Immunomodulation of the Cellular Immune Response by Human Cytomegalovirus

A thesis submitted in candidature for the degree of

DOCTOR OF PHILOSOPHY

by

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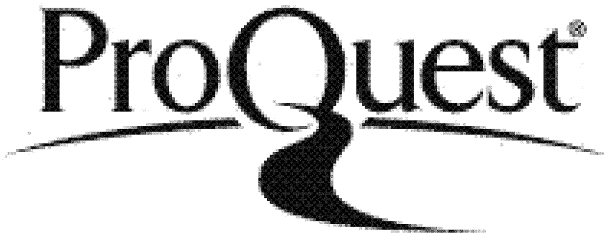

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SUMMARY

HCMV is a ubiquitious β herpesvirus that usually causes asymptomatic primary infection. Individuals infected with HCMV mount a strong immune response that suppresses persistent viral replication and is paramount for the prevention of HCMV disease. HCMV encodes a large number of immunomodulatory functions that modulate both the innate and adaptive arms of the immune system. The project undertaken in this thesis was to investigate and characterise immunomodulatory functions encoded by HCMV.

A novel and highly efficient method of Natural Killer (NK) cell cloning was developed to investigate modulation of the NK response by HCMV. This technology was utilised to further investigate the finding that CD94/NKG2A^{+} NK cells are inhibited by UL40-stabilised HLA-E. Analyses of polyclonal NK cell responses and NK clones showed that more CD94^{lo} than CD94^{hi} NK cells were activated by HCMV strain AD169 in comparison to uninfected targets. Flow cytometry showed that there was an increase in the frequency of NK cells expressing the activatory receptor CD94/NKG2C and a decrease in the frequency of cells expressing the inhibitory receptor CD94/NKG2A in HCMV seropositive individuals. The response of NK clones expressing CD94/NKG2A or CD94/NKG2C to targets infected with strain AD169 or RAdUL40 indicated that some CD94/NKG2A clones can be inhibited by gpUL40 while some CD94/NKG2C clones can be activated by gpUL40. Comparative analysis of NK responses to HCMV strain Towne, indicated that strain Towne encoded a novel NK modulatory function that differentially targeted the CD94^{lo} and CD94^{hi} NK cell subset in certain individuals.

HCMV strain Toledo is known to encode a powerful inhibitor of NK function. Analysis of polyclonal NK cell responses mapped this inhibitory function to gpUL141. In depth analysis of 98 NK clones demonstrated that gpUL141 inhibited a large proportion of NK cells and this was independent of CD94 expression.

The initial aim of this study was to characterise the immunomodulatory function of pp65, an HCMV protein that has previously been shown by others to abrogate recognition of HCMV infected cells by HCMV-IE1 specific CTL. Fluorescence microscopy showed that pp65 did not alter the localisation of IE1 and a yeast two-hybrid assay indicated that there was no direct interaction between these 2 proteins. A functional assay using IE1 specific CTL was not performed because sufficient numbers of peptide specific CTL could not be cultured. Nevertheless, this study has contributed to the characterisation of powerful HCMV immunomodulatory functions that selectively target NK cell subsets and are sufficient to alter frequencies of cells in the innate immune system. The results presented here enhance our understanding of HCMV pathogenesis, the regulation of NK cell function and the biology of the innate immune system.

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SUPPLIERS AND COMPANY ADDRESSES

Abbott Laboratories, Maidenhead, Berkshire, UK

Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK.

Applied Biosystems, Foster City, CA, USA.

ATCC Teddington, Middlesex,UK

Basic Chemical Company Basic Chemical Company, High Wycombe, UK.

Beckman Instruments Ltd., High Wycombe, Bucks, UK.

BD Pharmingen UK Ltd., Cowley, Oxford, UK.

Biochrom AG, Berlin, Germany.

Caltag Ltd., Silverstone, Towcester, UK.

CellGenix Technologie Transfer, Frieburg, Germany.

Cetus Corp., Emeryville, CA, USA.

Clontech laboratories Inc., Basingstoke, Hampshire, UK.

CP Pharmaceuticals Ltd., Wrexham, Clwyd, UK.

Cytomation Inc., Fort Collins, CO, USA.

DakoCytomation Ltd., Ely, Cambridge, UK.

Dynal Biotech UK, Bromborough, Wirral, UK.

Eurogentec Ltd., Hythe, Southampton, UK.

Fisher Scientific Ltd., Loughborough, Leics., UK.

GraphPad Software Inc., San Diego, CA, USA.

Greiner, Stonehouse, Gloucestershire, UK.

Hamamatsu, Hamamatsu City, Japan

Hybaid Ltd., Ashford, Middlesex, UK.

Hycor Biomedical Inc., Penicuik, Edinburgh, UK.

Invitrogen, Groningen, The Netherlands.

Improvision Ltd., Coventry, Warwickshire, UK.

Immunotech SA. Marseille, France.

Leica Camera AG, Solms,Germany.

Merck Pharmaceuticals, West Drayton, UK.

Miltenyi Biotec Ltd., Bisley, Surrey, UK.

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Qiagen, West Sussex, UK

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Santa Cruz Autogen Bioclear UK Ltd., Calne, Wiltshire, UK.

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Skatron AS Libergyn, Norway.

Sorvall Kendro Lab. Products Ltd., Bishops Stortford, Herts., UK.

Vector Laboratories, California, USA.

ABBREVIATIONS

°C	Degrees centigrade
^{51}Cr	Sodium Dichromate (radioactive)
μC	microCurie
μg	microgram
μl	microlitre
μM	micromolar
μm	micromoles

A

A_{420}	Absorbance at 420 nm
Aa	Amino acids
ABS	AB Serum
ADCC	Antibody-Dependent Cellular Cytotoxicity
Ad	Adenovirus
AIDS	Acquired immune deficiency syndrome
APC	Allophycocyanin
APS	Ammonium per sulphate
ANT	Adenine nucleotide translocator
ATCC	American Type Culture Collection
ATP	Adenosine Tri-phosphate
ATPase	Adenosine Tri-phosphatase

B

β_2M	β_2-microglobulin
BAC	Bacterial artificial chromosome
BCL	EBV transformed B-cell line
BDCRB	2-bromo-5,6-dichloro-1-(β-$_D$-ribofuranosyl)benzimidazole
BMT	Bone marrow transplant
Bp	Base pairs
BSA	Bovine serum albumin

C

CD	Cluster determinant
CID	Cytomegalic Inclusion Disease
CLTs	CMV latency associated transcripts
CPE	Cytopathic Effect
CPM	Counts Per Minute
CRACC	CD2-Like Receptor Activating Cytotoxic Cells
CREB	cAMP response element binding protein
crs	*cis* repression signal
CTL	Cytotoxic T-lymphocyte
CVID	Common Variable Immunodeficiency

D

DAPI	2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride

dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphospate
dH_20	Distilled water
DMEM	Dulbeccos modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNA-AD	DNA-activation domain
DNA-BD	DNA-binding domain
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DO	Drop out

E

E	Early (gene expression of HCMV)
EAT-2	EWS activated transcript-2
EBV	Epstein-Barr virus
ECACC	European collection of Animal Cell Cultures
E.coli	Escherichia coli
EDID	Ectodermal dysplasia with immunodeficiency
EDTA	Ethylene diamine tetraacetic acid
EGFR	Epidermal growth factor receptor
Ep-CAM	Epithelial cellular adhesion molecule
ER	Endoplasmic Reticulum
E:T	Effector:target

F

FACS	Fluorescence Activated Cell Sorting
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
FLICE	Caspase 8
FLIPs	FLICE-ligand inhibitory proteins
FLT3	FMS-Related Tyrosine Kinase 3
FLT3 Ligand	FMS-Related Tyrosine Kinase 3 Ligand
FSC	Forward Scatter

G

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCV	gancyclovir
GFP	Green fluorescent protein
gB	Glycoprotein B
gH	Glycoprotein H
gL	Glycoprotein L
Gln	Glutamate
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O

GPCR	G-protein-coupled 7-TM receptor
H	
HA	Haemagluttinin
HAART	Highly Active Antiviral Therapy
HCMV	Human Cytomegalovirus
HDAC3	Histone Deacetylase 3
HF	Human Fibroblasts
HFE	Hereditary hemochromatosis protein precursor (HLA-H)
HFFF	Human Foetal Foreskin Fibroblast
HHV-5	Human Herpesvirus 5
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
HSC	Haematopoeitic Stem Cell
Hsp70	Heat shock protein 70
I	
ICAM-1	Intercellular Adhesion Molecule-1
Ig	Immunoglobulin
IE	Immediate Early (gene expression in HCMV)
IE1	Immediate Early 1 (gene of HCMV)
IE2	Immediate Early 2 (gene of HCMV)
IFNα	Interferon alpha
IFNγ	Interferon gamma
IL2	Interleukin 2
IL7	Interleukin 7
IL10	Interleukin 10
IL15	Interleukin 15
IL21	Interleukin 21
IR	Internal Inverted Repeats
IRp60	Inhibitory receptor protein 60
ILT	Immunoglobulin-like Transcript
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
ITSM	Immunreceptor Tyrosine-based switch motif
IU	International Units
K	
kb	Kilobases
kDa	Kilodaltons
KIR	Killer cell Immunoglobulin-like receptor
L	
L	Late (gene expression in HCMV)
LAIR	Leukocyte-associated immunoglobulin-like receptor
LB	Lauria bertani
Leu	Leucine

LFA-1	Lymphocyte Function Associated antigen-1
LIR	Leukocyte Immunoglobulin-like receptor
LRC	Leukocyte Receptor Cluster
LTR	Long terminal repeat

M

mAb	Monoclonal Antibody
MCMV	Murine cytomegalovirus
mg	Milligram
ml	Millilitre
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
MICA	Major Histocompatibility Complex Class I Chain Related Gene A
MICB	Major Histocompatibility Complex Class I Chain Related Gene B
MIE	Major immediate early
MLR	Mixed Lymphocyte Reaction
MOI	Multiplicity of infection
MPC	Magnetic Particle Concentrator

N

$Na_2{}^{51}CrO_4$	Sodium dichromate (radioactive)
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride
ND	Not Done
NHS	National health service
NIEPs	Non-infectious enveloped particles
NK	Natural killer
NKC	NK gene complex (murine)
NKc	NK clone
NFAT	Nuclear factor of activated T cells
nm	Nanometres
NP40	Nonidet-P40
NS	Not Significant
NTBA	NK-, T-, and B-Cell Antigen

O

OD	Optical density
ONPG	o-Nitrophenyl-β-D-Galactopyranoside
ORF	Open reading frame
*Ori*Lyt	Viral origin of replication

P

p.i.	Post infection
PACS-1	Phosphofurin acidic cluster sorting protein I
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PBS-FCS	Phosphate buffered saline with 1% FCS
PBS(T)	Phosphate buffered saline (tween)
pCL1	15.3 kb positive control plasmid, encodes full length, wild type GAL4 protein (Yeast two hybrid).
PCR	Polymerase chain reaction
PE	Phycoerythrin
PE-Cy5.5	Phycoerythrin-Cy5.5
PE-Cy7	Phycoerythrin-Cy7
PEG	Polyethylene glycol
PEG/LiAc	Polyethylene glycol/lithium acetate solution
pfu	Plaque forming units
PG	Prostaglandin
pGAD424	6.6 kb cloning vector, used to generate fusions of a known protein with the GAL4-AD (Yeast two hybrid).
pGBT9	5.4 kb cloning vector, used to generate fusions of the bait protein with the GAL4 DNA-BD (Yeast two hybrid).
PHA	Phyto haemagglutinin
PHLS	Public health laboratories
Plk	Polo-like kinase
PML	Promyelocytic leukaemia
PMT	Photomultiplier Tube
POD	Promyelocytic Oncogenic Domain
Poly I:C	Polyinosinic acid-polycytidylic acid
pTD1	15 kb postive control plasmid used with pVA3. Encodes an AD/SV40 large T antigen fusion protein in pGAD3F
PTPC	Permeability Transition Pore complex
pVA3	6.4 kb positive control plasmid used with pTD1. Encodes a DNA-BD/murine 53 fusion protein in pGAD3F

R

RAd	Recombinant adenovirus
RAET	Related retinoic acid early transcript
RANTES	Regulated Upon Activation, Normal T Cell Expressed and Secreted
RNA	Ribonucleic Acid
RNase	Ribonuclease
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (media)
RT-PCR	Reverse transcription PCR

S

SAP	SLAM Associated Protein
SCGM	Stem Cell Growth Media
SDS	Sodium dodecyl sulphate
Ser	Serine
SH2D1A	SH2 Domain Protein 1A
SIGLEC	Sialic Acid-Binding Immunoglobulin-Like Lectin

SLAM	Signaling Lymphocyte Activation Molecule
SMC	smooth muscle cell
SSC	Side Scatter
SUMO	Small ubiquitin-like modifier

T

t	Elapsed time (in minutes) of incubation
TAP	Transporter associated with antigen processing
TBE	Tris borate EDTA
TCID50	Tissue culture infectious dose 50%
TcR	T Cell Receptor
TE	Tris-EDTA buffer
TEMED	N N N' N'-tetraethylenediamine
TGN	Trans Golgi Network
Thr	Threonine
TM	Transmembrane
TNF(R)	Tumour necrosis factor (receptor)
TR	Terminal Inverted Repeats
Tris	Tris (hydroxymethyl)methylamine
Trp	Tryptophan
Tyr	Tyrosine

U

UI	Uninfected
UL	Unique Long
ULBP	UL16-Binding Protein
TLR2	Toll-like Receptor-2
US	Unique Short
UV	Ultraviolet

V

V	0.1 ml x 5 (the concentration factor)
Vac	Vaccinia virus
vCIA	Viral caspase inhibitor of activation
VCXC1	Viral CXC chemokine 1
vIL10	Viral IL10
vMIA	Viral mitochondrial inhibitory of activation
v/v	Volume to volume ratio

W,X,Y,Z

w/v	Weight to volume ratio
x-gal	5-bromo,4-chloro-3-indolyl-β-D-galactopyranosidase
XLP	X-linked Lymphoproliferative Syndrome

INTRODUCTION

1 INTRODUCTION

1.1 THE HERPESVIRUS FAMILY

The Herpesviridae are a large family of enveloped double-stranded DNA viruses. To date, more than 150 members of the family have been identified and these have been classified into 3 subfamilies, the alpha (α)-, beta (β)- and gamma (γ)-herpesviruses (Roizman, 2001). Herpesviruses have been isolated from a wide range of species including mammals, birds and fish. The mammalian and avian herpesviruses have been estimated to have evolved from a common ancestor between 180 to 200 million years ago and evolution of the mammalian herpesviruses has been placed in a closely equivalent timeframe to the mammalian radiation, 60 to 80 million years ago. A number of cellular homologues have been identified in the Herpesvirus genome indicating that a major factor in the evolution of herpesviridae has been co-speciation with the host (McGeoch *et al.*, 1995).

Alphaherpesvirinae are neurotropic, have a relatively short reproductive cycle (~18 hours), a variable host range and the ability to cause efficient destruction of infected cells. In contrast, the gammaherpesvirinae have a limited host range, and are limited to the family or order of the natural host. Gammaherpesviruses are lymphotrophic and specific for either T or B lymphocytes. Members of the betaherpesvirinae are lymphotrophic and have a long reproductive cycle and persist for the lifetime of the host. Fatal infections would be counterproductive for viral transmission thus, the long term survival of the host, the ability of the virus to be transmitted and modulation of the immune system permits the virus to persist in populations over time. Human Cytomegalovirus (HCMV), and Human herpesvirus 6A, 6B and 7 (HHV-6A, HHV-6B and HHV-7) are members of the *Betaherpesvirinae* and like other herpesviruses, persist for the lifetime of their host. HCMV, formally designated HHV-5, is the prototypical betaherpesvirus and possesses the largest genome of any known herpesvirus (Roizman, 2001).

To date, 8 human herpesviruses have been identified, with representatives from all 3 subfamilies (Figure 1.1).

1.2 Discovery and Isolation of HCMV

In 1881, Ribbert *et al* (1904) noticed "protozoan like cells" in kidney sections of a still born infant, although he did not attribute the alteration in cellular morphology to a viral infection (Ribbert, 1904). Subsequently, the protozoan origin was dismissed and Goodpasture and Talbot suggested a viral aetiology (Goodpasture and Talbot, 1921). However, it wasn't until 1950's, that the human form of the virus, including the commonly used laboratory strain AD169, was first isolated (Rowe *et al.*, 1956, Smith, 1954, Smith, 1956, Weller *et al.*, 1957). The term "cytomegalovirus" was coined by Weller *et al* (1960) who also defined the characteristic cellular changes observed by HCMV infection (Weller *et al.*, 1960).

1.3 Virus Structure

The virion of HCMV is composed of 4 distinct structural components, the central core, capsid, tegument and the envelope and is typical of other members of the herpesvirus family (Figure 1.2).

The central core of the virion contains a 236 kb double stranded, linear DNA genome surrounded by a 100-nm diameter icosahedral nucleocapsid which in turn is surrounded by a protein-rich layer known as the tegument or matrix (Dolan *et al.*, 2004). The tegument is enclosed by a lipid bilayer (envelope), which contains a large number of viral glycoproteins. Seventy one HCMV-encoded proteins and 70 host cellular proteins have been associated with the virion (Varnum *et al.*, 2004). The tegument is estimated to comprise up to 50% of the protein content within the virion, with the remaining protein coming from the capsid (30%), envelope (13%) and 7% remaining undefined (Varnum *et al.*, 2004).

Figure 1.1 **Genetic relationship of the human herpesviruses.**

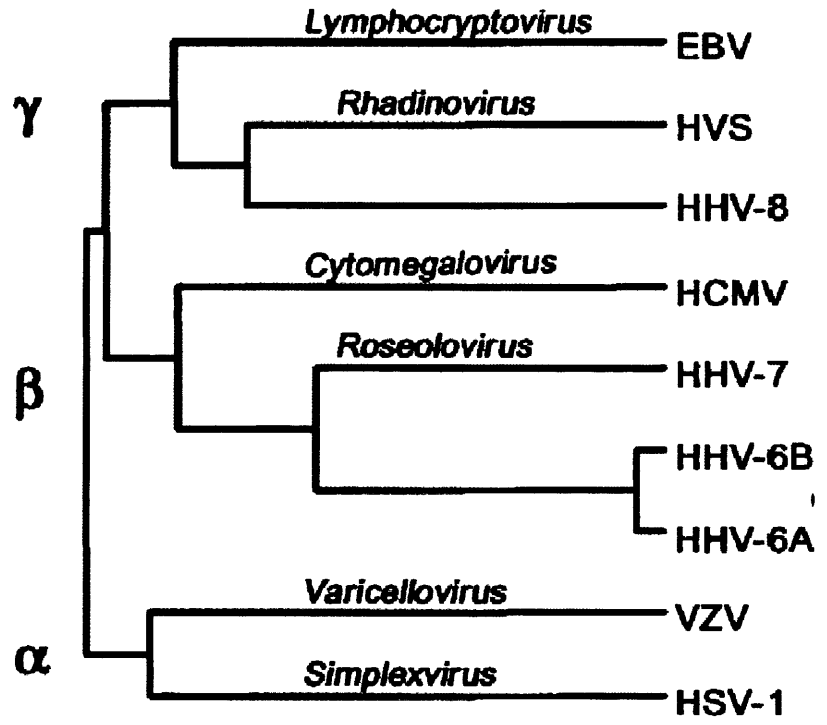

Figure 1.1. Genetic relationship of the human herpesviruses Vertical branch lengths are proportional to the distance between the sequences. α-, β- and γ-herpesvirus subfamilies are indicated. Relationships based on gH sequences, taken from Braun *et al.* (1997).

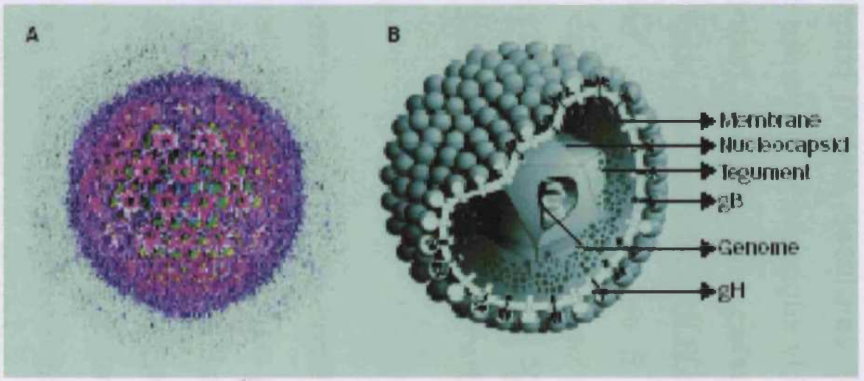

Figure 1.2 Human Cytomegalovirus. (A) Shaded surface representation of 18 x 10^{-3} m resolution 3-dimensional reconstruction of icosahedraly ordered portion of intact HCMV particle as viewed along a 3-fold symmetry axis. (B) Virtual 3-dimensional model, showing various components of HCMV. Taken from (Gandhi & Khanna, 2004).

1.3.1 Capsid

The capsid is icosahedral in structure consisting of 12 pentons, 150 hexons, and 320 triplexes (Chen *et al.*, 1999). The protein content of the capsid includes at least 5 proteins that are able to self-assemble into the icosahedral capsid structure. The major capsid protein pUL86, is the most abundant protein component of the capsid (960 copies) and forms the penton and hexons of the icosahedral capsid. The minor capsid proteins, pUL85 and pUL46 form the triplexes and are located between the pentons and hexons. The smallest capsid protein decorates the hexons of the capsid and is essential for the assembly of infectious virions (Chen *et al.*, 1999). HCMV-infected cells contain 3 capsid forms called A-, B- and C-capsids (Irmiere & Gibson, 1985). Type A capsids lack UL80 and are unable to stably package the viral genome. In contrast, type B capsids contain UL80 and are predominantly localised in the nucleus as precursors of the mature capsids (Mocarski, 2001). Type C capsids are fully mature nucleocapsids, which form when type B capsids become filled with DNA.

1.3.2 Tegument

The virion tegument is an amorphous coating of the capsid that maintains the association between the virion envelope and the capsid. The tegument is composed of approximately 25 proteins that are expressed in the cytoplasm (including ppUL32, pp150, ppUL99 and pp28) or in the nucleus (ppUL69) and appear to be added sequentially (Sanchez *et al.*, 2000). pp150 and pp65 are the most abundant tegument proteins, with pp150 estimated to constitute 20% of the virion mass (Gibson, 1996, Irmiere & Gibson, 1983, Jahn *et al.*, 1987). Whilst deletion of UL83 results in a growth modified viral phenotype, pp65 was shown to be dispensable for growth *in vitro* (Klages *et al.*, 1989, Schmolke *et al.*, 1995b). The tegument proteins pp71 and ppUL69 have been shown to regulate viral gene expression and modify host cell responses to HCMV infection, suggesting that many of the tegument proteins have functions unrelated to the structural role of the virion tegument.

1.3.3 Envelope Glycoproteins

The virion envelope is complex and many of the proteins comprising this structure remain undefined. Recent studies have suggested that the glycoproteins in HCMV are present in the following order of abundance; gM/gN>gB>gH/gL/gO (Britt & Boppana, 2004). These glycoproteins have been shown to exist as 3 highly conserved disulphide-linked complexes within the virion, gCI (gB), gCII (gM/gN), and gCIII (gH, gL, gO) (Gretch *et al.*, 1988).

gB, is a type 1 integral membrane protein and the mature form is extensively glycosylated. Many essential roles in HCMV infection including attachment to the extracellular heparin sulphate for initiation of infection, fusion of adjacent cells and transmission of infection from cell-to-cell involve gB (Compton *et al.*, 1993, Navarro *et al.*, 1993, Tugizov *et al.*, 1994). gB and gH are both targets for the humoral immune response. The abundance of gB in the virion envelop has led to it being chosen for a recombinant subunit vaccine (Britt *et al.*, 1990, Gonczol & Plotkin, 2001). Interestingly, data suggests that HCMV can vary the content of gH expression in the virion envelope, thereby avoiding virus neutralisation by the humoral immune response (Li *et al.*, 1995). gM and gN but not gO are required for production of infectious virus *in vitro* (Hobom *et al.*, 2000). In addition to these glycoproteins, HCMV encodes a substantial number of glycoproteins that do not have obvious homologous proteins in other herpesviruses eg gpUL132, gpTRL10, gpTRL11, and possibly gpULTRL12 (Britt & Boppana, 2004).

1.3.4 Host Proteins

The number of host proteins associated with the HCMV virion is difficult to determine, and the proteins identified are largely dependent on the method used. Additionally, it has been difficult to identify which proteins are essential for the virion because as virions are purified they lose their infectivity. However, a recent study has identified 71 host cellular proteins as being associated with the HCMV virion. These include proteins involved in cell

structure (α-actinin, vimentin), protein transport (clathrin, polyubiquitin B), transcription-translation (elongation factor 2) and signaling (α_1-casein kinase 2) (Varnum *et al.*, 2004).

1.3.5 Other Virion Constituents

The core of the HCMV virion comprises viral DNA and 2 polyamines (spermidine and spermine) (Gibson *et al.*, 1984). Elimination of the pool of spermidine by drug treatment has been shown to interfere with HCMV replication, resulting in lower viral titres and incomplete capsid forms, suggesting that spermidine and spermine are involved in DNA packaging or capsid envelopment (Gibson *et al.*, 1984).

Although HCMV is classically considered a DNA virus, 7 viral RNA transcripts have been detected within the virion of the laboratory strain of HCMV AD169 (Bresnahan & Shenk, 2000, Greijer *et al.*, 2000). The product of the virion RNA encoded by ORF UL21.5, pUL21.5 has recently been shown to function by blocking RANTES (regulated upon activation, normal T cell expressed and secreted), suggesting that this protein can modulate the host antiviral response even before a newly infecting viral genome has become transcriptionally active (Wang *et al.*, 2004). In addition to these viral RNAs, 2 cell RNAs, those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U1A, were found in the HCMV virion (Greijer *et al.*, 2000). Additionally, 2 viral RNAs were identified within the HCMV origin of replication, *ori*Lyt. One of these viral RNAs, termed vRNA-2 was shown to form a stable RNA-DNA hybrid structure in an *ori*Lyt-containing plasmid (Prichard *et al.*, 1998).

1.4 Dense Bodies and Non-Infectious Particles

HCMV infection of cells in culture generates 3 different types of particles, mature infectious virions (described above), dense bodies and non-infectious enveloped particles (NIEPs) (Irmiere & Gibson, 1983).

Dense bodies are uniquely characteristic of HCMV infection. They are non-replicating enveloped particles which lack DNA (Irmiere & Gibson, 1983). Recently, 31 viral proteins including 5 capsid proteins, 9 tegument proteins, 4 glycoproteins and 4 proteins involved in virus transcription and replication have been identified in dense bodies (Varnum *et al.*, 2004). Only a small number of host cell proteins were identified associated with dense bodies and these included GAPDH, annexin A2, β-actinin and the heat shock 70-kDa protein. UL83 (pp65) was shown to be significantly increased in dense bodies compared to virions (Varnum *et al.*, 2004).

NIEPs have been identified in all HCMV strains examined. They are morphologically distinguishable from the virion and dense bodies only by their core structure, and are composed of the same viral proteins as infectious virions but lack DNA (Irmiere & Gibson, 1983). Protein content of the NIEPs is more complex than dense bodies, and NIEPs are distinguished by the presence of a 35 kDa (35K) protein. This protein is only produced in infected cells, is phosphorylated and partitions with the nuclear fraction.

1.5 Viral Genome

The HCMV genome is among the largest of any characterised herpesvirus and is among the largest and most complex of animal virus genomes sequenced to date. It is organised into 2 covalently linked unique regions designated U_S and U_L, which are flanked by terminal (TR) and internal (IR) inverted repeats (TR_L and IR_L, TR_S and IR_S) yielding the overall genome configuration $TR_L - U_L - IR_L - IR_S - U_S - TR_S$. The $TR_L - U_L - IR_L$ sequence is known as the b sequence and the $IR_S - U_S - TR_S$ as the c sequence (Figure 1.3) (Davison *et al.*, 2003; Mocarski *et al.*, 2001).

Strain AD169 was sequenced in 1990 and was reported to be 229,354 bp encoding 208 ORFs (Chee, *et al.*, 1990). Comparison of the laboratory strains AD169 and Towne and the lesser passaged strain Toledo has shown that *in vitro* tissue culture has resulted in large genetic differences in the

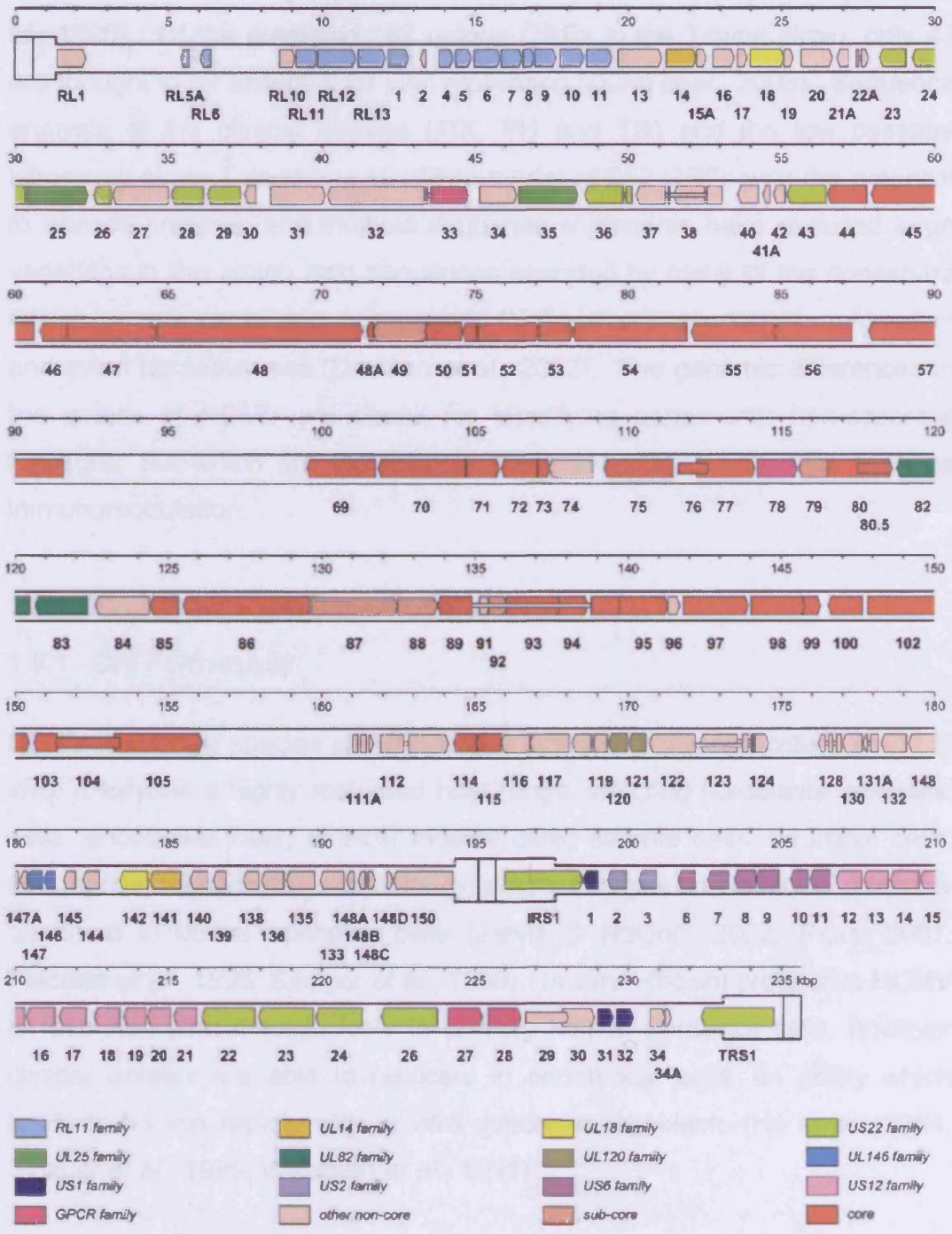

Figure 1.3 Consensus genetic Map of wild-type HCMV based on the strain Merlin Genome. Protein coding regions are indicated by coloured arrows, introns are shown as narrow white bars. Colours differentiate between genes on the basis of conservation across α-, β- and γ- herpesvirinae (core genes) or between the β- and γ-herpesvirinae (sub core genes), with subsets of the remaining non core genes grouped into gene families. Taken from (Dolan *et al.*, 2004).

laboratory strains of HCMV, the most notable being a 15-kb and a 13-kb deletion in the U_L/b' region in strains AD169 and Towne, respectively (Cha *et al.*, 1996). Of the predicted 162 unique ORFs in the Towne strain, only 45 are thought to be essential for viral replication (Dunn *et al.*, 2003). Sequence analysis of the clinical isolates (FIX, PH and TR) and the low passage laboratory strain Toledo has identified a total of 252 ORFs with the potential to encode proteins, and multiple sequence alignments have revealed large variations in the amino acid sequences encoded by many of the conserved ORFs. A core set of approximately 40 ORFs is conserved in all mammalian and avian herpesviruses (Davison *et al.*, 2002). The genomic differences in the strains of HCMV are useful for identifying genes with non-essential functions, but which are increasingly being associated with roles such as immunomodulation.

1.6 Viral Growth and Gene Expression

1.6.1 Cell Permissivity

HCMV is strongly species specific, and is thought to only replicate in man. *In vivo*, it exhibits a highly restricted host range, infecting fibroblasts, epithelial cells, endothelial cells, smooth muscle cells, stromal cells, neuronal cells, neutrophils, hepatocytes and monocyte/macrophages and is most commonly observed in ductal epithelial cells (Jarvis & Nelson, 2002, Pass, 2001, Plachter *et al.*, 1996, Sinzger *et al.*, 1996). *In vitro* efficient productive HCMV is restricted almost exclusively to primary human fibroblast cells, however clinical isolates are able to replicate in endothelial cells, an ability which tends to be lost rapidly with *in vitro* culture in fibroblasts (Ho *et al.*, 1984, Sinzger *et al.*, 1999, Waldman *et al.*, 1991).

HCMV replicates slowly in fibroblasts and cytopathic effect (CPE) is characterised by cell rounding and enlargement. In strain AD169 infected fibroblasts, rounding begins approximately 5 hours post infection (p.i) and continues through to 24 hours p.i. (Albrecht *et al.*, 1980). By 48 to 72 hours

p.i, nuclear inclusions consisting of nucleocapsids and perinuclear inclusions comprising a complex of nucleocapsids, dense bodies and Golgi apparatus appear (Pass, 2001). Clinical isolates produce a CPE characterised by cell rounding and the appearance of a kidney shaped nucleus (Pass, 2001).

1.6.2 Virus Entry

HCMV infection can be divided into 3 essential steps; virus attachment, penetration and translocation to the nucleus allowing for release of viral DNA. In the first step, the envelope glycoproteins, gM/gN and/or gB are thought to bind to heparin sulphate proteoglycans to mediate tethering at the target cell surface (Compton *et al.*, 1993). This interaction is rapidly followed by binding of epidermal growth factor receptor (EGFR) on the surface of the target cell by gB, either directly or with other cell receptors that associate with EGFR. This is followed by receptor clustering and fusion, which leads to the internalisation of the virion components in a pH-dependent manner (Adlish *et al.*, 1990, Compton *et al.*, 1992, Keay *et al.*, 1989, Taylor & Cooper, 1990, Wang *et al.*, 2003). A further binding event, however, is thought to be required for this final fusion involving the tripartite gCIII complex and unknown receptors (Theiler & Compton, 2001). This is then followed by rapid translocation of the nucleocapsids and tegument proteins into the nucleus where viral DNA is released. The importance of EGFR binding in HCMV entry is yet to be determined as it is absent from many HCMV-permissive cell types including haematopoietic cells, such as monocytes/macrophages, dendritic cells and neutrophils (Ewald *et al.*, 2003).

Roles for a number of other HCMV proteins in virus entry have been suggested. The HCMV encoded HLA class I homologue gpUL18, has been shown to form a complex with β_2m, suggesting an interaction with HLA class I on the target cell could have a role in virus entry (Chee *et al.*, 1990; Browne *et al.*, 1990). Additionally, HCMV glycoproteins have been associated with triggering of Toll-like receptor-2 (TLR2) suggesting a role for TLR2 in virus entry (Compton, 2004, Compton *et al.*, 2003). Recently, integrins have been

proposed as co-receptors for HCMV entry because gB binds certain β1-containing integrins through a disintegrin-like domain (Feire *et al.*, 2004).

1.6.3 Regulation of Gene Expression

During productive infection by HCMV, viral gene expression has been divided into 3 kinetic classes depending on the time of expression and sensitivity to inhibition of protein synthesis or viral DNA synthesis. These classes have been designated immediate early (IE), early (E) and late (L). Expression of IE genes occurs immediately after virus entry and is required for the expression of the early and late genes. Transcription of the HCMV genes is performed by the host cell RNA polymerase II and its basal transcription complex. To date, there is no evidence for an HCMV-encoded RNA polymerase (Mocarski, 2001).

1.6.3.1 Immediate Early Gene Expression

Immediate early genes are defined as those that are transcribed in the absence of *de novo* virus-encoded protein synthesis. The most abundant IE transcripts are those controlled by the MIE (*ie1*/*ie2*) promoter-enhancer, one of the strongest transcriptional enhancers yet identified in mammalian biology (Boshart *et al.*, 1985, Chee *et al.*, 1990a, Mocarski, 2001). IE1 and IE2 are the two major products of the MIE locus and are differentially spliced variants, which contain the same 85 amino acid terminal domain but splice either to UL123 for IE1, or to UL122 for IE2 (Spector, 1996, Stenberg, 1996). The IE1 and IE2 gene products cooperate to activate and upregulate viral E genes such as the major 2.7 and 1.2 kb transcripts of the UL segment of the genome and the UL112/113 transcripts (Spector, 1996).

IE1 encodes a 72 kDa, nuclear phosphoprotein and has been shown to be dispensable for growth in cultured cells infected at high MOIs, but required at

low input MOIs (Greaves & Mocarski, 1998). IE1 associates with, and disrupts PML-bodies, and this has been suggested to contribute to the progression of the lytic cycle (Ahn & Hayward, 1997, Ahn & Hayward, 2000, Kelly *et al.*, 1995, Wilkinson *et al.*, 1998). Recently, IE1 and IE2 have been shown to interact with the histone deacetylase 3 (HDAC3), which plays a role in transcriptional regulation. Furthermore, a lack of IE1 was shown to result in hypoacetylation and reduced transcription (Nevels *et al.*, 2004). Additionally, the region located between amino acids 400 and 491 have been shown to be involved in chromatin tethering (Reinhardt *et al.*, 2005).

IE2 is a 80 to 86 kDa phosphoprotein and has 2 core functions; to control the transition from IE to E, and possibly L, and as infection proceeds to the late phase, to ensure down modulation of IE1 and IE2 by binding to *cis* repression signal (*crs*) sequences (Cherrington *et al.*, 1991, Liu *et al.*, 1991, Mocarski, 2001, Pizzorno & Hayward, 1990). The HCMV strain Towne was cloned into a bacterial artificial chromosome (BAC) and deleted for IE2. This mutant was arrested in the early replication cycle and this was shown to be a consequence of the failure to express early genes (Marchini *et al.*, 2001). IE2 acts as a transcriptional regulator of cellular promoters such as the heat shock protein 70 (hsp70) and cyclin E promoters, as well as heterologous viral control elements (such as the HIV-1 long terminal repeat (LTR)) (Caswell *et al.*, 1996, Dal Monte *et al.*, 1997). It has also been shown to interact with the basal trancription factors TBP and TFIIIB and with cellular transcription factors such as CREB, AP-1 and Sp1 (Caswell *et al.*, 1993, Lang *et al.*, 1995, Scully *et al.*, 1995, Yurochko *et al.*, 1997). As such IE2 has subsequently been shown to play a role in many cell processes, including cell cycle control and apoptosis (Kalejta & Shenk, 2002, Zhu *et al.*, 1995).

Other IE genes include UL36-UL38, TRS1/IRS1 and US3. The products of UL36, and UL37 have been shown to encode the viral inhibitors of apoptosis, vICA and vMIA, respectively (discussed in section 1.12.1) (Goldmacher *et*

al., 1999, Skaletskaya *et al.*, 2001a). Whilst the TRS1 and IRS1 gene products have been shown to transactivate E promoters in co-operation with IE1 and IE2 and block interferon-induced dsRNA-activated antiviral pathways (Child *et al.*, 2004, Romanowski & Shenk, 1997). gpUS3 is involved in preventing HLA class I maturation by binding and retaining MHC class I heavy chains in the endoplasmic reticulum (discussed in section 1.12.3.1.2) (Jones *et al.*, 1996).

1.6.3.2 Early Gene Expression

Expression of early genes occurs prior to viral DNA replication and requires the presence of functional IE gene products (Fortunato & Spector, 1999). Early proteins involved in viral DNA replication include the catalytic and accessory protein subunits of the viral DNA polymerase UL54 and UL44, respectively (Kerry *et al.*, 1994, Rodems *et al.*, 1998). Other early proteins include the structural proteins gp48 and gB and the immune evasion proteins gpUS28 and gpUS11 (Gewurz *et al.*, 2001a, Jones *et al.*, 1995a, Mocarski, 2001, Smuda *et al.*, 1997). The most abundantly transcribed HCMV E gene, β2.7, may not encode a functional translation product and is non-essential for growth *in vitro* (McSharry *et al.*, 2003).

1.6.3.3 Late Gene Expression

Little is known about the regulation of late genes, however their expression requires the prior expression of IE gene products (Stinski, 1978). Late genes are believed to encode mainly structural virion proteins, for example UL83 which encodes pp65 and UL99 which encodes the tegument protein pp28 (Chee *et al.*, 1990a, Depto & Stenberg, 1989, Depto & Stenberg, 1992).

1.6.4 Viral DNA Replication

HCMV DNA replication begins at approximately 16 hours after infection and peaks at 60 to 80 hours p.i.. Human, murine and simian CMV genomes contain a single origin of replication, *ori*Lyt. The *ori*Lyt is mapped to a 3-4 kb region near the centre of the UL segment and is characterised by extensive

repeat sequences from which viral DNA replication occurs in a bi-directional fashion. This process requires 6 core replication proteins (ppUL84, IE2 and ppUL57, pUL70, pUL105 and pUL102) (Hamzeh *et al.*, 1990, Mocarski, 2001) (Masse *et al.*, 1992). In addition to these proteins, 4 gene loci (UL36-38, UL112/113, IRS1 and UL122/123) encode proteins that co-operate to activate the expression of the replication genes (Iskenderian *et al.*, 1996).

1.6.5 Latency and Reactivation

Latent infection is defined as a state in which no, or only a very limited set of viral genes are expressed in cells in which the virus persists. Evidence from several laboratories suggests that HCMV is latent in myeloid cell lineages but peripheral blood monocytes are thought to be the chief cells implicated in latency (Hahn *et al.*, 1998, Mendelson *et al.*, 1996). Endothelial cells have also been suggested as a site for viral latency, however there is no evidence to date to support a role in latency for this cell type (Danesh, 1999). The mechanism by which HCMV establishes latency is incompletely understood. A possible mechanism was proposed by (Sissons *et al.*, 2002). In this model, HCMV is carried in the myeloid lineage progenitor cells and is maintained in the cells at very low frequencies until peripheral blood monocytes differentiate into macrophages.

1.7 Epidemiology

HCMV infection rates are high in both developing and developed countries, with infection rates between 50 and 80% in developed countries and approaching 100% in developing countries (Griffiths *et al.*, 2000). HCMV is shed in breast milk, saliva, urine, cervical secretions and semen for months to years following HCMV infection and the virus is thought to be transmitted horizontally and vertically through contact with these fluids from seropositive individuals (Pass, 2001).

Foetal infection can occur following primary and recurrent maternal infection,

where recurrent infection may be reactivation or reinfection by a new virus strain. HCMV can be transmitted from mother to baby either transplacentally, or through shedding of the virus from the mother's vagina, cervix or via breast milk. Transmission via breast milk is thought to be the most common route of acquiring HCMV in neonates and 1 study showed that of 87 premature babies breast fed by mothers excreting HCMV, 33 became infected (Hamprecht *et al.*, 2001). Congenital HCMV disease is more common following primary infection, with 1 study reporting that up to 40% mothers who seroconverted during gestation transmitted the virus transplacentally to their child, whilst only 1% of children born to HCMV seropositive women who suffered a recurrent infection were born HCMV seropositive (Reynolds *et al.*, 1973, Stagno, 2001).

HCMV infection is the main cause of complications for seronegative and seropositive patients undergoing solid organ and bone marrow transplantation. For HCMV seropositive individuals, the immunosuppressive treatment required prior to surgery allows the virus to reactivate, and HCMV can be detected in the body secretions of approximately 50% of transplant patients usually within one month after surgery (Sia & Patel, 2000). Similarly, complications occur for HCMV seronegative individuals who receive transplanted material from HCMV seropositive donors (Pass, 2001).

1.8 Clinical Manifestations

HCMV rarely causes clinically relevant disease in the immunocompetent host, however HCMV infection accounts for 8% of all cases of mononucleosis (Nesmith & Pass, 1995). Clinical symptoms of this syndrome are similar to clinical manifestations of Epstein-Barr virus (EBV)-induced disease and include fever, malaise, lymphocytosis and prominent weight loss (Rodriguez-Bano *et al.*, 2004). HCMV hepatitis, thrombocytopenia, haemolytic anaemia, pneumonitis and encephalitis may also occur with or without mononucleosis (de la Hoz *et al.*, 2002). In contrast to immunocompetent hosts, HCMV is commonly the cause of disease in individuals whose immune system is not fully developed or is suppressed.

As such, HCMV is a relevant pathogen following congenital infection, in allograft recipients and AIDS patients.

1.8.1 Congenital Infection

While HCMV has been estimated to infect approximately 1% of babies born in the United States, only 10% of those infected at birth exhibit overt symptoms of disease. However, between 10% to 15% of asymptomatic HCMV infected neonates go on to later manifest mild to moderate neurological sequelae (Istas *et al.*, 1995, Stagno, 2001). Infants born to mothers who contract primary infection or reactivate during pregnancy are at the highest risk of developing congenital disease. In contrast, clinically apparent disease is rare in those born to mothers who are HCMV seropositive prior to conception (Fowler *et al.*, 1992). The most common complications reported associated with HCMV disease in neonates are jaundice, hepatosplenomegaly, and petechiae, and these may affect up to 66% of overtly infected infants (Bale *et al.*, 1990, Boppana *et al.*, 1992, Fowler *et al.*, 1992). Intrauterine growth retardation, thrombocytopenia, chlorioretinitis and hepatitis are also associated with *in utero* infection (Stagno, 2001).

1.8.2 Allograft Recipients

The major clinical manifestations of HCMV in infected solid organ recipients are fever, gastroenteritis, mononucleosis, chorioretinitis, malaise, arthralgias and macular rash. Life-threatening complications such as pneumonia and hepatitis occur in fewer patients (Pass, 2001). Up to 50% of patients undergoing allogeneic bone marrow transplantation develop symptoms of disease, pneumonitis being the most clinically significant manifestation (Rubie *et al.*, 1993). Immunosuppression by HCMV may confound these problems and allow infection with opportunistic pathogens (Rubin, 2001).

1.8.3 AIDS

HCMV disease is one of the most frequent opportunistic infections in patients with advanced HIV infection (Gallant *et al.*, 1992). Co-infection with HCMV

and HIV has been reported as an independent predictor of mortality in AIDS (Pass, 2001). HCMV IE1 and IE2 proteins have been shown to synergise with the HIV Tat protein, a transcriptional activator of the HIV-1 long terminal repeat indicating that dual infection with HCMV and HIV could play a role in HIV activation and acceleration of AIDS (Dal Monte *et al.*, 1997, McCarthy *et al.*, 1998).

HCMV retinitis is the most common presentation in AIDS patients and is seen in up to 25% of patients with AIDS prior to effective anti-viral therapy (Jacobson, 1997). Other frequently observed clinical manifestation include oesophagitis and colitis (Gallant *et al.*, 1992). Less common clinical manifestations include encephalitis, hepatitis, pneumonitis and gastritis (Cheung & Teich, 1999).

There are several drugs used in the prophylaxis and treatment of HCMV infection and these are summarised in Table 1.1.

1.9 The Immune Response to HCMV

1.9.1 Humoral Immunity

The importance of the humoral response is well illustrated in the murine model. Although primary MCMV infection could be controlled in B-cell deficient mice following reactivation, the virus load was found to be 100 – 1000 fold higher than in immunocompetent controls (Jonjic *et al.*, 1994).

During a primary infection in immunocompetent individuals anti-HCMV immunoglobulin (Ig) M antibodies are produced first, later followed by IgG class antibodies that persist for life. Antibodies to HCMV have been identified with specificity for many proteins including the envelope glycoproteins proteins gB, gH, and the structural proteins pp65 and pp150 (Britt, 1991, Landini & Michelson, 1988, Landini *et al.*, 1988). Evidence suggests that antibodies to the structural proteins do not induce neutralising

Table 1.1 Drugs used in the prophylaxis and treatment of Cytomegalovirus Infection

Drug	Mechanism of Action	Comment
Intravenous ganciclovir	Nucleoside analogue. Monophosphorylated by UL97. Competitive inhibitor of DNA polymerase UL54	First drug treatment licensed for treatment of HCMV.
Oral ganciclovir	As above	Low bioavailability
Intraocular ganciclovir	As above	Treatment of CMV retinitis in AIDS patients. Associated with retinal detachment and endopthalamus.
Oral valganciclovir	Ganciclovir prodrug; mechanism as above	Better bioavailability than oral ganciclovir.
Intravenous foscarnet	Pyrophosphate analogue. Competitive inhibitor of DNA polymerase UL54.	Resistance associated with mutations in UL54
Intravenous cidofovir	Acyclic nucleoside analogue. Inhibits HCMV replication	Highly toxic. Restricted almost exclusively for the treatment of HCMV in patients infected with HIV.
Intraocular fomiversen	Antisense oligonucleotide. Inhibits translation of IE2.	Treatment of HCMV retinitis in AIDS. Administered only if unresponsive to other treatments.
Maribavir	Benzimidazole analogue. Inhibits HCMV DNA synthesis and nuclear egress.	Resistance associated with mutations in UL27 and UL97.

antibodies, whereas antibodies specific for the glycoprotein antigens have neutralising activity (Schoppel *et al.*, 1998, Urban *et al.*, 1996). Anti-CMV IgG has been shown to mediate antibody-dependent cellular cytotoxicity (ADCC) by NK Cells, indicating a link between the antiviral activities of the humoral and cellular immune response (Lang *et al.*, 2004).

A role for antiviral antibodies in preventing severe HCMV infection and in modulating the clinical course of HCMV has been demonstrated for immunocompromised as well as immunocompetent individuals. Indeed, results of a study by Winston *et al* (1993) showed that the probability of transmitting the virus from mother to foetus was greatly increased if the antibody response to HCMV was of a low avidity and poor neutralising activity (Winston *et al.*, 1993). This has been supported by a number of other studies that have demonstrated substantial protection by maternal immunisation against symptomatic infection of newborns and secondary infection of mothers (Adler *et al.*, 1995, Fowler *et al.*, 1992, Tanaka *et al.*, 1991). The importance of humoral immunity in the control of HCMV is demonstrated by a reduction in the severity of disease in renal transplant patients after the administration of high titre anti-HCMV immunoglobulins (Snydman *et al.*, 1987). More recently, protection from severe HCMV disease in renal transplant patients has been directly correlated with the number of HCMV specific epitopes recognised by IgM antibodies present post-operatively in these patients (Muller *et al.*, 2002). The CMV specific humoral response was shown to occur in approximately 50% of bone marrow patients as early as 20 days after transplantation, and this was associated with improved survival rates (Schoppel *et al.*, 1998). However, the benefits of prophylactic treatment of allogeneic bone marrow transplants with immunoglobulins are unclear. In this respect, a meta-analysis of a number of trials has shown that treatment with HCMV specific immunoglobulins results in a reduction in HCMV disease and HCMV pneumonitis in both HCMV seropositive and seronegative recipients (Bass *et al.*, 1993). Additionally, treatment of patients with both gancyclovir and immunoglobulin was shown

to be more effective than the use of either agent alone for the treatment of established HCMV disease (Reed *et al.*, 1988). A role of humoral immunity in the control of HCMV disease in immunocompetent individuals was demonstrated by Alberola *et al* (2000) (Alberola *et al.*, 2000). This study showed patients who developed HCMV mononucleosis had lower levels of neutralising antibody titres in their sera after the early onset of symptoms than patients who presented with a milder clinical presentation of disease.

1.9.2 Adaptive Immunity

1.9.2.1 $CD8^+$ T-cells

Initial studies of MCMV showed that suppression of MCMV-specific cytotoxic T-cells (CTL) resulted in reactivation and dissemination of infection, indicating the requirement of these cells for recovery from MCMV infection (Gardner *et al.*, 1974, Quinnan *et al.*, 1978). The importance of these cells in the control of MCMV infection has also been demonstrated by adoptive transfer experiments that showed that CTLs with antiviral activity were expanded in the lungs, spleen and adrenal glands of MCMV infected mice. In contrast, $CD4^+$ cells were shown to have no antiviral effects, and were not required for $CD8^+$ T-cell function (Reddehase *et al.*, 1988). A nonapeptide from the immediate early protein (IE1) was identified as the target for a significant number of murine CTL and this led to the suggestion that IE1 specific CTL were immunodominant (Del Val *et al.*, 1988, Volkmer *et al.*, 1987). However, this was later questioned in a study which found that IE1 specific CTL accounted for only a minor number of cells expanded in an $\alpha\beta$ T-cell infiltrate in the lungs of MCMV infected mice (Holtappels *et al.*, 1998). The perforin/granzyme pathway has been identified as an important contributor to viral elimination in the salivary glands of MCMV infected mice. However, in the long term control of MCMV infection, neither this pathway nor the FasL/Fas cytolytic pathways appeared to be necessary (Riera *et al.*, 2001, Riera *et al.*, 2000).

In humans, the role of CTL *in vivo* for the control of HCMV infection was

demonstrated by the reconstitution of cytotoxic CTL by adoptive transfer of cytotoxic CTL clones into patients who had undergone bone marrow transplants. In follow up studies, these cells were shown to persist for up to 12 weeks and generate a vigorous virus-specific CTL response in the absence of viremia or HCMV disease (Walter *et al.*, 1995). However, the use of CTL clones in the clinic is restricted by difficulties in culturing large enough numbers of these cells for therapeutic use. Peggs *et al* (2003) therefore investigated the ability of CMV specific polyclonal T-cell lines to control HCMV reactivation in seropositive patients who had undergone haematopoetic stem-cell transplantation (HSC) (Peggs *et al.*, 2003). Of 16 patients who received polyclonal T-cells, 8 patients cleared viral DNA without antiviral drugs during the primary episode of reactivation. Additionally, only 2 of the 14 patients the authors could assess had a second episode of CMV reactivation. Furthermore, the authors estimated that pp65 peptide-specific CTL were expanded by 3 – 5 log *in vivo.*

In HCMV infection, CTLs specific for structural proteins, including tegument (pp65) and envelope glycoproteins (gB and gH) and the non-structural immediate early protein (IE1), have been identified (Boppana *et al.*, 1996; Kern *et al.*, 1999). CTL specific for pp65 and IE1 are often reported as being immunodominant and in one study pp65 specific CTL were shown to account for 50% of the donors $CD8^+$ CTL (Boppana & Britt, 1996, Hebart *et al.*, 2002, Lang *et al.*, 2002, Saulquin *et al.*, 2000). Wills *et al* (1996) investigated the contribution of pp65 specific CTL to the CTL repertoire and showed by limiting dilution analysis that pp65 specific cells were found in the highest frequency (Wills *et al.*, 1996). Further, using a HCMV deletion mutant of pp65 they showed that between 70% and 90% of CTL that recognise CMV were pp65 specific. High frequencies of HLA-A*0201 IE1 peptide VLEETSVML restricted $CD8^+$ T cells have been identified in HCMV seropositive donors that do not respond to the pp65 epitope NLVPMVATV (Khan *et al.*, 2002a). More recently, a wider range of proteins capable of eliciting CTL responses have been identified (Elkington *et al.*, 2003). These

proteins include structural, early/late antigens and HCMV-encoded immunomodulators (pp28, pp50, gH, gB, US2, US3, US6 and UL18). Significantly, more than 40% of CTL reactivity was estimated against antigens other than pp65 and IE1 which questions the previously thought immunodominance of the pp65 and IE1 specific proteins.

HCMV has been shown to greatly influence the phenotype and number of $CD8^+$ T cells in the peripheral blood. In this respect, Looney *et al* (1999) demonstrated a strong association with CMV seropositivity and increased number of $CD4^+$,$CD28^-$ and $CD8^+$,$CD28^-$ T cells, as well as with increased numbers of $CD3^+$ cells expressing CD56 (Looney *et al.*, 1999). Associations with HCMV serostatus and increased numbers of $CD8^+$, $CD57^+$ cells have also been reported (Gratama *et al.*, 1989, Wang *et al.*, 1995, Wang *et al.*, 1993). These cells were shown to be oligoclonally expanded and to proliferate in response to HCMV infected fibroblasts (Wang *et al.*, 1995). Furthermore, $CD8^+$ cells expressing intermediate to high levels of CD57 were shown to rapidly synthesis IFNγ and TNFα in response to HCMV peptides (Kern *et al.*, 1999). The introduction of tetramer technology allowed detailed analysis of HCMV specific $CD8^+$ T cells. HLA peptide tetramer staining of IE1 specific CTL showed that these cells have high CD57 and low expression of the costimulatory molecule CD28 (Khan *et al.*, 2002a). Additionally the authors showed that these cells were $CCR7^-$ and expressed high levels of intracellular perforin, suggesting that these cells were differentiated effector cells. Expression levels of CD45RA and CD45RO was shown to vary between donors, however although T cells are thought to progress from a CD45RA to a CD45RO and then back to a CD45RA phenotype, the functional significance of these different CD45 isoforms remains unclear. This phenotype has also been observed in pp65 specific CTL (Gillespie *et al.*, 2000). In this study, the authors demonstrated that CD28 was absent from large proportions of pp65 specific $CD8^+$ T cells in a number of donors. Additionally they showed that these cells often lacked expression of the costimulatory receptor CD27 and the lymph node homing

receptor CD62L, a phenotype associated with effector function (Hamann *et al.*, 1997). In this respect, the authors were able to show that for 3 donors tested, cell mediated cytotoxicity was restricted to tetramer positive cells which lacked CD28 expression (Gillespie *et al.*, 2000). It is well documented that the human $CD8^+$ T cell repetoire frequently has oligoclonal expansions which accumulate with age and these expansions have been correlated with persistent viral infections (Posnett *et al.*, 1994, Schwab *et al.*, 1997). HCMV specific $CD8^+$ CTL have been shown to have a restricted TcR usage and to accumulate with age, meaning that HCMV is now considered a major cause of the polarisation of the T cell repertoire in humans (Vargas *et al.*, 2001; Wills *et al.*, 1996; Khan *et al.*, 2004; (Khan *et al.*, 2002b).

1.9.2.2 $CD4^+$ T-cells

The role of $CD4^+$ T cells in control of HCMV was demonstrated by adoptive transfer of $CD4^+$ and $CD8^+$ T cell lines into patients who had undergone allogeneic SCT (Einsele *et al.*, 2002). In this study, the authors show that transfer of virus specific $CD4^+$ T cells was coincident with the expansion of $CD8^+$ T cells from precursors and a great fall in viral load. In healthy HCMV seropositive donors, typically 1 – 2% of circulating $CD4^+$ T cells are specific for HCMV and these expansions were shown to be stable over a period of 3 - 4 years (Sester *et al.*, 2002, Weekes *et al.*, 2004). The $CD4^+$ T cell response to a number of viral proteins was investigated in healthy virus carriers and strong proliferative responses in all donors were identified in response to pp65, with lesser responsiveness to gB, gH and the IE proteins (Beninga *et al.*, 1995). CMV specific $CD4^+$ cells were shown to be oligoclonal and to express low levels of CD62L and CCR7, indicating that these cells were antigen experienced mature effector cells (Sester *et al.*, 2002). More recently, studies have identified several pp65 specific β-interferon producing $CD4^+$ T cell epitopes (Li Pira *et al.*, 2004).

1.9.3 Innate Immunity

Natural killer (NK) cells do not require antigen-specific recognition to lyse target cells, and as such are capable of limiting viral infection prior to the

induction of the adaptive immune response. Studies in mice have provided the most compelling evidence that NK cells play a crucial role in the control of CMV infection. In a study by Shellam *et al* (1981), NK cell defective mice were shown to be more susceptible to lethal infection and developed higher virus titers than control mice (Shellam *et al.*, 1981). These results were supported by a second study that showed that NK cell depleted mice had higher viral titers, greater viral dissemination and more severe pathological changes compared to controls (Bukowski *et al.*, 1984). A subsequent study demonstrated that the adoptive transfer of NK cells into irradiated mice provided resistance to MCMV (Bukowski *et al.*, 1985). More recently, MCMV infection has been correlated with NK cell expansions (Sweet, 1999).

Studies performed over 10 years ago, revealed that mice with a gene, *Cmv*-1, demonstrate resistance to MCMV replication in the spleen (Scalzo *et al.*, 1990). *Cmv*-1 maps to the NK gene complex (NKC) on mouse chromosome 6, and encodes members of the Ly49 family (Scalzo *et al.*, 1992, Scalzo *et al.*, 1995). The activating NK cell receptor Ly49H, was later shown to be involved in resistance to MCMV *in vivo* (Brown *et al.*, 2001, Daniels *et al.*, 2001, Lee *et al.*, 2001). The ligand for Ly49H is encoded by the m157 ORF (Arase *et al.*, 2002, Smith *et al.*, 2002). Deletion of this ORF results in a virus that has increased virulence *in vitro* and that is unable to activate Ly49H NK cells (Bubic *et al.*, 2004). More recently the MCMV m155 ORF has been implicated in modulation of NK cell function. MCMV mutants for m155, were shown to be deficient in growth and to have lower viral tires in many organs (Abenes *et al.*, 2004). The m155 gene product was subsequently shown to function by down-regulating cell surface expression of H60, the high affinity ligand for the NK cell activating receptor, NKG2D (Lodoen *et al.*, 2004). These *in vivo* pathogenesis studies indicate that not only is the NK response crucial to controlling disease but the virus-encoded genes that modulate NK functions are important virulence determinants.

A number of studies have demonstrated a role for NK cells in the control of

HCMV infection. The first study, identified a 13 year old girl with an NK cell deficiency (no CD56 or CD16 expressing lymphocytes) who, when infected with HCMV and other herpesviruses developed a life threatening infection (Biron *et al.*, 1989). Since publication of the original report, the patient developed aplastic anaemia and died from complications related to bone marrow transplantation. Post mortem attempts to uncover a genetic aetiology for her syndrome were unsuccessful and showed that she had normal IL15 receptor genes (Orange, 2002). A patient with similar symptoms was recently described by Gazit, (2004). In this short report, the authors described a 7 year old patient who expressed the killer immunoglobulin like receptor (KIR) 2DL1 on every NK cell and frequently suffered from HCMV infection. Expression of other NK receptors, including KIR2DL2, KIR3DL1, CD94/NKG2A, LIR1 and NKp46 was normal. KIR2DL1 recognises type II HLA-C allotypes. A number of studies have suggested that HCMV downregulates HLA-A and HLA-B expression whilst maintaining HLA-C expression. As KIR2DL1 recognises HLA-C, the authors suggested that the frequency of HCMV infections in this donor was the result of the inability of the patients NK cells to respond to HCMV (Gazit *et al.*, 2004).

Patients suffering from ectodermal dysplasia with immunodeficiency (EDID) have impaired NK-κB signaling and are most commonly susceptible to mycobacterial infections, however 2 studies have reported susceptibility to herpesvirus infection (Doffinger *et al.*, 2001, Orange *et al.*, 2002). The study by Orange *et al* (2002) showed that 1 patient who had deficient NK cell cytotoxicity but normal antibody-dependent cellular cytotoxicity (ADCC) and frequency of NK cells had recurrent, invasive episodes of CMV despite protective levels of CMV-specific Ig (Orange *et al.*, 2002). *In vivo* administration of IL2 was able to induce NK cytotoxicity and control HCMV disease in this patient.

Common variable disease immunodeficiency (CVID) patients have a well documented humoral immune deficiency and many patients have decreased

or impaired NK cell cytotoxicity and ADCC (Witte *et al.*, 2000). Additionally, whilst NK cells are present in CVID they are often decreased in numbers and percentages and contain less IFNγ (Aspalter *et al.*, 2000). Individuals with CVID are most susceptible to bacterial and candidal infections, however a single patient with a particularly severe NK cell defect had invasive HCMV infection (Witte *et al.*, 2000)

1.10 Natural Killer Cell Receptors

NK cell lysis correlates with cell surface MHC class I expression, whereby cells with lower levels of MHC class I are lysed and those with normal levels of MHC class I are not, an observation which led to the "missing self" hypothesis (Karre *et al.*, 1986, Ljunggren & Karre, 1990, Yokoyama & Scalzo, 2002). Recently, a fine tuning of this hypothesis means that NK cell cytotoxicity is now thought of as a balance between activating and inhibitory NK receptor stimulation, the latter generally arresting the signaling cascades that trigger the former. The main NK cell receptors, along with their stimulatory or inhibitory function, and their ligands can be found in Table 1.2 and Figure 1.4.

1.10.1 CD94/NKG2 Receptors

CD94 and NKG2 are type II integral membrane glycoproteins encoded by genes clustered with other members of the C-type lectin family on human chromosome 12 (Houchins *et al.*, 1991, Yokoyama *et al.*, 1990). The CD94 and NKG2 chains form a dimer in which the CD94 chain is conserved and this chain is thought to determine the binding specificity of the dimer (Chang *et al.*, 1995, Farrell *et al.*, 2002). The NKG2 chain consists of a family of at least 6 family members NKG2A, NKG2B (a splice variant of NKG2A), NKG2C, NKG2D, NKG2E and NKG2F (Natarajan *et al.*, 2002, Plougastel & Trowsdale, 1997). These receptors may be inhibitory (NKG2A/B) or stimulatory (NKG2C or NKG2E), depending on whether the cytoplasmic chain is short or long, respectively. The inhibitory isoforms have a pair of

Table 1.2 NK Receptor and Ligand Specificity

Gene Name	Chromosome locolisation	Receptor Name	Function	Ligand Specificity
KIR2DL1	19q13.42	p58.1	Inhibitory	HLA-Cw2, 4, 5, 6
KIR2DL2	19q13.42	p58.2	Inhibitory	HLA-Cw1, 3, 7, 8
KIR3DL1	19q13.42	p70	Inhibitory	HLA-Bw4
KIR3DL2	19q13.42	p140	Inhibitory	HLA-A3, -All
KIR2DL4	19q13.42	p49	Inhibitory	HLA-G
LIR/ILT2	19q13.42	LIR1/ILT2	Inhibitory	HLA-G and broad HLA class I
LIR2/ILT4	19q13.42	LIR2/ILT4	Inhibitory	HLA-F
CD94	12p – 13	*	*	*
NKG2A	12p – 13	CD94/NKG2A	Inhibitory	HLA-E
KIR2DS1	19q13.42	p50.1	Activating	HLA-Cw2, 4, 5, 6
KIR2DS2	19q13.42	p50.2	Activating	HLA-Cw1, 3, 7, 8
KIR2DS4	19q13.42	p50.3	Activating	Unknown
NKG2C	12p12 – 13	CD94/NKG2C	Activating	HLA-E
LAIR1	19q13.42	P40//LAIR1	Inhibitory	Unknown
IRC1	17	Irp60	Inhibitory	Unknown
CMRF35	17q24	CMRF35	Inhibitory (?)	Unknown
Nkp46	19q13.42	NKp46	Activating	Unknown (sialic acid dependent)
1c7/NK-a1	6p21.3	NKp30	Activating	Unknown
Nkp44	6p21.3	NKp44	Activating	Unknown
NKG2D	12p12 – 13	NKG2D	Activating	MICA, MICB, ULBP
2B4	1q23.3	2B4	Activating & inhibitory	CD48
AIRM1	19q13.3	Siglec 7	Inhibitory	α2,8-linked diasialic acids
SLAM 6	1q23.1	NTB-A	Activating & inhibitory	Unknown
KLRF1	12p12 – 13	NKp80	Activating	Unknown
SLAM 7	1q23 – q24	CRACC	Activating	Unknown
CD59	11p13	CD59	Activating	Unknown

* Forms a heterodimer with different members of the NKG2 family, including NKG2A and NKG2C

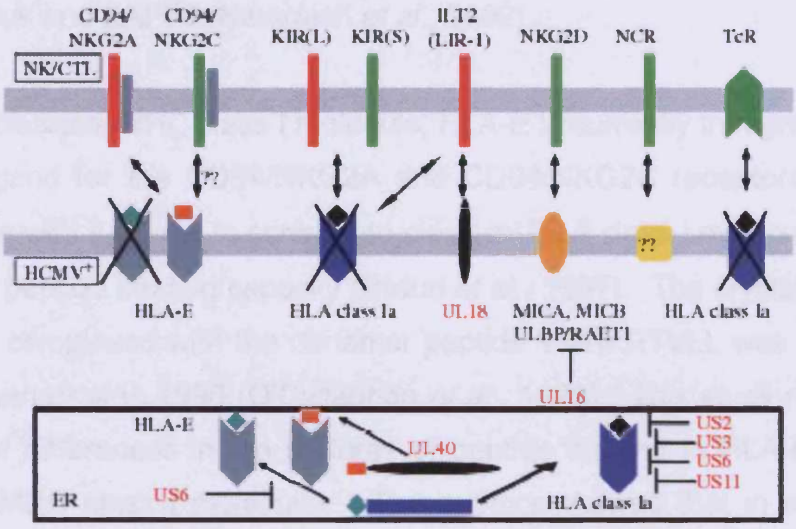

Figure 1.4 Receptor Recognition of MHC class I and related Molecules in HCMV. Putative interactions between stimulatory (red) and inhibitory (green) receptors expressed on NK cells and CTL, with their ligands in a HCMV infected cells (Taken from (Lopez-Botet *et al.*, 2004)).

inhibitory ITIM motifs in their cytoplasmic tail, which upon phosphorylation recruit SHP-1 and SHP-2 phosphatases to block cellular signaling (Carretero *et al.*, 1998). In contrast, the activating isoforms are associated with a dimeric ITAM containing signaling molecule DAP12 that initiates signaling through Syk and ZAP70 (Natarajan *et al.*, 2002).

The non-classical MHC class I molecule, HLA-E is currently thought to be the *in vivo* ligand for the CD94/NKG2A and CD94/NKG2C receptors. HLA-E associates with β_2m, but in contrast to classical HLA class I molecules, has a restricted peptide binding capacity (Braud *et al.*, 1997). The crystal structure of HLA-E complexed with the nonamer peptide VMAPRTVLL was solved by O'Callaghan *et al* in 1998 (O'Callaghan *et al.*, 1998). This study revealed a number of differences in the features of peptide binding to HLA-E and the classical MHC class I molecules. The authors showed that in contrast to classical MHC class I molecules, the amino acid side chains of peptides bound by HLA-E occupied all of the available binding pockets, indicating enhanced peptide selectivity. Similarly to classical MHC class I molecules, the peptide binding groove was shown to be hydrophobic, however the extent to which hydrophobic interactions were used to interact with peptides was far greater than that seen in classical MHC class I molecules. Efficient cell surface expression of HLA-E requires that class I leader peptides are loaded onto HLA-E in a transporter associated with antigen processing (TAP) dependent manner (Lee *et al.*, 1998a). Association of cell surface HLA-E with CD94/NKG2A results in an inhibition of NK-mediated cytoxicity (Borrego *et al.*, 1998, Braud *et al.*, 1998, Lee *et al.*, 1998b). Recently, in a study using mutants of HLA-E, Wada *et al* (2004) showed that both CD94/NKG2A and CD94/NKG2C bind to the top of the $\alpha_1\alpha_2$ domain HLA-E. The binding domain is thought to be mostly shared, however CD94/NKG2C could bind to 2 mutants that were not bound by CD94/NKG2A indicating that there are differences in the binding ability of these proteins (Wada *et al.*, 2004).

1.10.2 NKG2D

NKG2D is an activating receptor which is expressed on all NK cells, some T cells and on activated macrophages associated with the signal transducing adaptor protein DAP12 (Bauer *et al.*, 1999, Wu *et al.*, 1999, Li *et al.*, 2001). In contrast to the other NKG2 proteins, NKG2D is present on the surface of the cell as a homodimer and binds to the MHC class I related proteins (MIC) A and MICB, the UL16 binding proteins (ULBPs) 1,2,3 and the newly identified ULBP4 Cosman *et al.*, 2001, Jan Chalupny *et al.*, 2003, Natarajan *et al.*, 2002, Li *et al.*, 2001, Sutherland *et al.*, 2002). More recently the RAET1E and RAET1G have been identified as ligands for NKG2D (Bacon *et al.*, 2004). Unlike MHC class I molecules, MICA and MICB are expressed in response to stress, do not associate with β_2M and are stably expressed without presenting peptide (Groh *et al.*, 1996).

1.10.3 KIRs

The KIRs are type I membrane proteins encoded on chromosome 19 in a region called the leukocyte receptor complex (LRC) (Wende *et al.*, 1999). They contain either 2 or 3 extracellular domains (KIR2D or KIR3D, respectively) and are further grouped according to their function, which may be inhibitory or stimulatory. Inhibitory KIRs have a short cytoplasmic tail (L) and contain 1 or 2 ITIM motifs, whilst stimulatory KIRs have a long (S) cytoplasmic tail and associate with other molecules containing ITAMs (Colonna & Samaridis, 1995, Lanier *et al.*, 1998). The best characterised members of this family are the inhibitory KIR with specificity for HLA-C ligands. HLA-C molecules are divided into type I and type II allotypes. KIR2DL2 and KIR2DL3 bind type I HLA-C allotypes that are identified by an asparagine residue at position 80. In contrast, KIR2DL1 binds type II HLA-C allotypes that have a lysine residue at position 80 (Winter *et al.*, 1998). Other inhibitory KIR have specificity for determinants of HLA-B (KIR3DL1), HLA-A (KIR3DL2) and HLA-G (KIR2DL4) (Dohring *et al.*, 1996, Rajagopalan & Long, 1999). The first crystal structure determined of a KIR/HLA complex

was that of the inhibitory KIR2DL2 bound with HLA-Cw3 and a nonamer self peptide GAVDPLLAL (GAV) from importin-α (Boyington *et al.*, 2000). This revealed that KIR2DL2 and HLA-Cw3 were bound in a 1:1 stoichiometry, with the KIR bound across both the α1 and α2 helices of HLA in an orientation that was analogous to the TcR/HLA interaction. However, differing from the TcR interaction with HLA, which centred on the peptides in positions P4 - P7, the KIR/HLA interaction was shown to centre over the P7 – P8 positions in the C-terminus of the peptide (Garcia *et al.*, 1996). Despite this difference in binding, the KIR and TcR binding patterns were shown to overlap, and this was shown to preclude simultaneous binding on the same HLA molecule. The KIR2DL1/HLA-Cw4 complex has also been determined, and verifies the recognition principles observed in the KIR2DL2/HLA-Cw3 structure (Fan *et al.*, 2001).

A number of stimulatory KIR have been identified, and these include KIR2DS2 and KIR2DS1 which have specificity for type I and type II HLA-C allotypes, respectively. Recently, studies of KIR2DS4 have indicated that this KIR is able to weakly bind HLA-Cw4 in a highly restricted manner (Katz *et al.*, 2001). Interestingly, further studies have indicated that this KIR is able to functionally bind with an unidentified non-MHC class I protein expressed on the surface of melanoma cells (Katz *et al.*, 2004). To date, the only stimulatory KIR to be crystallised is KIR2DS2 and no crystal structure for stimulatory KIR complexed with HLA have been reported. The difference in the 3 dimensional structures of KIR2DS2 and its inhibitory counterpart, KIR2DL2 were shown not to be major, and to have been principally brought about by the presence of the hydroxyl group of Tyr^{45} and from the displacement of the Gln^{71} side chain. This study also showed that KIR2DS2 did not bind the HLA-Cw3 peptide complexes bound by KIR2DL3, leading the authors to suggest that KIR molecules cannot tolerate any variability in their 3 dimensional structure without altering their MHC class I recognition capacities (Saulquin *et al.*, 2003).

1.10.4 LIRs

LIRs are type I transmembrane proteins characterised by 4 extracellular Ig type domains and are clustered on chromosome 19 with KIR in the LRC (Wende *et al.*, 1999). LIRs are expressed on monocytes and B cells and at lower levels, on dendritic and NK cells (Borges *et al.*, 1997). The amino acid sequences in the extracellular regions of eight of these receptors show between 63 and 84% identity with LIR-1, the prototypic LIR (Borges *et al.*, 1997). LIRs are divided into 3 classes; those containing cytoplasmic ITIMs (LIR-1, -2, -3, -5 and –8), those that are ITIM negative (LIR-6, -7, ILT7, 8, 11) and those without a transmembrane domain (LIR-4). LIR-1, is the most broadly expressed LIR family member, present in monocytes and dendritic cells, as well as on T cells, the majority of B cells and a variable percentage of NK cells (Borges *et al.*, 1997, Cosman *et al.*, 1997). LIR-2 is not expressed on the surface of circulating B, T or NK cells, but is expressed on the surface of monocytes and some dendritic cells (Borges *et al.*, 1997, Fanger *et al.*, 1999). The ITIM containing LIRs have been shown to recruit SHP-1 and inhibit monocyte and lymphocyte function upon interaction with a broad range spectrum of class I molecules (Colonna *et al.*, 1997). LIR-1 domain 1 and domain 2 (D1D2) contacts the mostly conserved β_2m and α3 domains of HLA-A2 (Willcox *et al.*, 2003). The ITIM negative LIRs have short cytoplasmic domains that are thought to transmit inhibitory signals by coupling with the ITAM bearing adaptor molecules such as the Fc receptor γ chain (Nakajima *et al.*, 1999b). LIR-4 represents the only LIR identified without a transmembrane domain and this protein is thought to be secreted (Borges & Cosman, 2000, Cella *et al.*, 2000).

1.10.5 Natural Cytotoxicity Receptors

It is well established that NK cells preferentially lyse target cells that lack surface expression of MHC class I molecules, implying the existence of triggering receptors that recognise non-MHC ligands on these cells. The receptors responsible for this activation in humans have recently been

identified as the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 (Cantoni *et al.*, 1999b, Pende *et al.*, 1999, Pessino *et al.*, 1998, Sivori *et al.*, 1997, Vitale *et al.*, 1998). NCRs exhibit no homology with each other and their expression is restricted to NK cells. NKp30 is a polypeptide characterised by an extracellular region forming an Ig like domain connected to the transmembrane domain by a region rich in hydrophobic amino acids (Pende *et al.*, 1999). Analysis of murine genomic organisation has only identified an NKp30 pseudogene, however a functional NKp30 receptor with 88% homology to human NKp30 has been defined in *Macaca fascicularis* (De Maria *et al.*, 2001). The NKp30 ligand has not been identified, however a recent study suggests that it may be present on 293T cells (Flaig *et al.*, 2004). NKp46 is characterised by 2 extracellular Ig domains, followed by a stretch of amino acids connecting the ectodomain to the transmembrane domain (Pessino *et al.*, 1998). The murine orthologue of NKp46 shares 58% amino acid identity with human NKp46 and maps to chromosome 7, in a region that is syntenic to human chromosome 19 that encodes human NKp46 (Biassoni *et al.*, 1999). As for NKp30, a functional NKp46 receptor has been identified in *Macaca fascicularis*. *Macaca fascicularis* NKp46 shares 86% homology with human NKp46, and is recognised by a mAb specific for the human molecule. The receptor was shown to share both cellular distribution and function(s) with human NKp46 (Biassoni *et al.*, 2001). Both NKp30 and NKp46 associate with the CD3ζ adaptor proteins that contain ITAMs (Pende *et al.*, 1999, Pessino *et al.*, 1998). The third member of the NCR family, NKp44, is a glycoprotein that is thought to associate with ITAM bearing DAP12 molecules that become phosphorylated upon NKp44 cross-linking (Cantoni *et al.*, 1999b, Vitale *et al.*, 1998). Unlike the other NCR, NKp44 is selectively expressed by IL2 activated NK cells suggesting that it may contribute to increased efficiency of NK lysis by NK susceptible targets (Vitale *et al.*, 1998). Unlike NKp30 and NKp46, NKp44 does not appear to be expressed in *Macaca fascicularis* (Biassoni *et al.*, 2001).

Phenotypic and functional analysis has revealed that the surface density of

NCR varies between donors. Moreover, NCR surface density was shown to correlate with the magnitude of cytolytic activity against NK susceptible neuroblastoma and glioblastoma cell lines (Sivori *et al.*, 2000b). Furthermore, viremic HIV patients were shown to have lower surface density of NCR, and this correlated with a parallel decrease in cytolytic function (De Maria *et al.*, 2003). However, the ligands recognised by NCRs remain ill defined. The 3-dimensional structure of NKp44 was published in 2003 and showed that the inner surface of a prominent groove was lined by positively charged amino acids, suggesting that the ligands of NKp44 may contain sugar moieties (Cantoni *et al.*, 2003). In this respect, NKp44 and NKp46 but not NKp30 have been shown to recognise the viral glycoprotein haemagluttinin (HA) of influenza and the haemagluttinin-neuroamidase of Sendai viruses (Arnon *et al.*, 2001, Mandelboim *et al.*, 2001). In these studies, recognition of HAs required the sialyation of NKp44 and NKp46 oligosaccharides, however binding of NKp44 and NKp46 to HAs was thought to be dependent upon the carbohydrates expressed on the target cell rather than on the oligosaccharides of NKp44 and NKp46. Recognition of HA by NKp46 was subsequently shown to be strongly, though not exclusively mediated through the α(2,6)-terminal sialic acid expressed on NKp46. Additionally, deletion mutants demonstrated that the Thr^{225} residue of NKp46 was required for proper activation of NKp46 (Arnon *et al.*, 2004). More recently, membrane heparin sulphate proteoglycans (HSPGs) have been shown to have a role in the lysis of tumour cells by NKp30 and NKp46 expressing NK cells (Bloushtain *et al.*, 2004).

1.10.6 Other NK receptors

Leukocyte associated Ig-like receptors (LAIRs) 1 is a 32 kDa transmembrane protein with a leader sequence, an extracellular domain, a transmembrane domain, and a cytoplasmic region (Meyaard *et al.*, 1997). The LAIRs are expressed on most leukocytes including haematopoeitic progenitor cells (Meyaard *et al.*, 1997, Verbrugge *et al.*, 2003). The cellular ligands for LAIR-1 and LAIR-2 were shown not to be MHC class I molecules (Meyaard *et al.*,

1997). Recently, a functional murine LAIR-1 homologue has been identified (mLAIR1) which shares a 40% sequence identity with human LAIR-1. mLAIR-1 maps to the proximal end of mouse chromosome 7, in a region syntenic with human chromosome 19 where the LRC is located (Lebbink *et al.*, 2004).

IRp60 (inhibitory receptor protein 60) is a 60 kDa glycoprotein belonging to the Ig superfamily and is characterised by the presence of 3 ITIMs in its cytoplasmic tail. It is expressed on T-cell subsets, monocytes, granulocytes and on all NK cells. Cross-linking of IRp60 by a specific mAb was shown to strongly inhibit the spontaneous cytotoxicity of NK cells as well as the NK-mediated cytolytic activity induced via different non-HLA specific (including CD16, NKp46 and NKp44) and HLA-specific activating receptors (including CD94/NKG2C, KIR2DS1 and KIR2DS4) (Cantoni *et al.*, 1999a). Different to other members of the inhibitory receptors, Irp60 maps to chromosome 17.

Siglec 7 is a 75 kDa glycoprotein expressed on most NK cells and represents a novel member of the sialoadhesin family. Siglec 7 has 3 Ig like domains and 2 conserved classical ITIM motifs in its cytoplasmic portion that can be phosphorylated and associate with SHP-1 (Nicoll *et al.*, 1999). Recently, the binding preference of Siglec 7 has been identified as α2,8-linked diasialic acids, and binding of gangliosidase-GD3, which expresses these acids, has been shown to inhibit Siglec 7 expressing NK cells (Nicoll *et al.*, 2003).

CMRF35H is expressed on most leukocytes including monocytes, neutrophils, macrophages, dendritic cells, T-cell subsets and NK cells. It is a type I membrane protein and has a long tail which contains 4 ITIMs. As such CMRF35H is thought to have an inhibitory function *in vivo* (Green *et al.*, 1998).

1.10.7 NK cell Co-receptors

Other molecules expressed at the NK cell surface have been implicated in NK cell mediated cytotoxicity however, these molecules appear to function as

co-receptors rather than as true receptors.

1.10.7.1 2B4

2B4 was first identified in mice as a surface receptor expressed by NK, $CD8^+$ and γδ-T cells (Garni-Wagner *et al.*, 1993). Subsequently, human 2B4 was identified as a 70 kDa surface molecule, which bears 4 immunoreceptor tyrosine-based switch motifs (ITSMs), and is expressed on NK cells, monocytes, granulocytes and a subset of $CD8^+$ T-cells (Valiante & Trinchieri, 1993). Cross linking 2B4 with a mAB was shown to strongly enhance NK cell mediated cytotoxicity but to have no effect on $CD8^+$ T-cell cytotoxicity (Valiante & Trinchieri, 1993). The high affinity ligand for 2B4, CD48, is a GPI-linked member of the CD2 family expressed on lymphoid and myeloid cells (Brown *et al.*, 1998). However, in 2 different studies that investigated NK clone responses to CD48 transfected and non-transfected targets, CD48 expression was found to stimulate and inhibit NK clone function (Nakajima *et al.*, 1999a, Tangye *et al.*, 2000). Moreover, activation of $2B4^+$ NK cells was shown to be restricted to those NK cells that co-expressed high levels of NKp46 (Sivori *et al.*, 2000a). The function of 2B4 however, was shown not to be entirely dependent upon NKp46 expression as $2B4^+/NKp46^{dull}$ NK clones could be activated if the first signal was provided by sub-optimal doses of anti-CD16 or anti-NKp44 monoclonal antibodies (Sivori *et al.*, 2000a). The inhibitory ability of 2B4 was thought to be mediated by competition between SHP-1 and the SH domain containing protein (SAP or SH2D1A) for association with 2B4 ITSMs; association with SAP preventing the inhibitory signal mediated by SHP-1 (Biassoni *et al.*, 2001). The importance of SAP in 2B4 signaling is supported by studies of patients with X-linked lymphoproliferative disease (XLP). In this disease, patients either lack SAP or encode a non-functional protein, and this has been correlated with a reduction in 2B4 mediated cytotoxicity. The functional mechanism of this reduced cytotoxicity has recently been shown to be a result of abrogating the interaction between SAP and Fyn-T, an Src family kinase that phosphorylates 2B4 (Chen *et al.*, 2004). However, a recent study in mice

has shown that $2B4^+$ NK cells deficient in SAP were less able to clear the $CD48^+$ tumor cells than $2B4^-$ control cells, suggesting that 2B4 inhibitory signaling occurs regardless of the presence of SAP (Lee *et al.*, 2004b).

1.10.7.2 NTB-A

NTB-A is a 60 kDa glycoprotein found on the surface of all human NK, T and B cells. The cytoplasmic tail of NTB-A contains 3 tyrosine based motifs which after phosphorylation associate with SAP, SHP-1, SHP-2 and EWS-activated transcript-2 (EAT-2). Similar to the activation mediated by 2B4, stimulation of NTB-A requires high surface expression of NCRs. Also similar to 2B4, studies of XLP patients indicate that NTB-A can mediate an inhibitory signal that is dependent on functional SAP (Bottino *et al.*, 2001). Recently, the ligand for NTB-A was identified as NTB-A. The formation of NTB-A homodimers between target and NK cells was shown to enhance NK cell cytotoxicity and IFN-γ production (Flaig *et al.*, 2004). Interestingly, this study also showed that blocking the interaction of NTB-A on target and NK cells reduced NK cell proliferation, indicating that the homophilic interaction of NTB-A between NK cells may be important for NK cell proliferation (Flaig *et al.*, 2004).

1.10.7.3 CRACC

The CD2-like receptor activating cytotoxic cells (CRACC) is widely expressed on NK, T-cells, B-cells and mature dendritic cells. Although the cytoplasmic domains of CRACC contain 2 ITSMs that enable the recruitment of SAP and EAT-2, CRACC activation may occur in the absence of SAP (Morra *et al.*, 2001). In this respect, one group found that murine CRACC was unable to associate with SAP and EAT-2, whilst another group reported human CRACC associated with SAP in a 3-hybrid yeast system (Bouchon *et al.*, 2001, Tovar *et al.*, 2002). In human NK cells, ligation of CRACC by antibodies was observed to induce NK cell mediated cytotoxicity, however in keeping with the observation that CRACC might not interact with SAP, this response was not altered in NK cells from XLP patients (Bouchon *et al.*, 2001). Recently, 2 splice variants of CRACC have been identified, CRACC-

S and CRACC-L that are expressed on NK cells constitutively. CRACC-S lacks ITSMs and transfection of rat NK cells with CRACC-S did not alter their function. In contrast CRACC-L appeared to provide NK cells with an activatory function (Lee *et al.*, 2004a).

1.10.7.4 NKp80

NKp80 is as a member of the C-type lectin superfamily and is expressed on the surface of cells as an 80 kDa homodimer (Vitale *et al.*, 2001). The transmembrane region of NKp80 was shown to be non-polar, and hence no classical ITAM-containing polypeptides were shown to associate with this protein. NKp80 is expressed by nearly all fresh or activated NK cells and was identified on large granular lymphocyte expansion derived from patients with lymphoproliferative disease of granular lymphocytes (Vitale *et al.*, 2001). Like 2B4, the magnitude of the cytolytic response of NK clones expressing NKp80 was shown to correlate with the density of NKp46 expression (Vitale *et al.*, 2001). Homologues of NKp80 have been identified in *Macaca fascicularis* and *Macaca mulatta* (NCBI protein CAD19994 and CAF22244, respectively), however the signal transduction pathway and ligands of NKp80 remain unknown.

1.10.7.5 CD59

CD59 is an 18- to 25-kD GPI anchored glycoprotein which is expressed on all human peripheral blood leukocytes, erythrocytes, and several human cell lines (Meri *et al.*, 1990). CD59 is a most commonly thought of as a complement regulatory protein because it restricts the formation of membrane attack complexes (MAC) by binding to C8a and/or C9 in the assembling membrane of the MAC and interferes with C9 membrane insertion and polymerisation. Recently, a role for CD59 in the activation of NK cells has been reported (Marcenaro *et al.*, 2003). In this study, the authors demonstrated that CD59 is physically associated with NKp46 in human NK cells and was able to associate with NKp30 following mAb cross linking of this NCR. Importantly the authors showed that binding of CD59

with a CD59 mAb resulted in tyrosine phosphorylation of the CD3ζ chains associated with NKp30 or NKp46. This suggested that the stimulatory function of CD59 required direct interaction with the NCR signal transduction pathway. The authors found no association between CD59 and NKp44.

1.11 Immunomodulation by HCMV

HCMV is a large, complex DNA virus encoding many ORFs that express proteins dispensable for productive replication, suggesting that they may play a role during infection of the host. A significant number of these genes have been shown to modulate the host immune responses (Table 1.3).

1.11.1 Modulation and mimicry of the cell cycle and apoptosis

ppUL69 and pp71 are capable of imposing a G1/S-like block that dysregulates the expression of many cell cycle regulated gene products. Delivery of ppUL69 within the HCMV virion has been shown to impose a G1 block that is independent of synthesis of additional ppUL69 encoded by the infecting viral genome whereas pp71 accelerates progression through the G1 phase and activates the immediate early gene, IE2 (Hayashi *et al.*, 2000, Kalejta & Shenk, 2003). Activation of the major immediate early enhancer-promoter has been shown to be synergistically enhanced by binding to the coiled-coil region of Daxx in the PML nuclear bodies (Hofmann *et al.*, 2002, Ishov *et al.*, 2002). pp71 has also been shown to dysregulate the cell cycle by targeting for degradation members of the retinoblastoma family of proteins (p105, p107 and p130), which are general repressors of cell proliferation, (Kalejta & Shenk, 2003).

Four main HCMV gene products have been implicated in blocking apoptosis (IE1, IE2, pUL36/vICA and pUL37x1/vMIA). IE1 and IE2 have been shown to inhibit apoptosis by activating the PI3 kinase pathway via the cellular

Table 1.3 HCMV Genes and Proteins Involved in Modulation and Mimicry of the Immune Response

Target of Modulation and Mimicry	Gene Name	Protein	Function
Cell Cycle and Apoptosis	*UL69*	ppUL69	Imposes G1 block on cell cycle
	UL82	pp71	Dysregulates expression of cell cycle proteins.
	UL123	IE1	Activates PI3 kinase pathway, inhibiting apoptosis.
	UL122	IE2	Activates PI3 kinase pathway, inhibiting apoptosis. Leads infected cells into senescence.
	UL36	vCIA	Inhibitor of caspase activation, inhibiting Fas mediated apoptosis.
	UL37	vMIA	Inhibitor of mitochondrial membrane permeabilisation.
Leukocyte Behaviour	*US28*	gpUS28	Chemokine receptor. Binds CC chemokines and fractalkine. May assist viral dissemination.
	TRL11	gpTRL11	IgG Fc receptor (function unclear)
	UL119-118	gpUL119-118	IgG Fc receptor. (function unclear)
	UL111a	gpUL111a	IL10 homologue. Reduces MHC class I/II expression. Regulates lymphocyte proliferation.
	UL146	vCXC1	Viral CXC chemokine homologue. Potency similar to IL8
	UL147	UL147	Viral CXC chemokine(?). Function unclear.

Table 1.3 continued

Target of Modulation and Mimicry	Gene Name	Protein	Function
Cellular Immune system	*US2*	gpUS2	Down regulation of MHC class I molecules. Degrades MHC class I heavy chains.
	US3	gpUS3	Down regulation of MHC class I molecules. Retains class I heavy chains in the ER.
	US6	gpUS6	Down regulation of MHC class I molecules. Blocks TAP dependent translocation of proteins across the ER.
	US11	gpUS11	Down regulation of MHC class I molecules. Degrades MHC class I heavy chains.
	UL16	gpUL16	Bind MICB, ULBP1 and ULBP2, preventing NKG2D triggering NK cell activation.
	UL18	gpUL18	Binds LIR1. Ligand to HLA-G
	UL40	gpUL40	Leader sequence peptide stabilises HLA-E, which binds to CD94/NKG2A, inhibiting NK cell lysis.
	UL83	pp65	Abrogates IE1 antigen presentation.

kinase Akt (Yu & Alwine, 2002). Additionally, IE2 appears to lead infected cells into senescence after arresting the cell cycle and inhibiting apoptosis, and this is thought to occur through the p16 (INK4a) and p53 pathways (Noris *et al.*, 2002). UL36 encodes a viral inhibitor of caspase activation (vICA), which has been shown to protect cells from extrinsic apoptosis induced via the death receptors TNFR1, Fas/CD95 or Trail. Expression of vCIA inhibits Fas-mediated apoptosis by binding to the pro-domain of caspase-8 and preventing its activation. Interestingly, vCIA has no sequence homology with other suppressors of apoptosis, including the FLICE-ligand inhibitory proteins (FLIPs) with which its behaviour shares most homology (Skaletskaya *et al.*, 2001b). HCMV also encodes a viral mitochondrial inhibitor of apoptosis (vMIA) (encoded by the UL37 gene). This protein was initially thought to be targeted to, and inhibit the adenine nucleotide translocator (ANT), one of the key components of the permeability transition pore complex (PTPC), which controls the release of cytochrome c during apoptosis (Vieira *et al.*, 2001). Despite the similarity in behavior of vMIA with the anti-apoptotic members of the Bcl-2 family, vMIA was recently shown to inhibit apoptosis associated with PTPC by a different mechanism. In 2 independent studies, vMIA was shown to bind with Bax in the mitochondria forming a vMIA – Bax complex, preventing Bax-mediated mitochondrial membrane permeabilisation (Arnoult *et al.*, 2004, Poncet *et al.*, 2004).

1.11.2 Modulation and Mimicry of Leukocyte Behaviour

1.11.2.1 Receptors

HCMV encodes 4 G-protein-coupled 7-TM receptor (GPCR) homologue genes – US27, US28, UL33 and UL78 (Chee *et al.*, 1990b, Margulies *et al.*, 1996). Both US27 and US28, are homologues of human chemokine receptors and the product of US28, but not US27 binds to several CC chemokines, including RANTES, MIP1α, MCP1 and fractalkine (Gao & Murphy, 1994, Kledal *et al.*, 1998, Neote *et al.*, 1993). The interaction between US28 and the membrane bound chemokine fractalkine were shown to be strong enough to mediate adhesion of virus infected cells to the cell

surface (Kledal *et al.*, 1998). These strong binding characteristics were thought to demonstrate a role for US28 as a chemokine sink, reducing or eliminating inflammatory chemokines from the vicinity of the virus thus preventing attraction of leukocytes and reducing the intensity of the immune response (Haskell *et al.*, 2000). However, studies by Streblow *et al* (1999) demonstrated that arterial smooth muscle cell (SMCs) infected with HCMV could migrate further than control cells, and this could be reversed by knocking out US28 (Streblow *et al.*, 1999). These data have given rise to a model of CMV infected cell migration towards sites of inflammation as a means of virus dissemination. Recently, a role for US28 in co-ordinating viral gene expression through the constitutive activation of the transcription factors NFAT (nuclear factor of activated T cells) and CREB (cAMP response element binding protein) have been proposed (McLean *et al.*, 2004).

There is no evidence that US27, UL33 or UL78 are able to bind chemokines. Indeed, fluorescence microscopy has shown that whilst US27 and UL33 are expressed at the surface of the cell, the majority of these proteins are localised intracellularly, possibly correlating to endocytic organelles and may be incorporated into the viral membrane during the final stages of virus assembly (Fraile-Ramos *et al.*, 2002). Little work has been carried out to characterise the UL78 gene or protein. However, recent data indicates that the UL78 gene is conserved in a number of clinical isolates and is dispensable for growth in culture (Michel *et al.*, 2005).

HCMV expresses 2 separate IgG Fc receptors during infection of cultured fibroblasts. One is a 33-34 kDa membrane glycoprotein encoded by the TRL11 gene and can be detected as early as 12 hours p.i (Lilley *et al.*, 2001). The second is a 68 kDa glycoprotein encoded by UL119 – UL118 (Atalay *et al.*, 2002). Neither of these gene products exhibits significant amino acid identity with each other or any of the host or other herpesvirus encoded Fc receptors.

1.11.2.2 Cytokines and Chemokines

The HCMV gene UL111A encodes a viral homologue of IL10 (v-IL10) that shares only 25% amino sequence identity with human IL10 (Kotenko *et al.*, 2000). v-IL10, recovered from infected cell supernatants and *Escherichia coli*-derived recombinant v-IL10 were both shown to inhibit proliferation of, and cytokine synthesis by, mitogen stimulated peripheral blood mononuclear cells (PBMC) with the potency of human IL10. Also analogous to human IL10, v-IL10 was shown to reduce cell surface expression of MHC class I and MHC class II on PBMC (Spencer *et al.*, 2002).

The HCMV gene UL146 encodes a CXC chemokine (vCXC1) (Penfold *et al.*, 1999). vCXC1 is a 117 amino acid glycoprotein, that when secreted has been shown to attract neutrophils, inducing calcium mobilisation and degranulation with potency approaching that of human IL8. This is perhaps surprising considering the only conserved sequence between these 2 proteins is an amino terminal ELRCXC motif and similar spacing of 2 additional Cs that form disulphide bridges characteristic of all chemokines (Penfold *et al.*, 1999). Additionally, the sequence of vCXC1 in different virus strains varies immensely, although the critical ELRCXC motif and the additional Cs are preserved (Stanton *et al.*, 2005). Recombinant vCXC1 indicates that *in vivo* vCXC1 could modulate the behavior host cells bearing the CXC cognate receptor CXCR2, which includes mononuclear cells and progenitors that are potentially important for the spread of the virus. Adjacent to UL146 is UL147, also thought to encode a CXC chemokine although little information is known about this protein (Penfold *et al.*, 1999). Although present in all clinical isolates, UL146 and UL147 are absent from strains of AD169 (Figure 1.5).

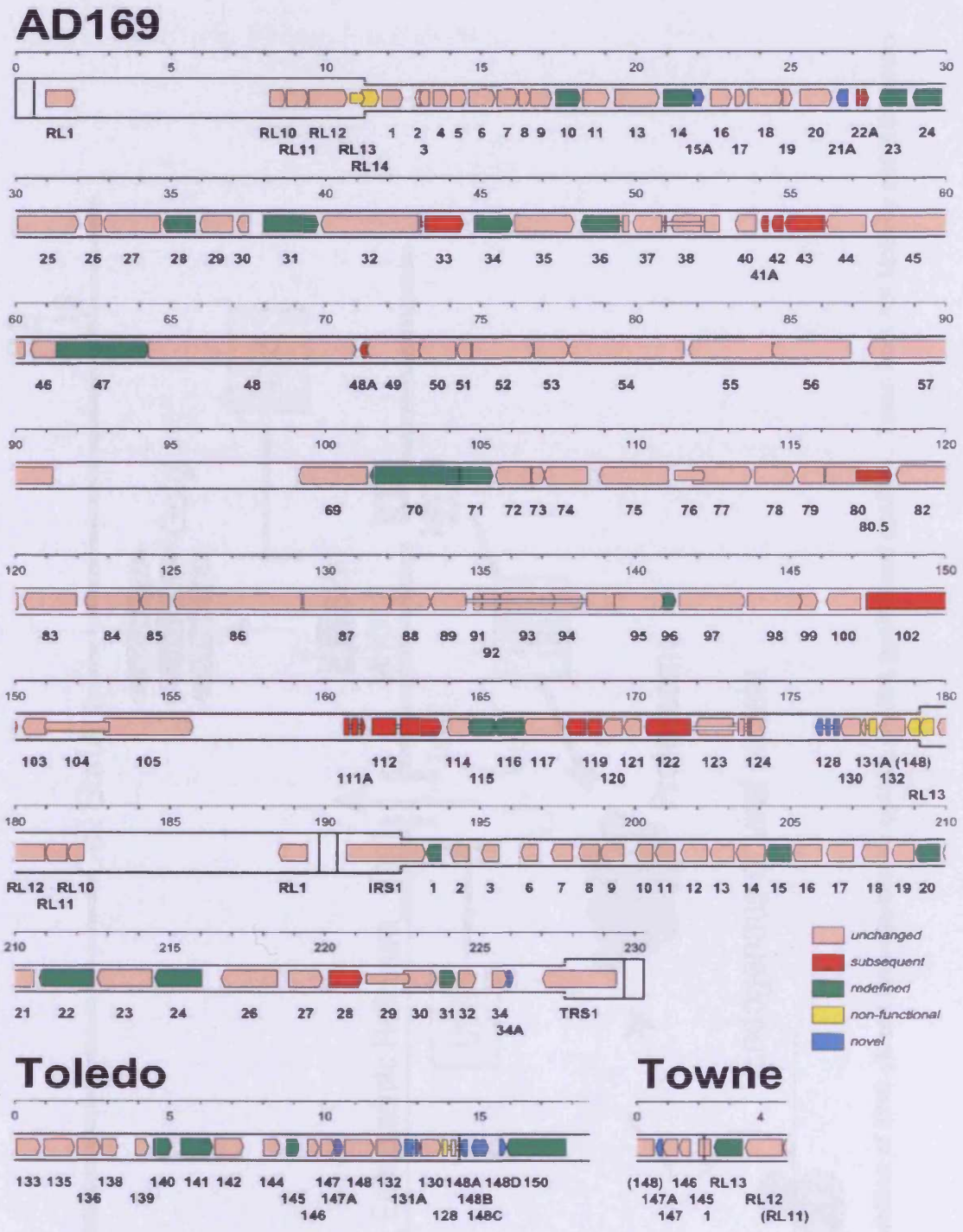

Figure 1.5 Consensus genetic Map of laboratory strains of HCMV. Protein coding regions are indicated by coloured arrows, introns are shown as narrow white bars. Colours indicate gene status in comparison with layouts deduced by Chee *et al* (1990) and Cha *et al* (1996) (Chee *et al.*, 1990) (Cha *et al.*, 1996). Taken from (Davison *et al.*, 2003).

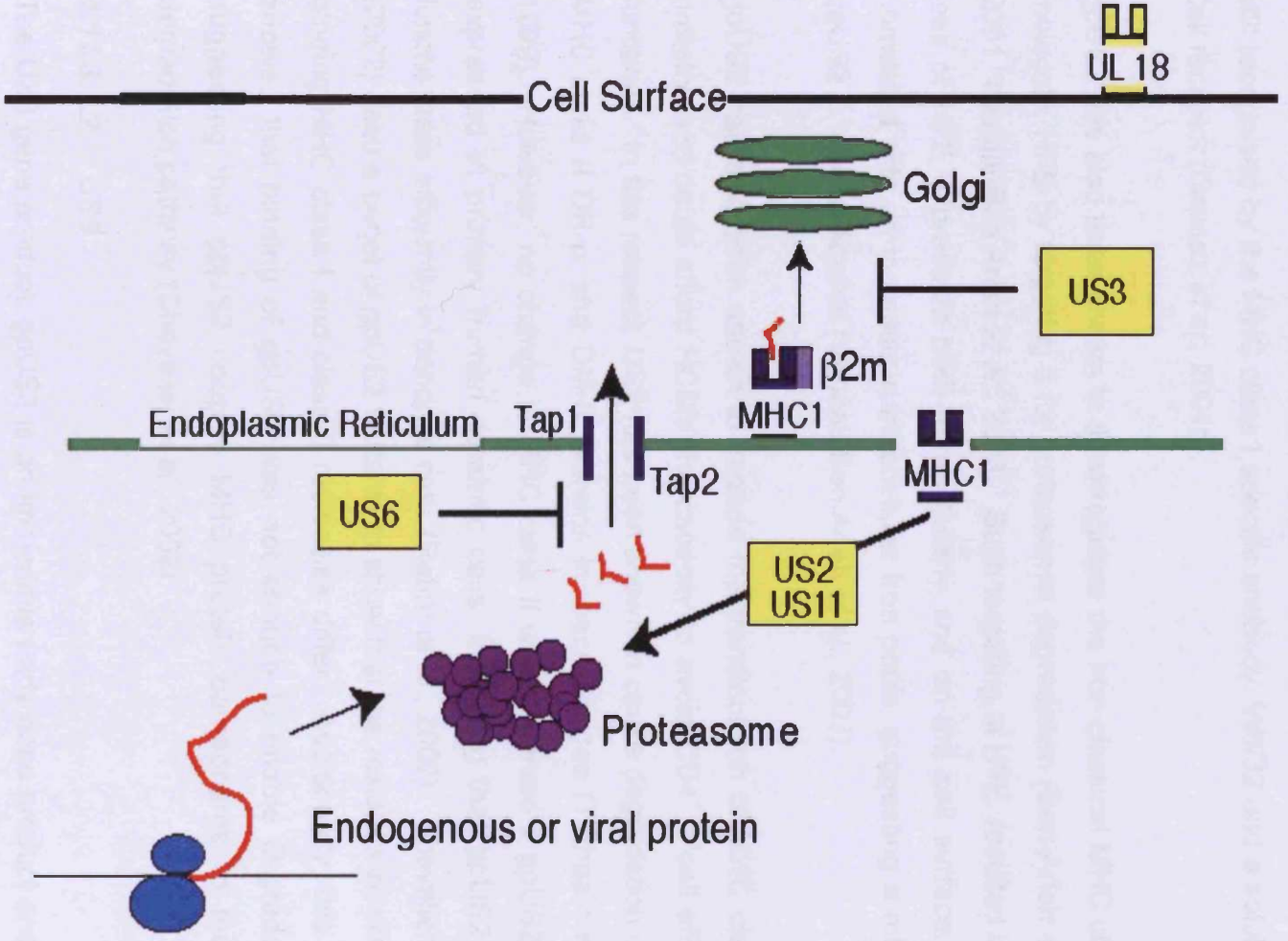

Figure 1.6 **Inhibition of MHC class I Presentation by gpUS2, gpUS3, gpUS6 and gpUS11.** Taken from A.K McElroy and S.S Morello.

class I occurs when gpUS2 and MHC class I are bound, it was not found to be required during the initial step of US2-mediated dislocation (Furman *et al.*, 2003). gpUS2 can differentiate between products of the MHC class I loci, tightly binding HLA-A2 allotypes but not HLA-B7, HLA-B27, HLA-Cw4 and HLA-E. Binding is thought to occur in a 1:1 stoichiometry, without altering the conformation of MHC class I; conjugates of US2 and MHC class I were still recognised by the MHC class I specific antibody, W6/32 and a soluble T cell receptor (Gewurz *et al.*, 2001b).

gpUS2 has also been shown to downregulate the non-classical MHC class I molecule, HFE by targeting it for proteosomal degradation (Ben-Arieh *et al.*, 2001, Vahdati-Ben Arieh *et al.*, 2003). Such targeting of HFE, resulted in the loss of HFE expression both intracellularly and on the cell surface, and correlated with an increase in intracellular iron pools, suggesting a role for gpUS2 in viral replication (Vahdati-Ben Arieh *et al.*, 2003).

gpUS2 has also been shown to impede the translocation of MHC class II proteins and could afford HCMV the capacity to avoid $CD4^+$ T cell effector functions. In this respect, US2 has been shown to cause degradation of the MHC class II DR-α and DM-α proteins in macrophages (Tomazin *et al.*, 1999). However, no change in MHC class II was observed if gpUS2 was expressed in primary human dendritic cells, indicating that gpUS2 may function less efficiently in dendritic cells (Rehm *et al.*, 2002). Chevalier *et al* (2002) used a panel of gpUS2 mutants to show that the residues involved in binding MHC class I and class II molecules differ. Additionally, this work showed that binding of gpUS2 was not enough to enable degradation, suggesting that gpUS2 couples MHC protein components to the ER degradation pathway (Chevalier *et al.*, 2002).

1.11.3.1.2 US3

The US3 gene product, gpUS3 is an immediate early gene product and was the first IE gene product to be identified having a function other than

transcription regulation (Ahn *et al.*, 1996, Jones *et al.*, 1996). gpUS3 resides in the endoplasmic reticulum (ER) where it associates with *de novo* synthesised MHC class I heavy chains complexed with β_2m preventing their egress from the endoplasmic reticulum (ER) to the Golgi apparatus without interfering with peptide loading (Ahn *et al.*, 1996, Jones *et al.*, 1996). gpUS3 is capable of downregulating surface expression of classical class I molecules (HLA-A, HLA-B and HLA-C) and has been implicated in the downregulation of the non-classical HLA-G molecule (Jun *et al.*, 2000). An ER retention signal in the luminal domain of gpUS3 maintains gpUS3 in the ER, however, the transmembrane domain is required for association with MHC class I molecules (Lee *et al.*, 2003). Following the observation that gpUS3 only associates transiently with MHC class I, Gruhler showed that a fraction of gpUS3 is trafficked to the Golgi where it is degraded, most likely by lysosomes (Gruhler *et al.*, 2000). In this respect it is interesting that 3 differentially spliced transcripts of US3 have been identified (unspliced, singularly spliced and doubly spliced). Only the unspliced transcript was shown to encode an MHC class I modulatory protein; the singularly spliced transcript was processed through the secretory pathway and the doubly spliced transcript localised to the Golgi apparatus (Liu *et al.*, 2002). Recently, gpUS3 has been shown to bind to and inhibit tapasin-dependent peptide loading of MHC class I molecules (Park *et al.*, 2004). As tapasin is only required for peptide loading of certain MHC class I molecules, this may explain why gpUS3 does not affect the expression of all MHC class I molecules equally (Park *et al.*, 2004).

gpUS3 has also been associated with inhibiting recognition of antigen by $CD4^+$ T cells (Hegde *et al.*, 2002). In this study, gpUS3 was found to interfere with the association of MHC class II $\alpha\beta$ complexes with the ER, hindering the sorting of MHC class II into the loading compartment and thus reducing the formation of peptide loaded MHC class II complexes.

1.11.3.1.3 US6

Transcription of US6 is detected a few hours p.i and reaches a maximum by 72 p.i in the late phase of viral replication (Hengel *et al.*, 1997). US6 encodes, gpUS6, a 21 kDa type 1 transmembrane protein with 1 N-linked glycosylation site (Hewitt *et al.*, 2001). Intracellular staining for gpUS6 demonstrates an ER-like localization pattern similar to transporter associated with antigen processing (TAP1/2) which gpUS6 was also shown to associate with (Hengel *et al.*, 1997, Lehner *et al.*, 1997). TAP1/2 is responsible for the translocation of proteins across the ER. The association of the ER luminal domain of gpUS6 with the ER luminal loops of TAP1/2 transmits a signal across the membrane to the nucleotide binding domains of TAP1, preventing binding of ATP by TAP1 and therefore protein translocation (Hewitt *et al.*, 2001, Kyritsis *et al*,. 2001). Through this mechanism, gpUS6 has been shown to inhibit expression of both classical and non-classical MHC class I molecules. The α-helical content of the protein was shown to be essential for this function of gpUS6, however, glycosylation was not required (Jun *et al.*, 2000). In HCMV-infected cells, IFNγ both inhibited gpUS6 synthesis and stimulated TAP1/2 gene expression to promote restoration of peptide translocation (Benz & Hengel, 2000).

1.11.3.1.4 US11

The product of US11, gpUS11 is a 32 kDa, ER resident, type I membrane glycoprotein which is expressed at early and late times during the viral cycle and is dispensable for growth and replication *in vitro* (Jones *et al.*, 1995b, Jones *et al.*, 1991, Wiertz *et al.*, 1996a). In US11^{+} cells, MHC class I heavy chains were shown to be degraded with a half-time of less than 1 minute and experiments with inhibitors indicated that the proteosome was the key protease involved in the degradation of MHC class I (Wiertz *et al.*, 1996a). gpUS11, like gpUS2 is capable of destabilizing HLA-A and HLA-B locus products but not heavy chains encoded by HLA-C and HLA-G alleles (Schust *et al.*, 1998). Extension of a chimeric HLA-G construct with the tail residues of HLA-A2 accounted for the constructs sensitivity to gpUS11 mediated

degradation thus, degradation of HLA alleles by gpUS11 was shown to rely on the presence of essential cytosolic tail residues within the HLA alleles (Barel *et al.*, 2003).

In contrast to US2, the α1/α2 domains of MHC class I heavy chains have been identified as being important for affinity of gpUS11 (Barel *et al.*, 2003). Additionally, gpUS11 truncation experiments have indicated that the transmembrane domain of gpUS11 is required to trigger dislocation of MHC class I heavy chains, indicating that gpUS2 and gpUS11 utilise different targeting mechanisms for MHC class I degradation (Furman *et al.*, 2002). Recently, the α helical domain of gpUS11 has been shown to form interhelical hydrogen bonds within the ER and this has been shown to be essential for mediating MHC class I heavy chain dislocation (Lilley *et al.*, 2003). Shamu *et al* (1999) found that heavy chains are ubiquitinated before they are degraded and that ubiquitinated MHC class I heavy chains are associated with membrane fractions, suggesting that ubiquitination occurs while the heavy chain is still bound to the ER membrane (Shamu *et al.*, 1999). In experiments using hamster cells, disruption of the E1 ubiquitin activating enzyme resulted in an accumulation of MHC class I heavy chain/gpUS11 complexes in the ER membrane, suggesting that ubiquitination is a prerequisite for gpUS11-dependent dislocation from the ER to the cytosol (Kikkert *et al.*, 2001). Later experiments indicated that polyubiquitination is required for gpUS11 dependent dislocation and suggested that polyubiquitination prevents the luminal domain of MHC class I heavy chains from moving back into the ER lumen (Shamu *et al.*, 2001). Recently, Derlin-1, the human homologue of yeast Der1p, a protein essential for the degradation of a subset of misfolded ER proteins has been shown to be essential for degradation of MHC class I molecules by gpUS11 and not gpUS2 (Lilley & Ploegh, 2004). This protein was shown to be a central component of p97-interacting membrane protein complex in the ER which links recognition of substrate and dislocation through p97 ATPase (Ye *et al.*, 2004).

1.11.3.2 Modulation of IE1 Specific CTL Function

Early data showed that most IE1 specific CTL clones were able to lyse HCMV infected autologous fibroblasts (Borysiewicz *et al.*, 1988). However, a small number of clones that lysed vac/IE1 targets did not lyse autologous HCMV infected fibroblasts, suggesting that some but not all IE1 epitopes presented were presented by strain AD169 infected cells (Borysiewicz *et al.*, 1988). A study by Gilbert *et al* (1996) showed that the tegument protein, pp65 abrogated IE peptide presentation on the surface of HCMV infected cells, rendering them refractory to IE1 specific T-cell lysis (Gilbert *et al.*, 1996b). In this study, the authors used vaccinia virus vectors to express pp65 (vac/pp65) and IE1 (vac/IE1) proteins in skin fibroblasts and showed that co-expression of vac/IE1 and vac/pp65 reduced the specific lysis of IE1 but not pp65 specific CTL clones in an autologous setting. As pp65 expression has been shown to result in the phosphorylation of several CMV proteins, the authors investigated whether pp65 could modify IE1 by phosphorylation (Britt & Auger, 1986). A recombinant vaccinia virus containing a pp65 mutant, lacking the carboxyl terminus of pp65, which contains conserved sequences of serine/threonine kinases, was therefore constructed (vac/Δpp65). Expression of full-length pp65, but not the Δpp65 mutant was shown to result in increased threonine-phosphorylation of IE1. Co-infection of fibroblasts with vac/Δpp65 but not vac/pp65 and vac/IE1 were recognised by IE1 specific CTL. From these data, the authors concluded that the IE1 specific CTL were unable to lyse cells expressing IE1 because pp65 phosphorylated IE1 threonine residues thus interfering with IE1 peptide presentation.

1.11.3.3 Modulation of NK Cell Cytotoxicity

1.11.3.3.1 UL18

Analysis of the sequence of strain AD169 identified the gene product of UL18, gpUL18, as encoding a type I transmembrane glycoprotein that exhibited sequence homology to MHC class I molecules of higher organisms

(Beck & Barrell, 1988). gpUL18 was subsequently shown to bind β_2M and peptide. However, unlike host MHC, gpUL18 is heavily glycosylated, containing 13 potential N-linked glycosylation sites, in contrast to the 1 - 3 sites found on host MHC class I (Beck & Barrell, 1988). Also, in contrast to host MHC class I, gpUL18 was shown to only bind with the leukocyte immunoglobulin like receptor, LIR1 (Cosman *et al.*, 1997). The crystal structure of LIR-1 indicates that binding to gpUL18 is mediated through similar interfaces as LIR-1 uses to interact with MHC class I molecules (Willcox *et al.*, 2003). LIR1 is widely expressed on monocytes and B cells but only on a minority of NK cells and was found to bind gpUL18 with a >1000 fold higher affinity than classical MHC class I molecules (Cosman *et al.*, 1997). This suggested that even at low levels of expression, gpUL18 could compete with host MHC class I for binding to LIR1 (Chapman *et al.*, 1999). The biological role for gpUL18, however, remains undefined. Initially, based on the similarity between gpUL18 and MHC class I molecules Fahnestock *et al* (1995) proposed that the viral glycoprotein could function as a molecular decoy for NK cells, inhibiting NK cell lysis (Fahnestock *et al.*, 1995). A number of subsequent studies have investigated this as a function of gpUL18. The first study, carried out by Reyburn *et al* (1997) indicated that gpUL18 could be as effective at inhibiting NK cell lysis as MHC class I, and that this inhibition was likely to be mediated through an interaction with CD94 (Reyburn *et al.*, 1997). However, subsequent studies demonstrated that gpUL18 could not protect target cells from lysis by CD94/NKG2A$^+$ NK clones (Tomasec *et al.*, 2000). In another study, gpUL18 expressing transfectants were found to enhance NK cell killing compared to control cells (Leong *et al.*, 1998).

1.11.3.3.2 UL16

The UL16 gene product, gpUL16, is a 50 kDa glycoprotein expressed from 24 hours p.i through to the late stages of virus's replication cycle. UL16 has been shown to be dispensable for viral growth *in vitro* (Kaye *et al.*, 1992). A role for gpUL16 in the subversion of the NK cell cytotoxic response was first

proposed by Cosman *et al* (2001) (Cosman *et al.*, 2001). Soluble gpUL16 is able to block the interaction of the activating receptor, NKG2D with its cognate ligands, UL16 binding proteins (ULBPs) and the MHC class I related protein (MIC) B. Subsequently, infection of fibroblasts with HCMV was shown to induce expression of all known NKG2D ligands, although MICA and ULBP3 were unable to reach the cell surface (Welte *et al.*, 2003). Retention of MICB was later shown to be mediated by binding of gpUL16 to MICB, preventing it from maturing and exiting the secretory pathway (Wu *et al.*, 2003). In the same year, a number of studies demonstrated that gpUL16 was capable of protecting cells from NK lysis. Addition of soluble gpUL16 was shown to competitively inhibit NKG2D activation by ULBPs and cells infected with a wild-type HCMV showed significant resistance to cytotoxic proteins, whereas infection with a UL16 deletion mutant increased susceptibility to NK lysis by as much as 70% (Odeberg *et al.*, 2003, Rolle *et al.*, 2003, Vales-Gomez *et al.*, 2003). Odeberg *et al* (2003) proposed a different mechanism for gpUL16 modulation of NK cell lysis, speculating that gpUL16 either with additional proteins or by itself can neutralise the action of cytolytic peptides in several different ways: (1) by preventing pore formation in the cell membrane, (2) by blocking the binding of pore forming proteins to the cell membrane, (3) by degrading the cytolytic proteins inside the cell, or (4) by inhibiting the intracellular signaling pathways induced by cytolytic proteins that cause apoptosis of the target cell (Odeberg *et al.*, 2003).

1.11.3.3.3 UL40

The HCMV UL40 gene is thought to inhibit NK cell activity indirectly via the inhibitory receptor CD94/NKG2A (Tomasec *et al.*, 2000). Binding of CD94/NKG2A to the non classical MHC class I molecule, HLA-E, was shown to deliver an inhibitory signal to the NK cell (Braud *et al.*, 1998, Lee *et al.*, 1998b). However, efficient surface expression of HLA-E requires the attachment of nonapeptides cleaved from the N-terminal of HLA-A, B and C molecules (Braud *et al.*, 1997). During HCMV infection, MHC class I is efficiently down regulated by proteins encoded within the US2 – US11

region, a mechanism which is thought to inhibit lysis by cytotoxic T cells but which through logical extension of the missing self hypothesis, has been proposed to facilitate NK cell mediated lysis (Hengel *et al.*, 1995, Ploegh, 1998). Degradation of MHC class I proteins may thus diminish the pool of nonapeptides required for efficient cell surface expression of HLA-E. Additionally, efficient cell surface expression of HLA-E requires that class I leader peptides are loaded onto HLA-E in a transporter associated with antigen processing (TAP) dependent manner, a pathway that is blocked by HCMV gpUS6 (Lee *et al.*, 1998a, Lehner *et al.*, 1997). To combat the downregulation of HLA-E, which would render HCMV infected cells susceptible to CD94/NKG2A^{+} NK cell lysis, the virus encodes an early protein (gpUL40) bearing a nonapeptide identical to that of HLA-C (VMAPRTLIL) (Tomasec *et al.*, 2000). Furthermore, this UL40-derived peptide is loaded onto HLA-E in a TAP independent manner, thus bypassing the inhibitory function of gpUS6 (Ulbrecht *et al.*, 2000). HCMV UL40 thus affords presentation of HLA-E on infected cells despite the depletion of cellular MHC class I molecules.

The potential role of gpUL40 in protection of HCMV infected cells from NK cell lysis is still debated. Wang *et al* (2002) showed that blocking of CD94 on NK cells rendered strain AD169 infected fibroblasts more susceptible to NK lysis and that an AD169 deletion mutant for UL40 was unable to protect infected cells from CD94^{hi} NK cell lysis (Wang *et al.*, 2002). In contrast, Carr *et al* (2002) demonstrated that CD94 expression could not alter the ability of NK clones to lyse HCMV infected cells, suggesting that there was no correlation with NK receptor expression and susceptibility to HCMV strain AD169 infected cells to NK killing (Carr *et al.*, 2002). While Falk *et al* (2002) showed that the activation of NK cells induced by MHC downregulation was dominant over the UL40 effect (Falk *et al.*, 2002).

1.12 AIMS OF THE PROJECT

HCMV encodes numerous factors that modulate the adaptive and innate cellular immune response. The aim of this project was to investigate the mechanisms underlying these immunomodulatory functions, particularly:

1. Modulation of IE1 specific CTL responses by pp65. This was first reported in 1996, but no further characterisation of this effect has been performed. The initial aim of this project was to culture IE1 and pp65 peptide specific CTL and confirm whether expression of pp65 abrogated recognition of IE1 by IE1 specific CTL and to investigate the mechanisms used by pp65 to interfere with IE1 antigen presentation.

2. Development of a novel method of NK cloning to allow the dissection of NK responses to different strains of HCMV and different immunomodulatory functions encoded by HCMV.

3. Investigation of the impact of HCMV infection on the frequency and numbers of NK cell subsets.

4. Comparison of NK responses to different strains of HCMV using polyclonal and NK clones in an attempt to identify novel NK immunomodulatory functions.

MATERIALS AND METHODS

2 METHODS

2.1 SOLUTIONS

Ampicillin solution — Ampicillin (Roche, Germany) was dissolved in H_2O at 50 mg/ml before being filter sterilised through a 0.22 µm filter and stored in aliquots at -20°C.

Bicarbonate Buffer — 2% (w/v) sodium bicarbonate in H_2O.

DABCO — 2% (w/v) 1,4-Diazabicyclo[2.2.2]octane (DABCO), 10% (v/v) phosphate buffered saline (PBS) (Oxoid, Hampshire) in glycerol.

DNA loading buffer — Orange G (5x)
30% glycerol in 2X TBE with 2.5 mg/ml Orange G loading dye.

Bromophenol Blue
50% glycerol in 2X TBE with bromophenol blue (0.025%) and xylene cyanol FF (0.05%).

Drop Out Supplement (10X)
0.64 g if DO supplement (either –Leu/-Trp or –Leu) was dissolved in 100 ml of H_2O. DO supplements were used in SD medium and agar in the Yeast Two Hybrid assay. The DO supplement selects for transformants that have the ability to make the essential amino acids that are missing from the supplement.

Freezing Mixture — 90% (v/v) Foetal Calf Serum (FCS) (Invitrogen, Netherlands) mixed with 10% dimethyl sulphoxide (DMSO) filtered through a 0.2 µm filter.

Kanamycin solution Kanamycin (Roche) was dissolved in H_2O at 50 mg/ml before being filter sterilised through a 0.22 µm filter and stored in aliquots at -20°C.

Lauria Bertani (LB) agar

As for LB but additional agar (Oxoid) was added to 1.5% (w/v). Ampicillin or Kanamycin was added to 50 µg/ml if required when the broth had cooled to 50°C.

LB broth 1% (w/v) tryptone (Oxoid), 1% (w/v) NaCl, 0.5% (w/v) yeast extract (Oxoid) were mixed in H_2O and autoclaved. Ampicillin or Kanamycin was added at 100 µg/ml if required when the broth had cooled to 50°C.

LB X-gal plates 40µl of X-gal solution was spread on pre-warmed LB agar plates and allowed to dry at room temperature.

Lithium Acetate solution

100 mM lithium acetate. Adjusted to pH 7.5 with dilute acetic acid and autoclaved.

MACS® Buffer 0.5% (w/v) Bovine Serum Albumin (BSA), 2 mM EDTA in PBS pH 7.2. Degassed prior to use by leaving to stand for 1 week.

o-Nitrophenyl-β-D-Galactopyranoside (ONPG)

4 mg/ml ONPG in Z buffer. Adjusted to pH 7.0. Prepared fresh before each use.

PBS 8% (w/v) NaCl, 0.2% (w/v) potassium chloride, 0.12% (w/v) anhydrous sodium hydrogen phosphate at pH 7.4.

PBS-T	PBS, 0.1% (v/v) Tween-20.
PEG/LiAc solution	40% (v/v) polyethylene glycol (PEG), 1 ml 10X Tris-EDTA (TE) buffer, 1ml 10X LiAc mixed in 10 ml H_2O. Prepared fresh just prior to use.
SD Medium	26.7 g Minimal SD Base (Clontech) was dissolved in H_2O and 100 ml sterile 10X Dropout (DO) supplement was added. The total volume was adjusted to 1 litre.
SD Selective Plates	46.7 g Minimal SD Agar Base was dissolved in H_2O and 100 ml sterile 10X Dropout (DO) supplement was added. The total volume was adjusted to 1 litre.
SOC	A mix of 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl and 2.5 mM KCl was autoclaved and 0.22 µm filter sterilised 2M $MgCl_2$ and 1M glucose added to a final concentration of 10 mM and 20 mM, respectively.
Superbroth	3% (w/v) tryptone, 2% (w/v) yeast extract, 1% (v/v) 3-N-morpholinpropanesuphonic acid was made up in H_2O.
TBE (10X)	108 g Tris, 55 g boric acid dissolved in deionised H_2O to a final volume of 920 ml and then 80 ml 0.5 M EDTA pH 8.0 added.
TE	10 mM Tris (pH 7.5), 1 mM EDTA sterilized and DNAases inactivated by autoclaving.

X-gal solution (40%)

40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 100 ml N, N-dimethyl formamide (DMF).

X-gal solution (20%)

20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in 100 ml DMF. For use in Yeast Two Hybrid.

YPD Agar — 70 g YPD Agar Medium (Clontech) was dissolved in H_2O and adjusted to pH 6.5. After cooling, glucose was added to a final concentration of 2% and total volume adjusted to 1 litre.

YPD Medium — 50 g YPD Medium (Clontech) was dissolved in H_2O and adjusted to pH 6.5. After cooling, glucose was added to a final concentration of 2% and total volume adjusted to 1 litre.

Z buffer — 16.1 g $Na_2HPO_4 \cdot 7H20$, 5.50 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO4 \cdot 7H2O$. Total volume adjusted to 1 litre and pH adjusted to pH 7.0. Autoclaved.

All reagents were obtained from Sigma-Aldrich Co. Ltd., Poole, or Fisher Scientific Ltd., Loughborough unless otherwise stated.

2.2 SUBJECTS

All subjects were normal, healthy individuals. HCMV serology was determined by measurement of IgG antibody levels to HCMV in plasma using the AXSYM HCMV IgG assay (Abbott Laboratories, Berkshire). The presence of 15 AU/ml of sample was indicative of past or current infection with HCMV, values lower than 15 AU/ml were considered negative for IgG antibodies to HCMV. HCMV serology testing was carried out by public health laboratories (PHLS), Cardiff. HLA-typing was performed by the Welsh Blood Transfusion Service (Rhydlafa, Llantrisant) or by mAb staining with mA2.1 (American Type Culture Collection, ATCC). The main subjects used in this study are summarised in Table 2.1.

2.3 MEDIA

2.3.1 Tissue Culture Media

Cell cultures were grown in either Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen), Dulbecco's modified Eagle Medium (DMEM) (Invitrogen), or CellGro Stem Cell Growth Media (SCGM) (Cell Genix) supplemented with L-glutamine (2 mM) (Invitrogen), penicillin (1 x 10^5 IU/ml) (Invitrogen), streptomycin (100 mg/ml) (Invitrogen), and either 10% (v/v) FCS, or 5 or 10% (v/v) human AB Serum. Media was warmed to 37°C before use. Different media were used in different experiments and shall be referred to in the text. A list of media used with definitions and varying serum constituents is given in Table 2.2.

Serum-free media, referred to as either RPMI-wash or DMEM-wash, and PBS were used to wash cells where necessary.

Table 2.1 List of Donors

HCMV Seronegative†					HCMV Seropositive†					
Donor	HLA-types				Donor	HLA-types				
D1	A*02				D3	A*11		Bw60	Bw62	
D4	A*02				D7	A*02	A*24	B*44		
D5	A*02				D8	A*02				
D6	A*02				D9	A*01	A*24	B*44	B*14	
D12	ND				D10	ND				
D13	A*03	A*11 C*06	B*07 C*07	B*37	D11	A*02	A*24	B*57		
D14	A*02	A*03	B*51	B*07	D18	ND				
D15	ND				D21	A*29 Cw4	A*31 Cw16	B*44	B*51	Bw4
D16	A*02	A*29 C*03	B*08 C*07	B*40	D30	A*01	A*25	B*08	B*39	
D19	A*02	A*03 C*05	B*44 C*08	B*14	D31	A*02				
D20	ND				D32	A*02		B*41	B*55	
D22	ND				D33	A*03	A*24	B*07	B*27	
D23	ND				D34	A*01		B*07	B*35	
D24	A*11	A*01 C*01	B*15 C*07	B*08	D36	ND				
D25	A*24	A*02 C*44	B*40 C*05	B*44	D37	ND				
D26	ND				D38	ND				
D27	ND				D39	A*02				
D28	ND									
D29	ND									
D35	ND									
D40	ND									
D41	ND									
D42	ND									
D43	A*02	A*68	B*44	B*14						

ND not done

HLA typing was performed using the by the Welsh Blood Transfusion Service or by mAb staining with mA2.1.

(†) HCMV serology was determined by measurement of IgG antibody levels to HCMV in plasma using the AXSYM HCMV IgG assay.

Table 2.2 Media Used for Cell Culture

Media	Constituents
RPMI-wash*	RPMI
DMEM-wash*	DMEM
RPMI-10†	RPMI, 10% FCS (v/v)
RPMI-AB/5†	RPMI, 5% Human AB Serum (v/v)
RPMI-AB/10‡	RPMI, 10% Human AB Serum (v/v)
DMEM-10†	DMEM, 10% FCS (v/v)
DMEM-20†	DMEM, 20% FCS (v/v)
SCGM-AB/5†	SCGM (CellGenix, Germany), 5% Human AB Serum (v/v)
PBS 2% AB	Phophate buffered saline, 2% AB Serum (v/v)
PBS FCS	Phophate buffered saline, 2% FCS (v/v)

(*) no supplements added to media
(†) Supplemented with 2 mM L-glutamine, 1 x 10^5 IU/ml penicillin and 100 mg/ml streptomycin
(‡) Supplemented with 2 mM L-glutamine, 1 x 10^5 IU/ml penicillin, 100 mg/ml streptomycin and 25mM HEPES buffer.

2.3.2 Human AB Serum

10 ml aliquots of pooled human AB serum were prepared, heat inactivated (56°C for 45 minutes) and stored at -20°C until required. AB serum batches were tested for their ability to maintain the proliferation of natural killer (NK) and T cell subsets by culturing peripheral blood mononuclear cells (PBMC) for 3 days in varying concentrations of IL2 (Cetus, USA) (for NK cells) or with phytohaemagglutinin (PHA) (5 μg/ml) for T-cells. The proliferation of cell in culture was assayed by measuring ^{3}H thymidine (Amersham, Bucks) incorporation (see section 2.11.2).

2.4 CELL LINES

All cell lines were screened for mycoplasma by Sian Llewelyn-Lacey using the VenorGeM® Mycoplasma PCR detection kit (Biochrom AG, Germany). In this procedure, samples of cell lines were probed for mycoplasma DNA by PCR. Only negative lines were used in these studies.

2.4.1 Established Cell Lines

Human Foetal Foreskin Fibroblasts's (HFFFs) (kindly provided by Dr G. Farrar, Porten Down), immortalised primary skin fibroblasts and 293 cells (kindly provided by Prof. R. Graham) were maintained in 175 cm^2 tissue culture flasks (Greiner, Stonehouse) in DMEM-10. K562 cells (ATCC) were maintained in 25 cm^2 tissue culture flasks (Greiner) in RPMI-10. All cell lines were grown at 37°C in humidified incubators in 5% CO_2.

2.4.2 Establishment of Primary Fibroblast Lines

Skin biopsies were taken by Stephan Siebert as described by Siebert *et al*, 2005. Establishment of primary skin fibroblasts lines was carried out by Eddie Wang and then immortalised by Sian Llewellyn-Lacey as described by (McSharry *et al.*, 2001). In brief, lignocaine (1%) (Novartis, Frimley) was injected intradermally to anaesthetise and bleb the biopsy area. A small biopsy was taken (~2mm^3), cut into 8 small fragments with a scalpel, placed

into 35 mm Primaria™ Easy Grip™ culture dishes (Becton Dickinson) and covered with a glass coverslip and cultured in DMEM-20. Media was changed at weekly intervals. When cells were confluent, they were transferred to tissue culture flasks and maintained in DMEM-10. Early passage numbers cryopreserved for later use (see section 2.4.7). Cells were maintained in the medium described above.

2.4.3 Passage of Adherent Cells

Confluent monolayers of cells were passaged by removing medium, washing the cell layer with PBS, and incubating the cells with 1 - 5 mls of Trypsin/EDTA (Invitrogen) (depending on the size of the flask) until the cells fell into suspension. After addition of DMEM-10 each fibroblast and 293 suspension were divided between 1:2 and 1:5 or when confluent.

2.4.4 NK Cell Cloning

NK clones were generated from fresh PBMC by single cell sorting $CD3^-$ $CD56^+$ cells into single wells of a 96 well plate (Greiner) (see section 2.4.3). The NK cells were cultured at 37°C and 5% CO_2 in SCGM-AB/5 with Interleukin 2 (IL2) (250 IU/ml), OKT3 (50 ng/ml) (ATTC) and 5 x 10^5 irradiated PBMC from 3 allogeneic individuals for 4 days. On day 4 half the media was removed and replaced with fresh medium containing IL2 (1000 IU/ml). From day 7 the NK clones were restimulated with weekly cycles of irradiated feeders as described above. Cells were cultured for 6-8 weeks, and restimulated with irradiated PBMC, IL2 and OKT3 every 7 to 10 days. Flow cytometry was used to analyse the NK cells for clonality by staining for the extracellular markers CD3, CD56 and CD94 (see section 2.7.1). Cells were accepted as being clonal if a single population of $CD3^-CD56^+$ and $CD94^+$ cells were observed. A more detailed description of the method is described in Chapter 5.

2.4.5 Stimulation of Peptide Specific Cytotoxic T Cells (CTL)

$CD8^+$ T cells were enriched as described in Section 2.5.4. The enriched $CD8^+$ T cells were plated out at 1 – 2 x 10^6 cells per well in a 24 well plate (Greiner) and cultured in RPMI-10 with the irradiated autologous $CD8^-$ negative fraction (stimulator:$CD8^+$ T cell, 3:1), peptide (10 μg/ml) (SevernBiotech) and IL2 (10 IU/ml). On day 4 and 6, 1 ml of media was removed and replaced with fresh media containing IL2 (20 IU/ml). On day 7 cells were harvested and plated out at 1 – 2 x 10^6 cells per well in a 24 well plate with irradiated autologous PBMC (stimulator:$CD8^+$ T cell, 3:1), peptide (10 μg/ml) and IL2 (10 IU/ml). On day 14 cells were stained with 7AAD and tetramer (Proimmune) and antibodies for CD8 and CD14 and analysed by flow cytometry (see section 2.7.3).

2.4.6 Expansion of Peptide Specific CTL

Peptide specific $CD8^+$ T cells were cultured as described in section 2.4.5 and enriched using MACS® (Miltenyi Biotec, Surrey) as described in section 2.5.4. Enriched peptide specific $CD8^+$ T cells were expanded by culture in a tilted (45°) T25 flask (Greiner) in 25 mls of RPMI-10 with 10 x 10^6 irradiated allogeneic PBMC from 3 donors, PHA (0.5 μg/ml) and IL2 (20 IU/ml). On day 5 half of the media was removed and replaced with fresh media containing IL2 (20 IU/ml). The cells were gently re-suspended and the flask was stood upright. On day 7, cells were stained with 7AAD and tetramer and with antibodies for CD8 and CD14 and analysed by flow cytometry (see section 2.7.3). The remaining cells were counted and plated out at 2 x 10^6 per well in a 24 well plate in RPMI-10 with IL2 (20 IU/ml). On day 12 half of the media was removed and replaced with fresh media containing IL2 (20 IU/ml). On day 14, cells were stained with 7AAD, tetramer and antibodies CD14 and analysed by flow cytometry (see section 2.7.3) and the cells were re-expanded with irradiated allogeneic PBMC, PHA and IL2 as described above.

2.4.7 Cryopreservation of Cells

Adherent cells were put into suspension as described in section 2.4.3. Suspended cells were then recovered by centrifugation and re-suspended in 1 ml of Freezing Mixture. The 1 ml aliquot was transferred to a cryovial (Greiner) and placed in a Nalgene 5100 Cryo 1°C freezing container (Merck, West Drayton) which contained room temperature isopropanol. This was then transferred to a -70°C freezer for 1-2 hours before transfer to liquid nitrogen for storage until required.

To resurrect cells from liquid nitrogen, they were thawed rapidly in a 37°C H_2O bath. Once thawed the cells were transferred to a 15 ml falcon (Greiner) and 10 ml of warm DMEM-20 was added dropwise. The cells were centrifuged at 250 x g for 5 minutes, resuspended and counted ready for use.

2.5 Isolation of PBMC and PBMC subsets

2.5.1 Isolation of PBMC

Blood was collected into tubes containing preservative-free heparin (Monoparin) (CP Pharmaceuticals Ltd., Clwydd) (5 IU/ml blood) and layered onto Histopaque-1077 in a 3:2 volume ratio (15 mls blood to 10 ml Histopaque) in a 30 ml universal container (Greiner). Tubes were centrifuged for 20 minutes at 1100 x g without braking. The interfacing layer of mononuclear cells was removed into a universal container and diluted with PBS. The cells were washed first at 1100 x g for 6 minutes followed by 2 slower washes of 350 x g for 4 minutes. The cells were re-suspended in appropriate media, counted and diluted to the required concentration for use. Generally 1 x 10^6 PBMC were collected per ml of blood from healthy donors.

2.5.2 Isolation of Polyclonal NK Cells by Dynal Bead Depletion

NK cells were enriched from PBMC using immunomagnetic beads. PBMC were isolated from 50 ml of fresh heparinised blood by histopaque density centrifugation (see section 2.5.1). Cells were then washed in 4°C PBS 2%

(v/v) AB serum for 3 minutes at 300 x g and divided into 1/3 and 2/3 aliquots. CD3-FITC (25 μl) was added to the 1/3 fraction and 50 μl to the 2/3 fraction. CD94-PE (25 μl) was added to the 2/3 fraction and samples were left for 30 minutes at 4°C in the dark. M-450 sheep anti-mouse IgG Dynalbeads (Dynal, Wirral) were prepared by washing 3 times in PBS 2 % (v/v) AB serum to remove excess azide. Cells were then washed twice for 4 minutes at 300 x g and a sample collected for flow cytometric analysis. The cells were added to the prepared M450 sheep anti-mouse IgG Dynal beads and incubated for 2 hours at 4°C in the dark with occasional agitation. The mixture was then placed in a Magnetic Particle Concentrator (MCP 1) (Dynal) to draw bead:cell conjugates to the side of the container. The supernatant was collected (negative selection) and the process was repeated to remove as many bead:cell conjugates as possible. The cells were counted and a sample collected for flow cytometric analysis. The cells were re-suspended at 1 x 10^6 cells/ml in SCGM-AB/5 with interferon alpha (IFNα) (1000 IU/ml) (Roferon, Roche). The suspension was then aliquoted into 24 well flat bottomed plates at 2 x 10^6 cells/well and kept in a 37°C incubator overnight. The number of NK cells was determined by flow cytometric gating for the $CD3^-CD56^+$ subset.

2.5.3 Isolation of NK Clones from Fresh PBMC by Single Cell sorting using the MoFlo

Single $CD3^-CD56^+$ NK cells were isolated from fresh PBMC as follows: 1 x 10^6 PBMC were incubated with appropriate concentrations of CD3 and CD56-specific antibodies in 200 μl of PBS-FCS and incubated for 30 minutes at 4°C. The resulting immunostained samples were washed twice in 1 ml of PBS 2% (v/v) AB serum, filtered with a 40 μm cell strainer and re-suspended at a final concentration of 1 x 10^6 cells/ml. $CD3^-CD56^+$ cells were sorted into single wells of a 96 well u-bottomed plate containing SCGM-AB/5, irradiated feeder cells, IL2 and OKT3 (see section 2.5.3).

2.5.4 Isolation of Cells from PBMC using MACS® Separation Columns

$CD8^+$ cells were isolated from fresh PBMC and tetramer positive T cells were isolated from peptide stimulated $CD8^+$ T cell cultures. 1 x 10^7 cells were re-suspended in cold MACS® buffer with 20 µl of anti-CD8 (for isolation of CD8 cells) or anti-PE (for isolation of tetramer positive cells) of MACS® beads and the cells were put on a spinning wheel for 15 minutes at 4°C. Bead:cell conjugates were then washed by adding 5 – 10 mls of cold MACS® buffer and centrifuging at 300 x g for 10 minutes. MACS® columns were prepared placing them on a MACS® Separator™ and rinsing with 500 µl (small columns) or 3 ml (large columns) of cold MACS® buffer. The bead:cell conjugates were re-suspended in 500 µl of cold MACS® buffer and put onto the column. The negative fraction was collected and the tubes were washed 3 times by rinsing with 500 µl (small columns) or 3 ml (large columns) of cold MACS® buffer. The column was removed from the MACS® Separator™ and the positive fraction eluted by running 500 µl of MACS® buffer down the column and applying a plunger.

2.5.5 Cell Counting

A 10 µl aliquot of cell suspension was diluted 1:1 with Trypan Blue and counted on a Glasstic ® slide counting chamber (Hycor Biomedical Inc., Edinburgh) under white light. Viable cells were identified by exclusion of the blue dye. The total number of cells present was determined by the following equation:

Tot No Cells = (No cells in grid) x (1 x 10^4) x (2) x (Tot vol of cells) (in mls)

2.6 VIRUSES

A list of the viruses used during this study are given in Table 2.3.

2.6.1 Propagation of Human Cytomegalovirus (HCMV) Stocks

HCMV was grown in HFFFs in 175 cm^2 tissue culture flasks. When HFFFs reached 70-80% confluency they were infected at an MOI of 0.1:1 in a

Table 2.3 List of Viruses.

HCMV	Source/Reference
AD169	Kindly provided by Gavin Wilkinson
Towne	Kindly provided by Ed Mocarski
Toledo	Kindly provided by Ed Mocarski
HCMV-ΔUL40	(Wang *et al.*, 2002)
RAd	
RAdpp65	Kindly provided by Gavin Wilkinson
RAdIE1	(Wilkinson & Akrigg, 1992)
RAdUL40	(Tomasec *et al.*, 2000)
RAdHLA-E	(Tomasec *et al.*, 2000)
RAd60	Empty vector control. Kindly provided by Carole Rickards
RAdUL141	(Tomasec *et al.*, 2005)
RAdUL141-GFP	(Tomasec *et al.*, 2005)
RAdUL141 control	(Tomasec *et al.*, 2005)
RAdUL141-GFP control	(Tomasec *et al.*, 2005)

minimal volume (4 ml) for 2 hours in a rocking incubator at 37°C. The innoculum was removed, cells washed in PBS and 30 ml of DMEM-10 was added. After 9 - 11 days when 80 - 90% cytopathic effect (cpe) was visible the media was collected, centrifuged at 700 x g for 3 minutes and the pellet frozen. This harvesting procedure was repeated until all the cells became infected. Infectious titre of virus were calculated as 50% tissue culture infectious dose ($TCID_{50}$) assay (see section 2.6.3).

2.6.2 Propagation of recombinant adenovirus stocks

Recombinant adenoviruses were grown in 293 cells in 175 cm^2 culture flasks. When 293 cells reached 70-80% confluency 25 µl of virus stock was added to each flask and the cells were incubated overnight at 37°C. The following day the innoculum was removed and fresh DMEM-10 was added. After 7-10 days, when maximum cpe was observed, the cells and their supernatant were harvested by centrifugation at 700 x g for 3 minutes. The infected cells were re-suspended in 5 ml PBS and an equal volume of arkalone-*P* (Basic Chemical Company, High Wycombe) was added. Samples were shaken to form an emulsion then centrifuged at 4000 x g for 5 minutes. The upper virus-containing layer was removed and 1 ml aliquots were stored at -80°C.

2.6.3 Virus Titration

Estimation of virus titre was carried out on HFFFs for HCMV and 293 cells for adenovirus by limiting dilution analysis. Cells were seeded into flat bottomed 96 well plates for adenoviral titrations and in 24 well plates for HCMV titrations at a confluency of 80% and allowed to adhere overnight. The following day a series of 10 fold dilutions were made from 10^{-2} to 10^{-10}. For adenoviral titrations 100 µl of each of the virus dilutions was added to each well and for HCMV 1 ml was added. After 24 hours and thereafter as necessary, the media was changed. After 28 days (HCMV) or 8 days (adenovirus) cells were observed microscopically for cpe and those exhibiting evidence of viral growth marked. Viral titres were calculated as $TCID_{50}$ units using the formula of Reed and Meunch (Reed & Meunch, 1938).

For convenience, $TCID_{50}$ were converted to pfu/ml using an accepted conversion factor [1 $TCID_{50}$ unit = 0.7 infectious units] (Davis *et al.*, 1990).

2.6.4 Infection of Cells for Use in Assays

HFFFs were infected with HCMV (multiplicity of infection (MOI) 10), or recombinant adenovirus (MOI 50). Immortalised skin fibroblasts were infected with HCMV (MOI 10) but required an MOI of 500 for efficient infection by recombinant adenovirus. Replication deficient adenoviruses were titred in permissive 293 cells (see section 2.6.3). Fibroblasts express lower levels of the Ad receptors than 293 cell, thus a higher concentration of virus was required to provide a similar level of infection. The amount of virus required to infect non-permissive fibroblasts was determined by titration using a reporter virus encoding green fluorescent protein (GFP) (Figure 2.1). For infection, semi confluent cells washed in PBS and the virus was added in a minimum volume of DMEM-10. Cells were incubated for 2 hours in a rocking incubator at 37°C, the innoculum removed, cells washed once in PBS and DMEM-10 was added.

2.6.5 Isolation of Towne DNA

HCMV was recovered by centrifugation by spinning at 23,000 x g for 30 minutes in an ultracentrifuge. The virus containing cells were re-suspended in TE supplemented with 1% (v/v) nonidet-P40 (NP-40) and vortexed for 30 seconds. The solution was made up to 0.5% (v/v) SDS and proteinase K (200 μg/ml) and incubated at 37°C overnight. The DNA containing solution was then extracted 3 times very gently with equal volumes of phenol:chloroform:isoamylalcohol 25:24:1 then phenol removed by a single extraction with an equal volume of chloroform:isoamylalcohol 24:1. The DNA was precipitated by adding sodium acetate pH 4.6 (0.1 volumes), 100% ethanol (2.6 volumes) and centrifuging at 10,000 x g for 30 minutes at 4°C. The supernatant was discarded and the DNA washed with 70% (v/v) ethanol. The 70% (v/v) ethanol was removed after centrifugation and the pellet allowed to air dry. An appropriate volume of TE was added and the DNA

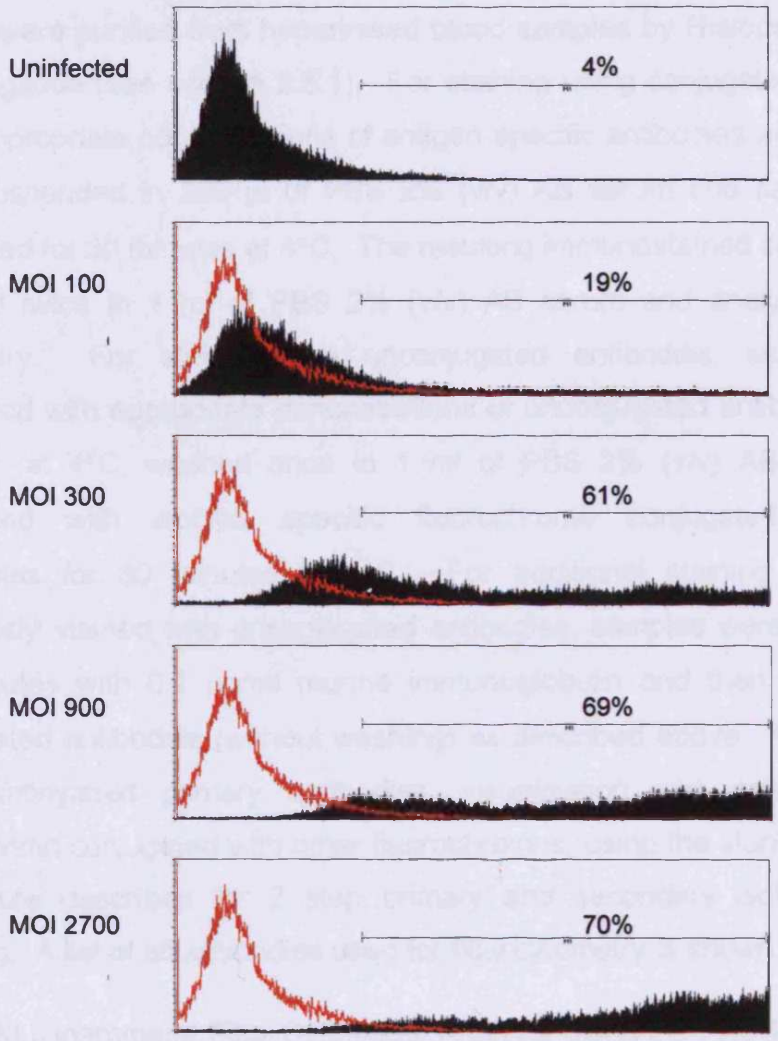

Figure 2.1 Titration of RAd-GFP in Skin Fibroblasts. D9 SF were infected with RAd-GFP, MOI ranged 100 – 2700 as described in section 2.6.4. Increasing the MOI over 300 did not significantly increase the percentage of GFP positive cells.

was allowed to dissolve at 4°C.

2.7 Flow Cytometry

2.7.1 General Staining Procedure

PBMC were purified from heparinised blood samples by Histopaque density centrifugation (see section 2.5.1). For staining using conjugated antibodies only, appropriate concentrations of antigen-specific antibodies were added to cells suspended in 200 µl of PBS 2% (v/v) AB serum and samples were incubated for 30 minutes at 4°C. The resulting immunostained samples were washed twice in 1 ml of PBS 2% (v/v) AB serum and analysed by flow cytometry. For stains using unconjugated antibodies, samples were incubated with appropriate concentrations of unconjugated antibodies for 30 minutes at 4°C, washed once in 1 ml of PBS 2% (v/v) AB serum and incubated with isotype specific fluorochrome conjugated secondary antibodies for 30 minutes at 4°C. For additional staining of samples previously stained with unconjugated antibodies, samples were blocked for 15 minutes with 0.1 µg/ml murine immunoglobulin and then stained with conjugated antibodies (without washing) as described above. After staining with biotinylated primary antibodies, visualisation was achieved using streptavidin conjugated with other fluorochromes, using the standard staining procedure described for 2 step primary and secondary isotype specific staining. A list of all antibodies used for flow cytometry is shown in Table 2.4.

2.7.2 Multiparameter Flow Cytometric Analysis Using the FACSCalibur

The FACSCalibur flow cytometer (Dakocytomation, Cambridge) uses 2 lasers that can detect a maximum of 4 fluorescent markers. Fluorochromes used and their peak excitation and emission spectra are shown in Table 2.5. Samples were prepared and stained as described in section 2.7.1. For multiparameter flow cytometry, unstained cells were used to set the negative gate. Single stains for each fluorescence were used to calculate compensations that occur because of overlap of the emmision spectra with other fluorochromes.

Table 2.4 List of Antibodies Used for Flow Cytometry

	Antibody	Supplier	Clone	Reference
Conjugated Antibodies	CD3-PE-Cy5.5	Caltag	S4.1	(Schlossman *et al.*, 1995)
	CD4-PE	BD Pharmingen	RPA-T4	(Schlossman *et al.*, 1995)
	CD8-FITC	Serotech	LT8	(Manninen & Saksela, 2002)
	CD8-PE-Cy7	Caltag	3B5	(Schlossman *et al.*, 1995)
	CD14-PE-Cy5.5	BD Pharmingen	M5E2	(Wright *et al.*, 1990)
Biotin conjugated Antibodies	CD56-biotin	Serotec	MEM-188	(Costa *et al.*, 1997)
Unconjugated primary antibodies	HNK-1	ATCC	J.CAM1.6	(Abo & Balch, 1981)
	CD3 (OKT3)	ATCC	mIgG2a	(Hoffman *et al.*, 1980)
	mA2.1	ATCC	mA2.1	(McMichael *et al.*, 1980)
	NKG2A	Immunotech	Z199	(Guma *et al.*, 2004)
	NKG2C	R & D Systems	134591	(Ortega *et al.*, 2004)
Streptavidin conjugated secondary	Strep-APC	Caltag	N/A	(Katakai *et al.*, 2002)
Isotype Specific Secondaries	Anti-mouse IgG_1 PE	Caltag	polyclonal	(Game *et al.*, 2003)
	Anti-mouse IgG_{2B} FITC	Caltag	polyclonal	(Debacq *et al.*, 2004)
	Anti-mouse IgM PerCP-Cy5.5	BD Pharmingen	R6-60.2	BD biosciences Pharmingen.
Polyclonal Antibody	Mouse polyclonal IgG	Serotec	polyclonal	(Joao *et al.*, 2004)

Table 2.5 Lasers, Excitation wavelength and Emmision Spectra of Flourochromes used for Flow Cytometry

Flourochrome	Laser (nm)	Emission
FITC	Argon Laser 488nm	525nm
PE	Argon Laser 488nm	575nm
PE-Cy5.5	Argon Laser 488nm	695nm
PE-Cy7	Argon Laser 488nm	767nm
APC	Diode Laser 633nm	660nm

Isotype specific controls conjugated to the relevant fluorochrome were used to identify false positives. Data was saved during acquisition. Lymphocytes were selected by standard forward scatter and side scatter criteria and the data was analysed either by dot plot analysis or by histogram analysis or a combination of both.

2.7.3 Tetramer Analysis

For tetramer staining 2 μl of tetramer (ProImmune, Cambridge) was added to cells re-suspended in 200 μl of PBS 2% (v/v) AB and samples were incubated for 30 minutes at 37°C. Cells were washed twice in PBS 2% (v/v) AB serum and cells were incubated with CD8-FITC (2 μl) and CD14-PE-Cy5.5 (2 μl) for 30 minutes at 4°C. Samples were washed twice in PBS 2% (v/v) AB and re-suspended in 200 μl of PBS 2% (v/v) AB. 7AAD (5 μl) was added and the cells were incubated for 20 minutes at room temperature in the dark before being analysed using the FACScalibur. 7AAD and CD14 were used to gate out dead cells and monocytes, respectively.

2.7.4 Multiparameter Flow Cytometric Analysis using the MoFlo

Multiparameter flow cytometry was carried out by Janet Fisher and Dr Terry Hoy on a MoFlo (DakoCytomation). The MoFlo has 2 lasers which emit in the blue (488nm) and red (633nm) ranges and detect the 5 fluorescent markers fluoroscein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Cy5.5 (PE-Cy5.5), phycoerythrin-Cy7 (PE-Cy7) and allophycocyanin (APC). Forward scatter (FCS), side scatter (SSC) and the 5 fluorescence's were detected by diodes after passage through or deflection from a number of filters and mirrors (Figure 2.2). For multiparameter flow cytometry, negatives for each fluorescence were required to set the negative gate. Additionally, single positive stains for each fluorescence were required to calculate compensations that occur because of spectral overlap of the emission spectra with other fluorochromes. Compensation due to spectral overlap was controlled by Cytomation Summit V3.1 software (Cytomation, USA) either prior to or post acquisition.

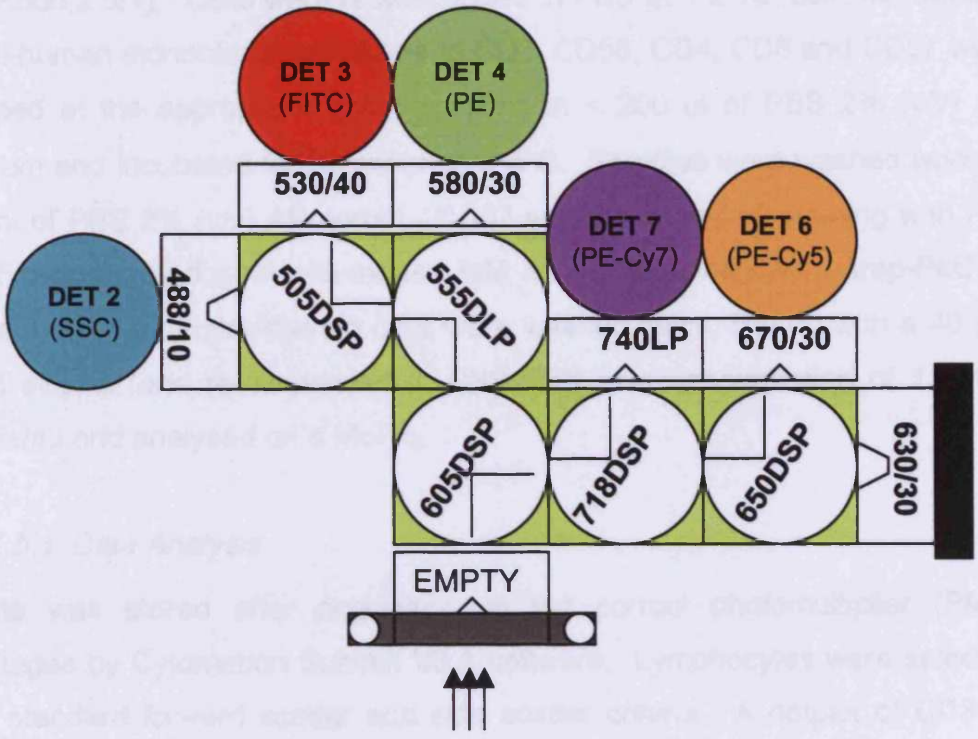

Figure 2.2 Z scheme for Detection of Five Fluorochromes using the MoFlo. Fluorochromes were excited with an argon (FITC, PE, PE-Cy5, PE-Cy7) or a diode laser (APC) (not shown). Dichroic mirrors reflect or transmit photons depending on the wavelength characteristics of the filter, for example the 740 LP (long pass) dichroic filter reflects wavelengths shorter than 740 nm and transmits photons longer than 740 nm. Band pass filters further purify the transmitted light, for example the 670/30 band-pass filter eliminates photons outside the range 655 – 685 nm.

2.7.5 Five Colour Fluorescence Staining

Antibodies used for 5 colour flow cytometry are shown in Table 2.4. A summary of the staining procedure is shown in Figure 2.3. PBMC were separated from heparinised blood using histopaque density centrifugation (section 2.5.1). Cells were re-suspended in PBS at 1 x 10^7 cells/ml. Mouse anti-human monoclonal antibodies to CD3, CD56, CD4, CD8 and CD57 were added at the appropriate concentrations in < 200 µl of PBS 2% (v/v) AB serum and incubated for 30 minutes at 4°C. Samples were washed twice in 1 ml of PBS 2% (v/v) AB serum. CD57 was visualised by staining with PE-Cy5.5-conjugated goat anti-mouse IgM and CD56-biotin with strep-PeCy7. The resulting immunostained cells were washed twice, filtered with a 40 µm cell strainer and re-suspended in PBS-FCS at a concentration of 1 x 10^6 cells/ml and analysed on a MoFlo.

2.7.5.1 Data Analysis

Data was stored after acquisition at the correct photomultiplier (PMT) voltages by Cytomation Summit V3.1 software. Lymphocytes were selected by standard forward scatter and side scatter criteria. A dotplot of CD3 vs CD56 was displayed and the $CD3^+CD56^+$ population was selected for further analysis. $CD3^+CD56^+$ cells were analysed for CD4 and CD8 positive cells and sub-populations were selected and analysed for CD57 expression. The percentage of cells expressing these markers was calculated from the plots.

2.8 Microscopy

2.8.1 Acetone and Methanol Fixation of Cells

Cells which had previously been seeded on to coverslips were washed in PBS and fixed by immersion in acetone/methanol (1:1) for 30 seconds.

2.8.2 Immunofluorescence

HFFFs were seeded onto coverslips and infected with RAdIE1 or RAdpp65 (MOI of 50) or HCMV strain Towne (MOI of 5). 24hr p.i the media was changed and the cells were incubated for the indicated times. The coverslips

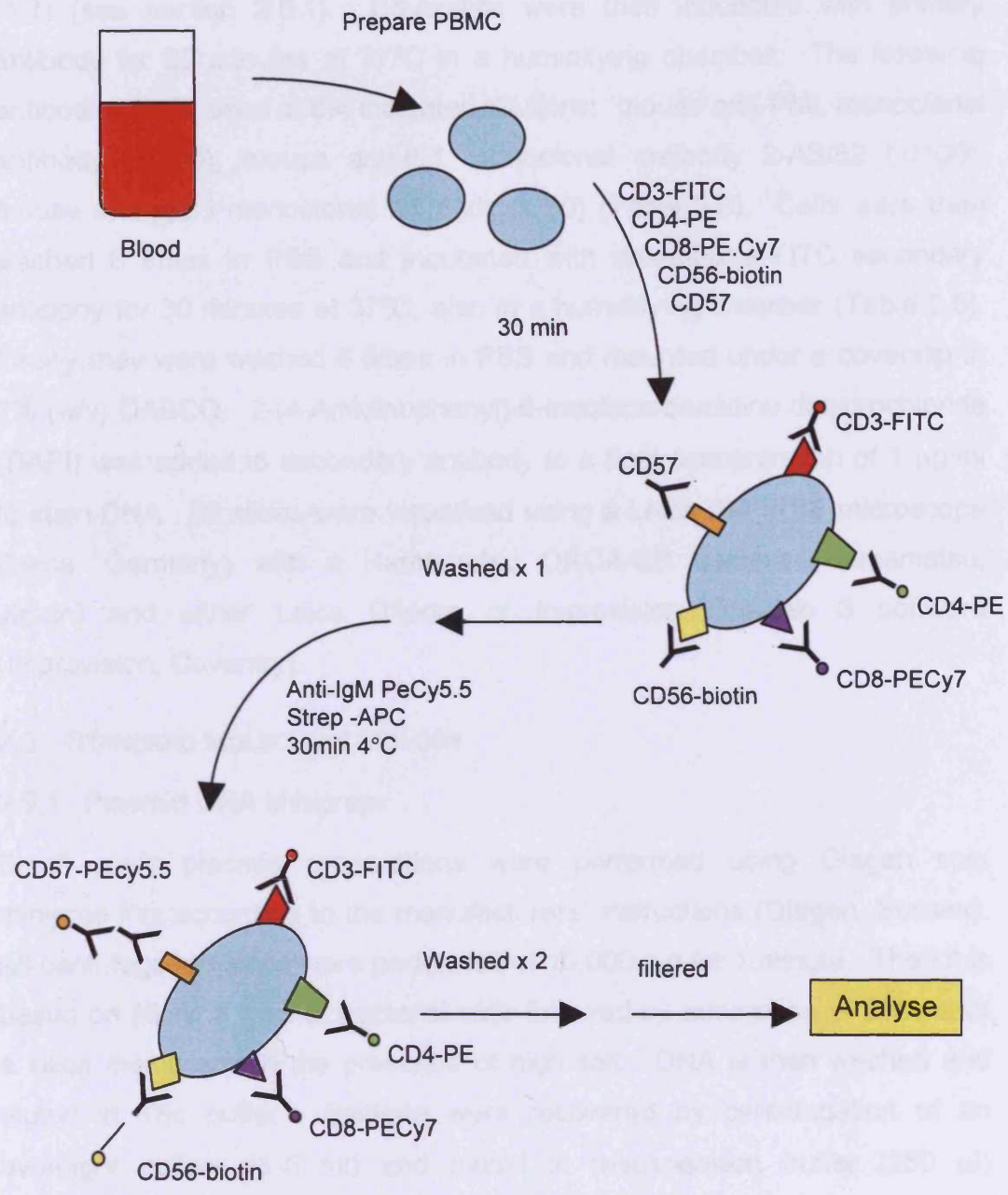

Figure 2.3 Staining Procedure for Five Colour Flow Cytometry. PBMC were prepared by Histopaque density centriguation and incubated with primary antibodies for 30 mins at 4°C. Prior to addition of the secondary antibody cells were washed once in PBS 0.1% (v/v) FCS. Cells were then incubated for 30 mins at 4°C before after which time they were washed twice in PBS 0.1% (v/v) FCS and filtered.

were harvested by first washing in PBS and then fixed in acetone/methanol (1:1) (see section 2.8.1). Coverslips were then incubated with primary antibody for 30 minutes at 37°C in a humidifying chamber. The following antibodies were used at the indicated dilutions: mouse anti-PML monoclonal antibody (1/100), mouse anti-IE1 monoclonal antibody 2-AS/52 (1/100), mouse anti-pp65 monoclonal antibody (1/50) (Table 2.6). Cells were then washed 6 times in PBS and incubated with anti-mouse FITC secondary antibody for 30 minutes at 37°C, also in a humidifying chamber (Table 2.6). Finally they were washed 6 times in PBS and mounted under a coverslip in 2% (w/v) DABCO. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was added to secondary antibody to a final concentration of 1 μg/ml to stain DNA. All slides were visualised using a Leica DM IRBE microscope (Leica, Germany) with a Hamamatsu ORCA-ER camera (Hamamatsu, Japan) and either Leica Qfluoro or Improvision Openlab 3 software (Improvision, Coventry).

2.9 Standard Molecular Biology

2.9.1 Plasmid DNA Minipreps

Small scale plasmid preparations were performed using Qiagen spin miniprep kits according to the manufacturers' instructions (Qiagen, Sussex). All centrifugation steps were performed at 10,000 x *g* for 1 minute. The kit is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica membrane in the presence of high salt. DNA is then washed and eluted in Tris buffer. Bacteria were recovered by centrifugation of an overnight culture (1-5 ml) and mixed in resuspension buffer (250 μl) containing RNase by pipetting. The lysis buffer (250 μl) was added and mixed by inversion and allowed to stand for 5 minutes. Neutralisation buffer (350 μl) was added and samples mixed immediately by inversion. A precipitate formed which was cleared by centrifuging at 13,000 x g for 10 minutes and the supernatant poured into a separation column. After centrifugation the pellet was discarded and wash buffer (750 μl) added. After standing for 1 minute, columns were centrifuged, and the flow-through again

Table 2.6 List of Antibodies used for Immunofluorescence Studies

	Antibody	Supplier	Clone	Reference
Primary Antibodies	2AS/52 IE1			
	Anti-pp65	BioDesign	1_L_11	(Tabi *et al.*, 2001)
	Anti-PML	Santa-Cruz Biotech	polyclonal	(Bjorndal *et al.*, 2003)
Secondaries	Anti-mouse FITC	Vector Laboratories, USA	No data	(Machelska *et al.*, 2004)

discarded. Columns were centrifuged for a second time to ensure all wash buffer was removed and then the DNA eluted into a fresh 1.5ml Eppendorf tube with elution buffer (30-50 μl). The purified plasmid DNA was stored at -20°C for future use.

2.9.2 Plasmid DNA Maxipreps

For large scale plasmid preparation and for transfection-quality DNA, Qiafilter plasmid maxipreps were utilized (Qiagen). In this procedure bacteria are subjected to alkaline lysis, and DNA bound to an anion-exchange resin under appropriate low salt and pH conditions. RNA, proteins, dyes and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. Following manufacturers instructions, a colony of bacteria containing the plasmid of interest was inoculated into 250 ml LB media containing antibiotics and incubated overnight at 37°C with shaking (160 rpm). Cultures were centrifuged at 6,000 x *g* for 15 minutes and the recovered cells re-suspended in 10 ml resuspension buffer containing RNase. Lysis buffer (10ml) was added, and samples mixed by inversion before being incubated for 5 minutes. Neutralisation buffer (10 ml) was added and the mixture poured into the barrel of a Qialfilter cartridge (containing a plug to prevent filtrate passing through). Solutions were incubated for a further 10 minutes to allow the precipitate to float to the surface, during which time equilibration buffer (10 ml) was added to a Qiagen tip-500 column, and the column allowed to empty by gravity flow. After the 10 minutes incubation, the plug was removed from the Qiafilter cartridge, a plunger inserted and the solution filtered through the Qiafilter such that the filtrate passed into the equilibrated Qiagen tip-500 column. Once the filtrate had passed through the column by gravity flow, 2 washes with wash buffer (30 ml) were performed and then the DNA was eluted into a fresh tube with elution buffer (10 ml). Isopropanol (0.7 volumes) were added to the eluate to precipitate DNA and the solution was centrifuged at 15,000 x *g* for 30 minutes, at 4°C. The DNA precipitate was washed with 70% (v/v) ethanol

and centrifuged again before drying by incubation at 37°C for 10 minutes. TE (400µl) was added to the tube and DNA dissolved by incubation at 37°C with shaking for 30 minutes. Solutions of DNA in TE were stored at -20°C.

2.9.3 DNA concentration estimation

Estimation of DNA concentration was carried out by 2 methods. DNA from plasmid preparations was analysed by a spectrophotometer. Absorbance of a DNA solution at 260 nm was compared to a control. Given that a solution of double stranded DNA with an optical density of 1.0 has a concentration of 50 µg/ml (using a 10 mm path length cell) the DNA concentration was calculated. Where DNA concentration was lower (e.g. after gel purification of a PCR product or vector) purified product was run on a TBE gel along with a smart-ladder marker (Eurogentec, Southampton) containing DNA fragments of defined size and abundance.

2.9.4 Restriction enzyme digestion

Restriction enzyme digestion of DNA was undertaken according to the manufacturers' instructions (NEB, Hertforshire). Reaction mixtures were set up with manufacturers supplied buffer (2.5 µl), restriction enzyme (20 units) and DNA template/distilled H_2O as required. Reaction mixtures were incubated at 37°C for 1-24 hours. Double digests were performed simultaneously in the manufacturers' recommended buffer or sequentially if the buffers were not compatible. Following digestion, DNA was separated by TBE agarose electrophoresis (see section 2.9.5) then gel extracted using a QiaQuick gel extraction kit (Qiagen).

2.9.5 Agarose gel electrophoresis

Size separation of DNA fragments was undertaken on 0.7% (v/v) TBE agarose gels by electrophoresis. The gel was poured into a gel tray and left to stand for 30 minutes. When the gel had set, the tray was placed into a gel tank and TBE running buffer added to cover the gel. Samples were mixed with loading buffer and pipetted into the gel wells, along with a Smart Ladder

marker containing DNA fragments of defined size and abundance. Gels were run at 70-100 V for between 1 and 3 hours depending on the resolution required, after which time the gel was removed and stained with ethidium bromide for 10 minutes. Bands were then visualised by ultraviolet (UV) illumination at 365 nm.

2.9.6 QIAquick Gel Extraction Kit

This kit is based on the principle that DNA adsorbs to a silica gel membrane in the presence of high salt while contaminants pass through (Qiagen). Impurities are washed away and the DNA eluted in Tris buffer. All centrifugation steps were carried out at 10,000 x *g* for 1 minute. Gel slices were weighed and 3 volumes (where 1g is equal to 1ml (1 volume) of buffer) of solubilisation buffer (QF) added. Following 10 minutes incubation at 50°C (or until gel slices had melted), 1 gel volume of isopropanol was added and solutions were applied to a separation column. Samples were centrifuged and the flow-through discarded. Buffer QF (500 µl) was added, samples centrifuged again, and the flow through discarded again. Wash buffer (750 µl) was added and samples allowed to stand for 5 minutes before centrifugation. The flow though was discarded and samples centrifuged again to remove all traces of wash buffer. Elution buffer (30 µl) was added to column and the sample was allowed to stand for 1 minute. Finally columns were placed in fresh collection tubes, centrifuged, and the eluate containing DNA stored for future use at -20°C.

2.9.7 Sodium Acetate/Alcohol Precipiation of DNA

10% (v/v) sodium acetate and 95% (v/v) ethanol was added to the sample and left for 15 minutes room temperature. The sample was centrifuged for 15 minutes 10,000 x g and the supernatant removed. Precipitated DNA was then washed in 70% (v/v) ethanol (250 µl) and spun for 15 minutes at 10,000 x g. The supernatant was removed and the DNA air dried in a 37°C incubator.

2.9.8 Ligation

Approximately 130 ng linearised vector DNA was mixed with sufficient insert to give molar ratios of insert:vector of 1:3, 1:1 and 3:1. T4 DNA ligase (1 unit) (Roche), manufacturers supplied buffer (1 μl), and H_2O were added to a total volume of 10 μl. Sticky-end ligations were carried out overnight at 16°C then a maximum of 10 ng total DNA was used in transformation.

2.9.9 PCR

PCR reactions were carried out using the proof reading Platinum® *Pfx* DNA polymerase (Invitrogen) with the manufacturers supplied buffer, dNTPs (1 μM) (Amersham), $MgSO_4$ (50 μM) and 20 pmol of each primer. The following thermocycling reaction was used:

Denaturation:	94°C for 2 minutes	
Denaturation:	94°C for 15 seconds	30 cycles
Annealing:	55°C for 30 seconds	
Elongation:	68°C for 30 seconds	
Hold:	4°C	

2.9.10 Sequencing using the Big Dye Reaction

Samples were sequenced using the 'Big Dye' terminator cycle sequencing kit (Perkin-Elmer, USA) and the dideoxy terminator cycle sequencing. Plasmid (200 ng) was mixed with terminator ready reaction mix (4 μl) and primer (3.2 pmol) and distilled H_2O was added to a total of 10 μl. The following thermocycling program, and a Hybaid PCR express thermocycler (Hybaid, Ashford) was used:

Denaturation:	96°C for 30 seconds	25 cycles
Annealing:	50°C for 15 seconds	
Elongation:	60°C for 4 minutes	

Following thermocycling, DNA was precipitated (see section 2.9.7) and the sequencing products were separated on an ABI model 377 sequencer (Applied Biosystems, USA).

2.9.11 Heat Shock of JM109 Bacteria

Ligation mixture (10 µl) was added to 1 vial of JM109's (Promega, Southampton) that had been thawed on ice and mixed gently. Bacteria were allowed to stand for 30 minutes on ice, then heat shocked by incubation in a pre-warmed H_2O bath at 42°C for 45 seconds. The bacteria were then returned to ice for 2 minutes and LB medium (800µl) was added. Tubes were incubated at 37°C with shaking for 30 minutes to 1 hour, after which time the bacteria were recovered by centrifugation by spinning for 5 seconds at 10,000 x g. The supernatant was decanted and 100-200 µl of fresh LB was added to each vial and plated out onto selective LB agar plates.

2.9.12 Glycerol Stocks

Plasmids of interest were stored as glycerol stocks. An overnight culture (500 µl) was added to an equal volume of 80% (v/v) glycerol in a cryovial. Vials were mixed vigorously and stored at $^{-}$80°C for later recovery.

2.10 YEAST TWO HYBRID

Yeast two hybrid is a powerful molecular technique to detect weak and transient protein-protein interactions that may not be detectable using standard biochemical techniques. A Matchmaker GAL4 two-hybrid system (Clontech) was set up in collaboration with E. Sherratt. To determine whether there was an interaction between pp65 and IE1, UL123 (IE1) was cloned into the GAL4 activating domain vector, pGAD424 by E. Sherratt and UL83 (pp65) was cloned into the GAL4 DNA binding domain vector. The principle steps in using the Clontech Matchmaker GAL4 Two-Hybrid System (Clontech, Basingstoke) are described in Figure 2.4.

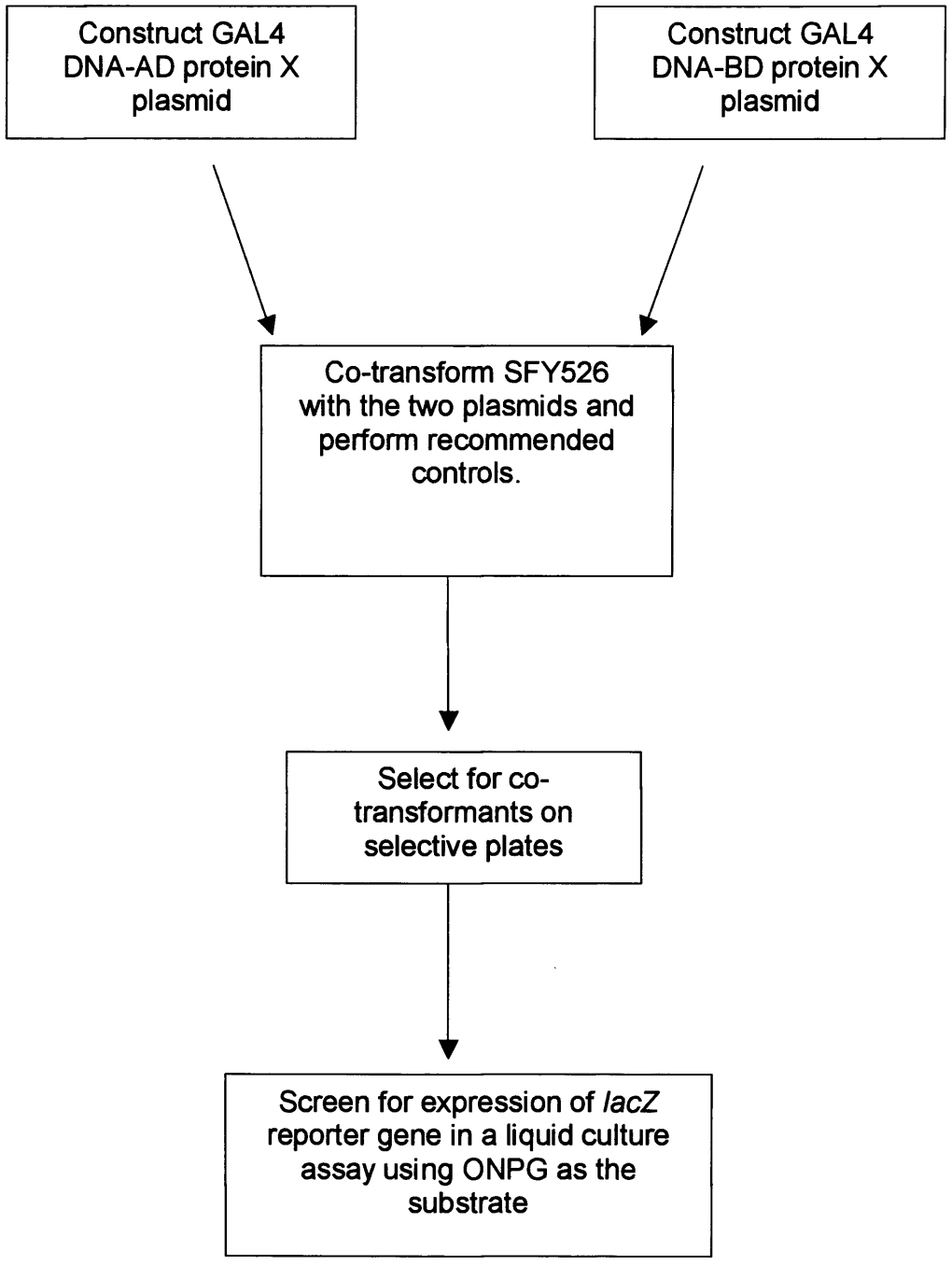

Figure 2.4 The Principle Steps in using the CLONTECH Matchmaker GAL4 Two-Hybrid System

2.10.1 Restriction Enzyme Digesion of pAL181 to Recover AD169 encoded pp65

The strain AD169 UL83 open reading frame (ORF) had previously been amplified by PCR and inserted under the control of the HCMV major immediate early promoter in the transient expression vector pMV100, thus generating pAL181 (Wilkinson & Akrigg, 1992). The UL83 ORF was excised from pAL181 by digestion with *Bam*HI (see section 2.9.4). The digested DNA was separated on a 0.7% (w/v) TBE agarose gel (see section 2.9.5), and DNA extracted using the QiaQuick Gel Extraction Kit (see section 2.9.6).

2.10.2 Preparation of GAL4 Vector DNA for Ligation with UL83

The GAL4 DNA Binding Domain vector, pGBT9 was digested with *Bam*HI to allow insertion of UL83 (Figure 2.5). To prevent pGBT9 from self-annealing prior to the addition of UL83 DNA alkaline phosphatase (1 μl) (Roche) was added and the digest incubated for 30 minutes at 37°C. The digested vector DNA was separated on a 0.7% (w/v) TBE agarose gel (see section 2.9.5), and DNA extracted using the QiaQuick Gel Extraction Kit (see section 2.9.6). UL83 was ligated into the GAL4 digests as described in section 2.9.8. UL83 was sequenced using the 'Big Dye' terminator cycle sequencing kit and dideoxy terminator cycle sequencing (see section 2.9.10). The sequence was assembled and aligned with strain AD169 UL83 sequence using the ClustalX Lasergene DNAstar software (Chee *et al.*, 1990a).

2.10.3 Transformation of GAL4-pp65 Plasmids

Transformations of ligated products were carried out by electroporation into chemically competent *E. coli* JM109 bacteria (see section 2.9.11). Multiple colonies were picked and grown overnight in LB and plasmid minipreps performed (see section 2.9.1). *Bam*HI restriction digestion was used to screen for colonies containing the correct plasmid insert (see section 2.9.4). Plasmid DNA was sequenced using the Big Dye reaction mix and GAL4 vector specific primers (see section 2.9.10). Vectors containing the inserts in

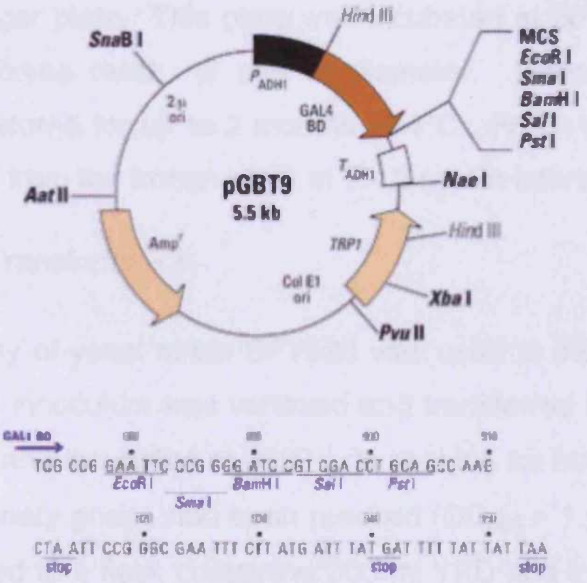

Figure 2.5 The GAL4 DNA Binding Domain Vector, pGBT9. pp65 was cloned into the BamH I site of the MCS, in the correct orientation and in the correct reading frame, to generate a hybrid protein that contains the sequence of the GAL4 DNA-Binding domain.

the correct orientation were selected and maxipreps performed using the Qiagen Spin Maxiprep Kit (see section 2.9.2).

2.10.4 Recovery of frozen Yeast strains and preparation of working stock plates

A glycerol stock of yeast strain SFY526, was partially defrosted and streaked onto a YPD agar plate. This plate was incubated at 30°C for 3-5 days until the yeast colonies reach ~2 mm in diameter. Plates were sealed with Parafilm and stored for up to 2 months at 4°C. Fresh working stock plates were streaked from the frozen stock at 1 – 2 month intervals.

2.10.5 Yeast Transformation

A single colony of yeast strain SFY526 was used to inoculate 1 ml of YPD medium. The innoculum was vortexed and transferred to a flask containing 50 ml of YPD and incubated at 30°C with shaking for between 16 - 18 hours until the stationary phase had been reached (OD_{600} > 1.5). The culture was then transferred to a flask containing 300 ml YPD and incubated at 30°C for 3 hours with shaking until the OD_{600} = 0.5 ± 0.1. The culture was then centrifuged at 1,000 x g for 5 minutes at room temperature and the supernatant discarded. The pellet was re-suspended by vortexing in TE (25-30 ml) and then centrifuged at 1,000 x g for 5 minutes at room temperature and the supernatant removed. The recovered cells were re-suspended in 1 X TE/LiAc (1.5 ml) and 100 µl of this solution was added to an Eppendorf containing DNA-Binding Domain (BD) vector construct (0.1 µg), Activation Domain (AD) vector construct (0.1µg) and herring testes carrier DNA (0.1 mg). The solution was mixed by vortexing at high speed and incubated at 30°C for 30 minutes with shaking (200 rpm) after which time DMSO (70 µl) was added and the solution mixed by gentle inversion. Samples were heat shocked at 42°C for 15 minutes and then chilled on ice for 1-2 minutes. Samples were briefly centrifuged, the supernatant removed and re-suspended in 1 X TE (500 µl). Two hundred microlitres of the transformation mixture was plated out on selective plates to select for colonies containing

both hybrid plasmids. Plates were incubated upside-down, at 30°C until colonies appeared. A list of transformations, including controls, and selective plates required is shown in Table 2.7.

2.10.6 Liquid Culture Assay using OPNG as Substrate

Liquid overnight cultures were prepared by inoculating 5 ml of SD medium supplemented with the appropriate DO supplement with 1 large (2–3 mm diameter) colony and vortexing to disperse the cells. The innoculum was incubated at 30°C for between 16 - 18 hours with shaking at 230-270 rpm. The overnight cultures were vortexed to disperse cell clumps and 2 ml was transferred to 8 ml of YPD. The fresh culture was incubated at 30°C for 3-5 hour with shaking (230 - 250 rpm) until the cells reached mid-log phase (OD_{600} of 1 ml = 0.5 - 0.8). The exact optical density (OD) was recorded and the cells were harvested by placing 1.5 ml of culture into each of 3 1.5 ml Eppendorfs and centrifuging at 10,000 x g for 30 seconds. The supernatants were removed and Z buffer (1.5 ml) was added to each tube. The cells were vortexed, centrifuged at 10,000 x g for 30 seconds, the supernatant removed and the recovered cells were re-suspended in Z buffer (300 μl) with vortexing to resuspend the cells. One hundred microlitres of the cell suspension was transferred to a fresh Eppendorf and frozen in liquid nitrogen for 1 minute. The frozen tubes were then placed in a 37°C H_2O bath for 1 minute. This freeze/thaw cycle was repeated 3 times to ensure all the cells had broken open. Z-buffer and β-mercaptoethanol (700 μl) and ONPG in Z buffer (160 μl) were added to the freeze-thawed cells and the timer started. Tubes were placed in a 30°C incubator and monitored for a positive interaction by the appearance of a yellow colour. As soon as a yellow colour appeared 1 M Na_2CO_3 (400 μl) was added to the reaction and the timer stopped. The time elapsed before the yellow colour was seen was recorded

Table 2.7 List of Constructs used in Yeast Two Hybrid

	DNA – Binding Domain	DNA – Activation Domain	Selective Plate*	Reference
Negative controls	pGBT9[†] (empty)	pGAD424[‡] (empty)	SD/-Leu/-Trp	(Holtz and Zhu., 1995)
	IE1–pGBT9	pGAD424 (empty)	SD/-Leu/-Trp	
	pGBT9 (empty)	PML–pGAD424	SD/-Leu/-Trp	
Positive control plasmid	-	pCL1[§]	SD/-Leu	(Fields & Song, 1989)
	pVA3[II]	pTD1[¶]	SD/-Leu/-Trp	(Li & Fields, 1993)
Literature Positive Control	IE1–pGBT9	PML–pGAD424	SD/-Leu/-Trp	(Ahn et al., 1998)
Test	pp65–pGBT9	IE1–pGAD424	SD/-Leu/-Trp	

(*) Selection plates were used to select for yeast transformed with plasmids that encode genes for leu2 (-Leu plates) and/or trp1 (-Trp plates)
(†) 5.4-kb cloning vector; used to generate fusions of the bait protein with the GAL4 DNA-BD
(‡) 6.6-kb cloning vector; used to generate fusions of a known protein (or a collection of random, unknown proteins) with the GAL4 AD.
(§) 15.3-kb positive control plasmid; encodes the full-length, wild-type GAL4 protein
(II) 6.4-kb positive control plasmid used with pTD1; encodes a DNA-BD/murine p53 fusion protein in pGBT9.
(¶) 15-kb positive control plasmid used with pVA3; encodes an AD/SV40 large T-antigen fusion protein in pGAD3F.

in minutes. Tubes were centrifuged for 10 minutes at 10,000 x g to pellet debris and supernatants were transferred to clean cuvettes. The OD_{420} of each sample was measured on a spectrophotometer which had been calibrated against the blank at A_{420}. The β-galactosidase units were calculated using the following formula;

β-galactosidase units = $1{,}000 \times OD_{420}/(t \times V \times OD_{600})$

where:
- t = elapsed time (in minutes) of incubation
- V = 0.1 ml x 5 (the concentration factor)
- OD_{600} = A_{600} of 1 ml of culture

1 unit of β-galactosidase is defined as the amount which hydrolyses 1 μmol of ONPG to *o*-nitrophenol and D-galactose per minutes per cell (Miller *et al.*, 1972; Miller, 1992).

2.11 Functional Assays

2.11.1 $Na_2{}^{51}CrO_4$ Release Cytotoxicity Assay for NK Cells

2.11.1.1 Virus Infection of Targets

Autologous immortalised skin fibroblasts targets were produced by infecting 4×10^5 semiconfluent cells with HCMV strain AD169 or Towne (MOI 10) in 1 ml of DMEM-10 in a 25 cm culture flask. Cells were incubated for 2 hours in a rocking incubator at 37°C, the innoculum was removed, cells washed once in PBS and 4 ml DMEM-10 was added. Targets were used at 72 hours p.i.

2.11.1.2 Preparation of Effector Cells

For assays using NK clones, cells were harvested and counted. When using enriched NK cells from PBMC, the number of NK cells was estimated by flow cytometry by gating on $CD3^-CD56^+$ cells. The cells were washed in RPMI and re-suspended in RPMI-AB/2 and dispensed into 96-well round bottomed plates. Where cell numbers allowed, serial dilutions were made in RPMI-AB/2 so that a number of E:T ratios were tested (in triplicate or quadruplicate).

2.11.1.3 Preparation of Targets

Primary skin fibroblasts were infected with HCMV strains AD169 or Towne as described in section 2.12.1.1. Following infection, cells were washed in PBS and incubated with 1 ml of Trypsin/EDTA then washed in RPMI-10 followed by PBS. Cells were recovered by centrifugation and labelled with $Na_2\,^{51}CrO_4$ (*150 μC*) (Amersham) for 60 minutes at 37°C then washed in RPMI-10 to remove excess $Na_2\,^{51}CrO_4$. RPMI-AB/5 (5 ml) was then added and cells were left to leach for 30-60 minutes at 37°C. Finally target cells were counted, washed and 1.5 x 10^3 or 2 x 10^3 cells were plated out in 100 μl of media in 96 well U-bottomed plates (Greiner) to give a final volume of 200 μl.

2.11.1.4 Cytotoxicity Assay

Cytotoxicity assays were performed in 200 μl in 96 well round bottomed plates using RPMI-AB/2. Spontaneous $Na_2\,^{51}CrO_4$ release was determined by incubating target cells with medium only. Maximum $Na_2\,^{51}CrO_4$ release was determined by addition of TritonX-100 5% (v/v) (100 μl). After addition of target cells plates were incubated at 37°C for 4 hours, after which time 20 μl of supernatant was harvested from each well and added to a beta plate which contained scintillation fluid (150 μl) (Perkin-Elmer). The plates were agitated for 15 minutes and counted in a 1450 Microbeta Trilux Liquid Scintillation Counter (Perkin-Elmer).

For cytotoxicity mean percent specific $Na_2\,^{51}CrO_4$ released for each E:T ratio was calculated using the standard equation:

$$\% \text{ specific lysis} = \frac{(\text{mean test cpm}) - (\text{mean spontaneous cpm})}{(\text{mean max cpm}) - (\text{mean spontaneous cpm})} \times 100$$

2.11.2 Proliferation Assays

NK cell proliferation assays were carried out on clones and on freshly sorted polyclonal NK cells. NK clones were cultured as described in section 2.4.4. Polyclonal NK cells were isolated as described in section 2.5.3. The NK cells were plated out in triplicate at 4 x 10^4 cells/well in a U-bottomed 96 well plate. ^{3}H thymidine (Amersham) (1 μC/well) was added to the clones in 96 well-U bottomed plates and the cells were incubated overnight at 37°C. The radiolabelled cells were harvested onto fibreglass Titertek filtermats, by a Skatron Titertek cell harvester (Skatron, Norway). Radioactive ^{3}H thymidine incorporation was measured on a 1450 Microbeta Trilux Liquid Scintillation Counter (Perkin-Elmer).

2.12 Mathematical Analysis

2.12.1 Calculation of Stimulation Index

The stimulation index was calculated by dividing the geometric mean of the test wells by the geometric mean of the control wells. A stimulation index of 4 or more was considered as positive for proliferation.

2.12.2 Identification of Proliferating NK Clones

To measure the proliferation of a clone in culture, a single clone was incubated with irradiated allogeneic feeder cells from 3 different donors. Control wells contained irradiated allogeneic feeder cells only. As the control wells did not contain a clone, they could not be used to account for the ^{3}H thymidine incorporation values of a clone that was not proliferating. The use of a stimulation index was therefore not appropriate for the identification of proliferating clones. To determine which wells were proliferating the geometric mean of 6 control samples was multiplied by 4 and subtracted from the test well. Test wells which gave a positive number were considered to be proliferating. To demonstrate differences in the rate of proliferation, proliferating wells were then categorised according to the amount of ^{3}H thymidine incorporated into the cells.

2.12.3 Statistical Analysis

To test the relationship between the ^{3}H thymidine incorporation of NK cells from the same individual cultured in 2 different media, a 1-way ANOVA was performed. A Tukey's multiple comparison post test was used to compare ^{3}H thymidine incorporation values for each media. A 2-way ANOVA was used to test the difference in ^{3}H thymidine incorporation when NK cells were cultured in 2 different media with varying concentrations of IL2. A Bonoferroni post test was used to compare values at each concentration of IL2. All ANOVA and post tests were carried out using Prism 4 software (GraphPad, USA). For categorical analysis of clones, a standard χ^2 test was performed. To test for differences in percentages a 2-tailed Student's t-test assuming unequal variance was performed. A 2-tailed Student's t-test assuming equal variance was used to compare differences in numbers that had not been expressed as a percentage. All χ^2 and T-tests were performed using Excel (Microsoft, USA). For all tests, a p value of less than 0.05 was taken as being significant.

RESULTS

3 MODULATION OF IE1 SPECIFIC CTL LYSIS BY pp65

A study by Gilbert *et al* (1996) showed that the tegument protein, pp65 abrogated IE peptide presentation on the surface of HCMV infected cells, rendering them refractory to IE1 specific T-cell lysis (Gilbert *et al.*, 1996b). As pp65 expression has been shown to result in the phosphorylation of several CMV proteins the authors investigated whether pp65 could modify IE1 by phosphorylation (Britt & Auger, 1986). Using a vaccinia virus expression system, the authors demonstrated that pp65 increased phosphorylation of IE1 threonine residues. From this data the authors suggested that phosphorylation of IE1 threonine residues could restrict access of the protein to the antigen-processing machinery or divert the protein to a different degradative pathway.

The suppression of IE1 presentation by MHC class I is potentially extremely important in a clinical setting, and specifically in the design of immunotherapies. However, this key viral immune evasion function had not been further characterised since it was first reported in 1996. An initial aim in this PhD was therefore to attempt to reproduce the effect so that the immune evasion mechanism could be independently corroborated, before going on to investigate the effect of pp65 on the presentation of IE1-derived epitopes in more depth. As a first step, IE1 (VLEETSVML) and pp65 (NLVPMVATV) peptide-specific CTL were cultured for use in a ^{51}Cr release assay. To identify potential donors, intracellular cytokine staining was used to detect IFNγ release in response to peptide. Subsequently, this approach was replaced with tetramer technology. Attempts at culturing HCMV-specific CTL were hindered by a shortage of seropositive donors with appropriate peptide-specific responses, combined with limited access to these donors. Out of 50 donors tested, only 17 were HCMV-seropositive. Of these 17 donors, only 7 exhibited IE1 and/or pp65 peptide specific CTL and at that time, autologous fibroblast lines were only available for D7 (Table 3.1).

Table 3.1 Donors with Peptide Specific CTL

	pp65 peptide*		IE1 peptide*	
Donor	NLVPMVATV (A2) †	TPRVTGGGAM (B7) †	VLEETSVML (A2) †	ELRRKMMYM (B8) †
D7	√		√	
D8	√			
D9				√
D31	√			√
D32	√			
D33		√		
D34		√		

(*) Peptide specific CTL were identified by staining cells with CD3, CD8 and tetramer.

(†) Tetramers specific for HLA-A2 (A2), HLA-B7 (B7) or HLA-B8 (B8) HCMV peptide epitopes.

Throughout the duration of the study a range of methods of antigen stimulation were systematically trialed in order to culture peptide-specific CTL:

Method 1. T-cell cloning using limiting dilution analysis; stimulation of PBMC with peptide coated irradiated autologous PBMC.

Method 2. Stimulation of $CD8^+$ cells with irradiated strain AD169 infected autologous fibroblasts.

Method 3. Stimulation of $CD8^+$ cells with peptide pulsed dendritic cells.

Method 4. Stimulation of $CD8^+$ cells with Hsp70-peptide pulsed dendritic cells.

Method 5. Stimulation of $CD8^+$ cells with peptide-coated, plate-adhered monocytes and immature dendritic cells.

Method 6. Stimulation of $CD8^+$ cells with peptide pulsed irradiated autologous PBMC.

Unfortunately, none of these approaches generated sufficient numbers of T-cells to enable functional ^{51}Cr release assays to be performed. Factors governing the efficient growth of $CD8^+$ CTL *in vitro* are not fully defined and thus it was not possible to clearly identify specific reasons why HCMV-specific CTL could not be expanded reliably. In addition to the methods described above, an *ex vivo* assay using tetramer-positive IE1 (VLEETSVML) peptide specific cells isolated by cell sorting was attempted. These cells lysed peptide coated autologous fibroblasts at 15% specific lysis in a 4 hour ^{51}Cr release assay but were unable to lyse fibroblasts infected with RAd60, RAdIE1 or RAdIE1 and RAdpp65. Method 6 most successfully expanded peptide-specific CTL. This method stimulated MACS® purified $CD8^+$ T-cells with peptide-coated irradiated autologous PBMC as described by Ayyoub *et al* (2003) and in Section 2.4.5 (Ayyoub *et al.*, 2003). Using this method, the frequency of $CD8^+$ T cells that were IE1 and pp65 specific were increased after 2 weeks, however this did not correlate with an increase in the number of peptide specific cells (Figure 3.1 and 3.2). The peptide specific CTL were purified by staining with tetramer and positively selected

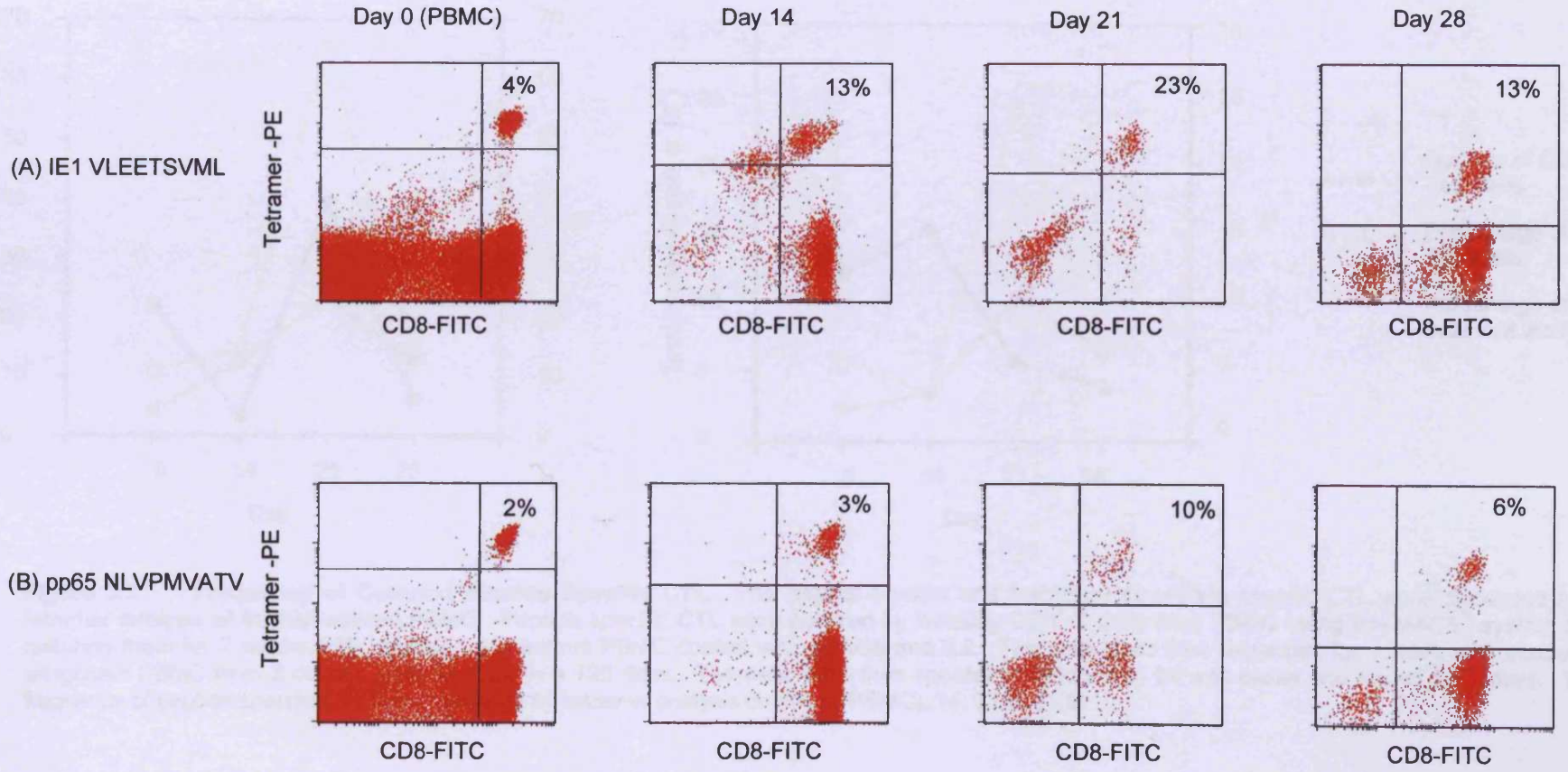

Figure 3.1 Culture of Peptide Specific CTL. IE1 and pp65 peptide specific CTL were cultured as described in section 2.4.6. The frequency of (A) IE1 VLEETSVML and (B) pp65 NLVPMVATV CTL was analysed using tetramers as described in section 2.7.3. The percentage of $CD8^+$, tetramer positive cells are shown.

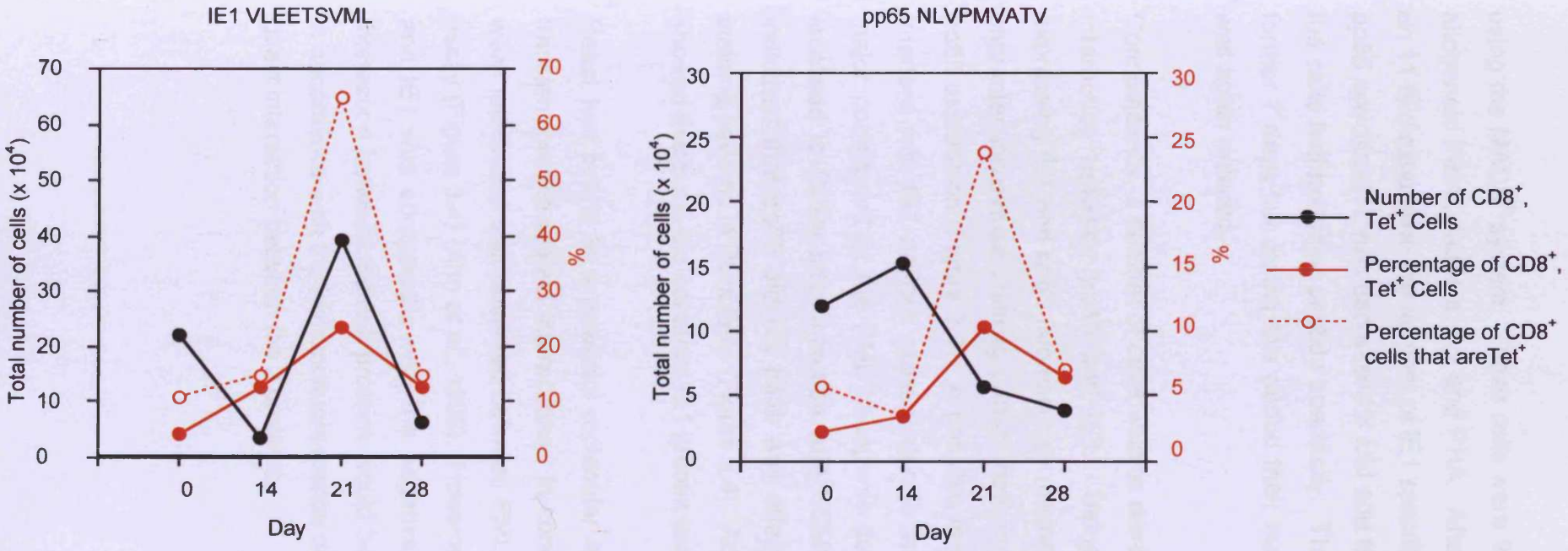

Figure 3.2 Frequency of Cultured Peptide Specific CTL. The original number and frequency of peptide specific CTL were calculated from tetramer analysis of freshly isolated PBMC. Peptide specific CTL were cultured by isolating $CD8^+$ T cells from PBMC using the MACS® system and culturing them for 2 weeks with irradiated autologous PBMC coated with peptide and IL2. The cells were then expanded for 1 week with irradiated allogeneic PBMC from 3 donors, PHA and IL2 in a T25 flask. The cells were then counted, plated out in 24 well plates and rested for 7 days. The frequency of peptide specific CTL was calculated by tetramer analysis on day 0 (PBMC), 14, 21 and 28.

using the MACS® system. These cells were then expanded using irradiated allogeneic PBMC feeders, IL2 and PHA. After 1 week of culture there was an 11 fold expansion the number of IE1 specific CTL however, the number of pp65 specific CTL had decreased 3 fold and tetramer analysis indicated that the cells had lost their peptide specificity. The cells were then rested for a further 7 days but during this period their numbers and peptide specificity was again reduced.

Concomitantly, a number of other studies were undertaken to investigate the interaction between pp65 and IE1. Using recombinant adenoviruses expressing IE1 and pp65 fluorescence microscopy showed IE1 exhibited its characteristic diffuse staining pattern both in the presence and absence of pp65 expression (Figure 3.3). A possible mechanism by which pp65 could interfere with IE1 antigen presentation is through interaction with PML a major constituent of the PML oncogenic domain (POD), because IE1 is localised to PODs after infection with HCMV. Fluorescence microscopy indicated that pp65 did not have any effect on PML's punctate nuclear staining patterns in fibroblasts (Figure 3.4). Additionally western blot analysis showed that pp65 did not affect IE1 protein levels (data not shown).

Yeast two hybrid is a powerful molecular technique to detect weak and transient protein-protein interactions. In concordance with the literature, a weak interaction was observed between PML and IE1 in a yeast two hybrid assay (Figure 3.4) (Ahn *et al.*, 1998). However the interaction between pp65 and IE1 was comparable with the negative control, suggesting that any interaction between these 2 proteins would be extremely weak. This data is in accordance with the immunofluorescence data, and are not supportive of a direct interaction between the 2 proteins.

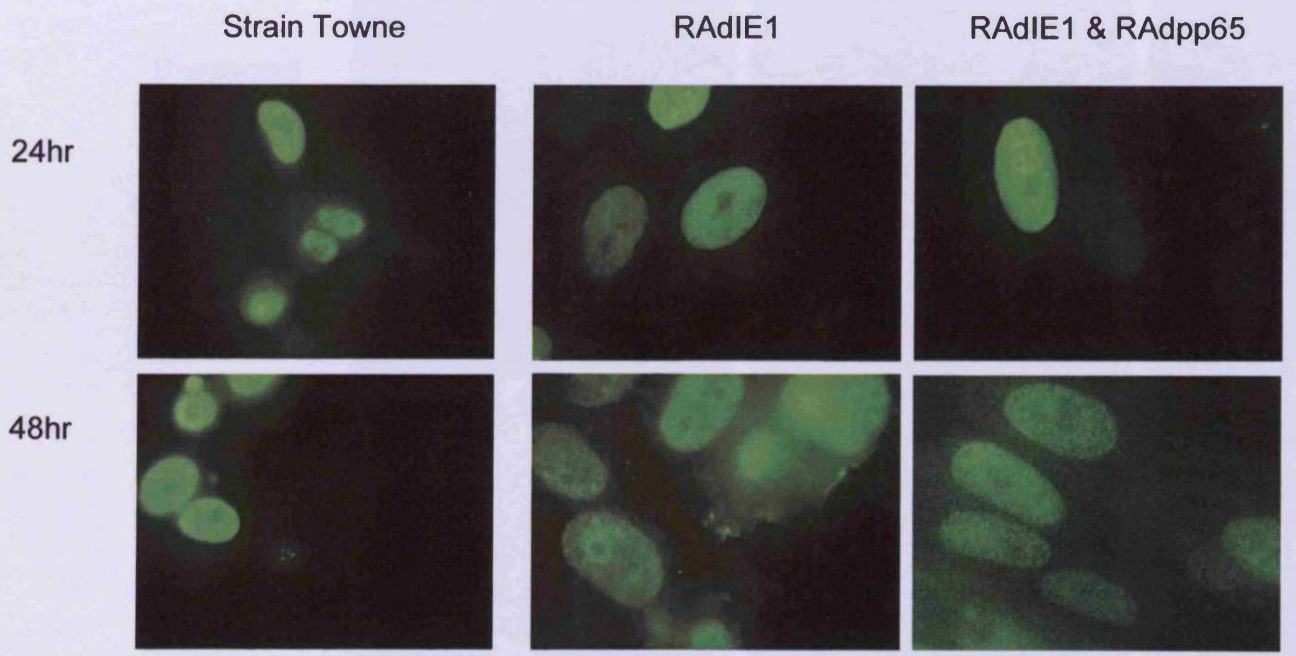

Figure 3.3 pp65 does not alter the localisation of IE1 in HFFFs. HFFFs were infected with strain Towne (MOI 10), or recombinant adenovirus (MOI 50) expressing RAdIE1 and/or RAdpp65 and stained for IE1 or pp65 at the timepoints indicated.

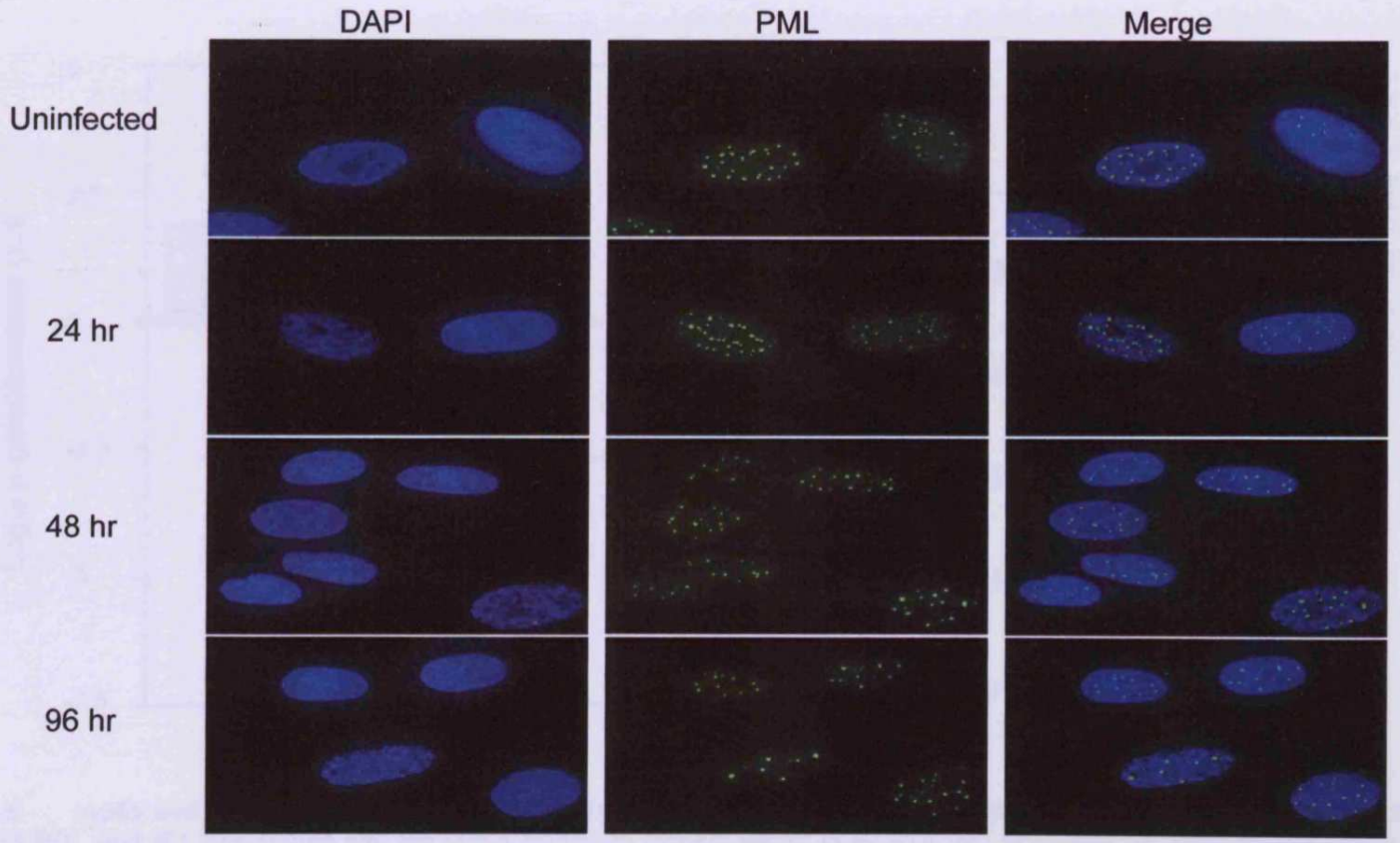

Figure 3.4 pp65 does not Alter the Localisation of PML in HFFFs. HFFFs were infected with RAdpp65 (MOI 50) for the time points indicated. Cells were fixed in acetone and methanol and stained with DAPI and pp65.

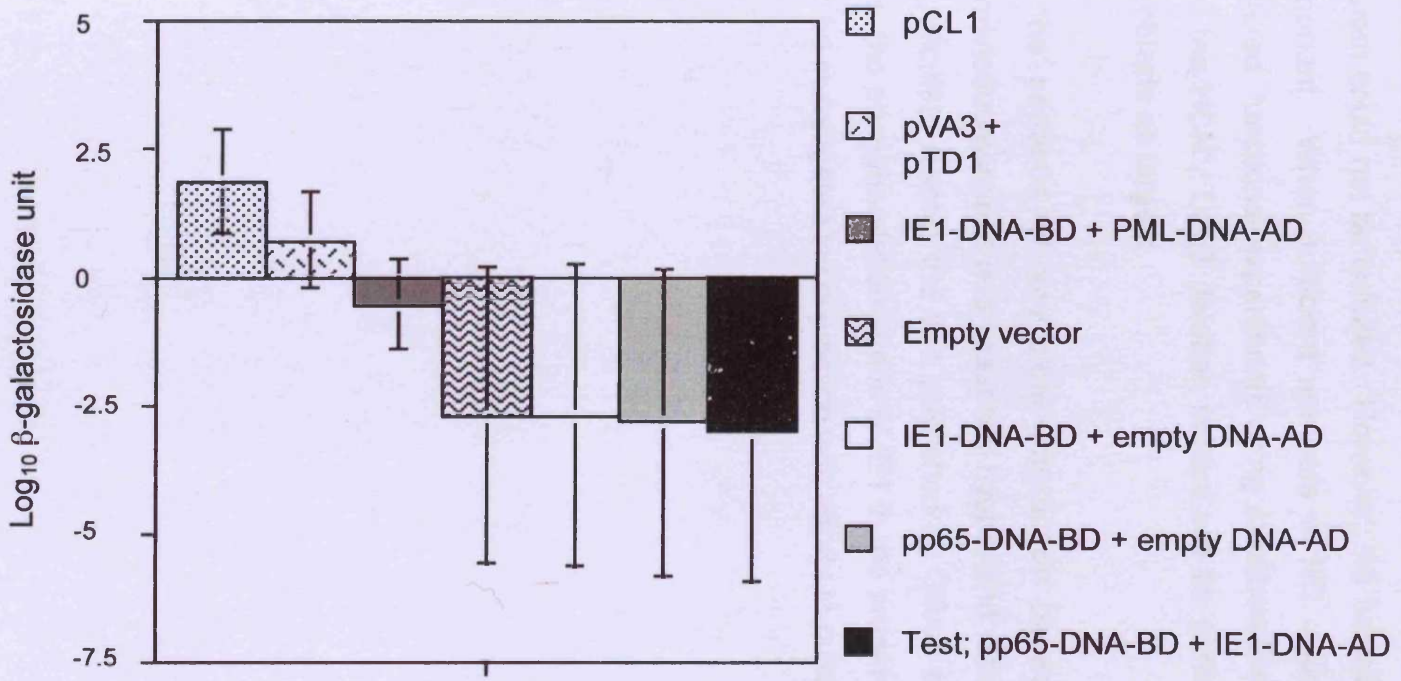

Figure 3.5 pp65 and IE1 do not Interact in a Yeast Two Hybrid System. pp65 was cloned into the GAL4 DNA binding domain vector pGBT9 (pp65-DNA-BD), and IE1 was cloned into the GAL4 activation domain vector pGAD424 (IE1-DNA-AD). A liquid culture assay using ONPG as the substrate was used to test for a positive interaction between the 2 proteins. pCL1 and pVA3 + pTD1 are positive controls. IE1-DNA-BD + PML-DNA-BD is the literature positive control. IE1-DNA-BD + empty DNA-BD, pp65-DNA-BD + empty DNA-AD and empty vector are negative controls. Results presented are a mean of a minimum of 3 samples, which were tested in either 1, 2 or 3 separate experiments.

3.1 SUMMARY

Methods for culture of HCMV specific T-cells were investigated throughout the PhD, however whilst a number of methods were trialed, sufficient numbers of cells to study the effects pp65 on IE1 in a functional read out system could not be cultured. However, the functional experiment remains important. When sufficient numbers of IE1 and pp65 specific CTL are cultured, functional experiments using adenovirus expressing IE1 and pp65 and the HCMV UL83 deletion mutant will be performed using autologous fibroblasts as targets.

In the absence of any clear interaction between the 2 proteins by immunofluorescence and yeast two hybrid, and without a functional assay, it is difficult to resolve the data published by Gilbert *et al* (1996). To address this, the phosphorylation status of IE1 in the presence and absence of pp65 will be investigated in conjunction with efforts to culture peptide specific CTL.

4 CORRELATIONS OF T-CELL SUBSETS WITH HCMV SEROSTATUS

4.1 INTRODUCTION

It is well established that infection with HCMV drives stable HCMV specific T-cell expansions (Khan *et al.*, 2002b, Wang *et al.*, 1995). Recently, HCMV has been shown to specifically inhibit the CD94/NKG2A$^+$ NK cell subset (Wang *et al.*, 2002). The premise of this study was that such specific targeting of an NK subset could alter its frequency in the HCMV seropositive host. Therefore, the distribution of CD94$^+$,CD3$^-$,CD56$^+$ NK cells in HCMV seropositive and seronegative donors was investigated. Whilst this study indicated that there was no difference in the distribution of CD94$^+$ NK cells in HCMV seropositive individuals (see Chapter 6.3.5.1), it was noticed that HCMV seropositive individuals had a greater frequency of CD3$^+$ cells that expressed CD56. CD56 is an N-cam and is usually expressed on NK cells but is also expressed on a small population of T-cells. In this chapter, the CD3$^+$,CD56$^+$ T-cell phenotype in HCMV seropositive and seronegative individuals was studied over a period of 8 months.

4.2 CD3$^+$,CD56$^+$ T-CELL FREQUENCY AND HCMV SEROSTATUS

PBMC isolated from 13 HCMV seropositive and 9 seronegative donors were stained for CD3 and CD56 and analysed by flow cytometry. HCMV seropositive individuals had a significantly higher proportion and number of CD3$^+$,CD56$^+$ cells compared to seronegative donors; 4.6 ± 1.9% (range 1.5 – 7.2%) versus to 2.2 ± 1.1% (range 0.9 – 5.1%) (p = 0.018) and 7.6 ± 3.2 x 10^5 cells per ml of blood (range 2.0 – 14.9 x 10^5) versus 3.2 ± 2.0 x 10^5 (range 0.8 – 6.8 x 10^5) (p = 0.018) respectively (Table 4.1).

4.3 CD3$^+$,CD56$^+$,CD4$^+$,CD8$^+$ T-CELL FREQUENCY AND HCMV SEROSTATUS

CD3$^+$ cells may be CD4 or CD8 positive. The proportion of CD3$^+$,CD56$^+$ cells in the periphery of HCMV seropositive (n = 12) and seronegative (n =

Table 4.1 HCMV serostatus and Correlations with $CD3^+$,$CD56^+$ T-cells

	HCMV Seronegative			HCMV Seropositive	
Donor	% PBMC $CD3^+CD56^{+*}$	Number $CD3^+CD56^{+\dagger}$ (x 10^5/ml)	Donor	% PBMC $CD3^+CD56^{+*}$	Number $CD3^+CD56^{+\dagger}$ (x 10^5/ml)
D1	1.1	1.3	D3	6.2	11.3
D4	3.7	4.5	D7	3.7	5.7
D5	3.1	5.3	D9	1.6	2.0
D6	1.0	0.8	D11	3.7	6.2
D12	2.0	3.0	D18	6.7	8.7
D13	1.1	1.5	D21	6.9	10.4
D14	1.0	0.9	D30	1.5	5.4
D15	3.9	6.0	D31	4.0	4.1
D16	2.4	5.2	D39	7.2	14.9
D19	5.1	6.8			
D24	0.9	1.0			
D40	1.0	1.0			
D41	2.3	4.3			
Mean ± SEM	2.2 ± 1.1[‡]	3.2 ± 2.0[§]		4.6 ± 1.9[‡]	7.6 ± 3.2[§]

(*) Percentage of PBMC as determined by flow cytometry.
(†) The number of positive cells in PBMC as determined from cell counts.
(‡) $p = 0.018$ (Student's t-test, assuming unequal variance).
(§) $p = 0.0038$ (Student's t-test, assuming equal variance).

18) individuals was therefore analysed for CD4 and CD8 positive cell frequency (Table 4.2). Interestingly the results of this second, larger study indicated that the proportion and number of $CD3^+$,$CD56^+$ cells was no longer significantly different between HCMV seropositive and seronegative individuals; 4.0 ± 2.0% (range 0.8 – 7.6%) versus 3.0 ± 1.4% (range 0.5 – 5.9%) and 0.7 ± 0.3 x 10^5 cells per ml of blood (range 0.1 – 1.2 x 10^5) versus 0.5 ± 0.3 x 10^5 (range 0.1 – 1.3 x 10^5), respectively. There was no association between HCMV serostatus and the proportion of $CD3^+$,$CD56^+$ cells that were single positive for CD4 or CD8 (Table 4.2). However, analysis of the $CD4^+$ and $CD8^+$ cells within the $CD3^+$,$CD56^+$ population revealed that HCMV seropositive individuals had significantly higher proportions and numbers of $CD3^+$,$CD56^+$ cells which were $CD4^+$,$CD8^+$; 12.1 ± 6.5% (range 1.2 – 24.3%) versus 6.4 ± 2.5% (range 2.7 – 15.1%) (p = 0.033) and 2.3 ± 1.9 x 10^4 cells per ml of blood (range 0.2 – 5.9 x 10^4) versus 1.0 ± 0.5 x 10^4 (range 0.2 – 2.7 x 10^4) (p = 0.017) (Table 4.3). Careful examination of the dot plots revealed HCMV seropositive individuals had significantly higher proportions and numbers of $CD3^+$,$CD56^+$ cells which were $CD8^{hi}$,$CD4^{lo}$ and $CD8^{hi}$, $CD4^{hi}$ (Figure 4.1 and Table 4.3). The proportion and number of $CD3^+$,$CD56^+$ cells which were $CD8^{hi}$,$CD4^{lo}$ was 7.1 ± 4.9% (range 0.4 – 18.9) versus 2.9 ± 1.2% (range 0.6 – 4.4%) (p = 0.034) and 1.4 ± 1.1 x 10^4 cells per ml of blood (range 0.1 – 3.3 x 10^4) versus 0.5 ± 0.2 x 10^5 (range 0.3 – 0.7 x 10^4), respectively (p = 0.0078). The proportion of $CD8^{hi}$,$CD4^{hi}$ cells was 2.4 ± 1.7% (range 0.5 – 6.9%) versus 0.7 ± 0.4% (range 0.0 – 1.6%) (p = 0.020) and the number of these cells was 0.4 ± 0.4 x 10^4 per ml of blood (range 0.1 – 1.8 x 10^5) versus 0.1 ± 0.1 (range 0 – 0.2 x 10^4) (p = 0.011) for HCMV seropostitive and seronegative donors respectively. The proportion and number of $CD3^+$,$CD56^+$ cells with a $CD8^{lo}$,$CD4^{hi}$ phenotype was small; 0.6 ± 0.4% (range 0.1 – 1.8%) versus 0.3 ± 0.2% (range 0 – 0.5%) and 0.1 ± 0.1 x 10^5 cells per ml of blood (range 0.0 – 0.3 x $10^{4)}$ versus 0.4 ± 0.4 x 10^4 (range 0 – 0.2 x 10^4) in HCMV seropositive and seronegative individuals respectively. There was no significant association with the proportions or numbers of the $CD8^{lo}$,$CD4^{hi}$ subset and HCMV serostatus.

Table4.2 HCMV Serostatus and Correlations with $CD3^+$,$CD56^+$ Cells and $CD3^+$,$CD56^+$,$CD4^+$ and $CD3^+$,$CD56^+$,$CD8^+$ T-Cells

	Donor	% $CD3^+CD56^{+*}$	Number $CD3^+CD56^{+\dagger}$ (x 10^5/ml)	% $CD3^+$, $CD56^+CD4^{+*}$	Number $CD3^+$,$CD56^+$ $CD4^{+\dagger}$ (x 10^4ml)	% $CD3^+$, $CD56^+CD8^{+*}$	Number $CD3^+CD56$ $CD8^{+\dagger}$ (x 10^4/ml)
Seronegative	D1	1.4	0.2	10.0	1.7	65.8	11.1
	D5	3.1	0.5	14.6	2.1	60.5	8.8
	D13	2.6	0.3	15.4	1.6	58.1	6.0
	D15	3.2	0.6	13.0	2.4	47.3	8.6
	D17	5.9	0.9	6.1	0.9	51.4	8.0
	D18	5.8	0.9	5.3	0.8	67.6	10.0
	D20	2.2	0.3	14.7	2.1	35.4	5.0
	D22	3.1	0.5	36.4	5.3	41.6	6.1
	D23	1.8	0.2	16.6	1.8	22.8	2.4
	D24	1.0	0.1	30.2	2.4	36.9	2.9
	D25	5.2	0.6	15.2	1.7	41.2	4.7
	D26	0.5	0.1	18.9	2.5	57.3	7.6
	D27	2.5	0.6	16.3	3.9	46.6	11.2
	D28	2.7	0.1	5.7	0.3	27.1	1.5
	D29	5.8	1.3	4.7	1.0	64.2	14.1
	D35	4.1	0.9	36.5	8.0	19.4	4.3
	D40	1.3	0.2	10.0	1.4	43.6	6.3
	D41	1.9	0.3	14.4	2.5	47.3	8.2
Mean ± SEM		3.0 ± 1.4	0.5 ± 0.3	15.8 ± 6.7	2.4 ± 1.2	46.3 ± 11.4	7.0 ± 2.7
Seropositive	D3	4.5	0.9	4.6	0.9	75.6	14.7
	D8	3.0	1.0	11.3	3.8	52.2	17.7
	D9	1.4	0.2	10.5	1.5	59.7	8.7
	D11	3.3	0.6	20.3	3.7	58.7	10.8
	D30	2.9	0.4	10.3	1.3	29.7	3.7
	D31	7.5	0.7	15.2	1.4	58.5	5.5
	D32	7.6	1.2	9.7	1.5	47.3	7.4
	D33	3.7	1.0	34.8	9.0	35.6	9.2
	D34	7.3	1.3	8.1	1.4	64.0	11.4
	D36	5.3	0.8	15.1	2.2	62.3	9.1
	D37	0.8	ND	15.3	ND	63.1	ND
	D42	0.9	0.1	17.9	2.2	59.7	7.3
Mean ± SEM		4.0 ± 2.0	0.7 ± 0.3	14.4 ± 5.3	2.6 ± 1.6	55.5 ± 9.6	8.8 ± 3.4

ND not done

(*) Percentage of PBMC as determined by flow cytometry.
(†) Number of cells in PBMC as determined from cell counts.

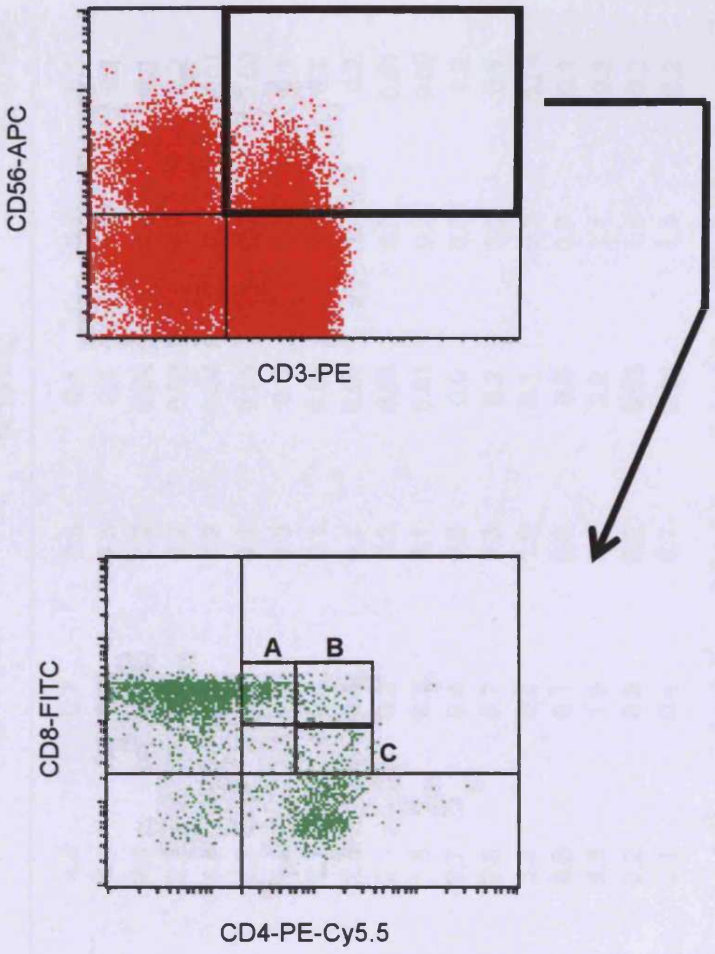

Figure 4.1 Expression Levels of CD4 and CD8 on $CD3^+$,$CD56^+$ cells. PBMC were stained with CD3, CD56, CD4 and CD8 antibodies and analysed by flow cytometry. The percentage of $CD3^+$,$CD56^+$ cells expressing $CD8^{hi}$,$CD4^{lo}$ (A), $CD8^{hi}$,$CD4^{hi}$ (B) and $CD8^{lo}$,$CD4^{hi}$ (C) were calculated by dot-plot analysis.

Table 4.3 HCMV Serostatus and Correlations with $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ T-cell subsets

	Seronegative Donor	% $CD8^+$,$CD4^{+*}$	Number $CD8^+$,$CD4^{+\dagger}$ (x 10^4/ml)	% $CD8^{hi}$,$CD4^{lo*}$	Number $CD8^{hi}$,$CD4^{lo\dagger}$ (x 10^4/ml)	% $CD8^{lo}$,$CD4^{hi*}$	Number $CD8^{lo}$,$CD4^{hi\dagger}$ (x 10^4/ml)	% $CD8^{hi}$,$CD4^{hi*}$	Number $CD8^{hi}$,$CD4^{hi\dagger}$ (x 10^4/ml)
	D1	6.0	1.0	4.4	0.7	0.5	0.1	0.7	0.1
	D5	8.0	1.2	4.3	0.6	0.5	0.1	0.9	0.1
	D13	4.4	0.5	2.0	0.2	0.2	0.02	0.8	0.1
	D15	15.1	2.7	2.7	0.5	0.2	0.03	0.9	0.2
	D17	3.2	0.5	1.5	0.2	0.2	0.04	0.1	0.01
	D18	7.0	1.0	2.2	0.3	0.1	0.01	0.2	0.03
	D20	7.9	1.1	4.2	0.6	0.0	0.0	1.0	0.1
	D22	4.6	0.7	2.7	0.4	0.1	0.01	0.8	0.1
	D23	4.5	0.5	2.5	0.3	0.1	0.01	1.4	0.2
	D24	2.7	0.2	2.1	0.2	0.3	0.03	0.2	0.01
	D25	9.8	1.1	1.5	0.2	0.1	0.01	0.2	0.02
	D26	4.0	0.5	2.7	0.4	0.0	0.0	0.0	0.0
	D27	8.1	2.0	2.8	0.7	0.8	0.2	0.3	0.1
	D28	5.7	0.3	3.9	0.2	1.0	0.1	0.7	0.04
	D29	2.9	0.6	0.6	0.1	0.0	0.0	0.7	0.1
	D35	11.8	2.6	8.5	1.9	0.7	0.2	1.1	0.2
	D40	5.8	0.8	2.2	0.3	0.2	0.03	1.6	0.2
	D41	3.9	0.7	2.1	0.4	0.1	0.01	1.1	0.2
Mean ± SEM		6.4 ± 2.5[§]	1.0 ± 0.5[‖]	2.9 ± 1.2[¶]	0.5 ± 0.3[#]	0.3 ± 0.2	0.4 ± 0.4	0.7 ± 0.4[**]	0.1 ± 0.1[††]

Table 4.3 continued

Seropositive Donor	% $CD8^{+},CD4^{+*}$	Number $CD8^{+},CD4^{+\dagger}$ ($\times 10^4$/ml)	% $CD8^{hi},CD4^{lo}$	Number $CD8^{hi},CD4^{lo}$ ($\times 10^4$/ml)	% $CD8^{lo},CD4^{hi*}$	Number $CD8^{lo},CD4^{hi\dagger}$ ($\times 10^4$/ml)	% $CD8^{hi},CD4^{hi*}$	Number $CD8^{hi},CD4^{hi\dagger}$ ($\times 10^4$/ml)
D3	1.2	0.2	0.4	0.1	0.1	0.03	0.6	0.1
D8	13.8	4.7	9.9	3.3	0.4	0.1	1.2	0.4
D9	6.0	0.9	3.7	0.5	0.2	0.03	1.0	0.2
D11	11.0	2.0	8.0	1.5	0.4	0.1	0.6	0.1
D30	5.0	0.6	2.4	0.3	0.1	0.01	0.5	0.1
D31	24.3	2.3	18.9	1.8	1.5	0.1	3.0	0.3
D32	15.6	2.4	6.2	1.0	0.1	0.01	1.9	0.3
D33	22.7	5.9	13.3	3.4	0.8	0.2	6.9	1.8
D34	22.8	4.1	14.9	2.7	1.8	0.3	5.4	1.0
D36	8.2	1.2	2.8	0.4	0.4	0.1	4.5	0.7
D37	10.3	ND	4.3	ND‡	1.1	ND	2.1	ND
D42	4.2	0.5	1.0	0.1	0.6	0.1	0.8	0.1
Mean ± SEM	12.1 ± 6.5§	2.3 ± 1.9‖	7.1 ± 4.9¶	1.4 ± 1.1#	0.6 ± 0.4	0.1 ± 0.1	2.4 ± 1.7**	0.4 ± 0.4††

(*) Percentage of PBMC as determined by flow cytometry.
(†) Number if cells in PBMC as determined from cell counts.
(‡) ND, not done
(§) $p = 0.033$ (Student's t-test, assuming unequal variance).
(‖) $p = 0.017$ (Student's t-test, assuming equal variance).
(¶) $p = 0.034$ (Student's t-test, assuming unequal variance).
(#) $p = 0.0078$ (Student's t-test, assuming equal variance).
(**) $p = 0.021$ (Student's t-test, assuming unequal variance).
(††) $p = 0.011$ (Student's t-test, assuming equal variance).

4.4 Further Phenotyping of $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ Cells

The $CD8^+$, $CD57^+$ cell subset has been shown to be specifically expanded in HCMV seropositive individuals (Gratama *et al.*, 1989, Wang *et al.*, 1995, Wang *et al.*, 1993). Five colour flow cytometry was therefore used to investigate whether CD57 was also present on the $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ cell subsets. PBMC were stained with CD3, CD56, CD4, CD8 and CD57 and analysed by flow cytometry on a MoFlo. PBMC were selected using standard FSC vs SSC gates and the proportion of $CD3^+$,$CD56^+$ cells which were $CD8^{hi}$,$CD4^{lo}$ or $CD8^{lo}$,$CD4^{hi}$ or $CD8^{hi}$,$CD4^{hi}$ were calculated (Table 4.4). Within each $CD4^+$, $CD8^+$ population the proportion of $CD57^+$ cells were calculated and the values were compared with HCMV serostatus.

In agreement with the initial findings, HCMV seropositive donors had a significantly higher proportion of $CD3^+$,$CD56^+$ cells than seronegative donors; 4.5 ± 1.7% (range 0.9 – 7.6%) versus 2.2 ± 0.9% (range 0.9 – 3.5%) (p = 0.039) (Table 4.4). However, the proportion of $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ cells were not different between HCMV seropositive and seronegative donors; (4.3 ± 3.2% and 4.4 ± 2.1% in HCMV seronegative and seropositive donors, respectively). At the time of this study, the Cytomation summit software restricted the acquisition of cell events to 1 x 10^6 per sample. Because the frequency of $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ cells was relatively small, this meant that the average of $CD57^+$ events acquired in this gate was too small for analysis; 297 ± 115 events for HCMV seronegative and 713 ± 438 events for seropositive individuals (Table 4.5). It was therefore not possible to acquire enough events to draw reliable conclusions regarding the frequency of the individual $CD4^+$,$CD8^+$ double positive populations or the frequency of these cells that were $CD57^+$. Acquiring multiple data sets and pooling data was also not a realistic alternative. For example, for donor D15 2 data sets were acquired from the same sample, and the total number of CD4, CD8 double positive cells were 431 (242 from the first set and 189 from the second set of data). These few events meant that less than 40 cells were acquired in each of the CD4,CD8 double positive populations. This data indicated that the

Table 4.4 Phenotyping of the $CD3^+$,$CD56^+$ T-cells using the MoFlo

		% Total PBMC*	% $CD3^+CD56^{+\dagger}$			
	Donor	$CD3^+CD56^+$	$CD4^+$ $CD8^+$	$CD8^{hi}$ $CD4^{lo}$	$CD8^{lo}$ $CD4^{hi}$	$CD8^{hi}$ $CD4^{hi}$
Seronegative	D5	1.1	4.5	0.8	2.1	0.7
	D13	1.8	2.4	0.1	0.4	0.2
	D14	2.5	1.2	0.5	0.1	0.2
	D15	3.5	0.9	0.1	0.1	0.1
	D19	2.1	1.3	0.5	0.4	0.1
	D20	2.1	2.4	0.2	0.3	0.3
	D24	0.9	3.3	0.1	0.7	0
	D25	4.7	8.2	3.4	0.4	0.4
	D26	1.5	14.7	6.7	0.3	0.9
Mean ± SEM		2.2 ± 0.9	4.3 ± 3.2	1.4 ± 1.6	0.5 ± 0.4	0.3 ± 0.2
Seropositive	D3	4.7	6.7	4.4	0.5	0.3
	D10	3.2	3.2	12.6	0.8	7.6
	D11	2.8	8.7	4.9	0.6	1.9
	D18	3.9	1.6	0.2	0.3	0.2
	D30	2.3	3.1	0.8	0.5	0.2
	D36	6.2	2.3	0.2	0.7	0.3
	D39	8.7	5.0	1.7	0.1	0.2
Mean ± SEM		4.5 ± 1.7	4.4 ± 2.1	3.5 ± 3.2	0.5 ± 0.2	1.5 ± 1.8

(*) Percentage of PBMC as determined by flow cytometry.
(†) Expressed as a percentage of $CD3^+$,$CD56^+$ positive cells as determined by flow cytometry.

Table 4.5 Numbers of Events Acquired during Phenotyping of the $CD3^+$,$CD56^+$ T-cells using the MoFlo

		Number of Acquired Events*			
	Donor	$CD3^+CD56^+$ $CD4^+$ $CD8^+$	$CD8^{hi}$ $CD4^{lo}$	$CD8^{lo}$ $CD4^{hi}$	$CD8^{hi}$ $CD4^{hi}$
Seronegative	D5	350	65	165	50
	D13	222	9	17	33
	D14	63	28	4	9
	D15	431	19	31	13
	D19	150	61	42	12
	D20	252	25	32	28
	D24	483	274	32	104
	D25	440	180	21	19
	D26	281	128	5	18
Mean ± SEM		297 ± 115	88 ± 71	39 ± 29	32 ± 20
Seropositive	D3	923	608	59	48
	D10	1521	601	39	364
	D11	483	274	32	104
	D18	353	38	71	48
	D30	123	32	18	6
	D36	362	30	52	119
	D39	1229	410	32	51
Mean ± SEM		713 ± 438	285 ± 219	43 ± 15	106 ± 78

(*) The number of acquired events as determined by flow cytometry.

method was not reliable as many more events would be needed to be acquired for each donor for a significant analysis.

4.5 Variations in the Frequency of $CD3^+$,$CD56^+$ Cells

During this investigation a number of the donors were analysed in each study and Figure 4.2a showed that the frequency of $CD3^+$,$CD56^+$ cells varied with time. This variation in frequency may explain why only 2 of 3 studies showed that HCMV seropositive individuals had more $CD3^+$,$CD56^+$ cells and only 1 of 2 studies showed significant differences in the $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ subset. Interestingly, Figure 4.2b showed that if all of the data gathered from the 3 studies is pooled, HCMV seropositive individuals had more $CD3^+$,$CD56^+$ cells than seronegatives ($p = 0.0084$).

4.6 Summary

These data indicate that the proportion of $CD3^+$,$CD56^+$ T-cells fluctuated with time but that there was an association between increased frequency of $CD3^+$,$CD56^+$ T-cells and HCMV seropositivity. Additionally, the data also indicated that there may be an association with an increased frequency of $CD8^{hi}$,$CD4^{lo}$ and $CD8^{hi}$,$CD4^{hi}$ cells and HCMV seropositivity. However, due to technical constraints further analyses of these data was not achieved.

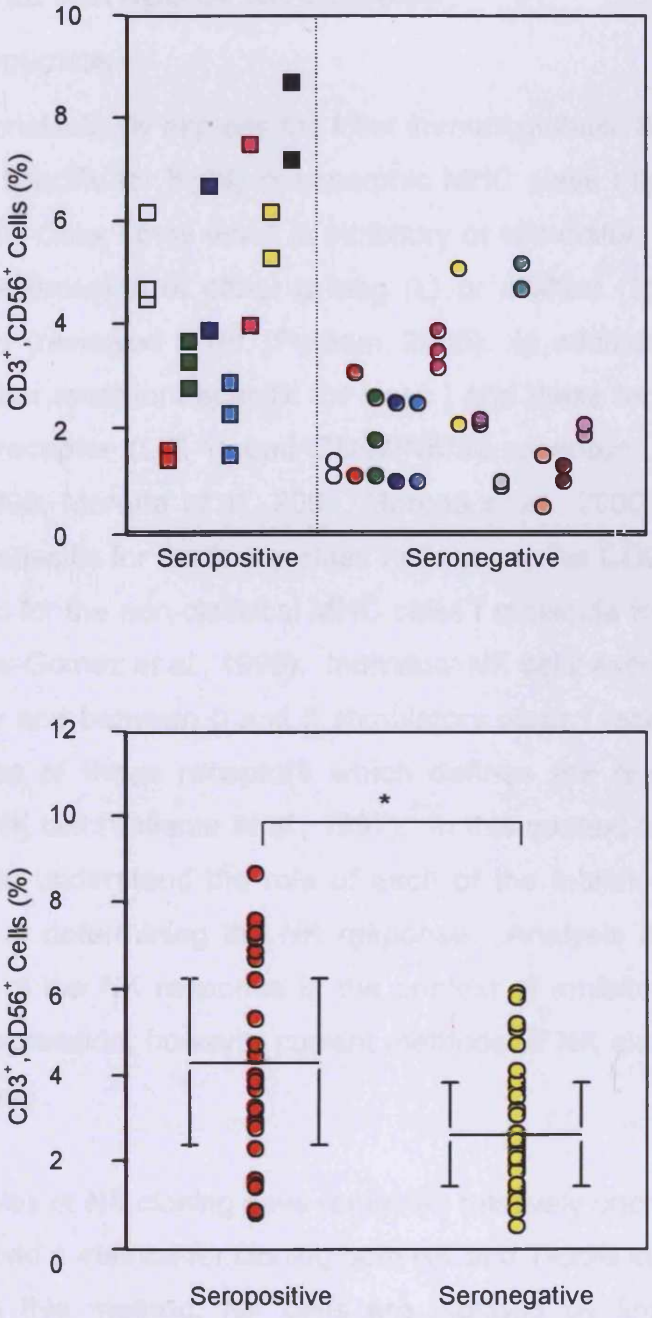

Figure 4.2 The Percentage of $CD3^+$,$CD56^+$ Cells in the Peripheral Blood of HCMV Seronegative and Seropositive Individuals. PBMC were stained with CD3 and CD56 and analysed by flow cytometry. (a) The percentage of $CD3^+$,$CD56^+$ cells varied over time. Results from each donor at different time points are presented by different colours in vertical lines. (b) Combining the percentage of $CD3^+$,$CD56^+$ cells acquired at each time point indicated that HCMV seropositive donors (red circles) had more $CD3^+$,$CD56^+$ cells than HCMV seronegative donors (yellow circles). * $p = 0.00084$.

5 A NOVEL METHOD OF NK CLONING

5.1 INTRODUCTION

NK cells constitutively express the killer immunoglobulin-like receptors (KIR), which are specific for highly polymorphic MHC class I ligands. Ligation of KIR by MHC class I may result in inhibitory or stimulatory activity depending upon the expression of either a long (L) or a short (S) cytotplasmic tail, respectively (reviewed in ref. (Parham, 2005). In addition to KIRs, NK cells express other receptors specific for class I and these include the leukocyte inhibitory receptor (LIR-1) and CD94/NKG2 receptors (reviewed in refs. (Lanier, 1998, Moretta *et al.*, 2001, Moretta *et al.*, 2000). Unlike the KIRs which are specific for particular class I allotypes, the CD94/NKG2 receptors are specific for the non-classical MHC class I molecule HLA-E (Braud *et al.*, 1998, Vales-Gomez *et al.*, 1999). Individual NK cells express between 1 and 6 inhibitory and between 0 and 5 stimulatory class I receptors and it is the combination of these receptors which defines the responsiveness of a particular NK cell (Valiante *et al.*, 1997). In this context it is becoming more important to understand the role of each of the inhibitory and stimulatory molecules in determining the NK response. Analysis of NK clones allow dissection of the NK response in the context of inhibitory and stimulatory receptor expression, however current methods of NK cloning are inefficient and laborious.

The principles of NK cloning have remained relatively unchanged since Yssel first described a method for cloning both NK and T-cells in 1984 (Yssel *et al.*, 1984). In this method, NK cells are isolated by limiting dilution and stimulated with irradiated EBV-LCL, PBMC and PHA. NK cells are then cultured in Yssel's medium, a serum free medium containing bovine serum albumin, transferrin, insulin, linoleic- oleic- and palmitic acid, penicillin and streptomycin. Whilst Yssel's medium was shown to be better than RPMI for NK clone culture, NK cloning using these methods are still inefficient (Spits & Yssel, 1996). More recently, Carlens *et al* (2000) have demonstrated that

the commercially available serum free media, SCGM is capable of supporting the expansion of polyclonal NK cells within PBMC (Carlens *et al.*, 2001, Carlens *et al.*, 2000). Additionally they demonstrated that the culture of PBMC *in vitro* in SCGM with the CD3 specific antibody, OKT3 boosted the number of NK cells within PBMC however, they did not investigate these conditions in a NK cloning environment. In this chapter, a novel method of NK cloning using SCGM, OKT3 and irradiated allogeneic PBMC was examined.

5.2 Culture of NK Cells in SCGM and RPMI

To compare the efficacy of SCGM with RPMI for culture of NK cells in the absence of other cell types, the ability of SCGM and RPMI to maintain the proliferation of FACs purified $CD3^-$,$CD56^+$ polyclonal NK cells over a 7 day period was tested in a ^3H thymidine incorporation assay (Figure 5.1). For all donors, ^3H thymidine incorporation values were greatest in SCGM + IL2. A 1-way ANOVA and subsequent pair-wise comparison of sample means via the Tukey HSD test, indicated that for all donors ^3H thymidine incorporation was greater in SCGM + IL2 than SCGM – IL2 ($p < 0.01$). For donors D11 and D12, ^3H thymidine incorporation was significantly greater in SCGM + IL2 than in RPMI + IL2 ($p < 0.01$). Addition of IL2 to RPMI did not significantly alter ^3H thymidine incorporation for donors D12 and D11 over the timecourse. However, IL2 was necessary for ^3H thymidine incorporation as polyclonal NK cells cultured in media without IL2 was lowest for D2 and D12 in both SCGM and RPMI.

5.3 Effects of IL2 Concentration on NK cell Proliferation

IL2 is essential for NK proliferation, however it was necessary to define the optimal concentration of IL2 for polyclonal NK cell proliferation using SCGM media. Polyclonal NK cells from 3 different donors were sorted from fresh PBMC and cultured in SCGM or RPMI with serial dilutions of IL2 from 125 IU/ml to 2000 IU/ml. ^3H thymidine incorporation assays were performed on

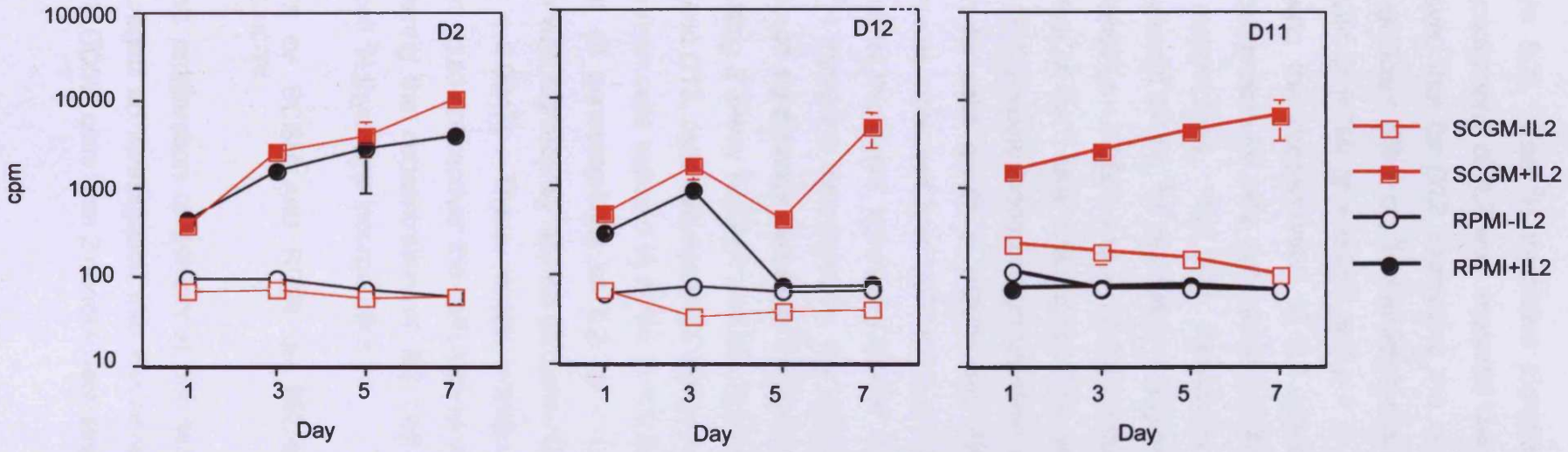

Figure 5.1 Comparison of Proliferation by Polyclonal NK Cells Cultured in SCGM or RPMI. Polyclonal NK cells were isolated from PBMC by FACS for $CD3^-$,$CD56^+$ cells and used in standard ^{3}H-thymidine incorporation assay following culture in either SCGM or RPMI supplemented with 5% AB serum ± 500 IU/ml IL2 at the different time points indicated. Results from 3 different donors are shown and represent mean ± SEM of triplicate or quadruplicate wells.

day 3 (Figure 5.2). The ^{3}H thymidine incorporation for each media at different concentrations of IL2 was analysed using a 1-way ANOVA. This analysis showed that for D12, increasing the concentration of IL2 had no statistically significant effect on ^{3}H thymidine incorporation for cells cultured in either SCGM or RPMI (p = 0.050 and p = 0.75, respectively) whilst for D13, increasing the concentration of IL2 significantly increased the ^{3}H thymidine incorporation for cells cultured in SCGM and RPMI (p = 0.039 and p = 0.016, respectively). For D14, increasing the concentration of IL2 significantly altered D14's ^{3}H thymidine incorporation for cells cultured in SCGM and RPMI ($p < 0.0001$ and p = 0.043, respectively). Tukey's multiple comparison test of the 1-way ANOVA for D14, indicated that there was no difference in ^{3}H thymidine incorporation between sequential increases in IL2 concentration for cells grown in SCGM and RPMI, except for when the concentration was increased from 1000 IU/ml to 2000 IU/ml of IL2. This data suggested that for this donor, between 125 and 1000 IU/ml IL2 could support high levels ^{3}H thymidine incorporation, but increasing IL2 concentration to 2000 IU/ml could significantly decrease ^{3}H thymidine incorporation. Analysis of the data using a 2-way ANOVA and the Bonferroni test indicated that for donors D12 and D13, cells cultured in SCGM incorporated significantly more ^{3}H thymidine than cells cultured in RPMI ($p < 0.0001$ for both) and this was significant at all concentrations of IL2 ($p < 0.05$). D14 ^{3}H thymidine incorporation was significantly altered for cells cultured in SCGM compared to RPMI (p = 0.0047). These results indicated that increasing the IL2 concentration could not recover the deficit observed between the two media and that altering the concentration of IL2 had different effects on each donor's NK cell ^{3}H thymidine incorporation.

5.4 Effects of SCGM and RPMI on NK clone Proliferation and Cytotoxicity

The improved proliferation of polyclonal NK cells in SCGM compared to RPMI encouraged an investigation into the benefits of SCGM for NK cell cloning. $CD3^{-}$,$CD56^{+}$ cells from 2 donors were single cell sorted from PBMC

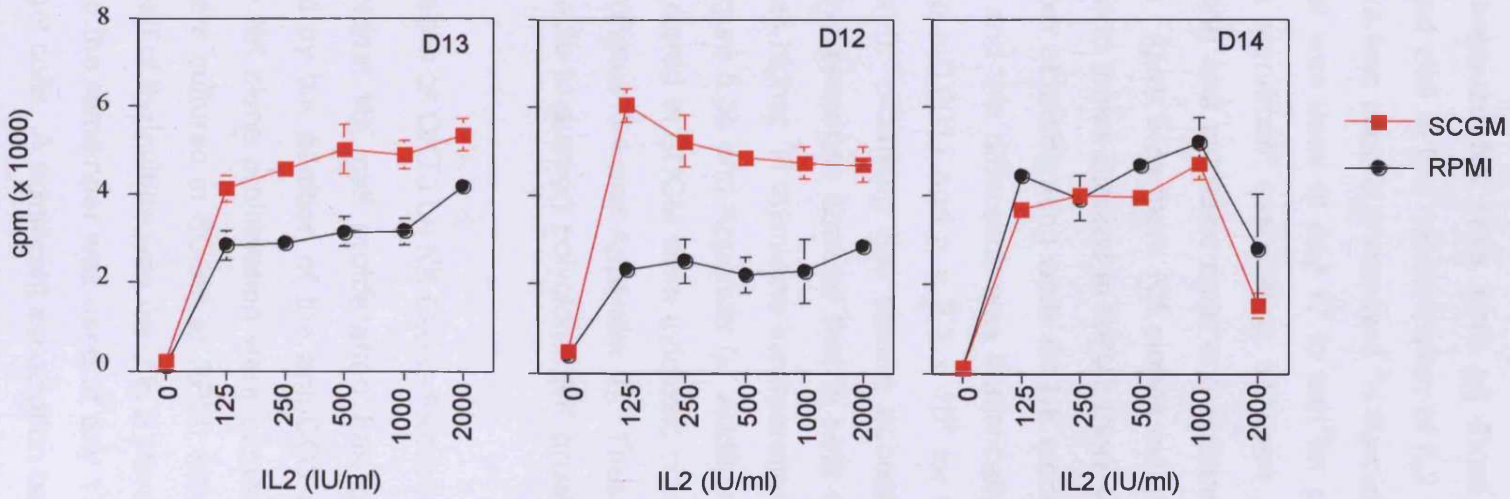

Figure 5.2 Comparison of Proliferation by Polyclonal NK Cells Cultured in SCGM or RPMI with Different Concentrations of IL2. Polyclonal NK cells were isolated from PBMC by FACS for $CD3^-$, $CD56^+$ cells and used in standard ^3H-thymidine incorporation assay following culture for 3 days in SCGM supplemented with 5% AB serum and IL2 at the concentrations indicated. Results from 3 different donors are shown and represent mean ± SEM of triplicate or quadruplicate wells.

into the central 60 wells of a 96 well U-bottomed plate, the outer right hand 6 wells of each plate were filled with media without clones to serve as a control. The cells were cultured in SCGM or RPMI containing irradiated allogeneic feeders from 3 donors and 500 IU/ml IL2. 500 IU/ml of IL2 was chosen because NK cells from all three individuals previously tested proliferated well at this concentration of IL2 (Figure 5.2). On day 10 half of the culture was used in a standard ^{3}H thymidine incorporation assay and the remainder was used at day 17 to test for cytotoxicity against K562 target cells. A significant association between increased proportions of both proliferating and cytotoxic cells and culture in SCGM was observed. As shown in Figure 5.3a more NK clones cultured in SCGM were proliferating compared to those cultured in RPMI; there was at least a 400% increase in the number of proliferating wells for NK clones cultured in SCGM compared to RPMI and this difference was statistically significantly different for both donors (p = 0.0011 and p = 2.3 x 10^{9} for D7 and D9, respectively) (See Appendix I). Ranking the clones according to the magnitude of the proliferative response showed that in both donors more clones cultured in SCGM had higher ^{3}H thymidine incorporation values than clones cultured in RPMI (Figure 5.3b and Appendix I). Additionally, at least 12 times as many clones cultured in SCGM were cytotoxic compared to those clones cultured in RPMI (Figure 5.4 and Appendix II). These data illustrated that SCGM is not only able to support polyclonal NK growth but also NK clone growth in culture.

5.5 Effects of OKT3 on NK Clone Proliferation and Cytotoxicity

As polyclonal NK cell proliferation has previously been shown to be enhanced by the addition of the anti-CD3 antibody, OKT3, the effects of OKT3 on NK clone proliferation were explored (Carlens *et al.*, 2000). NK clones were cultured in SCGM or RPMI with or without 50ng/ml OKT3. On day 10, half of the culture was used in a standard ^{3}H thymidine incorporation assay and the remainder was used at day 17 to test for cytotoxicity against K562 target cells. A significant association between increased proportions of both proliferating and cytotoxic cells and culture in SCGM was observed. As

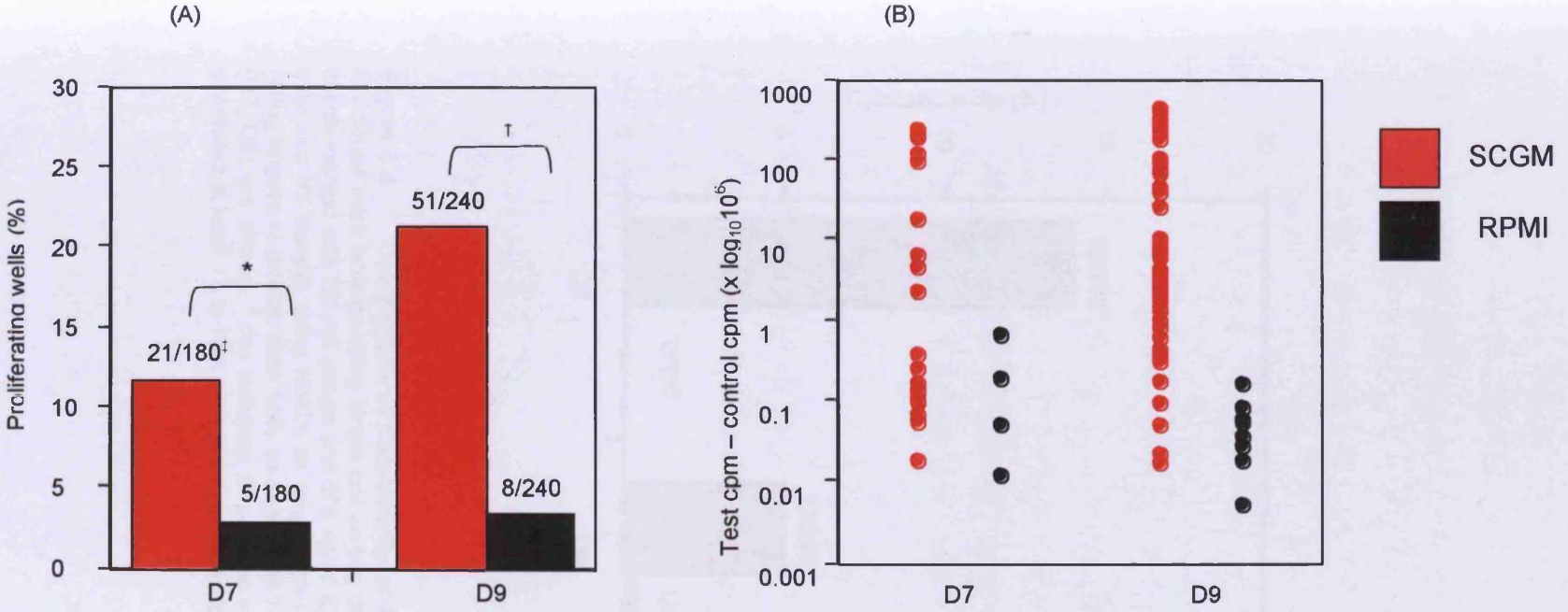

Figure 5.3 Comparison of Proliferation of NK Clones Cultured in SCGM or RPMI. NK clones were isolated using single cell sorting and used in a standard ^{3}H-thymidine incorporation assay following culture in either SCGM or RPMI supplemented with 5% AB serum, 500 IU/ml IL2 and irradiated allogeneic feeders for 10 days. Positive wells were identified as described in section 2.12.2. Results from 2 donors (D7, D9) are shown. (A) SCGM generated between 4 and 6 x more positive proliferating wells compared with RPMI. (B) The proliferating clones identified in (A) were ranked according to the magnitude of the proliferative response. More positive wells cultured in SCGM had higher incorporation values of ^{3}H-thymidine than wells cultured in RPMI. χ^2 tests showed significant differences between the indicated results at * $p = 0.0011$ and † $p = 2.3 \times 10^{-9}$.
‡ Number of positive wells and total wells tested. See Appendix I.

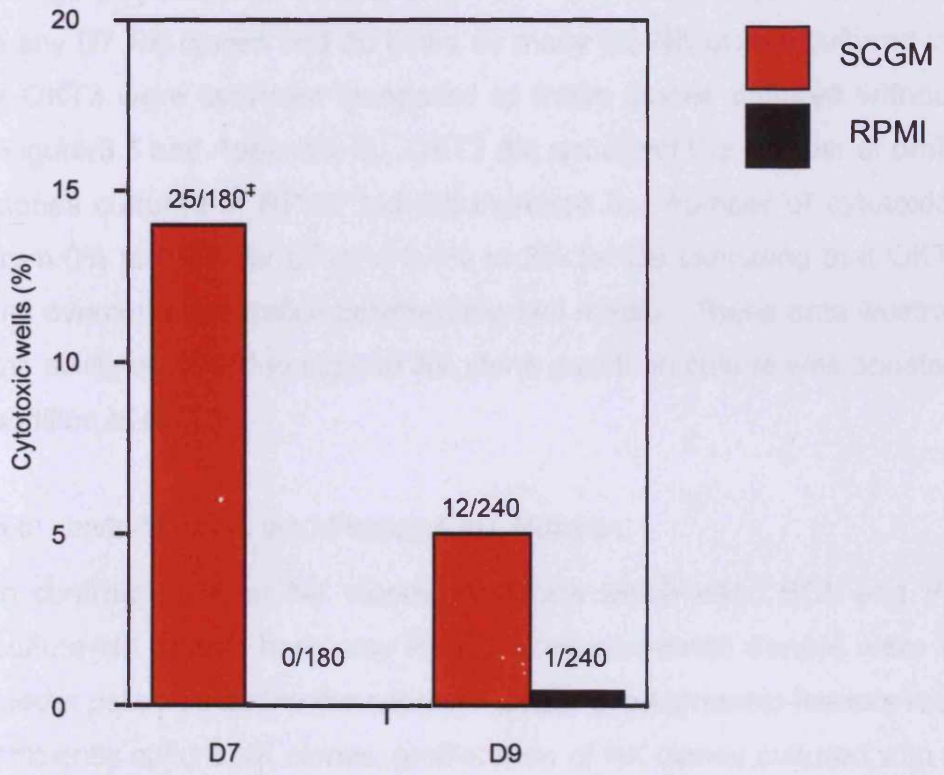

Figure 5.4 Comparison of Cytotoxicity of NK Clones Cultured in SCGM or RPMI. NK clones were isolated using single cell sorting direct and cultured in either SCGM or RPMI supplemented with 5% AB serum and 500 IU/ml IL2 and irradiated allogeneic feeders before cytotoxic NK assays using K562s as targets on day 17. Positive wells were identified as lysing targets at greater than 10% as described in section 2.11.1.4. Results from 2 donors (D7, D9) are shown. ‡ the numbers of positive wells are indicated above the bar. SCGM generated at least 12 to 14 x more positive cytotoxic wells compared with RPMI.

shown in Table 5.1 the addition of OKT3 in increased the number of proliferating clones cultured in SCGM by 157% for D7 and 78% for D9 (Appendix I). Categorising the proliferating clones cultured in SCGM according to the magnitude of the proliferative response showed that the addition of OKT3 increased the number of wells proliferating in the highest category by 400% for D7 and 314% for D9. Additionally, at least 14 times as many D7 NK clones and 30 times as many D9 NK clones cultured in SCGM + OKT3 were cytotoxic compared to those clones cultured without OKT3 (Figure 5.5 and Appendix II). OKT3 did not affect the number of proliferating clones cultured in RPMI, but did increase the number of cytotoxic clones from 0% to 0.6% for D7 and 0.4% to 2% for D9 indicating that OKT3 could not overcome the deficit between the two media. These data illustrated that the ability of SCGM to support NK clone growth in culture was boosted by the addition of OKT3.

5.6 Investigation into Feeder Cell Number

In contrast to other NK cloning methods which used BCL and PBMC to culture NK clones here only PBMC from allogeneic donors were used as feeder cells. To define the optimal number of allogneneic feeders required to efficiently culture NK clones, proliferation of NK clones cultured with different numbers of allogeneic feeder PBMC was investigated. NK clones were cultured in SCGM with 500 IU/ml IL2 and 50 ng/ml OKT3 with irradiated allogeneic PBMC from either 1, 2 or 3 different donors. On day 10 half of the culture was used in a standard ^{3}H thymidine incorporation assay and the remainder was used at day 17 to test for cytotoxicity against K562 target cells. Table 5.2 showed that NK clone proliferation was greatest when a combination of 2 irradiated allogeneic donors was used (See Appendix IV). 69% more NK clones were proliferating when cultured with allogeneic PBMC from 2 donors compared to 1 (p=0.0011). However, increasing the number of allogeneic donors from 1 to 3 only increased the number of proliferating wells by 35%, and this was not significantly different. Categorising the clones according to the magnitude of the proliferative response showed that

Table 5.1 Comparison of Proliferation of NK Clones Cultured in SCGM or RPMI with or without OKT3

	Positive Wells*			
Proliferation cpm x 1000	SCGM†	SCGM+OKT3‡	RPMI§	RPMI+OKT3‖
D7				
<50	15/21 (71%)	22/54 (40%)	5/5 (100%)	1/2 (50%)
51-100	1/21 (5%)	9/54 (17%)	0/5 (0%)	0/2 (0%)
101-150	2/21 (10%)	7/54 (13%)	0/5 (0%)	0/2 (0%)
151-200	1/21 (5%)	6/54 (11%)	0/5 (0%)	0/2 (0%)
201+	2/21 (10%)	10/54 (19%)	0/5 (0%)	1/2 (50%)
Total Positive*	21/180¶	54/180¶	5/180#	2/180#
D9				
50<	37/51 (72%)	31/91 (34%)	4/8 (50%)	0/0 (0%)
51-100	6/51 (12%)	15/91 (16%)	3/8 (38%)	0/0 (0%)
101-150	0/51 (0%)	9/91 (10%)	0/8 (0%)	0/0 (0%)
151-200	1/51 (20%)	7/91 (8%)	1/8 (12%)	0/0 (0%)
201+	7/51 (14%)	29/91 (31%)	0/8 (0%)	0/0 (0%)
Total Positive*	51/240**	91/240**	8/240#	0/240#

(*) Positive wells were identified as described in section 2.12.2
(†) SCGM media supplemented with 500 IU/ml IL2.
(‡) SCGM media supplemented with 500 IU/ml IL2 and 50 ng/ml OKT3.
(§) RPMI media supplemented with 500 IU/ml IL2.
(‖) RPMI media supplemented with 500 IU/ml IL2 and 50 ng/ml OKT3.
(¶) The percentage of wells proliferating in each category were compared using the χ^2 test. Proliferation of cells cultured in SCGM were not significantly different to cells cultured in SCGM + OKT3 ($p = 0.19$).
(#) The percentage of wells proliferating in each category could not be compared using the χ^2 test as there were too few samples in each category.
(**) The percentage of wells proliferating in each category were compared using the χ^2 test. Proliferation of cells cultured in SCGM were significantly different to cells cultured in SCGM + OKT3 ($p = 0.00022$).

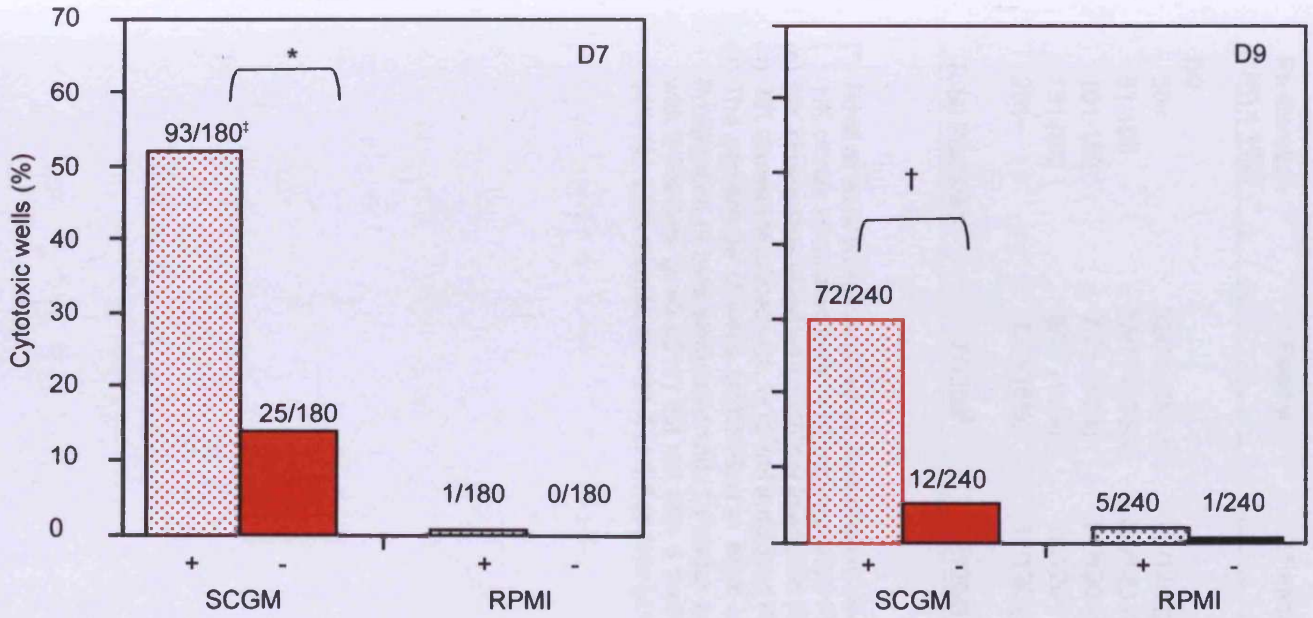

Figure 5.5 Comparison of Cytotoxicity of NK Clones Cultured in SCGM or RPMI with or without OKT3. NK clones were isolated using single cell sorting and cultured in either SCGM or RPMI supplemented with 5% AB serum, 500 IU/ml IL2 and irradiated allogeneic feeders with (+) or without (-) 50 ng/ml OKT3 before cytotoxic NK assays using K562s as targets on day 17. Positive wells were identified as lysing targets at greater than 10% as described in section 2.11.1.4. Results from 2 donors (D7, D9) are shown. OKT3 generated between 14 and 30 x more positive cytotoxic wells for cells cultured in SCGM. Atleast 5x more positive cytotoxic wells were identified for cells cultured in RPMI with OKT3. χ^2 tests showed significant differences between the indicated results at * $p = 2.3 \times 10^{-14}$ and † $p = 7.6 \times 10^{-14}$. ‡ Number of positive wells and total wells tested. See Appendix II.

Table 5.2 Comparison of Proliferation of NK Clones Cultured with Irradiated PBMC Feeders Derived from Different Numbers of Allogeneic Donors.

	Positive Wells*		
Proliferation cpm x 1000	1 Feeder	2 Feeders[a]	3 Feeder[¤]
D9			
50<	32/71 (45%)	28/120 (23%)	26/96 (27%)
51-100	18/71 (26%)	20/120 (17%)	20/96 (21%)
101-150	7/71 (10%)	21/120 (18%)	18/96 (19%)
151-200	8/71 (11%)	19/120 (16%)	16/96 (17%)
201+	6/71 (8%)	32/120 (26%)	16/96 (17%)
Total Positive*	71/300[‖]	120/300[‖]	96/300[‖]

(*) Positive wells were identified as described in section 2.11.2
( ) NK clones stimulated with $1x10^6$/ml irradiated PBMC from 1 donor
(a) NK clones stimulated with $1x10^6$/ml irradiated PBMC from 2 different donors
(¤) NK clones stimulated with $1x10^6$/ml irradiated PBMC from 3 different donors
(‖) The percentage of wells proliferating in each category were compared using the χ^2 test. Proliferation of cells stimulated with 1 Feeder was significantly different to cells stimulated with 2 Feeders (p =0.0011) but not with 3 feeders (p = 0.055). There was no difference between cells stimulated with 2 or 3 Feeders (p = 0.51).

most of the clones stimulated with 2 allogeneic feeders were in the highest category (26%), compared to the lowest percentage of clones stimulated with 1 feeder (8%). In contrast, most of the clones stimulated with 1 feeder were in the lowest category (45%) compared to 23% of clones stimulated with 2 feeders. Clones stimulated with 3 allogeneic feeders were distributed a across the proliferative range. These data are supported by the cytotoxicity data which showed that more cytotoxic clones were generated when NK clones were stimulated with 2 donors compared to 1 or 3 allogeneic donors (Figure 5.6 and Appendix III).

5.7 The Novel Method Expands NK Clones

Using this novel method of NK cloning a panel of at least 140 clones from 4 different individuals were cultured. These clones were maintained in culture for a period of up to 10 weeks. Flow cytometric analysis for CD3 and CD56 was used to confirm that the NK cell phenotype and that the cells were clonal. Nine clones were excluded from further analysis because flow cytometric analysis indicated either a double population of $CD56^+$ cells or of $CD94^+$ cells, indicating that the cultures were not clonal. A further 17 NK clones were not able to kill the MHC class I negative cell line, K562 at a percentage specific lysis of greater than 20% and were excluded from analysis. Seven cultures contained contaminating T-cells and were excluded from analysis. The remaining 116 NK clones were used for subsequent experiments.

5.8 Summary

Collectively, this data indicated that NK clones grow most efficiently in SCGM supplemented with 500 IU/ml IL2, 50 ng/ml OKT3 and stimulated with PBMC from more than 1 allogeneic donor. It is a major extension of previous work, which showed that SCGM is more efficient than RPMI for expansion of polyclonal NK cells within a PBMC culture (Carlens *et al.*, 2001, Carlens *et al.*, 2000). This system has re-defined the conditions required for NK cell clone growth and is a simple and highly efficient method for of NK cloning.

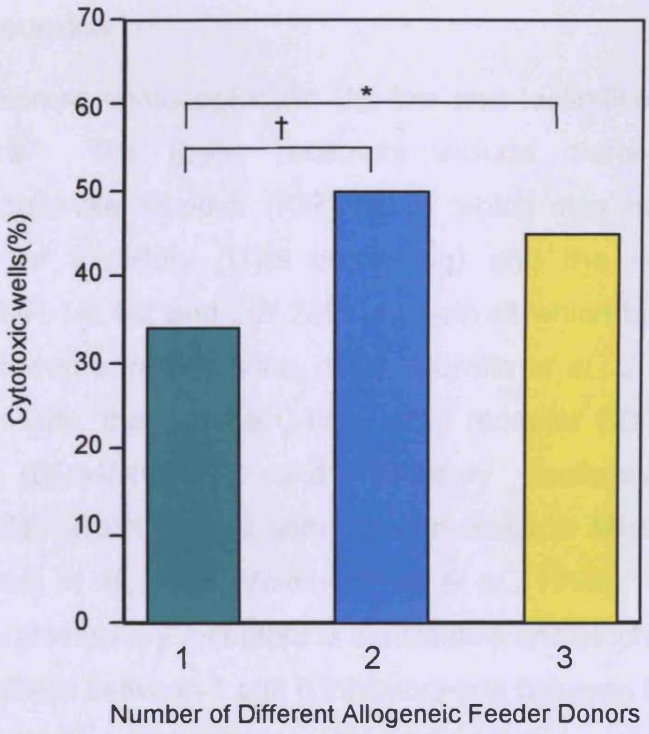

Figure 5.6 Comparison of Cytotoxicity of NK Clones Cultured with Irradiated PBMC Feeders Derived from Different Numbers of Allogeneic Feeder donors. NK clones were isolated using single cell sorting direct and cultured in SCGM supplemented with 5% AB serum, 500 IU/ml IL2 and 50 ng/ml OKT3. Different numbers of donors were used for irradiated allogeneic feeders. Cytotoxic NK assays using K562s as targets were performed on day 17. Positive wells were identified as lysing targets at greater than 20% as described in section 2.11.1.4. χ^2 tests showed significant differences between the indicated results at * $p = 0.0082$ and † $p = 0.000056$.

6 INVESTIGATION OF NK RESPONSES TO HCMV STRAIN AD169

6.1 INTRODUCTION

NK cells express immunoglobulin (Ig)-like and lectin-like receptors at their cell surface. The Ig-like receptors include members of the killer immunoglobulin-like receptor (KIR) family which may be activating (ITAM containing) or inhibitory (ITIM containing) and the leukocyte inhibitory receptors (LIR-1/ILT-2 and LIR-2/ILT-4), both of which bind to MHC class I alleles (reviewed in refs. (Lanier, 1998, Moretta *et al.*, 2001, Moretta *et al.*, 2000). Similarly, the surface C-type lectin receptor CD94/NKG2 has both activating (CD94/NKG2C) and inhibitory isoforms (CD94/NKG2A, CD94/NKG2B) which interact with the non-classical MHC class I molecule HLA-E (Braud *et al.*, 1998, Vales-Gomez *et al.*, 1999). The expression of activating and inhibitory receptors is constitutive and stochastic. A single NK cell may express between 1 and 6 inhibitory and between 0 and 5 stimulatory class I receptors and will express at least 1 inhibitory receptor that is specific for self MHC class I (Valiante *et al.*, 1997). The selective expression of inhibitory and activating KIR by different NK cells means that whilst one NK cell may potentially be activated by the loss of 1 HLA allele, another may be inhibited.

HCMV has developed sophisticated mechanisms to evade NK cell lysis. The HCMV MHC class I homolog gpUL18 exhibits high affinity for LIR-1, however the role of gpUL18 in virus infection remains to be resolved (Chapman *et al.*, 1999, Cosman *et al.*, 1997). Additionally, the HCMV glycoprotein UL16 binds ULBP-1, ULBP-2 and MICB, preventing ligation by the NK-activating receptor NKG2D, representing another potential mechanism of NK evasion (Cosman *et al.*, 2001). Finally the HCMV UL40 protein contains an HLA-E stabilising leader sequence that results in increased HLA-E expression at the cell surface (Tomasec *et al.*, 2000). The physiological significance of the CD94/NKG2A interaction with HLA-E stabilised by the UL40 leader sequence is still debated. Wang *et al,* (2002) showed that blocking of CD94 on NK

cells rendered strain AD169 infected fibroblasts more susceptible to NK lysis and that a mutant HCMV lacking UL40 was not protected from $CD94^{hi}$ NK cell lysis (Wang *et al.*, 2002). In contrast, Carr *et al,* (2002) showed that blocking of CD94 did not alter the ability of NK clones to lyse HCMV infected cells and demonstrated no correlation with NK receptor expression and susceptibility to HCMV strain AD169 infected cells to NK killing (Carr *et al.*, 2002). Falk *et al,* (2002) showed that the activation of NK cells induced by MHC downregulation was dominant over the UL40 effect (Falk *et al.*, 2002). Central to the understanding of these contradictory data is the resolution of how HCMV is able to both activate and inhibit NK cells.

In this study, the response of NK cells to HCMV strain AD169 in relation to their CD94 expression has been dissected. As a commercial antibody to CD94/NKG2C became available part way through the study, the frequency of CD94/NKG2C and CD94/NKG2A subsets in HCMV seropositive and seronegative individuals and their responses to HCMV UL40 stabilised HLA-E was investigated.

6.2 Isolation of Polyclonal NK cells and culture of NK clones

6.2.1 Isolation of Polyclonal NK cells

Freshly isolated polyclonal NK cells allow analysis of the general behavior of NK cells with minimal manipulation through tissue culture. Freshly isolated polyclonal NK cells were therefore initially used to examine the differential ability of $CD94^{hi}$ and $CD94^{lo}$ NK cells to lyse strain AD169 infected targets in comparison to their ability to lyse uninfected targets. $CD3^-$, $CD56^+$ polyclonal NK cells were enriched from fresh PBMCs by Dynal bead depletion of $CD3^+$ cells. $CD3^-$, $CD56^+$, $CD94^{lo}$ polyclonal NK cells were prepared by depletion of $CD94^+$ as well as $CD3^+$ cells. As these cells exhibit little NK activity in the absence of stimulation the cultures were stimulated with 1000 IU/ml of IFNα overnight to induce cytotoxicity (Borysiewicz *et al.*, 1985).

6.2.2 Analysis of NK clones

Using the novel method for NK cloning, a panel of 110 NK clones from 4 different individuals was generated. Clones were categorised as being $CD94^{lo}$ or $CD94^{hi}$ depending on the CD94 fluorescence channel at which separation of the two populations could be observed in their donor's PBMC as shown in Figure 6.1a. Figure 6.1b shows how the clones were categorised according to their ability to lyse uninfected fibroblasts compared to strain AD169 infected fibroblasts in an autologous setting. The 4 categories were inhibition, activation, kill/no change and no kill/ no change. For a clone to be categorised as being inhibited it had to lyse uninfected fibroblasts at or greater than 10% and lyse strain AD169 infected fibroblasts at or greater than 10% less than uninfected cells. For a clone to be categorised as being activated by strain AD169, it had to lyse uninfected fibroblasts at or greater than 10% and lyse strain AD169 infected fibroblasts at or greater than 10% more than uninfected cells. Kill/no change clones were identified as those which were able to lyse uninfected fibroblasts at or greater than 10% but there was less than 10% difference in the ability to lyse strain AD169 infected fibroblasts. A no kill/no change clone lysed both uninfected and strain AD169 infected cells less than 10%. A 10% difference was taken as being significant because this was greater than 3 standard deviations of the mean % specific lysis of a plate read 4 times on the 1450 Microbeta Trilux Liquid Scintillation Counter (Table 6.1). This analysis was also used to analyse $CD94^{hi}$ and $CD94^{lo}$ NK responses to strain AD169 versus Towne infected fibroblasts and $CD94/NKG2A^{hi}$ and $CD94/NKG2C^{hi}$ NK clone responses to uninfected versus strain AD169 infected fibroblasts, strain AD169 versus strain AD169 ΔUL40 infected fibroblasts and RAd60 versus RAdUL40 infected fibroblasts. The frequency of clones in each category was tested statistically using a χ^2 test.

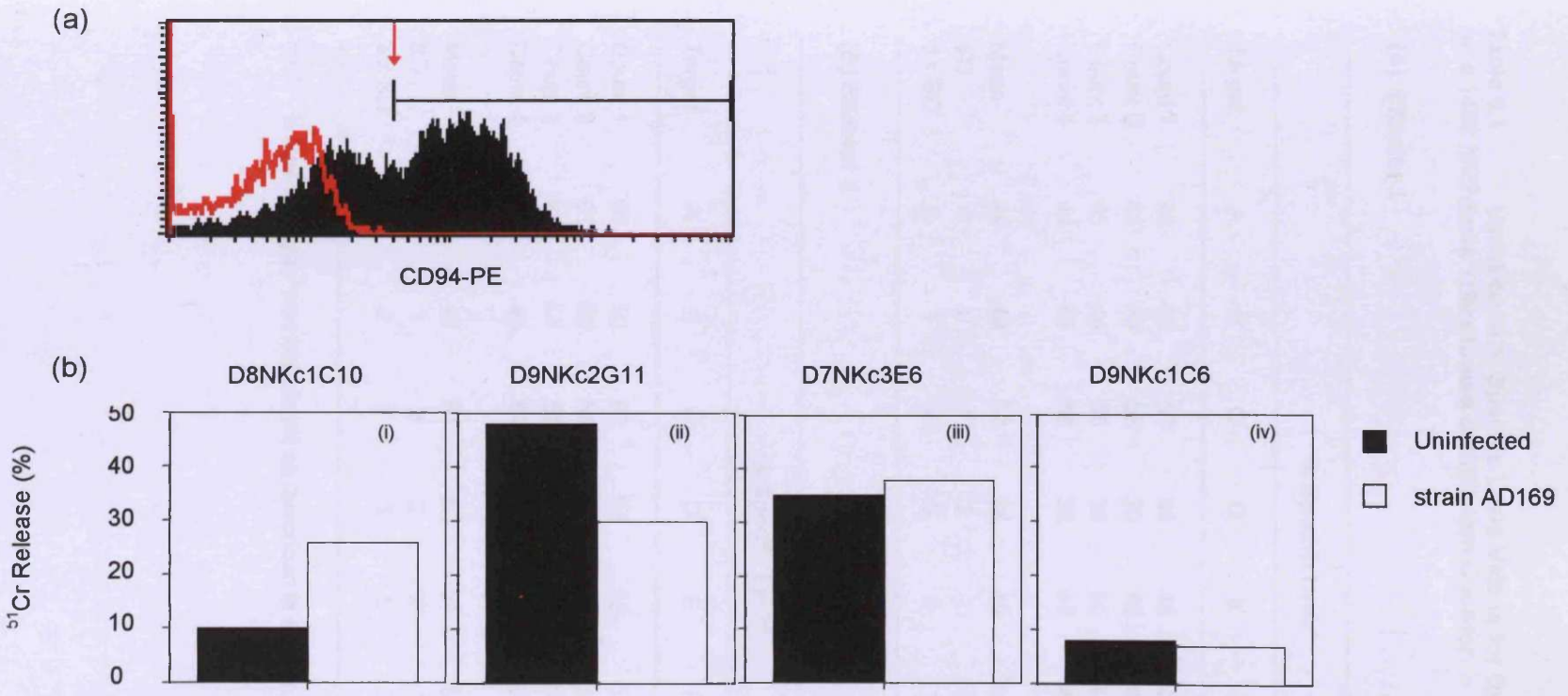

Figure 6.1. Categorsing NK Clone Phenotype and NK Clone Responses to Uninfected and strain AD169 Infected Autologous Fibroblasts. NK clones were single cell sorted from PBMC by FACS for $CD3^-$, $CD56^+$ cells and used in standard ^{51}Cr release assays with uninfected or strain AD169 infected (MOI 10, 72hr p.i) fibroblasts as targets. (a) PBMC were stained with anti CD3, CD56 and CD94 and gated for $CD3^-$, $CD56^+$ and then for CD94. The CD94 flourescence channel at which separation of the two populations could be observed (red arrow) was used to categorise NK clones as being $CD94^{hi}$ or $CD94^{lo}$. An isotype control is shown in red. (b) NK clones were categorised as (i) activated, (ii) inhibited, (iii) kill/no change or (iv) no kill/no change according to their ability to lyse strain AD169 infected compared to uninfected targets.

Table 6.1 Variation in % Specific Lysis Values for the Same Plates Read 4 Times on a 1450 Microbeta Trilux Liquid Scintillation Counter.

(A) Effector 1

	% Specific Lysis*							
Target	A	B	C	D	E	F	G	H
Count 1	45	48	57	36	49	47	55	33
Count 2	44	49	58	39	48	47	54	35
Count 3	45	48	58	39	50	46	55	36
Count 4	44	49	55	36	48	46	51	35
Mean	44	49	57	38	49	46	54	35
SD	0	0	1	2	1	0	2	1
3 x SD	1	1	4	5	3	1	5	3

(B) Effector 2

	% Specific Lysis*							
Target	A	B	C	D	E	F	G	H
Count 1	63	50	62	54	53	57	63	47
Count 2	61	50	59	52	51	56	60	45
Count 3	63	48	59	51	49	56	61	45
Count 4	61	48	64	52	49	57	60	47
Mean	62	49	61	52	51	56	61	46
SD	1	1	2	2	2	1	1	1
3 x SD	4	3	7	5	5	2	4	3

(*) % Specific Lysis was calculated as described in section 2.12.1

6.3 NK Responses to HCMV strain AD169

6.3.1 $CD94^{lo}$ Clones are Activated by HCMV Strain AD169

A total of 25 $CD94^{lo}$ and 85 $CD94^{hi}$ NK clones were cultured from 4 donors (See Appendix VI). Analysis of $CD94^{lo}$ and $CD94^{hi}$ responses to uninfected versus strain AD169 infected targets showed that 44% (11/25) of the $CD94^{lo}$ clones were activated by strain AD169 infected targets compared to 11% (9/85) of $CD94^{hi}$ NK clones (Figure 6.2 a). Conversely, more $CD94^{hi}$ NK cells (25% (21/85)) were inhibited by strain AD169 infected targets than $CD94^{lo}$ NK cells (8% (2/25)) (Figure 6.2 b). The numbers of clones in each category were tested using a χ^2 test and were statistically significantly different (p = 0.0014).

6.3.2 NK Clone Responses to Uninfected and HCMV strain AD169 Infected Targets Vary Between Individuals

Whilst pooled NK clone responses show that more $CD94^{lo}$ NK clones were activated by strain AD169, considerable variation was observed between different individuals (Table 6.2). Comparison of the variation in responses of $CD94^{hi}$ and $CD94^{lo}$ NK clones was only possible for donors D7 and D9 as too few $CD94^{lo}$ clones were cultured from donors D3 and D8. These data showed that in contrast to D9, both $CD94^{hi}$ and $CD94^{lo}$ NK clones derived from donor D7 were activated by strain AD169 infected targets with 69% (9/13) and 58% (7/12), respectively, being activated by strain AD169 compared to no $CD94^{lo}$ and 30% (3/10) of $CD94^{hi}$ NK clones from D9. Interestingly in the pooled data, the $CD94^{hi}$ NK clones which were activated by strain AD169 were all derived from D7 and 28% (7/25) of the activated $CD94^{lo}$ NK clones were also derived from D7. Importantly, even with omission of donor D7's NK clone response from the pooled data, more $CD94^{lo}$ clones were activated by strain AD169 and this withstood statistical analysis (p = 0.000014) (Table 6.3). In contrast, D3 NK clones were generally unresponsive; all of D3 $CD94^{hi}$ NK clones and 50% (1/2) of $CD94^{lo}$

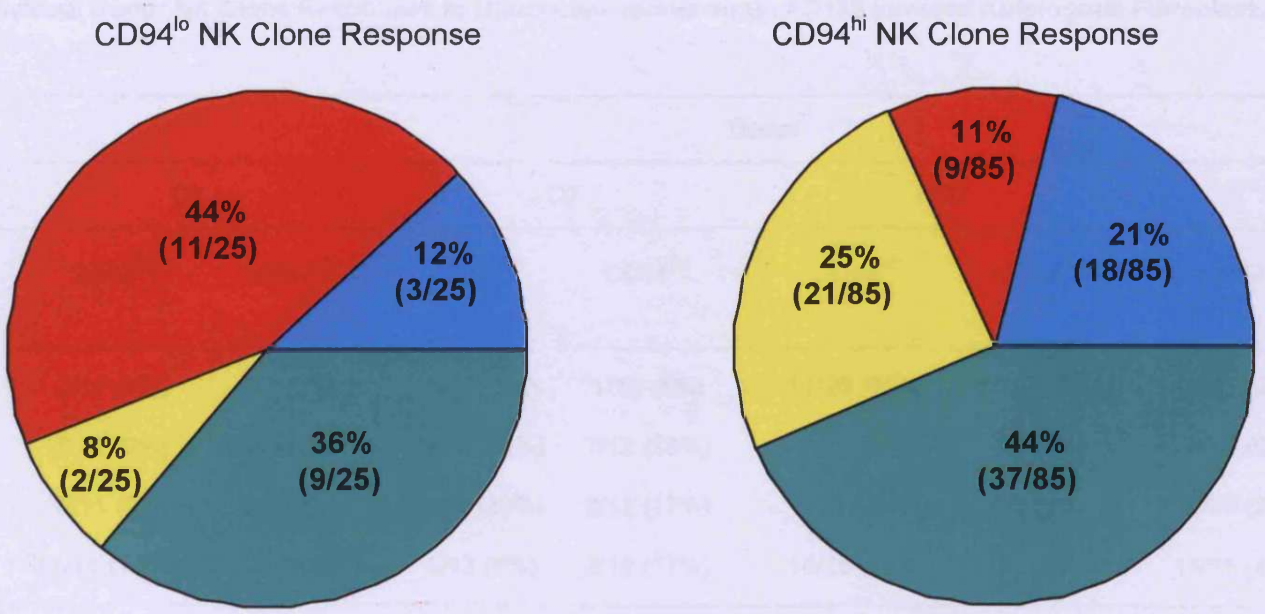

Figure 6.2. Comparison of CD94^{hi} and CD94^{lo} NK Responses to Uninfected versus strain AD169 Infected Autologous Fibroblasts. NK clones from 4 individuals were divided into CD94^{hi} and CD94^{lo} categories. NK clones were further categorised as being activated (red), inhibited (yellow), kill/no change (blue), or no kill/no change (green). More CD94^{lo} clones were activated by strain AD169 compared to CD94^{hi} clones (p = 0.0014). See Appendix V.

Table 6.2 Individual Donor NK Clone Responses to Uninfected versus strain AD169 Infected Autologous Fibroblasts

	Donor							
	D3		D7		D8		D9	
NK Clone Response*	$CD94^{hi\dagger}$	$CD94^{lo\dagger}$	$CD94^{hi\dagger}$	$CD94^{lo\dagger}$	$CD94^{hi\dagger}$	$CD94^{lo\dagger}$	$CD94^{hi\dagger}$	$CD94^{lo\dagger}$
Inhibition	0/11 (0%)	0/2 (0%)	0/13 (0%)	1/12 (8%)	11/26 (42%)	1/1 (100%)	10/35 (29%)	0/10 (0%)
Activation	0/11 (0%)	1/2 (50%)	9/13 (69%)	7/12 (58%)	0/26 (0%)	0/1 (0%)	0/35 (0%)	3/10 (30%)
Kill/No Change	0/11 (0%)	0/2 (0%)	3/13 (23%)	2/12 (17%)	5/26 (25%)	0/1 (0%)	10/35 (29%)	1/10 (10%)
No Kill/No Change	11/11 (100%)	1/2 (50%)	1/13 (8%)	2/12 (17%)	10/26 (38%)	0/1 (0%)	15/35 (43%)	6/10 (60%)

(*) NK clones were categorised as described in section 6.2.2 and figure 6.1
(†) PBMC were stained with anti CD3, CD56 and CD94 and gated for $CD3^-$,$CD56^+$ and then for $CD94^+$ cells. NK clones were categorised as being $CD94^{hi}$ or $CD94^{lo}$.according to the CD94 fluorescence channel at which separation of the two populations could be observed.

Table 6.3 Combined NK Clone Responses to Uninfected and strain strain AD169 Infected Autologous Fibroblasts

NK Clone Response*	Total NK Clones		NK Clones excluding D7	
	$CD94^{hi\dagger}$	$CD94^{lo\dagger}$	$CD94^{hi\dagger}$	$CD94^{lo\dagger}$
Inhibition	21/85 (25%)	2/25 (8%)	21/72 (29%)	1/13 (8%)
Activation	9/85 (11%)	11/25 (44%)	0/72 (0%)	4/13 (30%)
Kill/No Change	18/85 (21%)	3/25 (12%)	15/72 (20%)	1/13 (8%)
No Kill/No Change	37/85 (44%)	9/25 (36%)	36/72 (50%)	7/13 (54%)
p value[II]	0.0014		0.000014	

(*) NK clones were categorised as described in section 6.2.2 and Figure 6.1

(†) PBMC were stained with anti CD3, CD56 and CD94 and gated for $CD3^-$,$CD56^+$ and then for $CD94^+$ cells. NK clones were categorised as being $CD94^{hi}$ or $CD94^{lo}$.according to the CD94 fluorescence channel at which separation of the two populations could be observed

(II) *p* values calculated using a χ^2 test.

NK clones were unable to lyse either uninfected or strain AD169 infected targets.

6.3.3 Comparison of NK Clone Responses and Polyclonal NK Cell Cultures

The reliability of cloned NK cells to provide an insight into *in vivo* NK responses to HCMV was tested by comparison of the clonal data with the polyclonal NK assays. Polyclonal NK cells were isolated from PBMC using Dynal beads. Using this method between 83% and 95% of $CD3^+$ and 60% and 95% of $CD3^+$,$CD94^+$ cells were depleted from PBMC (Table 6.4). As shown in Table 6.5, D7 polyclonal and $CD94^{lo}$ polyclonal NK cells were activated by strain AD169 infected targets and this pattern was observed over a range of E:T ratios (Figure 6.3). This activation pattern was reflected in D7 NK clone data, which showed that most of the $CD94^{hi}$ clones (69% 9/13) and most of the $CD94^{lo}$ clones (58% 7/12) were activated by strain AD169 infected targets.

D9 16/5/3 polyclonal data showed that D9 $CD94^{lo}$ polyclonal NK cells were able to lyse both uninfected and strain AD169 infected targets but were unable to respond differently to each target. In contrast, D9 total NK cells were inhibited by strain AD169 suggesting that $CD94^{hi}$ NK cells were inhibited by strain AD169. Interestingly, D9 27/5/4 data suggested that both $CD94^{hi}$ and $CD94^{lo}$ NK cells were unresponsive towards strain AD169 infected targets. However, statistical analysis showed that a decrease in % specific lysis of 7% for D9 27/5/4 total NK cells and 3% for $CD94^{lo}$ NK cells were both statistically significant (p = 0.0078 and 0.036, respectively). Whilst a 7% difference may be real, the difference of 3% is likely to be a result of variation within the system. Therefore, D9 16/5/3 and possibly D9 27/5/4 polyclonal data supported the $CD94^{hi}$ NK clone data which showed that 29% (10/35) of $CD94^{hi}$ clones were inhibited by strain AD169. However, in contrast to the $CD94^{lo}$ NK clone data, neither of the polyclonal assays indicated that $CD94^{lo}$ clones were activated by strain AD169.

Reliable comparisons of $CD94^{hi}$ and $CD94^{lo}$ NK clone responses with

Table 6.4 Calculation of Polyclonal NK Cell Purity after Isolation using Dynal Beads

	% Pre DP-% Post DP*			% Depletion†		
	Total‡	$CD94^{lo}$§		Total‡	$CD94^{lo}$§	
Donor	CD3	CD3	CD94	CD3	CD3	CD94
D3 15/7/3	65 ↓3	65 ↓ 3	42 ↓ 5	95%	95%	88%
D7 10/6/4	52 ↓ 7	51 ↓ 16	45 ↓12	87%	69%	73%
D9 16/5/3	74 ↓ 6	74 ↓ 5	64 ↓ 13	92%	93%	80%
D9 27/5/4	66 ↓ 11	66 ↓ 10	62 ↓ 25	83%	85%	60%
D10 16/6/4	74 ↓ 3	77 ↓ 6	60 ↓ 18	96%	92%	70%
D11 23/6/4	63 ↓ 6	65 ↓ 12	62 ↓ 9	90%	82%	85%

(*) First value represents the % of indicated cells present prior to depletion and second value represent the % of cells present after depletion as measured by flow cytometry.

(†) Calculated from % of cells present prior to depletion and the % of cells present after depletion as measured by flow cytometry.

(‡) Total NK cells represent polyclonal NK cells isolated from PBMC by dynal bead depletion and activated overnight in IFN-α (see section 2.5.2).

(§) $CD94^{lo}$ polyclonal NK cells isolated from PBMC by dynal bead de pletion and activated overnight in IFN-α (see section 2.5.2).

Table 6.5 Polyclonal and CD94^{lo} Polyclonal NK Responses to Uninfected and strain AD169 Infected Autologous Fibroblasts

	% Specific Lysis ± SEM*							
	Total†				CD94^{lo}‡			
Donor	E:T	UI	strain AD169	p§	E:T	UI	strain AD169	p§
D3 15/7/3	30	7 ± 1	13 ± 1	0.00077	60	5 ± 1	8 ± 2	NS
D7 10/6/4	44	10 ± 1	35 ± 1	0.000025	68	5 ± 0.2	34 ± 2	0.0044
D8 29/5/3	22	17 ± 1	7 ± 1	0.000037	35	21 ± 6	12 ± 1	NS
D9 16/5/3	45	39 ± 4	20 ± 4	0.0062	54	24 ± 1	25 ± 2	NS
D9 27/5/4	44	20 ± 1	13 ± 1	0.0078	64	15 ± 1	12 ± 1	0.036
D10 16/6/4	60	23 ± 3	16 ± 1	NS	30	13 ± 1	11 ± 1	NS
D11 23/6/4	66	6 ± 1	5 ± 1	NS	42	2 ± 1	4 ± 2	NS

NS, not significant (p >0.05).

(*) % Specific Lysis was calculated as described in section 2.10.1.4.
(†) Total represents polyclonal NK cells isolated from PBMC by dynal bead depletion and activated overnight with IFN-α (see section 2.5.2).
(‡) CD94^{lo} polyclonal NK cells enriched from PBMC by dynal bead depletion and activated overnight in IFN-α (see section 2.4.2).
(§) p values compare killing of uninfected versus strain AD169 infected targets (Student's t-tests, assuming unequal variance).

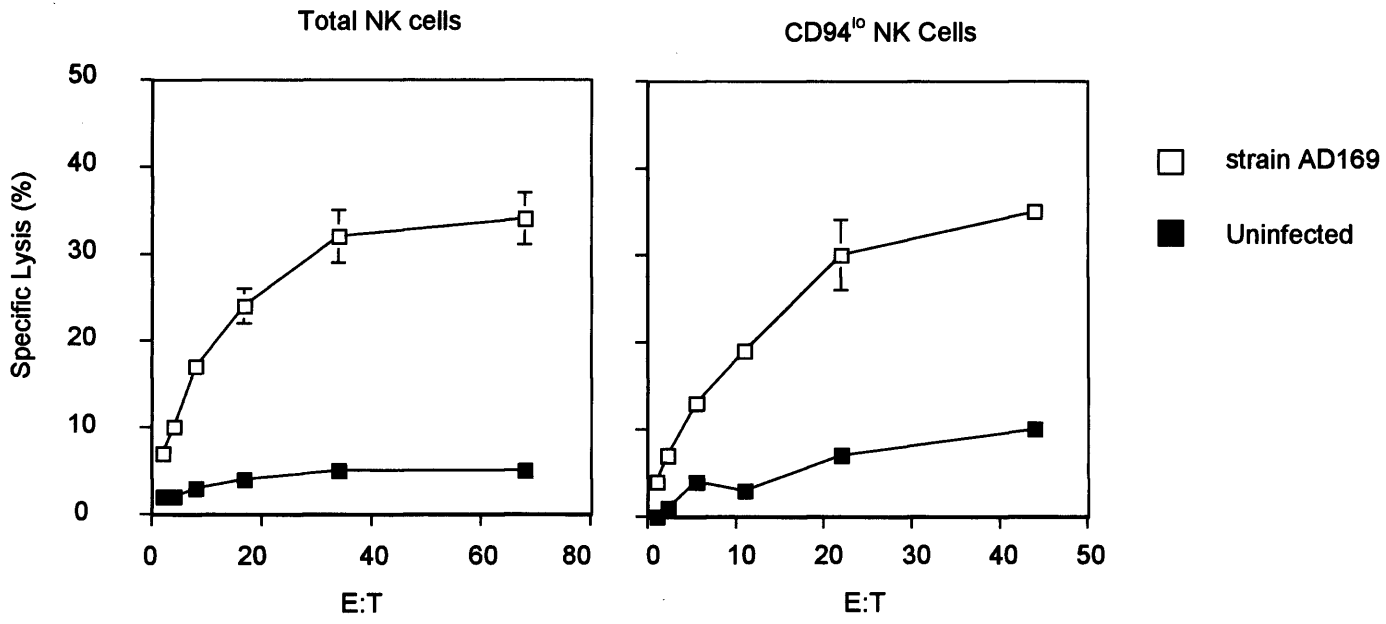

Figure 6.3. D7 Polyclonal NK Cell Response to Uninfected and strain AD169 Infected Autologous Fibroblasts. Polyclonal and $CD94^{lo}$ polyclonal NK cells were generated from PBMC by dynal bead depletion and activated overnight with IFN-α (see section 2.5.2). Cells were used in a ^{51}Cr release assay with uninfected and strain AD169 infected autologous fibroblasts as targets and % Specific Lysis ± SEM was calculated as described in section 2.11.1.4

polyclonal data for donors D3 and D8 was not possible because of the small number of clones. However, it was interesting to note that 42% (11/26) of the $CD94^{hi}$ clones and the single $CD94^{lo}$ NK clone cultured from D8 were inhibited by strain AD169 and this pattern was reflected in this subject's polyclonal data, which showed that both total NK cells and $CD94^{lo}$ polyclonal NK cells were inhibited by strain AD169 (Table 6.5).

6.3.4 HCMV Serostatus and Correlations with CD94/NKG2C and CD94/NKG2A expression

Initial studies were performed on NK clones and polyclonal NK cells that were segregated into $CD94^{hi}$ and $CD94^{lo}$ expressing cells. However, CD94 is found on the cell surface as a heterodimer with an NKG2 chain. CD94/NKG2 receptors have both activating (CD94/NKG2C) and inhibitory isoforms (CD94/NKG2A, CD94/NKG2B), which interact with the non-classical MHC class I molecule HLA-E (Braud *et al.*, 1998, Lanier *et al.*, 1998, Vales-Gomez *et al.*, 1999). Whilst, these studies were being carried out an antibody for the activating CD94/NKG2C receptor became commercially available allowing for further study of $CD94^{hi}$ NK cells in the context of CD94/NKG2C and CD94/NKG2A expression. In the following sections, the CD94/NKG2C and CD94/NKG2A heterodimers shall be referred to as NKG2C and NKG2A, respectively.

6.3.4.1 Expansion of $CD94/NKG2C^{+}$ cells in HCMV Seropositive donors

PBMC isolated from 9 HCMV seropositive and 13 HCMV seronegative donors were stained with CD3, CD56 and CD94 antibodies and analysed by flow cytometry. A Student's t-test showed that there was no significant difference in the proportion or number of NK cells or the proportion or number of $CD94^{hi}$ NK cells in the PBMC of HCMV seropositive or seronegative individuals (Table 6.6).

Table 6.6 The Percentage and Number of NK Cells and CD94^{hi} NK Cells in HCMV Seropositive and Seronegative Donors

	HCMV Seronegative					HCMV Seropositive			
	% PBMC*		Number (x10^5/ml)†			% PBMC*		Number (x10^5/ml)†	
Donor	CD3$^-$CD56$^+$	CD3$^-$CD56$^+$ CD94^{hi}	CD3$^-$CD56$^+$	CD3$^-$CD56$^+$ CD94^{hi}	Donor	CD3$^-$CD56$^+$	CD3$^-$CD56$^+$ CD94^{hi}	CD3$^-$CD56$^+$	CD3$^-$CD56$^+$ CD94^{hi}
D1	14.2	58.9	17.0	10.0	D3	14.8	41.6	27.0	11.2
D4	8.6	59.2	10.4	6.2	D7	33.5	45.7	52.0	23.8
D5	18.7	69.0	32.2	22.2	D9	11.7	74.9	14.6	11.0
D6	18.7	36.8	14.6	5.1	D11	16.1	57.2	27.1	15.5
D12	16.2	34.8	24.3	8.5	D18	8.1	58.6	10.4	6.1
D13	3.6	41.5	4.8	2.0	D21	18.6	43.4	27.9	12.1
D14	13.7	57.6	12.9	7.4	D30	7.3	62.8	26.1	16.4
D15	14.0	58.2	21.6	12.6	D31	5.3	87.3	5.5	4.8
D16	9.2	44.6	19.8	8.8	D39	5.2	41.7	10.8	4.5
D19	10.2	27.3	25.5	7.0					
D24	11.8	83.5	13.4	11.2					
D39	10.9	46.9	20.2	9.5					
D40	6.7	49.9	6.6	3.3					
Mean±SEM	13.6 ± 18.7	51.4 ± 12.0	17.2 ± 6.3	8.7 ± 3.4		13.4 ± 6.5	57.0 ± 12.4	22.4 ± 10.7	11.7 ± 4.7

(*) Percentage of PBMC as determined by flow cytometry
(†) The number of positive cells PBMC as determined from cell counts.

The percentage of NK cells expressing NKG2C and/or NKG2A was calculated by staining PBMC with CD3, CD56, NKG2C and NKG2A antibodies and analysing the cells by flow cytometry. The proportion of NK cells expressing NKG2A and NKG2C were calculated as described in Figure 6.4 and the number of positive cells was determined from cell counts. HCMV seropositive donors had significantly higher proportions and numbers of $NKG2C^+$ cells compared to seronegative donors; 14.5 ± 8.2% versus 4.2 ± 1.5% (p = 0.032) and 2.3 ± 1.3 x 10^5 cells per ml of blood versus 0.7 ± 0.3 x 10^5 (p = 0.011), respectively (Figure 6.5 and Table 6.7). Interestingly, whilst HCMV seropositive donors had a significantly lower proportion of $NKG2A^+$ cells compared to seronegative donors (29.9 ± 8.2% versus 42.9 ± 8.6% (p = 0.025) the numbers of $NKG2A^+$ cells were not significantly altered (5.1 ± 2.2 x 10^5 cells per ml of blood versus 6.5 ± 1.3 x 10^5) for HCMV seropostive and seronegative subjects, respectively. However, the ratio of NKG2A:NKG2C positive cells was significantly lower in the HCMV seropositive group (4.2 ± 3.5% versus 12.4 ± 4.9%, respectively; p = 0.0041). The overall sum of the proportion of NKG2A and NKG2C positive was 44.3 ± 5.1% and 47.0 ± 9.5% for HCMV seropositive and seronegative, respectively. Within the HCMV seropositive group, those individuals who had low proportions of $NKG2C^+$ cells had high proportions of $NKG2A^+$ cells. These data suggested that in certain HCMV seropositive individuals, there was an expansion of $NKG2C^+$ NK cells with a concomitant decrease in $NKG2A^+$ NK cells whilst, the total proportion of $NKG2A^+$ and $NKG2C^+$ cells was maintained.

6.3.5 CD94/$NKG2C^+$ and CD94/$NKG2A^+$ NK clone Response to HCMV strain AD169

The expansion of $NKG2C^+$ cells in HCMV seropositive individuals, suggested a causal role for the virus. A role for strain AD169 and specifically UL40 in the activation of $NKG2C^+$ NK cells and inhibition of $NKG2A^+$ cells was therefore investigated.

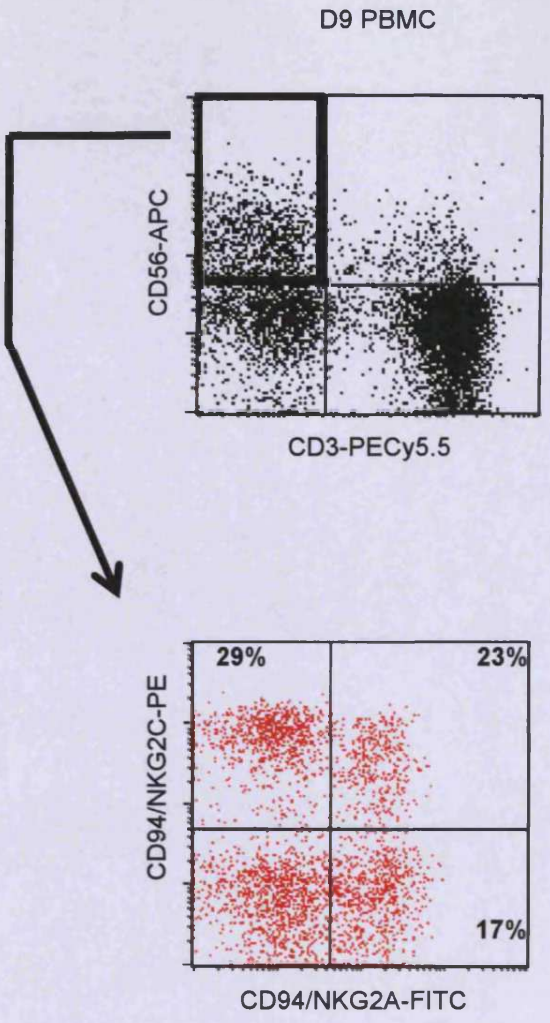

Figure 6.4 Determination of the Percentage of CD94/NKG2A and CD94/NKG2C positive NK cells in PBMC. PBMC were stained with CD3, CD56, CD94/NKG2A and CD94/NKG2C antibodies and analysed by flow cytometry. The percentage of NK cells expressing CD94/NKG2A and CD94/NKG2C was determined by selecting for $CD3^-$, $CD56^+$ cells and then for CD94/NKG2A and CD94/NKG2C expression by dot plot.

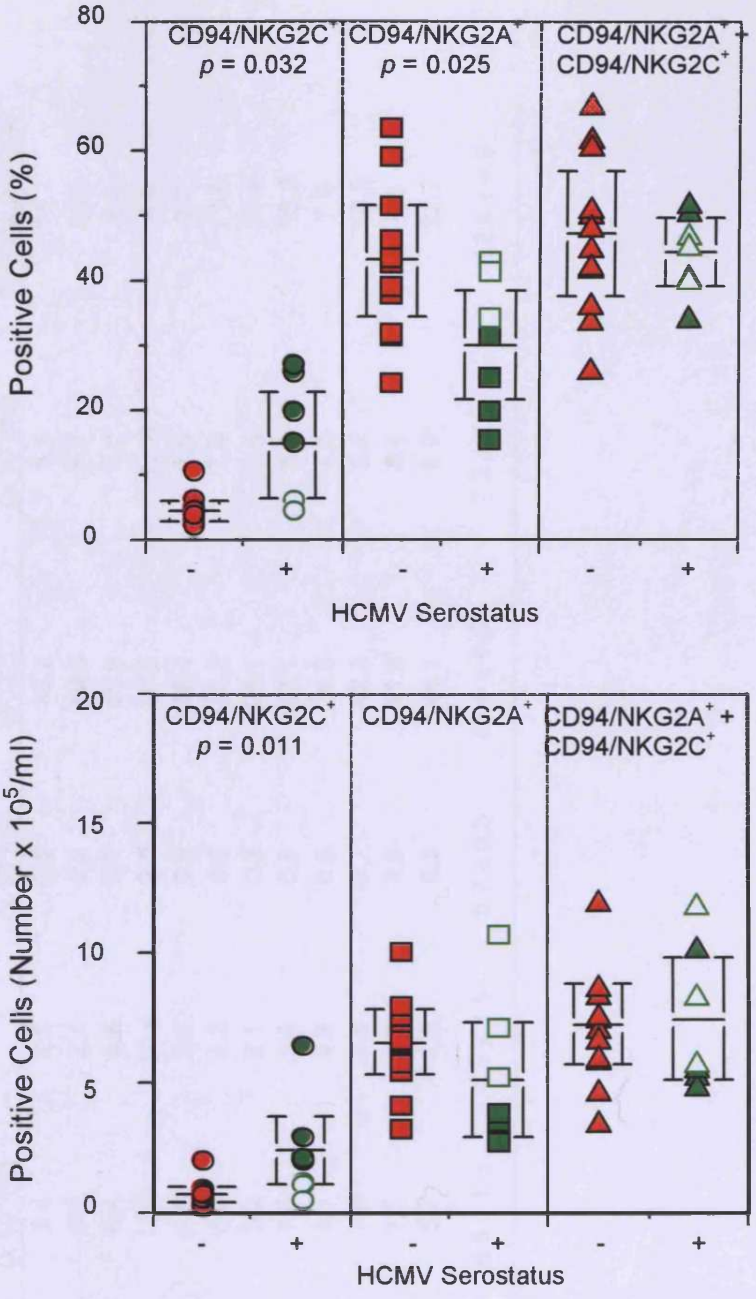

Figure 6.5 CD94/NKG2A$^+$ and CD94/NKG2C$^+$ Cells in HCMV Seropositive and Seronegative Donors. (a) The proportion of CD94/NKG2A$^+$ and CD94/NKG2C$^+$ cells were calculated as described in Figure 6.4 and (b) the number of positive cells in the PBMC were determined from cell counts. HCMV seropositive donors (+) had more CD94/NKG2C$^+$cells (green circles) than HCMV seronegative (-) donors (red circles). Concomitantly, HCMV seropositive donors had less CD94/NKG2A$^+$ cells (green squares) than HCMV seronegative donors (red squares). The combined proportion and number of CD94/NKG2A$^+$ and CD94/NKG2C$^+$ cells did not differ between HCMV seropositive (green triangles) and seronegative donors (red triangles). The distribution of CD94/NKG2A$^+$and CD94/NKG2C$^+$ was bimodal; HCMV seropositive donors with low proportions and numbers of CD94/NKG2C$^+$ cells (open green circles) had larger proportions and numbers of CD94/NKG2A$^+$ cells (open green squares).

Table 6.7 CD94/NKG2A$^+$ and CD94/NKG2C$^+$ Cells in HCMV Seropositive and Seronegative Donors.

	Donor	%NKG2A*	No. NKG2A† (x 10^5/ml)	% NKG2C*	No. NKG2C† (x 10^5/ml)	%NKG2A & NKG2C*	No. NKG2A & NKG2C† (x 10^5/ml)	Ratio NKG2A:NKG2C
Seronegative	D5	31.1	5.4	2.8	0.5	33.9	5.9	11.1
	D13	58.7	6.5	2.1	0.2	60.8	6.7	28.0
	D14	46.1	6.4	4.8	0.7	50.9	7.0	9.6
	D15	51.4	10.0	10.4	2.0	61.8	12.0	4.9
	D17	42.5	6.6	5.9	0.9	48.4	7.6	7.2
	D18	46.0	6.8	4.2	0.6	50.2	7.4	11.0
	D19	24.0	3.2	2.1	0.3	26.1	3.5	11.4
	D24	63.2	7.8	3.9	0.5	67.1	8.3	16.2
	D25	31.7	4.1	4.3	0.6	36.0	4.6	7.4
	D35	38.7	7.9	3.6	0.7	42.3	8.6	10.8
	D40	37.7	7.5	4.2	0.8	41.9	8.4	9.0
	D43	43.2	5.7	1.9	0.3	45.1	6.0	22.7
Mean ± SEM		42.9 ± 8.6	6.5 ± 1.3	4.2 ± 1.5	0.7 ± 0.3	47.0 ± 9.5	7.2 ± 1.6	12.4 ± 4.9

Table 6.7 continued

	Donor	%NKG2A*	No. NKG2A† (x 10^5/ml)	% NKG2C*	No. NKG2C† (x 10^5/ml)	%NKG2A & NKG2C*	No. NKG2A & NKG2C† (x 10^5/ml)	Ratio NKG2A:NKG2C
Seropositive	D3	19.7	2.8	14.7	2.1	34.4	4.9	1.3
	D7	41.1	10.7	4.4	1.1	45.5	11.8	9.3
	D8	15.1	3.8	25.5	6.4	40.6	10.2	0.6
	D9	25.0	2.7	26.7	2.9	51.7	5.6	0.9
	D34	42.9	5.2	4.3	0.5	47.2	5.8	10.0
	D38	31.2	3.2	19.6	2.0	50.8	5.3	1.6
	D39	34.1	7.2	6.0	1.3	40.1	8.4	5.7
Mean ± SEM		29.9 ± 8.5‡	5.1 ± 2.2	14.5 ± 8.2§	2.3 ± 1.3‖	44.3 ± 5.1	7.4 ± 2.3	4.2 ± 3.5

(*) Percentage of PBMC as determined by flow cytometry.
(†) Number of cells in PBMC as determined from cell counts.
(‡) $p = 0.025$ (Student's t-test, assuming unequal variance).
(§) $p = 0.032$ (Student's t-test, assuming unequal variance).
(‖) $p = 0.011$ (Student's t-test, assuming equal variance).

6.3.6 Flow Cytometric Analysis as a Method for Categorising NK Clone Phenotype

Eighteen NK clones cultured from donor D9 were stained for CD3, CD56, and CD94 and CD3, CD56, NKG2C and NKG2A and analysed by flow cytometry. The mean fluorescence channel at which the $NKG2A^+$ and $NKG2C^+$ populations could be separated in D9 PBMC was used to categorise the clones into $NKG2C^+$, $NKG2A^+$, NKG2C/NKG2A double positive and NKG2C/NKG2A double negative NK clones (Figure 6.6). All the clones expressed high levels of CD94 as determined by CD94 fluorescence at which $CD94^{hi}$ and $CD94^{lo}$ populations could be separated in D9 PBMC as described in Figure 6.1. Interestingly, dot plot analysis demonstrated that clone D9NKc10A had a striking $NKG2C^+$, $NKG2A^+$ double positive population which differed from the other clones (Figure 6.7).

6.3.7 Analysis of the CD94/$NKG2C^+$ and CD94/$NKGA^+$ NK Clone Responses to UL40

The NK clone response to HCMV strain AD169 in the context of HCMV UL40 stabilisation of HLA-E was tested using uninfected autologous skin fibroblasts as targets or autologous skin fibroblasts infected with either HCMV strain AD169, strain AD169 ΔUL40, RAd60 or RAdUL40 as targets in a standard ^{51}Cr release assay (Table 6.8). As these experiments were carried in the final months of this PhD there was only time to study 1 donor. As such, donor D9 was chosen for this study because previous experiments had shown that large numbers of NK clones could be cultured from this donor. The response of each clone was then categorised using the method described in section 6.2.2 and Figure 6.1, except an additional category of no kill/activation was used where an NK clone killed the first target at less than 10% but killed the second target at greater than 10% more than the first target.

Using this method of analysis, 2/9 of the $NKG2C^+$ clones were activated by strain AD169 (D9NKc3E10 and D9NKc7G8), compared to 0/5 of the $NKG2A^+$

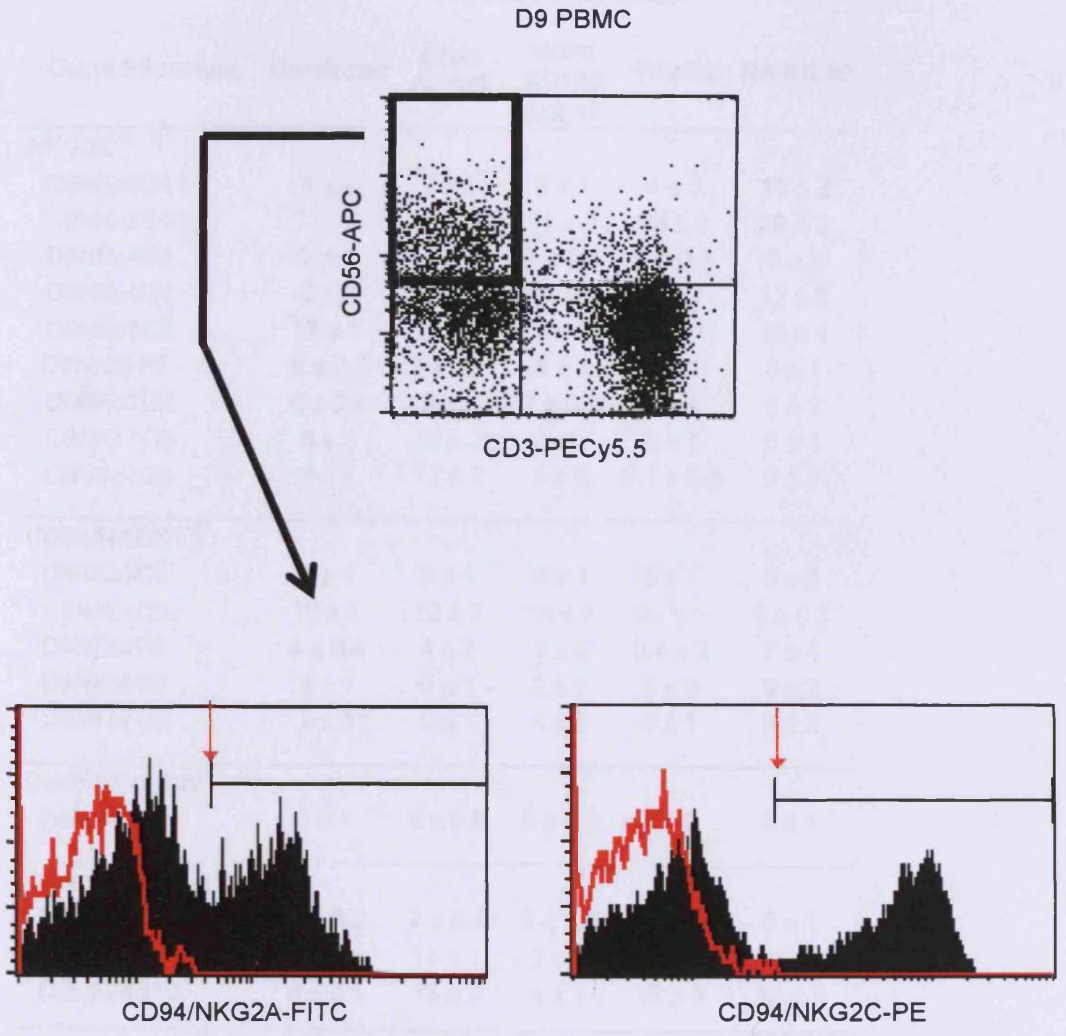

Figure 6.6 Expression Levels of CD94/NKG2A and CD94/NKG2C in D9 PBMC. PBMC were stained with anti-CD3, CD56, CD94/NKG2A and CD94/NKG2C and gated for $CD3^-$,$CD56^+$ then for CD94/NKG2A or CD94/NKG2C. Clones with a mean fluorescence of greater than the fluorescence channel at which the seperation of the 2 populations could be observed (red arrow) were categorised as high expressing; those with a mean fluorescence of less than this value were categorised as low expressing.

Table 6.8 Responses of NK Clones Expressing Different Levels of CD94/NKG2A and CD94/NKG2C to UL40

	% Specific Lysis ± SEM*				
Clone Phenotype	Uninfected	strain AD169	strain AD169 ΔUL40	RAd60	RAdUL40
NKG2C+†					
D9NKc3D11	1 ± 2	7 ± 1	2 ± 1	4 ± 1	11 ± 2
D9NKc3E10	11 ± 2	31 ± 2	11 ± 3	23 ± 3	29 ± 2
D9NKc4B3	2 ± 1	8 ± 3	7 ± 0.1	0 ± 0.4	3 ± 2
D9NKc4G1	0 ± 1	5 ± 1	0.1 ± 2	8 ± 2	12 ± 2
D9NKc5C2	16 ± 1	12 ± 2	9 ± 1	10 ± 1	16 ± 1
D9NKc5F8	5 ± 0.2	5 ± 0.2	3 ± 1	2 ± 1	8 ± 1
D9NKc7G2	0 ± 0.4	0 ± 1	2 ± 0.3	0 ± 1	0 ± 2
D9NKc7G5	5 ± 2	14 ± 2	0 ± 2	0 ± 1	0 ± 1
D9NKc7G8	2 ± 2	13 ± 2	5 ± 2	0.1 ± 0.8	5 ± 2
CD94/NKG2A+‡					
D9NKc3C8	4 ± 1	2 ± 1	4 ± 1	3 ± 1	3 ± 2
D9NKc4G5	12 ± 1	12 ± 2	10 ± 2	25 ± 1	6 ± 0.3
D9NKc1F9	4 ± 0.4	4 ± 2	3 ± 2	0.4 ± 2	7 ± 1
D9NKc6D2	3 ± 1	0 ± 1	2 ± 1	2 ± 0	9 ± 2
D9NKc7G9	2 ± 1	0 ± 1	4 ± 2	1 ± 1	9 ± 2
Double Positive§					
D9NKc10A	2 ± 1	0 ± 0.9	0 ± 0.3	1 ± 1	2 ± 1
Double Negative**					
D9NKc3C10	0 ± 0.2	2 ± 0.4	2 ± 0.7	0 ± 2	0 ± 1
D9NKc3F10	0 ± 0	14 ± 1	2 ± 2	2 ± 2	12 ± 2
D9NKc4B10	6 ± 0.4	13 ± 2	4 ± 1	15 ± 3	13 ± 1

Responding clones are highlighted in red

(*) % Specific Lysis was calculated as described in section 2.11.1.4.

(†) $NKG2C^{hi}$ clones expressed a median NKG2C fluorescence of >50 and a median NKG2A fluorescence of <50.

(‡) $NKG2A^{hi}$ clones expressed a median NKG2A fluorescence of >40 and a median NKG2C fluorescence of <50.

(§) Double positive clones expressed a median NKG2C fluorescence of > 50 and a median NKG2A fluorescence of >40.

(**) Double negative clones expressed a median NKG2C fluorescence of <50 and a median NKG2A fluorescence of <40.

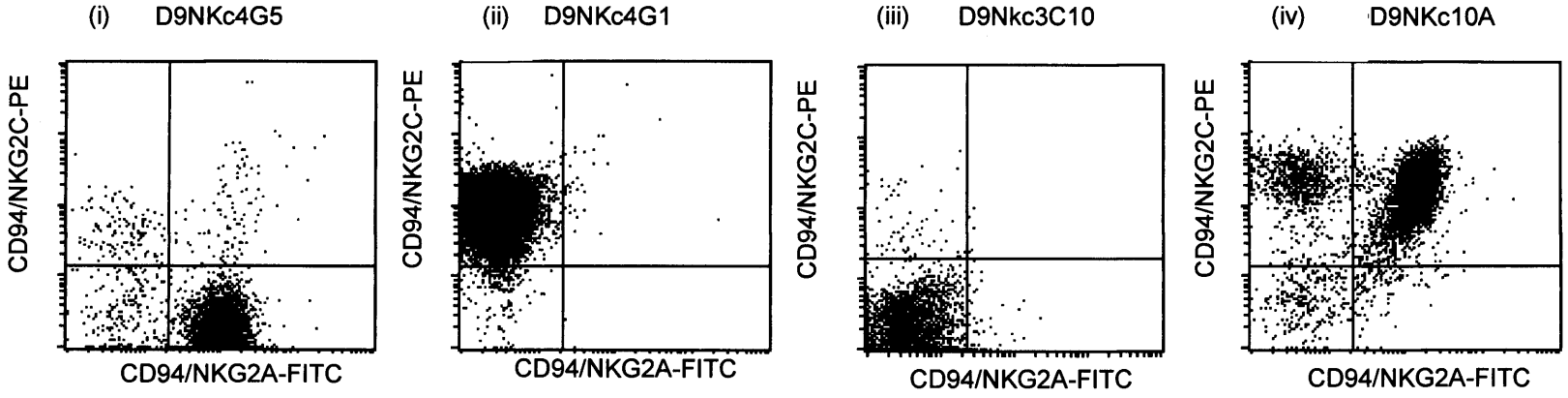

Figure 6.7 Demonstration of Differential CD94/NKG2A and CD94/NKG2C expression in Different NK Clones. NK clones were stained with CD3, CD56, CD94/NKG2A andCD94/NKG2C antibodies and analysed by flow cytometry. $CD3^-$, $CD56^+$ cells were gated and analysed for CD94/NKG2A and CD94/NKG2C expression and categorised as (i) $CD94/NKG2A^+$ (ii) $CD94/NKG2C^+$ and (iii) double negative. Clone NKc10A showed a distinctive $CD94/NKG2A^+$, $CD94/NKG2C^+$ double stain (iv).

clones and 1/3 of the double negative clones (D9NKc3F10). The double positive clone, D9NKc10A was unaffected by strain AD169. No clones were inhibited by strain AD169. In response to the strain AD169 ΔUL40 virus, 2/4 of the NKG2C$^+$ clones which lysed strain AD169 infected targets were inhibited by AD169 ΔUL40 virus, whilst the only NKG2A$^+$ clone (D9NKc4G4) that lysed strain AD169 infected targets was unaffected by strain AD169 ΔUL40. The double negative clone, D9NKc3F10 lysed strain AD169 infected targets, and was inhibited by strain AD169 ΔUL40. No clones were activated by strain AD169 ΔUL40.

Interestingly, whilst only 1/5 of the NKG2A$^+$ clones (D9NKc4G5) lysed the RAd60 control target, this clone was inhibited by RAdUL40. Flow cytometry data showed that this clone had the highest mean NKG2A fluorescence and the lowest NKG2C fluorescence of the NKG2A$^+$ clones, giving an NKG2A:NKG2C ratio of 97:1, compared to ratios of 19:1 (D9NKc3C8), 4:1 (D9NKc6D2), 3:1 (D9NKc1F9) and 2:1 (D9NKc7G9) in the remaining NKG2A^{hi} clones. As the remaining NKG2A$^+$ clones were unable to lyse the control adenovirus target, it was impossible to tell whether these clones could be inhibited by RAdUL40. The NKG2C$^+$ clones were unaffected by RAdUL40 expression, however, as described for the NKG2A$^+$ clones, only 2/9 of these clones lysed the control virus, therefore it is also impossible to tell whether these clones were inhibited by NKG2C expression. A similar problem was observed for the double negative clones as only 1/3 of these clones lysed the RAd60 control target (D9NKc4B10). However, 1/3 of the double negative clones was activated by RAdUL40 (D9NKc3F10). The double positive clone was unable to lyse the RAd60 control target and was categorised as no kill/no change in response to RAdUL40. Interestingly, the double negative clone D9NKc3F10 was the only clone to be activated by strain AD169, inhibited by strain AD169 ΔUL40 and activated by RAdUL40.

Lysis of control targets at less than 10%, made it impossible to determine whether these clones could be inhibited by the test target. In this respect,

72% (13/18) D9 NK clones were categorised as no kill/no change with respect to uninfected versus strain AD169 infected targets (Table 6.8). This level of unresponsiveness was not limited to this experiment or this donor; of the donors used to initially study NK responses to uninfected versus strain AD169 infected targets, 92% (12/13) of D3, 8% (3/25) of D7, 37% (10/27) of D8 and 47% (21/45) of D9 NK clones were categorised as no kill/no change (Table 6.2). Even though D7 had the lowest number of NK clones categorised as no kill/no change, D7 was not chosen to study NK responses to gpUL40 because the lysis of targets by this donor did not reflect the patterns observed for the other donors. These data demonstrate the need to expand this experiment, by increasing the number of donors and by increasing the total numbers of clones analysed.

6.3.8 Summary

The polyclonal NK cell responses to uninfected versus strain AD169 infected fibroblasts are consistent with previous reports which showed that surface stabilisation of HLA-E by the HCMV UL40 leader sequence resulted in increased HLA-E expression at the cell surface, providing protection against $NKG2A^+$ NK cell lysis during HCMV infection (Tomasec *et al.*, 2000, Wang *et al.*, 2002). The finding that HCMV seropositive individuals have expansions of NK cells expressing the activating receptor $NKG2C^+$ and a reduction in the frequency of NK cells expressing the inhibitory receptor, $NKG2A^+$ suggested that HCMV may influence the NK cell repertoire. The focus of the study was therefore to investigate the functional importance of $NKG2A^+$ and $NKG2C^+$ NK cells with respect to HCMV and specifically gpUL40. To this end, 18 NK clones from D9 were analysed. This study showed that 2/9 $NKG2C^+$ clones were activated by strain AD169 infected targets, and 1 of these was inhibited by strain AD169 ΔUL40 but was unchanged by RAdUL40. In contrast, 1 $NKG2A^+$ clone was inhibited by RAdUL40. Interestingly, 1 NKG2A and NKG2C double negative NK clone was cultured (D9NKc3F10) and this was the only clone to be activated by strain AD169, inhibited by strain AD169 ΔUL40 and activated by RAdUL40, suggesting that gpUL40 may act through

a receptor distinct from NKG2A or NKG2C.

7 NK RESPONSES TO DIFFERENT STRAINS OF HCMV

7.1 INTRODUCTION

NK cytotoxicity is regulated by the balance of activating and inhibitory receptors that are expressed on the surface of the NK cell and bind to MHC class I molecules. Normally, signals received from inhibitory NK receptors binding to cognate MHC class I molecules are dominant over the signals generated by binding of activating receptors. However, in HCMV infection 4 proteins act in concert to downregulate MHC class I expression, thereby avoiding recognition by cytotoxic T-cells (Karre, 1997). This massive downregulation of MHC class I by HCMV has been proposed to facilitate NK cell mediated lysis of infected cells (Tortorella *et al.*, 2000). However, responses of individual NK cell clones and lines to fibroblasts infected with HCMV vary greatly, ranging from inhibition to activation (Carr *et al.*, 2002). The mechanisms that determine whether an NK cell is activated or inhibited by HCMV infection remain unclear, however a number of HCMV genes have been identified which provide HCMV with a sophisticated array of mechanisms for avoiding NK cell mediated lysis (Cosman *et al.*, 1997, Cosman *et al.*, 2001). One of these genes encodes HCMV gpUL40, and leader sequences of this protein have been shown to stabilise HLA-E surface expression (Tomasec *et al.*, 2000). Ligation of UL40 stabilised HLA-E has been shown to inhibit CD94/NKG2A$^+$ NK cells (Wang *et al.*, 2002).

Sequencing of clinical isolates and laboratory strains of HCMV has revealed that *in vitro* tissue culture has resulted in large genetic differences in the laboratory strains of HCMV, the most notable being a 15-kb and a 13-kb deletion in the U_L/b' region in strains AD169 and Towne respectively (Cha *et al.*, 1996). In contrast to these strains, clinical isolates of HCMV and the less extensively cultured Toledo strain provide substantially more protection against NK cell mediated cytolysis ((Cerboni *et al.*, 2000, Wang *et al.*, 2002) and Table 7.1).

Table 7.1 Polyclonal NK Cell Responses to Uninfected, HCMV Strain AD169 and Strain Toledo Infected Autologous Fibroblasts

			% Specific Lysis ± SEM*			
Donor	Target	E:T	Uninfected	AD160	Toledo	$p^{\dagger}$
D3	D3-SF	20:1	40 ± 6	21 ± 1	1 ± 1	0.00005
D3	D3-SF	30:1	10 ± 1	13 ± 1	1 ± 1	0.0002
D7	D7-SF	18:1	20 ± 2	18 ± 3	1 ± 4	0.0005
D7	D7-SF	20:1	10 ± 2	16 ± 2	0 ± 1	0.002
D7	D7-SF	25:1	56 ± 6	37 ± 1	4 ± 2	0.000002
D7	D7-SF	25:1	ND	33 ± 1	0 ± 1	0.0000001
D9	D9-SF	45:1	40 ± 5	20 ± 5	3 ± 1	0.01
D9	D9-SF	39:1	40 ± 4	14 ± 1	2 ± 1	0.00008

Assays were performed by E.C.Y Wang and *P.* Tomasec.

ND, not done

(*) % Specific Lysis ± SEM was calculated as described in section 2.11.1.4.
(†) *p* values compare killing of strain AD169 and strain Toledo infected targets (Student's t-test, assuming unequal variance).

The previous chapter concentrated on NK responses in the context of UL40 stabilisation of HLA-E using strain AD169 infected fibroblasts. In this chapter, the NK response to the laboratory strains AD169 and Towne are compared, and the NK clone response to HCMV strain Toledo is dissected.

7.2 Comparison of NK Responses between HCMV strain Towne and strain AD169

Genetic differences between strains of HCMV highlight non-essential genes found within the HCMV genome. Increasingly, these non-essential genes are being associated with roles in immunomodulation, an important factor in the biology of HCMV. As HCMV strain Towne encodes an extra 2 kb in the U_L/b' region which is not found in HCMV strain AD169, the potential role of this region to contribute to NK modulatory functions was investigated using polyclonal NK cells and NK clones.

7.2.1 Inhibition of $CD94^{lo}$ NK Clones by HCMV strain Towne

Twenty six $CD94^{lo}$ and 84 $CD94^{hi}$ NK clones were cultured from 4 donors and tested for their ability to lyse fibroblasts infected with HCMV strain AD169 or Towne as described in section 6.2.2. Table 7.3 showed that 15% (4/26) of $CD94^{lo}$ NK cells were inhibited by strain Towne compared to 5% (4/84) of $CD94^{hi}$ NK clones whilst there was little difference in the percentages of clones which were categorised as kill/no change and no kill/no change. This suggested that even though equal frequencies of $CD94^{hi}$ and $CD94^{lo}$ NK clones are able to respond differently to strain Towne infected fibroblasts, more $CD94^{lo}$ than $CD94^{hi}$ NK clones were inhibited by strain Towne infected fibroblasts. Table 7.2 showed that the difference in responsiveness towards strain Towne infected fibroblasts ranged from the minimum cut off of 10% to a difference of 24% in percentage specific lysis. This data suggested that HCMV strain Towne encoded a novel NK immunomodulatory function that had the capability to strongly effect NK cell responsiveness and that specifically inhibited larger numbers of $CD94^{lo}$ NK cells.

Table 7.2 NK Clone Responses to HCMV Strain AD169 versus Strain Towne Infected Autologous Fibroblasts

	Total NK Clones	
NK Clone Response	$CD94^{hi}$*	$CD94^{lo}$*
Inhibition†	4/84 (5%)	4/26 (15%)
Activation‡	1/84 (1%)	1/26 (4%)
Kill/No Change§	38/84 (46%)	10/26 (39%)
No Kill/No Change‖	41/84 (48%)	11/26 (42%)

(*) PBMC were stained with anti CD3, CD56 and CD94 and gated for $CD3^-$,$CD56^+$ and then for CD94. The median CD94 fluorescence channel at which separation of the 2 populations could be observed was used to categorise the clones as being $CD94^{hi}$ or $CD94^{lo}$.

(†) Clone lysed strain AD169 fibroblasts ≥10% and strain Towne infected fibroblasts ≥ 10% less than strain AD169 fibroblasts.

(‡) Clone lysed strain AD169 fibroblasts ≥10% and strain Towne infected fibroblasts ≥ 10% more than strain AD169 fibroblasts.

(§) Clone lysed strain AD169 fibroblasts ≥10% and strain Towne infected fibroblasts <10% more than strain AD169 fibroblasts.

(‖) Clone lysed strain AD169 fibroblasts <10% and strain Towne infected fibroblasts at <10%.

Table 7.3 NK Clones that Responded Differently to HCMV strain AD169 and strain Towne Infected Autologous Fibroblasts

			% Specific Lysis ± SEM*		
Response	NK Clone	E:T	Strain AD169	Strain Towne	% Difference
	$CD94^{hi}$				
Inhibited	D7.NKc4B7	21 to 1	58 ± 1	34 ± 6	24%
	D7.NKc3F5	49 to 1	41 ± 1	26 ± 2	15%
	D7.NKc6D11	30 to 1	41 ± 1	28 ± 4	13%
	D9.NKc4B2	18 to 1	19 ± 2	8 ± 0.3	11%
	$CD94^{lo}$				
	D7.NKc2E2	30 to 1	21 ± 1	11 ± 0.3	10%
	D7.NKc3G2	30 to 1	42 ± 6	32 ± 2	10%
	D9.NKc2G11	39 to 1	26 ± 4	5 ± 2	21%
	D9.NKc3F2	24 to 1	11 ± 1	1 ± 1	10%
Activated	$CD94^{hi}$				
	D7.NKc6G3	30 to 1	60 ± 1	79 ± 1	19%
	$CD94^{lo}$				
	D7.NKc1D4	58 to 1	25 ± 4	46 ± 2	21%

(*) % specific lysis ± SEM was calculated as described in section 2.11.1.4

7.2.2 Variation of NK Clone Responses to HCMV strain AD169 and strain Towne Vary Between Individuals

As described in chapter 6, different donors can show variation in the patterns of NK responses to HCMV infected compared to uninfected autologous targets. Similarly, Table 7.4 showed that NK clone responses from individual donors responded differently to strain AD169 compared to strain Towne infected fibroblasts. In particular, differential responses were observed from NK clones derived from donors D7 and D9. In contrast, donors D3 and D8 did not respond differently to strains AD169 or Towne infected cells. These data suggested that the NK modulatory function(s) encoded by strain Towne act dominantly only in certain donors and that strain Towne is capable of eliciting both a stimulatory and inhibitory signal.

7.2.3 Polyclonal NK Cell Responses to Strain AD169 and Strain Towne Infected Fibroblasts

Polyclonal NK cells isolated from PBMC and stimulated overnight with IFNα allow NK cell responses to be investigated with minimal manipulation through tissue culture. Analysis of polyclonal NK cell responses to strain Towne and strain AD169 infected fibroblasts indicated that the polyclonal NK response to strain Towne infected targets varied between donors. Taking a difference of 10% in percentage specific lysis as being significant, D9 16/5/3 $CD94^{lo}$ polyclonal cells were inhibited by strain Towne whilst $CD94^{lo}$ polyclonal NK cells from the other donors showed no difference in response to strain AD169 and strain Towne infected fibroblasts (Table 7.5). This suggested that strain Towne could modulate $CD94^{lo}$ NK cell responses in a subset of donors. For donors D7 and D9 27/5/4, the total polyclonal cells were activated by strain Towne compared to strain AD169 infected fibroblasts, whilst $CD94^{lo}$ polyclonal NK responses were unchanged. This indicated that this difference in % specific lysis was dependent upon $CD94^{hi}$ NK cells, and suggested that $CD94^{hi}$ NK cells from these donors could be activated by

Table 7.4 Individual Donor NK Clone Responses to HCMV Strain AD169 and Strain Towne Infected Autologous Fibroblasts

	Donor							
	D3		D7		D8		D9	
NK Clone Response	$CD94^{hi}$	$CD94^{hi}$	$CD94^{hi*}$	$CD94^{hi*}$	$CD94^{hi}$	$CD94^{lo}$	$CD94^{hi}$	$CD94^{lo}$
Inhibition†	0/11 (0%)	0/2 (0%)	3/13 (23%)	2/12 (17%)	0/25 (0%)	0/1 (0%)	1/35 (3%)	2/11 (18%)
Activation‡	0/11 (0%)	0/2 (0%)	1/13 (8%)	1/12 (8%)	0/25 (0%)	0/1 (0%)	0/35 (0%)	0/11 (0%)
Kill/No Change§	1/11 (10%)	1/2 (50%)	8/13 (61%)	6/12 (50%)	11/25 (44%)	0/1 (0%)	18/35 (51%)	3/11 (27%)
No Kill/No Change‖	10/11 (90%)	1/2 (50%)	1/13 (8%)	3/12 (25%)	15/25 (56%)	1/1 (100%)	16/35 (29%)	6/11 (55%)

(*) PBMC were stained with anti CD3, CD56 and CD94 and gated for $CD3^{-}$,$CD56^{+}$ and then for CD94. The median CD94 fluorescence at which separation of the two populations could be observed was used to categorise NK clones as being $CD94^{hi}$ or $CD94^{lo}$.
(†) Clone lysed strain AD169 fibroblasts ≥10% and strain Towne infected fibroblasts ≥ 10% less than strain AD169 fibroblasts.
(‡) Clone lysed strain AD169 fibroblasts ≥10% and strain Towne infected fibroblasts ≥ 10% more than strain AD169 fibroblasts.
(§) Clone lysed strain AD169 fibroblasts ≥10% and strain Towne infected fibroblasts <10% more than strain AD169 fibroblasts.
(‖) Clone lysed strain AD169 fibroblasts <10% and strain Towne infected fibroblasts at <10%.

Table 7.5 Polyclonal and CD94^{lo} Polyclonal NK Responses to HCMV Strain AD169 and Strain Towne Infected Autologous Fibroblasts

	% Specific Lysis ± SEM*							
	Total†				CD94^{lo}‡			
Donor	E:T	Strain AD169	Strain Towne	p§	E:T	Strain AD169	Strain Towne	p§
D3 15/7/3	30	13 ± 1	9 ± 2	0.045	60	8 ± 2	4 ± 1	NS
D7 10/6/4	44	35 ± 1	45 ± 1	0.0024	68	34 ± 2	37 ± 3	NS
D8 29/5/3	22	7 ± 1	6 ± 0.4	NS	35	12 ± 1	4 ± 0.3	0.0027
D9 16/5/3	45	20 ± 4	21 ± 2	NS	54	25 ± 2	15 ± 2	0.0094
D9 27/5/4	44	13 ± 1	25 ± 1	0.0062	64	12 ± 1	20 ± 2	0.042
D10 16/6/4	60	16 ± 1	20 ± 2	NS	30	11 ± 1	16 ± 1	0.011
D11 23/6/4	66	5 ± 1	16 ± 3	0.043	42	4 ± 2	7 ± 1	NS

NS, not significant (p >0.05)

(*) % Specific Lysis was calculated as described in section 2.10.1.4.

(†) Total NK cells represent polyclonal NK cells isolated from PBMC by dynal bead depletion and activated overnight in IFN-α (see section 2.4.2).

(‡) CD94^{lo} polyclonal NK cells isolated from PBMC by dynal bead depletion and activated overnight with IFN-α (see section 2.4.2.).

(§) p values compare killing of strain AD169 versus strain Towne infected targets (Student's t-tests, assuming unequal variance).

strain Towne. Together, these data indicated that HCMV strain Towne encoded a factor that was capable of modulating both $CD94^{lo}$ and $CD94^{hi}$ NK cell responses and these effects were either more distinct or restricted to particular donors.

A Student's t-test was used to identify statistically significant differences in the percentage specific lysis of each of the targets. This analysis suggested that in contrast to total polyclonal NK cells, D8 and D9 16/5/3 $CD94^{lo}$ polyclonal NK cells were inhibited by strain Towne. There was no difference in the lysis of strain AD169 and strain Towne infected targets by D7 and D11 $CD94^{lo}$ polyclonal NK cells, however the total polyclonal NK cells were activated by strain Towne infection, indicating that $CD94^{hi}$ NK cells were activated by strain Towne. Statistical analysis also suggested that D3 and D10 total polyclonal NK cells behaved differently towards strain Towne infected fibroblast in contrast to $CD94^{lo}$ polyclonal NK cells which lysed strain AD169 and Towne infected fibroblasts with the same efficiency. Specifically, the data indicated that D3 total polyclonal NK cells were inhibited by strain Towne, and D11 total NK cells were activated by strain Towne. However, for D3, the difference in specific lysis of strain Towne infected fibroblasts compared to strain AD169 infected fibroblasts was the same for both polyclonal and $CD94^{lo}$ polyclonal NK cells, suggesting that there was no difference in the ability of $CD94^{lo}$ and total polyclonal NK cells to lyse strain Towne infected targets, and that the statistical difference may be an artefact of the method.

These data indicated that caution must be taken when comparing differences in % specific lysis using a student's T test. Using this method of analysis statistically significant differences may be identified because of the closeness of the triplicate values counted on the β plate counter, rather than because of true differences between the mean % specific lysis values. Statistical analysis may therefore be useful as a method of identifying trends but a difference in 10% specific lysis may be a clearer identifier of real differences

in NK cell responses.

Using a 10% difference as being significant, analysis of polyclonal NK cell responses to strain Towne and strain AD169 infected fibroblasts showed that $CD94^{lo}$ NK cells can be inhibited and $CD94^{hi}$ NK cells can be activated by strain Towne infected fibroblasts compared to strain AD169 infected fibroblasts. This suggested that HCMV strain Towne encoded a novel immunomodulatory function that was capable of affecting $CD94^{hi}$ and $CD94^{lo}$ cells and this is either more pronounced in certain donors or was restricted to certain individuals. Together, these data suggested that strain Towne encoded an NK modulatory function that affected enough NK cells to demonstrate differences in the % specific lysis of strain AD169 and strain Towne infected fibroblasts of up to 12% in polyclonal NK cell cultures.

7.2.4 Comparison of Polyclonal and NK Clone Responses to Strain AD169 and Strain Towne Infected Fibroblasts

NK clones allow the magnitude and specificity of an immunomodulatory effect to be dissected but may not indicate how total NK cells behave in response to a stimulus. The NK clone responses to strain AD169 and strain Towne were therefore compared with the individual's polyclonal NK cell responses. D9 16/5/3 $CD94^{lo}$ polyclonal data showed that $CD94^{lo}$ polyclonal NK cells were inhibited by strain Towne infected fibroblasts, whilst the total polyclonal NK cell population did not behave differently to strain AD169 and strain Towne infected cells. This data agreed with D9 NK clone data that showed that $CD94^{lo}$ clones were inhibited by strain Towne and that most $CD94^{hi}$ NK clones were unresponsive to strain Towne. In contrast, D7 clone data suggested that there was no difference in the $CD94^{lo}$ or $CD94^{hi}$ NK cell response to strain Towne, a pattern discordant with D7 polyclonal data which showed that $CD94^{lo}$ NK cells did not respond differently to strain Towne infected targets while, $CD94^{hi}$ NK cells were activated by strain Towne. Comparison of D3 and D8 clonal and polyclonal data was not possible because of the small number of clones cultured from each of these

individuals.

Using the 10% rule defined in section 6.2.2 and Figure 6.1, the polyclonal data suggested that $CD94^{lo}$ NK cells were inhibited by strain Towne whilst $CD94^{hi}$ NK cells were activated by strain Towne infected fibroblasts. As described in Table 7.4, the capability to respond differently to strain AD169 and strain Towne infected fibroblasts may be donor dependent. The overall differences in the results between the NK clone and polyclonal data may therefore be a consequence of using different donors for these experiments.

7.2.5 Summary

In this study, data indicated that HCMV stain Towne encoded a novel NK modulatory function that was not found in strain AD169. There are a number of differences between HCMV strains AD169 and Towne. Most significant is an extra 2 kb encoded by strain Towne that is not found in strain AD169, however other differences in the genetic sequences of these viruses have occurred following culture *in vitro*. Analysis of polyclonal and NK clone responses indicated that a novel NK modulatory function was encoded by strain Towne that had differential but significant effects on both $CD94^{lo}$ and $CD94^{hi}$ NK cells. Analysis of NK clones allowed the magnitude of the modulatory function to be estimated. This analysis showed that whilst only a relatively small number of clones responded differently to strain Towne compared to strain AD169 infected fibroblasts, the difference in % specific lysis in response to these targets could be as much as 24%. This data also suggested that more $CD94^{lo}$ NK clones were inhibited by strain Towne than $CD94^{hi}$ NK clones. The polyclonal data indicated that $CD94^{lo}$ NK cells from a single donor were inhibited by strain Towne and no donors' $CD94^{lo}$ NK cells were activated by strain Towne. In contrast to the NK clone data, $CD94^{hi}$ polyclonal NK cells were activated by strain Towne compared to strain AD169 infected fibroblasts. This suggested HCMV strain Towne encoded a factor that was able to induce a considerable alteration in responsiveness in specific subsets of NK cells and that modulation of the NK cell response may

be donor specific.

7.3 Comparison of NK Responses between HCMV strain AD169 and strain Toledo

HCMV strain Toledo provides substantially more protection against polyclonal NK cell mediated lysis than strains AD169 and Towne (Cerboni *et al.*, 2000, Wang *et al.*, 2002) and Table 7.1). The HCMV protein gpUL141, encoded within the U_L/b' region, which has been deleted from strains AD169 and Towne, was identified by Dr *P.* Tomasec as mediating protection from polyclonal NK lysis (Table 7.6).

In this study, NK clones were cultured and analysed as described in section 6.2, with help from Cora Griffin and used in ^{51}Cr assays using HCMV strain Toledo and replication-deficient adenovirus vectors containing the complete gene of UL141 alone (RAdUL141) or in combination with GFP (RAdUL141-GFP).

7.3.1 Inhibition of NK Clones by HCMV Strain Toledo

Nineteen NK clones from 2 different donors, 4 from D3 and 13 from D8 were used in ^{51}Cr release assays against uninfected, strain AD169 and strain Toledo infected autologous fibroblasts (Table 7.7 and Appendix VII). Ten clones were able to lyse uninfected fibroblasts at greater than 10% specific lysis and 8 of these clones were inhibited by strain Toledo by 10% or more. Of the 10 clones that were able to lyse strain AD169 infected fibroblasts, 5 of these clones were inhibited by strain Toledo by 10% or more. This data supported the previous observation that polyclonal NK cells were inhibited by strain Toledo compared to strain AD169 infected fibroblasts. Furthermore this data also indicated that inhibition by strain Toledo compared to strain AD169 infected targets could be as much as 17%. Together this data showed that infection of fibroblasts with strain Toledo resulted in high levels of NK inhibition compared to lysis of strain AD169 infected fibroblasts.

Table 7.6 Polyclonal NK Cell Responses to RAdUL141-GFP

			% Specific Lysis ± SEM*		
Donor	Target†	E:T	Control RAdUL141-GFP	RAdUL141-GFP	p‡
D3	D3-SF	20 to 1	30 ± 2	13 ± 5	0.01
D3	D3-SF	12 to 1	17 ± 5	11 ± 1	NS
D3	HFFF	12 to 1	22 ± 8	8 ± 2	NS
D7	D7-SF	18 to 1	20 ± 2	8 ± 2	0.0007

Assays were performed by E.C.Y Wang and P. Tomasec.

NS, not significant

(*) % Specific Lysis ± SEM was calculated as described in section 2.10.1.4.
(†) Target cells were autologous skin fibroblasts (-SF) or HFFFs.
(‡) p values compare killing of strain AD169 and strain Toledo infected targets (Student's t-test, assuming unequal variance).

Table 7.7 NK Clone Responses to HCMV Strain Toledo

		% Specific Lysis ± SEM*			
NK Clone	E:T	Uninfected	AD169	Toledo	$p^{\dagger}$
D3.NKc1C11	7 to 1	0 ± 0.5	0 ± 0.4	3 ± 2	NS
D3.NKc3C11	11 to 1	0 ± 1	1 ± 1	1 ± 1	NS
D3.NKc5E11	40 to 1	1 ± 1	15 ± 3	4 ± 1	0.025
D3.NKc5F8	20 to 1	0 ± 1	0 ± 2	0 ± 1	NS
D3.NKc3B5	7 to 1	3 ± 2	12 ± 4	9 ± 1	NS
D3.NKc3C9	7 to 1	4 ± 3	9 ± 2	5 ± 1	0.013
D8.NKc1C10	58 to 1	48 ± 2	30 ± 2	13 ± 2	0.00033
D8.NKc1E11	21 to 1	29 ± 2	10 ± 3	4 ± 0.4	NS
D8.NKc1F11	44 to 1	24 ± 2	15 ± 2	3 ± 1	NS
D8.NKc2F3	25 to 1	38 ± 3	13 ± 3	4 ± 1	0.0094
D8.NKc2G9	5 to 1	2 ± 0.4	3 ± 1	3 ± 0.3	NS
D8.NKc3C8	26 to 1	8 ± 1	5 ± 1	3 ± 1	NS
D8.NKc3D9	11 to 1	11 ± 1	15 ± 1	8 ± 1	NS
D8.NKc3E9	32 to 1	35 ± 1	25 ± 2	9 ± 1	0.025
D8.NKc4F3	25 to 1	48 ± 2	25 ± 0.3	10 ± 2	0.048
D8.NKc4E10	44 to 1	13 ± 3	8 ± 1	6 ± 1	NS
D8.NKc5E7	14 to 1	7 ± 3	2 ± 0.2	2 ± 0.3	NS
D8.NKc5C11	63 to 1	36 ± 2	17 ± 2	13 ± 1	0.039
D8.NKc1B11	33 to 1	20 ± 5	5 ± 1	4 ± 0	NS

NK clones were cultured and assayed with the help of Cora Griffin.

NS, not significant $p > 0.05$

(*) % Specific Lysis ± SEM was calculated as described in section 2.10.1.4.
(†) *p* values compare killing of AD169 and Toledo infected targets (Student's t-test, assuming unequal variance).

7.3.2 NK Clone Responses to RAdUL141 and RAdUL141-GFP

Pete Tomasec showed that polyclonal NK cell lysis was also inhibited by gpUL141 (Table 7.6). To dissect the NK response to gpUL141, 99 NK clones from 4 different donors were cultured and used in ^{51}Cr release assays using autologous fibroblasts infected with recombinant adenovirus vectors expressing gpUL141 (Appendix VII). Two different replication deficient adenovirus vectors, RAdUL141 and RAdUL141-GFP, were made. To test whether there was any difference in the ability of NK clones to lyse each of the constructs, 8 NK clones from 2 different donors were tested for their ability to lyse RAdUL141 and RAdUL141-GFP infected targets (Table 7.8). The NK clone responses to RAdUL141 and RAdUL141-GFP were not consistent between the 2 viruses, with 7/8 of NK clones inhibited by RAdUL141 compared to 2/8 by RAdUL141-GFP compared to their empty vectors. However, none of the clones were activated by RAdUL141 or RAdUL141-GFP, indicating that although individual clones could respond differently to RAdUL141 and RAdUL141-GFP, the general pattern of response was maintained. An important consideration in this analysis is that only 8 NK clones were used in this study and the responses to each of the viruses was tested at different time points and with different E:T ratios. The differences between the NK clone responses to each of the viruses may therefore have reflected differences in the activation status and number of effector cells used.

7.3.3 NK Clones are Inhibited by gpUL141

Thirty nine NK clones from 3 donors (D3, D7 and D8) were used in ^{51}Cr release assays against RAdUL141 infected targets, and 60 clones from 3 donors (D3, D8 and D9) against RAdUL141-GFP infected targets (Appendix VII). NK clone responses were categorised as described in Section 6.2.2. This analysis showed that 71% (27/38) of clones were inhibited by RAdUL141 and 45% (27/60) of clones were inhibited by RAdUL141-GFP (Figure 7.1). No clones were activated by RAdUL141 and only 3% (2/60) of clones were activated by RAdUL141-GFP infected targets. 29% (10/38) of

Table 7.8 NK Clone Responses to Replication-deficient Adenovirus Vectors Containing UL141 and UL141-GFP

		% Specific Lysis ± SEM*		
NK Clone	E:T	RAdUL141 Control	RAdUL141	$p^{\dagger}$
D3.NKc5E11	24 to 1	19 ± 1	5 ± 1	0.00060
D3.NKc5F8	55 to 1	55 ± 1	25 ± 3	0.0039
D3.NKc3C9	100 to 1	9 ± 0.4	3 ± 1	0.00038
D8.NKc1C10	8 to 1	21 ± 1	7 ± 1	0.00053
D8.NKc1E11	40 to 1	18 ± 1	16 ± 2	NS
D8.NKc1F11	32 to 1	19 ± 1	9 ± 1	0.0022
D8.NKc3C8	35 to 1	24 ± 1	8 ± 1	0.00029
D8.NKc4E10	23 to 1	30 ± 3	11 ± 2	0.0023

		% Specific Lysis ± SEM*		
NK Clone	E:T	RAdUL141-GFP Control	RAdUL141-GFP	$p^{\dagger}$
D3.NKc5E11	32 to 1	7 ± 1	5 ± 1	NS
D3.NKc5F8	20 to 1	16 ± 2	10 ± 1	NS
D3.NKc3C9	24 to 1	32 ± 0.3	26 ± 3	NS
D8.NKc1C10	27 to 1	39 ± 0.1	28 ± 0.1	0.0043
D8.NKc1E11	23 to 1	33 ± 4	30 ± 2	NS
D8.NKc1F11	19 to 1	23 ± 1	17 ± 1	0.0029
D8.NKc3C8	20 to 1	21 ± 1	19 ± 3	NS
D8.NKc4E10	20 to 1	14 ± 2	13 ± 2	NS

NK clones were cultured and assayed with the help of Cora Griffin.

NS, not significant $p > 0.05$

(*) % Specific Lysis ± SEM was calculated as described in section 2.10.1.4.
(†) *p* values compare killing of AD169 and Toledo infected targets (Student's t-test, assuming unequal variance).

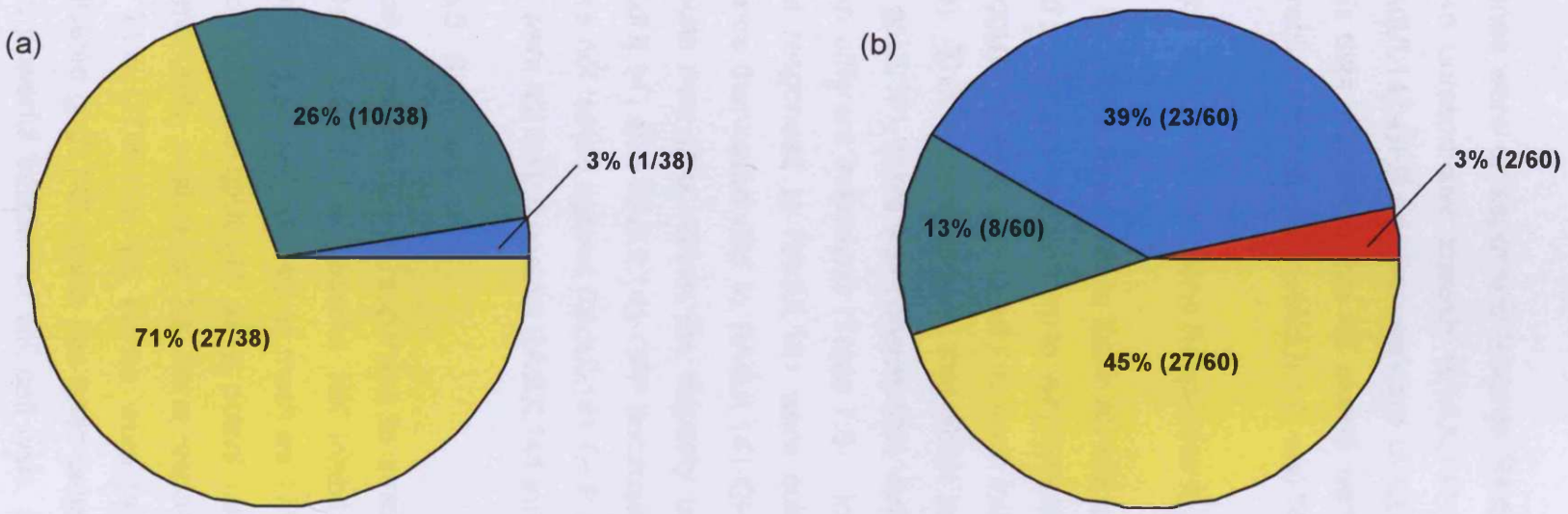

Figure 7.1 NK Clone Responses to RAdUL141 and RAdUL141-GFP. NK Clones from 3 individuals were used in ^{51}Cr release assay using (a) RAdUL141 or (b) RAdUL141-GFP as targets. Clones were categorised as being inhibited (yellow), kill/no change (blue), no kill/no change (green) or activation (red). More clones were inhibited by RAdUL141 and RadUL141-GFP. See Appendix VII.

clones were unresponsive towards RAdUL141 and 52% (31/60) of clones were unresponsive towards RAdUL141-GFP. Inhibition by RAdUL141 and RAdUL141-GFP was independent of CD94 expression levels (Figure 7.2). This data suggested that NK clones were generally inhibited by autologous fibroblasts infected with RAdUL141 and RAdUL141-GFP.

7.3.4 Individual NK Clone Responses to gpUL141

As NK clone responses to strain AD169 and strain Towne infected fibroblasts had previously been shown to vary between donors, the NK clone responses to gpUL141 were separated into each individual's NK clone response (Table 7.9). This analysis showed that whilst the pooled NK clones were inhibited by gpUL141, there was considerable variation between NK clone responses from different individuals (Table 7.9). In particular, most of the NK clones that responded to RAdUL141 were cultured from D7 whilst most of the clones that responded to RAdUL141-GFP were cultured from D9. These results may also explain the disparity between the total NK responses to RAdUL141 and RAdUL141-GFP because NK clones cultured from donor D7 were not tested against RAdUL141-GFP and NK clones cultured from donor D9 were not tested against RAdUL141 infected cells.

7.3.5 Summary

Analysis of NK clone responses to strain Toledo demonstrated that strain Toledo encoded a powerful NK inhibitory function that was capable of inhibiting NK cell lysis by as much as 17%. Previous work in the laboratory had identified gpUL141 as a potent inhibitor of polyclonal NK cell lysis. Remarkably, analysis of NK clone responses to gpUL141 showed as many as 71% (27/38) of NK clones were inhibited by gpUL141. These data indicated gpUL141, which has been deleted from strain AD169 and Towne, is a powerful inhibitor of NK cell lysis, inhibiting NK cells from all donors tested.

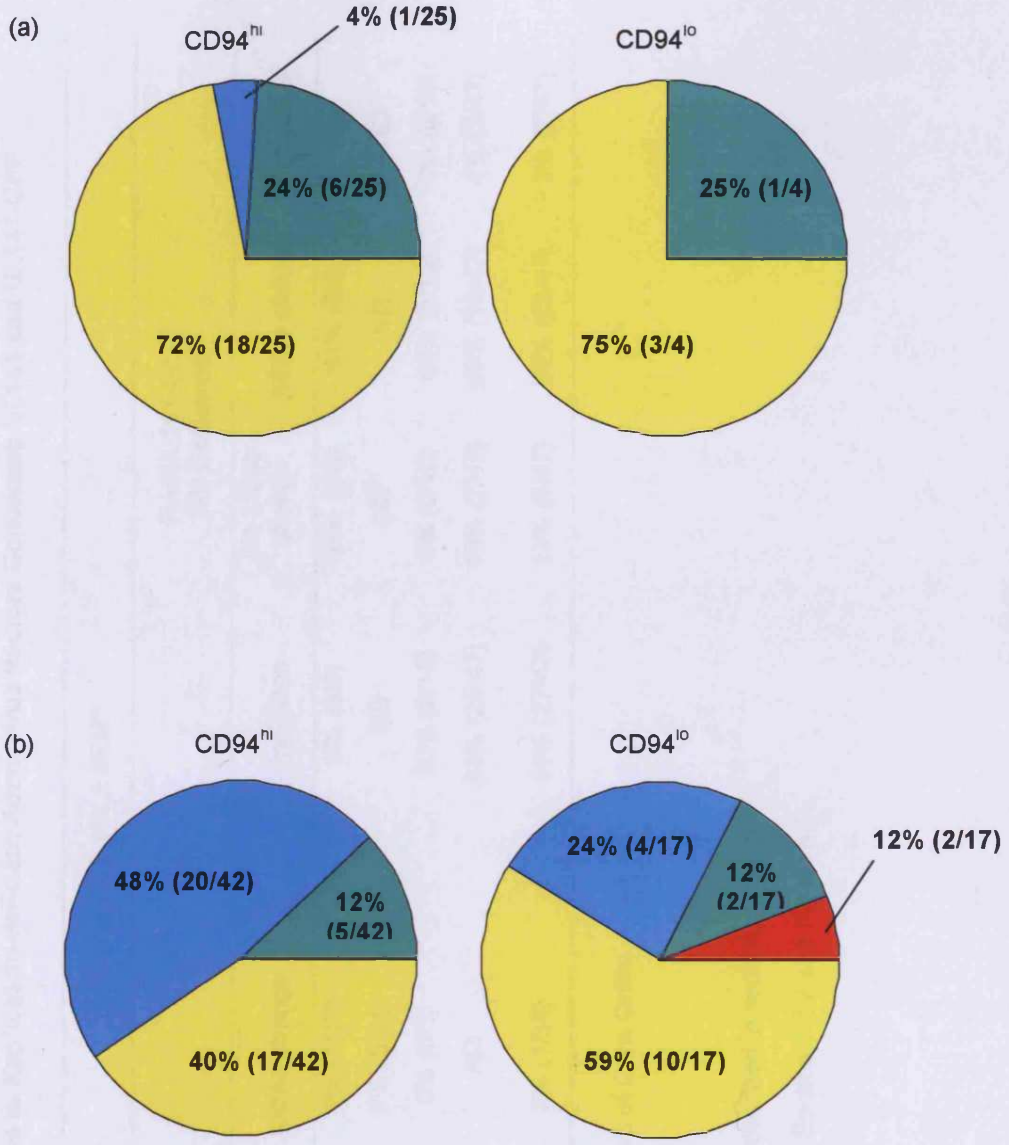

Figure 7.2 Comparison of CD94^{hi} and CD94^{lo} Clone Responses to RAdUL141-GFP and RAdUL141. NK clones were divided into CD94^{hi} and CD94^{lo} categories as described in section 6.2.2. Clones were categorised as being inhibited (yellow), kill/no change (blue), no kill/no change (green) or activation (red). (a) NK clone responses to RAdUL141 (b) NK clone responses to RAdUL141-GFP. NK clone responses to UL141 were independent of CD94 expression.

Table 7.9 Individual Donor NK Responses to Replication-deficient Adenovirus Vectors Containing UL141 and UL141-GFP

	% Specific Lysis ± SEM*						
	RAdUL141 NK Response†			RAdUL141-GFP NK Response†			
Donor	Inhibition	No kill/no change	Kill/no change	Inhibition	No kill/no change	Kill/no change	Activation
D3	25% (3/12)	67% (8/12)	8% (1/12)	0% (0/3)	33% (1/3)	67% (2/3)	0
D7	90% (19/21)	10% (2/21)	0% (0/21)	ND	ND	ND	ND
D8	100% (5/5)	0% (0/5)	0% (0/5)	20% (2/10)	0% (0/10)	80% (8/10)	0% (0/10)
D9	ND	ND	ND	53% (25/47)	15% (7/47)	28% (13/47)	4% (2/47)
Total	71% (27/38)	26% (10/38)	3% (1/38)	45% (27/47)	13% (8/47)	39% (23/47)	3% (2/47)

NK clones were cultured and assayed with the help of Cora Griffin.

ND, not done.

(*) % Specific Lysis ± SEM was calculated as described in section 2.10.1.4.

(†) NK clones were categorised as described in Section 6.2.2 and Figure 6.1.

DISCUSSION

8 DISCUSSION

8.1 MODULATION OF IE1 SPECIFIC CTL LYSIS BY PP65

Although early studies indicated IE1-specific CTL may not always be capable of recognising HCMV-infected cells (Borysiewicz *et al.*, 1988), it was not until 1996 that Gilbert and co-workers proposed that direct phosphorylation by pp65 impeded IE1 antigen presentation, either by restricting its access to the antigen processing machinery or by diverting it to a different degradation pathway (Gilbert *et al.*, 1996). Importantly, this observation has yet to be reproduced by another research group, nor has the group that published the finding pursued the mechanism further. This lack of corroborative data has led to scepticism regarding the role of pp65 in modulation of IE1 antigen presentation.

The proposed phosphorylation of IE1 by pp65 implies a direct interaction occurs between the 2 proteins following infection with HCMV. As pp65 is delivered into the cytosol and then trafficks to the nucleus it has the potential to interact with the *de novo* synthesised IE1 protein in both intracellular compartments (Schmolke *et al.*, 1995). However in this study, a yeast two-hybrid assay was unable to identify specific binding between pp65 and IE1, indicating that if pp65 forms a complex with IE1, it does so indirectly by interacting with a protein intermediary. Interestingly, pp65 has been shown to interact with the Ser/Thr kinase, Polo like kinase-1 (Plk 1), suggesting a mechanism whereby pp65 may indirectly phosphorylate IE1 (Gallina *et al.*, 1999). Additionally, pp65 did not alter IE1 localisation in fibroblasts infected with an HCMV UL83 deletion mutant (not shown) nor when expressed by using adenovirus recombinants, fibroblasts infected with RAdIE1 relative to co-infection with RAdIE1 and RAdpp65. Neither *de novo* expression of pp65 by recombinant adenovirus or pp65 delivered with the HCMV virion appeared to affect intracellular IE1 localisation.

pp65 has also been shown to suppress the induction of IFN-responsive

genes (Browne & Shenk, 2003). IFNs are important modulators of the antigen processing pathway, upregulating MHC and TAP gene products in addition to proteosome subunits and regulators (Ayalon *et al.*, 1998, Fruh *et al.*, 1994). This observation raises the possibility that inhibition of IE1 specific T-cell lysis by pp65 may have been mediated indirectly by the suppression of the IFN responsive genes, rather than a direct effect of pp65 phosphorylation of IE1. This possibility is further supported by the observation that efficient lysis of HCMV infected fibroblasts by IE1 specific CTL is dependent on pretreatment with IFNγ (Khan *et al.*, 2002). A first step in dissecting the role of pp65 in IE1 antigen presentation should therefore include a repetition of the immunoprecipitation experiments performed by Gilbert to clarify the phosphorylation status of IE1 in the presence and absence of pp65. These experiments only report on the gross level of phosphorylation and could be further developed using deletion mutants of IE1 to map the site of phosphorylation. In light of the frequency of serine (S) and threonine (T) residues in IE1 and their presence in identified IE1 peptide epitopes (CVE**T**MCNEY, YILEE**TS**VM, VLEE**TS**VML, **S**LL**S**EFCRV, CVE**T**MCNEY), mapping which residues are phosphorylated in IE1 may provide a valuable insight into the function of IE1 phosphorylation by pp65.

The initial aim of this PhD was to culture IE1 and pp65 specific CTL to test whether pp65 could abrogate recognition of IE1 epitopes by IE1 specific CTL. The use of replication-deficient adenovirus vectors to drive IE1 and pp65 expression permits transgene expression in the absence of background expression from the vector. The initial study by Gilbert and colleagues utilised lytic vaccinia virus vectors, which can be expected to perturb many cellular processes including MHC class I antigen presentation. The use of adenovirus vectors would thus represent a significant improvement over the 1996 study. However, sufficient CTL were not available at any stage to complete the study. A number of reasons could account for these difficulties. Initial attempts were made to culture peptide specific CTL from donors whom were known to be HCMV seropositive and HLA-A2 positive. However, it was

only found by staining retrospectively with tetramers that the original donors did not possess the CTL of the specificities the protocol was designed to culture. Additionally, other groups within the department that routinely cultured large numbers of CTL were unable to culture CTL at this time. The difficulties experienced in culturing IE1 and pp65 CTL during this period could therefore have been a consequence of unsuitable donors (those without IE1 (VLEETSVML) or pp65 (NLVPMVATV) specific CTL) or problems with the media, incubators or plasticware that were available within the department. With the purchase of IE1 and pp65 tetramers, a number of suitable donors were identified although at a much lower frequency than expected. Fifty donors were tested for HCMV serostatus and only 17 of these were HCMV seropositive, of these tetramers identified IE1 and/or pp65 peptide specific CTL in 7 of these donors. In addition, autologous fibroblasts lines were only available for 1 of theses donors – an essential prerequisite for the planned use of recombinant adenoviruses to drive expression of IE1 and pp65. Further investigations were also frustrated by the irregular availability of these donors. A series of experiments was carried out to investigate different media that had been designed for optimal CTL growth and multiple batches of human AB serum were tested for their ability to maintain CTL proliferation in culture. However, none of these approaches provided a method for the efficient culture of IE1 specific CTL.

A method developed by Ayyoub *et al* (2003), which stimulates enriched $CD8^+$ cells with peptide coated autologous PBMC has recently been adopted by Steve Man's group within the department (Ayyoub *et al.*, 2003). Using this method, this group has successfully cultured Melan A and retinoblastoma (Rb) peptide specific CTL. However, whilst Melan A specific CTL were cultured in parallel using this protocol, it was not able to culture sufficient IE1 and pp65 peptide specific CTL from D7. This suggests that either this method or this donor is unsuitable for culture of IE1 and pp65 specific CTL. In this respect, more CMV specific $CD8^+$ cells have been shown to express CD57 and LIR-1 than other $CD8^+$ cells in the periphery, and this has been

associated with decreased proliferative potential and increased propensity for apoptosis upon stimulation with a CD3 mAb (Brenchley *et al.*, 2003, Ince *et al.*, 2004, Young *et al.*, 2001). Furthermore, HCMV specific CTL have reduced telomerase activity and shorter telomeres contributing to finite replicative potential (Gillespie *et al.*, 2000, Valenzuela & Effros, 2002). As $CD57^+$ cells have been shown to accumulate with age and LIR-1 expressing T-cells have been shown to increase in frequency with time, donors that have been infected with HCMV for a short period of time may have a greater proportion of HCMV peptide specific CTL which do not express these late differentiation antigens. The difficulty in culturing HCMV specific CTL from this donor may therefore have been a consequence of the phenotype of D7's HCMV specific CTL, a donor who is known to have been HCMV seropositive for at least 15 years. It may therefore be easier to generate long-term CTL lines from donors with lower levels of CD57 and LIR-1 on their HCMV specific $CD8^+$ T cells.

8.1.1 Concluding Remarks

IE1 specific CTL are frequent in HCMV seropositive individuals but are not always capable of recognising HCMV infected targets. The reason for this remains to be resolved. The possibility that pp65 selectively interferes with IE1 antigen presentation cannot be excluded, however a yeast two hybrid assay indicated that pp65 does not interact directly with IE1 and fluorescence microscopy has shown that pp65 does not alter the localisation of IE1 in fibroblasts. As sufficient numbers of IE1 and pp65 specific CTL were not cultured, it was not possible to functionally test whether pp65 abrogated IE1 antigen presentation. A number of reasons may account for this, which include lack of suitable donors and possible problems with media, human AB serum and other hardware available within the laboratory. The differentiated phenotype of HCMV specific CTL, and expression of LIR-1 may also have contributed to these difficulties.

8.2 Correlations of T-Cell Subsets with HCMV Serostatus

Analysis of $CD3^+$,$CD56^+$ cell frequency in HCMV seropositive and seronegative individuals indicated that HCMV seropositive have more $CD3^+$,$CD56^+$ cells than seronegative individuals. The percentage of $CD3^+$,$CD56^+$ cells which were single positive for CD4 or CD8 did not differ between HCMV seropositive and seronegative individuals. However, HCMV seropositive individuals had higher proportions of $CD3^+$,$CD56^+$ cells which were CD4 and CD8 double positive. This CD4 and CD8 double positive population could be divided into $CD8^{hi}$,$CD4^{lo}$ cells, $CD8^{lo}$,$CD4^{hi}$ cells and $CD8^{hi}$,$CD4^{hi}$ cells and HCMV seropositive individuals had higher proportions and numbers of $CD8^{hi}$,$CD4^{lo}$ and $CD8^{hi}$,$CD4^{hi}$ but not $CD8^{lo}$,$CD4^{hi}$ cells. As previous studies had demonstrated that $CD8^+$,$CD57^+$ cells are expanded in HCMV seropositive individuals and the majority of responding $CD8^+$ cells are $CD57^+$ the expression of CD57 on $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ cells was investigated (Gratama *et al.*, 1989; Kern *et al.*, 1999). Unfortunately sufficient numbers of events could not be acquired with the Cytomation Summit Software to allow proper analysis of this phenotype and further phenotyping of $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ cells was no longer continued.

A number of papers have characterised the $CD4^+$,$CD8^+$ population as a whole, whilst only a few investigators have concentrated on the specific cell subsets defined above. However, none of these papers have addressed the $CD4^+$,$CD8^+$ cells as a subset within the $CD3^+$,$CD56^+$ population, and only a number have identified them within the $CD3^+$ population. For the purpose of this discussion, I will discuss the results presented by these researchers on $CD4^+$,$CD8^+$ cells and their subsets and at the end I will discuss the relevance of these cells to the $CD3^+$,$CD56^+$,$CD4^+$,$CD8^+$ subsets which were identified in this study.

$CD4^+$,$CD8^+$ cells constitute between 1 and 3% of PBMC in normal healthy individuals and abnormal expansions of these cells have been described for individuals suffering with a number of autoimmune diseases including

myasthenia gravis and multiple sclerosis (Buckley *et al.*, 2001, Munschauer *et al.*, 1993). They have also been shown to be expanded in individuals suffering from Behcets Syndrome, Infectious Mononucleosis, Kawasaki Disease and HIV (Hirao & Sugita, 1998, Ortolani *et al.*, 1993, Valesini *et al.*, 1985, Weiss *et al.*, 1998). Unfortunately, most investigators have not attempted to distinguish the phenotypes of the $CD4^+$,$CD8^+$ cells expanded within these individuals, however *in vitro* studies have provided elements to understand the function of these cells in health and disease. Two studies have attempted to investigate the *in vivo* function of these cells with respect to viral disease and 1 study has investigated the function of these cells in normal individuals (Nascimbeni *et al.*, 2004, Patel *et al.*, 1989, Weiss *et al.*, 1998).

$CD4^+$,$CD8^+$ cells have been shown to be expanded in HIV and HCV seropositive donors (Nascimbeni *et al.*, 2004, Weiss *et al.*, 1998). In both studies, these cells were shown to be cytotoxic T-lymphocytes that are likely to have been expanded in response to specific antigens. The $CD4^+$,$CD8^+$ cells expanded in HCV seropositive donors were further divided into $CD8^{hi}$,$CD4^{lo}$ and $CD8^{lo}$,$CD4^{hi}$ phenotype and were suggested to represent an effector memory ($CCR7^-$, $CD45RO^+$) and a central memory phenotype ($CCR7^+$, $CD45RO^+$), respectively (Nascimbeni *et al.*, 2004). However, the authors detected no other differences in the activation (CD69, CD56), lymph node and tissue homing (CD62L, CXCR3), differentiation (CD27, CD28), senescence (CD57), and maturation (CD1a) markers and therefore grouped these two subsets as $CD4^+$,$CD8^+$ cells for further analysis. These cells were shown to contain similar levels of granzyme A and perforin as $CD8^+$ cells indicating they cells can potentially display cytotoxic functions against viral and vaccine antigens. Interestingly, the authors suggest that $CD4^+$,$CD8^+$ cells are capable of producing more IFNγ and TNFα in response to virus infected lysates and HLA-A2 restricted viral peptides than either $CD4^+$ and $CD8^+$ cells alone. However, with respect to HCMV, their data showed only a 0.4% and a 0.05% increase in production of TNFα and IFNγ in response to

HCMV infected lysate by $CD4^+$,$CD8^+$ cells compared to $CD4^+$ and $CD8^+$ cells. Furthermore, the authors did not show that these subjects were seropositive for HCMV, HLA-A2 positive or able to bind the NLVPMVATV tetramer. The observed increase in IFNγ production of 5.42% by $CD4^+$,$CD8^+$ cells compared to a 0.87% increase by $CD8^+$ cells in response to the NLVPMVATV peptide may therefore represent an artefact of the method, which stimulated cells not only with peptide but also with mAb against the co-stimulatory molecules CD28 and CD49d.

Nascimbeni *et al* (2004) established the number of *in vivo* divisions of the $CD4^+$,$CD8^+$ cells by analysing the cells for T-cell Receptor Excision Circles (TRECs) content and telomere length. $CD4^+$,$CD8^+$ had shorter telomeres than $CD4^+$ and $CD8^+$ populations, indicating that this population had undergone more divisions. TRECs are episomal DNA products which are generated during TcR rearrangement and are not amplified during mitosis, therefore the lower the number of TRECs the larger the number of cellular divisions (Hazenberg *et al.*, 2002). TREC copy number in $CD4^+$,$CD8^+$ cells was found to be at least half that of $CD4^+$ cells and $CD8^+$ cells, however as cell proliferation is logarithmic, a decrease in TREC copy number by half is not that significant.

The study carried out by Patel *et al* (1989) differs from those described above in that $CD4^+$,$CD8^+$ clones, not PBMC were used for analysis (Patel *et al.*, 1989). These clones were shown to be αβ TcR positive, $CD3^+$ and able to lyse the classic NK target K562. Lysis of K562 by $CD4^+$,$CD8^+$ cells is interesting because this cell line is MHC class I negative, suggesting that activation of $CD4^+$,$CD8^+$ cells is not MHC class I dependent. In contrast to the $CD4^+$,$CD8^+$ cells described by Weiss *et al*, the $CD4^+$,$CD8^+$ clones secreted IL2. Patel used 5 clones from 1 donor and this may not give a reliable estimate of $CD4^+$,$CD8^+$ cells *in vivo*. In this respect, it may be important that recent understanding of CD8 and CD4 cytokine responses has moved away from the idea that distinct subsets of cells secrete particular

cytokines towards more probabilistic principles (Kienzle *et al.*, 2004). This leaves the possibility that a few $CD4^+$,$CD8^+$ clones may express IL2 whereas $CD4^+$,$CD8^+$ cells in general do not and this may explain these contrasting data.

Together these data suggest that $CD4^+$,$CD8^+$ cells are distinct from $CD4^+$ and $CD8^+$ cells. However, these cells may still exhibit the phenotypic and functional characteristics of cytotoxic T lymphocytes but the exact nature of their specificity remains unknown. As these cells are known to comprise of at least 3 distinct phenotypic populations it is perhaps not surprising that some of the results discussed here are contradictory. As shall be discussed below, each population may have very different phenotypic and functional profiles.

The T-cell subset expressing $CD8^{hi}$,$CD4^{hi}$ phenotype appear at very low frequencies in normal individuals and have been reported at higher frequencies in some auto-immune diseases such as psoriasis vulgaris and in HCV infected individuals (Munschauer *et al.*, 1993, Sala *et al.*, 1993). The presence of $CD8^{hi}$,$CD4^{hi}$ cells in the skin of patients with atopic dermatitis has also been described (Bang *et al.*, 2001). In this study, 26 ± 6.5% of T-cells derived from skin punches were shown to be $CD8^{hi}$,$CD4^{hi}$ after 1 week of culture *in vitro.* However, the starting percent of $CD8^{hi}$,$CD4^{hi}$ cells could not be determined as a comparison for expansion. Phenotypic analysis of the cultured cells indicated that the CD4 and CD8 staining pattern was similar to that of freshly isolated thymocytes. According to this analysis the authors suggested that these cells were circulating as a result of a defect in thymic selection of single positive cells. However, the $CD8^{hi}$,$CD4^{hi}$ cells lack CD1a, a cell surface biomarker for premature thymocytes. Additionally, stimulation of thymocytes through their TcR receptor and CD28 leads to apoptosis (Ebert *et al.*, 2000). These data are inconsistent with $CD8^{hi}$,$CD4^{hi}$ these being released prematurely from the thymus and having a role in autoimmune diseases.

$CD8^{hi}$,$CD4^{hi}$ cells have been shown to be stably expanded over a period of 17 months in 1 patient with HCV (Sala *et al.*, 1993). These cells were phenotyped and shown to express CD3, CD56, CD57, CD45RA and CD45RO and to have a monoclonal TcR β band. A role for these cells in HCV is supported by more recent reports that have shown a close correlation with the frequency of activated $CD4^{+}$,$CD8^{+}$ cells and HCV viral kinetics in the chimpanzee (Nascimbeni *et al.*, 2004). $CD8^{hi}$,$CD4^{hi}$ cells are expanded in multiple sclerosis and corticosteroid treatment reduces their frequency, and it has been inferred that these cells are partly responsible for the disorder (Munschauer *et al.*, 1993). Stable $CD4^{hi}$,$CD8^{hi}$ cell expansion have also been described for a healthy 64 year old male, whose HCMV serology was unknown (Kay *et al.*, 1990). Phenotyping of these cells indicated that they were negative for the thymocyte antigen CD6, and expressed CD3 at the same level as $CD8^{+}$ cells. These cells were not clonal and had little or no NK activity. Together these data indicated that $CD8^{hi}$,$CD4^{hi}$ cells are found in both normal and diseased patients. However, these papers give little indication of their function and in light of this it is difficult to speculate a reason for their expansion in HCMV seropositive individuals.

$CD8^{hi}$,$CD4^{lo}$ cells were expanded in HCMV seropositive donors, however no studies have demonstrated a role of $CD8^{hi}$,$CD4^{lo}$ cells in HCMV or any other disease. Kitchen *et al* (2004) showed that ligation of CD4 on $CD8^{hi}$,$CD4^{lo}$ cells by an anti-CD4 antibody or IL-16 resulted in an increase in IFNγ production by these cells (Kitchen *et al.*, 2004). IL16 is an inflammatory cytokine secreted by a number of cell types, including bronchial epithelial cells which can be infected by HCMV (Kitchen *et al.*, 2002). As such, ligation of CD4 on $CD8^{hi}$,$CD4^{lo}$ cells by IL16 suggests a role for $CD8^{hi}$,$CD4^{lo}$ cells in the control of HCMV (Kitchen *et al.*, 2004). In this scenario, secretion of IL16 by HCMV infected cells would attract $CD8^{hi}$,$CD4^{lo}$ cells which would secrete IFNγ. Secretion of IFNγ by $CD8^{hi}$,$CD4^{lo}$ cells may then have direct or indirect antiviral effects, including the upregulation of Fas on infected cells, rendering them more susceptible to $CD8^{+}$ T-cells expressing FasL. Expression of CD4

may also act as a co-stimulatory molecule by binding to MHC class II on the surface of the infected cell.

While no significant difference in the number or proportion of $CD8^{lo}$,$CD4^{hi}$ cells in HCMV seropositive or seronegative donors was found in this study, a role for these cells in control of HCMV disease has been proposed (Suni *et al.*, 2001). A number of studies have demonstrated that $CD8^{lo}$,$CD4^{hi}$ cells express the CD8α not the CD8αβ chain which is found on $CD8^{hi}$ expressing cells (Colombatti *et al.*, 1998, Patel *et al.*, 1989). Whilst the CD8α chain is capable of binding to class I MHC molecules through the α3 domain, expression of the CD8αβ heterodimer greatly enhances CD8 function, resulting in TCR-CD8 adducts with high affinity for MHC-peptide (Arcaro *et al.*, 2001). It may therefore not be surprising that in cytotoxicity assays using CMV pulsed autologous B cells and DCs as targets, the $CD4^{hi}$,$CD8^{lo}$ population was more cytotoxic than the $CD4^{+}$ population (Suni *et al.*, 2001). In this study, the authors used intracellular cytokine staining to show that in 1 donor the $CD4^{hi}$,$CD8^{lo}$ population contributed 39% of the total CMV specific $CD4^{hi}$ cell cytokine response, even though this population constituted only 17% of the $CD4^{hi}$ population. Additionally using monoclonal antibodies and inhibitors specific to MHC class I and II, the authors demonstrated that production of IFNγ in response to HCMV lysate was dependent upon MHC class II not MHC class I presentation. A role for CD8 expression in activation of these cells is still possible as antibodies specific to MHC class I inhibited production of IFNγ by 17% on $CD4^{hi}$,$CD8^{lo}$ cells, and this contrasts with a massive 70% reduction in $CD8^{+}$ cells. In this regard it may be significant that infection of cells, including dendritic cells by HCMV downregulates MHC class I expression, thus making class II restricted cytotoxicity important in the control of disease (Soderberg-Naucler *et al*, 1999). In this scenario low expression of CD8 may enhance interaction with the APC by serving as an adhesion and co-stimulatory molecule by binding to MHC class I remaining on the surface of the infected cell.

Unfortunately, most investigators have not defined the expression of CD56 on $CD4^+$,$CD8^+$ cells. However, one study showed that between 4% and 30% of adult $CD8^+$ cells express CD56 (Pittet *et al.*, 2000). According to these authors, these cells displayed an effector phenotype with 62% of the cells expressing CD57 and 65% expressing CD45RA. Furthermore, analysis of TcR usage indicated that for the 3 individuals tested, the $CD8^+$,$CD56^+$ cells were clonally expanded, suggesting that the high frequency of these cells was the result of specific antigen stimulation *in vivo*. A strong correlation was shown with CD56 expression and cytotoxicity and this is likely to reflect the higher levels of perforin and granzyme B in the $CD8^+$,$CD56^+$ population. Unfortunately the authors did not test the individuals for HCMV serology. These data support the notion that $CD3^+$,$CD56^+$ which are double positive for CD4 and CD8 may represent a cytotoxic phenotype and participate in the control of viral infection *in vivo.*

8.2.1 Concluding Remarks

The data discussed here indicates that $CD4^+$,$CD8^+$ cells may be involved in the control of a number of viral infections, including HCV and HCMV and are expanded in response to a variety of autoimmune diseases. $CD4^{hi}$,$CD8^{hi}$ cells are found in both normal and diseased patients (Kay *et al.*, 1990, Munschauer *et al.*, 1993, Nascimbeni *et al.*, 2004, Sala *et al.*, 1993). However, these papers have given little insight into their function, making speculation regarding the reasons for their expansions in HCMV seropositive individuals difficult. In contrast, $CD4^{hi}$,$CD8^{lo}$ cells have been shown to be expanded in HCMV seropositive individuals and to lyse target cells in a MHC class II restricted manner, however the data presented here showed there was no association between HCMV serostatus and the frequency of these cells (Suni *et al.*, 2001). No role for $CD8^{hi}$,$CD4^{lo}$ cells has been demonstrated with respect to HCMV, however CD4 may act as a co-stimulatory molecule, leading to increased secretion of IFNγ and its associated anti-viral effects, thus indicating a potential role for these cells in HCMV infection (Kitchen *et al.*, 2004). In light of this review of the literature,

it could be speculated that at least a proportion of $CD3^+$,$CD56^+$ cells and their subsets represent HCMV specific cytotoxic cells which have been expanded in HCMV seropositive individuals.

Further studies to investigate the role of these cells in the seropositive individual in the context of HCMV could include; flow cytometric analysis for intracellular perforin and granzymes; intracellular cytokine staining for IFNγ in response to HCMV peptides and/or lysates; proliferation in response to HCMV peptides an/or lysates. In light of the variation in proportion and numbers of these cells with time it would also be interesting to investigate a correlation with HCMV serology and the frequency of these cells. Additionally, analysis of the distribution of these cells in elderly HCMV seropositive donors could also provide a further insight into the importance of these cells in the context of HCMV infection.

8.3 Novel Method of NK Cloning

Most current NK cloning techniques are based on a method developed by Yssel *et al* in 1984 for expanding antigen specific cytotoxic and helper T-cells (Yssel *et al.*, 1984). However, the NK cloning efficiency using this method is usually only around 2 – 8% (Spits & Yssel, 1996). In this study, a novel and highly efficient system for NK cloning was developed that has enabled at least 110 NK clones from 4 donors to be cultured.

There are a number of important differences between the methods commonly used for NK cloning and the method described here. Firstly, in contrast to current techniques, which stimulate NK clones with irradiated EBV-LCL, PHA and OKT3, a mixture of irradiated allogeneic PBMC supplemented with IL2 and OKT3 was used in this study (Carr *et al.*, 2002, Litwin *et al.*, 1993, Roncarolo *et al.*, 1991). Binding of OKT3 to T cells in the irradiated PBMC may have a similar role to that proposed for PHA and EBV-LCL, stimulating the irradiated PBMC to produce IL2 and perhaps other growth and/or co stimulatory factors, that enhance NK clone growth (Spits & Yssel, 1996). However, unlike PHA that has non-specific potent agglutinating and mitogenic activities, OKT3 specifically stimulates T cells by binding to CD3 on their surface (Leavitt *et al.*, 1977). NK clones proliferated the most when stimulated with a combination of allogeneic PBMC feeder cells, suggesting that factors secreted by the allo-response are able to stimulate and maintain the growth of NK clones. As mixed lymphocyte reactions are dependent upon allogeneic responses, it unexpected that a combination of 2 allogeneic donors was capable of supporting NK clone proliferation better than a combination of 3 allogeneic donors. One possibility for this apparently contradictory observation is that by using a combination of 3 allogeneic feeders large amounts of cytokines may have been released which may have overstimulated the NK clone and led to its death.

The second major difference in the methods was the media used to culture NK clones. In light of recent data that indicated that the commercially

available serum free medium, SCGM was capable of supporting the expansion of polyclonal NK cells within PBMC the ability of this medium to maintain NK clone growth was investigated (Carlens *et al.*, 2001, Carlens *et al.*, 2000). There was at least a 400% increase in the number of proliferating wells for clones cultured in SCGM compared to RPMI, suggesting that SCGM was not only able to support polyclonal NK growth but also NK clone expansion in culture. For maintenance and expansion of T and NK cells, Yssel's medium or RPMI is usually used (Litwin *et al.*, 1993, Robertson *et al.*, 1993, Wada *et al.*, 2004). Yssel's medium is a serum free media that was shown to be more efficient than RPMI supplemented with either human or foetal calf serum for generating and expanding T and NK cell clones (Spits & Yssel, 1996). The ability of Yssel's medium and SCGM to support NK clone growth have not been compared during this study, however cloning efficiencies in Yssel's medium are very low (typically 2 – 5%), indicating that SCGM is superior for the culture of NK clones. Yssel's medium is prepared with Iscove's modified Dulbecco's medium supplemented with sodium bicarbonate, bovine serum albumin, transferrin, insulin, linoleic-, oleic- and palmitic acid (Yssel *et al.*, 1984). The formulation of SCGM has not been fully disclosed. The information available cites it does not contain any growth factors, antibiotics or undefined supplements and that it is nutritionally complete, providing a balanced environment for haematopoietic stem and progenitor cells, T cells and NK cells. Like Yssel's medium SCGM contains albumin, insulin and transferrin however, the roles of these factors in NK cell proliferation and function were not discussed by Yssel or the manufacturer's of SCGM. Transferrin is a growth factor, which has previously been shown to enhance NK cell cytotoxicity, however it has been shown to have no effect on mature or IL2 induced NK like cells (Shau *et al.*, 1986). This suggests that transferrin is important for the development of mature NK cells, rather than their expansion. Arachidonic acid, the major precursor of several classes of signal molecules (prostaglandins, prostacyclins, thromboxanes and leukotrines) is derived from linoleic acid. Inclusion of linoleic acid, which cannot be endogenously synthesised by mammals may therefore be

important for NK cell proliferation and function. In this respect, it is interesting that inhibition of phopholipase A2 (PLA2), the enzyme responsible for converting phospholipids to arachidonic acid, inhibited NK activity (Rola-Pleszczynski *et al.*, 1985). However, evidence suggests that prostaglandins (PGs) inhibit NK cell activity whilst the leukotrine, LTB4 enhances NK cell function against K562 targets (Hall & Brostoff, 1983, Herman & Rabson, 1984, Stankova *et al.*, 1992). Interestingly, monocytes stimulated with polyinosinic acid-polycytidylic acid (poly I:C) produce PGs and this was reversed by treatment with the prostaglandin synthase inhibitor, indomethacin (Koren *et al.*, 1981). Stimulation of lymphocytes with poly I:C increases secretion of IFN, a positive stimulator of NK cell activity. Linoleic acid, poly I:C and the prostaglandin synthase inhibitor, idomethacin could therefore support NK activity by reorientating the metabolism of arachidonic acid away from prostaglandin production, towards leukotrine production and by increasing IFN production. These factors may therefore be some of the undefined components of SCGM. Thromboxanes are also derived from arachidonic acid however, the role of thromboxanes in NK function remain unclear. Inhibitors of thromboxane A2 have been shown to both enhance and inhibit lymphocyte proliferation in response to mitogens (Defreyn *et al.*, 1982, Gordon *et al.*, 1981). However, it is most likely that inhibition of thromboxane synthesis enhances production of PGs and it is these that inhibit mitogen induced proliferation and NK activity (Rola-Pleszczynski *et al.*, 1985). Serum albumin was included in both Yssel's medium and SCGM and has been shown to have a stimulatory effect on NK activity (Goren & Nelken, 1981, Ito *et al.*, 1987). The mechanism by which albumin enhances NK function remain unclear, however it may be linked with the ability of albumin to bind free fatty acids (Soltys & Hsia, 1977, Spector *et al.*, 1969). Similarly, insulin was included in both media. Studies in type I diabetes have indicated that a severe lack of insulin and an excess of glucagons inhibits the desaturation of precursor fatty acids to arachidonic acid, indicating a role for insulin in the production of PGs, prostacyclins, thromboxanes and leukotrines (Metz, 1983).

FMS-like tyrosine kinase 3 ligand (FLT3L), c-KIT ligand and IL7 have been shown to drive precursor $CD34^+$ HSCs to express IL2Rβ, CD38 and transcripts for IL15Rα but not CD7, CD16, CD56, NKG2A and several NK cell receptors (Yu *et al.*, 1998). Considering that most mature ($CD56^+$) NK cells are negative for FLT3 a role for FLT3L in maintaining and expanding $CD3^-$,$CD56^+$ NK clones in culture is doubtful (Lyman & Jacobsen, 1998). This is supported by a study by Shaw *et al* (1998) who showed that co-culture of mouse splenocytes (which contained mature NK cells) with FLT3 and had no effect on NK function (Shaw *et al.*, 1998). In contrast, human $CD3^-$,$CD56^{hi}$ NK cells constitutively express c-KIT. Binding of c-KIT ligand to c-KIT expressing $CD56^{hi}$ cells was shown to upregulate bcl-2 preventing apoptosis, thereby indicating a role for c-KIT ligand in the survival of $CD56^{hi}$ cells (Carson *et al.*, 1994b). In a later study, the authors demonstrated that c-KIT ligand had differential effects on $CD56^{hi}$,$CD16^+$ and $CD56^{hi}$,$CD16^{lo}$ NK cells (Carson *et al.*, 1997). CD16 is a low affinity FcγR that binds to antibody coated targets and signals through ITAMs to direct Antibody-dependent cellular cytotoxicity (ADCC). Most $CD56^{hi}$ NK cells (50 – 70%) lack expression of CD16 and the remaining express only low levels of this receptor. Interestingly, incubation of $CD56^{hi}$,$CD16^+$ with c-KIT ligand induced a proliferative response which was not observed for the $CD56^{hi}$,$CD16^{lo}$ NK subset. Furthermore, co-stimulation of $CD56^{hi}$,$CD16^+$ and $CD56^{hi}$,$CD16^{lo}$ cells with IL12, IL2 and c-KIT ligand induced both subsets to produce IFNγ (Carson *et al.*, 1997). Interestingly, c-KIT ligand has been shown to potentiate MLR (Bluman *et al.*, 1996). Therefore, c-KIT ligand may specifically support the proliferation of $CD56^{hi}$,$CD16^+$ NK cells and the MLR, and thus support NK clone growth in culture. Culture of HSCs with IL7 was shown to generate NK cell precursors (NKPs). Murine NKPs have been shown to express the IL7 receptor (IL7Rα) however, expression of this receptor decreases as the cell proceeds from NKP to immature to mature NK cell. In humans, IL7, may play a role in the survival of NK cells by enhancing bcl-2 (Armant *et al.*, 1995). It has also been shown to increase proliferation and cytotoxicity of $CD56^{hi}$ but not $CD56^{lo}$ NK cells (Dadmarz *et al.*, 1994).

Interestingly, IL7 has also been shown to induce the upregulation of CD56 on $CD56^+$ NK cells, indicating a role in the maturation of human NK cells (Naume *et al.*, 1992).

IL15 represents another cytokine that could maintain and expand NK clone growth. Binding of IL15 to components of the IL2 receptor was shown to activate NK cells in a similar but not identical manner to that of IL-2 (Carson *et al.*, 1994a). Furthermore, a role for IL15 in the survival of mature NK cells *in vivo* was demonstrated in adoptive transfer experiments where NK cells transferred to IL15 deficient mice were rapidly lost (Ranson *et al.*, 2003). Survival of NK cells was subsequently shown to be mediated by the maintenance of the anti-apoptotic factor Bcl-2 by IL15 (Ranson *et al.*, 2003) (Cooper *et al.*, 2002). As the IL2 receptor is expressed by $CD56^{hi}$,$CD16^+$ and $CD56^{hi}$,$CD16^{lo}$, the inclusion of IL15 in SCGM would benefit both subsets of NK cells.

IL21 has also been implicated in proliferation of NK cell *in vitro*. This cytokine is most closely related to IL2 and IL15, and binds to the IL21 receptor (Parrish-Novak *et al.*, 2000). Culture of mature $CD56^+$ cells with IL21 for 1 – 2 days has been shown to enhance the lytic activity against K562 targets, however this was not as efficient as that mediated by IL2 or IL15 (Parrish-Novak *et al.*, 2000). Treatment of resting murine NK cells with IL21 resulted in an increase in size and granularity and the upregulation of cell surface markers associated with activated NK cells. Interestingly, these phenotypic changes correlated with an enhancement of cytolytic ability and IFN γ and IL10 secretion (Brady *et al.*, 2004). However, IL21 was unable to support their viability, thus limiting their duration of activation, and IL21 was shown to block IL15 induced expansion of resting NK cells (Kasaian *et al.*, 2002). Treatment of activated NK cells with IL21 boosted their effector function however it concurrently reduced the viability of these cells (Kasaian *et al.*, 2002). These data suggest that IL21 unlike the closely related cytokines IL15 and IL2, does not necessarily promote the survival or

activation of NK cells but rather that IL21 induces a terminal differentiation or maturation program.

FLT3L, c-KIT ligand, IL7, IL15 and IL21 are all cytokines and thus are growth factors, and as such would not have been included in SCGM. FLT3L, is produced by T-cells and IL21 is expressed exclusively by activated $CD4^+$ T cells, as such FLT3L and IL21 may be present in the media as a consequence of the MLR (Lyman *et al.*, 1994, Parrish-Novak *et al.*, 2000). c-KIT ligand has been shown to be relatively abundant in human serum and potentiates MLR which could increase the secretion of factors which support NK clone growth (Bluman *et al.*, 1996, Langley *et al.*, 1993). The main sources of IL7 are stromal and epithelial cells however IL7 has been shown to be produced by mature dendritic cells (Fernandez *et al.*, 1994, Sorg *et al.*, 1998, Tang *et al.*, 1997). IL7 has not been detected in normal lymphocytes. This would indicate that IL7 would not be present in significant quantities in SCGM during NK cloning. The main sources of IL15 are monocytes and macrophages, suggesting that this cytokine may be present in SCGM during NK cloning (Carson *et al.*, 1995, Kennedy & Park, 1996). As these factors would also have been present in RPMI, which was not as efficient for culturing NK clones, it is likely that they would work in concert with SCGM to provide an optimum environment for culture of NK clones.

8.3.1 Concluding Remarks

A novel and highly efficient method of NK cloning that uses SCGM media supplemented with IL2 and a stimulating protocol using irradiated allogeneic PBMC and OKT3 only has been described. This contrasts to the methods currently in use, which stimulate NK cells with a mixture of allogeneic PBMC and EBV-LCL and culture cells in IMDM supplemented with fatty acids, insulin, albumin, transferrin and PHA. SCGM contains a number of factors found in Yssel's medium (insulin, transferrin and albumin), which support NK growth and cytotoxicity. However, the factor responsible for the superiority of SCGM was not identified because the formula for SCGM has not been

disclosed. A number of factors included in Yssel's medium such as fatty acids may have been included in SCGM and may be partly responsible for the enhanced capability of SCGM to maintain NK clone growth in culture compared to RPMI. SCGM may also contain indomethacin and poly I:C which would favour the metabolism of arachidonic acid into leukotrines that are beneficial for NK cells and increase the production of IFN. The cytokines Flt3 ligand, c-KIT ligand, IL15, IL21 will not have been included in SCGM because they are growth factors, however they may be released by feeder cells and in combination with SCGM may potentiate NK clone growth and maintenance in culture.

8.4 NK Responses to HCMV

Activation of NK cells in response to tumour and allogeneic cells has been explained by the absence of self MHC class I on the surface of the target cell. In these systems, NK cells are able to lyse target cells because self MHC class I antigens do not engage the inhibitory receptors on the NK cell surface. Many viruses, including HCMV reduce or perturb MHC class I expression in the cells they infect, a state which could render infected cells susceptible to NK cell attack by mechanisms like those that permit recognition of tumour and allogeneic cells (Karre, 1997). In recent years, the identification of both activating and inhibitory NK receptors specific for each MHC class I allele and the identification of HCMV proteins with the potential to interact with these receptors has indicated that the loss of MHC class I surface expression may no longer be the dominating factor determining NK cell activity.

8.4.1 NK Responses to HCMV Strain AD169

Down regulation of classical MHC class I molecules by HCMV may be counteracted by a stabilisation of the surface expression of the non-classical MHC class I molecule, HLA-E, by the HCMV strain AD169 gene gpUL40 leader sequence, VMAPRTLIL (Tomasec *et al.*, 2000). HLA-E has been shown to interact with CD94/NKG2A and CD94/NKG2C on the surface of NK cells and has been shown to transmit an inhibitory and stimulatory NK signal, respectively (Braud *et al.*, 1998, Lanier *et al.*, 1998). However, the functional significance of NK cell inhibition by gpUL40 stabilised HLA-E in the context of MHC class I is downregulation is currently under discussion.

To assess the importance of HLA-E:CD94/NKG2 interactions in the context of HCMV infection, freshly isolated polyclonal NK cells and 110 NK clones from 4 donors, have enabled a detailed dissection of NK responses in the context of CD94 expression. These clones showed that more $CD94^{lo}$ NK clones are activated by strain AD169 infected targets and that more $CD94^{hi}$

NK clones were inhibited by strain AD169. Additionally, $CD94^{hi}$ polyclonal NK cell cultures were inhibited by AD169 infected targets in 2 out of 4 donors. A CD94/NKG2C antibody became commercially available near the end of this study and this has allowed us to gather preliminary data regarding NK2GA and NKG2C expression in PBMC of seropositive and seronegative donors and to dissect clonal responses to strain AD169 and AD169 ΔUL40 and a recombinant adenovirus expressing RAdUL40. These studies indicated that HCMV seropositive individuals have a higher frequency of NK cells expressing NKG2C and a lower frequency of NKG2A compared to seronegative donors whilst there was no difference in the cumulative frequency of $NKG2A^+$ and $NKG2C^+$ NK cells. Analysis of NK clones expressing NKG2A and/or NKG2C indicated that more $NKG2C^+$ were activated by strain AD169 than $NKG2A^+$ clones. The only $NKG2A^+$ clone that was able to lyse the control RAd60 target was inhibited by RAdUL40, whereas, no $NKG2C^+$ clones were activated by RAdUL40. Additionally 1/3 of the double negative clones was activated by strain AD169, inhibited by strain HCMV ΔUL40 and activated by RAdUL40. Thus the response of NK cells to HCMV gpUL40 does not appear to be a simple activation by $NKG2C^+$ clones and inhibition by $NKG2A^+$ clones.

The role of NKG2A in recognition of HCMV infected cells has been studied using polyclonal systems (Carr *et al.*, 2002, Tomasec *et al.*, 2000, Wang *et al.*, 2002). Interestingly there appear to be inconsistencies between the results. In the first study, fibroblasts infected with recombinant adenoviruses engineered to express either HLA-E or gpUL40, were used to show that gpUL40 stabilisation of HLA-E could confer substantial protection against lysis by NKL ($NKG2A^+$, $LIR1^+$) and that blocking this interaction with the anti-CD94 antibody DX22, rendered cells susceptible to NK lysis (Tomasec *et al.*, 2000). The relevance of this in the context of HCMV strain AD169 infection was later demonstrated by Wang *et al* (2002) who showed that strain AD169 inhibited lysis of the NK line DEL ($NKG2A^+$, $LIR1^+$) and this was reversed by blocking of CD94 or using strain AD169 ΔUL40 (Wang *et al.*, 2002). These

authors also demonstrated that in 2/10 assays, lysis of strain AD169 infected fibroblasts by polyclonal NK cells could be increased by more than 10% by blocking with anti-CD94. Furthermore, the authors showed that in 6/6 assays, there was no difference in the lysis of strain AD169 infected fibroblasts by polyclonal and CD94 depleted NK cells whilst in 5/6 experiments using polyclonal NK cells and 3/6 experiments using NK lines, strain AD169 ΔUL40 infected fibroblasts increased the lysis, suggesting that ligation of NKG2A was sufficient to provide protection from lysis against strain AD169 infected fibroblasts. In contrast, experiments by Carr *et al* (2002) suggested that ligation of HLA-E on HCMV infected cells by NKG2A does not contribute to enhanced resistance of HCMV infected cells to NK lysis (Carr *et al.*, 2002). In this experiment, polyclonal NK cell cultures from 2 donors were sorted into $NKG2A^{hi}$ and $NKG2A^{lo}$ populations and assessed for killing of strain AD169 infected and uninfected autologous fibroblasts in the presence and absence of the anti-CD94 antibody (DX22). For both donors $NKG2A^{hi}$ but not $NKG2A^{lo}$ NK cells were inhibited by strain AD169 infected autologous fibroblasts, whilst blocking of CD94 in donor 1 appeared to reduce the ability of $NKG2A^{hi}$ NK cells to lyse uninfected fibroblasts and in donor 2, blocking of CD94 increased the lysis of uninfected fibroblasts by $NKG2A^{lo}$ NK cells. This suggested that ligation of HLA-E on HCMV infected cells by NKG2A does not contribute to enhanced resistance of HCMV infected cells to NK lysis. A possible reason for differences in these results may be attributed to the systems used. Native HLA-E expression is low on fibroblasts, therefore in the study by Tomasec *et al* (2000) HLA-E expression was driven by recombinant adenovirus (Tomasec *et al.*, 2000). Since the assays performed by Carr *et al* (2002) were performed on native HLA-E expression, the different results may be attributable to differences in the expression levels of HLA-E and gpUL40 (Carr *et al.*, 2002). The differences between the observations of Carr *et al* (2002) and Wang *et al* (2002) however are harder to reconcile (Carr *et al.*, 2002, Wang *et al.*, 2002). gpUL40 is transcribed through early and late phases of HCMV infection, and is expressed more strongly at 52 hours than 24 hours post infection

(Tomasec *et al.*, 2000). It is therefore possible that the lower MOI (5 vs 10) and shorter time post infection (48 vs 72 hours) used by Carr *et al* (2002) may have contributed to the disparity between these two papers (Carr *et al.*, 2002). Alternatively, the low number of donors assayed by Carr *et al* (2002) may explain the differences in the two studies. In this respect, Wang *et al* (2002) performed each experiment in triplicate for 2 of 3 donors tested, the results of which were consistent in 2 of 3 of each experiment.

Falk *et al* (2002) studied the MHC class I and HLA-E expression of fibroblasts infected with HCMV mutants lacking the gpUL40 gene and/or the US2 – 11 region (Falk *et al.*, 2002). In this study, the authors showed that the MHC class I and HLA-E expression was equivalent in strain AD169 versus strain AD169 ΔUL40 and in strain AD169 ΔUS2 - 11ΔUL40 versus strain AD169 ΔUS2 – 11 infected fibroblasts, indicating that the down regulation of MHC class I was independent of gpUL40 and that gpUL40 was unable to maintain HLA-E surface expression in the context of HCMV infection. Furthermore, there was no difference in the susceptibility to lysis of strain AD169 and strain AD169 ΔUL40 infected fibroblasts by the $NKG2A^+$ NK line, NKL, whilst infection with ΔUS2 – 11 viruses conferred resistance to NKL mediated lysis. Blocking NKL cells with CD94 and NKG2A specific mAb resulted in increased lysis of uninfected human fibroblasts and HLA-E expressing K562, whilst blocking the $NKG2A^-$ B.3NK had no effect on lysis of target cells, indicating that NKL were dominantly regulated through CD94/NKG2A interactions. A number of factors may account for the discrepancies between this study and those by Tomasec *et al* (2000) and Wang *et al* (2002) (Tomasec *et al.*, 2000, Wang *et al.*, 2002). Firstly, the US2 – US11 deletion mutant lacked the whole set of genes that downregulate MHC class I expression. Therefore, the experiments by Falk *et al* (2002) were unable to discriminate to what extent individual US molecules may differentially influence HLA-E expression and target susceptibility to $NKG2A^+$ cells along the viral replication cycle. In this respect, a recent study has demonstrated that US6 downregulates

expression of all MHC class I molecules, whilst US2 and US11 preserved expression of HLA-E (Llano *et al.*, 2003). As these US genes are expressed in different amounts during the viral replication cycle and analysis of HLA-E by Falk *et al* (2002) was only carried out at 72 hours p.i, the possibility that HLA-E is expressed at earlier stages in the viral replication cycle cannot be ruled out. The studies by Tomasec *et al* (2000), Falk *et al* (2002) and Wang *et al* (2002) used the NKG2A$^+$ and LIR-1$^+$ NK line, NKL (Falk *et al.*, 2002, Tomasec *et al.*, 2000, Wang *et al.*, 2002). LIR-1 is an inhibitory receptor which broadly interacts with MHC class I molecules, therefore in the study by Falk *et al* (2002) and Wang *et al* (2002), which compared the responses of NKL to fibroblasts with depleted surface MHC class I, the inhibitory signal provided by LIR-1 would have been lost, thus complicating experiments in which infected fibroblasts became sensitive to NKL. In contrast, the adenoviral system used by Tomasec *et al* (2000) did not decrease MHC class I expression and specifically increased HLA-E expression, therefore the response of NKL to gpUL40 stabilised HLA-E was independent of a LIR-1 effect. Additionally, whereas in the study by Falk *et al* (2002), strain AD169 fibroblasts appeared to be relatively resistant to lysis by NKL, the study by Wang *et al* (2002) showed that strain AD169 infected fibroblasts were readily lysed by this line, suggesting that in each study the responsiveness of NKL varied. The study by Wang *et al* (2002), used a different strain of AD169 and infected cells at an MOI of 10 compared to an MOI of 2-4 used by Falk *et al* (2002). The possible genetic differences between strains and infection at different MOI may also contribute to the differences between the results of the 2 studies. Finally, the conclusions drawn by Falk *et al* (2002) regarding the ability of NKG2A$^+$ NK cells to lyse HCMV infected targets were based on a partially HLA-matched system, whilst the conclusions drawn by Wang *et al* (2002) were drawn from an allogeneic (NKL) and an autologous system. Considering the complexity and incomplete understanding of how NK activation is controlled, an autologous system is preferred when investigating NK cell responses.

Analysis of a large numbers of NK clones has allowed us to dissect NK responses in the context of their receptor expression. NK clone responses to HCMV infected autologous fibroblasts can most simply be categorised into 4 groups with respect to uninfected and AD169 infected autologous fibroblasts; inhibition, activation, kill/no change and no kill/no change. The total frequency of autologous NK clones which were activated by strain AD169 was 18% compared to 21% of clones which were inhibited by strain AD169, despite reduction of MHC class I expression of > 70% in HCMV infected cells. The remaining clones did not differentiate between infected and uninfected targets as there was no difference between the lysis of these target cells. These data show that human hosts can make NK cell responses to autologous uninfected and HCMV infected fibroblasts and that the virus is capable of activating and inhibiting NK cell responses, thus indicating that NK cell activation in the context of HCMV infection is not dominated by the down regulation of HLA class I. Segregation of the NK clones into $CD94^{hi}$ and $CD94^{lo}$ expressing cells indicated that more $CD94^{hi}$ clones were inhibited by strain AD169 (21/85 25%) compared to those being activated (9/85 11%). In contrast, more $CD94^{lo}$ clones were activated by strain AD169 (11/25 44%) and than were inhibited (2/25 8%) and these results were supported by analysis of polyclonal NK cell reponses. Additionally, the response of 9 $NKG2C^{+}$ and 5 $NKG2A^{+}$ NK clones to strain AD169, strain AD169 ΔUL40 and RAdUL40 was investigated. These data indicated that $NKG2C^{+}$ and NKG2A/C double negative clones can be activated by AD169. As the $NKG2A^{+}$ NK clones were unable to lyse uninfected fibroblasts it was not possible to ascertain whether NKG2A expression could inhibit NK responses to AD169. The role of gpUL40 in this response however remains unclear as activation by AD169 does not always correlate with an inhibition by strain AD169 ΔUL40, and an activation by RAdUL40. Concomitantly, inhibition by RAdUL40 did not correlate with inhibition by strain AD169 and activation by strain AD169 ΔUL40. Nevertheless, this data support the findings of previous investigators who used polyclonal NK cells and NKL to show that ligation of gpUL40 stabilised HLA-E by CD94 is able to overcome the

activating signal brought about by the downregulation of MHC class I by HCMV (Tomasec *et al.*, 2000, Wang *et al.*, 2002). However, these data appear to contrast with a number of other publications (Carr *et al.*, 2002, Falk *et al.*, 2002). Whilst Carr *et al* (2002) agree that the ability of clones to lyse strain AD169 infected fibroblasts ranged broadly, they found no correlation with the NK response and expression of inhibitory KIR, LIR-1, CD94/NKG2A or with the number of different inhibitory KIR expressed per clone (Carr *et al.*, 2002). However they did find that within their group of clones that preferentially killed strain AD169 infected cells there was a lower frequency of expression of the CD94/NKG2A receptor than within the group of clones that preferentially killed uninfected cells. Several factors are likely to account for the differences in these conclusions. The study by Carr *et al* (2002) categorised 36 clones from 2 donors according to the percentage difference in lysis of uninfected and strain AD169 infected autologous cells by subtracting the percent specific lysis of strain AD169 infected targets from the percent specific lysis of uninfected targets. Clones that could preferentially kill strain AD169 infected targets therefore had a positive percentage difference and clones which preferentially killed uninfected cells had a negative percentage difference. Using this method Carr *et al* (2002) identified 15 clones that preferentially killed strain AD169 infected fibroblasts and 17 clones that preferentially killed uninfected fibroblasts. However, when analysed by the criteria used for analysis in this thesis, 3/36 (8%) of clones were activated by strain AD169 and 6/36 (16%) were inhibited. Interestingly, all of these clones were $NKG2A^+$, suggesting that the inhibitory signal is not always dominant. In contrast to this data the $NKG2A^+$ clones analysed in this thesis were generally unresponsive and 2/9 of the $NKG2C^+$ clones were activated by strain AD169 infected fibroblasts. These data indicate that the factors governing NK cell responses are complex. It is therefore unfortunate that at the time of the study by Carr *et al* (2002) the NKG2C antibody was not available.

Of note is the identification of a number of alleles for HLA-E. Two of these

alleles, HLA-E*0101 (HLA-E^R) and HLA-E*0103 (HLA-E^G) which differ in the amino acid at position 107 were recently investigated. Interestingly, HLA-E^G has been shown to be insensitive to US6 in transfected K562 cells, whilst HLA-E expression was downregulated by US6 in the HLA-E heterozygous cell line RPMI 8866, suggesting that the HLA-E^R, but not the HLA-E^G is degraded by US6 (Llano *et al.*, 2003, Ulbrecht *et al.*, 2003). Furthermore, whilst the steady state protein levels of the 2 alleles was shown to be similar in B-cells, the cell surface expression of HLA-E^R was lower than that for HLA-E^G and this correlated with differences in thermal stabilities and stability of HLA-E coupled to peptide (Strong *et al.*, 2003). Therefore an individuals HLA-E haplotype may alter the susceptibility of HCMV infected cells to $NKG2C^+$ and $NKG2A^+$ NK cell lysis. In this respect, it is interesting that the HLA-E^R allele was used in the studies by Tomasec *et al* (2000) and Falk *et al* (2002) whilst the HLA-E haplotype of fibroblasts used in the study by Wang *et al* (2002) were not defined. Whether these 2 alleles interact differently with $NKG2A^+$ NK cells has not been investigated, however, a number of studies have indicated that the peptide loaded into HLA-E alters the affinity of HLA-E for NKG2A and NKG2C molecules (Wada *et al.*, 2004, Vales-Gomez *et al.*, 1999, Llano *et al.*, 1998). Surface plasmon resonance showed that both NKG2A and NKG2C proteins bound to HLA-E^G molecules that were assembled with HLA-B7, -B58, -Cw3, -Cw4, -Cw7 and HLA-G leader sequence derived peptide nonamers. Interestingly, NKG2C bound to HLA-E-peptide complexes with a much lower affinity than NKG2A, indicating that if NKG2A and NKG2C were to compete for the ligand, the inhibitory receptor is more likely to bind. Importantly, this study and a functional study which expressed HLA-E in 721.221 cells have suggested that only HLA-E assembled with HLA-G peptide leader sequence, VMAPRTLFL would be sufficient to trigger a response by an $NKG2C^+$ NK cell whilst the leader sequence derived from HLA-Cw3 VMAPRTLIL, which is the same as the gpUL40 sequence, would be insufficient to trigger $NKG2C^+$ NK cells. In contrast, HLA-G VMAPRTLFL and HLA-Cw3 VMAPRTLIL were estimated to be sufficient to trigger an inhibitory signal through $NKG2A^+$ (Llano *et al.*,

1998, Vales-Gomez *et al.*, 1999). Interestingly, the surface expression of HLA-E assembled with the HLA-G peptide did not differ from HLA-E assembled with other nonamers, suggesting that activation of NKG2C by HLA-E assembled with HLA-G was likely to be a consequence of increased affinity (Llano *et al.*, 1998). These data were later supported by a study which determined the region involved in the interaction between NKG2A and NKG2C with HLA-E (Wada *et al.*, 2004). In this study, a series of alanine substitution mutants within the α1, α2 and α3 domain of HLA-E^G were investigated for their ability to bind to C1498 mouse lymphoma cells transfected with human CD94 together with human NKG2A or NKG2C. Using this method, the authors concluded that NKG2A and NKG2C bound to the top of the α1/α2 domain of HLA-E. Interestingly, mutation of aspartic acid residue 69 or histidine residue 155 to alanine significantly impaired HLA-E binding to NKG2A but had a minimal effect on NKG2C. As these 2 residues are located in the middle of the α1 and α2 helices which sandwich the HLA-E bound peptide, the authors raised the possibility that HLA-E loaded with some peptides may have the capacity to selectively engage NKG2C but not NKG2A.

Three $CD94^{hi}$ NK clones were identified that were NKG2A/NKG2C double negative. CD94, can be expressed on the cell surface in the absence of NKG2 proteins, whereas in the absence of CD94, NKG2A proteins are retained in the ER and do not reach the cell surface (Brooks *et al.*, 1997). Therefore these $CD94^+$ clones may be NKG2 negative or CD94 may be expressed on their surface as a heterodimer with an NKG2 chain other than NKG2A or NKG2C. Interestingly, 1 of these NKG2A/NKG2C double negative clones was activated by strain AD169, inhibited by strain AD169 ΔUL40 and activated by RAdUL40, suggesting that this clone is activated by gpUL40. Activation of this NKG2A/NKG2C double negative clone by gpUL40 could suggest that there is a ligand for gpUL40, either in the context of CD94/NKG2 or through a novel mechanism involving the gpUL40 protein. As the NKG2C and NKG2E genes are highly homologous across the

carbohydrate recognition domain (92% nucleotide and 90% amino acid identity) where ligand interaction occur, it is possible that this clone was activated by binding of NKG2E to gpUL40 stabilised HLA-E (Miyashita *et al.*, 2004). This is further supported by D7 polyclonal and clone data. D9 clone data was unique, and showed that 9/13 (69%) of $CD94^{hi}$ and 7/12 (58%) of $CD94^{lo}$ NK clones were activated by AD169, whilst only 1 clone ($CD94^{lo}$) was inhibited by strain AD169. This pattern was also seen in polyclonal NK cells cultured from this donor. In light of the flow cytometric data which showed that in this donor, the frequency of NKG2C (4%) and of NKG2A (41%) was similar to HCMV seronegative individuals, this would suggest that the NK clone response of donor D7 to strain AD169 is controlled by a mechanism other than interaction of CD94/NKG2C with gpUL40 stabilised HLA-E.

Together these data suggest that $NKG2A^+$ NK cells are inhibited by HLA-E loaded with leader sequences derived from gpUL40, whilst $NKG2C^+$ NK cells may be unaffected by gpUL40. This leaves the question "why do we see an expansion of $NKG2C^+$ NK cells in the blood of HCMV seropositive individuals?" unanswered. An alternative explanation from that investigated here may be found in previous work showing that leader sequences derived from HLA-G are sufficient to stimulate $NKG2C^+$ clones. HLA-G is a non-classical MHC class I molecule which is found primarily on human trophoblasts and a subpopulation of thymic epithelial cells (Le Bouteiller *et al.*, 1999). Expression of HLA-G in this context has been implicated in the inhibition of the cellular immune response through the recognition of LIR-1, LIR-2 and KIR2DL4. Additionally, translation of HLA-G has been shown to occur in a number of tumour cells, and has been associated with protection from NK and CTL mediated lysis. More recently, HCMV reactivation has been shown to induce translation of HLA-G in macrophages and dendritic cells and has been suggested to help these cells escape the cellular immune response and aid in viral dissemination (Onno *et al.*, 2000). The ability of the US2 – US11 genes to downregulate HLA-G remains unclear and has not been investigated in macrophages or dendritic cells. It is therefore possible

that NKG2C$^+$ NK cells are expanded in HCMV seropositive individuals in response to HLA-E loaded with HLA-G peptides on the surface of macrophages and dendritic cells.

There is no literature regarding the acquisition of NKG2A and/or NKG2C expression on NK cells. However 1 study showed that the frequency of NKG2A$^+$ NK cells and their median level of surface expression is stable and unaffected by infection over a period of a year (Shilling *et al.*, 2002). This suggests that the alteration in NKG2A and NKG2C expression levels by HCMV is the result of a major and persistent influence over the immune system. The expansion of NKG2C$^+$ cells in HCMV seropositive individuals has also recently been reported by another group (Guma *et al.*, 2004). However, in contrast to this study they did not identify a concurrent decrease in NKG2A expression, nor have they presented data regarding the functional significance of this expansion. If it is assumed that NKG2C$^+$ clones are expanded because they are able to respond strain AD169 (either through gpUL40 stabilised HLA-E or a novel mechanism), examination of the polyclonal and clone data suggests that the change in frequency of NKG2A and NKG2C can occur over a relatively short period of time. In this respect, the original clone data for D9 indicated that CD94^{hi} clones were either inhibited or unable to respond to strain AD169 infected fibroblasts, whilst a second cloning experiment 1 year later showed that none of the clones were inhibited and 3/18 were activated by strain AD169. The NKG2A and NKG2C expression levels on the first batch of clones is unknown, however in the second batch 2/3 of the activated cells were NKG2C$^+$ and none of them were NKG2A$^+$. This would suggest that in the year between the first and second batch of NK cloning, donor D9's NKG2C$^+$ NK cells were expanded. The change in polyclonal NK responses over time also supports a role for NKG2C cells being expanded with time. Additionally, flow cytometry data shows, from the time of the second batch of cloning that 25% of D9's NK cells were NKG2A$^+$ and 27% were NKG2C$^+$ which differs greatly from the mean frequency in HCMV seronegative donors (43% and 4%, respectively).

However, considering approximately equal proportions of D9's NK cells expressed NKG2A as expressed NKG2C, it is important to note that a disproportionate number of clones were NKG2C$^+$ (50% 9/18) compared to NKG2A$^+$ (28% 5/18), indicating that this cloning protocol may preferentially expand NKG2C$^+$ clones. Alternatively, the difference in frequency of NKG2A$^+$ and NKG2C$^+$ clones may reflect the small number of clones cultured.

The cumulative frequency of NKG2A and NKG2C positive NK cells is the same in HCMV seropositive and seronegative individuals, suggesting 2 possible mechanisms for the expansion of NKG2C$^+$ cells in HCMV seropositive individuals; NKG2A$^+$ cells could be deleted and replaced with NKG2C$^+$ cells or NKG2A$^+$ cells could develop into NKG2C$^+$ cells and lose expression of NKG2A. The data presented here is currently unable to reconcile this question. However, flow cytometric analysis of PBMC indicates that some NK cells are able to express both NKG2A and NKG2C. Monitoring the frequency of these cells with time may give some indication of the role of these cells *in vivo* – whether they are part of a homeostatic mechanism for maintaining the frequency of NKG2A and NKG2C positive cells or whether they have a functional role for themselves. As it is possible to clone double positive NK cells, it would be interesting to monitor whether this is a stable phenotype or reflects an activation state. As there have been no reports regarding NKG2A/NKG2C heterodimers and CD94 has only been found as a heterodimer with 1 NKG2 chain, the exact nature of this clone is unknown. Immunoprecipitation experiments could give us some indication of the interactions of NKG2A and NKG2C in this clone however, the low number of cells cultured for this clone meant that only a single cytotoxicity assay could be performed.

8.4.1.1 Concluding Remarks

Data presented here demonstrated that more CD94^{hi} clones were inhibited by AD169 (21/85 25%) compared to those being activated (9/85 11%) whilst,

more CD94^{lo} clones were activated by AD169 (11/25 44%) than were inhibited (2/25 8%). These results were supported by polyclonal NK cell data. With the availability of an NKG2C antibody, the response of NK clones to AD169 in the context of NKG2A and NKG2C expression was investigated. This data showed that 2/9 NKG2C clones were activated by AD169 whilst no NKG2A^{lo} clones responded to strain AD169. However, as only 1 of the NKG2A^{+} clones was capable of lysing uninfected fibroblasts it was not possible to assess whether strain AD169 was capable of inhibiting lysis by NKG2A^{+} clones. The NK clone responses to AD169, AD169 ΔUL40 and RAdUL40 indicated that the response of NK clones to gpUL40 was not always inhibition by NKG2A^{+} cells and activation NKG2C^{+} cells. Furthermore, evidence in the literature suggests that ligation of NKG2C by HLA-E stabilised by the gpUL40 nonamer may not be sufficient to activate NK cell lysis whereas the HLA-G leader sequence maybe. As such, the observed expansion of NKG2C^{+} cells in HCMV seropositive individuals may be a result of persistent reactivation of HCMV in macrophages and monocytes which has been shown to upregulate the translation of HLA-G in these cells.

The mechanism by which frequency of NKG2C NK cells are expanded and NKG2A NK cells are decreased remains unanswered. As the cumulative frequency of NKG2A and NKG2C cells in the peripheral blood of HCMV seropositive and seronegative individuals is maintained it is possible that NKG2A^{+} cells could be deleted and replaced with NKG2C^{+} cells or NKG2A^{+} cells could develop into NKG2C^{+} cells and lose expression of NKG2A. This remains to be resolved.

8.4.2 NK Responses to Strain Towne

Sequencing of clinical isolates and laboratory strains of HCMV has revealed that *in vitro* tissue culture has resulted in large genetic differences between different laboratory strains of HCMV and with wild type strains of HCMV. The most notable difference in laboratory strains is a 15-kb and a 13-kb deletion in the U_L/b' region in strains AD169 and Towne, respectively (Cha *et al.*, 1996). Analysis of immune responses to these genetically different strains of HCMV provides a tool for identification of genes encoding immunomodulatory function. The capability of $CD94^{hi}$ and $CD94^{lo}$ NK clones to lyse strain Towne infected fibroblasts compared to their ability to lyse strain AD169 infected fibroblasts was investigated, and showed that more $CD94^{lo}$ NK clones were inhibited by strain Towne. In contrast, the polyclonal NK cell responses to strain AD169 and strain Towne infected fibroblasts suggest that strain Towne is capable of inhibiting $CD94^{lo}$ and activating $CD94^{hi}$ NK cells and that the response may be dependent upon the donor. This was reflected in the NK clone data when clone responses were assigned to individual donors. For example, the clone data showed that both D9 $CD94^{hi}$ and $CD94^{lo}$ NK cells were inhibited by strain Towne, whilst equal proportions of D7 $CD94^{hi}$ and $CD94^{lo}$ NK cells were activated and inhibited by strain Towne infected fibroblasts. This would suggest that the NK function encoded by strain Towne does not target a principal NK regulatory pathway.

The NK modulatory function encoded by HCMV strain Towne but not strain AD169 may be encoded within the extra 2-kb found in strain Towne but not in strain AD169. Alternatively, a mutation in one of the genes found in both viruses may have provided a gene found in strain Towne with a gain of function. The 2-kb region found in strain Towne but not in AD169 encodes 4 genes, UL146, UL147, UL147A and UL148. There has been no published work regarding the proteins encoded by ORFs UL147A and UL148 however, both of these proteins have been predicted to encode a hydrophobic amino acid region, therefore suggesting that these proteins are membrane resident. The products of UL146 and UL147 in Toledo exhibit size and limited

sequence similarity to α (CXC) chemokines and have been designated vCXC-1 and vCXC-2, respectively (Penfold *et al.*, 1999). The sequence homology between UL146 and UL147 in strain Toledo and strain Towne suggests that the function of these proteins is preserved in the Towne strain of HCMV. Both vCXC-1 and vCXC-2 have sequence motifs reminiscent of IL8, including putative signal peptides, cysteine spacing and in the case of vCXC-1, an ELR-CXC motif that is know to be important in receptor binding (Penfold *et al.*, 1999). The function of vCXC-1 was investigated further and it was shown to induce migration, cytoplasmic calcium mobilisation, and degranulation of neutrophils with a similar efficacy to IL8. Furthermore, treatment of neutrophils with vCXC-1 prevented other ELR-CXC chemokines (NAP-2, gro-α, gro-β, gro-γ and ENA-78) to induce a second calcium flux, whilst IL8 and to a lesser extent GCP-2 were able to induce a second calcium flux. The authors also demonstrated that vCXC-1 was able to bind to CXCR2 (receptor for all ELR-CXC chemokines) but not to CXCR1 (predominantly an IL8 receptor). These data therefore suggest that vCXCR-1 is a functional CXC chemokine. There have been no published studies with regard to the impact of vCXCR-1 on NK cell function. However, the CXC chemokine receptors, CXCR1 and CXCR2 have been identified on the surface of NK cells by a number of groups and NK cells have been reported to migrate in response to IL8 (Campbell *et al.*, 2001, Chuntharapai *et al.*, 1994, Morohashi *et al.*, 1995, Sebok *et al.*, 1993, Taub *et al.*, 1995). Interestingly, the study by Campbell *et al* (2001) showed that $CD16^+$ NK cells expressed CXCR1 and CXCR2, and were strongly attracted by IL8, whilst $CD16^-$ NK cells did not express CXCR1 or CXCR2 and were less responsive to IL8 (Campbell *et al.*, 2001). The CC chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) and the CXC chemokines CXCL10 (ICP10) and CX3CL1 (fractalkine) have all been implicated in activating NK cell cytotoxicity (Yoneda *et al.*, 2000). The only study investigating the effects of CXC chemokines on NK cell cytolysis used IL8 (Taub *et al.*, 1995). This study found that IL8 failed to enhance NK killing of K562 and was only capable of inducing degranulation of NK cells at high

concentrations (100 ng/ml) in selected donors (Taub *et al.*, 1995). The ability of vCXC-1 to prevent other ELR-CXC chemokines (NAP-2, gro-α, gro-β, gro-γ and ENA-78) to induce a second calcium flux would suggest that if any of these were capable of enhancing NK cytotoxicity, binding of vCXC-1 would inhibit this action. Thus providing a model by which vCXC-1 could inhibit NK cell lysis of HCMV infected fibroblasts. Therefore, $CD94^{lo}$ NK cells may be inhibited by strain Towne, because binding of vCXC-1 to $CD94^{lo}$ NK cells in preference to $CD94^{hi}$ NK cells could prevent activation of $CD94^{lo}$ NK cells by blocking the CXCR2 receptor. This would require that $CD94^{lo}$ but not $CD94^{hi}$ NK cells express the CXCR2 receptor. In this respect, it is interesting that CXCR2 is expressed on $CD16^{+}$ but not $CD16^{-}$ NK cells and $CD16^{+}$ NK cells have been shown to express lower levels of CD94 (Campbell *et al.*, 2001). Thus vCXC-1 may have a 2 functions in HCMV infection; (1) to attract neutrophils to the site of infection enhancing viral dissemination and (2) to block the activation of $CD94^{lo}$ NK cells by binding to CXCR2 on their surface and preventing binding of NK stimulatory CXC chemokines. Unfortunately, the authors did not characterise vCXC-2 and no further studies regarding this protein have been published. It is therefore difficult to attribute a role for this putative CXC chemokine in the modulation of NK cell activity.

Analysis of polyclonal NK cell responses to strain Towne showed that in only 1/5 assays $CD94^{lo}$ NK cells which were able to lyse strain AD169 infected fibroblasts at greater than 10% were inhibited by strain Towne. In contrast 3/6 assays indicated that total NK cells were activated by strain Towne when $CD94^{lo}$ NK cells showed no difference in the ability to lyse these targets. This suggests that $CD94^{hi}$ NK cells are activated by strain Towne in these donors. The polyclonal NK cells used in these assays are isolated from PBMC and stimulated overnight in tissue culture with IFNα prior to being used in cytoxicity assays. Therefore in contrast to the NK clones they have been minimally manipulated through tissue culture. This difference in manipulation may explain the disparity between the polyclonal and NK clone results. In this respect the mean frequency of $CD94^{hi}$ NK cells in donor

PBMC was 57% whilst 85% of the NK clones were CD94^{hi}. This suggests that the NK cloning method may support the culture of particular subsets of NK cells.

8.4.2.1 Concluding Remarks

Polyclonal and NK clone responses to strain Towne compared to strain AD169 infected fibroblasts suggests that strain Towne encodes an immunomodulatory function that is not found in strain AD169. The extra 2 kb found in the U_L/b' region of strain Towne and not in strain AD169 potentially encodes this function, however gain of function mutation in gene in strain must also be considered. Of the 4 genes encoded by the 2-kb found in strain Towne, only UL146 and UL147 have been studied to date, and this has indicated that these protein are CXC chemokines. The chemokines have been designated vCXC-1 and vCXC-2, respectively. vCXC-1 was further characterised and was shown to have a potent IL8 like activity and bind to the CXCR2 receptor. As this receptor has been shown to be expressed on NK cells, vCXC-1 may be implicated in inhibiting activation of NK cells by preventing calcium mobilisation by other CXC chemokines. The potential roles of UL147A and UL148 in modulating NK cell responses remain unclear as there is no published data regarding the protein products encoded by these ORFs. However, the proteins encoded by these ORFs have a hydrophobic region, suggesting they are transmembrane proteins. The disparity between responses of cloned NK cells and polyclonal NK cells may be a consequence of differences in the culture of the cells, the number of clones analysed or donors used in the study.

The 4 genes found in strain Towne but not in strain AD169 have been cloned into the pShuttle CMV vector pAL449, which will allow us to make replication deficient adenovirus vectors using the AdEasy adenoviral vector system. These viruses will be used to screen for potential immunomodulatory functions using polyclonal NK cells and NK clones.

8.4.3 NK Responses to Strain Toledo

Previous work in the laboratory has demonstrated that autologous fibroblasts infected with the Toledo strain of HCMV reproducibly inhibited lysis of 3 different donor's polyclonal NK cells compared to the lysis of strain AD169 infected fibroblasts. This suggested that the extra 15-kb at the right end of the unique long region in Toledo encoded a gene or a series of genes which were capable of inhibiting a principle target of the NK regulatory pathway. In this study 50% (5/10) clones capable of lysing strain AD169 infected autologous targets were inhibited by strain Toledo. In a systematic search of the U_L/b', Dr P Tomasec identified the product of the HCMV gene UL141, encoded in this region, as mediating at least some of this protection from polyclonal NK cell lysis. Analysis of NK clone responses to 2 different replication deficient adenovirus vectors (RAdUL141 and RAdUL141-GFP), showed that gpUL141 was able to inhibit up to 71% (27/38) of NK clones. This effect was independent of CD94 expression, indicating that gpUL141 was not functioning through stabilisation of HLA-E. In order to identify a possible mechanism for the inhibitory action of gpUL141, Dr P Tomasec undertook a large scale screening programme of a broad range of NK cell receptor ligands by flow cytometry on gpUL141 transfected, and RAdUL141 or HCMV infected cell lines (Tomasec *et al.*, 2005). This study revealed that the NK cell activating receptor, CD155 was down regulated on cells infected with the HCMV strains Toledo and TB40E-Lisa, which encode gpUL141, whereas cells infected with strain AD169 or TB40E-Bart (both negative for UL141) upregulated CD155. Furthermore, infection with RAdUL141 or transfection of cells with a UL141 expressing plasmid downregulated CD155, indicating that the expression of gpUL141 was sufficient and necessary for the downregulation of CD155. The mechanism by which gpUL141 was able to downregulate CD155 remains unclear. However, the study by Tomesec *et al.*, (2005) suggests that gpUL141 prevents the maturation and cell surface expression of CD155.

CD155 is a ligand for the activating receptor CD226 (DNAM-1), which has

been reported to be expressed on almost all NK cells. Recently, interaction between $CD226^+$ NK cells with CD155 has been shown to enhance the lysis of a number of tumour cells (Castriconi *et al.*, 2004, Pende *et al.*, 2005a, Pende *et al.*, 2005b). CD112 (nectin-2) is another ligand for CD155, and has been implicated in the activation of NK cell lysis of myeloid leukemias and the melanoma cell line, FO-1 (Pende *et al.*, 2005a, Pende *et al.*, 2005b). Surface expression of CD112 was not affected by gpUL141 in fibroblasts or 293 HEK cells (Tomasec *et al.*, 2005). In normal tissues CD155 binds to proteins involved in cell-matrix adhesion (vitronectin and Tctex-1) and cell-cell junctions (nectin-3) (Lange *et al.*, 2001, Mueller *et al.*, 2002, Mueller & Wimmer, 2003). In virus infected and tumour cells, cell-cell and cell-matrix interactions become disrupted potentially making CD155 available to interact with CD226 resulting in NK cell activation and cytolysis. The importance of CD155 downregulation by HCMV in evading NK cell lysis has been highlighted by studies of tumour cells. In this respect, a recent study has shown that most tumour target cells expressing CD155 and CD112 have reduced expression of MHC class I molecules, suggesting that in the absence of effective MHC class I:NK cell inhibitory receptor interaction, engagement of these receptors by CD226 is sufficient to stimulate NK cell lysis (Pende *et al.*, 2005a). As CD155 has been associated with cell-cell interactions, Tomasec *et al.,* (2005) tested whether gpUL141 affected adhesion between NK cells and their targets but were able to detect only marginal differences (Tomasec *et al.*, 2005). This suggests that the primary role of gpUL141 is mediated by impeding signaling through the activating receptor CD226, rather than by reducing adhesion between NK cells and their targets.

The function of CD226 has been shown to be dependent upon the formation of a complex with LFA-1. Cross linking of LFA-1 has been shown to augment tyrosine phosphorylation of Fyn, which in turn is likely to phosphorylate CD226, initiating $CD226^+$ NK cell cytotoxic function (Shibuya *et al.*, 1999). It is well established that the LFA-1 ligand, ICAM-1 is

upregulated during HCMV infection, thus implying that HCMV infection has the potential to activate lysis by $CD226^+$ NK cells (Ito *et al.*, 1995, Sedmak *et al.*, 1994). However, whether or not CD226 mediated cytotoxicity essentially requires LFA-1 ligation remains unclear, and current studies have suggested that it may depend on the activation status of the NK cell (Tahara-Hanaoka *et al.*, 2004).

A second gene, UL142 found in this region has been shown to encode a protein with homology to MHC class I (personal communication with Mark Wills and presented at conferences throughout 2005). Fibroblasts infected with a UL142 HCMV deletion mutant were shown to be more susceptible to polyclonal NK lysis. Furthermore, expression of UL142 using a recombinant adenovirus inhibited NK clone lysis at a frequency of 1/10 to 1/15 in an autologous system.

8.4.3.1 Concluding Remarks

These results suggest that the extra 15-kb found in strain Toledo but not in strain AD169 may encode a number of proteins that are involved in modulating the NK cell response to HCMV. One of these proteins, gpUL141 is a potent inhibitor of NK cell cytotoxicity. This inhibition is mediated through the downregulation of surface CD155 expression, a ligand for the NK cell activating receptor CD226. The mechanism by which CD155 is down regulated by gpUL141 is not completely understood, however it appears that gpUL141 prevents CD155 from maturing and reaching the cell surface. Elucidation of the mechanism used by gpUL141 to abrogate the maturation of CD155 should therefore be a subject for future study. CD226 is also expressed on T cells, monocytes, megakaryocytes and B cell subsets. It would therefore be interesting to investigate the effects of gpUL141 on these cell subsets. In light of the important role of T cells in control of HCMV infection it would be especially interesting to study the effects of gpUL141 on T cell cytolysis.

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9 REFERENCES

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APPENDIX

Appendix I D7 and D9 NK Clone Proliferation

D7 Plate 1	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm) *										Control†	Mean Control‡
Test Well Values§	6686	66	23	112	60	92	61	160	25	106	19	
	65	409	105	31	30	74	169	66	183	309	81	
	54	58	45	470	100	75	687	203288	4849	560	125	
	309	31	52	20	97	110628	163	226	2525	277	280	
	93	60	88	46	133	102	28	10	268	378	78	
	72	266	368	47	13	19	61	289	72	191	56	79
Test Well Value/4x (Mean Control Value)	6372	-248	-291	-202	-254	-222	-253	-154	-289	-208		
	-249	95	-209	-283	-284	-240	-145	-248	-131	-5		
	-260	-256	-269	156	-214	-239	373	202974	4535	246		
	-5	-283	-262	-294	-217	110314	-151	-88	2211	-37		
	-221	-254	-226	-268	-181	-212	-286	-304	-46	64		
	-242	-48	54	-267	-301	-295	-253	-25	-242	-123		

(*) ^{3}H Thymdine Incorporation values
(†) Control wells contained feeder cells only
(‡) Geometric mean of control wells
(§) Test wells containing a single NK clone and feeder cells

SCGM-OKT3

D7 Plate 1	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	6686	66	23	112	60	92	61	160	25	106	19	
	65	409	105	31	30	74	169	66	183	309	81	
	54	58	45	470	100	75	687	203288	4849	560	125	
	309	31	52	20	97	110628	163	226	2525	277	280	
	93	60	88	46	133	102	28	10	268	378	78	
	72	266	368	47	13	19	61	289	72	191	56	79
Test Well Value/4x (Mean Control Value)	6372	-248	-291	-202	-254	-222	-253	-154	-289	-208		
	-249	95	-209	-283	-284	-240	-145	-248	-131	-5		
	-260	-256	-269	156	-214	-239	373	202974	4535	246		
	-5	-283	-262	-294	-217	110314	-151	-88	2211	-37		
	-221	-254	-226	-268	-181	-212	-286	-304	-46	64		
	-242	-48	54	-267	-301	-295	-253	-25	-242	-123		
D7 Plate 2												
Test Well Values	93	11	49	41	252	64	20	34	138	31	72	
	87	10	44	13	23	28	25	127	72	36	54	
	12	223	31	34	41	64	90	94	163	31	21	
	51	36	30	15	152	199	46	118	22	84	52	
	12	335	25	18	30	35	30	224	66	48	134	
	23	114	21	56	57	39	142	42	148	88	70	58
Test Well Value/4x (Mean Control Value)	-141	-223	-185	-193	18	-170	-214	-200	-96	-203		
	-147	-224	-190	-221	-211	-206	-209	-107	-162	-198		
	-222	-11	-203	-200	-193	-170	-144	-140	-71	-203		
	-183	-198	-204	-219	-82	-35	-188	-116	-212	-150		
	-222	101	-209	-216	-204	-199	-204	-10	-168	-186		
	-211	-120	-213	-178	-177	-195	-92	-192	-86	-146		

D7 Plate 3	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	111	19	11	90	115	188276	224	154	22	39	211	
	111	358	135	57	13	41	27	20	18	33	11	
	101	27	35	39	148	111	114034	96	54	54	44	
	17	386	236	91709	65	17802	118	111	72	22	90	
	28	34	57	230	157	21	35	101	98	397	42	
	51	21	79	108	94	46	21	27	143	222440	228	66
Test Well Value/4x (Mean Control Value)	-156	-248	-256	-177	-152	188009	-43	-113	-245	-228		
	-156	91	-132	-210	-254	-226	-240	-247	-249	-234		
	-166	-240	-232	-228	-119	-156	113767	-171	-213	-213		
	-250	119	-31	91442	-202	17535	-149	-156	-195	-245		
	-239	-233	-210	-37	-110	-246	-232	-166	-169	130		
	-216	-246	-188	-159	-173	-221	-246	-240	-124	222173		

SCGM+OKT3

D7 Plate 1	SCGM+OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	19	27	44	16	54	89	20	38	27	16	72	
	15	38	32	26	23	15	12	22	179	31	17	
	49	19	21	47	88	57	107	58	37	29	27	
	33	44	10	18	39	82	24	65	45	14		
	82	77	18	29	321	36	21	80	20	10		
	53	12	28	17	26	10	65	17	13	19	35	33
Test Well Value/4x (Mean Control Value)	-112	-104	-87	-115	-77	-42	-111	-93	-104	-115		
	-116	-93	-99	-105	-108	-116	-119	-109	48	-100		
	-82	-112	-110	-84	-43	-74	-24	-73	-94	-102		
	-98	-87	-121	-113	-92	-49	-107	-66	-86	-117		
	-49	-54	-113	-102	190	-95	-110	-51	-111	-121		
	-78	-119	-103	-114	-105	-121	-66	-114	-118	-112		

D7 Plate 2	SCGM+OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	27	1873	364	930	2308	2699	147377	1133	1996	1148	2479	
	2155	33396	580	5835	225127	135006	2251	863	1961	1839	1705	
	1133	1299	536	1610	2220	53470	110537	1694	1407	181164	1591	
	1286	545	1424	973	831	1306	645	180970	1108	1771	1307	
	2120	1157	1704	819	745	1992	743	27313	493	27452	1323	
	1948	2441	1403	6261	1488	704	3215	723	14087	2764	2809	1189
Test Well Value/4x (Mean Control Value)	-5380	-5279	-6788	-6222	-4844	-4453	140225	-6019	-5156	-6004		
	-4997	26244	-6572	-1317	217975	127854	-4901	-6289	-5191	-5313		
	-6019	-5853	-6616	-5542	-4932	46318	103385	-5458	-5745	174012		
	-5866	-6607	-5728	-6179	-6321	-5846	-6507	173818	-6044	-5381		
	-5032	-5995	-5448	-6333	-6407	-5160	-6409	20161	-6659	20300		
	-5204	-4711	-5749	-891	-5664	-6448	-3937	-6429	6935	-4388		
D7 Plate 3												
Test Well Values	3305	108214	781	1328	2621	185841	6380	347174	3847	2102	1981	
	1100	2099	1117	3086	1963	1673	1520	864	2800	90964	3193	
	2428	15615	2260	368838	124349	2232	2688	2846	1203	1635	1590	
	2165	2001	1003	2039	60713	5348	3271	2033	981	1798	2899	
	61102	1287	155395	745	2605	2302	292830	2557	47303	2090	2446	
	4941	949	2529	2533	997	1273	104967	2077	213889	847	2087	2302
Test Well Value/4x (Mean Control Value)	-5903	99006	-8427	-7880	-6587	176633	-2828	337966	-5361	-7106		
	-8108	-7109	-8091	-6122	-7245	-7535	-7688	-8344	-6408	81756		
	-6780	6407	-6948	359630	115141	-6976	-6520	-6362	-8005	-7573		
	-7043	-7207	-8205	-7169	51505	-3860	-5937	-7175	-8227	-7410		
	51894	-7921	146187	-8463	-6603	-6906	283622	-6651	38095	-7118		
	-4267	-8259	-6679	-6675	-8211	-7935	95759	-7131	204681	-8361		

RPMI-OKT3

D7 Plate 1	RPMI-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	19	27	44	16	54	89	20	38	27	16	72	
	15	38	32	26	23	15	12	22	179	31	17	
	49	19	21	47	88	57	107	58	37	29	27	
	33	44	10	18	39	82	24	65	45	14		
	82	77	18	29	321	36	21	80	20	10		
	53	12	28	17	26	10	65	17	13	19	35	33
Test Well Value/4x (Mean Control Value)	-112	-104	-87	-115	-77	-42	-111	-93	-104	-115		
	-116	-93	-99	-105	-108	-116	-119	-109	48	-100		
	-82	-112	-110	-84	-43	-74	-24	-73	-94	-102		
	-98	-87	-121	-113	-92	-49	-107	-66	-86	-117		
	-49	-54	-113	-102	190	-95	-110	-51	-111	-121		
	-78	-119	-103	-114	-105	-121	-66	-114	-118	-112		
D7 Plate 2												
Test Well Values	41	44	18	21	18	17	20	41	14	77	-	
	24	22	19	48	11	41	15	24	20	11	10	
	94	20	21	20	27	29	16	27	16	30	30	
	59	49	37	19	22	8	17	16	32	24	26	
	61	20	29	59	15	14	10	20	23	9	20	
	63	15	11	74	20	34	23	27	19	12	23	20
Test Well Value/4x (Mean Control Value)	-41	-38	-64	-61	-64	-65	-62	-41	-68	-5		
	-58	-60	-63	-34	-71	-41	-67	-58	-62	-71		
	12	-62	-61	-62	-55	-53	-66	-55	-66	-52		
	-23	-33	-45	-63	-60	-74	-65	-66	-50	-58		
	-21	-62	-53	-23	-67	-68	-72	-62	-59	-73		
	-19	-67	-71	-8	-62	-48	-59	-55	-63	-70		

D7 Plate 3	RPMI-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	55	18	28	34	21	44	12	48	33	110	51	
	70	64	41	764	27	37	57	36	12	14	19	
	25	69	37	100	39	42	24	24	16	23	24	
	43	91	43	40	11	13	23	9	17	31	26	
	14	177	16	34	43	42	18	21	31	13	41	
	17	51	10	17	6	5	10	29	8	52	40	32
Test Well Value/4x (Mean Control Value)	-71	-108	-98	-92	-105	-82	-114	-78	-93	-16		
	-56	-62	-85	638	-99	-89	-69	-90	-114	-112		
	-101	-57	-89	-26	-87	-84	-102	-102	-110	-103		
	-83	-35	-83	-86	-115	-113	-103	-117	-109	-95		
	-112	51	-110	-92	-83	-84	-108	-105	-95	-113		
	-109	-75	-116	-109	-120	-121	-116	-97	-118	-74		

RPMI+OKT3

D7 Plate 1	RPMI+OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	162	552	269	439	459	271	116	649	327	187	382	
	382	605	478	238545	175	945	629	133	273	508	787	
	195	164	208	535	300	330	449	310	156	762	506	
	94	194	474	578	305	207	391	50	310	461	348	
	239	348	390	94	478	390	241	123	283	121	758	
	338	100	429	364	159	380	253	469	481	212	283	474
Test Well Value/4x (Mean Control Value)	-1734	-1344	-1627	-1457	-1437	-1625	-1780	-1247	-1569	-1709		
	-1514	-1291	-1418	236649	-1721	-951	-1267	-1763	-1623	-1388		
	-1701	-1732	-1688	-1361	-1596	-1566	-1447	-1586	-1740	-1134		
	-1802	-1702	-1422	-1318	-1591	-1689	-1505	-1846	-1586	-1435		
	-1657	-1548	-1506	-1802	-1418	-1506	-1655	-1773	-1613	-1775		
	-1558	-1796	-1467	-1532	-1737	-1516	-1643	-1427	-1415	-1684		

D7 Plate 2	RPMI+OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	320	89	445	108	287	424	101	73	222	131	140	
	347	487	170	204	241	208	595	74	266	540	196	
	257	487	175	263	222	374	355	246	748	397	604	
	390	138	198	396	331	279	148	321	232	436	678	
	640	210	97	215	400	558	636	188	956	453	839	
	425	104	176	229	575	499	336	230	353	178	266	367
Test Well Value/4x (Mean Control Value)	-1154	-1385	-1029	-1366	-1187	-1050	-1373	-1401	-1252	-1343		
	-1127	-987	-1304	-1270	-1233	-1266	-879	-1400	-1208	-934		
	-1217	-987	-1299	-1211	-1252	-1100	-1119	-1228	-726	-1077		
	-1084	-1336	-1276	-1078	-1143	-1195	-1326	-1153	-1242	-1038		
	-834	-1264	-1377	-1259	-1074	-916	-838	-1286	-518	-1021		
	-1049	-1370	-1298	-1245	-899	-975	-1138	-1244	-1121	-1296		
D7 Plate 3												
Test Well Values	133	102	123	112	590	388	245	77	305	288	117	
	3006	438	574	818	373	558	224	360	98	457	579	
	110	251	765	826	377	260	182	512	813	489	257	
	626	260	201	595	286	185	561	340	378	471	477	
	573	304	391	271	158	352	312	413	210	138	188	
	198	199	136	657	230	343	95	221	102	589	152	249
Test Well Value/4x (Mean Control Value)	-862	-893	-872	-883	-405	-607	-750	-918	-690	-707		
	2011	-557	-421	-177	-622	-437	-771	-635	-897	-538		
	-885	-744	-230	-169	-618	-735	-813	-483	-182	-506		
	-369	-735	-794	-400	-709	-810	-434	-655	-617	-524		
	-422	-691	-604	-724	-837	-643	-683	-582	-785	-857		
	-797	-796	-859	-338	-765	-652	-900	-774	-893	-406		

D9 SCGM-OKT3

D9 Plate 1	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	399	129	247	181	447	963	386	119	45614	307	428	
	141	179	1486	177	206657	1233	667	293	346	3527	170	
	398	167	188	278	82	172	98	233	216	195	569	
	8529	1836	403	112	175	533	258	95	182	151	300	
	139	294	80	173	119	124	1415	121	809	3077	195	
	153	145	593	209	154	7464	363	177	317	546	141	264
Test Well Value/4x (Mean Control Value)	-659	-929	-811	-877	-611	-95	-672	-939	44556	-751	-630	
	-917	-879	428	-881	205599	175	-391	-765	-712	2469	-888	
	-660	-891	-870	-780	-976	-886	-960	-825	-842	-863	-489	
	7471	778	-655	-946	-883	-525	-800	-963	-876	-907	-758	
	-919	-764	-978	-885	-939	-934	357	-937	-249	2019	-863	
	-905	-913	-465	-849	-904	6406	-695	-881	-741	-512	-917	
D9 Plate 2												
Test Well Values	364	228	5274	2056	126	625	141	2053	128	428		
	172	318	38102	257	161	437	149	6700	140	62		
	330	267	297	293	131	202	238	133	160	124		
	130	191	92094	1150	139	143	523	121	197	243		
	655	3440	582	100269	37980	174	686	203	92247	562		
	372	7766	1654	291	373	319	275	126	107	2766		264
Test Well Value/4x (Mean Control Value)	-692	-828	4218	1000	-930	-431	-915	997	-928	-628		
	-884	-738	37046	-799	-895	-619	-907	5644	-916	-994		
	-726	-789	-759	-763	-925	-854	-818	-923	-896	-932		
	-926	-865	91038	94	-917	-913	-533	-935	-859	-813		
	-401	2384	-474	99213	36924	-882	-370	-853	91191	-494		
	-684	6710	598	-765	-683	-737	-781	-930	-949	1710		

D9 Plate 3	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	353	914	2721	150	437	246732	785	72546	26287	571		
	389	4682	247	147	169	414	479	423	937	232		
	241	312	121	9262	1362	402	273214	945	275143	1078		
	430	156	143	297	309	508	415	114	649	446		
	45	694	223	326	172	218	3893	225	160	76		
	559	65701	2349	70	153	99	164	405	132	90		264
Test Well Value/4x (Mean Control Value)	-703	-142	1665	-906	-619	245676	-271	71490	25231	-485		
	-667	3626	-809	-909	-887	-642	-577	-633	-119	-824		
	-815	-744	-935	8206	306	-654	272158	-111	274087	22		
	-626	-900	-913	-759	-747	-548	-641	-942	-407	-610		
	-1011	-362	-833	-730	-884	-838	2837	-831	-896	-980		
	-497	64645	1293	-986	-903	-957	-892	-651	-924	-966		
D9 Plate 4												
Test Well Values	148	456	227	186	4409	237	444	354	395	460		
	605	203	157	304	335	155	219	5006	354	813		
	121	5020	115	363	1072	351	1406	409607	2845	575		
	624	237	134	305	305565	2429	378619	968	455	392		
	84905	559	344	325	200	155	215	57	643	10867		
	271	178	331	776	1107	153	72	322	174796	422		264
Test Well Value/4x (Mean Control Value)	-908	-600	-829	-870	3353	-819	-612	-702	-661	-596		
	-451	-853	-899	-752	-721	-901	-837	3950	-702	-243		
	-935	3964	-941	-693	16	-705	350	408551	1789	-481		
	-432	-819	-922	-751	304509	1373	377563	-88	-601	-664		
	83849	-497	-712	-731	-856	-901	-841	-999	-413	9811		
	-785	-878	-725	-280	51	-903	-984	-734	173740	-634		

X

SCGM+OKT3

D9 Plate 1	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	392364	51638	327995	56964	63031	4093	2668	6139	15692	224086	6195	
	135595	1991	4890	33502	4246	4622	4186	263334	158240	2446	3988	
	3395	145689	18048	1133	4792	229278	3361	6216	458134	4860	4068	
	82757	4868	16356	2248	5089	5300	3874	25679	71317	1190	1460	
	2949	274223	350955	4081	228589	3586	1742	4950	81110	2705	1250	
	51457	3761	8206	2828	331173	122001	2985	1232	2113	3519		2836
Test Well Value/4x (Mean Control Value)	381020	40294	316651	45620	51687	-7251	-8676	-5205	4348	212742		
	124251	-9353	-6454	22158	-7098	-6722	-7158	251990	146896	-8898		
	-7949	134345	6704	-10211	-6552	217934	-7983	-5128	446790	-6484		
	71413	-6476	5012	-9096	-6255	-6044	-7470	14335	59973	-10154		
	-8395	262879	339611	-7263	217245	-7758	-9602	-6394	69766	-8639		
	40113	-7583	-3138	-8516	319829	110657	-8359	-10112	-9231	-7825		
D9 Plate 2												
Test Well Values	4173	204655	40414	20248	112163	31511	7037	12246	14782	419173		
	84230	13569	6278	219384	2639	4379	2507	8206	3002	808		
	4243	22928	2245	12828	2421	190306	4319	4346	334561	2386		
	3903	443564	3743	107736	5668	4722	4629	3129	5072	5363		
	3862	37467	57802	4583	2778	284163	3200	2285	3821	4096		
	5258	3302	34391	7957	3019	2548	3769	3465	2700	3194		2836
Test Well Value/4x (Mean Control Value)	-7171	193311	29070	8904	100819	20167	-4307	902	3438	407829		
	72886	2225	-5066	208040	-8705	-6965	-8837	-3138	-8342	-10536		
	-7101	11584	-9099	1484	-8923	178962	-7025	-6998	323217	-8958		
	-7441	432220	-7601	96392	-5676	-6622	-6715	-8215	-6272	-5981		
	-7482	26123	46458	-6761	-8566	272819	-8144	-9059	-7523	-7248		
	-6086	-8042	23047	-3387	-8325	-8796	-7575	-7879	-8644	-8150		

D9 Plate 3	SCGM-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	4538	1879	4533	3996	3387	63319	172421	4990	4196	5360		
	359065	63331	4822	2589	194249	4463	112254	3565	4712	177928		
	3853	4107	2881	4518	6137	5252	224068	53200	4493	4998		
	12015	5633	2746	65173	4742	27316	4120	2028	3764	3472		
	3652	247116	4227	2088	4003	227876	275951	1831	130474	4897		
	4215	3166	3346	3294	3393	2662	2277	3935	2910	4083		2836
Test Well Value/4x (Mean Control Value)	-6806	-9465	-6811	-7348	-7957	51975	161077	-6354	-7148	-5984		
	347721	51987	-6522	-8755	182905	-6881	100910	-7779	-6632	166584		
	-7491	-7237	-8463	-6826	-5207	-6092	212724	41856	-6851	-6346		
	671	-5711	-8598	53829	-6602	15972	-7224	-9316	-7580	-7872		
	-7692	235772	-7117	-9256	-7341	216532	264607	-9513	119130	-6447		
	-7129	-8178	-7998	-8050	-7951	-8682	-9067	-7409	-8434	-7261		
D9 Plate 4												
Test Well Values	137381	4999	349034	82283	64039	115975	1772	4168	427966	4487		
	2673	23246	87864	6195	3100	81239	5353	3351	10701	447127		
	214066	24801	163811	3988	3292	8403	83913	101513	461846	2119		
	3898	37425	8359	4068	9731	177200	2081	6650	1709	3376		
	18886	4317	14552	1460	47685	26648	221235	4770	2307	453932		
	45130	40621	4526	1250	4160	4144	252550	239258	3959	3314		2836
Test Well Value/4x (Mean Control Value)	126037	-6345	337690	70939	52695	104631	-9572	-7176	416622	-6857		
	-8671	11902	76520	-5149	-8244	69895	-5991	-7993	-643	435783		
	202722	13457	152467	-7356	-8052	-2941	72569	90169	450502	-9225		
	-7446	26081	-2985	-7276	-1613	165856	-9263	-4694	-9635	-7968		
	7542	-7027	3208	-9884	36341	15304	209891	-6574	-9037	442588		
	33786	29277	-6818	-10094	-7184	-7200	241206	227914	-7385	-8030		

RPMI-OKT3

D9 Plate 1	RPMI-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	115	76	23	11	128	31	25	164	34	50	38	
	69	60	21	46	24	35	51	20	26	111	68	
	22	18	75	51	77	13	22	22	81	57	82	
	87	215	31	48	26	176	30	25	52	50	35	
	12	145	16	120	31	111	83	41	111	50	44	
	16	33	124	23	48	74	100	8	19	155	12	40
Test Well Value/4x (Mean Control Value)	-44	-83	-136	-148	-31	-128	-134	5	-125	-109	-121	
	-90	-99	-138	-113	-135	-124	-108	-139	-133	-48	-91	
	-137	-141	-84	-108	-82	-146	-137	-137	-78	-102	-77	
	-72	56	-128	-111	-133	17	-129	-134	-107	-109	-124	
	-147	-14	-143	-39	-128	-48	-76	-118	-48	-109	-115	
	-143	-126	-35	-136	-111	-85	-59	-151	-140	-4	-147	
D9 Plate 2												
Test Well Values	242	25	77	17	33	22	65	52	16	77		
	39	26	16	27	14	91	82	79	18	44		
	28	23	119	40	100	26	11	142	21	27		
	25	12	51	24	16	154	43	25	29	8		
	47	75	144	79	10	21	41	34	95	21		
	120	19	98	10	50	38	32	27	21	11		40
Test Well Value/4x (Mean Control Value)	83	-134	-82	-142	-126	-137	-94	-107	-143	-82		
	-120	-133	-143	-132	-145	-68	-77	-80	-141	-115		
	-131	-136	-40	-119	-59	-133	-148	-17	-138	-132		
	-134	-147	-108	-135	-143	-5	-116	-134	-130	-151		
	-112	-84	-15	-80	-149	-138	-118	-125	-64	-138		
	-39	-140	-61	-149	-109	-121	-127	-132	-138	-148		

D9 Plate 3	RPMI-OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	186	31	17	17	28	35	138	38	26	92		
	61	61	20	63	53	30	194	89	94	57		
	29	81	12	21	45	6	36	30	41	22		
	20	47	10	42	86	43	32	21	17	21		
	324	30	42	30	27	16	88	23	12	27		
	9	12	55	16	112	25	9	79	104	22		40
Test Well Value/4x (Mean Control Value)	27	-128	-142	-142	-131	-124	-21	-121	-133	-67		
	-98	-98	-139	-96	-106	-129	35	-70	-65	-102		
	-130	-78	-147	-138	-114	-153	-123	-129	-118	-137		
	-139	-112	-149	-117	-73	-116	-127	-138	-142	-138		
	165	-129	-117	-129	-132	-143	-71	-136	-147	-132		
	-150	-147	-104	-143	-47	-134	-150	-80	-55	-137		
D9 Plate 4												
Test Well Values	20	11	39	99	143	35	25	19	41	124		
	54	41	32	68	66	58	64	17	12	52		
	24	110	156	22	51	23	50	55	21	44		
	64	24	47	16	17	50	81	53	11	76		
	36	15	72	38	64	67	26	28	213	24		
	29	36	44	24	32	54	32	4	17	73		40
Test Well Value/4x (Mean Control Value)	-139	-148	-120	-60	-16	-124	-134	-140	-118	-35		
	-105	-118	-127	-91	-93	-101	-95	-142	-147	-107		
	-135	-49	-3	-137	-108	-136	-109	-104	-138	-115		
	-95	-135	-112	-143	-142	-109	-78	-106	-148	-83		
	-123	-144	-87	-121	-95	-92	-133	-131	54	-135		
	-130	-123	-115	-135	-127	-105	-127	-155	-142	-86		

RPMI+OKT3

D9 Plate 1	RPMI+OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	351	559	530	691	1048	808	671	335	376	749	1600	
	710	1000	691	832	832	772	840	549	655	662	941	
	780	636	436	1007	1065	1462	366	583	875	1028	977	
	947	965	509	691	898	915	806	1143	1024	403	1105	
	1354	969	687	1397	1175	1043	938	582	836	345	1599	
	901	169	410	359	681	662	1161	1232	1009	148	1287	1223
Test Well Value/4x (Mean Control Value)	-4541	-4333	-4362	-4201	-3844	-4084	-4221	-4557	-4516	-4143	-4541	
	-4182	-3892	-4201	-4060	-4060	-4120	-4052	-4343	-4237	-4230	-4182	
	-4112	-4256	-4456	-3885	-3827	-3430	-4526	-4309	-4017	-3864	-4112	
	-3945	-3927	-4383	-4201	-3994	-3977	-4086	-3749	-3868	-4489	-3945	
	-3538	-3923	-4205	-3495	-3717	-3849	-3954	-4310	-4056	-4547	-3538	
	-3991	-4723	-4482	-4533	-4211	-4230	-3731	-3660	-3883	-4744	-3991	
D9 Plate 2												
Test Well Values	612	857	976	872	879	559	461	910	752	561		
	401	676	1465	776	739	1474	1266	786	916	680		
	810	735	1086	1252	1241	910	969	837	1118	364		
	1979	475	921	1607	301	869	1006	823	507	634		
	1044	1187	492	796	918	444	481	873	391	353		
	509	881	856	1502	533	520	785	620	1144	485		1223
Test Well Value/4x (Mean Control Value)	-4280	-4035	-3916	-4020	-4013	-4333	-4431	-3982	-4140	-4331		
	-4491	-4216	-3427	-4116	-4153	-3418	-3626	-4106	-3976	-4212		
	-4082	-4157	-3806	-3640	-3651	-3982	-3923	-4055	-3774	-4528		
	-2913	-4417	-3971	-3285	-4591	-4023	-3886	-4069	-4385	-4258		
	-3848	-3705	-4400	-4096	-3974	-4448	-4411	-4019	-4501	-4539		
	-4383	-4011	-4036	-3390	-4359	-4372	-4107	-4272	-3748	-4407		

D9 Plate 3	RPMI+OKT3 ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	808	1191	478	1139	932	916	880	841	1168	821		
	1365	822	1607	772	628	1012	48	1185	671	496		
	1041	930	753	1348	1082	1117	767	1010	1083	936		
	530	730	884	859	721	642	597	506	1009	1383		
	792	702	1461	860	745	624	488	344	460	1026		
	457	595	897	624	753	557	1197	835	710	1264		1223
Test Well Value/4x (Mean Control Value)	-4084	-3701	-4414	-3753	-3960	-3976	-4012	-4051	-3724	-4071		
	-3527	-4070	-3285	-4120	-4264	-3880	-4844	-3707	-4221	-4396		
	-3851	-3962	-4139	-3544	-3810	-3775	-4125	-3882	-3809	-3956		
	-4362	-4162	-4008	-4033	-4171	-4250	-4295	-4386	-3883	-3509		
	-4100	-4190	-3431	-4032	-4147	-4268	-4404	-4548	-4432	-3866		
	-4435	-4297	-3995	-4268	-4139	-4335	-3695	-4057	-4182	-3628		
D9 Plate 4												
Test Well Values	1061	456	384	766	1275	751	594	884	546	755		
	856	841	828	424	1046	901	474	454	456	865		
	1145	502	457	359	991	846	687	422	262	409		
	296	290	732	906	211	289	355	150	416	1076		
	433	525	445	379	347	452	476	418	387	504		
	154	209	308	308	327	207	990	644	663	989		1223
Test Well Value/4x (Mean Control Value)	-3831	-4436	-4508	-4126	-3617	-4141	-4298	-4008	-4346	-4137		
	-4036	-4051	-4064	-4468	-3846	-3991	-4418	-4438	-4436	-4027		
	-3747	-4390	-4435	-4533	-3901	-4046	-4205	-4470	-4630	-4483		
	-4596	-4602	-4160	-3986	-4681	-4603	-4537	-4742	-4476	-3816		
	-4459	-4367	-4447	-4513	-4545	-4440	-4416	-4474	-4505	-4388		
	-4738	-4683	-4584	-4584	-4565	-4685	-3902	-4248	-4229	-3903		

Appendix II Chapter 5; NK Clone Cytotoxicity in SCGM and RPMI ± OKT3

												Control wells*	Mean Min†	Mean Max‡
^{51}Cr Values	98	481	80	77	97	86	82	75	80	82	42	99		
	96	81	67	82	63	56	67	106	74	90	78	82		
	83	102	67	63	67	60	64	136	599	251	145	92		
	944	93	73	69	69	56	536	58	65	255	80	96		
	931	79	68	74	68	94	70	67	57	91	117	84		
	932	93	71	138	86	73	82	85	83	71	76	83	92	936
% Specific Lysis§		46	-1	-2	1	-1	-1	-2	-1	-1	-6	1		
		-1	-3	-1	-3	-4	-3	2	-2	0	-2	2		
		1	-3	-3	-3	-4	-3	5	60	19	6	0		
		0	-2	-3	-3	-4	53	-4	-3	19	-1	0		
		-2	-3	-2	-3	0	-3	-3	-4	0	3	-1		
		0	-3	5	-1	-2	-1	-1	-1	-3	-2	-1		

(*) Wells contain feeder cells only
(†) Mean ^{51}Cr release values of wells containing K562s and media (Spontaneous ^{51}Cr release)
(‡) Mean ^{51}Cr release values of wells containing K562s and 5% Tryton-X 100
(§) % Specific Lysis was calculated as described in section 2.10.1.4

D7 SCGM-OKT3

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	98	481	80	77	97	86	82	75	80	82	42	99		
	96	81	67	82	63	56	67	106	74	90	78	82		
	83	102	67	63	67	60	64	136	599	251	145	92		
	944	93	73	69	69	56	536	58	65	255	80	96		
	931	79	68	74	68	94	70	67	57	91	117	84		
	932	93	71	138	86	73	82	85	83	71	76	83	92	936
% Specific Lysis		46	-1	-2	1	-1	-1	-2	-1	-1	-6	1		
		-1	-3	-1	-3	-4	-3	2	-2	0	-2	2		
		1	-3	-3	-3	-4	-3	5	60	19	6	0		
		0	-2	-3	-3	-4	53	-4	-3	19	-1	0		
		-2	-3	-2	-3	0	-3	-3	-4	0	3	-1		
		0	-3	5	-1	-2	-1	-1	-1	-3	-2	-1		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	68	80	100	70	77	84	98	87	84	69	110	74		
	81	83	47	79	67	92	57	79	76	61	72	75		
	71	100	110	58	53	52	65	67	74	89	61	82		
	906	92	77	58	59	57	56	96	79	70	115	82		
	970	88	254	36	64	77	65	71	65	57	70	78		
	848	86	70	53	68	81	71	84	76	90	109	69	73	908
% Specific Lysis		1	3	0	0	1	3	2	1	-1	4	0		
		1	-3	1	-1	2	-2	1	0	-1	0	0		
		3	4	-2	-2	-3	-1	-1	0	2	-1	1		
		2	0	-2	-2	-2	-2	3	1	0	5	1		
		2	22	-4	-1	0	-1	0	-1	-2	0	1		
		2	0	-2	-1	1	0	1	0	2	4	-1		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	67	88	68	73	80	123	743	89	81	87	70	80		
	81	75	93	62	60	73	63	71	70	64	65	65		
	93	86	83	58	59	84	59	570	64	82	77	84		
	800	80	88	74	501	72	357	71	59	72	70	71		
	809	82	57	52	54	55	50	75	87	59	80	74		
	793	76	62	76	108	80	75	81	97	94	673	70	80	800
% Specific Lysis		1	-2	-1	0	6	92	1	0	1	-1	0		
		-1	2	-3	-3	-1	-2	-1	-1	-2	-2	-2		
		1	0	-3	-3	1	-3	68	-2	0	0	1		
		0	1	-1	58	-1	38	-1	-3	-1	-1	-1		
		0	-3	-4	-4	-4	-4	-1	1	-3	0	-1		
		-1	-3	-1	4	0	-1	0	2	2	82	-1		

D7 SCGM+OKT3

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	86	86	117	634	96	167	768	140	561	688	613	91		
	85	142	462	604	83	95	114	520	63	67	66	87		
	92	476	631	720	545	742	603	584	616	503	265	100		
	858	854	707	659	589	596	635	660	552	461	183	62		
	878	81	145	743	731	614	641	121	770	548	63	113		
	711	621	91	85	75	78	74	69	83	82	561	75	88	816
% Specific Lysis		0	4	75	1	11	93	7	65	82	72	0		
		7	51	71	-1	1	4	59	-3	-3	-3	0		
		53	75	87	63	90	71	68	73	57	24	2		
		105	85	78	69	70	75	79	64	51	13	-4		
		-1	8	90	88	72	76	5	94	63	-3	3		
		73	0	0	-2	-1	-2	-3	-1	-1	65	-2		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	82	96	85	89	94	115	102	694	93	94	85	81		
	78	99	512	102	153	646	554	76	77	85	101	82		
	80	98	86	57	51	68	533	645	85	79	622	103		
	834	94	68	56	64	59	64	70	573	77	104	86		
	808	96	59	82	67	72	63	62	230	96	556	81		
	797	113	101	80	512	87	142	152	108	591	93	79	80	813
% Specific Lysis		2	1	1	2	5	3	84	2	2	1	0		
		3	59	3	10.0	77	65	-1	0	1	3	0		
		2	1	-3	-4	-2	62	77	1	0	74	3		
		2	-2	-3	-2	-3	-2	-1	67	0	3	1		
		2	-3	0	-2	-1	-2	-2	20	2	65	0		
		5	3	0.0	59	1	8	9.8	4	70	2	0		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	75	86	556	110	86	98	519	138	585	288	83	88		
	85	79	70	81	47	65	77	156	74	72	468	80		
	79	105	422	75	511	445	101	90	225	75	179	78		
	933	71	53	57	80	422	129	62	51	62	68	79		
	912	472	97	486	76	89	79	586	76	423	75	76		
	938	159	72	70	79	79	88	559	87	623	73	80	80	928
% Specific Lysis		1	56	4	1	2	52	7	60	25	0	1		
		0	-1	0	-4	-2	0	9	-1	-1	46	0		
		3	40	-1	51	43	3	1	17	-1	12	0		
		-1	-3	-3	0	40	6	-2	-3	-2	-1	0		
		46	2	48	0	1	0	60	0	40	-1	0		
		9	-1	-1	0	0	1	57	1	64	-1	0		

D7 RPMI-OKT3

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	81	59	115	115	92	112	109	102	118	121	105	121		
	98	98	89	98	81	101	88	96	96	121	85	114		
	113	60	103	83	53	104	81	101	93	80	106	111		
	1207	79	105	93	50	82	75	105	83	86	100	96		
	1213	94	77	101	58	105	101	97	78	101	98	108		
	1283	97	90	104	80	115	95	85	96	102	96	123	97	1234
% Specific Lysis		-3	2	2	0	1	1	0	2	2	1	2		
		0	-1	0	-1	0	-1	0	0	2	-1	1		
		-3	0	-1	-4	1	-1	0	0	-2	1	1		
		-2	1	0	-4	-1	-2	1	-1	-1	0	0		
		0	-2	0	-3	1	0	0	-2	0	0	1		
		0	-1	1	-2	2	0	-1	0	0	0	2		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	100	119	104	95	125	87	114	103	102	101	112	105		
	80	114	108	97	91	83	102	91	109	95	117	113		
	87	117	72	131	102	104	94	95	107	101	124	117		
	772	112	101	125	117	106	101	92	117	92	108	118		
	692	133	111	109	115	108	123	94	100	95	117	120		
	633	121	102	109	128	128	72	109	113	137	119	101	89	669
% Specific Lysis		5	2	1	6	0	4	2	2	2	4	3		
		4	3	1	0	-1	2	0	3	1	5	4		
		5	-3	7	2	2	1	1	3	2	6	5		
		4	2	6	5	3	2	0	5	0	3	5		
		7	4	3	4	3	6	1	2	1	5	5		
		5	2	3	6	6	-3	3	4	8	5	2		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	84	119	117	109	97	86	99	124	105	96	114	99		
	86	117	121	99	258	107	104	95	95	101	103	107		
	89	102	106	113	102	115	92	96	119	126	120	129		
	705	121	97	93	85	124	140	122	118	109	128	130		
	591	134	112	107	99	124	126	85	95	108	121	89		
	725	115	91	104	105	108	112	103	113	100	84	95	86	674
% Specific Lysis		6	5	4	2	0	2	6	3	2	5	2		
		5	6	2	29	4	3	1	1	2	3	4		
		3	3	5	3	5	1	2	6	7	6	7		
		6	2	1	0	6	9	6	5	4	7	7		
		8	4	4	2	6	7	0	1	4	6	0		
		5	1	3	3	4	4	3	5	2	0	1		

D7 RPMI+OKT3

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	86	109	132	127	121	119	101	95	111	89	120	106		
	137	107	130	146	777	107	116	95	106	97	122	83		
	110	120	136	135	114	99	103	87	106	114	120	120		
	1205	139	110	107	108	119	124	117	123	101	110	109		
	1242	123	104	112	107	120	114	88	116	165	126	88		
	1060	124	98	124	101	142	126	128	139	126	324	117	111	1169
% Specific Lysis		0	2	2	1	1	-1	-2	0	-2	1	0		
		0	2	3	63	0	0	-2	0	-1	1	-3		
		1	2	2	0	-1	-1	-2	0	0	1	1		
		3	0	0	0	1	1	1	1	-1	0	0		
		1	-1	0	0	1	0	-2	0	5	1	-2		
		1	-1	1	-1	3	1	2	3	1	20	1		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	123	139	101	104	105	108	121	130	119	119	103	140		
	131	124	144	301	122	125	120	113	121	116	111	109		
	129	104	129	115	134	124	110	122	109	96	132	103		
	1209	119	130	100	93	110	103	121	120	103	104	113		
	1163	141	112	111	109	110	109	152	123	105	98	115		
	1234	125	113	126	113	122	115	89	138	126	105	114	128	1202
% Specific Lysis		1	-2	-2	-2	-2	-1	0	-1	-1	-2	1		
		0	2	16	-1	0	-1	-1	-1	-1	-2	-2		
		-2	0	-1	1	0	-2	-1	-2	-3	0	-2		
		-1	0	-3	-3	-2	-2	-1	-1	-2	-2	-1		
		1	-1	-2	-2	-2	-2	2	0	-2	-3	-1		
		0	-1	0	-1	-1	-1	-4	1	0	-2	-1		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	115	126	114	147	119	135	122	136	145	111	131	141		
	189	853	118	151	132	100	134	127	120	104	124	77		
	111	127	111	103	127	104	146	112	114	150	136	145		
	1493	140	115	129	108	136	108	102	120	144	96	146		
	1402	126	119	115	118	136	136	109	130	134	107	138		
	1432	149	139	118	116	120	159	92	117	130	133	114	138	1442
% Specific Lysis		-1	-2	1	-1	0	-1	0	1	-2	-1	0		
		55	-2	1	0	-3	0	-1	-1	-3	-1	-5		
		-1	-2	-3	-1	-3	1	-2	-2	1	0	1		
		0	-2	-1	-2	0	-2	-3	-1	0	-3	1		
		-1	-1	-2	-2	0	0	-2	-1	0	-2	0		
		1	0	-2	-2	-1	2	-4	-2	-1	0	-2		

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Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	658	29	27	31	26	34	50	35	37	158	82	37		
	677	35	35	53	29	254	58	114	22	28	162	22		
	680	36	36	31	43	33	41	25	33	28	36	38		
	55	193	186	26	20	41	54	30	55	31	25	39		
	40	30	25	30	35	37	42	57	44	64	123	28		
	36	30	34	54	38	20	155	26	63	35	56	30	44	672
% Specific Lysis		-2	-3	-2	-3	-2	1	-1	-1	18	6	-2		
		-1	-1	1	-2	33	2	11	-3	-2	19	-1		
		-1	-1	-2	0	-2	0	-3	-2	-2	-1	-1		
		24	23	-3	-4	0	2	-2	2	-2	-3	24		
		-2	-3	-2	-1	-1	0	2	0	3	13	-2		
		-2	-2	2	-1	-4	18	-3	3	-1	2	-2		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	583	30	32	171	57	22	26	34	78	29	34	3		
	613	37	40	241	98	33	46	42	161	35	25	5		
	572	24	30	45	35	49	43	36	23	17	31	5		
	107	37	39	261	37	35	28	190	35	23	34	2		
	92	98	50	28	200	80	32	37	38	150	27	2		
	57	46	167	135	39	27	42	27	21	25	66	1	85	590
% Specific Lysis		-11	-11	17	-6	-13	-12	-10	-1	-11	-10	-16		
		-10	-9	31	3	-10	-8	-9	15	-10	-12	-16		
		-12	-11	-8	-10	-7	-8	-10	-12	-14	-11	-16		
		-10	-9	35	-10	-10	-11	21	-10	-12	-10	-17		
		3	-7	-11	23	-1	-11	-10	-9	13	-12	-17		
		-8	16	9.9	-9	-12	-9	-12	-13	-12	-4	-17		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	631	42	77	78	48	25	198	52	112	110	20	1		
	576	26	160	52	37	31	28	32	36	35	61	1		
	712	52	45	44	119	89	34	375	26	265	33	5		
	78	38	34	30	56	32	31	33	35	36	25	1		
	100	23	28	30	31	27	15	62	17	22	98	1		
	74	37	90	89	40	25	28	24	33	31	29	2	84	640
% Specific Lysis		-8	-1	-1	-6	-11	21	-6	5	5	-12	-15		
		-10	14	-6	-8	-10	-10	-9	-9	-9	-4	-15		
		-6	-7	-7	6	1	-9	52	-10	33	-9	-14		
		-8	-9	-10	-5	-9	-10	-9	-9	-9	-11	-15		
		-11	-10	-10	-10	-10	-12	-4	-12	-11	3	-15		
		-8	1	1	-8	-11	-10	-11	-9	-10	-10	-15		

Plate 4

												Control wells	Mean Min	Mean Max
^{51}Cr Values	627	19	34	31	32	136	31	33	101	26	25	2		
	608	60	27	28	19	22	29	31	83	24	53	0		
	613	67	103	36	35	37	38	40	245	82	36	3		
	98	37	32	40	42	306	103	200	29	34	88	2		
	114	202	31	27	36	31	28	25	29	39	39	0		
	97	41	20	23	34	25	16	39	23	182	32	2	103	616
% Specific Lysis		-16	-13	-14	-14	6	-14	-14	0	-15	-15	-20		
		-8	-15	-15	-16	-16	-14	-14	-4	-15	-10	-20		
		-7	0	-13	-13	-13	-13	-12	28	-4	-13	-19		
		-13	-14	-12	-12	39.6	0	19	-14	-13	-3	-20		
		19	-14	-15	-13	-14	-15	-15	-14	-12	-12	-20		
		-12	-16	-16	-13	-15	-17	-12	-16	15	-14	-20		

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Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	571	322	265	277	276	105	28	36	95	233	320	0		
	650	330	53	74	149	39	57	43	251	264	33	2		
	623	152	198	386	45	32	210	36	34	228	40	2		
	63	284	46	197	94	37	22	25	259	275	30	2		
	74	54	361	191	46	221	47	37	45	119	72	5		
	92	322	54	53	28	271	241	36	35	50	49	2	76	615
% Specific Lysis		46	35	37	37	5	-9	-7	3	29	45	-14		
		47	-4	0	13	-7	-4	-6	32	35	-8	-14		
		14	23	58	-6	-8	25	-7	-8	28	-7	-14		
		39	-6	22	3	-7	-10	-10	34	37	-9	-14		
		-4	53	21	-6	27	-5	-7	-6	8	-1	-13		
		46	-4	-4	-9	36	31	-7	-8	-5	-5	-14		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	599	232	360	99	356	318	270	126	304	45	270	2		
	601	369	167	75	334	63	58	99	235	44	191	2		
	405	36	338	42	107	42	244	34	32	185	45	2		
	89	32	240	49	238	57	55	45	184	32	251	1		
	90	37	194	198	48	38	228	86	177	31	29	1		
	46	23	33	119	129	141	35	41	22	29	19	2	80	813
% Specific Lysis		34	62	5	61	53	42	11	50	-7	42	-16		
		64	20.0	0	56	-3	-4	5	35	-7	25	-16		
		-8	57	-7	7	-7	37	-9	-9	24	-7	-16		
		-9	36	-6	35	-4	-4	-7	24	-9	38	-16		
		-8	26	27	-6	-8	33	2	22	-10	-10	-16		
		-11	-9	9.6	12	14	-9	-7	-12	-10	-12	-16		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	527	27	62	38	25	28	367	262	48	43	60	1		
	571	185	299	40	27	317	308	259	40	43	266	0		
	629	56	28	32	83	196	41	325	147	50	327	2		
	76	173	63	52	230	42	261	48	36	91	180	3		
	42	23	92	27	26	24	129	94	26	116	45	0		
	55	157	33	28	15	56	24	21	57	29	37	4	58	576
% Specific Lysis		-6	1	-4	-6	-6	60	39	-2	-3	0	-11		
		25	47	-3	-6	50	48	39	-3	-3	40	-11		
		0	-6	-5	5	27	-3	52	17	-1	52	-11		
		22	1	-1	33	-3	39	-2	-4	6	24	-11		
		-7	7	-6	-6	-6	14	7	-6	11	-2	-11		
		19	-5	-6	-8	0	-6	-7	0	-6	-4	-10		

Plate 4

												Control wells	Mean Min	Mean Max
^{51}Cr Values	642	171	71	244	173	174	219	79	38	269	61	2		
	568	58	172	212	34	41	299	104	43	122	283	6		
	661	232	218	178	26	30	118	126	89	278	54	5		
	107	23	226	58	31	140	202	65	104	35	39	0		
	147	181	64	101	163	249	151	293	47	35	288	2		
	66	146	229	41	24	28	40	351	225	35	36	1	107	624
% Specific Lysis		12	-7	27	13	13	22	-5	-13	31	-9	-20		
		-9	13	20.4	-14	-13	37	-1	-12	3	34	-19		
		24	22	14	-16	-15	2	4	-3	33	-10	-20		
		-16	23	-9	-15	6	18	-8	-1	-14	-13	-21		
		14	-8	-1	11	28	9	36	-12	-14	35	-20		
		8	24	-13	-16	-15	-13	47	23	-14	-14	-20		

RPMI-OKT3
Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	625	64	67	56	63	53	48	59	55	67	63	60		
	650	71	58	78	72	61	33	64	65	66	71	70		
	659	67	91	60	55	63	71	61	59	72	62	75		
	68	87	91	67	50	57	77	54	60	63	88	73		
	66	80	72	65	55	68	63	55	67	68	78	58		
	60	44	42	85	45	56	70	59	55	63	68	80	65	645
% Specific Lysis		0	0	-1	0	-2	-3	-1	-2	0	0	-1		
		1	-1	2	1	-1	-5	0	0	0	1	1		
		0	5	-1	-2	0	1	-1	-1	1	0	2		
		4	5	0	-3	-1	2	-2	-1	0	4	1		
		3	1	0	-2	1	0	-2	0	1	2	-1		
		-4	-4	4	-3	-1	1	-1	-2	0	1	3		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	654	61	101	94	77	88	74	68	63	64	59	1		
	693	88	81	64	78	76	92	70	48	75	59	4		
	627	83	73	100	87	84	66	72	62	69	55	2		
	58	111	73	80	98	85	61	96	48	77	79	2		
	74	88	93	94	71	80	115	89	80	57	58	0		
	74	92	87	96	77	81	67	86	64	53	66	3	69	658
% Specific Lysis		-1	5	4	1	3	1	0	-1	-1	-2	-11		
		3	2	-1	2	1	4	0	-4	1	-2	-11		
		2	1	5	3	3	0	1	-1	0	-2	-11		
		7	1	2	5	3	-1	5	-4	1	2	-11		
		3	4	4	0	2	8	3	2	-2	-2	-12		
		4	3	5	1	2	0	3	-1	-3	0	-11		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	585	93	89	87	68	64	74	40	72	54	107	4		
	609	100	96	102	67	64	79	62	62	69	74	4		
	602	100	105	86	68	79	62	68	72	71	56	5		
	89	103	94	95	67	77	77	62	65	53	47	0		
	112	95	93	90	78	72	68	54	44	78	79	1		
	104	81	83	93	47	52	78	58	65	73	62	4	102	599
% Specific Lysis		-2	-3	-3	-7	-8	-6	-12	-6	-10	1	-20		
		0	-1	0	-7	-8	-5	-8	-8	-7	-6	-20		
		0	1	-3	-7	-5	-8	-7	-6	-6	-9	-19		
		0	-2	-1	-7	-5	-5	-8	-7	-10	-11	-20		
		-1	-2	-2	-5	-6	-7	-10	-12	-5	-5	-20		
		-4	-4	-2	-11	-10	-5	-9	-7	-6	-8	-20		

RPMI+OKT3
Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	672	73	80	86	90	107	108	76	85	76	81	2		
	636	95	70	64	111	77	129	65	80	81	91	2		
	639	78	97	89	78	108	80	65	66	83	101	1		
	83	89	93	77	104	97	96	106	87	71	97	2		
	86	55	88	70	102	104	101	122	80	75	96	3		
	80	93	78	94	105	100	90	99	95	68	92	0	83	649
% Specific Lysis		-2	-1	1	1	4	4	-1	0	-1	0	-14		
		2	-2	-3	5	-1	8	-3	-1	0	1	-14		
		-1	2	1	-1	4	-1	-3	-3	0	3	-14		
		1	2	-1	4	2	2	4	1	-2	2	-14		
		-5	1	-2	3	4	3	7	-1	-1	2	-14		
		2	-1	2	4	3	1	3	2	-3	2	-15		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	767	71	82	82	61	102	74	75	83	73	78	0		
	615	87	73	79	77	109	105	101	83	98	79	2		
	580	98	111	67	81	95	83	99	83	81	76	3		
	76	102	137	75	69	81	89	76	106	107	114	1		
	78	150	82	74	98	93	64	96	86	83	100	1		
	99	85	78	92	65	76	85	99	86	81	86	2	84	654
% Specific Lysis		-2	0	0	-4	3	-2	-2	0	-2	-1	-15		
		0	-2	-1	-1	4	4	3	0	2	-1	-14		
		2	5	-3	-1	2	0	3	0	-1	-1	-14		
		3	9	-2	-3	-1	1	-1	4	4	5	-15		
		12	0	-2	2	2	-4	2	0	0	3	-15		
		0	-1	1	-3	-1	0	3	0	-1	0	-14		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	700	75	75	66	77	69	65	75	59	60	69	66		
	788	71	58	74	69	65	84	69	57	56	57	73		
	613	59	53	70	75	64	62	84	66	83	78	59		
	58	93	72	68	57	57	65	77	50	49	55	67		
	69	69	77	77	76	78	73	74	63	65	57	63		
	70	81	82	87	81	68	91	70	64	58	55	71	66	700
% Specific Lysis		1	1	0	2	1	0	1	-1	-1	1	0		
		1	-1	1	1	0	3	1	-1	-2	-1	1		
		-1	-2	1	1	0	-1	3	0	3	2	-1		
		4	1	0	-1	-1	0	2	-2	-3	-2	0		
		1	2	2	2	2	1	1	0	0	-1	0		
		2	3	3	2	0	4	1	0	-1	-2	1		

Plate 4

												Control wells	Mean Min	Mean Max
^{51}Cr Values	573	69	59	70	84	62	53	74	74	100	72	1		
	603	72	75	75	55	68	67	70	77	95	92	2		
	666	66	62	75	75	65	78	73	79	78	111	3		
	58	57	79	69	62	76	70	95	86	76	94	1		
	69	57	72	60	53	70	71	68	69	81	79	2		
	68	54	45	57	85	54	56	62	71	83	81	2	65	614
% Specific Lysis		1	-1	1	3	-1	-2	2	2	6	1	-12		
		1	2	2	-2	1	0	1	2	5	5	-11		
		0	-1	2	2	0	2	1	3	2	8	-11		
		-1	3	1	-1	2	1	5	4	2	5	-12		
		-1	1	-1	-2	1	1	1	1	3	3	-11		
		-2	-4	-1	4	-2	-2	-1	1	3	3	-11		

Appendix III Chapter 5; NK Clone Cytotoxicity – Investigation into Number of Allogeneic Feeder Donors

												Control wells*	Mean Min†	Mean Max‡
^{51}Cr Values	23	34	45	150	114	76	36	176	164	49	85	80		
	22	32	196	47	49	42	165	53	154	280	166	75		
	26	34	92	48	34	55	50	44	220	61	213	44		
	292	38	32	38	66	271	42	35	215	45	66	64		
	282	48	34	119	37	49	228	46	63	47	63	65		
	328	209	47	24	40	89	39	215	31	38	54	66	24	301
% Specific Lysis§		4	8	46	33	19	4	55	51	9	22	20		
		3	62	8	9	7	51	11	47	93	51	19		
		4	25	9	4	11	9.5	7	71	13	68	7		
		5	3	5	15	89	7	4	69	8	15	15		
		9	4	34	5	9	74	8	14	8	14	15		
		67	8	0	6	24	6	69	3	5	11	15		

(*) Wells contain feeder cells only
(†) Mean ^{51}Cr release values of wells containing K562s and media (Spontaneous ^{51}Cr release)
(‡) Mean ^{51}Cr release values of wells containing K562s and 5% Tryton-X 100
(§) % Specific Lysis was calculated as described in section 2.10.1.4

One Allogeneic Feeder

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	23	34	45	150	114	76	36	176	164	49	85	80		
	22	32	196	47	49	42	165	53	154	280	166	75		
	26	34	92	48	34	55	50	44	220	61	213	44		
	292	38	32	38	66	271	42	35	215	45	66	64		
	282	48	34	119	37	49	228	46	63	47	63	65		
	328	209	47	24	40	89	39	215	31	38	54	66	24	301
% Specific Lysis		4	8	46	33	19	4	55	51	9	22	20		
		3	62	8	9	7	51	11	47	93	51	19		
		4	25	9	4	11	9.5	7	71	13	68	7		
		5	3	5	15	89	7	4	69	8	15	15		
		9	4	34	5	9	74	8	14	8	14	15		
		67	8	0	6	24	6	69	3	5	11	15		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	48	278	56	160	518	514	456	57	51	67	56	75		
	59	507	248	78	234	78	81	55	508	180	450	128		
	76	79	69	70	74	83	63	202	102	486	500	85		
	1285	484	90	82	168	134	61	76	414	322	706	104		
	2932	206	2005	2198	1895	1520	279	2611	2129	243	282	385		
	3143	6	2	44	89	5	9	7	34	14	9	4	61	2453
% Specific Lysis		9	0	4	19	19	17	0	0	0	0	1		
		19	8	1	7	1	1	0	19	5	16	3		
		1	0	0	1	1	0	6	2	18	18	1		
		18	1	1	4	3	0	1	15	11	27	2		
		6	81	89	77	61	9	107	86	8	9	14		
		-2	-2	-1	1	-2	-2	-2	-1	-2	-2	-2		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	30	38	35	303	249	172	49	38	44	343	427	55		
	25	34	41	15	294	40	350	355	30	64	1994	100		
	24	44	48	42	47	32	53	55	40	119	732	55		
	344	55	44	162	45	213	217	41	77	311	357	60		
	344	232	47	295	164	318	301	39	39	199	707	83		
	307	44	167	56	222	45	342	49	47	762	202	79	26	332
% Specific Lysis		4	3	91	73	48	7	4	6	104	131	9		
		3	5	-4	88	4	106	108	1	12	644	24		
		6	7	5	7	2	9	9	4	30	231	9		
		9	6	44	6	61	62	5	17	93	108	11		
		67	7	88	45	96	90	4	4	57	223	19		
		6	46	9.7	64	6	103	7	7	241	58	17		

Plate 4

												Control wells	Mean Min	Mean Max
^{51}Cr Values	23	38	24	84	35	222	32	150	31	326	40	32		
	23	36	47	85	132	35	37	42	33	33	101	33		
	28	27	233	92	44	134	231	235	37	42	38	49		
	336	129	36	335	302	34	249	387	48	29	380	49		
	354	31	198	247	47	286	231	50	34	212	70	60		
	374	231	28	141	150	170	41	141	27	45	79	50	25	355
% Specific Lysis		4	0	18	3	60	2	38	2	91	5	6		
		3	7	18	33	3	4	5	3	3	23	12		
		1	63	20	6	33	63	64	4	5	4	9		
		32	3	94	84	3	68	110	7	1	108	11		
		2	53	67	7	79	63	8	3	57	14	8		
		63	1	35	38	44	5	35	1	6	16	11		

Plate 5

												Control wells	Mean Min	Mean Max
^{51}Cr Values	22	24	211	120	209	42	187	226	35	57	54	41		
	27	24	176	174	249	262	34	45	51	42	129	58		
	23	79	45	36	51	99	204	324	308	66	813	51		
	320	61	201	85	208	35	48	41	34	300	367	55		
	365	235	62	267	54	46	170	76	45	144	201	47		
	253	151	251	37	38	36	217	168	48	97	409	56	24	313
% Specific Lysis		0	65	33	64	6	56	70	4	11	10.4	6		
		0	53	52	78	82	3	7	9	6	36	12		
		19	7	4	9	26	62	104	98	15	273	9		
		13	61	21	64	4	8	6	3	96	119	11		
		73	13	84	10.4	8	51	18	7	42	61	8		
		44	79	5	5	4	67	50	8	25	133	11		

Two Allogeneic Feeders

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	27	183	36	148	31	568	26	100	216	199	31	26		
	26	45	32	37	33	38	216	237	310	36	43	41		
	32	31	30	266	30	364	264	45	30	229	243	69		
	373	36	133	138	239	48	37	26	212	526	157	50		
	389	57	200	51	42	313	197	228	66	126	457	61		
	286	158	43	188	169	314	289	310	331	118	376	62	28	349
% Specific Lysis		48	2	37	1	168	-1	22	58	53	1	-1		
		5	1	3	1	3	58	65	88	2	5	4		
		1	1	74	1	105	73	5	1	63	67	13		
		2	33	34	66	6	3	-1	57	155	40	7		
		9	53	7	4	89	53	62	12	30	134	10		
		40	5	50	44	89	81	88	94	28	108	10		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	26	149	38	249	286	279	233	318	147	287	102	40		
	28	40	254	32	284	71	131	49	254	204	444	31		
	26	45	62	253	208	70	121	31	59	42	75	30		
	401	279	39	417	89	50	206	61	250	47	113	49		
	408	41	193	53	46	131	305	39	276	73	347	28		
	426	201	319	210	53	264	67	350	52	371	55	44	27	412
% Specific Lysis		32	3	58	67	66	54	76	31	68	20	3		
		3	59	1	67	12	27	6	59	46	108	1		
		5	9	59	47	11	25	1	8	4	13	1		
		66	3	101	16	6	47	9	58	5	22	6		
		4	43	7	5	27	72	3	65	12	83	0		
		45	76	48	7	62	10.5	84	7	89	7	5		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	36	35	272	136	378	277	265	37	37	51	125	47		
	34	38	45	71	62	57	176	273	108	291	307	85		
	24	230	41	333	32	51	36	184	290	320	42	48		
	411	301	32	334	280	319	365	403	299	289	48	36		
	364	287	39	80	303	69	75	295	220	66	283	47		
	394	64	24	300	50	305	57	168	271	53	51	46	31	390
% Specific Lysis		1	67	29	97	69	65	2	2	5	26	4		
		2	4	11	9	7	40	67	21	72	77	15		
		55	3	84	0	5	1	43	72	81	3	5		
		75	0	84	69	80	93	104	75	72	5	1		
		71	2	14	76	11	12	74	53	9.7	70	4		
		9	-2	75	5	76	7	38	67	6	5	4		

Plate 4

												Control wells	Mean Min	Mean Max
^{51}Cr Values	22	52	148	38	304	35	117	206	30	33	139	27		
	34	297	100	291	88	58	370	311	229	436	41	50		
	28	88	63	354	66	385	51	46	53	61	34	43		
	400	305	304	236	66	72	64	46	55	52	38	41		
	370	75	65	363	250	467	52	58	42	338	38	40		
	406	324	42	52	50	43	56	239	211	352	314	50	28	392
% Specific Lysis		7	33	3	76	2	24	49	1	1	30	0		
		74	20	72	16	8	94	78	55	112	4	6		
		16	9.6	90	10.4	98	6	5	7	9	2	4		
		76	76	57	10.4	12	9.9	5	7	7	3	4		
		13	10.2	92	61	121	7	8	4	85	3	3		
		81	4	7	6	4	8	58	50	89	79	6		

Plate 5

												Control wells	Mean Min	Mean Max
^{51}Cr Values	28	19	189	36	31	34	29	35	32	27	53	42		
	31	37	169	35	44	96	63	41	195	215	386	55		
	27	30	43	319	201	54	74	251	65	241	339	58		
	325	32	229	335	303	47	211	226	36	326	43	41		
	365	188	201	216	352	292	116	50	176	406	42	72		
	368	25	234	327	251	343	222	46	30	371	321	49	29	353
% Specific Lysis		-3	49	2	1	2	0	2	1	-1	8	4		
		3	43	2	5	21	11	4	51	58	110	8		
		0	4	90	53	8	14	69	11	66	96	9		
		1	62	95	85	6	56	61	2	92	4	4		
		49	53	58	100	81	27	7	45	116	4	13		
		-1	63	92	69	97	60	5	0	106	90	6		

Three Allogeneic Feeders

Plate 1

												Control wells	Mean Min	Mean Max
^{51}Cr Values	43	38	31	120	23	165	34	23	50	40	294	46		
	42	163	31	170	185	146	75	31	38	42	67	126		
	28	32	217	207	82	43	19	33	220	22	341	52		
	389	268	37	58	37	53	42	41	35	29	43	35		
	480	267	189	200	128	376	51	284	219	33	60	43		
	456	294	301	44	44	318	25	372	413	258	133	36	38	442
% Specific Lysis		0	-2	20	-4	32	-1	-4	3	1	63	2		
		31	-2	33	36	27	9	-2	0	1	7	22		
		-1	44	42	11	1	-5	-1	45	-4	75	4		
		57	0	5	0	4	1	1	-1	-2	1	-1		
		57	37	40	22	84	3	61	45	-1	6	1		
		63	65	2	2	69	-3	83	93	55	24	0		

Plate 2

												Control wells	Mean Min	Mean Max
^{51}Cr Values	28	194	288	210	383	369	352	35	40	31	59	25		
	40	269	343	40	59	108	85	275	37	249	339	53		
	30	55	281	280	39	173	48	67	38	50	52	51		
	456	30	40	247	328	98	52	61	142	48	269	43		
	463	319	374	51	71	269	235	47	41	57	54	39		
	459	197	39	313	41	224	41	201	266	66	77	32	33	459
% Specific Lysis		38	60	42	82	79	75	1	2	0	6	-2		
		55	73	2	6	18	12	57	1	51	72	5		
		5	58	58	1	33	4	8	1	4	5	4		
		-1	2	50	69	15	5	7	26	4	55	2		
		67	80	4	9	55	47	3	2	6	5	1		
		39	1	66	2	45	2	39	55	8	10.4	0		

Plate 3

												Control wells	Mean Min	Mean Max
^{51}Cr Values	35	31	55	71	54	36	118	60	34	220	51	64		
	19	147	275	44	360	295	340	186	295	443	368	81		
	22	258	58	264	301	45	132	39	42	127	57	57		
	424	39	43	222	359	294	337	264	50	372	108	69		
	423	40	44	314	45	154	329	69	31	85	36	45		
	409	56	194	82	290	197	339	297	39	47	43	49	25	419
% Specific Lysis		1	8	12	7	3	24	9	2	49	7	10		
		31	63	5	85	69	80	41	69	106	87	14		
		59	8	61	70	5	27	3	4	26	8	8		
		3	4	50	85	68	79	61	6	88	21	11		
		4	5	73	5	33	77	11	1	15	3	5		
		8	43	14	67	44	80	69	3	6	4	6		

Plate 4

												Control wells	Mean Min	Mean Max
^{51}Cr Values	25	88	41	236	144	59	31	295	30	67	42	102		
	25	67	32	45	56	315	34	120	338	362	195	36		
	27	32	22	35	230	160	104	28	56	46	60	30		
	373	202	29	122	47	269	188	39	44	357	223	40		
	398	51	43	395	404	224	88	212	113	379	284	40		
	401	39	142	201	43	124	237	284	303	45	172	20	26	391
% Specific Lysis		17	4	58	32	9	1	74	1	11	4	21		
		11	2	5	8	79	2	26	86	92	46	3		
		2	-1	3	56	37	21	1	8	6	9	1		
		48	1	26	6	67	44	4	5	91	54	4		
		7	5	101	104	54	17	51	24	97	71	4		
		4	32	48	5	27	58	71	76	5	40	-2		

Appendix IV Chapter 5; NK Clone Proliferation – Investigation into Number of Allogeneic Feeder Donors

Plate 1	1 Feeder ^{3}H Thymidine Incorporation (cpm)*										Control†	Mean Control‡
Test Well Values§	3157	6972	15184	303236	13799	6911	58520	6031	5105	5153	9780	
	4460	29581	9657	15400	8504	35367	16994	34234	12966	4691	1964	
	7393	7368	8305	8597	8825	7749	7736	15874	6189	20639	1372	
	7327	8770	8802	16459	95956	7555	4808	65780	6194	6425	3224	
	5700	6293	10233	6838	6382	21073	6774	11398	5740	2968	6491	
	9972	11165	109197	5051	3080	4407	97193	6461	4469	5476	4705	4589
Test Well Value/4x (Mean Control Value)	-15200	-11385	-3173	284879	-4558	-11446	40163	-12326	-13252	-13204		
	-13897	11224	-8700	-2957	-9853	17010	-1363	15877	-5391	-13666		
	-10964	-10989	-10052	-9760	-9532	-10608	-10621	-2483	-12168	2282		
	-11030	-9587	-9555	-1898	77599	-10802	-13549	47423	-12163	-11932		
	-12657	-12064	-8124	-11519	-11975	2716	-11583	-6959	-12617	-15389		
	-8385	-7192	90840	-13306	-15277	-13950	78836	-11896	-13888	-12881		

(*) ^{3}H Thymdine Incorporation values
(†) Control wells contained feeder cells only
(‡) Geometric mean of control wells
(§) Test wells containing a single NK clone and feeder cells

One Allogeneic Feeder

Plate 1	1 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	3157	6972	15184	303236	13799	6911	58520	6031	5105	5153	9780	
	4460	29581	9657	15400	8504	35367	16994	34234	12966	4691	1964	
	7393	7368	8305	8597	8825	7749	7736	15874	6189	20639	1372	
	7327	8770	8802	16459	95956	7555	4808	65780	6194	6425	3224	
	5700	6293	10233	6838	6382	21073	6774	11398	5740	2968	6491	
	9972	11165	109197	5051	3080	4407	97193	6461	4469	5476	4705	4589
Test Well Value/4x (Mean Control Value)	-15200	-11385	-3173	284879	-4558	-11446	40163	-12326	-13252	-13204		
	-13897	11224	-8700	-2957	-9853	17010	-1363	15877	-5391	-13666		
	-10964	-10989	-10052	-9760	-9532	-10608	-10621	-2483	-12168	2282		
	-11030	-9587	-9555	-1898	77599	-10802	-13549	47423	-12163	-11932		
	-12657	-12064	-8124	-11519	-11975	2716	-11583	-6959	-12617	-15389		
	-8385	-7192	90840	-13306	-15277	-13950	78836	-11896	-13888	-12881		
Plate 2												
Test Well Values	579	1347	1161	2256	2315	1601	1009	754	1180	1126	1721	
	531	27127	2950	5263	20925	19803	5080	789	14645	1894	1735	
	609	11274	11748	3132	19047	2367	1844	1206	2845	2625	5289	
	1575	2248	4086	1496	3142	3774	2226	1358	1913	11446	17414	
	6183	25887	3247	1623	2289	2687	2428	1987	5450	10108	3223	
	3631	2167	18791	5941	1807	2323	2741	47223	28564	3578	1569	5501
Test Well Value/4x (Mean Control Value)	5122	-19055	-16742	-1080	-2202	-16925	-21216	-7360	-20111	-20270		
	-10731	-10257	-18873	-2958	-19638	-20161	-20799	-19160	-19380	-16716		
	-19757	-17919	-20509	-18863	-18231	-19779	-20647	-20092	-10559	-4591		
	3882	-18758	-20382	-19716	-19318	-19577	-20018	-16555	-11897	-18782		
	-19838	-3214	-16064	-20198	-19682	-19264	25218	6559	-18427	-20436		
	-12980	-19283	-18832	2155	-18852	-328	-20303	-21415	-11715	-18645		

Plate 3	1 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	5030	5177	334865	131863	24994	8403	8178	8709	50349	102705	8798	
	7153	7835	8540	173730	10201	70474	94469	8340	8334	112624	8481	
	7837	8443	8516	10152	10025	9609	10775	11822	11639	61888	8489	
	91535	7550	50648	9163	141081	190305	10741	31510	31441	122123	10125	
	256973	8464	47123	29047	107959	53646	10113	12301	124016	48251	12341	
	8470	98951	8244	231571	8279	184131	10063	8909	63821	208027	10799	9839
Test Well Value/4x (Mean Control Value)	-34325	-34178	295510	92508	-14361	-30952	-31177	-30646	10994	63350		
	-32202	-31520	-30815	134375	-29154	31119	55114	-31015	-31021	73269		
	-31518	-30912	-30839	-29203	-29330	-29746	-28580	-27533	-27716	22533		
	52180	-31805	11293	-30192	101726	150950	-28614	-7845	-7914	82768		
	217618	-30891	7768	-10308	68604	14291	-29242	-27054	84661	8896		
	-30885	59596	-31111	192216	-31076	144776	-29292	-30446	24466	168672		
Plate 4												
Test Well Values	3843	5446	87976	7543	11302	5247	203065	8092	193571	7107	6955	
	5110	5206	6071	26397	4075	8288	4998	5645	5596	36374	7965	
	3738	14237	7099	4773	4224	92673	11176	4636	5344	5120	6751	
	3820	4000	45023	6184	3268	37615	33370	5830	5054	12600	6725	
	2925	5529	89330	7719	15477	70525	7273	5619	104772	15810	5778	
	53979	5043	5616	70665	16192	4308	3744	3457	4582	5350	4020	6366
Test Well Value/4x (Mean Control Value)	-21620	-20017	62513	-17920	-14161	-20216	177602	-17371	168108	-18356		
	-20353	-20257	-19392	934	-21388	-17175	-20465	-19818	-19867	10911		
	-21725	-11226	-18364	-20690	-21239	67210	-14287	-20827	-20119	-20343		
	-21643	-21463	19560	-19279	-22195	12152	7907	-19633	-20409	-12863		
	-22538	-19934	63867	-17744	-9986	45062	-18190	-19844	79309	-9653		
	28516	-20420	-19847	45202	-9271	-21155	-21719	-22006	-20881	-20113		

Plate 5	1 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	8106	178427	197085	63598	11073	17120	107913	8579	6254	6332	8366	
	5970	21150	17022	120449	203394	13722	11199	11008	7670	8060	7489	
	11322	13414	10336	11241	33043	220768	272316	260767	11045	23019	8861	
	11557	48774	168893	10435	8324	11015	9954	9537	15418	15885	9959	
	178091	9454	188826	9435	7788	64922	9835	8910	12079	10587	9881	
	303788	18156	7661	7268	7893	49014	10568	7370	7626	24273	9581	9023
Test Well Value/4x (Mean Control Value)	-27985	142336	160994	27507	-25018	-18971	71822	-27512	-29837	-29759		
	-30121	-14941	-19069	84358	167303	-22369	-24892	-25083	-28421	-28031		
	-24769	-22677	-25755	-24850	-3048	184677	236225	224676	-25046	-13072		
	-24534	12683	132802	-25656	-27767	-25076	-26137	-26554	-20673	-20206		
	142000	-26637	152735	-26656	-28303	28831	-26256	-27181	-24012	-25504		
	267697	-17935	-28430	-28823	-28198	12923	-25523	-28721	-28465	-11818		

Two Allogeneic Feeders

Plate 1	2 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	163930	6203	55465	6493	168482	10758	59	6342	92	6178	11954	
	6449	7754	12800	9307	8291	157087	107	21810	9781	8492	13672	
	6119	7169	15079	5021	38485	246716	266	9942	160274	66986	14748	
	5223	98528	47164	18034	5748	9362	225	171166	30509	9701	7475	
	7259	9714	4109	5957	226332	189219	484	12574	18085	18586	8118	
	177469	7359	282445	244427	72400	147484	143	231465	1163	89807	8805	10795
Test Well Value/4x (Mean Control Value)	120749	-36978	12284	-36688	125301	-32423	-43122	-36839	-43089	-37003		
	-36732	-35427	-30381	-33874	-34890	113906	-43074	-21371	-33400	-34689		
	-37062	-36012	-28102	-38160	-4696	203535	-42915	-33239	117093	23805		
	-37958	55347	3983	-25147	-37433	-33819	-42956	127985	-12672	-33480		
	-35922	-33467	-39072	-37224	183151	146038	-42697	-30607	-25096	-24595		
	134288	-35822	239264	201246	29219	104303	-43038	188284	-42018	46626		
Plate 2												
Test Well Values	40242	10135	131262	134457	45107	312024	243035	48064	529	399582	12109	
	9556	397494	9536	140054	6055	16013	10464	86567	44052	298197	10815	
	8242	7271	111674	89420	57909	105373	9800	84965	10346	302247	7938	
	12799	7879	342530	17423	14655	108072	10919	10392	8823	9149	8748	
	10634	120721	7487	10017	24326	113578	10386	329904	11678	189865	7649	
	288772	99199	389812	9052	271119	9408	79299	11249	314386	9202	8114	9229
Test Well Value/4x (Mean Control Value)	3327	-26780	94347	97542	8192	275109	206120	11149	-36386	362667		
	-27359	360579	-27379	103139	-30860	-20902	-26451	49652	7137	261282		
	-28673	-29644	74759	52505	20994	68458	-27115	48050	-26569	265332		
	-24116	-29036	305615	-19492	-22260	71157	-25996	-26523	-28092	-27766		
	-26281	83806	-29428	-26898	-12589	76663	-26529	292989	-25237	152950		
	251857	62284	352897	-27863	234204	-27507	42384	-25666	277471	-27713		

Plate 3	2 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	7799	247826	85742	305174	238312	58112	6849	8906	8992	14147	7726	
	7357	12731	10255	8720	8076	10900	279957	9328	142765	14815	7318	
	169781	5293	187825	5943	9443	8082	144176	116102	80197	8926	10113	
	171996	5851	206783	11480	231903	15386	31958	65908	222511	9142	8926	
	115983	6035	135711	68213	9484	6140	7443	223690	9458	263868	8629	
	12031	4483	157063	6777	217177	5137	65287	176291	9059	245014	7607	8387
Test Well Value/4x (Mean Control Value)	-25747	214280	52196	271628	204766	24566	-26697	-24640	-24554	-19399		
	-26189	-20815	-23291	-24826	-25470	-22646	246411	-24218	109219	-18731		
	136235	-28253	154279	-27603	-24103	-25464	110630	82556	46651	-24620		
	138450	-27695	173237	-22066	198357	-18160	-1588	32362	188965	-24404		
	82437	-27511	102165	34667	-24062	-27406	-26103	190144	-24088	230322		
	-21515	-29063	123517	-26769	183631	-28409	31741	142745	-24487	211468		
Plate 4												
Test Well Values	6197	98103	6436	294063	6224	342501	238411	8264	9550	80059	16509	
	189461	8316	8311	257366	6514	158544	132769	242334	123822	11227	15580	
	8602	6293	281309	6816	262541	9429	9643	9199	8678	10313	17965	
	296175	232775	9129	8128	8314	7418	6313	10864	9211	10736	13028	
	7871	8749	120413	166415	65457	6851	5795	8116	14219	8822	12022	
	8392	7455	7293	6414	5869	5874	7853	126530	303956	209801	10101	14201
Test Well Value/4x (Mean Control Value)	-50606	41300	-50367	237260	-50579	285698	181608	-48539	-47253	23256		
	132658	-48487	-48492	200563	-50289	101741	75966	185531	67019	-45576		
	-48201	-50510	224506	-49987	205738	-47374	-47160	-47604	-48125	-46490		
	239372	175972	-47674	-48675	-48489	-49385	-50490	-45939	-47592	-46067		
	-48932	-48054	63610	109612	8654	-49952	-51008	-48687	-42584	-47981		
	-48411	-49348	-49510	-50389	-50934	-50929	-48950	69727	247153	152998		

Plate 5	2 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	10365	234666	9268	7307	5907	7015	6994	6635	6998	7080	6400	
	5502	159643	6585	6441	7457	6745	5915	11593	7335	10458	8236	
	8252	180143	14355	9449	7879	10027	48627	8254	33552	102078	9110	
	8946	200197	90302	96121	7669	16968	73414	8285	217016	9919	13086	
	22795	216439	55794	47965	42695	10245	8912	57963	242521	8071	9203	
	7783	189615	277811	262199	13378	76052	6797	7828	304225	200475	9883	9320
Test Well Value/4x (Mean Control Value)	-26914	197387	-28011	-29972	-31372	-30264	-30285	-30644	-30281	-30199		
	-31777	122364	-30694	-30838	-29822	-30534	-31364	-25686	-29944	-26821		
	-29027	142864	-22924	-27830	-29400	-27252	11348	-29025	-3727	64799		
	-28333	162918	53023	58842	-29610	-20311	36135	-28994	179737	-27360		
	-14484	179160	18515	10686	5416	-27034	-28367	20684	205242	-29208		
	-29496	152336	240532	224920	-23901	38773	-30482	-29451	266946	163196		

Three Allogeneic Feeders

Plate 1	3 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	6935	6394	3730	5278	107889	8331	6275	5670	6730	70276	6375	
	15444	7676	59980	21167	7200	7646	6057	6758	5022	3215	7346	
	8521	56116	98787	8794	5608	7139	7493	109364	6366	58234	7059	
	179805	7794	5451	5358	3964	4089	5045	5261	5341	5727	7145	
	228413	183091	167139	97155	200494	7156	117974	44023	5227	4994	6829	
	129289	132805	6781	6543	148702	6138	220381	38897	162227	10328	7119	6979
Test Well Value/4x (Mean Control Value)	-20980	-21521	-24185	-22637	79974	-19584	-21640	-22245	-21185	42361		
	-12471	-20239	32065	-6748	-20715	-20269	-21858	-21157	-22893	-24700		
	-19394	28201	70872	-19121	-22307	-20776	-20422	81449	-21549	30319		
	151890	-20121	-22464	-22557	-23951	-23826	-22870	-22654	-22574	-22188		
	200498	155176	139224	69240	172579	-20759	90059	16108	-22688	-22921		
	101374	104890	-21134	-21372	120787	-21777	192466	10982	134312	-17587		
Plate 2												
Test Well Values	158725	234873	182505	224716	176085	135911	7653	2925	6806	173436	9035	
	232042	250853	9973	9694	78900	7726	126058	3115	7650	166025	7894	
	13238	30479	16739	7614	58343	7338	6444	4002	7232	8138	3254	
	7778	11932	285338	13922	9263	10822	7666	6402	4610	240929	8549	
	175359	334334	8113	10050	11553	24769	5292	5978	7264	7020	7005	
	32947	12556	117988	8854	56743	7391	270936	248576	7984	6402	5194	6822
Test Well Value/4x (Mean Control Value)	131438	207586	155218	197429	148798	108624	-19634	-24362	-20481	146149		
	204755	223566	-17314	-17593	51613	-19561	98771	-24172	-19637	138738		
	-14049	3192	-10548	-19673	31056	-19949	-20843	-23285	-20055	-19149		
	-19509	-15355	258051	-13365	-18024	-16465	-19621	-20885	-22677	213642		
	148072	307047	-19174	-17237	-15734	-2518	-21995	-21309	-20023	-20267		
	5660	-14731	90701	-18433	29456	-19896	243649	221289	-19303	-20885		

Plate 3	3 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	6625	13154	7906	7329	7690	134427	5827	6550	7284	6878	8280	
	18550	170253	7847	32622	8765	78164	10186	67888	51334	184383	10369	
	24995	8204	13037	103371	5513	12993	7838	5523	7434	7520	9496	
	10892	13415	10895	90700	13593	22266	105812	5682	154421	7461	10421	
	6385	8790	201243	6999	7388	93462	5410	5566	6295	5843	11166	
	4681	59620	7642	218303	11576	229305	78728	6293	5300	6636	8169	9650
Test Well Value/4x (Mean Control Value)	-31976	-25447	-30695	-31272	-30911	95826	-32774	-32051	-31317	-31723		
	-20051	131652	-30754	-5979	-29836	39563	-28415	29287	12733	145782		
	-13606	-30397	-25564	64770	-33088	-25608	-30763	-33078	-31167	-31081		
	-27709	-25186	-27706	52099	-25008	-16335	67211	-32919	115820	-31140		
	-32216	-29811	162642	-31602	-31213	54861	-33191	-33035	-32306	-32758		
	-33920	21019	-30959	179702	-27025	190704	40127	-32308	-33301	-31965		
Plate 4												
Test Well Values	6447	6023	62271	17968	6121	7239	128757	7205	6564	7292	11380	
	77163	8209	7811	7265	191555	7697	7099	32855	204784	52273	9614	
	5702	7972	5341	12836	11099	8425	7575	6769	6648	7714	10431	
	175298	5402	5377	5367	39134	66445	7237	6159	242647	11139	11326	
	134281	6713	11647	55197	6344	6657	15196	255500	266950	229745	7456	
	5048	63814	28072	4652	116424	153231	190207	19889	5845	10113	5039	9208
Test Well Value/4x (Mean Control Value)	-30384	-30808	25440	-18863	-30710	-29592	91926	-29626	-30267	-29539		
	40332	-28622	-29020	-29566	154724	-29134	-29732	-3976	167953	15442		
	-31129	-28859	-31490	-23995	-25732	-28406	-29256	-30062	-30183	-29117		
	138467	-31429	-31454	-31464	2303	29614	-29594	-30672	205816	-25692		
	97450	-30118	-25184	18366	-30487	-30174	-21635	218669	230119	192914		
	-31783	26983	-8759	-32179	79593	116400	153376	-16942	-30986	-26718		

Plate 5	3 Feeder ^{3}H Thymidine Incorporation (cpm)										Control	Mean Control
Test Well Values	153103	49560	7745	11784	10410	12233	41865	39891	19439	8790	12279	
	55059	10166	30881	10641	207757	67327	402256	19301	12891	263350	17238	
	57516	8677	12371	19924	288991	27736	265952	218197	11358	11437	14569	
	7517	139340	12123	112188	15583	16176	49365	125096	12605	14262	17899	
	4866	70069	8394	11722	10896	6172	226001	11840	12940	10139	14812	
	172709	8618	5293	109059	11091	10684	78966	12760	49782	219280	12788	14931
Test Well Value/4x (Mean Control Value)	93380	-10163	-51978	-47939	-49313	-47490	-17858	-19832	-40284	-50933		
	-4664	-49557	-28842	-49082	148034	7604	342533	-40422	-46832	203627		
	-2207	-51046	-47352	-39799	229268	-31987	206229	158474	-48365	-48286		
	-52206	79617	-47600	52465	-44140	-43547	-10358	65373	-47118	-45461		
	-54857	10346	-51329	-48001	-48827	-53551	166278	-47883	-46783	-49584		
	112986	-51105	-54430	49336	-48632	-49039	19243	-46963	-9941	159557		

Appendix V Chapter 6; NK Clone Responses to Uninfected and Strain AD169 Infected Autologous Fibroblasts

			% Specific Lysis ± SEM	
	Median CD94 Fluorescence	NK:T	Uninfected	Strain AD169
CD94hi				
D3.NKc1C11	457	7 to 1	0 ± 0.5	0 ± 0.4
D3.NKc3C11	293	11 to 1	0 ± 1	1 ± 1
D3.NKc5E11	267	46 to 1	1 ± 1	0 ± 1
D3.NKc5F8	542	20 to 1	0 ± 1	0 ± 2
D8.NKc1C10	178	58 to 1	48 ± 2	30 ± 2
D8.NKc1E11	337	21 to 1	29 ± 2	10 ± 3
D8.NKc1F11	235	44 to 1	24 ± 2	15 ± 2
D8.NKc2F3	500	25 to 1	38 ± 3	13 ± 3
D8.NKc2G9	294	5 to 1	2 ± 0.4	3 ± 1
D8.NKc3C8	365	26 to 1	8 ± 1	5 ± 1
D8.NKc3D9	305	11 to 1	11 ± 1	15 ± 1
D8.NKc3E9	470	32 to 1	35 ± 1	25 ± 2
D8.NKc4F3	209	25 to 1	48 ± 2	25 ± 0.3
D8.NKc4E10	236	44 to 1	13 ± 3	8 ± 1
D8.NKc5E7	319	14 to 1	7 ± 3	2 ± 0.2
D8.NKc5C11	225	63 to 1	36 ± 2	17 ± 2
D8.NKc7F8	470	8 to 1	10 ± 5	3 ± 1
D8.NKc8G2	365	26 to 1	21 ± 2	10 ± 2
D8.NKc9B4	407	34 to 1	0 ± 1	0 ± 1
D8.NKc9E3	1263	9 to 1	6 ± 1	0 ± 1
D8.NKc9G3	575	9 to 1	9 ± 1	2 ± 0.1
D8.NKc10C11	328	36 to 1	54 ± 4	12 ± 3
D8.NKc11D6	372	7 to 1	7 ± 2	0 ± 3
D8.NKc11F5	457	15 to 1	12 ± 3	8 ± 1
D8.NKc11F8	257	19 to 1	17 ± 1	6 ± 2
D8.NKc12E9	594	20 to 1	9 ± 3	1 ± 1
D8.NKc12E9	500	8 to 1	3 ± 1	1 ± 1
D8.NKc12F3	991	14 to 1	26 ± 5	4 ± 1
D8.NKc13E8	604	35 to 1	0 ± 1	1 ± 0.4
D8.NKc7B2	145	20 to 1	40 ± 3	16 ± 1
D9.NKc1C3	215	12 to 1	28 ± 2	18 ± 2
D9.NKc1C6	154	7 to 1	8 ± 1	7 ± 2
D9.NKc1C11	882	8 to 1	29 ± 6	10 ± 3
D9.NKc1E3	195	27 to 1	39 ± 2	33 ± 4
D9.NKc1E4	350	52 to 1	19 ± 1	13 ± 1
D9.NKc2B2	271	32 to 1	58 ± 4	46 ± 2
D9.NKc2B3	362	5 to 1	5 ± 2	7 ± 1
D9.NKc2E3	233	15 to 1	28 ± 1	18 ± 2
D9.NKc3B8	150	6 to 1	0 ± 0.3	4 ± 1

			% Specific Lysis ± SEM	
	Median CD94 Fluorescence	NK:T	Uninfected	Strain AD169
CD94hi				
D9.NKc3D4	567	13 to 1	3 ± 1	8 ± 1
D9.NKc4B2	505	18 to 1	30 ± 3	19 ± 2
D9.NKc4B3	380	8 to 1	2 ± 1	7 ± 1
D9.NKc4C3	365	10 to 1	27 ± 3	26 ± 1
D9.NKc4E8	196	6 to 1	15 ± 2	11 + 1
D9.NKc4F10	356	44 to 1	30 ± 2	19 ± 0.1
D9.NKc5F2	244	3 to 1	3 ± 1	4 ± 0.4
D9.NKc5G7	496	52 to 1	16 ± 3	15 ± 2
D9.NKc6G3	155	14 to 1	11 ± 2	10 ± 1
D9.NKc7B11	217	26 to 1	25 ± 3	21 ± 2
D9.NKc7E2	311	8 to 1	7 ± 2	6 ± 2
D9.NKc7E10	276	63 to 1	2 ± 2	0.1 ± 0.2
D9.NKc7E11	910	8 to 1	12 ± 1	6 ± 1
D9.NKc7G5	389	22 to 1	3 ± 1	1 ± 1
D9.NKc8B6	552	12 to 1	19 ± 2	8 ± 1
D9.NKc8E3	250	25 to 1	4 ± 2	13 ± 1
D9.NKc9G10	248	2 to 1	4 ± 1	3 ± 1
D9.NKc10D4	939	15 to 1	7 ± 2	7 ± 1
D9.NKc8G6	103	12 to 1	7 ± 3	2 ± 1
D9.NKc9C2	141	11 to 1	47 ± 1	26 ± 3
D9.NKc9C6	150	38 to 1	39 ± 0.4	36 ± 1
D9.NKc10G5	107	18 to 1	0 ± 1	1 ± 1
D9.NKc7F8	106	15 to 1	34 ± 5	18 ± 2
D7.NKcD10	673	91 to 1	30 ± 2	48 ± 3
D7.NKc4B7	281	21 to 1	21 ± 3	58 ± 1
D7.NKc5D6	449	58 to 1	0 ± 1	14 ± 2
D7.NKc4F3	167	29 to 1	6 ± 1	30 ± 4
D7.NKc3F5	279	49 to 1	17 ± 3	41 ± 1
D7.NKc3D5	205	44 to 1	0 ± 1	0 ± 1
D7.NKc6D11	311	30 to 1	11 ± 2	41 ± 1
D7.NKc6G3	155	30 to 1	83 ± 0.1	60 ± 1
D7.NKc4E7	1165	30 to 1	51 ± 6	52 ± 3
D7.NKc2B11	757	40 to 1	7 ± 2	38 ± 1
D7.NKc5B4	802	28 to 1	11 ± 2	12 ± 2
D7.NKc3E6	1032	62 to 1	35 ± 4	38 ± 1
D7.NKc5B8	638	87 to 1	10 ± 3	38 ± 4

			% Specific Lysis ± SEM	
	Median CD94 Fluorescence	NK:T	Uninfected	Strain AD169
CD94lo				
D3.NKc3B5	66	7 to 1	3 ± 2	13 ± 4
D3.NKc3C9	42	7 to 1	4 ± 3	9 ± 2
D8.NKc1B11	63	33 to 1	20 ± 5	5 ± 1
D9.NKc2G5	91	28 to 1	8 ± 1	4 ± 0.4
D9.NKc2G11	69	39 to 1	10 ± 2	26 ± 4
D9.NKc3B8	93	11 to 1	4 ± 3	9 ± 1
D9.NKc3B11	83	19 to 1	4 ± 1	1 + 0.4
D9.NKc3C2	69	5 to 1	5 ± 2	4 ± 1
D9.NKc3F2	15	24 to 1	0 ± 1	11 ± 1
D9.NKc4C11	79	34 to 1	10 ± 1	27 ± 1
D9.NKc4E3	47	8 to 1	3 ± 1	0.3 ± 1
D9.NKc5D2	21	14 to 1	4 ± 1	9 ± 1
D9.NKc7D7	37	7 to 1	22 ± 4	18 ± 1
D7.NKc3C7	41	23 to 1	2 ± 0.3	9 ± 1
D7.NKc2E2	72	69 to 1	26 ± 11	39 ± 6
D7.NKc3G2	50	30 to 1	24 ± 8	42 ± 6
D7.NKc2B2	23	30 to 1	21 1	21 ± 1
D7.NKc3F5	80	30 to 1	11 ± 1	23 ± 1
D7.NKc1G2	79	30 to 1	12 ± 2	43 ± 3
D7.NKc1D4	28	58 to 1	31 ± 2	25 ± 4
D7.NKc1F7	41	26 to 1	13 ± 3	3 ± 1
D7.NKc2C6	27	25 to 1	4 ± 1	9 ± 2
D7.NKc6C9	65	25 to 1	0 ± 1	10 ± 4
D7.NKc6D6	67	41 to 1	0 ± 1	12 ± 2
D3.NKc4G7	59	25 to 1	31 ± 3	41 ± 3

Appendix VI Chapter 7; NK Clone Responses to Strain AD169 and Strain Towne Infected Autologous Fibroblasts

			% Specific Lysis ± SEM	
	Median CD94 Fluorescence	NK:T	Strain AD169	Strain Towne
CD94hi				
D3.NKc1C11	457	7 to 1	0 ± 0.4	1 ± 0.8
D3.NKc3C11	293	11 to 1	1 ± 1	1 ± 1
D3.NKc5E11	267	40 to 1	15 ± 3	10 ± 2
D3.NKc5F8	542	20 to 1	0 ± 2	2 ± 1
D8.NKc1C10	178	58 to 1	30 ± 2	30 ± 1
D8.NKc1E11	337	21 to 1	10 ± 3	5 ± 0.2
D8.NKc1F11	235	44 to 1	15 ± 2	7 ± 1
D8.NKc2F3	500	25 to 1	13 ± 3	24 ± 1
D8.NKc2G9	294	5 to 1	3 ± 1	5 ± 1
D8.NKc3C8	365	26 to 1	5 ± 1	3 ± 1
D8.NKc3D9	305	11 to 1	15 ± 1	13 ± 1
D8.NKc3E9	470	32 to 1	25 ± 2	17 ± 1
D8.NKc4F3	209	25 to 1	25 ± 0.3	22 ± 3
D8.NKc4E10	236	44 to 1	8 ± 1	3 ± 1
D8.NKc5E7	319	14 to 1	2 ± 0.2	3 ± 0.1
D8.NKc5C11	225	63 to 1	17 ± 2	19 ± 1
D8.NKc7F8	470	8 to 1	3 ± 1	4 ± 1
D8.NKc8G2	365	26 to 1	10 ± 2	11 ± 1
D8.NKc9B4	407	34 to 1	0 ± 1	1 ± 1
D8.NKc9E3	1263	9 to 1	0 ± 1	4 ± 1
D8.NKc9G3	575	9 to 1	2 ± 0.1	6 ± 4
D8.NKc10C11	328	36 to 1	12 ± 3	17 ± 2
D8.NKc11D6	372	7 to 1	0 ± 3	7 ± 3
D8.NKc11F5	457	15 to 1	8 ± 1	8 ± 2
D8.NKc11F8	257	19 to 1	6 ± 2	4 ± 0.2
D8.NKc12E9	594	20 to 1	1 ± 1	1 ± 1
D8.NKc12F3	991	14 to 1	4 ± 1	6 ± 0.1
D8.NKc13E8	604	35 to 1	1 ± 0.4	4 ± 3
D8.NKc7B2	145	20 to 1	16 ± 1	9 ± 1
D9.NKc1C3	215	12 to 1	18 ± 2	10 ± 1
D9.NKc1C6	154	7 to 1	7 ± 2	2 ± 0.2
D9.NKc1C11	882	8 to 1	10 ± 3	8 + 1
D9.NKc1E3	195	27 to 1	33 ± 4	26 ± 1
D9.NKc1E4	35	52 to 1	13 ± 1	5 ± 2
D9.NKc2B2	271	32 to 1	46 ± 2	43 ± 1
D9.NKc2B3	362	5 to 1	7 ± 1	4 ± 1
D9.NKc2E3	233	15 to 1	18 ± 2	17 ± 1
D9.NKc3C4	42	16 to 1	10 ± 0.4	5 ± 1
D9.NKc3C11	118	6 to 1	6 ± 1	6 ± 1
D9.NKc3D4	567	13 to 1	8 ± 1	1 ± 1

			% Specific Lysis ± SEM	
	Median CD94 Fluorescence	NK:T	Strain AD169	Strain Towne
CD94hi				
D9.NKc4B2	505	18 to 1	19 ± 2	8 ± 0.3
D9.NKc4B3	388	8 to 1	7 ± 1	3 ± 1
D9.NKc4C3	365	10 to 1	26 ± 1	25 ± 2
D9.NKc4E8	196	6 to 1	11 + 1	13 ± 2
D9.NKc4F10	356	45 to 1	18 ± 2	26 ± 2
D9.NKc5F2	244	3 to 1	4 ± 0.4	3 ± 1
D9.NKc5G7	496	52 to 1	15 ± 2	10 ± 2
D9.NKc6G3	155	14 to 1	10 ± 1	7 ± 1
D9.NKc7B11	217	26 to 1	21 ± 2	15 ± 1
D9.NKc7E2	311	8 to 1	6 ± 2	2 ± 1
D9.NKc7E10	276	63 to 1	0.1 ± 0.2	2 ± 2
D9.NKc7E11	100	8 to 1	6 ± 1	7 ± 1
D9.NKc7G5	389	22 to 1	1 ± 1	3 ± 0.3
D9.NKc8B6	552	21 to 1	1 ± 1	2 ± 1
D9.NKc8E3	250	25 to 1	13 ± 1	10 ± 1
D9.NKc9G10	248	2 to 1	3 ± 1	3 ± 1
D9.NKc10D4	939	30 to 1	7 ± 1	8 ± 1
D9.NKc10E2	233	14 to 1	37 ± 0.1	37 ± 1
D9.NKc8G6	103	12 to 1	2 ± 1	3 ± 1
D9.NKc9C2	141	11 to 1	26 ± 3	31 ± 2
D9.NKc9C6	150	38 to 1	36 ± 1	29 ± 1
D9.NKc10G5	107	18 to 1	1 ± 1	0 ± 3
D9.NKc7F8	106	15 to 1	18 ± 2	22 ± 1
D7.NKcD10	673	91 to 1	48 ± 3	51 ± 1
D7.NKc4B7	281	21 to 1	58 ± 1	34 ± 6
D7.NKc5D6	449	58 to 1	14 ± 2	5 ± 4
D7.NKc4F3	167	29 to 1	30 ± 4	23 ± 3
D7.NKc3F5	279	49 to 1	41 ± 1	26 ± 2
D7.NKc3D5	205	44 to 1	0 ± 1	5 ± 1
D7.NKc6D11	311	30 to 1	41 ± 1	28 ± 4
D7.NKc6G3	155	30 to 1	60 ± 1	79 ± 1
D7.NKc4E7	1165	30 to 1	52 ± 3	61 ± 5
D7.NKc2B11	757	40 to 1	38 ± 1	40 ± 3
D7.NKc5B4	802	28 to 1	12 ± 2	10 ± 1
D7.NKc3E6	1032	62 to 1	38 ± 1	36 ± 4
D7.NKc5B8	638	87 to 1	38 ± 4	31 ± 6
D3.NKc2B4	107	30 to 1	0 ± 0	3 ± 1
D3.NKc.3D8	685	30 to 1	0 ± 0	2 ± 0
D3.NKc.3F11	500	26 to 1	0 ± 1	4 ±1
D3.NKc.2E8	850	37 to 1	0 ± 0	2 ± 1
D3.NKc.1F12	450	30 to 1	8 ± 1	27 ± 1
D3.NKc.6E12	178	30 to 1	3 ± 1	0 ± 0

			% Specific Lysis ± SEM	
	Median CD94 Fluorescence	NK:T	Strain AD169	Strain Towne
CD94lo				
D3.NKc3B5	66	7 to 1	13 ± 4	9 ± 4
D3.NKc3C9	42	7 to 1	9 ± 2	5 ± 1
D3.NKc4G7	59	25 to 1	41 ± 3	42 ± 1
D8.NKc1B11	63	33 to 1	5 ± 1	4 ± 0.4
D9.NKc2G5	91	28 to 1	4 ± 0.4	2 ± 1
D9.NKc2G11	69	39 to 1	26 ± 4	5 ± 2
D9.NKc3B8	93	11 to 1	9 ± 1	4 ± 1
D9.NKc3B11	83	19 to 1	1 + 0.4	0.2 ± 2
D9.NKc3C2	69	5 to 1	4 ± 1	2 ± 1
D9.NKc3F2	15	24 to 1	11 ± 1	1 ± 1
D9.NKc4C11	79	50 to 1	12 ± 1	8 ± 1
D9.NKc4E3	47	8 to 1	0.3 ± 1	1 + 0.4
D9.NKc5D2	21	14 to 1	9 ± 1	3 ± 1
D9.NKc7D7	37	7 to 1	18 ± 1	14 ± 1
D9.NKc7G8	44	27 to 1	36 ± 3	36 ± 2
D7.NKc3C7	41	23 to 1	9 ± 1	1 ± 1
D7.NKc2E2	72	30 to 1	21 ± 1	11 ± 0.3
D7.NKc3G2	50	30 to 1	42 ± 6	32 ± 2
D7.NKc2B2	23	30 to 1	21 ± 1	23 ± 3
D7.NKc3F5	80	30 to 1	23 ± 1	24 ± 2
D7.NKc1G2	79	30 to 1	43 ± 3	38 ± 2
D7.NKc1D4	28	58 to 1	25 ± 4	46 ± 2
D7.NKc1F7	41	26 to 1	3 ± 1	9 ± 1
D7.NKc2C6	27	25 to 1	9 ± 2	4 ± 1
D7.NKc6C9	65	25 to 1	10 ± 4	7 ± 1
D7.NKc6D6	67	41 to 1	12 ± 2	4 + 0.3

Appendix VII Chapter 7; NK Clone Responses to Strain Toledo and Recombinant Adenovirus Expressing gpUL40

			% Specific Lysis ± SEM						
Clone	Median CD94 Fluoure - scence	NK:T	RAd141 Control	RAd141	RAd141-GFP Control	RAd141 -GFP	UI	AD169	Toledo
D3.NKc5E11	267	32 to 1			9 ± 1	8 ± 1			
		24 to 1	19 ± 1	5 ± 1					
		40 to 1					1 ± 1	15 ± 3	4 ± 1
D3.NKc5F8	542	20 to 1			21 ± 2	20 ± 1	0 ± 1	0 ± 2	0 ± 1
		55 to 1	55 ± 1	25 ± 3					
D3.NKc3C9	42	7 to 1					4 ± 3	9 ± 2	5 ± 1
		24 to 1			20 ± 1	14 ± 1			
		100 to 1	9 ± 0.4	3 ± 1					
D3.Nkc1C11	457	7 to 1					0 ± 0.5	0 ± 0.4	3 ± 2
D3.Nkc3C11	293	11 to 1					0 ± 1	1 ± 1	1 ± 1
D8.Nkc1B11	63	22 to 1			49 ± 3	35 ± 2			
		33 to 1					20 ± 5	5 ± 1	4 ± 0
D8.Nkc1C10	178	8 to 1	21 ± 1	7 ± 1					
		27 to 1			39 ± 0.1	28 ± 0.1			
		58 to 1					48 ± 2	30 ± 2	13 ± 2
D8.Nkc1E11	337	21 to 1					29 ± 2	10 ± 3	4 ± 0.4
		23 to 1			33 ± 4	30 ± 2			
		40 to 1	18 ± 1	16 ± 2					
D8.Nkc1F11	235	19 to 1			23 ± 1	17 ± 1			
		32 to 1	19 ± 1	9 ± 1					
		44 to 1					24 ± 2	15 ± 2	3 ± 1
D8.Nkc2F3	500	20 to 1			31 ± 3	23 ± 2			
		25 to 1					38 ± 3	13 ± 3	4 ± 1
D8.Nkc3C8	365	20 to 1			21 ± 1	19 ± 3			
		26 to 1					8 ± 1	5 ± 1	3 ± 1
		35 to 1	24 ± 1	8 ± 1					
D8.Nkc3E9	470	18 to 1			11 ± 1	10 ± 3			
		32 to 1					35 ± 1	25 ± 2	9 ± 1
D8.Nkc4F3	209	10 to 1			39 ± 1	21 ± 1			
		25 to 1					48 ± 2	25 ± 0.3	10 ± 2
D8.Nkc4E10	236	20 to 1			14 ± 2	13 ± 2			
		23 to 1	30 ± 3	11 ± 2					
		44 to 1					13 ± 3	8 ± 1	6 ± 1
D8.Nkc5C11	235	50 to 1			34 ± 1	40 ± 3			
		63 to 1					36 ± 2	17 ± 2	13 ± 1
		66 to 1	36 ± 3	23 ± 3					
D8.Nkc2G9	294	5 to 1					2 ± 0.4	3 ± 1	3 ± 0.3
D8.NKc3D9	305	11 to 1					11 ± 1	15 ± 1	8 ± 1

			% Specific Lysis ± SEM					
Clone	Median CD94 Fluoure - scence	NK:T	RAd141 Control	RAd141	RAd141-GFP Control	RAd141 -GFP	UI	AD169	Toledo
D8.NKc5E7	319	14 to 1					7 ± 3	2 ± 0.2	2 ± 0.3
D3.NKc3B5	66	7 to 1					3 ± 2	12 ± 4	9 ± 4
D9.NKc1C3	215	12 to 1			30 ± 3	15 ± 3			
D9.NKc1C6	154	7 to 1			10 ± 1	0 ± 3			
D9.NKc1C11	882	8 to 1			23 ± 3	3 ± 2			
D9.NKc1E3	195	27 to 1			42 ± 5	19 ± 4			
D9.NKc1E4	35	52 to 1			37 ± 4	17 ± 4			
D9.NKc2B2	271	32 to 1			64 ± 3	27 ± 8			
D9.NKc2B3	362	5 to 1			8 ± 3	0 ± 5			
D9.NKc2E3	233	15 to 1			29 ± 6	26 ± 2			
D9.NKc3B8	ND	6 to 1			13 ± 1	5 ± 4			
D9.NKc3C4	42	16 to 1			23 ± 2	12 ± 1			
D9.NKc3C11	118	6 to 1			23 ± 2	15 ± 3			
D9.NKc3D4	567	30 to 1			48 ± 2	34 ± 3			
D9.NKc4B2	505	18 to 1			27 ± 4	17 ± 4			
D9.NKc4B3	38	8 to 1			28 ± 2	17 ± 2			
D9.NKc4C3	365	10 to 1			30 ± 3	25 ± 8			
D9.NKc4E8	196	6 to 1			18 ± 2	16 ± 6			
D9.NKc4F10	356	75 to 1			41 ± 1	20 ± 1			
D9.NKc5F2	244	30 to 1			29 ± 2	13 ± 3			
D9.NKc5G7	496	78 to 1			30 ± 3	20 ± 0.1			
D9.NKc6G3	155	14 to 1			9 ± 3	5 ± 3			
D9.NKc7B11	217	26 to 1			23 ± 4	20 ± 7			
D9.NKc7E2	311	8 to 1			11 ± 4	8 ± 1			
D9.NKc7E10	276	63 to 1			1 ± 2	2 ± 2			
D9.NKc7E11	91	8 to 1			23 ± 2	11 ± 2			
D9.NKc7G5	389	22 to 1			3 ± 2	4 ± 2			
D9.NKc8B6	552	12 to 1			28 ± 4	11 ± 1			
D9.NKc8E3	250	25 to 1			15 ± 4	7 ± 1			
D9.NKc9G10	248	2 to 1			10 ± 4	7 ± 2			
D9.NKc10D4	939	30 to 1			1 ± 0.1	2 ± 1			
D9.NKc10E2	233	10 to 1			19 ± 1	9 ± 3			
D9.NKc8G6	103	140 to 1			28 ± 4	12 ± 1			
D9.NKc9C2	141	11 to 1			44 ± 2	30 ± 5			
D9.NKc9C6	150	5 to 1			21 ± 3	17 ± 3			
D9.NKc10G5	107	18 to 1			1 ± 2	7 ± 2			
D9.NKc7F8	106	15 to 1			35 ± 0.1	20 ± 2			
D9.NKc2G5	91	28 to 1			10 ± 3	10 ± 3			
D9.NKc2G11	69	38 to 1			70 ± 4	41 ± 4			
D9.NKc3B8	93	11 to 1			12 ± 1	4 ± 1			
D9.NKc3B11	83	19 to 1			1 ± 3	28 ± 1			

			% Specific Lysis ± SEM						
Clone	Median CD94 Fluoure - scence	NK:T	RAd141 Control	RAd141	RAd141-GFP Control	RAd141 -GFP	UI	AD169	Toledo
D9.NKc3C2	69	5 to 1			6 ± 4	12 ± 2			
D9.NKc3F2	15	24 to 1			27 ± 2	8 ± 0.4			
D9.NKc4C11	79	34 to 1			45 ± 6	20 ± 1			
D9.NKc4E3	47	8 to 1			6 ± 5	13 ± 2			
D9.NKc5D2	21	14 to 1			35 ± 2	19 ± 1			
D9.NKc7D7	37	7 to 1			30 ± 3	17 ± 2			
D9.NKc7G8	44	16 to 1			34 ± 2	21 ± 2			
D9.NKc5E11	ND	21 to 1			11 ± 0.1	11 ± 2			
D7.NKc2B2	23	30 to 1	37±2	9±1					
D7.NKc3F5	80	30 to 1	48±3	13±2					
D7.NKc1G2	79	30 to 1	45 ± 3	11 ± 2					
D7.NKc6D11	311	30 to 1	35 ± 3	0 ± 0					
D7.NKc6G3	155	30 to 1	70 ± 1	52 ± 6					
D7.NKc4E7	1165	30 to 1	62 ± 3	28 ± 3					
D7.NKc2G2	ND	30 to 1	49 ± 3	23 ± 2					
D7.NKc2E2	316	30 to 1	41± 4	11± 1					
D7.NKc5D6	449	58 to 1	10 ±3	1 ± 1					
D7.NKcD10	673	91 to 1	60 ± 3	15 ± 3					
D7.NKc2B11	757	40 to 1	50 ± 4	14 ± 2					
D7.NKc5B4	802	28 to 1	24 ± 1	7 ± 1					
D7.NKc3E6	1032	62 to 1	46 ± 3	31 ± 4					
D7.NKc5B8	638	87 to 1	41 ± 8	13 ± 1					
D7.NKc1C9	ND	112 to 1	49 ± 5	38 ± 5					
D7.NKc2F3	ND	30 to 1	26 ± 3	6 ± 1					
D7.NKc2F9	ND	30 to 1	34 ± 2	12 ± 3					
D7.NKc1F11	ND	30 to 1	4 ± 3	3 ± 1					
D7.NKc2G10	ND	30 to 1	52 ± 3	25 ± 2					
D7.NKc2E6	ND	30 to 1	6 ± 3	2 ± 0					
D7.NKc2G11	69	30 to 1	40 ± 4	18 ± 1					
D3.NKc3D8	685	30 to 1	24 ± 2	12 ± 5					
D3.NKc2F7	ND	32 to 1	16 ± 2	11 ± 1					
D3.NKc2E8	850	37 to 1	1 ± 1	0 ± 1					
D3.NKc2E12	227	20 to 1	5 ± 1	0 ± 0					
D3.NKc2D12	126	31 to 1	7 ± 1	0 ± 1					
D3.NKc6E12	178	30 to 1	6 ± 1	0 ± 1					
D3.NKc1C8	ND	28 to 1	3 ± 2	2 ± 0					
D3.NKc1G10	698	20 to 1	2 ± 1	0 ± 0					
D3.NKc3C11	293	25 to 1	4 ± 0	11 ± 1					

Appendix VIII Publications

Published

Tomasec, P., Wang, E.C., Davison, A.J., Vojtesek, B., Armstrong, M., Griffin, C., McSharry, B.P., Morris, R.J., Llewellyn-Lacey, S., Rickards, C., Nomoto, A., Sinzger, C., & Wilkinson, G.W.

Downregulation of natural killer cell-activating ligandCD155 by human cytomegalovirus UL141.

Nature Immunology (2005).

Submitted for Publication

Morris, R.J., Chong, L.C., Wang, E.C.Y.

Development and Characterisation of a Novel, High-efficiency System of Natural Killer Cell Cloning.

Submitted to Journal of Immunological Methods, March 2005.

nature
immunology

Downregulation of natural killer cell–activating ligand CD155 by human cytomegalovirus UL141

Peter Tomasec[1,6], Eddie C Y Wang[1,6], Andrew J Davison[2], Borivoj Vojtesek[3], Melanie Armstrong[1], Cora Griffin[1], Brian P McSharry[1], Rebecca J Morris[1], Sian Llewellyn-Lacey[1], Carole Rickards[1], Akio Nomoto[4], Christian Sinzger[5] & Gavin W G Wilkinson[1]

Natural killer (NK) cells are crucial in the control of cytomegalovirus infections in mice and humans. Here we show that the viral UL141 gene product has an immunomodulatory function that is associated with low-passage strains of human cytomegalovirus. UL141 mediated efficient protection of cells against killing by a wide range of human NK cell populations, including interferon-α-stimulated bulk cultures, polyclonal NK cell lines and most NK cell clones tested. Evasion of NK cell killing was mediated by UL141 blocking surface expression of CD155, which was previously identified as a ligand for NK cell-activating receptors CD226 (DNAM-1) and CD96 (TACTILE). The breadth of the UL141-mediated effect indicates that CD155 has a key role in regulating NK cell function.

Human cytomegalovirus (HCMV) is a ubiquitous human herpesvirus normally associated with subclinical primary infection followed by lifelong asymptomatic carriage during which the innate and adaptive immune responses act in concert to limit the consequences of infection. However, HCMV infections can cause severe disease in people whose immune systems are immature (such as those with congenital cytomegalic inclusion disease) or compromised (such as transplant recipients or patients with AIDS). The interplay between the virus and the immune system is central to our understanding of HCMV pathogenesis. People with defects in natural killer (NK) cell function are extremely sensitive to herpesvirus infections and to HCMV in particular[1].

NK cell cytotoxicity is regulated by a fine balance of signals received by activating and inhibitory receptors. Interactions between endogenous human leukocyte antigen (HLA) class I molecules and NK cell inhibitory receptors normally dominate over signals received from activating receptors. The HCMV genome encodes four proteins that act in concert to downregulate HLA class I expression and thus would be predicted to render HCMV-infected cells susceptible to NK cell recognition[2]. However, responses of individual NK cell clones and lines to fibroblasts infected with HCMV vary greatly, ranging from activation to inhibition[3]. The mechanisms that determine whether activation or inhibition dominates during HCMV infection remain unclear, but the resistance of HCMV-infected cells to a proportion of NK cells can be attributed to specific virus genome–encoded products that mediate NK cell–evasion functions. A peptide derived from the leader sequence of HCMV gpUL140 stimulates cell surface expression of the nonclassical HLA class I molecule HLA-E independent of the transporters associated with antigen processing; HLA-E binds the NK cell inhibitory receptor complex CD94-NKG2A to suppress cytotoxicity mediated by $CD94^{+}NKG2A^{+}$ NK cells[4,5]. Several human UL16-binding proteins (ULBPs) were identified and named for their affinity for the HCMV protein UL16. ULBP1–ULBP3, major histocompatibility complex class I–related chain A (MICA) and MICB each bind the activating receptor NKG2D to stimulate NK cell functions. The protein gpUL16 acts by sequestering MICB, ULBP1 and ULBP2 in the endoplasmic reticulum. By impeding cell surface expression of these NKG2D ligands, UL16 suppresses NK cell recognition[6–8]. UL18 has long been known to be an HLA class I homolog with affinity for the inhibitory leukocyte immunoglobulin-like receptor 1 (also called immunoglobulin-like transcript 2), but its function in HCMV evasion of NK cell recognition remains controversial[9].

HCMV has the largest genome (236 kilobases (kb)) of any characterized human virus, and most of its genes are nonessential for replication in fibroblasts *in vitro*[10]. Extensive *in vitro* passage of laboratory strains results in an accumulation of genetic defects, the most notable being the 15-kb and 13-kb deletions of strains AD169 and Towne, respectively, affecting one end of the long unique (U_L) region commonly referred to as the U_L/b' sequence[11]. When assessed as potential vaccine candidates, both strains showed reduced virulence *in vivo*[12]. We were interested in the observation that cells infected with HCMV clinical isolates or the Toledo strain, which has been less extensively cultured *in vitro*, routinely provided substantially more protection against NK cell–mediated cytolysis than could be achieved with high-passage laboratory isolates[5,13]. The U_L/b' sequence deleted

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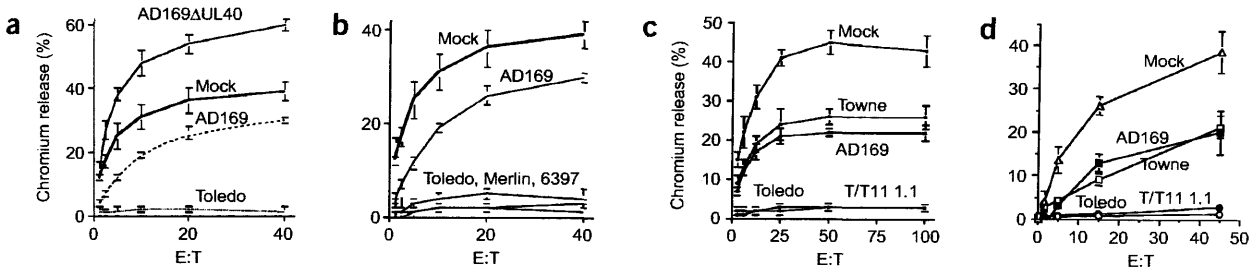

Figure 1 Resistance to NK cell attack maps to the HCMV U_L/b' sequence. (**a**,**b**) Human fetal foreskin fibroblasts (HFFFs) were mock infected (Mock) or were infected for 72 h with HCMV strains AD169, Toledo, Merlin or 6397 or a strain AD169ΔUL40 deletion mutant (multiplicity of infection, 10). NKL cells were used as effectors in allogeneic chromium release assays. (**c**) HFFFs were infected with strains AD169, Towne, Toledo or the recombinant virus Towne/Tol11 1.1 (T/T11 1.1) and NKLs were used as effectors in allogeneic NK cell assays. (**d**) Autologous assay with D9SF targets (primary skin fibroblast from donor 9) infected as described in **c** with IFN-α-activated bulk cultures derived from D9 PBMCs (D9NKb) as effectors. Results are mean ± s.d. of triplicate or quadruplicate cultures and are representative of at least three and up to eight independent experiments. E:T, effector:target ratio.

from strain AD169 is predicted to contain 23 genes that include many with potential immunomodulatory functions[14–16]. In a systematic search, we have identified and characterized an extremely efficient HCMV NK evasion function that mapped within the U_L/b' sequence to UL141.

RESULTS

NK cell protection associated with HCMV U_L/b'

We initially investigated the susceptibility of cells infected with different HCMV strains to NK cell–mediated cytolysis with the transformed NK cell clone NKL as effectors against allogeneic primary human fetal foreskin fibroblasts or primary skin fibroblast targets. Consistent with previous findings, more protection against NK cell–mediated cytolysis was achieved by infection with the laboratory strain AD169 than with mock-infected cells, whereas infection with an HCMV UL40 deletion mutant (AD169ΔUL40) enhanced killing[5] (**Fig. 1a**). However, nearly complete NK cell inhibition was achieved after infection with HCMV clinical isolates (6397 or Merlin) or the Toledo strain (**Fig. 1a,b**). Because certain NK cell receptors are sensitive to interactions with allogeneic HLA class I molecules, we also set up assays with matched NK cell effectors and skin fibroblasts derived from the same volunteer donors. We used three different types of NK cell effectors: bulk cultures depleted of T cells and activated with interferon-α (IFN-α), representing the broad NK cell response (D#NKb; in these designations, '#' indicates the donor number); polyclonal NK cell lines (D#NKp); and NK cell clones derived from single-cell sorting (D#NKc). With NKL (**Fig. 1c**) and bulk cultures from three separate donors (**Fig. 1d** and **Table 1**), minimal killing was detectable against strain Toledo–infected cells, whereas cells infected with strain AD169 or strain Towne showed intermediate killing or killing comparable to that of mock-infected cells. To further characterize this enhanced protection, we tested a panel of 15 NK cell clones. Of these, nine clones capable of killing mock-infected targets (more than 10% killing) showed less killing against targets infected with strain Toledo than against those infected with strain AD169 (**Table 1**). Indeed, eight clones showed more than 10% killing against targets infected with strain AD169 compared with only two clones showing similar killing efficiency against cells infected with strain Toledo. Thus, results from NK cell bulk cultures, polyclonal lines and individual NK cell clones are all consistent with strain Toledo exhibiting an additional strong inhibitory function effective against most NK cells.

Table 1 Susceptibility of autologous HCMV-infected cells to NK cell–mediated lysis

NK cell	Target	NK:target	Mock	+ AD169	+ Toledo	*P*
Primary T cell–depleted PBMC bulk cultures						
D3NKb.1	D3SF	20:1	40 ± 6	21 ± 1	1 ± 1	0.00005
D3NKb.2	D3SF	30:1	10 ± 1	13 ± 1	1 ± 1	0.0002
D7NKb.1	D7SF	18:1	20 ± 2	18 ± 3	1 ± 4	0.0005
D7NKb.2	D7SF	20:1	10 ± 2	16 ± 2	0 ± 1	0.002
D7NKb.3	D7SF	25:1	56 ± 6	37 ± 2	4 ± 2	0.000002
D7NKb.4	D7SF	25:1	ND	33 ± 1	0 ± 1	0.0000001
D9NKb.1	D9SF	45:1	40 ± 5	20 ± 5	3 ± 1	0.01
D9NKb.2	D9SF	39:1	40 ± 4	14 ± 1	2 ± 1	0.00008
NK clones						
D3NKc5E11	D3SF	46:1	1 ± 1	1 ± 1	2 ± 1	NS
D3NKc5E11	D3SF	40:1	1 ± 1	15 ± 3	4 ± 1	0.03
D3NKc5F8	D3SF	20:1	1 ± 1	1 ± 2	1 ± 1	NS
D8NKc1C10	D8SF	58:1	48 ± 2	30 ± 2	13 ± 2	0.0003
D8NKc1E11	D8SF	21:1	29 ± 2	10 ± 3	4 ± 1	NS
D8NKc1F11	D8SF	44:1	24 ± 2	15 ± 2	3 ± 1	0.0008
D8NKc2F3	D8SF	25:1	38 ± 3	13 ± 3	4 ± 1	0.009
D8NKc2G9	D8SF	5:1	2 ± 1	3 ± 1	3 ± 1	NS
D8NKc3C8	D8SF	26:1	8 ± 1	5 ± 1	3 ± 1	NS
D8NKc3D9	D8SF	11:1	11 ± 1	15 ± 1	8 ± 1	NS
D8NKc3E9	D8SF	32:1	35 ± 1	25 ± 2	9 ± 1	0.03
D8NKc4F3	D8SF	25:1	48 ± 2	25 ± 1	10 ± 2	0.04
D8NKc4E10	D8SF	44:1	13 ± 3	8 ± 1	6 ± 1	NS
D8NKc5E7	D8SF	14:1	7 ± 3	2 ± 1	2 ± 1	NS
D8NKc5C11	D8SF	63:1	36 ± 2	17 ± 2	13 ± 1	NS

Data are presented as percent chromium release ± s.e.m. D#SF, primary skin fibroblast; the number (#) designates the donor. ND, not done. *P* values compare killing of strain AD169–infected targets versus strain Toledo–infected targets (*t*-tests, assuming unequal variance); NS, not significant ($P > 0.05$).

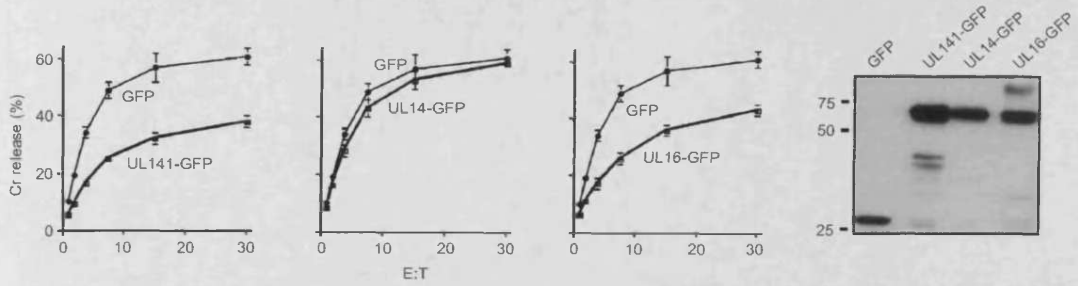

Figure 2 UL141 induces protection against NK cell attack. Cytotoxicity assay with NKLs as the effectors against stable HEK 293 cell lines expressing UL141-GFP, UL14-GFP or UL16-GFP fusion proteins or GFP alone. To ensure homogeneous expression, we sorted cell lines on the basis of GFP fluorescence before the assay. Far right, immunoblot with a GFP-specific rabbit polyclonal antibody, indicating fusion proteins were being expressed by the transfected cell lines. Left margin, molecular sizes (in kDa). Data are representative of three experiments.

One or more NK cell–evasion function(s) must have been lost from the high-passage HCMV laboratory strains either through the deletion of the U_L/b' sequence or as a consequence of mutation elsewhere on their genomes. HCMV Towne/Toll1 1.1 corresponds to a recombinant strain Towne virus in which the U_L/b' from strain Toledo has been inserted[17]. The addition of this Toledo sequence in the strain Towne genome was sufficient to confer protection from NK cell killing comparable to that achieved by infection with strain Toledo alone (**Fig. 1c,d**). Thus, the 13-kb U_L/b' transferred from strain Toledo may contain one or more gene(s) that inhibit(s) NK cell recognition.

UL141 and NK cell–evasion function

To screen for a NK cell evasion function, we transiently transfected human embryonic kidney (HEK) 293 cell lines with plasmids encoding open reading frames (ORFs) UL133–UL150. The ORFs cloned were as defined in the original analysis of the strain Toledo U_L/b' sequence[11]. The ORFs were expressed as green fluorescent protein (GFP) fusion proteins to track expression. Although none of this panel of transfected cells showed resistance to NK cell cytolysis, the assay was restricted by the use of a single NK cell line, DEL[5]. Sequencing of the UL133–UL150 PCR products identified a small number of errors in the original strain Toledo U_L/b' sequence that affected gene usage. A reannotated, revised version of the strain Toledo U_L/b' sequence was therefore generated[18] (GenBank accession number AY446871). We cloned the corrected version of the strain Toledo UL141 ORF[11,14] as a GFP fusion construct and sorted the transfected HEK 293 cell line for GFP expression, expanded the population *in vitro* and used these in assays within 3 weeks. Using NKL cells, HEK 293 cells expressing the UL141-GFP fusion protein showed resistance to NK cell–mediated cytolysis (**Fig. 2**). A UL16-GFP fusion protein was also capable of eliciting protection compared with UL14-GFP or control cells expressing GFP alone. UL16 is a recognized HCMV NK cell–evasion gene

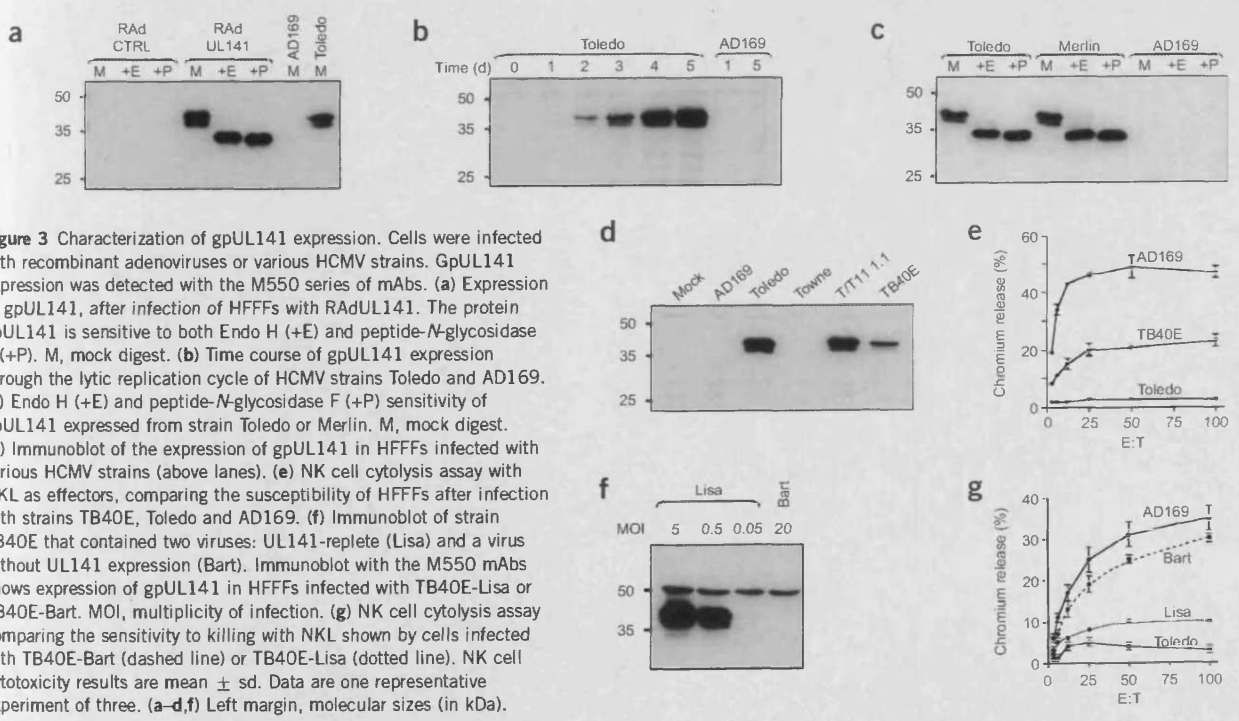

Figure 3 Characterization of gpUL141 expression. Cells were infected with recombinant adenoviruses or various HCMV strains. GpUL141 expression was detected with the M550 series of mAbs. (**a**) Expression of gpUL141, after infection of HFFFs with RAdUL141. The protein gpUL141 is sensitive to both Endo H (+E) and peptide-*N*-glycosidase F (+P). M, mock digest. (**b**) Time course of gpUL141 expression through the lytic replication cycle of HCMV strains Toledo and AD169. (**c**) Endo H (+E) and peptide-*N*-glycosidase F (+P) sensitivity of gpUL141 expressed from strain Toledo or Merlin. M, mock digest. (**d**) Immunoblot of the expression of gpUL141 in HFFFs infected with various HCMV strains (above lanes). (**e**) NK cell cytolysis assay with NKL as effectors, comparing the susceptibility of HFFFs after infection with strains TB40E, Toledo and AD169. (**f**) Immunoblot of strain TB40E that contained two viruses: UL141-replete (Lisa) and a virus without UL141 expression (Bart). Immunoblot with the M550 mAbs shows expression of gpUL141 in HFFFs infected with TB40E-Lisa or TB40E-Bart. MOI, multiplicity of infection. (**g**) NK cell cytolysis assay comparing the sensitivity to killing with NKL shown by cells infected with TB40E-Bart (dashed line) or TB40E-Lisa (dotted line). NK cell cytotoxicity results are mean ± sd. Data are one representative experiment of three. (**a–d,f**) Left margin, molecular sizes (in kDa).

Table 2 Expression of gpUL141 increases resistance to NK cell–mediated lysis

NK cell	Target	NK:target	Rad-GFP	RAdUL141-GFP	*P*
Fresh IFN-α-stimulated whole PBMCs					
D3PBMC	HFFF	25:1	35 ± 1	20 ± 2	0.0006
Primary T cell–depleted PBMC bulk cultures					
D3NKb1	D3SF●	20:1	30 ± 2	13 ± 5	0.01
D3NKb2	D3SF●	12:1	17 ± 5	11 ± 1	NS
D3NKb2	HFFF	12:1	22 ± 8	8 ± 2	NS
D7NKb1	D7SF●	18:1	20 ± 2	8 ± 2	0.0007
Primary T cell– and CD94$^+$ cell–depleted PBMC bulk cultures					
D3NKb1(ΔCD94)	D3SF●	40:1	31 ± 3	11 ± 2	0.0008
D7NKb1(ΔCD94)	D7SF●	22:1	16 ± 4	7 ± 3	0.01
Expanded primary polyclonal NK lines					
D7NKp3.2	D7SF●	40:1	18 ± 4	3 ± 3	0.009
D7NKp3.2	HFFF	40:1	20 ± 3	8 ± 2	0.004
NK clones					
D8NKc1C10	D8SF●	8:1	21 ± 1	7 ± 1	0.0005
D8NKc1E11	D8SF●	40:1	18 ± 1	16 ± 2	NS
D8NKc1F11	D8SF●	32:1	19 ± 1	9 ± 1	0.002
D8NKc3C8	D8SF●	35:1	24 ± 1	8 ± 1	0.0003
D8NKc4E10	D8SF●	23:1	30 ± 3	11 ± 2	0.002
D8NKc5C11	D8SF●	66:1	36 ± 3	23 ± 3	0.02

Data are presented as percent chromium release ± s.e.m. ●, autologous targets; D#NKb(ΔCD94), bulk cultures depleted of CD94$^+$ NK cells (the number (#) indicates donor number). *P* values compare killing of RAd-GFP–infected targets versus RAd-UL141–GFP–infected targets (*t*-test, assuming unequal variance); NS, not significant ($P > 0.05$).

product[6], whereas UL14 is an uncharacterized homolog of UL141. The GFP fusion proteins had comparable expression in the sorted cells used in the assay (**Fig. 2**). UL141 was thus identified as potentially encoding a product that mediates NK cell–evasion function.

Inhibition of a wide range of NK cells by gpUL141

We constructed replication-deficient adenovirus vectors (RAds) containing the complete gene encoding UL141 alone (RAdUL141) or in combination with GFP (RAdUL141-GFP) to provide efficient expression of glycoprotein UL141 (gpUL141) in fibroblast targets (**Fig. 3a**). In NK cell assays, RAdUL141-GFP-infected cells showed notably reduced cytolysis compared with that of control cultures exposed to infection with an equivalent adenovirus recombinant encoding GFP alone (**Table 2**). We readily found inhibition with NKL cells (data not shown), NK cell bulk cultures from two different volunteers and five of six NK cell clones regardless of whether targets were allogeneic or autologous (**Table 2**). We further tested the specificity of gpUL141 by investigating its effects on bulk cultures depleted of CD94$^+$ NK cells; UL40 inhibits a specific subset of CD94$^+$NKG2A$^+$ NK cells by the upregulation of HLA-E[4,5]. We noted gpUL141 inhibited complete and CD94-depleted NK cell bulk cultures with similar efficiency (**Table 2**) and had no differential effect on CD94^{hi} and CD94^{lo} NK cell clones (data not shown). Indeed, gpUL141 inhibited 67% (82 of 123) of the NK cell clones tested in an autologous setting by a margin of more than 10% in NK cell cytolysis assays, whereas three clones were stimulated (**Table 3**). The gpUL141-inhibited killing was thus independent of CD94 expression and was readily detected with freshly isolated NK cell bulk cultures and most NK cell clones.

Characterization of the UL141 gene product

UL141 is highly conserved between HCMV isolates, encoding a protein containing a potential signal peptide, a hydrophobic transmembrane domain and three potential *N*-linked glycosylations sites (**Supplementary Fig. 1** online). A protein fold–recognition program indicates that gpUL141 as defined originally[10] contains immunoglobulin-like regions[19]. Analysis of the updated sequence[14,18] indicated the presence of an immunoglobulin-like β-sandwich domain at residues 15–114 in the mature protein (**Supplementary Fig. 2** online). We expressed a truncated, secreted form of gpUL141, in which the predicted C-terminal cytoplasmic and transmembrane domains were replaced with an affinity tag (*Strep*-tag). This protein was expressed in abundance with a replication-deficient adenovirus vector and purified with a Streptactin Macroprep column (**Supplementary Fig. 3** online). We determined the N-terminal amino acid sequence of purified soluble gpUL141 to identify the cleavage site for signal peptidase. The gpUL141 sequence started with the D residue specified by codon 37 in the ORF[14]. We also used purified, secreted gpUL141 as an immunogen to generate specific monoclonal antibodies. During productive HCMV infection, the UL141 protein could be readily detected at 24 h after infection and continued to accumulate into the late phase of the replicative cycle (**Fig. 3b** and **Supplementary Fig. 4** online). We detected gpUL141 as a glycoprotein doublet with apparent molecular masses of 37 kDa and 40 kDa that after digestion with endo-*N*-acetylglucoseaminidase H (Endo H) or peptide-*N*-glycosidase F yielded a single 34-kDa product (**Fig. 3a,c**), consistent with the predicted molecular mass of 34.7 kDa (302 residues). Sensitivity to Endo H treatment was consistent with retention of gpUL141 in the endoplasmic reticulum, and this result was supported by immunocytochemistry (**Supplementary Fig. 5** online). Expression of gpUL141 was readily detected with clinical HCMV isolate Merlin, strain Toledo or the recombinant HCMV Towne/Tol11 1.1 but not with the laboratory strains AD169 or Towne, which lack the U_L/b' nucleotide sequence (**Fig. 3c,d**).

UL141 frameshift mutation in HCMV strain TB40E

During screening of gpUL141 expression with different strains of HCMV, the amount of gpUL141 detected with the passaged strain TB40E-infected cells was much lower than with any other virus (**Fig. 3d**). Furthermore, infection with strain TB40E elicited

Table 3 Effect of UL141 expression on cytotoxicity mediated by individual NK cell clones derived from multiple donors

Donor	UL141 expression inhibited lysis[a]	UL141 expression increased lysis[a]	UL141 expression, no change[b]
D3	4 of 16	0 of 16	12 of 16
D7	21 of 24	0 of 24	3 of 24
D8	9 of 16	1 of 16	6 of 16
D9	48 of 67	2 of 67	17 of 67
Total	82 of 123	3 of 123	38 of 123

Data represent autologous assays; each event represents a single experiment with an individual clone.
[a]Increase or decrease in cytolysis of more than 10% chromium release. [b]Increase or decrease in cytolysis of less than 10% chromium release.

intermediate NK cell resistance relative to infection with strains AD169 and Toledo (**Fig. 3e**). TB40E contained two viral species: a virus containing an intact copy of the UL141 gene (defined as the clonal strain TB40E-Lisa here) and a natural mutant thereof (called TB40E-Bart here). We cloned these separately by plaque purification (**Fig. 3f**). DNA sequence analysis showed that strain TB40E-Lisa and TB40E-Bart were identical throughout U_L/b', except that TB40E-Bart had a two–base pair (bp) frameshift insertion at codon 63 of UL141 and a 1,347-bp deletion removing all of UL144 and all but the six C-terminal codons of UL145 (**Supplementary Fig. 1** online). Cells infected with strain TB40E-Bart ($UL141^-$) were reproducibly more sensitive to NK cell–mediated lysis than were those infected with strain TB40E-Lisa or Toledo using NKL, D7NKb or D9NKb as effectors against allogeneic or autologous targets (**Fig. 3g** and data not shown). Infection with TB40E-Bart resulted in values for NKL killing close to those noted with strain AD169 (**Fig. 3g**). These results are consistent with gpUL141 providing a chief contribution to the differences in NK cell inhibition noted between strains Toledo and AD169. However, functions for UL144 or UL145 cannot be excluded in experiments comparing TB40E-Bart and TB40E-Lisa.

GpUL141 downregulates cell surface expression of CD155

We attempted to identify the molecular target of gpUL141 by screening a broad range of previously identified NK cell receptor ligands by flow cytometry on UL141-transfected or RAdUL141-infected cell lines. The protein gpUL141 did not alter cell surface expression of HLA class I, MICA, MICB or a range of integrins or other adhesion molecules or the binding of NKp30-Fc and NKp46-Fc fusion proteins (data not shown). CD155 (also called poliovirus receptor or nectin-like molecule 5) and CD112 (also called nectin-2) are both cell surface ligands for the NK cell–activating receptors CD226 (also called DNAX accessory molecule 1) and CD96 (also called T cell–activation increased late expression)[20,21]. Infection with strains Toledo or TB40E-Lisa efficiently downregulated CD155 expression, as determined by the D171 monoclonal antibody (mAb), whereas infection with strain AD169 or TB40E-Bart (both $UL141^-$) upregulated CD155 (**Fig. 4**). The NK cell cytotoxicity of strain TB40E-Bart-infected cells was partially inhibited by blocking with a mAb to CD155 (**Supplementary Fig. 6** online). Furthermore, infection with adenovirus or transfection with a plasmid driving expression of gpUL141 downregulated cell surface CD155, demonstrating that expression of gpUL141 or a UL141-GFP fusion protein was sufficient and necessary for this effect (**Fig. 4**). In contrast, HCMV gpUL16, UL16-GFP and the two additional ORFs deleted in TB40E-Bart (UL144 and UL145) had no effect on CD155 expression (**Fig. 4** and **Supplementary Fig. 7** online). UL141 did not affect CD112 expression on human fibroblasts or HEK 293 cells (data not shown). GpUL141 thus acts specifically to downregulate cell surface expression of the NK cell–activating receptor CD155. Soluble tagged gpUL141 inhibited binding of the D171 mAb to CD155 (**Supplementary Fig. 8** online), suggesting a direct interaction between CD155 and gpUL141.

We analyzed the effect of gpUL141 on CD155 expression with immunofluorescence studies of permeabilized cells. We used adenovirus and HCMV recombinants that also contained an unfused GFP gene as an infection tracker. Similarly, in transfected cells, specific fluorescence of UL141-GFP or UL16-GFP fusion proteins identified expressing cell populations. When expressed with an adenovirus vector, HCMV infection or DNA transfection, UL141 correlated exclusively with a loss of CD155 staining when detected with mAb D171. In contrast, UL141 had no obvious effect on the signal when we used the alternative 5D1 mAb to CD155 (**Fig. 5**). The disparate results

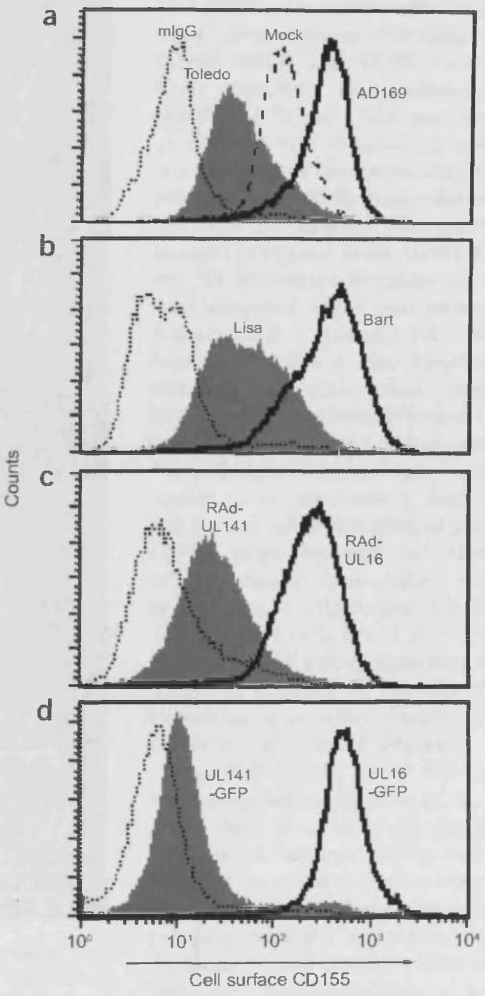

Figure 4 UL141-mediated downregulation of cell surface CD155 detected by flow cytometry. Flow cytometry with mAb D171 of cell surface CD155 on mock-infected cells or cells after infection with HCMV strains AD169 ($UL141^-$) or Toledo (**a**), after infection with TB40E-Bart ($UL141^-$) or TB40E-Lisa (**b**), after infection with adenovirus vectors encoding UL141 or UL16 (**c**), and in continuous cell lines expressing UL141-GFP or UL16-GFP fusion proteins (**d**). Dotted lines, staining with an isotype-matched control antibody. mIgG, mouse IgG.

obtained with the two CD155 mAbs were resolved in immunoblot experiments, which demonstrated that gpUL141 was compatible with CD155 expression. However, gpUL141 blocked CD155 maturation from an Endo H–sensitive, intracellular 69-kDa form to an Endo H–resistant cell surface 70- to 80-kDa species[22] (**Fig. 6**). The D171 mAb is conformation specific[23] and seemed to not recognize the immature 69-kDa CD155 form that accumulated when gpUL141 was present. In contrast, the 5D1 mAb was capable of recognizing immature forms[24]. With HCMV strains encoding UL141, mature CD155 was absent, whereas the immature Endo H–sensitive CD155 was synthesized in abundance. With HCMV strains lacking UL141, the mature 75-kDa form of CD155 was present, and an additional slower migrating and Endo H–resistant CD155 species was detected (**Fig. 6**). As CD155 has been associated with cell-cell interactions, we tested whether UL141 affected adhesion between NK cells and their

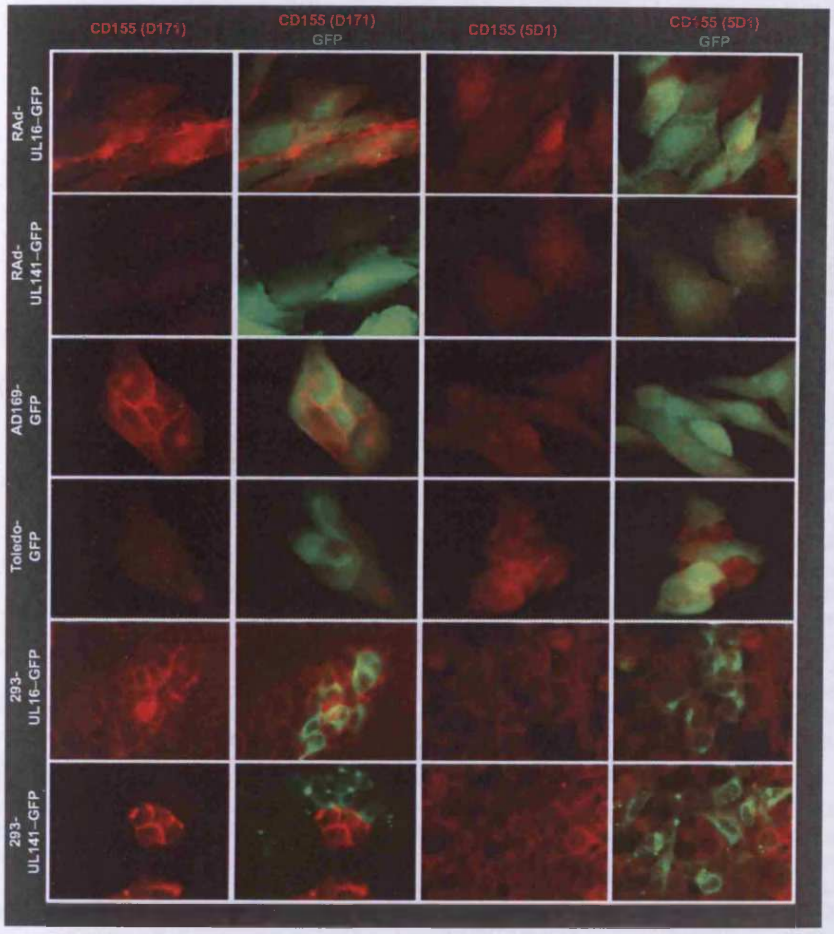

Figure 5 UL141-mediated downregulation of cell surface CD155 detected by fluorescence microscopy. CD155 expression monitored by staining with the monoclonal antibodies D171 or 5D1 in the presence or absence of UL141. The gpUL141 was expressed with an adenovirus vector (RAdUL141-GFP), infection with strain Toledo-GFP in HFFFs or in UL141-GFP–transfected HEK 293 cells. Similarly, gpUL16 was expressed with an adenovirus vector (RAdUL16-GFP) or in UL16-GFP-transfected cells. HCMV strain AD169 is UL141 negative. HCMV and adenovirus vectors contain an intact GFP gene to identify infected cells. In HEK 293 cells, gpUL16-GFP and gpUL141-GFP fusion proteins traffick to the endoplasmic reticulum and concentrate in intracytoplasmic foci.

targets, but noted only marginal differences (**Supplementary Fig. 9** online). When expressed alone or in the context of a productive HCMV infection, gpUL141 thus acts to retain CD155 as an immature form associated with the endoplasmic reticulum to inhibit its cell surface expression and therefore prevent its interaction with an activating ligand on NK cells.

DISCUSSION

The HCMV genome shows an inherent instability during *in vitro* culture[18,25]. The spontaneous deletion of 13–15 kb from HCMV laboratory strains correlates with reduced virulence *in vivo* and increased susceptibility of HCMV-infected cells to NK cell–mediated cytolysis *in vitro*. Downregulation of surface HLA class I strips HCMV-infected cells of a key set of NK cell inhibitory ligands and thus renders them potentially vulnerable to NK cell attack. Should activating ligands become dominant, the balance would shift toward priming of NK cell killing. However, HCMV has responded to this strong selective pressure. Multiple NK cell inhibitory functions have been identified in studies with strain AD169: UL16 downregulates activating NK cell ligands MICB and ULBP1 and ULBP2, UL40 upregulates the inhibitory NK cell ligand HLA-E, and UL18 may itself act as an inhibitory NK cell ligand. However, infection with HCMV clinical isolates consistently bestowed substantially more effective protection from NK cell attack than did the highly passaged laboratory strain AD169, indicating that NK cell–evasion functions had been lost from laboratory strains. Here we have identified UL141 as a powerful NK cell evasion function that has a chief function in the enhanced protective effect noted with HCMV clinical isolates. Whether UL141 is the only gene in the UL/*b*′ region to inhibit NK cells cannot be concluded from these studies, as we used only a single NK cell line for our initial screening of the UL133–UL150 genes. Treatment of TB40E-Bart-infected targets, which showed disruptions in the UL141, UL144 and UL145 genes, with the D171 mAb to CD155 only partially blocked NK cell killing, indicating either an inefficient blocking of the CD155-CD226 interaction or an added function for either UL144 or UL145 in NK inhibition.

Regardless of this, UL141 was capable of suppressing killing of 67% of all NK cell clones tested, consistent with targeting of a principal NK cell–regulatory pathway by the gene. We calculated this value using a rigorous definition of inhibition (more than 10% reduction in specific target lysis). Insight into the broad NK cell inhibitory effect of UL141 came from the identification of its cellular target, CD155. CD155 is a ligand for the activating receptor CD226, which has been reported as being present on almost all NK cells[20], and was expressed universally on all the NK cell bulk cultures and clones evaluated here (data not shown). UL141 showed specificity for CD155 in that it did not influence the expression of CD112 (nectin-2), another reported ligand for CD226. Furthermore, the UL141-related gene HCMV UL14 had no detectable effect on either CD112 or CD155 (data not shown). The efficiency with which UL141 inhibits NK cell function indicates the interaction of CD226 with CD155 has a major role in the regulation of NK cell function[26].

Parallels exist between the action of UL141 and the HCMV-encoded NK cell inhibitor UL16. Both are proteins residing in the endoplasmic reticulum that act to promote intracellular retention of NK cell–activating ligands. HCMV contains many gene families thought to be generated through gene duplication, but the lack of sequence similarity between the UL16 and UL141 proteins suggests they may act through different mechanisms. Perhaps the most important functional distinction is that UL16 targets stress-induced NKG2D ligands, whereas UL141 targets a ligand that is constitutively expressed by a wide range of cell types. The function of CD155 as a constitutive activating ligand is likely to stimulate NK cell recognition if surface HLA class I is downregulated, as noted during HCMV infection as well

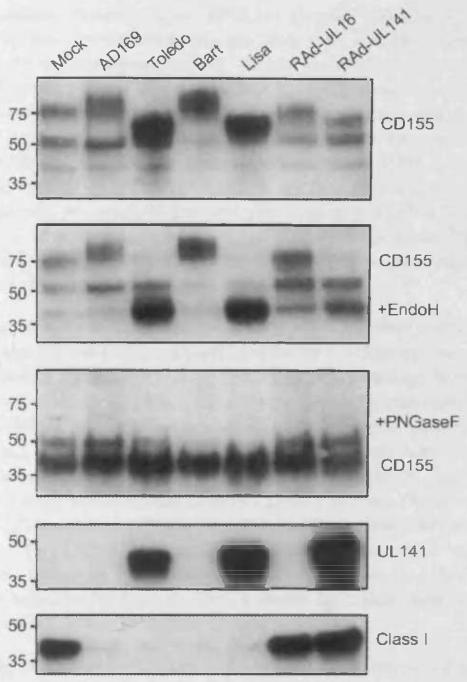

Figure 6 UL141 inhibits maturation of CD155. Immunoblots of extracts of cells infected with viruses with the 5D1 mAb to CD155, the gpUL141-specific M550 mAbs or the HC-10 mAb to HLA class I. The sensitivity of the CD155 protein to Endo H and peptide-*N*-glycosidase F (PNGaseF) digestion is analyzed in parallel. HLA class I (Class I) downregulation is a surrogate marker of efficient HCMV infection. Left margin, molecular sizes (in kDa).

as during other viral infections and cell transformation. UL141 suppressed NK cell function when expressed in either stably transfected cells or with the use of a replication-deficient adenovirus vector, neither of which downregulates HLA class I expression or upregulates CD155. In autologous assays, constitutive CD155 expression may therefore have been sufficient to stimulate NK cell killing of fibroblasts expressing physiological amounts of HLA class I. Such spontaneous killing may be accentuated by *in vitro* cell culture. In tissues, heterophilic interactions between membrane-bound CD155 and nectin-3 contribute to cell-cell and cell-matrix adhesion and restrict these proteins to adhesion junctions. During *in vitro* culture, virus infection[27] or cell transformation, cell-cell and cell-matrix interactions become disrupted, potentially making CD155 available to interact with CD226 resulting in NK cell activation and cytolysis.

The function of CD226 is dependent on its forming a complex with the adhesion molecule LFA-1 on the effector cell. Activation of NK cell effectors through CD226 thus also indicates involvement in effector-target adhesion through the interaction of LFA-1 on effectors with the adhesion molecule ICAM-1 on the HCMV-infected target; it is well established that ICAM-1 is upregulated during HCMV infection[27,28]. Furthermore, CD155 interacts with another nectin family member, CD96 (TACTILE), to stimulate adhesion between NK cells and their targets and thus promote NK cell–mediated cytotoxicity[21]. Given those observations, we tested whether UL141 affected adhesion between NK cells and their targets but were unable to detect any changes. UL141 therefore seems to act mainly by impeding signaling through the activating receptor CD226 rather than by radically reducing adhesion between NK cell effectors and their targets.

The function of UL141 may yet prove to transcend its function in impeding NK cell recognition, as CD226 is also expressed on T cells, monocytes, megakaryocytes and B cell subsets[20,29–31]. The best characterized functions of CD155 are associated with its roles in cell-cell adhesion and cell motility. CD155 interacts with Tctex-1, a subunit of the dynein motor complex linked to endocytosis and retrograde transport[31,32], is found at the leading edges of migrating cells[33] and has been linked to fibroblast migration[34] and monocyte transendothelial migration[35]. UL141 suppression of CD155 function has the potential to affect multiple cell processes and may thus have even more influence on the biological interaction of HCMV and its host.

METHODS

Cells. Human fetal foreskin fibroblasts, primary skin fibroblasts from volunteer donors, human alveolar epithelial carcinoma A549 cells, human embryonic retinoblast 911 cells and human embryonic kidney HEK 293 cells were all grown in DMEM supplemented with 10% FCS (Invitrogen). Primary human fibroblasts were preferred targets in NK cell assays because they support productive HCMV infection. Skin fibroblast cultures from biopsies were immortalized with human telomerase reverse transcriptase[36]. The established NK cell clone NKL and the methods used for generating NK cell lines and T cell–depleted, IFN-α-activated bulk cultures have been described[5,37]. NK cell cloning was accomplished by single-cell sorting with clones stimulated weekly with irradiated allogeneic peripheral blood mononuclear cells (PBMCs), mouse mAb OKT3 to CD3, and IL-2 (1,000 IU/ml). All NK cell clones were maintained in SCGM medium (Cellgro) supplemented with 5% human AB serum and were allowed to 'rest' from feeder stimulation for at least 1 week before use in assays. This project was approved by the Bro Taf Local Research Ethics Committee. Prior consent was obtained from all blood and skin biopsy donors.

Cell lines. UL14, UL16 and UL141 were amplified by PCR from HCMV genomic DNA and were inserted directly into the pcDNA3.1/CT-GFP-TOPO vector (Invitrogen) such that the genes were expressed as GFP fusion (C-terminal) proteins. The primers used were as follows: for strain Toledo UL141, 5′-ATCATGTGCCGCCGGGAGTCG-3′ (forward) and 5′-GCCTCTTCATCTTT CTAACACC-3′ (reverse), for strain AD169 UL16, 5′-CCGGCATGGAGCGTCG CCGA-3′ (forward) and 5′-GGTCCTCGGTGCGTAACCGCT-3′ (reverse); and for strain AD169 UL14, 5′-CCGGCATGGAGCGTCGCCGA-3′ (forward) and 5′-GGTCCTCGGTGCGTAACCGCT-3′ (reverse). The identity of all PCR-amplified sequences was confirmed by sequencing. HEK 293 cells were transfected with the cloned GFP fusion products and cell lines were selected with 2 mg/ml of geneticin (Invitrogen) and cell sorting. Purity of the cloned populations were more than 90%.

Virus. The HCMV strain AD169, deletion mutant AD169ΔUL40, strain Merlin, strain TB40E, strain Toledo encoding GFP (TOLΔβ2.7) and strain AD169 encoding GFP (RCMV288) have been described[5,18,36], and strains Towne, Toledo and recombinant Towne/Toll1 1.1 were provided by E. Mocarski[17] (Stanford University, Palo Alto, California). For NK cell cytolysis assays, fibroblasts were infected for 72 h with HCMV (10 plaque-forming units per cell). Replication-deficient adenovirus recombinants were constructed with the AdEasy-1 vector system provided by B. Vogelstein[38] (Johns Hopkins Oncology Center, Baltimore, Maryland). HCMV genes were amplified by PCR, then were subcloned into the transfer vectors pShuttle-CMV or pAd-Track-CMV. The adenovirus recombinants, referred to by their transgenes in the text, have the following 'formal' designations: RAd592 has no transgene insert; RAd502 encodes GFP alone; RAd588 encodes both UL16 and GFP; RAd522 encodes both the complete strain Toledo UL141 and GFP genes; RAd587 encodes strain Toledo UL141 alone; RAd550 encodes GFP and strain Toledo UL141 with the transmembrane and cytoplasmic domains replaced with a streptactin-binding tag (*Strep*-tag; IBA). For RAd550, the *Strep*-tag was inserted by PCR amplification of the UL141 gene with the forward primer 5′-GGGGTACCATCATGTGCCGCCGGGAGTCG-3′ and the reverse primer 5′-CCGCTCGAGTTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTCCGAG TGGCCCAGGGAGACATC-3′ (Invitrogen).

Protein analysis. Soluble tagged gpUL141 protein (designated P550) was prepared by infection of A549 cells for 72 h with RAd550 (multiplicity of infection, 10) in serum-free medium. Culture supernatant was collected and secreted P550 was purified on a Streptactin Macroprep column (IBA) according to the manufacturer's instructions. N-terminal sequencing was done on an Applied Biosystems Procise Sequencer (LSUMC Core Laboratories, USA). Purified P550 was also used to generate mouse mAbs M550.2, M550.3 and M550.4 (all isotype IgG1) to gpUL141. A mixture of M550.2, M550.3 and M550.4 was used for immunoblots, and a combination of M550.3 and M550.4 was used for immunocytochemistry. Glycosylation was analyzed by digestion with Endo H or peptide-*N*-glycosidase F according to the manufacturer's instructions (New England Biolabs).

Flow cytometry and immunohistochemistry. A FACSCalibur with CELLQuest PRO software (Becton Dickinson) was used for flow cytometry, and cell sorting was provided by the Cardiff University Central Biotechnology Service with a Cytomation MoFlo sorter. Before immunocytochemistry, cells were fixed with paraformaldehyde and were permeabilized with detergents. Fluorescence was detected and analyzed with a Leica DMIRBE microscope with Improvision Openlab software. Antibodies used included mouse mAb to CD155 (D171, ab3142; Abcam); another mouse mAb to CD155 (5D1; ref. 24); goat antibody to mouse IgG conjugated to AlexaFluor 594 (A-11020; Molecular Probes) or to AlexaFluor 647 (A-21237; Molecular Probes); mouse mAb to calnexin (MAB3126; Chemicon International); and concavalin A–AlexaFluor 488 (C-11252; Molecular Probes); the Zenon mouse IgG1 fluorescein labeling kit was also used (Z-25045; Molecular Probes).

Other reagents. Other antibodies and reagents used were as follows: rabbit anti-GFP (sc-8334; Santa-Cruz), goat anti-rabbit–horseradish peroxidase (170-6515; Bio-Rad), goat anti-mouse–horseradish peroxidase (170-6516; Bio-Rad), HC-10 mouse mAb to HLA class I heavy chain, Streptactin–horseradish peroxidase (2-1502-001; IBA), mouse mAb to Streptag (2-1507-001; IBA), the VYBRANT CFDA SE Cell Tracer Kit (V-12883, Molecular Probes) and the Cell Trace Far Red DDAO-SE kit (C-34553, Molecular Probes).

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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