THE EFFECT OF ADENOVIRUS E1A ON THE HUMAN IMMUNOPROTEASOME AND MHC COMPLEX

BY

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A thesis submitted to the

University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

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August 2011

ABSTRACT

Adenovirus E1A (AdE1A) is a viral oncoprotein that targets many cellular proteins and pathways, mainly those involved in transcriptional regulation. Proteasomes represent the major non-lysosomal mechanism responsible for protein degradation. Following interferon-γ treatment, three proteasome subunits are replaced by immunosubunits LMP2, LMP7 and MECL-1 producing immunoproteasomes. The proteasome and immunoproteasome generate peptide antigens for MHC class I presentation to cytotoxic T-cells. In this study, the effect of AdE1A on human immunoproteasomes as well as MHC class I and class II cell surface expression was examined.

It was found that AdE1A interacts with the immunoproteasome subunit MECL-1 through its N-terminal and CR3 regions. AdE1A also down-regulated all three immunosubunit expressions during adenovirus infection, transformation and AdE1A transfection, with the exception of Ad5-transformed cells where immunosubunit expression remained unchanged. Furthermore, MHC class I expression remained unaffected in the same three backgrounds. However, in the Ad12 transformants MHC class I was generally reduced prior to IFNγ treatment but was expressed after. MHC class II surface expression, in contrast, was down-regulated in all cases, except in Ad5 infected cells. Similarly, AdE1A reduced IFNγ-stimulated STAT1 phosphorylation and transcriptional response to IFNγ. And finally, T-cell recognition of target cells was reduced in the presence of AdE1A.

In conclusion, AdE1A targets the human immunoproteasome, both through direct binding and down-regulation of expression. It also targets the expression of MHC class I and class II surface expression.

To my family

Acknowledgments

I would like to thank my supervisors Dr. Roger Grand and Dr. Jane Steele for giving me the opportunity to carry out this study and the Medical School of the University of Birmingham which funded this work for three years. I thank Roger for his help and support throughout the years, teaching me through the different techniques. I also thank him for spending a great deal of time reading and correcting this manuscript. I also thank Jane for her support and constructive advice on the immunology aspect of the study and for her guidance and encouragement.

I would also like to express my gratitude to Gordon Ryan for his help and advice and who kindly allowed me to use his reagents when money was running thin! I will always be indebted to him. I also thank Ankit Rao, Jill Brooks, Claire Shannon-Lowe, Jailal Ablack and Cristina Areste for providing me with plasmids, cells and reagents.

Deep appreciation to my lab colleagues Natalie, Rakesh, Ellis, Shaun, Georgia as well as Garry and Iga (who now left the School) for their friendship and support. I have really enjoyed working with them over the three years. I also thank Ania, Shan, Angelo, Emma Yates, Emma Prescott, Alice, Esther, Suzy and Hardeep for their friendship and whom I had a great pleasure of getting to know.

Lastly, my greatest thanks go to my family. I thank my parents and my brother for their eternal moral support and love. I am immensely indebted to them.

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Abbreviations

µCi	Micro Curie
µg	Microgram
µl	Microlitres
aa	Amino acid
Ad	Adenovirus
AdE1A	Adenovirus early region 1A
AdE1B	Adenovirus early region 1B
AdE3	Adenovirus early region 3
APC	Antigen presenting cell
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CBP	Cyclin ANP response element binding protein
CD8	Cluster of differentiation 8
cm	Centimetre
CR	Conserved region
CtBP	C-terminal binding protein
C-terminal	Carboxyl-terminal
CTL	Cytotoxic T-cells
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E	Early Region
E2F	AdE2 early transcription factor
EBV	Epstein Barr Virus
ECL	Enhanced chemiluminescence
EDTA	Sodium ethylene tetraacetate
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GAS	Gamma-activated site
GST	Glutathione-S-transferase

HAT	Histone acetyltransferase
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDAC	Histone de-acetylase
HER	Human Embryonic retinoblasts
Hr	Hour
IFN	Interferon
IFN	Interferon
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRF-1	Interferon regulatory factor
Kb	Kilo base
kDA	Kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
L-broth	Luria broth
LMP2	Low molecular mass polypeptide 2
LMP2A	Latent membrane protein 2A
LMP7	Low molecular mass polypeptide 7
M	Molar
mAB	Monoclonal antibody
MECL-1	Multicatalytic endopeptidase Complex-like 1
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimole
mRNA	Messenger ribonucleic acid
M.W.	Molecular Weight
ng	Nanogram
N-terminal	Amino-terminal
P/CAF	p300/CBP associated factor
$p21^{WAF1/CIP1}$	Cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$
$p27^{Kip1}$	Cyclin-dependent kinase inhibitor $p27^{KIP1}$
p53	p53 tumour suppressor
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PBS-T	Phosphate buffered saline-tween 20
pRB	Retinoblastoma protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
S.D.W	Sterile distilled water
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
SV40	Simian Virus 40
TAF	TBP-associated factor
TAP	Transporter associated with antigen processing
TBE	Tris-borate-EDTA buffer
TBP	TATA binding protein
TE	Tris EDTA buffer
TEMED	N'-N'-N'-N' tetramethyl-ethylenediamine
TF	Transcription factor
TFIIA	Transcription factor II A
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl)-aminoethane
Triton X-100	Octyl phenoxy polyethoxyethanol
TRRAP	Transactivation/transformation-domain-associated protein
v/v	Ratio of volume per volume
w.t.	Wild type
w/v	Ratio of weight per volume
w/w	Ratio of weight per weight
YY1	Ying Yang 1
µF	microfarads

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a disease that generally occurs as a result of damage to genes encoding components of the highly regulated pathways of cell proliferation and cell death. This involves genetic mutations that lead to the stabilisation and gain of function of oncogenes or the inactivation and hence loss of function of tumour suppressors. Evidence shows that cancer is a multistep process where the accumulation of multiple mutations in key regulatory genes that control cell proliferation, differentiation and survival, drives a normal cell to a "cancer state" (Vogelstein and Kinzler 1993; Hartwell and Kastan 1994; Hahn and Weinberg 2002). There are more than 100 distinct types of cancer and during their development, most cancer cells have acquired six capabilities; namely self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, unlimited replication, sustained angiogenesis and local tissue invasion leading to metastasis (Hanahan and Weinberg 2000; Hanahan and Weinberg 2002; Hanahan and Weinberg 2002).

1.2 DNA tumour viruses

DNA tumour viruses are a diverse group of viruses that can cause transformation and in some cases cancer by targeting common cellular proteins involved in tumour suppressor activity and/or growth regulation. It has been found that 15-20% of global cancer incidence is linked to viral infection (zur Hausen, 1991; Pagano *et al.,* 2004; McLaughlin-Drubin and Munger, 2008). Examples of DNA tumour viruses include adenovirus (Berk 2007), Hepatitis B virus, HBV (Seeger *et al.,* 2007), human papillomaviruses, HPV (Howley and Lowy 2007; Beaudenon *et al*., 1986, de Villiers *et al*., 2004). Other DNA tumour viruses also include certain members of the Herpes family of viruses such as Epstein-Barr virus, EBV (Epstein *et al.,* 1965, de-The *et al*., 1978; Kieff and Rickinson 2007), Cytomegalovirus, CMV (Mocarski *et al.,* 2007) and Kaposi's sarcoma-associated herpesvirus, KSHV (Ganem 2007; Arvanitakis *et al*., 1997; Bais *et al*., 1998), as well as

members of the polyomavirus family, for instance, Merkel Cell polyomavirus (Houben et al., 2009), simian virus 40 (SV40; Ahuja *et al*. 2005), BK and JC (John Cunningham) viruses (Imperiale and Major 2007).

DNA tumour viruses have long been useful tools in the study of cellular transformation and understanding the biology of cancer. Two important tumour suppressor proteins retinoblastoma (pRB) and p53 are targeted by oncoproteins produced by most DNA tumour viruses. In addition, other cellular regulatory and transcriptional proteins are also deregulated through the activities of their tumour antigens. To state a few examples, the p53 pathway is targeted by HPV E6. HPV E6, in cooperation with a cellular protein termed E6 associated protein (E6-AP), target the p53 protein for very rapid ubiquitin-dependent degradation (Scheffner *et al*., 1993), as a result eliminating the transcriptional repression and pro-apoptotic effect of p53. p53 is also targeted by adenovirus E1B 55k and E4ORF6 for ubiquitin dependent degradation (Querido *et al.,* 2001; Harada *et al.,* 2002; Blanchette *et al*., 2008; Steegenga *et al*., 1998). Additionally, HPV, adenoviruses and SV40 drive quiescent cells into the proliferative state by inactivating pRB signalling through their oncoproteins thus allowing replication of the viral genome (Dyson *et al.,* 1989; Munger *et al*., 1989, Massimi and Banks, 1997). EBV latent membrane protein 1 (LMP1) induces the expression of the anti-apoptotic genes Bcl-1 and A20, thus inhibiting p53 induced apoptosis (Fries *et al*., 1996) and also repressing p53 dependent DNA repair, subsequently affecting genome stability, as a result contributing to oncogenesis (Liu *et al*., 2005). The SV40 T antigen binds and inactivates the transcriptional transactivation function of p53 (Mietz *et al*., 1992; Lane and Crawford, 1979; Linzer and Levine, 1979). It also binds and inactivates Rb protein thus inhibiting its function as a promoter of cell cycle arrest (Lee and Cho 2002). The LANA protein expressed by KSHV can bind p53 hence reducing the activation of p53 mediated gene expression increasing resistance to p53-dependent apoptosis (Friborg et al., 1999). LANA also binds to pRB, functionally inactivating it, as demonstrated by the increased upregulation of E2F in LANA-transfected cells (Radkov *et al.,* 2000). Direct mechanism of how the HBV virus contributes to

hepatocellular carcinoma is not entirely known. However, there is evidence that integration of HBV DNA into the cell genome (Esumi *et al.,* 1986), as well as the anti-DNA repair and anti-cell cycle control activities of the viral protein HBx (Lee *et al.,* 1995; Sitterlin *et al.,* 2000; Kim *et al.,* 1991) may contribute to the oncogenesis. Merkel Cell polyomavirus large T-cell antigen targets pRB function by directly binding to it as it possesses an LXCXE motif (Feng *et al.,* 2008). Similarly, BK and JC viruses, express the small and large T-antigens, that target pRB and p53 proteins, deactivating them and eventually forcing the cell to enter the S-phase (Imperiale and Major 2007; Moens *et al.,* 2007; Caracciolo *et al.,* 2006). And very recently, cytomegalovirus (CMV) has been implicated in mucoepidermoid carcinoma of salivary glands (Melnick *et al.,* 2011), and hence labelled a DNA tumour virus. The adenovirus E1A protein is also known to bind to the transcriptional regulators p300, p400, CtBP and pRB family members p107 and p130 (Turnell and Mymryk 2006), overall promoting cell growth. More details of adenovirus and its biological activity will be discussed in the following sections. These different viral manipulations eventually lead to the promotion of viral replication in a lytic infection or interference with the cell's ability to regulate and control its division and growth during transformation.

1.3 Adenovirus (Ad)

Adenoviruses are medium sized, non-enveloped icosahedral viruses that are composed of a nucleocapsid and a DNA genome (Berk 2007). They were first discovered and characterised in the 1950s (Row *et al*., 1953, Hilleman and Werner 1954, Huebner *et al.,* 1954) and named because they were initially found in human adenoid tissue (Enders *et al*., 1956). Adenovirus is a frequent cause of acute respiratory tract as well as gastrointestinal tract infections, and this is especially common in children. Studies show that 5-15% of acute upper respiratory tract infections and about 5% of lower respiratory

tract infections are caused by adenoviruses (Avila *et al.,* 1989; Edwards *et al*., 1985; Gardner, 1968; Hong *et al*., 2001; Kim *et al*., 2000).

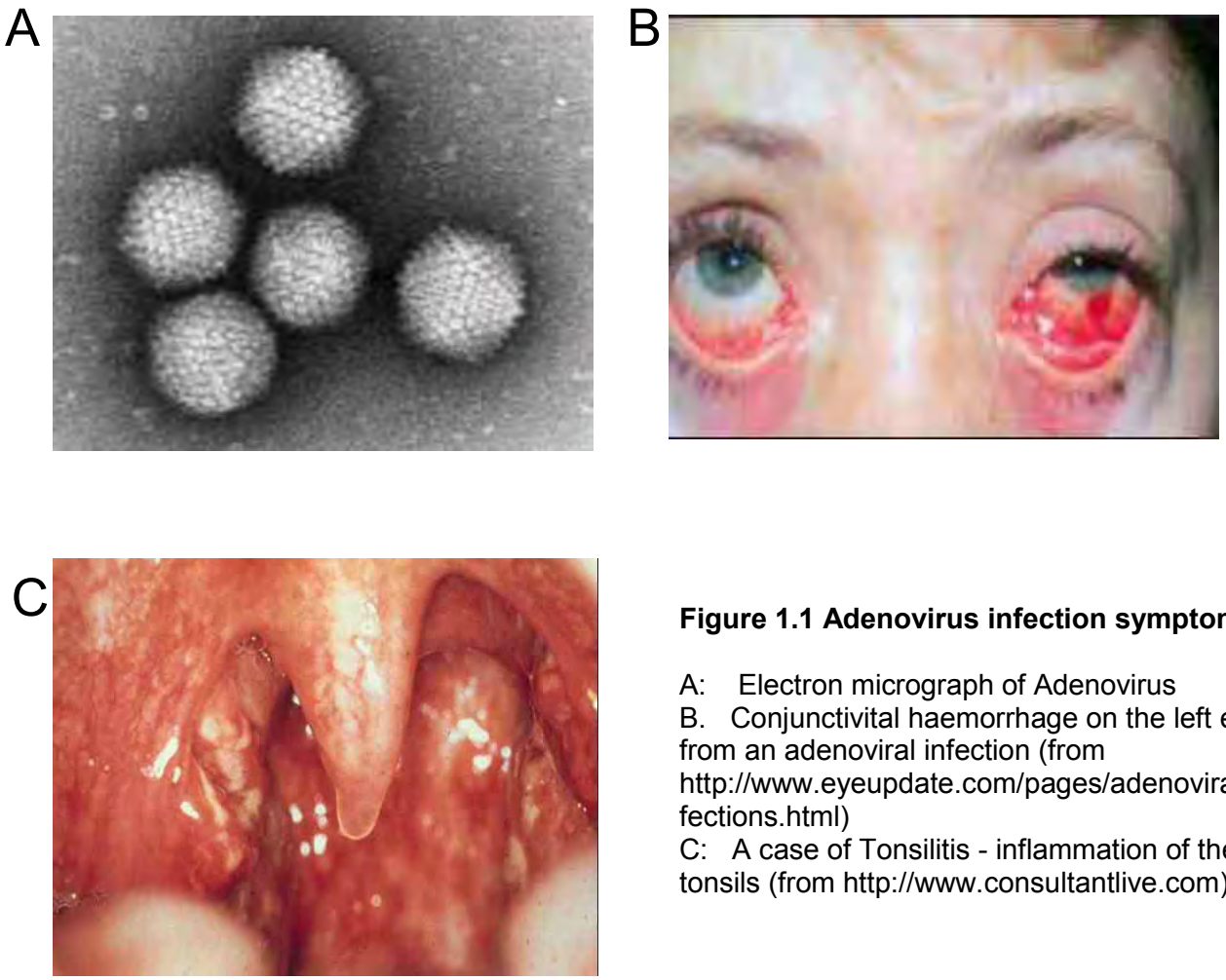


Figure 1.1 Adenovirus infection symptoms

A: Electron micrograph of Adenovirus
B. Conjunctivital haemorrhage on the left eye from an adenoviral infection (from http://www.eyeupdate.com/pages/adenoviral_infections.html)
C: A case of Tonsilitis - inflammation of the tonsils (from http://www.consultantlive.com)

While these infections are generally of a mild nature in immunocompetent individuals, they can cause severe forms of diseases such as childhood bronchiolitis, asthma and chronic obstructive pulmonary disease (COPD) in adults as well as acute febrile pharyngitis and pharyngeal-conjunctival fever in infants (Matsuse *et al.,* 1992; Macek *et al.,* 1994; Elliot *et al.,* 1995; Vitalis *et al.,* 1998). Other infections caused by adenoviruses include acute follicular conjunctivitis, keratoconjunctivitis cystitis, tonsilitis and gastroentitis (Figure 1.1). Adenovirus infection can be transmitted via respiratory droplets, conjunctival and faecal-oral route with incubation periods ranging from 2 to 14 days.

1.3.1 Classification

The family *Adenoviridae* is comprised of five clades (groups of viruses sharing a common ancestor) (Davison *et al.,* 2003). Members of the *Mastadenovirus* genera infect mammals including humans; *Aviadenovirus* genera infects birds; *Atadenovirus* genera (possess unusually high A + T content) infects reptiles, birds, a marsupial and mammals; *Siadenovirus* genera infects reptile and birds; and finally a new proposed clade that infects a sturgeon. There are now over 50 human adenovirus serotypes based on their resistance to neutralization by anti-sera (Rosen 1960) and DNA sequencing. These different serotypes are in turn classified into six species (Table 1.1) on the basis of their ability to agglutinate red blood cells in haemagglutination reactions testing the binding of the central shaft of the viral fibre protein to erthrocytes (Hierholzer, 1973; Ben Israel and Kleinberger, 2002). The reaction is inhibited using antisera that are specific for viruses of the same type but not inhibited by antisera to viruses of different types (Rosen 1960). Adenovirus serotype 5 (Ad5) and its closely related serotype 2 (Ad2) from group C and adenovirus serotype 12 (Ad12) from group A have been the primary focus of most studies surrounding adenoviruses.

1.3.2 The biology of adenoviruses

As discussed earlier in the chapter, adenoviruses frequently cause acute respiratory tract as well as gastrointestinal tract infections generating immune responses to inflammation. Additionally, some adenoviruses can also induce tumours in animals by a process comparable to cellular transformation in vitro (Green and Pina 1964). Table (1.1) shows the different adenovirus serotypes and their appropriate sub-groups. Across these serotypes, there is a varying degree of oncogenicity. For instance, group A adenoviruses such as Ad12 are highly oncogenic (Trentin *et al.,* 1962; Yabe *et al.,* 1962, 1964), with the ability to induce tumours in new born rodents within four months. On the other hand, group B adenoviruses are weakly oncogenic, and finally group C, E and F viruses are not

known to be oncogenic. However, it has been shown that the cells transformed by non-oncogenic adenoviruses can cause tumours in immunocompromised host (Gallimore 1972, Gallimore *et al.,* 1977, Van der Eb 1977), indicative of the host immune system rejecting those transformed by the non-oncogenic adenoviruses. Importantly, cultured rodent cells can be transformed by both tumourigenic and non-tumourigenic adenoviruses, for example Ad12 and Ad5 respectively. The adenovirus E1 genes that encode for AdE1A and AdE1B proteins are required for the transforming capability of the virus (Gallimore *et al.,* 1974; Graham *et al.,* 1974). Both AdE1A, in cooperation with AdE1B, de-regulate cell growth by targeting key cellular regulators that control transcription, cell cycle progression and DNA synthesis. This will be discussed in the Section 1.3.5. However, alternative novel mechanism for adenovirus-mediated transformation was discovered in members of species D adenoviruses, namely Ad9 and Ad10. Both viruses were found to exclusively cause estrogen-dependent mammary tumours in rats (Ankerst and Jonsson 1989; Ankerst *et al.,* 1974; Javier *et al.,* 1991). Unlike the other adenovirus serotypes like Ad5 and Ad12 where the cellular transformation and tumourigenesis is mediated by AdE1A and AdE1B oncoproteins; Ad9 adenovirus relies instead on the viral E4 region-encoded reading frame 1 (E4-ORF1) oncoprotein (Thomas *et al.,* 1999; Javier 1994). In fact, Ad9 lacks the requirement for E1 region-encoded gene products for tumourigenesis (Thomas *et al.,* 1999; Javier 1994). The 12kDa E4-ORF1 protein transforms by activating the protein kinase mammalian target of rapamycin (mTOR) (O'Shea 2005). Activation of mTOR leads to a stimulation of protein synthesis and entry to the S phase (Hay and Sonenberg 2004; Martin and Hall 2005; Lane et al., 1993). E4-ORF1 activates mTOR by strongly activating phosphatidylinositol 3-kinase (PI3K) through associations with PDZ-domain containing proteins at the plasma membrane (Frese *et al.,* 2003).

Research into adenoviruses therefore has helped us understand the mechanisms of transformation and oncogenesis. Through dissecting the processes by which adenovirus manipulate and exert their effect on the host cell system, we have been able to

understand much better the processes of cellular gene expression and regulation, DNA replication, cell cycle control and apoptosis.

Adenoviruses have also been a useful tool in the study and application of gene therapy. Recombinant virus vectors have been produced where they have been made replication defective by the deletion of the early genes (E1 or E1/E3 region), allowing space for the insertion of foreign DNA into the adenoviral genome (Bett *et al.,* 1994).

Table 1.1 Classification of human adenoviruses

Species	Types	Oncogenicity
A	12, 18, 31	High
B	3, 7, 11, 14, 16, 21, 34, 35, 50	Moderate
C	1, 2, 5, 6	Low or none
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	Low or none (mammary tumours)
E	4	Low or none
F	40, 41	Not reported

(Modified from Berk 2007)

1.3.3 Virus structure

Adenoviruses are the largest of the non-enveloped viruses. They are of icosahedral shape and medium sized around 70-100nm in diameter. This structure is composed of 20 triangular surfaces and 12 vertices (Horne *et al*., 1959; Cusack, 2005; Saban *et al*., 2005) The virus particles consist of 13% DNA and 87% protein with no membrane or lipid (Green and Pina, 1963). The surrounding protein shell (capsid) is composed of 252 subunits, namely 240 hexons and 12 pentons (Ginsberg *et al*., 1966, van Oostrum and Burnett 1985) (Figure 1.2). Within this capsid and the core proteins, there is a double

stranded linear DNA genome which is around 34-36kb in size depending on the serotype. Also in the capsid are nine viral proteins and four other proteins connected with the a double stranded DNA including polypeptides V, VII, mu and terminal protein. These proteins may serve as a histone-like around which the viral DNA is wrapped and also as a bridge between core and capsid (Maizel, Jr. *et al*., 1968; Russell *et al*., 1968; Robinson *et al*., 1973, Anderson *et al*., 1989; Mirza and Weber 1982; Chatterjee *et al*., 1986; Hosakawa and Sung 1976).

The adenovirus genome contains five early transcription units (E1A, E1B, E2, E3 and E4), three delayed early units (E2L, IX and IVa2), one major late unit (ML), and also the VA genes (Shenk 1996). With the exception of the latter, which are transcribed by RNA polymerase III, all of the remaining genes are transcribed by host RNA polymerase II. Due to differential splicing, each of these viral genes encode multiple mRNAs with different sedimentation coefficients. AdE1A is the first protein to be expressed, followed by E4. The expression of those two protein leads to the transcriptional activation of E1B and E2 respectively.

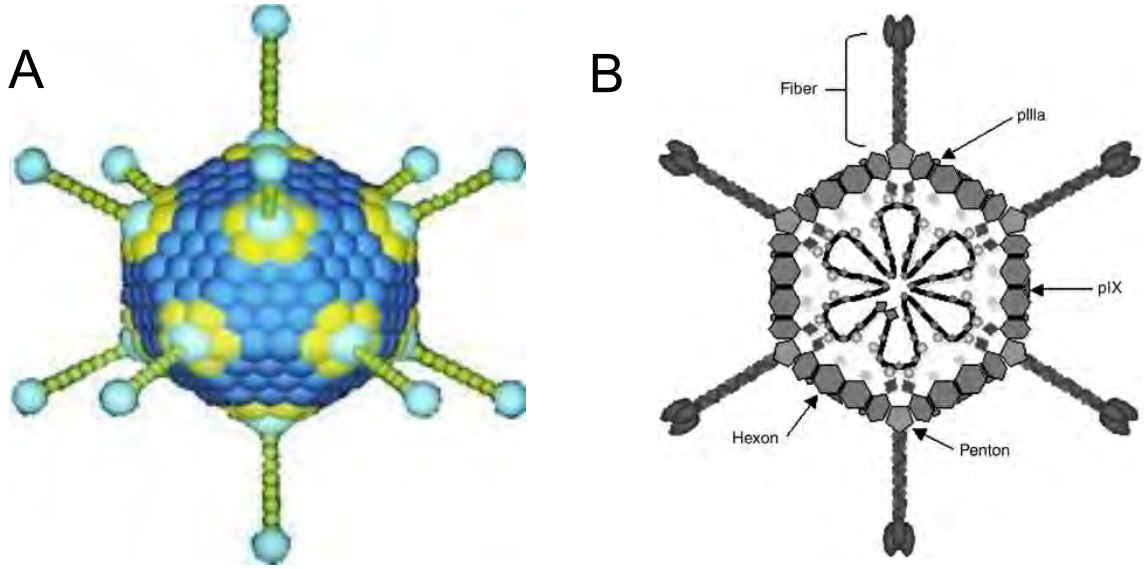


Figure 1.2. Adenovirus Structure

A. A three-dimensional image on the structure of an intact adenovirus particle (from http://www.macroevolution.net/)
B. Generalised schematic cross-section of wild type adenovirus capsid, depicting the structural components and DNA genome (from Glasgow *et al.,* 2006: Transductional targeting of adenovirus vectors for gene therapy).

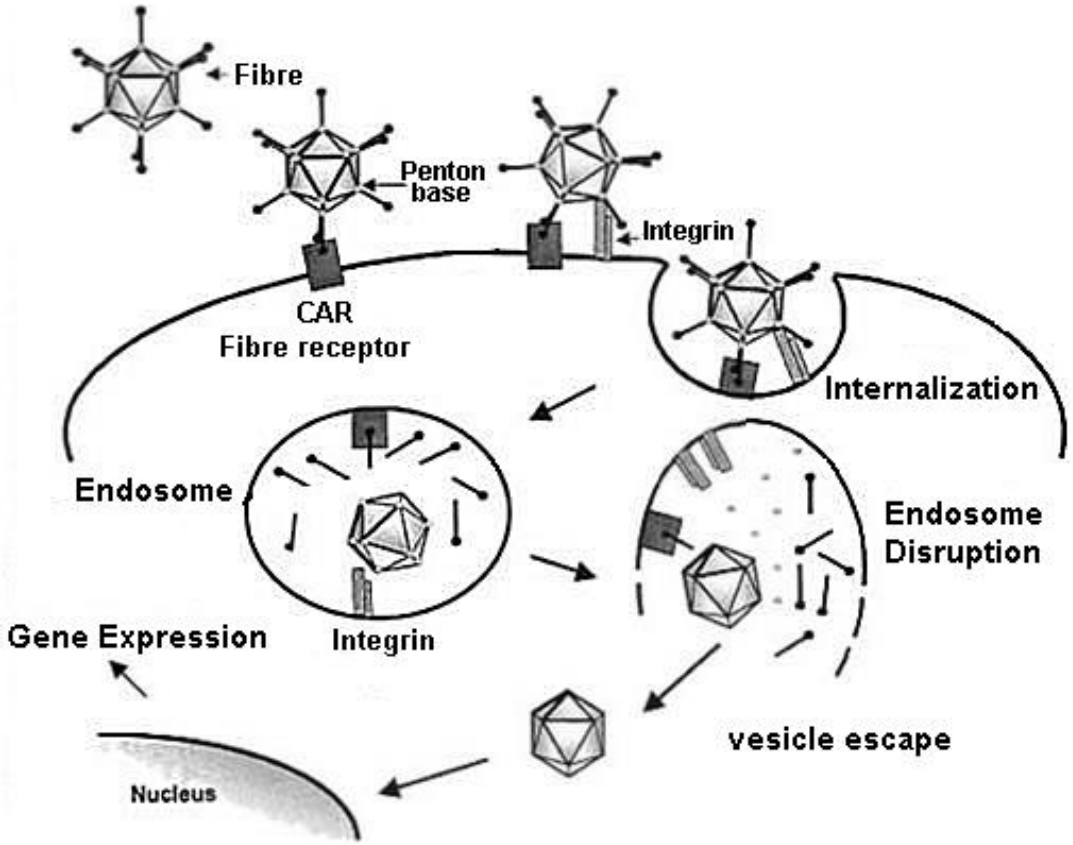
1.3.4 Replication

The different stages in the successful infection of a permissive cell involves virus attachment, endocytosis, production of progeny, virion release and cell lysis (Figure 1.3A). A typical productive infection of cultured cells takes 20-30hrs following a very high multiplicity of infection, however, in vivo host infection is very much prolonged as the level of viral exposure is much lower, in comparison to direct infection of cultured cells. The adenovirus first binds to cell surface receptors on the target cell. Contact begins when the adenovirus fibre protein attaches to cellular receptors, for instance the Coxsackievirus B3 and Adenovirus receptor (CAR) (Lonberg-Holm and Philipson, 1969; Shenk 1996), while the penton base protein binds to vitronectin-binding integrins (Wickham *et al.,* 1993). Both interactions are required for virus attachment and endocytosis (Meier and Greber 2003). The virus-receptor complex is internalised by endosomes, and in sequential uncoating steps the virus particle is released into the cytoplasm and is eventually transported into the nucleus by association with the cellular microtubules (Dales and Chardonnet 1973; Luftig and Weihing 1975). The viral capsids inject the viral DNA through the nuclear pore. Thirty minutes after internalisation, most of the viral DNA reaches the inside of the nucleus. Naked adenovirus DNA is incorporated by host cell histones and core adenovirus proteins into the chromatin. The viral DNA is transcribed by the host cell machinery to express both the early and late genes (Figure 1.3B). The early region genes code for non-structural, regulatory proteins (E1A, E1B, E2, E3 and E4), that re-programme host cell transcription to create a favourable environment for virus replication (Shenk 1996, Burgert *et al.,* 2002). E1A promotes cell cycle progression and DNA synthesis by targeting cellular proteins involved in transcription regulation (refer to section 1.3.5.1). E1B functions include (during infection) targeting and degradation of cellular proteins that may negatively affect viral replication such as p53 and MRN complex (refer to section 1.3.5.2). The E2 gene comprises of E2A and E2B regions that encode for proteins vital for DNA replication (Challberg and Kelly 1979; Hay *et al.,* 1995; Berk 2007). E2B encodes for precursor terminal protein (80kDa) and viral DNA polymerase, AdPol (140kDa), while E2A gene

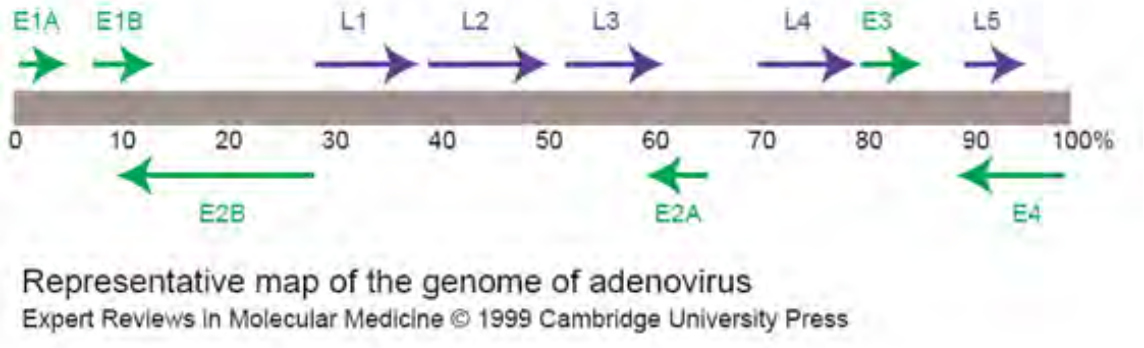
product is a 72kDa nuclear ssDNA binding protein, DBP (Challberg *et al.,* 1982; Cleat and Hay 1989; Ikeda *et al.,* 1982; Stillman *et al.,* 1981; Stuiver and van der Vliet 1990). All three proteins collaborate in viral DNA replication. Due to alternative splicing events, the E4 gene produces an array of mRNAs which in turn encodes several different proteins (Virtanen *et al.,* 1984). For instance, both E4-ORF1 and E4-ORF4 activate mTOR protein kinase in the absence of mitogenic and nutrient signaling (O'Shea *et al.,* 2005). Activation of mTOR leads to a stimulation of protein synthesis and entry to the S phase (Hay and Sonenberg 2004; Martin and Hall 2005; Lane *et al.,* 1993). E4-ORF4 can also activate mTOR independently of E4-ORF1 (O'Shea 2005). E4-ORF3 interferes with the function of MRN complex inhibiting cellular DNA damage response (Evans and Hearing 2005; Stracker *et al*., 2002). E4-ORF6 is also involved in the inhibition of the DNA damage response (Boyer *et al.,* 1999; Carson *et al.,* 2003). It does this by interacting with E1B to cause the proteasomal degradation of the MRN complex and p53 (Stracker *et al.,* 2002) (see also section 1.3.5.2).

10-14hr following high multiplicity infection, the late phase of adenovirus infection begins. The late phase genes (major late promoter) code for structural proteins and others involved in the packaging of viral DNA. The host cells' machinery is redirected towards the packaging and assembly of progeny viruses. These are released following the weakening of the cytoskeleton by the viral proteinases, eventually followed by cell death initiated by adenovirus death protein (ADP) (Shenk 1996; Tollefson *et al.,* 1996).

A



B



Representative map of the genome of adenovirus
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Figure 1.3 : Adenovirus, replication and genome

A Diagram depicting replication cycle of an adenovirus: Adenovirus binds to the cell receptors on the surface of the host cell, entering the cell via endocytosis. The virus escapes the endosome then enters the nucleus. Transcription of the early and late viral genes occur, and regulatory and structural proteins are expressed. New viral progeny are assembled and they exit the cell through cell lysis (From Wagner E., Hewlett M.J., Bloom D.C., Camerini D. 2008. Basic Virology. Blackwell Publishing., M.A. 452 pp).

B Gene expression profile of the adenovirus genome. E refers to early region genes while L for the late proteins.

1.3.5 Adenovirus E1 region

The adenovirus E1 region constitutes two transcriptional units that encode the major proteins that are crucial for the progression of infection, namely E1A and E1B. These are discussed in detail below.

1.3.5.1 Adenovirus early region 1A

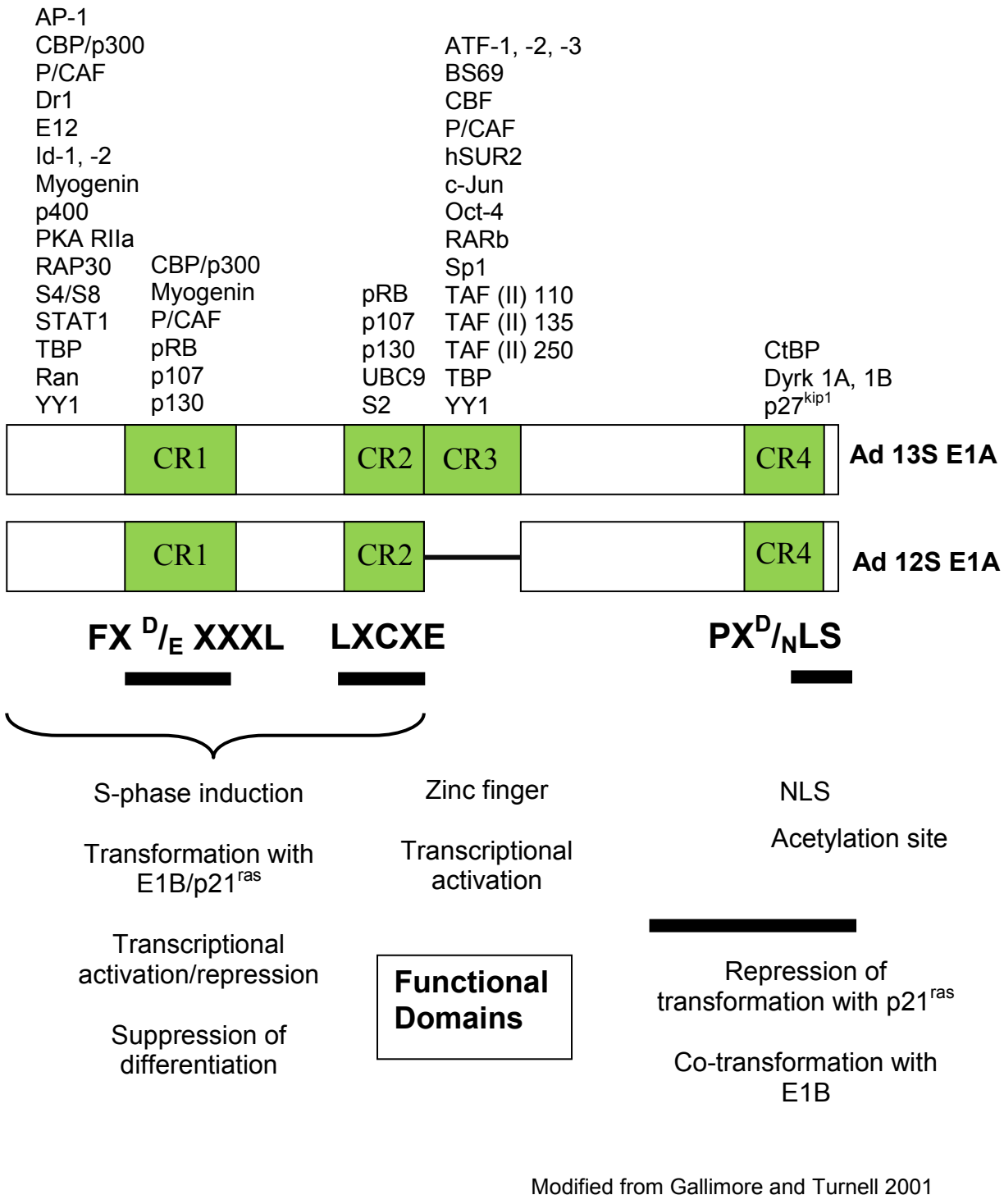
Adenovirus early region 1A (AdE1A) is first viral protein to be expressed following infection. The AdE1A gene is situated within the first 1500bp of the viral genome. It is essential for viral infection and is involved in the induction of cell cycle progression, DNA synthesis and the positive and negative regulation of cellular transcription (Frisch and Mymryk 2002; Gallimore and Turnell 2001; Ben Israel and Kleinberger 2002). It also regulates the expression of other adenovirus early region genes (Nevins 1981; Berk *et al.,* 1979; Jones and Shenk 1979). Additionally, with a cooperating oncogene like AdE1B or mutated *Ras* it has the capacity to transform both primary human and rodent cells (Ruley 1983).

As a result of differential splicing of the primary transcript, AdE1A exists in various isoforms with mRNAs 12S and 13S being the most abundant and transcribed early in infection while 9S, 10S and 11S are present at minor levels and accumulate at later stages of infection (Stephens and Harlow 1987; Ulfendahl *et al*., 1987; Boulanger and Blair 1991). The 12S and 13S mRNAs encode 243 and 289 amino acid proteins, respectively in Ad2/Ad5. Both proteins are identical in all serotypes except that in 13SAdE1A there is an additional conserved region 3 (CR3) that is situated towards the C terminus; this region acts as a transcriptional activation domain (Berk *et al.,* 1979; Jones and Shenk 1979; Ablack *et al.,* 2010). The size of AdE1A is about 26 to 32kDa in molecular weight, with the pI being around 4-5 depending on the serotype (Bayley and Mymryk 1994) and is localised roughly equally between the cytoplasm and nucleus (Rowe *et al.,* 1983; Grand and Gallimore 1984) It is degraded rapidly with a half-life of 20-80mins

in infected cells (Spindler and Berk 1984). AdE1A functions primarily through a complex series of protein-protein interactions targeting various regulatory host proteins involved in cell cycle progression, DNA synthesis, apoptosis and differentiation by activating or repressing their expression at a transcriptional level. For instance, transcriptional co-repressors such as the Rb family and CtBP, co-activators such as p300 and CBP, and proteins involved in chromatin modification such as TRRAP and p400 all interact with AdE1A. These are discussed in detail in section 1.3.5.1.2. AdE1A is possibly the most studied viral oncogene, and it had an important role in the identifying and understanding cellular proteins crucial in the various regulation processes in the cell.

1.3.5.1.1 Structure of AdE1A

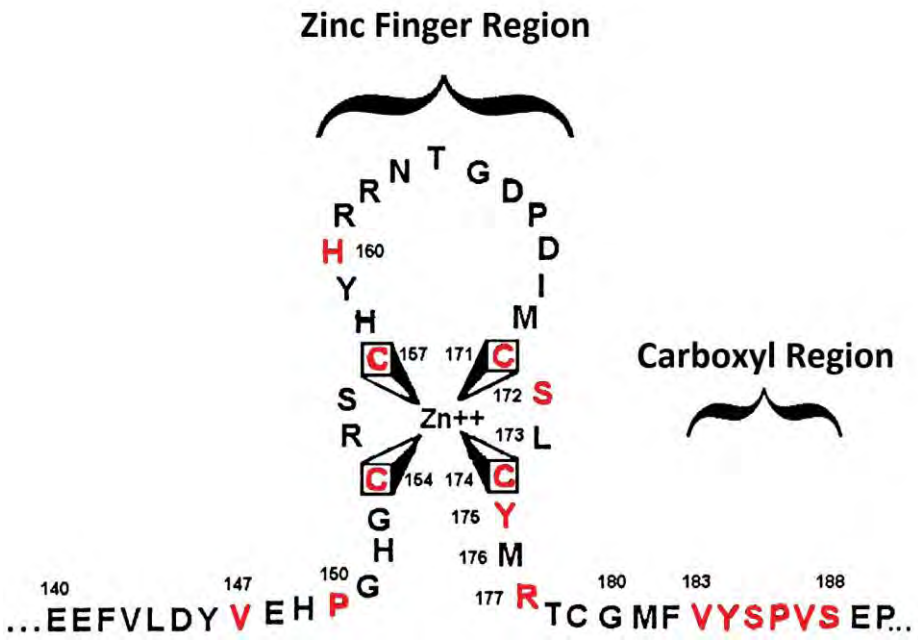
Comparison of AdE1A amino acid sequences from the different adenovirus serotypes of human and simian origin has helped identify 4 highly conserved regions CR1, CR2, CR3 and CR4 (Kimelman *et al*., 1985; Avvakumov *et al*., 2002, 2004). These regions are distributed throughout the protein and comprise about half the residues (Avvakumov *et al.,* 2002, 2004). They encompass amino acid positions 42-72, 113-137, 144-191, and 240-288 respectively in the Ad513SE1A protein (Figure 1.4). Between the different serotypes, CR1 is found to be the most highly conserved while CR4 is the least. There is also an N-terminal region, amino acids 1-39 (in Ad5), known to be weakly conserved between the different adenovirus serotypes with the exception of a conserved three amino acid sequence ILE at residues 18-20 (Gedrich *et al*. 1992), as well as M1, R2, L4 and L7 residues (Rasti *et al.,* 2005; Avvakumov *et al.,* 2002) that exists within a predicted *α*-helical structure that spans residues 13-29 in Ad2/5 (Rasti *et al.,* 2005; Pelka *et al.,* 2008; Grand and Molloy 2009). The conserved regions, in addition to the N-terminal region, are the binding sites for almost all of AdE1A's binding partners.



Modified from Gallimore and Turnell 2001

Figure 1.4. Representation of 12S and 13S AdE1A showing conserved regions, associated cellular binding proteins and functional domains.
Schematic diagram displaying the positions of the regions conserved across the different serotypes: CR1, CR2, CR3 and CR4 as well as the N-terminus region. The interacting cellular proteins for the corresponding region are also shown, in addition to the structural motifs (zinc finger) and other features, Nuclear localisation region (NLS) and acetylation site. Also depicted are the functional domains of AdE1A that involve in transcriptional repression/activation, transformation and repression of differentiation.

Studies on AdE1A secondary structure noted the possibility of one or two *α*-helices in CR1, and two within CR3 and one in CR4 (Avvakumov *et al.,* 2002; Pelka *et al.,* 2010; Grand and Molloy 2009). The CR3 of the 13S E1A has two domains: a zinc finger structure (residues 147-177) (Culp *et al.,* 1988) and a carboxyl domain (183-188) – Figure 1.5. Mutational analysis revealed that 16 residues within the CR3 are crucial for its transactivation function. The four cysteine residues binding the Zn^{2+} atom: C154, C157, C171 and C174; H160 which is part of the zinc finger loop; S172, Y175 and R177 that are positioned at the C terminal side of the loop; V147 and P150 are in the N-terminus of the zinc finger region and finally the six residues at 183-188 that form part of the carboxyl region of the CR3 (Webster and Ricciardi 1991).



Modified from Sanchez *et al.,* 2000

Figure 1.5 Schematic representation of CR3 of adenovirus E1A 13S 289R
An illustration of the CR3 of 13S AdE1A, showing the zinc finger and carboxyl region; also shown are the four cysteine residues that bind the Zn^{2+} and the crucial residues involved in its transactivation function (in red).

Ad5E1A also has multiple phosphorylation sites, at serine residues 89, 219, 132, 185, 188 and 231 (Whalen *et al.,* 1997). It was observed that mutations at these sites caused modest effects on AdE1A functions which may indicate that phosphorylation could regulate AdE1A activity to some extent (Whalen *et al.,* 1996, 1997; Mal *et al.,* 1996). However, the exact biological function of these has yet to been determined. AdE1A can also be acetylated at a lysine residue (Zhang *et al.,* 2000; Molloy *et al.,* 2006; Madison *et al.,* 2002). One report has found that acetylation of lysine residue 285 and 261 (in Ad5E1A and Ad12E1A respectively) and lysine residue 239 may interfere with its association with the transcriptional co-repressor CtBP (carboxyl-terminal binding protein) (Zhang *et al.,* 2000; Molloy *et al.,* 2006). However, this was contradicted by another study that showed that acetylation of lysine 239 does not disrupt binding to CtBP (Madison *et al.,* 2002) however it affects its distribution between the nucleus and cytoplasm; with acetylated AdE1A being enriched in the latter and excluded from the nucleus (Madison *et al.,* 2002). Similarly, at the carboxyl terminus of AdE1A, there is a pentapeptide (Lys-Arg-Pro-Arg-Pro) which serves as a signal for the import of E1A to the nucleus (Lyons *et al.,* 1987). Despite this, it has been found that E1A proteins are present equally in both cytoplasm and nucleus (Rowe *et al.,* 1983, Grand and Gallimore 1984; Turnell *et al.,* 2000).

AdE1A has evolved to contain a number of motifs which can mimic the binding sites of certain cellular proteins (See table 1.2). It has been presumed that AdE1A binds to a particular host protein with a motif that is similar or identical to the host protein's original partner thus displacing or preventing the normal interaction, eventually leading to the deregulation of that pathway. An example of this is the binding of AdE1A to the retinoblastoma (Rb) protein through the LXCXE motif, which is also present in the transcription factor E2F (Fattaey *et al.,* 1993; Ikeda and Nevins 1993). These interactions will be discussed in the next section.

Table 1.2: AdE1A motifs present in cellular proteins

Sequence	AdE1A Location	Examples of Cellular proteins with the motif	Binding protein
YxxxExAxS/TLLxxxL	NTR	APC5, APC7	CBP/p300
LxxLIxxxL	NTR	NCoR	Thyroid hormone receptor
LLxxLxxLL	NTR/CR1	ACTR, TIF-2, Ets-1, Ets-2	CBP
LxD/ELY/F	CR1	E2Fs	Rb, p107, p130
FxD/ExxxL	CR1	APC5, APC7	CBP/p300
LxCxE	CR2	CtIP, cyclinD, MDM2, BRM, BRG1	Rb, p107, p130
PxDLS	CR4	CtIP, ZF217, HDAC, Net, ZEB, Ikaros, FOG-2	CtBP1 + 2

Modified from Grand and Molloy 2009

As mentioned previously, the oncogenicity of adenoviruses depend on the serotype. Ad12 is known to be oncogenic whereas Ad2/Ad5 is not. The sequences of both serotypes are about 50% identical; however there is an alanine-rich region within Ad12 E1A between CR2 and CR3, that is not present in Ad5 E1A, known as the oncogenic spacer region, and thus appears be partly responsible for the ability of Ad12 E1A to induce tumours (Telling and Williams 1994, Jelinek *et al.,* 1994, Williams *et al.,* 1995).

With regards to the secondary and tertiary structure of AdE1A, there is so far no published structure. Several failed attempts have been made by various labs using crystallography or nuclear magnetic resonance (NMR). As a result, it was concluded that AdE1A is largely disordered (Pelka *et al.,* 2008). The AdE1A sequence has a high percentage of proline residues that are distributed throughout the primary sequence, which probably prevents extensive secondary structure formation (reviewed by Frisch and Mymryk 2002, Pelka *et al.,* 2008). Additionally, other studies have hypothesized that some structure may be limited to parts of the conserved regions. For instance, in Ad4E1A, the protein is unfolded apart from the CR3 and a short region in CR4. In Ad3, Ad5, Ad9 and Ad12, structure was

limited to the N terminus and CR3. This disordered structure is one feature exhibited by hub proteins. Most cellular proteins interact with one or two other proteins, however, a minority bind to tens or even hundreds of other proteins to form a network hub with multiple interaction partners (Pelka *et al.,* 2008). This unfolded conformation may allow AdE1A to bind to multiple proteins where the intrinsic disordered structure allows the flexibility for multiple protein interactions. A virally-encoded hub protein like AdE1A can reprogramme aspects of cell function and behaviour by disrupting the cellular protein interaction network (Pelka *et al.,* 2008).

1.3.5.1.2 Interactions of AdE1A

AdE1A protein is crucial for both infection and adenovirus-mediated transformation. AdE1A itself does not bind to DNA even though some ionic interactions between phosphodiester backbone of DNA and a basic region at residues 201-218 were reported. (Avvakumov *et al.,* 2002). There have also been suggestions that AdE1A associates with other cellular DNA binding domains and hence is recruited to various promoters (Liu and Green 1994) although supporting evidence is limited. It binds to key proteins involved in transcriptional regulation and cell growth such as co-activators, co-repressors, cell cycle regulatory proteins and parts of the transcriptional machinery (reviewed in Berk 2005; Gallimore and Turnell 2001; Frisch and Mymryk 2002). Some of these interactions will be discussed in the next section. Transcriptional activation is mediated largely by the CR3 region of 13SAdE1A while transcriptional repression is mediated by the N-terminal region of both 12S and 13S AdE1A (Pelka *et al.,* 2009). The N-terminus and the highly conserved regions (CR1, CR2, CR3 and CR4) all contribute towards the AdE1A-mediated transformation. Figure (1.4) shows the different array of cellular proteins and their binding sites within AdE1A. Nearly 30 different cellular proteins bind within this sequence of less than 300 amino acids, as a result sabotaging the cell's ability to control cell cycle, transcription and gene expression. The majority of AdE1A targets are located in the nucleus with a few exceptions such as the cytoplasmic proteins Sug1 (S8) and S4 (Grand

et al., 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004) that are components of the proteasome and RACK1 (a protein kinase C scaffolding protein) (Sang *et al.,* 2005) and IRS-4 (Shimwell *et al.,* 2009). Due to its ability to bind to multiple proteins and complexes with its multiple binding sites, AdE1A has been classified as a "hub" protein (Pelka *et al.,* 2008; Keskin and Nussinov 2007, Grand and Molloy 2009) that can undergo both simultaneous and consecutive interactions (Pelka *et al.,* 2008). By reprogramming host cell transcription, AdE1A not only creates an environment suitable for viral replication but also, in combination with AdE1B and mutant activated *Ras*, can potentially immortalise and transform cells. It has been shown that Ad5E1A and Ad12E1A expression alone can promote transformation in baby rat kidney cells (BRKs) however these quickly die through apoptosis (Shiroki *et al.,* 1979; Houweling *et al.,* 1980; Gallimore *et al.,* 1985). The collaboration of AdE1B aids stable transformation when co-expressed with AdE1A as it inhibits E1A-induced apoptosis (Bernards *et al.,* 1986; White and Cipriani, 1990; Rao *et al.,* 1992; Debbas and White 1993; Lowe and Ruley, 1993).

1.3.5.1.3 Transcriptional activation by AdE1A

AdE1A promotes DNA synthesis and entry into S-phase in quiescent cells by interactions with at least two cellular protein families, the retinoblastoma tumour suppressor protein pRB and CBP/p300 (Howe *et al.,* 1990). AdE1A binds to CBP/p300 through both its N-terminal and the CR1 region while it binds pRB through CR1 and CR2 region (Barbeau *et al.,* 1992; Dyson and Harlow 1992; Cobrinik 2005). CR2 contains an LXCXE motif, a sequence conserved in oncoproteins from small DNA tumour viruses such as HPV E7 proteins and SV40 large T antigen (Whyte *et al.,* 1988, Dyson *et al.,* 1989, 1990, 1992; Munger el al 1989; Chellappan *et al.,* 1992; Knudsen and Wang 1998). E2F binds to the pRB pocket via its own LXCXE motif. In normal cells, pRB in its dephosphorylated state, inhibits DNA replication and cell cycle entry by binding to and inhibiting the transcription factor E2F (Nevins *et al.,* 1997; Dyson 1998; Nevins 2001; Trimarchi and Lees 2002). The activity of E2F family of transcription factors can push a cell into S-phase. High affinity

binding of E1A through its LXCXE motif to the same “pocket domain” that pRB binds to in E2F displaces pRB releasing E2F (Nevins *et al.,* 1997; Ghosh and Harter 2003) to become active eventually pushing the cell into the cell cycle by activating the transcription of genes involved in DNA synthesis such as cyclin-dependent kinase CDK2 and cyclins E and A (Figure 1.6).

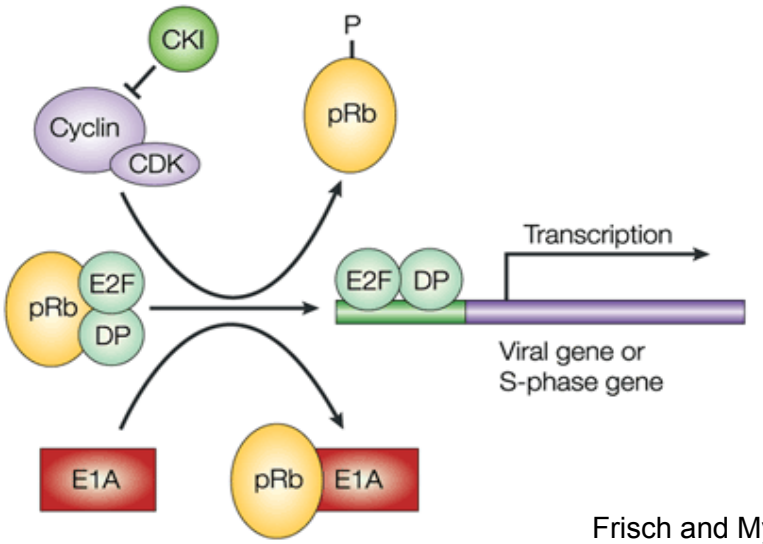


Figure 1.6 AdE1A disrupts retinoblastoma (pRB) and E2F interaction
At the G0 phase, the hypophosphorylated pRB binds to the transcription factor E2F/DP preventing it from activating transcription. For the progression through the cell cycle, cyclin-cyclin dependent kinases (CDKs) phosphorylate pRB hence releasing E2F to activate transcription of target genes required for the S-phase. This is counteracted by CDK inhibitors that bind to cyclin-CDK complex to prevent it from phosphorylating pRB. The presence of AdE1A disrupts this regulated cell cycle control by binding to hypophosphorylated pRB and thus disrupting its interaction with E2F, releasing the latter to initiate transcription.

AdE1A also interacts with the Rb homologous proteins p107 and p130 through CR1 and CR2 (LXCXE motif) to relieve transcriptional repression (Ewen *et al.,* 1991, Cobrinik *et al.,* 1993, Hannon *et al.,* 1993; Li *et al.,* 1993). p107 and p130, together with pRB, form a retinoblastoma family of transcriptional regulators, also known as “pocket proteins” (Classon and Dyson 2001). p107 and p130 are structurally and functionally related to pRB, one prominent shared function between all three proteins being the negative

regulation of E2F (Grana *et al.,* 1998; Classon and Dyson 2001). Similar to pRB, the phosphorylation status of p107 and p130 is modulated during the cell cycle (Grana *et al.,* 1998; Classon and Dyson 2001), where the hyperphosphorylated forms do not associate with E2F (Beijersbergen et al., 1995; Mayol et al.,1996; Xiao et al.,1996). As with pRB, AdE1A binds to p107 and p130 through its CR1 and CR2 regions (Dyson *et al.,* 1992), disrupting their binding to E2F. Furthermore, AdE1A also blocks hyperphosphorylation of p107 and p130, without affecting the phosphorylation status of pRB (Parreno *et al.,* 2000). It was suggested that this selective targeting of p107 and p130 hyperphosphorylation may indicate that both proteins may have an additional function that is not shared by pRB (Parreno *et al.,* 2000), but whose inactivation is important for the biological effect of AdE1A (Parreno *et al.,* 2000). Perhaps, this function may potentially be as a CDK inhibitor (Zhu *et al.,* 1995), as it has been shown that p107 and p130 form stable complexes with cyclin A/E-dependent kinases (Ewen *et al.,* 1992; Faha *et al.,* 1992; Lees *et al.,* 1992).

Besides inhibiting E2F activity, pRB also recruits histone-directed deacetylases (HDACs) to the promoter of its target genes (Lai *et al.,* 1999; Brehm *et al.,* 1998; Magnaghi-Jaulin *et al.,* 1998). When histones are deacetylated, they bind tightly to DNA, blocking accessibility to transcription factors hence blocking DNA synthesis (Luo *et al.,* 1998, Ferreira *et al.,* 1998, Magnaghi-Jaulin *et al.,* 1998). Though HDACs also have an LXCXE motif, they appear to bind pRB through the bridging protein RBP1 (Suryadinata *et al.,* 2010). Hence AdE1A interaction with pRB also influences histone and chromatin structure.

Another role of AdE1A in chromatin structure control includes association with CBP/p300 protein through its CR1 domain (Stein *et al.,* 1990; Barbeau *et al.,* 1992; Wang *et al.,* 1993; Arany *et al.,* 1995). CBP/p300 is a multi-domain protein known to possess HAT (histone acetyltransferase) activity (Chan and La Thangue 2001; Vo and Goodman 2001; Iyer *et al.,* 2004). It acetylates various proteins such as p53 (Gu and Roeder 1997; Ito *et al.,* 2001), MyoD (Polesskaya and Harel-Bellan 2001) and histones (Bannister and Kouzarides 1996; Ogryzko *et al.,* 1996). The acetylation of histone proteins causes the

loosening of DNA from the structure, hence rendering it more accessible to transcription factors. Ad12 AdE1A stimulates this HAT activity and recruits CBP/p300 for promoter activation (Fax *et al.,* 2000). This activation of HAT together with AdE1A's interaction with pRB, leads to the activation of genes required for S-phase entry. CBP/p300 is also a recruiter of basal transcription machinery including RNA polymerase II, TFIIB and TBP (Nakajima *et al.,* 1997, Kee *et al.,* 1996, Yuan *et al.,* 1996, Kwok *et al*., 1994) leading to activation of the target genes. However research from other laboratories has shown that AdE1A can also inhibit CBP/p300 activity (Frisch and Mymryk 2002) (Refer to 1.3.5.1.4). There is still no agreement on the exact effect of AdE1A on CBP/p300 and little understanding as to how inhibition of HAT activity promotes entry into S-phase. However, it has been reported that inhibition of p300 expression leads to a marked increase in Myc expression as well as activation of cyclin E and cyclin A/cyclin dependent kinase (CDK) activity, in turn driving G0 cells into S-phase. This shows that AdE1A inhibition of p300 function may drive entry into S-phase due to the increase in Myc expression and cyclin/CDKs (Kolli *et al.,* 2001, reviewed by Berk 2005). However, the mechanism of inhibition of p300 and subsequent increase in Myc expression is still not clear. Additionally, CBP/p300 is reported to promote transcription of genes involved in cellular differentiation (Goodman and Smolik 2000), suggesting that AdE1A inhibition of CBP/p300 may inhibit the expression of genes involved in differentiation and cell cycle exit (Frisch and Mymryk 2002). Other chromatin-remodelling complexes associated with AdE1A are p400 (Fuchs *et al.,* 2001; Flinterman *et al.,* 2007; Tworkowski *et al.,* 2008) which contains a DNA helicase domain, P/CAF (Lang and Hearing 2003) and TRAPP which is a component of three HAT complexes TIP60, hGen5 and P/CAF HAT complexes in human cells (Deleu *et al.,* 2001; Fuchs *et al.,* 2001; Nikiforov *et al.,* 2002; Sterner and Berger 2000).

The TATA box is a 25 to 30bp DNA sequence found in the promoter region of many viral and cellular genes. It has been reported that approximately 24% of human genes have the TATA sequence as part of their core promoter (Yang C *et al.,* 2007). It is therefore, one of

the most important elements of transcriptional activation. It is now known that 13S AdE1A can promote transcription through the TATA box by interacting with TBP (TATA-binding protein) which is a transcription factor that binds directly to the TATA sequence. AdE1A binds TBP through its N-terminal domain and CR3. TBP along with other TBP-associated factors (TAFs), make up the basal transcription factor IID (TFIID), the first protein complex to bind to DNA during the formation of the pre-initiation transcription complex at a promoter of a gene (Horikoshi *et al.,* 1991, Lee *et al.,* 1991, Geisberg *et al.,* 1994). The tumour suppressor protein p53 can bind to TFIID causing transcription repression. It binds to a domain in TBP that overlaps with the AdE1A binding site. Thus AdE1A interaction probably displaces p53, disrupting p53 mediated repression (Horikoshi *et al.,* 1995).

CR3, the transactivation domain of AdE1A, can also interact with various DNA-binding transcription factors such as the Activating transcription factor - 2 (ATF-2), Specificity protein - 1 (SP1), Upstream stimulatory factor (USF) (Liu and Green 1990, 1994) and TBP associated factor (TAF) (Mazzarelli *et al.,* 1997; Geisberg *et al.,* 1995). This enables AdE1A to stimulate transcription from a variety of genes that lack a common promoter element (Liu and Green 1990, 1994).

AdE1A is also found to bind to a cellular transcription factor Yin Yang 1(YY1) (Lewis *et al.,* 1995). YY1 is a multifunctional, zinc finger-containing, transcription factor that represses transcription if bound upstream of heterologous basal promoters. Two regions within YY1 are required for its repression activity: the N terminal (residues 54-260) and the carboxyl region (residues 332-414). It has been shown that AdE1A binds to those two regions, affecting YY1 function and relieving transcriptional repression (Lewis *et al.,* 1995). AdE1A binds YY1 through its N terminal and CR3 regions. Another transcriptional repressor Dr1 functions by binding to TBP, preventing it from associating with TFIIA (involved in RNA polymerase II dependent transcription of DNA). Through its N terminal region, AdE1A binds to Dr1 disrupting its association with TBP, releasing it to associate with TFIIA (Inostroza *et al.,* 1992, Kraus *et al.,* 1994; Shenk 1996).

The chromatin remodelling factors TRRAP and p400 are also targeted by AdE1A during cellular transformation (Fuchs *et al.,* 2001; Deleu *et al.,* 2001). In fact, in addition to disrupting Rb and p300/CBP function, AdE1A must also recruit TRRAP to transform cells (Deleu *et al.,* 2001). TRRAP is an adaptor protein that links Myc with histone acetylases (McMahon *et al.,* 2000). Myc is an oncoprotein transcription factor that regulates the expression of 15% of genes including ones involved in cell division, cell growth and apoptosis (Gearhart *et al.,* 2007). Myc recruits TRRAP to target chromatin and induces localised histone acetylation (Bouchard *et al.,* 2001; Frank *et al.,* 2001) activating the transcription of Myc-target genes. As with Myc, AdE1A also binds TRRAP through its N-terminus, involving it in cell transformation (Deleu *et al.,* 2001). AdE1A can also regulate Myc via the chromatin regulator p400. In normal cells, Myc is present at low levels and its expression highly regulated, through, for instance, ubiquitin-mediated proteolysis (Gregory and Hann 2000). AdE1A stabilises Myc by interfering with its ubiquitylation (Tworkowski *et al.,* 2008; Chakraborty and Tansey 2009). In addition AdE1A interaction with p400 promotes interaction with Myc hence the formation of more Myc-p400 complexes (Chakraborty and Tansey 2009; Tworkowski *et al.,* 2008), and these complexes in turn go on to activate Myc-target genes (Chakraborty and Tansey 2009).

In both infected and transformed cells, AdE1A also binds to MED23 (also called SUR-2), a part of the Mediator that is a multiprotein complex involved in the transcriptional activation (Boyer *et al.,* 1999, Wang and Berk 2002). AdE1A binds to the Mediator through the CR3 zinc finger region and recruits it to activate transcription. 12S AdE1A, lacking CR3, does not bind to Mediator complex. Studies from AdE1A transformed cell lines, for instance 293, showed that much of the AdE1A bound to the Mediator is also in a stable complex that includes RNA polymerase II, which led to the belief that AdE1A-CR3/Mediator interaction stabilised Mediator/polymerase interaction, and contibutes to transcriptional activation (Wang and Berk 2002). Similarly, AdE1A, through the CR3 domain, promotes the assembly of transcription preinitiation complexes (PICs) (Roeder 1998) that includes RNA polymerase II and its general transcription factors (Cantin *et al.,* 2003), and this

assembly is stimulated by AdE1A by its association with Mediator complex (Cantin *et al.,* 2003).

Another role of AdE1A contributing to the initiation of the cell cycle is their binding to the CDK inhibitors (CKIs) p21 (Chattopadhyay *et al.,* 2001) and p27 (Mal *et al.,* 1996; Alevizopoulos *et al.,* 1998; Nomura *et al.,* 1998) preventing them from binding to CDK-cyclin complexes therefore promoting the cell cycle. CKIs repress the activity of CDK-cyclin complexes which are regulators of cell cycle progression, helping drive each stage through the cell cycle in response to a variety of growth factors (Pines 1999). Another way that AdE1A targets the CKI p21 is by inhibiting its transactivation by p53 via sequestering CBP/p300 that are required for p53 function (Somasundaram and El-Deiry 1997).

1.3.5.1.4 Transcriptional repression by AdE1A

AdE1A, in particular 12S, can also repress transcription of many cellular genes, such as those involved in cell differentiation and p53 expression. The N-terminus and the CR1 region are crucial for AdE1A transcriptional repression (Song *et al.,* 1995), and a number of proteins are involved in mediating this repression. In the previous section, it was shown that AdE1A associates with CBP/p300 for transcriptional activation. In contrast, it was also found by other laboratories to inhibit CBP/p300 activity (Howe *et al.,* 1990; Stein *et al.,* 1990; Mymryk *et al.,* 1992; Eckner *et al.,* 1994; Lundbad *et al.,* 1995; Giles *et al.,* 1998; Lipinski *et al.,* 1999; Hamamori *et al.,* 1999; Giordano and Avantaggiati 1999). Through the N-terminal region and CR1, AdE1A binds the TRAM domain (transcriptional adaptor motif) of the CBP/p300 (O'Connor *et al.,* 1999) inhibiting its HAT activity (Chakravarti *et al.,* 1999; Ait-Si-Ali *et al.,* 1998), as well as disrupting its ability to recruit the basal transcription components, hence repressing transcription (Arany *et al.,* 1995, Goodman and Smolik 2000). Additionally, as CBP/p300 is also known to possess HAT activity, this binding to AdE1A can also disrupt this function, preventing accessibility of target promoters to transcription factors, leading also to repression of transcription activity (Bannister and Kouzarides 1996; Yang *et al.,* 1996; Ogryzko *et al.,* 1996, Ait-Si-Ali *et al.,*

1998, Reid *et al.,* 1998; Chakravarti *et al.,* 1999). P/CAF (CBP/p300-associated factor) is a mammalian acetyl transferase that interacts with CBP/p300 (Yang *et al.,* 1996). The N-terminal and CR3 regions of AdE1A bind to P/CAF through its HAT domain (Reid *et al.,* 1998) thus disrupting its association with CBP/p300 and inhibiting its HAT activity along with P/CAF mediated transcriptional activation (Figure 1.7B) (Reid *et al.,* 1998; Chakravarti *et al.,* 1999). However, in addition to histones, P/CAF also acetylates p53 in response to DNA damage which leads to the enhancement of p53 binding to promoters of genes such as those encoding p21 (CDK inhibitor) (Liu *et al.,* 1999; Sakaguchi *et al.,* 1998). It also acetylates MyoD (a protein involved in the regulation of muscle differentiation), inducing MyoD dependent gene expression and p21 expression finally leading to cell cycle arrest (Puri *et al.,* 1997; Sartorelli *et al.,* 1999). AdE1A binding to P/CAF therefore represses cell cycle exit and cell differentiation.

The tumour suppressor protein p53, which is commonly mutated in many cancers, is also targeted by AdE1A. It is known that CBP/p300 binding to p53 is required for its function, (Lill *et al.,* 1997, Levine 1997, Prives and Hall 1999, Lowe 1999) as well as CBP/p300 and p53 both being able to activate transcription synergistically (Gu *et al.,* 1997). Additionally, CBP/p300 also regulates p53 allowing to accumulate stability by directly interacting with the protein (Lowe and Ruley 1993, Querido *et al.,* 1997a, Chiou and White 1997, Yuan *et al.,* 1999). p53 is normally unstable and degraded by the ubiquitin-proteasome pathway. CBP/p300 mediates binding between p53 and MDM2 which is an E3 ubiquitin ligase that recruits E2, an ubiquitin conjugating enzyme, that, in turn, ubiquitinates p53 for degradation by the 26S proteasome. As a result of binding to CBP/p300, AdE1A represses p53 transactivation (Song *et al.,* 1995), and by disrupting p53 ubiquitination, prevents its degradation, hence p53 allowing to accumulate in the cell (Figure 1.7A). Similarly, AdE1A induces cellular p53 levels through the tumour suppressor $p19^{ARF}$ (de Stanchina *et al.,* 1998). AdE1A inactivates Rb and this leads to the accumulation of $p19^{ARF}$, which in turn leads to the prevention of MDM2-mediated proteolytic degradation

and thus accumulation of p53 protein (Pomerantz *et al.,* 1998; Zhang *et al*., 1998). Rising p53 levels leads to cell cycle arrest and apoptosis.

We have seen that AdE1A either blocks CBP/p300-dependent transcription or activates it through redirecting its HAT activities to other regulatory proteins like pRB and cellular promoters. AdE1A might mediate these functions through the use of different binding proteins in a large multi-protein complex with CBP/p300 (Gallimore and Turnell 2001). Another transcriptional co-activator with HAT properties is P/CAF (CBP/p300-associated factor) (Yang *et al.,* 1996).

Another AdE1A target for transcriptional repression is the TATA binding protein TBP. It was noted in the previous section that 13S AdE1A can promote transcription through the TATA box by interacting with TBP transcription factor. TBP along with other TBP-associated factors (TAFs), make up the basal transcription factor IID (TFIID), the first protein complex to bind to DNA during the formation of the pre-initiation transcription complex at a promoter of a gene (Horikoshi *et al.,* 1991, Lee *et al.,* 1991, Geisberg *et al.,* 1994). On the contrary, AdE1A can also repress transcription by binding to TBP through its N-terminal region. It has been reported that the N-terminal region in the 12S AdE1A can bind independently to TBP (Geisberg *et al.,* 1994; Song *et al.,* 1997). However, this binding affinity is five times greater in 13S AdE1A than in 12S AdE1A. AdE1A binding to the TBP prevents it binding to the TATA box thus interrupting transcription initiation.

Both TBP and CBP/p300 are required in p53 transactivation, so AdE1A association with both protein enables it to control and regulate p53 activity and transactivation. Complete repressive activity of AdE1A requires the association with both TBP and CBP/p300 (Boyd *et al.,* 2002).

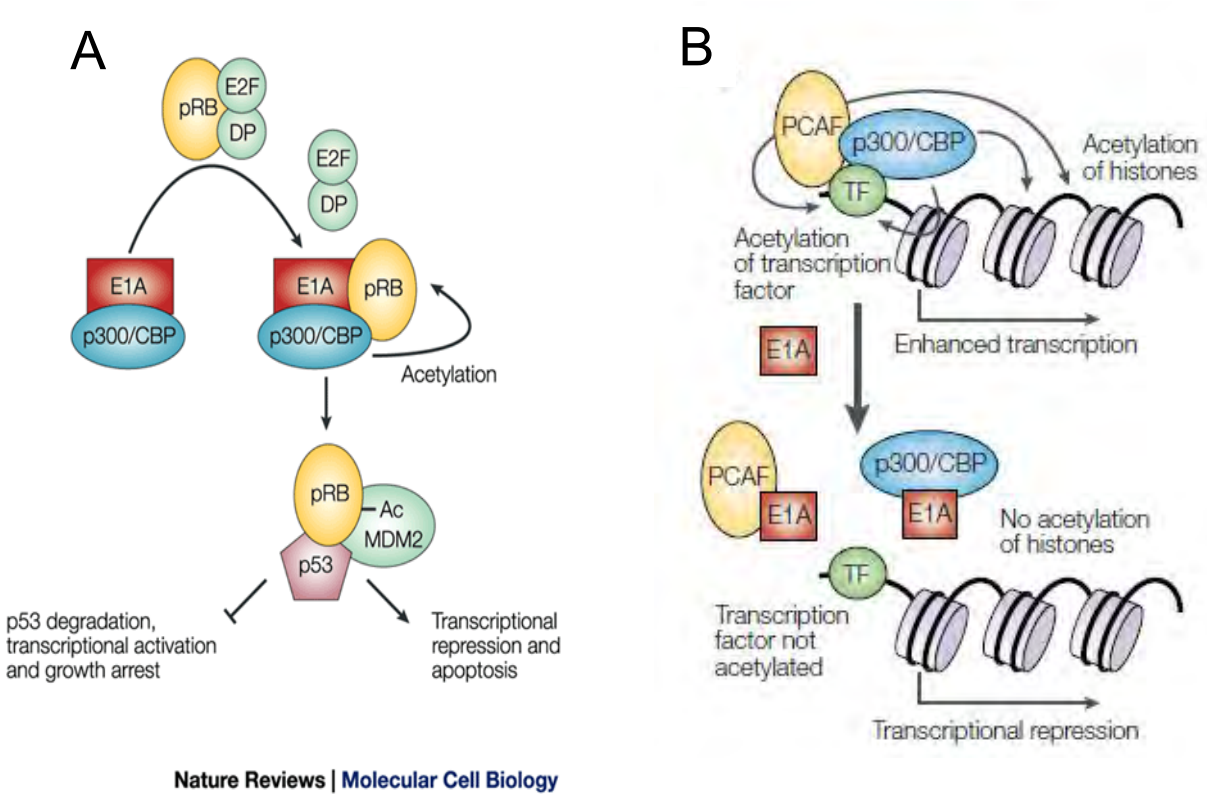


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Figure 1.7 AdE1A interactions with CBP/p300.
A AdE1A frees E2F transcription factor from pRB. Then in cooperation with CBP/p300 acetylates p53. This promotes its association with MDM2 oncoprotein, which then in turn binds and forms a complex with p53. This protects p53 from degradation, as well as its transcriptional activation and its function in growth arrest. However it still can induce apoptosis and transcriptional repression.
B P/CAF and CBP/p300 aid transcription by acetylating histone proteins allowing accessibility by transcription factors (TF) which may in turn be also acetylated to stabilise protein-protein binding to optimise transcription. The presence of AdE1A inhibits P/CAF and CBP/p300 function, hence blocking this process and inhibiting transcriptional initiation by TFs.

AdE1A is also shown to interact with the C-terminal binding protein (CtBP) (Boyd *et al.,* 1993; Schaeper *et al.,* 1995; Poortinga *et al.,* 1998); causing a negative effect on the transforming activity of AdE1A. CtBP is a 48kD transcriptional co-repressor; it represses transcription by recruiting proteins that modify the chromatin structure (Turner and Crossley 2001; Chinnadurai 2007). It interacts with proteins that contain the PXDLS motif (Turner and Crossley 2001; Chinnadurai 2007), including AdE1A which possesses this motif close to its C-terminus at residues 234-240 and 279-286 of Ad5 E1A 12S and 13S

respectively (Molloy *et al.,* 1998, 2000; Schaeper *et al.,* 1995). Binding of AdE1A to CtBP causes an inhibition of AdE1A-mediated transactivation. CtBP binding to the PXDLS motif of 12SAdE1A causes the inhibition of CR1 transcriptional activity (Sollerbrant *et al.,* 1996). CtBP also binds to the CR3 region of the 13SAd5E1A, thus repressing Ad5E1A CR3-dependent transcriptional activation (Bruton *et al.,* 2008). It has been hypothesized that this action of CtBP may be required for subtlety of transcriptional regulation of AdE1A (Bruton *et al.,* 2008). Additionally, the effect of CtBP's interaction with AdE1A is context-dependent; for instance, deletion of the CtBP binding motif PXDLS increases frequency of AdE1A/activated *Ras*-transformation, whereas it reduces AdE1A/AdE1B-mediated transformation (Boyd *et al.,* 1993; Subramanian *et al.,* 1989, 1991; Douglas and Quinlan 1995). The reasons behind this contrasting function of CtBP are yet to be determined.

1.3.5.2 Adenovirus early region 1B

The second protein that is expressed by the AdE1 transcription unit (besides AdE1A) is the adenovirus early region 1B (AdE1B). Due to alternative mRNA splicing, AdE1B gene encodes at least five different gene products, but the two major ones are the 19K and 55K proteins (Sieber and Dobner 2005).

Compared to AdE1A, the functions of AdE1B-55K are less straightforward as it exhibits differing roles depending on the setting of either infection or transformation (Blackford and Grand 2009). During infection, AdE1B-55K activity can be divided into early and late functions. The early functions include the targeting and degradation of cellular proteins that may negatively affect viral replication such as members of the cellular DNA damage response, i.e. p53, DNA ligase IV and the MRN complex (heterotrimeric protein complex comprising of Mre11, Rad50 and Nbs1) (Baker *et al.,* 2007; Querido *et al.,* 2001; Stracker *et al.,* 2002). This occurs due to the interaction of AdE1B-55K and Ad E4 open reading frame 6 (E4-ORF6) protein, to form an E3 ubiquitin ligase complex, comprising the cellular proteins cullin 5, RING-box1 and elongins B and C (Harada *et al.,* 2002; Querido *et al.,*

2001) – Figure 1.8A. This ligase complex ubiquitylates target proteins for degradation by proteasomes. The late functions involve the inhibition of cellular mRNA export and promotion of viral mRNA export and translation (Blackford and Grand 2009). E1B-55K/E4-ORF6 complex ubiquitylates cellular proteins that are involved in the mRNA transport, leading to their degradation by the proteasome (Berk 2005).

During AdE1-mediated transformation, AdE1B-55K cooperates with AdE1A (in the absence of E4-ORF6) to enhance cellular transformation (Houweling *et al.,* 1980; Bernards *et al.,* 1983; Gallimore *et al.,* 1985; Yew and Berk, 1992). In AdE1 transformed cells AdE1B-55K protein is found to bind to p53 (figure 1.8B) resulting in the inhibition of p53 transactivation and apoptosis (White 1996). Most importantly, AdE1B inhibits apoptosis induced by AdE1A (White *et al.,* 1991). As mentioned earlier in the chapter, AdE1A stabilises p53 by disrupting its ubiquitylation, and degradation by the 26S proteasomes. This causes p53 levels to rise, which can induce apoptosis when DNA is damaged (Grand *et al.,* 1994, Teodoro and Branton 1997; Prives 1998; Lowe and Ruley 1993; Debbas and White 1993). To prevent this, AdE1B-55K binds and inactivates p53 (Zantema *et al.,* 1985b, van den Heuvel *et al.,* 1990, Grand *et al.,* 1995; Martin *et al.,* 1998) by inhibiting its association with its promoters hence repressing p53 activated transcription. Additionally, the binding with AdE1B-55K causes the p53 to be translocated to the cytoplasm (Zantema *et al.,* 1985), neutralising the transcriptional activity of p53. Thus, during viral infection, p53 is degraded by the E1B-55K/E4-ORF6 E3 ubiquitin ligase, whereas in transformed cells, p53 is inhibited without being degraded by direct binding and relocalisation.

Ad5E1B-55K is a cytoplasmic protein as it has no nuclear localisation signal (NLS) (Zantema *et al.,* 1985), requiring binding to E4-ORF6 to be transported to the nucleus during infection (Dobbelstein *et al.,* 1997; Goodrum *et al.,* 1996; Ornelles and Shenk 1991) . However, it has been revealed that Ad5E1B-55K may possess a well-defined nuclear export signal (NES), similar to that of human immunodeficiency virus type 1 Rev protein (Dobbelstein *et al.,* 1997; Dosch *et al.,* 2001; Kratzer *et al.,* 2000). On the other

hand, Ad12 E1B 54K already has NLS so it is present in the nucleus independently of Ad12 E4-ORF6 (Grand *et al.,* 1999). This may explain the nuclear and cytoplasmic localisations of the Ad12E1B-55K and Ad5E1B-55K respectively in AdE1 transformed cells (Zantema *et al.,* 1985; Blair-Zajdel and Blair 1988).

The smaller AdE1B-19K is a homologue of Bcl2, (Rao *et al.,* 1992; Farrow *et al.,* 1995). In normal cell conditions, Bcl2 binds to the pro-apoptotic Bax on the mitochondrial and endoplasmic reticulum membranes, keeping it inactive. High levels of nuclear p53 triggers apoptosis by transcriptionally activating Bax and other related pro-apoptotic proteins, AdE1B-19K binds to Bax keeping it inactivated to prevent AdE1A induced apoptosis during infection (Chen *et al.,* 1996; Han *et al.,* 1996a). It also blocks p53-independent apoptosis mediated by tumour necrosis factor-α (TNF- α) and Fas ligand (Hashimoto *et al.,* 1991).

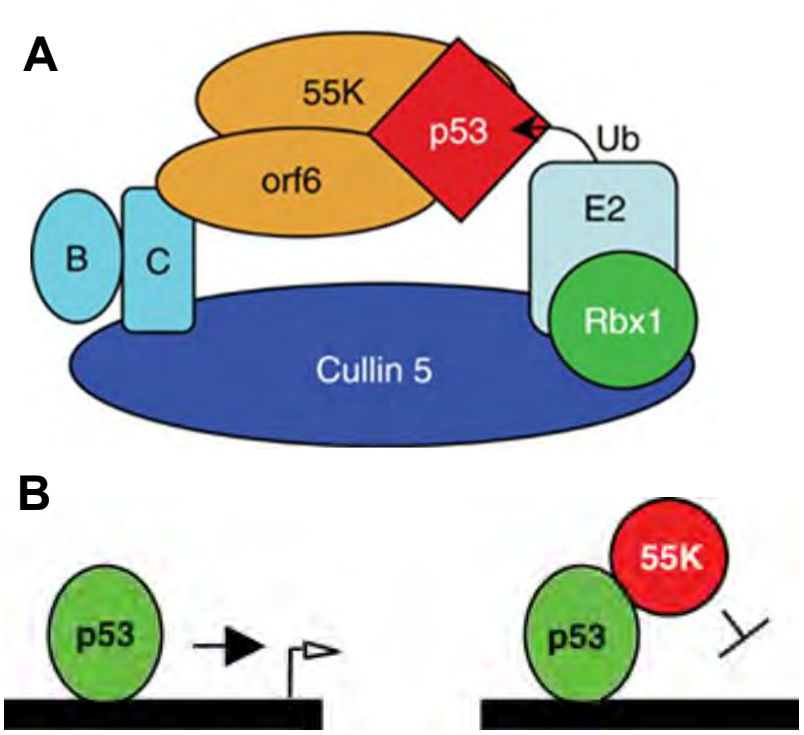


Figure 1.8 Inhibition of p53 function by AdE1B-55K .

A. During infection, AdE1B-55K, E4-ORF6, Cullin 5, Elongin BC, Rbx1complex to form an E3 ubiquitin ligase that targets and ubiquitylates p53 and other proteins for degradation by the proteasome.

B. AdE1B-55K binds to p53 and hence blocks transcriptional activation. AdE1B-55K prevents p53 interaction with transcriptional co-activators. It also increases the affinity of p53-DNA binding sites

1.3.5.3 Adenovirus early region 3

The adenovirus early transcription unit 3 (AdE3) encodes three proteins with immunosubversive functions: E3-19K, E3-14.7K and E3-10.4K/14.5K (RID, receptor

internalisation and degradation). They are not essential for viral replication although they play an important role in facilitating the establishment and persistence of adenovirus infection in vivo. They reduce the recognition of infected cells by the host immune system, allowing the viability of the cell while the viral replication continues.

E3-19K down-regulates cell surface MHC class I expression by inhibiting its transport from the endoplasmic reticulum to the plasma membrane, where it normally presents peptides for recognition to cytotoxic T lymphophcytes (CTL) (Burgert *et al*., 1987; Wold *et al.,* 1999, reviewed by Horwitz 2004). E3-gp19K also inhibits the processing of peptides by tapasin, as a result reducing the amount of peptide presented by MHC class I in infected cells (reviewed by Horwitz 2004; Bennett *et al*., 1999). AdE3-14.7k is a 128 amino acid protein that inhibits TNF-induced apoptosis (Gooding *et al.,* 1988). E3-10.4K/14.5K (RID) is a protein complex, consisting of RIDα and RIDβ polypeptides (Tollefson *et al.,* 1991), which was found to downregulate fas receptors of the death ligands, FAS-L and TRAIL by internalisation into cell and degradation in lysosomes, thus prohibiting apoptosis (Shisler *et al*., 1997, Tollefson *et al.,* 1998). It also inhibits TNF-induced apoptosis (Gooding *et al.,* 1991), degrades the epidermal growth factor receptor (EGFR) from the cell surface (Tollefson *et al.,* 1991; Carlin *et al.,* 1989) as well as inhibiting TNF-induced NF-Kb signal transduction (Friedman and Horwitz 2002). Both AdE3-14.7K and RID inhibit TNF-α-induced secretion of arachidonic acid, a 20 carbon unsaturated fatty acid involved in the inflammatory immune response (Zilli *et al.,* 1992; Krajcsi *et al.,* 1996). The three E3 proteins prevent the infected cell's recognition by the immune system. There is an additional Ad E3 protein called the AdE3-11.6K (also known as the adenovirus death protein, ADP) (Tollefson *et al.,* 1996). This protein is not expressed at the early promoter, at the same time as the other E3 proteins, but synthesized later in infection from the major late promoter, seemingly during the death and lysis of the cell when the newly formed virus progeny are released (Tollefson *et al.,* 1996).

All the mechanisms of E3 protein function described above, was largely derived from the study of adenovirus serotype 2 and 5 of species C. Various E3 proteins from other

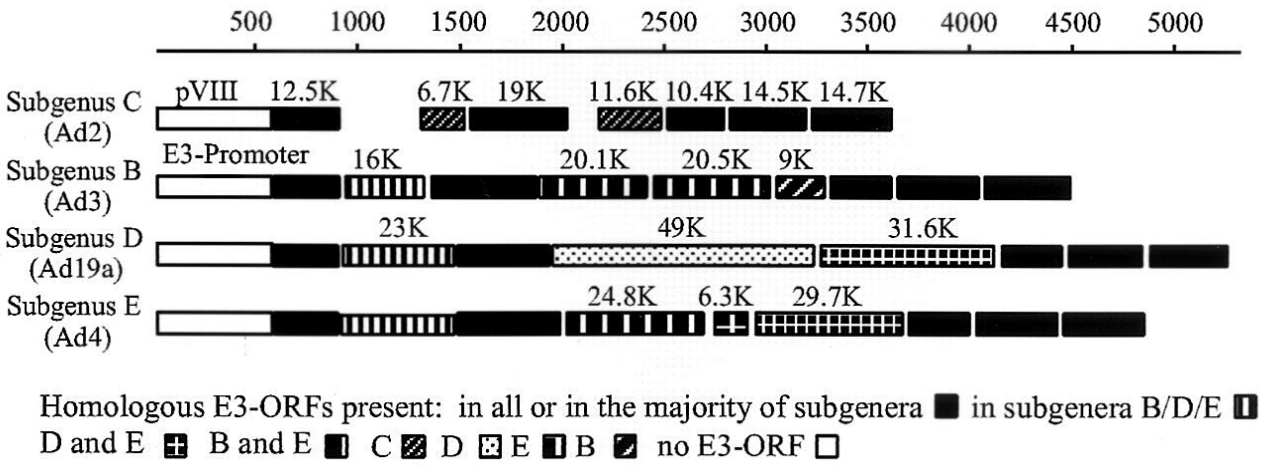
adenovirus serotypes have been identified (see Table 1.3, Figure 1.9) (Windheim *et al*., 2004). However, the functionality of only four of these proteins have been confirmed so far, namely, 19K, 10.4K, 14.5K and 49K. With the exception of adenoviruses from species A and F, E3-19K is expressed in all human adenoviruses (Blusch et al., 2002). E3-19K from Ad7, Ad35, Ad5, Ad2, Ad37 and Ad4 and their interactions with MHC class I molecules were examined (Fu *et al.,* 2011). The E3-19K proteins of species B, C, D and E display allele-specific interactions with varying binding affinities with MHC class I molecules. The said species showed stronger binding to HLA-A molecules compared to HLA-B, and no interactions with HLA-C molecules (Fu *et al.,* 2011). Binding was strongest between Ad4 E3-19K and HLA-A MHC class I and weakest between Ad37 E3-19K and HLA-A (Fu *et al.,* 2011). It was also shown that the binding affinity negatively correlate with levels of MHC class I surface expression in infected cells (Fu *et al.,* 2011). Another protein E3-49K is expressed by Ad19 of the species D. This species is associated with epidemic keratoconjuntivitis (EKC) (Deryckere and Burgert 2002). A novel immunomodulatory function is demonstrated by this protein. E3-49K may not act on infected cells but rather on cells of the immune system. After synthesis, E3-49K is extensively modified at the Golgi then enters the secretory pathway where they are packed in secretory vesicles destined for the plasma membrane (Windheim *et al.,* 2004). Proteolytic cleavage takes place releasing the N-terminal ectodomain at either the cell surface, in secretory vesicles or endosomes. The protein is secreted followed by binding to NK cells (Windheim et al., 2004).

Table 1.3: E3 proteins from the different species of adenovirus

Protein	Species	Serotype
19K	B C D E	Ad3 Ad2 Ad19 Ad4
16K	B B B	Ad3 Ad7 Ad35
22K	D	Ad19
23K	E	Ad4
6.7K	C	Ad2
20.1K	B	Ad3
20.5K	B	Ad3
24.8K	E	Ad4
49K	D	Ad19
11.6K	C C C C	Ad2 Ad5 Ad2 Ad5
9K	B B	Ad3 Ad7
31.6K	D	Ad19
29.7K	E	Ad4

Protein	Species	Serotype
31.6K	D	Ad19
29.7K	E	Ad4
29.4K	A	Ad12
19.4K	F	Ad40
30.7K	A	Ad12
31.6K	F	Ad40
10.4K	A B C D E F	Ad12 Ad3 Ad2 Ad19 Ad2 Ad40
14.5K	A B C D E F	Ad12 Ad3 Ad2 Ad19 Ad4 Ad40
14.7K	A-E	
12.5K	A-E	
6.3K	E	Ad 4

Modified from Windheim *et al.,* 2004



Windheim and Burgert 2002

Figure 1.9: Compositions of the E3 region
From species C(Ad2), B (Ad3), D (1d19) and E (Ad4). The top scale indicates size of base pairs. ORFs are denoted as bars and drawn to scale. Shading code is shown below the figure.

1.3.6 The effect of AdE1A on components of the antigen presentation machinery.

It is known that viruses have evolved mechanisms to prevent the detection of infected or transformed cells by the host immune system, producing ways to evade recognition by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (Alcami and Koszinowski 2000). These mechanisms involve targeting multiple components of the antigen processing machinery such as proteasomes, transporter associated with antigen processing (TAP) and the MHC complexes (Alcami and Koszinowski 2000). 20S proteasome and immunoproteasomes are discussed in section 1.4.

1.3.6.1 Major histocompatibility complex (MHC) class I and II and transporter associated with antigen processing (TAP)

On the surface of CTLs, T cell receptors (TCRs) recognise antigenic peptides presented by the major histocompatibility complex (MHC) molecules on the target cells (Kindt *et al.,* 2007). Proteins are continuously synthesized and destroyed in cells. Those proteins can be cellular proteins or those derived from pathogens. MHCs are heterodimeric glycoproteins whose function is to display fragments of processed proteins on the surface to be recognised by the surveilling immune cells, such as CTLs (Janeway *et al.,* 2004). If the CTLs recognise the presented peptide/MHC complex, they initiate an immune response against the cell (Corse *et al.,* 2011). Due to the large diversity of microbes in the environment, MHC has evolved to be able to present a wide range of peptides. This is because the MHC locus is polygenic and highly polymorphic (Janeway *et al.,* 2004). MHC genes are codominantly and concomitantly expressed. There are two classes of MHC molecules: MHC class I and II. MHC class I proteins are present on the surface of most nucleated cells while MHC class II are present constitutively on the surface of only immune cells, specifically on antigen presenting cells like dendritic cells. MHC class I

comprises an α chain that is composed of three domains: α1, α2 and α3; and, in association wih β2-microglobulin, they protrude from the cell surface (Figure 1.10A) (Bjorkman and Parham 1990; Janeway *et al.,* 2004). The whole structure is anchored on the cell membrane through a hydrophobic transmembrane region in the α3 domain. MHC class I specifically presents peptides of cytosolic or viral origin to CD8+ CTLs (Bjorkman and Parham 1990). After a viral infection, the host cell presents non-self peptides through the MHC class I complex present on the cell surface where CTLs recognise the peptide fragment derived from pathogens. The CTLs in turn become activated stimulating an immune response in the process (Pamer and Cresswell 1998).

MHC class II proteins are composed of α and β poly-peptide chains, each one consisting of two domains: α1 and α2, β1 and β2 (Figure 1.10A). Each polypeptide chain is anchored to the cell membrane via a hydrophobic transmembrane region (Janeway *et al.,* 2004). MHC class II is present on the surface of antigen presenting cells like dendritic cells and macrophages; however, they can be induced in most other cells by exposure to interferon gamma (IFNγ). MHC class II presents processed peptides of extracellular origin (e.g. endocytosed bacterial pathogens) to CD4+ T cells (helper T lymphocytes) (van den Hoorn et al., 2011). Extracellular proteins are endocytosed, digested in the lysosome then loaded onto MHC class II molecules before being transported to the cell surface (van den Hoorn et al., 2011).

Antigenic peptides of viral or cellular origin that are loaded onto the MHCs are generated by the proteasomes in the cytoplasm (see section 1.4). These peptides are then pumped into the lumen of the endoplasmic reticulum (ER) by an active transport mechanism carried out by a protein complex termed “transporter associated with antigen processing” (TAP) (Lankat-Buttgereit and Tampe 1999), which utilises ATP for its function. TAP consists of two subunits: TAP1 and TAP2. TAP is essential for antigen presentation as cells mutant for TAP have their levels of surface MHC class I greatly reduced (Van Kaer *et al.,* 1992). In the ER lumen, with the aid of several chaperones that assemble the newly formed MHC class I, and tapasin (TAP-associated glycoprotein) to aid loading, peptides

associate with the newly synthesized MHC class I molecules. The MHC class I and peptide complex are then finally transported to the cell surface to be presented to CD8+ CTLs (Figure 1.10B)

1.3.6.2 The effect of AdE1A on the antigen processing components

AdE1A targets multiple components of this antigen processing machinery, and the extent of this effect determines adenovirus oncogenicity. The level of cell surface MHC class I molecules are down-regulated in Ad12 E1A transformed human (Bottley *et al.,* 2005; Vasavada *et al.,* 1986) and rodent cells (Schrier *et al.,* 1983, Ackrill and Blair 1988), but not in cells transformed by the non-oncogenic Ad2/5. The biological function of this was demonstrated by the inability of influenza specific CTLs to recognise and lyse influenza virus infected Ad12 transformed mouse cells in comparison to the less resistant Ad5 transformed cells (Yewdell *et al.,* 1988). Thus, reduced levels of MHC class I expression enables Ad12 transformed cells to evade the immune system. This effect is reversed by either the treatment of the cells with IFNγ or transfection of heterologous class I heavy chain gene (Yewdell 1988; reviewed by Blair and Blair-Zajdel 2004). The overall effect of Ad12 E1A on the level of surface MHC is at the level of transcription (Proffitt *et al.,* 1994; Ackrill and Blair 1988). Similarly, Ad5 transformed but not Ad12 transformed cells are eradicated by E1A specific CTLs in mice (Kast *et al.,* 1989).

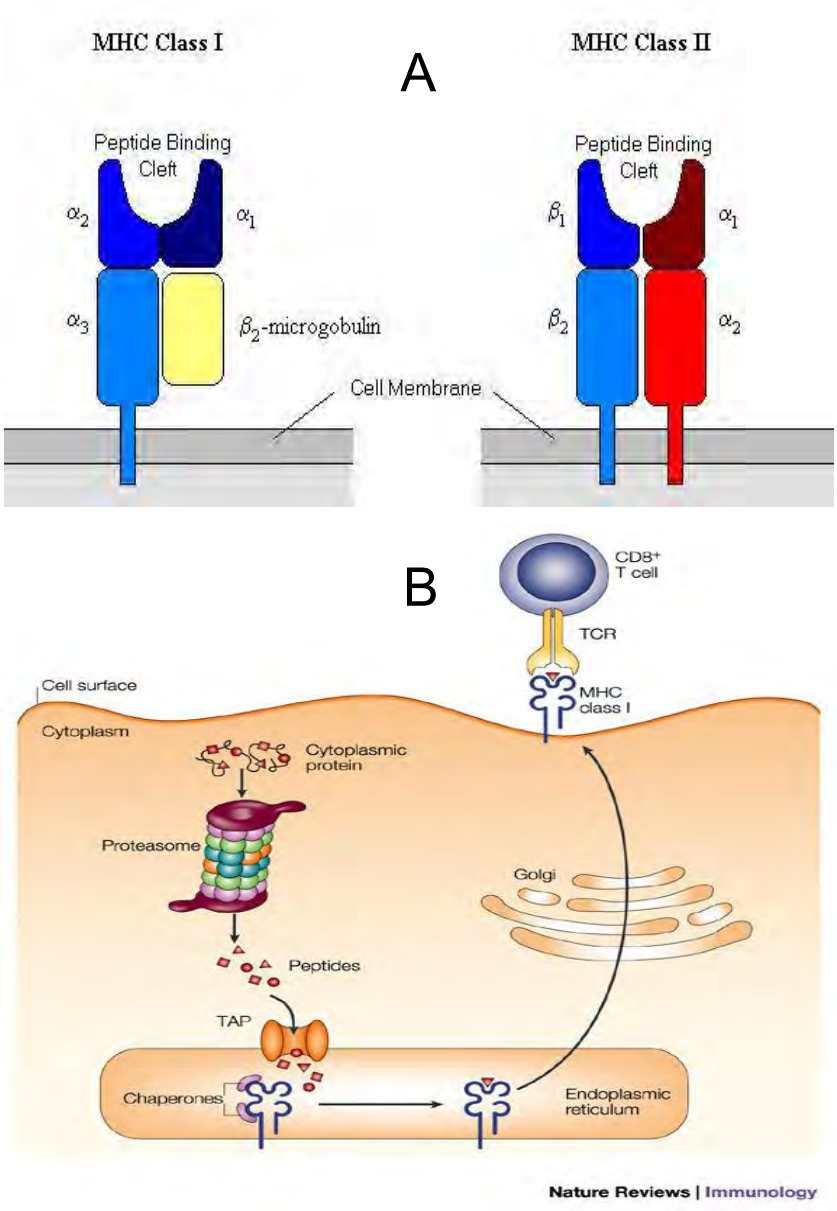


Figure 1.10: Components of the antigen processing machinery.

A Schematic diagram showing the structures of the major histocompatibility complex (MHC) class I and MHC class II (from Immunobiology: the immune system in health and disease (6th edition) by Janeway *et al.* 2004.

B Proteins of viral or cellular origin are degraded by proteasomes. They are then pumped into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex. In the ER, with the aid of chaperones, the peptides are loaded into newly synthesized MHC class I molecules, then eventually transported to the surface to be recognised by cytotoxic CD8+ T-cells (CTLs) (from Yewdell *et al.,* 2003)

This is because in Ad5 transformed cells, antigenic peptides can be derived from a non-conserved C-terminal region of the E1A (residues 232-247 of 13S Ad5 E1A) and presented to the CTLs by the MHC class I. However, it was speculated that in Ad12 E1A the corresponding peptide inefficiently binds or fails to be recognised by CTL. This and the reduced level of surface MHC class I may by responsible for the oncogenic nature of Ad12 transformed cells (reviewed by Blair and Blair-Zajdel 2004).

Ad12 E1A also inhibits the expression of other genes from the MHC locus, namely TAP1, TAP2, and the immunoproteasome subunits LMP2 and LMP7 in Ad12 transformed cells

(refer to section 1.5 for details), but not in those transformed by Ad5 (Rotem-Yehudar *et al.,* 1994, 1996; Vertegaal *et al.,* 2003).

With regards to the effect of AdE1A on the level of surface MHC class II, very few studies have been undertaken so far. A study by Ackrill *et al.,* 1991 showed that Ad5E1A represses MHC class II expression due to it inhibiting the induction by IFNγ (see section 1.5).

1.4 The Immunoproteasome

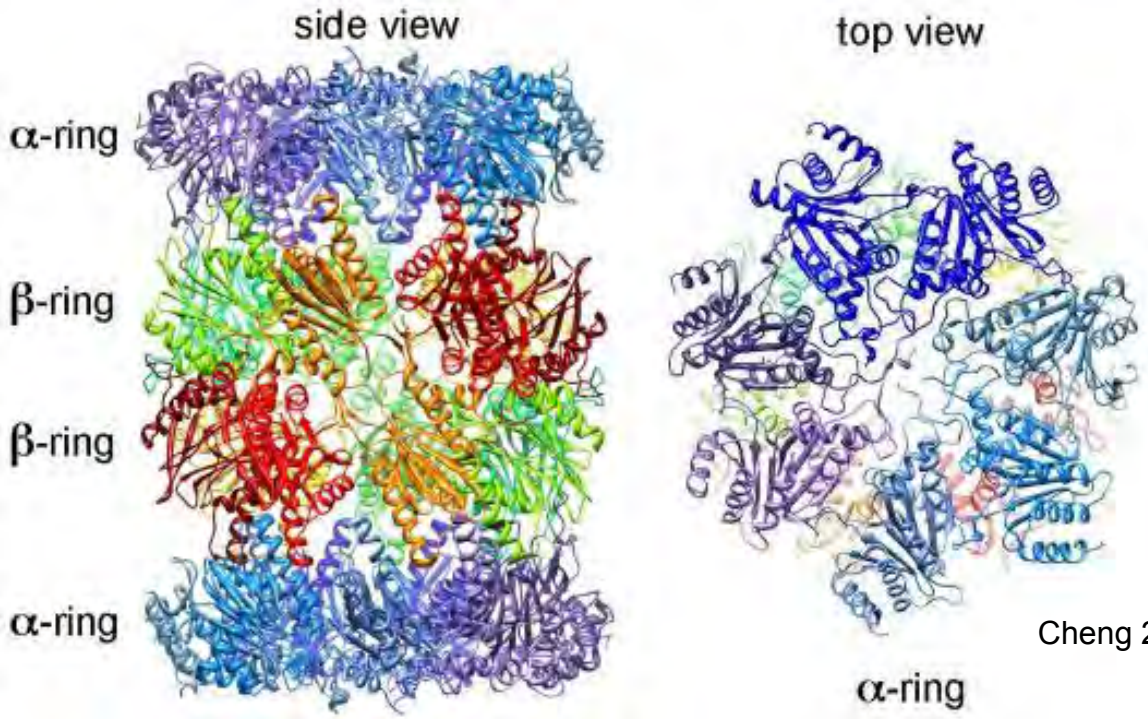
1.4.1 The 20S proteasome

Proteasomes are proteolytic complexes that are involved in regulated degradation of intracellular proteins, and they are also involved in the production of peptides for antigen presentation via the MHC class I pathway (Groettrup *et al.,* 1996; York *et al.,* 1999; Yewdell *et al.,* 1999). The proteasome exists in two forms: the 20S complex that degrades proteins, independently of ubiquitin and is based on a proteolytic mechanism involving a threonine binding site (Figure 1.10D) (Dahlmann 2005; Coux *et al.,* 1996). The 26S proteasome, which comprises one 20S core particle and one or two 19S regulatory caps, degrades ubiquitinated target substrates (Figure 1.12).

The 20S proteasome has a barrel shaped structure consisting of four stacked rings: two outer α and two inner β rings (Figure 1.11A) . Each ring has seven subunits (α1-7 or β1-7) and proteolysis occurs in the central channel (Lowe *et al.,* 1995; Groll *et al.,* 1997; Baumeister *et al.,* 1998; Coux *et al.,* 1996). The molecular structure of the α and β subunits of the 20S proteasome consist of two layers of five-stranded β-sheets that are sandwiched between two helices at either side (Figure 1.11B) (Dahlmann 2005). However, the N-terminus of the α-subunits has an extra helix that fills the cleft between the two layers of β-sheets. Similarly, the helices mediate interaction between the α and β as well as β and β subunits (Dahlmann 2005). Amino acids at the extreme N-terminal region of the α-subunits form a lattice that gate the central pore of the α-ring (Figure

1.11A). *In vivo*, the 19S complex docks to the 20S proteasome in a ATP-dependent process, where it then controls access to the lumen (see below).

The proteolytic activity of the 20S proteasome is due to three β subunits: β1, β2 and β5. β-subunits lack the extra helix and their N-terminal threonine is positioned at the open cleft between two layers of β-sheets (Dahlmann 2005). The N-terminal threonine is exposed to the inner lumen of the proteasome (Figure 1.11C), hence forming an active site, serving as a catalytic nucleophile and primary proton acceptor (Figure 1.11D). Although these subunits have a common catalytic mechanism, they have different substrate specificities. β1 possesses "caspase-like" activity which cleaves after acidic residues, β2 has "trypsin-like" activity that cleaves after basic residues and β5 "chymotrypsin like activity" that cleaves after hydrophobic amino acids (reviewed in Rivett and Hearn 2004, Groettrup *et al.,* 2001). Each of these subunits has their active sites facing inside the lumen of the cylinder where the proteolytic activity takes place (Baumeister *et al.,* 1998; Groll *et al.,* 1997). This is to protect other cytosolic proteins from uncontrolled degradation. The access to the lumen is controlled by the 19S cap, a proteasome regulatory sub-complex with ATPase activity that is involved in functions such as unfolding and translocating protein substrates and ubiquitin recognition. The 19S complex is composed of 20 subunits which constitute a "lid" and a "base". The six ATPases are incorporated in the "base" that is in direct contact with the outer α rings of the 20S proteasome and two other subunits involved in the binding of ubiquitin-like domains (Hendil and Hartmann-Petersen 2004). The 19S cap, in combination with the 20S proteasome core forms the complete 26S proteasome (Figure 1.12) (Marteijn *et al.,* 2006; Baumeister *et al.,* 1998).



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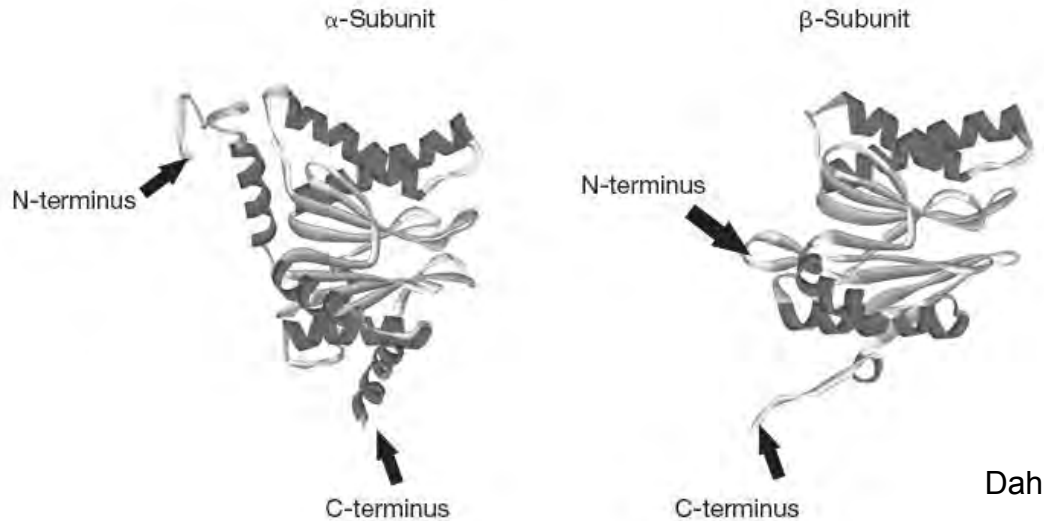

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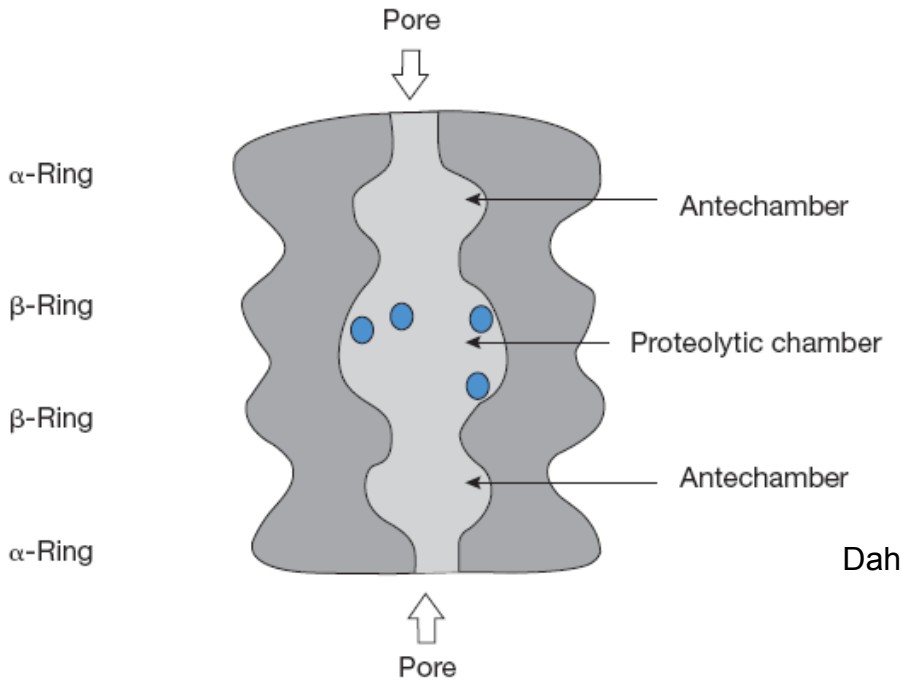

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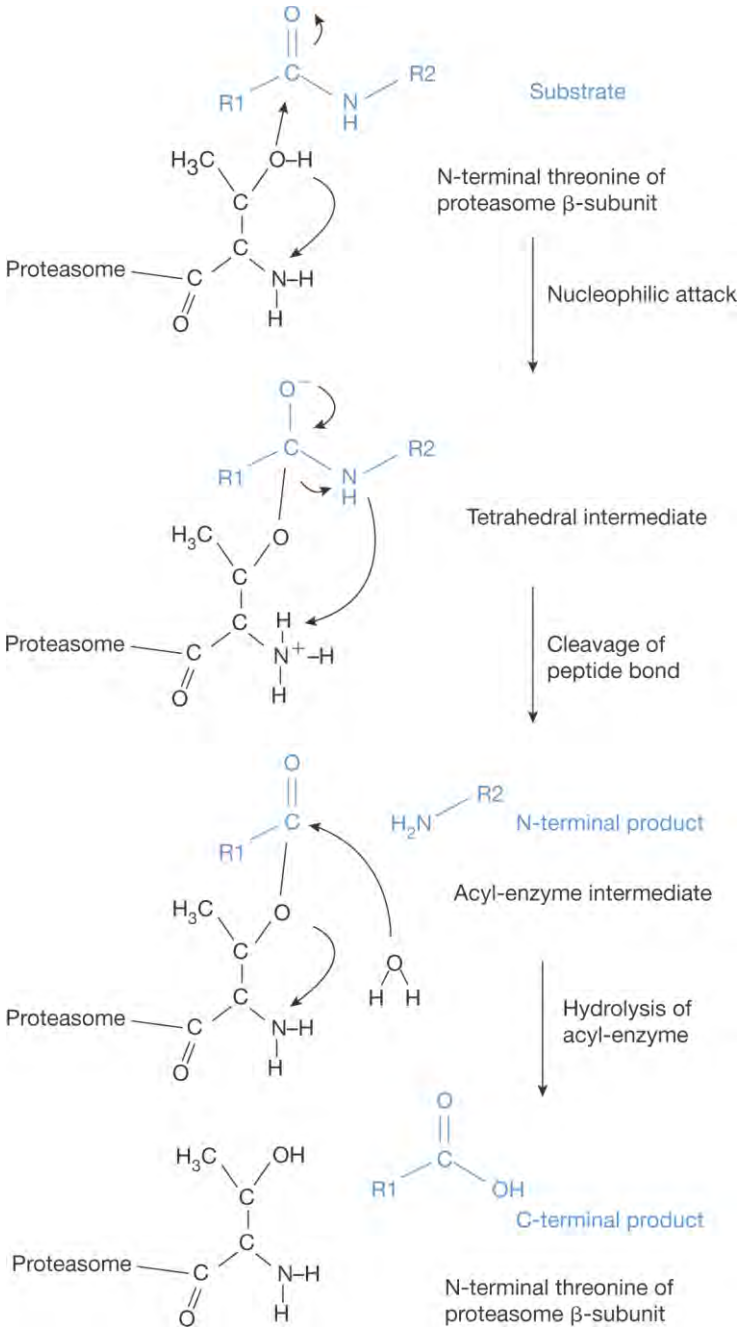
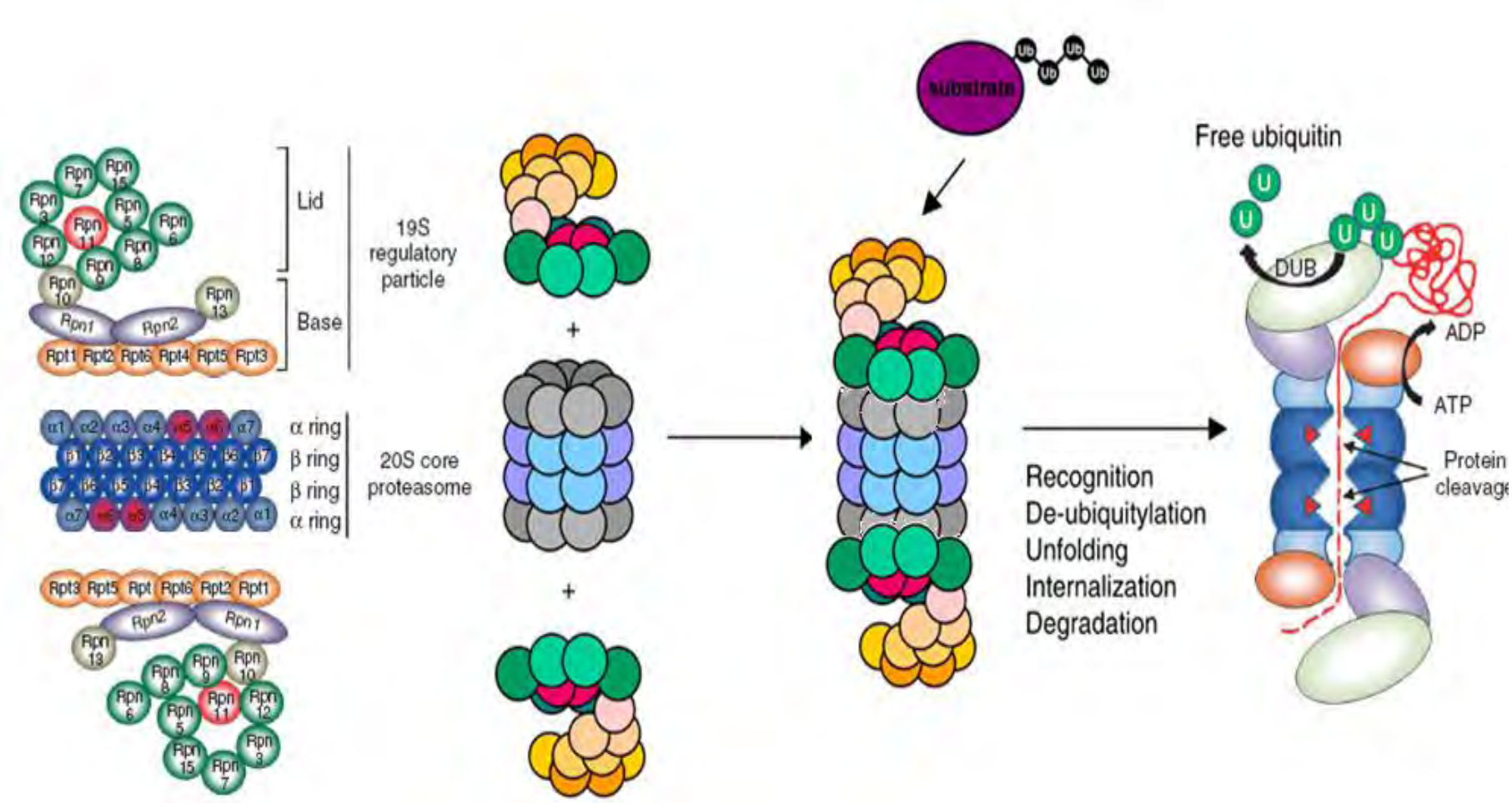


Dahlmann 2005

Figure 1.11: Structure and catalytic mechanism of the 20S proteasome
A. ribbon diagram of the yeast 20S proteasome. **B**. Schematic drawing of an α- and β-subunit of eukaryotic 20 S proteasomes. **C.** Longitudinal view of the 20S proteasome. The N-terminal threonine residues of the β-subunits are shown in blue circles. These are exposed to the lumen for catalysis of peptide bind hydrolysis. Substrate entry and exit is through the ring pores (Dahlmann 2005). **D.** The N-terminal threonine of a proteasome β-subunits faces the lumen of the proteasome cylinder. It acts as a catalytically active nucleophile. The hydroxy group attacks the carbonyl group of the substrate peptide bond, while its proton is delocalized to the threonine amino group. As a result, a tetrahedral intermediate is formed, followed by peptide-bond cleavage and release of the N-terminal fragment of the substrate protein. Hydrolysis of the acyl-enzyme intermediate releases the C-terminal substrate fragment (Dahlmann 2005).



Modified from Brooks 2010 and Marteijn *et al.,* 2006

Figure 1.12: Structure of the 26S proteasome

26S proteasome degrades proteins that have been ubiquitinated. They consist of the 20S proteasome core and the 19S sub-complex lid. The catalytic domain is within the 20S core while the 19S recognises ubiquitylated proteins, unfolds them while also removing the ubiquitin.

1.4.1.1 AdE1A and the 20S proteasome

AdE1A also targets parts of the 20S proteasome, binding to the ATPase (S4 and S8) and non-ATPase (S2) components of the 19S regulatory complex (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004) leading to the decrease in their ATPase activity. Through its CR3 transactivation domain, AdE1A also binds to several α subunits of the 20S proteasomes (Rasti *et al.,* 2006). In addition, the expression of the chaperone tapasin and the immunoproteasome components MECL-1, PA28α and PA28β was much lower in Ad12 transformed rat cells compared to Ad5 transformed cells enabling escape from recognition by CTLs as the generation of peptides and loading onto MHC complex require those components (Vertegaal *et al.,* 2002).

AdE1A also recruits 20S proteasomes and the 19S ATPase, S8, a component of 19S ATPase proteins independent of 20S (APIS) to viral promoters to aid transcription of early region proteins (Rasti *et al.,* 2006). They bind specifically to the AdE1A transactivation domain CR3 enhancing its ability to stimulate transcription. Both S8 and 20S proteasome are required for the process.

1.4.2 The Immunoproteasome

1.4.2.1 Formation and assembly

In the presence of Interferon gamma (IFNγ) (resulting from cellular stimulation during host infection), each of the three catalytic subunits of the 20S proteasome β1, β2 and β5 are replaced by their inducible homologous subunits LMP2 (β1i), MECL1 (β2i) and LMP7 (β5i) respectively, to form the immunoproteasome (Hisamatsu *et al.,* 1996) – (Figure 1.13).

Immunoproteasomes are expressed constitutively in cells of lymphoid origin such as spleen, lymph node and thymus (Stohwasser *et al.,* 1997) as well as immune cells such as dendritic cells. Immunoproteasomes are enriched at the endoplasmic reticulum (ER) (Brooks *et al.,* 2001), where they may provide peptides directly to the TAP complex. The peptides are then pumped into the ER lumen, finally binding to the MHC. Even though the

efficiency of the immunoproteasome to degrade proteins is the same as the 20S proteasome, the former has different cleavage preferences (see next section), and hence a different spectrum of peptides are produced which may potentially enhance antigen presentation (Groettrup *et al.,* 2001; Ehring *et al.,* 1996; Gaczynska *et al.,* 1996; Cascio *et al.,* 2001; Cerundolo *et al.,* 1995).

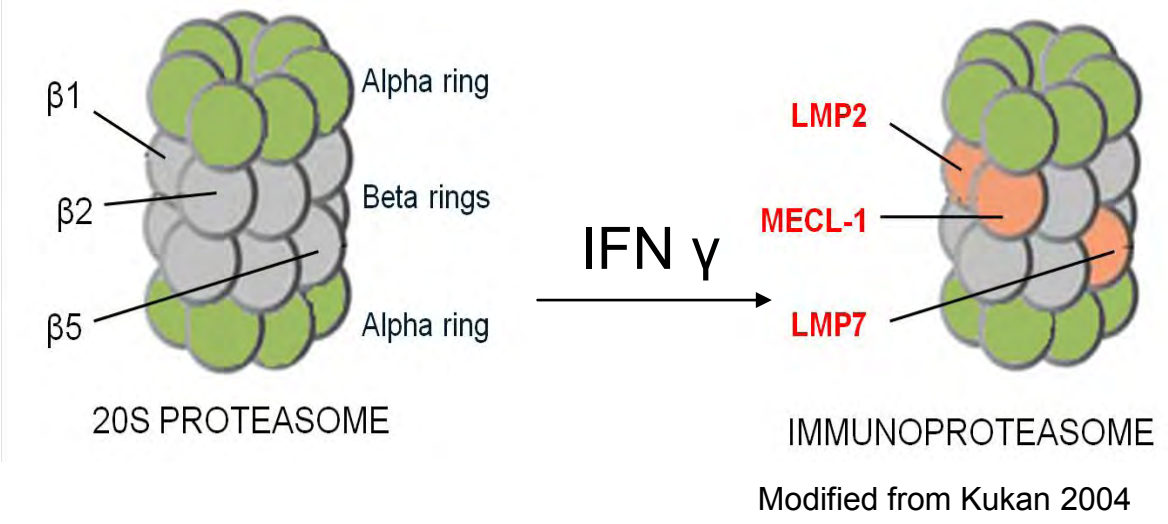
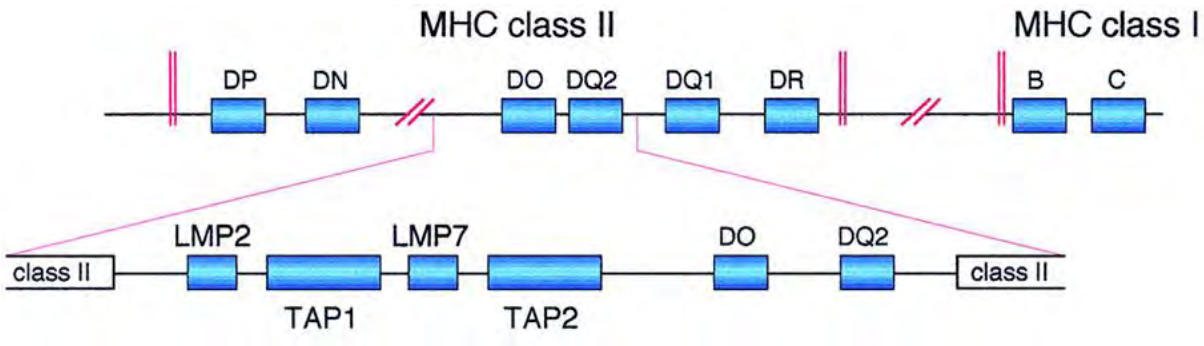


Figure 1.13: The formation and structure of the immunoproteasome
Upon IFNγ exposure, the constitutive 20S catalytic subunits β1, β2 and β5 are replaced by their IFNγ inducible homologes LMP2, LMP7 and MECL1 respectively. The immunoproteasome has different cleavage preferences.

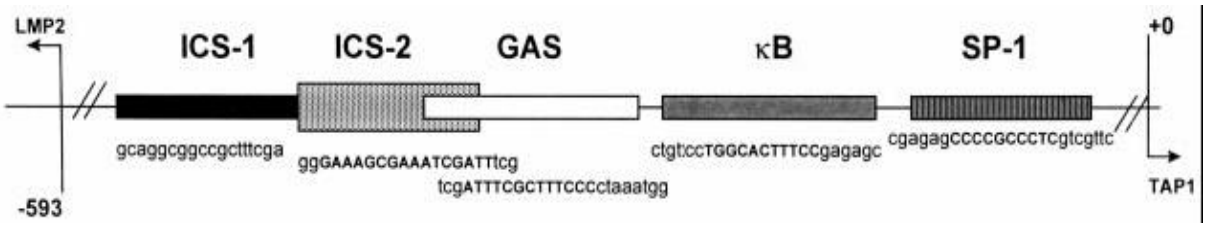
LMP2 and LMP7 genes are located adjacent to the TAP1 and TAP2 genes in the MHC locus (Figure 1.14) (reviewed in Rivett and Hearn 2004; Glynne *et al.,* 1991; Martinez and Monaco 1991; Ortiz-Navarrete et al 1991), whereas the MECL-1 gene is found separately in a locus on human chromosome 16q22.1 (Larsen et al 1993).

TAP1 and LMP2 genes are located in close proximity to each other separated by their common 593bp bidirectional promoter; this may potentially indicate coordinate regulation (Wright *et al.,* 1995) (Figure 1.15). The expression of the three immunosubunits is mediated by IRF-1 after IFNγ stimulation (Namiki *et al.,* 2005; Foss and Prydz 1999; Chatterjee-Kishore *et al.,* 2000) – (see section 1.5).



Lankat-Buttgereit and Tampé 2002

Figure 1.14: The MHC locus: The location of the LMP and TAP genes within the MHC locus II. Boxes represent the approximate position of the genes.



Chatterjee-Kishore *et al.,* 1998

Figure 1.15: The LMP2/TAP1 bidirectional promoter: a 593bp bi-directional promoter shared by LMP2 and TAP1. The diagram also shows transcription factor binding sites.

The assembly of immunoproteasomes and the mechanisms by which the inducible immunosubunits replace their constitutive homologues is poorly understood. However, evidence so far supports the cooperative model for assembly. It has been shown that the incorporation of MECL1 is strictly and mutually dependent on LMP2 but not LMP7 (Groettrup *et al.,* 1997), and LMP2 is also not incorporated in the absence of MECL1 (Griffin *et al.,* 1998; Kingsbury *et al.,* 2000, Groettrup *et al.,* 1997). This idea of interdependent cooperation is further supported by the observation that both LMP2 and

MECL1 are direct neighbours in the β-ring (Dahlmann *et al.,* 1999). LMP7 seems to incorporate into immunoproteasomes independently of the other two subunits (Kingsbury *et al.,* 2000), and is separated from LMP2 and MECL1 by two β-type subunits: β3 and β4 (Dahlmann *et al.,* 1999). Additionally, LMP7 is required for maturation, where it removes the amino-terminal propeptide of MECL1 and LMP2 to liberate their active sites. Due to the independent nature of LMP7, it may be possible that in certain cells, populations of proteasomes contain LMP7 together with the two other constitutive subunits, β1 and β2 (Gaczynska *et al.,* 1994; Boes *et al.,* 1994). The functional significance of these proteasomes is not known, although, there are suggestions the resulting diversity of "mixed proteasome" populations could translate into a larger array of peptides produced (reviewed in Groettrup *et al.,* 2001).

IFNγ exposure also induces PA28 (also known as 11S), a protein regulator complex that enhances the activity of the proteasome core in both the 20S and the immunoproteasome (Baumeister *et al.,* 1998; Groettrup *et al.,* 1995; Dick *et al.,* 1996). Like the 19S complex in the 26S proteasome, it controls access to the lumen; however, it is in an ATP-independent manner and it mediates the degradation of non-ubiquitinated short peptides. There have also been reports of PA28 promoting immunoproteasome assembly (Preckel *et al.,* 1999), although this was not supported by other reports which showed that PA28 is not required for assembly and that its effect is independent of the proteasome (reviewed by Van den Eynde and Morel 2001; Schwarz *et al.,* 2000; van Hall *et al.,* 2000).

1.4.2.2 Catalytic activity and antigen processing

The immunosubunits have different proteolytic activities in comparison to the 20S proteasome. Trypsin-like (cleavage after basic residues) and chymotrypsin-like activities (cleavage after hydrophobic residues) are stimulated whereas caspase-like activity (cleavage after acidic residues) is suppressed (Aki *et al.,* 1994; Driscoll *et al.,* 1993; Gaczynska *et al.,* 1993). This is possibly due to the incorporation of LMP2 that possesses

chymotrypsin-like activity to substitute for the caspase-like β1 subunit (Groettrup *et al.,* 1995; Gaczynska *et al.*, 1994; Schmidtke *et al.,* 1998). TAP and MHC class I molecules are reported to strongly prefer peptides with carboxyl terminal, hydrophobic and basic residues over acidic ones, making the immunoproteasome-processed peptides more favourable for presentation. However, the view that the immunoproteasome is more efficient at producing peptides for presentation has been challenged. A study using LMP2 and LMP7 knockout mice showed that while presentation of certain epitopes was reduced, others were still processed and presented (Fehling *et al.,* 1994; Van Kaer *et al.*, 1994), revealing that the immunoproteasome is not a necessity for antigen presentation. In fact, the majority of MHC class I epitopes known are processed by the standard proteasome. Similarly, some epitopes are processed more effectively by the 20S proteasome than the immunoproteasome (Gaugler *et al.,*1994; Morel *et al.,*1999; Van den Eynde *et al.,* 1995). Examining the protein origins of these epitopes reveals that the immunoproteasome epitopes are derived mainly from infectious agents like viruses, whereas 20S proteasome epitopes are derived from self proteins (reviewed by Van den Eynde and Morel *et al.,* 2001). Examples of such epitopes and their origins are summarized in Table 1.4 and 1.5. So in conclusion, the notion that immunoproteasomes are more efficient at producing epitopes for MHC class I presentation remains correct as far as epitopes derived from infectious organisms in particular. This appears to make sense, in view of its presence in antigen presenting cells and in response to IFNγ produced during infection (reviewed by Van den Eynde and Morel *et al.,* 2001). However, it was recently suggested that immunoproteasomes may also play a primary role in the maintenance of protein homeostasis and preservation of cell viability under inflammatory conditions of IFN-induced oxidative stress by rapidly degrading nascent oxidant-damaged proteins (Seifert *et al.,* 2010).

Table 1.4 Epitopes preferentially processed by cells carrying immunoproteasomes - Van den Eynde and Morel (2001)

Protein	MHC restriction	Peptide sequence	Amino acid positions and epitopes	References
Viral (mouse)				
Influenza NP (strain A/NT/60/68)	H-2D^{b}	TRGVQI **ASNENMDAM** ESSTLE	366-374	Cerundolo *et al.,* 1995
Influenza NP (strain A/PR/8/34)	H-2D^{b}	TRGVQI **ASNENMETM** ESSTLE	366-374	Van Kaer *et al.,* 1994
LCMV NP	H-2L^{d}	KIMRTE **RPQASGVYM** GNLTAQ	118-126	Schwarz *et al.,* 2000
Mo MuLV gag	H-2D^{b}	CCSIVL **CCLCLTVFL** YLSENM	75-83	Van Hall *et al.,* 2000
Adenovirus E1B-19K	H-2D^{b}	YKISKL **VNIRNCCYI** SGNGAE	192-200	Sijts *et al.,* 2000
Viral (human)				
Influenza M1	HLA-A2	LSPLTK **GILGFVFTL** TVPSER	58-66	Luckey *et al.,* 1998; Gileadi et al., 1999
HIV-1 RT	HLA-A2	KQNPDI **VIYQYMDDL** YVGSDL	346-354	Sewell et al., 1999
HBV core Ag	HLA-A68	PNAPIL **STLPETTVVRR** RGRSPR	141-151	Sijts et al., 2000
Self (Mouse)				
H-Y	H-2D^{b}	n.d.	n.d.	Fehling et al., 1994
Tumoral (human)				
MAGE-A3	HLA-B60	ALSRKV **AELVHFLLL** KYRARE	114-122	Schultz, van der Bruggen (unpublished data)

Table 1.5 Epitopes poorly processed by cells carrying immunoproteasomes- Van den Eynde and Morel (2001)

Protein	MHC restriction	Peptide sequence	Amino acid positions and epitopes	References
Self (human)				
RU1	HLA-B51	ETGSTA **VPYGSFKHV** DTRLQN	34-42	Morel et al., 2000
Melanoma differentiation (human)				
Melan-A^{MART1}	HLA-A2	SYTTAE **EAAGIGILTV** ILGVLL	26-35	Morel et al., 2000
gp100^{Pmel17}	HLA-A2	SSSAFT **ITDQVPFSV** SVSQLR	209-217	Morel et al., 2000
Tyrosinase	HLA-A2	HNALHI **YMDGTMSQV** QGSAND	369-377	Morel, Van den Eynde (unpublished)

1.4.3 The Relationship between viral proteins (other than AdE1A) and the immunoproteasome.

Due to their role in antigen processing and presentation, proteasomes are known to be targeted by various viruses that are capable of establishing persistent infections. Proteasomes are not only involved in the production of peptides for antigen presentation, but they also involved in the degradation of key cellular regulatory proteins such as p53 and Rb (Maki *et al.,* 1996; Sdek *et al.,* 2005; Ciechanover *et al.,* 1991; Boyer *et al.,* 1996; Wang *et al.,* 2001). The interaction between viral proteins and the proteasomes can affect not only recognition of the infected cell by the immune system but also the events that lead to cell transformation and apoptosis. Interactions between viral proteins and components of the 20S/26S proteasome as well as immunoproteasome have been studied. As mentioned in the previous section, immunoproteasomes are more efficient than 20S proteasomes in processing antigens of viral origin, so they will form potential targets for viral manipulations as part of a strategy to evade the immune system.

The E7 protein of the high-risk human papillomavirus (HPV) type 18 is reported to repress the bidirectional promoter that encodes the expression of the immunoproteasome subunit LMP2 as well as TAP1 (Georgopoulos *et al.,* 2000), hence affecting immunoproteasome composition and activity. Similarly, the low-risk HPV 6b E7 protein also repressed the TAP1/LMP2 promoter; potentially explaining the ability of low-risk HPV to induce benign tumours (Georgopoulos *et al.,* 2000).

Research into the effect of human immunodeficiency virus type 1 (HIV-1) p24 on antigen presentation showed that the viral protein down-regulated the expression of PA28α, MECL1 and LMP7 in primary dendritic cells. In addition, PA28β and MECL1 expression was reduced in the dendritic cell line JAWS II (Steers *et al.,* 2009), as a result interfering with the immunoproteasome complex. However, this effect was reversed by pre-treatment of cells with IFN γ. Furthermore, HIV-1 Tat protein was found to bind to LMP2 and LMP7, in addition to α4 and α7 (therefore interfering with the binding of PA28 to the

alpha ring) plus six β subunits of the constitutive 20S proteasome leading to the inhibition of immunoproteaosme and 20S proteasome activity (Apcher *et al.,* 2003).

NS3, a non-structural protein of the Hepatitis C virus (HCV) also affects immunoproteasome activity by directly binding to the immunsubunit LMP7, repressing its peptidase activity, potentially interfering with the processing of viral antigens to MHC class I (Yee-Ling Khu *et al.,* 2004).

1.5 Interferon gamma and the JAK/STAT1 signalling pathway

Interferons are cytokines that are released by lymphocytes in response to viral and bacterial infections. They are known as interferons for their ability to "interfere" with viral replication. They have antiviral, antiproliferative and immunomodulatory effects, stimulating the immune system, leading to the expression of anti-viral genes. They activate immune cells such as macrophages and natural killer cells, enhance antigen presentation to CTLs and increase the ability of uninfected cells to resist infection.

There are two types of interferons: type I and type II. Type I interferon includes IFN α and IFN β. Most types of cells express type I interferons. On the other hand, type II IFN has one member: Interferon gamma (IFNγ). IFNγ is produced only by cells of the immune system such as natural killer cells as well as CD4+ and CD8+ T-cells. All IFNs mediate their effect by association with type-specific cell surface receptors that set into motion a complex series of signalling pathways that lead to the eventual expression of IFN stimulated genes (ISG).The pathway through which IFNγ activates gene transcription is called the Janus kinase/signal transducer and activator of transcription-1 (JAK/STAT1) pathway (Figure 1.16). IFNγ is a homodimeric soluble cytokine that has a crucial role in activating an array of immune responses.

It does not have a marked structural homology with type I interferons (Pestka *et al.,* 1987, 2004, 1997; Chen *et al.,* 2004; Platanias and Fish 1999; Parmar and Platanias 2003). It

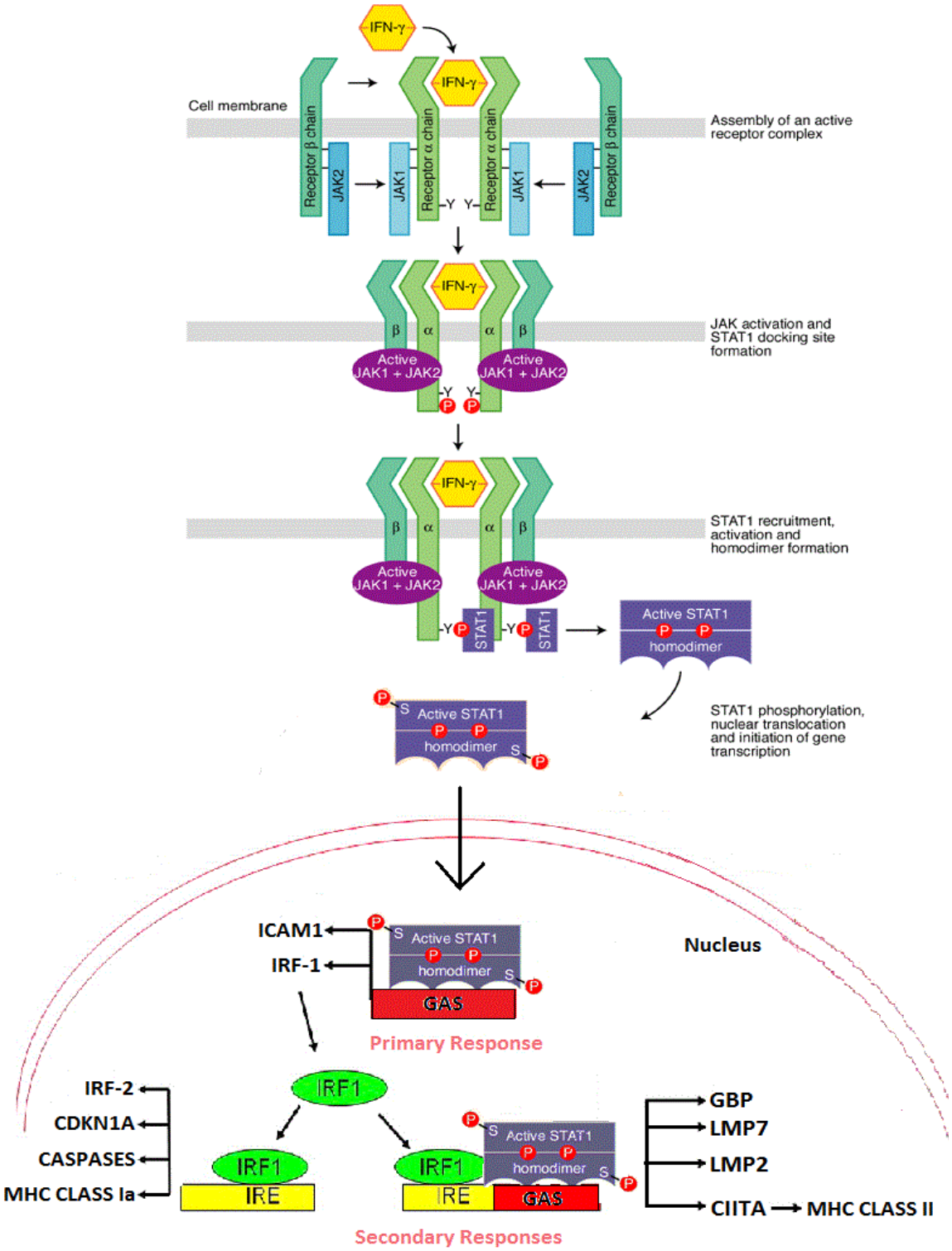


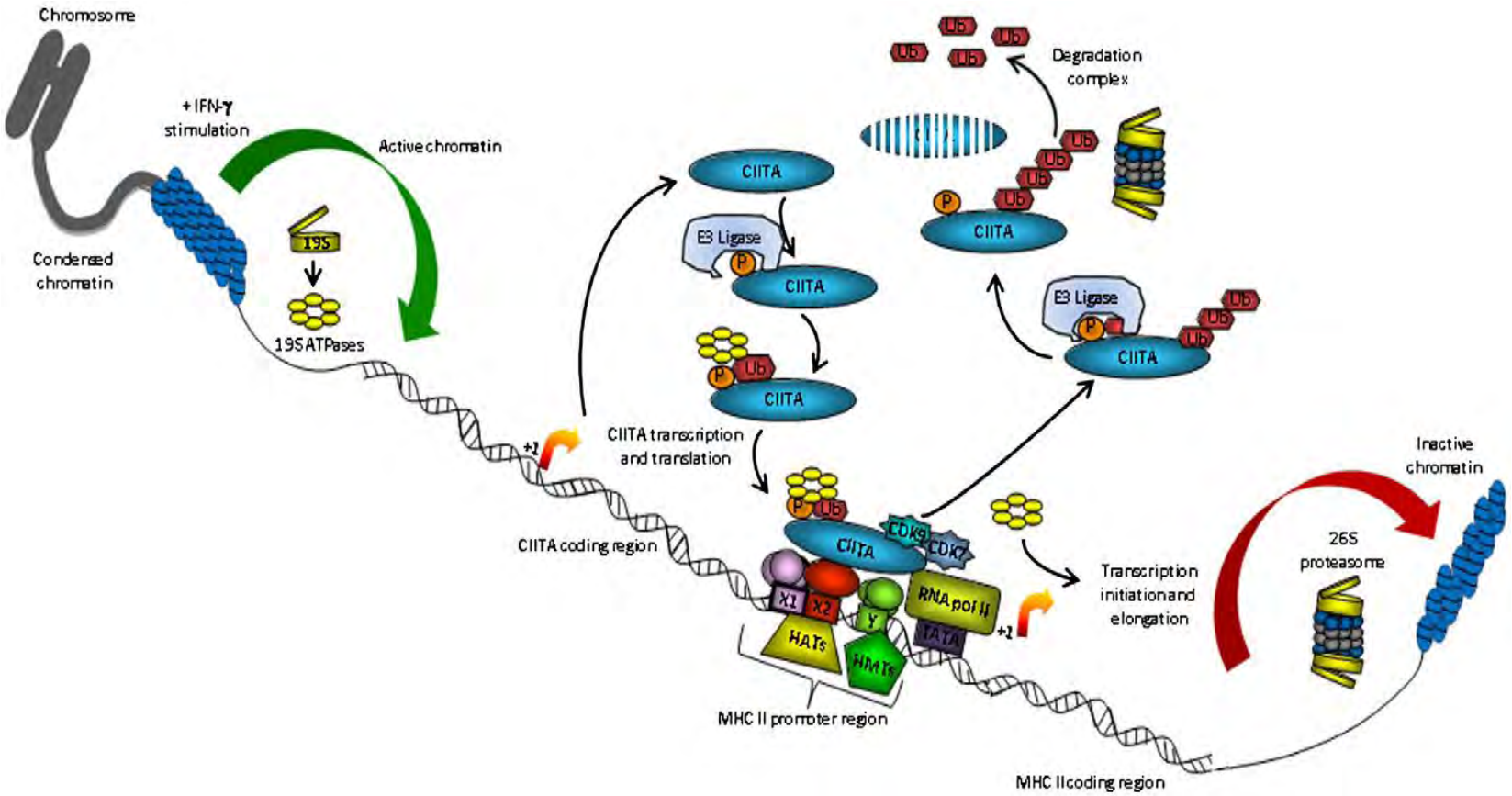
Figure 1.16: The JAK/STAT1 pathway.
The binding of IFNγ ligand to its receptor causes the activation of the JAKs, which results in the phosphorylation of a residue at the cytoplasmic tail of the IFNγ receptors. This then serves as a docking site for STAT1 proteins which in turn get phosphorylated and activated. The STAT1 dimerse and translocate to the nucleus and initiates the transcription from gene promoters that GAS element (primary response). IRF-1 become expressed and in the secondary response, IRF-1 activates transcription various genes, with or without association with the STAT1 homodimers *(modified from Shawn P. Murphy et al., 2009 and Melanie Newport: http://journals.cambridge.org/fulltext_content/ERM/ERM5_06/S1462399403005908sup004.htm)*

binds to the cell surface IFNγ receptor (IFNγR) (Pestka *et al.,* 1997; Bach *et al.,* 1997) through which it activates the JAK/STAT signalling pathway.

The IFNγR consists of a heterodimer of two chains: α (IFNγR1), and β chain (IFNγR2). These are 90 and 60-67kDa glycoproteins respectively that mediate binding with IFNγ (Farrar and Schreiber 1993; Soh *et al.,* 1994; Hibino *et al.,* 1991; Celada 1988; Bach *et al.,* 1997). IFNγR1 and IFNγR2 are not associated with each other in unstimulated cells; however, they are constitutively bound to members of the Janus family of protein tyrosine kinases, JAK1 and JAK2 that mediate IFNγR signalling activation following ligand binding. JAK1 and JAK2 bind to specific residues/motifs on the cytoplasmic tails of the IFNγR1 and IFNγR2 respectively (Farrar *et al.,* 1991; Kotenko *et al.,* 1995; Kaplan *et al.,* 1996). Binding to the ligand leads to oligomerisation with two IFNγR1 molecules associating with one IFNγ homodimer. This is then followed by the recruitment of the two IFNγ R2 chains to the complex (Kotenko *et al.,*1995; Windsor *et al.,* 1996; Marsters *et al.,* 1995; Greenlund *et al.,* 1993, 1994; Bach *et al.,* 1996; Fountoulakis *et al.,* 1992). This association brings the two inactive JAKs into close proximity where they transactivate one another through phosphorylation. It has been suggested that JAK2 first autophosphorylates, followed by phosphorylation of JAK1 (Bach *et al.,* 1997; Briscoe *et al.,* 1996; Igarashi *et al.,* 1994). Subsequently, the activated JAK1 phosphorylates a tyrosine residue pair present near the C-terminus of each of the IFNγR1 chains (Y440) (Farrar *et al.,* 1992; Igarashi *et al.,* 1994; Greenlund *et al.,* 1994; Hershey *et al.,* 1990), which is within a recognition sequence – $_{440}$YDKPH$_{444}$ – that serves as a docking site for STAT1, a member of the Signal Transducers and Activators of Transcription family of latent cytoplasmic proteins (Farrar *et al.,* 1992; Greenlund *et al.,* 1994, 1995; Hein *et al.,* 1995). After STAT1 docks and binds to the sequence in the IFNγR complex, it becomes activated by phosphorylation of tyrosine residue Y701 by JAK2 (Shuai *et al.,* 1992, 1993; Schindler *et al.,* 1992). After phosphorylation, STAT1 homodimerises and translocates to the nucleus where it binds to a nine nucleotide consensus sequence TTNCNNNAA, which is also known as GAS (gamma-activated site) element (Tau and Rothman 1999, Darnell

et al., 1994; Schindler and Darnell 1995, Sekimoto *et al.,* 1997; Tessitore *et al.,* 1998). This sequence is present in the regulatory DNA sequences of more than 200 genes. Thus the binding of STAT1 homodimers activates the expression of a whole array of proteins that are involved in immune effects of IFNγ (Boehm *et al.,* 1997) – Figure 1.16. Such genes include the transcription factor interferon regulatory factor 1 (IRF-1), a master regulator for the concerted expression of immunoproteasome subunits (Namiki *et al.,* 2005, Boehm *et al.,* 1997; Stark *et al.,* 1998). IRF-1, in turn, activates several genes, such as those encoding MHC class Ia, by binding to IFNγ response elements (IRE) or cooperates with STAT1 to form a complex that binds to IRE/GAS elements of promoters in TAP1, TAP2 , immunoproteasome subunits and CIITA (MHC class II) to activate transcription.

CIITA is the MHC class II master regulator, which, in collaboration with 19S ATPases, is recruited to the MHC class II promoter where it orchestrates various transcription factors and co-factors leading to the initiation of transcription (Bhat and Greer 2011) – Figure 1.17. In addition, even though TAP1 and LMP2 share a bidirectional promoter, both proteins have differential cellular expression. TAP1 is expressed constitutively and LMP2 is induced following exposure to IFNγ. This is because the binding of either IRF-1 or STAT1 to the IRE/GAS element of promoter is sufficient to initiate transcription of TAP1, both proteins are required for LMP2 transcription (Chatterjee-Kishore *et al.,* 1998).



Bhat and Greer 2011

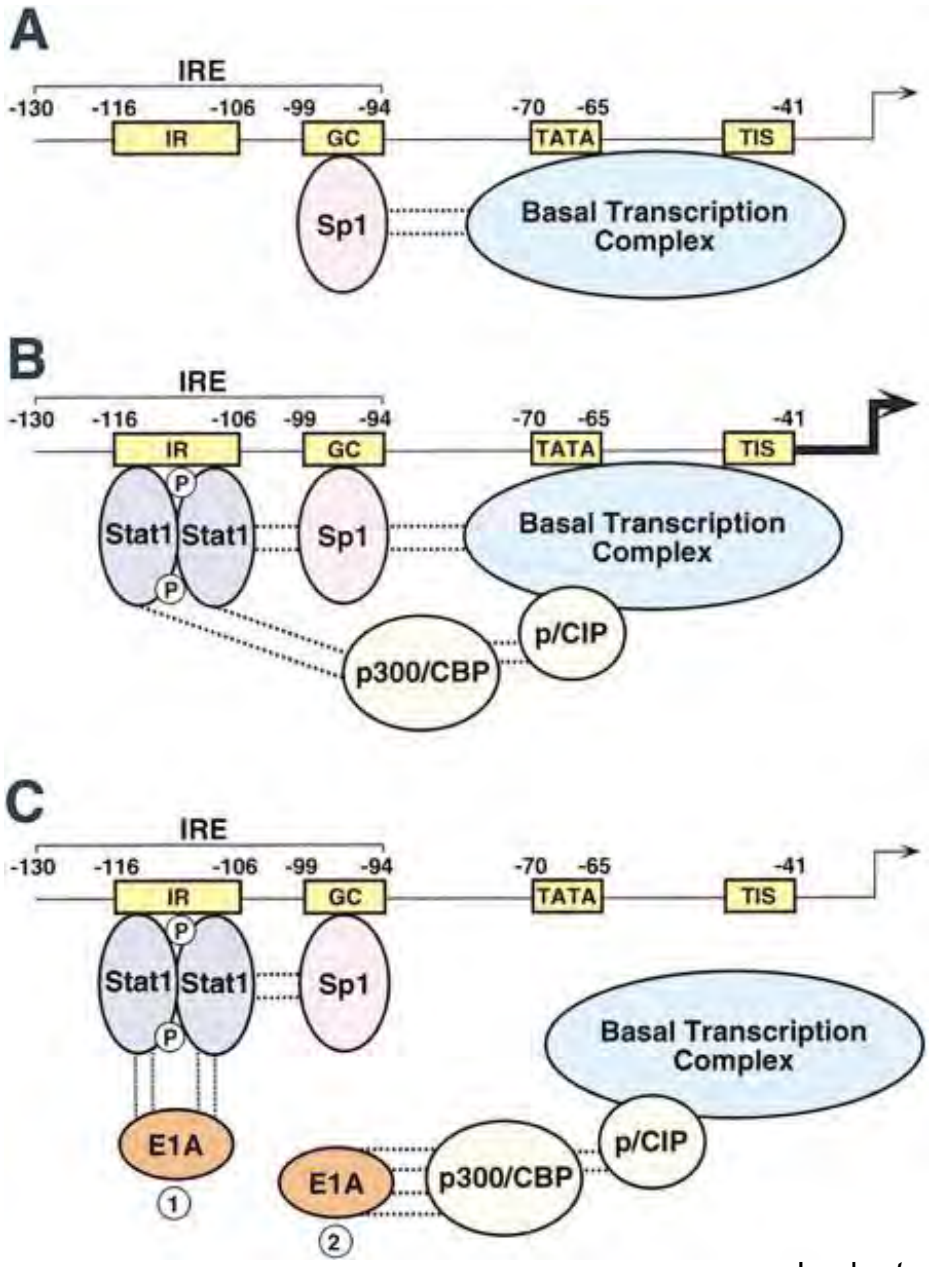
Figure 1.17: Regulation of MHC class II transcription by the 26S proteasome
After IFNγ stimulation, 19S proteasome binds to promoters and recruits histone modifying enzymes to open chromatin to allow access for transcription factors. MHC class II regulator CIITA which is now induced (from Figure 1.12) is phosphorylated at S280 and this results in its mono-ubiquitination. CIITA then, with a collaboration from 19S ATPases is recruited to the MHC class II promoter where it coordinates the assembly of transcription factors and co-factors to initiate transcription. The APIS complex detaches from CIITA and transcription elongation continues. After prolonged cytokine stimulation, CIITA is poly-ubiquitinated and degraded by the 26S proteasome.

1.5.1 The effects of AdE1A on IFNγ signalling

As mentioned previously, IFNγ stimulates the immune response and enables uninfected cells to resist infection, as well as increasing the recognition of infected cells by the immune system. Viruses, including adenoviruses, have evolved a mechanism to counteract this response in order to support viral replication and disrupt the antiviral action of IFNγ (Anderson and Fennie 1987; Kalvakolanu *et al.,* 1991; Katze *et al.,* 2002). Most adenovirus resistance to IFNγ is attributable to AdE1A (Anderson and Fennie 1987; Kalvakolanu *et al.,* 1991; Ackrill *et al.,* 1991; Gutch and Reich 1991).

In some studies, in AdE1A expressing cells, the level of STAT1 protein was particularly reduced (Leonard and Sen 1996). During the course of early infection, AdE1A directly binds to STAT1 homodimer through its N-terminus, thus disrupting transcriptional initiation and its association with CBP/p300 (Look *et al.,* 1998) (Figure 1.18). AdE1A also represses STAT1 activity by targeting CBP/p300. Upon IFNγ stimulation, the activated STAT1 homodimer binds to the GAS sequence and interacts with CBP/p300 which in turn recruits the basal transcription complex (Zhang *et al.,* 1996). In section 1.3.5.1.3 / 4, it was stated that CBP/p300 is a target of AdE1A, affecting the transcription of many genes. In fact, both STAT1 and AdE1A bind to the same domain on CBP/p300 (Zhang *et al.,* 1996), so there may be a direct competition between STAT1 and AdE1A for binding with CBP/p300, therefore affecting the anti-viral effect of IFNγ (Zhang *et al.,* 1996; Look *et al.,* 1998) (Figure 1.18). Phosphorylation of STAT1, which leads to the formation of activated STAT1 homodimer prior to transcription initiation, is inhibited by AdE1A at the later stages of infection (Look *et al.,* 1998). These multiple strategies employed by AdE1A to inhibit STAT1 function may be due to the fact that STAT1 is crucial as a trigger of immune responses in mucosal epithelial cells, which are the natural hosts of adenovirus (Look *et al.,* 1998). It is notable however that adenoviruses often target particular pathways in more than point.

The inhibiting effect of AdE1A on the IFNγ pathway, affects the expressions of MHC class II, immunoproteasomes, PA28 and other proteins involved in IFNγ mediated immune response.



Look *et al.,* 1998

Figure 1.18: Suppression of STAT1 function by AdE1A
A Under normal (unstimulated) conditions, the transcription factor Sp1 binds to the GC box and recruits the basal transcription factors that bind to the TATA box, eventually leading to transcription initiation (TIS=transcription initiation site).
B Upon IFNγ stimulation, the STAT1 homodimer binds to inverted repeat (IR) interacting with Sp1 and CBP/p300, that in turn associates with the basal transcription complex (via p/CIP).
C The presence of AdE1A disrupts these processes by directly binding to STAT1 and CBP/p300.

1.6 Aims

It has been known for some time that AdE1A targets multiple components of the antigen processing machinery. Most of these studies have focused on rat cell models; only a few have been carried out in human cells.

The aim of this project is to examine the interaction of AdE1A (from both the oncogenic Ad12 and non-oncogenic Ad5) with the immunoproteasome components and the biological significance of this on antigen presentation in human cells. We will also examine the effect of adenovirus infection on MHC class I and class II cell surface expression as well the effect of AdE1A transfection. The specific aims are:

1. To examine the binding of AdE1A to the immunoproteasome subunits LMP2, LMP7 and MECL-1; mapping sites of interaction on AdE1A and comparing these with 20S proteasome components.
2. To examine the effect of AdE1A on the ability of immunoproteasomes to hydrolyse peptide substrates.
3. To determine the effect of AdE1A and adenovirus infection on MHC class I and class II expression.
4. To examine how the interaction of AdE1A with the immunoproteasome and other cellular targets affects antigen presentation to T-cells.
5. To determine the effect of AdE1A on the ability of cells to respond to IFNγ.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture techniques

2.1.1 Cell culture media and solutions

All tissue culture reagents were presterilised and purchased from Sigma Aldrich unless otherwise stated. All reagents were stored at 4°C and pre-warmed at 37°C unless otherwise specified. Cell lines were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine. Before use, all media were supplemented with 10% foetal calf serum (FCS) unless otherwise stated.

2.1.2 Cell lines

Table 2.1: Human cell lines used in this study.

Name	Description	Notes
Tumour cell lines		
A549	Small cell lung carcinoma cell line	
12S (8) & 12S(10)	A549 expressing Ad5 E1A 12S	Rasti *et al.,* 2005
13S (G418)	A549 expressing Ad5 E1A 13S	Rasti *et al.,* 2005
293	Ad5 E1 transformed human embryo kidney cells (HEK)	Graham *et al.,* 1977
911	Ad5 E1 transformed human embryo retinoblasts	Fallaux *et al.,* 1996
H1299	Non small lung carcinoma cell line	
HCT116	Colon carcinoma cell line	
HER lines	Human embryonic retinoblast expressing Ad12E1, Ad2E1A + mutant ras, Ad5E1A + mutant ras or Ad12E1A + mutant ras	Gallimore *et al.,* 1986, Byrd *et al.,* 1982
T47D	Human ductal breast epithelial tumor cell line	
Primary cells		
ICS5491	Tonsil epithelial cells (keratinocytes)*	
B237	Skin Fibroblast cells (HLA-A02, HLA-B4)#	

*Kindly donated by Claire Shannon-Lowe, University of Birmingham
#Liquid nitrogen stock, adult lab donor

2.1.3 CD8$^+$ T- Cells

EBV-specific HLA class I restricted CD8$^+$ cytotoxic T lymphocyte clones that were specific to the following peptides generated from the LMP2A protein: HLA A*0201-restricted epitopes, CLGGLLTMV (CLG), FLYALALLL (FLY); and HLA B*4001-restricted epitope, IEDPPFNSL (IED) (Lautscham *et al.,* 2001, 2003). These were kindly donated by Dr Jill Brooks, University of Birmingham. The T cells were cultured in 24-well tissue culture plates in a medium that contains 10% B-cell serum, 1% human serum, 30% MLA144 in RPMI 1640 supplemented with L-glutamine and penicillin/streptomycin. Cells were fed once a week by replacing half the medium with the above containing instead 60% MLA144.

2.1.4 Cryopreservation of cells

Cells were usually frozen at a concentration of 10 – 30 x 10^6/ ml /cryovial. Cells were first harvested by trypsinisation and resuspended in appropriate media. The total number of cells was determined using a haemocytometer. After the number of cryovials required have been calculated, the cells were pelleted by centrifugation at 1600 rpm for 5 minutes then resuspended in warm FCS (pre-incubated at 37°C) at twice the density required and the tube was placed at 4°C. When the cell suspension has chilled, an equal volume of freezing medium – FCS with 25% v/v dimethyl-sulphoxide (DMSO) – was added to the cells dropwise and with gentle agitation (the cells now being at the correct density for freezing). One millilitre aliquots of the resulting solution was transferred to cryovials which in turn placed in a “Mr Frosty” freshly filled with isopropanol. The container was transferred to the -80°C freezer, and finally after 24 hrs, the cryovials were transferred to a liquid nitrogen storage tank.

2.1.5 Recovery of cells from liquid nitrogen

To recover cells from nitrogen, the cell suspension were thawed rapidly in a 37°C waterbath. The contents of the cryovials were transferred to a centrifuge tube, and this was followed by the dropwise addition of 10 mls of the appropriate culture medium containing 10% FCS. The cells were then pelleted by centrifugation at 1600rpm and resuspended in fresh culture medium and re-plated and incubated at 37°C.

2.1.6 Maintenance of cell culture

Cells were grown in humidified incubators in a 95% air and 5% carbon dioxide environment at 37°C. Adherent cells were maintained in DMEM containing 10% FCS on 10cm dishes. To subculture adherent cells, when cells reached confluency, medium was removed and cells washed twice with PBS. Two millilitres of trypsin solution was added and dishes left at room temperature for a minute. Trypsin solution was removed and dishes were incubated at 37°C as necessary. Detachment of the adherent cells was confirmed by microscopy, after which 10 mls of media containing FCS was added to deactivate the trypsin. After an even suspension of cells was obtained, the cells were re-plated at a required density by dividing it between the appropriate number of dishes.

Suspension cells were grown in 75 cm^2 tissue culture flasks and subcultured by removing 50% of the cell culture volume then adding an equal volume of RPMI-1640.

2.1.7 Viral infection

Cells were infected when cultures were approximately 90% confluent. Aliquots of virus was diluted with 0.4 ml serum free DMEM. Culture medium was removed from the cells and 0.4 ml of the virus-containing medium was added to each 10cm dish or 0.2 ml virus to each 6 cm dish, at an infectivity of 20 plaque forming units (p.f.u) per cell. The dishes were incubated at 37°C and rocked at 15 minute intervals to ensure the even dispersal of the virus. Two hrs after the initiation of the virus infection, excess virus was removed and

10 ml of fresh complete media was added to the cells which were subsequently incubated at 37°C until the harvesting time point. All the cells were grown and maintained in DMEM containing 10% FCS.

2.2 Cell biology techniques

2.2.1 Transient transfection of mammalian cells using Lipofectamine 2000

Twenty four hours prior to transfection, cells were plated onto 6 cm tissue culture dishes such that they would reach 80-90% confluency by the time of transfection. Transfection solution was prepared by incubating 5 µl of Lipofectamine 2000 transfection reagent (*Invitro*gen) with 200 µl of Optimem media (serum-free) for five minutes at room temperature (5 µl of Lipofectamine 2000 was used for every 1 µg of DNA). One microgram of plasmid DNA was diluted into 200 µl of Optimem media. The transfection reagent and DNA mixture were then combined, mixed gently and incubated at room temperature for 20 minutes to allow DNA-liposome complexes to form. Meanwhile, medium from each dish was removed and replaced by 4 mls of fresh DMEM containing 10% FCS. The transfection mixture was slowly added to the medium, followed by gentle rocking of the dish to mix in the transfection medium. The cells were then incubated at 37°C. Transfected cells were harvested at appropriate time after transfection and assayed for protein expression or reporter gene activity. The DNA plasmids used for transfection in this study are listed in Table 2.3.

2.2.2 Electroporation

One million cells were used per electroporation with *in vitro* transcribed mRNA (section 2.3.11). Cells were harvested by trypsinisation and the total number determined using a haemocytometer. The cells were then washed twice with Optimem media by centrifugation at 1600 rpm for 5 minutes. One million cells were resuspended in 300µl Optimem. One microgram of mRNA was added to the cell suspension and gently mixed

then transferred to 4 mm electroporation cuvettes (Cell Projects/Geneflow). Cells were pulsed at 300 V voltage, 500 µF capacitance and resistance of ∞. Electroporation equipment was Gene Pulser Xcell from Biorad. Three hundred microlitres of pre-warmed media containing 10% FCS was added to the electroporated cells and slowly transferred to a tube containing an appropriate volume of pre-warmed media that gives the required cell density for the assay to follow. Any remaining cells were cultured in a 6 cm tissue culture dish and harvested after 24 hrs to check for protein expression.

2.2.3 Flow cytometry analysis

Flow cytometry was used to stain for MHC class I and class II on the cell surface of cells that had been either infected or transformed with adenovirus or electroporated with *in vitro* transcribed mRNA encoding E1A. The effect of AdE1A on the level of MHC class I and II prior to and after, interferon gamma (IFNγ) treatment at 400 U/ml was examined.

Cells were harvested by trypsinisation and washed twice with ice-cold PBS and resuspended in 5 mls of PBS. About 5 x 10^4 - 5 x 10^5 cells from each treated and untreated sample were retained for MHC class I/II staining and the remaining were pelleted and resuspended in 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol for protein analysis. Cells for staining were pelleted and resuspended in 20 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). For blocking reaction, 5 µl of mouse polyclonal IgG (Serotec) was added, gently tapped to mix, followed by incubation on ice for 30 minutes. A volume of 75 µl of FACS buffer was added to the cells and mixed well. Fifty microlitres of untreated cells were dispensed into 2 wells of a 96 well v-bottomed plate (for the IgG2a and untreated MHC I/II), and another 50µl of the IFNγ treated in a third well. Three microlitres of each of IgG2a isotype control and MHC class I/II antibodies was added to the appropriate wells, gently mixed and left to incubate at room temperature for 30 minutes. Subsequently, in case of conjugated antibodies, the cells were washed twice with FACS buffer, and finally resuspended in 400 µl of PBS to be

analysed by flow cytometry. Unconjugated antibodies require an additional step involving an appropriate conjugated secondary antibody and further 30 minutes incubation at room temperature.

2.2.4 20S Proteasome/Immunoproteasome assay

The caspase-like, chymotrypsin-like and trypsin-like activity of the proteasome/immunoproteasome was examined in the presence or absence of AdE1A *in vitro*, using the fluorogenic substrates (Biomol) Z-LLE-AMC, Suc-LLVY-AMC and Bz-VGR-AMC. The substrates were kept at a stock concentration of 10 mM in DMSO. This was diluted to 200 µM using the assay buffer 50 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, prior to being used in an assay. Purified human immunoproteasomes (Biomol) were diluted to a working dilution of 6.25 µg/ml. To set up the assays, 50 µl of each of the diluted proteasome and appropriate substrate were mixed in an microfuge tube, and to observe the effect of AdE1A, 5 µg of the protein was added to the reaction. Negative controls consisted of 50 µl of the substrate solution and 50 µl of assay buffer. The tubes were incubated at 37°C for 30 minutes. Forty microlitre duplicates of each reaction were transferred into individual wells in a 96-well fluorescence plate on ice then the reaction was stopped by adding 200 µl per well of stopping buffer, 100 mM sodium chloroacetate in 30 mM sodium acetate, 70 mM acetic acid, pH 4.3. The fluorescence of each sample was measured on “umbelliferone 360 nm/460 nm” on the Victor^2 plate reader (Wallac).

2.3 Molecular biology techniques

2.3.1 Bacterial strains

Table 2.2: Bacterial strains used in the study

Strain	Genotype
BL21-codon Plus (DE3)-RIL (Stratagene)	E.coli B F^- *omp*T hsdS ($ra^-m^-a^-$) *dcm*$^+$ Tet^r *gal* λ (DE3) *end*A Hte [*arg*U *ile*Y *leu*W Cam^r] DH5α
DH5α (Invitrogen)	*sup*E44, Δ*lac*U169 (Φ80 *lac*ZΔM15) *hsd*R17 *rec*A1*end* A1 gyrA96 *thi*-1 *rell*A1

2.3.2 Media and buffers

Luria Broth (LB): 10 g/L bactotryptone (Difco), 5 g/L bacto-yeast extract (Difco) and 10 g/L NaCl, and was sterilised prior to use.

LB-agar plates: LB was supplemented with 1.5% agar and sterilised. Prior to pouring ampicillin was added to a concentration of 100 µg/ml.

2.3.3 Antibiotics

Ampicillin was made up as a stock solution of 100 mg/ml in sterile water; filter sterilised through a 0.2 µm filter and stored at 4°C.

2.3.4 Long term storage of bacterial cultures

A volume of 0.5ml of the 500ml overnight bacterial culture was added to 0.5 ml of 80% glycerol in sterile distilled water, gently mixed and frozen at -80°C in cryovials.

2.3.5 Plasmid DNA

Table 2.3: DNA constructs used in the study

Gene	Vector	Application	Source
12S Ad5 E1A	pcDNA3.1+	Transfection *In vitro* transcription of RNA	Prof. Joe Mymryk, University of Western Ontario
13S Ad5 E1A	pcDNA3.1+	Transfection *In vitro* transcription of RNA	Prof. Joe Mymryk, University of Western Ontario
12S Ad12 E1A	pcDNA3.1+	Transfection *In vitro* transcription of RNA	Jailal Ablack, University of Western Ontario
13S Ad12 E1A	pcDNA3.1+	Transfection *In vitro* transcription of RNA	Jailal Ablack, University of Western Ontario
12S Ad5 E1A L19/20A	pcDNA3.1+	Transfection *In vitro* transcription of RNA	Rasti *et al.,* 2005, 2006
12A Ad5 E1A L19/20A	pcDNA3.1+	Transfection *In vitro* transcription of RNA	Rasti *et al.,* 2005, 2006
LMP2A-HA	pCMV.TnT	*In vitro* transcription of RNA	Dr Steve Lee, University of Birmingham
pGAS-luc	Reporter plasmid	Luciferase assay	Dr Cristina Areste, University of Birmingham
pGL3-Basic	Reporter plasmid	Luciferase assay	Dr Cristina Areste, University of Birmingham
pGL3-Control	Reporter plasmid	Luciferase assay	Dr Cristina Areste, University of Birmingham
pGL3-Renilla	Reporter plasmid	Luciferase assay	Dr Cristina Areste, University of Birmingham

2.3.6 Measuring DNA concentration

DNA concentration was measured using a NanoDrop® spectrophotometer.

2.3.7 Transformation of bacteria

Transformation of DNA constructs into BL21 and DH5α competent cells was performed as follows (DNA constructs are displayed in Table 2.3). The cells were initially thawed on ice and 25 µl aliquots were combined with approximately 50 ng of DNA, and mixed with gentle pipetting. Following incubation on ice for 30 minutes, samples were heat shocked at 42°C for 1 minute and cooled on ice for 5 minutes. After the addition of 250 µl of LB, the transformation mixture was incubated at 37°C for 1 hr at 220 rpm in a shaking incubator. Cells were then pelleted at 13000 rpm for 1 minute, 175 µl of the supernatant removed and pellet resuspended in the remaining 100 µl of LB broth which was in turn spread onto L-agar plates containing ampicillin. After drying, the plates were incubated overnight at 37°C and analysed the next morning.

2.3.8 Large scale preparation of DNA

DNA was purified using the Qiagen plasmid maxi kit following the protocol supplied.

Five millilitres of LB containing 100 µg/ml of ampicillin was inoculated with a single colony of bacteria containing the desired plasmid, and left at 37°C for 9 hrs at 220 rpm in a shaking incubator. The culture was then used to inoculate 250 ml of LB containing 100 µg/ml of ampicillin, and grown overnight. The following morning the bacterial cells were pelleted by centrifugation at 6000 g for 15 minutes at 4°C, then resuspended in 10 ml of Buffer P1 (resuspension buffer) that had RNase A pre-added to it. Ten millilitres of Buffer P2 (lysis buffer) was then added and mixed thoroughly by vigorously inverting the sealed tube 4-6 times, and incubated at room temperature for 5 min. Ten millilitres of pre-chilled Buffer P3 (neutralisation buffer) was subsequently added and mixed immediately and thoroughly by vigorously inverting again 4-6 times, followed by incubation on ice for 20 minutes. The lysate was centrifuged at 20,000g for 30 minutes to pellet the cell debris. The supernatant, which contains the plasmid DNA, was centrifuged again at 20,000 x g for 15 minutes to remove any remaining debris. Meanwhile, the QIAGEN-tip 500 (binding

column) was equilibrated by the addition of 10 ml of Buffer QBT and the column allowed to empty by gravity flow. After centrifugation, the supernatant was loaded onto the QIAGEN-tip 500 and allowed to pass through the resin by gravity flow. Once the column has emptied, it was washed twice with 30 ml Buffer QC (washing buffer), allowing the column to empty by gravity flow each time. The plasmid DNA was eluted by adding 15 ml of Buffer QF (pre-warmed to 65°C). The DNA in the elutant was precipitated by adding 10.5 ml of isopropanol, then mixed and centrifuged immediately at 5000 x g for 60 minutes at 4°C. The supernatant was removed and the resulting DNA pellet was washed with 5 ml of 70% ethanol, then again centrifuged at 5000 x g for 60 minutes at 4°C. The ethanol supernatant was carefully removed and the DNA pellet allowed to air-dry for 5-10 minutes. Finally, the pellet was redissolved in the appropriate volume of sterile nuclease-free distilled water.

2.3.9 Restriction enzyme digestion

The digest was carried out for 1.5 hrs at 37°C with restriction enzymes as in the manufacturer's protocol. DNA plasmid constructs were digested so they were linear prior to the process of in-vitro translation of mRNA.

XbaI and ClaI (Roche)

For 1 µg digestion of DNA in a 20 µl mix: 2 µl 10 x restriction enzyme buffer, 0.2 µl of acetylated BSA (10 µg/µl), 1 µg of DNA, 0.5 µl restriction enzyme (10 u/µl), were made up to a total volume of 20 µl with sterile, nuclease-free water. The reaction was scaled up as required.

XhoI (Roche)

Digests carried out in a volume of 50 µl that contained 2 µg of DNA, 5 µl of 10 x buffer, and 25 units of the restriction enzyme.

The digests were analysed by agarose gel electrophoresis as described below.

2.3.10 Agarose gel electrophoresis

Analysis of DNA and RNA was performed using agarose gels prepared with 50 ml of TBE (0.89 M Tris Borate pH 8.3, 20 mM Na_2EDTA) (Geneflow), 0.8% w.v agarose, and 1 µl of SYBR® Safe DNA gel stain (Invitrogen). DNA samples were diluted 1/6 with 6X gel buffer [30% v/v of glycerol, 0.25% w/v of bromophenol blue and 0.25% w/v of xylene cyanol FF in SDW] and then loaded. Gel electrophoresis was performed in 1X TBE at 60 V for approximately 40 minutes, depending on the size of the DNA/RNA to be analysed. DNA/RNA was visualised by exposure to UV light.

2.3.11 In vitro transcription of RNA

RNA for electroporation was *in vitro* transcribed from 4 µg of DNA construct plasmids listed in Table 2.3. It comprises of several steps:

(a) Plasmid DNA linearisation: The first step involved linearising the plasmid using the appropriate restriction enzyme that cuts further downstream in the insert (see section 2.3.8). The linearised plasmid was visualised by 0.8% agarose gel electrophoresis to confirm digestion. The DNA was then purified using the High Pure PCR Product Purification Kit (Roche), following the manufaturer's instructions: The total volume of DNA was adjusted to 100 µl using nuclease free water, followed by the addition of 500 µl of Binding Buffer. The whole mixture was then loaded onto a High Pure filter tube which was centrifuged at maximum speed for 60 seconds at room temperature. The flowthrough was discarded. This was followed by two washes with 500 µl and 200 µl of Wash Buffer, centrifuging at 13000 g for 1 minute at room temperature each time and discarding the flowthrough. Finally the purified, digested DNA was eluted in 100 µl of Elution Buffer. The

next step was then to precipitate the DNA and resuspend in sterile nuclease-free water to obtain the appropriate concentration (see below).

(b) DNA precipitation: To the 100 µl of DNA solution, 10% (10 µl) of 3 M sodium acetate (pH 5.5) (Ambion) followed by 250% (250 µl) of ethanol was added and tube incubated at -20°C for 30 minutes. To pellet the DNA, the tube was centrifuged at 13000 rpm for 15 minutes at 4°C, and the supernatant was carefully removed and discarded. A volume of 500 µl of 70% ethanol was slowly added and the tube centrifuged again at 13000 rpm for 3 minutes at 4°C. The supernatant was discarded. The pellet was left to air-dry in the hood for 5-10 minutes, after which the DNA was resuspended in 18 µl of sterile nuclease-free water. The DNA is now ready for the in vitro transcription reaction.

(c) RNA *in vitro* transcription reaction: This reaction enables the synthesis of mRNA from the linearised DNA plasmid template. The pcDNA3 plasmids used contain T7 promoter, hence enabling *in vitro* transcription using the T7 RNA polymerase. The *in vitro* transcription reaction was assembled at room temperature using the components of the mMESSAGE mMACHINE® T7 kit (Ambion): 18 µl of cleaved DNA, 10 µl of 2 x NTP/CAP, 6 µl of 10 x reaction buffer, 6 µl enzyme mix in a total volume of 60 µl. The NTP/CAP buffer contains ATP, CTP, UTP, GTP and cap analog. This allows 5' capping of the newly formed mRNA. This step is crucial for creating mature and stable mRNA for translation. The reaction buffer contains mainly salt, buffer and dithiothreitol creating conditions for optimal enzymatic activity. The main constituents of the enzyme mix are buffered 50% glycerol containing T7 RNA polymerase and RNase inhibitor. All the components were mixed by flicking the tube and micro-centrifuging briefly. The reaction mix was incubated in a 37°C water bath for 2 hrs. In order to remove the DNA template, 3 µl of TURBO DNase was added and the reaction incubated again for a further 15 minutes.

(d) RNA clean up: RNA was recovered using the RNeasy® mini kit (Qiagen) following the manufacturer's protocol. The sample volume was adjusted to 100 µl with RNase-free water then 350 µl of lysis buffer RLT (pre-mixed with β-mercaptoethanol) was added, and mixed thoroughly. A volume of 250 µl of 100% ethanol was also added to the diluted RNA and mixed thoroughly by pipetting. The whole mixture was loaded onto an RNeasy mini column (that was placed in a 2 ml collection tube). Buffer RLT is a guanidine isothiocyanate-containing lysis buffer. Both RLT and ethanol create conditions that promote selective binding of RNA to the RNeasy mini column membrane. The column was centrifuged for 15 seconds at 10000 rpm. The collection tube with the flowthrough was discarded and replaced. This was followed by two washes with 500 µl of wash buffer RPE (pre-added with ethanol) at 10000 rpm for 15 seconds and 2 minutes, discarding the flow through each time. The empty column was placed in a new collection tube and spun again for 1 minute at maximum speed to remove any traces of wash buffer RPE. Finally the RNA was eluted twice with the same 30 µl of RNase free water by centrifuging for 1 minute at 10000 rpm each time. RNA yield was determined by spectrophotometry (NanoDrop).

(e) Polyadenylation of RNA: A reaction mix was assembled as follows: 5 µl reaction buffer (USB), 20 µg of RNA, 1.25 µl of 10 mM ATP (USB), 1 µl of 600 U poly (A) polymerase (USB), and RNase-free water to make up the volume to 25 µl. The reaction was mixed well and incubated at 37°C in a water bath for 20 minutes. The polyadenylation reaction enables the addition of the poly (A) tail (consisting of multiple adenosine monophosphates) to the mRNA molecule. The tail protects mRNA from degradation by enzymes in the cytoplasm as well as aiding export from the nucleus, and translation (Guhaniyogi and Brewer 2001).

(f) Polyadenylated mRNA purification: The above reaction mix was made up to 300 µl with RNase-free water, and an equal volume of phenol chloroform was added and mixed by

gentle vortexing. The tube was centrifuged at 13000 rpm for 10 minutes at 4°C to separate the layers. The top aqueous layer that contained the purified RNA was carefully aspirated and transferred to a fresh tube. This RNA was then ethanol-precipitated by adding 10% (v/v) 3M sodium acetate (pH 5.5) (Ambion) and mixed. This was followed by the addition of 250% (v/v) ethanol and incubated at -80°C for 30 minutes. The tube was centrifuged at 13000 rpm for 20 minutes at 4°C, and the supernatant carefully removed and discarded. The RNA pellet was washed with 500 µl of 70% ethanol and centrifuged again at 13000 rpm for 5 minutes, and the supernatant carefully discarded. The pellet was left to air dry in the hood and finally the purified polyadenylated mRNA was resuspended in 20 µl of RNase free water. RNA yield was determined again and RNA aliquoted and stored at -80°C.

2.3.12 Luciferase assays

Typically, 0.5-2 µg of the relevant DNA constructs was transfected into H1299 cells in 6-well tissue culture plates using Lipofectamine 2000 according to the manufacturer's instructions (see Table 2.3). "Empty" pcDNA3.1 vector was used as a negative control for the transfections. All luciferase assays were carried out using the Dual-Luciferase® Reporter assay system (Promega) using white opaque 96-well optiplates (PerkinElmer), following the manufacturer's instructions. After 48 hrs (including the 24 hrs IFNγ treatment), the cells were washed once with PBS, removing all traces of the rinse solution. 500 µl of Passive Lysis Buffer (PLB) was dispensed into each well, and the plates left to rock gently for 15 minutes at room temperature. Each of the lysates was subsequently transferred to a tube. Twenty microlitres of each sample was pipetted into the white optiplate wells in triplicates, followed by the addition of 100 µl of LARII substrate. The firefly luciferase activity was measured using the luminometer (Victor^2, Wallac). To measure the *Renilla* luciferase activity, a further 100 µl Stop & Glo® Reagent was added into each well then activity determined again by the luminometer. Luciferase readings are normalized by Renilla. The Renilla is a control reporter that is co-transfected into cells in

addition to the luciferase reporter. It has a different substrate to Luciferase thus allowing the evaluation of transfection efficiency. The data are normalised by dividing each luciferase reading by the Renilla values; in that way the variation in transfection efficiency is incorporated in the observed readings.

2.3.13 In vitro translation of radio-labelled protein

Eukaryotic *in vitro* translation was carried out using the Promega TNT T7/SP6 coupled rabbit reticulocyte system following the manufacturer's instructions. Briefly, the reaction consisted of 25 µl reticulocyte lysate, 2 µl reaction buffer, 1 µl T7 RNA polymerase, 1 µl of 1 mM amino acid mixture minus methionine, 2 µl of [^{35}S]-methionine (1000 Ci/mmol at 10mCi/ml; Amersham Biosciences), 0.5 µl RNasin ribonuclease inhibitor (40 U/µl), 1µg of DNA template and nuclease free water to a final volume of 50 µl. The reaction was incubated at 30°C for 90 minutes, quickly centrifuged and then stored at -80°C.

2.3.14 GST fusion protein production and purification

pGEX 4T-1 expression constructs containing the coding sequence of interest downstream of a region that encodes glutathione S-transferase (as displayed in Table 2.3) were transformed into BL21 cells and plated out as previously described. The following day, two colonies were used to inoculate 15 mls of LB containing 100 µg/ml ampicillin. This starter culture was incubated overnight at 37°C with shaking at 200 rpm. This was then used to inoculate 500 mls of LB in the presence of 100 µg/ml ampicillin; the cultures were placed in a 200 rpm shaker at 37°C for approximately 1.5 hrs, and the optical density subsequently monitored. Once the bacteria had reached an appropriate absorbance of 0.6-0.8 (indicating that they were in the log phase of growth), the culture was cooled to 30°C and incubated for 3 hrs in the presence of 0.02% w/v isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma). The bacterial cells were then pelleted at 5000 rpm

for 15 minutes at 4°C, and stored at -80°C until ready to be used in lysis and protein purification.

In order the purify the GST-fusion proteins, the bacterial cell pellet was lysed in 20 mls of lysis buffer containing PBS, 1 mM EDTA (pH 8) and 1% v/v Triton X-100. The lysate was sonicated 3 times on ice for 45 seconds, at two minute intervals. The lysate was then centrifuged at 15000 rpm for 10 minutes at 4°C to remove the cell debris, and the supernatant transferred to a fresh tube and then centrifuged again at 18000 rpm for 30 minutes at 4°C. The supernatant was mixed with 2 mls of a suspension of 1:1 lysis buffer and glutathione-agarose beads (Sigma), and rotated for 2 hrs at 4°C. The beads were centrifuged at 3000 rpm and the supernatant transferred to a fresh tube (to which fresh beads were added and the whole process repeated), while the original beads were washed three times in lysis buffer and twice with wash buffer (PBS, 2 mM EDTA, pH 8). In order to elute the GST-fusion proteins, the glutathione agarose beads were incubated twice with 2 ml of 20 mM glutathione and 50 mM Tris solution pH 8 at 4°C for 1 hr with rotation each time. Each of the eluants containing the GST-fusion protein was transferred to dialysis tubing, which was previously hydrated in distilled water. Overnight dialysis was carried out at 4°C in 5 litres of 0.1 M NaCl, 50 mM Tris, 0.1 mM dithiothreitol (DTT) (Sigma), adjusted to pH 7.3. The dialysed GST-fusion protein was transferred to a new tube and its protein concentration determined by Bradford assay and quantified against a standard curve. The purity of the proteins was assessed by SDS-PAGE electrophoresis and Coomasie blue staining. The protein was stored in aliquots at -80°C.

2.4 Protein chemistry techniques

2.4.1 Preparation of cultured adherent cell samples for protein analysis by Western blotting

Tissue culture medium was removed and cells were washed twice in 5ml of cold saline. Cells were lysed in buffer containing 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-

mercaptoethanol, and then detached from the tissue culture dish with a plastic cell scraper and transferred to a microfuge tube. Harvested cells were sonicated for 10 seconds. Protein concentration was determined and samples stored at -20°C.

2.4.2 Protein determination by Bradford assay

A small volume (2-10 µl) of the protein sample was carefully diluted and mixed with 1ml of Bradford reagent (BioRad) which was pre-diluted 1:5 with distilled water. The resultant mixture was vortexed to mix. The absorbance was measured in a spectrophotometer at λ 595 nm against the distilled water blank. Protein concentrations were determined by comparison with a standard calibration curve prepared from known quantities of bovine serum albumin (0-10 µg).

2.4.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Immunoprecipitates, GST pull-down samples, or 50 µg of whole cell lysates were electrophoresed on 11% polyacrylamide gels made from 30% bisacrylamide stock solution (Severn Biotech), 0.1 M Tris, 0.1 M Bicine (N,N-bis [2-hydroxy-ethyl-glycine]) pH 8.3, 0.1% SDS and 0.3% v/v TEMED (N,N,N',N'-tetramethyl-ethylenediamine) in a total volume of 50 ml. After the addition of 300 µl of 10% ammonium persulphate solution (APS), the gel was poured into the apparatus (Hoefer) and left to polymerise. A well-forming comb was inserted into the gel before polymerisation occurred. Once the gel has set the comb was removed, the wells were rinsed with distilled water and immersed in running buffer (0.1 M bicine, 0.1 M Tris pH 8.3, 0.1% w/v SDS). Samples were diluted with an equal volume of Laemmli sample buffer (25% v/v glycerol, 62.5 mM Tris pH 6.8, 5% β-mercaptoethanol, 2% w/v SDS and 0.01% w/v bromophenol blue), boiled for 5 minutes and applied to the gel. Pre-stained molecular weight markers were also applied to the gel. The samples were typically electrophoresed overnight, at 10 mA in running buffer.

2.4.4 Alkaline urea gel electrophoresis (urea-PAGE)

Immunoprecipitated samples for immunodetection of AdE1A were fractionated in the absence of SDS on 15% polyacrylamide gels, containing 7 M urea, 15.2 mM Tris, 96 mM glycine, pH 8.5, 0.1 ml TEMED and 0.25 ml 10% APS in a total volume of 50 ml. The gel was poured into the apparatus and overlaid with a thin layer of water-saturated butan-2-ol (1:3). When the gel had fully polymerised, the butan-2-ol was washed off with water. Before loading the samples, the gel was pre-run for 1 hr at 25 mA in a running buffer of 15.2 mM Tris, 96 mM glycine, pH 8.5. 50 µl of the immunoprecipitate or 25 µg of the protein lysate was diluted in an equal volume of urea buffer (9 M urea, 50 mM Tris-HCl pH 7.4, 150 mM β-mercaptoethanol and 0.03% bromophenol blue). Samples were loaded onto the gel and electrophoresed at 15 mA for 4 hrs, followed by Western blotting. AdE1A migrates into the urea gel based on its negative charge (a preponderance of acidic amino acids) and relatively low molecular weight. Before, the urea gel is used for separation of low-molecular-weight acidic proteins, which have marked differences in charge (pI) or conformation (Grand and Gallimore 1984). As high molecular weight proteins such as antibodies (Ab) are not able to migrate into the gel, urea gels are a good way for analysing some proteins when the heavy chain of the Ab against the target protein is of similar molecular weight to the protein of interest.

2.4.5 Staining of polyacrylamide gels

After electrophoresis, gels were stained in 0.1% w/v Coomasie brilliant blue R-250 (Sigma) in methanol/glacial acetic acid/water (3:1:6 v/v) on a shaker for 20 minutes at room temperature, and subsequently destained in methanol/glacial acetic acid/ water (3:1:6 v/v) as required.

2.4.6 Staining of proteins on nitrocellulose membranes

To evaluate even loading and efficient transfer of proteins after electroblotting, nitrocellulose membranes were stained with Ponceau S stain (0.1% w/v Ponceau S (Sigma) and 3% w/v trichloroacetic acid (TCA)) for 1 minute. Excess stain was washed off with distilled water. After visualisation of protein bands, the dye on the membrane was removed with Tris-buffered saline with Tween 80 (TBS-T) (0.1% v/v Tween^{TM} 80, 8 g/L sodium chloride and 0.02 M Tris-HCl pH 7.6) and the proteins on it were subjected to immunoblot analysis.

2.4.7 Detection of radioactive proteins on gels

Following electrophoresis, gels were stained with Coomasie Blue as previously described. The gel was then soaked in the Amplify^{TM} reagent (Amersham Bioscience) for 30 minutes with agitation. Amplify^{TM} reduces exposure times for gels containing [^{35}S]-labelled samples. The gel was dried under vacuum at 80°C for 1 hr and exposed for autoradiography at -20°C.

2.4.8 In vitro GST pulldown assays

The binding capacity of [^{35}S]-labelled LMP2, LMP7 and MECL-1 for GST-12SAd5E1A, 13SAd5E1A and 13SAd12E1A, and their corresponding GST-tagged mutant fragments were assayed by GST-pulldown assay. Typically, 20 µg of GST-fusion protein was incubated with 10-20 µl of [^{35}S]-labelled protein on ice for 2 hrs in PBS, 1 mM EDTA, 1% Triton x100. The volumes were then equalised by adding 1 ml of lysis buffer and protein complexes were isolated by incubation with 20µl glutathione-agarose beads for 1 hr with rotation at 4°C. Beads were then washed with 1 ml of lysis buffer 3 times followed by a further two washes in PBS, 2 mM EDTA, pH 8. GST-protein complexes were then eluted with 60 µl of buffer containing 20 mM glutathione and 50 mM Tris pH 8, on ice for 1 hr with

occasional mixing. The beads were pelleted and supernatant containing the protein complexes transferred to a fresh tube. A further 40 µl of the elution reagent was added and tube left on ice for a further 30 minutes. The beads were pelleted again and supernatant pooled together with the previous 60 µl elution. Lastly, the eluate was mixed with Laemmli sample buffer and boiled for 2 minutes in preparation for SDS-PAGE.

2.5 Immunological techniques

2.5.1 Antibodies

Table 2.4: Antibodies used in the study.

Name	Species	M.W. (K)	Application	Dilution	Source
Anti-LMP2	Rabbit polyclonal	23	WB	1:2000	AbCam
Anti-LMP7	Rabbit polyclonal	20	WB	1:2000	AbCam
Anti-MECL-1	Rabbit polyclonal	29	WB	1:10000	Dr Marcus Groettrup, University of Constance
Anti-20S (α1,2,3,5,6 and 7 subunits)	Mouse monoclonal	29,32	WB, IP	1:2000	Biomol
Anti-Ad5 E1A (M73/M58)	Mouse monoclonal	45	WB	1:2000	E. Harlow *et al.,* 1985
Anti-Ad12 E1A	Mouse monoclonal	45	WB	1:10	In-house
Anti-Ad5 E1B (2A6)	Mouse monoclonal	58	WB	1:10	A. Levine *(*Sarnow *et al.,* 1982*)*
Anti-Ad12 E1B (XPH9)	Mouse monoclonal	58	WB	1:10	Merrick *et al.,* 1991
Anti-β actin	Mouse	45	WB	1:20000	Sigma
Anti-mouse HRP	Goat IgG	-	WB	1:2000	Dako
Anti-rabbit HRP	Swine IgG	-	WB	1:3000	Dako
Anti-human HLA ABC (class I)	Mouse monoclonal	-	Flow cytometry		Serotec
Anti human HLA II DP DQ DR RPE (class II)	Mouse monoclonal	-	Flow cytometry		Serotec

IgG2a Isotype control PE	Mouse monoclonal	-	Flow cytometry		R & D systems
of CD107a-FITC antibody	Mouse monoclonal	-	Flow cytometry		BD Pharmingen

2.5.2 Western blotting

Following electrophoresis, cell lysates were electrophoretically transferred to a nitrocellulose membrane. A blotting cassette was set up containing a piece of nitrocellulose membrane pre-soaked in blotting buffer (0.05 M Tris, 0.19 M glycine and 20% v/v methanol). The membrane was placed onto a sheet of 3 MM Whatman filter paper on a blotting pad and then the gel was placed onto the nitrocellulose membrane. The gel was then overlaid with another sheet of pre-soaked 3 MM Whatman filter paper and a second blotting pad. The blotting cassette was placed in a Hoefer transblot electrophoresis apparatus that was filled with blotting buffer with the nitrocellulose towards the anode. Blotting was carried out for 6-7 hrs at 20-30 volts. After transfer, the membrane was stained with Ponceau S stain as previously described, in order to visualise the transferred proteins. The membrane was then washed for 10 minutes in Tris-buffered saline with Tween 80 (TBS-T) (0.1% v/v Tween^{TM} 80, 8g/L sodium chloride and 0.02 M Tris HCl pH 7.6) to remove the Ponceau S stain. The nitrocellulose membrane was incubated on an orbital shaker for 1 hr at room temperature in blocking agent (5% w/v skimmed dried milk, in PBS) to block non-specific binding sites. Primary antibodies were diluted in TBS-T with 5% w/v skimmed milk, and added to the blots in polythene bags, which were heat sealed and incubated for 5 hrs at room temperature or overnight at 4°C on a rocking platform. The membrane was rinsed and washed with 6 x 5 minute washes in TBS-T. Blots were then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase, which was diluted in TBS-T with 5% w/v skimmed milk and incubated for 1-2 hrs at room temperature on a rocking platform. After a further 6 x 5

minute washes in TBS-T, the antigen-antibody complex was visualised using the enhanced chemiluminescence (ECL) detection reagent (Immobilon^{TM} Western, Millipore). The membranes were soaked in 1:1 mixture of ECL detection solution for one minute. The membranes were wrapped, protein side up, in a Saran wrap and exposed to X-ray film for an appropriate period of time. Exposure times, ranging from a few seconds to 5 minutes were used, depending on the protein being detected or the primary antibody.

2.5.3 Immunoprecipitation of proteins

Cells were lysed in 0.5 ml of immunoprecipitation (IP) buffer containing 50 mM Tris pH 7.4, 0.825 M NaCl and 1% v/v NP-40. Cell lysates were centrifuged at 35000 rpm for 20 minutes at 4°C. Protein complexes were then immunoprecipitated by adding an appropriate antibody, typically 10 µl, and mixed by rotation overnight at 4°C. The resulting protein-antibody complexes were then mixed for a further hour with 20 µl of packed protein G agarose beads (Sigma). Immunocomplexes bound to the beads were then centrifuged and washed three times with 1 ml of IP buffer, prior to resuspending in Laemmli sample buffer and boiling for 5 minutes in preparation for alkaline urea gel electrophoresis.

2.5.4 Enzyme linked immunosorbent assay (ELISA)

ELISA was used to measure IFNγ release from T cells incubated with target cells expressing the appropriate epitope in the absence or presence of AdE1A. The assay was first set up by incubating the appropriate T-cells with target cells (fibroblasts) that were electroporated with mRNA encoding the LMP2A epitope (from Epstein Barr Virus) in the presence or absence of mRNA encoding AdE1A. In one well, $1x10^3$ CD8+ T-cells to $5x10^4$ electroporated fibroblasts were used in the assay and 1 µg of RNA was used to electroporate 10^6 fibroblasts. Background levels were monitored by incubating each of the targets and T cells alone with only media. The cells were incubated in 96-well round-

bottomed plates overnight at 37°C. On the same day as this was being set up, Maxisorp plates (Nunc) were coated with 50 µl anti-human IFNγ antibody (Thermo-Scientific) that was diluted 1/1360 to 0.75 µg/ml in coating buffer (0.1 M Na_2HPO_4 adjusted to pH 9 with 0.1 M NaH_2PO_4), and left overnight (covered in foil) at 4°C. The next day, the coating antibody was flicked off and 200 µl of blocking buffer (1%BSA in PBS, filtered, 0.05% Tween 20) was added to each well and left to incubate at room temperature for 1 hr (covered in foil). In the meantime, standards using two-fold dilutions of IFNγ from 2000 pg/ml to 31.25 pg/ml in ELISA medium (RPMI1640, 10% FCS) plus ELISA medium alone were prepared for the purpose of generating a standard curve. The Maxisorp plates were washed six times with PBS-T (PBS/0.05% v/v Tween 20) using a water bottle to squirt each well thoroughly. Subsequently, 50 µl of standard and test supernatants from the cultures in the round-bottomed plates were carefully dispensed into each well of the Maxisorp plates and then left to incubate at room temperature for 2-4 hrs. After this, the plates were washed again 6 times with PBS-T followed by the addition of 50 µl of biotin-labelled anti-human IFNγ antibody that has been diluted with blocking buffer to 0.36 µg/ml. The plates were left to incubate at room temperature for 1 hr, and then washed 6 times with PBS-T followed by the careful addition of 50 µl of Extravidin Peroxidase (Sigma) diluted 1/1000 with blocking buffer and plates were left incubating at room temperature for 30 minutes. The plates were washed 8 times with PBS-T for the last time and 100 µl of TMB substrate (tebu-bio laboratories) was dispensed in each well. The plates are left at room temperature for 20 minutes. The reaction was stopped by the addition of 100 µl of 0.1 M hydrochloric acid. The absorbance was finally read at 450 nm on a plate reader (Victor^2, Wallac).

2.5.5 Chromium release assay

On designated work stations for radioactive use, chromium release assays were carried out in conjunction with ELISA as confirmation of T-cell killing. 24 hrs after fibroblasts had

been electroporated, they were labelled with ^{51}Cr Chromium (sodium chromate in PBS). Approximately 5x10^5 fibroblasts were first washed once with 10 ml RPMI-1640 with centrifugation at 1600 rpm for 5 minutes. To the pellet of cells, 20 µl of ^{51}Cr was added and then placed in an incubator for 90 minutes at 37°C with gentle shaking to mix every 15 minutes. Meanwhile, during this incubation time, the appropriate T-cells were prepared by resuspending them at two concentrations of 2.5 x 10^5/ml and 1.25 x 10^5/ml. The labelled fibroblasts were washed twice with RPMI-1640 containing 10% FCS and then resuspended at 2.5 x 10^4/ml. One hundred microlitres of this cell suspension was pipetted into each well (5000 cells/well) in triplicate in a V-bottomed 96-well assay plate. One hundred microlitre of the previously prepared T-cells were dispensed into the plated fibroblasts (2.5 x 10^4/well and 1.25 x 10^4/well). For spontaneous (background) and maximum release, targets were plated alone with 100 µl media and with 100 µl of 1% SDS respectively (in place of T-cells). Additional controls also include, using peptide-loaded chromium-labelled LCLs. Here chromium labelling also includes addition of the appropriate peptide while the remaining method stayed the same. The plate was centrifuged for 3 minutes at 1000 rpm and then incubated for 6 hrs at 37°C. A volume of 100 µl of the supernatant was harvested from each well and transferred into LP2 tubes, racked in an empty 96-well plate. The ^{51}Cr Chromium release was counted using a gamma counter.

2.5.6 CD107a staining

CD107a staining was conducted simultaneously with the ELISA (section 2.5.4). Some electroporated fibroblasts from the ELISA assay were retained for this experiment. This assay was used to measure the level of CD107a on the surface of T-cells. CD107a is a functional marker of CTL degranulation following stimulation (Betts and Koup 2004). CTLs degranulate after recognition of a peptide-MHC class I complex on a target cell. It comprises several steps:

(a) Peptide loading of LCL control: Approximately 2 x 10^6 of the appropriate HLA-matching LCLs were washed twice with serum-free RPMI-1640 and resuspended in 200 µl of the same media. The cell suspension was split into two 15 ml tubes, and 100 µg/ml of the appropriate peptide was added to one tube and an equivalent volume of DMSO to the other. Both were incubated at 37°C for 90 minutes with occasional shaking (every 15 minutes). During the 90 minute incubation time, T-cells and fibroblasts (targets) electroporated with the appropriate mRNA were prepared (see below). At the end of the incubation, LCLs were washed with 10% FCS RPMI-1640 and resuspended at 5 x 10^5 – 1 x 10^6/ml (1:1 ratio with T-cells).

(b) Preparation of T-cells: T-cells were harvested and washed twice with 10% FCS RPMI-1640, then resuspended at 5 x 10^5 – 1 x 10^6/ml. Fifty microlitres of this was aliquoted to the required number of wells (in triplicates) to a 96-well V-bottomed plate (2.5 x 10^4 – 5 x 10^4 T-cells per well). Unused wells were filled with 200 µl of media.

(c) Electroporation of fibroblasts: About 1 x 10^6 fibroblasts were electroporated as described in section 2.2.2. The cells were finally suspended at 5 x 10^5 – 1 x 10^6/ml (1:1 ratio with the T-cells). Fifty microlitres were aliquoted from this to each well.

(d) Setting up the plates: Fifty microlitres of T-cells were aliquoted to the required number of wells (in triplicates). Five microlitres of CD107a-FITC antibody (BD Pharmingen) was added to each well containing the T-cells (with the exception of the compensation wells). This was followed by the addition of 50 µl LCLs, and fibroblasts to each of the appropriate wells. Five microlitres of monensin was added to each well and mixed. A summary of the plate plan is illustrated in Table 2.5 below. Finally, the plate was centrifuged for 3 minutes at 1000 rpm and incubated at 37°C for 12 hrs.

(e) Flow cytometry: After the 12 hr incubation, the cells were washed twice with 150 µl of FACS buffer (1% BSA, 0.02% sodium azide in PBS) and then finally resuspended in 50 µl of the same solution. Five microlitres of CD3 FITC (for the compensation control) or CD3 PE (for the experimental samples) was added and plate left to incubate at room temperature for 30 minutes. The cells were washed twice again with FACS buffer, then finally resuspended in 600 µl PBS. The samples were analysed via flow cytometry.

The plate plan illustrating the different additions is summarized in Table 2.5 below.

	T cells only			T cells + Targets					
	Controls and compensation			Experimental					
Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Purpose	**Background**	**PE (FL2)**	**FITC (FL1)**	**CD107a Background**	**Mock**	**LMP2a**	**LMP2a + E1A**	**LCL**	**LCL +PEPTIDE**
Surface Stain									
CD107a-FITC (µl)	0	0	0	5	5	5	5	5	5
Monensin (µl)	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
12 hrs Incubation at 37°C									
CD3-PE (µl)	0	5	0	5	5	5	5	5	5
CD3-FITC (µl)	0	0	5	0	0	0	0	0	0

CHAPTER 3

INTERACTION OF AdE1A WITH THE IMMUNOPROTEASOME COMPONENT MECL-1

3.1 Introduction

Adenovirus early region 1A (AdE1A) functions primarily through a complex series of protein-protein interactions targeting various cellular regulatory proteins involved in cell cycle, DNA synthesis, differentiation, apoptosis, transcription and antigen processing (Berk 2005; Gallimore and Turnell 2001, Mymryk 1996). AdE1A exists in various isoforms with mRNAs 12S and 13S being the most abundant. These encode 243 and 289 amino acid 12S and 13S AdE1A proteins respectively in Ad2/Ad5. Both proteins are identical with the exception of a 46 amino acid conserved region 3 (CR3) region that is present in 13SAdE1A. Comparison of AdE1A amino acid sequences from the different adenovirus serotypes of human and simian origin have revealed the presence of 4 highly conserved regions known as CR1, CR2, CR3 and CR4 (Kimelman *et al.,* 1985; Avvakumov *et al.,* 2002, 2004). These encompass amino acid 42-72, 113-137, 144-191 and 240-288 respectively in the Ad513SE1A; and there is also an N-terminal region, amino acids 1-39, which is less conserved but equally important in mediating interactions. The conserved regions and the N-terminal region mediate nearly all of the interactions of AdE1A with cellular binding partners. Cellular protein targets bind to one or more of these regions, hindering their normal activity and creating a suitable environment for viral replication.

Immunoproteasomes are a subtype of 20S proteasomes whose expression can be induced following IFNγ exposure of cells. They are more efficient than 20S proteasomes at presenting to $CD8^+$ T-cells peptides of viral origin. Past studies of the relationship of AdE1A and immunoproteasomes have shown that there is a down-regulation of immunosubunit expression in Ad12 transformed rat cells compared to those transformed by Ad5. (Vertegaal *et al.,* 2003; Rotem-Yedudar *et al.,* 1996). A further study revealed down-regulation of LMP2 transcription by adenovirus AdE1A (Chatterjee-Kishore *et al.,* 2000). Research into AdE1A inhibition of immunosubunits hinted at disruption of the JAK/STAT pathway, specifically the repression of STAT1 activity, however these studies did not examine whether if AdE1A also targets immunosubunits through direct binding

(Look *et al.,* 1998). In this chapter, we consider the relationship of AdE1A and immunosubunit interaction in detail, to investigate if AdE1A directly binds to the immunoproteasome subunits LMP2, LMP7 and MECL1, and if that is the case, what regions on AdE1A are involved in this interaction. We also examine AdE1A binding to the catalytic β1, β2 and β5 subunits of the 20S proteasome.

3.2 Results

3.2.1 Co-immunoprecipitation of AdE1A with the immunoproteasome

To examine AdE1A binding to the immunoproteasome, a co-immunoprecipitation (IP) experiment (as in section 2.5.3) was carried out using H1299 cells that had been treated with IFNγ for 48 hrs prior to Ad5 infection for a further 24 hrs. Protein complexes were immunoprecipitated with LMP7 antibody. Immunoprecipitates were run on urea gels in the absence of SDS then blotted for AdE1A. The results show that AdE1A co-immunoprecipitated with the immunoproteasome and the proteasome (Figure 3.1) as has been shown previously (Grand *et al.,* 1999; Turnell *et al.,* 2000).

3.2.2 *In vitro* binding of AdE1A to MECL-1

In vitro binding of AdE1A to the individual immunoproteasome subunits was also tested. The binding capacity of *in vitro* translated [^{35}S]-labelled LMP2, LMP7 and MECL-1 for GST tagged 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A was analysed by GST pull-down assay (as in section 2.4.8). Twenty microgram of GST-fusion protein was incubated with 10-20 µl of the *in vitro* translated [^{35}S]-labelled immunosubunits. GST- pulldown was carried out using glutathione-sepharose beads; and the bound proteins were eluted with 25 mM glutathione solution; the resulting eluate was resolved by SDS-PAGE. The relative binding capacity of each immunosubunit was visualised by autoradiography (section 2.4.7). The results show that [^{35}S]-labelled MECL-1 but not [^{35}S]-labelled LMP2 or [^{35}S]-

labelled LMP7 bound to GST -12SAd5E1A, GST-13SAd5E1A and GST-13SAd12E1A (Figure 3.2).

3.2.2.1 Sites of interaction on Ad5E1A for MECL-1

In light of the above observation that showed AdE1A binding specifically to MECL-1, we next investigated what regions of Ad5E1A are involved in this interaction. The binding of *in vitro* translated [^{35}S]-labelled MECL-1 to GST-tagged fragments from across the length of Ad5E1A was analysed by GST-pulldown assay. The GST fragments were amino acids 1-40, 41-80, 81-140, 141-185, 186-289 (which encompass the N-terminal region, CR1, CR2, CR3 and CR4 respectively) as well as GST alone and GST-13SAd5E1A as negative and positive controls respectively. The results (Figure 3.3) indicated that [^{35}S]-labelled MECL-1 binds strongly to the N-terminal region (amino acid 1-40) and CR3 region (amino acids 141-185) of Ad5E1A. The amino acids within these two regions that have an important role in this binding were further mapped (see 3.2.2.2 and 3.2.2.3 below).

3.2.2.2 Mapping the binding sites within the Ad5E1A N-terminal region

The sites of interaction for MECL-1 in the N-terminal region of AdE1A were further defined, by assessing the role of each residue in the N-terminal region in this interaction. Twenty-two GST-12SAd5E1A proteins with a series of point mutations (Figure 3.4) across the N-terminal region (Rasti *et al.,* 2005) were incubated with *in vitro* translated [^{35}S]-labelled MECL-1 in a pulldown assay. The raw autoradiography results are shown in Figure 3.5A. These were analysed by densitometry (Figure 3.5C). The results show that mutations at mainly hydrophobic residues: L20A, L19/20A, D21A, L23A, I24A and L28A have severely disrupted binding of the N-terminal region to MECL-1 (Figure 3.5C). Whereas R2G, I5G, C6A, H7A, G8A, V10A, I11A, T12A, E14A, A16G, S18G, E25A, E26A, V27A, A29G and D30A bound to [^{35}S]-labelled MECL-1 as well as wild type 12SAd5E1A (Figure 3.5A).

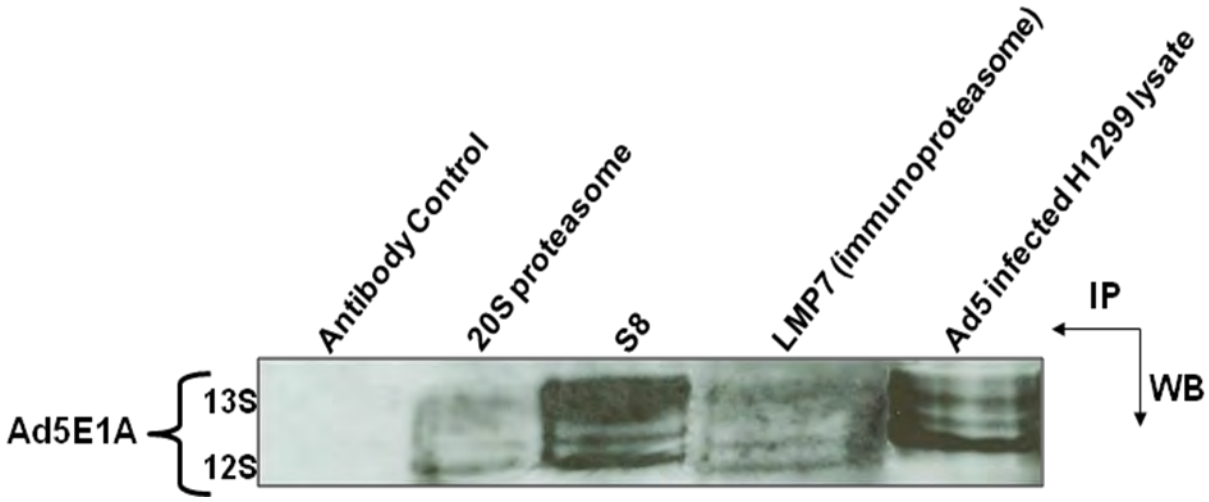


Figure 3.1 Ad5E1A binds to the immunoproteasome
H1299 cells were treated with IFNγ for 24 hrs followed by infection by Ad5 virus for a further 24 hrs. Immunoprecipitation was carried out with the antibodies shown in the figure, 20S proteasome antibody: anti-α1, 2, 3, 5, 6 and 7 (mouse), anti-S8 antibody (rabbit) and anti-LMP7 (rabbit). Immunoprecipitated proteins were run on urea gels in the absence of SDS, then blotted for Ad5E1A.

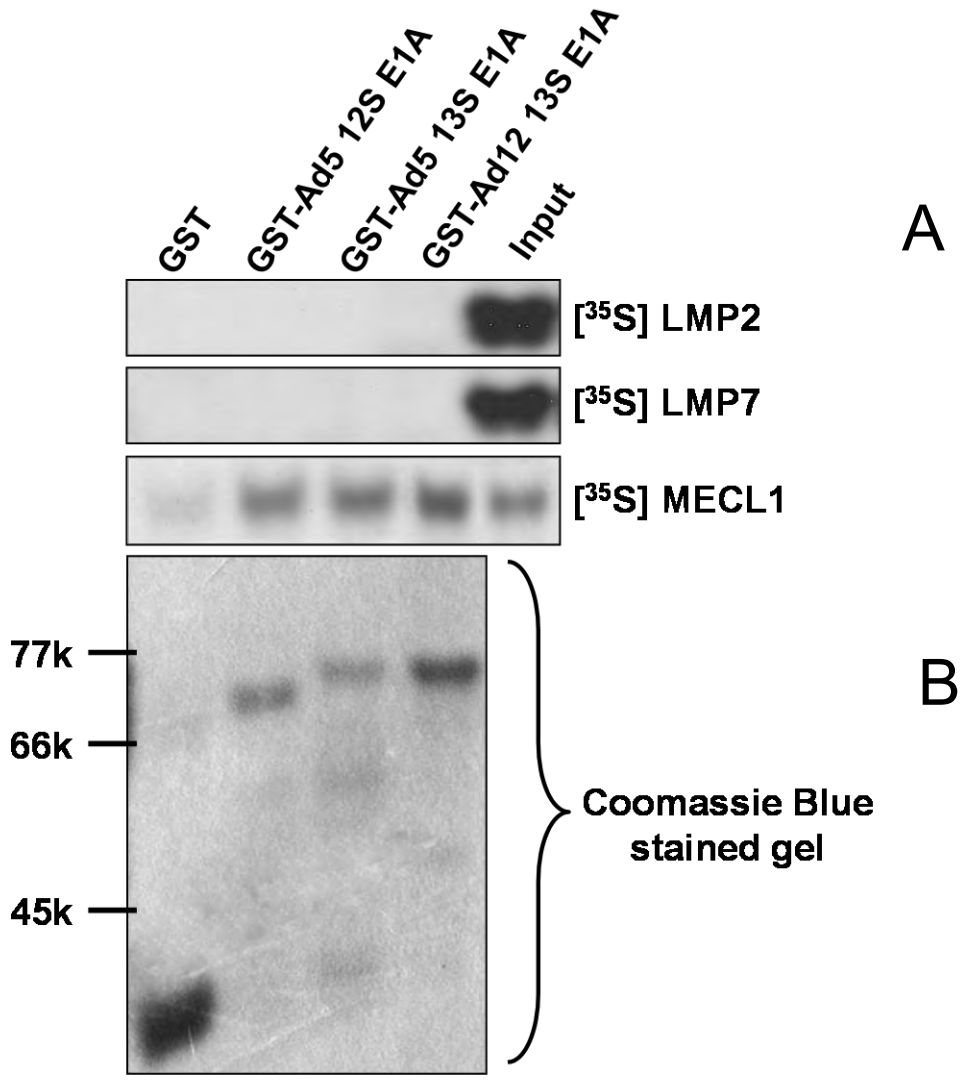


Figure 3.2 AdE1A binds to MECL-1 but not LMP2 or LMP7
Ten microlitres of [^{35}S]-labelled LMP2, LMP7 and MECL-1 were incubated with 20 µg of GST tagged 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A on ice for 2 hrs in PBS, 1 mM EDTA, 1% Triton X100. GST pull down assay was conducted as described in section 2.4.8. The eluate along with 5% of each input was run on an SDS-PAGE gel. The gel was stained with Coomassie Blue **(B)**, soaked in Amplify™ Reagent (Amersham bioscience) for 30 minutes with agitation and dried under vacuum at 80°C for 1 hr and exposed for autoradiography at -20°C **(A)**. M.W. of LMP2 = 24kDa, LMP7 = 23kDa, MECL-1 = 29kDa.

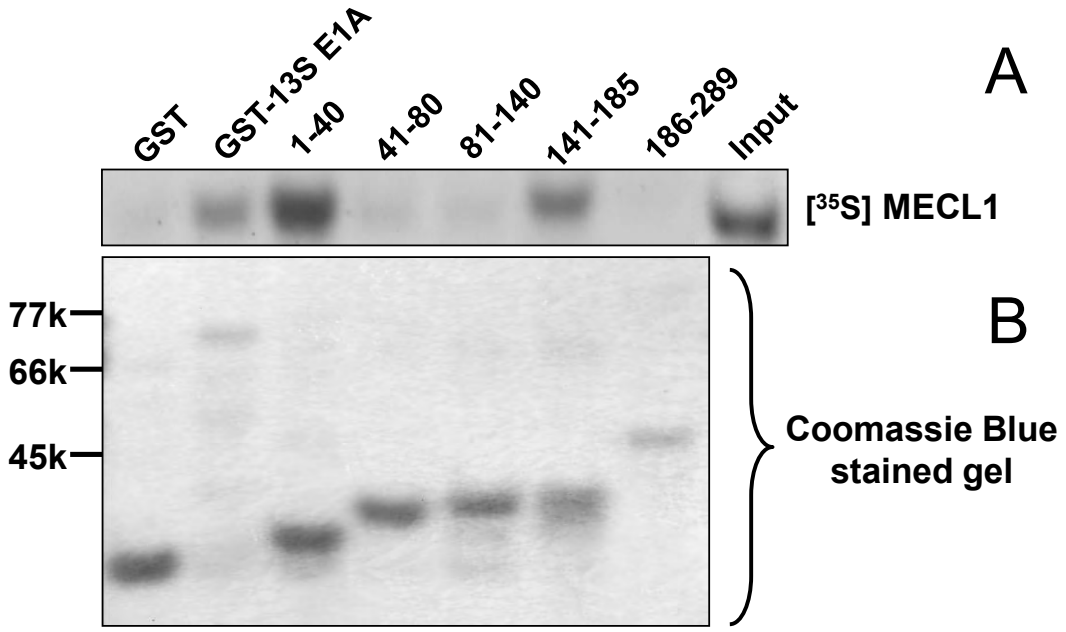


Figure 3.3 MECL-1 binds to the N-terminal and CR3 regions of Ad5E1A
Ten microlitres [^{35}S]-labelled MECL-1 was incubated with 20 µg of GST-tagged fragments from across the different regions of Ad5E1A in a pulldown assay as described in section 2.4.8. 5% of the input was run on the SDS-PAGE gel **(A)** Autoradiography results from the **(B)** Coomassie Blue stained gel.

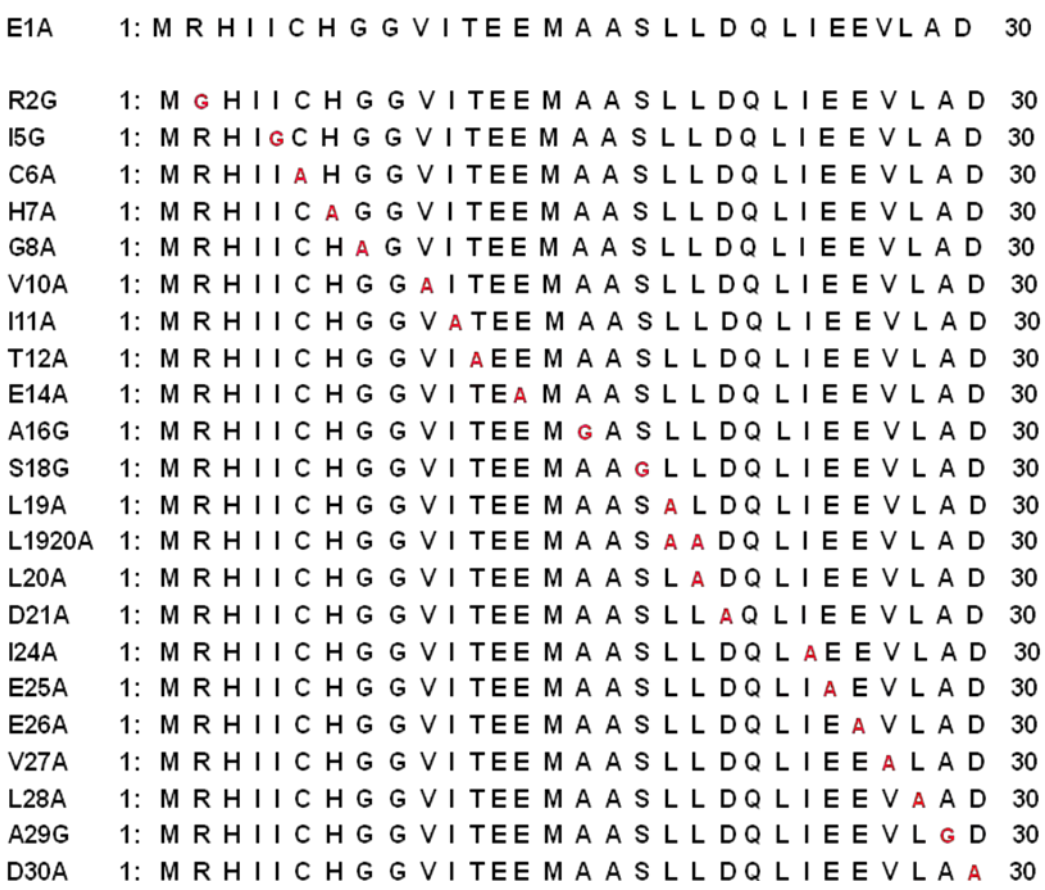
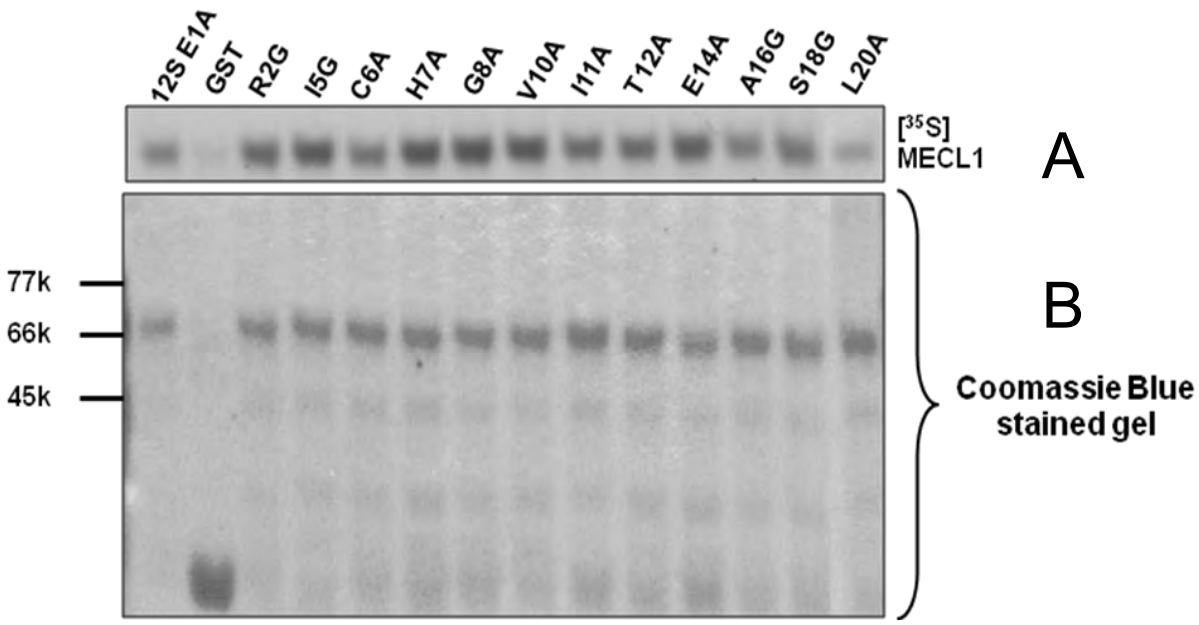
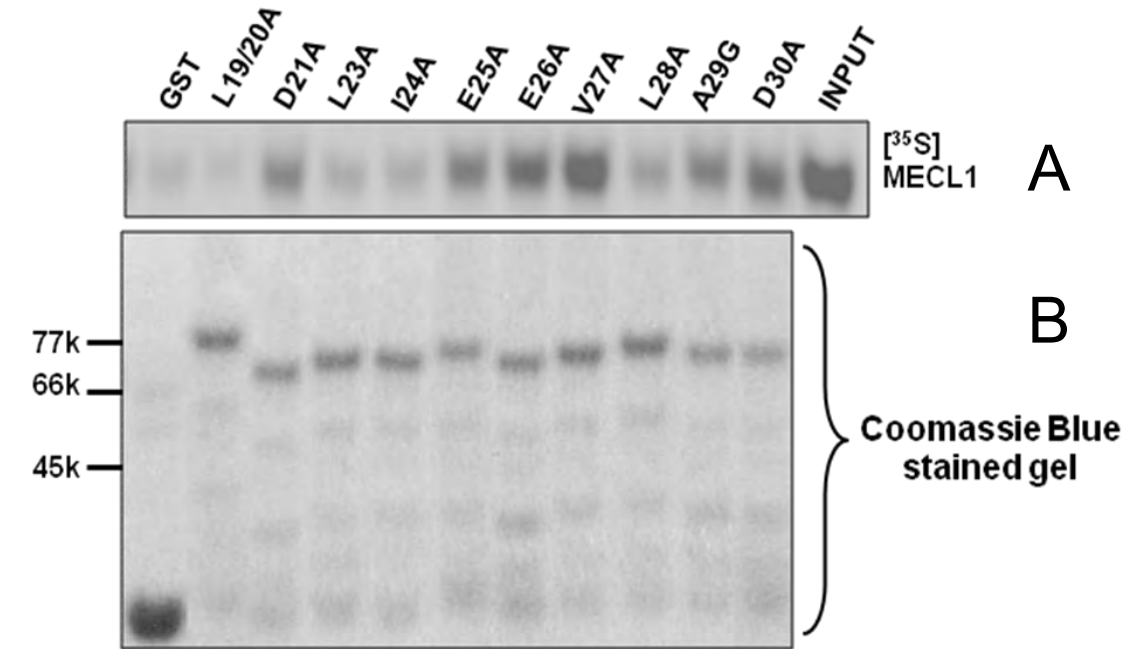


Figure 3.4 Diagram showing the positions of the point mutations at the N-terminal of Ad5 E1A used in the study.
Generated by Rasti *et al.,* (2005).


12S E1A
GST
R2G
I5G
C6A
H7A
G8A
V10A
I11A
T12A
E14A
A16G
S18G
L20A
[35S]
MECL1
A
77k
66k
45k
B
Coomassie Blue
stained gel


GST
L19/20A
D21A
L23A
I24A
E25A
E26A
V27A
L28A
A29G
D30A
INPUT
[35S]
MECL1
A
77k
66k
45k
B
Coomassie Blue
stained gel

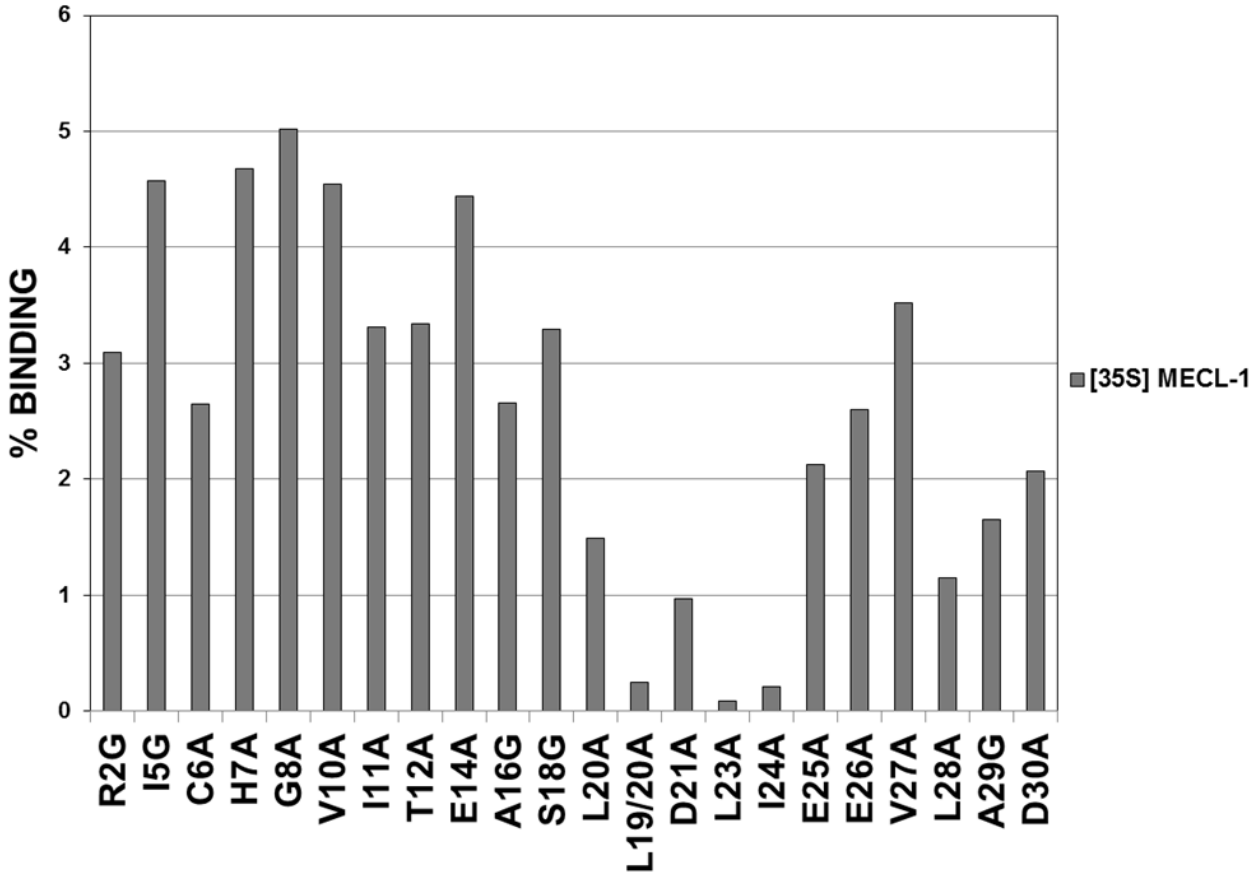


Figure 3.5 Mapping the binding site for MECL-1 in the Ad5E1A N-terminal region

Ten microlitres of [^{35}S]-labelled MECL-1 was incubated with 20 µg of GST-12SAd5E1A with a series of point mutations in the N-terminal region in a pulldown assay as briefly described in section 2.4.8. 5% of the input was also run on the SDS-PAGE gel **(A)** Autoradiography results from the **(B)** Coomassie Blue stained gel. The lower panel is a continuation of the upper panel list of mutants. **(C)** Densitometric scan of the autoradiograph results shown in (A).

3.2.2.3 Mapping the binding sites within the Ad5E1A CR3 region.

A series of deletion mutants of GST-Ad5E1A CR3 (Figure 3.6A, Rasti *et al.,* 2006) were incubated with *in vitro* translated [^{35}S]-labelled MECL-1 in pulldown assays carried out as section 2.4.8. All mutations have to varying degree affected the binding to MECL-1. Deletion mutants GST-CR3 Δ180-184 and GST-CR3 Δ188-204 had about two-fold less binding to MECL-1 than mutants GST-CR3 Δ 139-160, GST-CR3 Δ 161-168 and GST-CR3 Δ169-177 (Figure 3.7A). An additional CR3 fragment that is mutated at one of the Zn^{2+} binding residues (in order to disrupt the zinc finger structure of CR3 - see section 1.3.5.1.1) was used to determine if this motif plays any role in the interaction with MECL-1. The results reveal that this mutation slightly affected binding in comparison to *w.t.* CR3 (GST-CR3). However, this was not as evident as GST-CR3 Δ 139-160 (Figure 3.7B). In a further experiment, *w.t.* GST-AdE1A CR3 regions derived from different adenovirus serotypes (Figure 3.6B) were also used with [^{35}S]-labelled MECL-1 in a pulldown assay. The results show that CR3 regions from Ad9 and Ad12 bind about 3-fold and 2-fold more strongly (respectively) to MECL-1 in comparison to CR3s from Ad4, Ad3 and Ad40 (Figure 3.8).

3.2.3 *In vitro* binding of AdE1A to the constitutive homologues β1, β2 and β5

AdE1A binding to the 20S proteasome was examined. HCT116 lysates (in the absence of IFNγ) were incubated with GST-13SAd5E1A and GST-13SAd12E1A proteins and pulldown assays carried out were using glutathione-sepharose beads followed by elution of binding proteins with glutathione. The eluates were run on an SDS-PAGE gel and blotted with anti-20S antibody (which recognises α1, 2, 3, 5, 6 and 7 subunits). The results showed that both AdE1A proteins bound to the 20S proteasome, with GST-13SAd12E1A binding considerably more strongly than GST-13SAd5E1A (Figure 3.9). In a further experiment, the binding capacity of *in vitro* translated [^{35}S]-labelled β1, β5, and β2 (homologues of LMP2, LMP7 and MECL-1 respectively) for GST tagged 12SAd5E1A,

13SAd5E1A, and 13SAd12E1A were also assessed in a pulldown assay. In contrast to the immunoproteasome, where AdE1A showed preferential binding to MECL-1, but not LMP2 or LMP7; the pulldown assay on the homologues shows that 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A bind preferentially to β1 and β5 but very weakly to the MECL-1 homologue β2 (figure 3.10A and B). The binding to MECL-1 was much weaker compared to the other β subunits.

Results from the above pulldown assays were analysed by densitometry in order to compare the AdE1A binding affinities between the homologues. Perhaps, a more accurate comparison can be deduced from the densitometric data as the capacity of the binding is quantitated. As we observe from Figure 3.10C, 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A all bound to MECL-1 but not LMP2 and LMP7. The trend observed from comparing the binding of AdE1A to the three 20S β subunits was rather different. All AdE1A proteins bound preferentially to β1 and β5, but weakly to β2, with the exception of 13SAd12E1A where it bound most strongly to β5, while binding to β1 and β2 equally but at a lower capacity than that of β5. 12SAd5E1A and 13SAd5E1A interacted more strongly to MECL-1 than to the three β subunits. However 13SAd12E1A binds equally to MECL-1 and β5. Similarly, comparing binding to MECL-1 and its homologue β2, there is approximately a 3 fold reduction in binding of the 13SAd12E1A and 6 fold reduction of 12SAd5E1A with β2 compared to MECL1. The reduction for 13SAd5E1A is very marked, with approximately 8 fold more protein bound to MECL1 than to β2.

3.2.3.1 Sites of interaction on Ad5E1A for β1 and β5

In the light of the above findings that revealed AdE1A binding to β1 and β5, the sites of interaction on Ad5E1A were investigated to compare binding to the immunosubunit MECL-1. *In vitro* translated [^{35}S]-labelled β1 and β5 were incubated with GST-tagged fragments from across the length of Ad5E1A in a pulldown assay (as above). As with MECL-1, the GST fragments were 1-40, 41-80, 81-140, 141-185, 186-289 as well as GST alone and GST-13SAd5E1A as negative and positive controls, respectively. Consistent

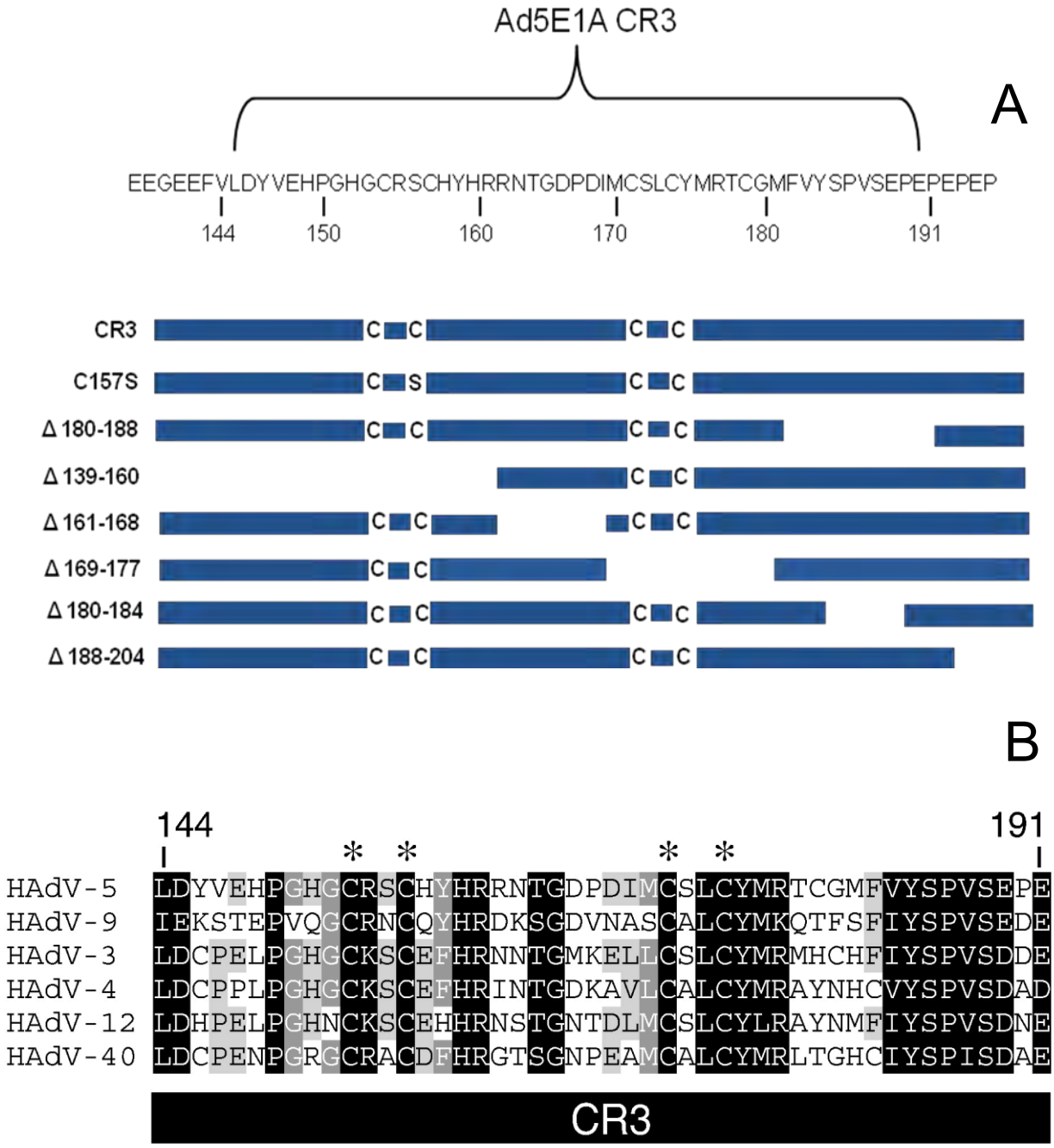


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Figure 3.6 Schematic representation of the Ad5E1A CR3 deletion mutants and AdE1A CR3 from different serotypes used in the study
(A) Ad5 CR3 region as defined by Avvakumov *et al.,* 2002. Mutants as described by Rasti *et al.,* (2005). The cysteine residues constitute the Zn^{2+} binding site. **(B)** GST-AdE1A CR3 fragments from different adenovirus serotypes used in this study, and their sequence alignments. Numbers represent amino acid positions in Ad5E1A (HAdV-5). Adenovirus serotype is stated on the left. Darker shading indicate greater conservation. Asterisks denote cysteine residues that co-ordinate with zinc.

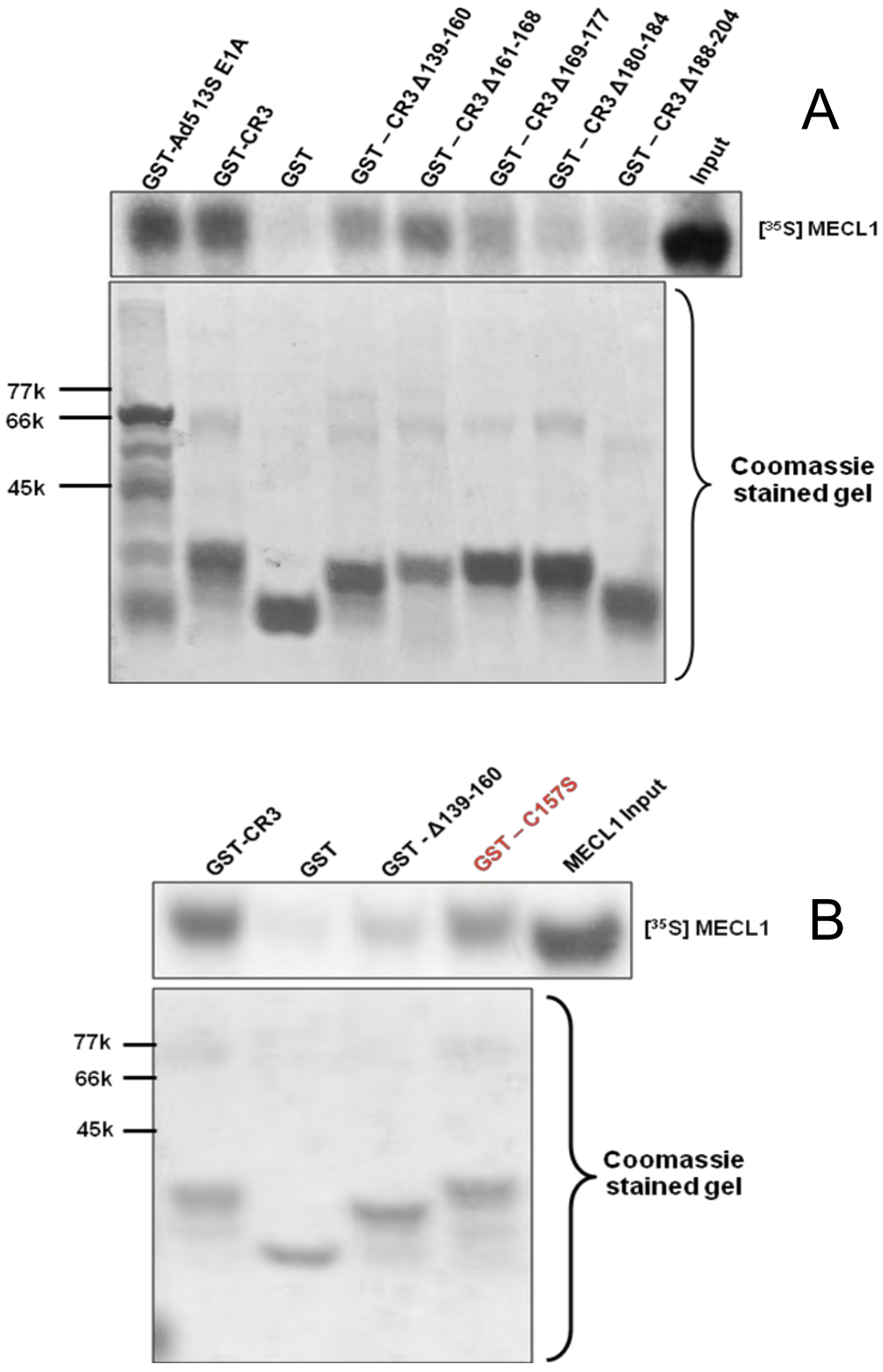


Figure 3.7 Mapping the binding site for MECL-1 in the Ad5E1A CR3 region.
Ten microlitres of [^{35}S]-labelled MECL-1 was incubated with **(A)** Twenty microgram of GST-Ad5E1A with a series of deletion mutations in the CR3 region or **(B)** a mutant in the zinc finger region within CR3, in a GST-pulldown assay as described in section 2.4.8. 5% of the input was also run on the SDS-PAGE gel. The upper panel of each figure represents the autoradiography results, and the lower panel shows the Coomassie stained gel prior to being dried under vacuum.

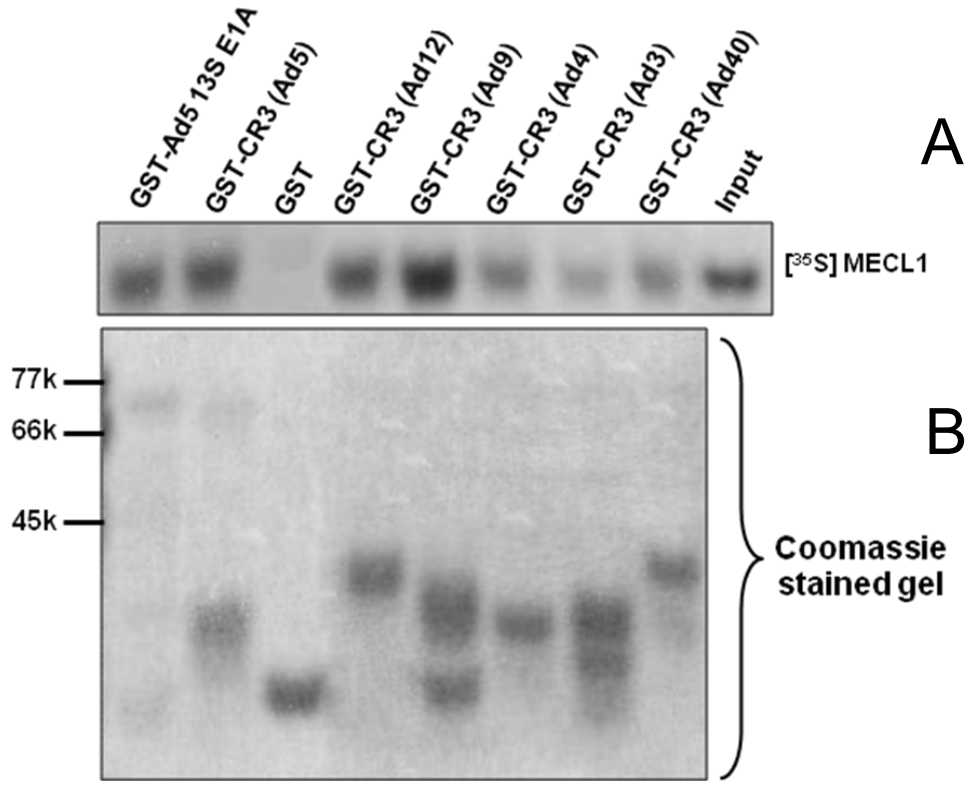


Figure 3.8 MECL-1 interaction with CR3 regions from different adenovirus serotypes.

Ten microlitres of [^{35}S]-labelled MECL-1 was incubated with 20 µg of GST-AdE1A CR3 from different adenovirus serotypes in a pulldown assay as described in section 2.4.8. 5% of the MECL-1 input was also run on the SDS-PAGE gel. **(A)** represents the autoradiography results, and **(B)** shows the Coomassie stained gel prior to being dried under vacuum.

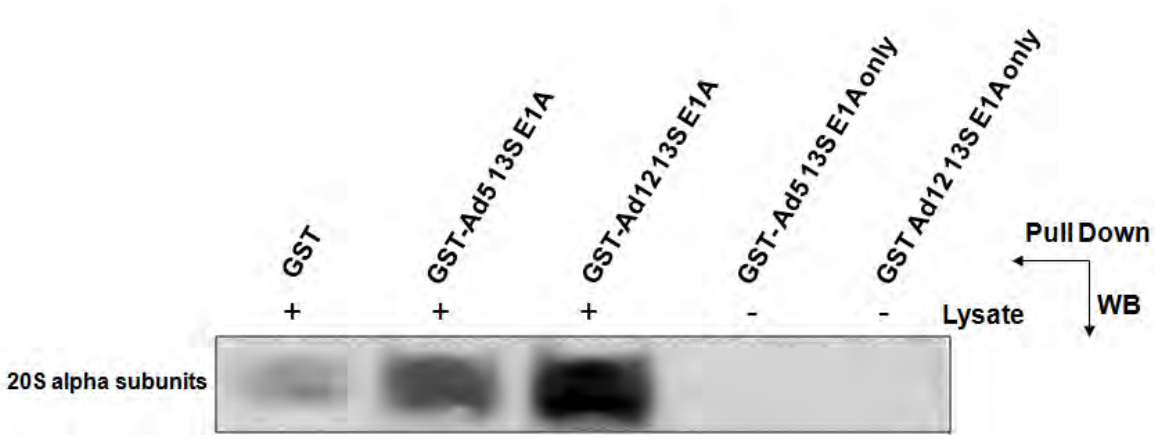


Figure 3.9 Binding of AdE1A to the 20S proteasome.
Twenty microgram of GST tagged 13S Ad5E1A and13S Ad12E1A were added to HCT116 lysates and a GST pull down carried out as described in section 2.4.8. The final eluate was analysed by Western Blotting for the presence of 20S proteasome α subunits. M.W. of 20S alpha units = 25-30kDa.

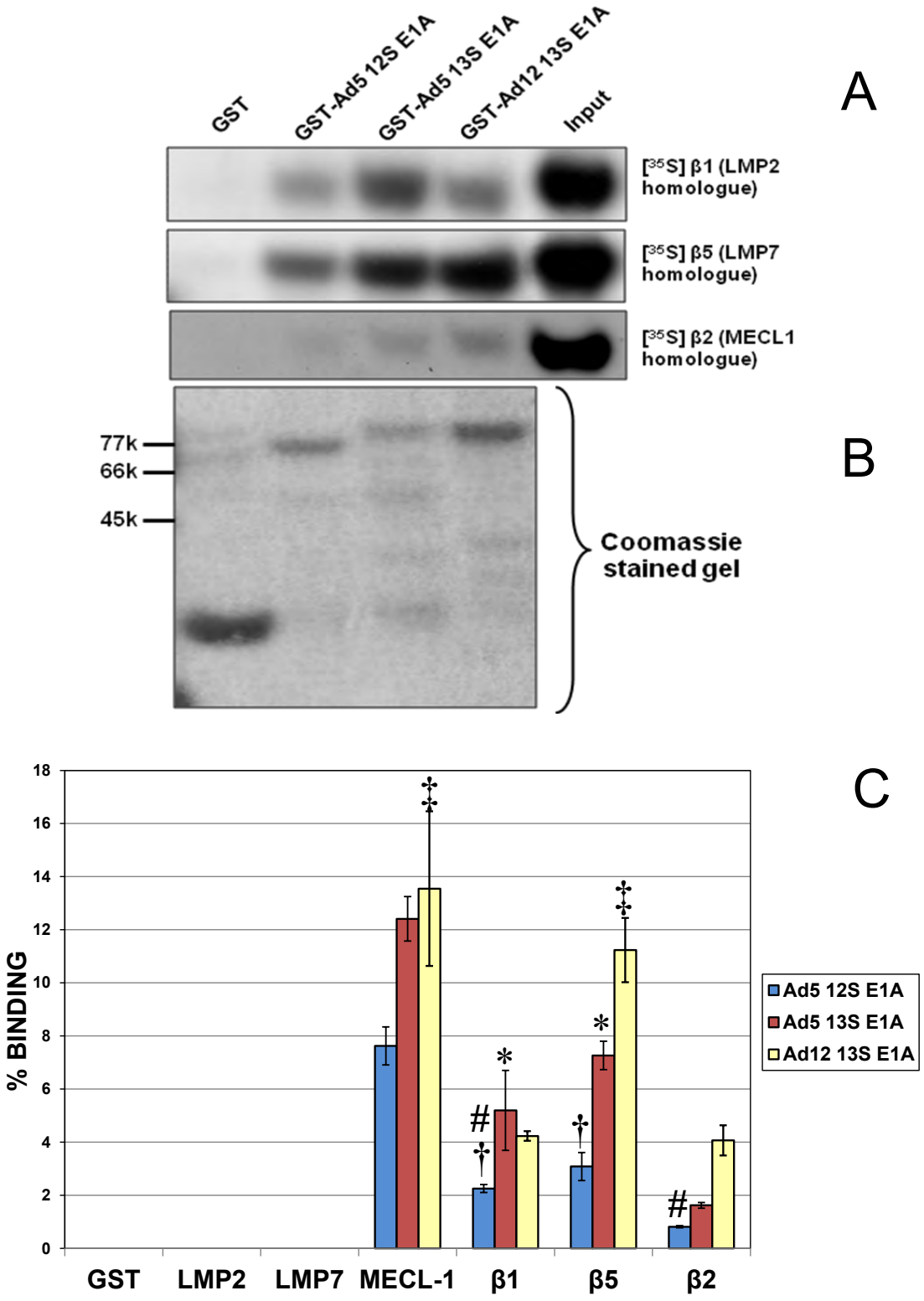


Figure 3.10 AdE1A binds to β1 and β5, but not β2 components of the 20S proteasome.

Ten microlitres of [^{35}S]-labelled β1, β2 and β5 were incubated with 20 µg of GST tagged 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A in a pulldown assay as described in section 2.4.8. 5% of each input was also run on the SDS-PAGE gel. **(A)** represents the autoradiography results, and **(B)** shows the Coomassie stained gel prior to being dried under vacuum. **(C)** Densitometry results comparing the different binding capacities of AdE1A to immunosubunit and the constitutive 20S proteasome catalytic subunits. Data are means +/- SEM from three repeats. ‡ = † = * = P > 0.05, # = P <0.05

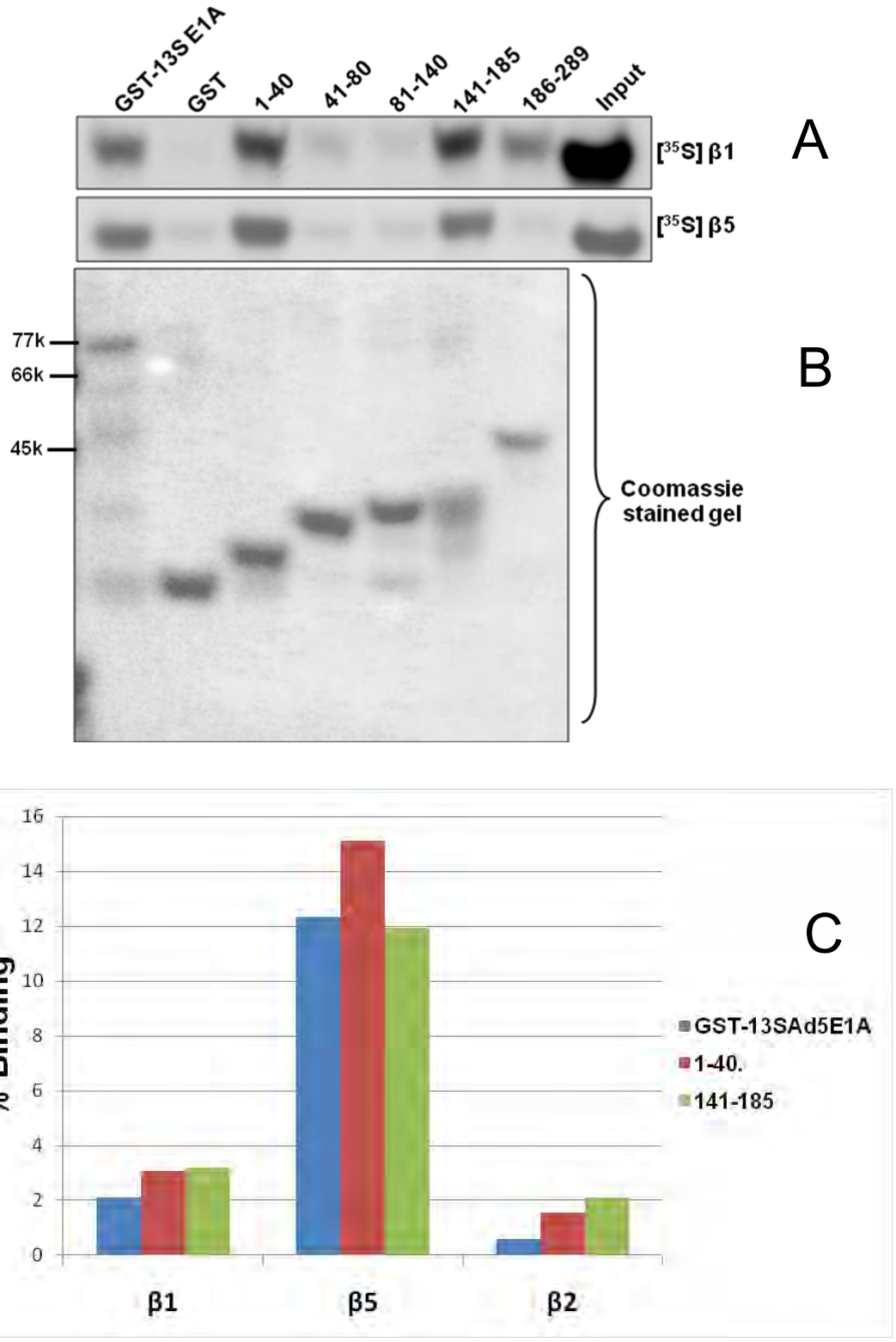


Figure 3.11 20S proteasome components β1 and β5 bind to the N-terminal and CR3 regions of Ad5E1A

Ten microlitres [^{35}S]-labelled β1 and β5 was incubated with 20 µg of GST-tagged fragments from across the different regions of AdE1A in a pulldown assay as described in section 2.4.8. 5% of the input was also run on the SDS-PAGE gel. **A)** represents the autoradiography results, and **(B)** shows the Coomassie stained gel prior to being dried under vacuum. **(C)** Densitometry results comparing the different binding capacities of AdE1A N-terminal and CR3 fragments to constitutive 20S proteasome catalytic subunits.

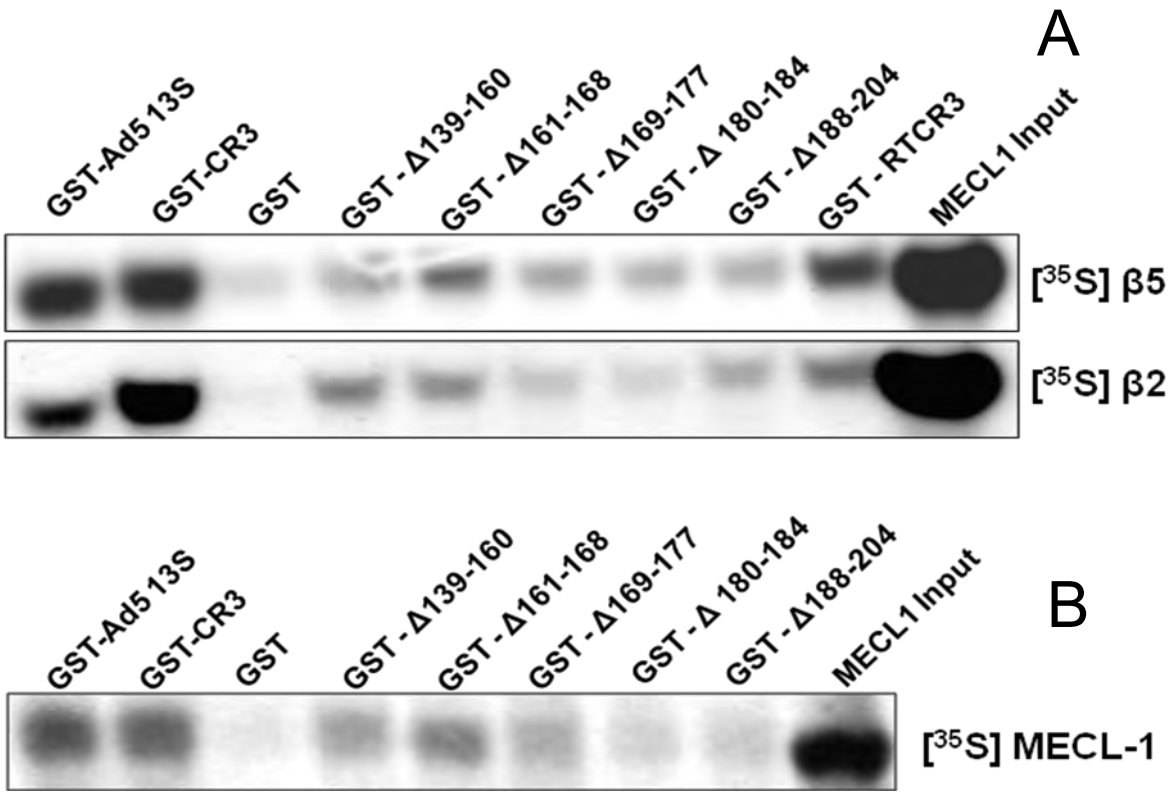


Figure 3.12 Mapping the binding site for β2 and β5 in the Ad5E1A CR3 region.
(A) Ten microlitres of [^{35}S]-labelled β2 and β5 was incubated with 20 µg of GST-Ad5CR3 carrying a series of deletions in a pulldown assay as briefly described in section 2.4.8. 5% of the input was also run on the SDS-PAGE gel. (B) pulldown assay using the same mutants with [^{35}S]-MECL-1.

with MECL-1, both β1 and β5 bound strongly to the N-terminal and CR3 regions of Ad5E1A (Figure 3.11).

3.2.3.2 Mapping the binding sites for β1 and β5 within the Ad5E1A CR3 region

Amino acid residues critical for the interaction of AdE1A CR3 with β2 and β5 were also mapped. β2 was included in order to compare its binding sites to those of its IFNγ inducible homologue MECL-1. A series of deletion mutants of GST-Ad5E1A CR3 (Figure 3.6) were used with [^{35}S]-labelled β1 and β5 in a pulldown assay. In comparison to *w.t.* Ad5E1A CR3, there was a varying degree of reduced binding to β2 and β5 in all of the mutants; with GST-Δ169-177 and GST-Δ180-184 mutants binding the weakest to β2, and GST-Δ 169-177, GST-Δ 180-184 and GST-Δ188-204 mutants being least binding to β5 (Figure 3.12).

3.3 Discussion

Studies to date onto the relationship of AdE1A with immunoproteasomes mainly focused on immunosubunit expression in adenovirus transformed cells (Vertegaal *et al.*, 2003; Rotem-Yedudar et al., 1996) and to a limited level, in AdE1A transfected cells (Chatterjee-Kishore *et al.,* 2000). Direct interaction of AdE1A to components of the 26S proteasome was rather discovered, for instance, binding to the ATPase (S4 and S8) and non-ATPase (S2) components of the 19S complex (Turnell *et al.,* 2000; Zhang *et al.,* 2004) or binding to several α subunits of the 20S proteasome (Rasti *et al.,* 2006). However, there have been no studies to date exploring the binding of AdE1A to the immunoproteasome, hence for the first time, this topic was addressed in this chapter.

Firstly, the binding of AdE1A to the immunoproteasome was examined. In a co-precipitation assay using antibodies directed at the immunosubunit LMP7, direct binding of AdE1A to the immunoproteasome was suggested (Figure 3.1). Immunoproteasomes are known to be efficient at generating peptides of viral origins and hence previous

research has shown that they are also a target for other viral proteins such as HIV-1 Gag p24 (Steers *et al.,* 2009), HIV Tat (Areste and Blackbourn 2006), Hepatitis C virus non-structural protein (NS3) (Khu *et al.,* 2004), EBV lytic cycle (De Leo *et al.,* 2010), human T-cell leukemia virus type 1 (HTLV-1) Tax protein (Hemelaar *et al.,* 2001) and now also adenovirus AdE1A.

Given that AdE1A binds to the immunoproteasome, whether this interaction was specific to one or more of the immunosubunits was further investigated. In a GST pulldown assay utilizing [^{35}S]-labelled LMP2, LMP7 and MECL-1 and GST tagged 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A, it was observed that AdE1A in all cases bound preferentially to MECL-1 rather than to LMP2 and LMP7 (Figure 3.2). The MECL-1 gene is found separately from LMP2 and LMP7 in a locus on human chromosome 16q22.1 (Larsen *et al.,* 1993). The reason for AdE1A specifically targeting MECL-1 is not clear, although, one study has shown that it is also targeted by HIV-1 tat protein that binds directly to MECL-1 and LMP7 leading to the inhibition of immunoproteasomal activity (Apcher *et al.,* 2003). Other studies, however, have shown that other viral proteins target MECL-1 expression rather than directly binding to it, for instance, HIV-1 p24 which inhibits MECL-1 (as well as LMP7) expression in dendritic cell (Steers *et al.,* 2009). Additionally, it was also targeted by Epstein-Barr virus where its expression was also repressed during the lytic cycle (De Leo *et al.,* 2010).

In MECL-1-deficient mice, there was a slight reduction in the incorporation of LMP2 into immunoproteasomes, (Basler *et al.,* 2006), as well as abrogating trypsin-like activity (Salzmann *et al.,* 1999). During immunoproteasome assembly, LMP2 and MECL-1 were incorporated first, and this incorporation was mutually dependent (Groettrup *et al.,* 1997). Together LMP2 and MECL-1 form a pre-proteasome that favours incorporation of LMP7, which, in turn, is required for efficient maturation to immunoproteasomes (Griffin *et al.,* 1998). This cooperative model (proposed by Griffin *et al.,* 1998) is thought to ensure the generation of homogeneous immunoproteasomes containing all the inducible subunits. In view of these observations, it can be hypothesized that AdE1A targeting MECL-1 might

interfere with immunoproteasome composition and hence affect the overall proteasomal activity leading to a modulated array of peptides presented in a manner that avoids adenovirus detection. Since incorporation of MECL-1 is inter-dependent with LMP2, the binding of AdE1A to MECL-1 may hinder the incorporation of not only MECL-1, but LMP2 as well. As a result, no pre-proteasomes favourable to LMP7 incorporation are generated. Hence, a hypothesis can be drawn that suggests AdE1A binding to MECL-1, may be sufficient to create a domino effect that eventually inhibits immunoproteasome formation. This, in addition to the repression of immunosubunit expression at transcriptional level (see section 4.2.1), might have a devastating effect in the functioning of the immunoproteasome. However, in order to test this hypothesis, further experiments need to be conducted into the effect of AdE1A on the proposed assembly of immunoproteasomes. This can be done via metabolic pulse-chase labelling of proteasomes and immunoproteasomes. These can then by analysed by two dimensional gels after immunoprecipitation (Griffin *et al.,* 1998).

The regions within AdE1A that are involved in MECl-1 binding were investigated. The results of a pulldown assay using [^{35}S]-labelled MECL-1 and GST-tagged fragments from across the different regions of AdE1A have shown that MECL-1 binds to the N-terminal and CR3 regions of AdE1A (Figure 3.3). These regions play a very important role in mediating the interaction of AdE1A with many host proteins (Gallimore and Turnell 2001). The N-terminal region also interacts with other proteins involved in the antigen processing machinery such as STAT1 (Look *et al.,* 1998), S4 and S8 (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004) as well as other crucial proteins involved in transcription, such as CBP/p300 and TBP (Berk 2005). The CR3 region is involved in binding several α subunits of the 20S proteasome (Rasti *et al.,* 2006) among others. These two regions are already involved in the targeting of proteasomes and other components of the antigen processing machinery, in addition to the immunoproteasome that is now shown in the present study. These binding sites were mapped more closely to determine the role of particular residues within those two regions in the interaction with MECL-1. At the N-

terminal region, the data have shown that the mutations L20A, D21A, L23A, I24A, L28A and L19/20A have reduced binding capacities, with the double mutant L19/20A most impaired (Figure 3.5). A study by Rasti *et al.,* (2005) used the same set of N-terminal region mutants in order to assess their binding to a number of cellular proteins namely CBP/p300, P/CAF, hGCN5, S4, S8, TBP and Ran. They found different binding patterns across the different proteins, with the double-mutant L19/20A completely eliminating the interaction with all of the seven proteins (Rasti *et al.,* 2005). Consistent with data from Rasti *et al.,* study, the same mutations that affected the binding of MECL-1 to the N-terminal region, have also significantly disrupted the binding to all of cellular proteins. All five mutations (L20A, L23A, I24A, L28A and L19/20A) have either eliminated or showed a maximum of 25% binding to each of CBP/p300, P/CAF, hGCN5, S4 and S8, with the exception of TBP and Ran where the L28A mutation showed a binding of 75-100% and 50-75% respectively (Rasti *et al.,* 2005). Other mutations had different binding patterns, with TBP and Ran showing closest similarity to MECL-1. Past mutagenesis studies have shown that R2, L20 (Ad5) and L19 (Ad12) are absolutely conserved between the different serotypes (Lipinski *et al.,* 1999; Wang *et al.,* 1993; Avvakumov *et al.,* 2004), highlighting their functional importance. Other mutations that affected binding with the seven proteins were C6A and I11A (Rasti *et al.,* 2005), however these did not show any significant decrease in binding capacity with MECL-1. Hence, we can deduce from the above data that MECL-1 may share the same binding sites at the N-terminal region as the seven proteins examined by Rasti *et al.,* 2005. However, *in vitro* GST pulldown assays may not necessarily reflect the situation in an *in vivo* setting, so it would be ideal to undertake the experiment in cells that express the mutant AdE1As and MECL-1.

The binding sites within the CR3 region were also mapped. GST pulldown using [^{35}S]-labelled MECL-1 and GST-Ad5E1A CR3 with a series of deletion mutations (Figure 3.6), namely GST-CR3 Δ139-160, GST-CR3 Δ161-168, GST-CR3 Δ169-177, GST-CR3 Δ180-184 and GST-CR3 Δ188-204 was undertaken (Figure 3.7A). It was found that all of the deletions have, to various extents, impacted on the binding capacity of CR3, with the

weakest binding seen with deletions Δ180-184 and Δ188-204, which may suggest an MECL-1 binding site around residues 180-204. The other deleted residues (Δ139-160, Δ161-168 and Δ169-177) may play a role in stabilising the interaction of MECL-1 with the region 180-204, or may be (more likely) required for the stabilisation of the conformation of the whole region. Further studies with a series of point mutations across the CR3 region might clarify or narrow down the binding regions of the AdE1A CR3 region. Research by Rasti *et al.,* (2006) used the same deletion mutants to study binding to the ATPase component S8 and the 20S α proteasome subunits. Their results have shown that the S8 binding site extends from residues 169-188 and the 20S proteasome binding site extends from residues 161-177. This suggests that MECL-1 binds to a different region of CR3 from S8 and 20S proteasome. The same group have also found that mutations of the zinc finger motif within the CR3 had no effect on S8 binding, whereas it disrupted binding to the 20S proteasome. In this study, we observed that mutation of the zinc finger region did slightly affect (but did not eliminate) binding to MECL-1, indicating that it may play a role in this interaction (Figure 3.7B). Similarly, MECL-1 bound, with different affinities, to CR3 from different serotypes of adenovirus, signifying a conserved function of AdE1A (Figure 3.8). This was also consistent with data on S8 and 20S proteasomes (Rasti *et al.,* 2006). Sequence comparison of AdE1A CR3 from the different serotypes is illustrated in Figure 3.6B. Residues 180-204 that was shown to affect binding of Ad5E1A CR3 to MECL-1 covers the region VYSPVSE (Ad5) which is conserved in all of the different serotypes.

The immunosubunits LMP2, LMP7 and MECL-1 replace the constitutive subunits β1, β5 and β2 respectively after exposure of cells to IFNγ (Hisamatsu *et al.,* 1996). So it would be of interest to investigate if AdE1A also binds to catalytic components of the 20S proteasome in addition to the immunoproteasome, which has not been addressed so far by previous research. Past studies have shown binding of AdE1A to components of the 20S and 26S proteasome (Rasti *et al.,* 2005, 2006, Turnell *et al.,* 2000, Zhang *et al.,* 2004). Preliminary data using GST tagged 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A with IFNγ untreated HCT116 lysates in a GST pulldown assay, and Western blotting the

eluates for 20S proteasome alpha subunits, has shown binding of AdE1A to the 20S proteasome (Figure 3.9). Nevertheless, from this experiment, it cannot be determined if this interaction is through the 19S components or the α and β subunits of the 20S proteasome. To expand on this, experiments assessing the binding of AdE1A to the individual β1, β2 and β5 were conducted. In another GST pull down assay, [^{35}S]-labelled β1, β2 and β5 were used with GST tagged 12SAd5E1A, 13SAd5E1A, and 13SAd12E1A. It was shown that, in contrast to the immunosubunits where AdE1A have shown preferential binding to MECL-1, here the AdE1A has bound strongly to β5 and β1, (LMP7 and LMP2 homologues, respectively), whereas it bound only weakly to β2 (MECL-1 homologue) (Figure 3.10). This may indicate a different strategy of AdE1A targeting the 20S proteasome. β1, β2 and β5 possess caspase-like, trypsin-like and chymotrypsin-like activity respectively (Groettrup *et al.,* 2001). When cells are exposed to IFNγ, this causes the catalytic β subunits to be substituted by LMP2, MECL-1 and LMP7 respectively forming the immunoproteasome (Hisamatsu *et al.,* 1996). This substitution results in the enhancement of the chymotrypsin-like activity and the abrogation of the caspase-like activity (Groettrup *et al.,* 2001). This may hint that the chymotrypsin activity has an important role in the generation of peptides for antigen processing. It is not yet known whether this interaction with β5 or any of the other β subunits leads to an increase or decrease in catalytic activity. On one hand, if it was to be presumed that the aim of AdE1A is to inhibit activity, then it can be hypothesized that since β5 possesses a chymotrypsin-like activity, AdE1A may target this in order to reduce the presentation of viral epitopes. However, this does not explain why AdE1A targets MECL-1, the immunosubunit that exhibits trypsin-like activity but not the chymotrypsin activity of LMP7. So it could be suggested that this may just be a general targeting of the total cellular proteasomal activity by AdE1A, rather than specific catalytic activities, in order to adjust or modulate the array of peptides presented to CTLs, thus playing a role in disrupting antigen recognition. On the other hand, if AdE1A interaction causes an increase in catalytic activity, then it can be hypothesized that AdE1A may recruit this to aid either the expression of viral proteins or

the degradation of DNA transcription regulators. For instance, the human T-cell leukemia virus type 1 (HTLV-1) Tax protein was found to interact with and enhance the activity of immunoproteasomes (Hemelaar *et al.,* 2001). The group proposes that this may be involved in the regulation of transcription control (Hemelaar *et al.,* 2001), such as the degradation of the nuclear pool of IκBα, a regulatory protein that inhibits the activity of the transcription factor NF-κB (Arenzana-Seisdedos *et al.,* 1995, 1997). Further experiments into the proteolytic activity of the 20S proteasomes and immunoproteasomes in the presence or absence of AdE1A are addressed in the chapter 5.

The regions of AdE1A involved in the interaction with β1, β2 and β5 were examined. A GST pulldown assay using [^{35}S]-labelled β1, β2 and β5 and GST-tagged fragments from across the different regions of AdE1A have shown that, just like MECL-1, all three β catalytic subunits bind to the N-terminal and CR3 regions of AdE1A (Figure 3.11). The amino acid residues in CR3 involved in the interaction were mapped by a further GST pull assay using [^{35}S]-labelled β2 and β5 and the same GST-tagged CR3 deletion mutants used with MECL-1. In this case, the weak binding β2 was used in order to compare its interaction, with that of its homologue MECL-1. The data have revealed that both β2 and β5 interact with similar set of residues. AdE1A binding capacity to both subunits has been significantly reduced by the deletions Δ169-177, Δ180-184 and Δ188-204, with β2 showing a more marked decrease in binding with deletions Δ169-177 and Δ180-184 (Figure 3.12). Furthermore, comparing the binding site of β2 to MECL-1, it was seen that binding to MECL-1, has been more affected with the deletions at Δ180-184 and Δ188-204, whereas β2 displayed less binding to mutants with deletions Δ169-177 and Δ180-184. This may indicate that AdE1A has different binding sites for both the homologues β2 and MECL-1. These data are however, not sufficient to draw definitive conclusions about the nature of AdE1A binding to the β subunits and MECL-1. Further investigations need to be carried out into to specific residues involved, such as using CR3 mutants with a series of point mutations across the sequence, similar to those employed for the N-terminal region.

Also, it would be interesting to examine if AdE1A binds to each of the subunits independently or if more than one subunit can bind to AdE1A at the same time.

Finally, results from the above GST-pulldown assays were analysed by densitometry in order to compare binding of the different subunits. The data in Figure 3.13 show that β5 binds to AdE1A more strongly than the other constitutive subunits. All of the β subunits have shown strongest binding to 13S Ad12E1A followed by 13S Ad5E1A and 12SAd5E1A, with the exception of β1 that displayed stronger binding to 13S Ad5E1A followed by 13S Ad12E1A and 12SAd5E1A. MECL-1 binds more strongly to AdE1A than to the other three constitutive subunits. 13S Ad12E1A and 13S Ad5E1A had similar binding affinities for MECL-1, followed by 12SAd5E1A. 13S AdE1A binds more strongly than 12SAdE1A. This may be due to the presence of CR3 in 13S AdE1A, which in combination with the N-terminal region make up two binding sites for the subunits. Generally, there was a stronger binding to Ad12E1A than to Ad5E1A.

In this chapter, it was demonstrated that AdE1A also binds preferentially to MECL-1, rather than LMP2 and LMP7. MECL-1 binds to the N-terminal region and CR3 regions of AdE1A. Through a series of GST pulldown assay, important residues involved in this interaction were mapped for both the N-terminal and CR3 regions. Additionally, the binding of AdE1A to the constitutive subunits β1, β2 and β5 was also addressed. It was found that AdE1A binds most strongly to β5 and β1 and the least binding to β2. These three catalytic subunits also bind, as MECL-1, to the N-terminal and CR3 regions of AdE1A. Further mapping of the binding sites within the CR3 region have shown different residues involved for the interactions with the β subunits and MECL-1.

In the next chapter, the effect of AdE1A binding on the expression of the immunoproteasome subunits was examined.

CHAPTER 4

REGULATION OF IMMUNOPROTEASOME AND MHC EXPRESSION BY AdE1A

4.1 Introduction

AdE1A is essential for viral replication and induction of cell cycle progression as well as having an involvement in transformation and oncogenesis. Through a series of protein-protein interactions, it targets multiple host regulatory proteins to activate or repress transcription, inhibit differentiation, promote progression into S phase as well as causing apoptosis (Berk 2005; Gallimore and Turnell 2001, Mymryk 1996). AdE1A is expressed in various isoforms as a result of differential splicing of the primary transcript, 12S and 13S mRNA are the most abundant and are transcribed earlier during infection whereas 9S, 10S and 11S are at low levels and accumulate later during infection (Stephens and Harlow 1987; Ulfendahl *et al.,* 1987). Most of the biological functions of AdE1A can be attributable to the protein products of the 12S and 13S RNA that encode 243 and 289 amino acid oncoproteins respectively in Ad2/Ad5. The proteins are identical apart from the presence of the additional 48 amino acid conserved region (CR3) in 13SAdE1A. The AdE1A conserved regions CR1, CR2, CR3, CR4 and the less conserved N-terminal region all contribute to the function of AdE1A in deviating cell cycle regulation and transcription.

AdE1A is already known to target a number of cellular proteins monitoring the cell cycle and transcription such as retinoblastoma tumour supressor (Rb) (and its related proteins p107 and p130) as well as CBP/p300 (Frisch and Mymryk 2002; Gallimore and Turnell 2001). It is thought that AdE1A can induce quiescent cells into S-phase by interacting with at least these two sets of proteins (Howe *et al.,* 1990). While AdE1A exerts its effect on host transcription pathway, it can also target components of the antigen processing pathways such as the proteasomes and major histocompatibility complex (MHC) class I and II (Rasti *et al.,* 2005, 2006; Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004).

Proteasomes are proteolytic complexes that degrade intracellular proteins, as well as processing peptides required for loading onto MHC molecules. The 20S proteasome

consists of 28 α and β subunits that are arranged in a barrel-like structure (refer to Figure 1.11)(Lowe *et al.,* 1995; Groll *et al.,* 1997; Baumeister *et al.,* 1998; Coux *et al.,* 1996). It combines with the 19S regulatory complex to form the 26S proteasome (Marteijn *et al.,* 2006; Baumeister *et al.,* 1998). Only three subunits are responsible for the proteolytic activity of the 20S proteasome namely β1, β2 and β5 (Rivett and Hearn 2004; Groetrrup *et al.,* 2001). Upon exposure to IFNγ, these three constitutively expressed subunits are substituted by their inducible homologues LMP2, LMP7 and MECL-1 respectively (Hisamatsu *et al.,* 1996). Immunoproteasomes have different cleavage specificity and thus produce a different array of peptides in comparison to 20S proteasomes and it is thought to be more efficient at processing peptides of viral origin (Groettrup *et al.,* 2001; Ehring *et al.,* 1996; Gaczynska *et al.,* 1996; Cascio *et al.,* 2001; Cerundolo *et al.,* 1995).

MHCs are heterodimer glycoproteins that exist on the surface of cells where they display fragments of processed proteins (peptides) for cytotoxic T cells (CTLs) and natural killer cells (NK) recognition (Flutter and Gao 2004); resulting in initiation of an immune response. MHC class I is expressed on the surface of all cells (York and Rock 1996) whereas MHC class II is expressed on antigen presenting cells of the immune system, such as dendritic cells and macrophages (Benoist and Mathis 1990; Steimle *et al.,* 1994); it can be induced in other cells by exposure to IFNγ (Steimle *et al.,* 1994). Whilst MHC class I presents peptides of viral or cytosolic origin, MHC class II mainly displays peptides processed from endocytosed extracellular proteins such as bacterial pathogens and viral antigens (Hegde *et al.,* 2003).

AdE1A targets the ATPase (S4 and S8) as well as the non-ATPase components (S2) of the 19S complex (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004) possibly affecting its ATPase activity. AdE1A also binds to multiple α subunits of the 20S proteasome (Rasti *et al.,* 2006) potentially affecting its function. With regard to the immunoproteasome, research so far indicates that AdE1A down-regulates LMP2 transcription partly by interference with components of the JAK/STAT1 pathway that is responsible for the induction of cellular response to IFNγ (Chatterjee-Kishore *et al.,* 2000).

LMP2, LMP7 and MECL-1 expression was also found to be down-regulated in Ad12 transformed but not Ad5 transformed rat cells (Vertegaal *et al.,* 2003; Rotem-Yedudar *et al.,* 1996).

In rat cells that have been transformed with AdE1A, downregulation of MHC class I was observed in Ad12 transformed cells but not in Ad2/5 (Schrier *et al.,* 1983; Ackrill and Blair 1988), similar results were also observed in a small panel of human cells transformed with AdE1A (Bottley *et al.,* 2005; Vasavada *et al.,* 1986). Ad12 is oncogenic, inducing tumours in new-born rodents whereas Ad2/5 is not. A study by Ackrill *et al.,* (1991) examined the effect of AdE1A on MHC class II expression; it was observed that transfected AdE1A represses MHC class II expression by inhibiting the cellular response to IFNγ. The effect of AdE1A on the immunoproteasome has not been studied extensively and as previously mentioned, research so far only indicates repression of LMP2 at a transcriptional level.

In this chapter, this will be explored further to study the effect of AdE1A on the immunosubunit expression to include both LMP7 and MECL-1, as well as LMP2 and to observe this in the setting of adenovirus infection, AdE1A transfection and in established adenovirus transformed human cells. Additionally, as the effect of AdE1A on MHC class I surface expression has been heavily focused on transformed cells lines, mainly of rodent origin, this will be further examined to include a wider panel of adenovirus transformed human cells as well as during adenovirus infection and AdE1A transfection. The cellular response to IFNγ in the presence of transfected AdE1A and adenoviral infection will also be investigated.

4.2 Results

4.2.1 AdE1A down-regulates expression of the proteasome immunosubunits

4.2.1.1 Adenovirus infection

To assess the effect of adenovirus infection on cellular immunosubunit levels, H1299 cells were infected (for 24 hrs) with Ad5, Ad12 and Ad5 ΔE3 at 20 plaque forming units (p.f.u) per cell. In order to induce immunosubunit expression, some of the cells were treated with 300 U/ml IFNγ for a further 24 hrs after which 30 µg of whole cell lysates were analysed for protein expression by western blotting using rabbit polyclonal LMP2 or LMP7 antibodies (Abcam) and rabbit polyclonal MECL-1 antibody (Figure 4.1). Immunosubunit expression was clearly induced following IFNγ in the mock infected cells. However, in comparison to mock infected cells, all of Ad5, Ad12 and Ad5 ΔE3 inhibited IFNγ-mediated induction of LMP2, LMP7 and MECL-1. Additionally, basal expression of the immunosubunits (prior to IFNγ treatment) was also sharply reduced following infection, as clearly observed with LMP2. The deletion of the adenovirus E3 gene has little effect on this inhibition. The E3 gene, like E1A, encode for proteins with immunosubversive functions, such as the down-regulation of MHC class I (Burgert *et al.,* 1987; Wold *et al.,* 1999) and inhibition of tapasin (Bennett *et al.,* 1999); hence the inclusion of an adenovirus with deleted E3 gene in this study will allow the ruling out its involvement in the observed experimental data.

These results show that infection prior to IFNγ treatment inhibited immunosubunit expression, so a further experiment was carried out to look at the effect of adenovirus infection in IFNγ pre-treated cells. In this case, it was found that the expression of LMP2, LMP7 and MECL-1 remain unaffected following infection with Ad5, Ad12 and Ad5 ΔE3 (Figure 4.2). It was also noticed that Ad5E1A and Ad12E1A expression was reduced in IFNγ pre-treated cells compared to the ones that were infected first. Furthermore, an additional time point was added so that Ad5 infection was extended to 48 hrs following IFNγ treatment and the same procedure repeated as above. It was found that at 24 hrs,

there was no effect on immunosubunit expression by infection; however, at 48 hrs a significant decrease in LMP2, LMP7 and MECL1 expression was observed in comparison to the mock infected cells (Figure 4.3). In order to examine whether this reduction of LMP2, LMP7 and MECL1 expression can also be seen after infection with other adenovirus serotypes, H1299 cells were infected with Ad3, Ad4, Ad5, Ad7, Ad9 and Ad12 for 24 hrs followed by a further 24 hrs of IFNγ treatment. Cell lysates were analysed by western blotting for LMP2, LMP7 and MECL1 expression. A significant decrease in IFNγ-mediated immunosubunit induction was observed in all the serotypes with the exception of Ad11 (Figure 4.4). Viral infection was confirmed by the presence of structural proteins in cell lysates as specific antibodies are not available for most of these viruses.

In view of these results that have shown the inhibition of immunosubunit expression by AdE1A, the effect of infection with adenoviruses that had their AdE1A gene deleted was subsequently examined. Two ΔE1A Ad5 mutant viruses, *dl*312 and *dl*343 (Stanton *et al.*, 2008; Winberg and Shenk 1984; Hearing and Shenk 1985), were used to infect H1299 cells for 24, 48 and 72 hrs followed by IFNγ treatment for a further 24 hrs. Infection by the mutant virus did not affect the induction of immunosubunit expression (Figure 4.5A) when compared to the mock infected cells (Figure 4.5B). There was an increasing expression of each of the subunits as infection progressed. The progression of infection with the adenoviruses that lack AdE1A was very slow compared to *w.t.* virus. Viral infection was confirmed by western blotting for Ad5E1B55K. AdE1A facilitates the expression of the other early genes including AdE1B. However, it is not an absolute requirement, because at a higher virus dose, or in this case, a prolonged incubation time at 24hrs, the early region genes will eventually be expressed although at a slower rate (Nevins 1981).

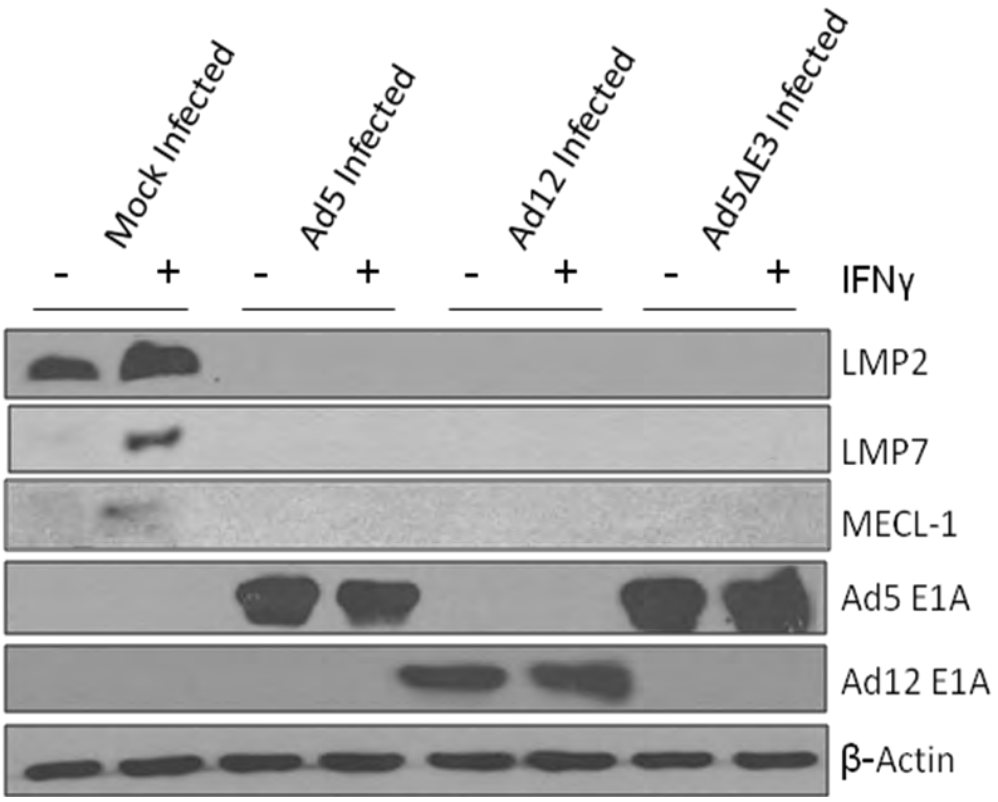


Figure 4.1 Adenovirus infection down-regulates LMP2, LMP7 and MECL1 expression.
H1299 cells were infected with 20 p.f.u. per cell for 24 hrs, followed by treatment with 300U/ml IFNγ for another 24 hrs. The cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. 30 µg of this lysate was analysed by Western blotting to determine immunosubunit and E1A levels.

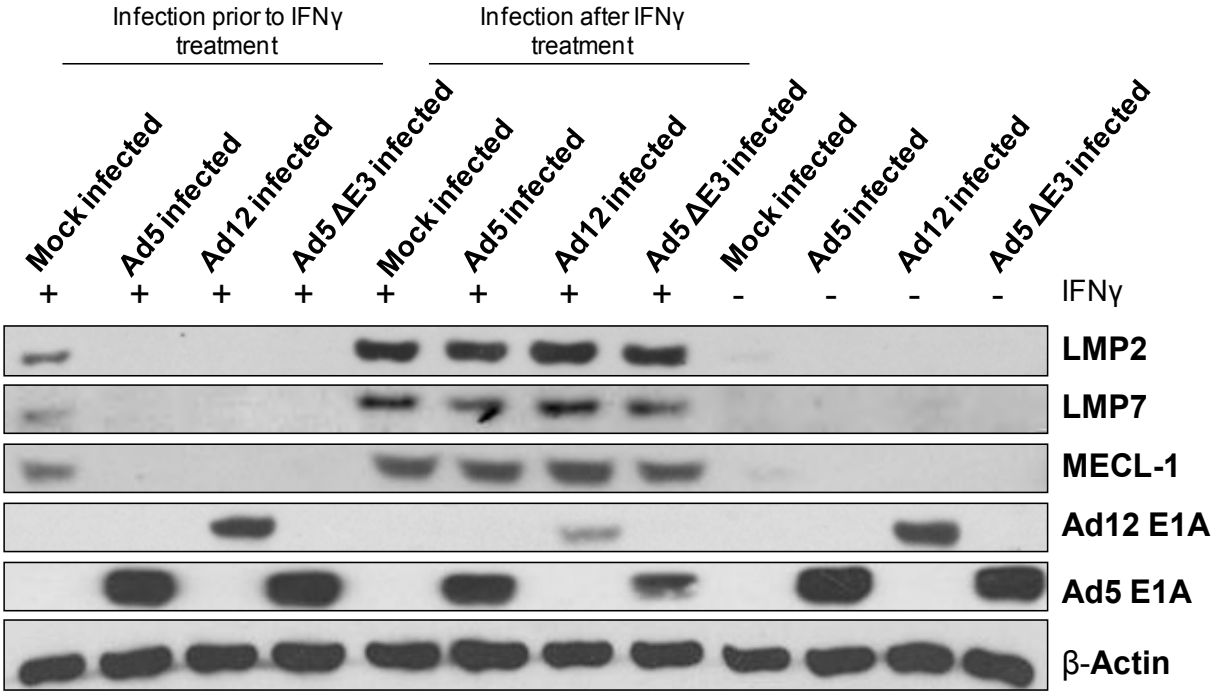


Figure 4.2 Adenovirus infection has no effect on immunosubunit expression of IFNγ pre-treated cells at 24 hrs

H1299 cells were treated with 300 U/ml IFNγ for 24 hrs then infected with 20 plaque forming units (p.f.u) per cell for another 24 hrs and vice versa. The cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. 30 µg of this lysate was analysed by Western blotting to determine immunosubunit and E1A expression.

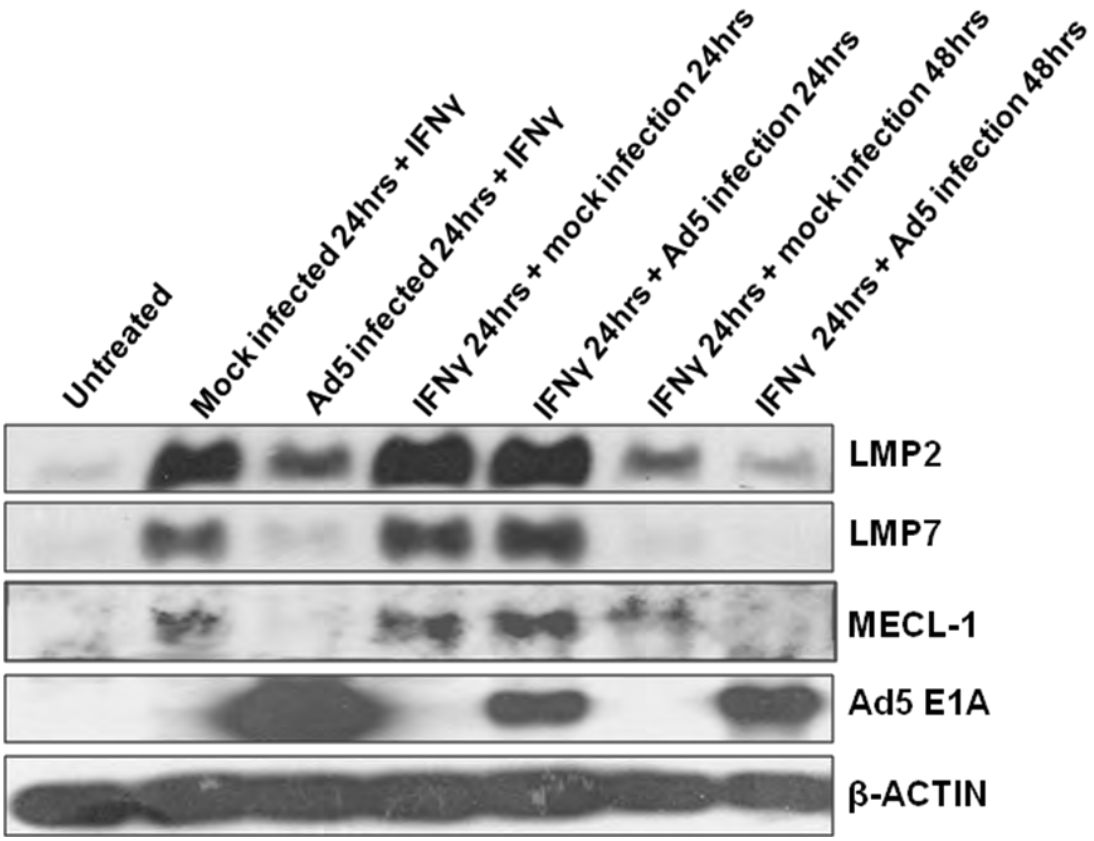


Figure 4.3 Prolonged adenovirus infection (48hrs) reduces immunosubunit expression in IFNγ pre-treated cells
H1299 cells were treated with 300 U/ml IFNγ for 24 hrs then infected with 20 plaque forming units (p.f.u) per cell for another 24 hrs or 48 hrs and vice versa (for 24 hr infection). The cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. 30 µg of this lysate was analysed by Western blotting to determine immunosubunit and E1A expression.

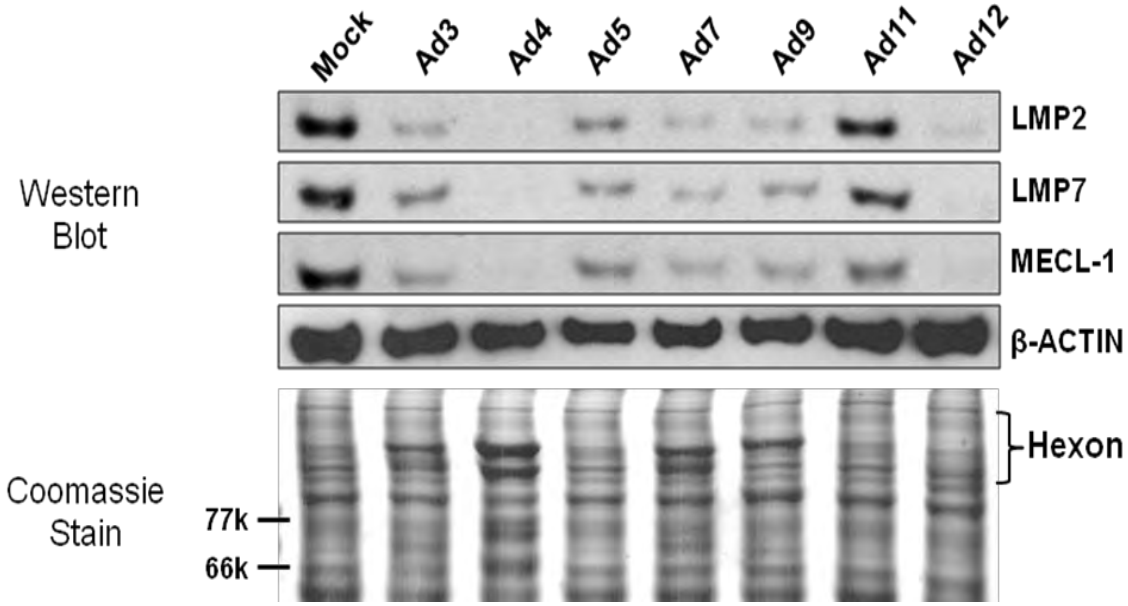


Figure 4.4 Infection with different adenovirus serotypes down-regulate immunosubunit expression
H1299 cells were infected with 20 p.f.u. per cell of each virus for 24 hrs, followed by treatment with 300 U/ml IFNγ for a further 24 hrs. The cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was either analysed by Western blotting to determine immunosubunit levels (upper panel) or analysed by PAGE and stained with 0.1% (w/v) Coomassie Brilliant Blue (lower panel) to illustrate hexon expression.

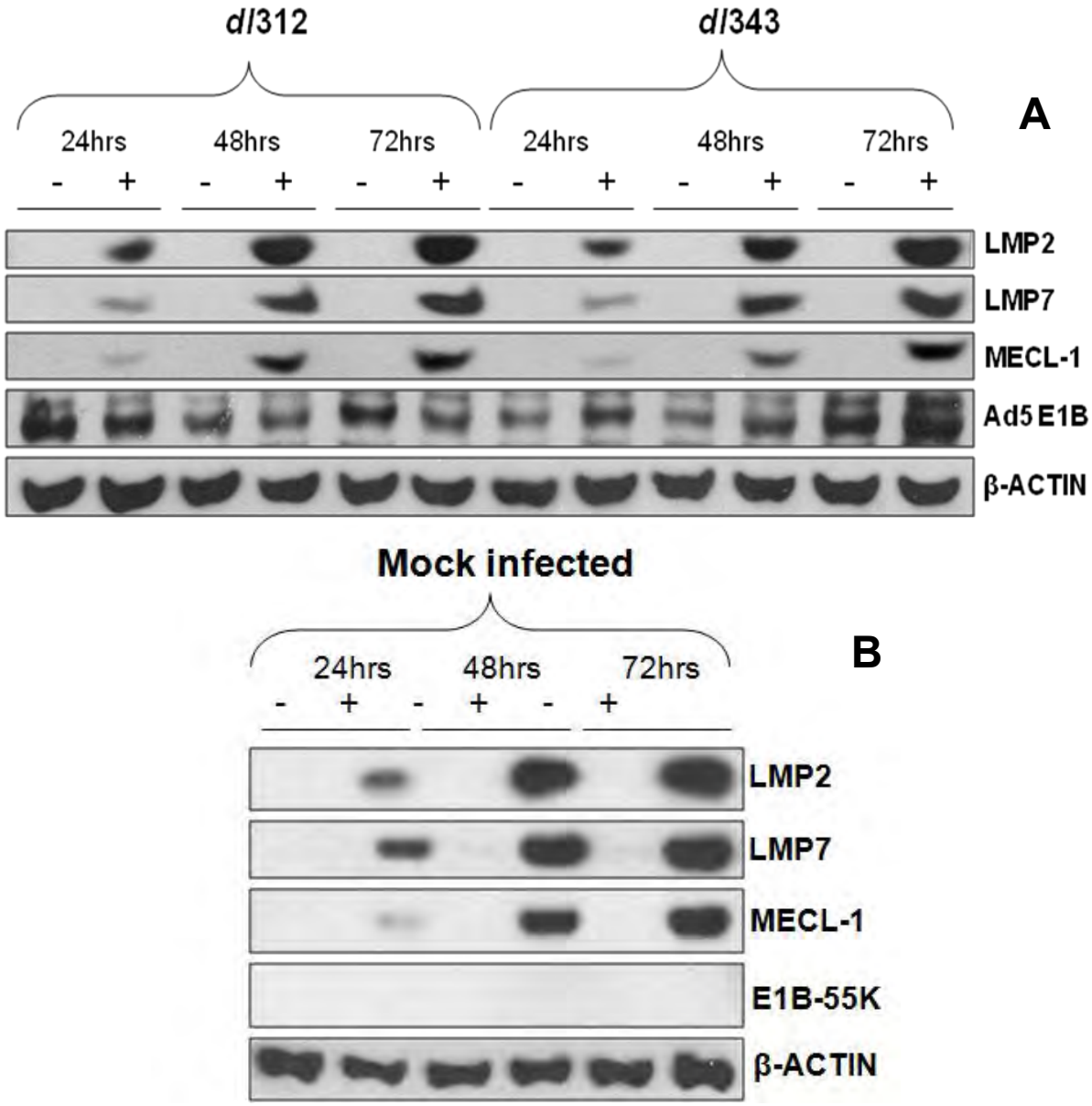


Figure 4.5 ΔE1A mutant viruses do not down-regulate immunosubunit expression
H1299 cells were either mock infected **(B)** or infected with 20 p.f.u. of the mutant viruses per cell for 24 hrs **(A)**, followed by treatment with 300 U/ml IFNγ for another 24 hrs. The cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was analysed by Western blotting to determine immunosubunit and E1A levels.

4.2.1.2 The effect of transfection of AdE1A on immunoproteasome expression

In order to investigate whether AdE1A transfection alone can show similar results to those obtained by viral infection, H1299 cells were transfected with plasmids encoding 12S and 13S Ad5E1A and Ad12E1A for 24 hrs. The cells were then treated with 300 U/ml of IFNγ for a further 24 hrs after which 30 µg of whole cell lysates were analysed for protein expression by western blotting using rabbit polyclonal LMP2 or LMP7 antibodies and rabbit polyclonal MECL-1 antibody. pcDNA3 was used for the transfection control transfection.

In pcDNA3 transfected cells, there was a large induction of LMP2, LMP7 and MECL1 expression following IFNγ treatment. However, in cells that have been transfected with 12S and 13S Ad5 and Ad12 E1A, this IFNγ-mediated induction was considerably reduced (Figure 4.6 A and B). This was particularly marked in case of MECL1 where little to no expression was visible after the transfection of Ad5 or Ad12E1A, although its worth noting that the MECL-1 antibody is of low titre. Efficiency of transfection was in excess of 80% in most cases as shown in Table 4.1.

In order to examine if this reduction varied in an AdE1A dependent manner, increasing amounts of pcDNA3 control and 13S Ad5 E1A (0.1 µg, 0.3 µg 1 µg and 2 µg) were transfected into cells and the expression of LMP2, LMP7 and MECL1 determined by western blotting. The results have shown that there was a direction correlation between in immunosubunit expression and increasing amount of Ad5 E1A (Figure 4.7) with 2 µg of 13S Ad5 E1A causing the greatest inhibition. This repression was again most marked in MECL1 with very little expression at 1µg in comparison to the corresponding LMP2 and LMP7 western blots.

Construct	Transfection efficiency (%)
12SAd5E1A	96.1
13SAd5E1A	90.2
12SAd12E1A	84.8
13SAd12E1A	72.9

Table 4.1: Transfection efficiency of AdE1A constructs

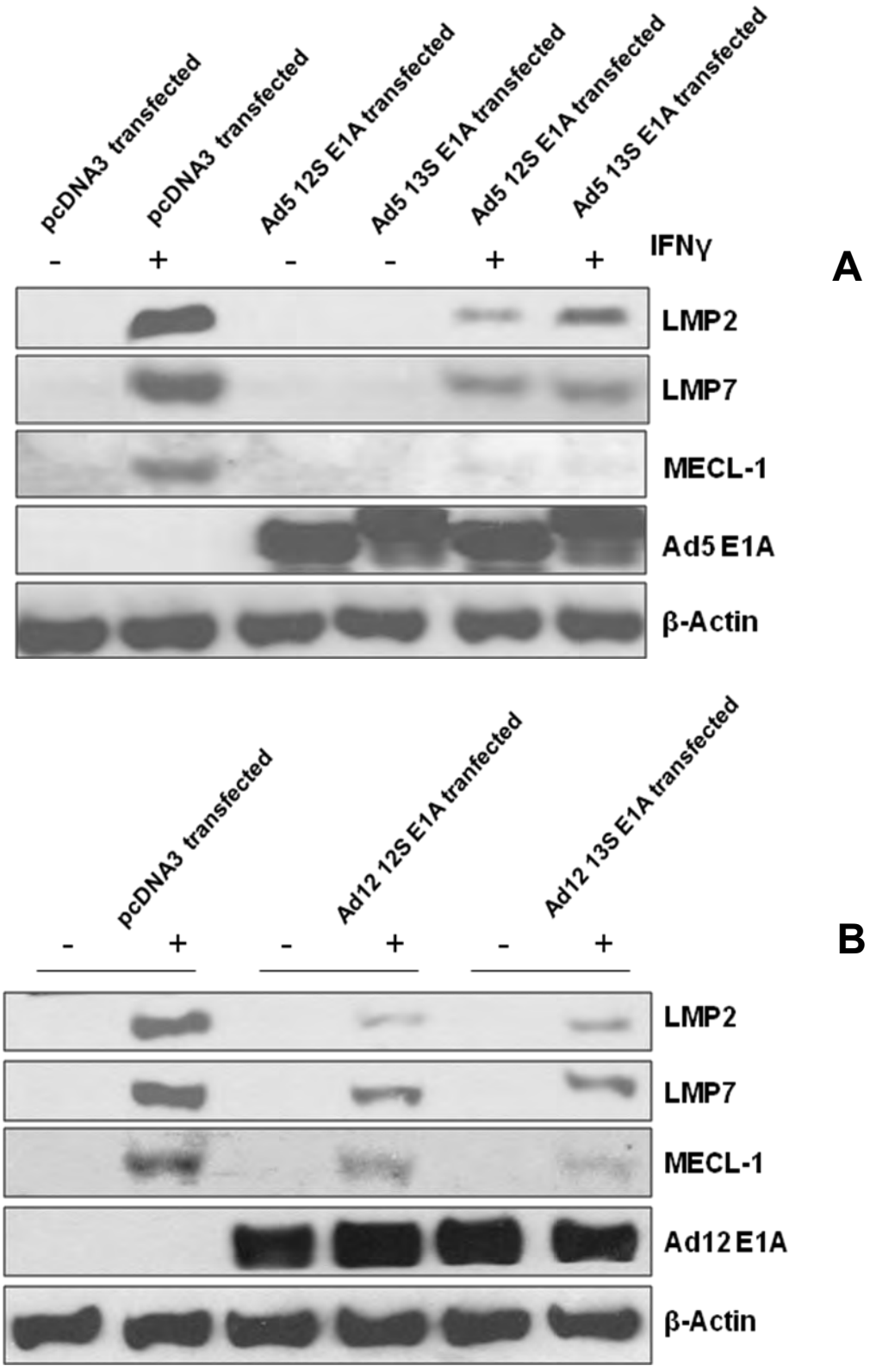


Figure 4.6 Transfection with AdE1A reduces IFNγ-induced immunosubunit expression.
H1299 cells were transfected with 1 µg each of 12S and 13S Ad5 and Ad12 E1A constructs for 24 hrs, followed by a further 24 hrs of IFNγ treatment. Cells were lysed in 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was analysed for protein expression by Western blotting. **A.** Transfection of Ad5E1A **B.** Transfection of Ad12E1A.

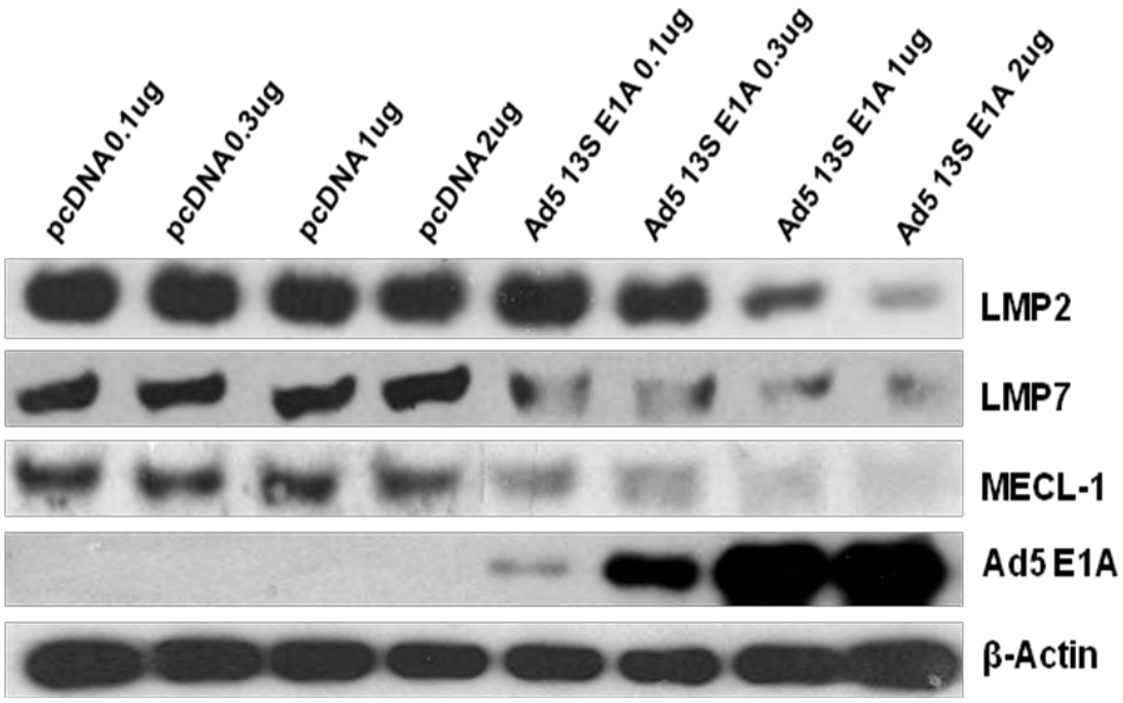


Figure 4.7 Immunosubunit down-regulation by Ad5E1A varies with AdE1A expression
H1299 cells were transfected with 0.1, 0.3, 1 and 2 μg of 13S Ad5E1A construct for 24 hrs, followed by a further 24 hrs of IFNγ treatment. Cells were lysed in 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was analysed for protein expression by Western blotting.

4.2.1.3 Immunoproteasome subunit expression in established adenovirus transformed human embyonic retinoblast (HER) cell lines

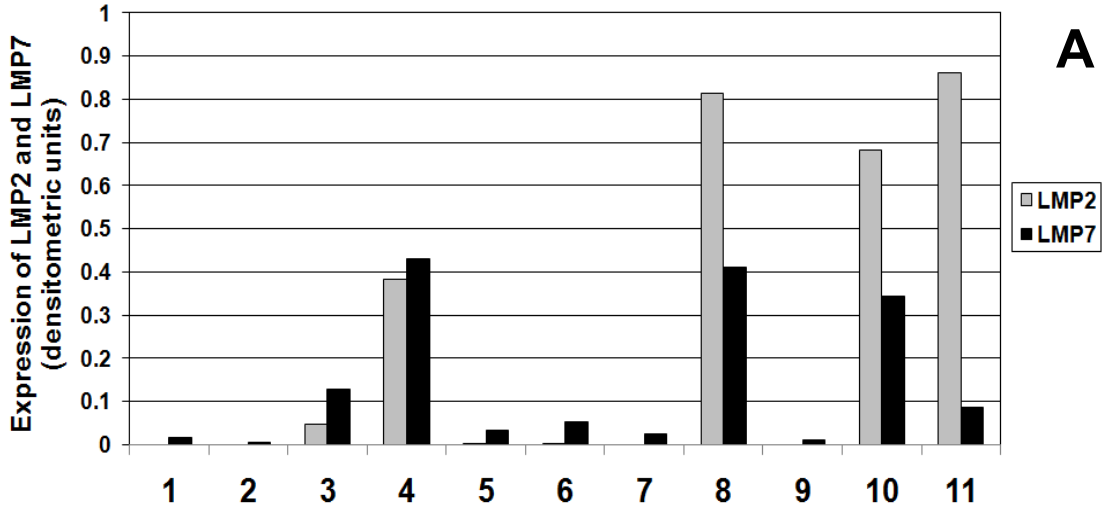
The relative levels of immunosubunits in a large panel of established adenovirus transformed human cell lines was examined (Figure 4.8A). The cell lines express various combinations of Ad5 and Ad12 E1A, Ad5 and Ad12 E1B and mutant ras (Figure 4.8B – lower panel). Cells were treated with 600 U of IFNγ for 48 hrs then lysed with 9 M urea lysis buffer. Thirty microgram of the whole cell lysates were analysed for expression of LMP2, LMP7 and MECL1 by western blotting. Before IFNγ treatment, there was little to no expression of LMP2, LMP7 and MECL1 in any of the cell lines (Figure 4.8B – upper panel), however, upon exposure to IFNγ, there was an upregulation of the expression of immunosubunits LMP2 and LMP7 in cell lines that express generally (not exclusively) Ad5E1A but not Ad12E1A (Figure 4.8A). The presence of activated mutant H-ras, N-ras or Ad12E1B had no effect on this trend. There was no expression of MECL1 observed in either Ad5 and Ad12 E1A expressing cell lines (Figure 4.8B).

A549 cells that constitutively express either 12SAd5E1A (12S10) or 13SAd5E1A (13S and 13S G418) were also analysed for immunosubunit expression (Figure 4.9). There was a strong induction of immunosubunit expression after IFNγ treatment of A549, whereas there was little or no expression in all of the Ad5E1A expressing A549 cells.

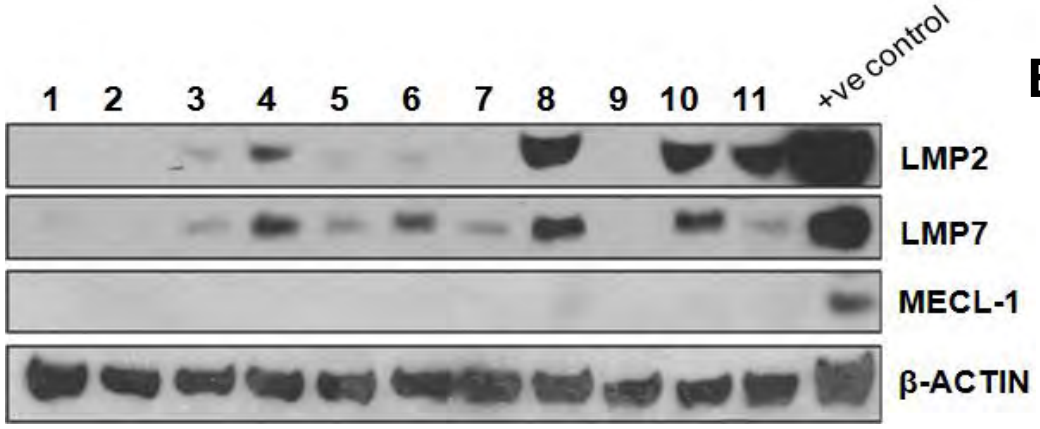
4.2.2 Adenovirus E1A and MHC Class I and Class II expression

In view of the observation that AdE1A negated the IFNγ-induced expression of immunoproteasome subunits and the well established role of Ad12E1A in the down-regulation of MHC class I in Ad transformed rat cells, an examination of the effect of AdE1A on the expression of MHC class I and MHC class II in human cells, following viral infection, transient transfection and cell transformation was undertaken.

Effect of AdE1A on immunosubunit expression in established adenovirus transformed human embryonic retinoblasts



Ad5 E1A			X	X				X		X	X
Ad5 E1B								X		X	
Ad12 E1A	X	X			X	X	X		X		
Ad12 E1B	X	X			X		X				
Mutant Ras			X	X		X					X



1 - Ad12E1HER12
2 - Ad12E1HER3
3 - Ad5E1A+NrasHER286.1
4 - Ad5E1A+NrasHER313.B
5 - Ad12E1HER11
6 - Ad12E1A+NrasHER414.B
7 - Ad12E1HER5
8 - Ad5E1HER911
9 - Ad12E1AHER 283
10 - Ad5E1HER359A2
11 - Ad2E1A+Nas HER 313A

Figure 4.8 Immunosubunit expression is down-regulated in Ad12 transformed but not in Ad5 transformed cells
Adenovirus transformed human embryonic retinoblast cell lines (HER) were treated with 600 U/ml of IFNγ for 48 hrs. Cells were then lysed in 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. The "positive control" is IFNγ-treated H1299 cells Thirty microgram of each lysate was analysed for immunosubunit expression by Western blotting. **A.** Relative level (to actin) of immunosubunit expression (by densitometric analysis of Western blots). **B.** Western blot result (upper panel). Details of the numbered cell lines are listed in lower panel.

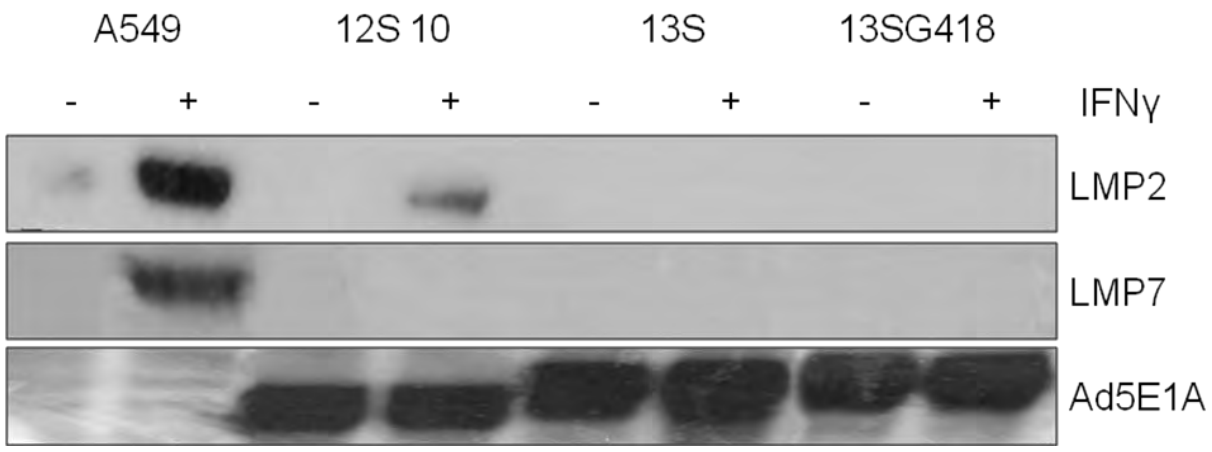


Figure 4.9 Immunosubunit expression in A549 cells expressing Ad5E1A
A549 cell lines expressing 12S and 13S Ad5E1A were treated with 300 U/ml of IFNγ for 24 hrs. Cells were then lysed in 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty miocrogram of this lysate was analysed for immunosubunit expression by Western blotting.

4.2.2.1 The effect of adenovirus infection on MHC Class I and Class II expression

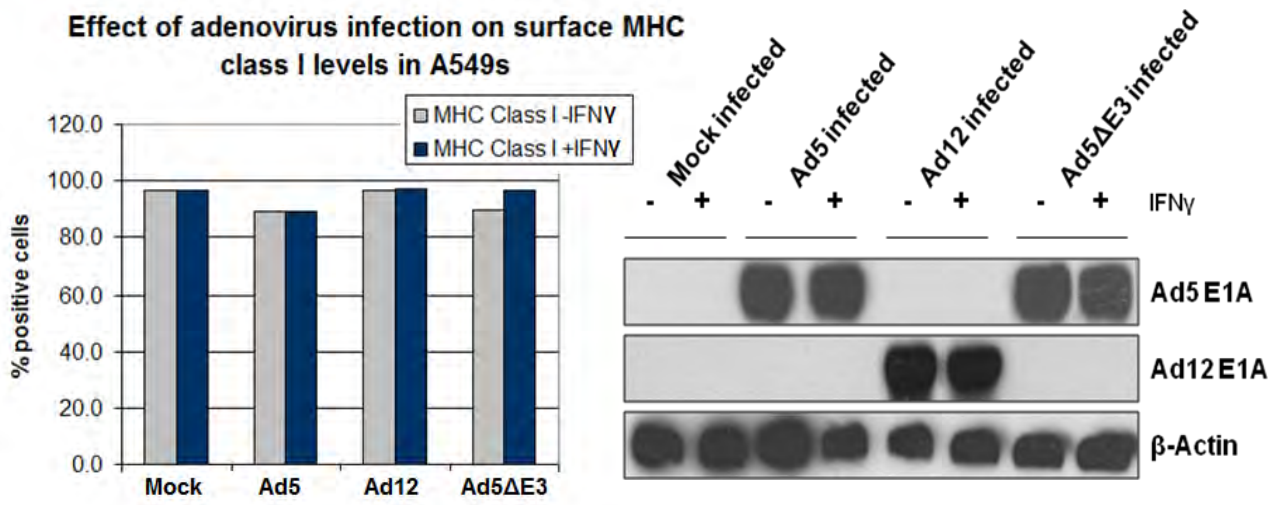
To examine the effect of viral infection on surface expression of MHC class I and II, primary tonsil epithelial cells, as well as various human tumour cell lines, were infected with Ad5, Ad12 and Ad5 ΔE3 for 24 hrs then treated with 300 U/ml IFNγ for a further 24 hrs before being harvested, stained with mouse anti-human MHC class I and II antibodies, and analysed by flow cytometry.

The results revealed that MHC class I levels remain unaffected after Ad5, Ad12 and Ad5 ΔE3 infections, regardless of IFNγ treatment. This was seen in A549, HCT116, primary tonsil epithelial cells and T47D samples (Figure 4.10A-D) where over 90% of cells were positive for MHC class I even after infection. Similarly, MHC class II levels after infection was examined in tonsil epithelial cells and the T47D cell line. Both T47D and tonsil epithelial cells were used in this study because they highly up-regulate their surface MHC class II levels upon IFNγ treatment. The results showed that infection with Ad12 caused down-regulation of MHC class II expression in comparison to Ad5 (Figure 4.11A and B). In the tonsil cells, MHC class II surface expression was reduced from 65% in mock infected cells to approximately 22% in Ad12 infected cells and 45% in Ad5 infected cells. However deletion of the E3 gene relieved the inhibition resulting in 66% MHC class II expression (Figure 4.11B). In T47D cells, Ad12 infection reduced MHC class II from 76% to 29%, whereas Ad5 and Ad5 ΔE3 infections did not affect expression significantly - 69% and 72% respectively (Figure 4.11B).

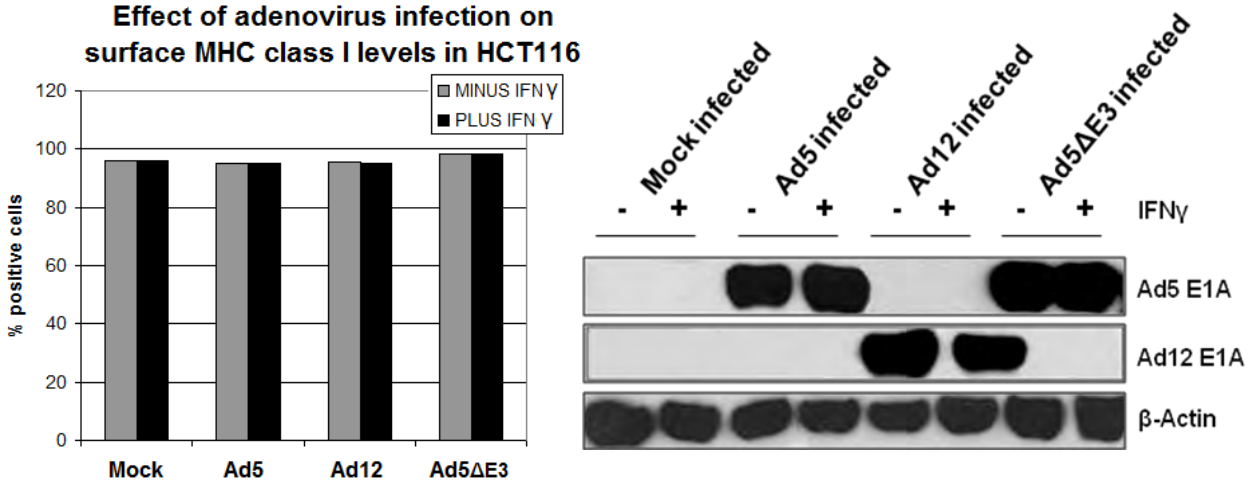
4.2.2.2 The effect of AdE1A transfection on MHC Class I and II expression

Further experiments examining the effect of AdE1A transfection (via mRNA electroporation) on MHC class I and II surface expression were also undertaken. mRNA was generated from 12SAd5E1A, 13SAd5E1A, 12SAd12E1A, 13SAd12E1A, 12SAd5E1A

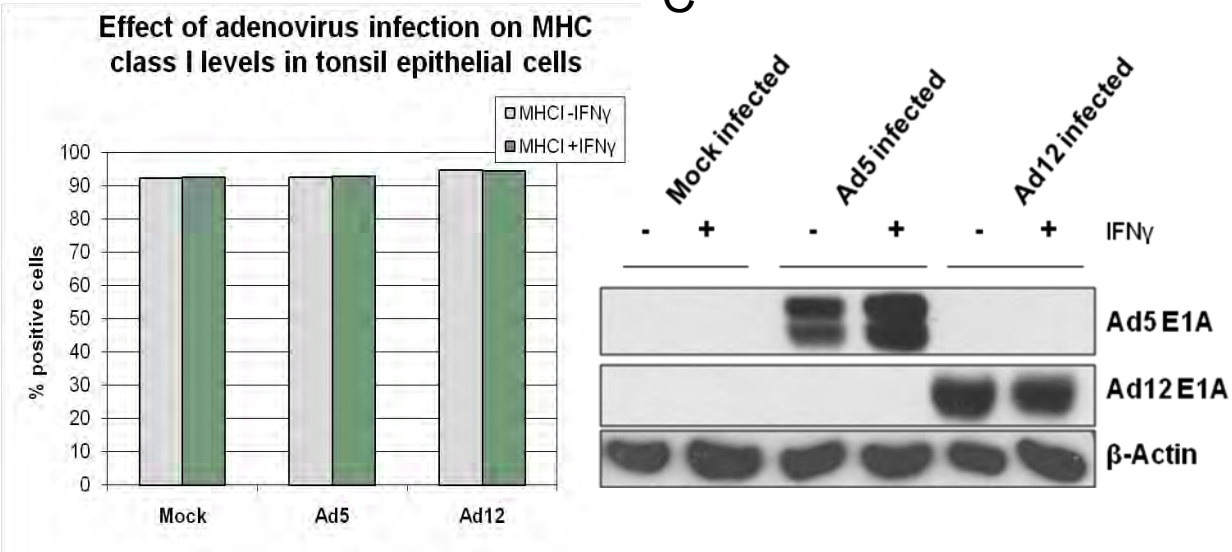
A


Effect of adenovirus infection on surface MHC class I levels in A549s
MHC Class I -IFNγ
MHC Class I +IFNγ
% positive cells
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Mock
Ad5
Ad12
Ad5ΔE3
Mock infected
Ad5 infected
Ad12 infected
Ad5ΔE3 infected
- + - + - + - +
IFNγ
Ad5 E1A
Ad12 E1A
β-Actin

B


Effect of adenovirus infection on surface MHC class I levels in HCT116
MINUS IFN γ
PLUS IFN γ
% positive cells
120
100
80
60
40
20
0
Mock
Ad5
Ad12
Ad5ΔE3
Mock infected
Ad5 infected
Ad12 infected
Ad5ΔE3 infected
- + - + - + - +
IFNγ
Ad5 E1A
Ad12 E1A
β-Actin

C


Effect of adenovirus infection on MHC class I levels in tonsil epithelial cells
MHCI -IFNγ
MHCI +IFNγ
% positive cells
100
90
80
70
60
50
40
30
20
10
0
Mock
Ad5
Ad12
Mock infected
Ad5 infected
Ad12 infected
- + - + - +
IFNγ
Ad5 E1A
Ad12 E1A
β-Actin

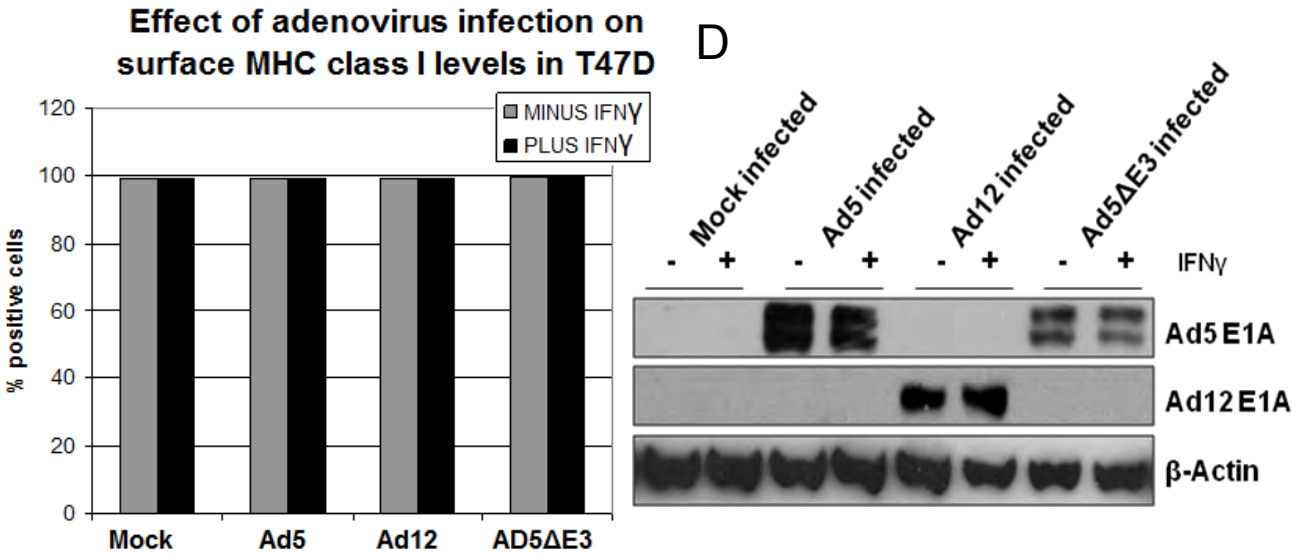


Figure 4.10 Adenovirus infection has no effect on MHC class I expression.
A549, HCT116, tonsil epithelial cells and T47D cells were infected with 20 p.f.u. of Ad5, Ad12 and Ad5 ΔE3 per cell for 24 hrs, followed by treatment with 300 U/ml IFNγ for another 24 hrs. They were then harvested by trypsinisation, washed in cold PBS and finally resuspended in 50 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). They were stained with 5 µl of mouse anti-human MHC class I antibody for 30 minutes, washed twice with FACS buffer, then resuspended in 400 µl PBS prior to being analysed by flow cytometry (left panel). For western blot analysis, the cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was analysed by Western blotting to determine immunosubunit and E1A levels (right panel). **A.** A549 cells **B.** HCT116 cells **C.** Tonsil epithelial cells **D.** T47D cells

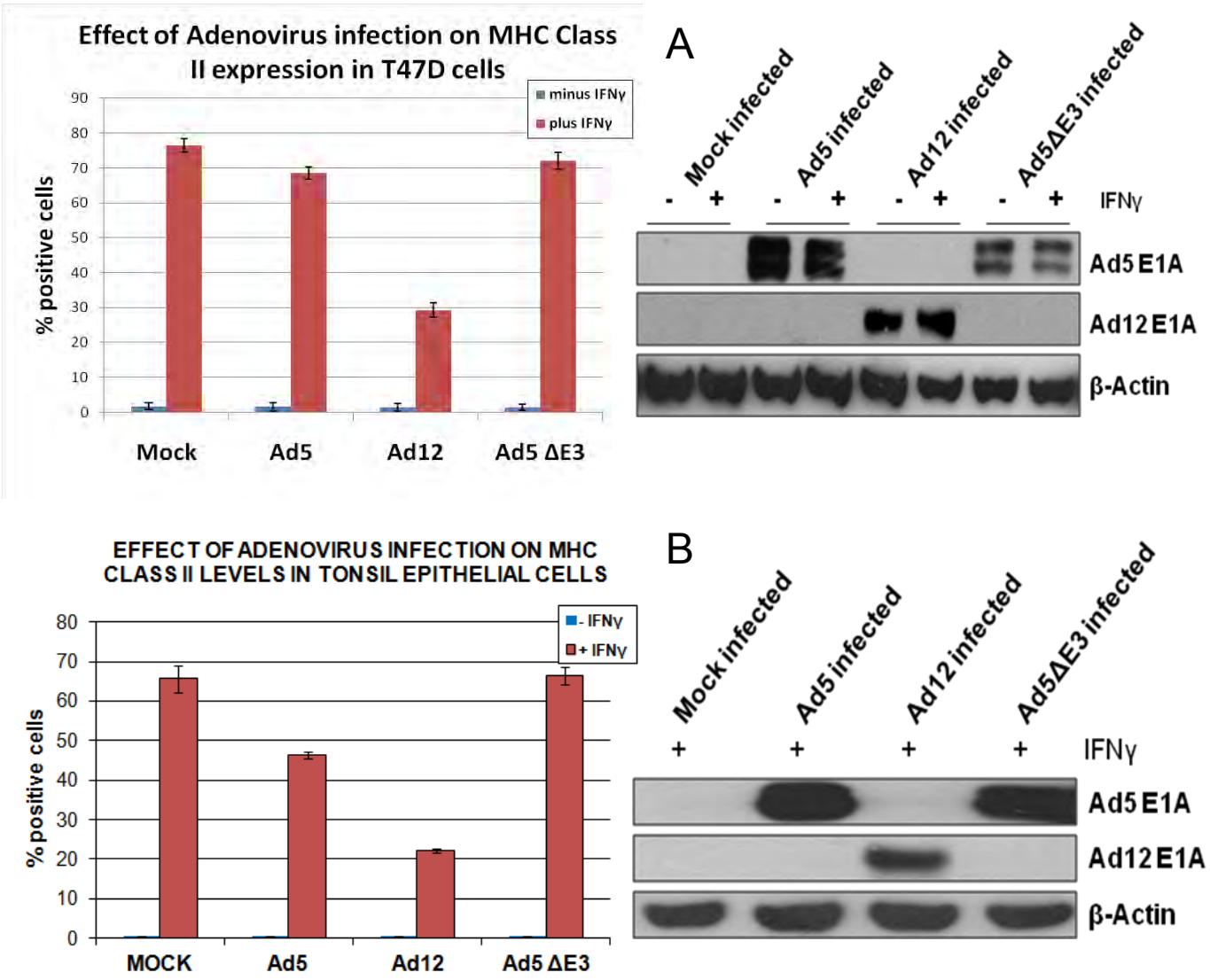


Figure 4.11 Ad12 infection down-regulates MHC class II expression.
T47D and tonsil epithelial cells were infected with 20 p.f.u. of Ad5, Ad12 and Ad5 ΔE3 per cell for 24 hrs, followed by IFNγ treatment for another 24 hrs. They were then harvested by trypsinisation, washed in cold PBS and finally resuspended in 50 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). They were stained with 5 µl of mouse anti-human MHC class II antibody for 30 minutes, washed twice with FACS buffer, then resuspended in 400 µl PBS prior to being analysed by flow cytometry (left panel). For western blot analysis, the cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was analysed by Western blotting to determine immunosubunit and E1A levels (right panel). **A.** T47D cells **B.** Tonsil epithelial cells. Data are means +/- SEM from two independent experiments.

mutant (L19/20S) and 13SAd5E1A mutant (L19/20S) through *in vitro* transcription (see section 2.3.11). mRNA was electroporated into T47D cells and primary fibroblasts.

The cells were grown for 12 hrs followed by another 12 hrs of 300 U/ml IFNγ treatment. They were then harvested and stained for MHC class I or class II prior to analysis by flow cytometry. Electroporation of cells with mRNA yielded high transfection efficiency and high cell viability compared to electroporating with DNA (Van Tendeloo *et al.,* 2001). Additionally, T47D cells were resistant to transfection using lipofectamine so electroporation was seen as a preferable option to achieve maximum transfection efficiency. However, the disadvantage lies in achieving equal protein expression between the different transfections, so we had to take into account the different levels of AdE1A expression when interpreting the data. With regard to the effect of AdE1A transfection on MHC class I levels in primary fibroblasts, data showed that surface levels remained unaffected after transfection with Ad5 and Ad12 E1A (Figure 4.12); whereas in the case of MHC class II, the results (Figure 4.13A) reveal that the transfection of 12S and 13S Ad5E1A caused an inhibition of MHC class II induction. The raw data showed 55% MHC class II expression in mock electroporated cells compared to 7% and 22% in 12S and 13S Ad5E1A expressing cells respectively. Transfection with the double mutants 12S and 13S Ad5E1A L19/20S relieved this inhibition with 37% and 40% MHC class II expression respectively. Examining the western blot of the corresponding lysates from each transfection, there is unequal expression, with both wild type and mutant 12S Ad5E1A being expressed about 3 times more than the mutant and wild type 13S Ad5E1A (Figure 4.13C). This may partly explain the added inhibition by 12S Ad5E1A in comparison to 13SAdE1A. Nevertheless, the double mutations in both cases affected AdE1A's ability to inhibit MHC class II induction in contrast to their wild type counterparts. Similarly, transfection with 12S and 13S Ad12E1A reduced MHC class II expression from 55% in the mock transfected cells to 33% and 42% in 12S and 13S Ad12 E1A transfected cells respectively (Figure 4.13B). However, this was found to be statistically insignificant.

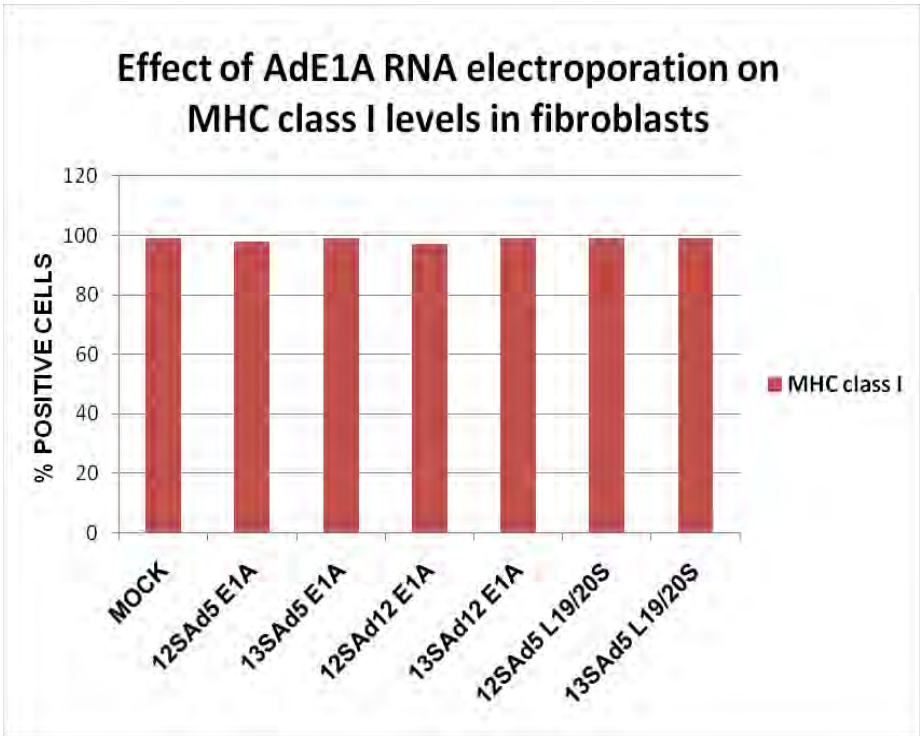


Figure 4.12 AdE1A transfection does not affect MHC class I cell surface expression
Primary fibroblasts cells were electroporated with the appropriate AdE1A mRNA for 12-16 hrs. They were then harvested by trypsinisation, washed in cold PBS and finally resuspended in 50 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). They were stained with 5 µl of mouse anti-human MHC class I antibody for 30 minutes, washed twice with FACS buffer, then resuspended in 400 µl PBS prior to being analysed by flow cytometry. This data is representative of repeated experiments.

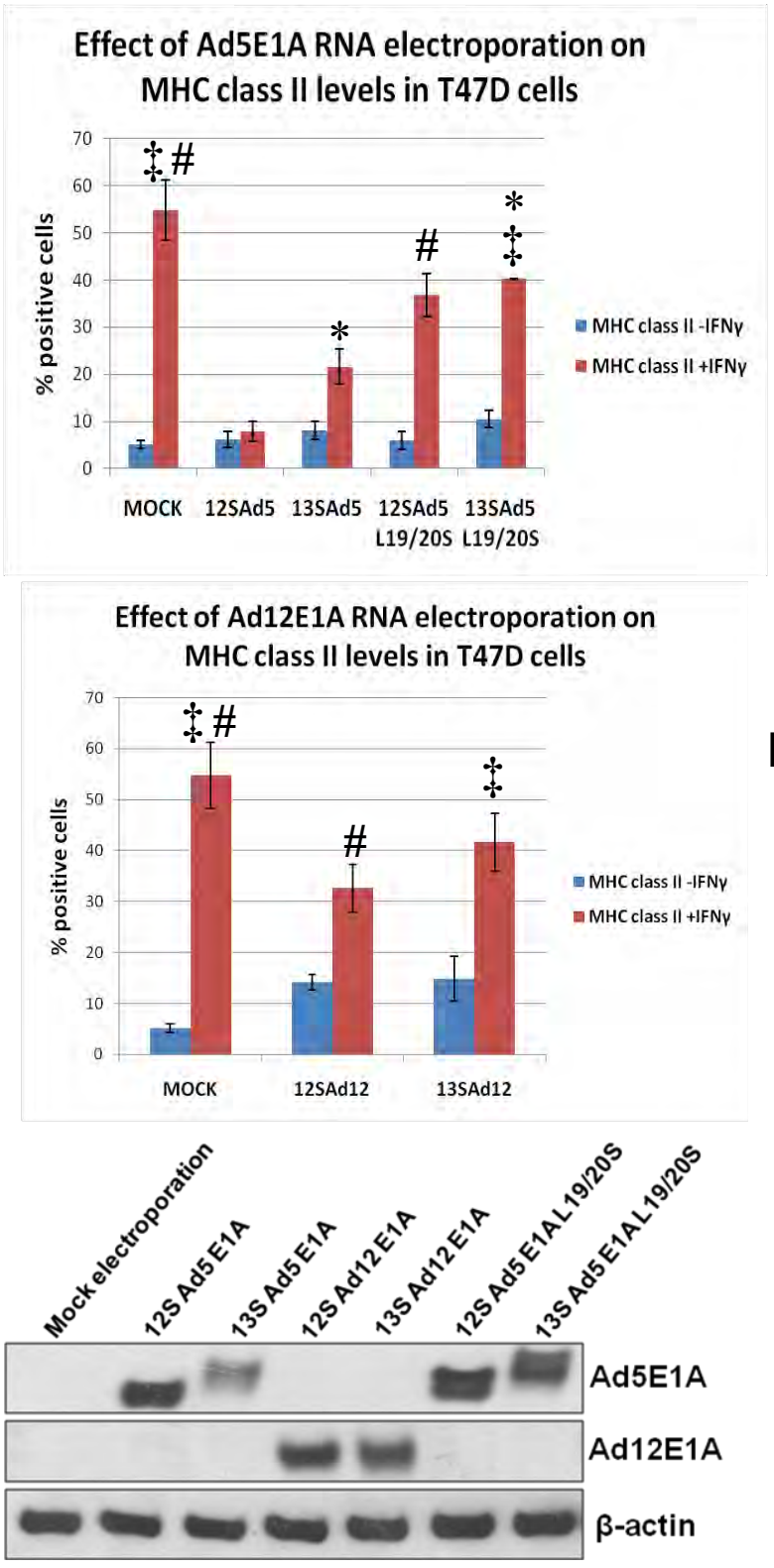


Figure 4.13 AdE1A transfection down-regulated MHC class II cell surface expression

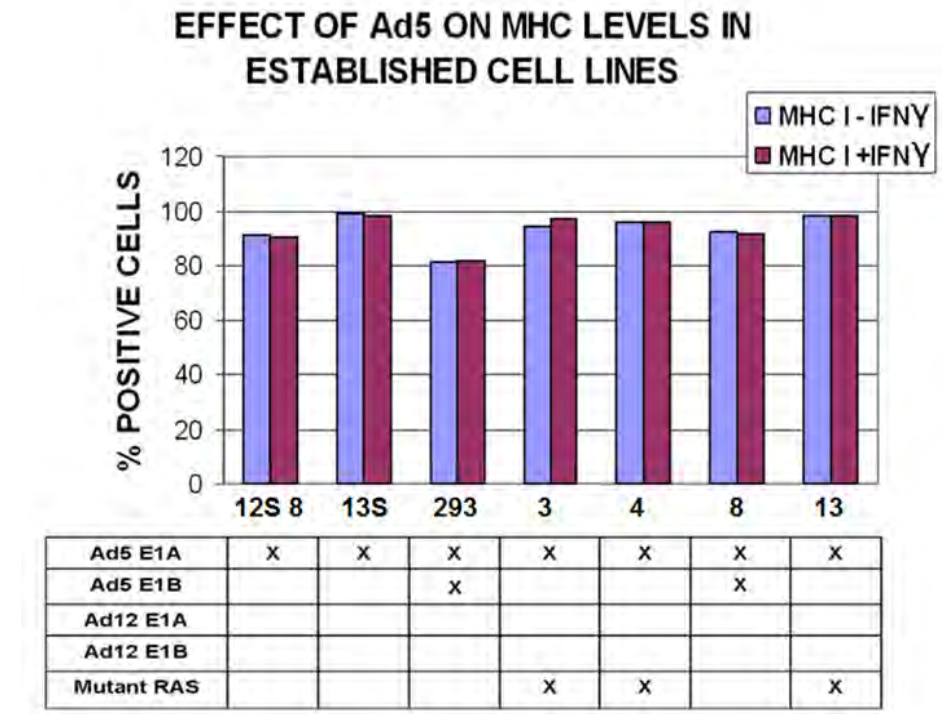
T47D cells were electroporated with the appropriate AdE1A mRNA for 12 hrs followed by another 12 hrs of IFNγ treatment. They were then harvested by trypsinisation, washed in cold PBS and finally resuspended in 50 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). They were stained with 5 µl of mouse anti-human MHC class II antibody for 30 minutes, washed twice with FACS buffer, then resuspended in 400 µl PBS prior to being analysed by flow cytometry. **A.** Ad5E1A transfected cells. **B.** Ad12E1A transfected cells. **C.** Western blot analysis of transfected lysates. Data are means +/- SEM from three repeats. **#, ‡ (P >0.05), * (P<0.05).**

AdE1A and its effect on MHC class I and class II as well as the immunosubunits is summarised in Table 4.2.

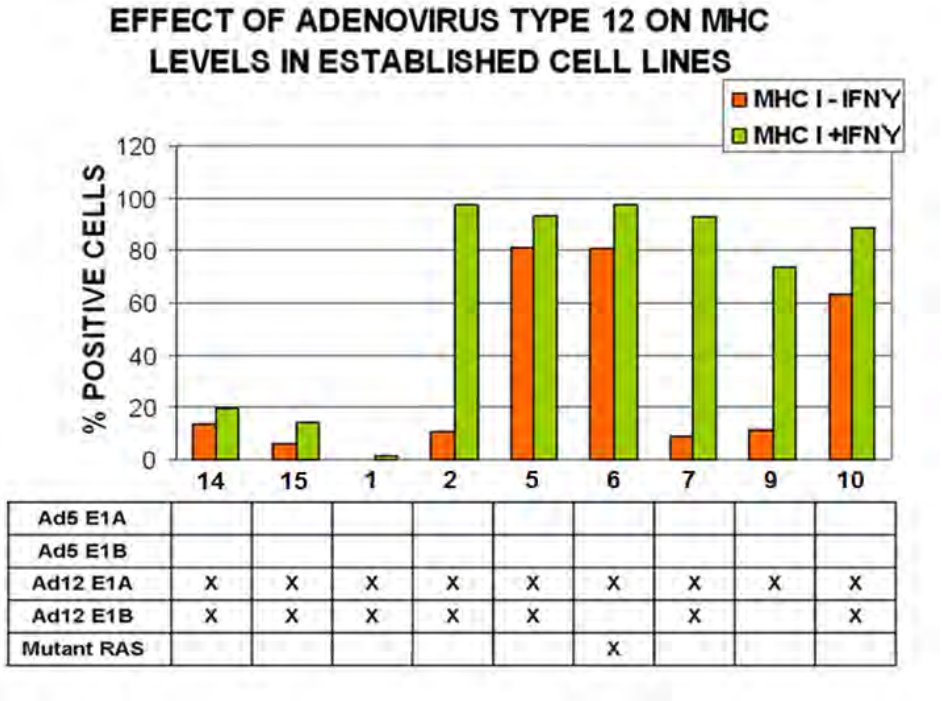
4.2.2.3 The effect of AdE1A on MHC Class I and II levels in adenovirus transformed cell lines

An investigation of the effect of AdE1A on the level of surface MHC class I and II in the adenovirus transformed human retinoblastoma (HER) cell lines was carried out to determine if there is a correlation with the immunosubunit expression. The cell lines were either untreated or treated with 600 U/ml of IFNγ for 48 hrs after which they were harvested and stained with mouse anti-human MHC class I or II antibodies (Serotec). Samples were then analysed by flow cytometry. It was seen that MHC class I was highly expressed on the surface of all the Ad5 transformed cell lines irrespective of IFNγ treatment (Figure 4.14A). However, for Ad12 transformed cells, the situation is rather more complex (Figure 4.14B). These responses can be divided into three groups. In the first, very low levels of surface MHC class I were expressed irrespective of IFNγ such as in cell lines Ad12E1 HER12 (1), Ad12E1 HER2 (14) and Ad12E1 HER10 (15). It was especially marked in cell line Ad12E1 HER12 (1), where there is no expression at all prior to IFNγ treatment. In the second group, expression of MHC class I is very low prior to IFNγ treatment but drastically increased afterwards - cell lines Ad12E1 HER3 (2), Ad12E1 HER5 (7) and Ad12E1A HER283 1 (9). Finally in the third group, MHC class I was highly expressed regardless of IFNγ treatment - Ad12E1 HER 11 (5), Ad12E1A +N ras HER 414 B.2 (6) and Ad12E1A HER283 +Ad12E1B 54k (10), as was seen with the Ad5 transformed cells.

Considering MHC class II, none of the Ad5 or Ad12 HER transformants showed any surface expression with or without IFNγ treatment; however, appreciable MHC class II was detected on the surface of certain human tumour cell lines used as a positive control (Figure 4.15).



A



B

Figure 4.14 MHC class I expression in adenovirus transformed HERs
Adenovirus transformed human embryonic retinoblast cell lines (HER) were treated with 600 U/ml of IFNγ for 48 hrs. They were then harvested by trypsinisation, washed in cold PBS and finally resuspended in 50 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). They were stained with 5 µl of mouse anti-human MHC class I antibody for 30 minutes, washed twice with FACS buffer, then resuspended in 400 µl PBS prior to being analysed by flow cytometry. **A**. Cell surface MHC class I in Ad5 transformed HERs . **B**. Cell surface MHC class I in Ad12 transformed HERs. For the specific cell lines, refer to the legend on figure 4.8.

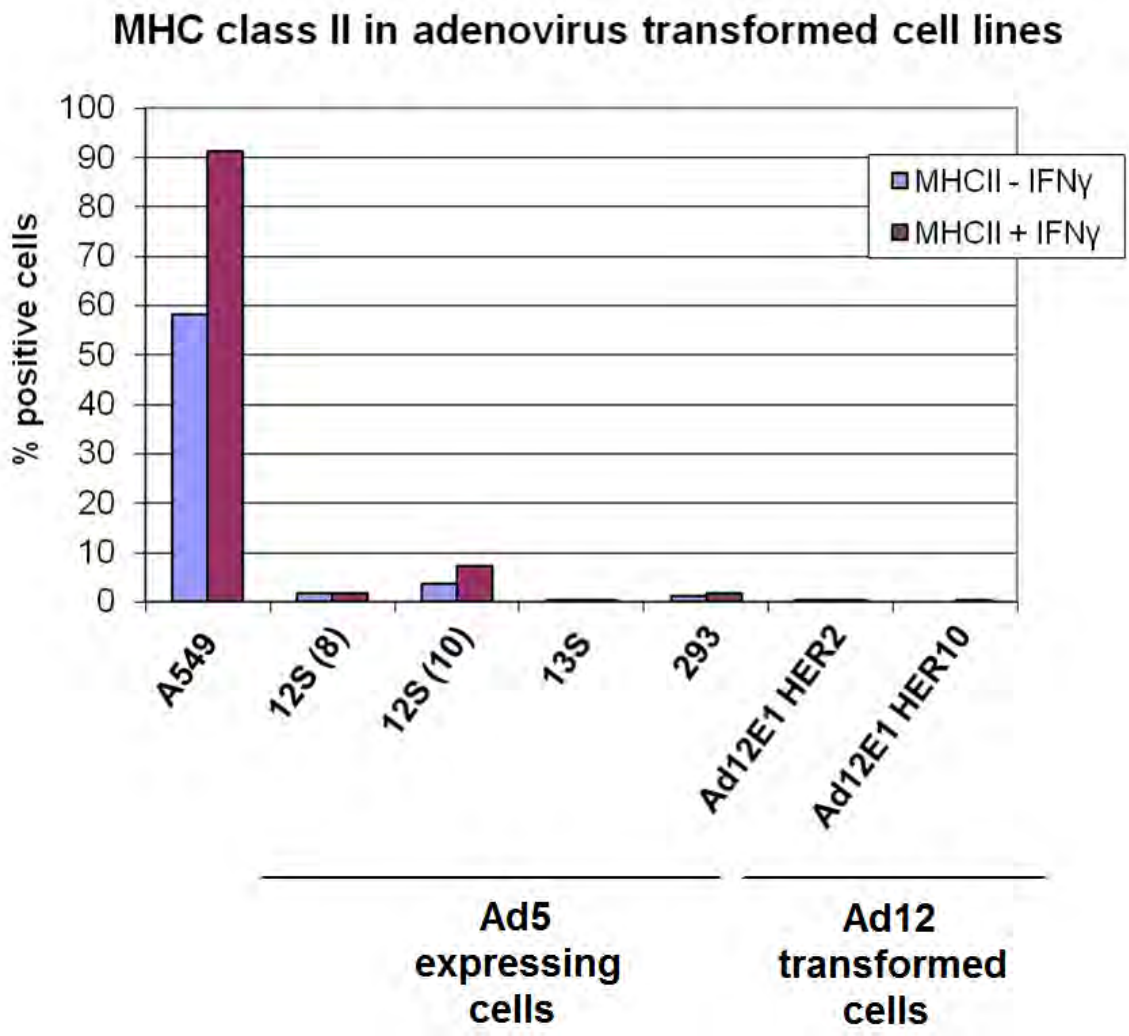


Figure 4.15 MHC class II expression in adenovirus transformed HERs and A549 cells expressing Ad5E1A

Adenovirus transformed human embryonic retinoblast cell lines (HER) and A549 cells were treated with 600 U/ml of IFNγ for 48 hrs. They were then harvested by trypsinisation, washed in cold PBS and finally resuspended in 50 µl FACS buffer (1% BSA, 0.02% sodium azide in PBS). They were stained with 5 µl of mouse anti-human for 30 minutes, washed twice with FACS buffer, then resuspended in 400 µl PBS prior to being analysed by flow cytometry. A549 cell line does not express AdE1A.

	MHC class I		MHC class II	
	Ad5	*Ad12*	*Ad5*	*Ad12*
Adenovirus transformation	=	↓	↓	↓
Adenovirus infection	=	=	=	↓
AdE1A transfection	=	=	↓	↓

	LMP2		LMP7		MECL-1	
	Ad5	*Ad12*	*Ad5*	*Ad12*	*Ad5*	*Ad12*
Adenovirus transformation	=	↓	=	↓	↓	↓
Adenovirus infection	↓	↓	↓	↓	↓	↓
AdE1A transfection	↓	↓	↓	↓	↓	↓

Table 4.2 Summary of the effect of AdE1A on MHC class I, class II and the immunosubunits in transformed, infected and transfected cells. "↓" signifies down-regulation, '=' signifies no change.

4.2.3 AdE1A reduces the transcriptional response to IFNγ in human cells

4.2.3.1 Luciferase assays

To study the effect of AdE1A on the cellular IFNγ response, a reporter plasmid construct - pGAS-luciferase (section 2.3.11) was used. As explained in detail in section 1.5 (JAK/STAT1 pathway), upon IFNγ stimulation, receptors dimerise at the cell surface causing their phosphorylation. This acts as a docking site for STAT1 which, in turn, dimerises and translocates to the nucleus where it can activate transcription of IFNγ inducible genes by binding to promoters that contain the GAS element. pGAS-luciferase reporter constructs can be used to monitor the induction of STAT1 as they possess a STAT1 enhancer element upstream of the firefly luciferase reporter gene (*luc*). Upon STAT1 homodimer binding to the promoter, luciferase enzyme is expressed, which upon substrate addition, enables the activity levels to be measured via a luminometer. The analysis is carried out on cell lysed with passive lysis buffer (PLB, Promega).

4.2.3.1.1 Adenovirus-infected cells

H1299 cells were first transfected with pGAS-luc for 24hrs followed by 6 hr infection with Ad5, Ad12 or Ad5 ΔE3. Cells were then treated with IFNγ for a further 24 hrs. As above, cells were lysed in PLB and luciferase activity measured.

There was very low basal pGAS activity prior to addition of IFNγ. After treatment, there was a massive upregulation of luciferase activity to 210 units in mock infected H1299 cells. Upon infection with Ad5 and Ad5 ΔE3, luciferase activity remained unaffected, however, inhibition of this activity was observed in Ad12 infected cells where it fell by 50% to around 100 units (Figure 4.16).

4.2.3.1.2 AdE1A transfected cells

Finally, the effect of AdE1A transfection on the cellular IFNγ response was studied. pGAS-luc constructs were co-transfected with or without 12SAd5E1A, 13SAd5E1A, 12SAd12E1A, 13SAd12E1A, 12SAd5E1A mutant (L19/20S) and 13SAd5E1A mutant

(L19/20S), for 24 hrs followed by another 24 hrs of IFNγ treatment. pcDNA3 was used as a negative control. The results revealed that AdE1A strongly inhibited luciferase activity, and therefore the IFNγ response, in all cases (Figure 4.17). There was up to 10 fold reduction in luciferase activity observed. Double mutations at residues 19 and 20 did not significantly affect this repressive activity of AdE1A.

4.2.3.1.3 Adenovirus transformed human cells

A number of Ad5 and Ad12 transformed cell lines (from 4.2.2.3) as well as A549 expressing 12SAd5E1A, were transfected with pGAS-luc for 24hrs followed by a further 24 hrs of IFNγ treatment or they were left untreated. The cells were lysed with PLB (Promega). 100 µl of LARII substrate (Promega) was added to this lysate in white optiplate wells. The firefly luciferase activity was measured by luminometer. Data was optimised using transfection of Renilla as a control.

The results showed that the cellular response to IFNγ was greatly reduced in all of the Ad5 and Ad12 transformed cells as well as 12SAd5E1A expressing A549 cells when compared to the original A549 cell line (Figure 4.18). Repression ranged from 85% to 62% down-regulation in Ad12E1 HER2 and Ad5 12S 10 A549 cells respectively.

4.2.3.2 The effect of AdE1A on STAT1 phosphorylation

In light of the above data that showed inhibition of the cellular IFNγ response by AdE1A, the effect of AdE1A on the phosphorylation of STAT1, which is a crucial component of the JAK/STAT1 signalling pathway, was investigated. Lysates from the transfections and infections described above were analysed by western blotting for the expression of phosphorylated (on Serine 727) and non- phosphorylated STAT1. Infection with Ad5, Ad12 and Ad5ΔE3 significantly reduced the level of phosphorylated STAT1 (phospho-STAT1) in comparison to the mock infected H1299 cells (Figure 4.19A). The level of total STAT1 however remained unchanged. Similarly, transfection with 13SAd5E1A down-regulated phospho-STAT1 levels after IFNγ treatment compared to pcDNA3 transfected

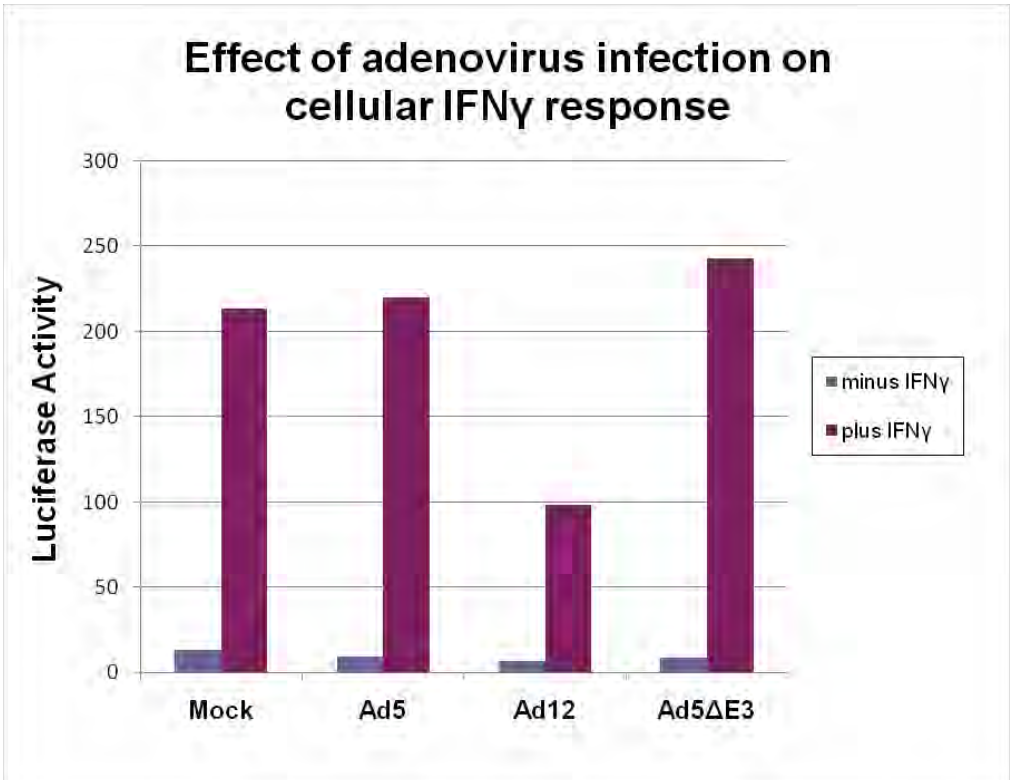


Figure 4.16 Cellular response to IFNγ in adenovirus infected cells.
H1299 cells were transfected with pGAS-luc for 24 hrs followed by 6 hrs of mock, Ad5, Ad12 and Ad5 ΔE3 infection. Cells were then treated with IFNγ for a further 24 hrs. Cells were lysed in passive lysis buffer (Promega). LARII substrate was added to 20 µl of this lysate and luciferase activity measured. The normalized luciferase activity was represented as ratio of Firefly luciferase activity (pGAS-luc) over Renilla luciferase activity (pGL3-Renilla).

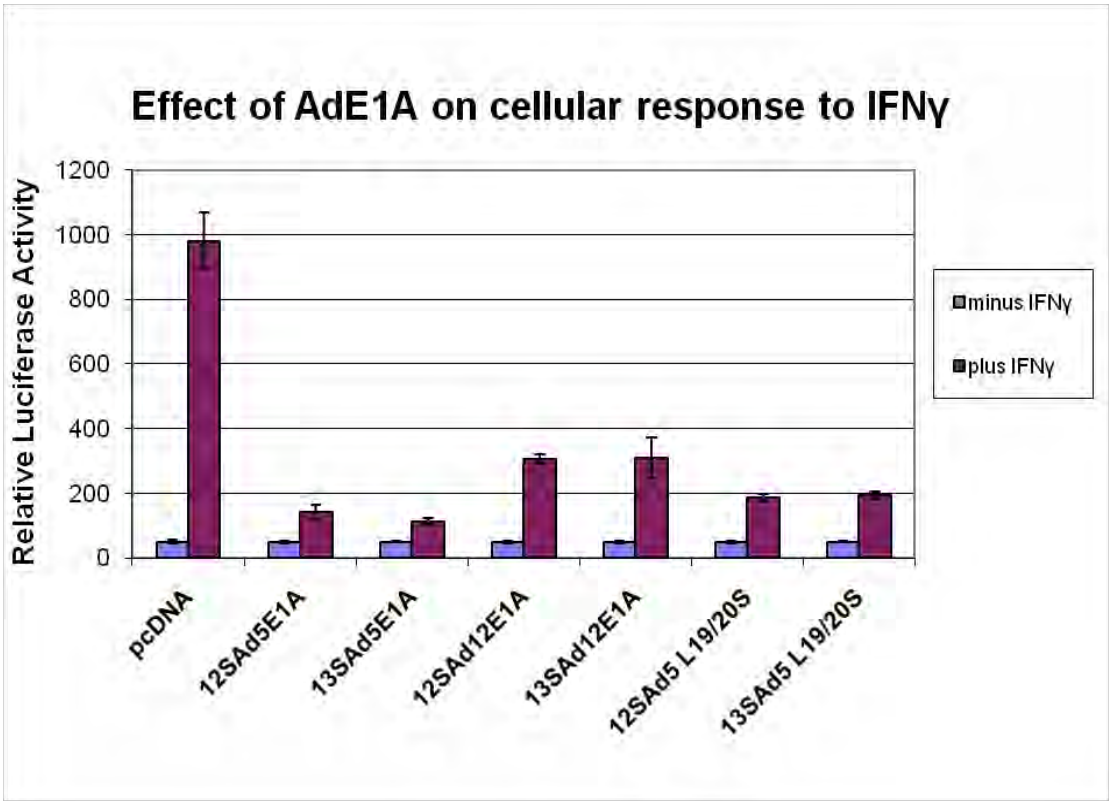


Figure 4.17 Cellular response to IFNγ in AdE1A transfected cells.
H1299 cells were transfected with 1 µg of pGAS luciferase reporter constructs and 1 µg of the appropriate plasmid construct for 24 hrs, followed by a further 24 hrs of IFNγ treatment. Cells were lysed in passive lysis buffer (Promega). LARII substrate was added to 20 µl of this lysate and luciferase activity measured. The normalized luciferase activity was represented as ratio of Firefly luciferase activity (pGAS-luc) over Renilla luciferase activity (pGL3-Renilla).

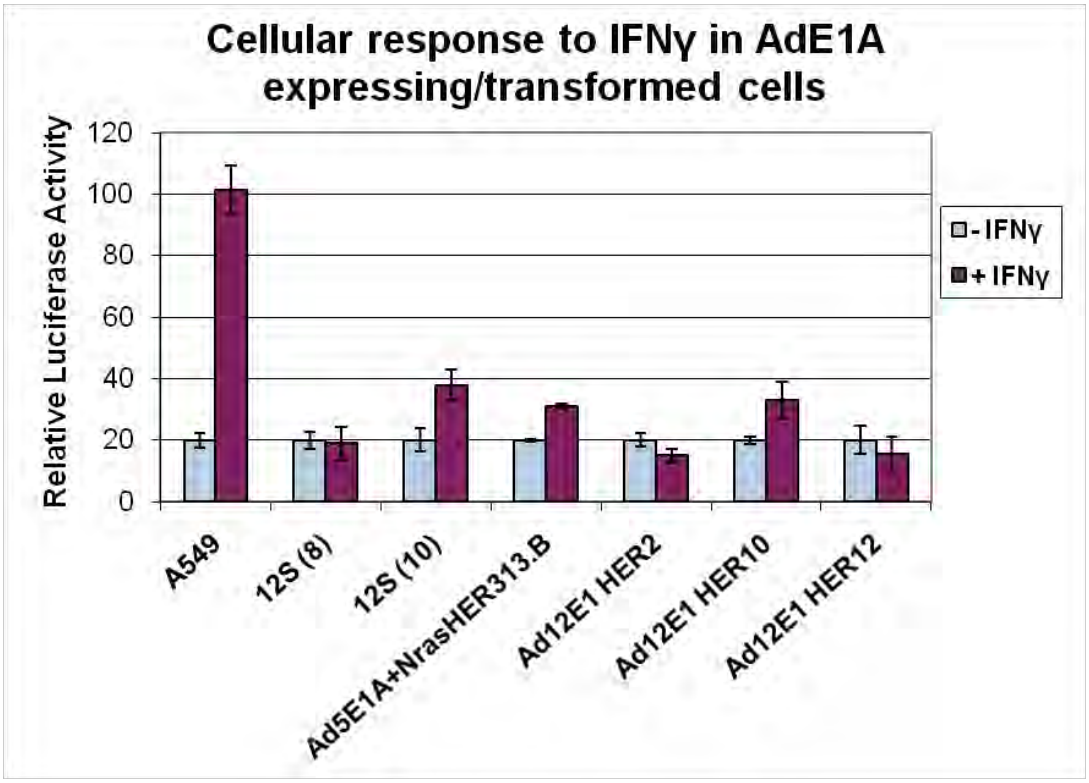


Figure 4.18 Cellular response to IFNγ in adenovirus transformed HERs and A549 cells expressing Ad5E1A

Cells were transfected with 1 µg of pGAS luciferase reporter constructs for 24 hrs, followed by a further 24 hrs of IFNγ treatment. Cells were lysed in passive lysis buffer (Promega). LARII substrate was added to 20 µl of this lysate and luciferase activity measured by luminometer. The normalized luciferase activity was represented as ratio of Firefly luciferase activity (pGAS-luc) over Renilla luciferase activity (pGL3-Renilla).

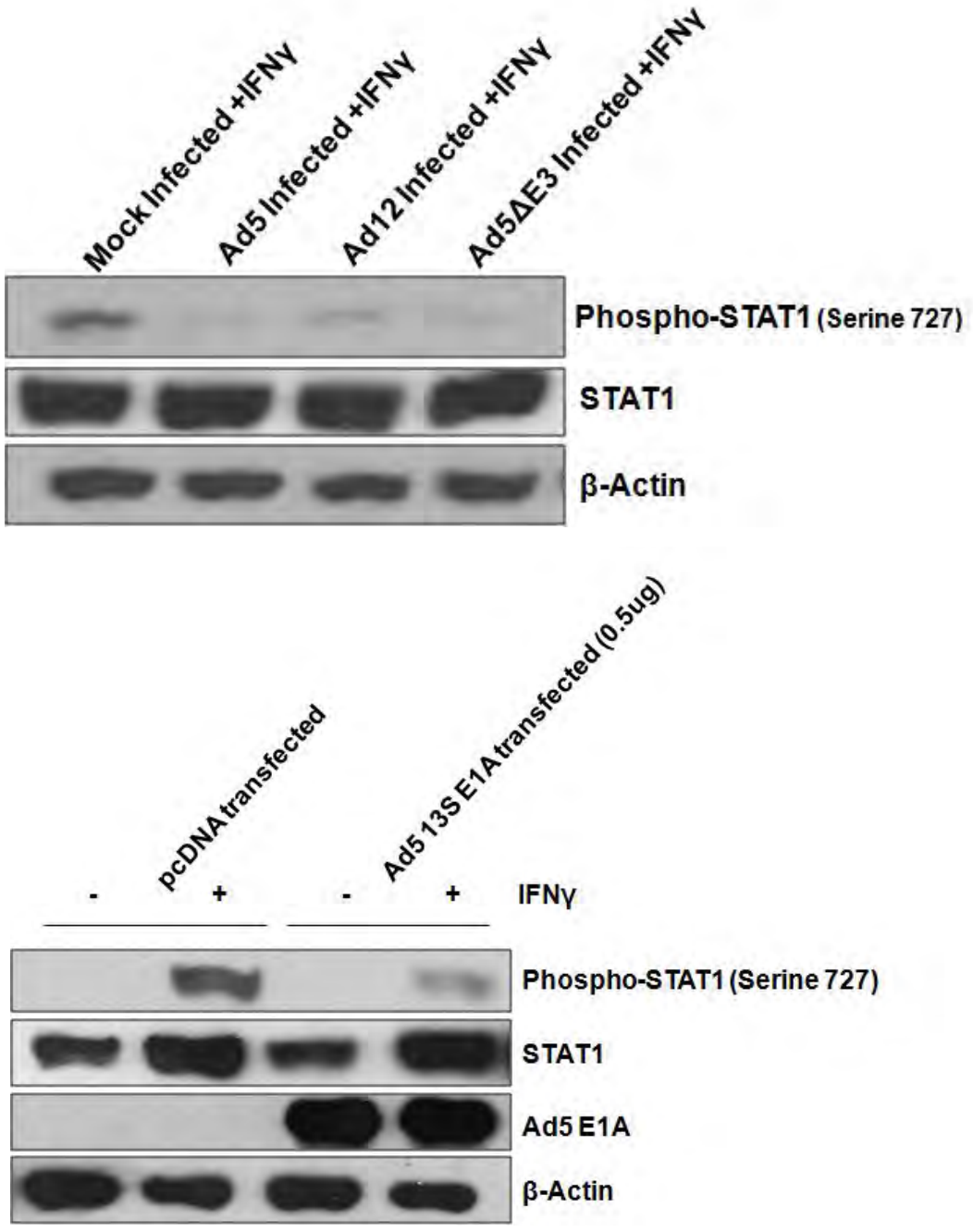


Figure 4.19 AdE1A inhibits the phosphorylation of STAT1 (Serine 727)
H1299 cells were either **A.** infected or **B.** transfected with adenovirus or Ad5E1A respectively for 24 hrs, followed by IFNγ treatment for another 24 hrs. Cells were lysed with 9 M urea, 50 mM Tris (pH 7.3) and 0.15 M β-mercaptoethanol. Thirty microgram of this lysate was analysed by Western blotting to determine proteins levels.

cells (Figure 4.19B); and there is a slight up-regulation of STAT1 levels after treatment with IFNγ.

4.3 Discussion

In this chapter, the effect of AdE1A on three components of the antigen processing machinery: the immunoproteasomes, MHC class I/II and the JAK/STAT1 pathway was examined. This was studied in three systems: in adenovirus transformed cells, AdE1A transfected cells and following adenovirus infections. AdE1A is already known to target various key cellular proteins involved in the regulation of transcription and cell cycle progression (Berk 2005; Gallimore and Turnell 2001). There is also well established evidence that AdE1A targets the proteasome components of the antigen processing machinery (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004; Rasti *et al.,* 2000; Chatterjee-Kishore *et al.,* 2000). In this chapter, we have expanded on these studies to give a further insight into AdE1A interaction with the antigen processing and immune evasion mechanisms.

Adenovirus infection and Immunosubunit expression: Investigations into the effect of adenovirus infection on LMP2, LMP7 and MECL1 levels were conducted. Research up to now, has focused mainly on adenovirus transformed cells and to a limited extent, in the case of AdE1A transfection. It appears that no studies have looked at changes in the immunosubunit levels during adenovirus infection. For this study, examining Ad5, Ad12 and Ad5 ΔE3 infection of H1299 has revealed that all three immunosubunits were strongly down-regulated after infection (Figure 4.1). The observation that the same level of inhibition was observed during *w.t.* and Ad5 ΔE3 infection demonstrates that the viral E3 proteins had little effect (section 1.3.5.3). AdE3 transcription unit encodes at least four proteins: E3-gp19K, E3-14.7K, E3-10.4K/14.5K and E3-11.6K. They have various immunosubversive functions such as down-regulation of MHC class I (Burgert *et al.,* 1987; Wold *et al.,* 1999) and inhibition of tapasin (Bennett *et al.,* 1999), in a manner such that

recognition of the infected cell by the immune system is negated. The purpose of the inclusion of E3 deletion mutant in this study is to rule out its involvement in the observed experimental data. There is also a fifth E3 protein called E3-49K, that has only recently been discovered, and only expressed by Ad19a adenovirus of the subgenus D, causing epidemic keratoconjunctivitis in AIDS patients (Blusch *et al.,* 2002; Windheim and Burgert 2002). However, its function is yet to be determined.

In the experiment shown in Figure 4.1, cells were infected with adenovirus prior to IFNγ treatment, or treated with IFNγ prior to adenovirus infection. Interestingly, it was found that for IFNγ pre-treated cells, infection with Ad5, Ad12 or Ad5 ΔE3 had no effect on the expression of LMP2, LMP7 and MECL-1 at 24hrs (Figure 4.2). The level of infection (as indicated by the AdE1A expressed) was significantly reduced. This is consistent with a previous study that demonstrated that IFNγ pre-treatment inhibits adenovirus replication (Mistchenko and Falcoff 1987). Perhaps, unsurprisingly, treatment with IFNγ increases the cell's defence mechanism making it more resistant to viral replication, hence hindering the ability of adenovirus to affect immunosubunit expression. However, prolonged infection to 48hrs began to inhibit the immunosubunit expression in comparison with mock infected cells (Figure 4.3).

It is well established that adenovirus targets components of the antigen processing machinery such as MHC class I, 20S proteasome, TAP proteins and, as it has been shown here, also targets the immunoproteasome. A previous study has revealed that the immunoproteasome processed the adenovirus early region 1B 19K protein (E1B)-derived epitope $E1B_{192\text{-}200}$ with increased efficiency leading to detection of the virus (Sijts *et al.,* 2000). This group produced a tetracycline-regulated mouse cell line that allowed titratable formation of immunoproteasomes. When the immunoproteasomes were highly expressed prior to infection, it produced optimal cytotoxic T cell (CTL) activation hence the recognition of the infected cell by the immune system. In the light of the above data, one can conclude that AdE1A may preemptively target immunoproteasome expression early in

infection, affecting its activity and hence avoiding recognition by the immune system. Interestingly, in addition to its role in producing epitopes of viral origin for MHC class I presentation, recent findings show that immunoproteasomes may also have a role in the maintenance of protein homeostasis and preservation of cell viability in response to interferon-induced oxidative stress (Seifert *et al.,* 2010). This might be prejudicial to viral replication and is a subject for future investigation.

AdE1A transfection and Immunosubunit expression: The effect of AdE1A on LMP2, LMP7 and MECL1 expression following transfection into cells was also examined. Data so far revealed down-regulation of LMP2 transcription by adenovirus AdE1A (Chatterjee-Kishore *et al.,* 2000). In this study, this was extended to examine LMP7 and MECL1. The non small lung carcinoma cell line (H1299) was found to be highly inducible by IFNγ to express LMP2, LMP7 and MECL1. Transfection experiments followed by western blotting analysis revealed that there was an inhibition of expression of all three immunosubunits in all of 12SAd5E1A, 13SAd5E1A, 12SAd12E1A and 13SAd12E1A transfections (Figure 4.6). Similarly, this inhibition varied in proportion to the amount of AdE1A expressed in the cell (Figure 4.7), where the higher the AdE1A expression, the greater the inhibition, supporting the proposition that AdE1A was responsible for this down-regulation. Furthermore, both 12SAdE1A and 13SAdE1A have caused inhibition, indicating that CR3 is not solely responsible. As mentioned before, these data contradict the outcome from the previous experiment conducted using 12SAdE1A and 13S AdE1A expressing A549 cells, which showed inhibition in the 13SAd5 expressing cell line only. However, research by Routes *et al.,* (1996) has revealed that E1A mutants that do not express CR3 were able to impair IFN-stimulated gene expression whereas mutants with deletions in the CR1 and N-terminal region were unable to block IFN-stimulated gene expression, supporting the premise that CR3 is not involved. Later experiments also point towards this inhibition being due to AdE1A interfering with the JAK/STAT1 pathway rather than being as a result

of direct AdE1A interaction with the immunosubunits; this will be discussed later in this chapter. Similarly, Ad5 and Ad12 have also both demonstrated similar levels of inhibition.

Adenovirus transformation and Immunosubunit expression: Prior to this study, research on transformed rat cells had suggested down-regulation of LMP2, LMP7 and MECL1 expression in Ad12 transformed but not in Ad5 transformed cells (Vertegaal *et al.,* 2003, Rotem-Yedudar *et al.,* 1996). However, there had been no studies investigating immunosubunit expression in human adenovirus transformed cells. To investigate this further, a panel of transformed human retinoblast (HER) cell lines with different expression profiles of AdE1A, AdE1B and activated (mutant) ras was used (Gallimore *et al.,* 1986; Byrd *et al.,* 1982, 1988). Western blot analysis on these lines has shown that LMP2, LMP7 and MECL1 expression was down-regulated in the lines that express Ad12E1A but not Ad5E1A (Figure 4.8A). The other proteins, namely AdE1B and ras, did not contribute to this trend. This observation was consistent with the previous studies on transformed rat cells which also showed that Ad12 transformed lines had downregulated immunosubunit expression in comparison to Ad5 transformed lines (Vertegaal *et al.,* 2003, Rotem-Yedudar *et al.,* 1996). A549 cells that constitutively express either 12SAd5E1A (12S 10) or 13SAd5E1A (13S and 13S G418) were analysed for immunosubunit expression following IFNγ treatment (Figure 4.9). There was an inhibition of immunosubunit expression with both 12SAd5E1A and 13SAd5E1A expressing cells. However, this contradicts data from the panel of transformed HER cell lines that showed down-regulation only in the Ad12 E1 transformed cells. The reason for this is not clear. However, the 12S 8 and 12S 10 cells used in the present study were of very high passage in comparison to the HER cell lines. Long-term culturing of the cells may have altered the cellular protein expression profile and clone stability due to potential cumulative mutations rather than it being due to the effect of AdE1A. Perhaps earlier passages of these cell lines may be used in a future experiment to re-examine immunosubunit expression.

Subsequently, our study also looked at the role of AdE1A in the regulation of cell surface levels of MHC class I and MHC class II. MHC class I is expressed constitutively on the surface of all cells (York and Rock 1996) whereas MHC class II is expressed mostly on antigen presenting cells of the immune system (Benoist and Mathis 1990; Steimle *et al.,* 1994); while it can be induced in other cells by exposure to IFNγ (Steimle *et al.,* 1994). Like the immunoproteasome subunits, MHC class II expression is induced via the JAK/STAT1 pathway (Gough *et al.,* 2008) (section 1.5, Figure 1.13). Activation of the JAK/STAT1 pathway leads to the expression of the master MHC class II regulator CIITA, which in turn initiates the transcription of MHC class II with the aid of 19S proteasome ATPases and other transcription factors (Bhat and Greer 2011). This transcription is illustrated in Figure 1.14.

Adenovirus infection and MHC class I and II surface expression: The effect of adenovirus infection on MHC class I and class II expression was investigated. Past studies have indicated that infection of cells with Ad5 down-regulated MHC class I expression (Burget *et al.,* 1985, 1987; Wold *et al.,* 1999; Lippe *et al.,* 1991). This has been attributed to adenoviral E3-gp19K inhibiting glycosylation and transport of newly synthesized MHC class I from the endoplasmic reticulum to the plasma membrane (Burgert *et al.,* 1985, 1987; Wold *et al.,* 1999; Lippe *et al.,* 1991). In the present study, this was revisited to include an Ad5 mutant virus that has the E3 gene deleted in addition to Ad5 and Ad12 *w.t.* virus infections. Infection of A549, HCT116, tonsil epithelial cells and T47D cells (Figures 4.10 A-D) with Ad5, Ad12 and Ad5 ΔE3 viruses had no effect in the surface levels of MHC class I with or without the treatment of IFNγ. This result conflicts with previous data that showed down-regulation of MHC class I by *w.t.* Ad5 (Burget *et al.,* 1987; Wold *et al.,* 1999; Lippe *et al.,* 1991). However, not all the studies were contradictory; for example, work by Routes and Cook (1990), using ten fibroblastic, epithelial (including A549) and lymphoid cell lines that have been infected by Ad2/5 adenovirus revealed that, with the exception of the Ad5E1A transformed 293, all of the cell lines maintained their surface

MHC class I expression until cell death is imminent. The reason for this is still not clear but these workers suggest that subclones of certain cell lines may be more sensitive to the effects of E3-19K than others. Similarly, in some mouse cells (which are semi-permissive for Ad infection), Ad5 infection resulted in an increased transcription rate of MHC class I mRNA (Rosenthal *et al.,* 1985) which may mask the inhibitory effect of E3-19K. Additionally, infection of mouse cells with mouse adenovirus type 1 (MAV-1) did not affect the expression of 10 different mouse class I MHC allotypes (Kring and Spindler 1996). The exact role of AdE1A in MHC class I regulation was also studied by looking into AdE1A transfection. This will be addressed later in this discussion.

There appears to have been no previous studies examining the effect of adenovirus infection on MHC class II levels on the cell surface. Only one study (that looked at the accumulation of HLA class II mRNA in Ad5E1A transfected cells) reported an inhibition in the induction of MHC class II genes (Ackrill *et al.,* 1991). In the present study, T47D cells and tonsil epithelial cells were mock, Ad5, Ad12 and Ad5 ΔE3 infected and then stimulated with IFNγ prior to analysis by flow cytometry. In both cases, a strong reduction of cell surface MHC class II expression by Ad12 was observed, compared to Ad5 and Ad5 ΔE3 (Figure 4.11). T47D cell infection with Ad5 and Ad5 ΔE3 left MHC class II expression unaffected. In tonsil epithelial cells there was a small but significant decrease in cell surface MHC class II level upon Ad5 infection. The expression was restored in the Ad5 ΔE3 infection (Figure 4.11). Tonsil epithelial cells are primary cells so they may be more sensitive to the effects of infection compared to the tumour cell line T47D. The restoration of MHC class II upon Ad5 ΔE3 may indicate E3 involvement; however, there is no research to date implicating E3 in MHC class II down-regulation. Oncogenic Ad12 down-regulating MHC class II expression during infection may correlate with its capacity to inhibit MHC class I expression in the transformed cell lines, however, this result was not in agreement with the outcome in the adenovirus transformed and AdE1A transfected cell lines, where MHC class II expression was low in all cases.

AdE1A transfection and MHC class I and II surface expression: In adenovirus infections and Ad transformed cell lines, AdE1A may or may not cooperate with other viral proteins to give the observed effect. The involvement of other proteins can be ruled out by examining AdE1A transfection in isolation. mRNA electroporation is a technique which is highly efficient (with high viability) in gene delivery, and is superior to lipofection or electroporation of plasmid cDNA (Van Tendeloo *et al.,* 2001), although it is more labour intensive. Primary fibroblasts and the IFNγ inducible T47D cell line were used to study the effect of the electroporation of AdE1A on MHC class I and class II, respectively. Previous research has been heavily focused on MHC class I levels in adenovirus transformed and infected cells. In the present study, previous findings were complemented by examining AdE1A transfection and the effect this may have on MHC class I and class II expression. Primary human fibroblasts were electroporated with mRNA encoding Ad512SE1A, Ad513SE1A, Ad1212SE1A, Ad1213SE1A, Ad512SE1A mutant (L19/20S) and Ad513SE1A mutant (L19/20S). The surface level of MHC class I remained unchanged following electroporation (Figure 4.12). With the exception of Ad12 transformed cells, so far it was observed that AdE1A transfection, infection and Ad5 transformation had no effect on surface MHC class I levels (summary in Table 4.2). MHC class I is expressed constitutively in all somatic cells. This may indicate lack of AdE1A involvement in MHC class I repression or it may indicate that AdE1A cannot affect expression when MHC is expressed constitutively but may do so in the case of inducible expression as evidenced in MHC class II expression in T47D cells. Electroporation of T47D cells gave a different MHC class II profile. AdE1A significantly down-regulated MHC class II surface expression in Ad512SE1A and Ad513SE1A transfected cells, although the introduction of a double mutant at residues 19 and 20 relieved the inhibition (Figure 4.13A). Although mRNA electroporation is highly efficient, one disadvantage is that obtaining equal expression between the different mRNA contructs is very difficult. RNA is highly sensitive to degradation and exhibits different levels of expression depending on its stability. So the western blot data (Figure 4.13C) was taken into account when considering

the effect of AdE1A on MHC class II levels. No significant down-regulation of MHC class II expression was observed in Ad1212SE1A and Ad1213SE1A transfected cells. Perhaps, it would be interesting to repeat this experiment using an alternative method of delivering the AdE1A genes, so as to ensure this is not due to problems with mRNA quality or electroporation efficiency.

The double mutations in residues 19 and 20 of AdE1A are located in the N-terminal region, which is important in mediating the transcriptional regulation effects of AdE1A. This region was also found to be involved in binding of AdE1A to STAT1 (Look *et al.,* 1998) a component of the JAK/STAT1 pathway- see section 1.5, impairing the IFN-mediated gene expression (Routes *et al.,* 1996). This may suggest that the double mutations at the N-terminal region have affected the ability of AdE1A to bind to STAT1 and hence reduced its repression of MHC class II. The activation of JAK/STAT1 pathway leads to the expression of various genes crucial for initiation of immune response and the resistance of cells to viral infection. Proteins expressed in response to IFNγ include MHC class II and the immunoproteasome subunits LMP2, LMP7 and MECL1. In fact, the down-regulation of LMP2, LMP7 and MECL1 as well as MHC class II observed in this study may be attributed to AdE1A targeting of JAK/STAT1 pathway via STAT1. The effect of AdE1A on the phosphorylation of STAT1 will be discussed below.

AdE1 transformation and MHC class I and II surface expression: Prior to this study, there has been an extensive research on MHC class I levels in adenovirus transformed rodent cell lines, where it was established that Ad12 transformed cells have down-regulated surface MHC class I levels in comparison to Ad5 transformed cells (Schrier *et al.,* 1983; Ackrill and Blair 1988; Friedmann and Ricciardi 1988). Limited studies on human cells transformed with AdE1 have also been presented. They showed similar results with MHC class I surface expression greatly reduced in Ad12 transformed cells (Bottley *et al.,* 2005; Vasavada *et al.,* 1986). Bottley *et al.,* (2005) utilized three Ad5 and three Ad12 transformed human retinoblast (HER) cell lines whereas in the study by Vasavada *et al.,*

1986, adenovirus transformed human embryonic kidney (HEK) cells were used. In the present study, the panel of Ad5 and Ad12 transformed HER cell lines was enlarged to include those expressing different combinations of AdE1A, AdE1B or mutant (activated) ras. Consistent with previous data, it was observed that there was no down-regulation of MHC class I in any of the Ad5 transformed human cells irrespective of the presence of IFNγ (Figure 4.14A). However, in the case of the Ad12 transformed cells, the situation is more complex (Figure 4.14B). The majority of the lines (6 out of 9) expressed very low MHC class I levels prior to IFNγ treatment as in the case of cell lines Ad12E1 HER12 (1), Ad12E1 HER3 (2), Ad12E1 HER5 (7), Ad12E1A HER283 1 (9), Ad12E1 HER2 (14) and Ad12E1 HER10 (15). This was in agreement with past data that showed similar results (Bottley *et al.,* 2005; Vasavada *et al.,* 1986). Additionally, three lines had their MHC class I fully restored following IFNγ treatment - Ad12E1 HER3 (2), Ad12E1 HER5 (7) and Ad12E1A HER283 1 (9) - while the rest showed little or limited induction. This induction by IFNγ is consistent with a previous study that showed similar results in transformed rat cell lines (Ackrill and Blair 1990, Eager *et al.,* 1989). In the remaining three cell lines MHC class I is highly expressed regardless of IFNγ: Ad12E1 HER 11 (5), Ad12E1A +N ras HER 414 B.2 (6) and Ad12E1A HER283 +Ad12E1B 54k (10). It seems that in these three cell lines Ad12E1A did not suppress MHC class I expression. The reason for this is not clear but it may be attributed to the differences between the different sub-clones of HER cells in terms of sensitivity to AdE1 expression.

There appears to have been no research so far investigating the effect of adenovirus transformation on MHC class II expression. In this study, for the first time, MHC class II surface level expression in Ad5 and Ad12 transformed cell lines were addressed. Four Ad5E1A expressing, two Ad12 transformed cell lines and the A549 tumour cell line were analysed for MHC class II cell surface expression in the absence or presence of IFNγ (Figure 4.15). In contrast to MHC class I, MHC class II surface expression levels were

very low in both Ad5 and Ad12 transformed lines in comparison to the A549 cell line which had high expression of MHC class II, especially after IFNγ treatment.

Retrospectively examining the effects of AdE1A on the surface MHC class I and class II levels against the backgrounds of adenovirus transformation, infection and AdE1A transfection have revealed some inconsistencies. Data so far from the present study, with regards to the effect of AdE1A on MHC class I, class II and the immunosubunits is summarised in Table 4.2. MHC class I levels in Ad12 transformed cells were down-regulated whereas it remained unchanged in cells infected or transfected by Ad12 virus and Ad12E1A respectively. Each of the three systems offer very different settings, and thus not strictly comparable. For instance, adenovirus infection involves the co-operation of different viral proteins; transfection enables the lone expression of AdE1A within a cell; and cell transformation occurs when cells fail to support lytic infection and there is chance of integration and expression of the early region AdE1A and AdE1B genes (Ricciardi 1999). Additionally, in each case, the same set of viral proteins may not be expressed. For instance, during Ad5 infection, E3 impairs the transportation of class I molecules to the cell surface (Wold and Tollefson 1999; Williams *et al.,* 2004) leading to the down-regulation of MHC class I, whereas in Ad5 transformants, no E3 is expressed so the levels of MHC class I remain unchanged (Williams *et al.,* 2004). Hence, different outcomes on MHC levels can be observed with the same serotype. Another study addressing the effect of AdE1A on insulin receptor substrate 4 (IRS-4), have shown that IRS-4 was only detected in 13SAd5E1A-expressing A549 cells but not in Ad12E1A and 12SAd5E1A expressing cells (Shimwell et al 2009). However, A549s transfected or infected with AdE1A and Ad5 w.t. virus respectively had no effect on their IRS-4 expression (Shimwell et al 2009). It was found that low passage 13SAd5E1A-expressing A549s do not express IRS-4 but over time in culture expression was markedly increased. It was suggested that stable but not transient expression of 13SAd5E1A is required for IRS-4 overexpression (Shimwell et al 2009). This concept may be applied to this study with regards to MHC

class I expression. It can be hypothesized that stable expression of Ad12E1A in the transformed cells caused down-regulation of MHC class I surface expression, while it remains unchanged in the infected and transfected cells. In addition, certain aspects of the experiments may be improved in future work to investigate if the same outcome will be observed. For instance, during flow cytometry analysis, dual staining (for AdE1A and MHC class I/II) may be suggested in order to examine MHC surface levels in selected AdE1A positive cells after infection and transfection. The fact that no change in MHC class I levels was observed in all Ad5/Ad12 transfected and infected cells may be due to the fact that any significant effect on MHC class I levels is diluted within a whole population of AdE1A-negative cells. Attempts were made, in this study, at dual staining of MHC class I and AdE1A for flow cytometry. However, there were problems obtaining optimum cell permeabilisation that enabled intracellular staining with anti-AdE1A antibodies. Cell permeabilisation methods included using 0.5% saponin and IntraPrep™ Permeabilization Reagent kit (Beckman Coulter).

On the other hand, infection by Ad5 has not caused any effect on MHC class II surface expression, whereas there was down-regulation after transfection and transformation. As mentioned above, this may also be attributed to the differences between the three backgrounds; however, additional experiments using increasing viral titre may be required to investigate this further.

Comparing Ad5 and Ad12, the latter is highly oncogenic whereas the former is not (Trentin *et al.,* 1962; Yabe *et al.,* 1962, 1964). This correlates with Ad12 ability to down-regulate MHC class I in the adenovirus transformed cells (Schrier *et al.,* 1983; Ackrill and Blair 1988; Friedmann and Ricciardi 1988) enabling the cell to escape immune system recognition and thus contributing to the induction of tumours in new-born rodents (Trentin *et al.,* 1962; Yabe *et al.,* 1962, 1964). In the present study, it was found that Ad12 transformation also down-regulated LMP2 and LMP7 immunosubunits after transformation whereas Ad5 did not have any effect. Past studies have attributed Ad12 oncogenicity to a 20 amino acid alanine-rich segment that is unique to the group A viruses (Telling and

Williams 1994). This “oncogenic spacer” region in situated between CR2 and CR3 and it is missing entirely in Ad5E1A. The group have shown an experiment where they produced a chimeric virus where the spacer region in Ad12 is replaced by the equivalent cassette from Ad5. This chimeric virus was defective for tumour induction in rats hence showing that this spacer region is an oncogenic determinant of Ad12 (Telling and Williams 1994). However, it is not entirely clear how this region influences oncogenicity, but it is thought that it may not be acting alone; as further studies with chimeric viruses have revealed that, though the spacer region is essential for tumourigenicity, it was not responsible for MHC class I down-regulation (Williams *et al.,* 2004).

AdE1A, cellular response to IFNγ and STAT1 phosphorylation: Previous studies have shown that STAT1 is reduced in AdE1A expressing cells although this does not appear to be the case here (Leonard and Sen 1996). STAT1 binds to the pGAS promoter and associates with CBP/p300 which in turn recruits the basal transcription complex (Zhang *et al.,* 1996) to initiate transcription. Through its N-terminal region, AdE1A binds to STAT1 disrupting its association with CBP/p300 and hence inhibiting transcriptional initiation (Look *et al.,* 1998). Another way AdE1A targets STAT1 function is by binding to CBP/p300 (Zhang *et al.,* 1996). Both AdE1A and STAT1 bind to the same domain of CBP/p300. This limits STAT1 from associating with CBP/p300 (Zhang *et al.,* 1996; Look *et al.,* 1998). In short, considering that the response to IFNγ via the JAK/STAT1 pathway is what up-regulates expression of the immunosubunits and MHC class II, and having been shown by past studies that AdE1A targets STAT1; one may conclude that the down-regulation of the immunosubunit and MHC class II expression observed in the present study may be due to AdE1A interference with the progression of the JAK/STAT1 pathway. To test the hypothesis that AdE1A affects the cell’s ability to respond to IFNγ, luciferase assays using a construct possessing the pGAS promoter were conducted, in transformed, infected and transfected cells. In transformed cells (Figure 4.18) there was a large decrease in IFNγ response in all of the Ad5 and Ad12 transformed cells compared to the tumour cell line

A549. However, in H1299 cells infected with Ad5, Ad12 and Ad5 ΔE3, it was shown that pGAS activity was reduced with Ad12 infection while it remained unchanged in mock, Ad5 and Ad5 ΔE3 infected cells (Figure 4.16). This result seems to correlate with the effect of AdE1A on MHC class II surface expression (Figure 4.11), hence it can be concluded that the reduction in MHC class II expression observed in that experiment was due to the inhibition of the cellular response to IFNγ. This however, contradicted the data from the transfected and transformed cells (Figure 4.17 and 4.18) where cellular IFNγ response was inhibited with both Ad5 and Ad12E1A (see below).

H1299 cells that were electroporated with mRNA have shown a large reduction of IFNγ response in the presence of Ad512SE1A, Ad513SE1A, Ad1212SE1A, Ad1213SE1A, Ad512SE1A mutant (L19/20S) and Ad513SE1A mutant (L19/20S) (Figure 4.17). In contrast to the previous data that showed weakening of AdE1A inhibition of MHC class II as a result of the double mutations at the N-terminal region, here the same mutants have demonstrated equal inhibition as the wild type AdE1A. This may indicate that AdE1A could inhibit MHC class II expression via other mechanisms. To complement the above data, the lysates from the experiments were analysed by Western Blotting to examine the effect on STAT1 phosphorylation. The data revealed that in both adenovirus infected and Ad5E1A transfected cells, while unphosphorylated STAT1 levels remained constant, there was an inhibition of phosphorylation of STAT1 (Figure 4.19 A and B). This is consistent with previous research on STAT1 and AdE1A (Look *et al.,* 1998).

In this chapter, the role of AdE1A in affecting parts of the antigen processing pathway, specifically on the expression of immunoproteasomes and MHC class I and II has been examined. The data showed that AdE1A down-regulates the expression of the immunosubunits LMP2, LMP7 and MECL1 in infected and transformed cells, whereas in transformed cells only Ad12 seemed to control expression. MHC class I surface expression remained unaffected following transformation, transfection and viral infection, except in Ad12 transformed cell lines where there was a down-regulation. MHC class II

surface expression was reduced following transformation, infection and transfection with both Ad5 and Ad12 AdE1A with the exception of Ad5 infection where MHC class II expression remained unchanged. And finally, AdE1A inhibited the ability of cells to respond to IFNγ by targeting STAT1. All this is just further evidence of the role of AdE1A in disrupting the antigen processing machinery in order to hinder recognition by the immune response. Data from this chapter is summarized in Table 4.2.

CHAPTER 5

THE EFFECT OF AdE1A ON IMMUNOPROTEASOME ACTIVITY AND ANTIGEN PRESENTATION

5.1 Introduction

The replacement of the constitutively expressed β1, β2 and β5 of the 20S proteasome by the IFNγ-inducible LMP2, MECL-1 and LMP7 protein subunits respectively to form the immunoproteasome (Hisamatsu *et al.,* 1996) leads to an adjustment of the cleavage preferences leading to a different spectrum of peptides being produced, that may potentially enhance antigen presentation to CTLs (Groettrup *et al.,* 2001; Ehring *et al.,* 1996; Gaczynska *et al.,* 1996; Cascio *et al.,* 2001; Cerundolo *et al.,* 1995). β1, β2 and β5 possess "caspase-like" activity which cleaves after acidic residues, "trypsin-like" activity that cleaves after basic residues and "chymotrypsin-like" activity that cleaves after aromatic and hydrophobic amino acids respectively (Groettrup *et al.,* 2001). Replacement by the immunosubunits, results in the enhancement of the "chymotrypsin-like" activity and the abrogation of the "caspase-like" activity (Groettrup *et al.,* 2001, Gaczynska *et al.,* 1994; Scmidtke *et al.,* 1998). Immunoproteasomes are more efficient at generating epitopes derived mainly from viral proteins (reviewed by Van den Eynde and Morel 2001), which may partly explain their expression being only in the presence of IFNγ. However, recent research has revealed that immunoproteasome may have other roles. A study by Seifert *et al.,* 2010 have shown that immunoproteasomes contributes in the maintenance of protein homeostasis and thus preservation of cell viability upon IFN-induced oxidative stress. Immunoproteasomes are known be targeted by viruses. Previous research had shed light on the various viral proteins that have suppressed individual immunosubunits at the transcriptional level, potentially affecting the total immunoproteasome composition and activity, as in for instance, HPV type 18 (Georgopoulous *et al.,* 2000) and HIV-1 p24 (Steers *et al.,* 2009). Others have targeted immunosubunits by direct binding such as in NS3 non-structural protein of the hepatitis C virus, that binds to LMP7 (Yee-Ling Khu *et al.,* 2004); and HIV-1 tat protein that can bind directly to LMP7 and MECL-1, as a result repressing its catalytic activity (Apcher *et al.,* 2003).

AdE1A is a multifunctional viral protein involved in the interaction with multiple cellular proteins involved in the regulation of transcription, DNA synthesis, cell differentiation and cell cycle progression (Gallimore and Turnell 2001, Frisch and Mymryk 2002) as well as aspects of the antigen processing machinery such as the TAP proteins (Proffitt and Blair 1997; Rotem-Yehudar 1994, 1996) MHC class I (Bottley *et al.,* 2005; Vasavada *et al.,* 1986; Schrier *et al.,* 1983; Ackrill and Blair 1988) and the immunoproteasome (Rotem-Yehudar 1996; Vertegaal et al 2003; Chatterjee-Kishore *et al.,* 2000). In chapter 3, the repression of the immunosubunit expression by adenovirus protein AdE1A was observed in all of the infected, transfected and adenovirus transformed cells. Subsequently, in chapter 4, it was seen that AdE1A binds to the immunoproteasome, specifically to the immunosubunit MECL-1. The AdE1A regions as well as the binding sites and residues involved in this interaction were mapped. Further to these observations, we have attempted to examine the effect of AdE1A's interaction on the proteolytic activity of immunoproteasomes. Research to date on AdE1A and the immunoproteasome was mainly focused on its effect on immunosubunit expression at a transcriptional level and some limited studies on its direct binding to the immunosubunits. In this study, complementary to the findings in chapter 3 and 4, the effect of AdE1A on the catalytic activity of the immunoproteasome will be examined, an area not addressed by previous research. The effect of AdE1A on the caspase, trypsin and chymotrypsin-like activities of the immunoproteasome will be assessed as well as the effect on the antigen presentation to cytotoxic T cells (CTLs). This will be determined using ELISA assays and CD107A staining.

5.2 Results

5.2.1 The effect of AdE1A on the proteolytic activities of the immunoproteasome

5.2.1.1 *In vitro* proteasome assays

The caspase-like, chymotrypsin-like and trypsin-like activity of the immunoproteasome was examined in the presence or absence of AdE1A *in vitro* using specific fluorogenic substrates (refer to protocol on section 2.2.4). Two hundred μM of each substrate in the presence or absence of varying amounts of AdE1A was added to 6.25 μg/ml of purified human immunoproteasome (obtained from Biomol). Negative controls include: irrelevant GST protein and immunoproteasome; as well as substrate solution and assay buffer only (50 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$) to test any background activity. After 30 minutes at 37°C, the total reaction was transferred to a 96-well fluorescence plate where the fluorescence was measured using the Victor plate reader. The results show that at high concentrations of GST-Ad5E1A as well as the irrelevant GST protein (1 μg, 3 μg and 10 μg), there was no significant change in the trypsin-like activity in comparison to the immunoproteasome on its own; whereas there was a strong (6-fold) reduction in chymotrypsin activity upon the addition of not only Ad5E1A, but GST protein as well (Figure 5.1A). The level of inhibition was generally the same across the different quantities of Ad5E1A and GST. Caspase activity was very low in comparison to trypsin and chymotrypsin activity in the presence of both Ad5E1A and GST protein (there was little or no caspase activity in the presence of 1 μg, 3 μg and 10 μg GST-Ad5E1A). The same experiment was repeated, except that smaller amounts of untagged Ad12E1A were used (0.5 μg, 2.5 μg and 4 μg). Here, the results have shown that in comparison to the lone immunoproteasome, there was no change in trypsin activity upon varying the quantity of both GST and Ad12E1A, however, there was about a six-fold reduction in chymotrypsin activity across all of the different quantities of Ad12E1A along with the GST

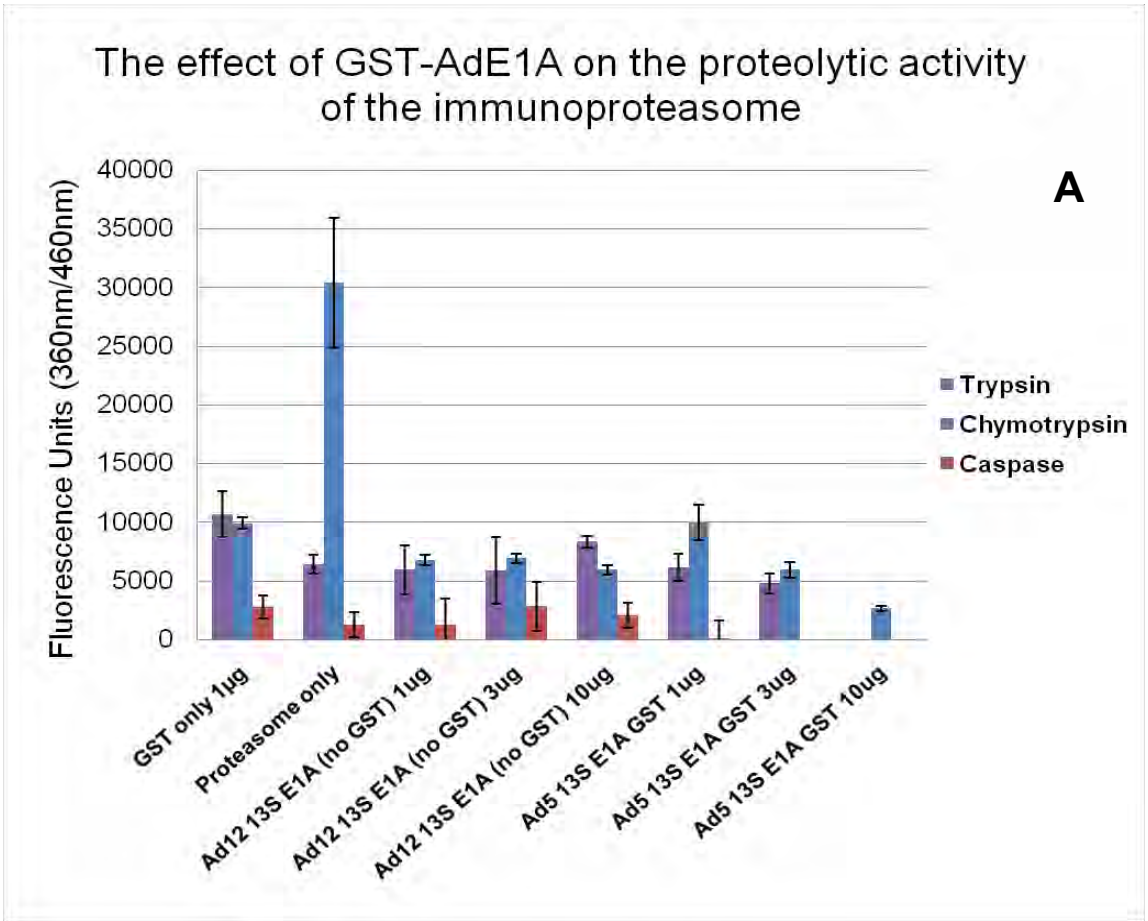
proteins (Figure 5.1B). Interestingly, there may be an inhibition of caspase activity specifically upon addition of Ad12E1A in comparison to GST (Figure 5.1B).

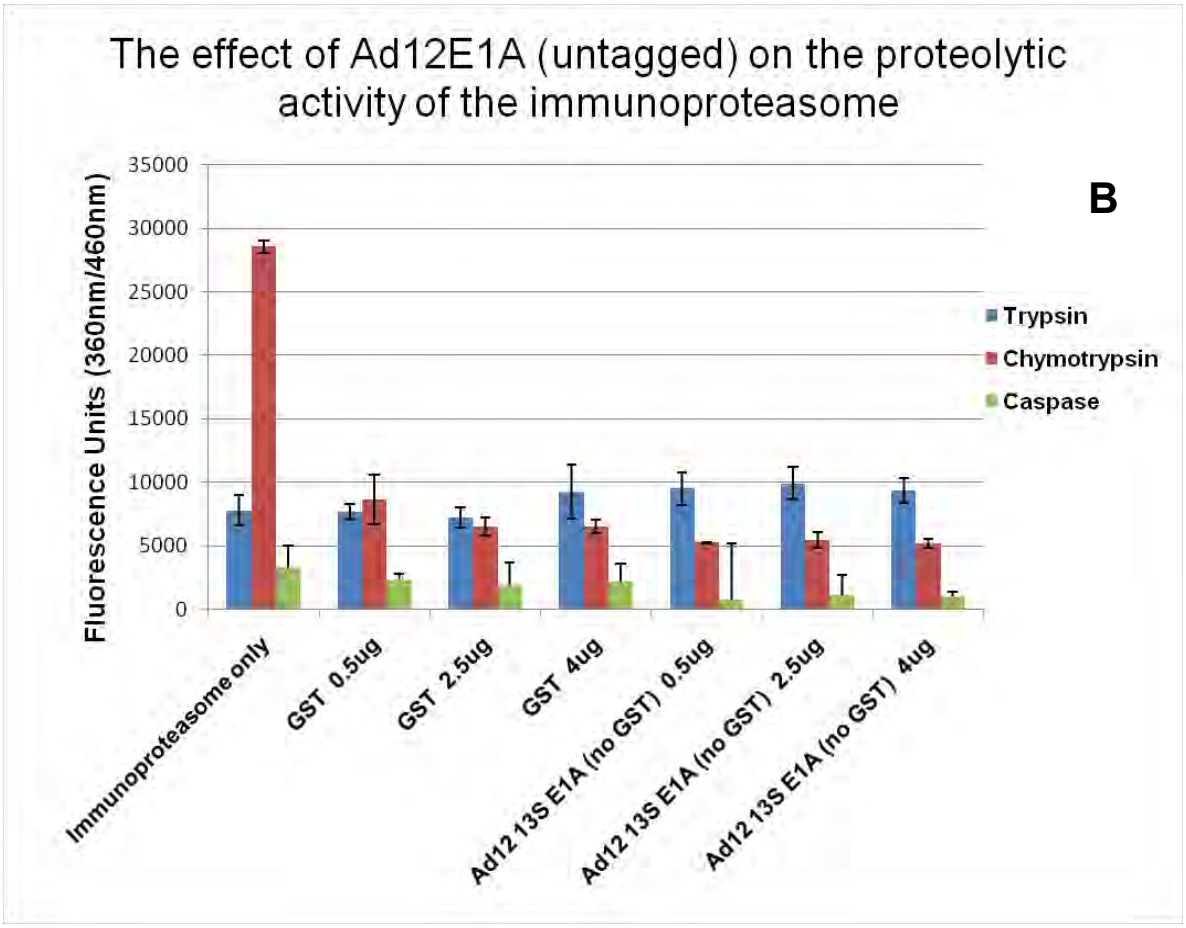
A further experiment focusing on only chymotrypsin activity and AdE1A was undertaken. Additionally, concern over protein over-saturation causing the inhibition of proteolytic activity by even GST was addressed by using smaller amounts of AdE1A and GST (0.01 µg, 0.05 µg, 0.1 µg and 0.2 µg). With increasing amount of both AdE1A and GST, there was a corresponding reduction in the chymotrypsin activity (Figure 5.1C). There was slight added reduction in chymotrypsin activity in all of the different amounts of GST protein, with the exception of at 0.01 µg, where 13S Ad12E1A caused more reduction in chymotrypsin activity at around 21000 compared to 29000 for the same amount of GST.

The proteolytic inhibition by GST protein alone is difficult to explain, as it was originally meant to act as a negative control in the assays. The reason why GST inhibited the chymotrypsin activity is not known. Another control protein, for instance bovine serum albumin (BSA) was used in place of GST protein, but that too inhibits the proteolytic activity of the immunoproteasome (data not shown)

5.2.1.2 Enzyme-linked immunosorbent assay (ELISA)

In the light of the above results, an alternative method to assess the effect of AdE1A on the activity of immunoproteasomes was sought. Immunoproteasomes are crucial components of the antigen processing machinery as they mediate degradation of antigens which are eventually presented by MHC class I to CTLs at the cell surface. The Epstein Barr virus (EBV) system is very well characterised with defined CTL epitopes and corresponding antigen specific $CD8^+$ T-cell clones (Lautscam *et al.,* 2001, 2003; Lee at al 1993, 1997; Houssaint *et al.,* 2001; Khanna and Burrows 2000; Khanna *et al.,* 1996; Meij *et al.,* 2002).


The effect of GST-AdE1A on the proteolytic activity of the immunoproteasome
A
Fluorescence Units (360nm/460nm)
40000
35000
30000
25000
20000
15000
10000
5000
0
Trypsin
Chymotrypsin
Caspase
GST only 1ug
Proteasome only
Ad12 13S E1A (no GST) 1ug
Ad12 13S E1A (no GST) 3ug
Ad12 13S E1A (no GST) 10ug
Ad5 13S E1A GST 1ug
Ad5 13S E1A GST 3ug
Ad5 13S E1A GST 10ug


The effect of Ad12E1A (untagged) on the proteolytic activity of the immunoproteasome
B
Fluorescence Units (360nm/460nm)
35000
30000
25000
20000
15000
10000
5000
0
Trypsin
Chymotrypsin
Caspase
Immunoproteasome only
GST 0.5ug
GST 2.5ug
GST 4ug
Ad12 13S E1A (no GST) 0.5ug
Ad12 13S E1A (no GST) 2.5ug
Ad12 13S E1A (no GST) 4ug

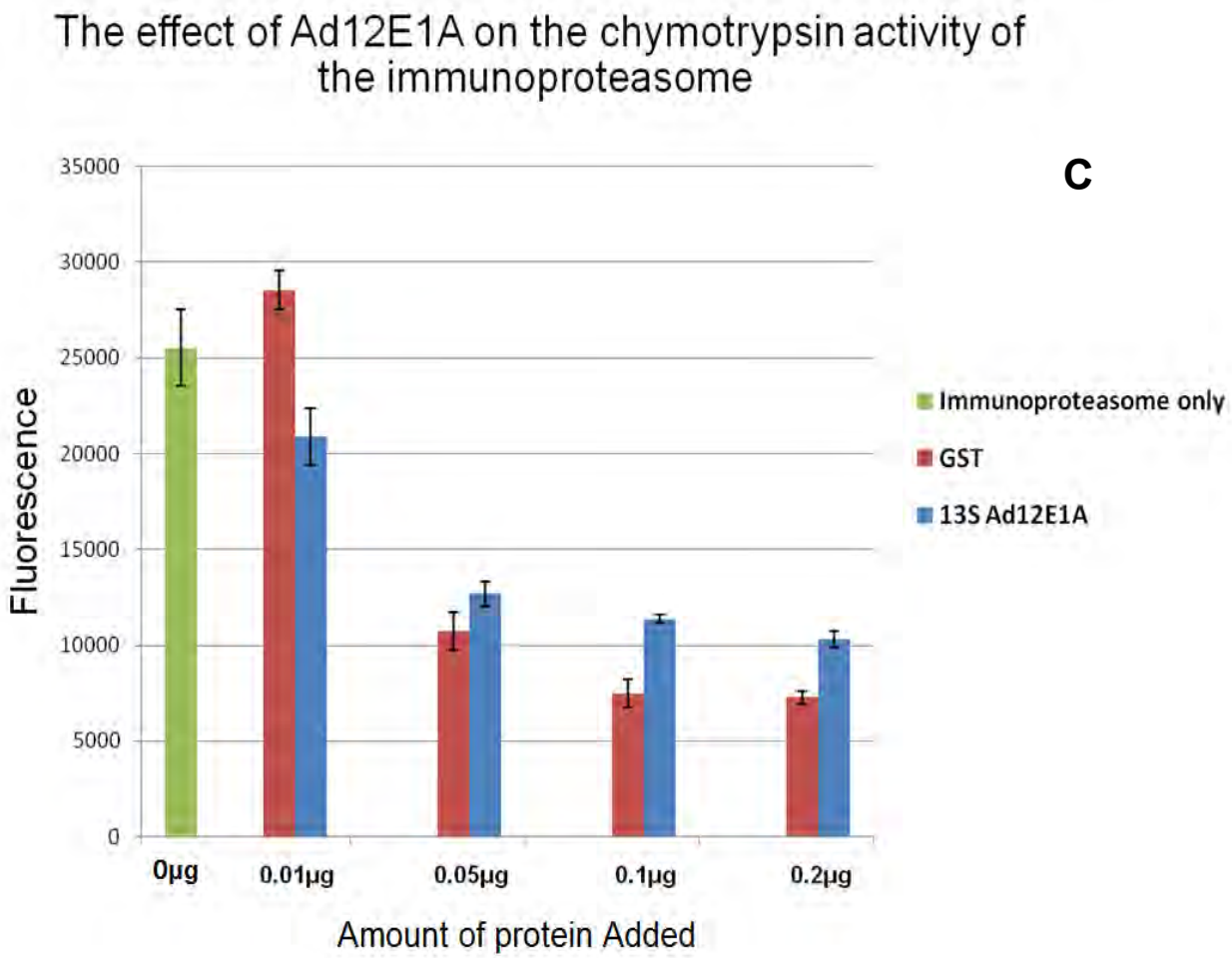


Figure 5.1 The effect of AdE1A on the catalytic activity of the immunoproteasome

In vitro proteasome assay involved using fluorogenic substrates Z-LLE-AMC, Suc-LLVY-AMC and Bz-VGR-AMC to examine the caspase, chymotrypsin and trypsin activity of the immunoproteasome. Assay was conducted as described in section 2.2.4. Fluorescence was measured by "umbelliferone 360 nm/460 nm" on a Victor plate reader. **A.** GST-13S Ad5E1A quantity range 1-10 µg **B.** 13S Ad12E1A (untagged) quantity range 0.5-4 µg **C.** 13SAd12E1A (untagged) quantity range 0.01-0.2 µg (chymotrypsin-like activity only). GST was included as a control.

EBV-derived CTL epitopes with known routes through the antigen processing pathway were used in an ELISA testing antigen presentation in the presence or absence of AdE1A. For this study, the epitopes are derived from the EBV-encoded latent membrane protein 2A (LMP2A). LMP2A is a multiple membrane-spanning molecule that is composed of short cytosolic NH2- and COOH-terminal domains that flank 12 transmembrane domains that are connected by loops that project into the ER (Lautscham *et al.,* 2001; Miller *et al.,* 1995; Longnecker 2000). The structure of LMP2A and the CTL epitope positions within its sequence is demonstrated in Figure 5.2. EBV infects resting B-cells driving them to become activated proliferating lymphoblasts (Thorley-Lawson 2001). LMP2A is one of the viral proteins that enable EBV to exploit pathways of B-cell differentiation (Thorley-Lawson 2001). Epitopes derived from LMP2A are either TAP-dependent or independent. This was determined using a particular human T2 cell line where TAP1 and TAP2 genes were deleted but which still managed to process and present LMP2A protein indicating TAP-independent pathway (Lee *et al.,* 1996; Khanna *et al.,* 1996). An additional epitope, FLY, was found to be TAP-independent and only generated in the presence of immunoproteasomes upon IFNγ exposure (Lautscham 2003).

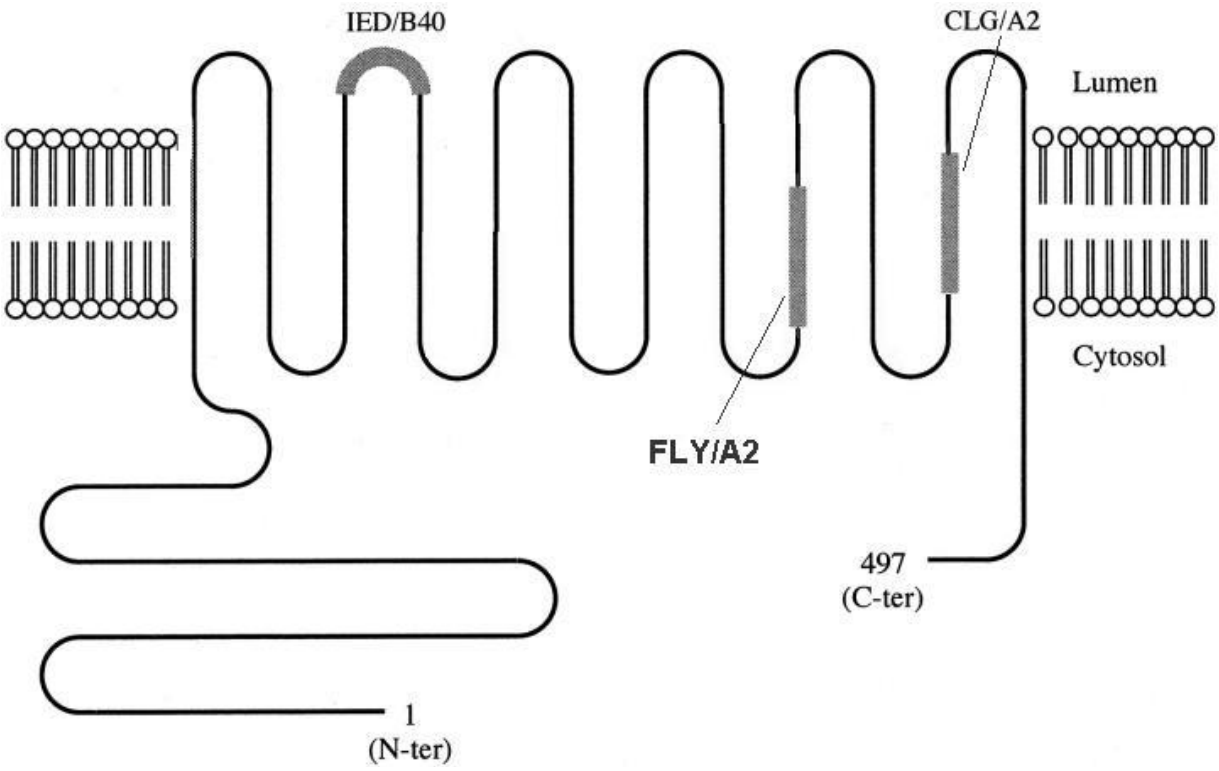
Primary fibroblasts were transfected or co-transfected with mRNA encoding LMP2A in the presence or absence of AdE1A. CTLs specific for the epitope peptides **CLG**GLLTMV (CLG), **FLY**ALALLI (FLY) and **IED**PPFNSL (IED) (all being HLA A*0201-restricted epitopes) were used (Lautscham *et al.,* 2001, 2003). IED is a TAP-dependent, proteasome-dependent epitope, CLG is a TAP-independent, proteasome-dependent epitope and finally FLY is a TAP-independent, immunoproteasome-dependent epitope (Figure 5.2B). AdE1A mRNAs co-transfected with LMP2A include 12SAd5E1A, 13SAd5E1A, 12SAd12E1A, 13SAd12E1A, 12SAd5E1A L19/20S and 13SAd5E1A L19/20S. Transfected fibroblasts were incubated with the appropriate T-cells, and the amount of IFNγ released was measured by ELISA assay (as explained in section 2.5.4). In the case of the FLY epitope, the fibroblasts were either pre-treated with 400 U/ml of IFNγ for 72 hrs before transfecting with RNA and incubating with CTLs, or transfected first

with the appropriate mRNAs for 12 hrs followed by another 12 hrs of IFNγ treatment, before exposure to CTLs.

The results have shown that in comparison to LMP2A alone, there was a significant decrease of CTL recognition of both IED and CLG epitopes after 12 hrs (Figure 5.3A). IED-specific CTL activity has significantly reduced from 2902 pg/ml IFNγ release absorbance units to 952, 1183, 816 and 637 pg/ml in the presence of 12SAd5E1A, 13SAd5E1A, 12SAd12E1A and 13SAd12E1A respectively, whereas in the 12S and 13S L19/20S Ad5E1A mutants, this inhibition was relieved to 1976 and 1399 pg/ml respectively (Figure 5.3A). The same pattern was also observed with the CLG-specific CTLs where, in comparison to LMP2A alone, there was also a drastic inhibition of CTL activity from 1396 pg/ml IFNγ-release to 237, 481, 254, and 145 pg/ml in the presence of 12SAd5E1A, 13SAd5E1A, 12SAd12E1A and 13SAd12E1A respectively. This inhibition was again reduced with the 12S L19/20S Ad5E1A mutants where the value was 766 pg/ml (Figure 5.3A). However, the mutation did not affect the inhibition capability of 13S L19/20S Ad5E1A mutant. The ELISA data was also expressed relative to actin (Figure 5.3 C and D). The data pattern however remained unchanged compared to Figure 5.3A.

With regards to the FLY-specific CTLs, there was difficulty in acquiring recognition of the transfected fibroblasts. There was little or no CTL activity observed regardless of whether the IFNγ treatment was carried out before or after electroporation. The FLY-specific CTLs were functional as results from incubation with peptide loaded LCLs have shown strong CTL activity (Figure 5.4A), which may indicate that the problem is due to fibroblast antigen presentation. Interestingly, the efficiency of electroporation was much reduced in the event of IFNγ pre-treatment of fibroblasts (Figure 5.4B).

A

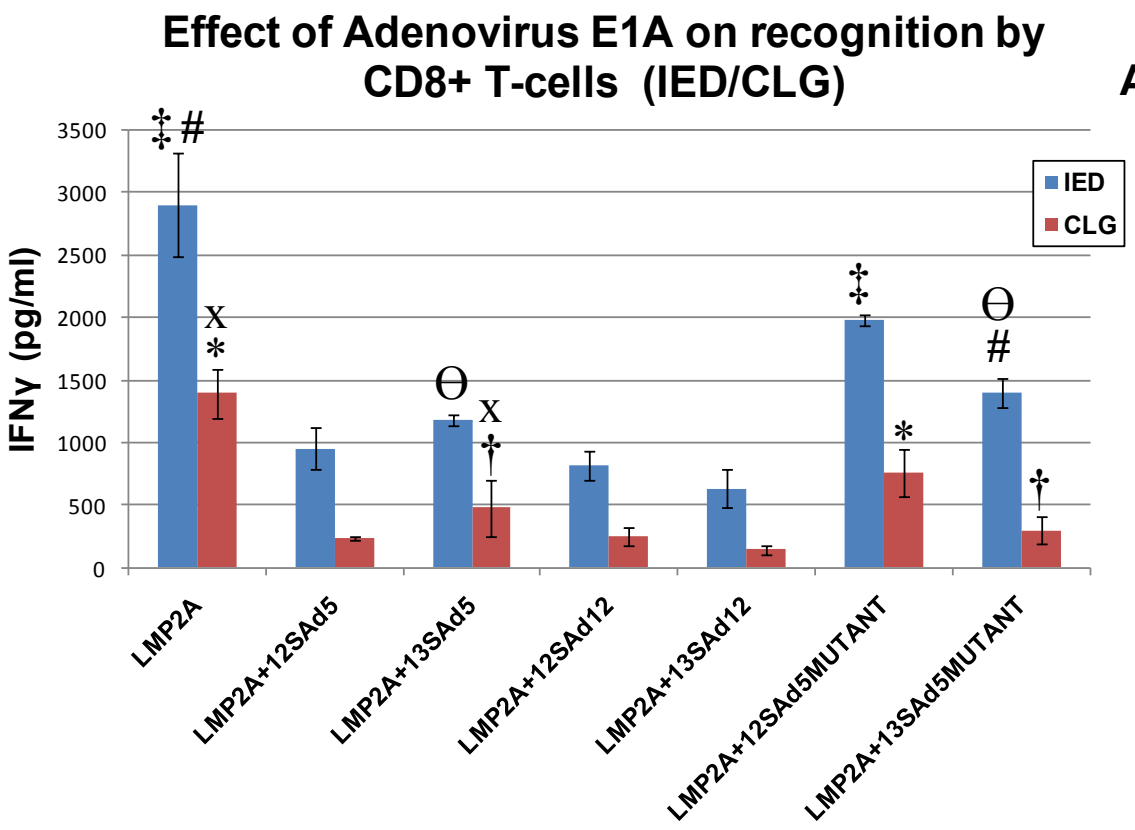


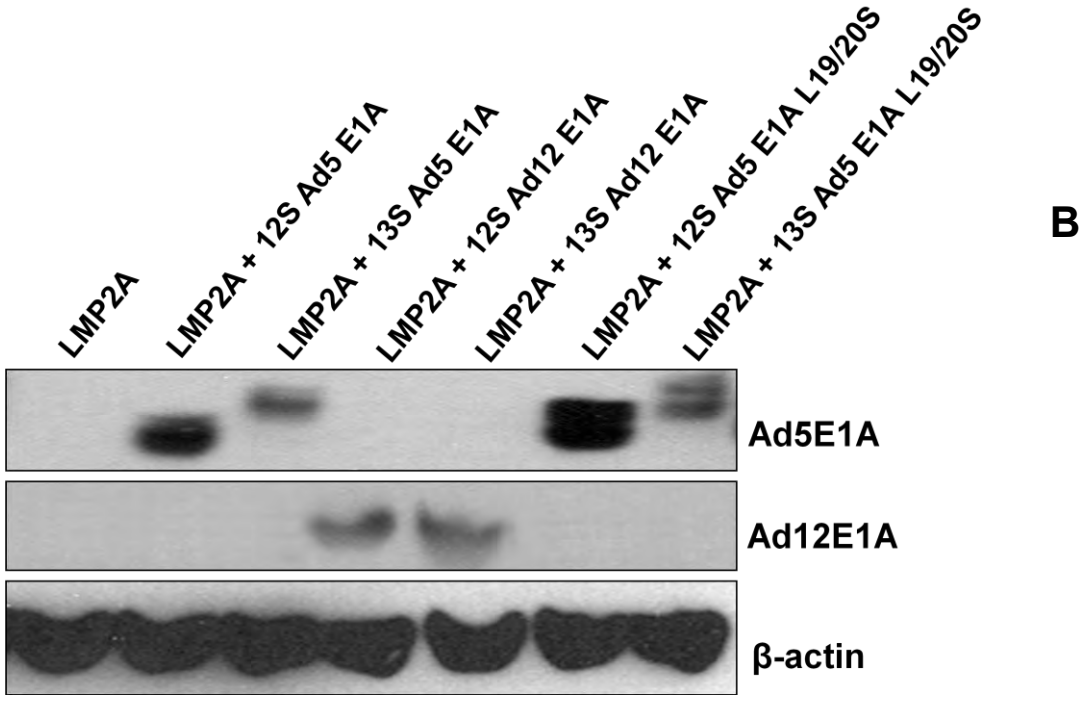
Modified from Lautscham *et al.,* 2001

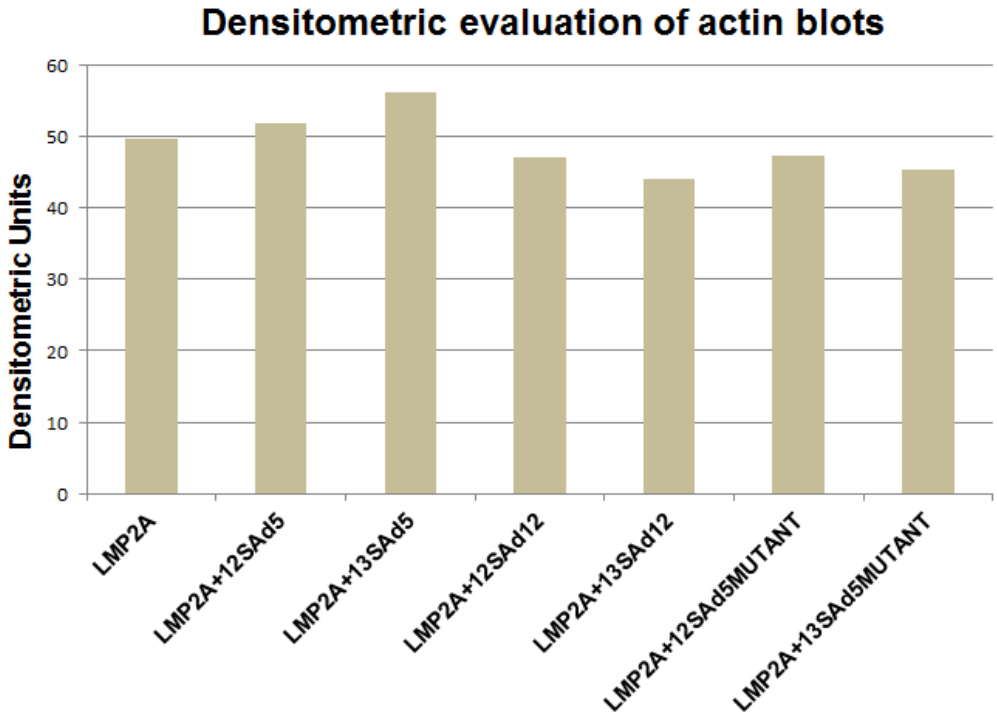
B

Epitope	Proteasome-dependent	Immunoproteasome-dependent	TAP-dependent
CLGGLLTMV (CLG)	Yes	No	No
IEDPPFNSL (IED)	Yes	No	Yes
FLYALALLI (FLY)	No	Yes	No

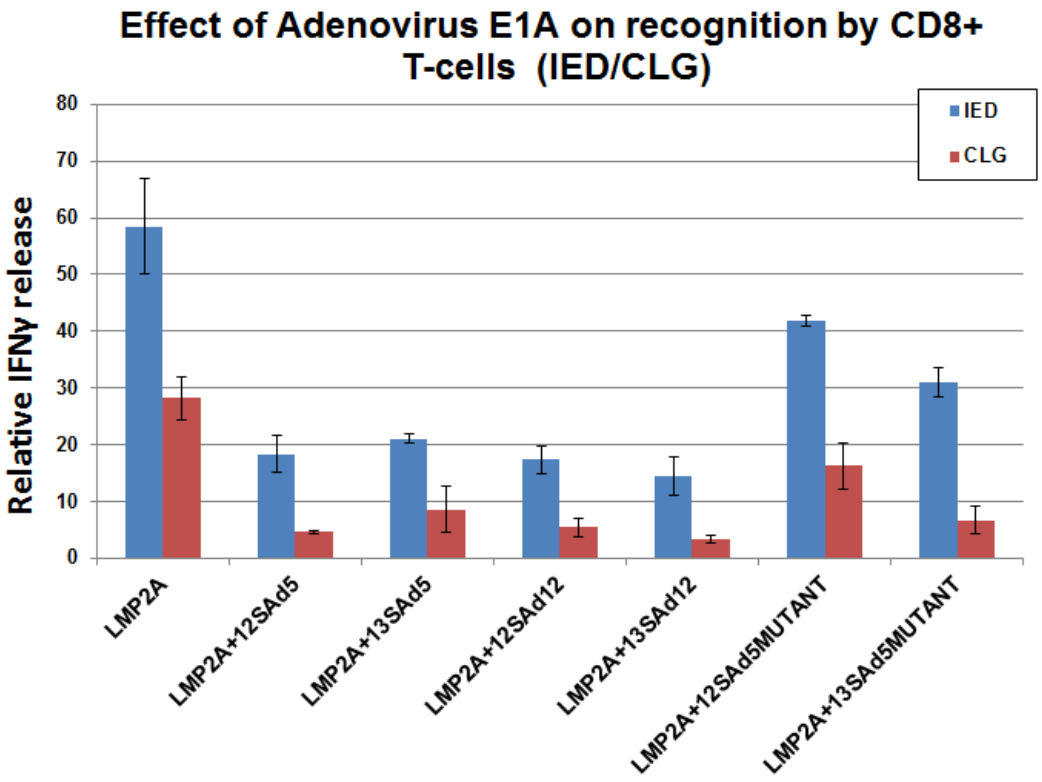
Figure 5.2 Diagrammatic representation of LMP2A in the cell membrane
A. Shaded boxes represent the positions of the CTL epitopes sequences. Each epitope is identified by the first three letters of their amino acid sequence and their corresponding HLA class I restricting alleles. **B.** Table summarizing the proteasome, immunoproteasome and TAP dependencies of the epitopes used in the study.


Effect of Adenovirus E1A on recognition by CD8+ T-cells (IED/CLG)
A
IFNγ (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
IED
CLG
LMP2A
LMP2A+12SAd5
LMP2A+13SAd5
LMP2A+12SAd12
LMP2A+13SAd12
LMP2A+12SAd5MUTANT
LMP2A+13SAd5MUTANT


B
LMP2A
LMP2A + 12S Ad5 E1A
LMP2A + 13S Ad5 E1A
LMP2A + 12S Ad12 E1A
LMP2A + 13S Ad12 E1A
LMP2A + 12S Ad5 E1A L19/20S
LMP2A + 13S Ad5 E1A L19/20S
Ad5E1A
Ad12E1A
β-actin



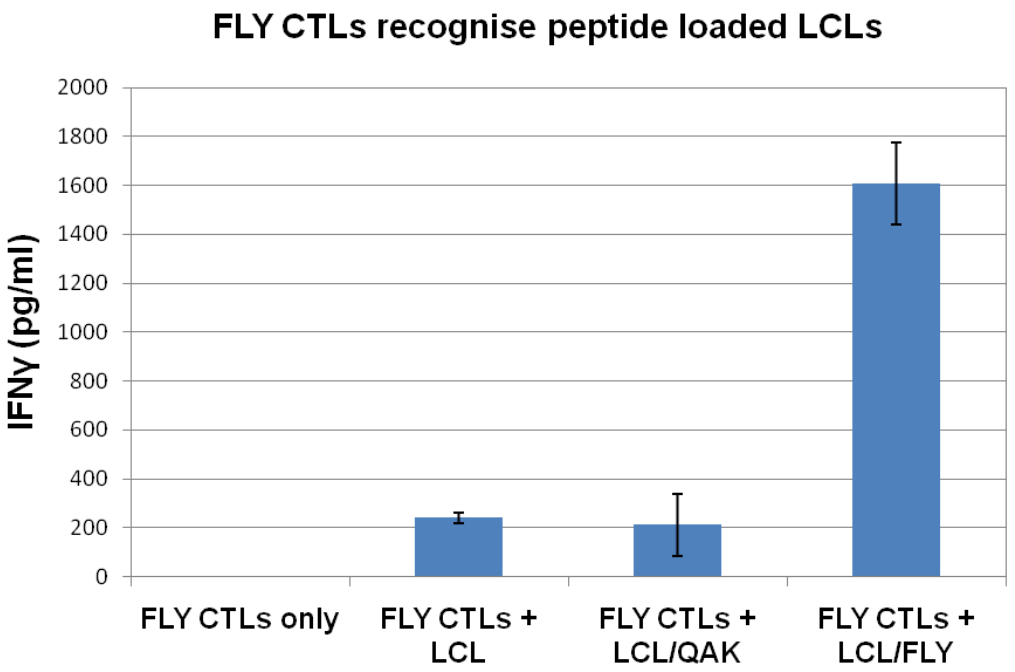
C



D

Figure 5.3 AdE1A reduces target cell recognition by cytotoxic CD8$^+$ T-cells (CTL)

A. ELISA assay measuring CTL activity by the amount of IFNγ released. ELISA assays were undertaken according to protocol in section 2.5.4. Absorbance measured at 450 nm. ***, Θ, x, #, ‡ (P<0.05), † (P>0.05).** . Data are means +/- SEM from three repeats. **B.** Western blot analysis of 50 µg target cell lysates electroporated with mRNAs encoding the listed proteins. **C.** Densitometric evaluation of the actin blot from B. **D.** CTL activity relative to actin.



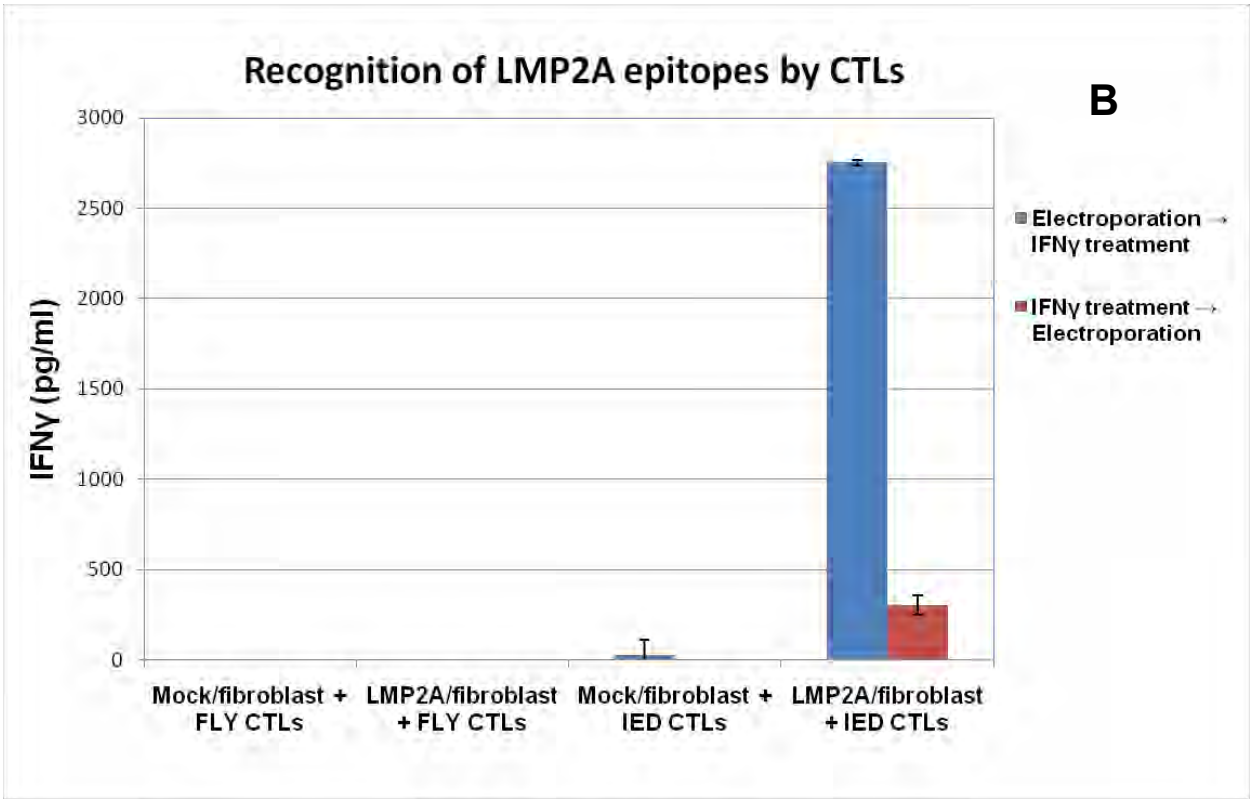


Figure 5.4 Testing IED and FLY CTL recognition of the appropriate epitopes
ELISA assay measuring CTL activity by the amount of IFNγ released. ELISA assays were undertaken according to protocol in section 2.5.4. **A.** FLY T-cells were either incubated on their own or in the presence of FLY/control peptide-loaded LCLs in order to test specificity. **B.** Fibroblasts were either electroporated first followed by 12 hrs of 400 U/ml IFNγ treatment or treated with IFNγ for 24hrs then electroporated; prior to use in ELISA. . Data are means +/- SEM from three repeats.

5.2.1.3 CD107a staining of T-cells (CTLs)

CTL target recognition leads to IFNγ release and cell killing. The ELISA assay described above assesses CTL activity by measuring IFNγ release. This, however, is not a confirmation of cell killing, which is verified via the widely used chromium release assay. It uses the concept of labelling cells with Chromium (^{51}Cr) followed by incubation with the appropriate CTLs; in the event of killing, chromium is released into the surrounding medium and the amount of radioactivity measured using a gamma counter, hence greater cell killing leads to increased radioactivity readings. This procedure was attempted; however, it was unsuccessful because the cells were labelled 24 hrs after electroporation by which time LMP2A expression was greatly reduced. Additionally, electroporation of the fibroblasts following labelling gave rise to high background values as electroporation generates pores in the cell membrane; so chromium release assay in this case was not feasible.

Another way to assess cytotoxic killing is by CD107a staining. CD107a is a membrane glycoprotein that is a functional marker of CTL degranulation following stimulation (Betts and Koup 2004). CTLs degranulate after recognition of a peptide-MHC class I complex on a target cell. Hence, for the next experiment, fibroblasts were transfected by mRNA electroporation with LMP2A mRNA with or without 12SAd5E1A, 13SAd5E1A, 12SAd12E1A, 13SAd12E1A, 12SAd5E1A L19/20S and 13SAd5E1A L19/20S mRNAs. This experiment was carried out simultaneously with the ELISA experiment described above. The results, from the IED-specific CTLs, reveal that there was a reduction in CD107a-expressing cells in the presence of AdE1A. CD107a expression was reduced from 50.6% (LMP2A on its own) to 31.8%, 22.9%, 18.9% and 8.25% in the presence of 12SAd5E1A, 13SAd5E1A, 12SAd12E1A and 13SAd12E1A respectively (Figure 5.5). Double mutants 12SAd5E1A L19/20S and 13SAd5E1A L19/20S reduced CD107a expression to 25.5% and 15.5% respectively. Despite this, the western blot data (Figure 5.2B) may need to be taken into account when considering the exact effect of AdE1A on CD107a expression due to the variability in expression following electroporation with the

different constructs of mRNA (refer to discussion below). CD107a staining was intended to cover all three CTL clones representing different antigen processing pathways, i.e. IED, CLG and FLY-specific CTLs (TAP-dependent, proteasome-dependent, TAP-independent, proteasome-dependent and TAP-independent, immunoproteasome-dependent epitopes respectively). However, CLG-specific CTLs had very low viability at the outset and not enough viable cells remained at the end of the CD107a staining to analyse via flow cytometry. Yet, due to the high sensitivity of the ELISA assay, results were obtained from as few as 1000 CTLs despite the low viability. As explained previously, no experiments could be carried out with the crucial FLY-specific CTLs due to problems with recognition of the transfected cells by CTLs.

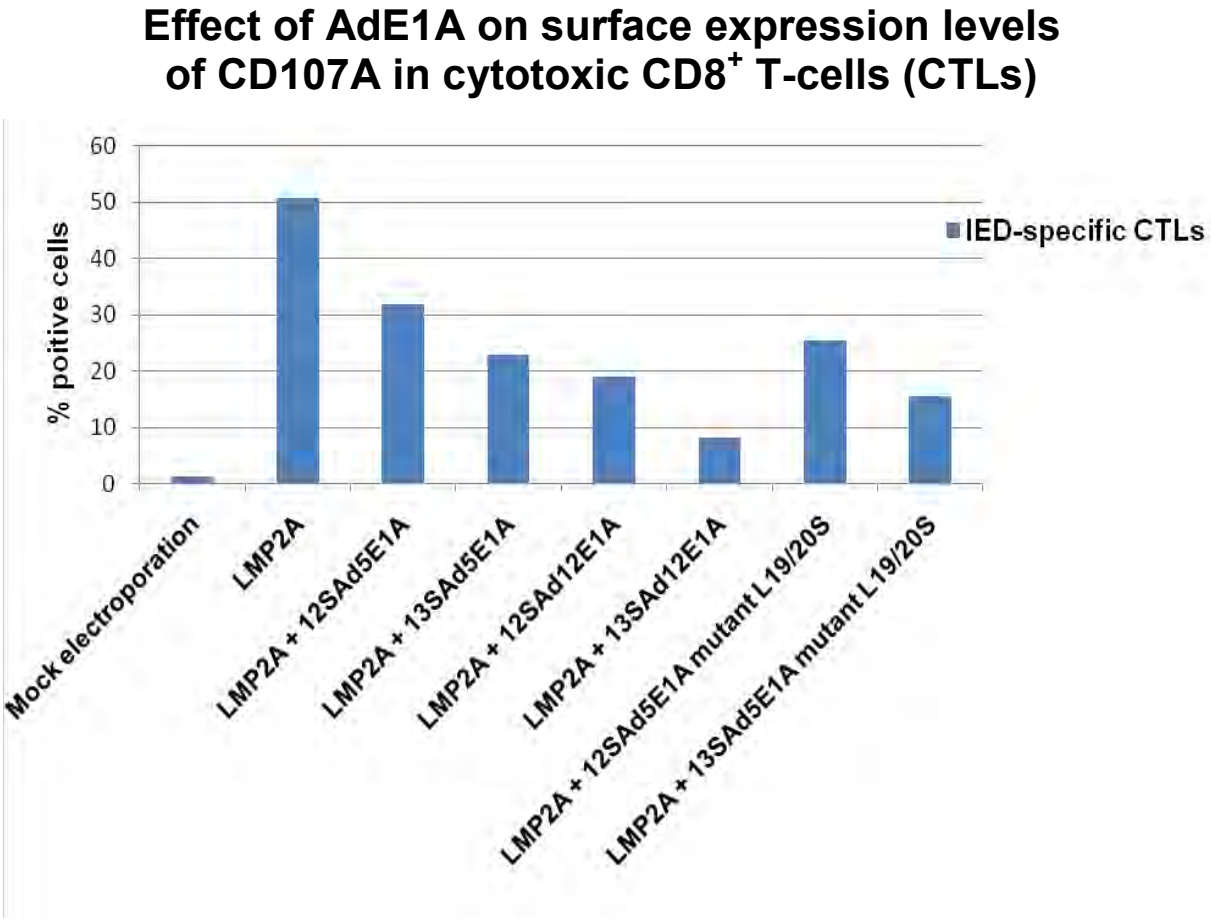


Figure 5.5 AdE1A reduces target cell recognition by CTLs
One thousand IED-specific T cells were incubated with 50000 fibroblasts electroporated with mRNA encoding the listed proteins. They were left at 37°C for 12 hrs after which cells were stained for surface CD107a molecules according to protocol in section 2.5.6. For Western blot analysis of fibroblast lysates, refer to Figure 5.3B. Experiment undertaken once.

5.3 Discussion

In the previous two chapters, it was shown that AdE1A inhibits immunoproteasome expression as well as binding to the MECL-1 immunosubunit. In this chapter, the effect of this binding on the catalytic function of the immunoproteasome was addressed, investigating the trypsin, chymotrypsin and caspase activity as well as the overall effect on the presentation of antigens to CTLs.

Prior to this study, research into the effect of AdE1A on the immunoproteasome mainly focused on the down-regulation of expression of the individual immunosubunits. This was seen in adenovirus transformed human and mouse cells (Vertegaal *et al.,* 2003; Rotem-Yedudar *et al.,* 1996) and in AdE1A transfected cells (Chatterjee-Kishore *et al.,* 2000). Results from the present study so far have expanded on both these aspects (chapter 4). Subsequently, for the first time, the effect of adenovirus infection on the level of immunosubunit expression was also examined (chapter 4). In chapter 3, the direct binding of AdE1A to immunoproteasomes and the binding sites involved in this interaction were studied. Nevertheless, the effect of AdE1A on the catalytic activity of the immunoproteasome and the potential repercussions of this for antigen processing has never previously been examined. There is data, however, of other viruses affecting the proteolytic activities of the immunoproteasome. For instance, hepatitis C virus NS3 non-structural protein binds to the LMP7 immunosubunit, as a result repressing its activity and that of the immunoproteasome (Yee-Ling Khu *et al.,* 2004). HIV-1 Tat protein binds to both MECL-1 and LMP7 and is able to disrupt its proteolytic activity (Apcher *et al.,* 2003). It has been hypothesized that the inhibitive effect of various viral proteins either in terms of immunosubunit expression at the transcriptional level or by direct binding to any of the immunosubunits skews the stoichiometry of the immunoproteasomes leading to modulation of overall enzymatic activity. Attempts were made to test this theory in this study by examining the direct effect of AdE1A on immunoproteasome activity.

In vitro immunoproteasome assays assessing the trypsin, chymotrypsin and caspase activity (via appropriate substrates) in the presence or absence of AdE1A were undertaken. This experiment, however, had a major setback. Even though there seems to be down-regulation of chymotrypsin activity in the presence of AdE1A, the same outcome was also observed with control GST protein (Figure 5.1A and B) and BSA that were used as negative controls, indicating that this was not an AdE1A-related inhibition. It is not known why the negative control proteins also reduced the proteolytic activity. Attempts were made to reduce the amount of AdE1A protein in a titratable manner to address concerns of over-saturation, however, the presence of either AdE1A or GST at a concentration as low as 0.05 µg also inhibited the chymotrypsin activity (Figure 5.1C).

To overcome this problem, an *in vivo* approach was undertaken whereby the effect of AdE1A on the presentation of known epitopes (derived from the EBV protein LMP2A) (Figure 5.2), to their corresponding antigen specific $CD8^+$ T-cell clones was studied. Three EBV-derived CTL epitopes with known pathways through the antigen processing machinery were used (Figure 5.2A and B) and these are: **CLG**GLLTMV (CLG), TAP-independent, proteasome-dependent epitope; **IED**PPFNSL (IED), TAP-dependent, proteasome-dependent epitope (Lautscham *et al.,* 2001) and **FLY**ALALLI (FLY), TAP-independent, immunoproteasome-dependent epitope (Lautscham *et al.,* 2003). Previous studies have shown that AdE1A, specifically from Ad12, down-regulates TAP1 and TAP2 expression in adenovirus transformed cells (Rotem-Yehudar *et al.,* 1994, 1996; Vertegaal *et al.,* 2003); hence using EBV-derived epitopes that may be either TAP dependent or independent should allow us to identify if any of the experimental outcomes may be due to potential inhibition of the TAP proteins rather than an effect on the proteasome/immunoproteasome directly. This should also provide further insight into the role of the TAP proteins in antigen presentation. Similarly, study of the FLY epitope should make it possible to study the effect of AdE1A on the immunoproteasome and antigen presentation without the involvement of the TAP proteins.

Primary fibroblasts were transfected or co-transfected with mRNA encoding the EBV protein LMP2A in the presence or absence of AdE1A. CTL activity was measured by the ELISA assay that quantifies the amount of IFNγ released. The data show that the presence of 12SAd5E1A, 13SAd5E1A, 12SAd12E1A and 13SAd12E1A reduced IED epitope presentation as demonstrated by reduced CTL activity in comparison to when LMP2A is transfected alone (Figure 5.3A). Mutations L19/20S of Ad5E1A have relieved this inhibition to some extent, suggesting that the N-terminal region of AdE1A is involved in this repression. Similar data were also observed with the CLG epitope, indicating that this is not due to AdE1A inhibition of TAP protein expression, but rather potentially due to AdE1A affecting the 20S proteasome. However, as with the previous experiments in chapter 4 studying the effect of AdE1A on surface MHC class II expression, there was great difficulty in achieving equal levels of AdE1A protein expression following electroporation with the different constructs of mRNA. As mentioned before, although mRNA electroporation is highly efficient, it is highly susceptible to degradation and exhibits different levels of expression depending on its stability; so again the western blot data (Figure 5.3B) was taken into account when considering the effect of AdE1A on antigen presentation. The graph (Figure 5.3A) displays the raw data and was thus not adjusted according to the expression shown on the Western Blots (Figure 5.3B). The above results may be partly due to AdE1A targeting the 20S proteasome. It is already known that AdE1A targets 20S and 26S proteasome by binding to the ATPase (S4 and S8) and non-ATPase (S2) components of the 19S complex (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004) as well as several α subunits of the 20S proteasome (Rasti *et al.,* 2006). Nevertheless, it may be premature to come to this conclusion without further experiments to rule out involvement of other components of the antigen processing machinery. Other components that may be targeted by AdE1A; for instance calreticulin, calnexin and ERp57, chaperone proteins present in the endoplasmic reticulum that are involved in the folding and assembly of MHC class I molecules (Zhang and Williams 2006), as well as tapasin, a glycoprotein involved in mediating interaction between MHC

class I and the TAP complex leading to the loading of peptides onto the newly assembled MHC class I molecule (Lankat-Buttgereit and Tampé 2002; Zhang and Williams 2006). Tapasin expression was in fact found to be reduced in Ad12 transformed rat cells (Vertegaal *et al.,* 2002) so, potentially, affecting CTL recognition. Introduction of minigene constructs that express minimal epitopes (Lautscham *et al.,* 2001), hence bypassing the role of proteasomes in order to study the proteasome independent, TAP dependent/independent pathways, may provide further understanding of other antigen processing components involved.

The double mutations at residues 19 and 20 have affected AdE1A's ability to repress CTL recognition of target cells. These double mutations are located at the N-terminus of AdE1A, a region which is important in mediating its transcriptional regulation effects. In chapter 3, it was shown that the same mutations also affected the ability of AdE1A to repress surface MHC class II repression. These mutations have also eliminated AdE1A binding to CBP, p300, TATA binding protein (TBP), S4, S8, hGcn5, P/CAF and Ran proteins (Rasti *et al.,* 2005). This signifies its important role in mediating the interactions of the AdE1A N-terminal region. In this study (chapter 4), it was found that the N-terminal region was involved in binding to the catalytic subunits of the 20S proteasome. If it was to be concluded that reduction in CTL recognition in the presence of AdE1A was due to 20S proteasome inhibition then it may be possible to partly relate this to the binding of AdE1A to the catalytic β subunits of the 20S proteasome.

The EBV-derived CTL epitope FLY is immunoproteasome-dependent and TAP-independent, which makes it ideal to study AdE1A's effect on immunoproteasome activity and the overall antigen presentation. However, there was considerable difficulty in acquiring recognition of the transfected fibroblasts. These problems seem to stem from the expression of protein or presentation of the epitope as the FLY-specific CTLs were found to be functional after incubation with peptide loaded LCLs (Figure 5.4A) Fibroblasts were either pre-treated with IFNγ for 48 hrs followed by electroporation with LMP2A mRNA, then plated out with T-cells or they were electroporated first then immediately

treated with IFNγ for 12 hrs before being washed and incubated with T-cells in culture plates. Either way, no CTL activity or IFNγ release was recorded. From optimisation experiments, it was found that LMP2A expression declines after 24 hrs, whereas immunoproteasome expression and assembly may depend on the cell line, and since primary fibroblasts are not natural antigen presenting cells, the expression and assembly of immunoproteasomes may take longer. So the issue here may lie with getting optimum LMP2A, AdE1A and immunoproteasome expression at about the same time in the cell. However, this may be difficult as expression from the very unstable mRNA makes LMP2A half-life very short and continuous IFNγ treatment for a longer period may be required to replace all the 20S proteasomes with immunoproteasomes; additionally, the half-life of immunoproteasomes is known to be shorter than that of 20S proteasomes (Heink *et al.,* 2005). An alternative way to deliver the LMP2A and AdE1A genes through a recombinant vaccinia virus (Lautscham et al., 2001, 2003) was ruled out due to concerns that using a virus might produce some side-effects that may affect the accuracy of the data. However, on retrospective examination, this may have been a better option, and thus a possibility for a future experiment. Interestingly, cells that were pre-treated with IFNγ prior to electroporation have reduced presentation of epitopes to CTLs (Figure 5.4B). This may indicate that IFNγ treatment affects electroporation efficiency.

In order to assess CTL activity, CD107a staining was carried out. CD107a is a functional marker of CTL degranulation (Betts and Koup 2004) that occurs after CTLs recognise peptide-MHC class I complexes on the target cell and become stimulated. CD107a staining was complementary to the ELISA. The same electroporated cells were used for both experiments so the release of IFNγ in the ELISA can be linked to CTL degranulation or cytotoxic killing. The data revealed that, compared to LMP2A alone, there was a significant decrease in CD107a expression in the presence of 12SAd5E1A, 13SAd5E1A, 12SAd12E1A and 13SAd12E1A (Figure 5.4). As with the ELISA, the western blot data (Figure 5.2B) should be taken into account when considering AdE1A's effect on the CD107a expression. There seems to be an increased inhibition of CTL activity by

13SAd5E1A and 13SAd12E1A compared to the corresponding 12S proteins. As AdE1A binds to the catalytic subunits of the 20S proteasome through its N-terminal and CR3 regions, and since 12SAdE1A proteins do not contain CR3, this may point to reduced interaction, hence weaker impact of the 12S than 13SAdE1A proteins.

In contrast to the ELISA, the double mutations at residues 19 and 20 did not have an effect on the ability of AdE1A to reduce CD107a staining. The double mutants have caused a reduction in CD107a expression nearly equal to *w.t.* AdE1A. This means that the double mutations affect IFNγ release from CTLs, preventing the stimulation of an immune response, while at the same time not interfering with the CTLs degranulation. CD107a staining, unlike the ELISA assay, is less sensitive and requires significant numbers of viable T-cells to analyse by flow cytometry. The CLG-specific CTLs had very low viability at the outset and unfortunately, at the end of the staining, not enough viable cells were acquired for flow cytometry analysis, so data from this was unfortunately not obtained.

Further experiments are still required to study the effect of AdE1A on immunoproteasome activity and antigen presentation. With the limited time available for this project, not all aspects have been covered in this study, and this may be a topic for a future investigation. For instance, the inclusion of the FLY epitope in both ELISA and CD107a staining is crucial to understand the effect of AdE1A on an immunoproteasome dependent antigen processing pathway. The experiments may be duplicated by using two different CTL clones/epitopes for each pathway. This would ensure consistency of the observed data. The effect of AdE1A on other components of the antigen processing machinery need to be investigated further by including minigenes as mentioned above. The quest for the appropriate negative control to equalise mRNA load has involved using mRNA encoding GFP and luciferase. However, GFP is a fluorescent protein and this interfered with flow cytometry analysis and luciferase protein on the other hand increased CTL background activity in the absence of LMP2A. Perhaps in a future experiment, an mRNA control

encoding actin may be attempted. Additionally, it would also be of interest to examine the effect of AdE1A on the composition of the immunoproteasome.

In this chapter, the effect of AdE1A on immunoproteasome activity and antigen presentation was examined. Results from the ELISA and CD107a assays have shown that the presence of AdE1A reduced target cell recognition by CTLs. This may be down to AdE1A hindering 20S proteasome activity leading to reduced epitope generation but further tests need to be carried out to confirm this observation.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK

AdE1A is the first viral protein to be expressed following infection. It targets multiple cellular proteins and pathways, specifically those that are involved in transcription (Frisch and Mymryk 2002, Gallimore and Turnell 2001). It has recently been shown that AdE1A also targets components of the antigen processing machinery, such as the 20S proteasome (Rasti 2005, 2006) which is the source of the major non-lysosomal proteolytic activity in the cell; ATPase and non-ATPase components (S4/S8 and S2 respectively) of the 19S complex (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004;); MHC class I surface expression in Ad12 transformed cells (Bottley *et al.,* 2005; Vasavada *et al.,* 1986; Schrier *et al.,* 1983; Ackrill and Blair 1988) and tapasin (reviewed by Horwitz 2004; Bennett *et al*., 1999, Vertegaal *et al.,* 2002). In light of these findings, in this study, it was decided to expand this further to include an examination of the effect of AdE1A on the immunoproteasome as well as MHC class I and class II surface expression in response to transfection, viral infection and in adenovirus transformed cells. The cellular response to IFNγ and the recognition of target cells by CTLs in the presence of AdE1A was also addressed. Additionally, previous research into this area had heavily focused on the use of rodent cells so this study was also an opportunity to study such responses in human cells.

6.1 Interaction of AdE1A with the immunoproteasome components

Chapter 3 examined whether AdE1A binds directly to the immunoproteasome. Studies to date focused mainly on immunsubunit expression in adenovirus transformed cells and to a limited level, in the case of AdE1A transfection, but not on AdE1A binding. It was shown in this study that AdE1A binds to the immunoproteasome (Figure 3.1), specifically to MECL-1, not LMP2 or LMP7 (Figure 3.2). This was followed by a series of pull down assays to identify residues within these two regions of AdE1A that are important in the interaction (Figure 3.5 and 3.7). Similarly, AdE1A also binds to the 20S proteasome (Figure 3.9), and interestingly its interaction was stronger to β1 and β5 (homologues to LMP2 and LMP7

respectively) than the MECL-1 homologue β2 (Figure 3.10). In addition, as with MECL-1, the β subunits also bind to the N-terminal region and CR3 regions of AdE1A. The N-terminal region and CR3 play an important role in mediating the interactions of AdE1A with cellular proteins (Gallimore and Turnell 2001). There is already evidence that those two regions are involved in the interaction with many components of the antigen processing machinery so it is not surprising that this may also include the immunosubunits and their constitutive homologues in the proteasome. It has already been established that the N-terminal region interacts with STAT1 (Look *et al.,* 1998), S4 and S8 (Turnell *et al.,* 2000; Zhang *et al.,* 2004) as well as other proteins that are involved in transcription, for instance CBP/p300 and TBP (Rasti *et al.,* 2005). On the other hand, the CR3 region is involved in the binding to several α subunits of the 20S proteasome (Rasti *et al.,* 2006) as well as transcriptional regulators such as Mediator (Wang and Berk 2002; Ablack *et al.,* 2010). Additionally, the binding sites for MECL-1 have been defined within these AdE1A regions to identify specific residues involved in the interaction. The residues identified were similar to those studied by Rasti *et al.,* (2004, 2005) that affected binding to several other cellular proteins. The study of the point mutations across the N-terminal region has provided more information on the interaction with MECL-1 and the β subunits by defining the role of each residue, however, the CR3 deletion mutants only defined the role of short regions rather than specific amino acids. The data (Figure 3.7 and 3.11) showed that all the deletion mutants to varying extent have affected the interaction with MECL-1 and the β subunits,

6.2 Regulation of immunoproteasome and MHC expression by AdE1A

The effect of AdE1A on the expression of the immunoproteasome components LMP2, LMP7 and MECL-1 and on cell surface MHC class I and class II expression was studied. It was seen that AdE1A down-regulates the expression of all immunosubunits during transfection and viral infection (Figure 4.1, 4.6 and 4.7), whereas in adenovirus

transformed cells this was more pronounced in Ad12 transformed cells compared to those transformed by Ad5 (Figure 4.8). The latter data were in agreement with previous studies that indicated down-regulation of the immunosubunits in Ad12 transformed rat cells (Vertegaal *et al.,* 2003, Rotem-Yedudar *et al.,* 1996). This study, for the first time, looked at the effect of AdE1A transfection and viral infection on immunosubunits levels; there has been little research to date examining this, with the exception of one study that found repression of LMP2 transcription following AdE1A transfection (Chatterjee-Kishore *et al.,* 2000). In most somatic cells, the immunoproteasome components and MHC class II are expressed in response to IFNγ via the JAK/STAT1 pathway (refer to section 1.5). The cellular response to IFNγ was found to be inhibited by AdE1A transfection (figure 4.17), during Ad12 infection (figure 4.16) and in adenovirus transformed cells (figure 4.18). This may partly be due to AdE1A interfering with the phosphorylation of STAT1 protein (figure 4.19). Previous finding have revealed that AdE1A targets STAT1 inhibiting its function (Zhang *et al.,* 1996; Look *et al.,* 1998; Leonard and Sen 1996). The same reasoning can also be applied to the inhibition of MHC class II surface expression by AdE1A (figure 4.11, 4.13 and 4.15). The fact that AdE1A double mutations at residue positions 19 and 20 in the N-terminal region affect the ability of AdE1A to inhibit MHC class II surface expression (figure 4.13) indicate this being a case for repression of STAT1 phosphorylation. STAT1 binds to the N-terminal region of AdE1A (Look *et al.,* 1998), and mutations at those residues have proved to abrogate binding to several other cellular proteins (Rasti *et al.,* 2005), hence indicating their importance in mediating interactions. However, this was not consistent with the data from figure 4.17, which showed the same mutants down-regulating cellular response to IFNγ at a level equal to w.t., hence not corresponding to the outcome on MHC class II levels (figure 4.13). To understand if this down-regulation of both immunosubunit and MHC class II is due to interference with STAT1 phosphorylation, or AdE1A targeting multiple points of the JAK-STAT pathway or AdE1A binding to other transcriptional components at the promoter is a requirement for future study. This may also include assessing the binding affinity of the AdE1A double mutant to STAT1. If it is

proven not to bind, it would be interesting to investigate if it affects immunosubunit expression after transfection into cells. Furthermore, IRF-1 protein is found to be crucial in initiating and regulating transcription of LMP2, LMP7 and MECL-1 (White *et al.,* 1996; Namiki *et al.,* 2005; Foss and Prydz 1999), so the relationship of AdE1A to this protein can also be investigated. MHC class I is constitutively expressed in all somatic cells. Results from this study have shown that surface MHC class I expression was unaffected following AdE1A transfection and viral infection (Figure 4.10A-D, 4.12), whereas the outcome was more complex in adenovirus transformed cells where MHC class I was expressed regardless of IFNγ in the Ad5E1A expressing cells. However, in the Ad12 transformants, MHC class I was generally reduced prior to IFNγ treatment but was expressed afterwards (Figure 4.14B). The outcome from the viral infection experiments has contradicted previous data that showed down-regulation of MHC class I following Ad5 infection (Burget *et al.,* 1987; Wold *et al.,* 1999; Lippe *et al.,* 1991). However, it was in agreement with one study by Routes and Cook (1990) that showed similar results to this study. The exact effect of AdE1A on MHC class I expression has yet to be clarified.

Retrospectively examining the effects of AdE1A on the surface MHC class I and class II levels against the backgrounds of adenovirus transformation, infection and AdE1A transfection have revealed some inconsistencies (Table 4.2). In Ad12 transformed cells, MHC class I levels were down-regulated while it remained unchanged in cells infected or transfected by Ad12 virus and Ad12E1A respectively. Each of the three systems offer very different setting, and thus are not strictly comparable. For instance, during Ad5 infection, the transportation of class I molecules to the cell surface is impaired by E3 (Wold and Tollefson 1999; Williams *et al.,* 2004) leading to the down-regulation of MHC class I, whereas in Ad5E1 transformants, no E3 is expressed so the levels of MHC class I remain unchanged (Williams *et al.,* 2004). Hence, different outcomes on MHC levels can be observed with the same serotype. Additionally, a study addressing the effect of AdE1A on insulin receptor substrate 4 (IRS-4), has shown that IRS-4 was only detected in 13SAd5E1A-expressing A549 cells but not in Ad12E1A and 12SAd5E1A expressing cells

(Shimwell *et al.,* 2009). However, no effect on IRS-4 expression was observed in A549s transfected or infected with AdE1A and Ad5 w.t. virus respectively (Shimwell *et al.,* 2009). In addition, it was found that early passage 13SAd5E1A-expressing A549s do not express IRS-4 but over time in culture expression was markedly increased. It was suggested that stable but not transient expression of 13SAd5E1A is required for IRS-4 overexpression (Shimwell *et al.,* 2009). This concept may be applied to this study with regard to MHC class I expression. It can be hypothesized that stable expression of Ad12E1A in the transformed cells caused down-regulation of MHC class I surface expression, whereas there was no change in the infected and transfected cells. Furthermore, certain aspects of the experiments may need to be improved. For instance, during flow cytometry analysis, dual staining (for AdE1A and MHC class I/II) may be suggested in order to examine MHC surface levels in selected AdE1A positive cells after infection and transfection. The fact that no change in MHC class I levels was observed in all Ad5/Ad12 transfected and infected cells may be due to the fact that any significant effect on MHC class I levels is diluted within a whole population of non-AdE1A positive cells. On the other hand, infection by Ad5 has not caused any effect on MHC class II surface expression, whereas there was down-regulation after transfection and transformation. An experiment involving a viral titration may be required to investigate this further.

Comparing Ad5 and Ad12, the latter is highly oncogenic whereas the former is not (Trentin *et al.,* 1962; Yabe *et al.,* 1962, 1964). This correlates with Ad12 ability to down-regulate MHC class I in the adenovirus transformed cells (Schrier *et al.,* 1983; Ackrill and Blair 1990; Friedmann and Ricciardi 1988) enabling the cell to escape immune system recognition and thus contributing to the induction of tumours in new-born rodents (Trentin *et al.,* 1962; Yabe *et al.,* 1962, 1964). In the present study, it was found that Ad12 transformation also down-regulated LMP2 and LMP7 immunosubunits after transformation whereas Ad5 did not have any effect. Past studies have attributed Ad12 oncogenicity on a 20 amino acid alanine-rich segment that is unique to the serotype (Telling and Williams 1994). This “spacer” region in situated between CR2 and CR3 and is missing entirely in

Ad5E1A. However, it is not clear how this region influences oncogenicity, but it is thought that it may not be acting alone; as other studies have revealed that, though the spacer region is essential for tumourigenicity, it was not responsible for MHC class I down-regulation (Williams *et al.,* 2004).

6.3 The effect of AdE1A on immunoproteasome activity and antigen presentation

In the light of the above, results from chapter 3 and 4 showed that AdE1A down-regulates immunosubunit expression as well as being involved in direct binding to MECL-1; the aim of chapter 5 was to investigate if this has any repercussions on the catalytic activity of the immunoproteasome. Initially, an *in vitro* experiment, in which purified immunoproteasomes were incubated with fluorogenic substrates (that assess the trypsin, caspase and chymotrypsin activity), in the absence or presence of AdE1A, was undertaken. However, AdE1A as well as irrelevant proteins used as controls, (namely GST and BSA) all caused inhibition of the chymotrypsin activity while the caspase and trypsin activities remain unchanged (Figure 5.1A-C). The same outcome was also observed with protein quantities as low as 0.05 µg. To overcome this problem, an *in vivo* approach was undertaken whereby the effect of AdE1A on the presentation of known epitopes, from the well-characterised EBV system, to their corresponding antigen specific $CD8^+$ T-cell clones was studied. Three EBV-derived CTL epitopes with known pathways through the antigen processing system were used and these are: **CLG**GLLTMV (CLG), TAP-independent, proteasome-dependent epitope; **IED**PPFNSL (IED), TAP-dependent, proteasome-dependent epitope (Lautscham *et al.,* 2001) and **FLY**ALALLI (FLY), TAP-independent, immunoproteasome-dependent epitope (Lautscham *et al.,* 2003). Previous studies have shown that AdE1A, specifically from Ad12, inhibits TAP1 and TAP2 expression in adenovirus transformed cells (Rotem-Yehudar *et al.,* 1994, 1996; Vertegaal *et al.,* 2003); hence using EBV-derived epitopes that may be either TAP dependent or independent

should allow us to identify if any of the experimental outcomes may be due to inhibition of the TAP proteins rather than the effect on the proteasome/immunoproteasome alone, additionally this could provide further insight into the role of the TAP proteins in antigen presentation. Similarly, study on the FLY epitope will make it possible to study the effect of AdE1A on the immunoproteasome and the resulting outcome on antigen presentation without the involvement of the TAP proteins. All three epitopes are derived from the EBV LMP2A protein. In ELISA and CD107a staining experiments carried out in the presence or absence of AdE1A, it was shown that AdE1A inhibits antigen presentation of IED and CLG epitopes (Figure 5.2 and 5.3). The observation that there was also a reduction in the TAP-independent CLG recognition by CTLs (ELISA) and that the MHC class I expression remains unchanged (Figure 3.14) suggests that this may be as a result of AdE1A targeting the 20S proteasome. Nevertheless, it may be premature to come to this conclusion without further investigations to rule out involvement of other components of the antigen processing machinery (see future work). Attempts were made to study the effect of AdE1A on the presentation of the immunoproteasome-dependent FLY epitope, but there was difficulty in acquiring recognition of the transfected fibroblasts by CTLs. An alternative way of delivering the LMP2A or AdE1A gene via a recombinant vaccinia virus (Lautscham *et al.,* 2001, 2003) was ruled out as there were concerns that the use of a virus may have its side-effects and hence affecting the accuracy of the data. However, it may have been a better option, and hence an option for a future experiment.

6.4 Role of AdE1A in the context of infection and immune evasion

Like all successful human viruses, adenoviruses have evolved multiple ways to evade the host's immune system. Soon after infection, the early region genes that code for non-structural, regulatory proteins (E1A, E1B, E2, E3 and E4) are expressed. These re-programme host cell transcription to create a favourable environment for virus replication

(Shenk 1996, Burgert *et al.,* 2002). Evidence so far shows that AdE1A and AdE3 have major roles in immune system invasion.

In this study, it was found that AdE1A down-regulates immunosubunit expression as well as having a profound effect on surface MHC class I and class II levels in at least one of the backgrounds of infection, transfection or in adenovirus transformed cells. This may affect the recognition of the infected cell by the host immune system. However, another adenovirus early protein, AdE3, also plays a primary role in immune evasion. The adenovirus early transcription unit 3 (AdE3) encodes at least four proteins: E3-gp19K, E3-14.7K, E3-10.4K/14.5K and E3-11.6K. They are not essential for viral replication although they play an important role in facilitating the establishment and persistence of adenovirus infection. They reduce the recognition of infected cells by the host immune system, allowing the viability of the cell while viral replication continues. E3-gp19K down-regulates surface MHC class I expression by inhibiting its transport from the endoplasmic reticulum to the plasma membrane (Burgert *et al*., 1987; Wold *et al.,* 1999, reviewed by Horwitz 2004). E3-gp19K also inhibits the loading of peptides by tapasin, as a result reducing the amount of peptide presented by MHC class I in infected cells (reviewed by Horwitz 2004; Bennett *et al*., 1999). AdE3-14.7k is a 128 amino acid protein that inhibits TNF-induced apoptosis (Gooding *et al.,* 1988). E3-10.4K/14.5K (RID) is a protein complex, consisting of RIDα and RIDβ polypeptides (Tollefson *et al.,* 1991), which was found to downregulate fas receptors of the death ligands, FAS-L and TRAIL by internalisation into the cell and degradation in lysosomes, thus inhibiting apoptosis (Shisler *et al*., 1997, Tollefson *et al.,* 1998). It also inhibits TNF-induced apoptosis (Gooding *et al.,* 1991), degrades the epidermal growth factor receptor (EGFR) from the cell surface (Tollefson *et al.,* 1991; Carlin *et al.,* 1989) as well as inhibiting TNF-induced NF-Kb signal transduction (Friedman and Horwitz 2002). The three E3 proteins prevent the infected cell's recognition by the immune system, although there is an additional Ad E3 protein called the AdE3-11.6K (also known as the adenovirus death protein, ADP) (Tollefson *et al.,* 1996). This protein is not expressed at the early promoter, at the same time as the other E3 proteins, but

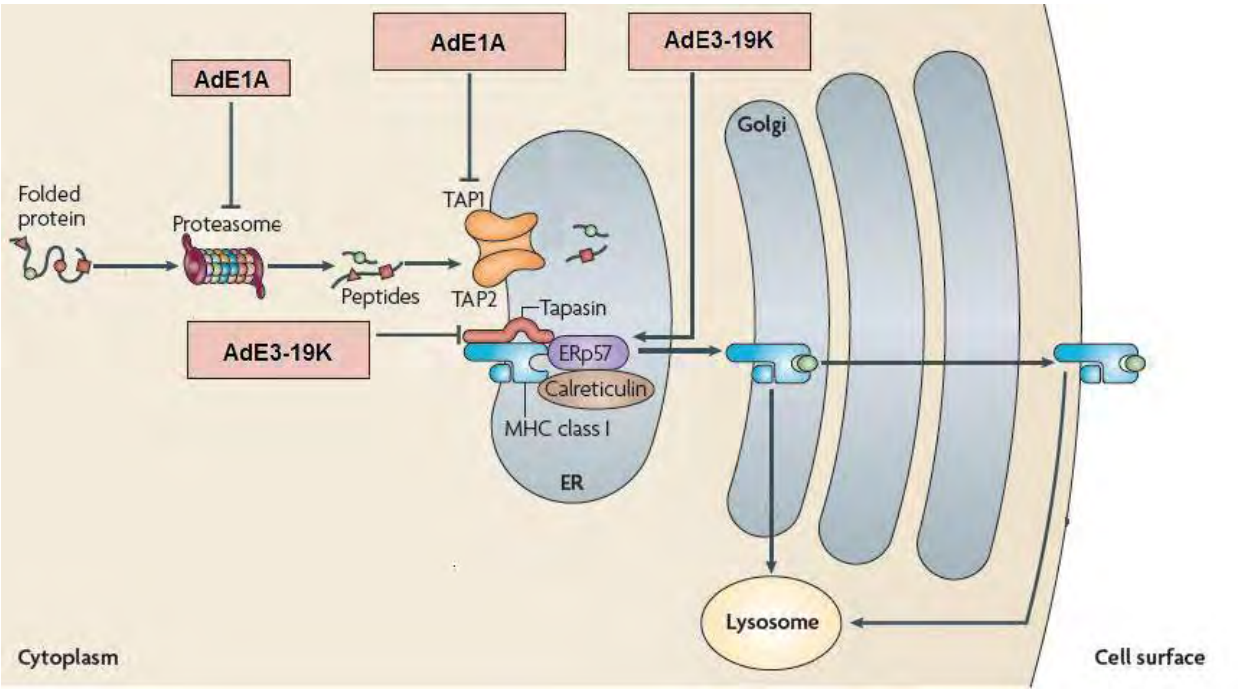
synthesized later in infection from the major late promoter, seemingly during the death and lysis of the cell when the newly formed virus progeny is released (Tollefson *et al.,* 1996).

AdE1A on the other hand, targets different components of the antigen processing machinery. Previous studies showed that it binds to the 20S proteasomes (Rasti *et al.,* 2005; 2006), as well as the ATPase and non-ATPase components (S4 and S8; S2 respectively) of the 19S complex (Grand *et al.,* 1999; Turnell *et al.,* 2000; Zhang *et al.,* 2004) and STAT1 proteins (Zhang *et al.,* 1996; Look *et al.,* 1998; Leonard and Sen 1996). Research has also found that the levels of MHC class I, TAP1, TAP2, tapasin, PA28α and PA28β expression is reduced in Ad12E1 transformed cells in comparison to those transformed by Ad5, hence enabling them to escape recognition by CTLs as the generation of peptides and loading onto MHC complex requires those components (Vertegaal *et al.,* 2002, 2003; Rotem-Yehudar *et al.,* 1994). In this study, this was expanded to reveal that AdE1A also inhibited immunoproteasome expression during infection, transfection and in Ad12 adenovirus transformed cells. It also down-regulated MHC class II cell surface expression in those conditions.

Evading detection by the host's immune response is an essential strategy for viruses, if they are to persist in host cells, enabling them to manipulate the host cell systems without detection by the host immune response. As with many viruses, to tackle this, adenoviruses have evolved a strategy to target multiple components of the antigen processing machinery. It does this by recruiting both AdE1A and AdE3; both proteins targeting a different set of components within the machinery. However, there is an overlap, in terms of their effect on MHC class I surface expression. This multiple targeting provides adenoviruses with backup proteins that may still disrupt antigen processing in the event of either viral protein being inhibited, adding an extra challenge to the host immune system. Thus, it can be presumed that both AdE1A and AdE3 complement each other in their inhibition of the antigen processing and presentation system (Figure 6.1). This

multiple targeting by several viral proteins is not unique to adenoviruses as will be seen below.

Strategies for immune evasion by viruses are very diverse. The processes behind the disruption of antigen processing and presentation components such as MHC class I, MHC class II, TAP proteins and proteasome vary between viruses, and each of these components are targeted in multiple ways. For the purpose of this discussion, we will compare and contrast the effect on surface MHC class I and class II expression between adenoviruses and other viruses.



Modified from Hansen and Bouvier 2009

Figure 6.1 MHC class I antigen presentation pathway targeted by adenovirus proteins
Schematic diagram illustrating components of the MHC class I antigen processing machinery that are targeted by AdE1A and AdE3.

As already mentioned, adenovirus down-regulates surface MHC class I expression mainly through the viral protein AdE3, specifically the E3-gp19K, that down-regulates surface MHC class I expression by inhibiting its transport from the endoplasmic reticulum to the plasma membrane (Burgert *et al*., 1987; Wold *et al.,* 1999, reviewed by Horwitz 2004). It also inhibits the loading of peptides by tapasin as a result reducing the amount of peptide

presented by MHC class I in infected cells (reviewed by Horwitz 2004; Bennett *et al*., 1999). Previous studies have shown that surface MHC class I expression is repressed in Ad12 transformed rodent cells in comparison to Ad5 transformed cells (Schrier *et al.,* 1983; Ackrill and Blair 1990; Friedmann and Ricciardi 1988). Limited research on transformed human cells also showed similar results (Bottley *et al.,* 2005; Vasavada *et al.,* 1986), and this was further expanded in this study. Previous research also revealed that Ad5 infection down-regulates MHC class I surface expression (Burget *et al.,* 1987; Wold *et al.,* 1999; Lippe *et al.,* 1991), however this was contradicted in this study, where no effect was observed. Its still unclear whether AdE1A has any direct effect on MHC class I levels on its own as transfection experiments in this study showed no disruption to surface MHC class I expression.

Other viruses use different ways to tackle MHC class I expression. HIV-1 recruits at least three viral proteins to target surface MHC class I expression. Each of these has a different method of disrupting the expression and function of MHC class I. For instance, HIV-1 Nef protein co-operates with another protein (phosphofurin acidic cluster sorting protein-1) to cause endocytosis and recycling of surface MHC class I back into the Golgi network (Schwartz *et al.,* 1996; Piguet *et al.,* 2000; Swann *et al.,* 2001). Similarly, HIV-1 Tat protein represses MHC class I promoter activity (Howcroft *et al.,* 1993; 1995; Weissman *et al.,* 1998; Carroll *et al.,* 1998). The human pathogen Kaposi's sarcoma-associated herpesvirus (KSHV) encodes two viral proteins, K3 and K5, which are able to down-regulate surface MHC class I expression by faciltating their endocytosis (Coscoy and Ganem 2000; Coscoy *et al.,* 2001; Stevenson *et al.,* 2000), followed by their ubiquitination and proteolytic degradation (Coscoy *et al.,* 2001; Lorenzo *et al.,* 2002). On the other hand, human cytomegalovirus (HCMV) recruits five viral proteins that all assist in evading MHC class I presentation: US2, US3, US6, US11 and UL18. UL18 is a MHC class I homolog that is capable of binding to β2m and peptide hence competing with MHC class I (Beck and Barrell 1988; Browne *et al.,* 1990; Fahnestock *et al.,* 1995). US2 and US11 cause the ejection into the cytoplasm and proteasomal degradation of MHC class I H chains (Wiertz

et al., 1996). US3 possesses an ER retention sequence and thus binds to MHC class I and prevents its transport to the cell surface (Jones *et al.,* 1996; Lee *et al.,* 2003; Ahn *et al.,* 1996). And finally US6 inhibits TAP, therefore inhibiting loading onto MHC class I (Ahn *et al.,* 1997; Hengel *et al.,* 1997; Lehner *et al.,* 1997). A summary of the different ways MHC class I is targeted by viruses is presented in Table 6.1.

Viral proteins can be processed and presented to $CD4^+$ T-cells via MHC class II molecules. There were no reports studying the effect of adenovirus on MHC class II. This was addressed for the first time in this study, where we found that AdE1A did down-regulate surface MHC class II expression during transfection, infection and in adenovirus transformed cells. However, it has previously been shown that adenovirus disrupts the JAK/STAT1 pathway, which is responsible for the induction on MHC class II expression (and other proteins involved in immune response) following cellular exposure to IFNγ, and specifically targeting STAT1 function (Look *et al.,* 1998; Leonard and Sen 1996; Zhang *et al.,* 1996); all this potentially affecting surface MHC class II expression. Various other viruses also seem to target the JAK/STAT1 pathway, and a higher proportion of those disrupting STAT1 protein (Hegde *et al.,* 2003). Table 6.2 shows different viruses and their respective proteins.

Infection of mouse embryonic cells with adenovirus and analysis of antigen presentation with Ad5-specific CTL have revealed that immunoproteasome-containing cells processed the adenovirus early 19k 1B protein (E1B)-derived epitope $E1B_{192\text{-}200}$ with increased efficiency leading to detection of the virus (Sijts *et al.,* 2000). This group produced a tetracycline-regulated mouse cell line that allowed titratable formation of immunoproteasomes. When the immunoproteasomes were highly expressed prior to infection, it produced optimal cytotoxic T cell (CTL) activation hence the recognition of the infected cell by the immune system. In the light of the above data, one can conclude that AdE1A may preemptively target immunoproteasome expression early in infection,

Table 6.1 Selected viral proteins that interfere with antigen presentation (Petersen *et al.*, 2003)

Mechanism	Virus	Protein
Down-regulates MHC class I β_2m transcription	HIV-1	Tat
Reduces the MHC class I mRNA level	Bovine papillomavirus	E5
Inhibits phagocytosis by DCs and thereby interferes with cross-presentation, but also induces DC maturation and surface MHC class I up-regulation	HIV-1	Secreted Tat
Blocks 11S regulator association with the proteasome	HIV-1	Tat
Binds TAP in the ER an inhibits peptide translocation	HCMV	US6
Prevents TAP transport of peptides into the ER	HIV-1	Unknown
Prevents TAP association with tapasin	Adenovirus	E3/19K
Competes for β_2m and peptide	HCMV	UL18
Reduce level lessens the availability of epitopes from other viral proteins	HIV-1	Rev
Delays MHC class I egress from the ER	HCMV	US10
Retains MHC class I molecules in the ER	Adenovirus	E3/19K
Binds MHC class I in the ER and prevents its egress	HCMV	US3
Blocks the transport of MHC class I molecules from the ER into the Golgi	MCMV	gp40 (m152 product)
Lowers the surface level of MHC class I by facilitating MHC class I/APLP-2 interaction	Adenovirus	E3/19K
Reduces the quantity of MHC class I protein	Bovine papillomavirus	E5
Binds MHC class I in the assembly complex and causes rapid turnover of MHC class I	Murine γ-herpesvirus 68	mK3
Increases MHC class I turnover	HIV-1	Vpu
Ejects MHC class I molecules into the cytoplasm	HCMV	US2 and US11
Redirects MHC class I molecules to lysosomes	MCMV	gp48 (m06 product)
Retains MHC class I in the Golgi	Bovine papillomavirus	E5
Increases endocytosis of MHC class I from the cell surface via an allele-specific mechanism	HIV-1	Nef
Facilitates MHC class I in the ER and remains associated with it at the cell surface	KSHV	K3 and K5
Complexes with MHC class I in the ER and remains associated with it at the cell surface	MCMV	gp34 (m04 product)

Table 6.2: Viral inhibition of the JAK/STAT signal transduction, expression of CIITA or induction of MHC class II gene expression (Hegde *et al.*, 2003)

Virus	Protein	Mechanism
Poxviruses		
Several species	e.g. T7, B8-R	Soluble homologs of IFN-gR that sequester immune IFN-γ
Vaccinia	VH1	Dephosphorylation of activated STAT1
Herpesviruses		
Herpes simplex virus 1	Unknown	Phosphorylation of Jaks and STAT1 affected
Human cytomegalovirus	Unknown	Loss of Jak1; inhibition subsequent to nuclear translocation of STAT1 dimer
Murine cytomegalovirus	Unknown	Inhibition subsequent to nuclear translocation of STAT1 dimer
Epstein-Barr virus	BZLF-1	Reduction in transcription of IFN-gR1
Varicella zoster virus	Unknown	Reduction in Jak2 and STAT1 levels
Paramyxoviruses		
Simian virus 5	V	Proteasome-mediated degradation of STAT1
Mumps virus	V?	Destabilization of STAT1
Sendai virus	C	Reduced synthesis and phosphorylation of STAT1; destabilization of STAT1
Human parainfluenzavirus 3	Unknown	STAT1 phosphorylation affected?
Nipah virus	V	Complex formation with STAT1 and sequestration in the cytoplasm
Adenoviruses	E1A or E1A-dependent events	Reduction in IFN-gR2 levels; inhibition of function of STAT1when bound by E1A; decrease in STAT1 levels; inhibition of general transcription
Other viruses		
HIV	Unknown	Reduced transcription of NF-YA
HIV	Tat	Binds cyclin T1 to inhibit CIITA–pTEFb interactions
Hepatitis C virus	Core	Decrease in STAT1 expression
Murine polyoma virus	Large T	Binds to Jak1
Ebola virus	Unknown	Inhibition before STAT1 dimer formation
Hepatitis B virus	Polymerase?	Effects on transactivation function of STAT1?

Abbreviations: CIITA, class II transactivator; IFN-gR, interferon-g receptor; Jak, Janus kinase; NF-YA, nuclear factor-Y subunit A; pTEFb, positive transcription elongation factor-b; STAT1, signal transducer and activator of transcription 1.

inhibiting its activity and hence avoiding recognition by the immune system. However, it is important to note that efficient production of MHC class I antigens of viral origin may not be the main function of immunoproteasomes. It was recently revealed that they may also have a major role in the maintenance of protein homeostasis and preservation of cell viability under conditions of IFN-induced oxidative stress by rapidly degrading nascent oxidant-damaged proteins (Seifer *et al.,* 2010). It would be interesting to investigate the effect of AdE1A on this aspect of immunoproteasome function in the future.

6.5 Future work

- Future experiments may include studying if AdE1A associates with components of MHC class I molecules or its chaperones. This can be done by Native Band Shift assay that analyses shifts by new bands in the event of complex formation, as described by Fu *et al.,* (2011). Alternatively, GST-tagged AdE1A can be used in a pull-down assay with cell lysates followed by blotting for constitutents of MHC class I molecule.
- Binding sites of MECL-1 within the CR3 region of AdE1A can be investigated by generating point mutations across the CR3 in order to obtain further insight into the role of each residue. Another future experiment may also include a pull-down assay using the N-terminal point mutants and the catalytic β subunits to identify their binding sites and comparing it to that of MECL-1.
- Other components of the antigen processing machinery that may be targeted by AdE1A need to be investigated. AdE1A's effect on chaperone proteins present in the endoplasmic reticulum that are involved in the folding and assembly of MHC class I molecules such as calreticulin, calnexin and ERp57, (Zhang and Williams 2006) can be studied. Additionally, association of AdE1A with tapasin, a glycoprotein involved in mediating interaction between MHC class I and the TAP complex leading to the loading of peptides onto the newly assembled MHC class I

molecule (Lankat-Buttgereit and Tampé 2002; Zhang and Williams 2006) can also be addressed. Tapasin expression was in fact found to be reduced in Ad12 transformed rat cells (Vertegaal *et al.,* 2002), so potentially affecting CTL recognition.

- With regard to antigen presentation to CTLs, introduction of minigene constructs that express minimal epitope (Lautscham *et al.,* 2001) hence bypassing the role of proteasomes in order to study the proteasome independent, TAP dependent/independent pathways may provide further understanding of other antigen processing components involved if applied to the adenovirus system.
- Further experiments are still required to study the effect of AdE1A on immunoproteasome activity and antigen presentation. With the limited time available for this project, a number of aspects have not been covered, which may be a topic for future investigation. For instance, the inclusion of the FLY epitope in both ELISA and CD107a staining is crucial to understand the effect of AdE1A on an immunoproteasome dependent antigen processing pathway. Furthermore, the experiments may be duplicated by using two different CTL clones/epitopes for each pathway. The quest for the appropriate negative control to equalise mRNA load have involved using mRNA encoding GFP and luciferase enzyme. However, GFP is a fluorescent protein and this interfered with flow cytometry analysis and luciferase protein on the other hand increased CTL background activity in the absence of LMP2A. Perhaps, in the future, mRNA encoding for actin could be attempted as the negative control.
- An alternative way of delivering LMP2A and AdE1A genes using a recombinant vaccinia virus (Lautscham *et al.,* 2001, 2003) may be attempted in a future experiment. This will avoid using the highly unstable and laborious mRNA.

6.6 Summary

Summarising the findings of this PhD project, it was found that AdE1A targets the immunoproteasome. AdE1A was shown to bind to the immunoproteasome as well as the 20S proteasome directly, preferentially binding to MECL-1 (in the immunoproteasome) and β1 and β5 (of the 20S proteasome). The binding sites of these subunits were in the N-terminual and CR3 regions of AdE1A, and through a series of pull-down assays, the binding sites within these two regions have been determined. AdE1A also down-regulates the expression of the immunosubunits LMP2, LMP7 and MECL-1 during infection and transfection. In adenovirus transformed cell lines, the immunosubunit expression was reduced more in Ad12 transformed cells than in the Ad5 transformants. Additionally, MHC class I expression was not affected following transfection and infection, however in adenovirus transformed cells, it was generally down-regulated to a greater extent in Ad12 expressing cells prior to IFNγ treatment; MHC class I expression was however restored in most of the cell lines following IFNγ treatment. With regards to MHC class II, there was little or no expression in adenovirus transformed as well as in Ad5 and Ad12 E1A expressing A549 cells; nevertheless, there was significant down-regulation upon transfection with AdE1A and during viral infection with Ad12. The phosphorylation of STAT1 and cell's ability to respond to IFNγ was also inhibited. ELISA assay and CD107a staining to measure CTL activity in response to target cells have revealed that the presence of AdE1A significantly reduced T-cell recognition, hinting at the interference with proteasomal activity.

CHAPTER 7

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