotypes, natural cytotoxic activities and LAK activities in PBMCs of HCV infected patients, HCV resolvers and healthy controls groups. Our results show that a variety of NKRs are expressed by human T cells and non-T lymphocytes. Both NK cells and NKR^+ T cells numbers are depleted in the blood of chronic HCV patients. In addition to decreases in numbers of these cells, we found that NK cells

in patients with chronic HCV infection have altered phenotypes. Expression of CD161 and CD16 by NK cells was found less frequently in patients with chronic HCV compared to healthy controls. In contrast, KIR2DL1 was more frequently expressed by NK cells in chronic HCV patients. Expression of KIR3DLI/S1 and CD56 was similar in all study groups. The frequencies of T cells expressing CD161 and CD56 were also decreased in chronic HCV patients compared to controls. In contrast the frequencies of T cells expressing KIR3DL1/S1 and CD16 were found to be similar in all study groups and the frequencies of T cells expressing KIR2DL1 were increased in chronic HCV patients compared to controls.

We also found that natural cytotoxicity and LAK activity by PBMCs against K562 and Daudi target cells are impaired in patients with chronic HCV infection compared to HCV resolvers and controls This impaired activity is not solely due to the decreases in NK cell numbers or NKR^+ T cells numbers.

Our results are different from those of Duesberg *et al* (2001) who found that natural cytotoxicity, LAK activity and ADCC were not impaired in a cohort of 29 patients with chronic HCV disease compared to controls. These differences may relate to differences in target cells used (HT 29 and Raji) or methodologies (we used the chromium release assay but Duesberg and coworkers used a flow cytometric assay for the assessment of cytotoxicity). Alternatively the different results may be due to differences in the patients. While Duesberg and coworkers studied patients infected with genotypes 1a, 1b, 2a/2c and 3a, we studied patients infected with genotypes 1 and 3.

We were surprised to find that the frequencies of circulating NK cells and NKR^+ T cells were significantly lower in the blood of patients who spontaneously resolved HCV compared to controls. This suggests that the virus may be responsible for depleting these cell numbers and that they do not expand after elimination of the virus, rather than reduced NK and NKR^+ T cell numbers predisposing individuals to chronic HCV infection. We can not exclude the possibility that lower NK and NKR^+ T cell counts in the chronic HCV and HCV resolver groups are due to differences in cell preparation and storage, since PBMCs from HCV patients and resolvers were prepared at St. Vincent's University Hospital while PBMCs from controls were prepared at NUI Maynooth. However, identical methods and reagents were used at both centres and NK cell and NKR^+ T cell numbers were similar in PBMCs prepared from healthy control subjects prepared at the two laboratories (data not shown).

The reduction in cytotoxic activity in patients with chronic HCV infection may be a factor that underlies susceptibility to chronic HCV disease, the result of viral infection of NK cells and NKR^+ T cells, or the induction of factors that might alter NK cell function. Impaired NK cytotoxic activity in chronic HCV groups and HCV resolvers may be attributed to the effect of suppressive cells and/or factors present in PBMCs of infected patients. Corado *et al* (1997) used PBMCs depleted of monocytes and B cells and purified NK cells as effectors in cytotoxicity assays.

They found that in spite of the depletion of monocytes and B cells, NK cytotoxic activity was still significantly lower compared with that of healthy controls.

Therefore, monocytes and B cells or their soluble factors do not seem to be implicated in the reduced NK activity. Also they found that short term *in vitro* stimulation with IL-2 partially restores NK cell cytotoxic activity in chronic HCV. HCV might impair NK cell activity by reducing the amount of IL-2 produced, a cytokine that stimulates NK cell response (Corado *et al* 1997).

The possibility that HCV might alter NK cell function has also been suggested in experiments in which PBMCs from healthy individuals were exposed to virus-containing serum obtained from HCV infected individuals. This resulted in the inhibition of NK mediated cytotoxicity (Corado *et al.* 1997). The same group found that impairment of NK cytotoxic activity was specific to HCV infection because the cytotoxic function of PBMCs incubated with serum from patients with hepatitis A virus (HAV) or hepatitis B virus (HBV) infections or autoimmune hepatitis was comparable to that of PBMCs incubated with serum from healthy controls.

It has been shown that the major HCV envelope protein E2 can inhibit, via cross linking CD81, NK cell production of IFN-γ, non-MHC restricted cytotoxic activity, activation marker expression, cytotoxic granule release and proliferation (Tseng and Klimpel 2002, Crotta *et al.* 2001). Thus, cross linking of CD81 on NK cells results in inhibition of NK cell function. In contrast, cross linking of CD81 on T cells or B cells result in costimulation. These different outcomes of cross linking CD81 on NK cells and other cells due to the activation of distinct signalling pathways (Crotta *et al* 2001). This finding provides a mechanism by which HCV might interfere with NK responses at the early stages of infection resulting in the generation of a persistent infection.

HCV may also impair NK functions at the level of DC activation. DCs augment effector functions of NK cells, but the mechanisms are not fully understood. Jinushi *et al* (2003) show that human monocyte derived DCs enhance IFN-γ production, CD69 expression, and K562 cytolytic ability of NK cells when DCs are prestimulated with various maturation stimuli such as IFN-α or lipopolysaccharide (LPS). They found that NK cell activation mediated by LPS-stimulated DCs was dependent on IL-2, but that IFN-α stimulated DC mediated activation was not. IFN-α was found to induce the expression of the MHC class I related chains, MICA and MICB, which are ligands for the stimulatory receptor NKG2D found on NK cells. IFN-α-mediated activation of NK cells was dependent on the MICA/B-NKG2D interaction and could be blocked using mAbs against MICA/B or by inhibition of direct cell to cell contact using transwell plate systems. DCs recovered from chronic HCV infected patients showed defects in the induction of MICA/B and impaired ability to activate NK cells in response to IFN-α stimulation. These findings suggest that MICA/B induction on DCs may be one of the mechanisms by which IFN-α activates NK cells; this impairment might affect IFN-α responsiveness in HCV infection.

The results of the present study could have important implications for our understanding of host immune response to HCV. Depleted NK function and insufficient production of IFN-γ by NK cells could alter the development of Th1 responses and favor the development of enhanced Th2 responses Interestingly, an imbalance between Th1 versus Th2 responses has recently been described in chronic HCV infected individuals (Sarih *et al* 2000, Valiante *et al* 2000). Sarih *et*

al (2000) investigated the levels of Th1 cytokines (IL-2, IL-12, IFN-γ) and Th2 cytokines (IL-10, IL-4) and their response in peripheral blood from HCV infected patients. They found that IFN-γ and IL-12 levels were lower in HCV infected subjects as compared to controls They also found no difference in IL-2 and IL-10 production. In contrast, Deignan *et al.* (2002) found that IFN-γ level was significantly increased in liver of patients with HCV infection. Golden-Mason *et al* (2003) also found that IL-18, IFN-γ and IL-10 levels were significantly increased in the livers of patients with HCV infection.

There is evidence that the NK cell mediated and CTL mediated cytotoxic systems regulate the functions of each other (Kos and Edgar 1996). The existence of a dynamic balance between NK cell and CTL systems may be essential to host defenses. If CTL precursors are not able to recognize target cells due to their expression of suboptimal levels of MHC class I molecules, NK cells can provide a protective cytotoxic backup system, although of limited efficiency. However, if CTL precursors efficiently recognize their targets and undergo activation, NK cells are signalled to limit their activity, allowing the CTLs to be dominant cells mediated cytotoxic system fighting an infection. Decreased NK function in HCV patients may impact on these interactions leading to dysfunction of the immune system.

Future studies using hepatic NK cells from large numbers of patients are required to determine whether the observations of the present study are also found in the liver. These studies should include the use of purified NK cells and NKR^+ T cells so that the contributions of other cell types to the impairment in cytotoxicity in HCV disease can be assessed. Cytotoxicity against broader range of target cells

lines should also be assessed. Future studies on cytokine production by NK and NKR^+ T cells from controls and patients with chronic HCV infection may also lead to the identification of mechanism of HCV persistence. An analysis of the effects of culturing NK cells from patients with chronic HCV infection and HCV resolvers with IFN-α may lead to the identification of mechanism by which the response to IFN-α is defective in some individuals Finally, a study of changes in NK activity and/or numbers during the course of disease or response to treatment may provide further information on this issue. An improved understanding of NK and NKR^+ T cell may ultimately lead to the identification of cellular or molecular targets for therapeutic intervention for HCV disease.

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