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Deficiency of Natural Killer Receptor Expressing Cells in Haemophagocytic Lymphohistiocytosis

A thesis submitted for the degree of Doctor of Philosophy

by

Jean Dunne FIBMS

Trinity College
University of Dublin

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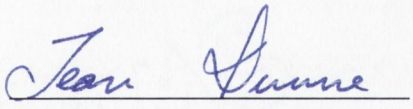

Jean Dunne

‘because they are unfathomable, one can only describe them vaguely by their appearance’

Lao Tsu (Tao Teh Ching)

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List of abbreviations

Activation induced cell death	AICD
Allophycocyanin	A-PC
Antigen presenting cells	APC
Avian myeloblastosis virus	AMV
Central nervous system	CNS
Cerebrospinal fluid	CSF
Chronic persistent hepatitis	CPH
common γ chains	γc
Complimentary DNA	cDNA
Concanavalin A	ConA
Count per minute	CPM
Cyclosporin A	Cs A
Cytomegalovirus	CMV
Cytotoxic T lymphocytes	CTL
Dendritic cells	DC
Deoxyribonucleic acid	DNA
Deoxyribonucleoside triphosphates	dNTPs
EBV nuclear antigens	EBNA
EBV-lymphoma associated haemophagocytic syndrome	EBV-LAHS
Endoplasmic reticulum	ER
Enzyme-linked immunosorbent assays	ELISA
European Bioinformatics	EBI
Epstein Barr Virus	EBV
6-carboxyfluorescein	FAM
Familial haemophagocytic lymphohistiocytosis	FHL
Fas ligand	FasL
Fcγ Receptor III	FcγRIII
Fluorescein isothiocyanate	FITC
Friedman Nonparametric Repeated Measures Test	Friedman RMT
Fulminant infectious mononucleosis	FIM

Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Granulocyte-macrophage colony stimulating factor	GM-CSF
Granzyme A	GrmA
Granzyme B	GrmB
Haemophagocytic lymphohistiocytosis	HLH
Hanks balanced salt solution	HBSS
Hematopoietic progenitor cells	HPC
Hepatitis B virus	HBV
Herpes simplex virus	HSV
Human immunodeficiency virus	HIV
Ig super family	IgSF
Ig-like transcript 2 / leucocyte inhibitory receptor 1	ILT2/ LIR-1
IL-15 receptor	IL-15R
Immunoreceptor tyrosine-based activation motifs	ITAM
Immunoreceptor tyrosine-based inhibition motifs	ITIM
Infectious mononucleosis	IM
Interferon gamma	IFN-γ
Interferon regulatory factor 1	IRF-1
Interleukin-15	IL-15
Interleukin-2	IL-2
Intestinal intraepithelial lymphocytes	IELs
Intravenous immunoglobulin	IVIG
Janus kinase	Jak
Killer cell Ig-like receptor	KIR
Killer cell lectin-like receptor family	Klra
Killer-activating receptor-associated protein	KARAP
KiloDalton	kD
Knockout	KO
Langerhans' cell histiocytosis	LCH
Latent membrane proteins	LMP
Lipopolysaccharide	LPS
Lymphokine activated killer	LAK

Lymphoma associated haemophagocytic syndrome	LAHS
Major histocompatibility complex	MHC
Matched unrelated donors	MUD
Median fluorescence intensity	MFI
Mixed lymphocyte culture	MLC
Monoclonal antibodies	mAb
Mononuclear cells	MNC
Murine gamma herpes 68	MHV-68
National Center for Biotechnology Information	NCBI
Natural cytotoxicity receptors	NCR
Natural killer cell	NK cell
Natural killer receptor positive	NKR^+
Natural killer T cell	NKT cell
Nonobese diabetic	NOD
Peridin chlorophyll protein	PerCP
Peripheral blood mononuclear cells	PBMC
Phorbol myristate acetate	PMA
Phosphatidylinositol 3-kinase	PI3K
Phosphotyrosine binding domains	PTB domains
Phycoerytherin	PE
Phytohaemagglutinin	PHA
Pokeweed mitogen	PWM
Polymerase chain reaction	PCR
Protein tyrosine kinase	PTK
Recombinase activating gene	RAG
Relative quantification values	RQV
Reverse transcriptase	RTase
Ribonucleic acid	RNA
Saline Sodium Phosphate EDTA	SSPE
Signal transducers and activators of transcription	STAT
Signalling lymphocytic activation molecule	SLAM
SLAM associated protein	SAP

Src-homology 2	SH2
Staphylococcal enterotoxin B	SEB
Stimulation index	SI
T cell receptor	TCR
6-carboxy tetramethylrhodamine	TAMARA
Transporter associated with antigen processing	TAP
Tris/Acetate/EDTA	TAE
Tritiated	^{3}H
Tumour necrosis factor alpha	TNFα
Virus / infection associated HLH	VAHS or IAHS
Whole blood	WB
X-linked lymphoproliferative	XLP
X-linked severe combined immunodeficiency	X-SCID
α-Galactosylceramide	α-GalCer
β2 microglobulin	β2M

Publications and presentations

Papers

Dunne J, Lynch S, O'Farrelly C, Hegarty JE, Feighery C, Doherty D. Selective expansion and partial activation of human NK cells and NK receptor-positive T cells by IL-2 and IL-15. *The Journal of Immunology*, 2001, 167:3129-38

Dunne J, Lynch S, Smith O, Doherty DG, Whelan A, McMahon C, Gardiner CM, Naughten E, Feighery C. Natural killer and natural killer receptor positive T cell deficiency in haemophagocytic lymphohistiocytosis (manuscript in preperation).

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Joint congress of the British Society for Immunology (BSI) and the British Society for Allergy and Clinical Immunology (BSACI), Harrogate, UK, December 2000 (poster presentation).

Dunne J, Lynch S, Whelan A, Smith O, Doherty DG, McMahon C, Gardiner CM, Naughten E, Feighery C. Reduced response of $CD8\alpha^+$ lymphocytes to rIL-15 stimulation in NK deficiency associated with haemophagocytic lymphohistiocytosis. *Joint congress of the British Society for Immunology (BSI) and the British Society for Allergy and Clinical Immunology (BSACI), Harrogate, UK, December 2000 (poster presentation)*

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Dunne J, Lynch S, O'Farrelly C, Feighery C, Doherty DG. Cytotoxic natural T cells induced from human peripheral lymphocytes by interleukin-15
Irish Society for Immunology, Annual Scientific Meeting Dublin, 1999 (poster presentation).

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Summary

Two children, patient A and patient B, were diagnosed on clinical and histological grounds to have haemophagocytic lymphohistiocytosis (HLH) (Henter *et al.* 1991a; Arico *et al.* 2001a). Clinical presentation of HLH consists of prolonged fever, failure to thrive, irritability, and hepatosplenomegaly (Henter *et al.* 1991a). Parental consanguinity indicated a diagnosis of familial haemophagocytic lymphohistiocytosis (FHL) in the case of patient A. Following the development of an Epstein-Barr virus (EBV)-driven lymphoma the diagnosis of patient B was altered to EBV-lymphoma associated haemophagocytic syndrome (EBV-LAHS). The two young patients lacked NK cells and had reduced $CD8^+$ T cells at presentation. They both suffered from recurrent infections most notably with *Herpes* family viruses.

This study was designed to identify the deficiency that resulted in recurrent infection and haemophagocytic lymphohistiocytosis in these two patients. To do this we first examined the phenotype and function of freshly isolated lymphocyte populations. Flow-cytometry with four-colour staining was used to assess lymphocyte populations and to look at natural killer associated receptor expression. Changes in peripheral lymphocyte populations were studied over time and in response to therapy. Humoral immunity, including specific IgG, was studied in order to establish the efficacy of B cell function. Proliferation in response to mitogens was used to confirm T cell function. Natural cytotoxicity was assessed using K562 and Daudi cells as targets. These studies indicated that both patients were deficient in NK and NKR^+ T cells and natural cytotoxicity. In order to establish whether patient A expressed any NK associated receptor PCR was used to examine expression of mRNA for NKG2A. Interleukin-15 (IL-15) and its receptor complex, comprising the α, β and common γ (γc) chains, have been shown to play a central role in the development and function of natural killer (NK) cells and natural killer receptor positive (NKR^+) T cells in mice (Liu *et al.* 2000; Fehniger *et al.* 2001). We examined the expression of IL-15 and its specific receptor IL-15Rα using polymerase chain reaction (PCR) and sequencing studies. Upregulation of IL-15 mRNA in response to stimulation was studied using TaqMan™ PCR. Flow-cytometry with intracellular- and surface-staining was used to demonstrate IL-15 protein in monocytes

from patient A and healthy controls. Having established that both patients expressed mRNA for IL-15 and IL-15Rα, we then went on to investigate the effect of this cytokine on NK and NKR^+ T cells. We examined the effects of culture with IL-2 and IL-15 on the cytolytic function, natural killer receptor expression, CD25 and CD122 receptor expression, cytokine production, perforin expression and proliferation of NK and NKR^+ T cells in healthy controls. Studies into the effect of culture with IL-15 or IL-2 on CD25 and CD122 receptors were carried out for both patients. Due to restricted access to samples from patients A, the cytolytic function, NKR expression and proliferation of NK and NKR^+ T cells in response to IL-15 only were examined. With the exception of cytokine production, all other studies carried out on cells from healthy controls were carried out on PBMC from patient B.

These two patients differed in their response to IL-15. PBMC from FHL patient A responded to prolonged culture with IL-15 with expansion of NKR^+ T and NK cells and spontaneous cytotoxicity against K562 cells. PBMC from Patient B showed a less than normal expansion of NKR^+ T and NK cells as well as low spontaneous cytotoxicity in response to IL-15. The reduced cytotoxic function of IL-15 expanded cells may be the result either of low numbers of effector cells or dysfunction due to impaired NKR expression. Peripheral $CD8^+$ T cells from this patient were shown to express perforin, as were the IL-15 expanded NKR^+ T and NK cells. The reduced numbers and function of NK and NKR^+ T cells may contribute to the pathogenesis of HLH in these two patients. Reduced spontaneous/NK-like killing mediated by perforin expressing cells may lead to susceptibility to viral and bacterial infections as well as dysregulation of T cell activation (Biron *et al.* 1999; de Saint Basile 2001). The decreased response of NK and NKR^+ T cells to IL-15 may predispose patient B to recurrent *Herpes* family viral infections as this cytokine orchestrates the immune response to these viruses (Gosselin *et al.* 1999). This may be especially important at sites of immune clearance such as the liver, spleen and lymph nodes. These are the sites of haemophagocytic infiltration seen in HLH.

While the clinical and laboratory findings in HLH have been linked to the biological effects of several inflammatory cytokines (Henter *et al.* 1991b and Takada *et al.* 1999), and deficiency of lymphocyte cytotoxicity (Stepp *et al.* 1999), the role IL-15 and NKR^+ T cells has not previously been investigated.

1. Introduction

1.1. Haemophagocytic Lymphohistiocytosis: clinical and pathological findings

1.1.1. Haemophagocytic Lymphohistiocytosis

Patients with haemophagocytic lymphohistiocytosis (HLH) show persistent high-grade fever, pancytopaenia, coagulation abnormality, liver dysfunction, and proliferation of benign histiocytes with haemophagocytosis in the bone marrow, lymph nodes, spleen and liver (Henter *et al.* 1991c; Cline 1994). HLH embraces both sporadic and familial haemophagocytic syndromes, with or without evidence of an infective agent. HLH is an important differential diagnosis in infants with prolonged fever and hepatosplenomegaly. This disorder is often rapidly fatal but sucessful treatment regimens have been described (Henter *et al.* 1998; Durken *et al.* 1999). Diagnosis of HLH presents a challenge since none of the clinical or laboratory findings are exclusively diagnostic and in the absence of specific markers, the condition may go undiagnosed (Arico *et al.* 2001a). Childhood haemophagocytosis is a feature of X-linked lymphoproliferative (XLP) syndrome, the 'accelerated' phases of Chediak-Higashi and Griscelli syndromes as well as Familial and sporadic HLH (Arico *et al.* 2001b; Dufourcq-Lagelouse *et al.* 1999a; Imashuku 1997; Henter *et al.* 1991a). Secondary HLH includes a wide range of disorders from virus / infection associated HLH (VAHS or IAHS) to malignant neoplasia, mostly lymphoma, associated haemophagocytic syndrome (LAHS). LAHS includes several types of lymphoma, NK-cell/T-cell type is often associated with Epstein-Barr virus (EBV) infection and is seen mainly in young children. B-cell lymphoma-associated haemophagocytic syndrome is diagnosed more frequently in the elderly (Imashuku 1997). In addition, nonmalignant

histiocyte disorders such as Langerhans' cell histiocytosis (LCH) must be included as part of a differential diagnosis (Henter *et al.* 1991a). Despite recent advances in genetic analysis of these disorders, within each syndrome the genetic abnormalities may be diverse (Graham *et al.* 2000).

The main histopathological feature of HLH is a non-malignant diffuse infiltration by lymphocytes and macrophages, most typically with haemophagocytosis, into visceral organs, lymph nodes, bone marrow, and central nervous system (CNS) (Henter *et al.* 1991c). This appears to reflect a massive activation of T lymphocytes and cells of the macrophage-histiocyte lineage (Hansmann *et al.* 1989). Haemophagocytosis is an essential, though not specific cytological finding, most commonly found in the spleen and lymph nodes, and less frequently in the bone marrow (Henter *et al.* 1998). During the course of disease most patients have clinical manifestations of central nervous system involvement; however cerebrospinal fluid (CSF) pleocytosis is not always present (Haddad *et al.* 1997). As shown in figure 1.1 the histiocytes appear activated and sometimes exhibit haemophagocytosis mostly affecting erythrocytes but occasionally platelets and leukocytes. The organs most frequently affected are the spleen, liver, lymph nodes, bone marrow and CNS (Henter *et al.* 1998). The early diagnosis of HLH may be complicated since examination of bone marrow at this stage will usually show only moderate hyperplasia without haemophagocytosis or other diagnostic features (Henter *et al.* 1998). Often an innocous report of 'a reactive marrow without evidence of malignancy' is given. At later stages of disease bone marrow cellularity may be severly decreased causing difficulties in differential diagnosis between HLH and aplastic bone marrow disorders (Henter *et al.* 1998). Spleens affected by the disease show expansion of the red pulp with heavy inflitration of mononuclear cells usually with histiocytes demonstrating active phagocytosis. The white pulp is usually severely diminished in size and lymphocytes are depleted. A similar picture is seen in lymph nodes where the follicles are small and sparse and lymphocytes are depleted especially at later stages of disease (Hansmann *et al.* 1989). In the liver a dense infiltration, consisting of lymphocytes and a few histiocytes, is often present in the portal tracts and to a lesser extent in the hepatic lobules. This histological picture is similar to chronic persistent hepatitis. Since chronic hepatitis is rarely seen in infancy, this type of infiltration is

indicative of HLH (Henter *et al.* 1991c). Kupffer cells are usually moderately increased and occasionally exhibit haemophagocytosis (Henter *et al.* 1998). In the CSF a moderate pleocytosis is frequently seen and is predominantly due to small lymphocytes, a few monocytes may be seen but haemophagocytosis is rare (Haddad *et al.* 1997).

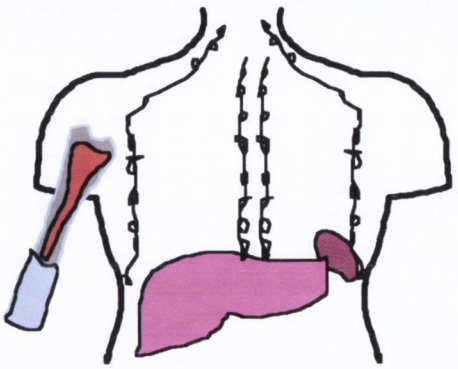
The diagnostic guidelines presented by the FHL Study Group of the Histiocyte Society, together with T- and NK-cell functional analysis go some way to facilitating diagnosis of HLH (Henter *et al.* 1991a; Egeler *et al.* 1996). However, where possible, genetic analysis allows for discrimination of disorders with a haemophagocytic component, which have diverse causes and severity spectrums.

Table 1.1. Diagnostic guidelines for Haemophagocytic Lymphohistoicytosis

Clinical and laboratory criteria
Fever (duration > 7 days with peaks > 38.5°C). Splenomegaly (> 3cm below the coastal arch)
Cytopaenia (affecting ≥ 2 of 3 lineages in the peripheral blood not caused by a hypocellular or dysplastic bone marrow):
Haemoglobin (<90 g/l). Platelets (<100 x 10^9/l). Neutrophils (<1.0 x 10^9/l)
Hypertriglyceridemia and/or hypofibrinogenemia (fasting triglycerides ≥ 2.0 mmol/l or ≥ 3SD of the normal value for age, fibrinogen ≤ 1.5 g/l or ≤ 3SD)
Histopathologic criteria: Haemophagocytosis in bone marrow or spleen or lymph nodes. No evidence of malignancy
All the above criteria are required for the diagnosis of Hemophagocytic Lymphohistiocytosis (HLH). The diagnosis of Familial Hemophagocytic Lymphohistiocytosis (FHL) is justified in the presence of a family history of HLH and all criteria listed above. Parental consanguinity is suggestive of FHL.
Comments
1. If haemophagocytic activity is not proven at the time of presentation, further search for haemophagocytosis is encouraged. If the bone marrow specimen is not conclusive, material should be obtained from other organs, especially lymph nodes or spleen. Serial marrow aspirates over time may be helpful
2. The following findings may provide strong supportive evidence for the diagnosis: (a) Spinal fluid pleocytosis (frequently <50 x 10^6 cells /l; mainly mononuclear cells); (b) Histological picture in the liver resembling chronic persistent hepatitis; (c) Low natural killer cell activity.
3. Other abnormal clinical and laboratory findings in HLH may be: lymph node enlargement, skin rash, cerebro-meningial symptoms, jaundice, edema - especially periorbital, increased spinal fluid protein content, elevated levels of transaminases, hypoproteinemia, hyponatremia, increased very low density lipoproteins, and decreased high-density lipoproteins.
Hyperferritinemia and increased number of soluble interleukin-2 receptor have been reported.

As proposed by Henter *et al.* 1991a (*Seminars in Oncology*)

A.



B.

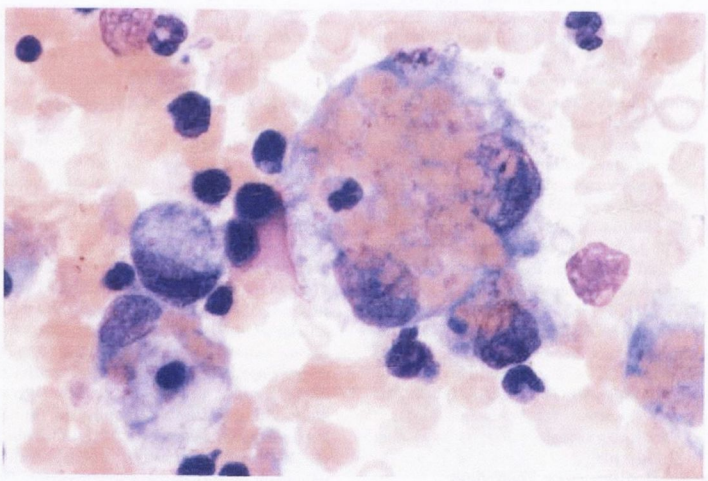


Fig. 21-38. Hemophagocytic syndrome. This photomicrograph depicts benign-appearing histiocytes with phagocytized red blood cells, white blood cells, even platelets. This particular patient had EBV-associated T cell lymphoma with marked hemophagocytosis in the marrow. (Romanowsky stain, × 600.)

Figure 1.1. Haemophagocytic lymphohistiocytosis showing sites of infiltration and active haemophagocytosis.
Sites of histiocytic infilatration in HLH include the liver, spleen, bone marrow and lymph nodes (A). Romanowsky stained bone marrow biopsy from a patient with EBV-associated T cell lymphoma showing active phagocytosis of lymphocytes and platelets by histiocytes (B).

1.1.2. FHL clinical and laboratory findings

Familial haemophagocytic lymphohistiocytosis (FHL) is a rare, often fatal disease, which presents most frequently in early infancy (Arico *et al.* 1996), and rarely in childhood (Boutin *et al.* 1988). In a European study its incidence has been estimated to be approximately 1 in 50, 000 births (Henter *et al.* 1991c). The pathogenesis of this disease remains enigmatic, but genetic analysis supports an autosomal recessive mode of inheritance (Gencik *et al.* 1984). Homozygosity mapping has revealed two loci associated with FHL - on 9q21.3-22 (FHL1) and 10q21-22 (FHL2) - later studies have provided evidence for additional genetic heterogeneity (Dufourcq-Langelouse *et al.* 1999b; Ohadi *et al.* 1999; Graham *et al.* 2000). In eight unrelated 10q21-22-linked FHL patients, nine independent mutations in exons 2 and 3 of the perforin gene were detected (Stepp *et al.* 1999). Premature stop codons were demonstrated in four patients, and missense mutations were found in the other four patients. Staining of patient cells for perforin revealed a complete or nearly complete absence of this protein, while the frequency of granzyme B in $CD8^+$ T cells was similar in control and patient samples. Further studies indicate that in 20% of FHL patients mutations were detected in the perforin gene, 10% were linked to 9q21.3-22 and in the majority of cases no gene was yet identified (Goransdotter-Ericson *et al.* 2001). The onset of disease is varied and non-specific, with symptoms including irritability, failure to thrive, anorexia and diarrhoea, however patients usually develop fever, hepatosplenomegaly and pancytopaenia. Up to 47% of cases show neurologic abnormalities (Janka 1983: Hirst *et al.* 1994, Henter *et al.* 1991c; Haddad *et al.* 1997) and in some cases neurological symptoms may predominate (Henter *et al.* 1992). Common laboratory findings include cytopaenia, hypertriglyceridaemia, hyperferritinemia and hypofibrinogenaemia (Henter *et al.* 1998). The most prominent histopathological feature is the multivisceral accumulation of activated lymphocytes and non-Langerhans histiocytes with active phagocytosis mainly of erythrocytes (Janka *et al.* 1983: Henter *et al.* 1998). Organ involvement is variable with liver, spleen, skin and lymph nodes most frequently affected (Fig. 1.1): bone marrow may be affected in less than 40% of cases, so a negative bone marrow does not rule out this diagnosis (Henter *et al.* 1991c; Ost *et al.* 1998). A positive family history

with FHL in first or second-degree relatives, or parental consanguinity strongly suggests a diagnosis of FHL.

1.1.3. Immune abnormalities in FHL

Hypercytokinaemia with elevated serum levels of inflammatory cytokines - such as tumour necrosis factor alpha (TNFα), interferon gamma (IFN-γ), and interleukin-6 (IL-6) - are striking features of HLH (including FHL) and indicate uncontrolled activation of T lymphocytes and macrophages (Henter *et al.* 1991b; Fujiwara *et al.* 1993; Imashuku *et al.* 1996). In FHL patients T cell activation has been demonstrated by the increased expression of HLA DR^+ T cells in blood and tissues (Henter *et al.* 1991c). The phenotype of activated T cells was shown to be mainly $CD8^+$, and high serum levels of CD8 and IL-2 receptor were also detected.

An important and consistent finding in FHL is impaired natural killer (NK) cell activity (Arico *et al.* 1988; Perez *et al.* 1984; Egeler *et al.* 1996), which has been demonstrated despite normal numbers of NK cells in the periphery. Arico *et al.* (1988) also demonstrated reduced IL-2 induced killing of Daudi cells in four FHL patients in clinical remission.

In the study carried out by Egeler *et al.* (1996), lymphocyte proliferation in response to mitogens including concanavalin A (ConA), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) were shown to be decreased in haemophagocytic lymphohistiocytosis. Most patients also had reduced cytotoxic T cell function - assessed using Epstein-Barr virus (EBV) transformed B lymphoblastoid cells as targets.

Investigations into the mediators of T cell-cytotoxicity have demonstrated that both perforin and Fas-Fas ligand mechanisms operate in short term *in vitro* assays (Kagi *et al.* 1994a). In FHL patients induction of Fas/Fas ligand-mediated cell death has been shown to be normal (Fadeel *et al.* 1999). Stepp *et al.* (1999) have demonstrated a link between

perforin gene defects and reduced anti-CD3 dependent cytotoxicity in some FHL patients.

Table 1.2. T lymphocyte and macrophage activation markers in FHL

Lymphocyte activation	Macrophage activation
Expression of HLA DR, CD25, Fas (CD95)	High levels of IL-1, TNF-α, IL-6, neopterin
High levels of soluble CD8 and CD25	Haemophagocytosis
High levels of IFN-γ secretion	

Adapted from Dufourcq-Lagelouse *et al.* 1999b

1.1.4. X-Linked Lymphoproliferative disease

X-linked lymphoproliferative (XLP) disease is a familial disorder with a rapidly fatal course in response to EBV infection (Purtilo *et al.* 1985). The disease is characterized by proliferation of lymphocytes and histiocytes, variable hepatic abnormalities and agamma- or hypergammaglobulinaemia. Clinically XLP is characterized by three major phenotypes: fulminant infectious mononucleosis (FIM) (50%), B cell lymphomas (20%), or dys-gammaglobulinaemia (30%) (Howie *et al.* 2000). The majority of malignant lymphomas are extra-nodal non-Hodgkin lymphomas, usually of the Burkitt type, and most involve the ileocaecal region of the intestine. Uncontrolled lymphocyte proliferation, organ infiltration, and T cell cytotoxic activity lead to multi-organ failure. Hepatic necrosis and bone marrow failure constitute the most common events associated with death in these patients. Mortality in XLP patients is 100% by the age of 40 (Morra *et al.* 2001). A defect in the adaptor proteins containing Src-homology 2 (SH2) also known as signalling lymphocytic activation molecule (SLAM) associated protein (SAP) is the cause of the X-linked lymphoproliferative disease (Coffey *et al.* 1998; Sayos *et al.* 1998; Nichols *et al.* 1998) SAP affects T lymphocyte intracellular signalling pathways. Different SAP/SH2D1A mutations have been identified in XLP patients leading to deletions, interference with mRNA transcription or splicing, and nonsense or missense mutations leading to premature stop codons. No correlation between mutations and

clinical phenotype has been found in this disease, with identical mutations manifesting different phenotypes (Sumegi *et al.* 2000). Only 50% to 60% of patients diagnosed with XLP have mutations in SAP/SH2D1A (Morra *et al.* 2001). Familial cases most often demonstrate SAP/SH2D1A mutations with no mutations demonstrable in 25 males with sporadic XLP (Sumegi *et al.* 2000; Morra *et al.* 2001). Therefore a positive family history is key in determining whether SAP is altered in patients with a clinical presentation compatible with XLP.

In normal children EBV infection is usually sub-clinical. Adolesents or adults who develop clinical infectious mononucleosis (IM) have a T cell response to infected B cells lasting for 2 to 3 months (Callan *et al.* 1998). Studies of the immune response in XLP patients with fulminant infectious mononucleosis suggest that abnormal T and B cell proliferation occurs in response to EBV-induced lymphoblasts (Sullivan *et al.* 1980). This polyclonal T and B cell proliferation infiltrates many organs, leading to bone marrow failure with a haemophagocytic component. The failure to eliminate EBV-transformed B cells in XLP does not appear to be caused by a B cell-specific defect (Jager *et al.* 1988). Variable defects in both T and NK cells have been reported (Sullivan *et al.* 1980). In some cases NK cell numbers are low, and other patients have normal numbers of NK cells but these cells have lost the ability to lyse appropriate target cells (Benoit *et al.* 2000). A direct causal relationship between XLP and EBV infection has not been established and the lymphomas or dys-gammaglobulinaemia may develop in patients without evidence of prior EBV infection (Strahm *et al.* 2000; Brandau *et al.* 1999). These findings indicate that the XLP gene, SAP/SH2D1A, has a more fundamental role in T / B cell homeostasis.

1.1.5. Viral infections associated with onset of FHL and HLH

In some, though not all cases of FHL, onset of disease activity has been shown to be associated with a variety of viral infections (Henter *et al.* 1993; Hoang *et al.* 1998). In the study by Henter *et al.* 1993, 22 of 32 children with HLH had clinical and laboratory signs of infection. EBV, cytomegalovirus (CMV) and parvoviral infections were demonstrated in 10 of 32 children tested using serology, virus isolation and DNA

hybridization. Thus viral infection may elicit a bout of familial haemophagocytic lymphohistiocytosis in genetically predisposed individuals. Therefore it cannot be used as the sole criterion for distinguishing FHL from an acquired form of haemophagocytic lymphohistiocytosis termed viral- or infection- associated haemophagocytic syndrome (VAHS or IAHS). In this condition a similar disease picture may be seen, however VAHS is a potentially self-limiting disorder seen mainly in the immunosuppressed (Risdall *et al.* 1979). EBV-associated VAHS may develop in patients with underlying immunodeficiency (Ohshima *et al.* 1999). In the case of treatable immunoglobulin deficiency the course may be relatively benign (Bethune *et al.* 2001). Where the immunodeficiency is untreatable the outcome of VAHS may be rapidly fatal (Purtilo *et al.* 1985). Non-familial haemophagocytic lymphohistiocytosis, frequently associated with EBV infection, has been described in a number of Asian children (Su *et al.* 1994 and 1995). In contrast to the normal pattern of infection of B cells in IM, EBV infection of $CD8^+$ T cell has been demonstrated in the periphery of some of these EBV-HLH cases (Kasahara *et al.* 2001). Patients with this, often fatal form of infectious mononucleosis, meet the diagnostic criteria for HLH. In these cases a diagnosis of FHL or X-linked lymphoproliferative disease has been ruled out due to a lack of family history of the disease and/or no parental consanguinity (Henter *et al.* 1991a; Chen *et al.* 1991).

1.1.6. Differential diagnosis in HLH

Disorders with pronounced macrophage activation include FHL and VAHS, Langerhans cell histiocytosis (LCH, Histiocytosis X), non-LCH histiocytic disorders, malignant histiocytic disorders, X-linked lymphoproliferative disorder and Chediak-Higashi syndrome (Imashuku, 1997). The differential diagnosis of HLH includes a wide range of diseases including infectious mononucleosis and other viral infections, septicaemia, syphilis, Leishmaniasis, encephalitis, hepatitis, systemic juvenile arthritis, degenerative cerebral disorders, neurometabloic disorders, aplastic anaemia, myelodysplastic syndromes and severe combined immunodeficiency.

1.1.7. Genetic basis for haemophagocytic lymphohistiocytosis

The HLH syndrome group includes, among others, FHL and the 'accelerated' phases of Chediak-Higashi syndrome, Griscelli disease and XLP disorder. The molecular basis for these four inherited HLH associated diseases have been characterized and are outlined in table 1.3. These hereditary disorders of the immune system are characterized by the occurance of similar T cell and macrophage activation. Perforin gene defect has been demonstrated in a proportion of FHL patients, although in the majority of cases the genetic defect is as yet unknown (Goransdotter-Ericson *et al.* 2001). The defects in Chediak-Higashi syndrome and Griscelli disease affects intracellular trafficking (Spritz *et al.* 1998; Pastural *et al.* 1997; Menasche *et al.* 2000). A defect in the gene for the adaptor protein SAP/SH2D1A - which affect T lymphocyte intracellular signalling pathways - is the cause of the X-linked lymphoproliferative disease (Coffey *et al.* 1998; Sayos *et al.* 1998; Nichols *et al.* 1998). The diagnostic criterion for HLH is outlined in Table 1.1, and is characterized by hyperactivation and proliferation of T cells and macrophages (Arico *et al.* 1996; Henter *et al.* 1998). In addition defects in cytotoxic NK function is a consistent feature of HLH.

Table 1.3. Genetic diseases with defective control of lymphocyte/macrophage activation (Adapted from Dufourcq-Lagelouse *et al.* 1999b)

Disease	Phenotype	Gene
Familial Haemophagocytic lymphohistocytosis (FHL)	Constitutive 'haemophagocytic syndrome'	FHL1 9q21.3-22 FHL2 10q21-22
X-linked lymphoproliferative syndrome (XLP)	EBV induced 'haemophagocytic syndrome' Agammaglobulinaemia Lymphoma	SH2DIA or SAP
Chediak-Higashi syndrome (CHS)	Albinism Giant granulation Defective cytotoxicity 'haemophagocytic syndrome'	CHS1 – human LYST- mice (Lysosomal trafficking regulator)
Griscelli disease	Albinism Defective cytotoxicity 'haemophagocytic syndrome'	Rab27a Myosin 5a

Abbreviations. SAP, SLAM associated protein known as SH2D1A.

1.1.8. Genetic defects leading to impairement of NK or cytotoxic activity

Reduced or absent NK cell cytotoxicity and/or defective T cell cytotoxicity is a consistent feature of conditions with associated haemophagocytic lymphohistiocytosis (Egeler *et al.* 1996; Baetz *et al.* 1995; Benoit *et al.* 2000; Harada *et al.* 1982). Variable genetic abnormalities result in cytotoxic dysfunction. Perforin gene defects, leading to absence of this important cytolytic mediator, result in decreased cytotoxicity in a proportion of FHL patients (Stepp *et al.* 1999). Giant granulation in cytotoxic cells is a

feature of Chediak-Higashi syndrome (Baetz *et al.* 1995). In this condition mutations in lysosomal trafficking (designated CHS1 in humans and LYST in mice) protein leads to defective release of granules containing melanin or cytolytic enzymes (Barbosa *et al.* 1996; Baetz *et al.* 1995; Barrat *et al.* 1996). Similarly in Griscelli syndrome, disrupted intracellular transport of melanosome or cytotoxic granules is due to defects in either Rab27a – a GTPase - or myosin 5a (Pastural *et al.* 1997; Menasche *et al.* 2000). Defective intracellular trafficking leads to the phenotypic manifestations of albinism and decreased cytotoxicity in both these syndromes, in Griscelli syndrome only RAB5a defects are associated with the haemophagocytic syndrome (Menasche *et al.* 2000).

Defects in intracellular signalling may result in reduced NK and T cell cytotoxicity seen in XLP patients. Exposure of XLP males to EBV leads to the development of fulminant infectious mononucleosis, lymphoma, hypo- or dys-gammaglobulinaemia and histiocytosis (Harada *et al.* 1982). Genetic analysis has revealed a number of mutations in the SH2D1A or SAP molecule in a proportion of patients (Coffey *et al.* 1998; Sayos *et al.* 1998; Nichols *et al.* 1998). This protein functions as a regulator in the signal transduction pathway of at least two receptors, namely, the SLAM protein, expressed on T and B cells, and 2B4, a natural cytotoxicity receptor (see below). SAP binding to SLAM has been shown to trigger tyrosine phosphorylation and is proposed to alter the profile of cytokine expression during T cell activation (Latour *et al.* 2001; Nichols *et al.* 2001). It is proposed that in the absence of SAP binding to SLAM, T cells from XLP patients secrete increased amounts of IFN-γ (Latour *et al.* 2001). 2B4 functions as an activating co-receptor in normal NK cells, however in NK cells from XLP patients it has been shown to have an inhibitory function (Parolini *et al.* 2000; Benoit *et al.* 2000). This alteration in function is postulated to be the result of association between 2B4 and the inhibitory phosphatase SHP-1 when the activatory SH2D1A/SAP is defective or absent (Moretta *et al.* 2001).

1.1.9. Treatment of FHL, non-familial HLH and XLP

FHL may be rapidly fatal in untreated patients. Long term remission has been accomplished by predominantly T-cell targeted immunosuppression with methylprednisolone, antithymocyte globulins and intrathecal methotrexate followed by maintenance therapy with cyclosporine A (Stephan *et al.* 1993; Henter *et al.* 1998), but ultimately many patients relapse (Jabado *et al.* 1997). Bone marrow transplant (BMT) from matched sibling donors has been shown to be sucessful, with recent reports of improved survival of patients with transplants from matched unrelated donors (MUD) (Baker *et al.* 1997; Durken *et al.* 1999).

Longitudinal study of patients with EBV-linked non-familial HLH showed moderate success using a treatment regimen containing etoposide with, in some cases, intravenous immunoglobulin (IVIG) and prednisolone. The overall survival of patients at 5 years was 40%, and disease free survival was 36% (Chen *et al.* 1998). Four of 22 patients had complete remission, while another four patients suffered a relapse followed by response to therapy and disease free survival. Three of 22 patients developed EBV-containing T-cell lymphomas: this led to death in the cases of two patients with survival accompanied by continuing disease in the third.

Following treatment of XLP patients with cytotoxic chemotherapy, with or without immunomodulatory agents, incomplete remission of short duration has been reported in a number of studies (Chen *et al.* 1995; Sullivan *et al.* 1985). Thus allogeneic BMT, which induces disease resolution, has been suggested as the treatment of choice (Teshima *et al.* 1996). However, a study from a Japanese group have demonstrated complete remission in 14 of 17 patients treated with immunochemotherapy consisting of steroids and etoposide, with or without immunomodulatory agents (Imashuku *et al.* 2000).

1.2. NK cell associated receptors on NK and T cells

1.2.1. Natural killer cells - introduction

NK cells were originally identified as large granular lymphocytes with the ability to lyse certain tumour cells and characterized by the absence of T cell receptor (TCR) and CD3 (Trinchieri 1989; Lanier *et al.* 1992). Human NK cells express CD56, a neural adhesion molecule (Lanier *et al.* 1989 and 1991). In response to viral infections these cells mediate perforin-dependent lysis, undergo proliferation, and produce cytokines including IFN-γ, TNF, granulocyte-macrophage colony stimulating factor (GM-CSF) and chemokines (Biron *et al.* 1999). In contrast to T and B cell responses to antigen, which typically require a proliferation phase, the innate NK response is immediate. NK cells contribute to defense against intracellular bacteria and parasites and they are critical for controlling several types of viral infection (Unanue *et al.* 1997; Scharton-Kersten *et al.* 1997; Biron *et al.* 1999; Biron *et al.* 2001). Despite the well described antitumour activity of NK cells *in vitro* and in certain *in vivo* models their role in defence against spontaneous neoplastic transformation remains less well established (Heberman *et al.* 1975; Seaman *et al.* 1987). NK lysis in of tumour cells may be indirectly enhanced by IFN-γ (Berthou *et al.* 2000). Recently the role of NK cells in Epstein-Barr-virus-associated lymphoproliferative disorders has been outlined (Robertson, 2001). The observations that NK cells lysed tumour targets which exhibited decreased levels of major histocompatibility complex (MHC) class I expression and mediated rejection of MHC-different bone marrow graft led to the formation of the 'missing self' hypothesis (Ljunggren *et al.* 1985; Bennett 1987; Ljunggren *et al.* 1990). This hypothesis stated that NK cells ignore potential targets expressing normal levels of MHC class I molecules and attack cells with reduced or absent self-MHC (Karre *et al.* 1986). Studies in mice have shown that NK cells attack otherwise normal cells that lack some or all self-MHC class I molecules (Bix *et al.* 1991; Liao *et al.* 1991). Since transformed and infected cells often downregulate or lose class I surface expression this hypothesis provides a rationale for NK cell function *in vivo* (Garrido *et al.* 1997; Tortorella *et al.* 2000). A molecular mechanism for missing self-recognition was established when several MHC class I-specific receptors were described that inhibit NK

cell function (Lanier, 1998a and b). An important feature of all inhibitory receptors is that each is expressed on a subset of NK cells that overlaps partially with the expression of other class I-specific inhibitory receptors. This varigeated pattern of receptor expression allows individual NK cells to discriminate among cells expressing different class I molecules. Thus downregulation of only one MHC class I molecule by a host cell will elicit a strong response by a subset of NK cells whose inhibitory receptor recognizes that particular molecule (Raulet *et al.* 2001). Missing self-recognition is only one of several modes of NK cell-target cell discrimination. Recognition of pathogen infected cells and tumours by NK cells is based on the expression of multiple cell-surface receptors that bind either MHC class I or non-MHC ligands and transduce either inhibitory or activating signals. A balance between activating and inhibitory receptors (Long, 1999) regulates the cytolytic activity of NK cells. The signals from several activating receptors are integrated and these in turn are regulated by the inhibitory receptors for MHC class I, based on the density and array of ligands and class I molecules expressed by the target cell (Lanier, 2000).

1.2.2. CD56 (N-CAM)

CD56 is a member of the Ig super family (IgSF) and is expressed in neural and muscle tissue as well as NK cells and some T cells (Lanier *et al.* 1989). It is a form of neural cell adhesion molecule I. Cells expressing CD56 can bind to each other (homotypic adhesion). However CD56 is not widely expressed on NK target cells and does not appear to play a role in cytotoxicity (Lanier *et al.* 1991)

1.2.3. Receptors activating NK cells

T and B cells recognise antigen by clonotypic antigen receptors however NK cells do not have antigen specific receptors but are able to detect changes in membrane glycoprotein expression in target cells. Their activities are controlled by receptors that mediate activation or inhibition upon ligation of surface molecules on target cells. One of these is CD16 (FcγRIII) the low affinity receptor for IgG (Daeron, 1997). The transmembrane-anchored CD16 isoform is a 70 kiloDalton (KD) glycoprotein of the

IgG superfamily expressed on NK cells, activated monocytes and a subset of T cells (Ravetch *et al.* 1989; Phillips *et al.* 1991; Lanier *et al.* 1985). Upon ligation of CD16 receptors, activated src-family tyrosine kinases (eg. lck) bind to and phosphorylate tyrosine residues contained within the immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domains of the high affinity IgE receptor (FcεRI-γ and ζ) (Wirtmueller *et al.* 1992; Vivier *et al.* 1991; O'Shea *et al.* 1991). Subsequently there is recruitment and phosphorylation of downstream signalling molecules including ZAP70 and MAP kinase (Leibson *et al.* 1997). CD16 mediated activation induces NK cells to secrete cytokines, mediate antibody-dependent cellular cytotoxicity and may induce apoptosis as a consequence of Fas ligand-induced cell death (Perussia *et al.* 1984; Ortaldo *et al.* 1995; Eischen *et al.* 1996).

In mice the NKR-P1 family of molecules, which includes NK1.1, mediates NK activation whilst the only known human NKR-P1 molecule NKR-P1A (CD161) does not although it is present on most NK cells (Brown *et al.* 1997; Lanier *et al.* 1997). In addition several other costimulatory and adhesion molecules including NKp46, CD2, CD11a/CD18 (LFA-1), CD69 and CD49d/CD29 (VLA-4) have been implicated in NK cell activation (Lanier *et al.* 1997; Moretta *et al.* 2001).

1.2.4. NK associated receptors involved in classical and non-classical MHC recognition mediate both inhibitory and activating functions

NK cells preferentially kill target cells lacking classical MHC class I molecules (HLA-A, B and C) on their cell surface (Ikeda *et al.* 1997). Two families of MHC class I receptors have been described: Immunoglobulin (Ig) superfamily receptors including the human killer cell Ig-like receptor (KIR) and the human Ig-like transcript 2 (ILT2)/ leucocyte inhibitory receptor 1 (LIR-1). As shown in Table 1.4, KIR and ILT2/LIR-1 recognise groups of class I allotypes but are not speicific for individual MHC class I peptide complexes. Two sub-families of KIR can be identified based on the number of IgG-like domains in the extracellular regions of the molecules. The KIR3D subfamily contains three Ig-like domains, whereas the KIR2D structures contain two Ig-like domains (Lanier, 1998a). Schematically illustrated in figure 1.2, KIRs with a similar

extracellular domain may be activating or inhibitory depending on their cytoplasmic domain. All inhibitory receptors are characterized by the presence of a long cytoplasmic domain containing two immunoreceptor tyrosine-based inhibition motifs (ITIM), and are expressed only on a subset of NK cells and activated cytotoxic T cells (Lanier, 1998b; Mingari *et al.* 1998a). Inhibitory KIRs include CD158a (formerly KIR2DL1 and p58.1), CD158b1/b2 (formerly KIR2DL2/3 and p58.2), CD158e1 (formlery KIR3DL1 and p70 or NKB1), and CD158k (formerly KIR3DL2 and p140) (Moretta *et al.* 2000a; Andre *et al.* 2001). KIRs containing short cytoplasmic tails (CD158h formly KIR2DS or p50 and CD158e2 formerly KIR3DS) lack ITIM sequences and are non-inhibitory (Lanier. 1998). These non-inhibitory receptors can mediate cell activation when combined with DAP-12, a di-sulphide-bonded homodimer containing an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail (Lanier *et al.* 1998b).

In mice, a killer cell lectin-like receptor family (Klra formerly Ly49) mediates activation or inhibition of NK function through the recognition of H-2 class-I molecules on potential target cells (Yokoyama. 1997; Lanier 1998b). In humans the C-type lectin receptor CD94 recognises the non-classical class I molecule HLA-E, which presents signal sequences derived from other MHC class I molecules (Braud *et al.* 1998; Borrego *et al.* 1998). Shown schematically in figure 1.2, CD94 lacks a cytoplasmic domain but can dimerize with the ITIM-containing NKG2A or NKG2B molecules or with the NKG2C molecule that contains no ITIM but associates with the ITAM containing DAP12 molecule (Lanier 1998b; Lanier *et al.* 1998a).

The inhibitory receptors prevent NK mediated lysis of self-MHC class I expressing cells. However NK cells lyse target cells that have lost or reduced MHC class I expression as frequently happens in tumours or virus infected cells (Garrido *et al.* 1997).

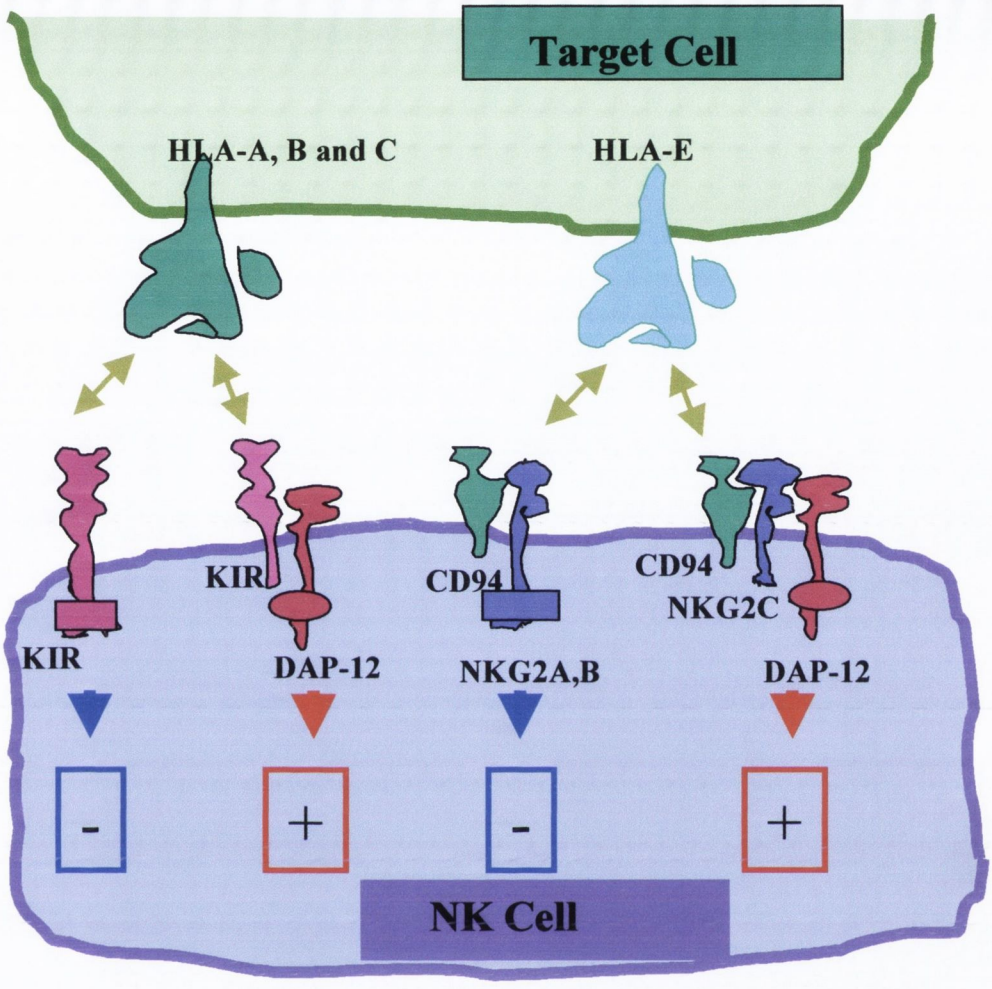


Figure 1.2. Natural killer associated activating and inhibitory receptors.

The double-ended arrows represent the interactions between HLA-class I molecules on target cells (top) with their receptors on NK cells (bottom). Oblong and oval symbols on the intracytoplasmic portions of NK receptor-associated molecules represent immunoreceptor tyrosine-based inhibitory and activatory motifs respectively (ITIMs and ITAMs). + and - denote activation and inhibitory signals transduced to the NK cell. Illustration adapted from Doherty *et al.* 2000

Table 1.4. Human MHC class I-specific inhibitory NK-cell receptors

Receptor	Molecular family	Chromosomal location	HLA specificity
CD158a (KIR2DL1)	IgSF	19q13.4	HLA-Cw 2,4,5,6
CD158b1/b2 (KIR2DL2/3)	IgSF	19q13.4	HLA-Cw 1,3,7,8
CD158e1 (KIR3DL1)	IgSF	19q13.4	HLA-Bw 4 alleles
CD158k (KIR3DL2)	IgSF	19q13.4	HLA-A3 A11
CD94/NKG2A	C-type lectin	12p12-p13	HLA-E
ILT2/LIR1	IgSF	19q13.4	Various HLA
CD158d (KIR2DL4)	IgSF	19q13.4	HLA-G and other alleles

Adapted from Moretta *et al.* 2000. Nomenclature from Andre *et al.* 2001.
Abbreviations IgSF, immunoglobulin superfamily; MHC, major histiocompatibility complex; NK, natural killer; ILT, immunoglobulin like transcripts; LIR, leukocyte inhibitory receptor.

1.2.5. Activating receptors involved in natural cytotoxicity

The observation that NK cell clones were heterogenous in their ability to lyse MHC class I$^-$ cells led to the search for receptors that turned on NK killing. The activation receptors, or 'natural cytotoxicity receptors' (NCR), induce NK cell cytotoxicity in an MHC independent fashion (Moretta *et al.* 2001; Bottino *et al.* 2000). NCRs are shown schematically in figure 1.3 and include NKG2D, NKp46, NKp30, NKp44, NKp80, and 2B4 and are expressed on resting and IL-2 activated NK cells (Moretta *et al.* 2000b).

Shown in figure 1.3, NKG2D is a C-type lectin that forms an activating receptor in association with the ITAM-containing adaptor molecule DAP10 (Wu *et al.* 1999) or KAP 10 (Chang *et al.* 1999). Most NCRs are expressed only on NK cells, in contrast, NKG2D is expressed by virtually all TCR$\gamma\delta^+$ and CD8$^+$ TCRα/β^+ cells well as NK cells (Moretta *et al.* 2001). The target cell ligands for NKG2D have been identified as the stress inducible molecules MICA and MICB (Wu *et al.* 1999; Bauer *et al.* 1999). These

molecules are encoded within the human MHC and are expressed on epithelial tumours including breast, ovary, colon, kidney, and lung carcinomas (Groh *et al.* 1999). The NKG2D/DAP10 complex has been shown to initiate NK and γδ T cell mediated cytotoxicity against transfectants and tumours expressing MICA and MICB (Wu *et al.* 1999; Bauer *et al.* 1999). While it has not yet been definitively demonstrated it is postulated that this complex may mediate the NK-like cytotoxicity of some $CD8^+$ $TCR\alpha/\beta^+$ cells (Moretta *et al.* 2001). The NKG2D mediated activation of TCR γδ cells in response to MICA/B requires TCR engagement (Bauer *et al.* 1999). Thus, NKG2D with MICA/B may potentially enhance diverse antitumour innate NK cell and antigen-specific T cell responses.

NCRs of the IgSF, including NKp46, NKp30 and NKp44, are coupled to the intracytoplasmic signal-transduction machinery via the ITAM containing CD3ζ and /or FcεRIγ (NKp46 and NKp30) or killer-activating receptor-associated protein (KARAP /DAP12) (NKp44) adaptor polypeptides (figure 1.3). The ligands for NCRs are not yet identified although NKp46 has been shown to bind the haemagglutinins of influenza and parainfluenza virus (Mandelboim *et al.* 2001). Both 2B4 and NKp80 have been shown to activate NK and T cells upon mAb-mediated receptor cross-linking (Nakajima *et al.* 1999; Moretta *et al.* 2001). 2B4 functions as a co-receptor with NKp46 and perhaps other molecules (Sivori *et al.* 2000). The activating function of 2B4 is linked to the signal transducing molecule SH2D1A (SAP) and evidence indicates that SHP-1 may mediate inhibition (Moretta *et al.* 2001). CD48 has been shown to be the major ligand for 2B4, suggesting a role in non-MHC-restricted cytotoxicity (Brown *et al.* 1998). Defective 2B4 mediated enhancement of NK cytotoxicity in XLP patients suggests that the association of SAP with 2B4 is necessary for optimal NK and lymphokine-activated killer cytotoxicity (Benoit *et al.* 2000; Nakajima *et al.* 2000).

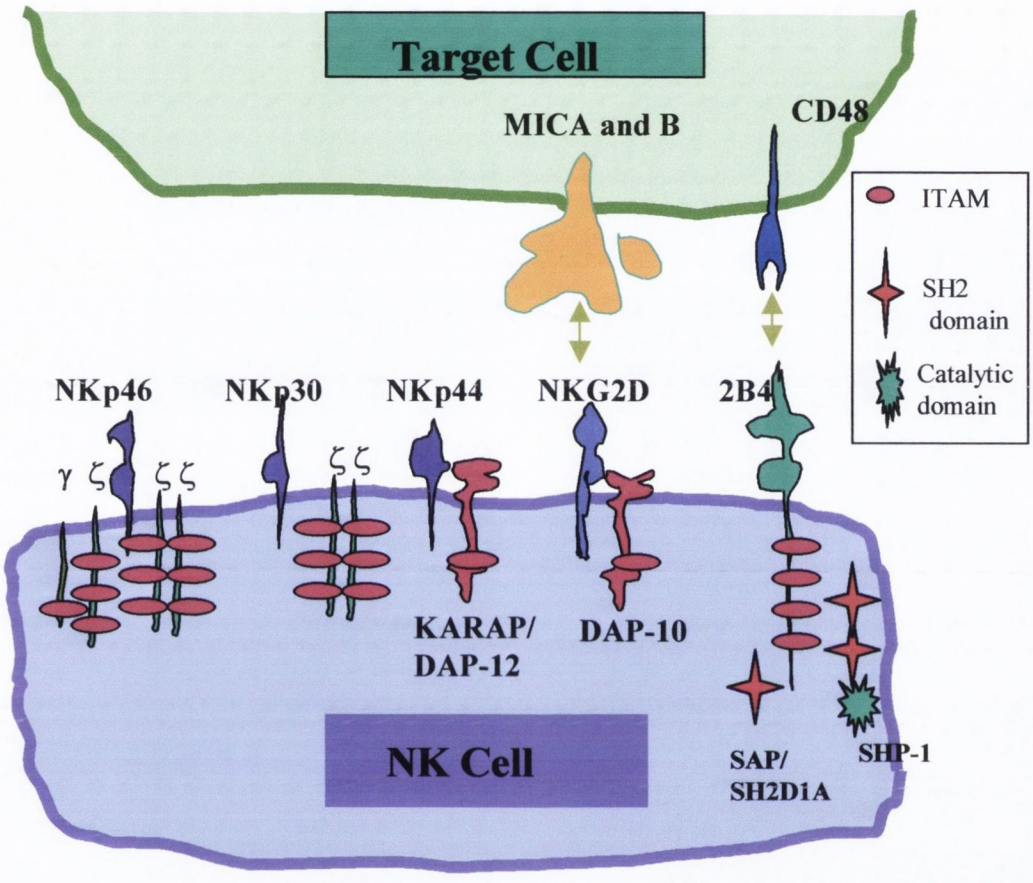


Figure 1.3. Natural killer activatory receptors with their distinct signal transducing molecules.

Triggering receptors NKp-30, -44 and -46 are coupled to different signal transducing adaptor proteins, including CD3ζ (ζ), FcεRIγ (γ), and KARAP/DAP12. DAP10/KAP10 associates with NKG2D. 2B4 has been shown to associate with SAP and SHP-1. Signalling is transduced through intracytoplasmic immunoreceptor tyrosine-based activatory motifs (ITAMs) (represented by oval shapes). CD48 has been shown to be a receptor for 2B4, and the stress inducible HLA-like molecules MICA and B interact with NKG2D. The ligands of other triggering receptors remain unidentified
Illustration adapted from Moretta *et al.* 2001

1.2.6. Control and maintenance of NK receptor expression

Little is known about the ontogeny of NK receptor expression in humans, although CD94 and KIR have been demonstrated in human foetal liver (Jaleco *et al.* 1997). In mice most NK cells express CD94/NKG2A at birth with a gradual decrease to approximately 50% in the adult (Salcedo *et al.* 2000; Sivakumar *et al.* 1999). This decrease in receptor expression in adults is not thought to be due to loss from receptor expressing cells (Salcedo *et al.* 2000). Since the CD94/NKG2A receptor is reactive with all known MHC haplotypes, it has been proposed that this receptor prevents most neonatal and prenatal NK cells from attacking self-cells (Salcedo *et al.* 2000; Sivakumar *et al.* 1999). *In vitro* studies of human progenitor cells have shown that culture with cytokines, particularly IL-15, induced development of NK cells expressing CD94/NKG2A receptors (Jaleco *et al.* 1997; Mingari *et al.* 1998b). Little or no KIR expression was induced in these cultures suggesting that additional signals are required for the initiation of KIR expression.

Evidence suggests that once the NK receptor gene is sucessfully activated, its expression is stably maintained in the cell, even as is undergoes multiple rounds of proliferation (Raulet *et al.* 2001). In human systems long-term NK clones sustain expression of specific KIR and CD94/NKG2 receptors for many cell generations (Moretta *et al.* 1990). Similarly in mice, receptor expression is maintained after transfer or in long-term culture (Salcedo *et al.* 2000). Both $CD8^+$ T and NK cells have been shown to downregulate expression of long inhibitory forms of KIR upon ligand binding to MHC class I (Huard *et al.* 2001; Huard *et al.* 2000a). In the absence of TCR engagement, the intensity of inhibitory KIR expression on $CD8^+$ T cells is reduced to 18% of the non-ligand bound level. However KIR expression can be *re*-induced *via* TCR engagement, possibly resulting in *de novo* synthesis (Huard *et al.* 2000c). Ligand binding reduces KIR expression on NK cell clones to between 80% and 40% of the density on non-ligand bound cells (Huard *et al.* 2001). It should be noted however that KIR expression is not reduced to negative levels on either NK or T cells. Downregulation of KIR does not result in reduced cytotoxic activity in T cells. In contrast KIR downregulation in NK

cells is associated with reduction in activating receptors (CD16, CD2 and 2B4) and both antibody-dependent and natural killing activity are reduced (Huard *et al.* 2001).

1.2.7. Self-tolerance and the NK cell

Studies of bone marrow graft rejection by irradiated mice have shown that NK cells can mediate alloreactivity (Bennett 1987). NK cells attack bone marrow cells that do not express host MHC molecules. In some instances NK cells positively recognise the presence of allogeneic MHC molecules (Ohlen *et al.* 1989). Tolerance can be induced in developing NK cells by exposure to either haematopoietic or non-haematopoietic class I$^-$ cells (Hoglund *et al.* 1991; Wu *et al.* 1997) The non-haematopoietic class I$^-$ cells have a more significant tolerance inducing effect (Wu *et al.* 1997). The dominant activity of ligand-negative cells in inducing tolerance is significant because it rules out simple positive selection as the sole mechanism of NK education. In the positive selection model, encounters with ligand positive cells would result in survival or functional maturation of NK cells expressing self-reactive inhibitory receptors, whereas ligand negative cells would have no effect. While NK cells attack MHC-different target cells, they exhibit tolerance for MHC-identical target cells. Self-tolerance by NK cells is probably imposed by a developmental process, however the mechanisms involved are poorly understood. Studies by *Valiante et al.* support the hypothesis suggests that 'at least one' inhibitory receptor specific for one self-MHC class I molecule is expressed on every NK cell. This hypothesis suggests that NK cells equipped with self-specific receptors, would be self-tolerant but also capable of attacking autologous cells that have downregulated only its specific class I molecule. Using cells from two donors each NK clone derived from a given donor was shown to express at least one inhibitory receptor specific for one of the donors MHC class I molecules, and each clone was inhibited from lysing self-target cells (Valiante *et al.* 1997). However studies in mice using PCR and surface staining suggest that not all NK cells express one of the known MHC inhibitory receptors (Kubato *et al.* 1999). While considerable differences in KIR repertoire between individuals have been observed no correlation with MHC allotype has been shown (Gumperz *et al.* 1996). In contrast in mice, MHC genes have been

shown to impact on the frequencies of freshly isolated NK cells expressing different inhibitory receptors (Held *et al.* 1996; Salcedo *et al.* 1997).

1.2.8. Invariant TCRαβ natural killer T (NKT) cells in mice and men

In mice, natural killer T (NKT) cells have been identified based on the expression of the NK cell marker NK1.1, Klra (Ly-49) and the IL-2 receptor β chain on $CD4^+CD8^-$ or $CD4^-CD8^-$ double negative T cells (MacDonald *et al.* 1995; Vicari *et al.* 1996). These cells are predominantly localized in the murine bone marrow (40% of $CD3^+$ cells) and liver (30-50%) in contrast to their low frequency in the peripheral blood, lymph nodes and spleen (less than 1% of $CD3^+$ cells) (Ohteki *et al.* 1994; Bendelac *et al.* 1997a). This tissue distribution would suggest that NKT cells exert their functions at specialized locations. In contrast to conventional T cells, murine $\alpha\beta^+NK1.1^+$ T cells frequently express an invariant T cell receptor (TCR) α-chain encoded by Vα14 and Jα281 in association with a limited number of Vβ chains (Lantz *et al.* 1994). NKT cells depend on the non-classical MHC class-I-like CD1 molecule and β2 microglobulin (β2M) for their development and they recognise glycolipids presented by CD1 (Bendelac *et al.* 1997b). IL-2 can support their proliferation and they can be induced to secrete IFN-γ, IL-4, IL-5, IL-10 and TGF-β by stimulation through the CD3 complex (Arase *et al.* 1996; Yoshimoto *et al.* 1995; Tamada *et al.* 1997). NKT cells exert regulatory functions, through their capacity to promptly release large quantities of IL-4 to orient responses in a Th2 direction and so influence antibody isotype switching (Yoshimoto *et al.* 1995; Bendelac *et al.* 1996). NK and NKT cells may be functionally linked *in vivo*, the activation of one leading to the activation of the other. This functional linkage has been demonstrated by activating NKT cells *in vivo* using α-Galactosylceramide (α-GalCer) (Carnaud *et al.* 1999). α-GalCer is functionally and chemically analogous to the natural glycolipids that have been purified from marine sponges on the basis of their anti-tumour properties against the mouse B16 melanoma (Morita *et al.* 1995). *In vivo* administration of α-GalCer results in the activation of NK cells by NKT cells in an IFN-γ dependent manner (Carnaud *et al.* 1999; Eberl *et al.* 2000). Administration of α-

GalCer has also been shown to upregulate CD69, a marker of activation, on B cells, $CD8^+$ T cells and to a lesser degree $CD4^+$ cells (Carnaud *et al.* 1999). However the activation of T and B cells appears to be transient with no proliferation (Eberl *et al.* 2000). Taken together it appears that the activation of NKT cells by this 'anti-tumour' glycolipid results in a cascade of cellular activation that involves elements of the innate and adaptive immune system. Studies of human lymphocytes have demonstrated a population of cytotoxic T lymphocytes that coexpress Vα24-JαQ, the homologue of the murine CD1-restricted Vα14-Jα281 TCR chain (Lantz *et al.* 1994; Porcelli *et al.* 1993). However their relationship to murine $NK1.1^+$ T cells is unknown. NKT cells have been shown to prevent type I diabetes in nonobese diabetic (NOD) mice and possibly also in humans (Naumov *et al.* 2001; Wilson *et al.* 1998). These cells may also have a role in the development and maintenance of systemic tolerance (Sonoda *et al.* 1999).

1.2.9. Natural killer receptor$^+$ (NKR^+) T cells

In contrast to murine NKT cells, natural-killer-receptor positive (NKR^+) T cells in humans are a heterogeneous population of T cells that are capable of potent cytotoxicity and production of multiple cytokines (Doherty *et al.* 1999; Ishihara *et al.* 1999). They express αβ or γδ TCR, as well as adhesion receptors and NK associated receptors. The NKRs expressed by these T cells include CD16, CD56, CD161 (NKR-P1A), NKG2D, 2B4 and receptors for MHC class I, including the activatory/inhibitory receptor CD94 and CD158e1 (KIR 3DL1), an immunoglobulin like receptor (KIR) (Moretta *et al.* 1997; Lanier *et al.* 1998b; Doherty *et al.* 2000). In mice and in humans when NKR^+ T cells express CD8 it is predominantly as CD8α chain in the absence of the β chain (Hammond *et al.* 1999; Bendelac *et al.* 1997a; Ishihara *et al.* 1999). As stated previously, β-2 microglobulin knockout ($\beta\text{-}2m^{-/-}$) mice fail to develop $NK1.1^+$ T cells, however $CD8\alpha^+\beta^-$ T cells expressing Klra (Ly49) were found to predominate in the liver of these mice (Emoto *et al.* 2000). In common with human NKR^+ T cells these cells were shown to be heterogeneous in their TCR α and β usage and produced IFN-γ but not IL-4 on *in vitro* stimulation (Emoto *et al.* 2000).

1.2.10. Inhibitory receptor expression on T cells

In humans individual KIR and CD94 receptors are normally expressed on up to 5% of peripheral blood T cells (Mingari *et al.* 1997; Speiser *et al.* 1999a). The majority of KIR^+ T cells are $CD8^+CD4^-$ (Phillips *et al.* 1995). In general, T cells that express inhibitory class I-specific receptors exhibit the phenotypic markers of activated T cells ($CD28^-$) (Speiser *et al.* 1999a; Mingari *et al.* 1996). In humans, KIR^+ CTL bear surface markers of previous activation (CD45RO and CD29) and lack markers for naive T cells (CD45RA and CD28) (Mingari *et al.* 1996). Prior and/or chronic activation may underlie inhibitory receptor expression. The majority of CTL clones do not express inhibitory receptors however KIR^+ and $CD94/NKG2A^+$ clones specific for human immunodeficiency virus (HIV) or melanoma antigens have been documented (De Maria *et al.* 1997; Ikeda *et al.* 1997; Speiser *et al.* 1999b). Interestingly, many of the KIR^+ T cells in humans are present as expanded monoclonal or oligoclonal populations (Mingari *et al.* 1996). Oligoclonal $CD8^+$ T cell populations have been proposed to represent T cells responsive to persistent antigens or self-antigens (Posnett *et al.* 1994). Non-conventional T cell subsets that express inhibitory receptors are thought to be specific for self-antigens. These include the $V\gamma9V\delta2$ population, specific for phospholipid antigen, and $TCR\alpha\beta^+$ CD1 restricted T cells, specific for the lipid antigens presented by CD1d (Halary *et al.* 1997). Self reactivity, as defined by the capacity to lyse class I deficient Daudi cells, was demonstrated only in some $V\gamma9V\delta2$ T cell clones, however Daudi reactive clones were more likely to express CD94/NKG2A receptors (Halary *et al.* 1997). These findings indicate that inhibitory receptors on T cells may function to prevent uncontrolled autoreactivity. This proposal is consistent with the theory that inhibitory receptor expression on T cells is generally a response to persistent antigenic stimuli.

1.2.11. Expression and upregulation of inhibitory receptors on T cells

Much evidence suggests that expression of inhibitory receptors on T cells typically occurs during or after activation of fully mature T cells. Recently matured conventional $CD4^+$ and $CD8^+$ T cells in the thymus do not express inhibitory receptors, nor do cord blood T lymphocytes from newborn humans, or T cells with a naive phenotype in the periphery of adults (Mingari *et al.* 1997; Speiser *et al.* 1999a). In mice expression of the Klra (Ly49) receptor by $CD8^+$ T cells is dependent on the expression of class I molecules by hematopoietic cells (Coles *et al.* 2000). This would suggest that inhibitory receptor expression requires class I antigen presentation. Upregulation of CD94/NKG2A expression on mature T cells has been demonstrated directly. Both IL-15 and TGF-β induce CD94/NKG2A expression on a large fraction of $CD8^+$ T cells undergoing antigenic stimulation *in vitro* (Mingari *et al.* 1998b; Bertone *et al.* 1999). However KIR are upregulated very poorly under the same conditions. These data suggest that CD94/NKG2A receptors can be upregulated rapidly by mature $CD8^+$ T cells of any specificity, whereas expression of KIR is limited to special conditions or antigen specificities. Maintenance of KIR expression levels on $CD8^+$ T cells has been demonstrated to require TCR engagement (Huard *et al.* 2000c). In the absence of TCR engagement inhibitory KIR expression is slowly downregulated by KIR-ligands expressed on antigen presenting cells. Thus inhibitory KIR expression on $CD8^+$ T cells *in vivo* may be maintained through continous exposure to antigen. This dynamic KIR expression may mediate self-tolerance by sparing self-reactive but potentially useful $CD8^+$ T cells. T cells, like NK cells, express inhibitory receptors in a variegated fashion, with extensive overlap in the expression of different receptors. The frequencies of human T cells expressing KIR varies, and individual KIR^+ T cells often co-express additional KIR, ILT2/LIR-1, or CD94/NKG2 receptors (Mingari *et al.* 1997; Speiser *et al.* 1999a). In mice stimulatory Klra (Ly49) receptor isoforms cannot be detected on T cells (Ortaldo *et al.* 1998; Coles *et al.* 2000). In contrast, human T cells have been shown to express stimulatory isoforms of KIR (Ferrini *et al.* 1994; Andre *et al.* 1999).

1.2.12. $CD56^+$ T cells

Of the NKR^+ T cells, $CD56^+$ T cells have been most extensively characterised with regard to phenotype and function. Studies of human $CD56^+$ T cells have shown that these cells can be induced to lyse NK-sensitive target cell lines *in vitro* (Lanier *et al.* 1987; Doherty *et al.* 1999). The activities of these $CD56^+$ T cells are regulated by inhibitory KIR and CD94 molecules (Mingari *et al.* 1995; Moretta *et al.* 1997). $CD56^+$ T cells can also be activated by TCR ligation or in response to cytokines in the microenvironment and stress-inducible proteins present on target cells (Doherty *et al.* 1999; Satoh *et al.* 1996; Bauer *et al.* 1999). A small proportion (<1%) of peripheral $CD56^+$ T cells express an invariant Vα24JαQ TCR α-chain which preferentially pairs with a Vβ11 β-chain and, in analogy to murine NKT cells, recognizes glycolipid antigens presented by CD1d (Norris *et al.* 1999; Takashi *et al.* 2000). However the majority of $CD8^+$ $CD56^+$ T cells in the periphery represent oligoclonally expanded populations with variable Vβ usage (Pittet *et al.* 2000). Upon activation, $CD56^+$ T cells can rapidly produce TNF-α, IFN-γ, IL-2 and /or IL-4 but little or no IL-5. The production of proinflammatory (Th1-type) and Th2-type cytokines suggests roles for these cells both in innate immunity and in the regulation of adaptive immune responses (Doherty *et al.* 1999; Exley *et al.* 1997; Prussin *et al.* 1997).

$CD56^+$ T cells account for a small percentage (~5%) of peripheral blood lymphocytes (PBL) but they can expand rapidly in response to certain pathogens and cytokines (Mingari *et al.* 1995; Doherty *et al.* 2000; Satoh *et al.* 1996). The coexpression of CD56 on peripheral $CD8^+$ T cells has been shown to correlate with cytotoxic function (Pittet *et al.* 2000). These NK-type CTLs, either express or upregulate perforin and granzyme B in response to stimulation with a combination of IL-2, IL-12 and IL-15 (Ohkawa *et al.* 2001). $CD56^+$ T cells are present in remarkably high numbers in the liver and bone marrow of healthy adults, accounting for 15-55% of all T cells in these organs (Doherty *et al.* 2000; Norris *et al.* 1999; Doherty *et al.* 1999). These cells constitute a minor population in the periphery, but they rapidly expand in response to infection and form one third of all hepatic T cells (Norris *et al.* 1999; Doherty *et al.* 1999).

1.2.13. NK and T cell common progenitor - the role of IL-15?

The existence of a common lymphocyte progenitor that commits to either the T/B- or NK-cell lineage is suggested by studies in mice. Deficiency of genes necessary for the development of lymphoid cells (eg. *Ikaros*) leads to failure to develop mature NK cells, T cells or B cells (Georgopoulos *et al.* 1994). However NK cells develop normally in mice deficient in the recombinase activating gene-2 (RAG2) which is involved in receptor rearrangement and is necessary for T and B cell development (Shinkai *et al.* 1992). Haematopoietic stem cells lacking lineage-specific membrane differentiation antigens (i.e. Lin-$CD34^+$ and Lin-$CD34^-$) are thought to be the common progenitors and have been identified in both human and mouse bone marrow, foetal liver, thymus and umbilical cord (Williams *et al.* 1998). Differentiation of NK progenitors depends on *in vitro* culture conditions and requires cytokines (e.g. IL-2, IL-7 and IL-15) and other haematopoetic growth factors (Williams *et al.* 1998). The pivotal role of IL-15 and IL-15 receptor (IL-15R) in the development of NK, NKR^+ T cell and γδ T cells, is demonstrated in gene knockout mice shown in Table 1.5. Most notably absence of genes for either IL-15Rα or IL-15Rβ resulted in deficiency of NK, NKR^+ T cell and γδ T cells. Indirect evidence of the requirement for IL-15 in development and function of NK, NKR^+ T cell and $CD8\alpha^+\beta^-$ T cells is given by studies in interferon regulatory factor 1 (IRF-1) knockout mice. In mice lacking the transcription factor IRF-1, mRNA for IL-15 was barely detectable, and these mice lack functional NK, NKR^+ T cell and $CD8\alpha^+\beta^-$ T cells (Ohteki *et al.* 1998). However culture of lymphocytes with IL-15 resulted in recovery of these lymphocyte subsets. Similarly IL-15 knockout mice exhibit reversible defects in NK and T cytoytic function (Kennedy *et al.* 2000).

1.3. IL-2, IL-15 and their receptor complexes

1.3.1. IL-2, IL-15 and their receptor complexes - introduction

IL-2 and IL-15 are structurally related cytokines with growth factor activity for T cells, B cells, NK cells and NKR^+ T cells (Grabstein *et al.* 1994; Waldmann *et al.* 1999; Waldmann *et al.* 1998). Both IL-2 and IL-15 are members of the four α helix bundle cytokine family and bind to receptors with shared signalling components, but they differ in their cellular sites of synthesis and their regulation of expression (Bamford *et al.* 1997; Waldmann *et al.* 1999). The genes for IL-2 and IL-15 are located on human chromosome 4q26-28 and 4q31 respectively (Fehniger *et al.* 2001). IL-2 is produced by activated T cells and binds to a heterotrimeric receptor (figure 1.4) consisting of the IL-2R α (CD25), β (CD122), and γ (CD132) chains. In contrast, IL-15 is produced in a variety of tissues including placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells and monocytes (Grabstein *et al.* 1994). IL-15 binds to two different receptor complexes: on lymphocytes a trimeric receptor (figure 1.4) consisting of the IL-15Rα chain and the β and γ chains shared by IL-2R (Waldman *et al.* 1998); and on mast cells a distinct receptor, IL-15RX (Tagaya *et al.* 1996).

1.3.2. IL-2, IL-15 and their receptor complexes – structures and functions

Both IL-2Rα and IL-15Rα are structurally similar, with shared fragmentary sequence matches, and have similar intron-exon organization in their genes, defining them as a new cytokine receptor family (Giri *et al.* 1995). Both genes are closley linked on human chromosome 10q14-15 (Anderson *et al.* 1995). IL-15Rα is more widely expressed than IL-2Rα, and has been demonstrated in T cells, B cells, macrophages, and in thymic and bone marrow stromal cells (Anderson *et al.* 1995). In addition IL-15Rα mRNA is widespread in such tissue as liver, heart, spleen, lung, skeletal muscle, and activated vascular endothelial cells (Giri *et al.* 1995).

IL-2/15Rβ (CD122) is expressed at low density on a sub-population of resting T cells and is upregulated by stimulation through the T cell receptor (TCR). Constitutive expression of IL-2/15Rβ has been demonstrated in a percentage of NK cells, monocytes, B cells, and neutrophils (Hodge *et al.* 2000; Espinoza-Delgado *et al.* 1990; Djeu *et al.* 1993).

Illustrated in figure 1.4, signalling through IL-2/15 receptor complex requires IL-2/15Rβ for binding the adaptor protein Shc and STAT proteins (Delespine-Carmagnat *et al.* 2000). The intracytoplasmic regions of the β and γc chains share domains of homology, called box 1 and box 2. These intra-cytoplasmic regions are responsible for constitutive association of the janus kinases (Jak) (Miyazaki *et al.* 1994). Three additional regions, illustrated schematically in figure 1.4, are defined in the IL-2/15Rβ cytoplasmic region, and these differentially affect IL-2/IL-15 signalling. A membrane proximal cytoplasmic region, termed the serine-rich region (S-region) is critical for IL-2/IL-15 induced proliferation and the prevention of apoptosis (Taniguchi 1995). This region has been shown to bind the *Syk* PTK of the *Syk/Zap70* family PTKs, which is postulated to induce *c-myc* expression and therefore influence the suppression of apoptosis (Miyazaki *et al.* 1995). The acidic or A region is associated with the protein tyrosine kinase (PTK) $p56^{lck}$. This region contains four phosphorylatable tyrosine residues at positions 338, 355, 358 and 361 and is thought to be involved in the pathway leading to *bcl-2*, *c-fos* and *c-jun* activation (Taniguchi, 1995; Gaffen *et al.* 1996; Ellery *et al.* 2000; Ellery *et al.* 2002). The C-terminal (H) domain regulates STAT5 activation through the phosphorylation of tyrosine residues at positions 392 and 510 (Taniguchi, 1995). Tyrosine phosphorylation is necessary for induction of STAT DNA binding activity and the promotion of gene transcription (Ellery *et al.* 2002).

The IL2R common γ chain (γc) is expressed on the surface of a proportion of monocytes, T cells and NK cells, however intracellular expression is high in these cell populations (Hodge *et al.* 2000). The γc chain is required for signal transduction and contains a membrane proximal region (PROX) and a carboxy terminus with which, as shown in figure 1.4, Janus kinase 3 (Jak3) associates (Johnston *et al.* 1995). Other

cytokines sharing the γc chain include IL-4, IL-7 and IL-9 (Kondo *et al.* 1993; Noguchi *et al.* 1993a).

The α, β and γ components of the IL2/15R have different affinities for their relevant cytokines. IL-2Rα binds IL-2 with low affinity while IL-15 Rα binds IL-15 with high affinity, however binding does not induce signal transduction (Giri *et al.* 1995). The IL2Rβγ complex has intermediate affinity for either IL-2 or IL-15, however for both cytokines the trimeric IL2Rαβγ or IL-15Rαβγ displays high affinity for the relevant cytokine (Waldman *et al.* 1998). Expression of both IL2Rβ and γ chains are required for IL-15 or IL-2 induced proliferation (Bamford *et al.* 1994).

1.3.3. IL-2, IL-15 and their receptor complexes - the Jak-STAT pathway

Because of the shared signalling components (IL-2/15Rβγc) the interaction of IL-2 and IL-15 leads to similar intracellular signalling events, shown schematically in figure 1.4 (Taniguchi *et al.* 1995; Johnston *et al.* 1995). Binding of IL-2 or IL-15 to the IL-2/15R complex triggers the activation of several tyrosine kinases and phosphorylation of multiple cellular components including the IL-2/15R β and γ chains (Taniguchi *et al.* 1995). This ultimatly results in regulation of gene expression through the Jak-STAT pathway (Beadling *et al.* 1994). Following the binding of IL-2 or IL-15 to their trimeric receptor phosphorylation of Jak1 and Jak3 kinases occurs. Jak1 associates with the IL-2/15R β (CD122) chain, and Jak3 associates with the γc (CD132) chain (Leonard *et al.* 1998). The activation of Jak proteins appears to be interdependent as no phosphorylation of Jak1 occurs in Jak3-deficient cells (Oakes *et al.* 1996). The phosphorylation of tyrosine residues in the IL-2/15R then serve as docking sites for signalling or adaptor proteins containing Src-homology 2 (SH2) or proteins with phosphotyrosine binding (PTB) domains such as the STAT proteins (Leonard *et al.* 1998). The phosphorylation of STAT 1, STAT 3 and STAT 5 (a and b isoforms) proteins, which all associate with IL-2/15R β, results in release from the receptor and dimerization (Delespine-Carmagnat *et al.* 2000). These dimerized forms of STATs can translocate to the nucleus where they modulate expression of target genes (Ihle *et al.* 1995).

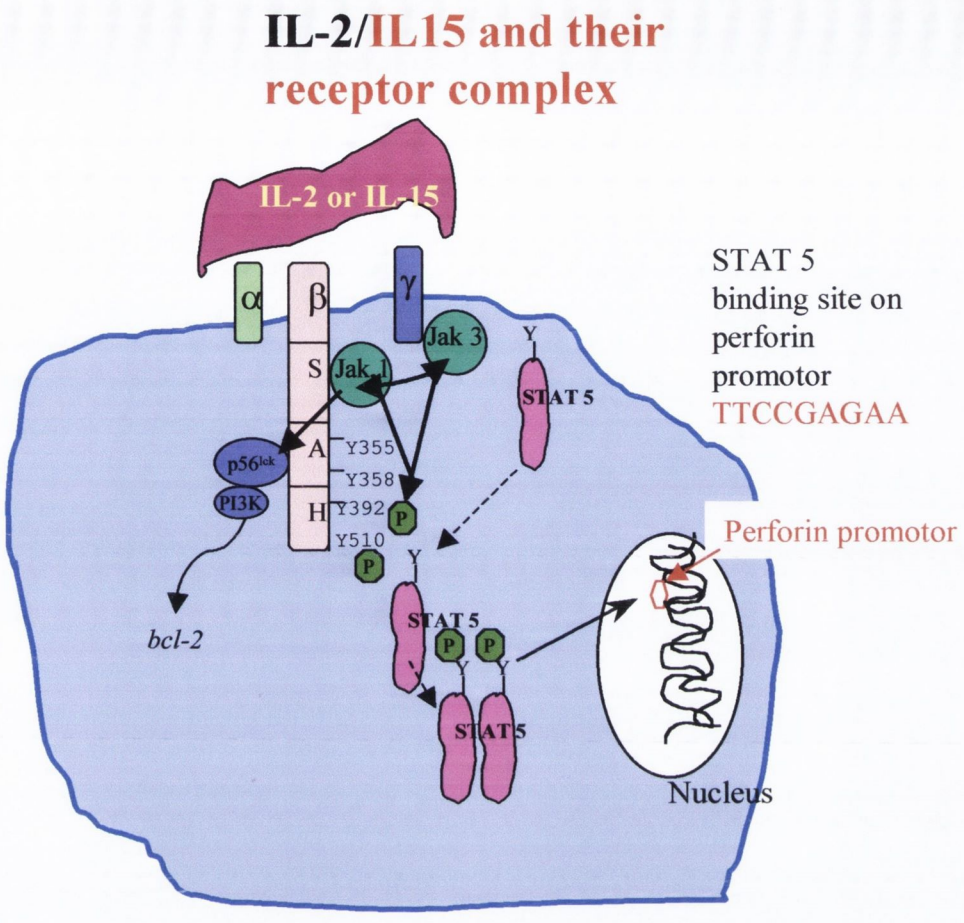


Figure 1.4. Intracytoplasmic signalling events resulting from IL-2/IL-15 binding to their specific receptor complexes.

IL-2/IL-15 receptor complexes include either the IL-2 or IL-15 specific α chains and the shared β and γ chains. Binding of cytokine to the receptor complex results in a cascade of intracellular signalling, including the Jak-STAT pathway. Activation of $p56^{lck}$ may be both Jak1/3 dependent and independent, and initiates activation of phosphatidylinositol 3-kinase (PI3K), leading to induction of anti-apoptotoic *bcl-2*. Phosphorylated STAT5 homo- or hetero-dimers (denoted by P) have been shown to bind to the perforin promotor region of DNA.
Illustration adapted from Leonard *et al.* 1998, Taniguchi 1995, Yu *et al.* 1999 and Ellery *et al.* 2000

The intermediate affinity receptor IL-2R ($\beta\gamma$) has been shown to have a role in anti-apoptotic signalling. Stimulation of resting T cells leads to activatation of $p56^{lck}$ (Ellery *et al.* 2000). Shown schematically in figure 1.4, this in turn activates phosphatidylinositol 3-kinase (PI3K), leading to induction of *bcl-2*. The activation of PI3K is Jak3 independent but requires the co-operation of Jak1 and the β subunit and may also involve the γc PROX subunit (Ellery *et al.* 2000).

Studies in knockout mice and human disease have revealed the essential roles of some of the IL-2/15R components and signalling proteins. Mutations in γc, which associates with Jak3, is the molecular basis of human X-linked severe combined immunodeficiency (X-SCID) (Noguchi *et al.* 1993b). These patients usually exhibit a deficiency of T and NK cells with normal B cells. A similar, though rarer, disease manifestation is seen in patients with autosomal defects in Jak3 gene, and also a patient with defective IL2/15R β expression (Macchi *et al.* 1995; Gilmour *et al.* 2001). Susceptibility to viral infections in STAT 1 knockout (KO) mice results from a selective defect in signalling in response to type I and II interferons (IFNs) (Durbin *et al.* 1996; Meraz *et al.* 1996). In contrast human studies have shown that increased susceptibility to mycobacterial but not viral infections can result from partial STAT 1 deficiency with impaired responses to IFNs (Dupuis *et al.* 2001). STAT3-KO mice exhibit a fatal lethality, this is consistent with the activation of STAT 3 by many cytokines (Leonard *et al.* 1998). Defects in IL-12 mediated signalling *via* STAT 1, 3 and 5 have been described in a patient with atypical mycobacterial infection (Golob *et al.* 2000). T cells from STAT 5a-deficient mice exhibit defective IL-2-induced receptor α chain expression and bone marrow derived macrophages have defective responses to granulocyte-macrophage colony stimulating factor (GM-CSF) (Feldman *et al.* 1997; Nakajima *et al.* 1997). Both STAT 5a- and STAT 5b-deficient mice have a partial decrease in NK cells, with more pronounced NK cell proliferation and cytotoxic defects associated with STAT 5b-deficiency (Imada *et al.* 1998). Interestingly STAT 5b-deficient splenocytes demonstrated decreased perforin mRNA expression in response to IL-2 and IL-15 (Imada *et al.* 1998). In addition, NK cells from STAT 5b-deficient mice exhibited lowered IL-2Rβ expression.

1.3.4. IL-15 has an essential role in development and functions of cells of innate immunity.

Studies *in vivo* and *in vitro* have demonstrated overlapping functions as well as distinct roles for IL-2 and IL-15 in lymphocyte development, homing, proliferation and survival. Comparative studies in knockout mice deficient in IL-2Rα, IL-15Rα, IL-2/15Rβ, IL-2 and IL-15 have demonstrated that IL-15/IL-15R signaling, but not IL-2/IL-2R signaling, is required for the development and homing of NK cells, NKR^+ T cells, subsets of $CD8^+$ T cells, γδ T cells and intestinal intraepithelial lymphocytes (IELs) (Table 1.5) (Liu *et al.* 2000; Suzuki *et al.* 1997; Ohteki *et al.* 1997; Lodolce *et al.* 1998). IL-15 has been shown to induce the differentiation of $CD34^+$ hematopoietic progenitor cells into functional $CD56^+$ NK cells (Mrozek *et al.* 1996). The bone marrow is essential for NK cell development (Kumar *et al.* 1979). The early phase of NK progenitor development requires stromal growth factors to induce an NK precursor with the phenotype $CD34^+$IL-2/15Rβ^+CD56^- (Yu *et al.* 1998). Synthesis of IL-15 by bone marrow stromal cells may thus be required for NK cell development and maturation (Mrozek *et al.* 1996).

IL-2 and IL-15 added *in vitro* promote the survival and proliferation of memory lymphocytes and the induction of cytolytic effector cells (Grabstein *et al.* 1994; Lodolce *et al.* 1998; Zhang *et al.* 1998; Carson *et al.* 1994). IL-2 has been shown to predispose activated T cells to die by apoptosis, however IL-15 promotes their survival (Bulfone-Paus *et al.* 1997). Exogenous IL-15 induces the proliferation, survival, and effector functions of resting NK cells, NKR^+ T cells, γδ T cells and IELs, suggesting that it has a more general role in the activation of innate and tissue-specific immune responses (Ohteki *et al.* 1997; Carson *et al.* 1994; Carson. *et al.* 1995; Carson *et al.* 1997; Garcia *et al.* 1998; Chu *et al.* 1999). In response to intracellular infection, IL-15 enhances the killing ability of NK cells and stimulates the synthesis and secretion of IFN-γ (Gosselin *et al.* 1999; Fawaz *et al.* 1999; Flamand *et al.* 1996). Results from one study indicated

that high concentrations of IL-15 markedly enhance both proinflammatory and anti-inflammatory cytokine production by activated monocytes (Alleva *et al.* 1997). Low levels of IL-15 have been shown to selectively suppress proinflammatory cytokine production thereby functioning as a potent autocrine regulator of macrophage proinflammatory cytokine production. (Alleva *et al.* 1997).

Table 1.5. NK-cell development in gene knockout mice

Knockout/ mutant gene	**NK-cell development / function**
IL-15	Deficient NK, NKT, memory $CD8^+$ T cell, decreased IEL TCR$\gamma\delta$ and CD8$\alpha\alpha$
IRF-1 (lack inducible IL-15)	Deficient NK cells lacking NK cytolytic effector function, deficient NKT and IEL CD8$\alpha\alpha$
IL-2	Phenotypically and functionally normal NK cells
IL-7	Phenotypically and functionally normal NK cells
IL-12p40	Normal number of NK cells; reduced NK cytotoxicity; decreased IFN-γ production induced by LPS
IL-18	Similar to IL-12p40-deficient mice
IL-15Rα chain	Absent NK cells, deficient in NKT, IEL $\gamma\delta$ T cells, peripheral $CD8^+$ T cells
IL-2/15Rβ chain	Deficient in NK, NKT and IEL $\gamma\delta$ T cells
γ-common chain	Deficient in NK, NKT and $\gamma\delta$ T cells, T and B cells deficient
Jak3	Deficient NK, and IEL $\gamma\delta$ T cells, T and B cells deficient
STAT5a	Deficient NK cells
STAT5b	Deficient NK cells with proliferation and cytotoxicity defects

Abbreviations: IRF-1 interferon regulatory factor 1: IEL intra-epithelial lymphocytes

Adapted from Liu *et al.* 2000 and Fehniger *et al.* 2001.

1.4. Immune response to herpes family viruses: cells of the innate and adaptive immune response

1.4.1. The anatomy of HSV infection - introduction

Herpes simplex viruses (HSV) are large and comprise a central core containing a linear, double stranded deoxyribonucleic acid (DNA) genome that encodes more than 70 gene products (Roizman *et al.* 1996). Surrounding this DNA core is a capsid containing the glycoprotein components, which are essential for entry into the host cell (Irurzun *et al.* 1997). Enveloped virus forms a low-affinity attachment with host cells followed by the interaction of multiple receptors with increasing affinity (Irurzun *et al.* 1997; Montgomery *et al.* 1996). These interactions are cell type specific, perhaps accounting for preferential infection of various tissues by different viral strains. Membrane fusion allows viral particles to enter into the cytosol, and, following entrance into the nucleus, three sets of viral proteins termed α, β, and γ regulate the course of viral replication (Roizman *et al.* 1996). In a process that is not completely resolved, mature virus is released from cells by budding from the plasma membrane. Cells that have completed the lytic cycle are destined to die. Primary HSV infection in humans usually occurs early in life, often without overt clinical manifestations. HSV infects epithelial cells in the mucosa or skin, then enters peripheral nerve endings and travels intraaxonally to the sensory ganglia (Wildly *et al.* 1982). It is in neurons that the virus establishes a latent infection, from which there may be periodic reactivation. Recurrent herpetic disease results from reactivation of HSV from latency in sensory neurons and axonal transport to the periphery. NK cells play a critical role in control of viral replication through their cytotoxic potential and secretion of anti-viral cytokines (Tanigawa *et al.* 2000; Karupiah *et al.* 1993). During the primary infection, a strong immune response arises composed of neutralizing antibodies and anti-viral CD4 and CD8 T cell response, which efficiently inhibits virus replication at mucosal sites and in the nervous system (Daheshia *et al.* 1998). Latency is the principal strategy used by the virus to evade host immune defences and for persisting indefinitely in the host. However other evasion strategies also exist during the primary infection and after reactivation and/or recurrence. By infecting the nervous system, the virus takes advantage of natural anatomical barriers to evade

immune defence. These include intraaxonal transport of virus particles, which renders virus invisible to antibody and cell mediated immune mechanisms, and the natural deficiency of MHC class I molecules on neurons which limits the activity of cytotoxic T cells. Using mouse models of HSV infection it has been shown that the initial stages of HSV infection are influenced by the activity of type I IFNs and NK cells, which serve to limit the spread of the virus to the nervous system (Tanigawa *et al.* 2000; Karupiah *et al.* 1993). As the adaptive immune response evolves, there is a clear role for T cells in the resolution of primary infection (Preston *et al.* 2000; Nash, 2000). Aside from the activities of CD4 and CD8 T cells as interrupters of virus spread, other powerful inhibitors of this process are neutralizing antibodies (Nash *et al.* 1987). The virus becomes a target for neutralizing antibodies as it moves from nerve endings to epithelial cells or *vice versa*. However once infection of epithelial cells takes place, then neutralizing antibodies are ineffective. In this instance T cell immune responses prevail. Resolution of primary infection in sensory ganglia is dependent on the presence of $CD8^+$ T cells (Simmons *et al.* 1992). These cells do not mediate cytotoxicity against infected ganglia but rather eliminate virus from the relevant cells. $CD8^+$ T cells have been shown to have a role in blocking reactivation of HSV from latency (Liu *et al.* 2001). IFN-γ may be a key antiviral mechanism in the resolution of HSV infections.

1.4.2. The anatomy of EBV infection

EBV was originally isolated in association with an unusual lymphoma described in children in West Africa (Burkitt 1962). Subsequently it was shown to be the causative agent in infectious mononucleosis (IM), and to be a B-cell-transforming virus that infects almost everyone (Diehl *et al.* 1968). EBV is a herpesvirus comprising a DNA genome in a protein core. One of the envelope glycoproteins, gp350/220 initiates the replication cycle by binding to the complement receptor type 2 (CR2 or CD21) on resting B cells (Frade *et al.* 1985). This binding triggers endocytosis of the virion into smooth walled vesicles and may have a role in the triggering of viral promotor during latency (Tanner *et al.* 1987; Sugano *et al.* 1997). Sucessful infection of B cells requires additional binding of viral glycoprotein to the polymorphic human leukocyte antigen HLA-DR (Li *et al.* 1997). Once inside the cell, the virus is uncoated and viral DNA

enters the nucleus where it forms a circularized extrachrosomal episome (Alfieri *et al.* 1991). The B cell is stimulated to divide and once transformation has occurred the number of episomes within the cell is amplified. Since no new virus is produced from the cell this is a true state of latency. However it is a very active form of latency that allows the virus to manipulate the cell. Through the expression of EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs) this virus can upregulate receptors, adhesion molecules, cytokines and apoptotic inhibitors (Hutt-Fletcher 2000). The lytic cycle has not been well defined due to the bias towards latency and the difficulty in inducing productive replication *in vitro*. Entry into the lytic cycle has been shown to occur in a small sub-population of B cells *in vitro*. This cycle is presumed to follow the general pattern of all herpes viruses with early genes controlling DNA synthesis and later genes encoding structural proteins (Kieff, 1996). While EBV has been shown to infect many cell types, including T cells and epithelial cells, there is no evidence for EBV transformation of T cells *in vitro* (Weiss *et al.* 1988). Infection of T lymphocytes has been demonstrated in relatively uneventful classic IM (Anagnostopoulos *et al.* 1995). In a preponderance of more severe cases the majority of EBV-infected peripheral blood cells have been found to be of T-cell origin. EBV-infected T cells have been demonstrated in VAHS with and without XLP. In some cases the proliferations were polyclonal, in others EBV-positive T cell lymphomas were identified (Kanegane *et al.* 1998; Su *et al.* 1994).

IM is the principal disease associated with primary EBV infection. Childhood infection is usually asymptomatic, however a third of infections occuring in adolesence or adulthood results in clinical IM (Niederman *et al.* 1970). In addition to virus specific antibodies, heterophile and autoimmune antibodies can be detected, these are probably produced by virus infected cells (Garzelli *et al.* 1984). Using sensitive PCR methods, infection of B cells with EBV has been demonstrated at between 1 and 10 per 10^6 cells in normal seropositive individuals (Miyashita *et al.* 1995). Whereas in acute primary infection the incidence of B cell infection is between 1 in 10^2 or 10^3 cells (Svedmyr *et al.* 1984). Clinical symptoms include fever, cervical lymphadenopathy, and exudative tonsillitis. The spleen is frequently enlarged and liver function tests may be abnormal. In a typical infection, more than 60% of circulating lymphocytes will be $CD8^+$ with an

activated phenotype (Uehara *et al.* 1992). A proportion of these may be expanded as a result of bystander activation with selective expansion of a few $CD8^+$ EBV-specific clones (Lynne *et al.* 1998). The clinical course usually runs for 2 to 3 weeks but may be prolonged for several months. As outlined above a small number of fatal cases of immunoblastic lymphomas occur predominantly in the context of immunodeficiency or immunosuppression (Purtilo *et al.* 1985; Craig *et al.* 1993).

1.4.3. Monocytes / macrophages – central role in innate and adaptive immunity

The term histiocyte embraces cells of the monocyte / macrophage series and Langerhans cell / dendritic cell series (Steinman *et al.* 1991). Both macrophages and Langerhans / dendritic cells arise from bone marrow $CD34^+$ stem cells, probably under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and TNF-α (Caux *et al.* 1992, 1993). The inclusion of Birbeck granules in Langerhans cells serves to distinguish these two cell types (Cline 1994). Macrophages develop from stem cells, which are commited to granulocyte and macrophage differentiation. These stem cells give rise to promonocytes that mature, in the bone marrow, to monocytes. After briefly circulating in the blood they enter the tissues to complete maturation (Cline 1994). Functionally monocytes and macrophages are 'professional phagocytes' that defend against microorganisms and rid the body of unwanted organic and inorganic particles by means of phagocytosis (Cline, 1994). In response to viral infection macrophages synthesize cytokines and bioactive mediators that have direct anti-viral activity, these include IFN-α/β, TNF-α, and nitric oxide (Laskin *et al.* 1995). Macrophages also produce cytokines that have indirect immunomodulatory functions including IL-1, IL-6, IL-12, IL-15, and IL-18, as well as the anti-inflammatory cytokines such as IL-10 (Heufler *et al.* 1992; Alleva *et al.* 1997; Dinarello, 1999; Laskin *et al.* 1995; Carson *et al.* 1995). Thus in response to various stimuli macrophages have a central role in the control of, and in response to, other cells of the innate and adaptive immune system through the cytokine profile produced. Cytokines such as IL-12, IL-15 and IL-18 exert their anti-viral effects through IFN-γ production and enhancement of NK and T cell proliferation and cytotoxicity (Trinchieri 1994; Trinchieri 1995; Carson *et al.* 1995;

Okamoto *et al.* 1999; Tomura *et al.* 1998). Combinations of macrophage and T cell derived cytokines such as IL-12 together with IL-2 have been shown to selectively expand NKR^+ T (Satoh *et al.* 1996). This selective expansion of particular cell types in response to cytokines may have a profound effect on clearence of infection. Macrophages also function as one of the 'professional' antigen presenting cells (APC). In order to initiate an immune response APCs present pathogen derived proteins in association with products of the major histocompatibility complex (MHC). Macrophages also play an important role in the recognition and clearence of apoptotic cells (Aderem *et al.* 1999). Apoptosis, or programmed cell death, is a process in the development of all multicellular organisms (Savill. 1997). Phagocytes efficiently dispose of enormous numbers of cells undergoing apoptosis due to continual turnover of tissue (Savill. 1997). Recognition of apoptotic cells is thought to be mediated by receptors on phagocytes that are specific for ligands not present on healthy cells (Aderem *et al.* 1999). Receptors that participate in phagocytosis of apoptotic cells by macrophages include scavenger receptors and CD14 (Aderem *et al.* 1999). A notable feature of this procedure *in vitro* is the absence of an inflammatory response (Fadok *et al.* 1998).

Class I-restricted antigen presentation is linked to the biosynthesis and intracellular trafficking of MHC molecules. Most nucleated cells transcribe and express class I genes. The class I heavy chains, together with the β-2 microglobulin light chains, are inserted into the endoplasmic reticulum (ER) (Williams *et al.* 1989). Cytosolic proteins targeted for destruction are attached to ubiquitin and rapidly destroyed by proteasome to yield peptides of 8 to 12 residues (Ciechanover. 1994). Binding of these short antigenic peptides is required for stability of MHC class I-complex. The peptides are translocated by an MHC-encoded transporter associated with antigen processing (TAP) (Shepherd *et al.* 1993). This transporter is physically linked to the class I molecule through the ER-resident protein tapasin, an arrangemant that facilitates peptide loading (Ortmann *et al.* 1997). Once loaded with peptide the stable class I complexs are released from the ER. They enter the secretory pathway and are displayed on the cell surface. Most class I molecules on the cell surface will be occupied by peptides derived from the cell's own proteins. Pathogen-derived peptides will be displayed in association with class I only

when these proteins gain access to the cytosol (Pamer *et al.* 1998). In addition, the uptake of dying infected cells by professional APCs has been shown to play a critical role in the priming of pathogen specific $CD8^+$ T cells (den Haan *et al.* 2001). A number of studies have shown that apoptotic infected cells efficiently provide antigen for cross-presentation. This cross-priming represents an antigen processing pathway overlap between cytosolic and phagosomal antigen (Pamer *et al.* 1998). The host benefits in those cases where the pathogen does not infect the APC, as well as where the pathogen inhibits presenting function after infection (den Haan *et al.* 2001).

The MHC class II-restricted pathway of antigen presentation is involved with proteins that are internalized by endocytosis. Peptide fragments are generated from these proteins by the action of endosomal and lysosomal proteases. An accesory molecule, the invariant chain Ii, delivers the MHC class II proteins to the endocytic pathway. The invariant chain is itself destroyed by proteases to yield a Ii remnant called CLIP (Chapman. 1998). Peptide loaded class II molecules are then transferred to the cell surface for recognition by CD4 cells (Pieters. 1997).

1.4.4. Viral immune escape strategies targeting the MHC class I molecules

Viral infections are eliminated by both the innate and adaptive cellular immune responses. To escape immune attack viruses have evolved a host of countermechanisms (Ploegh 1998; Tortorella *et al.* 2000). Viruses target the MHC class I antigen presentation pathways enabling them to escape recognition by CTL. Adenoviruses, EBV, HSV, HIV and CMV encode genes inhibiting the MHC class I-dependent antigenic presentation via different inhibitory strategies (Fruh *et al.* 1999). The downregulation of MHC class I expression by some viruses may be a mode of evasion of cytotoxic T cells of the adaptive immune response (Ploegh 1998). In addition down regulation of MHC class I together with expression of MHC homologues, such as UL18, by CMV and molluscum contagiosum virus have been put forward as another viral strategy to escape NK cell recognition (Chapman *et al.* 1998; Senkevich *et al.* 1998; Karre *et al.* 1997). HSV down-regulates peptide MHC class I expression via a small

cytoplasmic protein, ICP47, which inhibits peptide binding to TAP, thus inhibiting access of peptides to empty MHC class I molecules (Fruh *et al.* 1995). Consequently, MHC class I molecules remain empty in the endoplasmic reticulum (ER) or are transported as unstable complexes to the cell surface. By contrast human CMV uses multiple molecules which are expressed sequentially during infection (Ahn *et al.* 1996). One of these evasion molecules, US11 induces reverse translocation of MHC class I molecules from the ER to the cytoplasm and destruction of newly synthesized MHC class I heavy chains (Wiertz *et al.* 1996). Another is the glycoprotein US3 which retains newly synthesized MHC class I heavy chains in the ER (Jones *et al.* 1996).

The initial observation that the MHC homolog UL18 of human CMV blocked NK activity by triggering CD94/NKG2A was called into question by the finding that UL18 could enhance NK mediated killing (Reyburn *et al.* 1997; Leong *et al.* 1998). The role of MHC downregulation in triggering NK cytotoxicity remained unresolved. However a recent study has shown that HLA-C downregulation by HSV and CMV infected cells resulted in NK mediated lysis of the infected cells. Thus demonstrating a role for KIR in the recognition of virally infected cells (Huard *et al.* 2000a). Additionally, in mice, Klra8 (formerly Ly49H) stimulatory receptor has been shown to be essential for resistance to murine CMV (Lee *et al.* 2001). Another NK-complex locus *Rhs 1*, has been linked to control of herpes simplex virus infection in mice (Pereira *et al.* 2001).

1.4.5. Common role of effector mechanisms

Many of the effector mechanisms employed by cells of the immune system are non-exclusive to one cell type but are weapons in the arsenal of many cell types. In common with NK and $CD8^+$ T cells, at least some MHC class II-restricted $CD4^+$ T cells express perforin and /or FasL and are capable of cytolysis (Nakata *et al.* 1992; Yasukawa *et al.* 2000). IFN-γ is produced by Th1-type $CD4^+$ T cells, as well as NK cells, γδ-T cells and $CD8^+$ T cells (Gosselin *et al.* 1999; Biron *et al.* 1999; Butcher *et al.* 1996; Harty *et al.* 2000). TNF is produced by many cell types, and has a variety of effects on cells expressing one or both of the TNF receptors, ranging from activation to death (Ashkezai *et al.* 1998). Most of these molecules also play regulatory roles ranging from T cell homeostasis to enhancement of antigen presentation. The exact role of each cell type

and its' specific effector mechanism arsenal in response to infection is therefore difficult to identify in humans. Studies with gene knockout- and cell deficient-mice go some way to elucidating these individual roles, however the translation from mouse to human is always challenging.

1.4.6. Mechanisms of cytotoxicity - Perforin

Perforin mediated lysis is one of the major cytolytic effector functions in $CD8^+$ cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Clement *et al.* 1990; Podack. 1991; Kagi *et al.* 1994a; Yasukawa *et al.* 2000). Studies in perforin gene knockout mice have shown that perforin is critical in the clearence of some, but not all non-lytic viruses (Kagi *et al.* 1996). This 65 KDa protein, with sequence homology to complement components C6 to C9 is stored in cytoplasmic granules. Upon granule release in the presence of Ca^{++}, perforin monomers insert into the plasma membranes of target cells and polymerize into pore-forming aggregates (Liu *et al.* 1995). Shown schematically in figure 1.5, pores of perforin lead to osmotic lysis of the target cells, and allow granzyme to enter the target and induce apotosis (Shresta *et al.* 1998; Heusel *et al.* 1994). Granzyme B (GrmB) activates the caspase cascade in target cells and induces apoptosis associated with rapid DNA fragmentation (Heusel *et al.* 1994). Granzyme A (GrmA) induces DNA fragmentation in a caspase independent fashion and with delayed kinetics compared to GrmB (Shresta *et al.* 1999). Studies in perforin and granzyme deficient mice indicate that perforin, delivered by $CD8^+$ T cells, is not sufficient for cell lysis and that perforin-induced membrane alterations may be repaired in the absence of granzyme (Shresta *et al.* 1999).

The role of perforin as an effector in target cell lysis by CTLs and NK cells *in vitro* has been confirmed by studies in perforin deficient mice (Kagi *et al.* 1999; Kagi *et al.* 1994b). In addition perforin may be important in immune regulation through activation induced cell death (AICD) of CTLs and lymphocyte homeostasis (Spander *et al.* 1999; de Sainte Basile *et al.* 2001). Perforin has been shown to have a role in the regulation of antibody production, control of autoimmunity, and regulation of CTL responses to some viral infections (Spander *et al.* 1999; Stepp *et al.* 2000). As has been previously stated,

deficiency of perforin demonstrated in some FHL patients established perforin as an important regulator of the human immune response (Stepp *et al.* 1999). Several cytokines, including IL-2, IL-6, and IL-7 have been shown to enhance perforin expression in different systems (Smyth *et al.* 2001). Schematically illustrated in figure 1.4, the upregulation of perforin is mediated by the transcription factors STAT proteins which transfer signals generated by cytokine receptors from the cell surface to the nucleus (Yu *et al.* 1999). STAT proteins direct gene regulation by binding to a *cis*-acting STAT binding site (Darnell, 1997). Cytokines acting via STATs are therefore important in the regulation of NK and cytotoxic T cell lytic activity. While resting $CD8^+$ $\alpha\beta$ T cells have been shown to upregulate perforin mRNA expression, NK and $\gamma\delta$ T cells express significant levels of perforin mRNA constitutively and this cannot be further upregulated by IL-2 (Koizumi *et al.* 1991; Nakata *et al.* 1992).

1.4.7. Mechanisms of cytotoxicity - Fas/Fas ligand

Fas (CD95) is a member of the TNF receptor family containing an intracytoplasmic 'death domain' and when crosslinked with Fas ligand (FasL) (figure 1.5) transduces apoptotic signals into cells via the activation of the caspase enzyme cascade (Nagata *et al.* 1995; Cohen *et al.* 1997). Fas is expressed on immature thymocytes, activated T cells, and non-lymphoid cells in liver, ovary and heart (Drappa *et al.* 1993). FasL may be constitutively expressed or induced upon interaction with target cells (Berke. 1994). Engagement of TCR/CD3 complex on T cells induces increased FasL and most $CD4^+$ T cell-mediated cytotoxicity is Fas/ FasL mediated (Vignaux *et al.* 1995; Stalder *et al.* 1994). NK cells express mRNA for FasL, however induction of functional FasL requires stimulation with cytokines or CD16 crosslinking (Arase *et al.* 1995; Zamari *et al.* 1998; Oshimi *et al.* 1996). While perforin is the major lytic mediator of lymphokine activated killer (LAK) cells, FasL also play a significant role in short term killing assays (Lee *et al.* 1996).

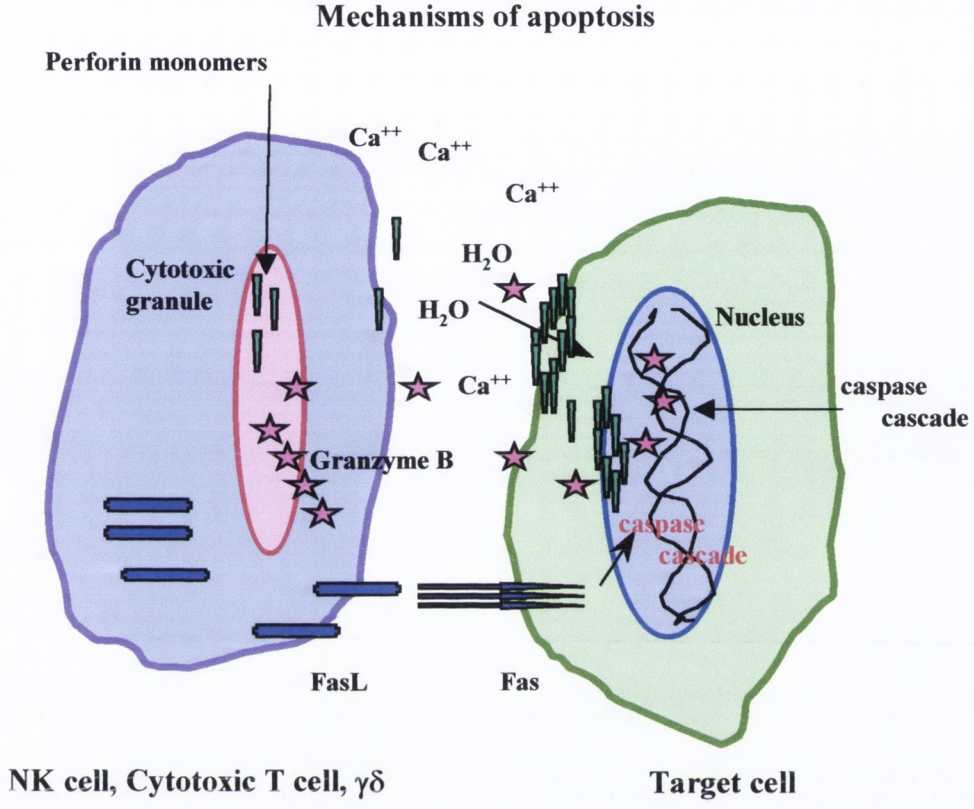


Figure 1.5. Mechanisms of apoptosis mediated by perforin/granzyme and Fas-Fas Ligand

Cytotoxic granules in NK and CTL cells contain perforin monomers ( ) and granzymes ( ) Surface expression of Fas ligand (FasL) ( ) can be upregulated on cytotoxic cells. In the presence of Ca^{++} perforin polymerizes and allows entry of granzyme A and B into the target cell. Granzyme A induces apoptosis in a caspase independent fashion. Both Fas-FasL interactions and granzyme B meidate apoptosis via the caspase cascade.
Illustration adapted from Kagi *et al.* 1996

1.4.8. Cytokines in resistance to viral infections

The body responds to viral infection by rapidly producing cytokines such as IFN-α / β (Biron *et al.* 1999). These cytokines are produced by most cell types and serve to limit viral spread via non-cytolytic pathways which interfere with steps in the viral life cycle. However once viral infection is established, clearance of virus can requires additional functions of the immune response. Destruction of infected cells by cytolytic effector cells of the innate (NK) or adaptive immune system (CTL) is often demonstrated *in vitro* and the accepted dogma is that this function is required for complete clearence of infected cells. However experimental evidence would indicate that the production of cytokines such as IFN-γ and TNF-α exerts a more important antiviral influence (Biron *et al.* 1999; Guidotti *et al.* 2001).

IFN-γ and TNF-α work by purging virus from infected cells noncytopathically using the cells own anti-viral mechanisms. The direct anti-viral effects of IFN-γ have been attributed to the transcriptional induction of genes for enzymes that influence cellular protein synthesis, namely RNA activated protein kinase, 2'-5' oligoadenylate synthetase and dsRNA specific deaminase (Boehm *et al.* 1997). These enzymes act *via* a variety of mechanisms to inhibit cellular as well as viral protein synthesis. The curing of hepatocytes of viral hepatitis illustrates this anti-viral activity. Hepatitis, resulting from infection with hepatitis B virus (HBV) is thought to occur through the destruction of infected hepatocytes by MHC class I-restricted $CD8^+$ T cells (Chisari 1997). Essentially all hepatocytes are infected, however virus can be eliminated with minimal hepatocyte lysis (Guidotti *et al.* 1996; Guidotti *et al.* 2001). Using perforin deficient mice, HBV specific $CD8^+$ T clones were shown to eliminate viral genome expression with no discernible hepatocyte destruction (Harty *et al.* 2000). The ability of wild-type $CD8^+$ T cells to inhibit viral infection was abrogated by neutralization of both IFN-γ and TNF indicating that these cytokines are the essential mediators of viral elimination (Guidotti *et al.* 1996). Murine gamma herpes 68 (MHV-68), the mouse model of human EBV infection, also illustrates the essential role of cytokines and cytokine receptors in control and elimination of viral infection (Ehtisham *et al.* 1993). IFN-γ deficient mice cleared lung infection and developed latent B cell infection in a manner only slightly less

effective than wild-type mice. However IFN-γ receptor deficient mice failed to clear lung infection and establishment of chronic infection was delayed (Dutia *et al.* 1997). This discrepancy in the nature of infection in IFN-γ and IFN-γ receptor-deficient mice is potentially interesting and may suggest the existence of alternate ligands for IFN-γ receptor. Herpes simplex virus infection has been shown to be more virulent in IFN-γ receptor-deficient mice than in IFN-γ -deficient mice (Cantin *et al.* 1999). Interestingly in humans impaired IFN-γ-mediated immunity have been shown to result in susceptibility to mycobacterial and salmonella infections rather than viral infections (Dupuis *et al.* 2000; Dupuis *et al.* 2001; Picard *et al.* 2002)

The nature of the local inflammatory exudate may also be influenced significantly by the cytokine mileau. IFN-γ and TNF in synergy with IL-1 from macrophages, activate transcription factors leading to induction of proinflammatory and immunomodulatory genes (Boehm *et al.* 1997; Tartaglia *et al.* 1992). These mediators induce upregulation of Class I and II expression, increase oxidative burst in phagocytes, and induce chemokines and chemoattractants (Boehm *et al.* 1997). Chemokines mediate the local formation of inflammatory exudates enriched in monocytes and activated T cells, with relatively few granulocytes (Issekutz *et al.* 1993). As has been stated previously, macrophage derived IL-12, IL-15 and IL-18 influence IFN-γ production (Trinchieri *et al.* 1994; Trinchieri. 1995; Carson *et al.* 1995; Okamoto *et al.* 1999; Tomura *et al.* 1998). However using human herpes virus infection as a model, only IL-15 has been shown to play an essential role in the subsequent production of IFN-γ by T and NK cells (Gosselin *et al.* 1999). Thus IL-15 may indirectly orchestrate the nature of the local inflammatory exudate in response to infection with the human herpes family of viruses.

1.4.9. Pathogen specific $CD8^+$ T cell responses

$CD8^+$ T cells are important mediators of adaptive immunity against certain intracellular infections with viral, protozoan, and bacterial pathogens. Clonal expansion of antigen specific $CD8^+$ T cells takes place when the TCR interacts with pathogen derived

peptides presented by MHC class I molecules on professional antigen presenting cells (APC) (Germain 1994). APC such as dendritic cells (DC) are capable of stimulating naïve T cells by virtue of their expression of co-stimulatory molecules (Banchereau *et al.* 1998). Priming of $CD8^+$ T cells occurs in response to antigens that gain access to the cytosol of APC and are processed by the endogenous MHC class I presentation pathway (Pamer *et al.* 1998). Thus $CD8^+$ T cells are activated in response to cytosolic infections with viruses, intracytoplasmic bacteria, and protozoa. $CD8^+$ T cells may also be cross-primed by antigen from infected cells which have been ingested by phagocytes (Pamer *et al.* 1998).

Proliferation and instigation of cytolysis and cytokine expression develops over time after initial stimulation of naïve cells. In the case of memory cells, mobilization of effector mechanisms is very rapid (Lalvani *et al.* 1997). Expression of NK associated receptors such as 2B4 may have a role in the control of proliferation (Kambayashi *et al.* 2001). $CD8^+$ T cells elaborate cytokines, including IFN-γ and TNF as well as chemokines that function to recruit and/or activate the microbiocidal activities of effector cells such as macrophages and neutrophils (Harty *et al.* 2000). As well as clearence of infection, $CD8^+$ T cells may also participate in mediating pathology through its arsenal of cytotoxic mediators. This dual role is illustrated by the findings that perforin-dependent, $CD8^+$ T cell-mediated cytolysis contributes to both the clearence of virus and clinical symptoms of central nervous system (CNS) disease in Theiler's virus infected mice (Kagi *et al.* 1994b; Dethlefs *et al.* 1997). Interestingly β-2 microglobulin deficient mice infected with Theiler's virus develop persistent CNS infection and demyelination but no overt CNS disease (Rodriguez *et al.* 1993). In the case of infection with murine gamma herpes 68 (MHV-68), the mouse model of human EBV infection, lytic mechanisms mediated by perforin do not appear to be important in clearence of acute lung infection or control of latently infected B cells (Usherwood *et al.* 1997). While $CD8^+$ T cell have an important role in elimination or control of latency in MHV-68 infection these activities are mediated through cytokines which are not exclusively produced by one cell type (Ehtisham *et al.* 1993). Similarly, in perforin deficient mice, HBV virus can be eliminated from hepatocytes without lysis of infected cells (Chisari 1997).

1.4.10. Pathogen specific $CD4^+$ T cell responses

The memory / effector subset of $CD4^+$ T cells comprise a heterogeneous group which play important roles in the induction and regulation of immune responsiveness (Ahmed *et al.* 1996). $CD4^+$ T cells orchestrate an acquired immune response by promoting intracellular killing by macrophages, antibody production by B lymphocytes, and clonal expansion of cytotoxic T lymphocytes. Cellular activation is triggered when the complex of peptides, derived from the breakdown products of endocytosed pathogens, and MHC class II molecules are presented to the T cell receptor (TCR) on $CD4^+$ T cells. Their functional heterogeneity allows for flexibility in response to diverse antigenic challenges. $CD4^+$ T cells co-ordinate immune responses against viruses and other pathogens via antigen-induced secretion of effector cytokines (Paul *et al.* 1994; Butcher *et al.* 1996; Emoto *et al.* 1999).

1.4.11. NK and NKR^+ T cells in viral infection – the role of IL-15

Murine studies have demonstrated the important role of NK cell cytotoxicity and IFN-γ production particularly in early response to infection (Biron *et al.* 1999). The contribution of NK cells to defense against human viral infections is supported by data from natural infections. Low NK cell cytotoxicity has been linked with increased human sensitivity to severe disseminating herpesgroup virus infections including those with human HSV, EBV in XLP, and CMV in Chediak-Higashi syndrome (Biron *et al.* 1989; Merino *et al.* 1986; Quinnan *et al.* 1982). However, as has been outlined above in the case of XLP and Chediak-Higashi syndrome, susceptibility to particular viral infections may be linked to defects affecting signalling or cytotoxic mediator release in both NK and T cells (Parolini *et al.* 2000; Barrat *et al.* 1996). In the case of severe disseminating herpes infection in a patient lacking NK cells there was no demonstrable IL-2 induced cytotoxic function (Biron *et al.* 1989). This finding may denote absence of both NK and T cells capable of IL-2 induced killing.

Studies in perforin deficient mice have demonstrated that NK cell cytotoxicity is induced during many viral infections, however this function accounts for only a proportion of the anti-viral effects mediated by NK cells (Kagi *et al.* 1996). Cytokines and chemokines produced by NK cells have an important role in anti-viral defence (Biron *et al.* 1999). NK cell IFN-γ production has been demonstrated in murine CMV (MCMV) and influenza virus infection (Orange *et al.* 1996a and b; Monteiro *et al.* 1998). During viral infection, NK cell IFN-γ production is dependent on virus induced IL-12 (Orange *et al.* 1996a). IL-15 has been shown to have a pivotal role in human natural killer cell responses to many viral infections. Upregulation of NK cytotoxicity in response to infection with, among others, HSV, EBV and influenza virus has been shown to be abrogated only by monoclonal-antibodies (mAb) to IL-15 with antibodies to other cytokines (IL-2, IL-12, IFN-γ and TNF-α) showing no observable effects (Gosselin *et al.* 1999; Fawaz *et al.* 1999; Flamand *et al.* 1996). Studies with human herpes virus infected (HSV and EBV) cells have shown that virus induced IL-15 triggers the synthesis of IFN-γ from both $CD4^+$ and NK cells (Gosselin *et al.* 1999). IFN-γ acts in both an autocrine and paracrine fashion to modulate NK anti viral-activity. (Gosselin *et al.* 1999). Thus IL-15 may indirectly orchestrate the nature of the local inflammatory exudate in response to infection with the human herpes family of viruses.

1.5. AIM

This study was designed to identify the deficiency that resulted in recurrent infection and haemophagocytic lymphohistiocytosis in these two patients. To do this we first examined the phenotype and function of freshly isolated lymphocyte populations. The two young patients lacked NK cells and had reduced $CD8^+$ T cells at presentation. They both suffered from recurrent infections most notably with *Herpes* family viruses. Studies of IL-15 and its receptor complex were undertaken in the light of the pivotal role of this cytokine / receptor in the development and function of NK and cytotoxic T cells, including NKR^+ T cells. In addition IL-15 has been shown to be important in control of infection with *herpes* family viruses through its role in IFN-γ induction.

Flow cytometry, using three and four colour staining, was used to identify the lymphocyte populations and to look at natural killer associated receptor expression. Humoral immunity, including specific IgG, was examined in order to establish the efficacy of B cell function. Proliferation in response to mitogens was used to confirm T cell function. Natural cytotoxicity was assessed using K562 and Daudi cells as targets in chromium release assays. These studies indicated that both patients were deficient in NK and NKR^+ T cells and natural cytotoxicity.

mRNA expression for the NK associated NKG2A receptor was measured for patient A and adult controls in order to establish whether the patient expressed this NK associated receptor.

Studies were carried out into the role of IL-15 and its receptors in normal controls and these two patients. We examined the expression of IL-15 and its specific receptor IL-15Rα in both these patients using PCR and sequencing studies. Flow-cytometry with intracellular staining was used to demonstrate IL-15 in monocytes from patient A. Upregulation of IL-15 mRNA in response to stimulation was examined using TaqMan^{TM} PCR. Having established that they both expressed mRNA for IL-15 and IL-15Rα, we then went on to investigate the effect of this cytokine on the cells deficient in both these patients, namely the NK and NKR^+ T cells. To do this we examined the

effects of culture with IL-2 and IL-15 on the cytolytic function, natural killer receptor expression, CD25 and CD122 receptor expression, cytokine production, perforin expression and proliferation of NK and NKR^+ T cells in healthy controls. Studies into the effect of culture with IL-15 or IL-2 on CD25 and CD122 receptors were carried out for both patients. Due to restricted access to samples from patients A, the cytolytic function, NKR expression and proliferation of NK and NKR^+ T cells in response to IL-15 only were examined. With the exception of cytokine production, all other studies carried out on cells from healthy controls were carried out on PBMC from patient B.

While the clinical and laboratory findings in HLH have been linked to the biological effects of several inflammatory cytokines (Henter *et al.* 1991b; Takada *et al.* 1999), and deficiency of lymphocyte cytotoxicity (Stepp *et al.* 1999), the role IL-15 and NKR^+ T cells has not been investigated.

2. Materials and Methods

2.1. Four colour staining and analysis for T cell subsets, B and NK cells

2.1.1. Reagents for four colour lymphocyte subset analysis

MultiTEST™ reagents (BD) incorporating fluorescein isothiocyanate (FITC) labelled monoclonal antibodies (mAb) specific for CD3, phycoerytherin labelled mAb (PE) to CD8, peridin chlorophyll protein (PerCP) labelled mAb against CD45 and allophycocyanin (A-PC) labelled mAb to CD4 with a second reagent incorporating anti-CD3-FITC, CD16+56-PE, CD45-PerCP and CD19-A-PC mAbs, were purchased from Becton Dickinson (BD, Oxford, UK). These reagents were used in conjunction with TruCOUNT™ (BD, Oxford, UK) tubes, beads in these tubes were used to calculate the number of cells per ml of sample.

2.1.2. Staining and flow cytometry for T, B and NK cells

Four colour staining for T, B and NK cells was carried out according to the manufacturer's instructions. Briefly, 20µl of antibody (Multitest™ BD, Oxford, UK) was incubated with 50µl of cells at $1x10^6$/ml or EDTA whole blood. After 15 minutes incubation 450µl of FACSlys™ solution (BD, Oxford, UK) (buffered diethylene glycol) was added to lyse erythrocytes. After a further 15 mins analysis was carried out using Multimate™ software (BD, Oxford, UK) for lymphocyte subsets on a FACSCalibur (BD, Oxford, UK) flow cytometer. Multimate software automatically selected lymphocytes on the basis of forward and side scatter as well as CD45 expression. 2000 events were analysed through this gate for CD3, CD4, CD8 and CD3, CD16+56 and CD19 expression. T cells were identified as $CD3^+$, B cells as $CD3^-CD19^+$ and NK cells as $CD3^-CD16/CD56^+$. Results were expressed as a percentage of the lymhocyte population and cells per ml.

2.2. NK and IL-2 induced cytotoxicity

2.2.1. PBMC separation

PBMC were separated from whole heparinised blood using centrifugation on Ficoll Hypaque gradient (Lymphoprep, Nycomed). After washing with Hanks Balanced Salt Solution (Gibco BRL) PBMC were resuspended in RPMI 1640 (Gibco BRL) supplemented with 10% pooled normal human serum, 1mM L-glutamine, penicillin 100 U/ml and streptomycin 100 mg/ml (medium).

2.2.2. Labelling of targets K562 and Daudi cells

Target K562 cells (an erythroleukemic line) were labelled for 1 hour with 100 mCi ^{51}Cr/ $1x10^6$ cells, washed in cold medium with 10% pooled human serum and diluted to a concentration of 4 x 10^4 cells per ml.

2.2.3. Killing of K562 and Daudi cells

For K562 killing, effector cell fractions were plated with targets at 50:1, 25:1, 12.5:1, 6:1 and 3:1: each ratio was carried out in triplicate. Spontaneous and maximum lysis were obtained by plating target cells with medium and a detergent, saponin (Sigma), respectively. Plates were centrifuged at 25g for 3 minutes and then incubated for 4 hours at 37°C in a 5% CO_2 atmosphere. They were then centrifuged at 380g for 10 minutes, and the supernatants from each well were analysed using a gamma counter for quantification of ^{51}Cr release. Percentage specific cytotoxicity was calculated using the mean counts per minute (cpm) in the following formula:

$$\frac{\text{CPM test - CPM spontaneous lysis}}{\text{CPM maximum lysis - CPM spontaneous lysis}}$$

For the lymphokine activated killing (LAK) assay Daudi cells were used as the target and PBMC were cultured for three days in medium containing 100 ng/ml IL-2 (R&D Systems).

2.3. Cell proliferation assays

2.3.1. Proliferation of PBMCs in response to mitogens Con A and PHA

Proliferation studies were carried out for both patients and a healthy control. PBMC were resuspended in medium at $1x10^6$/ml and 200 µl of this suspension was added to each well of a flat-bottomed microtitre plate (Nunc). Concanavalin A (ConA) (Sigma, UK) was added at 10 ng/ml and phytohaemagglutinin (PHA) (Sigma, UK) was added at 10 µg/ml to triplicates of cells and the plates were incubated in CO_2 at 37°C for 48 hours. The cell suspensions were pulsed with 0.3 µCi tritiated (^3H) thymidine (Amersham, Buckinghamshire, UK) per well and the plates were incubated for a further 16 hours in the same conditions as above. The content of each well was then harvested onto glass fibre filters (Denley Wellwash, Inotech) using a cell harvester (Inotech) fixed with methanol and dried at 70°C. Each filter was placed into a scintillation vial (Wheaton Scientific) containing 1 ml of scintillation fluid (Optiscint, HISAFE) and these were read using a 1409 DSA liquid scintillation counter (Wallac). The average count per minute (CPM) for each triplicate was used to calculate the fold increases in proliferation induced by mitogens using the following formula:

$$\frac{\text{CPM for mitogen stimulated cells}}{\text{CPM for unstimulated cells}}$$

2.3.2. Mixed lymphocyte culture

Mixed lymphocyte culture (MLC) was carried out in order to test the capacity of lymphocytes from patient A to respond to allogeneic cells. Isolated PBMC were cultured with irridiated cells from related and unrelated donors and the proliferation of lymphocytes was assessed by measurement of tritiated (^3H) -thymidine incorporation as outlined previously (Reinsmoen, 1993). This work was carried out in the Tissue Typing Laboratory of the Blood Transfusion Services Board (BTSB) in Dublin. Due to limited availability of sample this test was not carried out for patient B.

2.3.3. Proliferation of PBMCs in response to increasing concentrations of IL-2

In order to test the capacity of cells to respond to IL-2, PBMC from patient B and a healthy control were resuspended in medium at $1x10^6$/ml and 200 µl of this suspension was added to each well of a flat-bottomed microtitre plate (Nunc). IL-2 was added to triplicates of wells at concentrations of 0, 0.1, 1, and 10 ng/ml and the cells were incubated in CO_2 at 37°C for 6 days. The cell suspensions were pulsed with 0.3 µCi ^{3}H-thymidine per well and the plates were incubated for a further 16 hours in the same conditions as above. Cells were harvested following the protocol outlined in section *2.3.1.* The average CPM for each triplicate was used to calculate the fold increases in proliferation induced by the varying concentrations of IL-2 using the following formula:

$$\frac{\text{CPM for concentration of IL-2}}{\text{CPM for 0 ng/ml}}$$

2.4. Measurement of immunoglobulins and specific antibodies

2.4.1. Measurement of immunoglobulin, IgG specific subclasses, complement, classical and alternative pathway complement activity.

Serum immunoglobulins (IgG, IgA and IgM) and complement components C3 and C4, from both patients were measured on a Behring Laser Nephelometer using rabbit anti-human polyclonal antibodies (Dade Behring, UK). These antibodies bind the target protein and the complexes formed scatter a beam of light. The intensity of the scattered light is proportional to the concentration of the relevant serum protein. Results for patient's serum were evaluated with reference to a standard of known concentration. Specific IgG1 antibodies for tetanus toxoid and IgG1 and IgG2 for pneumococcus were measured using enzyme-linked immunosorbent assays (ELISA) using commercially available kits (Binding Site, UK). Diluted serum samples were added to antigen coated microwells. Antibody bound to the target antigen was visualised using peroxidase labelled rabbit anti-human IgG conjugate and tetramethylbenzidine (TMB) substrate. Total and alternative haemolytic complement pathways were measured using kits from Dade Behring (UK). In both of these methods, serum containing active complement components diffuses through agarose gel containing either sensitised sheep erythrocytes (total complement pathway) or chicken erythrocytes (alternative pathway). In both assays the activation of the complement cascade leads to lysis of the erythrocytes with a resulting clear zone of haemolysis. The complement activity of the sample was determined by measuring the zone of lysis and reading off a calibration curve. These tests were carried out in the Immunology Laboratory, St. James Hospital, Dublin.

2.4.2. Specific IgG for herpes, measels and CMV

Measurement of serum for specific antibodies to *Herpes simplex*, *Cytomegalovirus* (CMV), *Toxoplasma*, Mumps and Measles was carried out using ELISA tests in the Virus Reference Laboratory (University College Dublin, Ireland).

2.5. RNA extraction and PCR

2.5.1. RNA extraction

RNA extraction was carried out using cells at $1x10^{6-7}$/ml. PBMC used were either fresh or stimulated for up to 24 hours with lipopolysaccharide (LPS) (Sigma,10 μg/ml) and IFN-γ (R&D Systems, 100 ng/ml). Total cellular RNA was extracted from resting and stimulated PBMC (Chomenzynski *et al.* 1987) with all steps carried out on ice. Cells were washed in Hanks balanced salt solution (HBSS), and the supernatant was removed. The pellet was gently resuspended in a minimum volume and 1 ml of denaturing solution (Appendix II) per $1x10^7$ cells was added. The solution was gently mixed 7-10 times using a sterile pastette; 100 μl of 2 M sodium acetate (pH 4) was added and mixed thoroughly. The solution was divided into two sterile microfuge tubes and 500 μl of water saturated phenol (pH 4) followed by 200 μl of $CHCl_3$/ Isoamyl alcohol was added (Appendix II). This mixture was incubated on ice for 15 minutes, with intermittent mixing and subsequently centrifuged at 400g at 4°C for 12 minutes. The aqueous phase was transferred to a fresh tube and 500 μl of isopropanol added; this mix was incubated overnight at -70°C. After centrifugation at 400g for 8 minutes, the supernatant was removed and the RNA pellet was completely dissolved in 300 μl of denaturing solution, then 300 μl of isopropanol was added and the mix placed at -70°C for 20 minutes. Centrifugation at 400g was carried out for 10 minutes and the supernatant was removed and 500 μl of 75% ethanol was added (Appendix II). Following incubation on ice for 15 minutes and centrifugation at 400g for 20 seconds the supernatant was discarded and the RNA pellet was air dried for 15 minutes. The pellet was resuspended in RNase free water, and following quantification on a spectrophotometer at 260 nm the RNA concentration was adjusted to 1 μg per microliter, samples were stored at -70°C until reverse transcription was carried out.

2.5.2 Reverse transcription of total RNA

RNA samples were heated at 85°C for 2 minutes and then placed on ice. One microgram of RNA was then added to a 20 µl reaction mixture containing Tris-HCL pH 8.3, KCL (50nM), 40 mM $MgCl_2$, deoxyribonucleoside triphosphates (dNTPs) (Promega, Madison, Wi, USA) at 100 pM, Rnasin Ribonuclease Inhibitor (20 U) (Promega) and avian myeloblastosis virus (AMV) reverse transcriptase (RTase) at 15 U (Promega). The samples were mixed gently and maintained at room temperature for 8 minutes before incubation at 42°C for one hour. Control tubes contained no enzyme. The resulting complimentary DNA (cDNA) was stored at -70°C until PCR was carried out.

2.5.3. PCR amplification for NKG2A, IL-15 receptor alpha and β-actin

PCR amplification for NKG2A, IL-15Rα and β-actin was carried out using a reaction volume of 25µl which included 2µl cDNA in 10mM Tris HCL pH 9, 50mM KCL and 0.2 mM dNTPs and Taq polymerase, 2.5 U (Promega). The primer concentration varied for each receptor pair and was optimised to between 5 to 10 picomoles. The optimum $MgCl_2$ concentration was determined using titration curves and ranged from 1.5 for NKG2A and β-actin to 3 mM for IL-15Rα. All subsequent PCR reactions were performed at the optimised concentrations. For NKG2A and IL-15Rα, normal adult cDNA was included as positive controls for receptor expression. Negative controls were included in each run and incorporated all the reagents except the cDNA. Mineral oil was used to overlay the reaction mixture.

2.5.4. Designing primers and probes for PCR

The primers chosen for PCR were RNA specific and were designed to span the junctions of two exons, thus excluding amplification of genomic DNA. TaqMan probe and primers were designed using Primer Express software. The following guidelines were adhered to as far as possible:

- Keep the G-C content in 30-80% range
- Avoid runs of identical nucleotides. This is especially true for guanine, where runs of more than four Gs should be avoided.
- The melting point (Tm) should be 58-60 °C
- The five nucleotides at the 3' end should have no more than two G and/or C bases
- There should be no complementarity of primers
- For TaqMan PCR the forward and reverse primers should be as close as possible to the probe without overlapping it.

TaqMan Probes were designed using the following guidelines:

- Keep the G-C content in 20-80% range
- Avoid runs of identical nucleotides. This is especially true for guanine, where runs of more than four Gs should be avoided.
- Using Primer Express software, the Tm should be 65-67 °C
- Select the strand that gives the probe with more Cs than Gs

2.5.5. Visualization of PCR products

For the purposes of visualization, 5-15 µl of the PCR product from each reaction were mixed with 6x concentration loading buffer and loaded into wells in a 2% agarose gel (Ultra Pure Agarose, GIBCO, BRL, Life Technologies, Paisley, Scotland) containing ethidium bromide (Sigma). The gel was run in a Tris/Acetate/EDTA (TAE) buffer for 30 mins. The fluorescent bands were visualised under UV light and photographed using Polaroid. cDNA product size was compared to a base pair ladder (X174 DNA/HaeIII or 100bp DNA ladder, Promega, Madison, Wi, USA) which was included in each gel.

2.5.6. Detection of NKG2A mRNA

Primers were designed based on the published sequence of human NKG2A, Genbank Accession number X54867 and had the following sequences:

Table 2.1. Sequence of primers used for NKG2A and β actin amplification with PCR

Name	Primer sequence	Position in sequence
Forward 1 (F1)	5' GGA TGT GTG ACT TTC TGG GGA 3'	bp 107 - 127
Forward 2 (F2)	5' GCA CAA CAA TTC TTC CCT GAA TA 3'	bp 464-486
Reverse 1 (R1)	5' GCT AGG ATG TCT GTA CTT TAG 3'	bp 1040 - 1020
Reverse 2 (R2)	5' GCA ACC ACT ATT CTA CTT TCT GT 3'	bp 1179 - 1157
β actin forward	5'ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG 3'	bp 294 - 325
β actin reverse	5'CGT CAT ACT CCT GCT TGC TGA TCC ACA TGT GC 3'	bp 1131 - 1100

Forward and reverse primer pairs F1R1 and F2R2 were designed to yield products of 933 bp and 715 bp lengths respectively. PCR amplification was carried out on a Hybaid Omnigene (Hybaid, Teddington, Middlesex, UK) at 94°C 3 minutes - 94°C 40 secs, 55°C 40 secs, 72°C 1.5 minutes for 36 cycles then a final extension step consisting of 72°C for 10 minutes. Primers for β actin were were designed based on the published sequence of human β actin, Accession number X00351. These primers yielded a product of 837 bp and were included to check for the relative concentration of cDNA. Conditions of primer concentration and $MgCl_2$ concentration were optimised. PCR was carried out at various $MgCl_2$ concentrations: 0.5, 1, 1.5, 2, 3 and 4 mMolar. Results

from concentration curve indicated that the optimum $MgCl_2$ concentration was 1.5 mM for each NKG2A and β-actin primer pair

The PCR products were visualised on a 1 % agarose (Ultra Pure Agarose, GIBCO BRL, Scotland) gel incorporating ethidium bromide (Sigma). Product size was compared to the base pair ladder.

2.5.7. IL-15Rα primers for PCR

Primers were designed based on the published sequence of IL-15Rα, Genbank Accession U31628. The primers chosen were RNA specific and were designed to span the junctions of two exons, thus excluding amplification of genomic DNA. For both PCR and sequencing, overlapping primer pairs were synthesised using the Beckman Oligo 1000M DNA Synthesizer (Primers supplied by Clarke Stephenson at the Oligonucleotide Synthesis Unit, Queen's University, Belfast, Northern Ireland).

Table 2.2. Sequence of primers used for IL-15 receptor alpha (IL-15Rα) and β actin PCR amplification

Name	**Primer sequence**	**Position in sequence**
Forward 1 (F1)	5' CCG TGG AAC ACG CA 3'	bp 195 - 208
Forward 2 (F2)	5' CAA CAG CAG CTA TTG TCC 3'	bp 507 - 524
Reverse 1 (R1)	5' GGA CAA TAG CTG CTG TTG 3'	bp 524 - 507
Reverse 2 (R2)	5'CTT CAC TCC GGA CTT AGC 3'	bp 924 - 907
Reverse 3 (R3)	5' TAC CAC ATG TAT TCC AGG C 3'	bp 1202 - 1184
Reverse 4 (R4)	5' GTG TGC AGA GCA GC 3'	bp 1383 - 1370
Reverse 5 (R5)	5' AAT GGC ACT GAG TTG GAG 3'	bp 1480 - 1464
β actin forward	5' AAG AGA GGC ATC CTC ACC CT 3'	bp 222 - 241
β actin reverse	5' TAC ATG GCT GGG GTG TTG AA 3'	bp 439 - 420

2.5.8. PCR for IL-15 receptor alpha - conditions

PCR for IL-15Rα was carried out on a Hybaid Omnigene (Hybaid, Teddington, Middlesex, UK) using 36 cycles of 94°C for 40 secs, 55°C for 40 secs, 72°C for 1.5 minutes with an initial denaturation step of 94°C for 3 minutes and a final extension step consisting of 72°C for 10 minutes. Primer pairs used (table 2.2) for IL-15Rα amplification were: F1R4 yielding an amplified product of 1187 bp, F1R1 yielding an amplified product of 329 bp, F1R5 yielding an amplified product of 1285 bp. Primers for β actin, yielding a 217 bp product, were included to check for the relative concentration of cDNA.

Conditions of primer concentration and $MgCl_2$ concentration were optimised. PCR was carried out at various $MgCl_2$ concentrations: 0.5, 1, 1.5, 2, 3 and 4 mM. Results from the concentration curve indicated that the optimum $MgCl_2$ concentration was 3 mM for each IL-15Rα primer pair and 1.5 mM for the β actin primer pairs. Primer pairs were reconstituted with water to a concentration of 10 picomoles per microliter and relative levels checked on a 2 % agarose gel. Primers were aliquoted and stored at -70 °C. In subsequent PCR reactions 5 picomoles of primer were used in a 25 µl reaction volume. cDNA was reconstituted to a concentration of 10 µg/ml with levels checked on the spectrophotometer at 260nm for quantification and 280nm for preparation quality. 2µl of cDNA was used in a 25 µl reaction volume.

2.5.9. IL-15 receptor alpha - Sequencing

Sequencing of 63.5% of IL-15Rα precursor molecule including 91% of the extracellular domain, the entire transmembrane region and the cytoplasmic domain, was carried out for PCR product from patient A.

IL-15 Rα gene product was amplified using both the primer pairs F1R4 and F1R5 (table 2.2) on cDNA from a normal control and patient A. After PCR amplification, the target sequence was excised from the gel using a sterile scalpel blade. The cDNA was then purified using Wizard^R PCR Preps (Promega, Madison, Wi, USA), and 100-200 ng of cDNA was used for sequencing. Primers used for sequencing were F1, F2, R1, R2 and

R5 (table 2.2). Sequencing was carried out using an ABI PRISM 373 A automated sequencer (Applied Biosystems Inc., Foster City, CA., USA) at 'The National Pharmaceutical Biotechnology Sequencing Centre', Trinity College, Dublin. The samples were run on a 6% polyacrylamide buffered gel with 1X TBE gel at 33-Watts (W) and constant power for 12 hours. Automated fluorescence based sequencers present data in the form of electrophorograms, each coloured peak representing one nucleotide. Depending on the direction of the primer used, sequence data represents positive or negative strands. Editing of the sequence was carried by converting reverse strands to plus strands using SEQED software (version 1.0.3, Applied Biosystems Deleware) and then aligning overlapping sequences with ClustalW software from the European Bioinformatics (EBI) web-site http://www.ebi.ac.uk. Gaps in the sequence or unidentified nucleotides (appearing as N in the sequence data) were corrected in this way. The sequenced nucleotide product was than assembled as a text file in Word (Microsoft Word) and this was used for further identification of the sequence.

2.5.10. IL-15 receptor alpha sequencing - alignment of nucleotide and amino acid sequences

The PCR product was identified using the web-site: National Center for Biotechnology Information (NCBI) BLAST at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, which carried out a comparison between the sequence entered and sequences published in a number of databases including GENBANK. When the PCR product was identified the sequence was then compared with two of the published nucleotide sequences of IL-15Rα using multiple sequence alignment carried out with ClustalW software from the EBI web-site http://www.ebi.ac.uk. 'Translation' at the NCBI BLAST site was used to deduce the predicted amino acid sequence from the nucleotide sequence. NCBI BLAST for protein was then carried out to identify the protein product as human IL-15Rα.

2.6. IL-15 detection - TaqMan PCR and flow cytometry

2.6.1. TaqMan PCR for IL-15

TaqMan™ PCR reaction exploits the 5' nuclease activity of AmpliTaq Gold™ DNA Polymerase to cleave a TaqMan™ (Perkin Elmer, USA) probe during PCR. The TaqMan probe contains a reporter dye at the 5' end of the probe and a quencher dye at the 3' end. When the probe is intact the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence (figure 2.1A). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'-3' nucleolytic activity of AmpliTaq Gold DNA Polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. Cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. After the probe fragments have been displaced from the target, and polymerization of the strand continues (figure 2.1A). The 3' end of the probe is blocked to prevent extension during PCR. In our study accumulation of PCR product was detected directly by monitoring the increase in fluorescence of reporter dye using the ABI Prism 7200 Sequence Detection System.

2.6.2. Primers and probe for TaqMan PCR for IL-15 and IFN-γ

TaqMan Probe and Primers for IL-15 were designed based on the published sequence of human IL-15 Genbank Accession X91233, using Primer Express Software (Perkin Elmer).

TaqMan Probe and Primers for IFN-γ were designed based on the published sequence of human IFN-γ Genbank Accession X13274, using Primer Express Software.

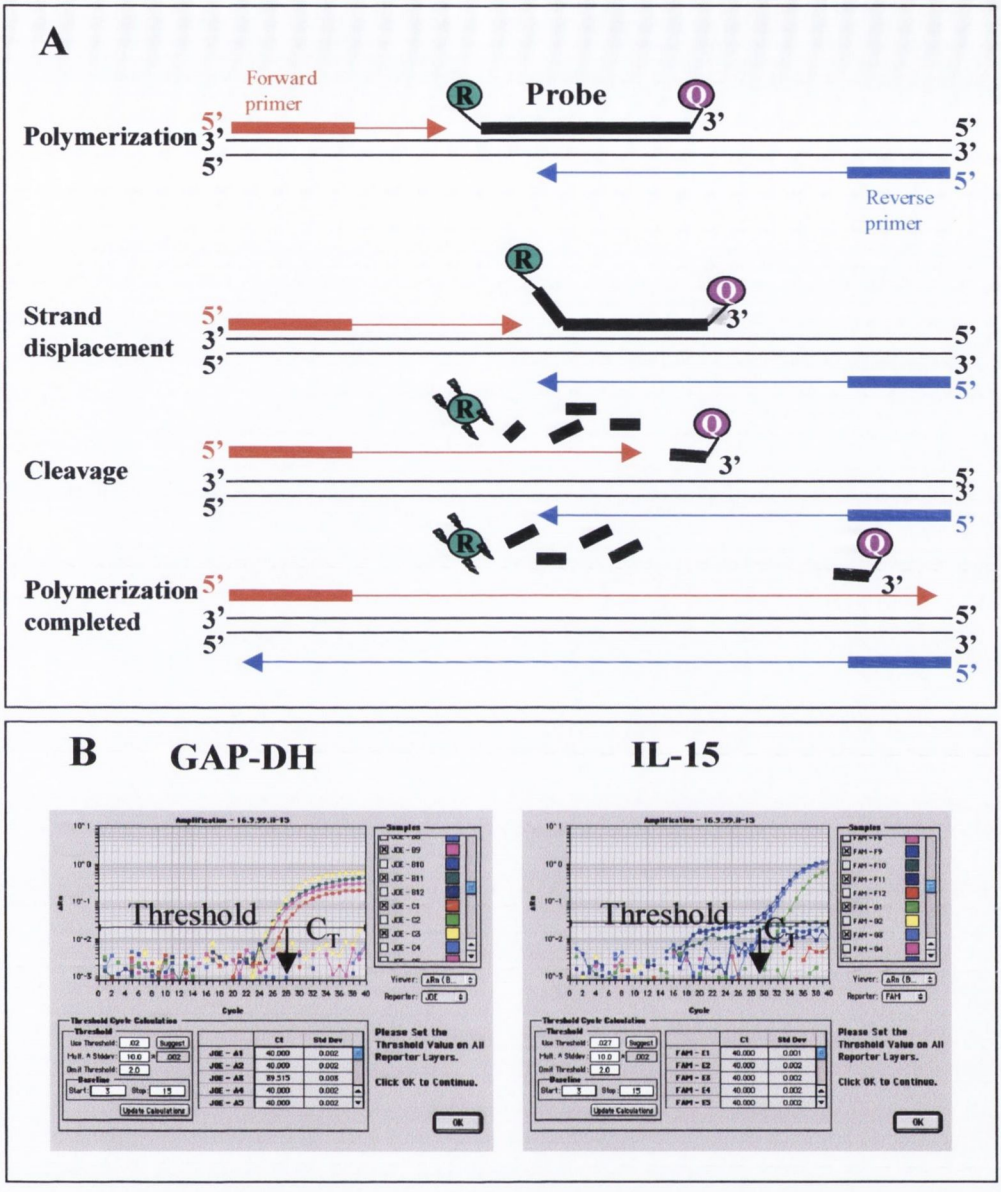


Figure 2.1. Basics of the 5'Nuclease assay for TaqMan PCR using Real Time Detection

A. The proximity of the reporter (R) and quencher (Q) dyes on the probe results in suppression of reporter fluorescence. During PCR, if the target sequence is present, the probe anneals between the forward and reverse primer sites. The 5'-3' nucleolytic activity of Ampli-Taq Gold DNA Polymerase cleaves the probe between the reporter and quencher, displacing the probe and allowing polymerization of the strand. The increase in fluorescence signal is measured. B. Real Time amplification plots for GAP-DH and IL-15 showing sample threshold cycles. The Threshold cycle (C_T) occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.

Table 2.3. Sequence of primers and probes for TaqMan PCR of IL-15 and IFN-γ

Primer/Probe	Sequence
IL-15 forward	5' TTT TCT AAC TGA AGC TGG CAT TCA 3'
IL-15 reverse	5' CCA GTT GGC TTC TGT TTT AGG 3'
IL-15 probe	5' TCT TCA TTT TGG GCT GTT TCA GTG CAG G 3'.
IFN-γ forward	5' TTC AGA TGT AGC GGA TAA TGG AAC 3'
IFN-γ reverse	5' GAG ACA ATT TGG CTC TGC ATT ATT T 3'
IFN-γ probe	5' TGT CAC TCT CCT CTT TCC AAT TCT TCA AAA TGC 3'

The probe contains a fluorogenic reporter dye FAM (6-carboxyfluorescein) covalently attached at the 5' end, and a quencher dye TAMARA (6-carboxy tetramethylrhodamine) covalently attached at the 3' end. TaqMan GAPDH Control Reagents (Perkin Elmer, USA), which detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, were used as reference or housekeeping genes allowing correction for minor variations in the level of mRNA in each sample.

2.6.3. TaqMan PCR conditions

Universal PCR Master Mix (Perkin Elmer, USA) a premix of all the components, except primers and probe, necessary to perform 5' nuclease assay was used with optimised primer concentrations and following universal thermal cycling parameters. The TaqMan Universal PCR Master Mix is a 2X concentration and contains AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optomized buffer components. Universal thermal cycling parameters were used on the ABI Prism 7700 Sequence Detector were as follows:

Table 2.4. Universal thermal cycling parameters for ABI Prism 7700

		Each of 40 cycles	
		Melt	Anneal / Extend
HOLD	HOLD	CYCLE	
2 minutes	10 minutes	15 seconds	1 minute
50°C	95°C	95°C	60°C

Sample RNA was reverse transcribed using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, Wi, USA) and the cDNA obtained was used at approximatly 20 ng/ reaction as the template for TaqMan PCR. Primer concentration was optimised for each primer pair, using 50, 300 and 900 nM of forward paired with 50, 300 and 900 nM of reverse primer in each of nine reaction mixes, which were run with at least four replicates. For both IL-15 and IFN-γ the final primer value was 300 nM forward with 300 nM reverse. The reaction components outlined in table 2.5 were made up to a volume of 25 µl were added to MicroAmp Optical 96-well reaction plates used with MicroAmp Optical Caps (Perkin Elmer, USA).

Table 2.5. Reaction components for TaqMan PCR

Reaction Component	**Volume(µl)**	**Final value**
TaqMan Universal PCR Master Mix (2X)	12.5	1X
Forward primer	x*	300 nM
Reverse primer	y*	300 nM
TaqMan probe	5	400 nM
DNA sample	2	20 ng
Water	5.5 - (x+y)	
Total	25	

* Note volume (µl) for x and y varied depending on the concentration of primer pair used

2.6.4. Calculation of results for TaqMan PCR

The TaqMan Universal master mix incorporates a passive reporter dye. The emmision intensity of the reporter dye is divided by the emission intensity of the passive reporter dye giving a ratio defined as Rn (normalised reporter). The value deltaRn is the difference in Rn between a reaction well containing all reaction components (Rn^+) and Rn of a reaction well containing all components but no cDNA template (Rn^-). Real Time Detection monitors the cycle at which a statistically significant increase in delta Rn is first detected, this is called the threshold cycle (C_T). On the graph reproduced in figure 2.1B, the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with exponential growth of PCR product. Relative quantification data was obtained by using a Comparative C_T Method (User Bulletin 2 for the ABI PRISM 770 Sequence Detection System). Quantity in a TaqMan reaction may be expressed as relative quantification values (RQV) in relation to a calibrator sample eg. zero time point in a time course experiment or resting versus stimulated samples. Relative quantification for each test was calculated using C_T results for resting control PBMC (n=5) as a calibrator. The $C_{\delta\delta T}$ for each test, relative to resting PBMC, was then calculated using the following formula:

$C_{\delta\delta T} = (C_T^{\text{IL-15}} - C_T^{\text{GAPDH}})$ - (mean of n=5 PBMC $C_T^{\text{IL-15}} - C_T^{\text{GAPDH}}$), where C_T is the threshold cycle. Relative Quantification Values (RQV) for IL-15 and IFN-γ mRNA were then calculated using the formula:

$RQV = 2^{-C_{\delta\delta T}}$.

2.6.5. Antibodies used for flow cytometric analysis of intracellular IL-15 expression

MAbs specific for CD3 and CD14 conjugated with PE and isotype-matched mouse IgG controls (PE) were purchased from Becton Dickinson (Oxford, UK). Unconjugated anti-IL-15 (four IgG1 clones) were kindly donated by R&D Systems (Abingdon, UK). Unconjugated mouse IgG1 mAb to vimentin was purchased from DAKO (Dakopatts,

Denmark) together with FITC-labelled rabbit-anti-mouse (RAM-FITC), mouse serum and isotype control IgG (mouse IgG1)

2.6.6. Intracellular staining for IL-15 in PBMC

Intracellular staining for IL-15 was carried out for a number of healthy controls (n=4) and for patient A. Prior to staining, PBMC were separated from whole heparinised blood according to the protocol outlined in section *2.2.1.*and were then resuspended at $1x10^6$/ml in medium. For the detection of IL-15, cells were fixed with 0.5 mls 4% paraformaldehyde/PBS (PFA/PBS) (Appendix I) at RT for 10 minutes, washed with 5% foetal calf serum in PBS (PBS-FCS buffer Appendix I) buffer, and permeabilized with 1 ml of 0.05% saponin in a PBS-FCS buffer (permeabilising buffer, Appendix I) at room-temperature (RT) for 10 minutes. Cells were pelleted and incubated with either control IgG1, anti-vimentin or anti-IL-15 mAbs (α, β, χ and δ clones at 5 µl anti-IL-15 in 50 µl permeabilising buffer) for 15 minutes at RT. Cells were washed at this and all subsequent points with permeabilising buffer. After washing cells were incubated with 100 µl of a 1:100 dilution of RAM-FITC at RT for 10 minutes. Cells were then washed and incubated with 100 µl of a 1:50 dilution of mouse serum at RT for 10 minutes. After further washing 5 µl of anti-CD3-PE or anti-CD14-PE were added and cells incubated for a further 10 minutes at RT. Finally cells were washed with PBS containing 0.01% bovine serum albumin (Sigma) and resuspended in 1% paraformaldehyde-PBS for analysis within 24 hours. Coexpression of cell surface molecules and intracytoplasmic cytokines was detected by two-colour flow cytometry (FACScan BD) and analyzed using CellQuest (BD) software.

2.6.7. Intracellular staining for IL-15 using whole blood

Because of the difficulty of obtaining sufficient specimen for isolation of PBMC from paediatric patients a technique for analysis of IL-15 expression using whole blood (WB) EDTA-specimens was developed. IL-15 expression was examined for healthy adult controls (n=3). Tests were carried out on whole blood with the erythrocytes lysed using FACSlyse solution (BD, Immunochemistry Systems, Oxford UK), a buffered diethylene

glycol solution, together with a whole blood technique that did not incorporate a lysing step. WB or FACSlysed-WB samples were fixed with 4% paraformaldehyde/PBS at RT for 10 minutes. An aliquot of these samples was used for surface staining for IL-15 together with CD3 or CD14 without permeabilization. For intracellular-IL-15 detection permeabilization and staining was carried out as outlined in section *2.6.6.* For the detection of IL-15 on the surface of cells the following procedure was carried out: cells were fixed using 4% paraformaldehyde/PBS and then washed with PBS-BSA buffer. These cells were then incubated with either control IgG1, anti-vimentin or anti-IL-15 mAbs (α–δ clones at 5 µl) for 15 minutes at RT. After washing cells were incubated with 100 µl of a 1:100 dilution of RAM-FITC at RT for 10 minutes. Cells were then washed and incubated with 100 µl of a 1:50 dilution of mouse serum at RT for 10 minutes. After further washing 5 µl of anti-CD3-PE or anti-CD14-PE were added and cells incubated for a further 10 minutes at RT. Finally cells were washed with PBS-BSA buffer and resuspended in 1% paraformaldehyde for analysis within 24 hours. Coexpression of cell surface molecules and intracytoplasmic cytokines was detected by two-colour flow cytometry (FACScan BD) and analyzed using CellQuest (BD) software.

2.6.8. Flow cytometric analysis of two colour staining for IL-15

Analysis of two colour staining was carried out within 24 hours on a FacSort (BD) using Cellquest software. Data for ungated events was collected based on 5,000 events within a lymphocyte gate defined using forward and side scatter characteristics. Surface expression of receptors and intracellular IL-15 expression in monocytes and lymphocytes was assessed by gating cells based on side scatter (SSC) and CD14 (for monocytes) or CD3 (for lymphocytes) expression. From this gate a histogram-overlay was generated showing the FL1 (FITC) median intensity fluorescence for control IgG1, vimentin and anti-IL-15 using clones α–δ. Histogram-overlays of control IgG with anti-IL-15 clone δ were generated for patient A and a healthy control.

2.7. Changes in phenotype following culture of PBMC with IL-2 or IL-15

The effects of IL-2 and IL-15 on percentage of cells expressing CD3, CD4, CD8α, CD8β, CD19, CD16, CD56, CD69, CD161, CD158a, CD158b, CD25, CD122, CD94, CD158e1 (KIR3DL1) and production of IFN-γ, IL-4 and IL-2 by stimulated peripheral blood NK ($CD3^-CD56^+$) cells, T ($CD3^+CD56^-$) cells and NKR^+ ($CD3^+CD56^+$) T cells was measured by a combination of cell-surface and intracytoplasmic mAb staining and analysis by flow cytometry. In addition the effects of culture of human control PBMC for up to three days with 10 μg/ml staphylococcal enterotoxin B (SEB) (Sigma, Poole, UK) on CD25 and CD122 expression in $CD3^+$ cells was examined.

2.7.1. Reagents for culture with IL-2 and IL-15

Human recombinant IL-2 and IL-15 were purchased from R&D Systems (Abingdon, UK). Bovine serum albumin, sodium azide, PMA, ionomycin, saponin and brefeldin A and SEB were purchased from Sigma (Poole, UK)

2.7.2. Cell separation and culture with IL-2 and IL-15

PBMC were separated from whole heparinised blood according to the protocol outlined in section *2.2.1.* and were then resuspended at $1x10^6$/ml in medium. Control PBMC were used to establish optimum concentrations of cytokine from concentration curves of 0, 0.1, 1, 10, 50, 100 and 500 ng/ml recombinant IL-2 or IL-15 for seven days in 5% CO_2. Time course studies were carried out using control cells in medium alone or supplemented with 100 ng/ml IL-2 and 25 ng/ml IL-15 for 1 to 7 days. A concentration of 100 ng/ml IL-2 and 25 ng/ml IL-15 for a period of seven days was established as the optimum conditions for cell expansion in response to these cytokines. Cells, from both patients and healthy controls, were cultured under optimum conditions and then analysed phenotypically and in functional assays.

2.7.3. Antibodies used for flow cytometric analysis of receptor expression, cytokine production and expression in response to IL-2 and IL-15

MAbs specific for CD3, CD4, CD8α, CD56, CD161, CD158a, IFN-γ and IL-2 conjugated with FITC, anti-CD56, CD4, CD8α, CD25, CD122 conjugated with PE, anti- CD3, CD8α, CD45 and CD19 conjugated with PerCP and isotype-matched mouse IgG controls (FITC, PE and PerCP) were purchased from Becton Dickinson (Oxford, UK). Unconjugated anti-CD3 (clone HIT3a) and anti-IL-4 FITC were obtained from Pharmingen (Oxford, UK). Unconjugated anti-CD94, anti-CD158e1 (KIR3DL1) and anti-CD8β were obtained from Coulter-Immunotech (Marseille, France). Dr. Lorenzo Moretta, Genova, Italy, kindly provided the CD158b mAb.

2.7.4. Cell surface labelling of whole blood and PBMC using both fluorescent labelled and unlabelled antibodies

Cell surface staining, using combinations of three fluorescent labelled antibodies or two fluorescent labelled mAbs with one unlabelled mAb, was carried out on whole blood (anticoagulant EDTA), fresh PBMC or PBMC cultured in medium alone or with IL-2 or IL-15 for seven days. Whole blood or PBMC at $1x10^6$/ml (100μl) was added to 10 μl of unlabelled monoclonal antibody and incubated in the dark at room temperature (RT) for 10 minutes. After washing in filtered phosphate buffered saline (PBS) (0.1 mol/L pH 7.2) indirect labelling was performed using 100 μl of 1:50 dilution FITC conjugated rabbit anti-mouse antibodies. Cells were incubated with label for 10 minutes at RT and washed as before. Samples were blocked with 100 μl of 1:50 normal mouse serum for 10 minutes at RT and washed once. Staining with labelled antibodies (FITC, PE and PerCP labelled) was carried out using 5 μl of labelled antibody added to cells and incubated for 10 minutes at RT, erythrocytes were lysed if necessary using FACS lysing solution for 10 minutes at RT. After washing, the cells were resuspended in 0.5% paraformaldehyde/PBS solution.

2.7.5. Three colour flow cytometric analysis for surface receptor staining

Analysis was carried out within 24 hours on a FacSort (BD) using Cellquest software for three-colour analysis. Data for ungated events was collected based on 5,000 events within a lymphocyte gate defined using forward and side scatter characteristics. To assess expression of PE and FITC labelled receptor on T cell subsets, lymphocytes were gated based on side scatter (SSC) and CD3 expression (PerCP). Dot plots of PE with FITC were then generated. For analysis of FITC labelled receptor expression on NK cells, lymphocytes were gated based on SSC and CD56 (PE) surface expression and a dot plot was generated showing PerCP (CD3) and FITC expression. NK cells were defined as those cells that expressed CD56 but not CD3.

2.7.6. Stimulation of cells and staining for intracellular cytokines

Control PBMC cultured with recombinant IL-2 and IL-15 were washed and resuspended in complete RPMI medium at a density of 0.5-1 $x10^6$ cells/ml. These cells were added to a 24 well culture plate (Nunc) at 0.5 ml/well at 37°C in 5% CO_2. Cells were stimulated with either 10 ng/ml phorbol myristate acetate (PMA) plus 1 μg/ml ionomycin or with plate bound anti-CD3 mAb together with 1 ng/ml PMA. Anti-CD3 (HIT3a clone) was bound to plates at 10 μg/ml by incubation for 6 hours at 37°C in 0.1 M Na_2HCO_3. Both freshly isolated PBMC and cells cultured for 7 days in medium only were treated similarly as controls. Brefeldin A (10 μg/ml), an inhibitor of protein translocation from the endoplasmic reticulum to the Golgi apparatus, was added to the cells for the last 4 hours. After stimulation or incubation without stimulators, cells were washed with PBS containing 0.07% BSA and 0.02% sodium azide and stained for 15 minutes at RT with anti-CD3-PerCP together with anti-CD56-PE or anti-CD8-PE or anti-CD4-PE. For the detection of IFN-γ, IL-2 and IL-4 (FITC conjugated mAbs) cells were fixed with 0.5 mls 4% paraformaldehyde at RT for 10 minutes, washed with PBS-BSA-azide buffer, and permeabilized with 1 ml of 0.2% saponin in PBS-BSA-azide buffer at RT for 10 minutes. Cells were pelleted and incubated with anti-cytokine mAbs (0.1 μg anti-IFN-γ FITC, anti-IL-2 FITC or anti-IL-4 FITC in 50 μl 0.2% saponin) for 30 minutes at RT. Finally cells were washed with PBS-BSA-azide buffer and resuspended in 1%

paraformaldehyde for analysis within 24 hours. Coexpression of cell surface molecules and intracytoplasmic cytokines was detected by three-colour flow cytometry (FACScan BD) and analyzed using CellQuest (BD) software.

Four colour analysis for T, B and NK lymphocyte subsets at time zero and following culture for up to seven days with varying concentrations of IL-2 or IL-15 was carried out according to the protocol in section *2.1.2*

2.7.7. Three colour flow cytometric analysis of permeabilised lymphocytes

Analysis of three colour staining was carried out within 24 hours on a FacSort (BD) using Cellquest software. Data for ungated events was collected based on 5,000 events within a lymphocyte gate defined using forward and side scatter characteristics. Surface expression of receptors and intracellular cytokine (IFN-γ, IL-2 or IL-4) in T cell subsets was assessed by gating lymphocytes based on side scatter (SSC) and CD3 (PerCP) expression, from this gate dot plots of PE (CD4, CD8 or CD56) and FITC (cytokine or perforin) were then generated. The analysis of receptor, intracellular cytokine or perforin expression in NK and $CD56^+$ (NKR^+) T cells was carried out by gating lymphocytes based on SSC and CD56 (PE) surface expression. Using this gate a dot plot was generated showing CD3 (PerCP) and FITC (IFN-γ, IL-2 or IL-4 or perforin) staining. NKR^+ T cells were defined here as those that expressed both CD3 and CD56, while NK cells were defined as those that expressed CD56 without CD3.

2.7.8. Assessment of cell expansions

The numbers of viable cells were determined before and after culture in the presence or absence of IL-2 or IL-15. Cells were first stained with ethidium bromide and acridine orange (EBAO) (Appendix I), green staining viable mononuclear cell (MNC) numbers were then determined using fluorescent microscopy (as outlined in the protocol in Appendix I). The proportions of MNC that were positive for combinations of CD3,

CD4, CD8α, CD8β, CD16, CD19, CD56, CD158a, CD158b, CD158e1 (KIR3DL1), CD161, CD25, CD122, CD94 and perforin were determined by mAb staining and flow cytometry. Absolute numbers of lymphocyte subpopulations were calculated from the MNC counts.

2.7.9. Statistics

Data was analysed using the INStat™ for Macintosh version 2.03. The Mann-Whitney Test, two-tailed unpaired, was used to compare the absolute numbers or percentages of each cell type at time zero with the numbers or percentages following culture for 7 days in medium or with either IL-2 or IL-15. For these tests a p value of <0.05 was considered significant.

2.8. $CD3^+$ and $CD3^-$ cytotoxic assays using IL-2 and IL-15 expanded PBMC

2.8.1. Magnetic bead separation of $CD3^+$ and $CD3^-$ fractions

In vitro expanded PBMC were separated into $CD3^+$ and $CD3^-$ fractions using anti-CD3 mAb-coated magnetic beads. After culture for seven days with and without recombinant IL-2 and IL-15 cells were washed and resuspended in 80µl of MiniMacs ™ buffer (Appendix II) / 10^7 cells. 20µl of anti-CD3 mAb coated magnetic beads (MACS Magnetic Microbeads, Miltenyi Biotec, Bergish Gladbach, Germany) was added to the cells. After mixing, these cells were incubated for 15 minutes at 6^0C, then 5 µl of CD3-FITC and CD56-PE (BD) were added and further incubated for 10 minutes. Cells were washed using x10 volume of buffer (Appendix II) and centrifuged at 150g for 10 minutes. Supernatant was removed completely and cells were resuspended in 500 µl of buffer per 10^8 cells. Positive selection column type MS/RS was placed in the field of a MACS magnetic separator and prepared by washing with 500 µl of buffer. The cell suspension was then added to the column and negative cells allowed to run through into a labelled collection tube (figure 2.2): the column was then rinsed with 3x500 µl of buffer. The column was removed from the separator and placed on a labelled collection tube, 1ml of buffer was added and the positive cells were firmly flushed through using the column plunger.

Both the $CD3^+$ and $CD3^-$ fractions were counted using a Neubauer haemocytometer chamber fractions were diluted in EBAO solution (protocol for cell counts in Appendix I). Surface staining for CD3 and CD56 and flow cytometry assessed the purity of each fraction.

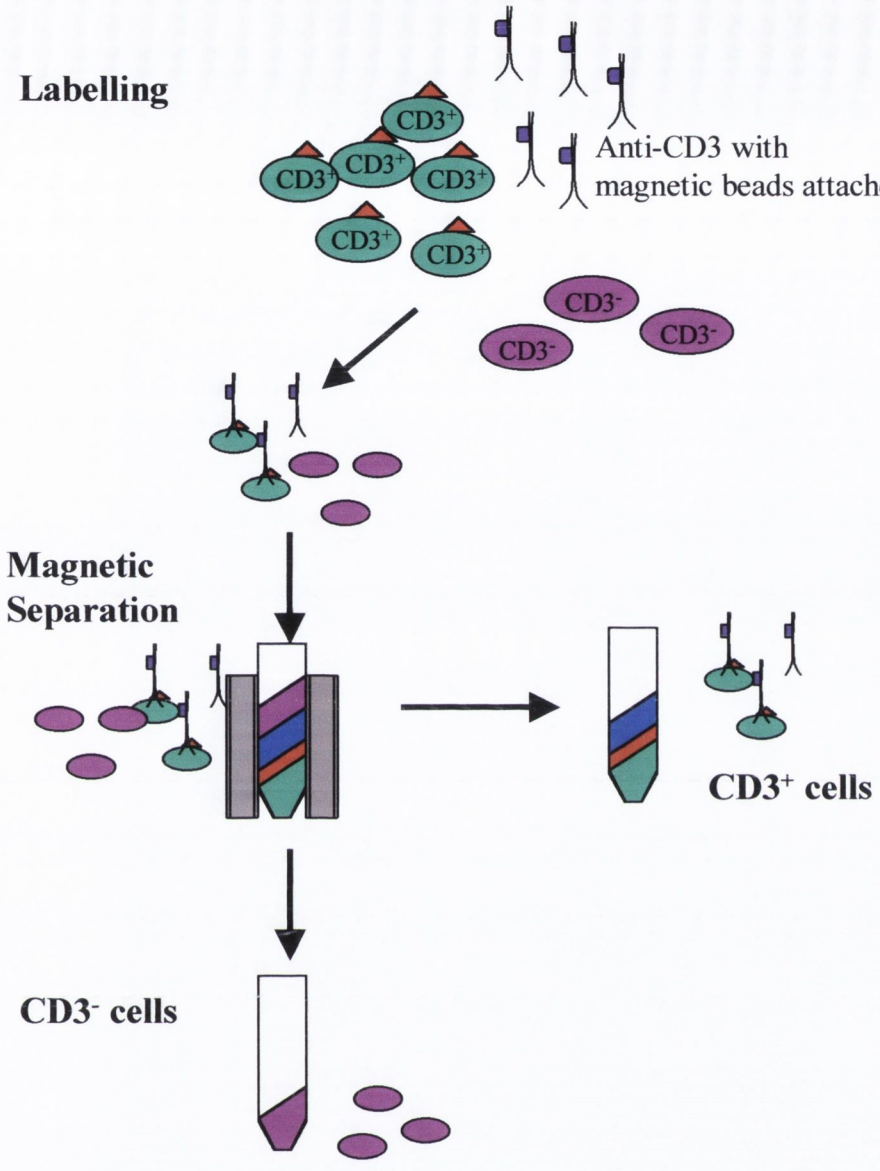


Figure 2.2. Magnetic bead separation of $CD3^+$ and $CD3^-$ populations

PBMC were mixed with anti-CD3-labelled magnetic beads. A positive selection column, type MS/RS, was placed in the field of a MACS magnetic seperator and the cell suspension was added to it. Negative cells ($CD3^-$) were allowed to run through the column into a labelled collecting tube. After removal of the column from the magnetic field, positive cells were collected into a labelled tube by flushing the column with 1 ml of buffer.

2.8.2. Cytotoxicity against K562 cells using $CD3^+$ and $CD3^-$ fractions

Lymphokine activated killing of K562 target cells by cytokine stimulated NK and NKR^+ T cells was assayed in a 4-hour ^{51}Cr release assay using $CD3^+$ and $CD3^-$ cells as effectors. The assay was carried out according to the protocol outlined in section *2.2.3.* using effector to target (E/T) ratios ranging from 1:1 to 1:250.

2.9. Intracellular staining for perforin using whole blood samples and PBMC

2.9.1. Donors for whole blood intracellular perforin study

Intracellular perforin expression was evaluated in ten donors using whole blood (WB) samples and separated mononuclear cells (MNC). In order to test the effect of anticoagulants used in conjunction with WB-lysis both EDTA and heparinised samples were taken from all ten donors. In the cases of four donors, the heparinised sample was further split and an aliquot was used for WB-lysis, another aliquot was washed before WB-lysis.

2.9.2. Reagents for intracellular perforin staining

PE labelled monoclonal antibodies (mAb) to CD4, CD8 and CD56, PerCP labelled mAb against CD3, together with PE and PerCP labelled isotype controls were purchased from Becton Dickinson (BD, Oxford, UK). FITC labelled mAb specific for perforin together with FITC labelled isotype control were obtained from Pharmingen (BD, Oxford, UK). Paraformaldehyde (2%) was made up in phosphate buffered saline (Sigma tablets) (Appendix I). Permeabilising buffer was made up with 0.05% Saponin (Sigma) and 5% foetal calf serum (FCS) (Sigma) in SSPE buffer (Sigma) (Appendix I).
Saline Sodium Phosphate EDTA (SSPE), a 20X concentrate buffer containing 0.2 M phosphate buffer pH 7.4, 2.98 M NaCl and 0.02 M EDTA (Sigma-Aldrich, Steinheim, Germany), was used at the manufacturers recommended dilution for washing whole blood and PBMC.

Erythrocytes were lysed using three methods:

(i) hypotonic lysis using distilled water and SSPE (Sigma) buffer concentrate;

(ii) lysis with FACSlyse solution (BD, Immunochemistry Systems), a buffered diethylene glycol solution;

(iii) Ammonium Chloride lysing solution made up of 500 ml of distilled water, 4.15 g NH_4Cl (BDH, Poole, UK) and 1 ml of 0.5 M EDTA (Sigma) (pH 8).

2.9.3. Whole blood samples and cell separation

Venous blood was obtained from healthy adult donors. The blood was collected in EDTA or heparinised vacutainers and used either directly in a WB assay, as washed whole blood or as isolated PBMC. In assays using washed whole blood, heparinised samples were washed with SSPE buffer and cells were resuspended at original volume. PBMC were separated from whole heparinised blood using centrifugation on Ficoll-Hypaque gradient (Lymphoprep, Nycomed). After washing with Hanks Balanced Salt Solution (Gibco BRL) PBMC were washed and resuspended at $1x10^7$ cells per ml in SSPE buffer (Sigma).

2.9.4. Intracellular perforin in whole blood and PBMC - staining, lysis and permeabilisation

Each sample was stained for surface receptor expression using 5 µl of anti-CD3-PerCP together with 5 µl of either anti-CD56-PE or anti-CD8-PE or anti-CD4-PE or isotype control. The antibodies were added to 200 µl of EDTA, heparinised or washed heparinised blood or 100 µl of PBMC at $1x10^7$/ml in polystyrene tubes (Falcon no 2052), and incubated in the dark at RT for 10 minutes. PBMC samples were then fixed and permeabilised as outlined below.

For WB-lysis, erythrocytes were lysed using three methods:

(a) 3 mls of distilled water was added to each tube and lysis carried out for 20 seconds, after which time SSPE buffer 20X concentrate was added to bring the solution to isotonic strength (150 µl of X20 concentrate).

(b) 2 mls of FACS lysing solution (BD) was added to each tube and incubated for 10 minutes at room temperature (RT).

(c) 3 mls of Ammonium Chloride lysing solution was added to each tube and incubated at 37°C for 5 minutes.

After centrifugation at 390g the supernatant was discarded and cells were washed with SSPE buffer.

Stained PBMC or WB-lysed cells were then resuspended in 2% paraformaldehyde/PBS (pH 7.2) solution for 10 minutes at RT, then washed in 2 mls of permeabilising buffer (Appendix I). Care was taken to resuspend cells after each washing step by vortexing gently. Cells were resuspended in 100 µl of permeabilising buffer and 5 µl of FITC labelled anti-perforin antibody or γ2-FITC control antibody was added to each tube. Staining was carried out for 15 minutes at RT in the dark after which cells were washed in permeabilising buffer and resuspended in 0.5 ml of Cellfix (BD).

Analysis was carried out within 24 hours on a FacSort (BD) using Cellquest software for three-colour analysis as outlined in section *2.7.7.*

2.9.5. Staining and analysis of intracellular perforin expression in IL-2 and IL-15 expanded lymphocytes

PBMC from six healthy controls were cultured for time periods of 1-7 days in medium alone or with IL-2 or IL-15 in optimised conditions as outlined in section *2.7.2.* Intracellular perforin expression was then measured together with CD3, CD4, CD8, and CD56 by a combination of cell-surface and intracytoplasmic mAB staining and analysis by flow cytometry. Staining was carried out as outlined in *2.9.4.* and analysis as outlined in *2.7.7.*

2.9.6. Statistics

Data was analysed using the software package INStat™ for Macintosh, version 2.03. The Friedman Nonparametric Repeated Measures Test (Friedman RMT) two tailed paired was used to assess the variation in both $CD3^+$ and $CD3^-CD56^+$ perforin

expression, in four healthy controls, using: (i) EDTA-WB, heparinised-WB, washed heparinised WB and PBMC and: (ii) three lysing techniques using EDTA-WB compared with PBMC. The Wilcoxon signed rank test, two-tailed, unpaired was used to compare the percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ and $CD3^-CD56^+$ perforin expression measured using EDTA whole blood hypotonic lysis versus PBMC preparations from ten healthy controls. The Mann-Whitney Test, two-tailed unpaired, was used to compare the absolute numbers of each cell type expressing perforin at time zero with the numbers following culture for 7 days with either IL-2 or IL-15. For these tests a p value of <0.05 was considered significant.

3. Results

3.1. Clinical, phenotypic and immune functional analysis of HLH patients.

3.1.1. Clinical Report

(Figure 3.1.A and B, table 3.1a and b)

The two children A and B presented aged nine months and eighteen months respectively, with gross failure to thrive, hepatosplenomegaly, and lymphadenopathy. Both patients had a history of serious recurrent bacterial and viral infections (table 3.1a and b). Histology of the cervical lymph node from patient A revealed proliferation of benign histiocytes with lymphocytic infiltration (figure 3.1A), and subsequently active haemophagocytosis was seen in a bone marrow aspirate. Lymph node biopsy from patient B showed infiltration of reactive histiocytes while a blood smear showed evidence of haemophagocytosis by monocyte like cells (figure 3.1B). Clinical and histological findings therefore indicated that these patients had haemophagocytic lymphohistiocytosis (HLH). Parental consanguinity led to a diagnosis of familial haemophagocytic lymphohistiocytosis (FHL) in these two cases (Henter *et al.* 1991a).

Following a recurrence of haemophagocytosis in 1997 patient A had been maintained on a regime of cyclosporin (Cs) A (Loechelt *et al.* 1994) immunosuppressive therapy to which he responded well, although he was hospitalised a number of times with infections including varicella, herpetic stomatitis and pneumonia. In 2000 he received a bone marrow transplant from a matched sibling donor which engrafted successfully and the patient remains clinically well.

Patient B presented with hepatosplenomegaly, lymphadenopathy and failure to thrive. Over the course of 18 months he suffered from recurrent infections including upper respiratory tract infections, herpetic stomatitis and molluscum contagiosum (viral

warts), a skin condition, which eventually resolved without treatment. At age 2 years and 9 months he developed an Epstein Barr virus (EBV) related lymphoproliferative disorder (figure 3.1 C and D) in the bowel which was resected and he is since clinically well. Although initially the diagnosis of patient B was FHL, this was subsequently changed to EBV-related lymphoma associated haemophagocytic syndrome (LAHS).

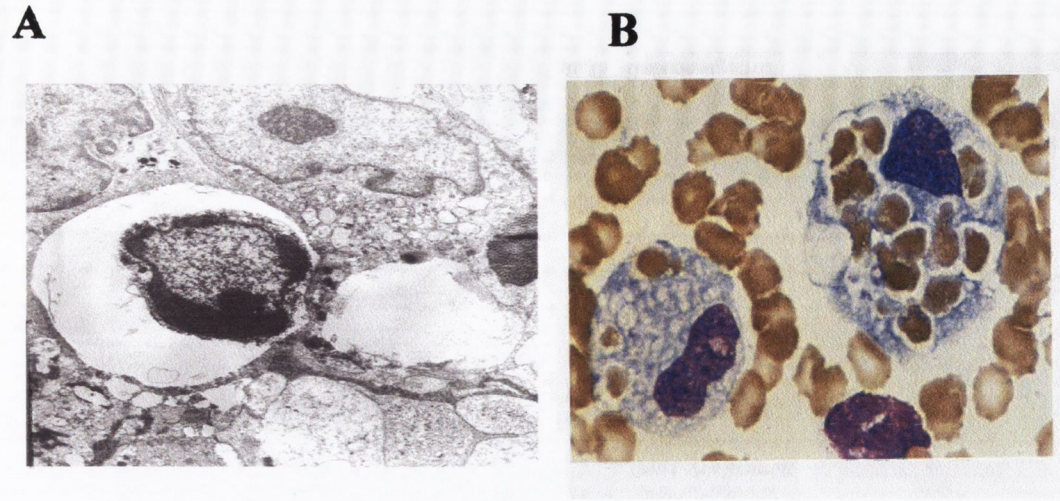


Figure 3.1. A, B. Active haemophagocytosis demonstrated in HLH patients.
Electron micrograph of a lymph node biopsy from patient A showing mononuclear cells phagocytosing lymphocytes and platelets (A). Monocytes actively phagocytosing erythrocytes demonstrated on a Romanoskwi stained whole blood preperation from patient B (B).

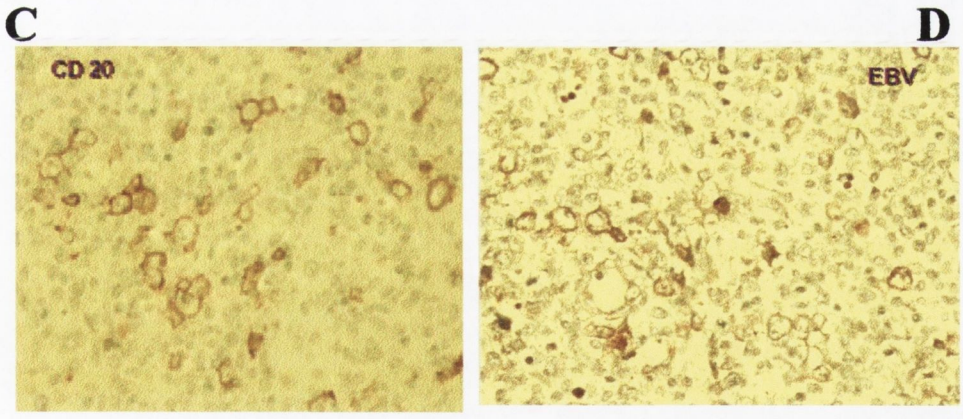


Figure 3.1.C, D. $CD20^{+}$ and EBV^{+} lymphocytes in lymphoma from HLH patient B.
Immunohistochemical staining of lymphoma resected from bowel of patient B showing many atypical lymphocytes positive for CD20 (C) and EBV (D).

Table 3.1a. Clinical and pathological findings for patient A

Age	Clinical condition	Findings
11 months	Failure to thrive, below 5th centile. Neck nodes x 3 months, lymphadenopathy and hepatosplenomegaly. Focal seborrhoeic rash, iron deficiency anaemia, WCC and platlets normal.	Cervical lymph node with paracortical proliferation of histiocytes and lymphocytic infiltration. Atypical reactive histiocytosis.
1-5 years	Recurrent episodes of upper respiratory tract infection (URTI), skin rash, anaemia, neutropaenia and hepatosplenomegaly.	Recurrent bacterial and viral infections including *Herpes simplex, Strep. pneumonia, Haemophilus influenza.*
5years 2months	Lymphadenopathy, hepatosplenomegaly, unwell. Bone marrow biopsy.	Bone marrow showing infiltrate of active haemophagocytic cells. Cyclosporin A therapy initiated.
6years 8months	Unwell skin rash and cold sore on nose.	*Varicella* and *Herpes simplex.*
7years	Pneumothorax.	Severe pneumococcal pneumonia.
7years 10months	Pyrexial, unwell, anaemia Hb 10.2 g/L.	Herpetic lesion on nose. Acyclovir therapy.
	Lymph node biopsy.	Evidence of haemophagoytosis.
8years	Matched related donor bone marrow transplant (BMT)	CMV reactivation and pneumonitis post BMT. Genetic evidence of engraftment.

Table 3.1b. Clinical and pathological findings for patient B

Age	Clinical condition	Findings
1year 8months	Failure to thrive, anaemia, lymphadenopathy and hepatosplenomegaly.	Liver biopsy with moderate peri-portal fatty change and portal histiocytic infiltration. Lymph node biopsy showing partial effacement of normal architecture by histiocytes/macrophages and a large palisade granuloma with central necrosis.
2years	Unwell.	Upper respiratory tract infection (URTI).
2years 4months	Unwell, skin rash.	URTI and molluscum contagiosum.
2years 6months	Diarrhoea and unwell	URTI.
2years 9months	Diarrhoea, vomiting, abdominal obstruction	Lymphoma in the abdomen with EBV demonstrated in-situ.

Table 3.2. Percentages and counts of T, B and NK cell cells in HLH patients and relatives of patient B compared to age matched controls. Results are expressed as percentages (%) of lymphocytes and cell counts per ml.

	Patient A aged 6 months	Patient B aged 18 months	Sibling C aged 7 years	Cousin D aged 6 years	Cousin E aged 2 years	Controls aged 0-11 months n=16*	Controls aged 1-6 years n=48*	Controls aged 2-7 years n=5**
$CD3^+$ %	78	63	77	68	70	58-67	62-69	64-80
$CD3^+ CD4^+$ %	68	59	55	43	57	38-50	30-40	26-62
$CD3^+ CD8^+$ %	7	4	22	23	16	18-25	25-32	13-37
$CD19^+$ %	23	36	21	28	27	19-31	21-28	13-25
$CD3^- CD56^+$ %	<1	<1	1	1	2	8-17	8-15	3-13
$CD3^+ CD56^+$ %	0.1	0.3	1	<1	1	NA	NA	1-3
$CD3^+$ /ml	2370	1382	2381	2660	3756	1700-3600	1800-3000	1840-3640
$CD3^+CD4^+$ / ml	2070	1303	1721	1679	3007	1700-2800	1000-1800	1248-2428
$CD3^+CD8^+$ / ml	210	77	643	918	852	800-1200	800-1500	432-1312
$CD19^+$ / ml	700	794	642	1099	1434	500-1500	700-1300	416-1116
$CD3^-CD56^+$ / ml	30	9	32	30	174	300-700	200-600	102-546
$CD3^+CD56^+$ / ml	3	9	30	30	60	NA	NA	34-103

*Ranges are shown as 25th to 75th percentiles (Erkeller-Yuksel *et al.* 1992)

**Ranges are shown as mean $\pm$ standard deviation for controls 2-6 yrs n=5. Testing was carried out in The Immunology Dept. St. James Hospital, Dublin.

NA indicates results not available.

3.1.2. $CD3^+CD8^+$, NK and NKR^+ T cells were reduced in both HLH patients

(Figure 3.2, table 3.2)

Determination of lymphocyte subsets was carried out on peripheral whole blood from the two patients and five age-matched controls, using labelled antibodies with flow cytometry. For the purposes of this study NK cells are defined as $CD3^-CD56^+CD16^+$ and NKR^+ T cells are $CD3^+$ cells which co-express one of the NK associated receptors including CD56, CD94, CD158a, b, e1, or CD161. Results for four-colour analysis of T cell subsets, B cells and NK and $CD56^+$ T cells are shown in figure 3.2A. Percentages and absolute counts for both patients and age-matched controls are shown in table 3.2 (Erkeller-Yuksel *et al.* 1992). The percentages and numbers of lymphocytes expressing the T cell marker CD3 and the $CD3^+CD4^+$ phenotype were within the expected range for patients A and B. The percentages of lymphocytes expressing the $CD3^+CD8^+$ phenotype were reduced in both patients, at 7 and 4% respectively, compared to age matched controls at 18-25%. The absolute counts of $CD3^+CD8^+$ cells were also reduced compared to age matched controls at 210 and 130 cells /ml for patients A and B respectively with an interquartile range of 800-1200 cells/ml for age matched controls. Normal percentages and numbers of B cells, defined by CD19 expression, were detected in patient A. Results for patient B show that while the percentage of B cells was raised at presentation (36% range = 19-31%) cell numbers were within the expected range. NK cells, defined as $CD3^-CD56^+CD16^+$ lymphocytes, were virtually absent from the periphery of patients A and B at <1% of lymphocytes and 30 and 9 cells/ml respectively (age matched controls 3-13% and 102-254 cells/ml). The natural killer receptor positive (NKR^+) T cells, defined here as $CD3^+CD56^+$ phenotypes, were detected at low levels and numbers in controls aged 2-7 yrs (Table 3.2) at 1-3% of lymphocytes and 34-103 cells/ml and were very reduced in patients A and B at 0.1 and 0.3% and 3 and 9 cells/ml respectively. Results for lymphocyte sub-sets in relatives of patient B, shown in table 3.2, indicate that two male cousins and a female sibling have reduced percentages and numbers of NK cells. The female sibling and one cousin have reduced numbers of NKR^+ T cells. The two males suffer from recurrent infections and the female is apparently unaffected.

A.

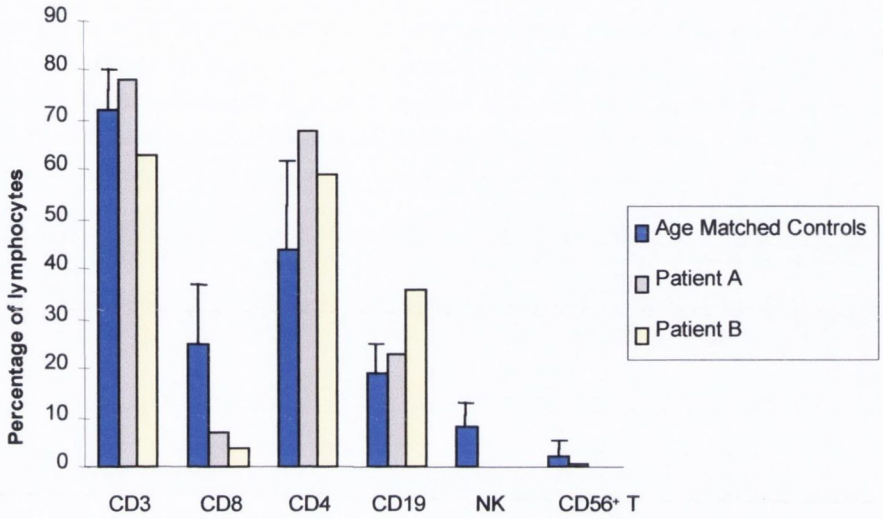


Figure 3.2. Analysis of peripheral blood lymphocyte subsets measured at initial presentation of patients A and B.

Percentages of lymphocytes staining with fluorescent labelled antibodies to CD3, CD3CD8, CD3CD4, CD3CD56, CD19 and CD56 without CD3 (NK cells) measured using four-coloured flow-cytometry. Results shown are for patients A and B at initial presentation together with results for age matched controls (n=3) showing mean+SD.

3.1.3. Increased CD16 but reduced CD18/CD11c expression on monocytes in HLH

(table 3.3)

Having established that $CD3^{-}CD56^{+}CD16^{+}$ lymphocytes were reduced at presentation in both of these patients with HLH, we decided to examine the expression of CD16 on other cell types. We measured CD16 and adhesion molecules, CD18 and CD11a, b and c, on monocytes using flow cytometry. Results in table 3.3 show that the percentage of $CD14^{+}$ monocytes expressing CD16 was increased in both patients compared to controls. The percentages of monocytes expressing CD18 were reduced in patients A and B at 62% and 67% respectively (expected range 94±5%) as were the percentages of monocytes expressing CD11c at 47% for patient A and 50% for patient B (expected range 94±5%). The expression of CD11a and b were normal in both patients.

Table 3.3. Expression of surface markers on monocytes in HLH patients and controls.

	$CD14^{+}$	$CD14^{+}$ $CD16^{+}$	$CD18^{+}$	$CD11a^{+}$	$CD11b^{+}$	$CD11c^{+}$
Patient A	94	20	62	94	94	47
Patient B	90	18	67	96	96	50
AMC* n=3	90±3	10±5	95±4	95±5	92±4	96±5
Adult controls n=5	92±3	11±5	94±5	96±3	92±5	94±5

Results are expressed as a percentage of monocytes

Control values are expressed as mean± standard deviation. AMC* refers to age matched controls

3.1.4. Changes in lymphocyte subsets in response to treatment and infection in NK deficient patients

(Figure 3.3.)

In order to monitor changes in lymphocyte subsets, CD3CD4, CD3CD8, NK and B cell percentages and numbers were measured over the course of treatment of these two young patients. Longitudinal studies of T cell subsets, B and NK cells, shown in figure 3.3A and C, indicate that in patient A an increase was observed in $CD3^+CD8^+$ percentages from 7% of lymphocytes at presentation to 29% in 1999 prior to bone marrow transplant (BMT). The percentage and numbers of lymphocytes expressing $CD3^+CD8^+$ were lower in patient A than age matched controls until the initiation of cyclosporin therapy (Table 3.2). Results for $CD3^+CD8^+$ in figures 3.3B (percentages) and D (cell counts/ml) show that for patient B, from presentation in 1998 to 2001, there has been some increase in both the percentage and number of $CD3^+CD8^+$. However, while the percentage of lymphocytes expressing $CD3^+CD8^+$ in 2001 was below the expected levels for age matched controls the number of $CD3^+CD8^+$ were within the expected range. Over time both percentages and numbers of $CD3^+CD4^+$ lymphocytes have remained within expected ranges in the two patients. Results for patient A show that, coincident with cyclosporin therapy, B cell percentages and numbers declined (fig 3.3A and C) to below the expected ranges. Results for patient B, shown in figure 3.3B and D, indicate that B cell percentages were high at presentation and except for a short period in 1998 - coincident with clinical exacerbation of HLH - percentages have remained high and numbers have risen steadily to 1494 cells/ml in January 2001. While other cell populations have shown changes over time, longitudinal studies did not show any increase in NK cell percentages or numbers in either patient A or B.

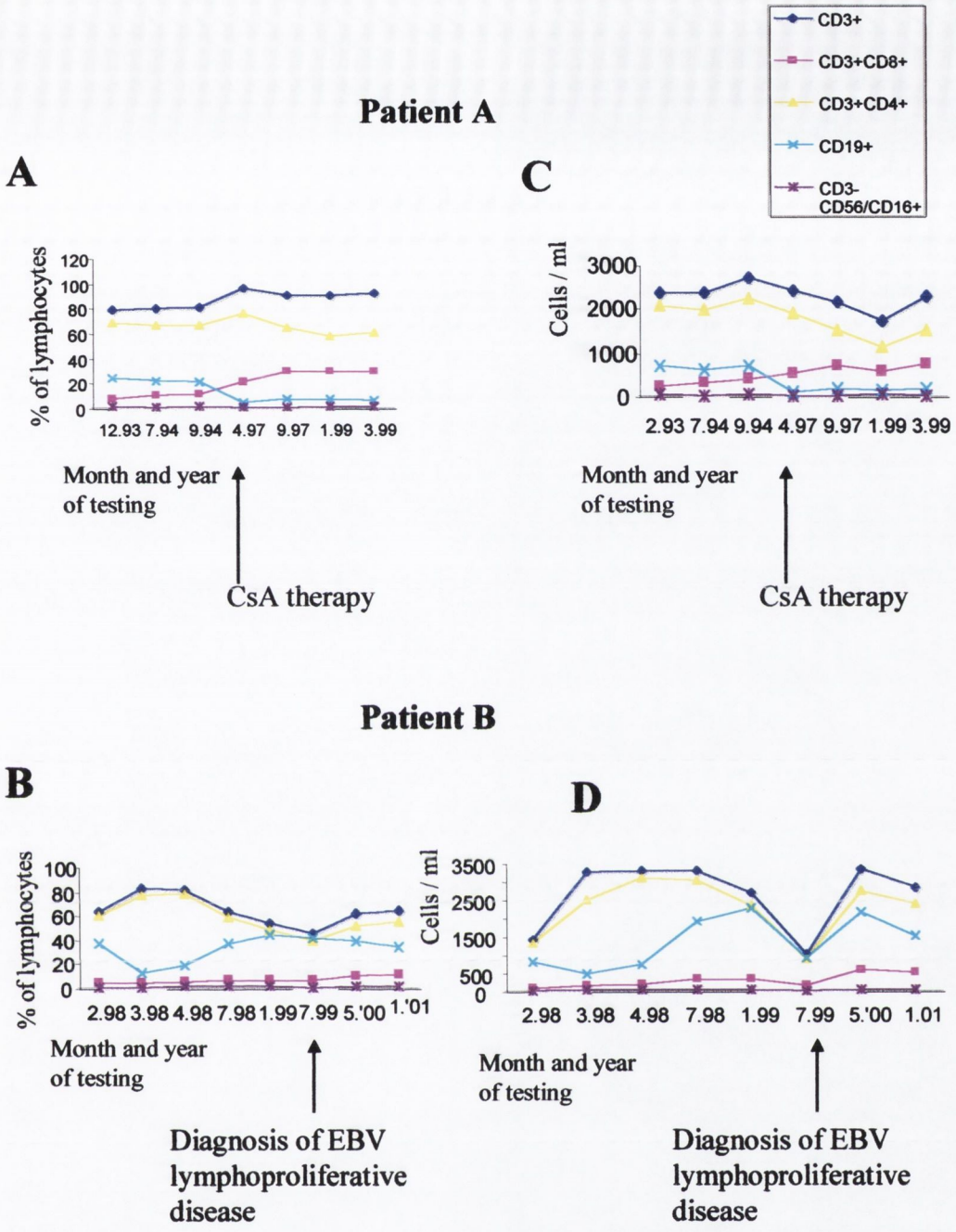


Figure 3.3. Longitudinal studies of lymphocyte subsets in patients A and B.

Percentages of lymphocytes (A) and counts (cells/ml) for each cell type (C) measured from 1993 to 1999 for patient A showing the date of initiation of cyclosporin therapy.
Percentages of lymphocytes (B) and counts (cells/ml) for each cell type (D) measured from 1998 to 2001 for patient B showing date of diagnosis of EBV lymphoproliferative disease

3.1.5. Effects of lysis and anticoagulant on intracellular perforin expression measured using whole blood

(Figure 3.4)

Perforin is an important mediator of cytotoxicity in NK cells, $CD3^+CD56^+$ cells and antigen specific $CD8^+$ and $CD4^+$ T cells (Yasukawa *et al.* 2000; Kagi *et al.* 1994a; Ortaldo *et al.* 1991). Defects in the perforin gene - leading to reduced or absent expression of this mediator - have been demonstrated in a proportion of patients with familial haemophagocytic lymphohistiocytosis (Stepp *et al.* 1999). The measurement of intracellular perforin expression in lymphocyte subsets was therefore considered to be an important aid in the diagnosis of FHL. In order to easily assess paediatric samples for intracellular perforin expression, a whole blood (WB) assay was developed. Intracellular perforin expression was measured using three-colour flow cytometry. The effect that various lysing techniques and anti-coagulants (EDTA and heparin) had on percentages of $CD3^+$ and $CD3^-CD56^+$ cells expressing perforin were compared. Lysis of erythrocytes with ammonium (NH_3) or Facslyse (BD) were found to be unsuitable (figure 3.4 B and E) as these resulted in a decrease in the observed percentages of $CD3^+$ cells expressing perforin with no alterations in NK cell perforin expression. Ammonium chloride lysis has been shown to alter granularity of lymphocytes and to inhibit NK-mediated lysis (Brander *et al.* 2001). Hypotonic lysis of erythrocytes for 20 seconds was the most suitable method, yielding intracellular perforin results comparable to those achieved with isolation of PBMC using a Ficoll gradient for both $CD3^+$ and $CD3^-CD56^+$ cells.

The effects of anti-coagulant on perforin expression using whole blood hypotonic lysis was also examined and compared to isolated PBMC. Heparin was found to cause a reduction in the measured intracellular perforin expression in $CD3^+$ cells, with reduction in the levels (data not shown) though not the percentages of perforin positive $CD3^-CD56^+$ cells (figure 3.4 C and F). While the measured percentages of both $CD3^+$ and NK cells expressing perforin was comparable for EDTA, washed heparinised cells and isolated PBMC, the level of intracellular perforin expression was reduced. This reduction is demonstrated in the histograms in figure 3.4 A and D, which compares the

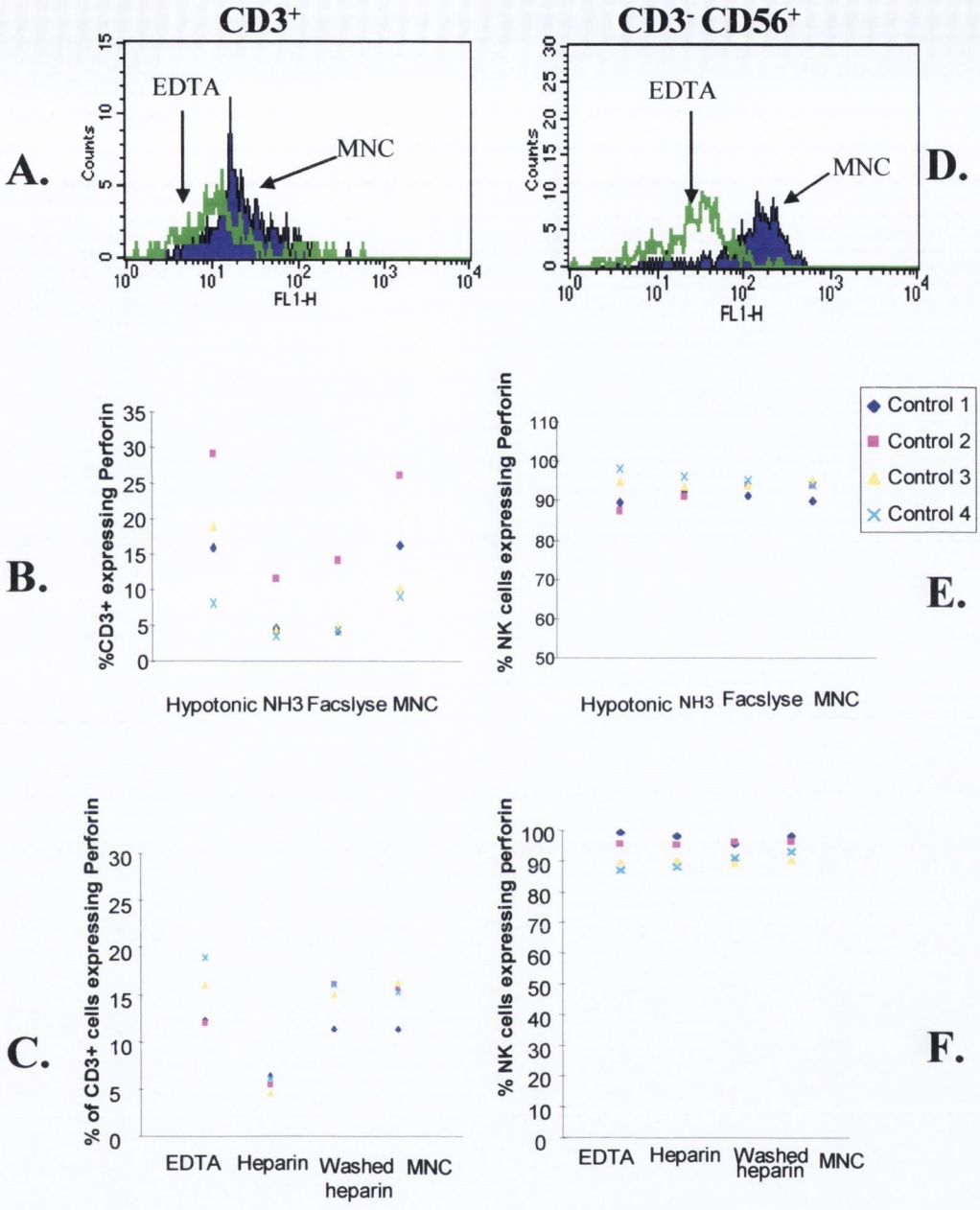


Figure 3.4. Effects of various methods of lysis and anti-coagulant on the measurement of intracellular perforin expression.

Histograms of flow cytometric detection of perforin expression in Ficoll-Hypaque isolated $CD3^+$ (A) and $CD3^-CD56^+$ (D) mononuclear cells (MNC) together with results for these cells using hypotonic lysis of whole blood with EDTA as an anticoagulant.
Effect of lysing erythrocytes using hypotonic lysis, ammonium (NH3) or Facslyse (BD) on measurement of intracellular perforin expression in $CD3^+$ (B) and $CD3^-CD56^+$ (E) cells compared to isolated mononuclear cell (MNC) levels. Comparison of the effects of anti-coagulants EDTA, heparin and washing of heparinised cells on the levels of perforin detected using hypotonic lysis compared to that measured in isolated MNC in $CD3^+$ (C) and $CD3^-CD56^+$ (F) cells. Results shown in B, C, E and F are for four healthy controls.

levels of intracellular perforin expression in $CD3^+$ and $CD3^-CD56^+$ using either EDTA whole blood or isolated mononuclear cells (MNC).

Perforin has been shown to be activated by heparin (Ishiura *et al.* 1988) and lysis using Facslyse (BD) causes blebs in the cell membrane, perhaps allowing perforin to leak out. NK cells express perforin at higher intracellular concentration than $CD3^+$ cells (figure 3.4 A and D and figure 3.24 A). The high level of perforin expression may protect NK cells from the effects of 'perforin leakage' during processing. Because of the lower intensity of perforin expression in T cells degranulation during processing is likely to have a profound effect on the observed percentages of T cells expressing perforin. These observations may explain why a recent study of intracellular perforin expression - where FACSlyse was used in a whole blood assay - reported lower percentages of adult $CD8^+$ and $CD56^+$ T cells expressing intracellular perforin than those reported in this present study (Kogawa *et al.* 2002).

3.1.6. The percentages of T cells and NK cells expressing perforin in normal controls

(Table 3.4)

Results for ten healthy controls, using EDTA-whole blood hypotonic lysis, in table 3.4, show that within the $CD3^+$ cell population a small percentage of $CD4^+$ cells (3.7 ± 2.1), a significant pecentage of $CD8^+$ cells (30.3 ± 13.5) and the majority of $CD56^+$ cells (82.3 ± 8.4) expressed perforin. The percentage of NK cells expressing perforin was high (93.9 ± 4.8). Similar results were obtained using PBMC and, except in the case of $CD4^+$ cells, results for whole blood and PBMC were not significantly different (table 3.4).

Table 3.4. Perforin expression in PBMC from healthy adult controls.

Cell Poplation	WB EDTA	PBMC	Wilcoxon signed rank p value
$CD3^+$	14.7±3.2	14.5±2.2	0.99
$CD3^+CD4^+$	3.7±2.1	2.9±2.0	0.0312
$CD3^+CD8^+$	30.3±13.5	30.9±14.1	1.000
$CD3^+CD56^+$	82.3±8.4	78.4±6.8	0.562
$CD3^-CD56^+$	93.9±4.8	92.7±6.5	0.687

Comparison of the percentages of lymphocyte sub-populations expressing perforin detected using whole blood-EDTA and isolated PBMC from 10 healthy controls. Results are expressed as percentages of the cell populations ± standard deviation. p value of <0.05 was considered significant

Isolated PBMC from patient B were tested for perforin expression. Results showed that 9.8% of $CD3^+$ cells and 44% of $CD3^+CD8^+$ cells expressed perforin. Since $CD3^-CD56^+$ cells were very reduced in this patient, perforin expression in this very tiny population was difficult to ascertain. Patient A had received a bone marrow transplant and expression of perforin in PBMC was not tested prior to transplantation.

3.1.7. Reduced NK and IL-2 induced cytotoxicity in HLH

(Figure 3.5)

Since both these patients were shown to be deficient in NK and $CD56^+$ T cells, we therefore examined the cytotoxic activity of these cells. NK cytotoxicity was measured using freshly isolated PBMC as the effector and K562, an erythroleukemic cell line, as the target cells. To further examine NK and NKR^+ T cell activity, killing of Daudi cells by IL-2 activated PBMC was carried out. In both patients NK function was absent (figure 3.5A) and IL-2 induced cytotoxicity was very reduced (figure 3.5B) compared to a healthy adult control. This test was repeated twice for each patient with the similar results.

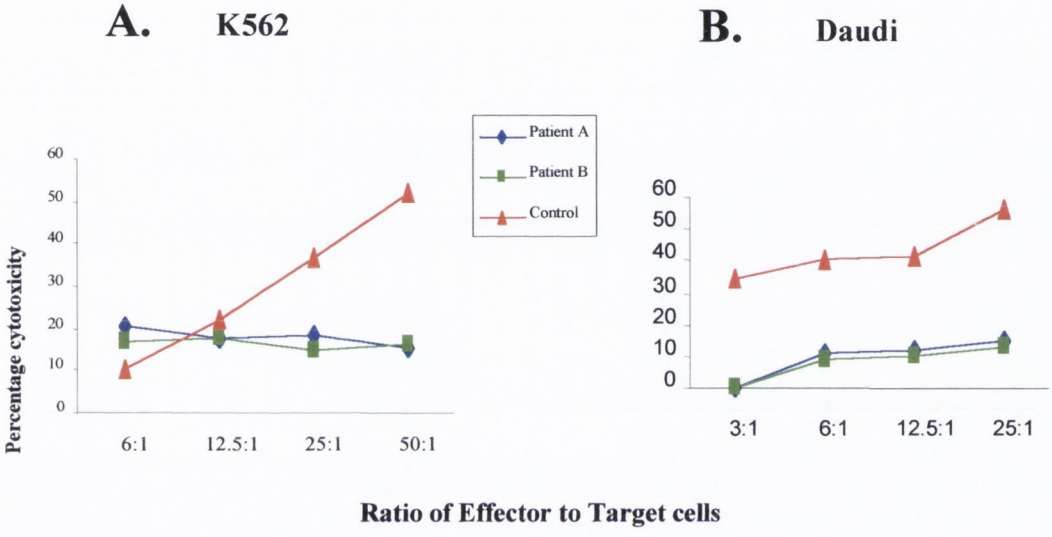


Figure 3.5. Cytotoxicity against K562 and Daudi cells measured at initial presentation of patients A and B.

A. Percentage specific cytotoxicity by PBMC from control and patient cells against K562 target cells.
B. Percentage specific cytotoxicity by IL-2 activated PBMC from control and patient cells against Daudi target cells.

3.1.8. HLH patient A has a reduced response in mixed lymphocyte culture (table 3.5)

In order to measure the ability of patient PBMC to mount an effective response to allogeneic cells we tested PBMC from patient A against unmatched irradiated target PBMC from his mother and two unrelated adult control subjects. Results in table 3.5 indicate that compared to control subjects, patient A had a reduced response to allogeneic unmatched cells in mixed lmphocyte culture (MLC). This test was carried out prior to cyclosporin A therapy. This test was not carried out for patient B.

Table 3.5. Mixed lymphocyte culture for patient A and controls.

	Patient A Target	Control 1 Target	Control 2 Target	Control 3 Target
Patient A (Effector)	1.0	0.7	1.2	2.6
Control 1 (Effector)	4.3	1.0	2.1	4.4
Control 2 (Effector)	7.0	3.7	1.0	6.9
Control 3 (Effector)	2.7	2.0	2.7	1.0

Results are expressed as stimulation indices (SI) for proliferation of effector cells in response to irradiated target cells. SI of >2 is expected against unmatched lymphocytes.

3.1.9. Normal lymphocyte proliferation in response to mitogens in HLH (Table 3.6)

Incorporation of tritiated (^{3}H) thymidine into lymphocytes was used to assess the proliferation of PBMC in response to mitogens. Results in table 3.6 demonstrate that PBMC from both patients proliferated in response to concanavalin (Con A) with a stimulation index for patient A at 36.5 and for patient B at 19.7. Cells from both patients proliferated in response to stimulation with phytohaemagglutinin (PHA) with a

stimulation index for patient A at 184 and for patient B at 83.2. A stimulation index of greater than 3 was deemed to be positive.

Table 3.6. Proliferation of lymphocytes in response to Con A and PHA

	Patient A	Patient B	Range and Units
Con A 10 μg/ml	36.5	19.7	>3 SI is positive
PHA 10 μg/ml	184	83.2	

Results are expressed as stimulation indices (SI)

3.1.10. Expression of mRNA for NKG2A demonstrated in HLH patient A

(Figure 3.6)

Flow cytometric analysis of PBMC from patient A had indicated that the NK associated receptor CD56 and CD57 (data not included) were not expressed on either $CD3^-$ or $CD3^+$ cells in the periphery. In order to further investigate NK associated receptors in this patient we examined expression of mRNA for the receptor NKG2A. At the time this study was undertaken no commercial monoclonal antibody to NKG2A was available. Two sets of sequence specific primers were used to demonstrate expression of mRNA for NKG2A in cells from patient A and an adult control. Results in figure 3.6 show that primer pairs I and II amplified sequences of 933 bp and 715 bp respectively, in both patient A and in an adult control.

3.1.11. Normal SAP and IL-2 receptor beta expression in HLH patient B

Following the development and excision of an EBV-driven lymphoma from the gastro-intestinal tract of patient B, investigations were undertaken to rule out X-linked lymphoproliferative (XLP) disorder. XLP patients suffer from unresolved infection with Epstein-Barr virus and this has been shown to be due to deficiency of a signalling protein known as SLAM associated protein (SAP) (Coffey *et al.* 1998; Nichols *et al.* 1998).

Figure 3.6. Expression of natural killer receptor NKG2A in patient A and a control subject.

mRNA was isolated from PBMC for patient A and a normal control and reverse transcribed to yield cDNA. Using two sets of primers for NKG2A, cDNA was amplified in a polymerase chain reaction (PCR) and visualized on an agarose gel. Negative controls were included in lanes 1 and 4, samples from patient A were placed in lanes 2 and 3 with control subject samples in lanes 5 and 6. Molecular weight markers were run in lane 7.

IL-2 receptor beta (IL-2Rβ) has been shown to be important in the development and regulation of NK and NKR^+ T cells (Suzuki *et al.* 1997; Suzuki *et al.* 1995; Ohteki *et al.* 1997). PBMC from patient B had been shown to express IL-2Rβ using mAb and flow-cytometry (figure 3.6 B), subsequently immunoblotting was undertaken to establish whether there was any alteration in the size of this receptor. Expression of SLAM associated protein (SAP) and IL-2Rβ was measured in PBMC from patient B, his NK cell deficient female sibling and a normal control. Testing was carried out in the Department of Immunology, Great Ormond Street Hospital, London following previously published protocols (Gilmour *et al.* 2000; Gaspar *et al.* 1998). Both patient B and his sibling were shown to express normal size SAP and IL-2Rβ on immunoblotting.

3.1.12. Normal humoral immunity in HLH patients

(Table 3.7)

Since these two NK deficient patients suffered from recurrent infections, both bacterial (*S. pneumonia* and *H. influenza*) and viral (*herpes simplex* and *varicella*), we were interested in assessing their humoral immunity. To do this we first examined serum levels of IgG, IgA and IgM in both patients: patient A had raised IgG and IgA levels, all other parameters measured were found to be within normal ranges (table 3.7). Classical complement function was examined using sensitized sheep red cells and alternative complement function using chicken erythrocytes: again serum from both patients responded normally in these assays (table 3.7). With ELISA testing for antibodies to bacterial antigens, serum from both patients had normal levels of specific IgG to tetanus, specific IgG1 and 2 against pneumococcus and specific IgG antibody to haemophilus influenza b. The level of antibodies to viral antigens in serum from both patients was also assessed using ELISA testing. Results in table 3.7 show that both patients had levels of specific antibody to *herpes*, measles and mumps indicating previous exposure and immunity. Patient B had specific antibodies to rubella, however serum from the two patients showed no response in these assays to CMV or toxoplasma indicating no previous exposure or a lack of humoral response to these pathogens. Serum from patient B was also tested for antibodies to EBV, which was negative, indicating no evidence of acute infection. EBV testing was not carried out for patient A.

Table 3.7. Humoral immunity in patients with HLH with age related ranges (mean ±SD)

	Patient A	Patient B	Range and units
IgG	17.6	13	5.1-13.5 g/L
IgA	2.04	0.7	0.26-1.47 g/L
IgM	0.55	0.73	0.34-1.35 g/L
Alternative -CH100	78	70	50-150 % of normal
Classical - CH100	720	630	330-770 CH100 Units
Anti-tetanus IgG	1.6	1.35	>0.43 IU/ml
Anti-pneumococcus	5.4	6.5	IgG mg/L
	1.9	2.65	IGg2 mg/L
Anti-Haemophilus Influenza b	1.1	1.76	>0.15 mg/L
Herpes simplex	Positive	Positive	
Mumps S	Positive	Positive	
Mumps V	Positive	Positive	
Measles	Positive	Positive	
CMV	Negative	Negative	
EBV	NA	Negative	
Rubella	Negative	Positive	
Toxoplasma	Negative	Negative	

3.2. Expression and function of IL-15 and its receptor components in HLH patients and controls

IL-15 and its receptor complex components have been shown to be important in the development and function of NK and NKR^+ T cells (Liu *et al.* 2000; Fehniger *et al.* 2001). The deficiencies of these cell types in the periphery of the two HLH patients led us to investigate IL-15-receptor and cytokine expression.

3.2.1. Expression of IL-15Rα chain mRNA in HLH patients and healthy controls

(Figures 3.7 and 3.8)

Since IL-15Rα is a high affinity receptor for IL-15 and forms a unique part of its receptor complex we examined expression of mRNA for this receptor in the two patients and two controls. Using two sets of sequence specific primers, expression of mRNA for IL-15Rα was demonstrated in PBMC from both patients and controls (figure 3.7A). Although there was a second, non-specific, band amplified with both sets of primers, identification of the sequenced products at 1285bp and 329bp was carried out using the web-site NCBI BLAST at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. The area of IL-15Rα gene precursor expression that was sequenced using PCR product from patient A is shown as blue and red highlighted nucleotides in figure 3.8.B and included 91% of the extracellular domain as well as the entire transmembrane and cytoplasmic domains. The sequenced nucleotide product from patient A was shown to be human IL-15Rα corresponding with between 99% and 100% identity to published sequences NM 002189, U31628 and others (figure 3.8 B and C). Single nucleotide polymorphisms in the sequence from patient A were shown to correspond to NM 002189 positions 529 (C to T), 567 (A to C) and 848 (A to G). The predicted amino acid (aa) sequence for product from patient A showed non-conserved substitutions when compared to human IL-15Rα precursor Accession number NP002189 (ref. NM002189 and U31628). There was an asparagine (N) to threonine (T) substitution at aa position 181 and an arginine (R) to glycine (G) at aa position 256.

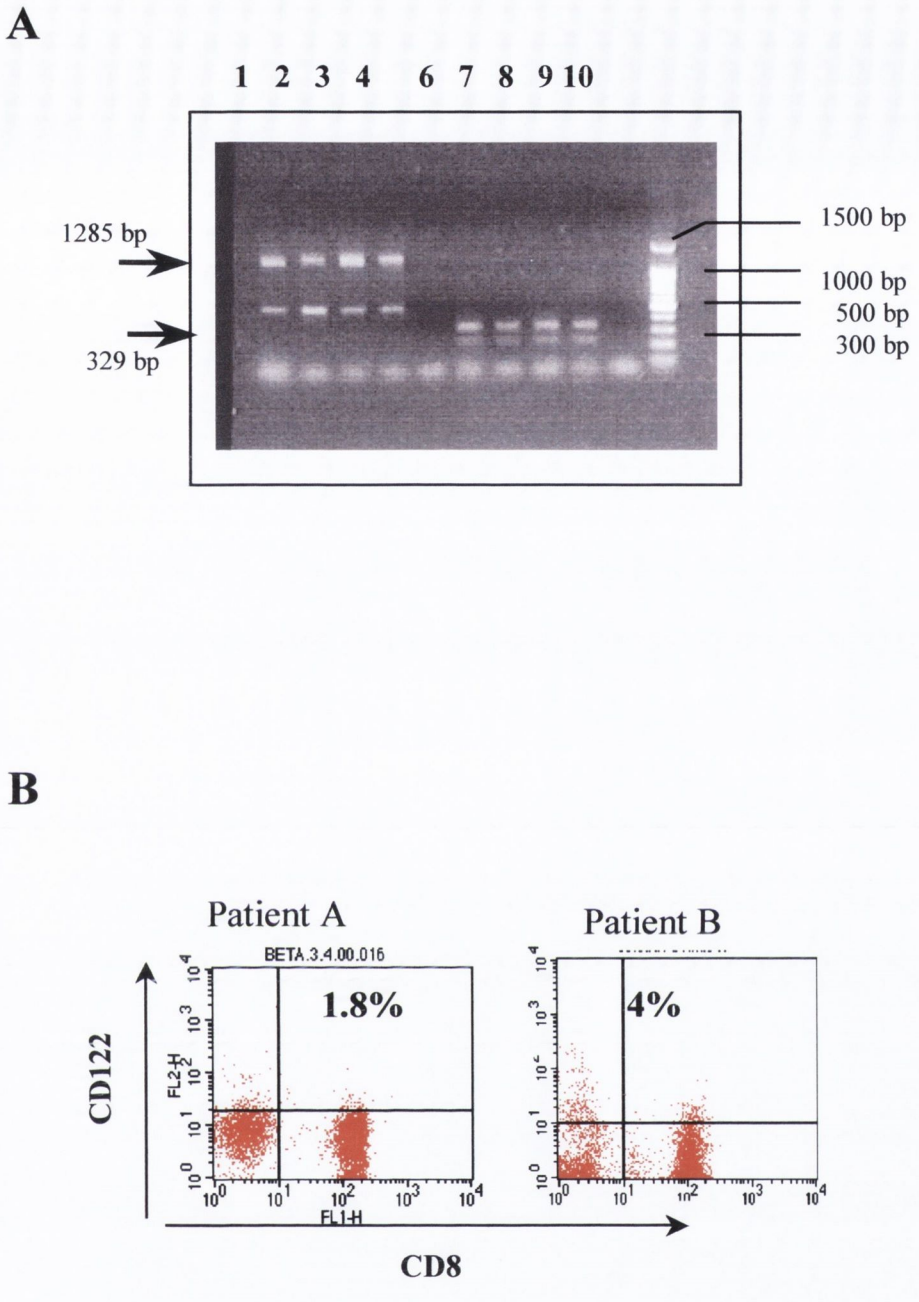
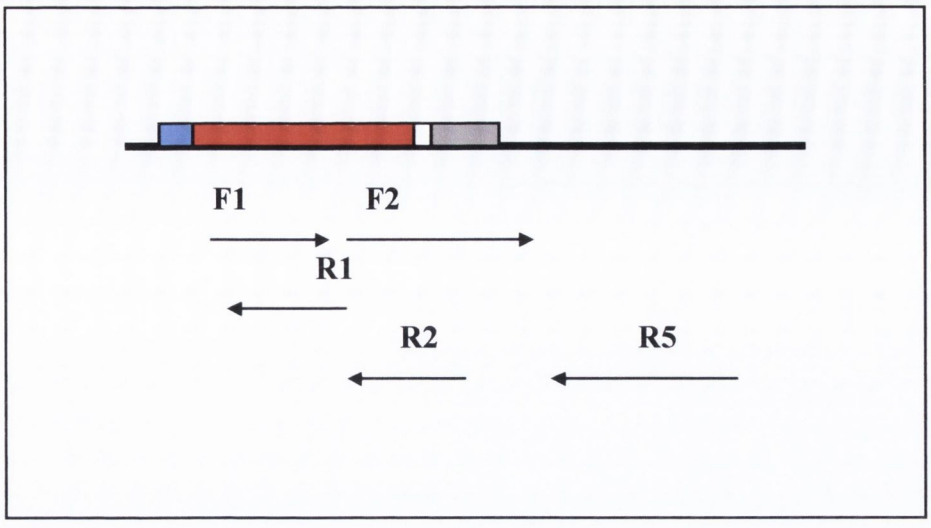


Figure 3.7. IL-15Rα and β (CD122) demonstrated in PBMC from HLH patients and controls.

PCR, using two sets of sequence specific primers for IL-15Rα yielding products of 1285 and 329 bp, was carried out for HLH patients A and B, and controls. PCR products visualized on an agarose gel with product from patient A in lanes 1and 7, patient B lanes 2 and 8 and controls lanes 3, 4, 9 and 10 (A). Dot plot showing staining for CD122 (IL-2/IL-15R-β) and CD8 on $CD3^+$ lymphocytes from patients A and B (B).

A



B

1 cccagagcag cgctcgccac ctccccccgg cctgggcagc gctcgcccgg ggagtccagc
61 ggtgtcctgt ggagctgccg ccatggcccc gcggcgggcg cgcggctgcc ggaccctcgg
121 tctcccggcg ctgctactgc tgctgctgct ccggccgccg gcgacgcggg gcatcacgtg
181 cctccccccc atgtccgtgg aacacgcaga catctgggtc aagagctaca gcttgtactc
241 cagggagcgg tacatttgta actctggttt caagcgtaaa gccggcacgt ccagcctgac
301 ggagtgcgtg ttgaacaagg ccacgaatgt cgcccactgg acaacccccа gtctcaaatg
361 cattagagac cctgccctgg ttcaccaaag gccagcgcca ccctccacag taacgacggc
421 agggggtgacc ccacagccag agagcctctc cccttctgga aaagagcccg cagcttcatc
481 tcccagctca aacaacacag cggccacaac agcagctatt gtccgggct cccagctgat
541 gccttcaaaa tcaccttcca caggaaccac agagataagc agtcatgagt cctcccacgg
601 cacccctct cagacaacag ccaagaactg ggaactcaca gcatccgcct cccaccagcc
661 gccaggtgtg tatccacagg gccacagcga caccactgtg gctatctcca cgtccactgt
721 cctgctgtgt gggctgagcg ctgtgtctct cctggcatgc tacctcaagt caaggcaaac
781 tcccccgctg gccagcgttg aaatggaagc catggaggct ctgccggtga ctgggggac
841 cagcagcaga gatgaagact tggaaaactg ctctcaccac ctatgaaact cggggaaacc
901 agcccagcta agtccggagt gaaggagcct ctctgcttta gctaaagacg actgagaaga
961 ggtgcaagga agcgggctcc aggagcaagc tcaccaggcc tctcagaagt cccagcagga
1021 tctcacggac tgccgggtcg gcgcctcctg cgcgagggag caggttctcc gcattcccat
1081 gggcaccacc tgcctgcctg tcgtgccttg gacccagggc ccagcttccc aggagagacc
1141 aaaggcttct gagcaggatt tttatttcat tacagtgtga gctgcctgga atacatgtgg
1201 taatgaaata aaaaccctgc cccgaatctt ccgtccctca tcctaacttg cagttcacag
1261 agaaaagtga catacccaaa gctctctgtc aattacaagg cttctcctgg cgtgggagac
1321 gtctacaggg aagacaccag cgtttgggct tctaaccacc ctgtctccag ctgctctgca
1381 cacatggaca gggacctggg aaaggtggga gagatgctga gcccagcgaa tcctctccat
1441 tgaaggattcaggaagaaga aaactcaact cagtgccatt ttacgaatat atgcgtttat
1501 atttatactt ccttgtctat tatatctata cattatatat tatttgtatt ttgacattgt
1561 accttgtata aacaaaataa aacatctatt ttcaatattt ttaaaatgca

Figure 3.8.A Nucleotide sequencing of IL-15Rα gene in patient A
Following PCR, using sets of sequence specific primers for IL-15Rα the target sequence was excised from an agarose gel and sequencing of the products was carried out for HLH patient A. Illustrated in A is a schematic diagram of IL15Rα, Genbank Accession U31628 with the signal sequence highlighted in blue, the extracellular domain in red, the transmembrane region in white and the cytoplasmic domain in grey. The direction and area of sequencing carried out, together with the forward (F) and reverse (R) primers used for sequencing are shown.
B. The published sequence of IL15Rα, Genbank Accession U31628 is shown with black arrows indicating exon-intron junctions. Nucleotides highlighted in red indicate the primers used in PCR and sequencing. Blue highlighted nucleotides indicate the areas sequenced.

C

Figure 3.8.C Nucleotide sequencing of IL-15Rα gene in patient A .....continued

Using primers specific for IL-15Rα, PCR was carried out on mRNA isolated from patient A. The nucleotide sequence of this product was identified as IL-15Rα. Sequence alignment showed 99% similarity to published sequences for IL-15Rα, Genbank Accession U31628 and NM02189 over the area 228bp to 939bp .

D

Figure 3.8.D Nucleotide sequencing of IL-15Rα gene in patient A …….continued

Using primers specific for IL-15Rα, PCR was carried out on mRNA isolated from patient A. The nucleotide sequence of this product was identified as IL-15Rα. Sequence alignment showed 100% similarity to published sequences for IL-15Rα, Genbank Accession U31628 and NM02189 over the area 1038bp to 1447bp.

3.2.2. Reduced expression of IL-2Rα (CD25) and β (CD122) in HLH patients compared to controls

(Figures 3.9 and 3.14C, table 3.8)

Flow cytometry revealed that, in normal adult controls, the IL-2 receptor α-chain, CD25, was expressed by a mean of 27% of freshly-isolated human $CD56^-$ T cells but only 2% and 7% of NK and $CD56^+$ T cells respectively (figure 3.9). In contrast, the IL-2/IL-15 receptor β-chain, CD122, was constitutively expressed by NK cells (99%) and most $CD56^+$ T cells (74%) but only 10% of $CD56^-$ T cells expressed this receptor (table 3.8, figure 3.9).

Freshly isolated lymphocytes from both patients lacked $CD56^+$ cells of either NK or T lineage. The percentage of $CD56^-$ T lymphocytes expressing CD25 was 11% in patient A and 15% in patient B. CD122 was expressed on 2% of $CD56^-$ T cells in patient A and on 3% in patient B. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells from both patients were shown to express CD25 and CD122 at lower than normal levels (figure 3.14C). In addition as has been stated earlier immunoblotting revealed normal sized IL-2Rβ (CD122) in PBMC from patient B. Immunoblotting for CD122 was not carried out for patient A Expression of the IL-2/IL-15 common γ-chain was not tested.

Table 3.8. CD25 and CD122 receptor expression in healthy adult controls.

Receptor	% NK cells	% $CD56^-$ T cells	% $CD56^+$ T cells
CD25	2.1 ± 0.2	26.9 ± 5.6	6.9 ± 3.5
CD122	99.8 ± 0.4	10.9 ± 1.9	73.8 ± 6.8

Distribution of lymphocyte receptors (% positivity ± SD) among freshly isolated NK cells ($CD3^-CD56^+$ cells), $CD56^+$ T cells and $CD56^-$ T cells.

All values are the means of at least 4 and up to 15 samples from normal adult controls

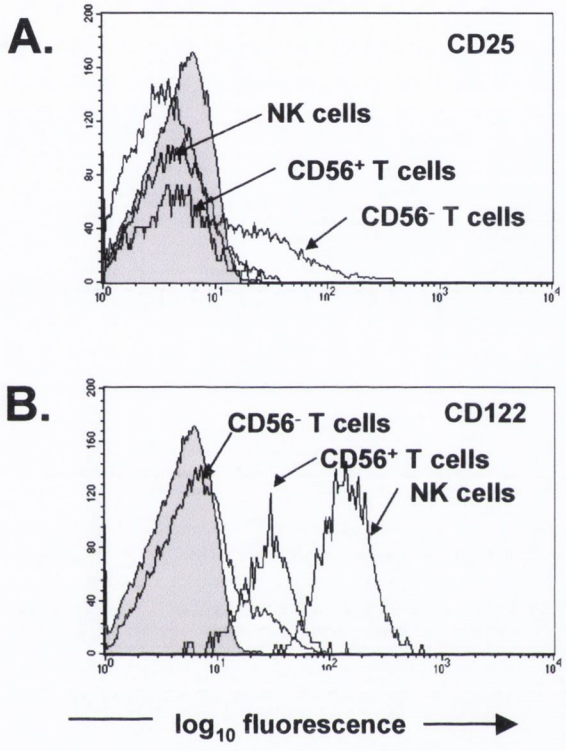


Figure 3.9. CD25 and CD122 expression in PBMC from healthy human controls

Expression of IL-2R α-chain, CD25 (A), and IL-2/IL-15Rβ-chain, CD122 (B), by human peripheral blood NK cells ($CD3^-CD56^+$), $CD56^-$ T cells and $CD56^+$ T cells. Staining by isotype matched anti-IgG mAbs is shown by the shaded histograms. Flow cytometry histograms are representative of eight experiments

3.2.3. IL-15 and IFN-γ mRNA expression in resting PBMC was similar in HLH patients and healthy controls

(Figure 3.10 and table 3.9)

Since IL-15 is important in the development and expansion of NK cells, we decided to test the capacity of PBMC from patients A and B to increase mRNA for IL-15 in response to stimulation by LPS and IFN-γ. The effects of IL-15 are mediated through control of IFN-γ production. TaqMan PCR (Perkin Elmer, USA) was used to measure baseline levels of mRNA for IFN-γ and IL-15 in two healthy adult controls and three age matched controls with recurrent infections, as well as the two patients. Relative quantification values (RQV) shown in table 3.9 indicate that compared to normal adults, the three age matched controls had normal levels of IL-15 mRNA but increased baseline levels of IFN-γ mRNA. In contrast both patients had normal levels of mRNA for both IL-15 and IFN-γ. Neither patient had a current infection at the time of testing.

Table 3.9. IL-15 and IFN-γ mRNA levels in HLH patients and controls.

	mRNA IL-15	mRNA IFN-γ
Control 1	1.5	1.2
Control 2	1.4	1.3
AMC 1	0.4	9.2
AMC2	1.3	11.9
AMC3	1.5	8.7
Patient A	1.9	1.3
Patient B	1.5	1.7

Relative Quantification Values (RQV) for IL-15 and IFN-γ mRNA levels normalized to resting PBMC (n=5) for adult controls, age-matched children with recurrent infections (AMC) and patients A and B

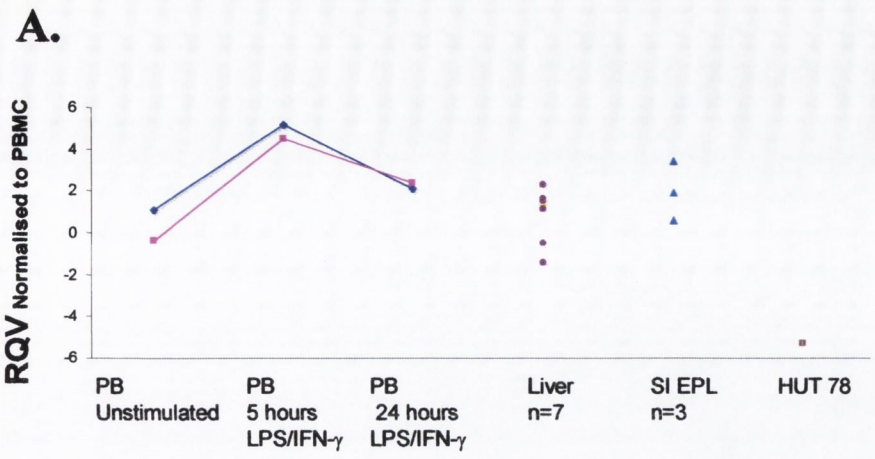


Figure 3.10.A. Upregulation of IL-15 mRNA following 5 and 24 hours stimulation with LPS and IFN-γ.

IL-15 mRNA levels were measured, using semi-quantitiative TaqMan PCR, in resting PBMC and following 5 and 24 hours stimulation with LPS/IFN-γ (A). Results shown are from two normal controls, included also are results from unstimulated liver samples from normal donor livers, lymphocytes isolated from small intestinal biopsies and a T cell line HUT 78 (A).

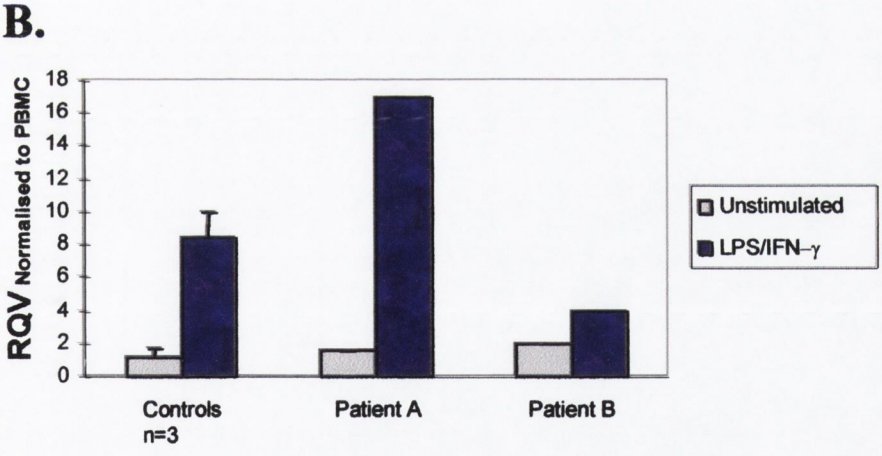


Figure 3.10. B. Upregulation of IL-15 mRNA following 5 hours stimulation with LPS and IFN-γ. – Results for HLH patients and healthy controls.

IL-15 mRNA levels in normal controls (n=3) and patients A and B measured in unstimulated PBMC and following 5 hours stimulation with LPS/IFN-γ (B).

In order to establish the optimal stimulation time for upregulation of mRNA for IL-15, semi-quantitative TaqMan (Perkin Elmer, USA) PCR was used to measure the levels of mRNA for IL-15 in unstimulated PBMC and following 5 and 24 hours stimulation with LPS and IFN-γ. Figure 3.10A shows results for two normal adult controls together with IL-15 mRNA levels tested in normal donor livers, small intestinal (SI) lymphocytes and a T cell line HUT78. Optimum increases in mRNA for IL-15 were achieved following 5 hours stimulation with LPS/IFN-γ. Liver and small intestine lymphocytes had variable levels of IL-15 mRNA, whereas HUT78 cells had very low levels when results were normalised against freshly isolated PBMC (n = 5).

TaqMan (Perkin Elmer, USA) PCR was then used to compare the levels of IL-15 mRNA in PBMC from the two HLH patients and three healthy adult controls. Quantitation was carried out using PBMC that were either freshly isolated or after incubation for 5 hours with LPS and IFN-γ. Results in figure 3.10B show that basal levels of IL-15 mRNA, normalized to resting PBMC, were similar in both patients and controls. Stimulation of control PBMC with LPS and IFN-γ dramatically increased Relative Quantification Values (RQV) of mRNA for IL-15 (unstimulated 1.2 ± 0.7 to 8.4 ± 1.5 at 5 hours). A similar increase was observed with PBMC from patient A (unstimulated 1.3 to 16.4 at 5 hours stimulation with LPS and IFN-γ). In contrast patient B showed a lower than normal response with a level of 1.2 for unstimulated PBMC increasing to 3.9 at 5 hours stimulation with LPS/IFN-γ.

3.2.4. IL-15 protein expression demonstrated in monocytes and lymphocytes from HLH patient A and healthy controls

(Figures 3.11 and 3.12)

Intracellular staining for IL-15 expression in monocytes and lymphocytes was carried out using a number of IgG1 anti-IL-15 mAb clones. Figure 3.11.A shows background and vimentin staining and compares the results from four anti-IL-15-clones α, β, χ and δ . The δ clone was shown to give the highest median intensity fluorescence (MFI) in

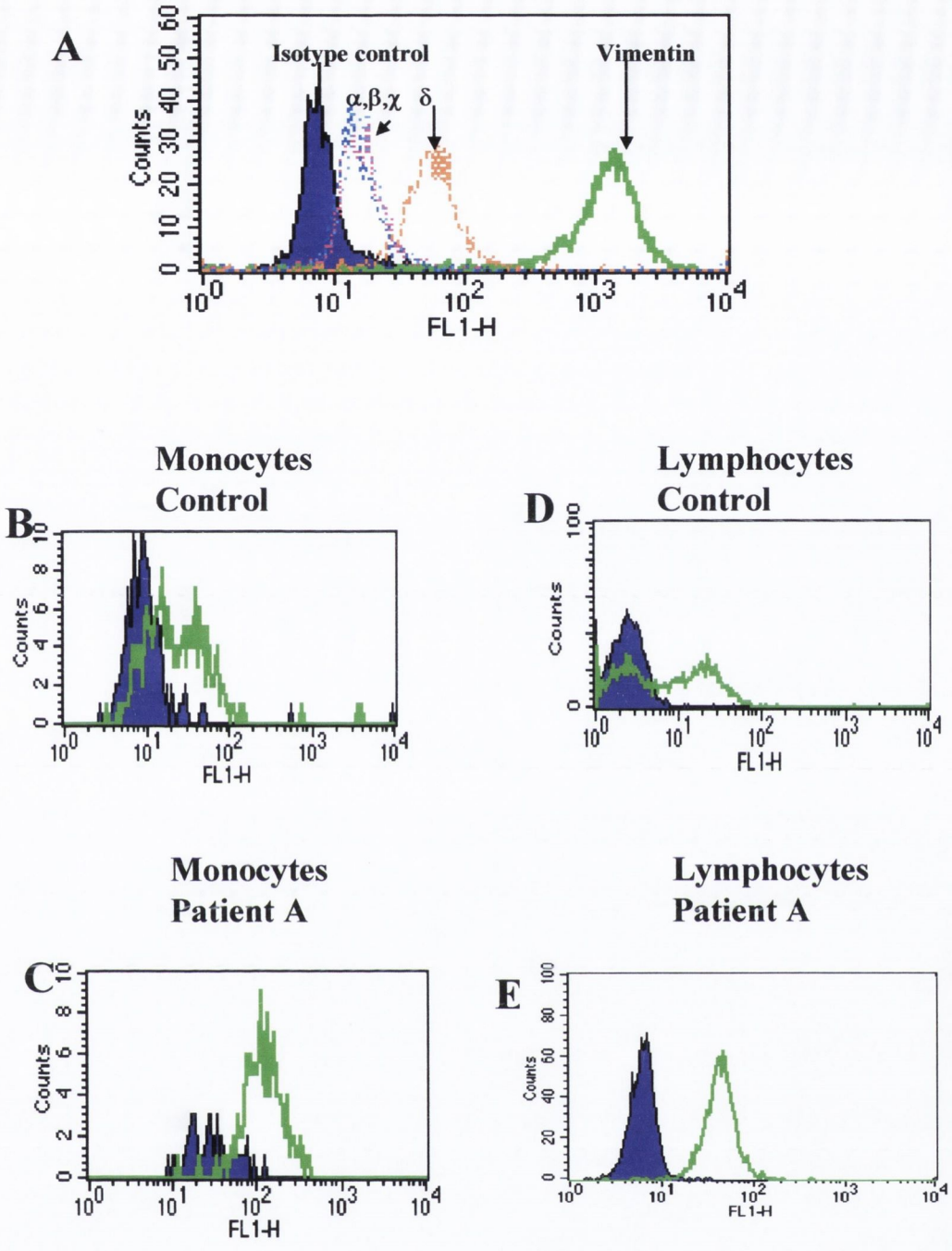


Figure 3.11. Expression of IL-15 protein in monocytes and lymphocytes.

Isolated PBMC from a healthy control and patient A were fixed with 4% PFA/PBS and permeabilized using 2% saponin PSB/BSA.The cells were stained for intracellular IL-15 expression and CD3 or CD14.

A.Histogram-overlay showing isotype control (filled curve) and vimentin staining in lymphocytes together with intracellular IL-15 detected using a number of IgG1 anti-IL-15 mAbs (labelled as α–δ). Histogram-overlays of isotype-control (filled curve) and intracellular IL-15 (δ mAb open curve) in monocytes (B) and lymphocytes (D) from a healthy control together with results for patient A monocytes (C) and lymphocytes (E).

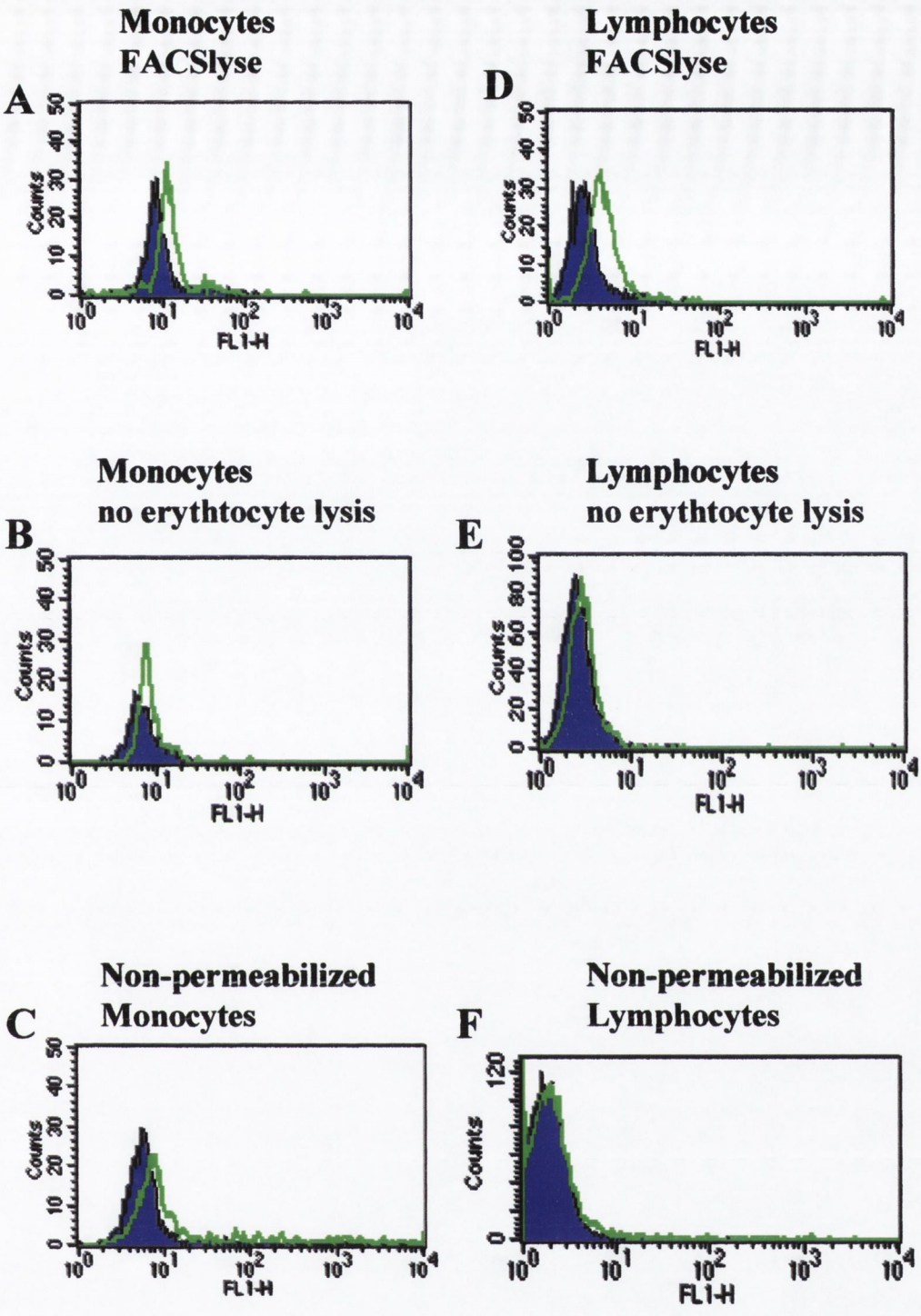


Figure 3.12. Expression of IL-15 protein in monocytes and lymphocytes demonstrated using whole blood.

Whole blood samples were fixed with 4% PFA/PBS either directly or following treatment with FACSlyse (A and D) to remove erythrocytes. The samples were then stained for IL-15 expression on the surface (C and F) or intracellulary (A, B, D and E). Histogram-overlays show isotype-control (filled curve) and intracellular IL-15 (δ mAb- open curve) demonstrated in permeabilized monocytes (A and B) and lymphocytes (D and E) from a healthy control. Constitutive surface expression of IL-15 was demonstrated on non-permeabilized monocytes (C) but not on lymphocytes (F) from the same subject. This experiment was repeated twice with the same results.

both lymphocytes and monocytes and was used to stain cells from patient A and a healthy control. While a number of earlier studies have demonstrated mRNA for IL-15 in monocytes, lymphocytes have only recently been shown to express this cytokine. The unexpected demonstration of intracellular-IL-15 protein in both lymphocytes and monocytes from patient A and a healthy control, shown in figure 3.11 B-E, mirrors results from a recent study (Neely *et al.* 2001). A whole blood staining technique was developed in order to demonstate surface and intracellular IL-15 expression on monocytes and lymphocytes. Results in figure 3.12 show surface and intracellular IL-15 expression in monocytes and lymphocytes from a healthy control. Non-permeabilized PBMC were used to demonstrate IL-15 adhered to the surface of monocytes (figure 3.12.C) but not lymphocytes (figure 3.12.F). Staining was carried out with and without erythrocyte lysis and was repeated three times with similar results. Using a commercial IgM isotype mAb to IL-15 and flow-cytometry *Neely et al.* were unable to demonstrated surface IL-15 on resting monocytes, however they did demonstrate constitutive IL-15 expression on monocytes using a sensitive ELISA test. In the present study, monocyte membrane-bound IL-15 was demonstrated using unseparated whole blood from healthy controls (figure 3.12.C). It should be noted that the specificity of anti-IL-15 mAbs was not checked by carrying out blocking experiments using IL-15.

3.2.5. HLH patients show differences in IL-2 and IL-15 induced levels of CD69 expression on CD3 cells

(Figure 3.13A)

CD69 is a differentiation antigen that is one of the earliest cell surface molecules expressed after activation of cells of haematopoetic origin (Testi *et al.* 1994). IL-15 has been shown to upregulate CD69 on T cells (Kanegane *et al.* 1996). Changes in expression of this receptor on T cells in response to stimulation with IL-2 or IL-15 were examined in both HLH patients and healthy controls.

Results in figure 3.13A demonstrate that using three-colour flow-cytometry CD69 is expressed on 5.6 $\pm$ 1.4% of unstimulated $CD3^+$ cells from normal controls. Culture of

healthy control PBMC for 24 hours with IL-2 at 100 ng/ml or IL-15 at 25 ng/ml resulted in a four-fold increase of CD69 expression on T cells. CD69 was expressed on 3.7% of unstimulated T cells from patient A: culture for 24 hours with IL-2 resulted in a 3 fold increase to 11.2% with IL-15 inducing a 4 fold increase to 15%. Unstimulated T cells from patient B expressed CD69 at 5.2%: culture with IL-2 for 24 hours induced a 1.4 fold increase to 7.2% and IL-15 induced a 1.5 fold increase to 8%. These results indicate that in short term culture IL-2 and IL-15 induced a normal fold increase in CD69 expression on T cells from patient A. Patient B exhibited a reduced fold increase in CD69 expression in response to these cytokines prompting further investigations of the proliferative response in this patient.

3.2.6. IL-2 induced proliferation of PBMC is normal in patient B

(Figure 3.13B)

Since T cells from patient B had a blunted response to IL-2 and IL-15 when CD69 upregulation was examined, we decided to look at the response of PBMC from this patient compared to controls in extended culture with IL-2. ^{3}H-thymidine incorporation was used to measure proliferation of isolated PBMC in response to varying concentrations of IL-2 (0, 0.1, 1 and 10 ng/ml) cultured for 6 days then pulsed with ^{3}H-thymidine and harvested 16 hours later. The stimulation index (SI) was calculated based on the counts per minute (cpm) achieved in the 0.1 - 10 ng/ml samples. Results shown in figure 3.13B indicated that patient B and a normal adult control exhibited a similar dose dependent response to IL-2 added once at time zero. Due to restricted access to samples, response to IL-15 was not measured for patient B. This test was not carried out for patient A.

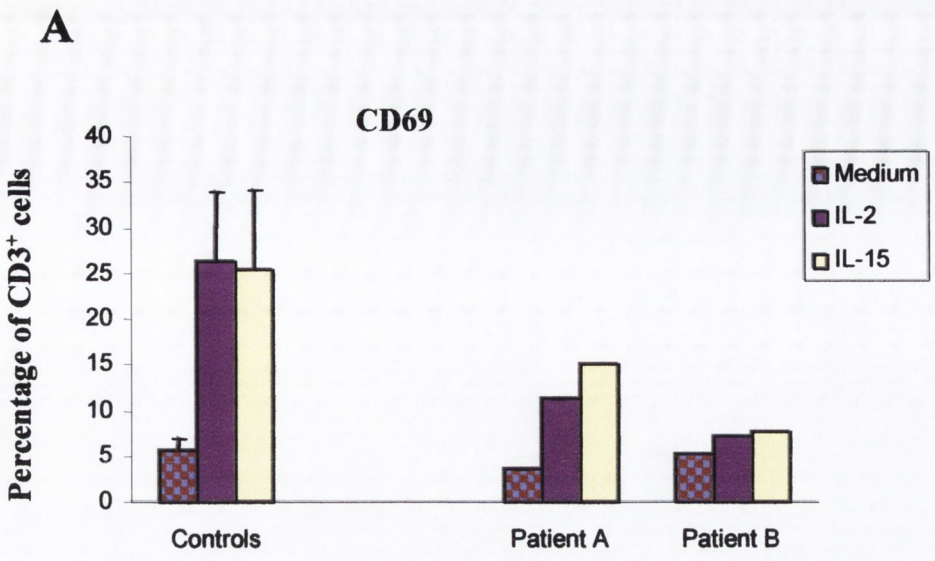


Figure 3.13.A. HLH patients exhibit variable T cell-activation and proliferation in response to IL-2 and IL-15 in short term culture

Upregulation of CD69 expression on $CD3^+$ cells in response to culture for 24 hours with IL-2 at 100 ng/ml and IL-15 at 25 ng/ml was observed. Results are shown as percentages of $CD3^+$ cells for normal adult controls (n=4) and patients A and B (A).

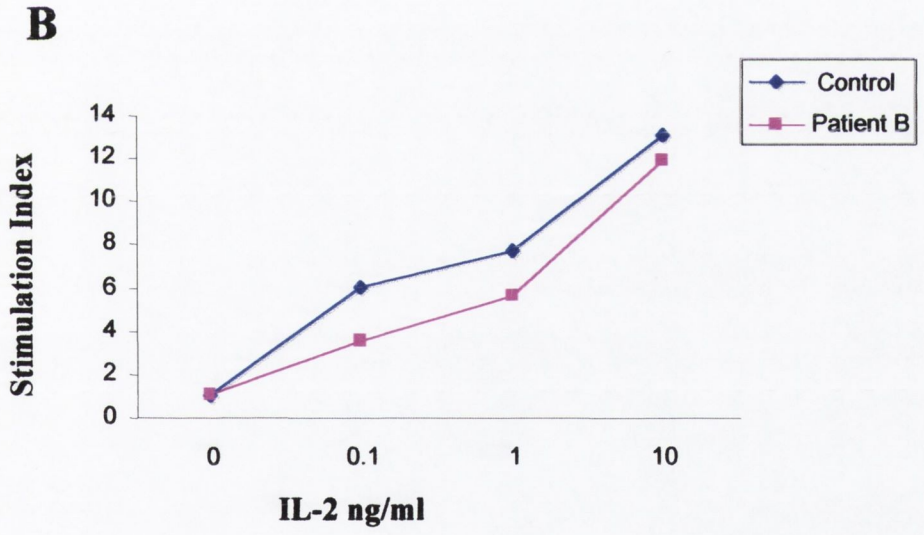


Figure 3.13.B. HLH patients exhibit variable T cell-activation and proliferation in response to IL-2 and IL-15 in long term culture

PBMCs from Patient B and an adult control were cultured for 6 days with various concentrations of IL-2 and then pulsed with ^{3}H-thiamidine 16 hours before harvesting. Stimulation indices in response to culture are shown in B.

3.3. Effects of IL-2 and IL-15 on IL-2/15Rα (CD25) and β (CD122) expression

While both the HLH patients had been shown to express receptor components for IL-2/IL-15R, there was a reduced level of expression on patient cells compared to controls. The upregulation of CD69 in response these cytokines was also low in both patients compared to controls, this reduction was more marked in cells from patient B. Mice deficient in intracytoplasmic components of the IL-2Rβ have been shown to have reduced NK and NKR^+ T cell development as well as reduced CD25 upregulation in response to IL-2 (Fujii *et al.* 1998; Ellery *et al.* 2000; Nakajima *et al.* 1997). In order to assess the functional integrity of IL-2/IL-15R we decided to examine the dynamics of expression of the unique IL-2Rα (CD25) and the shared CD122 receptor in response to IL-2 and IL-15 in CD4 and CD8 T cells. PBMC from both patients and controls were examined at time zero and following culture with cytokines for 24 hours. In order to study the effect of extended exposure to cytokines, PBMC from healthy controls were culture from 1 to 7 days.

3.3.1. IL-2 and IL-15 differ in their effects on CD25 and CD122 expression in cells from healthy adult controls and HLH patients

(Figure 3.14)

Dose-dependent changes in expression of CD25 and CD122 in response to culturing with IL-2 and IL-15 were observed using PBMC from 3 adult controls. Sample results from one control, shown in figure 3.14A, indicate that culture of PBMC for 24 hours with IL-2 ranging from 0 to 500 ng/ml resulted in a slight decrease in CD25 expression on $CD4^+$ T cells with no changes in expression on $CD8^+$ T cells. Culture of PBMC for 24 hours with concentrations of IL-15 varying from 0 to 100 ng/ml resulted in an increase in CD25 expression on $CD4^+$ T cells from 30% at 0 ng/ml to 40% peaking between 10 and 50 ng/ml IL-15. There was a dramatic increase in CD25 expression on $CD8^+$ T cells in response to culture with IL-15, from 2% at 0 ng/ml to 30% peaking between 10 and 50 ng/ml.

In contrast, as shown in figure 3.14B, control PBMC exhibited a reduction in CD122 expression on $CD4^+$ T cells and $CD8^+$ T cells, in a dose dependent fashion, following 24 hours incubation with either IL-2 or IL-15. The reduction in CD122 expression peaked at between 50 and 100 ng/ml IL-2 and 10 and 50 ng/ml IL-15.

Changes in expression of CD25 and CD122 for 5 adult controls and patients A and B are shown in figure 3.14C. Receptor expression was measured at time zero and after culture for 24 hours with medium, IL-2 at 100 ng/ml or IL-15 at 100 ng/ml. The percentage of $CD3^+CD8^+$ cells expressing CD25 was reduced in both patients at time zero, at 1% and 2% respectively compared to $8\pm3.3\%$ for adult controls. Expression of CD25 on $CD3^+CD4^+$ cells was within normal ranges ($20\pm4\%$) for both patients. Culture of PBMC for 24 hours in medium or IL-2 induced no changes in CD25 expression on T cells from either the patients or controls. Culture of PBMC for 24 hours with IL-15 resulted in increased CD25 expression on both $CD3^+CD8^+$ and $CD3^+CD4^+$ cells from patients and controls. The level of increased CD25 expression relfected the baseline levels of expression with both patients having a lower than normal $CD3^+CD8^+$ response (figure 3.14C).

Patients A and B had low baseline levels of CD122 expression on $CD3^+CD8^+$ cells at 2% and 5% respectively (normal adult range $20\pm5\%$). The percentage of $CD3^+CD4^+$ cells expressing CD122 was also low for patient A at 1% and normal for patient B at 3% compared to $5\pm3\%$ for adult controls. Culture of patient PBMC for 24 hours with IL-2 or IL-15 resulted in further reductions in the percentages of $CD3^+CD8^+$ and $CD3^+CD4^+$ cells expressing CD122 to less than 1% in all instances.

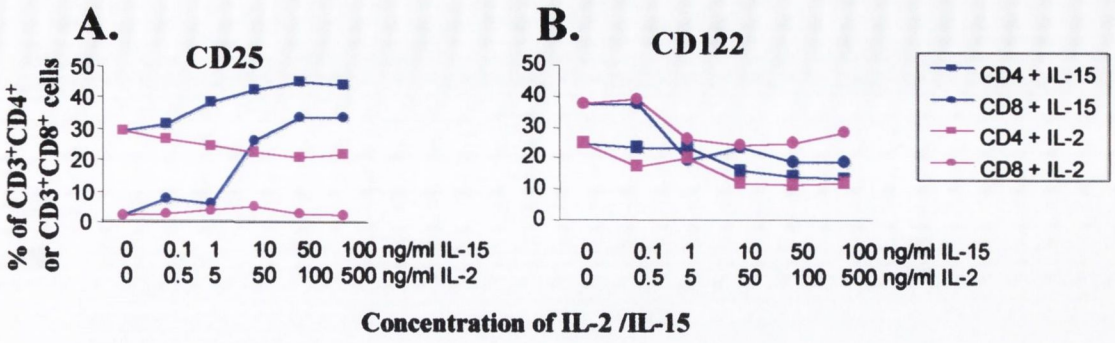


Figure 3.14. Changes in CD25 and CD122 expression in response to culture with IL-2 or IL-15.

Dose dependent changes in the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells expressing CD25 (A) and CD122 (B) following culture of PBMC from a sample healthy control with IL-2 or IL-15. This experiment was repeated on three control samples with similar results

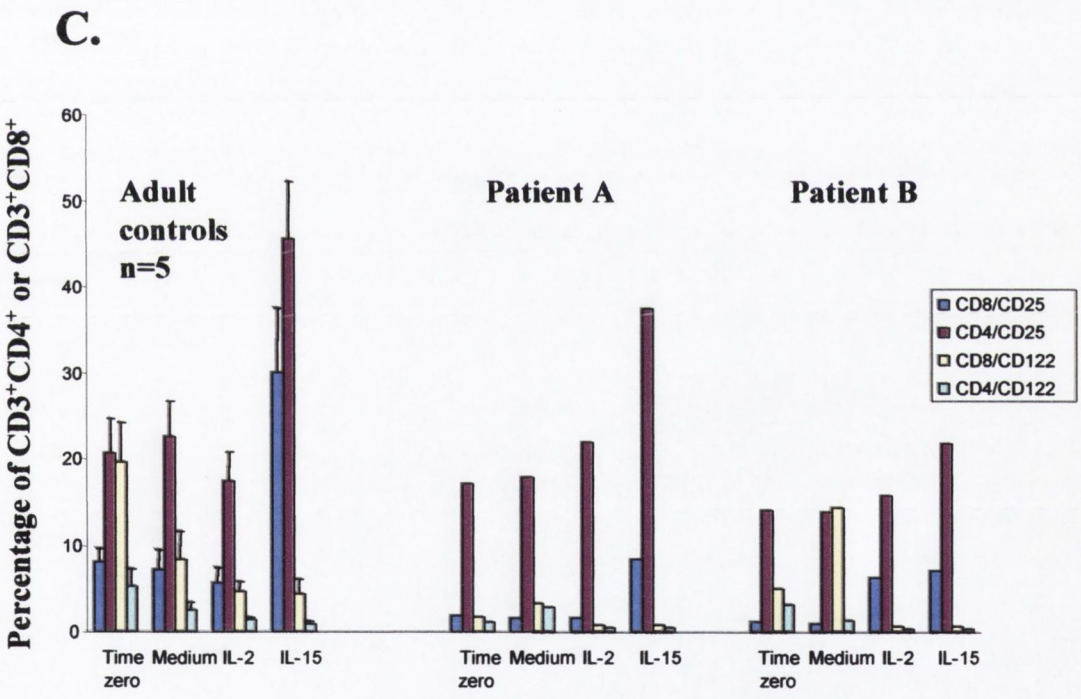


Figure 3.14.C. Changes in CD25 and CD122 expression in response to culture with IL-2 or IL-15.

Control and patient PBMC were examined for changes in expression of CD25 and CD122 on $CD3^+CD4^+$ and $CD3^+CD8^+$ in response to culture with medium, IL-2 at 100 ng/ml or IL-15 at 25 ng/ml for 24 hours. Results shown in C are mean percentages+SD for controls (n=5) and sample results for patients A, prior to CsA therapy, and patient B (C).

3.3.2. IL-2 and IL-15 induced increased CD25 but reduced CD122 expression in healthy control PBMC following culture for 7 days

(Figure 3.15)

Levels of CD25 and CD122 expression were measured in $CD3^+$ cells from 3 healthy adult controls using three colour flow cytometry, at time zero and over 1 to 7 days during culture in medium only or with IL-2 or IL-15. Changes in expression of these receptors on T cells in response to culture with staphylococcal enterotoxin B (SEB) for up to three days was also examined.

Results for three healthy adult controls shown in figure 3.15A indicate that for cells cultured in medium only there was no change in the percentage of $CD3^+CD8^+$ or $CD3^+CD4^+$ cells expressing CD25 at 24 hours, but there was a decline following 7 days culture compared to time zero. Culture of PBMC with IL-2 at 100 ng/ml for 24 hours resulted in a slight decrease in the percentage of both $CD3^+CD8^+$ and $CD3^+CD4^+$ cells expressing CD25. When the culture period with IL-2 was increased to between 5 and 7 days there was a significant increase in the percentage of $CD3^+CD8^+$ and $CD3^+CD4^+$ cells expressing CD25. Culture for 7 days with IL-2 resulted in CD25 expression on $15.5\pm3\%$ of $CD3^+CD8^+$ and $36\pm4\%$ $CD3^+CD4^+$.

IL-15 had a more marked effect on CD25 expression than IL-2 with different dynamics indicating early upregulation. Culture of PBMC with IL-15 at 25 ng/ml for 24 hours resulted in significant increases in the percentages of both $CD3^+CD8^+$ and $CD3^+CD4^+$ cells expressing CD25. When the culture period with IL-15 was extended to seven days $73\pm5\%$ of $CD3^+CD8^+$ and $79\pm3\%$ of $CD3^+CD4^+$ expressed CD25 (figure 3.15A).

Culture of PBMC with SEB, at 10 μg/ml for 24 hours, induced increases in the percentages of both $CD3^+CD8^+$ and $CD3^+CD4^+$ cells expressing CD25 similar to those seen with IL-15 and greater than those induced by IL-2 (figure 3.15A). Extending culture of PBMC with SEB to three days resulted in increases in CD25 expression on $CD3^+CD8^+$ cells to $62\pm6\%$ and on $CD3^+CD4^+$ cells to $75\pm3\%$. FACS analysis of cells cultured with SEB for 5 to 7 days yielded undeceipherable results with a lot of

background staining and altered forward and side scatter characteristics for lymphocytes and these results could not be presented here.

Culture of PBMC in medium only or with IL-2 or IL-15 for 24 hours resulted in a reduction in the percentage of $CD3^+CD8^+$ and $CD3^+CD4^+$ cells expressing CD122 (figure 3.15B). This reduced level of expression was maintained over 7 days culture with medium or IL-2, however with IL-15 there was some slight increase observed in $CD3^+CD4^+$ cells. Culture of PBMC with SEB also induced a reduction in CD122 expression on both $CD3^+CD8^+$ and $CD3^+CD4^+$ cells at 24 hours but not at day 3 (figure 3.15B).

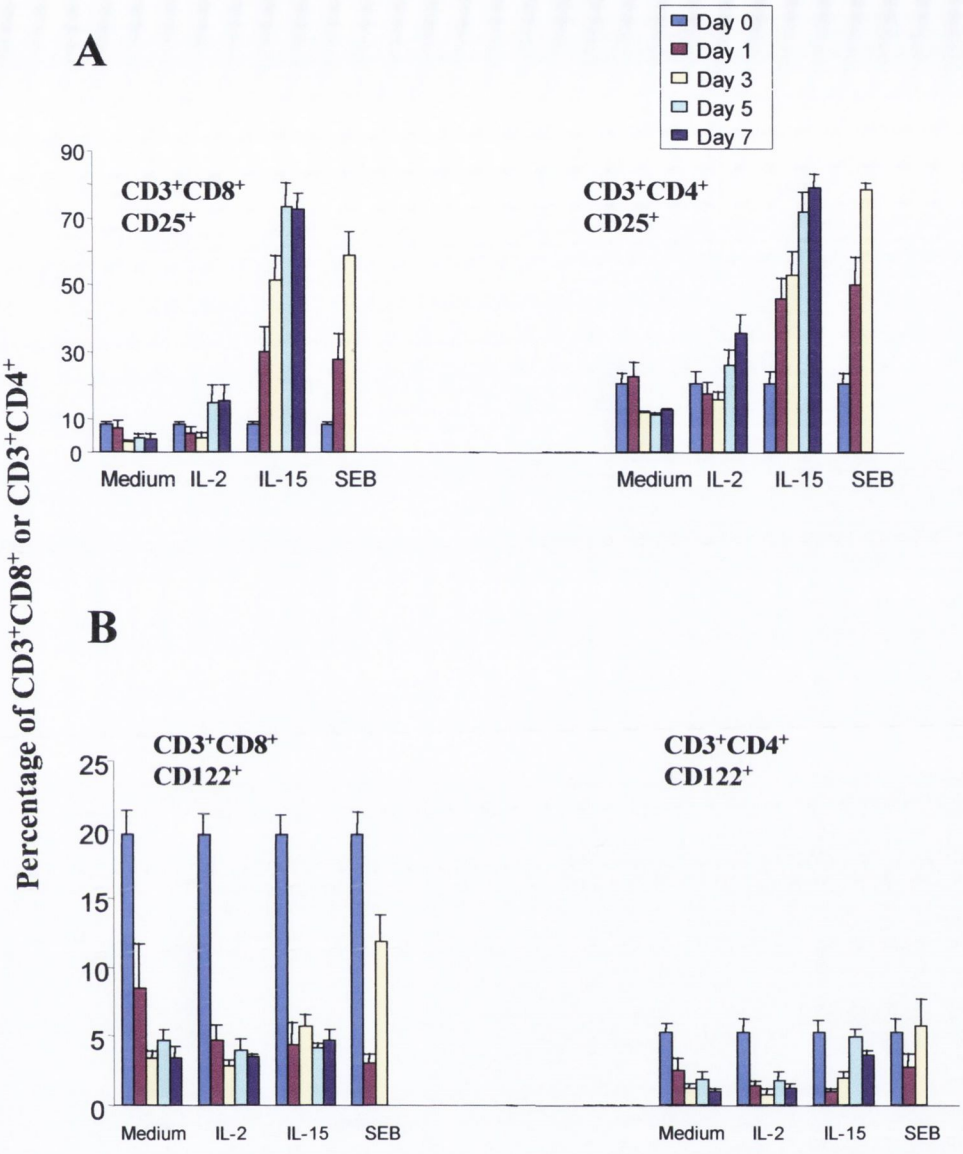


Figure 3.15. Alterations in expression of CD25 and CD122 on PBMC over time in culture medium or with IL-2, IL-15 or SEB added.

Time scale studies of changes in CD25 (A) and CD122 (B) on $CD3^+CD4^+$ and $CD3^+CD8^+$ cells induced by culture in medium or with IL-2 at 100 ng/ml or IL-15 at 25 ng/ml or staphylococcal enterotoxin B (SEB) at 10 μg/ml. Results, measured at time zero, days 1, 3, 5 and 7, are shown as means +SD of n=3 adult controls

3.4. Effects of IL-2 and IL-15 on lymphocyte populations over 7 days

IL-15 has been shown to be a growth factor for T cells, B cells, NK cells (Waldmann, 1998). In addition this cytokine has been shown to selectively induce the proliferation of antigen activated $CD3^+CD8^+$ cells expressing CD94/NKG2A (Mingari *et al.* 1998b). These functions together with the alteration in CD25 and CD122 expression observed following culture with IL-2 or IL-15 prompted further investigations into the effects of extended culture with these cytokines on lymphocyte subsets. Using PBMC from healthy controls and the two HLH patients the effects of these cytokines on resting B cells, NK cells and CD4, CD8 and CD56 expressing T cells was examined. In addition the effect of culture with IL-2 or IL-15 on the expression of natural killer receptors CD94, CD161, CD158 a, b, e1 and CD16 was examined.

3.4.1. IL-2 and IL-15 selectively expand healthy control NK, $CD56^+$ T and $CD8^+$ T cells, but HLH patients differ in their responses

(Figure 3.16)

Simple enumeration of cells indicated that the culturing of control PBMC (n=9) for 7 days in medium only, 100 ng/ml IL-2 or 25 ng/ml IL-15 resulted in mean 0.64-, 1.14- and 1.26-fold changes in total lymphocyte numbers, respectively. Culture in medium only resulted in reduction in all cell numbers compared to time zero levels (figure 3.16A). NK cells showed the largest fold changes to 0.2-, $CD56^+$ T cells 0.28-, $CD3^+CD8^+$ cells 0.56-, $CD3^+CD4^+$ 0.73- and CD19 0.64-fold time zero levels.

Figure 3.16A shows that culture for 7 days with IL-2 or IL-15 induced significant expansions of $CD3^+$, $CD3^+CD8^+$, $CD56^+$ T cells and NK cells but not $CD3^+CD4^+$ or B cells from healthy adult controls. $CD56^+$ T cells were consistently the most responsive to culture with cytokines. $CD3^+CD8^+$ cells had a more significant increase in response to IL-15 than in response to IL-2. NK cell number increases were similar in response to both IL-2 and IL-15.

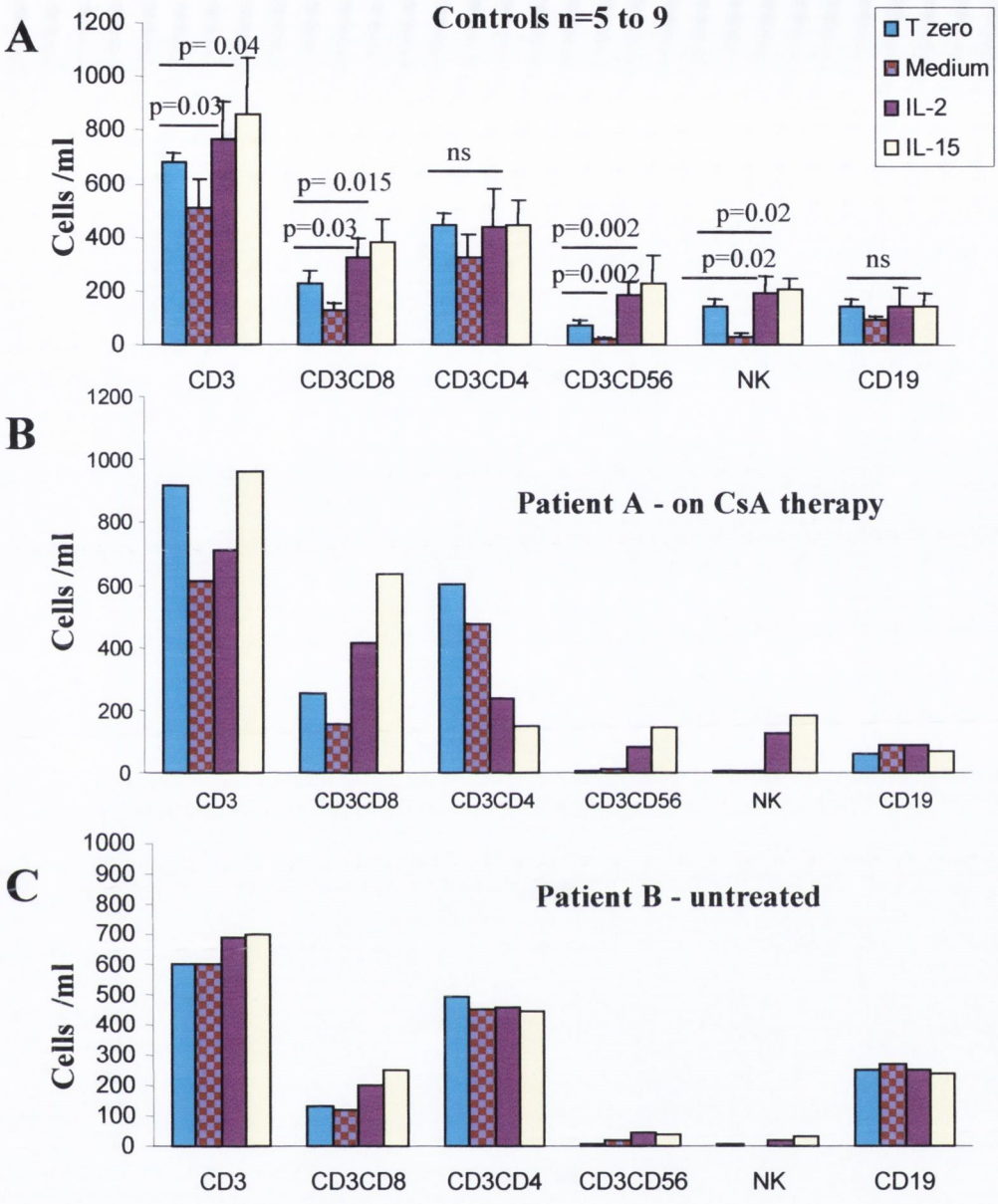


Figure 3.16. IL-2 and IL-15 induced changes in lymphocyte subset numbers in controls and HLH patients

Changes in absolute counts of $CD3^+$, $CD3^+CD8^+$, $CD3^+CD4^+$, $CD3^+CD56^+$, NK and B ($CD19^+$) cells measured at time zero and following 7 day culture in medium with and without IL-2 at 100 ng/ml or IL-15 at 25 ng/ml. Results shown are means+SD for healthy adult controls (n=5 to 9) (A), and means of cell counts for patient A , on CsA therapy (B) and patient B (C). Results for controls with and without cytokine were compared in non-parametric tests and p values are shown with lines indicating the parameters compared, ns = not significant, p value <0.05 was considered significant.

The two patients showed variable changes in lymphocyte numbers in response to culture with IL-2 and IL-15. Shown in figure 3.16B, PBMC from Patient A, who was on cyclosporin therapy, exhibited increases in $CD3^+CD8^+$, NK cells and $CD56^+$ T cells but not B cells in a similar pattern to that observed in normal adult controls. Culture with both IL-2 and IL-15 resulted in a marked reduction in $CD3^+CD4^+$ cell numbers in contrast to results from normal controls.

Results shown in figure 3.16C indicate that PBMC from patient B did not exhibit a normal pattern of response to IL-2 or IL-15. Numbers of $CD3^+CD8^+$ cells increased in response to both IL-2 and IL-15 although the numbers do not quite reach those achieved by control PBMC. NK cell and $CD56^+$ T cell expansion in response to these cytokines was much reduced in this patient compared to normal controls with IL-2 and IL-15 inducing 31 and 40 cells/ml respectively with normal ranges of 203 ± 48 and 225 ± 105 cells/ml respectively (figure 3.16C). In common with healthy controls $CD3^+CD4^+$ and CD19 cell numbers for this patients were not altered in response to prolonged culture of PBMC with cytokines.

3.4.2. Dose and time dependent expansions in NK and NKR^+ T by IL-2 and IL-15 in healthy controls

(Figure 3.17)

Since IL-2 and IL-15 selectively expanded both $CD3^+$ and $CD3^-$ cells expressing the natural killer receptor CD56 (figure 3.17), we decided to examine the effects of these cytokines on the expression of other NKRs in healthy control PBMC. Dose-dependent and time-dependent expansion of NKR^+ PBMC in response to culturing with IL-2 and IL-15 added once at time zero, indicated that maximal increases in the percentages of cells expressing these receptors occurred 7 days after additon of these cytokines (figure 3.17A). 10-50 ng/ml IL-15 (figure 3.17B) and 50-100 ng/ml IL-2 (results not shown) induced maximal expansion of cells expressing the NKRs including CD56, CD161, CD158e1 (KIR3DL1) and CD94.

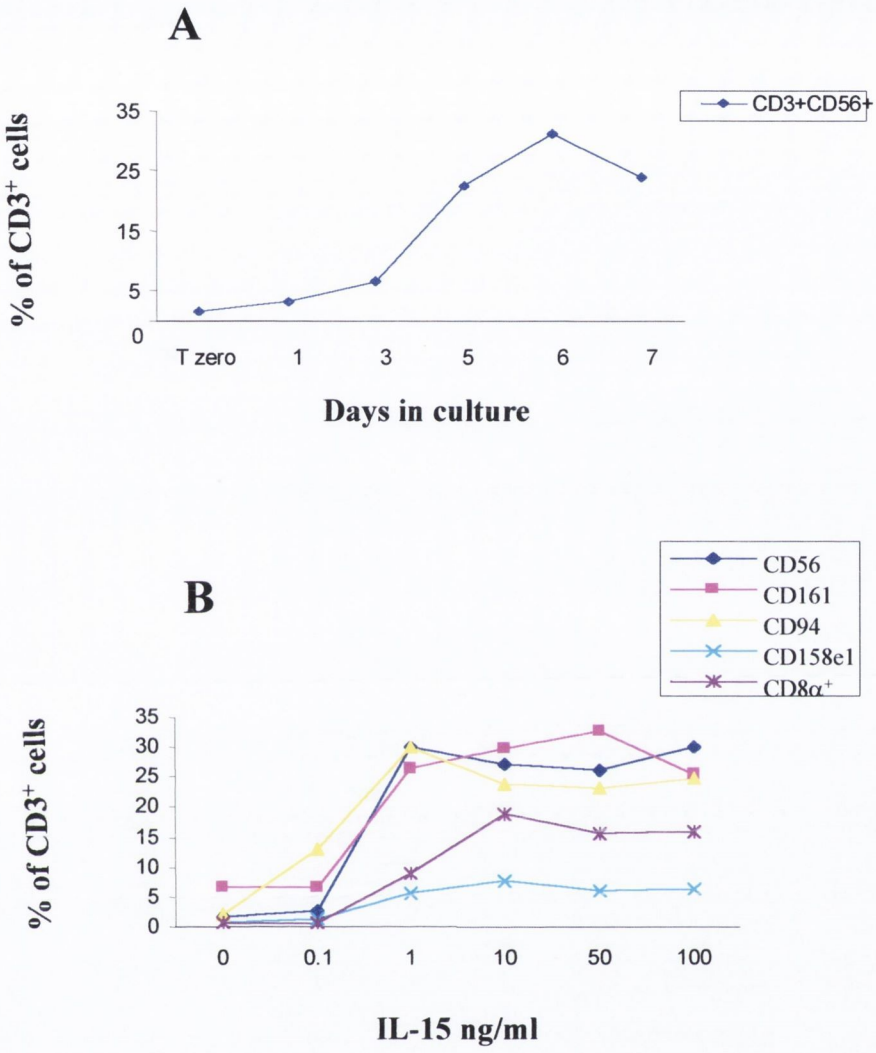


Figure 3.17. Effects of time and IL-15-concentration on NKR expression on T cells.

Following culture with IL-15 at 25 ng/ml, changes in the percentage of $CD3^+$ cells expressing CD56 were measured, using flow cytometry, at time zero and over 7 days (A). The changes in percentages of $CD3^+$ cells expressing NKRs CD56, CD161, CD94, CD158e1 and CD8α in response to culture for 7 days with varying concentrations of IL-15 are also shown (B).

3.4.3. IL-15 only partially corrected deficits in NK and NKR$^+$ T cells in HLH patients

(Figure 3.18)

Availability of samples from patient A was limited so only the effect of culture with IL-15 on the expression of some but not all of the NK associated receptors was studied. PBMC from both patient and controls were cultured for seven days with and without IL-15. Using flow cytometry we measured the expression of CD56, CD94, CD158e1 (KIR3DL1) and CD8$\alpha^+\beta^-$ on NK (CD3$^-$CD56$^+$) and CD3$^+$ cells at time zero, following culture in medium only and with IL-15 added at 25 ng/ml.

Results shown in figure 3.18A indicate that culture of PBMC from adult (n=5) and age matched controls (n=3) for seven days with IL-15 caused a marked expansion in the percentage of CD3$^+$ cells expressing CD94. There was a similar expansion of CD3$^-$ CD56$^+$ cells expressing CD94 in response to IL-15. The percentages of both CD3$^+$ and CD3$^-$ cells expressing CD94 were reduced in both patients at T zero compared to adult and age matched controls. In response to culture with IL-15, PBMC from FHL patient A, measured when on cyclosporin therapy, exhibited an increase in both CD3$^+$ and CD3$^-$ cell populations expressing CD94. PBMC from patient B exhibited only a small increase in the percentages of CD3$^-$ cell expressing CD94 following culture with IL-15 (figure 3.18D). Only some of the adult and age matched control samples showed increases in the percentages of CD3$^+$ and CD3$^-$CD56$^+$ cells expressing CD158e1 (KIR3DL1) following culture with IL-15 (figure 3.18B and E). Neither patient showed any increase in percentages of cells expressing this receptor following culture with IL-15. The percentages of CD3$^+$ cells expressing CD8α homodimer was increased following culture with IL-15 in control populations and in patient A on cyclosporin therapy, but only marginally increased in cells from patient B (figure 3.18C).

These results indicate that NK and NKR$^+$ T cell deficits found in freshly isolated PBMC from these two patients (time zero) could, in part, be corrected by IL-15 in one patient but not in the second patient.

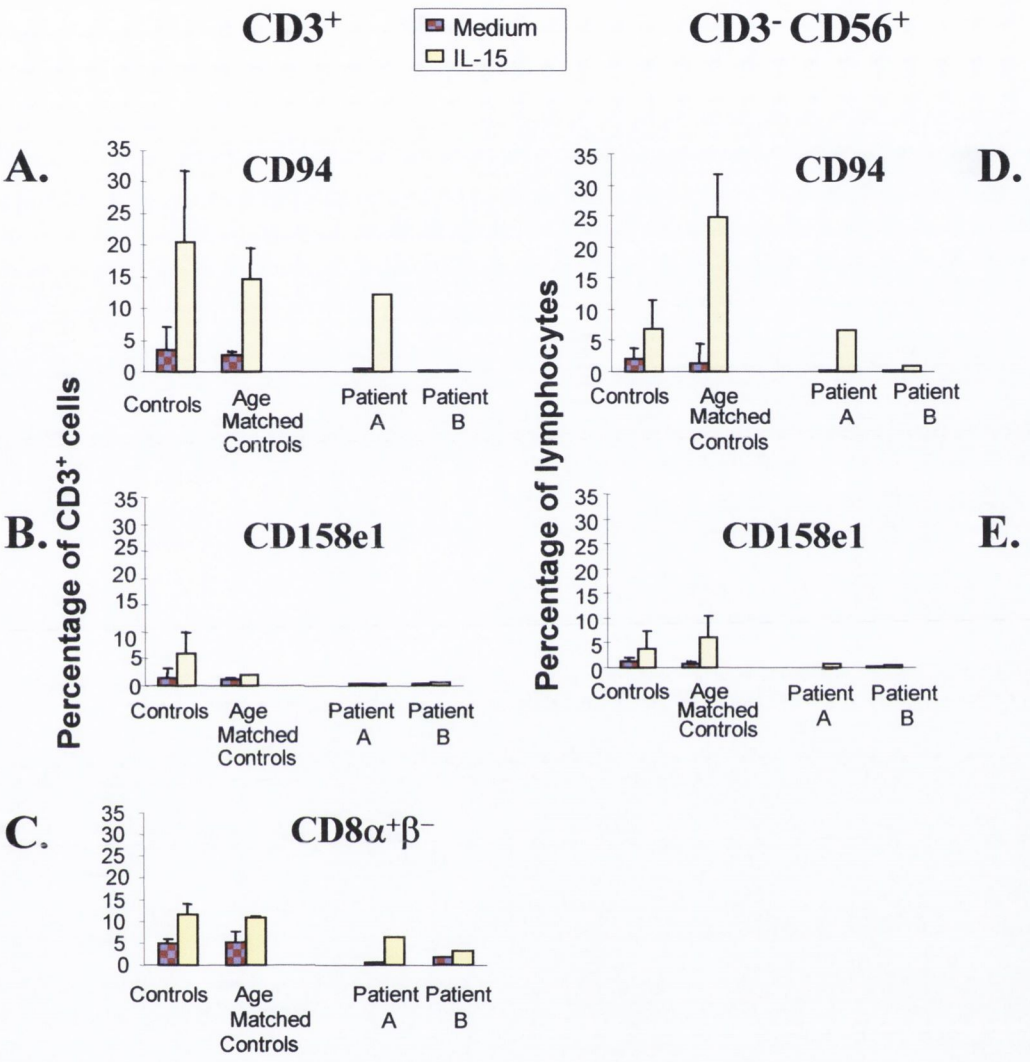


Figure 3.18. IL-15 induced changes in expression of NKRs on PBMC from controls and HLH patients.

Percentages of $CD3^+$ cells (A-C) and $CD3^-CD56^+$ (NK) cells (D and E) expressing NKRs - CD94, CD158e1 and CD8 $\alpha^+\beta^-$ - following culture for 7 days in medium only or with IL-15 added at 25 ng/ml. Results shown are means+SD for adult controls n=5, age matched controls n=3 and sample results for patients A, on CsA therapy, and patient B.

3.4.4. IL-2 and IL-15 selectively expanded human control NKR$^+$ T cells

(Figure 3.19)

The increases in proportions of $CD3^+$ cells expressing NKRs demonstrated in response to culture with IL-15 may be the result of either enhanced survival or selective expansion of these cell types. In order to prove selective expansion, measurement of the changes in numbers of NKR^+ T cells in response to cytokines was required. PBMC from patient B and controls (n = 4) were cultured for seven days with and without IL-2 or IL-15. Using flow cytometry we measured the expression of CD56, CD94, CD158e1 (KIR3DL1), CD158a, CD158b, CD161, CD16 and $CD8\alpha^+\beta^-$ on NK ($CD3^-CD56^+$) and T ($CD3^+$) cells at time zero, following culture in medium only and with IL-2 or IL-15 added. Cell counts were carried out on each sample and the numbers of cells expressing NKRs were calculated.

In response to culture in medium and IL-2 or IL-15 there were significant changes in numbers of $CD3^+$ cells expressing NKRs including CD158e1 (KIR3DL1), CD158a, CD158b, CD94, CD161, CD16 and $CD8\alpha$ homodimer, in all control individuals (n=4) relative to time zero levels (figure 3.19A). IL-15 had a consistently more marked effect than IL-2. Culture in medium without cytokines resulted in significant reductions in the numbers of $CD3^+$ cells expressing CD94 and CD161 – as has previously been shown for CD56 expressing T cells (see above). When cells from patient B were examined there was little or no IL-2 or IL-15 induced expansion in these T cell populations with the exception of CD16 expressing T cells (figure 3.19B). The data indicates that T cells from patient B express NKR^+ receptors, albeit at low levels, but there is a failure to expand these populations in response to IL-2 or IL-15, as occurs with normal control PBMC.

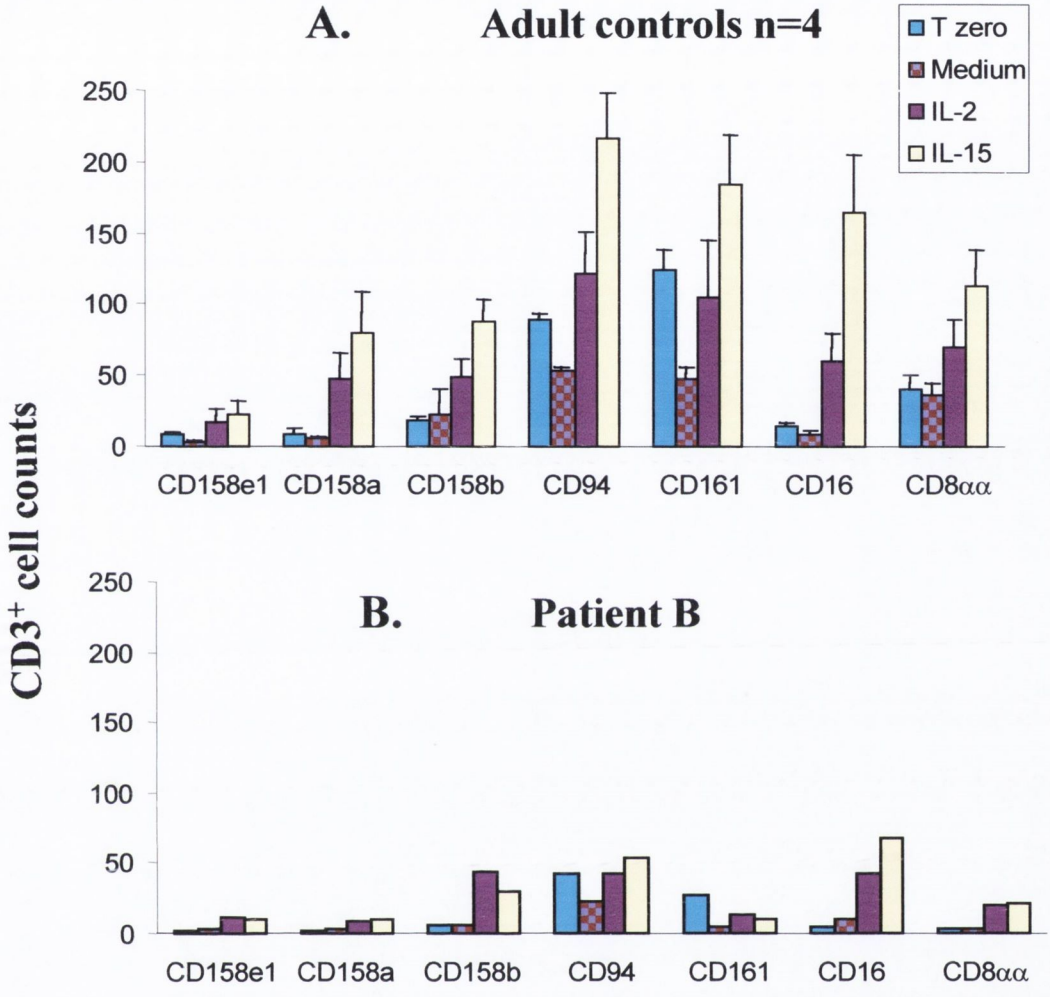


Figure 3.19. Effects of culture with IL-2 and IL-15 on numbers of NKR expressing T cells in controls and patient B.

Changes in absolute counts of $CD3^+$ cells expressing NKRs - CD158e1, CD158a and b, CD94, CD161, CD16 and CD8α - measured at time zero (T zero), and after culture for 7 days in medium only or with IL-2 at 100 ng/ml or IL-15 at 25 ng/ml. Results shown are means+SD for adult controls (n=4) (A) and sample results for patient B (B).

3.4.5. Culture of control PBMC with IL-2 or IL-15 induced the development of $CD3^+$ and $CD3^-$ cells capable of spontaneous cytotoxicity

(Figure 3.20)

To examine the effects of IL-2 and IL-15 on the induction of spontaneous cytotoxicity, PBMC from normal controls (n=4) were cultured for seven days with and without IL-2 at 100 ng/ml or IL-15 at 25 ng/ml. Cells were separated into $CD3^+$ and $CD3^-$ fractions using anti-CD3 mAb coated magnetic beads. The isolated $CD3^+$ and $CD3^-$ fractions were then tested for cytotoxicity against K562 cells in ^{51}Cr release assays.

Culture of control PBMC in medium for 7 days resulted in low specific cytotoxicity against K562 cells by both $CD3^+$ (<5%) and $CD3^-$ (<20%) cell fractions (figure 3.20A and B). The addition of either IL-2 or IL-15 to the medium resulted in increased specific cytotoxicity by $CD3^+$ cells (>70%) as well as $CD3^-$ cells (>65%).

3.4.6. IL-15 induced the development of $CD3^-$ and $CD3^+$ cells capable of normal spontaneous cytotoxicity from HLH patient A but not from patient B

(Figure 3.21)

The induction of spontaneous cytotoxicity by IL-15 was also examined in samples from patients A and B. PBMC from both patients were cultured for seven days with and without IL-15 at 25 ng/ml. Cells were separated into $CD3^+$ and $CD3^-$ fractions using anti-CD3 mAb coated magnetic beads. The isolated $CD3^+$ and $CD3^-$ fractions were then tested for cytotoxicity against K562 cells in ^{51}Cr release assays.

Figure 3.21A shows that cytotoxic function in both NK ($CD3^-$) and $CD3^+$ cell fractions was induced by IL-15 in patient A, with 67% and 74% specific cytotoxicity at E:T ratios

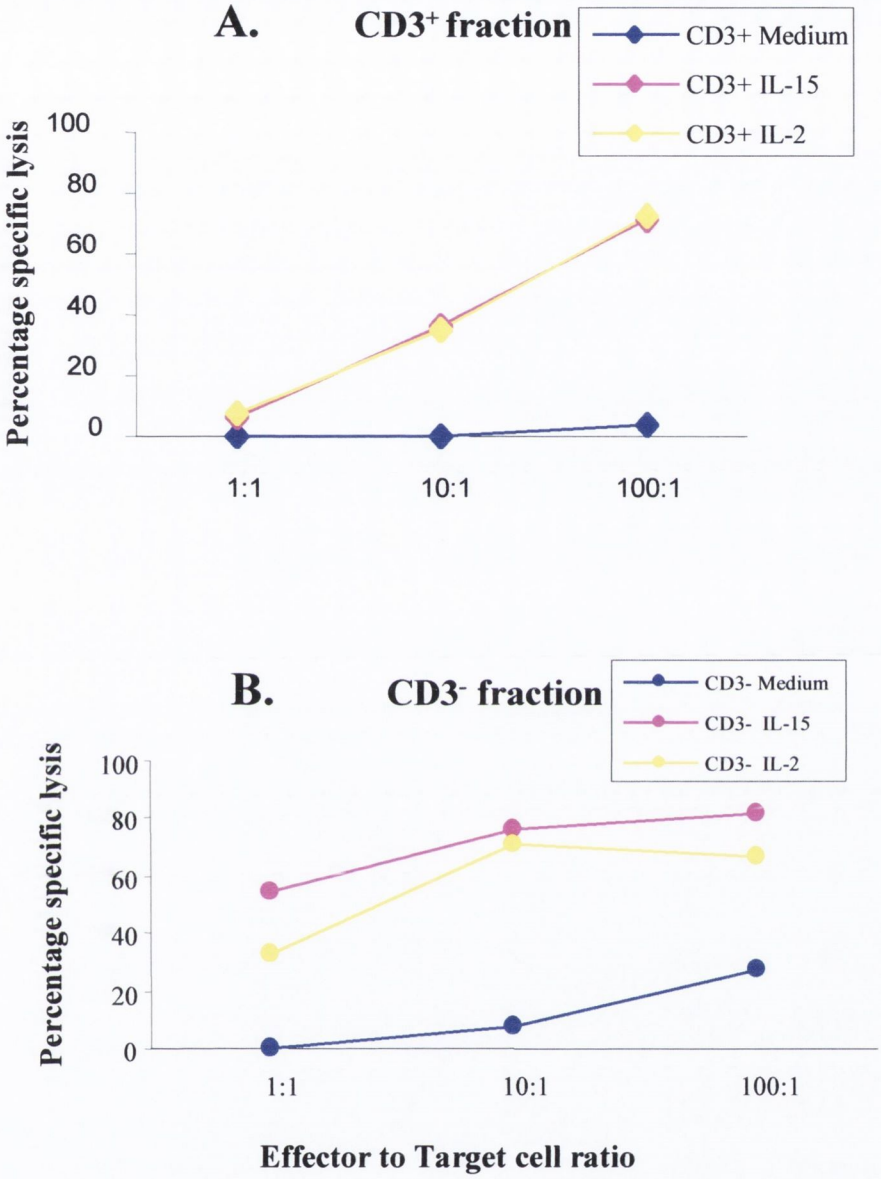


Figure 3.20. Effects of culture in medium with and without IL-2 or IL-15 on lysis of K562 cells by $CD3^+$ and $CD3^-$ cell fractions from healthy controls.

Lysis of K562 by $CD3^+$ (A) and $CD3^-$ (B) cell fractions was measured in a ^{51}Cr release assay, following culture for 7 days in medium, or with IL-2 at 100 ng/ml or IL-15 at 25 ng/ml. This experiment was repeated in n=6 adult controls, sample results from a healthy adult control are shown.

of 100:1. In contrast patient B showed only moderate induction of NK and $CD3^+$ cell cytotoxicity by IL-15, with only 30% specific lysis at E:T ratios of 250:1 (figure 3.21B). Thus the expansions of peripheral blood NK and NKR^+ T induced by IL-15 in patient A but not in patient B (figures 3.18 and 3.19) are mirrored by the induction of cytotoxic activity in these cells in patient A, which is impaired in patient B.

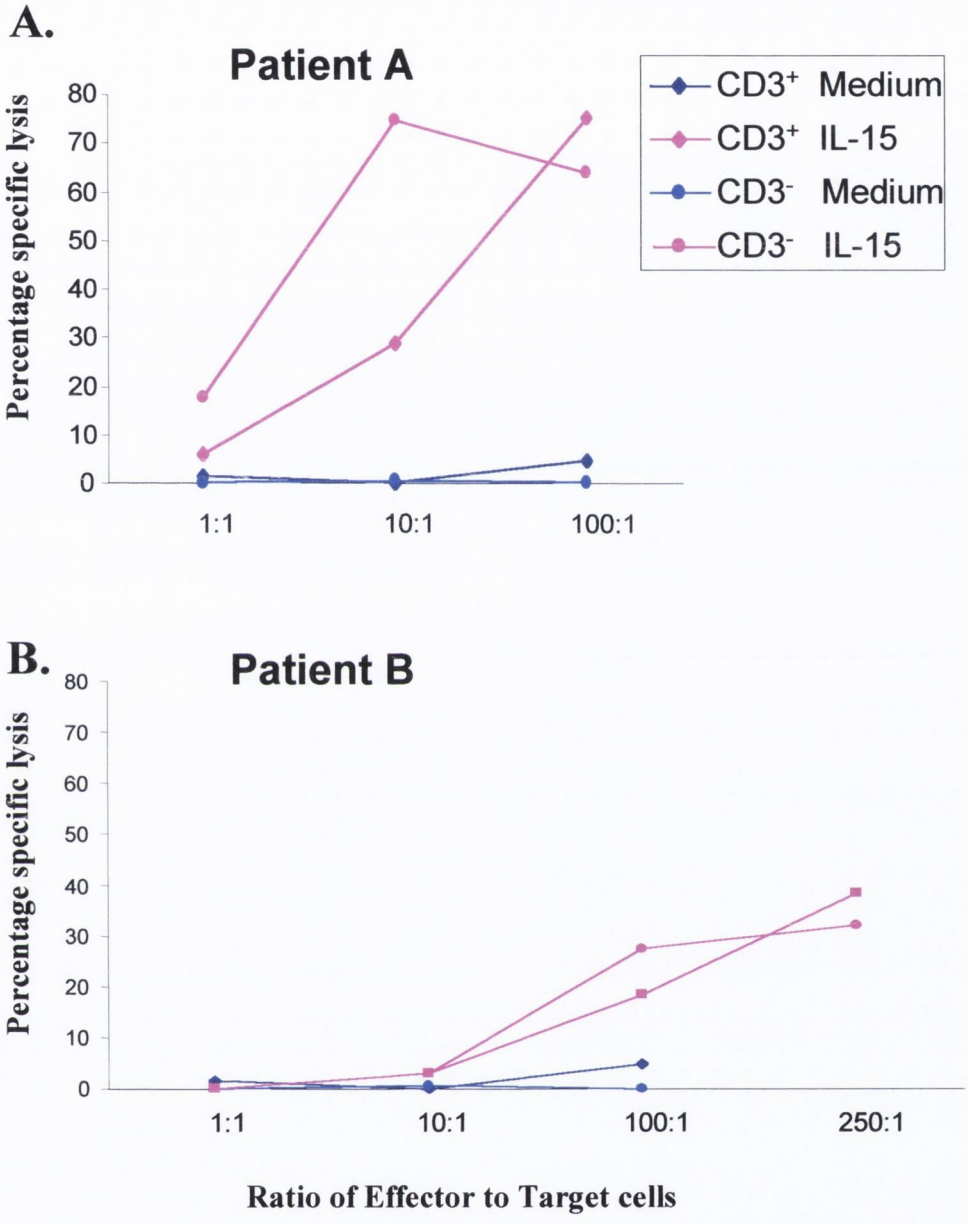


Figure 3.21. Effects of culture in medium with and without IL-15 on lysis of K562 cells by $CD3^+$ and $CD3^-$ cell fractions from HLH patients.

Lysis of K562 by $CD3^+$ and $CD3^-$ cell fractions following culture for 7 days in medium, or with IL-15 at 25 ng/ml, was measured in a ^{51}Cr release assay. Results from patient A (A) E:T ratios 1:1- 1:100, and patient B (B), E:T ratios 1:1- 1:250 are shown.

3.5. Effects of IL-2 and IL-15 on the mediators involved in response to infection - cytokines and perforin expression - in PBMC

In response to infection IL-15 has been shown to enhance the killing ability of NK cells and to stimulate the synthesis and secretion of IFN-γ (Gosselin *et al.* 1999; Fawaz *et al.* 1999; Flamand *et al.* 1996). In this study, using PBMC from healthy controls, we undertook to examine the cell types which express IFN-γ and IL-4 in response to culture with IL-2 or IL-15.

IL-2 and IL-15 have different sites of synthesis as well as distinct functions in lymphocyte expansion and homeostasis (Waldmann *et al.* 2001). We have previously shown that IL-15 induced upregulation of the CD25 receptor on T cells. Using PBMC from healthy controls we examined the effect of IL-15 on the survival and expansion of cells capable of IL-2 production.

Perforin is an important mediator of cytotoxicity in NK, NKR^+ T cells and antigen specific $CD4^+$ and $CD8^+$ T cells (Ortaldo *et al.* 1991; Yasukawa *et al.* 2000). Upregulation of mRNA for perforin has been demonstrated in response to IL-2 (Koizumi *et al.* 1991; Nakata *et al.* 1992). Absent or reduced perforin expression has been demonstrated in a number of FHL patients (Stepp *et al.* 1999). Patient B has been shown to express perforin in peripheral cells but there was reduced spontaneous cytotoxicity by IL-15 expanded populations from this patient. We therefore examined the requirement for and the effect of IL-2 and IL-15 on perforin expression by lymphocyte subsets in the periphery of healthy controls and in HLH patient B.

3.5.1. IL-2 and IL-15 do not induce cytokine production by resting human PBMC but augment cytokine production by activated PBMC

(Figure 3.22)

The effect of culturing control PBMC for 7 days in medium only, or with 100 ng/ml IL-2 or 25 ng/ml IL-15 on production of IFN-γ and IL-4 in the absence of stimulation, or upon stimulation with PMA and ionomycin or anti-CD3 mAb was examined by intracellular cytokine staining and flow cytometry. Results shown in figure 3.22B-E

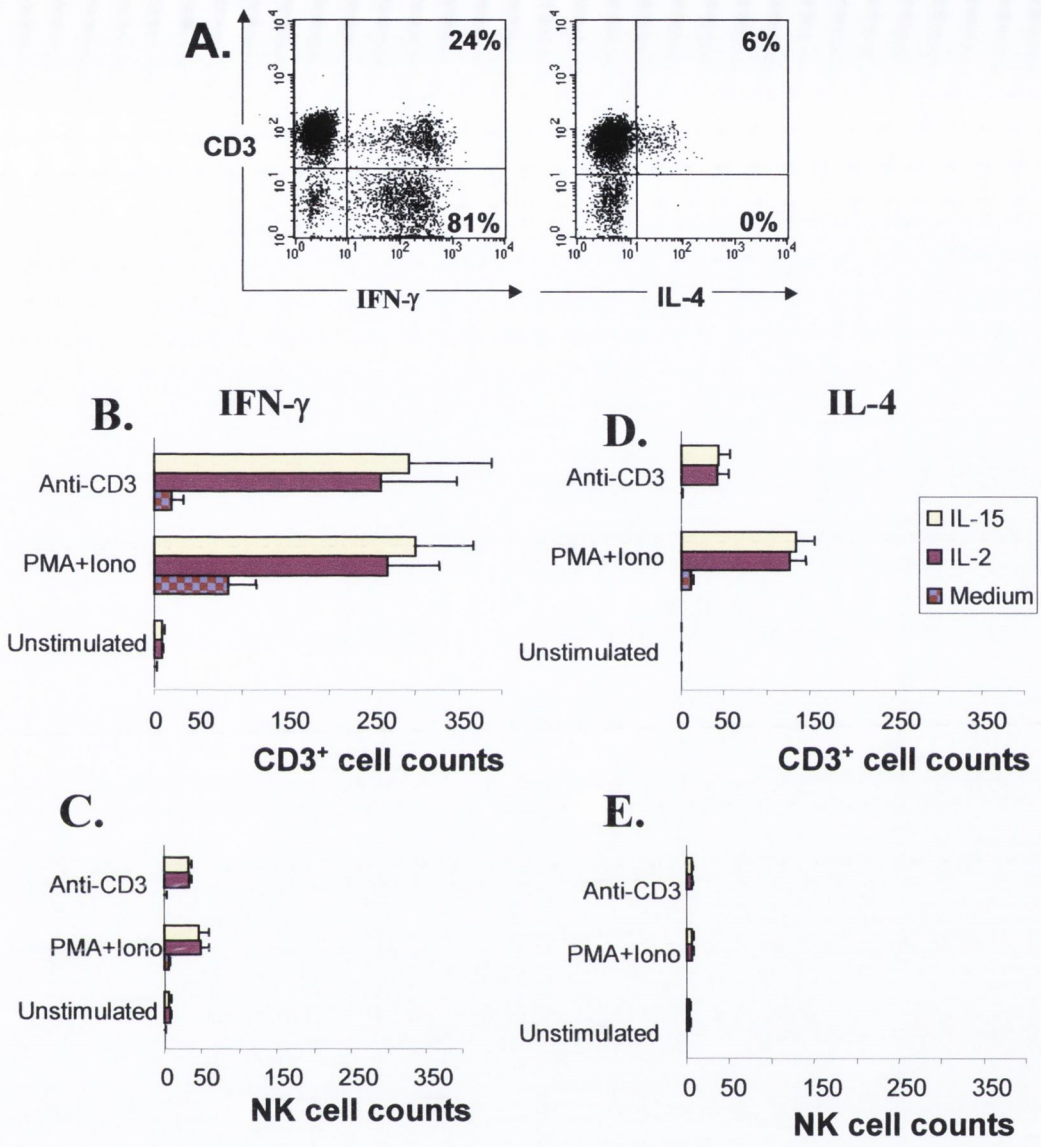


Figure 3.22. Effect of IL-2 and IL-15 on IFN-γ and IL-4 production by $CD3^+$ and NK cells.

Flow cytometric detection of CD3 expression and IFN-γ (left) and IL-4 (right) production by control PBMC following culture for 7 days with IL-15 followed by stimulation with PMA-ionomycin (A). The numbers in A indicate the percentages of $CD3^+$ (upper right) or $CD3^-$ cells (lower right) expressing cytokines. Absolute numbers of $CD3^+$ and NK cells producing IFN-γ (B and C) and IL-4 (D and E) following culture for 7 days in medium only or with 25 ng/ml IL-15 or 100 ng/ml IL-2 and either unstimulated or following 4 hours stimulation with PMA and ionomycin or plate bound anti-CD3. All results are means of four experiments +SD.

indicate that few cells produced IFN-γ or IL-4 in the absence of stimulation even after culture with IL-2 or IL-15. However, compared to PBMC cultured in medium only and stimulated with either PMA and ionomycin or anti-CD3 mAb, the numbers of $CD3^+$ cells capable of producing IFN-γ and IL-4 were dramatically increased by preculturing with IL-2 or IL-15 (figure 3.22B and D). Culture of PBMC with IL-2 or IL-15 also induced increased numbers of NK cells capable of expressing IFN-γ on stimulation with PMA ionomycin and suprisingly also with anti-CD3 mAb, but very few NK cells were shown to express IL-4 (figure 3.22B and D).

3.5.2. IL-2 and IL-15 augment cytokine production by PBMC by selectively expanding NK and $CD56^+$ T cells

(Figure 3.23)

The augmentation of NK and $CD3^+$ cell cytokine production by IL-2 and IL-15 (figure 3.22) could be due either to costimulation of IFN-γ and IL-4 production by PBMC or selective expansion of IFN-γ and IL-4 secreting cell subpopulations. To address this question we measured the percentages of NK cells, $CD56^-$ T cells and $CD56^+$ T cells incubated with medium only, IL-2 or IL-15 that produce IFN-γ and IL-4 upon stimulation.

Figure 3.23 shows that after incubation in medium alone, 47% of NK cells, 86% of $CD56^+$ T cells, but only 12% of $CD56^-$ T cells produce IFN-γ, and 1% of $CD56^-$ T cells and 13% $CD56^+$ T cells produce IL-4 upon stimulation with PMA and ionomycin. Slightly lower frequencies of these lymphocyte subpopulations produce cytokines upon stimulation with anti-CD3 mAb. When cells were cultured in medium only there was a reduction in the proportion of all cell types producing IFN-γ in response to anti-CD3 stimulation. It should be noted that cell numbers, particularly $CD3^+CD56^+$ and NK cells, were much reduced following culture with medium (figure 3.16). Culture with cytokines increased the proportions of $CD3^+$ cell types producing IFN-γ in response to anti-CD3 stimulation to that achieved with PMA-ionomycin. Interestingly, 20% of NK cells produced IFN-γ after anti-CD3 mAb stimulation, presumably due to factors produced by

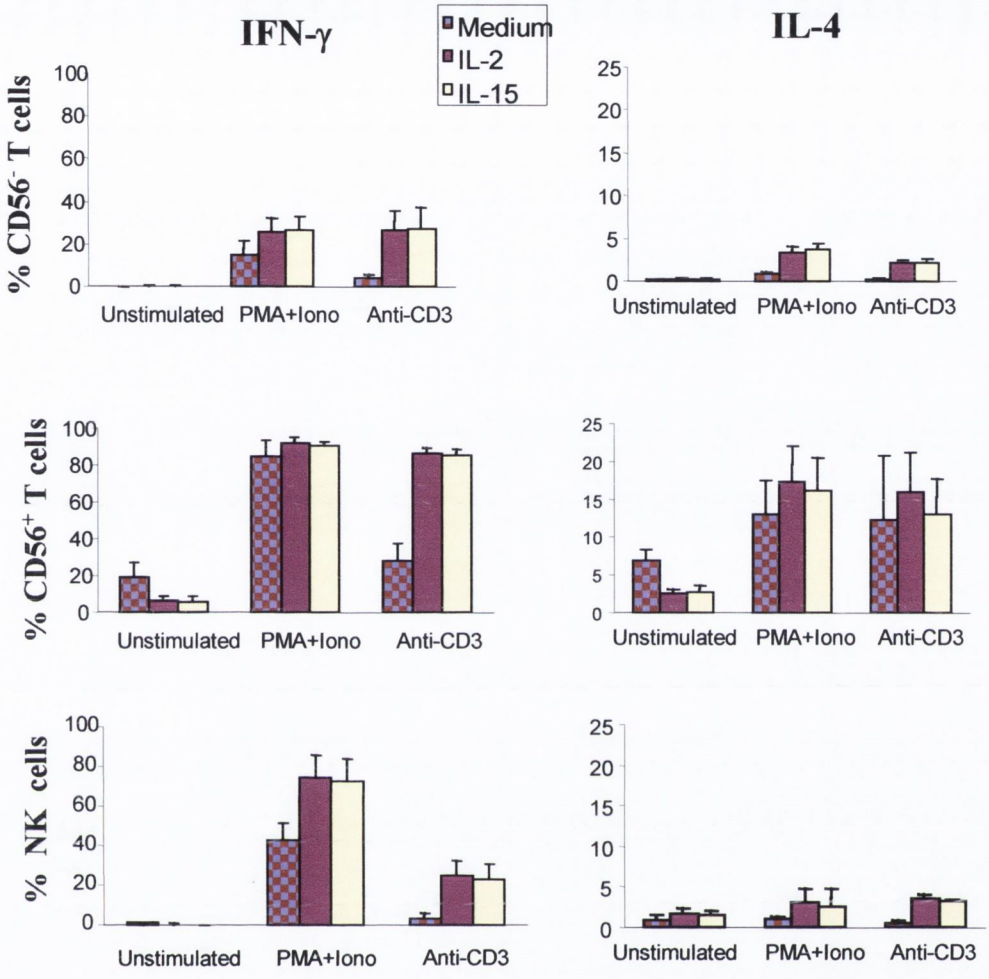


Figure 3.23. Effects of IL-2 and IL-15 on IFN-γ and IL-4 production by human $CD3^+CD56^+$, $CD3^+CD56^-$ and NK cells.

Intracellular IFN-γ (left) and IL-4 (right) production was measured on unstimulated, PMA-ionomycin or anti-CD3 mAb stimulated cells. The cells examined were from healthy controls (n=4) and had been cultured for 7 days in medium only or with IL-2 at 100 ng/ml or IL-15 at 25 ng/ml. Results shown are means+SD.

activated T cells or to stimulation of NK cells through binding of the Fc portions of anti-CD3 mAb to CD16.

These results show that greater proportions of $CD56^+$ T cells produce IFN-γ and IL-4 upon stimulation than NK cells and $CD56^-$ T cells, and that the augmentation of cytokine production by IL-2 and IL-15 seen in figure 3.22 was due to selective expansion of $CD56^+$ T cells and, to a lesser degree NK cells. Thus IL-2 and IL-15 do not induce or costimulate cytokine production by PBMC but they selectively expand IFN-γ and IL-4 producing NK and $CD56^+$ T cells.

3.5.3. IL-15 is a selective growth factor for cells capable of IL-2 expression upon anti-CD3 stimulation

(Table 3.10)

The effect of a single pulse of IL-15 added at time zero on intracellular IL-2 expression in response to stimulation with PMA-ionomycin or anti-CD3 was studied and results were compared with cells cultured in medium only for 7 days (table 3.10). Unstimulated lymphocytes, either $CD4^+$ or $CD8^+$, cultured for 7 days in medium only or with IL-15 added, were not shown to express intracellular IL-2. A proportion of $CD4^+$ and $CD8^+$ cells cultured for 7 days in medium only and stimulated with PMA-ionomycin were shown to be capable of IL-2 expression. The numbers of cells expressing intracellular IL-2 in response to PMA-ionomycin stimulation increased significantly when a single pulse of IL-15 was added to the culture medium at time zero. Culture of PBMC in medium only followed by anti-CD3 stimulation showed barely detectable numbers of $CD4^+$ and $CD8^+$ cells expressing intracellular IL-2. Addition of a single pulse of IL-15 with culture for 7 days followed by stimulation with anti-CD3 resulted in significant numbers of $CD4^+$ and $CD8^+$ cells with demonstrable intracellular IL-2.

Table 3.10. Intracellular IL-2 expression was demonstrated in increased numbers of $CD4^+$ and $CD8^+$ lymphocytes following culture with IL-15.

Stimulation	$CD4^+$ lymphocytes/ml		$CD8^+$ lymphocytes/ml	
	Medium	**IL-15**	**Medium**	**IL-15**
Unstimulated	0	2±0	0	1±1
PMA-ionomycin	118±23	254±26	31±2	119±28
Anti-CD3	4±1	108±14	3±1	71±14

Intracellular IL-2 was measured in cells that were cultured in medium only or with the addition of IL-15 at 25 ng/ml for 7 days. Cytokine expression was measured in cells that were either unstimulated or following stimulation with PMA-ionomycin or anti-CD3. Results shown are mean numbers of $CD4^+$ or $CD8^+$ cells per ml± standard deviation (SD) from four experiments with healthy control control cells. Column labels 'Medium' and 'IL-15' indicate culture for 7 days in medium only or the addition of IL-15 at 25 ng/ml followed by culture for 7 days respectively.

3.5.4. Sustained intracellular perforin expression requires IL-2 or IL-15

(Figure 3.24)

In order to examine whether sustained perforin expression required cytokine stimulation, PBMC from 2 healthy controls were cultured in medium only for up to 3 days. The percentage of $CD3^+$ and $CD3^-CD56^+$ cells expressing perforin were measured at time zero and days 1 to 3: these results are shown in figure 3.24B and C. A decline in the percentages of both $CD3^+$ and $CD3^-CD56^+$ cells expressing perforin were noted by day 2, which decreased further by day 3. The addition of IL-15 (figure 3.24B and C) or IL-2 (data not shown) resulted in upregulation of perforin expression with an increase to time zero levels following four days culture with IL-15.

The loss of perforin expression from cells cultured in medium only would indicate that sustained perforin expression requires IL-2 or IL-15 or other stimulation. Addition of either IL-2 or IL-15 to cells following three days culture in medium resulted in upregulation of perforin expression at 24 hours (data not shown) with further increases to time zero percentages following four days culture with cytokine. These results indicate that sustained perforin expression requires IL-2 or IL-15 and that a proportion of cells expressing perforin die off when cultured in medium without either of these cytokines.

3.5.5. IL-15 and IL-2 induced expansion in numbers of $CD56^+$ T expressing perforin in healthy controls but not in patient B

(Figure 3.25)

We have previously shown that prolonged culture with IL-2 and IL-15 selectively expanded $CD3^+CD8^+$, $CD3^+CD56^+$ and NK cells, these are also the cell types which express perforin. In order to assess the effects of cytokines on these populations, perforin expression was measured at time zero, following culture in medium and with IL-2 or IL-15 added.

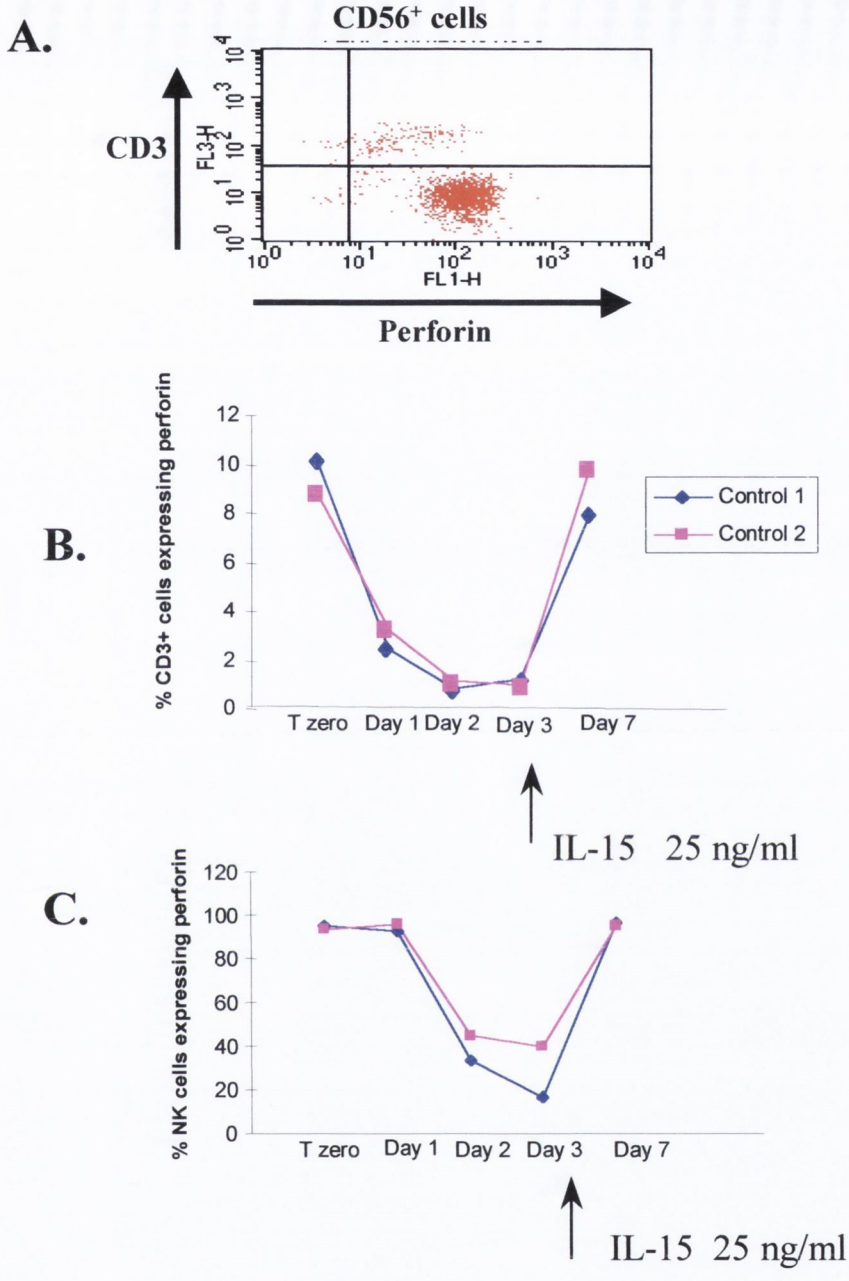


Figure 3.24. Is IL-2/ IL-15 required to maintain perforin expression in $CD3^+$ and $CD3^-$ $CD56^+$ cells?

Flow cytometric detection of CD3 and perforin expression in $CD56^+$ cells (A). Changes in perforin expression in $CD3^+$ (B) and $CD3^-CD56^+$ (NK) cells (C) following culture in medium for three days, with a subsequent addition of IL-15 at 25 ng/ml (indicated in diagrams by arrow) and culture for a further four days. The percentages of either $CD3^+$ and $CD3^-CD56^+$ (NK) cells expressing perforin are shown for two healthy controls.

Results for healthy controls (n=4) shown in figure 3.25 indicate that culture for 7 days with IL-15 had a more significant effect than IL-2 on increasing the numbers of $CD3^+$ cells expressing perforin compared to fresh PBMC (T zero). This population includes perforin expressing $CD8^+$ and $CD56^+$ T cells, both of which were significantly increased in response to cytokines. The increase in $CD56^+$ T cells was the most marked and comparison using a non-parametric test yielded a p value = 0.001 (<0.05 is significant) for cells cultured with IL-15 compared to T zero. The numbers of NK cells expressing perforin were increased in some individuals following culture with cytokine, but were not significantly increased overall. Perforin was expressed at a high level in freshly isolated NK cells. Following culture with cytokines for 7 days the level of intracellular perforin was lower in NK cells perhaps indicating that IL-2 and IL-15 selectively expand NK cells that express low levels of perforin.

As has been previously stated, using freshly isolated PBMC, patient B was shown to have near normal percentages of perforin positive $CD3^+CD8^+$ cells but lacked $CD3^+CD56^+$ and NK cells making measurement of perforin expression in these populations difficult. Cells from this patient showed some increase in the number of $CD3^+CD8^+$ cells expressing perforin following culture with IL-15 but not IL-2 (figure 3.25B). The numbers of NK and $CD3^+CD56^+$ cells expressing perforin increased slightly for patient B cells relative to T zero levels but did not reach the numbers achieved by normal PBMC (figure 3.25 C and D).

These results indicate that cells from normal controls respond to IL-15 and to a lesser extent IL-2, with expansion of the perforin positive $CD3^+$ and $CD3^-$ populations, with perforin expressing $CD3^+CD56^+$ cells showing the most significant expansion. The numbers of perforin positive $CD3^+CD8^+$ cells from patient B were near normal and increased following culture with IL-15. Neither IL-2 nor IL-15 induced significant expansion in the numbers of perforin positive NK and NKR^+ T cells from patient B, which were much reduced compared to normal controls.

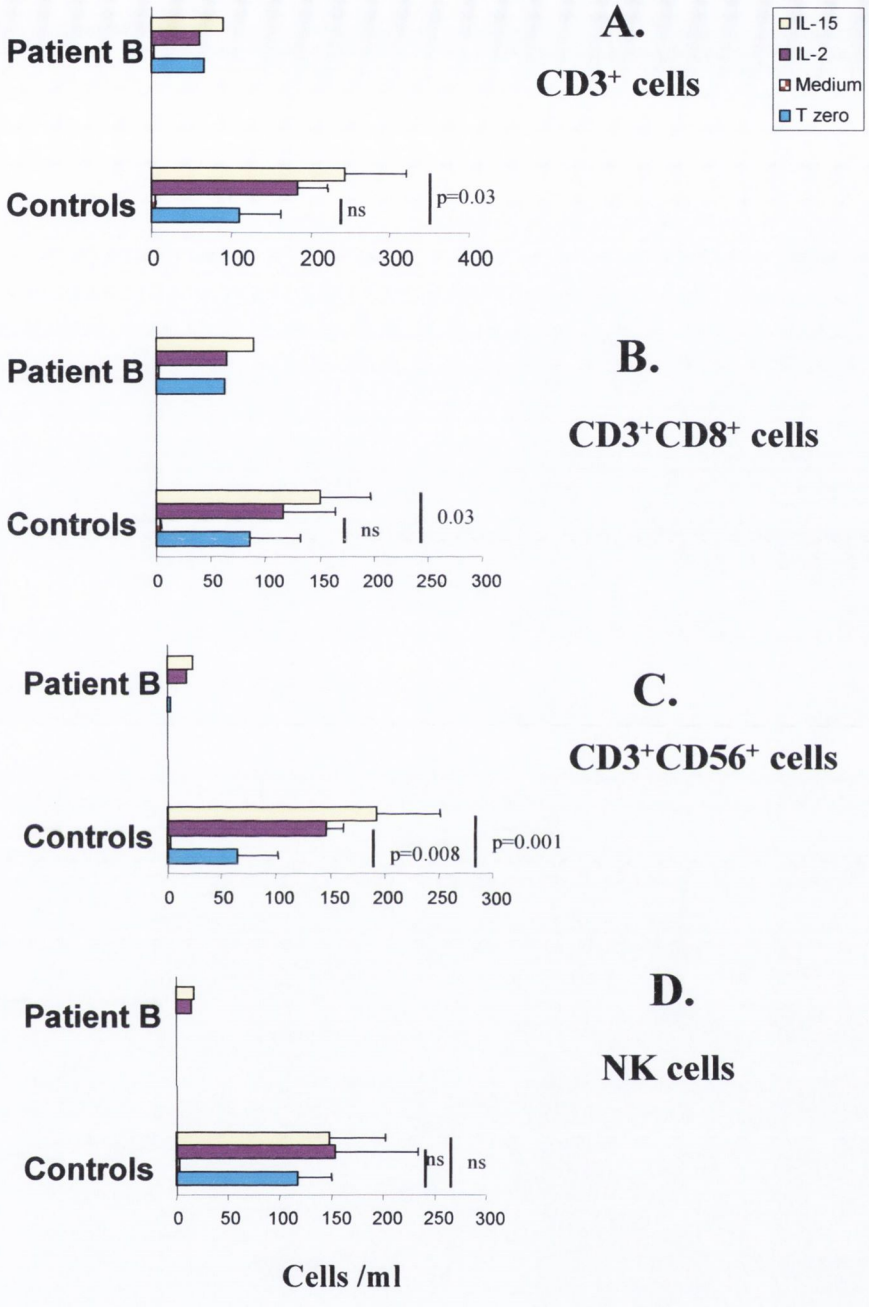


Figure 3.25. Expansion of perforin expressing human PBMC by IL-2 and IL-15.

Expansion of human peripheral blood $CD3^+$ (A), $CD3^+CD8^+$ (B), $CD3^+CD56^+$ (C) and NK ($CD3^-CD56^+$) (D) cells expressing perforin, measured at time zero (T zero) and following culture for 7 days in medium only or with IL-2 at 100 ng/ml or IL-15 at 25 ng/ml. Results shown are cells/ ml from HLH patient B and means +SD for healthy controls (n=4). p Values are shown with lines indicating the results compared in non-parametric tests, ns = not significant.

4. Discussion

4.1. Diagnosis of HLH

The clinical and histological findings in HLH are defined but the differential diagnosis may present difficulties and this rare condition is often not diagnosed until autopsy (Henter *et al.* 1991a; Imashuku 1997; Ost *et al.* 1998). Defects in cytotoxic function described in FHL patients can be attributed to perforin deficiency in a number of cases (Stepp *et al.* 1999), however in the majority of cases the cause of HLH is unknown. X-linked lymphoproliferative disorder is one of the conditions that may present with associated HLH, and this, together with a number of other conditions, must be considered in the differential diagnosis (Dufourq-Lagelouse *et al.* 1999b). The histological, phenotypic and functional studies presented here were carried out in order to establish a diagnosis for two HLH patients.

4.1.1. Deficiency of NK and $CD56^+$ T cells in association with HLH

Phenotypic studies indicated that the two patients with HLH were profoundly deficient in both NK cells ($CD56^+/CD16^+$) and in a population of NKR^+ T cells ($CD3^+CD56^+$). Furthermore, a functional deficiency of NK and NKR^+ T cells was demonstrated since PBMC from both patients were unable to lyse K562 cells and IL-2 induced killing of Daudi cells was also severely reduced. Natural killer cell deficiency in conjunction with recurrent *herpes* infection has previously been described by *Biron et al. 1989*.

Initially the clinical, histological and immunological findings in these patients were in keeping with a diagnosis of familial type HLH. Subsequently the diagnosis of patient B was altered to EBV-lymphoma associated haemophagocytic syndrome (EBV-LAHS). Earlier studies have reported decreased or absent T and NK cell cytotoxicity in FHL (Egeler *et al.* 1996). The finding of perforin gene abnormalities in a proportion of FHL cases further elucidated the essential role of perforin expressing cytotoxic cells in the pathogenesis of haemophagocytic lymphohistiocytosis (Stepp *et al.* 1999). While

cytotoxic dysfunction has been reported, NK and NKR^+ T cells deficiency in conjunction with haemophagocytic lymphohistiocytosis has not been described previously.

4.1.2. Differential diagnosis and the nature of HLH in NK and NKR^+ T cell deficient patients

The diagnosis of familial haemophagocytic lymphohistiocytosis (FHL) in patient A was supported by clinical and histological findings and this patient eventually received a bone marrow transplant from a matched sibling donor and is currently clinically well.

The diagnosis of patient B presented some difficulties as he had a less severe progression of disease that patient A. Immunohistochemical studies showed that patient B developed an EBV^+ lymphoma in the gastro-intestinal tract with the cells showing evidence of CD20 (B cell) expression. EBV together with lymphoma associated haemophagocytic syndrome (EBV-LAHS) has been described in a number of Asian children (Su *et al.* 1995). The pattern of EBV-infection of peripheral lymphocytes in a number of these cases showed a stronger association with $CD8^+$ T cell infection than $CD20^+$ cell infection (Kasahara *et al.* 2001; Ma *et al.* 2001). The similarities between patient B and the Asian children with EBV-LAHS prompted speculation on the exact diagnosis of this patient and the nature of his HLH. In order to rule out a diagnosis of XLP studies of SAP protein were carried out for patient B and his NK deficient sibling, both of who showed normal expression of this protein. While SAP protein has been shown to be absent in only 50% to 60% of XLP cases, generally these patients go on to develop bone marrow failure due to infiltration of polyclonal T and B cells into many organs (Morra *et al.* 2001; Sullivan *et al.* 1980).

The susceptibility of patient B to infection with *Herpes* family viruses may have been the important triggering mechanism in the development of haemophagocytosis. Failure to eliminate EBV-infected cells by SAP deficient XLP patients has been attributed to inhibition of NK cell function through 2B4, which acts as an inhibitory rather than an activatory receptor in these patients (Moretta *et al.* 2001). Studies in mice have

indicated that NK cells play an essential role in the control of EBV-driven lymphoma (Robertson, 2001). In the case of patient B the deficiency of NK and NKR^+ T cells, rather than functional inhibition of these cells, may account for the reduced capacity of this patient to eliminate EBV-infected cells. This failure in response to EBV infection may have given rise to the development of lymphoma and lymphocytic infiltration of bone marrow and lymph nodes.

Perforin deficiency has been described in some FHL patients (Stepp *et al.* 1999). Patient B and his NK deficient relatives were shown to express perforin in peripheral $CD8^+$ T cells, however there was an altered pattern of staining compared to controls due to deficiency of $CD56^+$ populations. A similar study by *Kogawa et al.* showed absence of perforin expression in $CD8^+$ and $CD56^+$ cells from 10q21-22 disrupted FHL patients, with other FHL patients showing normal pattern perforin expression (Kogawa *et al.* 2002). Taken together, the evidence suggests that patient B had EBV related lymphoma which precipitated the development of haemophagocytosis. The susceptibility of this patient to the development of this pathology may have resulted from NK / NKR^+ T cell deficiency.

4.1.3. Is the pathogenesis of HLH linked to deficiency of NK and NKR^+ T cells?

The pathogenesis of FHL remains obscure, it is possible that common viral infections could trigger the disease (Henter *et al.* 1993). As has been stated previously, a proportion of FHL patients lack perforin expression in cytotoxic lymphocytes (Stepp *et al.* 1999). Other disorders associated with haemophagocytosis, such as X-linked lymphoproliferation (XLP), Griscelli and Chediak-Higashi syndromes, manifest dysfunctional cytotoxicity either in relation to a single virus (EBV in XLP) or more globally due to failure to transport cytotoxic mediators such as perforin (Dufourcq-Lagelouse *et al.* 1999b). In the two cases presented here the development of HLH may be attributed to the absence of perforin expressing lymphocytes in the periphery and presumably at sites of immune clearence.

Of the two patients studied patient A exhibited a more severe disease pattern than Patient B, though treatment of patient A with cyclosporin (Loechelt *et al.* 1994) resulted in clinical remission. Both patients suffered from recurrent infections most notably *Herpes*, *Varicella* and *Pneumococcus pneumoniae*. Initial studies of peripheral blood cells from both of the HLH patients indicated that the lymphocytes capable of spontaneous cytotoxicity (NK cells, and NKR expressing T cells) were reduced. Longitudinal studies of both patients did not reveal any increases in numbers or percentages of NK and $CD3^{+}CD56^{+}$ cell populations. NK deficiency and low NK cell cytotoxicity has been linked to increased human sensitivity to severe disseminating *herpes* virus family including *HSV, EBV* and *CMV* (Biron *et al.* 1999). Deficiency of lymphocytes expressing NKRs may have resulted in susceptibility to recurrent *Herpes* family viral infections in these two patients. The male cousins of patient B also exhibited similar deficiencies together with susceptibility to recurrent viral infections but no evidence of HLH. The deficiency of these cells in the periphery may indicate a concurrent deficiency or elimination at sites of immune clearance such as the bone marrow, liver, spleen and lymph nodes. These are the sites of haemophagocytic infiltration demonstrated in patients with FHL (Ost *et al.* 1998).

Early clinical histories of both these patients described lymphadenopathy and hepatosplenomegaly. Histological studies revealed haemophagocytosis and lymphocyte infiltration of lymph nodes as well as haemophagocytosis in bone-marrow biopsies from both patients and peripheral blood of one of these patients. It is possible that the absence of spontaneous or NK-like cytotoxicity may have led to failure of early responses to viral infection together with the expansion of monocytes/antigen presenting cells (APC) in these two patients. Perforin expressing cells may have an important role in the control of histiocyte expansion in response to infection. Autologous APC have been shown to be killed by IL-2 activated NK and $CD8^{+}$ T cells in a perforin dependent fashion (Wilson *et al.* 1999; Parajuli *et al.* 1999). Perforin expressing populations, activated through natural cytotoxicity receptors, may play a role in downregulation of APC expansion (Spaggiari *et al.* 2001). In both of these patients the absence of cells capable of perforin mediated downregulation of APCs could then have resulted in the sustained

expansion of reactive monocyte/histiocyte populations demonstrated at sites of immune clearance.

Studies in perforin deficient mice have indicated that cytotoxic lymphocytes are important in restoring lymphocyte homeostasis following T cell expansion due to viral infection (de Saint Basile *et al.* 2001). It is postulated that a similar mechanism operates in human post-infection homeostasis and the persistance of activated lymphocytes in perforin deficient HLH patients may result from dysfunction in the perforin expressing populations (de Saint Basile *et al.* 2001). Activated lymphocyte populations were not demonstrated in the periphery of these two patients, as there was no increased CD25 expressing populations. This discrepancy may be explained by the differences between perforin deficiency seen together with normal numbers of T and NK cells, and the absence of NK and NKR^+ cells demonstrated in these two patients. Perforin deficient cells from FHL patients presumably have no defects in their cytokine responses and under continous cytokine onslaught from APC may contribute to the activated T cell phenotype and cytokine storm described in these FHL patients (Henter *et al.* 1991b).

In the present study, using healthy control cells, we have demonstrated that IL-2 or IL-15 activated $CD56^+$ T cells constitute the major population which express IFN-γ and IL-4 on CD3 crosslinking. Both patients were deficient in the cells shown to have greatest IFN-γ producing potential. In addition, the IL-15 induced expansion of these populations was reduced in patient A and very reduced in patient B. Thus the absence of activated phenotype T cells from the periphery of these two patients may be explained by deficiency of cytokine producing T and NK cells. The increases in infiltrating lymphocytes and histocytes demonstrated in lymph node and bone marrow biopsies from these two patients may further underline the important function of perforin expressing NKR^+ cells in controlling expansions of these cells at sites of immune clearence. It is interesting to note that activated monocytes/macrophages are the major source of IL-15 and in this study we have shown that this cytokine plays a major role in activation and expansion of perforin expressing NKR^+ cells (Bamford *et al.* 1996; Grabstein *et al.* 1994; Neely *et al.* 2001). Natural cytotoxicity receptors positive (NCR^+) cells have been shown to lyse autologous APCs and activated monocytes express

biologically active IL-15 (Spaggiari *et al.* 2001; Neely *et al.* 2001). Thus the effect of macrophage derived IL-15 on perforin expressing NCR^+ cells may constitute a feedback mechanism to control APC expansion.

Taken together with previous studies our results indicate that reduction in either numbers or function of perforin expressing NK and NKR^+ T cells may give rise to the characteristic lymphocytic infiltration and histiocyte expansion in HLH.

4.1.4. Activated monocytes from HLH patients may be trying to stimulate NK and NKR$^+$ cells that are not there.

CD16 is a low affinity receptor for IgG and is expressed on NK cells, activated monocytes and a subset of T cells (Maeda *et al.* 1996). The expression of this receptor has been shown to be increased in HLH (Emminger *et al.* 2001). Increased expression of Fcγ receptor CD16 on monocytes has been demonstrated in patients with septicaemia and in advanced HIV infection associated with bacterial and fungal infections (Thieblemont *et al.* 1995; Fingerle *et al.* 1993; Dunne *et al.* 1996). Monocytes from both patient A and patient B expressed increased CD16 and reduced CD18 and CD11c. The increases in CD16 expression in the two HLH patients may therefore be directly attributable to recurrent, unresolved infections.

The adhesion molecules CD18/CD11a, b and c are essential for normal functions of phagocytic cells *in vitro* and *in vivo* (Arnaout, 1990). The functions of CD11b/CD18 and CD11c/CD18 in monocyte phagocytosis are mediated through binding of complement derived iC3b, and are thought to be mutually redundant (Arnaout, 1990). Activation of monocytes has been shown to cause upregulation of CD11b, c/CD18 (Strassmann *et al.* 1985). Differentiation of monocytes into tissue macrophages is associated with reduction in CD11/CD18 (Clayberger *et al.* 1987). The activated monocyte phenotype observed in the periphery of these two HLH patients may have been induced by recurrent infection and preceeded monocyte migration into tissues. These maturing cells may have given rise to the expansions of activated histiocytes observed in these patients on bone marrow and lymph node biopsies.

The central role of macrophages in immunomodulation through cytokine production may also have contributed to the haemophagocytosis observed in the two patients presented here. Macrophage derived IL-12, IL-15 and IL-18 exert their anti-viral effects through control of IFN-γ production and enhancement of NK and T cell proliferation and cytotoxicity (Trinchieri *et al.* 1994; Carson *et al.* 1995; Okamoto *et al.* 1999; Tomura *et al.* 1998). In this study we have demonstrated that following culture with IL-2 or IL-15, the IFN-γ producing cells in healthy controls belong mainly to the CD56

expressing populations (NK and $CD3^{+}CD56^{+}$ cells). In a similar fashion IL-15 derived from activtivated monocytes/macrophages may orchestrate the expansion and/or activation of IFN-γ producing cells. The deficiency of these potential IFN-γ producing populations in the periphery of the two patients studied may have contributed both to susceptibility to viral infection and to expansion of the macrophage/histiocyte populations.

4.2. The possible role of IL-15/IL-15R in NKR^+ cell deficiency

4.2.1. NK deficient HLH patients A and B express IL-15 and IL-15R

In the present study we have examined the possible role of IL-15 and its receptor complex in NK and NKR^+ T cell deficiency. The variable effects of this cytokine on lymphocyte subsets has led us to a greater understanding of the role of this cytokine in the development of innate and adaptive immune response. IL-15Rα forms the unique component of the IL-15 receptor complex that includes IL-2/15Rβ (CD122) and the common gamma chain (Giri *et al.* 1994). Studies in knockout mice have shown that signalling through IL-15/IL-15R complex is required for the development and homing of NK cells, NKR^+ T cells, and subsets of $CD8^+$ T cells, γδ T cells and intestinal epithelial lymphocytes (IEL) (Suzuki *et al.* 1997; Ohteki *et al.* 1997; Lodolce *et al.* 1998; Liu *et al.* 2000; Kennedy *et al.* 2000). Given the important role this cytokine/receptor in the development of the lymphocyte populations shown to be deficient in the two HLH patients we were naturally interested to examine the expression of both IL-15 and IL-15Rα in PBMC.

Using PCR we first demonstrated the presence of mRNA for IL-15Rα in PBMC from healthy controls and both HLH patients. PCR product from patient A was further amplified and the nucleotide product sequenced and identified as IL-15Rα. The portions of the receptor that were sequenced were shown to have two amino acid substitutions at positions 181 and 256 (ref. NP002189) in patient A. These substitutions were not verified by a third set of sequencing experiments. Having established that both patients expressed IL-15Rα we then went on to examine CD122 expression. Comparison of results from patients and healthy adult controls indicated that both patients had reduced percentages of lymphocytes expressing CD122. Using freshly isolated PBMC from adult controls we have shown that a high percentage of NK and $CD3^+CD56^+$ populations express CD122. Since both of these patients were deficient in these populations the finding of decreased CD122 expression was perhaps not suprising.

In addition to its role in NK and NKR^+ T cell development and proliferation, IL-15 production is important in host defence against viral and intracellular bacterial infection (Flamand *et al.* 1996; Jullien *et al.* 1997; Gosselin *et al.* 1999). As stated earlier, the role of IL-15 in host defence may be mediated through control of IFN-γ expression in response to infection (Carson *et al.* 1995; Gosselin *et al.* 1999). TaqMan semi-quantitative PCR was used to measure expression of mRNA for IL-15 and IFN-γ. Both patients and controls expressed mRNA for IL-15, which increased on stimulation of PBMC with LPS/IFN-γ. The increase in mRNA for IL-15 was higher than normal in patient A (cyclosporin therapy) and reduced compared to normals in patient B (no therapy). This finding may have implications *vis a vis* the early response to viral and bacterial infection in these patients, although it should be noted that IL-15 expression is controlled at the levels of translation and intracellular trafficking (Waldmann *et al.* 1999; Fehniger *et al.* 2001). IL-15 protein has been demonstrated in both resting and activated human monocytes (Musso *et al.* 1999; Neely *et al.* 2001). Monocytes and lymphocytes from both patient A and healthy controls were shown to express IL-15, however due to technical difficulties monocytes from patient B have not been tested. It should be noted that blocking experiments using IL-15 are required in order to rule out non-specific staining by the anti-IL-15 mAbs used: these experiments have not been carried out. The demonstration of normal levels of mRNA for IFN-γ in both patient A and patient B was in contrast to the findings of increased levels in three age matched patients with recurrent infections.

4.2.2. IL-15 may have a profound effect on acquired immunity through expansion of T cells capable of producing and responding to IL-2

Both IL-2 and IL-15 have been shown to upregulate CD25 expression on human cell lines (Kumaki *et al.* 1996). The present study indicates that both of these cytokines have variable effects on the expression of at least two components of their own receptor-complexes, namely, CD25 which forms a unique part of the IL-2R, and CD122 which is a common receptor component for both IL-2 and IL-15 (Giri *et al.* 1994). Culture of healthy control PBMC for 24 hours with IL-2 resulted in reduction in the percentages of cells expressing the specific receptors CD25 and CD122 on both $CD3^+CD4^+$ and

$CD3^+CD8^+$ cells. This observed reduction may have been due to receptor-mediated endocytosis of the receptor-ligand complex. Endocytosis has been shown to be an early consequence of IL-2/IL-2R interaction, which acts to limit IL-2R signal transduction and so control the biological response to this cytokine (Duprez *et al.* 1992; Yu *et al.* 2000). Culture of healthy control PBMC with IL-15 for 24 hours induced a reduction in CD122 expression on both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells probably due to receptor-mediated endocytosis of the receptor-ligand complex. In contrast to its effects on expression of CD122, which forms a component of its own receptor complex (Giri *et al.* 1994), IL-15 was shown to induce increased expression of CD25 on both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells from healthy controls. These increases were most marked on $CD3^+CD8^+$ population.

In the present study we have shown that in addition to its role in 'priming' T cells for response to IL-2, IL-15 is an essential growth factor for cells capable of expressing intracellular IL-2 on anti-CD3 stimulation. A very small number of $CD4^+$ and $CD8^+$ cells cultured for 7 days in medium only and stimulated with anti-CD3 and ionomycin were shown to be capable of intracellular IL-2 expression. The numbers of cells expressing intracellular IL-2 in response to anti-CD3 stimulation increased significantly when a single pulse of IL-15 was added to the culture medium at time zero. IL-15 may be important in rescuing these IL-2 producing cells from apoptosis or it may act as a selective growth factor for these cells.

IL-15 would appear to be preparing or priming both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells for response to IL-2 and this function may be important in amplifying the T cell IL-2 induced responses. Support for this hypothesis comes from one *in vivo* study of the differential effects of IL-2 and IL-15 on cell proliferation in mice (Li *et al.* 2001). *Li et al.* showed that in response to IL-15 during the first five divisions, cycling T cells expressed IL-15Rα, CD122 and upregulated the common γ chain whereas CD25 was not expressed. After five cell divisions high levels of intracellular IL-2 were demonstrated in these T cells as well as upregulation of CD25 expression and downregulation of the common γ chain. The expansion of cells *in vivo* that are capable of both producing and responding to IL-2 may be important in T cell homeostasis, as

this cytokine has a role in downregulating T cell proliferation *in vivo* (Van Parijs *et al.* 1998).

4.2.3. IL-2 and IL-15 fail to induce normal levels of activation markers on PBMC from HLH patients

Comparison of patient PBMC with healthy adult controls indicated that the percentages of $CD3^+CD8^+$ cells expressing CD25 and CD122 were reduced on freshly isolated lymphocytes from both patient A and patient B. $CD3^+CD4^+$ cells from both patients expressed these receptors at near normal levels. Culture of PBMC for 24 hours with cytokines indicated that Patient A exhibited a normal $CD3^+CD4^+$ response to both IL-2 and IL-15 in contrast to patient B where the $CD3^+CD4^+$ response was blunted. Cells from both patients showed a much reduced level of IL-15 induced CD25 expression on $CD3^+CD8^+$ cells. Freshly isolated PBMC from healthy controls were shown to express CD122 on NK and $CD3^+CD56^+$ populations, both of which are deficient in the patients studied. The failure of upregulation of CD25 on $CD3^+CD8^+$ cells from these patients may stem from the deficiency of NKR^+ T cells expressing CD122 or may point to defects in STAT5 signalling or the IL2Rβ (Imada *et al.* 1998; Fujii *et al.* 1998).

CD69 is encoded by the natural killer complex (NKC) region on human chromosome 12p13.1 and is one of the earliest cell surface markers to be upregulated on haematopoetic cells in response to stimulation (Testi *et al.* 1994; Trowsdale *et al.* 2001). Stimulation through CD69 has been shown to trigger cytotoxicity and to induce NK cell proliferation and CD25 expression (Borrego *et al.* 1999). IL-15 has been shown to upregulate expression of this receptor on T cells (Kanegane *et al.* 1996). Both patient A and patient B had reduced expression of CD69 on freshly isolated PBMC compared to adult controls. Culture of PBMC with either IL-2 or IL-15 for 24 hours induced lower percentage of cells, but a normal fold increase in expression of this marker of activation on cells from patient A compared to healthy controls. In contrast, cells from patient B exhibited both reduced percentages and a reduced fold increase in CD69 expression on T cells after 24 hours incubation with IL-2 or IL-15. To further investigate the nature of this discrepancy, PBMC from patient B were cultured for 7 days in varying

concentrations of IL-2 and were shown to proliferate at the same rate as healthy adult control cells. The initial low levels of expression of CD69 on freshly isolated PBMC from the patients, together with reduced upregulation of this receptor may be explained by the general deficiency of NK associated receptor expressing cells in the periphery of these patients. As has been stated earlier, in humans the gene for CD69 is located on chromosome 12p13.1. This region is known as the natural-killer-complex and is associated with other C-type lectin genes associated with NK cells such as CD94 and NKG2 (Trowsdale *et al.* 2001). In order to throw further light on these results subsequent investigations focussed on the expression and upregulation of NK associated receptors in both healthy controls and patients in response to IL-2 and IL-15.

4.2.4. IL-2 and IL-15 induce the expansion of NKR expressing cytotoxic cells from healthy control PBMC

The selective induction of CD25 expression on cytotoxic populations, together with the stimulatory properties of IL-2 and IL-15 for different lymphocyte subpopulations prompted us to quantify the lymphocyte subpopulations present in human blood and to examine the effects of these cytokines on their proliferation, cytotoxic function and cytokine secretion *in vitro*. We found that the effects of IL-2 and IL-15 on resting human lymphocyte subpopulations were essentially the same, although at the concentrations used, IL-15 induced more marked effects on the NKR^+ populations than IL-2. Addition of either cytokine to human PBMC in the absence of prior activation resulted in selective expansions of NK cells and T cells expressing various NKRs, but not of conventional T cells or B cells. These cytokines induced moderate proliferation of NK cells and T cells expressing CD16, CD161, CD158a, CD158b, CD158e1 (KIR3DL1) and CD94, but $CD56^+$ T cells which almost invariably coexpress one or other of these NKRs, were the most responsive. These expansions were due both to proliferation, since cell numbers increased, and survival, since $CD56^+$ cells cultured in the absence of these cytokines were depleted. As reported previously (Carson *et al.* 1994; Warren *et al.* 1996; Caligiuri *et al.* 1990), only $CD56^{bright}$ NK cells proliferated

significantly in response to IL-2 or IL-15. $CD56^{bright}$ cells are absent among fresh T cells, but both IL-2 and IL-15 upregulated CD56 expression to levels comparable to those of $CD56^{bright}$ NK cells.

Our results indicate that, in the absence of antigenic stimulation, IL-2 and IL-15 selectively promote the survival and proliferation of NK cells and T cells that express CD8 and CD56. It remains to be determined whether these cytokine-induced expansions involve *de novo* induction of CD56 expression by CD56-negative cells. IL-2 or IL-15 promote the development of $CD56^+$ NK cells, but not T cells, from $CD56^-CD34^+$ hematopoietic stem cell precursors *in vitro* (Mrozek *et al.* 1996). The expression of other NKRs by $CD8^+$ T cells can be induced *de novo* or upregulated by T cell activation (Mingari *et al.* 1998b; Bertone *et al.* 1999). In mice, activation of IL-2/15Rβ^+CD8^+ T cells results in the induction of NK1.1 and Ly49 expression (Assarsson *et al.* 2000). KIR expression by human $CD8^+$ T cells can be upregulated by T cell activation (Huard *et al.* 2000c), and *de novo* expression of CD94 can be induced by IL-15 in the presence of TCR stimulation but not in the absence of TCR stimulation (Mingari *et al.* 1998b). We have found that IL-2 or IL-15, on their own, induced proliferation and upregulation (from $CD56^{dim}$ to $CD56^{bright}$) of CD56 expression by T cells in the absence of antigenic stimulation, but studies on clonal populations of resting $CD56^-$ T cells are needed to resolve whether *de novo* induction of CD56 expression by $CD56^-$ T cells occurs.

4.2.5. IL-2 and IL-15 may influence the innate and adaptive immune response through control of perforin expression by NK and T cells

Since IL-2 and IL-15 are potent stimulators of NK and $CD56^+$ T cell survival and proliferation, we have investigated whether these cytokines could activate these cells in cytotoxicity assays. Both $CD56^{dim}$ and $CD56^{bright}$ NK cells can spontaneously lyse a range of tumor cell lines *in vitro*, and IL-2 and IL-15 enhance this cytotoxicity (Lanier *et al.* 1997; Carson *et al.* 1994). $CD56^+$ T cells can be induced to kill K562 targets by preincubation with IL-2 (LAK activity) (Schmidt *et al.* 1986; Norris *et al.* 1999; Doherty *et al.* 1999). In the present study, IL-15 was found to have comparable effects to IL-2 in potently inducing cytotoxicity of K562 cells by both NK cells and $CD56^+$ T cells. Thus, IL-2 and IL-15 can similarly activate both proliferation and cytotoxicity by NK cells and $CD56^+$ T cells.

Perforin mediated lysis of target cells by $CD8^+$ T cells and NK cells has been shown to be important in the clearence of some viruses and in immune regulation through activation induced cell death of lymphocytes and lymphocyte homeostasis (Kagi *et al.* 1994b; Yasukawa *et al.* 2000; Spander *et al.* 1999; de Sainte Basile *et al.* 2001). Several cytokines including IL-2, IL-6 and IL-7 have been shown to enhance perforin expression in different systems (Smyth *et al.* 2001). In the present study we have shown that *in vitro,* continuous IL-2 or IL-15 stimulation was required for sustained perforin expression. Culture in medium without cytokines for only 24 hours resulted in demonstrable loss of perforin expression from both NK and T cells. Deprivation of IL-2/IL-15 stimulation for up to three days caused complete depletion of perforin, but addition of a single pulse of either of these cytokines for only 24 hours restored some perforin expression in T and NK cells. STAT proteins, including STAT3 and STAT5a/b, have been shown to bind to a perforin promotor region, indicating that this is the mechanism of IL-2/IL-15 induced perforin expression (Yu *et al.* 1999; Zhang *et al.* 1999). The observed requirement for cytokine stimulus for continued perforin expression *in vitro* prompts speculation on whether sustained IL-2/IL-15 stimulation is required *in vivo* for effector function of cytolytic cells.

We have demonstrated that IL-2 and IL-15 induce selective expansion of NK and T cells expressing CD8 and CD56. Perforin expressing NK cell numbers were sustained by prolonged culture with IL-2 or IL-15. The level of intracellular perforin expression was lower in these cultured cells than in freshly isolated NK cells, perhaps indicating expansion of a subpopulation of perforin^{Low} NK cells with concurrent loss of perforin^{High} NK cells. A single pulse of either of these cytokines followed by culture for 7 days elicited an increase in the numbers of perforin expressing $CD3^+CD8^+$ and $CD3^+CD56^+$ cells. The majority of freshly isolated $CD56^+$ T cells expressed perforin, and these cells showed the most significant increases in numbers in reponse to IL-2 or IL-15. Human effector cytolytic T lymphocytes (CTL) have been shown to co-express CD8, CD56 and intracellular perforin (Pittet *et al.* 2000). *In vivo* IL-2 and IL-15 may play pivotal roles in the oligoclonal expansion of CTLs in response to infection. The different sites of synthesis of these cytokines may further tune their individual contribution to the orchestration of the innate and adaptive immune responses.

4.2.6. The expansion of cytokine producing NK and $CD56^+$ T cells by IL-2 and IL-15 may influence both the innate and adaptive immune responses

NK cells and $CD56^+$ T cells can contribute to the activation and regulation of T cells, B cells and other cells of the adaptive immune system via the production of cytokines. NK cells can secrete IFN-γ, TNFα, and IL-5 while $CD56^+$ T cells can secrete IFN-γ, TNFα, IL-2 and IL-4 (Carson *et al.* 1995; Doherty *et al.* 1999; Warren *et al.* 1996). Thus, both cell types can induce Th1 (IFN-γ or TNFα) or Th2 (IL-4 or IL-5) cell activation. The selective secretion of Th1- or Th2-type cytokines by NK cells and murine NKT cells is controlled, in part, by cytokines in the microenvironment such as IL-2, IL-7, IL-10 and IL-12 (Leite-De-Moraes *et al.* 1997; Leite-De-Moraes *et al.* 1998). Our data indicate that neither IL-2 nor IL-15 induced the production of IFN-γ, IL-4 or IL-2 by resting PBMC but, as reported previously, both cytokines augmented the production of all of these cytokines in response to phorbol ester or anti-CD3 mAb

stimulation (Borger *et al.* 1999). This augmentation was not the result of an induction of cytokine production by NK cells or T cells, but was due to proliferation of $CD56^+$ T cells subpopulations that most frequently responded to these stimuli. Thus, while less than 20% of T cells cultured in medium alone could produce IFN-γ or IL-4 in response to phorbol ester stimulation or CD3-crosslinking, 50-100% of NK cells and $CD56^+$ T cells in the same cultures produced IFN-γ and/or IL-4. IL-2 or IL-15 did not enhance these proportions but induced the survival and proliferation of the most trigger-happy effectors, NK cells and $CD56^+$ T cells. Neither IL-2 nor IL-15 polarized lymphocyte activation to Th1- or Th2-type responses via the selective stimulation of IFN-γ or IL-4 production. The combination of IL-2 and IL-15 has been reported to selectively induce IL-5 production by resting human NK cells, and both cytokines can synergize with IL-12 and IL-18 in inducing IFN-γ production (Lauwerys *et al.* 2000; Khan *et al.* 1996; Carson *et al.* 1995). Therefore, IL-2 and IL-15 promote cytokine production by $CD56^+$ cells through expansion of cell numbers, but other cytokines are required to selectively activate Th1-type or Th2-type responses.

The selective responsiveness of cells to various cytokines is governed by their expression of the appropriate receptors. As previously reported, we found that the majority of NK cells express the IL-2/15Rβ chain, CD122, but not the IL-2Rα chain, CD25, suggesting that NK responses to IL-2 are mediated through a receptor complex consisting of CD122 and γc, which can participate in low-affinity binding of IL-2 (Caligiuri *et al.* 1990; Waldmann *et al.* 1999). The majority of NK cells expressed CD122. Similarly, $CD56^+$ T cells were found to express CD122 but not CD25, but interestingly, NK cells expressed approximately 10-fold higher levels of CD122 than $CD56^+$ T cells, yet $CD56^+$ T cells proliferated more vigorously than NK cells in response to IL-2 or IL-15. The CD122/γc receptor also mediates low-affinity IL-15 signaling, thus explaining the similarities of NK and $CD56^+$ T cell responses to both IL-2 and IL-15 (Carson *et al.* 1994). Resting $CD56^-$ T cells are negative for CD122 and do not respond to these cytokines. We did not carry out staining surface staining of cells for expression of IL-15Rα.

Although IL-2 and IL-15 differ in their cellular sites of synthesis and have distinct roles in lymphocyte development, we have found that these cytokines have almost identical effects on resting lymphocyte proliferation, cytotoxicity and cytokine secretion (Waldmann *et al.* 1999; Suzuki *et al.* 1997; Lodolce *et al.* 1998). IL-15 is produced by several nonlymphoid cell types and is likely to contribute to the initiation of innate immune responses via the partial activation of NK cells, which in turn regulate T cell differentiation into Th1 or Th2 cells. We and others have shown that IL-15 favours the proliferation of cells capable of both expressing and responding to IL-2 (Li *et al.* 2001). IL-2 is produced by activated T cells in the adaptive immune response and induces the proliferation of IL-2R-positive activated T cells, thus amplifying the response until a point when IL-2 induces apoptosis (Van Parijs *et al.* 1998). The partial activation of NK cells by IL-2 indicates that it also influences innate immune responses and this feedback mechanism may serve to enhance or substitute for the first-line defense mechanisms involving IL-15-secreting cells. $CD56^+$ T cells also display properties of innate lymphocytes, having invariant receptors for stimulatory ligands present on target cells and the capacity to rapidly kill tumor cells and produce cytokines (Mingari *et al.* 1996; Doherty *et al.* 2000; Bauer *et al.* 1999). Alternatively, they may constitute a subset of memory cells, expressing $CD45RA^-CD45RO^+CD28^-CD122^+$ phenotypes and homing chemokine receptors, the ability to rapidly acquire effector functions and a predominant localization in peripheral tissues (Moretta *et al.* 1997; Doherty *et al.* 1999; Campbell *et al.* 2001). A role for IL-15 in inducing proliferation of memory $CD8^+$ T cells has previously been reported and CD56 has been reported to be expressed by most IL-2-dependent antigen-specific CTL lines in long-term culture (Lanier 1998b). T cell expression of other NKRs including KIRs and CD94 can be induced by activation and KIR and CD94 induction correlates with the transition from effector to memory CTLs (Assarsson *et al.* 2000; Huard *et al.* 2000b; Young *et al.* 2001). Therefore, IL-2 and IL-15 appear to have roles in immune activation at the innate, adaptive and memory stages of an immune response.

4.2.7. IL-15 corrected deficiencies in NK and NKR$^+$ T cells in PBMC from HLH patient A but not from patient B

In order to investigate the potency of response to IL-2 and IL-15 in the HLH patients we studied the response of cells from these patients using phenotypic and functional studies. Patient A, who was receiving cyclosporin therapy, had a reduced but essentially normal response to culture of PBMC with IL-2 or IL-15. These cytokines induced expansion of CD8, CD8α, CD56, and CD94 expressing T cell as well as NK cells. These populations were shown to be deficient in freshly isolated PBMC from this patient. Isolated $CD3^+$ and $CD3^-$ cells from this patient and healthy controls were shown to spontaneously lyse K562 cell following 7 days culture with IL-15. In contrast while PBMC from patient B demonstrated proliferation in response to IL-2 and some increases in $CD3^+CD8^+$ cell numbers in response to both IL-2 and IL-15, the responses of NKR expressing cells to these cytokines was severly blunted. Following culture for 7 days with IL-15, isolated $CD3^+$ and $CD3^-$ cells from patient B were shown to lyse K562 cells, however the percentage lysis achieved was low in both populations at 30% compared to 65-80% for healthy controls. Increasing the effector to target ratio did not alter this low specific lysis.

Perforin has been shown to be the mediator of cytotoxicity against K562 cells in short term killing assays (Clement *et al.* 1990). PBMC from patient B were shown to express intracellular perforin both in freshly isolated lymphocytes and following culture for 7 days with either IL-2 or IL-15. The numbers of $CD3^+$ and $CD3^+CD8^+$ cells expressing this cytotoxic mediator were normal at time zero. Following culture with IL-2 or IL-15, intracellular perforin expression was demonstrated in both $CD3^+CD56^+$ and NK cells from patient B, however the numbers of these cell types were one-tenth of those in healthy controls. Since spontaneous cytotoxicity of isolated IL-15 expanded cells was not increased even when the effector to target ratios were increased to 250:1 it is possible that some defect in activating NKRs perhaps leading to impaired perforin delivery may also exists that this patient. Taken together the evidence would indicate that the reduced numbers of CD56 expressing T and NK cells seems to be the most

likely explanation for the reduced spontaneous lysis demonstrated in IL-15 expanded cells from patient B.

4.3. Cause and effect of NK and NKR$^+$ T cell deficiency

4.3.1. Musings on the causes of NK and NKR$^+$ T cell deficiency

In mice and men there is some evidence that NK and NKR$^+$ T cells may derive from a common progenitor cell (MacDonald *et al.* 1995; Plum *et al.* 1999) and hence the finding of a combined deficiency may be explained by a defect in the generation of these cell types. This defect may possibly arise either from failure in development due to stem cells deficiency, disruption in intra-cellular signalling, or deficiency in the cytokine mileau necessary for the development and proliferation of NK and NKR$^+$ T cells. A further possibility is that elimination of mature or precursor NK and NKR$^+$ T cells at sites of immune clearance such as the bone marrow, liver, spleen and lymph nodes could be responsible for the deficiency of these cells.

4.3.2. Is susceptibility to recurrent viral infections linked to NK and NKR$^+$ T cell deficiency?

Natural killer cells respond to viral infection through perforin-mediated killing of infected cells, proliferation, and the release of mediators including IFN-γ, TNF, GM-CSF and chemokines (Biron *et al.* 1999). Human NK deficiency and reduced NK function result in susceptibility to recurrent viral infections (Biron *et al.* 1999). Studies in mice have shown that the primary immune response to viral infection involves a massive proliferation of antigen specific CD8$^+$ T cell, with many of these expressing NK markers (Callan *et al.* 2000; Slifkta *et al.* 2000). Killing of infected targets by antigen specific CTLs is accompanied by the release of great quantities of mediators such as IFN-γ (Harty *et al.* 2000). Mouse TCRαβ invariant natural killer T (NKT) cells have been demonstrated to have an important role in the early activation of NK cells through IFN-γ production (Carnaud *et al.* 1999), in humans this function may be filled by NKR$^+$ T cells.

The elimination of some viral infections is closely related to the function of activating receptors expressed on NK and NKR^+ T cells. Engagement of activating receptors on these cells with either altered or foreign MHC-class I receptor, stress inducible MICA/B or other receptors on target cells results in the mobilization of cytotoxic mediators and killing of the target cell (Moretta *et al.* 2001; Bottino *et al.* 2000). Most humans are infected with *CMV, EBV* and other *Herpes* viruses but these infections are usually asymptomatic and exhibit latency. A single receptor type may mediate innate resistance to infections with *Herpes* family viruses. Studies in mice have shown that the lectin-like receptor Klra8 (formerly known as Ly49H) is essential in resistance to *CMV* infection (Lee *et al.* 2001). In common with human KIRs this receptor binds MHC class-IA molecules and, unlike most Klra, associates with DAP-12 resulting in activatory intracellular signalling (Lanier *et al.* 1998b). Susceptibility to *EBV* infection in XLP patients, resulting in uncontrolled IM or lymphoma, has been shown to be associated with deficiency of the activating protein SAP (SH2D1A) and to be mediated by inhibitory signalling through 2B4 (Moretta *et al.* 2001). An NK-complex locus *Rhs 1*, has been linked to control of herpes simplex infection in mice (Pereira *et al.* 2001). These studies demonstrate that failure to activate NKR expressing cells may result in susceptibility to recurrent or overwhelming infection with these commonly encountered viruses of the *Herpes* family. While deficiency of NK cells may result in susceptibility to viral infection, a concurrent deficiency of NKR^+ T cells may further contribute to this susceptibility.

4.3.3. Is the nature of the defect different in these two NKR^+ cell deficient HLH patients?

The variable response of PBMC from these patients to culture with IL-2 and IL-15 may reflect differences in the nature of the defect causing the observed deficiency of NKR expressing cells. Culture of PBMC from patient A with IL-15 induced functional NK and NKR^+ T cells. The demonstration of a normal baseline level of IL-15 mRNA together with an increased response by PBMC to LPS/IFN-γ induced mRNA expression from patient A would indicate that IL-15 mediated responses are not the primary cause of the deficiency of NKR expressing cells. It should be noted however that IL-15 is

regulated at post-transcription and secretion levels, and expression of mRNA for this cytokine does not indicate protein expression (Waldmann *et al.* 1999). The unexpected demonstration of intracellular IL-15 expression in resting lymphocytes as well as monocytes from normal controls was confirmed in a recent study (Neely *et al.* 2001). Monocytes from patient A were also shown to express IL-15 protein, ruling out simple deficiency of this cytokine as a cause of NKR^+ cell depletion in this patient. The generation of NK cells from progenitor cells in the bone marrow requires both early acting stromal factors such as ligands for c-kit and flt3 as well as IL-15. The early acting growth factors induce the development of a $CD34^{bright}$IL-2/15Rβ^+CD56^- precursor. This cell is then responsive to IL-15 for maturation into a functional $CD56^+$ NK cell (Yu *et al.* 1998). In analogy to the IL-15 induced development of functional NK and NKR^+ T cells from PBMC from patient A, NK and T cell deficiencies noted in the spleens of IL-15 KO mice can be corrected by in vivo administration of IL-15 (Kennedy *et al.* 2000). The expansion of functional NK and NKR^+ T cells from patient PBMC indicates that these cells were present either as precursors in the periphery or more likely at very low numbers. The mechansims controlling IL-15 secretion are not yet elucidated and while this cytokine is undetectable in the supernatants of stimulated cultures it has been shown to be bound to the surface of LPS-stimulated monocytes (Fehniger *et al.* 2001; Neely *et al.* 2001). It is therefore concievable that reduced or impaired IL-15 expression at sites which are important for extrathymic NKR^+ cell development could have resulted in the peripheral deficiency of NKR^+ cells and HLH symptoms in patient A. Since patient A has received a BMT with sucessful engraftment we shall not be able to further examine the nature of the defect which resulted in HLH.

PBMC from Patient B demonstrated upregulation of mRNA for IL-15 in response to stimulation. Although the level of upregulation was somewhat lower than that observed with cells from healthy controls this avenue of investigation was not pursued further. The blunted responses of NKR^+ cells to IL-2 and IL-15 observed in this patient points to a fundamental defect in either these cell types or in the cellular response mechanisms to these cytokines. The failure of expansion of cells in response to IL-2 or IL-15 observed in PBMC from patient B is unlikely to be due to low cell starting numbers, given the response of PBMC from patient A. The gross presence of the receptor components

CD25, CD122 and mRNA for IL-15Rα has been demonstrated in PBMC from this patient. The blunted responses of $CD56^+$ cells from this patient in response to cytokine stimulation could be the result of disruption of intracellular signalling through shared components of the IL-2/IL-15 receptor complexes. In this study we have demonstrated the pivotal role of these cytokines in selective expansion of the $CD56^+$ populations. We have also demonstrated the role of these cytokines in maintaining expression of the cytotoxic mediator perforin. Extra-thymic maturation may be important for NKR^+ cells and studies of cord blood cells have demonstrated that perforin expressing cells are mainly immature and mature NK cells, while perforin expressing T cells are present at low numbers (Berthou *et al.* 1995). The maturation and expansion of perforin expressing T cells may therefore be dependent on extrathymic sites such as the bone marrow and liver where $CD3^+CD56^+$ cells represent 15-55% of all T cells (Doherty *et al.* 2000).

A further possible explanation for the observed deficiencies would be a selective failure to upregulate the expression of NKRs including CD56, CD94, CD161 and KIRs. Patient B demonstrated a reduced upregulation of CD69 following 24 hours culture with IL-2. TCR-stimulation has been shown to upregulate NKR expression on $CD8^+$ T cells and together with IL-15 can induce *de novo* expression of CD94 (Huard *et al.* 2000c; Mingari *et al.* 1998b). In the present study we have shown increased percentages of control $CD3^+$ cells expressing CD56, CD69, CD94 and CD161 in response to culture for only 24 hours with IL-2 or IL-15. While this does not prove *de novo* expression in response to these cytokines it certainly indicates that both upregulation and expansion of cells contributes to the increases in cell numbers following long term culture with cytokines. Further studies would be required to demonstrate a role for IL-15 signalling in NKR upregulation.

In contrast to results seen with patient A, culture of PBMC from patient B with IL-15 did not correct the deficiencies of NK and NKR^+ populations. The numeric expansion of NKR^+ cells in response to IL-15 were much reduced, although the starting numbers were probably similar to those of patient A. The reduced proliferation of IL-2/IL-15 sensitive populations demonstrated in PBMC from patient B probably points to a

selective defect in intracellular signalling in the perforin and NKR expressing cytotoxic cell populations. Studies in KO mice would indicate that STAT 5b is a likely candidate gene however the translation from mice to man is always complex and there are some discrepancies between the cell activity in KO mice and our patient. In common with patient B, STAT5b KO mice have reduced CD122 expression as well as diminished NK proliferation and cytotoxicity in response to IL-2 and IL-15 (Imada *et al.* 1998). In addition to its effects on NK cells, deletion of the STAT 5b gene has wider growth and development implications as this protein also has a role in signal transduction by other cytokines, prolactin, growth hormones, erythropoietin and thrombopoietin (Leonard *et al.* 1998). The reduced proliferation observed in response to low doses of IL-2 in STAT 5a and STAT5b KO mice might be explained by defective upregulation of CD25 expression which has been demonstrated in lymphocytes from these mice (Imada *et al.* 1998). Since cells from patient B were shown to upregulate CD25 in response to both IL-2 and IL-15, a full deficiency of STAT5 proteins is unlikely to be the cause of NK and NKR^+ cell deficiency in this patient. Heterozygous mutation in the STAT 1 gene has been described in two patients with susceptibility to mycobacterial infection (Dupuis *et al.* 2001). While mutations in the STAT5 gene in humans has not been described previously it is possible that such a mutation may be present in patient B and his affected kindred.

Another possible candidate protein, which is important in NK cell development and function, is IL-2Rβ. Mice deficient in this receptor have abnormal development of intestinal intraepithelial lymphocytes and peripheral NK and NKR^+ T cells as well as dysregulated T cell activation and autoimmunity (Suzuki *et al.* 1997; Suzuki *et al.* 1995; Ohteki *et al.* 1997). Defective expression of this receptor was recently demonstrated in an NK cell deficient form of severe combined immunodeficiency (SCID) (Gilmour *et al.* 2001). The intracytoplasmic portion of IL-2Rβ consists of three functionally distinct regions S, A and H (Taniguchi 1995). Less global, selective defects in the development of splenic and peripheral NK cells and T cells bearing the γδ TCR have been demonstrated in mice lacking the intracytoplasmic H-region of IL-2Rβ (Fujii *et al.* 1998). IL-15 did not induce development of functional NK cells from the bone marrow of H-region KO mice, indicating the essential requirement for this region in NK cell

development. While the A region of IL-2Rβ was not essential for development of NK cells, it was shown to be required for the induction of cytotoxic function (Fujii *et al.* 1998). The importance of the A and H intracytoplasmic regions of IL-2Rβ in NK development and cytotoxicity probably resides in their interaction with the signal transduction machinery of the cell. Region A has been shown to be the site of interaction with $p56^{lck}$, PI3-kinase and Shc while the H region is essential for recruitment and activation of STAT3/STAT5 (Taniguchi *et al.* 1995; Ellery *et al.* 2000). The essential role of IL-2Rβ-A region for induction of NK cytotoxic function probably resides in the association of PI3-kinase with this region. Effective movement of NK derived perforin-granzyme B towards target cells is controlled by PI3-kinase (Jiang *et al.* 2000). PI3-kinase also plays a more general role in the control of growth through regulation of the anti-apoptotic pathways (Ellery *et al.* 2002). STAT5a/b has been shown to regulate NK cytotoxicity through interaction with the human perforin promotor (Yu *et al.* 1999). Gross expression of IL-2Rβ has been demonstrated in PBMC from patient B and his NK deficient sibling using both flow-cytometry and immunoblotting.

4.3.4. Conclusion

These two young patients were shown to have a phenotypic and functional deficiency of natural killer receptor expressing cells in their peripheral blood. There was no increase in numbers of these cells over the period of this study. The role of IL-2 and IL-15 in the selective expansion of cytotoxic and cytokine expressing NK and NKR^+ T cells has been elucidated using PBMC from healthy controls. The phenotypic and functional deficiencies of NKR^+ cells could be corrected in part by IL-15 in one of these HLH patients but not in the other, suggesting that the cause of selective cell deficiency is different in each of these patients.

4.3.5. Future work

Although patient A has received a bone marrow transplant further work may be carried out on stored mRNA samples. Sequencing of the IL-15 gene would be useful as this cytokine is controlled post-transcriptionally and while the patient has been shown to express mRNA for IL-15 definite secretion of this protein has not been demonstrated. In order to fully elucidate the nature of the defect in patient B and his NK deficient relatives further work is required to demonstrate whether the IL-2Rβ intracellular subunits and downstream signalling molecules are present and fully functional. It would be interesting to study the phosphorylation of STAT5a and b in response to IL-2 or IL-15 binding in PBMC and in isolated NK and NKR^+ cells from patient B and his relatives. The functioning of the anti-apoptotic pathway, mediated by *lck* interacting with phosphatidylinositol 3-kinase, would merit investigation. Sequencing of IL-2Rβ gene would also yield valuable information on the possible role of this protein in the observed failure of NKR^+ cells to proliferate in response to IL-2 and IL-15.

5. Appendices

Appendix I

5.1 Tissue culture

5.1.1. Ethidium Bromide Acridine Orange (EB/AO)

Stock solution

4 mls of 0.4% Ethidium Bromide stock solution (Sigma, UK)

10 mls of 0.1% 3,6, bis (Dimethyl amino acridine) (Acridine Orange) stock solution (Sigma, UK) 1 litre of normal saline

Store in the dark at 4°C

5.1.2. Medium

RPMI 1640 500ml (GIBCO BRL Paisley, UK) with pooled, heat inactivated, normal human serum made up to 10% with 1 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 mg/ml (GIBCO BRL Paisley, UK).

5.1.3. Tritiated (^{3}H) thymidine

0.1 ml of tritiated (^{3}H) thymidine (1 mCi/ml, Life Sciences) was diluted in 3.3 mls of Hanks Balanced Salt Solution (HBSS, GIBCO BRL Paisley, UK) to give a final concentration of 30 μCi/ml, 10 μl of this stock was added to each well containing 300 μl.

5.1. 4. Cell count

Cell count was carried out on a haemocytometer using a 1 in 20 dilution of cells in ethidium bromide / acridine orange solution (EBAO). Cells fluorscing green were taken as viable for the count and the volume of medium was adjusted to achieve the required concentration of PBMC.

5.2 Reagents for intracellular staining

5.2.1. PBS

Phosphate buffered saline (PBS) 0.1 mol/L pH 7.3 was made up using tablets from Oxoid, Hampshire, UK. Each tablet was sufficient to prepare 200mls of PBS.

5.2.2. SSPE buffer

Saline Sodium Phosphate EDTA (SSPE) is a 20X concentrate of containing 0.2 M phosphate buffer pH 7.4, 2.98 M NaCl and 0.02 M EDTA supplied by Sigma-Aldrich, Steinheim, Germany

5.2.3. PBS-FCS

5% foetal calf serum (FCS) in PBS was made up by adding 5 mls of FCS (GIBCO BRL Paisley, UK) to 95 mls of PBS

5.2.4. PBS-BSA-azide buffer

2% bovine serum albumin (BSA) in PBS was made up by adding 6.7 mls of 30 % BSA (Diamed AG, Cressier Sur Morat, Switzerland) to 93.3 mls of PBS with 0.01 M sodium azide (BDH, Poole, UK) added.

5.2.5. 2% paraformaldehyde in PBS

20g of paraformaldehyde (BDH, Poole, UK) was added to 1 liter of PBS. Solution was placed at 56°C overnight. Solution was allowed to cool then filtered and stored at 4°C

5.2.6. Permeabilizing buffer

For 0.05% saponin 0.25g of saponin (Sigma, UK) was added to 500mls of PBS or SSPE buffer with 5% foetal calf serum (FCS, GIBCO BRL) solution was mixed well.
Permeabilizing buffer must be prepared fresh daily.

Appendix II

5.3. Reagents for the isolation of RNA and Reverse Transcription

5.3.1. Denaturing solution

4M guanidium thiocyanate	(Sigma-Aldrich, Irvine, UK)
25 mM sodium citrate, pH 7	(Sigma-Aldrich, Irvine, UK)
0.1 M 2-mercapto ethanol (ME)	(Sigma-Aldrich, Irvine, UK)
0.5% *N*-lauroylsarcosine (Sarkosyl)	(Sigma-Aldrich, Irvine, UK)

A stock solution was prepared by dissolving 250g guanidium thiocyanate in a solution of 293 mls H_20, 17.6 mls of 0.75 M sodium citrate at pH 7, and 26.4 ml of 10% Sarkosyl at 60°C with stirring. This stock was stored at RT for 3 months.

5.3.2. Working solution

35 µl of 2-mercapto ethanol was added to 5 mls of denaturing solution, prepared fresh daily.

5.3. 3 M sodium acetate

16.42 g of anhydrous sodium acetate (BDH, Poole, UK) was added to 40 mls of H_20 (RNase free, Sigma-Aldrich, Taufkirchen, Germany) with 35 mls of glacial acetic acid (BDH, Poole, UK). The solution was adjusted to pH 4 with glacial acetic acid. Final volume was made up to 100 mls using H_20 (RNase free).

5.3.4. Water-saturated phenol

100 g of phenol crystals (BDH, Poole, UK) were dissolved in H_20 at 60°C. The upper aqueous phase was aspirated and the phenol stored under 20 mls of H_20 at 4°C for 1 month. Prepared water saturated phenol from BDH, Poole, UK, was also used.

5.3.5. Chloroform/ isoamylalcohol

49:1 (vol/vol) chloroform / isoamyl alcohol (Sigma-Aldrich, Irvine, UK)

5.3.6. Ethanol

75 % ethanol (BDH, Poole, UK) prepared with RNase free H_20 (Sigma-Aldrich, Taufkirchen, Germany)

5.3.7. 6x Loading Dye

(Promega, Madison, Wi, USA)

10% Ficoll 400

10 mM Tris HCL (pH 7.5)

50 mM EDTA

0.25% bromophenol blue

0.25% xylene cyanol FF

0.4% orange G

5.4. Mini-Macs Buffer

5.4.1. Preparation of Mini-Macs Buffer

PBS supplemented with 0.5% BSA and 5mM EDTA pH 7.2

Ethylenediametetra-acetic acid disodium salt (EDTA, BDH, Poole, UK) will only go into solution at pH 8 so a stock of 0.5M EDTA in water pH 8 should be prepared and autoclaved for subsequent preparation of buffer. The EDTA helps to prevent aggregation of the cells.

PBS was prepared by adding ten Oxoid PBS tablets to 194.7 mls distilled water, 2mls of EDTA 0.5M stock solution was added and the pH adjusted the to pH 7.2. The PBS/EDTA was autoclaved to sterilise and degas. 3.3 ml of filtered bovine serum albumin (30%) (Diamed AG, Cressier Sur Morat, Switzerland) was added to the PBS/EDTA and mixed gently by on a shaking incubator for 1 hour. Aliquots of complete mini-macs buffer were stored at $4^{o}C$.

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