MODULATION OF INNATE IMMUNE RESPONSES IN NASOPHARYNGEAL EPITHELIAL CELLS BY THE EPSTEIN-BARR VIRUS (EBV)-ENCODED LATENT MEMBRANE PROTEINS LMP2A AND LMP2B.

By

Stephen Fintan Murphy

A thesis submitted to The University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

Cancer Research UK Institute for Cancer Studies

College of Medical and Dental Sciences

The University of Birmingham

June 2010

Abstract

Latent membrane proteins (LMP) 2A and LMP2B are expressed in a wide variety of Epstein-Barr virus (EBV) associated malignancies. The exact function of the proteins remains to be elucidated although it has been shown that in B-cells, LMP2A functions as a surrogate B-cell receptor, preventing BCR signalling to ensure persistence of viral latency. Less is known about the function of both proteins in epithelial cells but they have been shown to modulate signalling through MAPK, PI3K and Akt, to promote cell motility and to alter the turnover of certain classes of immunogenic receptors. Upon viral infection of a host cell the innate immune system is activated to promote and sustain initiation of an anti-viral state. Viruses have developed immune evasion strategies to counteract the effects of this immune response and also to prevent recognition of viral antigens by the adaptive response. Here novel functions are described for LMP2A and LMP2B in modulation of innate immunity, which have implications for the role of these proteins in contributing to oncogenesis. The first wave of the innate immune response is mediated by Toll-like receptor (TLR) signalling that initialises the type I interferon response. Findings presented here display attenuation of signalling from these receptors upon expression of LMP2A. These data were observed only in LMP2A expressing cells while those expressing LMP2B showed little or no alteration of the TLR signalling network, indicating that these functions are controlled by the unique N-terminal signalling domain of LMP2A. Examination of the putative role of LMP2A and LMP2B in intra-cellular traffic was also undertaken and revealed a shared role for both proteins in increasing endosomal-lysosomal trafficking and lysosomal acidification, important for degradation and turnover of receptors. These data present novel mechanisms by which EBV may evade immune responses and in doing so, contribute to the progression of the EBV-associated epithelial cell malignancy, NPC.

Tiomnú:
Do mo chlann agus mo chairde;
mo shaol, mo chraoi, mo smaointe

Acknowledgements

Firstly I would like to thank Prof. Lawrence Young for the opportunity and support he has given me to pursue this research degree as part of his group, to the Medical school for funding me and to CRUK for funding much of the research.

I would also like to take this opportunity to express my gratitude and sincere thanks to Dr. Christopher Dawson for all his guidance, support and help throughout this research, and for the chance he took in hiring a long-haired Irish Bioinformatician! To Dr. Suzy Stewart, without whom I wouldn't know anything about lab-work, got both me and this project up and running and for her support when I joined the group, thank you. To Dr John O'Neil, Dr John Arrand and Dr Khilan Shah for stepping in and offering their assistance during my final months in the lab, thanks, it was good to know you were all there. A special thanks to Dr Tom and Katster for keeping me sane, or allowing me to be insane on a day to day basis, I'll never forget the laughter, the mistakes, Amy Winehouse, the magic drawer, KFC and your support, thank you both so much. Thanks also to Sonia for keeping the lab running, my "press" stocked and for not getting too angry with my untidiness! A big thank you to all lab members past and present for making my time in Birmingham pleasurable and memorable, "White-fang"-Lou (We'll always have Chewbacca), Mhairi(for your comments on the commas), Jia, Haide, Angela, Vicki, Rob and Olivia. I would also like to thank Dr. Sally Roberts and Dr. Elena Odinstova for their help with confocal microscopy.

To Sarah, Sven and Rafal, thanks for your friendship and for making B'ham my home. To Vicki, Clodagh, Aoif, John for your constant support and love despite the distance, thank you. To my girls, Jennifer, Caroline, Suzanne, Ruth and baby Lily, who have allowed me to pursue my dreams wherever they take me and for welcoming me back with open arms and loving hearts, thank you so much for all that you have given and taught me. Finally to my parents for all you have done, your guidance, your friendship, laughter and support, my deepest thanks.

Chapter 2 Materials and Methods

List of Figures

Chapter 1 Introduction

Chapter 3 Generation of a panel of LMP2 loop mutants in a lentiviral expression system.

Chapter 4 Modulation of innate immune signalling by the LMP2A and LMP2B Proteins.

List of Tables

Chapter 1 Introduction

Chapter 2 Materials and Methods

Chapter 3 Generation of a panel of LMP2 loop mutants in a lentiviral expression system.

Abbreviation	Meaning
2’5’OAS	2'5' oligoadenylate synthase
AIDS	Acquired Immune Deficieny Syndrome
AP1	Activator Protein-1
AP2	Adapter Protein-2
ASFV	African Swine Fever Virus
ATL	Adult T-cell Leukemia
ATP	Adenosine triphosphate
BART	Bam H1 rightward transcript
BCR	B-cell Receptor
BDV	Borna Disease Virus
BHV1	Bovine herpesvirus 1
BKV	BK virus
BL	Burkitts Lymphoma
BPV	Bovine papilloma virus
BVDV	Bovine viral diahorrea virus
CARD	Caspase Recruitment Domain
CBF1	Cell binding factor-1
CBP	Creb-Binding Protein
CDE	Clathrin Dependent Endocytosis
CIE	Clathrin Independent Endocytosis
CIN	Cervical Intraepithelial Neoplasia
CIS	Cytokine inducible SH2 containing protein
CNS	Central Nervous System
CR2	Complement Receptor 2
CSFV	Classical swine fever virus
CTAR	C-Terminal Activation Region
DC	Dendritic Cell
E2F	E2 Transcription Factor
EBER	Epstein-Barr virus encoded small RNAs
EBNA	Epstein-Barr enocded Nuclear Antigen
EBOV	Ebola Virus
EBV	Epstein-Barr Virus
EE	Early Endosome
EEA1	Early Endosomal Antigen 1
EGF	Epidermal Growth Factor
EIF2A	Eukaryotic translation initiation factor 2A
EMCV	Encephalomyocarditis virus
ERK	Extracellular Signal-Related Kinase
FR	Family of Repeats
GC	Gastric Carcinoma
GEF	GTP exchange factor
GSK3	Glycogen Synthase Kinase
GTP	Guanosine Triphosphate

HBV	Hepatitis B Virus
HBVx	HBV X protein
HCC	Hepatocellular carcinoma
HCMV	Human cytomegalovirus
HCV	Hepatitis C Virus
HD	Hodgkins Disease
HERVS	Human endogenous retroviruses
HeV	Hendra virus
HHV	Human Herpesvirus
HIV	Human Immunodeficiency virus
HL	Hodgins Lymphoma
HLA	Human Leukocyte Antigen
HPIV	Human parainfluenza virus
HPV	Human papilloma virus
HRS	Hodgkin and Reed Sternberg
HSV	Herpes simplex virus
HTLV1	Human T-cell Lymphotropic Virus
HTNV	Hantavirus
IARC	International agency for research of cancer
ICAM	Intracellular adhesion molecule
IFN	Interferon
IHC	Immunohistochemistry
IKK	IkB kinase
IL	Interleukin
IM	Infectious mononucleosis
IRAK	Interleukin receptor associated kinase
IRF	Interferon Regulatory Factor
ISG	Immune serum globulin
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimualted response element
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus-kinase
JCV	JC virus
JEV	Japanese encephalitis virus
JNK	c-Jun N-terminal kinase
KIR	Killer-cell immunoglobulin receptor
KS	Kaposi's sarcoma
KSHV	Kaposis's sarcoma human virus
LAMP	Lysosome associated membrane protein
LANA	Latency Associated Nuclear Antigen
LCL	Lymphoblastoid cell line
LD	Lymphocyte depleted
LE	Late endosome
LGTV	Langat virus

LMP	Latent membrane protein
LPS	Lipopolysaccharide
LRC	Lymphocyte rich classical
LRR	Leucine rich repeat
MA	Middle Antigen
MAL	MyD88-adapter like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral signalling protein
MCC	Merkel cell carcinoma
MCD	Multimeric castelmans disease
MCV	Merkel cell virus
MCMV	Murine cytomegalovirus virus
MDA5	Melanoma differentiation associated gene 5
MeV	Measles virus
MHV68	Murid herpesvirus 68
MKK	MAPK kinase
MPR	Mannose-6-phosphate receptor
MPRV	Mapuera virus
MPV	Murine papillomavirus
MuV	Mumps virus
MVB	Multivesicular body
MVE	Multivesicular endosome
MYXV	Myxoma virus
NBP1	Nef binding protein 1
NDV	Newcastle disease virus
NEMO	NFκB essential modifier
NFKB	Nuclear factor kappa B
NiV	Nipah virus
NK	Natural Killer
NLPHL	Nodular lymphocyte-predominant Hodgkins lymphoma
NPC	Nasopharyngeal carcinoma
NPC1	Niemann-Pick disease protein 1
OHL	Oral hairy leukoplakia
OV	Orf virus
PCR	Polymerase Chain Reaction
PI3K	Phophoinositide 3 kinase
PI3P	Phosphatidylinositol 3-phosphate
PKC	Protein Kinase C
PKR	Protein Kinase Receptor
PML	Promyelocytic leukaemia
PRD	Positive Regulatory Domain
PRR	Pattern Recognition Receptor
PTLD	Post-transplant lymphoproliferative disorder
RABV	Rabies Virus

RBP-JK	Recombination Signal-Binding protein J kappa
RE	Recycling endosome
RIG1	Retinoic acid inducible gene 1
RIP1	Receptor interacting protein 1
RLH	RIG-1 like helicases
RPV	Rinderpest virus
RSV	Respiratory syncytial virus
RTK	Receptor tyrosine kinases
SeV	Sendai Virus
SH2	Src homology 2
SNARES	Soluble NSF Attachment Protein Receptors
SOCS	Suppressors of Cytokine Signalling
STAT	Signalling Transducers and Activators of Transcription
SV40	Simian virus 40
TAB	TAK1 binding protein
TAK	Tat-associated kinase
TANK	TRAF family member associated NFκB activator
TBK	TANK-binding kinase
TCR	T-cell Receptor
TERT	Telomerase Reverse Transcriptase
TGFB	Transforming growth factor beta
TGN	Trans-Golgi Network
THOV	Thogoto virus
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF-R	Tumour Necrosis Factor Receptor
TR	Terminal Repeat
TRADD	Tumor necrosis factor receptor type 1-associated death domain
TRAF	TNF receptor associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR domain containing adaptor inducing IFNβ
TTV	TT virus
TYK	Tyrosine kinase
VACV	Vaccina virus
VCA	Viral capsid antigen
VSV	Vesicular stomatatis virys
VZV	Varicella Zoster Virus
XAP-1	X associated protein 1
XLA	X-Linked Agammaglobulinemia
XLP	X-linked lymphoproliferative syndrome

CHAPTER 1

INTRODUCTION

1.1 Biology of Cancer

Cancer accounted for 7.9 million or 13% of worldwide deaths in 2007 and it is estimated that 30% of all cancer is preventable (WHO). Cancer is a complex multi-step group of diseases, characterised by progression or regression of cell types, many of which have unique pathogenesis, aetiology and treatment prognosis. Ten years ago, Hanahan and Weinberg (Hanahan and Weinberg 2000), attempted to simplify the transformation of normal cells into malignant cancers in a robust "six-hit" model, which reflected the common cellular machinery used across all cell types to control, cell differentiation, growth and death.

These six hallmarks represent tenets of the transformation to the malignant state and it is understood that mechanisms controlling progression towards malignancy can be, and are, vastly different between different tumour types. Briefly the hallmarks include, self-sufficiency in growth stimulation, resistance or insensitivity to anti-growth signals, evasion of apoptosis, unlimited proliferative potential, localised angiogenesis and tissue invasion or metastasis (Hanahan and Weinberg 2000). Accumulation of abnormal genetic and epigenetic signals that control these areas of the cellular life-cycle results in progression to a malignant phenotype, with tumours displaying most, if not all of these hallmarks. These genetic anomalies can be the result of errors in DNA replication during cell division or inherited mutations. Environmental factors, including exposure to carcinogens, have also been shown to play a role in development of these mutations and an increase in human proximity and exposure to such agents has been postulated to account for increases in cancer incidence (Belpomme, Irigaray et al. 2007; Irigaray, Newby et al. 2007). Environmental carcinogens include forms of radiation, (UV, pulsed electromagnetic fields and radioactivity), chemicals (benzene) and microorganisms

(bacteria and viruses). Lifestyle choices such as bad diet, lack of exercise, smoking and alcohol consumption are other risk factors associated with cancer.

1.2 Viruses and cancer

The International Agency for research on cancer (IARC) estimates that approximately 15% of cancer worldwide have a viral aetiology (Liao 2006). Historically the association of viral particles with malignancy and disease is well characterised: M'Faydean and Hobday described the cell free transmission of oral dog warts with cell free extracts in 1898 (M'Faydean and Hodbay 1898). Human warts showed similar transmission capabilities (Ciuffo 1907). In 1910, Peyton Rous described the transmission capabilities of Rous sarcoma in chickens (Rous 1910). It wasn't until 1958 when Denis Burkitt described a novel West African endemic tumour known as Burkitt's Lymphoma (BL) (Burkitt 1958), that the possibilities of a transmissible agent in human disease emerged. A transmissible herpesvirus-like particle was isolated from BL biopsies by Anthony Epstein, Bert Achong, and Yvonne Barr (Epstein, Achong et al. 1964), and was identified as Epstein-Barr virus (EBV), the first virus to be associated with a human tumour.

The association between a virus and a given cancer cannot be readily assumed and various criteria have been suggested to allow clearer delineation of this relationship. In 1965, Hill described the criteria he thought necessary for a causative association to be placed between the environment and cancer. Briefly this included: strength of the association; consistency; specificity of association; temporal relevance; biological product; biological plausibility; coherence and experimental evidence (Hill 1965). More specifically tailored to viral association with cancer were the criteria set down by Evans and Mueller in 1990 (Evans and Mueller 1990), which were spilt into two sections, one comprising the epidemiological elements of the association and the other the virological. With respect to epidemiology, that the

geographical distribution of the tumour is the same as the virus, that viral markers are increased in cases compared to matched controls, that these markers precede tumour development and that there is a higher incidence of tumours with markers. Finally that tumour incidence decreases by prevention of viral infection. In terms of virology it must be shown that the virus can transform cells *in vitro*, that the viral genome is present in tumour and not in normal controls and that virus-induced tumours can be recapitulated in an experimental model (McLaughlin-Drubin and Munger 2008).

More recently, zur Hausen put forward four criteria to enable establishment of a causal role for infection in cancer as opposed to an association between virus and malignancy (zur Hausen 1999). These include plausibility and evidence that an infection is a risk factor for tumour development, presence and persistence of the genome of the microbe in cells of the tumour, the stimulation of cell proliferation in tissue culture cells by the viral genome *in vitro*, and induction of proliferation and phenotypic changes (Damania 2006). These criteria give an insight into the difficulty of definitively establishing causal links between infecting viruses and malignancy.

Thus far, members of three families of double stranded DNA viruses, the *Hepadnaviridae*, *Herpesviridae and Papillomaviridae*, and also members of two families of RNA viruses, the *Flaviviridae* and *Retroviridae,* have been identified as tumour viruses and the relationships described above are well established. Members from three additional families have been implicated as having the potential for oncogenic transformation and these include the *Circoviridae, Polyomaviridae* and additional members of the *Retroviridae.* In these cases, research is ongoing to increases the strength of association between viral infection and malignancy.

1.2.1 Hepadnavirinae

Hepatitis B virus (HBV) is associated with non-malignant hepatitis, cirrhosis and heptocellular carcinoma (HCC), which exhibits a 60-90% association in adults and 100% association in children (McLaughlin-Drubin and Munger 2008). HBV is a partially double stranded DNA virus with four identified open reading frames and respective proteins, C, P, S and X (Dayaram and Marriott 2008). HBVx protein is the major transforming viral oncoprotein and has been shown to exert its effects via proteasomal degradation, disruption of the activator protein 1 (AP-1) transcription factor (Masucci 2004), distortion of centriole formation and XAP-1-mediated DNA repair (de Oliveira 2007). HBV is a prime example of the efficacy of immunisation against a virus causing a marked decrease in both chronic carrier rates and incidence of the associated malignancy (Ni, Chang et al. 2001; Chang 2009).

1.2.2 Papillomaviridae

Human papilloma virus (HPV) is a non-enveloped double stranded DNA virus with around 100 types. Different types have been associated with both mucosal and cutaneous lesions with varying associated risk of malignancy. HPV 5 and 8 are associated with high-risk cutaneous skin cancers, in patients with the autosomal recessive disorder, Epidermodysplasia verruciformis (EV) (McLaughlin-Drubin and Munger 2008). Low risk viruses associated with non-malignant mucosal disease, e.g. genital warts, include HPV 6 and 11. HPV 16 and 18 are high-risk viruses that are commonly associated with squamous intraepithelial lesions such as cervical intraepithelial neoplasia, (CIN) and invasive cervical cancer (de Oliveira 2007). HPV16 proteins E6 and E7 have been shown to have oncogenic potential and can transform infected keratinocytes and are more effective when co-expressed than when expressed alone (Dayaram and Marriott 2008). E6 has been shown to disrupt growth by inducing p53 degradation and cell immortalisation via upregultaion of hTERT (de Oliveira 2007). E7 has

been shown to interact with the pRb family of proteins and modulates E2F regulated cell growth (Damania 2006). A vaccine is now available against the L1 capsid protein and affords protection against both high-risk HPV16 and HPV18, where infection is associated with 70% of all cervical cancers. It remains to be seen whether vaccination programmes can reduce incidence of cervical cancer, (Galani and Christodoulou 2009).

1.2.3 Retroviridae

Human T lymphtrophic virus-1 (HTLV-1) is a delta type complex retrovirus that is associated with adult T cell leukaemia (ATL) (Dayaram and Marriott 2008). It has been studied extensively in Japanese populations where it was first discovered in the late seventies. It is estimated that 10-20 million people are infected by the retrovirus with only 5% developing disease (Liao 2006), including tropic spastic paraperesis. It is endemic in Japan, South Africa and parts of the Caribbean. Tax is the major oncogenic protein of the virus and is shown to act via mechanisms including ubiquitin reprogramming (Rousset, Desbois et al. 1996) interacting with p300/CBP to inactivate p53 and alteration of chromosomal segregation via TAXIBP2 and RANBP1 (McLaughlin-Drubin and Munger 2008).

1.2.4 Flaviviridae

Hepatitis C virus (HCV), is an enveloped single stranded RNA virus with a genome of 9.4kb coding for approximately 9 open reading frames. HCV is causally linked to hepatitis, cirrhosis and malignant HCC (McLaughlin-Drubin and Munger 2008). It is estimated the 3% of the population is infected with the virus, for which there is no vaccine due to its highly mutagenic nature (Liao 2006). It establishes a persistent lifelong infection in its host and evades the immune system through a variety of mechanisms including PKR receptor inactivation by viral proteins E2 and NS3 (McLaughlin-Drubin and Munger 2008).

1.2.5 Viruses with oncogenic potential

A variety of viruses have been identified that have been implicated in malignant transformation. These include members of the double stranded DNA *Polyomoviridae,* BK virus (BKV) associated with prostate cancer, JC virus (JCV) with brain tumours (Damania 2006) and merkel cell virus (MCV) associated with merkel cell carcinoma (MCC) (Becker, Schrama et al. 2009). Simian virus 40 (SV40) is a controversial polyoma virus that has also been implicated in human oncogenesis; in particular, its ability to transform pleural mesotheliomas (de Oliveira 2007). Members of the *Retroviridae* with oncogenic potential include, Human Endogenous Retrovirus (HERVs) associated with seminomas, breast cancer and melanoma and the Human Mammary Tumour Virus (HTMV), which is implicated in breast cancer. Finally the single stranded DNA, *Circoviridae* family member, Torque Teno Virus (TTV) is implicated in gastrointestinal, lung and breast cancers (McLaughlin-Drubin and Munger 2008).

1.2.6 Herpesviridae

The *herpesviridae* family of viruses are large double stranded DNA viruses of which 8 have been identified in humans, designated the human herpes viruses 1-8 (HHV1-8). The typical herpesvirus virion is similar in architecture across the family and the human viruses are morphologically indistinguishable, consisting of a core with liner double stranded DNA surrounded by an icosahedral capsid 100-110nm in diameter with 162 capsomeres, an amorphous tegument and an envelope. Many of the family members also exhibit dual-tissue tropism, effectively replicating via the lytic cycle in one cell type and establishing a lifelong persistent latent infection in another. Family members differ in terms of gene content (70 to 100 genes), genome size (100-250 kb), arrangement of genes, host range, maintenance of latency and length of replicative cycle. Lytic reactivation can occur resulting in virion release and re-establishment of latency. Three further subfamily classifications, the

alphaherpesvirinae, *betaherpesvirinae* and *gammaherpesvirinae* further divide the family based on biological properties.

Alphaherpesvirinae are grouped by host range, are relatively short cycling, exhibit rapid spread in culture, efficiently destroy infected cells and persist by latent infection in glial or neuronal cells. Genera include: Herpes simplex virus types 1 and 2 (HSV-1, HSV-2) causing the common "cold sore" and genital herpes, and the Varicellovirus (VZV) responsible for chickenpox in children and shingles in adults.

Betaherpesvirinae generally exhibit a restricted host range, with long reproductive cycling, slow spread in culture and infection often results in enlargement of infected cells or cytomeglia. Persistence occurs in secretory glands, lymphoreticular cells, kidneys and other tissues. Genera include: Human cytomegalovirus (HCMV), and human herpes virus 6 (HHV-6) and 7 (HHV-7). HCMV establishes latency in haematopoietic progenitor cells and is thought to persistently infect epithelial and glandular cells (Roizman and Baines 1991). Although asymptomatic in the majority of hosts, lytic reactivation in the immunocompromised can be fatal.

Gammaherpesvirinae exhibit a narrow host range and can be further subdivided into two genera, the gamma-1-herpesviruses or lymphocryptoviruses, of which EBV is the prototypic member and the gamma-2-herpesviruses or rhadinoviruses, of which HHV-8 or Kaposi's sarcoma-associated human herpesvirus (KSHV) is a member. Viruses in this group are specific to T cells or B cells and replicate *in vitro* in lymphoblastoid cells, with some lytic infection of epithelial cells and fibroblasts.

1.2.7 KSHV as an oncogenic virus

KSHV or HHV-8, a member of the rhadinovirus subfamily of gammaherpesvirinae, is a double stranded DNA virus with a genome length of 165kb containing approximately 90 open

reading frames (McLaughlin-Drubin and Munger 2008). It is associated with the non-malignant Multicentric Castleman's Disease (MCD), malignant primary effusion lymphoma (PEL) and Kaposi's Sarcoma (KS). HIV is a cofactor of HHV-8-associated malignancy with disease occurring in immunocompromised AIDS patients. Classic, endemic and transplant-related KS have been described, as well as the epidemic AIDS-related cases (Damania 2006). The KSHV latency-associated nuclear antigen 1 (LANA1) has been shown to contribute to oncogenesis by modulation of p53 activity and attenuation of Fas-mediated apoptosis. KSHV also expresses viral homologues of human genes which act to prevent apoptosis, such as v-Flip, and evade the immune system, for example the secreted cytokines vCCL1(K6), vCCL2(K4), vCCL3(K4.1) (Damania 2006).

1.3 Epstein-Barr Virus

EBV was the first human tumour using electron microscopy from *in vitro* cultures derived from fresh BL biopsies when "herpesvirus like" particles were observed in a subset of cells. The virus was shown to be biologically distinct from the previously characterised herpesvirus family members and was named Epstein-Barr virus, (EBV) (Epstein, Achong et al. 1964). The first major experimental evidence highlighting the oncogenic potential of the virus was its ability to infect and transform normal resting B-cells into immortalised lymphoblastoid cell lines (LCLs) *in vitro.* It was found that when lymphocytes from EBV-positive donors were placed in culture and the T lymphocytes either removed or inhibited by cyclosporin-A treatment, spontaneous outgrowth of immortalised LCLs occurred. This can also be replicated upon direct infection of resting B lymphocytes with EBV (Rickinson, Rowe et al. 1984).

EBV is a widespread virus infecting over 95% of the world's population. Primary infection is also usually asymptomatic and generally occurs during early childhood. When primary infection occurs during late adolescence it often results in infectious mononucleosis, a

self-limiting lymphoproliferative disease. Fever, weight loss, malaise, pharyngeal inflammation and a haematological increase in numbers of lymphoblasts, mainly CD8 positive T cells, are classical symptoms (Henle, Henle et al. 1968; Henle and Henle 1969). From the first association between EBV and BL experimental evidence has amassed that implicates a link between the virus and other malignancies. These include, undifferentiated nasopharyngeal carcinoma (uNPC), a mucosal epithelial cell-derived tumour that is endemic in parts of South East China (Canton region) and with increased incidence in Greenland and North Africa (Henle, Henle et al. 1968; Henle, Henle et al. 1970; zur Hausen, Schulte-Holthausen et al. 1970). EBV is also associated with approximately 10% of lymphoepithelioma-like gastric carcinoma (Takada 2000), a subset of Hodgkins lymphoma, post-transplant lymphoproliferative disorder (PTLD), and rare T cell lymphomas (Pagano, Blaser et al. 2004).

1.3.1 EBV: Mode of infection

The dual-tissue tropism of EBV is borne out by its interactions with and infective capacity of B lymphocytes and epithelial cells. EBV persists latently as an extrachromosomal episome in a small percentage of B-lymphocytes (Rezk and Weiss 2007). Production of virion particles occurs via the lytic cycle in another cell type, most likely a specialised epithelial cell, given EBV's oral mode of transmission (Sixbey, Nedrud et al. 1984). Primary uptake of the virus is believed to occur by direct binding of the virus by these epithelial cells or by transfer from neighbouring B cells (Shannon-Lowe, Neuhierl et al. 2006). EBV replicates in these cells and lytic cycle results in release of the virus into the throat where it is capable of infecting mucosal B cells and establishing latency. This latent state facilitates growth-transformation that results in the expansion of LCL-like cells in the lymphoid tissue of the tonsil, particularly at extrafollicular areas, and the emergence of infected cells in the peripheral blood (Hislop, Taylor et al. 2007). EBV then persists in a subset of resting memory B cells, which are capable

of proliferating indefinitely and at a frequency of infection of approximately 1 in $1x10^5$ to $1x10^6$ cells (Thompson and Kurzrock 2004).

1.3.2 Viral entry to B-cells and epithelial cells

The EBV viral envelope consists of a complement of glycoproteins that are involved in viral cell entry, of which gp350/220 is one. Primary B-lymphocytes express the high affinity complement receptor 2 (CR2/CD21), which facilitates attachment of the virus via this gp350/220 glycoprotein (Fingeroth, Weis et al. 1984; Nemerow, Wolfert et al. 1985; Nemerow, Mold et al. 1987; Tanner, Weis et al. 1987). Upon this initial interaction, three additional envelope glycoproteins (gH/gp85, gL/gp25 and gp42) associate together, and in conjunction with the MHC class II cellular molecules, HLA-DR, DP and DQ, facilitate endocytosis of the virus and release into the cytoplasm (Li, Turk et al. 1995; Borza and Hutt-Fletcher 2002). EBV can enter normal B cells by endocytosis into large non-clathrin-coated vesicles; subsequently, the viral envelope fuses with the vesicular membrane and the nucleocapsid is released into the cytoplasm (Miller and Hutt-Fletcher 1992).

Although it is believed that the primary site for viral replication upon transmission of the virus is a specialised epithelial cell, this environment does not appear to be amenable to establishment of latency or lytic replication. Detection of EBV in epithelia has proved problematic and studies of tonsillar and desquamated epithelial cells from EBV-positive patients have failed to detect the virus. Virus has not been identified in epithelial cells in close proximity to uNPCs or gastric carcinomas (Niedobitek, Meru et al. 2001). However, the virus can be detected in pre-invasive NPC lesions (Pathmanathan, Prasad et al. 1995) and some gastric dysplasia (Gulley, Pulitzer et al. 1996).

In order to ensure dual-tissue tropism the virus has two mechanisms of infection, a complex of three glycoproteins as described above and a complex of two glycoproteins without

gp42 (Borza and Hutt-Fletcher 2002). It has been proposed that primary infection occurs in B-lymphocytes through interactions between CR2 and gp350/220 and HLA class II and gp42. gp42 is thought to be retained by intracellular HLA class II, and virus is then produced which lacks gp42. This virus is then capable of infecting epithelial cells via the gH and gL proteins, where it replicates, shedding gp42 positive virus which can then latently infect B-lymphocytes (Borza and Hutt-Fletcher 2002). This is intriguing when taken into account with a study by Molesworth et al. in 2000 (Molesworth, Lake et al. 2000) who showed that virus produced by epithelial cells contained more gp42 and was up to ten times more efficient at infecting B-cells than virus produced by B-cells, thus facilitating latent infection following primary infection of an epithelial cell.

CR2 is only expressed at a very low level on the surface of epithelial cells (Fingeroth, Diamond et al. 1999), and very low rates of infection are recorded upon exposure of epithelial cells to virus *in vitro* (Imai, Nishikawa et al. 1998). Artificial systems have been developed to allow infection of epithelia *in vitro* (Rickinson 2001), since the occurrence of EBV-positive epithelial-derived neoplasia is suggestive of *in vivo* infection. These systems incorporated the use of CR2 over-expression or bypassing its necessity for viral entry (Li, Young et al. 1992; Knox, Li et al. 1996; Gan, Chodosh et al. 1997). Spontaneous lytic reactivation can occur in these cases and this is thought to be linked to the differentiation status of the cell (Knox, Li et al. 1996). *In vitro,* co-cultivation of a monolayer of epithelial cells with the recombinant EBV-producing BL-derived cell line, Akata, have resulted in experimental systems which allow better study of viral interaction of epithelial cells (Takada and Ono 1989; Shimizu, Yoshiyama et al. 1996). This recombinant EBV has a selectable antibiotic resistance marker which allows for positive selection of infected cells (Chang, Tung et al. 1999).

1.3.3 Virion structure

EBV contains a double stranded DNA core surrounding a toroid shaped protein core. This is surrounded by an outer nucleocapsid comprising 162 capsomeres, an amorphous tegument and a viral envelope studded with glycoproteins (Rickinson 2001). The most abundant of these glycoproteins is gp350/220, which is necessary for virion binding to the CR2 receptor to facilitate infection and viral entry. The genome itself is approximately 172kb and was the first human herpesvirus genome to be fully sequenced using the B95.8 strain of the virus isolated from an IM patient and sequenced upon cloning the overlapping restriction endonuclease fragments of EcoR1 and BamH1 (Baer, Bankier et al. 1984). Nomenclature of the open reading frames was designated based on these fragments with a four letter and number acronym relating to their position and the direction of cloning, e.g. BZLF1, first fragment of the Z fragment, leftward reading (*Figure 1.1).*

The genome is divided into two unique regions by tandem repeats: Ul (unique long); and Us (unique short) (Hayward, Nogee et al. 1980). Internal repeat sequences are also found throughout the genome and include: IR1, comprising the glycine alanine repeat of the EBNA1 protein; the internal IR2-IR4 repeats within the Ul region; and the 500bp terminal repeat (TR), of which there are between 4-12 repeats (Kintner and Sugden 1979) and which can be used as an indicator of clonality (Raab-Traub and Flynn 1986). The origin of replication of the viral episome (*oriP*) consists of two EBNA1-binding elements, the family of repeats (FR) with 20 EBNA1-binding sites, and the dyad symmetry elements with only 4 binding sites (Wysokenski and Yates 1989; Frappier, Goldsmith et al. 1994). FR is involved in retention of the episome in the nucleus and the DS is the origin of replication. Replication from *oriP* occurs once during S-phase following EBNA1-binding and recruitment of cellular enzymes. The FR element blocks leftward replication, so genome replication only propagates in a rightward direction, therefore

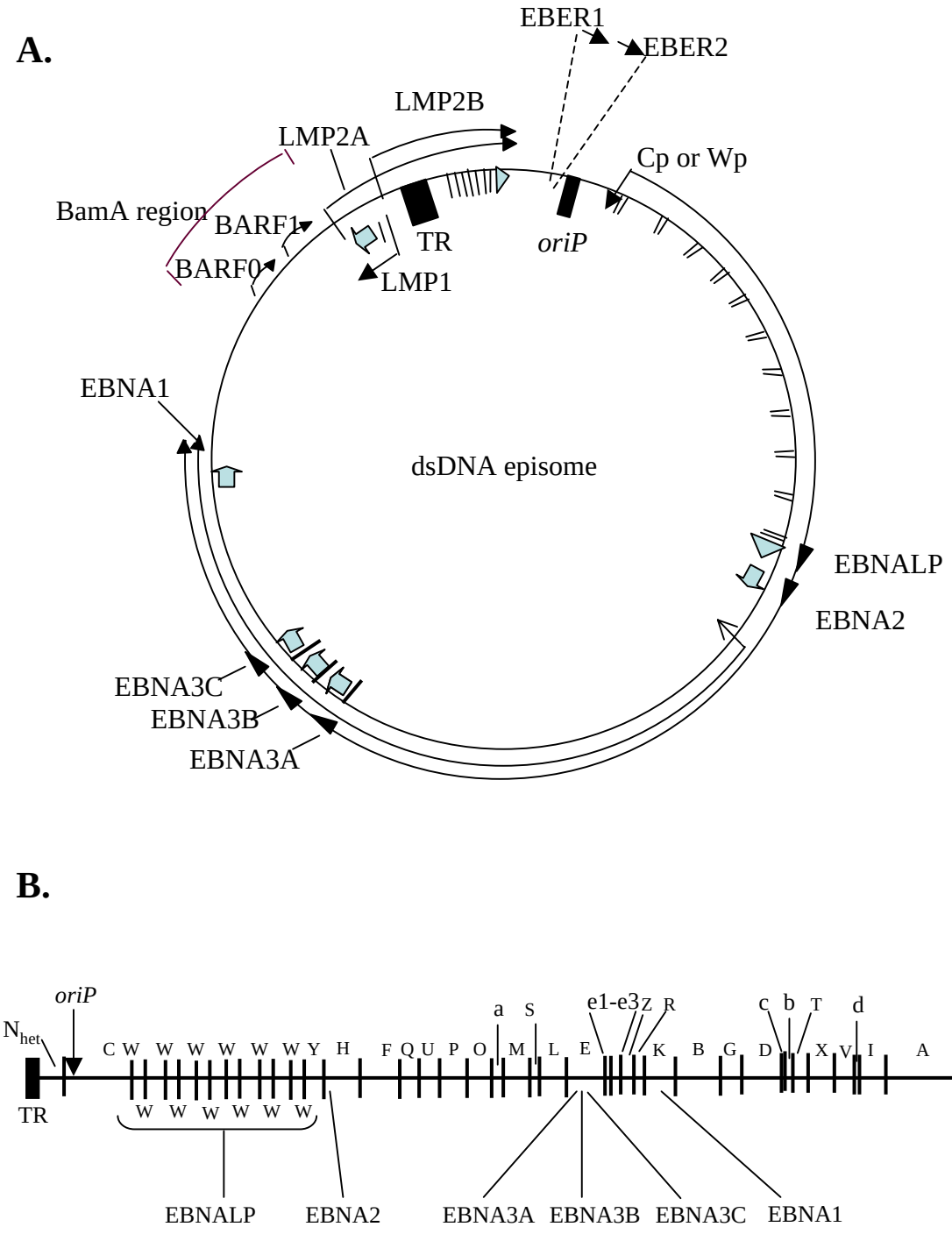
Figure 1.1 Structure of the EBV genome

(A) Viral Episome

Here a schematic of the EBV viral episome is displayed which is approximately 172kbp in length and codes for ~85 genes. The location of each of the latent ORFs are indicated including the various promoters controlling their expression. The nomenclature of the episome is designated from the BamHI fragments created during sequence of the EBV genome. Circularisation is facilitated by fusion of the 0.5kb TR (Terminal repeats) across which the LMP2 genes are transcribed. Maintenance of the episome is controlled by the *OriP,* which is also indicated.

(B) Linearised viral genome

Displayed here is a schematic of the linearised ORFs of the latent genes of EBV, the nomenclature designated by the BamHI restriction endonuclease map of the genome of the B95.8 EBV strain. Each region is named based according to size, wit A being the largest. The TR elements here are positioned at either end of the linearised genome. The W repeat element of EBNA-LP is also indicated. Both Figures are adapted from those in (Young and Rickinson 2004).



Adapted from Young and Rickinson, Nat. Rev Cancer; 2004: 4 (10) 757-768

beginning and ending at *oriP* (Dhar and Schildkraut 1991). *Figure 1.1* depicts a schematic representation of the EBV genome.

1.3.4 Strain Variation

Variation between EBV strains was originally identified on the basis of polymorphism in the EBNA2 gene, and subsequent identification of variation between EBNA3A, 3B and 3C facilitated division of EBV into two types: type 1 (EBV1) and type 2 (EBV2) (Dambaugh, Hennessy et al. 1984; Rowe, Young et al. 1989; Sample, Young et al. 1990). These two types show sequence homology of 50-80% (depending on the locus) with some allelic polymorphism amongst the aforementioned genes. Thus far, full sequences are available for two type 1 viruses, B95.8 and GD1 (Baer, Bankier et al. 1984; Zeng, Li et al. 2005), and one type 2 virus, AG876 (Dolan, Addison et al. 2006). Type 1 viruses (B95.8-like) have been shown to transform B-lymphocytes more efficiently *in vitro* than their type 2 (AG876-like) counterparts (Rickinson, Young et al. 1987). These EBNA2 polymorphisms appear to be the major determinant of the virus's transforming abilities (Cohen, Wang et al. 1989).

Epidemiological, serological and DNA sequencing studies reveal that both types are widespread in equatorial Africa and New Guinea and that type 1 is predominant in the West and South East Asia (Zimber, Adldinger et al. 1986). Other strain variants have been identified including the "f" polymorphism, which may protect against a predisposition to tumour development (Lung, Chang et al. 1990; Lung, Lam et al. 1991), and isolates carrying a 30bp deletion of the LMP1 gene, which is endemic in parts of south-east Asia. This particular LMP1 isolate displays increased transforming abilities in rodent fibroblasts, findings which have led to speculation that EBV isolates carrying this particular LMP1 isolate may predispose individuals to malignant disease (Li, Chang et al. 1996). EBV strain variation and comparisons between three strains, lineage and tree building based on differences within the EBNA proteins are reviewed elsewhere (McGeoch and Gatherer 2007).

1.3.5 Lytic cycle

The bi-phasic lifecycle of herpesviruses is divided into the latent cycle during persistent infection and productive lytic reactivation, during which viral replication occurs. Lytic replication is reliant on more EBV-encoded genes than the restricted number expressed during latency (Fixman, Hayward et al. 1995), is initiated from the *oriLyt* and multiple rounds of replication are usually involved (Hammerschmidt and Sugden 1988). The proteins involved are divided into three categories and are expressed sequentially: the *immediate early genes (IE),* mainly transsactivating proteins that control early gene expression; the *early genes (E)*, which include components of viral DNA replication; and the *late genes (L),* including structural proteins (Hislop, Taylor et al. 2007).

1.3.5.1 Immediate early genes (IE)

Induction of the lytic cycle begins with transcription of two genes: BZLF1 and Rta; the latter of which is transcribed from the BRFL1 locus. These are both transcribed from the Rp promoter, whilst BZLF1 can also be transcribed from the Zp promoter, and both can be activated by the BZLF1/ZEBRA protein itself (Flemington and Speck 1990). BZLF1 is a DNA-binding protein of approximately 36kDa and transactivates gene expression by binding to specific promoter elements, termed ZREs. These proteins can operate alone but it has been shown that maximal transactivation occurs when they act synergistically (Feederle, Kost et al. 2000). Lytic reactivation in latently infected cells is believed to be driven by BZLF1 expression and it has been shown that its transfection into the latently infected EBV-positive Raji BL cell line results in lytic gene expression (Chevallier-Greco, Manet et al. 1986; Biggin, Bodescot et al. 1987). Furthermore, Rta has been implicated in reactivation of the lytic cycle in epithelial cells *in vitro* (Zalani, Holley-Guthrie et al. 1996).

1.3.5.2 Early genes

BZLF1 and Rta act together to transactivate a number of genes including the thymidine kinase BXLF1, which acts via c-fos to downregulate the latent Cp promoter activity (Flemington and Speck 1990; Sinclair, Jacquemin et al. 1994; Zalani, Holley-Guthrie et al. 1996), and the DNA replication genes which comprise the core of the replication machinery. BMRF1, BALF2, BALF5, BSLF1, BBLF4 and BBLF2/3. BSLF1 and BMRF1 are transcriptional activators and induce other lytic cycle-associated genes. BHRF1 is a conserved 17kDa membrane protein that is related to Bcl-2 family of cellular proteins and may function to prevent apoptosis (Dawson, Eliopoulos et al. 1995).

1.3.5.2 Late genes

The late genes are transcribed following replication and encode viral structural proteins, including 11 glycoproteins. The classical membrane antigen complex (MA) consists of the BLLF1-encoded gp350 and the BXLF2-encoded gH/g85; these are involved in virus-cell interactions mediated by the CR2 cellular receptor. BZLF2 encodes the gp42 glycoprotein, which is important for viral entry into B-lymphocytes. Other structural proteins include p160 (BCLF1), three capsid proteins, p18, p23 and p40 (BFRF3, BLRF2 and BLRF1), and the tegument proteins, BNRF1 and BPLF1. The lytic cycle is reviewed extensively elsewhere (Tsurumi, Fujita et al. 2005).

1.3.6 Latent cycle

EBV establishes persistent latent infection in B-lymphocytes, which constitutes the second phase of its bi-phasic lifecycle. The B-lymphocyte is believed to be the site of viral persistence *in vivo* and this has been substantiated by analysis of the EBV strain of allogeneic bone marrow recipients prior to and post-transplant. Here it was shown that the recipient strain

was replaced with that of the donor, suggestive of a B-lympocytic reservoir for EBV (Gratama, Oosterveer et al. 1988). XLA (X-linked agammaglobulinaemia) patients cannot produce mature B-lymphocytes, are EBV-negative and are incapable of persistent EBV infection, further implicating these cells as the site of persistence (Young, Dawson et al. 1999). The pattern of gene expression during latency can change, which is heavily dependent on the differentiation status of the infected B-lymphocyte. Upon infection, B-cells become proliferating B-blasts characterised by the "growth programme" or *latency III type* pattern of EBV gene expression. This antigen activated B-blast can then enter the follicle, expand and undergo a germinal centre reaction. The latency programme is then switched to the "default" or *latency II* programme. Following this the B-cells leave the follicle and enter peripheral circulation as resting B-cells, entering the memory pool with a "latency programme" or *latency 0 type* pattern of expression, where expression of all viral proteins is restricted (Thorley-Lawson 2001). The pattern of viral gene expression is dependent on both the location of the infected B-cell and its differentiation status.

Examination of both EBV-associated malignancies and EBV-positive cell lines has lead to a greater understanding of these latency programmes and whilst these classifications are useful they are also fluid, and the virus has been shown to adapt to its cellular environment. BL cells displaying a classical *latency* I pattern of gene expression have been shown to drift overtime in culture to a *latency III* programme (Rowe, Lear et al. 1992). EBNA3 expression has also been identified in rare cases of an EBNA2-deleted BL, representing an unclassified latency programme (Kelly, Bell et al. 2002).

1.3.6.1 The growth programme/latency III

As mentioned previously, EBV infection has the ability to transform resting B-cells into proliferating LCLs, which are characterised by expression of a *latency III* pattern of gene

expression. Here, as in post-transplant lymphoproliferative disease (PTLD), all of the nine latent proteins are expressed, the six EBV nuclear antigens, EBNAs 1, 2, 3A, 3B, 3C and LP (leader protein), spliced from a single polycistronic transcript driven from the Cp/Wp promoter and the three latent membrane proteins, LMPs 1, 2A and 2B. In addition to these proteins, the virus also expresses two small non-polyadenylated RNAs, EBERs 1 and 2, and the differentially spliced RNAs from the Bam H1-A region of the genome, the BARTs, BARF0, BARF1 and the more recently described microRNAs (miRNAs). Three BHRF1 miRNAs have been shown to be expressed only in *latency III* (Cai, Schäfer et al. 2006). This pattern of gene expression activates the B-cell to becoming a proliferating B-blast (Thorley-Lawson 2001).

1.3.6.2 The default programme/latency II

This pattern of gene expression is observed in malignancies including NPC, EBV-positive Hodgkins lymphoma, T-cell lymphoma and NK-cell lymphoma (Young and Rickinson 2004). Gene expression here is restricted to Qp-driven EBNA1, LMP1, LMP2A, the EBERs, BARTs and BARF1 (Young and Rickinson 2004). A twenty member cluster of BART miRNAs is also expressed during *latency II* (Cai, Schäfer et al. 2006). A subdivision of *latency II* has been identified (*IIb*) in both lymphoid tissues of IM patients and EBV infected B-chronic lymphocytic leukaemia (B-CLL) cells *in vitro.* In these instances, LMP1 and LMP2A are not detected, but the six EBNA proteins are expressed (Klein, Kis et al. 2007). It is believed that this pattern of gene expression permits differentiation of B-cells into memory and enables maintenance of infection.

1.3.6.2 The restricted default programme/Latency I

Burkitt's lymphoma is characterised by a *latency I* pattern of gene expression which includes expression of the EBERs, BARTs and Qp promoter-driven EBNA1 (Rickinson and

Kieff 2001). The remaining five EBNA proteins and the LMPs are not detected. This expression programme often drifts to that of *latency III* in BL cells in culture (Blum, Lozanski et al. 2004).

1.3.6.4 The latency programme/Latency 0

This programme of viral latency is believed to allow persistence of infected B-cells in the memory pool and avoidance of immune surveillance (Thorley-Lawson 2001). As such, usually no proteins are detectable, although LMP2A is sometimes expressed. The EBER and BART transcripts are readily detected in these cells (Tierney, Steven et al. 1994), and EBNA1 may be expressed during division of these cells (Hochberg, Middeldorp et al. 2004).

1.4 EBV latent genes

1.4.1 EBNA1

EBV nuclear antigen I (EBNA1) is the most consistently expressed of all the EBV latent proteins. It is expressed in all forms of latency with the exception of the *latency 0* gene expression programme and is detectable in all malignancies associated with viral infection (Lee, Diamond et al. 1999; Rickinson 2001). Analyses of the EBNA1 protein have focused on its role in episomal maintenance. It is a DNA-binding protein of approximately 75kDa-encoded by the BamH1-K region of the viral genome, and comprises an 89 amino acid domain, a short basic residue-rich domain, a long hydrophilic carboxy terminus and a long Gly-Ala repeat element, which can be deleted without affecting EBNA1 function but may be detrimental to host immune evasion due to its role in the blockade of ubiquitin-mediated antigen presentation as demonstrated by domain swapping experiments (Levitskaya, Sharipo et al. 1997). It binds to elements within the *oriP* of the viral genome and facilitates synchronous replication with the host genome (Yates, Warren et al. 1985). EBNA1 can also modulate transcription of the viral

latent promoters (Lupton and Levine 1985), including that of LMP1 and the Cp promoter (Rickinson 2001), and may participate in cell growth transformation. This latter function is implicated by EBNA1's ability to form B-cell lymphomas when targeted to lymphoid cells in transgenic mice (Wilson, Bell et al. 1996), and this role may be extended to epithelial cells *in vitro,* where EBNA1 transformation of the NPC-derived HONE-1 cell line has been reported (Sheu, Chen et al. 1996). Cellular tolerance to EBNA1 in epithelial cells appears to be context-dependent (Jone et al., 2003), and whilst undifferentiated cells can tolerate EBNA1, its expression is associated with cellular toxicity in differentiation competent squamous epithelial cell lines (Jones, Smith et al. 2003). Differential promoter usage is a tenet of EBNA1 expression and these are utilised during the different latency programmes described above. The Cp/Wp promoter is silenced via methylation in *latency I and II* (Schaefer, Strominger et al. 1997) and EBNA1 expression is Qp promoter driven. During *latency III,* the Cp/Wp promoter is activated and the Qp promoter is silenced by EBNA1-binding (Nonkwelo, Skinner et al. 1996). The Fp promoter is believed to regulate EBNA1 expression during lytic cycle induction (Tsai, Liu et al. 1995).

1.4.2 EBNA2

EBV nuclear antigen 2 (EBNA2) is transcribed as part of the long polycistronic mRNA driven by the Cp/Wp promoter. Similar to EBNA1, EBNA2 is a nuclear phosphoprotein that transactivates transcription via interactions with the cellular DNA-binding protein CBF-1/RBP-Jκ. It has been shown to regulate viral latent promoters including those of LMP1, LMP2 and the Cp promoter, inducing Wp to Cp switching early in infection (Young and Rickinson 2004). EBNA2 expression is necessary for immortalisation of B-cells as demonstrated by the inability of the EBNA2-deleted P3HR1 EBV strain to transform B-lymphocytes and the restoration of

this upon EBNA2 reintroduction (Hammerschmidt and Sugden 1989; Rickinson 2001). EBNA2 has also been shown to mimic Notch (Zimber-Strobl and Strobl 2001).

1.4.3 EBNA-LP, 3A, B, C

EBV nuclear antigen leader protein (EBNA-LP) is not essential for transformation of B-lymphocytes but has been shown to be a coactivator of EBNA2 activity (Rickinson 2001) and is also involved in the progression of B-lymphocytes into the G1 phase of the cell cycle through interactions with p53 and the pRb tumour suppressor genes (Sinclair, Palmero et al. 1994). EBNA3A, 3B and 3C are transcriptional regulators that have been shown to target both cellular and viral latent genes including CD21, CD40, EBNA2 and LMP1. EBNA3A and 3C are essential for growth transformation of B-lymphocytes, whilst EBNA3B is dispensable (Robertson, Lin et al. 1996). The EBNA3 proteins negatively regulate EBNA2 function by interacting with the cellular transcriptional regulator protein CBF-1/RBP-Jκ (Robertson, Lin et al. 1996). EBNA3C can co-activate the LMP1 promoter in tandem with EBNA2 (Zhao and Sample 2000), and is also involved in cell cycle checkpoint deregulation (Radkov, Touitou et al. 1999).

1.4.4 Latent Membrane Protein 1 (LMP1)

Latent membrane protein 1 (LMP1) is the major transforming oncoprotein-encoded by EBV. It is transcribed from the BamH1-N region of the viral genome and codes for a 63kDa integral membrane protein, and is under the transcriptional control of either of two promoters: the L1-TR, activated in epithelial cells; and ED-L1, activated during infection of B-cells (Tsai, Lee et al. 1999). The oncogenic potential of LMP1 is borne out by its ability to induce transformation in the rodent fibroblast cell line, Rat-1, allowing anchorage-independent growth and tumour formation in athymic nude mice (Wang, Liebowitz et al. 1985). It is essential for

B-cell transformation *in vitro* (Kaye, Izumi et al. 1993) and acts as a constitutively active signalling molecule mimicking the activity of the tumour necrosis factor receptor (TNF-R) family member, CD40, engaging a variety of signalling pathways, mainly via the two domains of its cytosolic C-terminus, C-terminal activation regions 1 and 2 (CTAR1 and CTAR2). Through binding of members of both the TNF receptor associated factors (TRAFs) and TNFR1-associated death domain protein (TRADD) to these domains, LMP1 can mediate activation of various signalling pathways including Nuclear Factor kappa B (NFκB), c-Jun-N-terminal kinase (JNK), p38 MAPK, phosphatidylinositol-3-kinase (PI3-K), extra cellular signal-regulated kinase (ERK) and signal transducer activator of transcription (STAT). Activation of these pathways contributes to B-lymphocyte transformation, and fit well with LMP1's role as an activated CD40 receptor, given its role in B-lymphocyte development. LMP1 has also been shown to enhance epithelial motility and invasiveness *in vitro* (Dawson, Laverick et al. 2008) and to induce expression of genes involved in hyperproliferation and inflammation of *in vitro* keratinocytes (Morris, Dawson et al. 2008). LMP1 signalling and functions are reviewed extensively elsewhere (Eliopoulos and Young 2001; Morris, Dawson et al. 2009).

1.4.5 The EBV-encoded RNAs (EBERs)

The small non-polyadenylated RNAs, EBER1 and EBER2, are the most abundantly and consistently expressed EBV latent gene products. They are expressed in all EBV-associated malignancies and their abundance allows their use as a diagnostic marker for latent infection. The role of the EBERs in onocogenesis still remains unclear, although it has been shown that they are not essential for EBV-induced B-cell immortalisation (Swaminathan, Tomkinson et al. 1991) and that EBER2, but not EBER1, plays a role in B-cell transformation (Wu, Maro et al. 2007). It is likely that the EBERs play a role in viral immune evasion: they are recognised by

the cytosolic pattern recognition receptor (PRR), retenoic acid inducible gene 1 (RIG-1) (Samanta, Iwakiri et al. 2006), and have recently been shown to activate interferon (IFN) induction via toll-like receptor-3 (TLR3) activation in lymphocytes (Iwakiri, Zhou et al. 2009). An important role for the EBERs has been uncovered in the blockade of protein kinase R (PKR)-induced IFN responses. This is mediated by binding of the EBERs to PKR, and thus prevention of the phosphorylation and activation of the protein synthesis initiation factor, eIF2a (Nanbo and Takada 2002).

1.4.6 Bam-A Rightward Transcripts (BARTs)

The BamHI-A-encoded rightward transcripts (BARTs) were identified in 1989 (Hitt, Allday et al. 1989) and are set apart from the lytic cycle-associated transcripts encoded in this region by their direction of transcription. BARTs have been detected in NPC biopsies, EBV-positive BL and LCLs, suggestive of ubiquitous expression in latently infected epithelial cells and B-lymphocytes (Brooks, Lear et al. 1993). Although several open reading frames have been identified: BARF0, RK-BARF0, RPMS1 and A73 (Kusano and Raab-Traub 2001), the existence of putative protein products has been called into question and remains controversial. The BART intronic regions have been shown to generate the miRNAs and investigations into their role and the role of the BARTs is ongoing.

1.5 Latent membrane Protein 2 (LMP2)

1.5.1 Structure and transcription

The LMP2 genes are transcribed from across the terminal repeats of the viral episome and two mRNA species are generated: LMP2A and LMP2B (Laux, Perricaudet et al. 1988; Sample, Liebowitz et al. 1989; Salamon, Takacs et al. 2003). With the exception of the first exon, the genes share 100% identity. The unique exon of LMP2A encodes a 119 hydrophilic

amino acid sequence that constitutes the N-terminal signalling domain of the protein. The first exon of LMP2B is non-coding and translation is initiated at the ATG start codon located within the second exon. The proteins have molecular weights of 54kDa and 40Kda respectively. The LMP2A promoter is upstream of the gene, whilst LMP2B shares a bi-directional promoter with LMP1. It has been shown that the activity of the LMP2A promoter is regulated by DNA methylation and histone acetylation, with inactivation by methylation most pronounced in NPC-derived epithelial cell lines (Salamon, Takacs et al. 2003; Gerle, Korokani et al. 2007). Previous investigations have revealed that LMP2A promoter activity can be regulated by EBNA2 via CBF1 activity and the promoter has two consensus sequences, which are believed to mediate CBF1-binding (Zimber-Strobl, Suentzenich et al. 1991; Laux, Dugrillon et al. 1994; Zimber-Strobl, Strobl et al. 1994). In the absence of EBNA2 it is thought that LMP2A can autoregulate its own expression through manipulation of Notch signalling (Anderson and Longnecker 2008). Here it was also shown that LMP2B fails to regulate this activity and that point mutations within the N-terminal domain abrogated LMP2A promoter auto-activation by Notch.

The common regions of LMP2A and LMP2B encode the 12 hydrophobic transmembrane domains and the C-terminal cytoplasmic tails, which consist of 27 amino acids. Given the highly hydrophobic nature of the proteins it was postulated that they were multi-pass integral membrane proteins (Laux, Perricaudet et al. 1988; Longnecker and Kieff 1990). Some disparity exists in their exact localisation in B-lymphocytes compared to that observed in epithelial cells. LMP2A and LMP2B expression in B-cells is localised to the plasma membrane (Longnecker, Druker et al. 1991), but the proteins appear to be bound to internal membranes in epithelial cells, in endosomal perinuclear aggregates (Dawson, George et al. 2001; Lynch, Zimmerman et al. 2002; Allen, Young et al. 2005). The localisation of the proteins in both NPC-derived and HNSCC (Head and neck squamous cell carcinoma)-derived epithelial cells is

investigated in this thesis (results *Chapter 5*). The C-terminus of the proteins is believed to promote self-aggregation at the membrane and also to mediate LMP2B interference of LMP2A activity in B-lymphocytes (Rovedo and Longnecker 2007). Due to the extent of homology between the proteins it is likely that they possess similar functions, another aspect of LMP2A and LMP2B, which is investigated in this thesis (results *Chapters 3 and 5*).

1.5.2 N-terminal signalling domain

The 119 N-terminal signalling domain of the LMP2A protein is the most extensively studied region of both proteins. The region itself contains eight tyrosine (Y) residues, some of which become phosphorylated in B-cells (Longnecker, Druker et al. 1991) and epithelial cells (Scholle et al., 2001). Three of these residues have been identified as essential in the binding of non-receptor tyrosine kinases (non-RTKs) to the LMP2A protein. Two of these residues, Y74 and Y85, constitute the immunoreceptor tyrosine kinase activation motif (ITAM) and, upon phosphorylation, in concert with the Y112 residue, have been shown to bind to the Src homology (SH2) domain containing Syk tyrosine kinase. Syk is a member of the Src family of tyrosine kinases, of which Lyn is also a member. It has also been shown that Lyn can bind to the Y112 residue of LMP2A and mediate phosphorylation of the ITAM motif, and that all three of these residues are essential for LMP2A blockade of B-cell receptor signalling (BCR) (Miller, Lee et al. 1994; Fruehling, Lee et al. 1996

; Fruehling and Longnecker 1997). The five remaining tyrosine residues have been shown to be dispensable for LMP2A's effect on the BCR, but it has also been shown that the Y60 residue may bind Abl (Swart, Fruehling et al. 1999). The N-terminal tail of LMP2A also contains multiple serine/threonine residues, of which two (S15 and S102) can be phosphorylated by mitogen activated protein kinase (MAPK) *in vitro*, whilst LMP2A is identified a substrate for MAPK *in vivo* (Panousis and Rowe 1997). Finally, proline-rich motifs

are also found within the N-terminus and have been shown to interact with a number of WW domain containing, Nedd4-like E3 ubiquitin ligases, including AIP4/Ithcy, WWP2, Nedd4, and Nedd4-2 (Ikeda, Ikeda et al. 2000; Winberg, Matskova et al. 2000). These interactions result in targeting of LMP2A and LMP2A-bound proteins, such as Lyn, for ubiquitin-mediated degradation, thereby contributing to modulation of cell signalling. Ubiquitination has also been shown to be important for LMP2A activity and cellular localisation, possibly regulating its association with lipid rafts, via endocytosis and endosomal sorting to cholesterol-rich membrane regions (Ikeda and Longnecker 2007). It has also been shown that LMP2A may hijack the ubiquitin pathway to modulate cellular signalling from the Notch and Wnt (Portis, Ikeda et al. 2004).

1.5.3 LMP2 modulation of cell signalling

Although expressed in many of the viral latency programmes, both LMP2A and LMP2B been shown to be dispensable for EBV-induced transformation of B-lymphocytes (Longnecker, CL et al. 1992). However, LMP2A has been shown to contribute to the maintenance of latency via blockade of BCR signalling. This is achieved through physical exclusion of the BCR from lipid rafts and ubiquitin-mediated degradation of BCR-associated tyrosine kinases such as Lyn. Activation of the B-cell receptor results in recruitment of Src and Syk family tyrosine kinases to lipid rafts where signalling events can be initiated. In tandem with blocking normal B-cell signalling and thus B-cell development, LMP2A has also been shown to provide a “tonic” signal to protect B-lymphocytes from apoptosis. This allows development and persistence of B-cells in the absence of a normal BCR signal (Caldwell, Wilson et al. 1998; Caldwell, Brown et al. 2000; Longnecker and Portis 2003). LMP2A is constitutively active in non-transformed B-cells and can redirect B-cell development, as B-cells cannot progress through development checkpoints without PTK activity. Expression of

genes associated with B-cell development have also been shown to be downregulated in LMP2A-expressing cells when compared with their control counterparts. Microarray analysis has identified LMP2A-mediated downregulation of E2A, a transcription factor induced as a consequence of BCR activation and upregulation of Id2 and SCL, inhibitors of E2A. (Longnecker and Portis 2003).

1.5.3.1 MAPK signalling

MAPKs are serine/threonine kinases that under normal cellular controls are activated and signal via three distinct pathways: ERK-MAPK, JNK/SAPK and p38-MAPK, and are known to be modulated by LMP2A. MAPK signalling controls cellular events such as apoptosis, differentiation, migration and proliferation. Deregulation of these mechanisms by LMP2A may contribute to the onset and/or pathogenesis of EBV-associated malignancies. It has been demonstrated that LMP2A can regulate phosphorylation and activation of the MAPK-activated transcription factor, c-Jun and that this is prevented by inhibition of ERK signalling (Chen, Lu et al. 2002). Conversely, LMP2A is also a substrate for MAPK phosphorylation and is thought to be phosphorylated by ERK1 (Panousis and Rowe 1997). More recently it has been shown that LMP2A provides pre-B-cell receptor signalling via activation of ERK-MAPK (Anderson and Longnecker 2008).

1.5.3.2 PI3-K signalling

The surrogate BCR signal provided by LMP2A can be extended to include activation of phosphoinositide-3 kinase (PI3-K)/Akt signalling, which is responsible for cell growth, differentiation, motility, survival and intracellular trafficking under normal cellular conditions. It has been shown that upon expression of LMP2A, the PI3-K-activated serine/threonine

kinase, Akt, is constitutively phosphorylated (Swart, Ruf et al. 2000); however, this does not result in a concomitant increase in Akt-linked cell survival of B-lymphyocytes. Akt regulates the cell cycle proteins, glycogen synthase kinase 3 (GSK-3) and cyclin D. During normal epithelial cell differentiation, cells move up from layers of proliferating cells as they differentiate, and in doing so, lose contact with the extra-cellular matrix proteins. The activity of LMP2A in epithelial cells is dependent on tyrosine phosphorylation upon adhesion to these proteins (Scholle, Longnecker et al. 1999; Scholle, Longnecker et al. 2001). LMP2A has been shown to transform established epithelial cell lines and block the differentiation process in a PI3-K/Akt-dependent fashion (Scholle, Bendt et al. 2000). More recently it has been shown LMP2A-induced activation of PI3-K is mediated via Ras and that LMP2A expression is important for the clonal outgrowth of infected cells (Fukada and Longnecker 2007). Another indication of LMP2A's effect on PI3-K signalling is its ability to partially attenuate TGFβ1-mediated apoptosis, a phenomenon that is alleviated by the addition of PI3-K inhibitors (Fukada and Longnecker 2004).

1.5.3.3 NFκB signalling

LMP2A has been shown to modulate the NFκB signalling pathway in epithelial cells, and this modulation is another putative mechanism of its contribution to oncogenesis. The NFκB family of transcription factors control a variety of cellular pathways including cytokine release, cell growth and the inflammatory response. In 2004, Stewart and colleagues demonstrated modulation of NFκB activity by LMP2A in epithelial cell lines stably infected with a recombinant LMP2A-deleted EBV (Stewart, Dawson et al. 2004). Cells stably infected with an LMP2A-deleted EBV showed a reversal of NFκB inhibition, as demonstrated by production of the IL-6 cytokine, and a concomitant induction of STAT-mediated L1-TR promoter-driven LMP1 expression. These defects could be repaired by transient re-expression of LMP2A which

resulted in down-regulation of NF-kB and STAT activity and a concomitant repression of LMP1 promoter activity, (Stewart, Dawson et al. 2004).

Another investigation linking the effects of LMP1 and LMP2A demonstrated that co-expression of both viral proteins in epithelial cells augmented LMP1's ability to activate both the AP-1 and NFκB signalling pathways (Dawson, George et al. 2001). Moreover, LMP2A significantly increased the half-life of LMP1, possibly contributing to its dose-dependent effect in tumourigenesis. These events were mediated by the ITAM motif of the LMP2A protein since LMP2B co-expression with LMP1 did not yield the same results (Dawson, George et al. 2001). Findings suggest that this may be attributed to the regulation of TRAF2 expression by LMP2A (Guasparri, Bubman et al. 2008).

1.5.3.4 Cellular localisation

LMP2A and LMP2B's cellular localisation is believed to differ between epithelial cells and B-lymphocytes. Whereas LMP2A and LMP2B localise to the plasma membrane in B cells, they localise to perinuclear endosomes in epithelial cells (Allen, Young et al. 2005), with neither protein colocalising with the plasma membrane marker CD151 (Dawson, George et al. 2001). This latter study also demonstrated that LMP2A and LMP2B could alter the adhesion and migration of epithelial cells in a manner that was independent of PI3-K/Akt, ERK-MAPK and protein kinase C (PKC) activation, highlighting a function for the transmembrane domains of both proteins in modulating cell signalling (Allen, Young et al. 2005). LMP2A-mediated migration and invasion has also been observed in primary epithelial cells, further implicating the protein in epithelial malignancy (Pegtel, Subramanian et al. 2005). Further investigations into the role of LMP2B in B-cells revealed its ability to prevent LMP2A phosphorylation, and thus ubiquitin-mediated degradation of Lyn. LMP2B prevented LMP2A aggregation in lipid

rafts through competitive binding to the C-terminus of LMP2A (Rovedo and Longnecker 2007). Further investigations revealed that some, but not all of the membrane passes of LMP2A and LMP2B are necessary for membrane binding (Tomaszewski-Flick and Rowe 2007).

1.5.3.5 Other signalling cascades

LMP2A has also been implicated in modulating other cellular pathways, for example telomerase reverse transcriptase (hTERT) expression and telomerase activity. Telomerase is important for stabilisation of telomere shortening after rounds of cell division and its activation is linked to cellular immortalisation. A comparison of LMP2A-expressing cells with their parental counterparts revealed that telomerase activity and hTERT expression was inhibited by LMP2A, which although paradoxical, was hypothesised to maintain viral latency and suppress B-cell activation (Chen, Liu et al. 2005). Other investigations have identified a role for LMP2A in modulation of the Wnt/β-catenin signalling pathway with resultant effects on epithelial cell differentiation (Morrison, Klingelhutz et al. 2003; Morrison and Raab-Traub 2005).

Finally a recent publication from our laboratory revealed a novel function for LMP2A and LMP2B, in the modulation of IFN signalling. Here, type I IFN responses was attenuated when compared with their control counterparts, as demonstrated by a global downregulation of interferon stimulated genes (ISG) in NPC-derived epithelial cells *in vitro* (Shah, Stewart et al. 2009). This was postulated to be a result of the increased turnover and degradation of the type I IFN receptors in these cells (Shah, Stewart et al. 2009). The modulation of immune signalling and the increased level of cellular trafficking/lysosomal acidification are investigated further in this thesis (results *Chapters 4 and Chapter 5*).

1.6 EBV-associated disease

EBV persists as a latent infection in over 95% of the world's population. As mentioned previously EBV is associated with various diseases of both lymphoid and epithelial cell origin, an example of the dual-tissue tropism that characterises its lifecycle. These diseases, ranging from benign infections to the development of malignant tumours, are discussed below.

1.6.1 Diseases of lymphoid origin

1.6.1.1 Infectious mononucleosis

Primary infection with EBV is usually asymptomatic and occurs in early childhood. In Western countries, and particularly in areas of higher socio-economic background, infection may be delayed until adolescence or early adulthood where viral infection manifests as infectious mononucleosis (IM). This is a benign self-limiting lymphoproliferative disease encompassing a wide variety of symptoms including fever, fatigue, malaise, sore throat and nausea (Henle 1979/1968). The tonsils become enlarged due to both mild ulceration and virus-induced B-cell activation. Chronic mononucleosis can develop where disease symptoms persist for a prolonged period of time. IM is rarely fatal but in patients with the inherited condition X-linked lymphoproliferative syndrome (XLP), who are extremely sensitive to EBV infection, death can occur as a result of liver failure induced by lymphocytic infiltration and hepatic necrosis (Rickinson 2001).

1.6.1.2 Burkitt's Lymphoma (BL)

Historically, Burkitt's lymphoma was first characterised by Denis Burkitt in 1958, as a childhood malignancy that displayed unusual clinical features and a specific geographical distribution whose prevalence coincided with holoendemic malaria (Burkitt 1958). In 1964, Epstein and colleagues discovered that EBV was the transmissible particle commonly found in

BL cell lines. Burkitt's Lymphoma can be broadly divided into three categories based on geographical incidence: high incidence, classical or endemic Burkitt's (eBL), as first described by Burkitt in 1958, is endemic to areas of equatorial Africa and Papua New Guinea; low incidence or sporadic Burkitt's (sBL) found worldwide; and finally HIV-associated BL. The latter form of BL arises prior to severe immunosuppression by HIV and in tandem with the onset of full-blown AIDS (Powles, Matthews et al. 2000; Rochford, Cannon et al. 2005). It is estimated that approximately 30% of these tumours in adult AIDS patients are EBV-positive (Rickinson 2001).

The geographical incidence of eBL is limited to areas of Africa and Papua New Guinea that are also holoendemic for malaria. It is believed that co-infection of individuals in these regions with both the malaria causing parasite, *Plasmodium falciparum*, and EBV contributes to disease incidence and progression by the immunosuppression precipitated by malarial infection. eBL is the most commonly occurring childhood malignancy in these regions with incidence rates of 5-10 cases/100,000 (Murray and Young 2002), and although 95% of eBL are EBV-associated a definitive role for the virus in the pathogenesis and aetiology of the disease remains unclear (De-Thé 1979; Blum, Lozanski et al. 2004; Ferry 2006; Pattle and Farrell 2006). However, the clonal nature of viral isolates from eBL is well established, which implies that the malignant clone originated from a single virally infected cell (Raab-Traub and Flynn 1986). The sBL form of the disease shows no such geographical limitations and is detected worldwide; however, its association with EBV is much weaker than eBL with EBV positivity rates of approximately 20% in sBL (Lenoir, Vuillaume et al. 1985). BL has also been detected in areas of Brazil and North Africa with up to 87% EBV association (Anwar, Kingma et al. 1995; Araujo, Foss et al. 1999; Klumb, Hassan et al. 2004). Histologically, both forms are indistinguishable from one another expressing a similar pattern of cell surface markers, including decreased levels of cell adhesion molecules (ICAM-1, CD58), germinal centre

markers (CD77, CD10) and B-cell activation markers (CD21, CD23, CD40) (Rowe, Rooney et al. 1985; Gregory, Edwards et al. 1988).

MYC translocation is a genetic alteration that is present in all forms of BL regardless of geographical distribution or EBV status. This translocation places the *c-myc* proto-oncogene in close proximity to an immunoglobulin gene, a region of the B-cell genome that has high transcriptional activity (Klein and Klein 1985). This accounts for the high level of c-myc expression in BL tumours compared to the levels observed in resting B-cells (Nishikura, ar-Rushdi et al. 1983). The most frequently detected chromosomal abnormality is the reciprocal translocation between the long arm of chromosome 8, 8q24, and the long arm of chromosome 14, 14q32, occurring in 80% of cases. Alternative translocations occur at t(2:8) and t(8:22) (Zech, Haglund et al. 1976; Dalla-Favera, Bregni et al. 1982). This increased activation of *MYC* deregulates the cell cycle of affected cells, pushing them toward either proliferation or apoptosis depending on the presence or absence of growth-stimulating cytokines. Both HIV infection and malaria display high levels of B-cell stimulatory cytokines driving cells to proliferate and contributing to disease pathogenesis.

The role played by EBV in endemic BL remains under investigation, although some studies show that loss of EBV in BL results in a concomitant loss in its tumourigenic phenotype *in vitro*, which is restored upon re-infection with EBV (Ruf, Rhyne et al. 2000). In terms of EBV gene expression, BL typically displays a highly restricted pattern of viral gene expression termed latency I, where only Qp-driven EBNA1, the EBERs and the BARTs are expressed. *In vitro,* BL cell lines undergo phenotypic drift, with viral gene expression switching to the latency III programme of expression in tandem with an alteration of cell surface markers and a concomitant phenotypic alteration to a more lymphoblastoid-like one. Such switching may occur *in vivo,* with rare cases of EBNA2 and LMP1-positive BL documented also (Kennedy, Komano et al. 2003).

1.6.1.3 Hodgkin's disease

Hodgkin's disease (HD/HL) is the most common lymphoma in the Western world with a worldwide incidence of 2-3 cases/100,000. The most recent classification of the disease has divided it into classical and non-classical types with further subdivision of the classical type into four subtypes, based on morphological, phenotypic and molecular criteria (Harris 1999). Classical HL (cHL) includes the nodular-sclerosis (NS), mixed cellularity (MC), lymphocyte-rich classical (LRC) and lymphocyte-depleted (LD) subtypes; whilst nodular lymphocyte-predominant Hodgkin's lymphoma (NLPHL) represents the non-classical types. Although most HL is B-cell in origin some rare T-cell-derived HL has been described (Trümper, Brady et al. 1993; Kanzler, Küppers et al. 1996). The tumour itself is characterised by the presence of malignant multinucleate Reed-Sternberg (HRS) cells contained within hyperplastic lymph nodes and constitute only about 2% of the total tumour mass, the remainder consisting of a benign cellular infiltrate of lymphocytes, granulocytes and fibroblasts. HRS cells of cHL differ from their non-classical counterparts in that the former express CD15 or CD30 markers while the latter express the B-cell antigens CD20 or CD19 (Buettner, Greiner et al. 2005).

EBV is associated with approximately 50% of HL in the Western world: a rate that increases to 100% in the developing world (Jarrett, Gallagher et al. 1991), and also appears to be age dependent, with a higher frequency amongst patients under 10 and over 55 years of age (Ambinder, Browning et al. 1993). The clonal nature of the disease is well established implying progression of disease from a single virally infected cell (Anagnostopoulos, Herbst et al. 1989). The exact role of EBV in the pathogenesis of HL remains to be fully elucidated, but a direct role for EBV has been implicated in the MC subtype of cHL (Murray, Young et al. 1992; Young and Murray 2003). Increased antibody titres for EBV-associated antigens are detectable in HL patients, suggestive of lytic reactivation prior to symptom development (Johansson, Klein et al. 1970; Mueller, Evans et al. 1989). It has also been suggested that IM

patients are at an increased risk of HL (Bernard, Cartwright et al. 1987). EBV gene expression in HL is restricted to latency type II, and both immunohistochemistry and PCR analyses have confirmed expression of the EBERs, BARTs, Qp-EBNA1, LMP2A and surprisingly high levels of LMP1, given the absence of EBNA2 and its variable level in NPC (Pallesen, Sandvej et al. 1991; Deacon, Pallesen et al. 1993; Herbst, Samol et al. 1997). The level of LMP1 is thought to contribute to pathogenesis and has been the subject of investigations demonstrating that increased levels of IL-6 may be a result of LMP1-mediated modulation of NFκB signalling (Herbst, Samol et al. 1997). It has also been postulated that EBV contributes to B-lymphocyte survival of HL via LMP1 and LMP2A mimicking CD40 and BCR signalling, respectively (Rickinson 2001). Phenotypic differences associated with EBV status are also currently under investigation (Baumforth, Flavell et al. 2005).

1.6.1.4 Immunoblastic lymphomas

EBV-associated lymphoblastic B-cell lymphomas are lymphoproliferative diseases that become malignant as a result of chronic immunosuppression following organ transplantation or in immunocompromised patients, such as those suffering from AIDS or with the hereditary XLP syndrome (Thomas, Allday et al. 1991). These lymphomas are thought to arise as a result of a lack of T-cell control in these immunocompromised or immunosuppressed individuals (Carbone, Tirelli et al. 1993; Ambinder 2003). Post-transplant lymphoproliferative disorder (PTLD) includes diseases such as non-Hodgkins lymphoma, and both EBV-positive and negative cases have been documented. AIDS-related EBV-associated lymphomas develop predominantly in the central nervous system (CNS), but arise relatively rarely among AIDS patients (1-4%) (Niedobitek, Meru et al. 2001). A latency type III gene expression programme is displayed in EBV-associated forms, with all nine latent proteins being expressed, together with the BARTs and EBERs, comparable with EBV-transformed LCLs *in vitro*. Tumours of

this type are predominantly polyclonal but occasionally monoclonal tumours are identified, as defined by their immunoglobulin rearrangements. Monoclonal lesions have been shown to switch towards a latency II or latency I pattern of gene expression (Niedobitek, Mutimer et al. 1997), but the majority of tumours retain an LCL-like phenotype in terms of EBV gene expression and cell surface markers (Young, Alfieri et al. 1989; Gratama, Zutter et al. 1991).

1.6.1.5 T-Cell/NK-cell Lymphoma

Although the majority of EBV-associated lymphoid malignancies are of B-cell origin, rare infection of CD4 and CD8 positive T-cells and natural killer (NK) cells has been demonstrated in IM patients (Mitarnun, Suwiwat et al. 2002). Lymphoproliferative disorders with T-cell origin that have been identified as having an association with EBV include nasal T-cell lymphoma in patients with lethal midline granuloma (Harabuchi, Yamanaka et al. 1990), aggressive peripheral T-cell lymphoma (Dupuis, Emile et al. 2006), extranodal nasal type NK/T cell lymphoma, enteropathy-type T cell lymphoma, γδ T-cell lymphomas, (Arnulf, Copie-Bergman et al. 1998), and aggressive NK cell lymphoma (Rezk and Weiss 2007). Although extensive analyses of the pattern of latent gene expression has not been undertaken, evidence does suggest that nasal T-cell lymphoma displays a latency II pattern of expression, with peripheral T-cell lymphomas displaying a more restricted latency pattern (Young and Rickinson 2004). Given the lack of evidence surrounding the expression of the latent EBV proteins in these malignancies, it is unsurprising that the role for EBV in pathogenesis of these lymphomas remains to be elucidated.

1.6.2 Epithelial cell disease

1.6.2.1 Oral hairy leukoplakia

First described and characterised from HIV-positive patients in the 1980s, oral hairy leukoplakia (OHL) is a hyperkeratotic lesion of the tongue, which presents as white, poorly demarcated ridges on the lateral borders of the tongue that cannot be scraped off (Greenspan, Greenspan et al. 1985; Reichart, Langford et al. 1989). It is thought that emergence of the benign lesion is an indicator of progression to AIDS from HIV-positivity, which is suggestive of an association with immunosuppression as a contributing factor in this disease (Epstein, Sherlock et al. 1991). This is further highlighted by the fact that EBV shows little or no ability to replicate in the epithelia of the tongue in immunocompetent hosts. Histologically, little or no inflammatory infiltrate is detected in the underlying connective tissue, but the squamous epithelium of the tongue is notably hyperplastic (Greenspan, Greenspan et al. 1985). Reappearance of the lesion after treatment with the antiviral drug, acyclovir, is indicative of association with both productive and nonproductive EBV infection (Resnick, Herbst et al. 1988; Greenspan, De Souza et al. 1990), which is further evidence of the link between epithelial cell differentiation and viral replication (Sixbey, Nedrud et al. 1984; Walling, Brown et al. 2003). It would appear that OHL is the result of repeated re-infection of tongue epithelium rather than uncontrolled expansion of latently infected basal epithelial cells. The presence of multiple EBV strains are indicative of superinfection of EBV infected cells within the differentiating layers (Sandvej, Krenács et al. 1992; Webster-Cyriaque, Middeldorp et al. 2000). Given the inability to identify EBV infection in normal healthy oral mucosa, it is likely that immunosuppression is causally linked to the onset of OHL (Pegtel, Middeldorp et al. 2004; Frangou, Buettner et al. 2005).

Although analyses have revealed expression of the viral markers EBNA1, EBNA2, EBNA-LP and LMP1 in the suprabasal epithelium of these lesions (Murray, Niedobitek et al.

1996; Webster-Cyriaque and Raab-Traub 1998; Walling, Flaitz et al. 2001), these are shown to be localised to this layer and the consensus is that latent infection is not established. This is reinforced by the detection of immediate early (BZLF1, EA-D, BHRF1), and late (VCA, MA) proteins in terminally differentiating cells (Thomas, Felix et al. 1991; Young, Lau et al. 1991; Webster-Cyriaque and Raab-Traub 1998; Hayes, Brink et al. 1999). The exact role of EBV in this disease remains unclear. However the presence of LMP1 and EBNA2 in OHL lesions and the overexpression of cyclin B1 and IL-10 suggests that these may act to deregulate the cell cycle and increase cytokine production (Hayes, Brink et al. 1999),.

1.6.2.1 Gastric Carcinoma

Gastric carcinoma is one of the most common forms of cancer, with approximately 930,000 cases diagnosed in 2002 (Pisani, Bray et al. 2002), of which between 6 to 16 % were found to be EBV-positive (Takada 2000). A comprehensive review of EBV and its role in gastric carcinoma has been published recently (Fukayama, Hino et al. 2008). EBV is detected in 100% of the tumour cells of EBV-positive gastric carcinoma, and analyses have shown that tumour expansion occurs after infection with EBV and arises from a single virally infected cell. Two histologically distinct forms of EBV-associated GC have been identified and these vary substantially in incidence rate. Lymphoepithelioma-like GC is the rarer of the two, with over 80% of tumour cells detected as EBV-positive (Osato and Imai 1996; Takada 2000; Fukayama, Chong et al. 2001). It is characterised as a poorly differentiated carcinoma with a high level of lymphocytic infiltrate. The more common type of EBV-positive GC is an ordinary gastric adenocarcinoma, which is characterised as a moderately to poorly differentiated adenocarcinoma with varying levels of lymphocytic infiltrate. EBV is not detected in the tissue surrounding the tumour but is found in the dysplastic cells located at its periphery (Yuen, Chung et al. 1994). This may be indicative of dysplastic cells behaving in a more permissive

manner to EBV infection and allowing further development of the malignancy. This mode of late infection of cells by EBV is underlined by the fact that EBV is absent from pre-malignant lesions (Zur Hausen, van Rees et al. 2004).

A restricted pattern of viral gene expression is observed in EBV-positive gastric carcinoma, and is most similar to that displayed in HL and NPC, whereby Qp-driven EBNA1, the EBERs and BARF1 are all consistently detected and occasionally LMP2A (Takada 2000; Fukayama, Chong et al. 2001; Fukayama, Hino et al. 2008). Other latent proteins including EBNA2, the EBNA3s and LMP1 are not detected (Luo, Wang et al. 2005), and although these cells are latently infected, lytic reactivation can occur via expression of BZLF1 (Niedobitek, Herbst et al. 1992). BARF1 has been shown to control apoptosis and promote cell survival via modulation of the Bcl-2 family of anti-apoptotic proteins (Wang, Tsao et al. 2006). The BART microRNAs have been implicated in the pathogenesis of EBV-positive GC given their frequent detection both *in vitro*, in EBV associated GC (EBVaGC) cell lines, and *in vivo*, in tumour tissue samples (Chang, Lee et al. 2007). More recently the role of LMP2A in the pathogenesis of EBVaGC has been investigated: this was performed by cloning LMP2A from the EBV-positive SNU-719 cell line and stably expressing it in the EBV-negative AGS cell line. Upon stable and detectable expression, no differences were observed in the ability of LMP2A to effect colony formation of these cells in soft agar, compared to control counterparts. This is suggestive of a minor role for LMP2A in tumourigenesis (Seo, Jun et al. 2010), and could explain why it is not detected in some cases of EBVaGC. Finally the tumour suppressor gene, *p53*, is found to be frequently mutated in many EBV-negative GCs. In EBVaGC this is not the case: rather, *p53* shows weak expression indicating a role for EBV in the blockade of *p53* expression and function (Ojima, Fukuda et al. 1997; Leung, Chau et al. 1998).

1.6.3 Nasopharyngeal carcinoma (NPC)

EBV positive Nasopharyngeal carcinoma (NPC) is a lymphoepithelioma of the nasopharynx and is classified as a specific subtype of head and neck squamous carcinoma (HNSCC). Histologically, the world health organisation classifies the tumour into three types based on differentiation status. Type I is a differentiated keratinising squamous cell carcinoma; type II is a non-keratinising carcinoma; and type III is an undifferentiated carcinoma. EBV is most commonly associated with type II and III NPC, which are commonly grouped together as undifferentiated carcinomas (Shanmugaratnam 1978). Type I is the most rare and accounts for approximately 20% of all cases and does not have a consistent association with EBV (Desgranges, Wolf et al. 1975). Histologically, undifferentiated NPC is characterised by the presence of an EBV-negative non-malignant lymphocytic infiltrate, which surrounds latently infected, malignant epithelial cells (Chan, Teo et al. 2002; Chan and Lo 2002). The function of the infiltrate remains unclear although several investigations reveal that its presence may be due to EBV-induced antigen presentation, or perhaps that the lymphocytic tumour environment controls tumour growth and progression (Agathanggelou, Niedobitek et al. 1995; Tang, Rohwäder et al. 2007). It has been demonstrated that cells of the infiltrate express ligands that bind to receptors commonly found on the surface of the tumour cells (Tang, Rohwäder et al. 2007).

In a similar manner to endemic Burkitt's lymphoma, NPC shows a specific geographical distribution: it is the most common tumour in Southern China, particularly in Guangdong province, with incidence rates of between 25-30/100,000. In contrast, incidence rates of less than 1 in 100,000 occur in Europe and North America. Higher rates of incidence of 3-8/100,000 are also reported in areas of North Africa and among the Inuit populations of Alaska and Greenland (Busson, Keryer et al. 2004). Although strong familial correlation exists in endemic Chinese NPC, this is not displayed amongst North African cases (Busson, Keryer et

al. 2004). EBV's association with the disease and its role in the progression of oncogenesis have been studied since serological analyses in the late 60s and 70s revealed a putative link between the virus and the malignancy (Henle, Henle et al. 1970; zur Hausen, Schulte-Holthausen et al. 1970). Subsequent investigations demonstrated that EBV viral DNA, specifically EBNA1, was present in all tumour cells examined (Huang, Ho et al. 1974; Klein, Giovanella et al. 1974), regardless of the geographical origin of the tumour (Desgranges, Wolf et al. 1975).

In tandem with EBV infection, environmental and genetic factors are believed to play an important role in oncogenesis (Chan, Teo et al. 2002; Goldsmith, West et al. 2002). Various environmental factors have been implicated including phorbol esters in the drinking water, which can reactivate EBV, (Shao, Poirier et al. 1988; Raab-Traub 2002); ingestion of Cantonese salted fish which contains a high amount of the EBV lytic activator, nitrosamine (Mirvish 1995); ingestion of preserved vegetables and certain Chinese herbs; ingestion of badly preserved meat and rancid butter (North Africa) (Feng, Jalbout et al. 2007); and occupational exposure to pollutants. These factors have all been linked to increased levels of NPC (Li, Man et al. 2006). The rate of NPC in endemic areas of China does appear to be on the decline and this is attributed, at least in part, to the removal of salted fish from the diet of children in the region (Chan, Teo et al. 2004). Studies into the genetic susceptibility of the disease have highlighted potential genetic links. The association of various HLA types with the development and progression of NPC is reviewed elsewhere (Li, Fasano et al. 2009). In addition, a variant of the cytochrome P450 CYP2E1 gene also shows strong correlation with NPC among Chinese populations (Hildesheim, Anderson et al. 1997).

Analyses of genetic changes revealed a putative stepwise mechanism of disease development whereby it is hypothesised that accumulation of genetic alterations renders cells more permissive to latent EBV infection (Lo and Huang 2002). NPC tumours frequently

harbour chromosomal losses at multiple sites including: 3p, 9p, 9q, 11q, 14q and 16q, or chromosomal gains at 1q, 3q, 8q, 12p and 12q. Amongst these alterations, higher frequencies of loss at 3p/9p were observed in individuals from the high-risk region of China compared to control counterparts (Chan, To et al. 2000; Wong, Hui et al. 2003). These deletions are present in 80-100% of NPC, which is similar to the level of EBV infection, and is thus suggestive that EBV infection may not be the initiating event in NPC pathogenesis, but rather, occurs before the initiation of invasive growth (Niedobitek, Meru et al. 2001). NPC tumours are clonal in nature thereby supporting the notion that viral infection occurs prior to malignant proliferation (Raab-Traub and Flynn 1986). The exact nature of EBV's role in NPC pathogenesis remains unclear, with disease: development attributable to both genetic and epigenetic changes that can alter the function of genes involved in proliferation, growth, differentiation and latent EBV infection (Niedobitek, Meru et al. 2001). Nasopharyngeal intraepithelial lesion (NPIL), a preinvasive lesion with malignant potential, has been shown to be EBER-positive and BZLF1-negative, indicative of EBV infection prior to initiation of invasive carcinoma; however, it is rarely diagnosed since the disease is often presented at later stages (Cheung, Pang et al. 2004).

The vast majority of NPC tumours display a type II latency programme, with expression of Qp-driven EBNA1, the EBER and BART RNAs and variable expression of LMP1 and LMP2 (Brooks, Yao et al. 1992; Chen, Hu et al. 1995). Although LMP2A RNA is detectable in 100% of cases by RT-PCR, expression of LMP2A protein has been documented in approximately 50% of cases (Heussinger, Büttner et al. 2004). A similar situation is observed with LMP1 where protein expression varies widely between tumour samples; ranging anywhere between 15% and 60% (Niedobitek, Young et al. 1992). Although the majority of EBV positive tumour cells adopt a latent gene expression programme, a small proportion of NPC tumour cells have been reported to express immediate-early proteins, suggesting that a fraction of cells are semi-permissive for lytic reactivation (Cochet, Martel-Renoir et al. 1993).

The IE-protein BARF1 has been shown to increase the survival and proliferative ability of primary epithelial cells upon infection with an NPC-derived EBV strain (Danve, Decaussin et al. 2001). More recently it has been demonstrated that exogenous expression of SV40 promoter-driven BARF1 in the NPC-derived cell lines CNE-1 and HONE-1 results in cellular resistance to apoptosis and growth at high density as compared to their control counterparts (Seto, Ooka et al. 2008).

1.7 Innate Immunity

1.7.1 The TLRs

The innate immune system is "the first line of defence" against infection by microorganisms. Multiple signalling pathways are involved in its regulation and their activation results in a variety of cellular phenomena including inflammation, cell clearance by apoptosis, and NK cell killing (.A, Yanai et al. 2005; Kawai and Akira 2005; Uematsu and Akira 2007). In terms of viral infection, the host cells have a variety of responses to prevent the spread of infectious virus and to maintain the integrity of normal cellular function. Pattern recognition receptors (PRR) are cellular receptors that are responsible for mounting immune responses against invading pathogens (Takeda, Kaisho et al. 2003). These are divided into two classes: the toll-like receptors (TLRs) and the RIG-I-like helicases (RLHs) (Kawai and Akira 2008). The TLRs are members of the interleukin-1 receptor (IL-1R) superfamily and share homology within their cytoplasmic regions, specifically within the Toll/IL-1R (TIR) domain (Kawai and Akira 2006). The extracellular domain contains a characteristic leucine-rich repeat (LRR) and is the site of ligand recognition (Kawai and Akira 2005). Exhibiting high levels of evolutionary conservation, TLRs were first identified in *Drosophila melanogaster* (Lemaitre, Nicolas et al. 1996) and, to date, ten receptors have been identified in humans (TLRs 1-10) (Takeda, Kaisho et al. 2003).

Genetic analyses have revealed their respective ligands include bacterial components such as lipopeptide, peptidoglycan and lipoprotein (TLR1, TLR2 and TLR6, respectively), bacterial flagellin (TLR5), bacterial lipopolysaccharide (LPS – TLR4), and viral products and components including dsRNA (TLR3), ssRNA and imidazoquinoline-like molecules (TLR7/8) and microbial unmethylated CpG DNA (TLR9). However, as yet no ligand has been identified for TLR10 (Kawai and Akira 2006; West, Koblansky et al. 2006). Activation of TLR signalling results in induction of type I interferons, chemokines and inflammatory cytokines. TLR signalling links innate and adaptive immunity by initialising maturation of dendritic cells (DC), cells which are important for antigen presentation (Iwasaki and Medzhitov 2004; Blander and Medzhitov 2006; Husebye, Halass et al. 2006). In the context of viral infection, five TLRs have been shown to initiate innate immunity: TLR3, TLR4, TLR7/8 and TLR9 (Uematsu and Akira 2007; Randall and Goodbourn 2008.). Upon ligand recognition, signalling is initiated within endosomal compartments for all of these receptors, with the exception of TLR4, which can also signal from the plasma membrane (Fitzgerald, Palsson-McDermott et al. 2001; Kagan, Su et al. 2008).

1.7.2 TLR3 signalling

TLR3 signalling is initiated within the endosome (West, Koblansky et al. 2006), although the receptor has also been identified in alternative locations such as the plasma membrane (Matsumoto, Funami et al. 2003). Findings suggest that for signalling to occur, activation of adaptor molecules must occur within an acidified or partially acidified endosome compartments (Johnsen, Nguyen et al. 2006). The receptor recognises extra-cellular viral dsRNA which is internalised into endosomes via endocytosis (Alexopoulou, Czopik Holt et al. 2001; Marshall-Clarke, Downes et al. 2007). Upon ligand binding (poly(rI).poly(rC), TLR3 dimerises and becomes phosphorylated (Sarkar, Peters et al. 2004; Sen and Sarkar 2005)

through its TIR domain, subsequently recruiting the adaptor proteins TIR domain containing adaptor inducing IFNβ (TRIF) and PI3-K (Kawai and Akira 2005; Kawai and Akira 2006). These adaptor proteins mediate the signal through two distinct pathways of interferon induction: IRF3 and NFκB (O'Neill and Bowie 2007). The IRF3 pathway is the less well understood of the two but it is known that TRIF interacts with TNF receptor associated factor 3 (TRAF3) (Sato, Sugiyama et al. 2003; Häcker, Redecke et al. 2006), which subsequently recruits TRAF family member associated NFκB activator (TANK) (Sato, Sugiyama et al. 2003). The exact role of TANK is, as yet ill-defined, although it has been shown to associate with TANK-binding kinase (TBK1), which in addition to IκB kinase epsilon (IKKε – a member of the kinase complex involved in the control of NFκB) is responsible for directly phosphorylating IRF3 (Dragan, Hargreaves et al. 2007). Once phosphorylated, IRF3 forms homo/heterodimers with itself or IRF7, translocates to the nucleus and activates the transcription and production of IFNβ (Honda and Taniguchi 2006).

Activation of NFκB by TLR3 has been better characterised: TRIF recruits TRAF6 which initiates its ubiquitin ligase activity, enabling it to poly-ubiquitinate both itself and the receptor interacting protein 1 (RIP1) (Sato, Sugiyama et al. 2003; Häcker, Redecke et al. 2006). Once ubiquitinated, these proteins are recognised by and interact with TAK1-binding proteins 2 and 3 (TAB2 and TAB3), which leads to the recruitment of transforming growth factor B-activated kinase 1 (TAK1) to the complex (Sato, Sugiyama et al. 2003; Randall and Goodbourn 2008.). Recognition of poly-ubiquitinated RIP1 by the NFκB essential modifier (NEMO) component of the IKK complex brings it into close proximity with the TRIF-RIP1-TRAF6-TAB2-TAB3-TAK1 complex and facilitates phosphorylation of the IKKβ subunit by TAK1 (Kawai and Akira 2006; Sarkar, Elco et al. 2007). This leads to the downstream phosphorylation and ubiquitin-mediated degradation of IκBα, resulting in NFκB release,

processing and translocation of the transcriptional activators, usually p65 and p50, to the nucleus (Deng, Wang et al. 2000; Hayden and Ghosh 2004).

1.7.3 TLR7/8 and TLR9 signalling

TLRs 7 and 8 recognise endosomal viral ssRNA and the imidazoquinoline molecules which include imiquimod and R848; both signal from internal endosomes (Hemmi, Kaisho et al. 2002; Diebold, Kaisho et al. 2004; Nishiya, Kajita et al. 2005). These ssRNA products are commonly produced during the replication of Vesicular stomatitis virus (VSV), Sendai virus (SeV) (Kawai and Akira 2006), and activation of signalling from these TLRs can also occur upon infection and RNA release in the endosome of RNA viruses such as influenza A virus, without replication (Hemmi, Kaisho et al. 2002; Kawai and Akira 2008). Due to the restricted pattern of cellular expression of these TLRs, their activity has been extensively but not exclusively studied in plasmacytoid dendritic cells (pDCs), which express high levels of TLR7 (Cao and Liu 2007). TLR8 signalling is less well characterised, but it is widely thought that signalling activation is controlled in a similar manner to that of TLR7 given the similarities between their expression patterns and cognate ligands (Jurk, Heil et al. 2002). In the case of TLR7, upon recognition and binding of ligand, the adaptor molecule myeloid differentiation factor 88 (MyD88) is recruited to the receptor (McGettrick and O'Neill 2004), which interacts with the interleukin-1 receptor associated kinase (IRAK) proteins, IRAK1 and IRAK4 (Li, Strelow et al. 2002; Gottipati, Rao et al. 2007; Kim, Staschke et al. 2007), which facilitate activation of TRAF6 (Kawai and Akira 2005; Martin, Lee et al. 2007; Randall and Goodbourn 2008.). NFκB signalling is then initiated in a similar manner to the RIP1-TAB-TAK mechanism described for TLR3 signalling (Kawai and Akira 2006; Wullaert, Heyninck et al. 2006).

While the pathway to induction of IFN initiated from TLR3 depends heavily on phosphorylation and dimerisation of IRF3 in TLR7- and TLR8-expressing pDCs there are also high levels of endogenous IRF7 (Sato, Suemori et al. 2000). Here TLR7 engages the MyD88-IRAK-TRAF6 complex, described above, and binds directly to IRF7 (Kawai, Sato et al. 2004). IRF7 then becomes ubquitinated by TRAF6 in a RIP1-dependent manner, and is subsequently phosphorylated by IRAK1 (Caillaud, Hovanessian et al. 2005; Kawai and Akira 2005; Randall and Goodbourn 2008.). The complex then translocates to the nucleus where it can bind to promoter elements to activate transcription. IRF7 has been shown to be responsible for transcriptional activation of members of the IFNα cytokine (Au, Moore et al. 1998; Barnes, Richards et al. 2004; Honda, Yanai et al. 2005; Honda and Taniguchi 2006). Signalling downstream of TLR9 is identical to that of TLR7, although the ligand for this receptor is unmethylated microbial DNA (CpG DNA) which is derived from a variety of infective sources and is delivered to the receptor by retention in the endosome (Uematsu and Akira 2007; Kawai and Akira 2008).

1.7.4 TLR4 signalling

TLR4 is the PRR responsible for recognition of LPS, a component of the bacterial cell wall. In terms of viral infection it has also been shown to be involved in signalling by recognition of the F protein of Respiratory Syncitial Virus (RSV) (Kurt-Jones, Popova et al. 2000) and the gG protein of Vesicular Stomatitis Virus (VSV) (Georgel, Jiang et al. 2007). TLR4 is unique among the type I IFN-inducing TLRs in that it can signal through both MyD88-dependent and independent mechanisms (Takeda, Kaisho et al. 2003; Shen, Tesar et al. 2008). It does so through activation of a variety of signalling cascades but, unlike the previously described pathways, which demonstrate direct interaction between the activated receptor and the relevant adaptor molecule (TRIF or MyD88), signalling through TLR4

requires additional interacting proteins. Activation of IRF3 is mediated initially by binding of the TRIF-related adaptor molecule (TRAM) protein to TLR4 (Kagan, Su et al. 2008). This adaptor has been shown to be necessary for activation of TRIF-dependent induction of IRF3 and has dual localisation capabilities, shuttling between the endosomal and the plasma membrane. Upon interaction of TRAM with TRIF, signalling continues in a similar manner to that of TLR3, activating IRF3 via TBK1 and IKKε, and activating NFκB via RIP1 and TRAF6 (Kawai and Akira 2006). The second MyD88-dependent mode of signalling from this receptor occurs via the Mal protein (MyD88-adaptor like) (Fitzgerald, Palsson-McDermott et al. 2001). This protein interacts directly with the receptor and recruits the MyD88-IRAK-TRAF6 complex, thus facilitating signalling to proceed in manner to that observed upon activation of TLR7 (Randall and Goodbourn 2008.). *Figure 1.2* shows a schematic of TLR signalling.

1.7.5 The RIG-I-like helicases (RLHs)

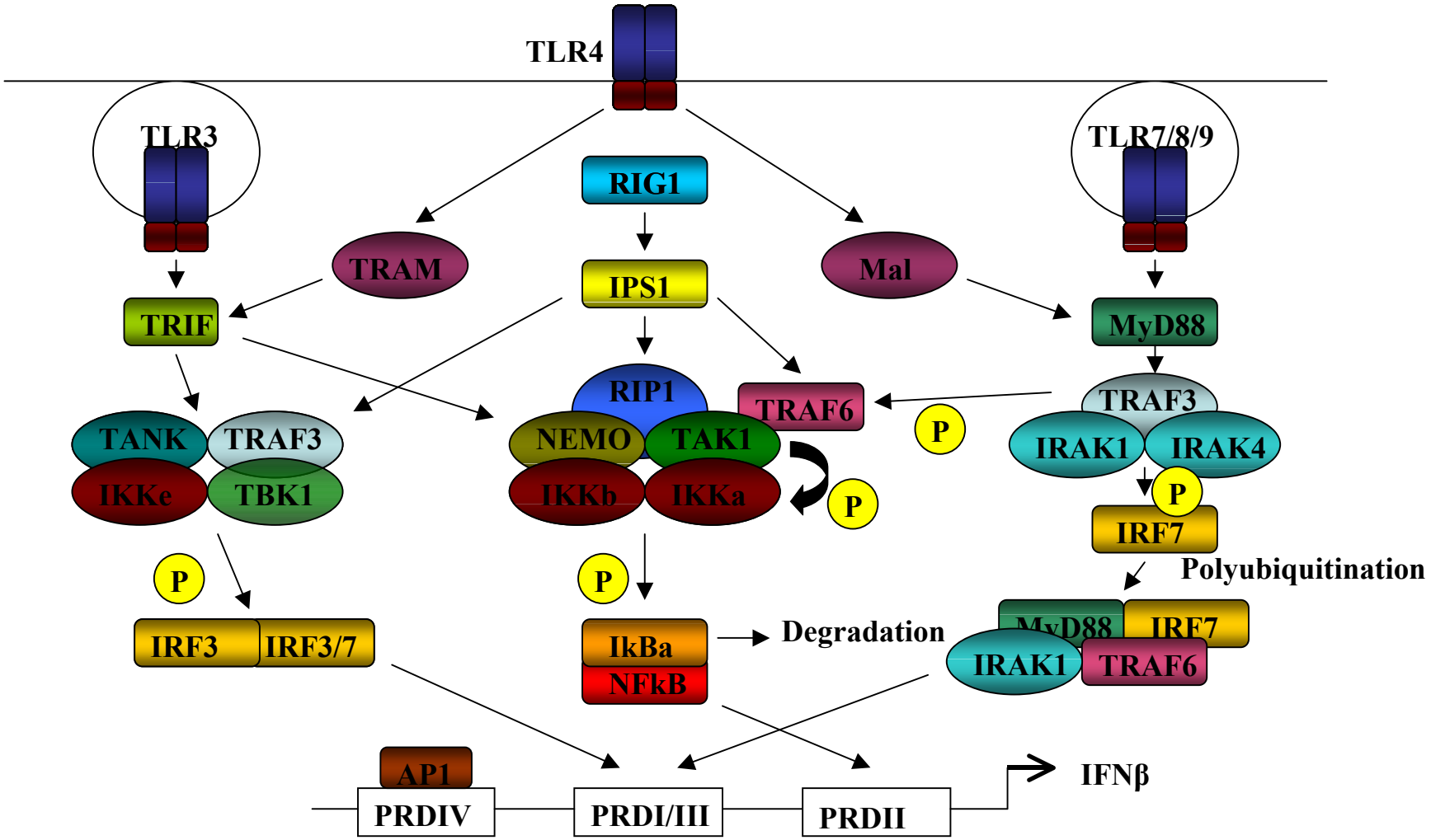
The RIG-I-like helicases (RLHs), retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5) are cytoplasmic sensory proteins that have been shown to recognise and bind cytosolic dsRNA (Yoneyama, Kikuchi et al. 2004; Kawai and Akira 2008). While detection of endocytosed dsRNA is performed by TLR3, the aforementioned sensory proteins are responsible for mediating an immune response upon viral replication within the cytosol of infected cells that may otherwise remain undetected by the endosomally located TLR3. Structurally these proteins share three common domains, two caspase-recruiting domains (CARD domains) and one DexD/helicase domain. The dsRNA is recognised by the helicase domain, whilst signalling is initiated from the CARD domains (Yoneyama, Kikuchi et al. 2004). In a manner similar to signalling from TLR3, stimulation of a response results in activation of the nuclear transcription factors, IRF3 and NFκB. In both cases, analyses have revealed that the adaptor protein involved in the initiation of these signals

is the interferon promoter stimulator-1 protein (IPS-1) also known as mitochondrial anti-viral signalling protein (MAVS) or virus-induced signalling adaptor (VISA) (Sun, Sun et al. 2006). Interactions between IPS-1, RIG-1 or MDA5 occur via their CARD domains and downstream signalling is thought to proceed in a manner similar to the TRIF-mediated pathways of TLR3/4, thereby leading to the formation of the enhanceosome and production of IFNβ, although these mechanisms remain to be fully elucidated (Randall and Goodbourn 2008.).

Whilst RIG-1 and MDA5 share significant structural homology, they appear to recognise infection by different virus types. RIG-1 is essential for ssRNA virus recognition, such as paramyxoviruses, influenza virus, VSV and Japanese encephalitis virus (JEV). MDA5 is also involved in recognising other RNA viruses, such as Encephalomyocarditis virus (EMCV), amongst others (Kawai and Akira 2008). A third member of the family has been identified: LGP2, which contains the helicase domain but not the signalling activatory CARD domains, therefore initially implicating it in the negative regulation of the two IFN-inducing receptors (Rothenfusser, Goutagny et al. 2005; Yoneyama, Kikuchi et al. 2005). A more recent investigation has proposed a role for LGP2 in the positive regulation of immune responses to viral infection (Satoh, Kato et al. 2010).

Figure 1.2 Innate immune signalling network.

Displayed is a schematic representation of the signalling from the type I interferon inducing Toll-like receptors (TLRs) and the RIG-1-like helicases (RLHs). (1) TLR3 signalling is initiated upon binding of dsRNA or poly (I:C) to the receptor, which dimerises, phosphorylates and recruits TRIF via its TIR domain. The adaptor protein TRIF then recruits either TRAF3 or TRAF6 to initiate activation of IRF3 or NFκB subunits. IRF3 is activated recruitment of TRAF3 by TRIF and subsequent interaction with TANK, TBK1 and IKKε. Phosphorylation of IRF3 occurs upon interaction with one of these proteins but an exact mechanism for this remains to be elucidated. P-IRF3 can then homo- or hetero-dimerise with IRF7, translocate to the nucleus and exert its effect. NFκB subunit activation is effected by interaction of TRIF with TRAF6, the IKK complex and phosphorylation and degradation of IκBα, which releases NFκB subunits. (2) TLR4 signalling is initiated upon binding of LPS to the receptor and recruitment of the TRAM and Mal intermediate proteins, TRAM activates signalling through interaction with TRIF as for TLR3. The Mal adaptor protein interacts with MyD88, facilitating recruitment of TRAF3 the IRAK proteins and subsequent phosphorylation, polyubiquitination and activation of IRF7. This complex can then move to the nucleus to activate transcription. NFκB activation id mediated through TRAF3 phosphorylation of TRAF6 and its interaction with the IKK complex. (3) TLR 7 8 and 9 signalling is initiated by binding of RNA and DNA molecules and proceeds in a MyD88 dependent manner as described for TLR4. (4) Signalling through the RLHs is initiated upon cytoplasmic recognition of RNA molecules which activate the receptor and facilitates recruitment of IPS1/VISA adapter protein. This intermediate interacts with TRAF3 and facilitates IRF3 activation in a similar manner to TLR3 signalling and with TRAF6 to activate NFκB subunits. Adapted from, (Kawai and Akira 2006; Randall and Goodbourn 2008.)


TLR4
TLR3
TLR7/8/9
RIG1
TRAM
IPS1
Mal
TRIF
MyD88
RIP1
TRAF6
TRAF3
TANK
TRAF3
NEMO
TAK1
IRAK1
IRAK4
IKKe
TBK1
IKKb
IKKa
P
P
P
P
P
IRF7
Polyubiquitination
IRF3
IRF3/7
IkBa
NFkB
Degradation
MyD88
IRF7
IRAK1
TRAF6
AP1
PRDIV
PRDI/III
PRDII
IFNβ

1.7.6 The IFNβ enhanceosome

Type I interferon induction, inflammatory cytokine release, and initialisation of the anti-viral state are all consequences of activated TLR signalling (Kawai and Akira 2006). One important feature is the production of IFNβ, a cytokine which is controlled through formation of the IFNβ "enhanceosome" (Yie, Senger et al. 1999). This is a multiprotein activator of transcription that binds to four distinct binding sites of the IFNβ promoter element: positive regulatory domains (PRD) I, II, III and IV, each of which has been shown to interact with a dimer of transcription factors (Panne 2008). The IRF homo/heterodimers bind to PRDI and PRDIII, whilst the AP-1 subunits bind to PRDIV, and the NFκB dimers bind to PRDII (Panne 2008). Translocation to the nucleus of the IRF and NFκB subunits is described in detail above. AP-1 is a dimeric transcription factor composed of two subunits of the Fos and Jun families, of which c-Jun and ATF-2 are thought to be responsible for mediating inflammatory responses (Vesely, Staber et al.; Ozanne, Spence et al. 2006). Activation of AP-1 upon TLR stimulation is controlled by the MAPK signalling pathways, which include the ERK, JNK and p38 signalling cascades. Signalling through these cascades is controlled by TAK1, which has been shown to phosphorylate MAPK kinase family members including, MKK3 and MKK6 (Sato, Sanjo et al. 2005).

Formation of the enhanceosome complex results in the recruitment of cellular co-activating molecules including CBP/p300 (Yoneyama, Suhara et al. 1998), and the subsequent transcriptional activation of the IFNβ promoter. In terms of IFNβ production, it has been shown that IRF3/IRF7-binding is indispensable whereas type I IFN induction has been documented in the absence of NFκB and AP-1 activity (Peters, Smith et al. 2002). Furthermore, it has also been reported that cooperative binding of NFκB subunits and IRF subunits to ISRE elements can result in the transcriptional activation of some chemokines involved in the inflammatory response (Honda and Taniguchi 2006). However, the correct

binding of the IRF components of the enhanceosome complex is fundamental to the production of IFNβ.

1.7.7 The IRF proteins

Activation of the IRF proteins is a fundamental component of the induction of the type I IFN response (Honda and Taniguchi 2006; Colonna 2007). As described above, there are multiple mechanisms that facilitate phosphorylation, dimerisation and translocation of these proteins to the nucleus where they can exert their effects. In TLR3 signalling, IRF3 is detrimental to the early phase of type I IFN induction, particularly IFNβ (Hiscott 2007). Although it shares a binding site with IRF7 within the IFNβ promoter and has been shown to form heterodimers with IRF7; this usually occurs after an initial wave of the innate immune response (Sato, Suemori et al. 2000). IRF3 expression is constitutive and in most cell types it is expressed at levels much higher than that of IRF7 (Wang, Zhang et al. 2008). Activation of TLR3 by dsRNA molecules is initially facilitated by utilisation of this intracellular pool of IRF3 and, following the initial wave, a positive feedback loop results in an increase of IRF7 expression (Honda, Yanai et al. 2005). This can then be phosphorylated and activated in a TBK1/IKKε-mediated manner and can contribute to or replace IRF3 activity in further IFN production. IRF5 has also been shown to be involved in inflammatory cytokine induction as a result of signalling from TLR3, 4, 7 and 9. This is mediated by binding of the protein to the interferon stimulated response element (ISRE) in the promoter region of activated genes (Barnes, Richards et al. 2004; Takaoka, Yanai et al. 2005; Balkhi, Fitzgerald et al. 2010). Furthermore, IRF8 has been implicated in TLR9-driven NFκB activity (Tsujimura, Tamura et al. 2004). Both IRF9 and IRF1 have been shown to be involved in controlling signalling from the IFNAR, which is activated para/autocrinely upon release of the IFNβ or IFNα cytokines.

1.7.8 The SOCS proteins

The suppressors of cytokine signalling (SOCS) family of proteins are involved in the negative regulation of interferon induction (Yoshimura, Naka et al. 2007), functioning to attenuate or limit IFN production in response to TLR or RIG-I/MDA-5 activation. The complex network of signalling cascades initiated by invading pathogens requires tight and rigorous control, which is mediated in part by these proteins. The family consists of eight members, Cytokine Inducible SH2 containing protein (CIS) and SOCS1-7 and roles have been defined for them in both innate and adaptive immunity, including inhibition of TLR and IFN signalling (Alexander and Hilton 2004). Structurally the proteins contain an SH2-interacting domain; a conserved carboxy-terminal domain (the SOCS-box) through which it is thought to mediate its effects in an E3 ligase-dependent fashion; and a kinase inhibitory domain (KIR), which is also important for attenuation of signalling responses (Zhang, Farley et al. 1999; Kobayashi, Takaesu et al. 2006). SOCS1 has been shown to inhibit TLR signalling through interactions with the NFκB subunit, p65, and the Mal adaptor protein, facilitating their ubiquitin-mediated degradation (Gingras, Parganas et al. 2004; Kobayashi, Takaesu et al. 2006). Type I interferon signalling is inhibited through direct interaction of the SOCS1 protein with the JAK tyrosine kinases (Yoshimura, Naka et al. 2007). Additionally the SOCS3 protein interacts with STAT3, which has been shown to control signalling from the pro-inflammatory IL-6 receptor (IL6R) and the anti-inflammatory IL-10R as well as the IL-12 receptor (IL-12R) (Yamamoto, Yamaguchi et al. 2003). It is thought that STAT3 activation of IL-6R is transient and operates mainly to promote an anti-inflammatory response through attenuation of TLR signalling through an indirect interaction with SOCS3 (Andrea, Frey et al. 2004). It has also been reported that SOCS3 can inhibit activation of both TRAF6 and TAK1 to modulate TLR signalling. Expression of both of these proteins is induced by TLR receptor signalling – an

example of the complex feedback loops utilised by innate immunity to regulate responses (Kile and Alexander 2001; Yoshimura, Naka et al. 2007).

1.7.9 Interferon signalling

The term "interferon" was first coined by Issacs in 1957 (Isaacs and Lindenmann 1957) to characterise a secreted cytokine that demonstrably "interfered" with viral replication. They are grouped into three types, which contribute to formation of the ant-viral state (Goodsell 2001; Randall and Goodbourn 2008.). Type I IFNs, which are induced by TLR, RIG-I and mda-5 activation, as described above, include IFNβ and the IFNα family, of which 13 have been identified in humans (Garcia-Sastre and Biron 2006). Type II has one member, IFNγ, which is secreted by activated T-cells and NK-cells as an indirect consequence of viral infection and serves to further promote the inflammatory response (Platanias 2005). The role of the type III interferons is less well characterised but they are known to be released as a result of viral infection and include IL-26, IL-28A and IL-28B (Uzé and Monneron 2007).

The type I interferons are recognised by the dimeric IFNα/β receptor which is composed of two subunits: IFNAR1 and IFNAR2, and signal through their respective associated tyrosine kinases, Tyk2 and Jak1 (Randall and Goodbourn 2008.). Upon ligand binding to the receptor, the two subunits dimerise enabling Tyk2-mediated phosphorylation of both STAT2 at tyrosine 690, which is associated with IFNAR2 prior to activation, and IFNAR1 at tyrosine 466. Jak1 phosphorylates STAT1 at tyrosine 701 and the phosphorylated STATs form a stable heterodimer, which can then interact and bind to IRF9/p48 to form the ISGF3 heterotrimeric transcription factor (Samuel 2001; Garcia-Sastre and Biron 2006; Murray 2007). The location of this IRF9 binding to STAT heterodimers was thought to be nuclear, given the nuclear localisation signal (NLS) that is revealed upon STAT dimerisation and the reversal of inhibition of STAT2 nuclear retention (Randall and Goodbourn 2008.).

However, recent evidence demonstrating that acetylation is necessary for IRF9 DNA-binding and that IFNAR2 also becomes acetylated thus recruiting the CBP cofactor, alludes to the formation of ISGF3 in the cytosolic compartment (Tang, Gao et al. 2007). The ISGF3 transcription factor binds to the ISRE element to activate transcription of over one hundred genes involved in the maintenance of an ant-viral state, including members of the IRFs, PKR, 2'5'-oligoadenylate synthase (OAS) and promyelocytic leukaemia nuclear bodies (PMLs). Upon activation by interaction with dsRNA, PKR can induce autophagy, which disrupts viral replication and initiates further immune responses. 2'5'OAS is also activated by dsRNA and is involved in production of RnaseL, and agent that degrades viral RNA (Platanias 2005; Tang, Gao et al. 2007). PML bodies are also involved in the regulation of viral replication and depletion of these bodies has been shown to increase replication of HSV-1 (Herpes simplex virus-1) (Boutell, Orr et al. 2003). Their exact function remains to be further elucidated and is reviewed elsewhere (Everett and Chelbi-Alix 2007).

IFNγ signalling is controlled in a similar manner: its cognate receptor becomes activated upon dimerisation of its two subunits, IFNGR1 and IFNGR2, which are associated with the tyrosine kinases JAK1 and JAK2, respectively (Goodbourn, Didcock et al. 2000). Once activated, JAK1 phosphorylates JAK2 and the IFNGR1 subunit, facilitating STAT1 binding, which results in homodimer formation and activation via phosphorylation. This homodimer can then bind to gamma-activation sequence (GAS) elements in the promoters of relevant genes, activating their transcription (Samuel 2001; Platanias 2005). Various other STAT dimers have been identified and these include STAT1 homodimers, STAT2 homodimers with IRF9, and STAT1-STAT2 heterodimers without IRF9, as a result of type I signalling. In response to type II interferon signalling, STAT1 homodimerises with IRF9 and the STAT molecules have also been found to be associated with various other proteins, including IRF1 (Honda and Taniguchi 2006).

1.8 Viral immune evasion

Viral infection triggers mechanisms, which serve to orchestrate host immune responses to initiate anti-viral responses, thereby limiting the spread of infectious virus. Viruses have developed strategies to circumvent these responses, be they short-term, as is the case for transient lytic infections, or more long-term, in the case of persistent viruses such as herpesviruses (Garcia-Sastre and Biron 2006). In addition to developing mechanisms to limit innate immune responses, viruses have also devised mechanisms to avoid detection by immunosurveillance. Such strategies include inhibition of interferon and TLR signalling, blockade of cytokine secretion, modulation of apoptosis and disruption of antigen presentation (Grandvaux, tenOever et al. 2002). In terms of the interferon response, viruses have acquired various methods of attenuating and blocking the establishment of an anti-viral state. Recent data highlighting a role of the EBV-encoded LMP2A/2B proteins have demonstrated that their expression leads to a downregulation of Type I interferon signalling and ISGF3 formation, which results in a global repression of ISG expression (Shah, Stewart et al. 2009). The importance of IRF3 in regulating these pathways has also been demonstrated in other viruses including the smallpox vaccinia virus and HCV which can both block IRF3 activation; the latter through the virally-encoded protein NS3/4A (Sen and Williams 2003). Blockade of IFR3 results in an attenuated IFN response and facilitates viral infection. The extensive and wide-ranging methods of viral disruption of interferon signalling are outlined in the Table below.

Table 1.1: Viral Inhibition of Interferon responses

Activity	**Virus**	**Protein/Gene**	**Mechanism**
Inhibition of TLR/RIG-1 signalling	HCV	NS3/4a	Inhibition of TLR response
	VACV	A52, A46	
	Paramyxo-viruses	V	Inhibition of RIG-1 and Mda5
	Influenza A virus	NS1	
	HCV	NS3/4a	Disruption of IPS-1 activity
	BDV	P	
	VACV	N1	
Inhibition of IRF3 activity	RABV	P	Inhibition of IRF3 activity by alteration of phosphorylation or degradation
	HTNV	G1	
	HHV6	E1	
	Rotaviruses	NSP1	
	BVDV	N Protein	
	CSFV	N Protein	
	HPV16	E6	
	HSV	ICP0	
	BHV1	BICP0	
	THOV	ML	
Inhibition of NFκB signalling	HPV	E7	Disruption of IKK complex formation/activity
	HCV	Core	
	VACV	Unknown	
	Adenovirus	E3	
	ASFV	A238L	Disruption of NFκB activity
	VACV	N1	
	MYXV	MNF	

Table 1.1: Viral Inhibition of Interferon responses

Activity	Virus	Protein/Gene	Mechanism
Downregulation of IFN receptor activity	HHV8	K3 K5	Receptor retention
	HCMV	Unknown	Interference with JAK
	HPV18	E6	
	HSV	U(L)41	
	JEV	NS5	
	LGTV	NS5	
	MeV	C, V proteins	
	MPV	T antigen	
	MuV	V protein	
Disruption of STAT activity	HPIV2	V protein	Degradation of STAT
	HPIV5	V protein	
	HSV	U(L)41	
	MuV	V protein	
	NDV	V protein	
	RSV	NSP2	
	SeV	C protein	
	SV41	V protein	
	SV5	V Protein	
	Adenovirus	E1A	STAT phosphorylation /sequestration
	EBOV	VP24	
	HBV	Unknown	
	HCMV	IE1	
	HCV	Core	
	HeV	V protein	
	HPIV3	Unknown	
	MeV	V protein	
	MPRV	V protein	
	NiV	V protein	
	RABV	P protein	
	RPV	P, V protein	
	VACV	VH1	
Inhibition of IFN induction/ transcriptional activity	HSV	UL41/UL13	Induction of SOCS proteins
	HCV	Core	
	HHV8	vIRFs 1-3	Interference of ISG promoter activity
	HBV	Capsid protein	
	Adenovirus	EIA	
	HHV8	vIRFs 1-3	Interference of IFNβ promoter
	EBV	EBNA2	Disruption of IFN induced transcription
	HPV16	E7	IGSF3 formation
Sequestration of IFN via soluble receptors	MYXV	M-T7	Bind IFNs and prevent signalling from cellular receptors

Table 1.1: Viral Inhibition of Interferon responses

Activity	Virus	Protein/Gene	Mechanism
Inhibition of PKR activity	Reovirus	Omega3	Binds and sequesters dsRNA preventing activation
	Rotavirus	NSP3	
	VACV	E3L	
	OV	OV20.0L	
	Influenza virus	NS1	
	HSV	US11	
	HCMV	TRS1	
	MCMV	m142/143	
	EBOV	VP35	
	EBV	SM	
	Adenovirus	VAI RNA	Binds to PKR without activation
	EBV	EBER RNA	
	HIV	TAR RNA	
	Baculovirus	PK2	Binds to and inhibits PKR
	HCV	NS5A, E2	
	HSV	US11	
	HIV	Tat	
	Poliovirus	Unknown	Degrades PKR
	Influenza virus	Unknown	Induction of p58IPK, inhibitor
Inhibition of eIF2a phosphorylation	VACV	K3L	Inhibition of eIF2a phosphorylation
	HSV	ICP34.5	
Inhibition of 2’5’OAS activity RnaseL	Reovirus	Omega3	Binds and sequesters dsRNA preventing activation
	Rotavirus	NSP3	
	VACV	E3L	
	OV	OV20.0L	
	Influenza virus	NS1	
	HSV	US11	
	HCMV	TRS1	
	MCMV	m142/143	
	EBOV	VP35	
	EBV	SM	
	EMCV	Unknown	RnaseL inhibitor induction
	HIV	Unknown	
	HSV	Unknown	2’5’OAS antagonist expression

The above Table is adapted from the following review articles (Alcami and Koszinowski 2000; Randall and Goodbourn 2008.), and demonstrates the wide variety of mechanisms of ant-viral immune evasion, highlighting the evolution of viral genes tailored to disrupt normal cellular defences. In addition to modulation of the interferon response, viruses have evolved

mechanisms to disrupt apoptosis, cytokine production and release; these have been reviewed extensively elsewhere (Levy and Garcia-Sastre 2001). Importantly in the context of this thesis, viruses have been shown to modify normal cellular trafficking to disrupt antigen presentation by MHC molecules. The following Table highlights some of the viral proteins that have been shown to exert such effects.

Table 1.2: Viral inhibition of MHC antigen presentation.

Activity	**Virus**	**Protein/Gene**	**Mechanism**
MHC class I inhibition	Adenovirus	E3/19K	Retention of MHC I molecules in (endoplasmic reticulum)
	HCMV	US3	
		US2, US11	Relocation of MHC I
		m4	MHC I binding
		m6	Lysosomal degradation of MHC I
	MCMV	m152	Retention of MHC I in Golgi
	HHV8	K3, K5	Downregulation of MHC from surface
	MHV68	K3, K5	
	HIV	Nef	Endocytosis of MHC
		Vpu	Destabilisation of MHC
MHC class II inhibition	Adenovirus	E1A	Prevents activation of MHCII expression
	HCMV	US2	Degradation of MHC II
	HSV	ORF14	MHC II binding
	HPV	E5, E6	Acidification of endosomes
	BPV	E5, E6	Disruption of AP complexes
	HIV	Nef	Disruption of processing
Inhibition of TAP (transporters associated with antigen processing)	HSV	ICP-47	Inhibits peptide:TAP binding
	HCMV	US6	Inhibits TAP transport
Inhibition of antigen production	EBV	EBNA1	Inhibits proteasomal degradation (Gly-Ala repeat)

The above Table is adapted from a selection of review articles (Bonnerot, Briken et al. 1997; Alcami and Koszinowski 2000; Donaldson and Williams 2009; Hansen and Bouvier 2009). In addition to demonstrating the ability of viral proteins to alter antigen presentation, this Table also highlights the mechanisms by which viruses can affect intracellular trafficking, a subject which is investigated in more detail later (*Chapter 5*).

1.9 Endosomal-lysosomal trafficking

The endosomal-lysosomal trafficking network is involved in a variety of cellular processes including receptor signalling, immune surveillance and sensing of the extracellular milieu. Vast quantities of membrane are constantly endocytosed into the cell, along with tethered proteins. These are subject to membrane recycling, undergo degradation or participate in cell signalling responses. Due to the pleiomorphic and dynamic nature of the network, analysis has been problematic, although advances in cell imaging and isolation of markers for specific compartments are aiding increases in our level of understanding. It is known that the family of small Rho GTPases have a significant role to play in the regulation of intracellular traffic (Zerial and McBride 2001; Rodriguea-Boulan, Kreitzer et al. 2005). *Figure 1.3* depicts a schematic representation of the trafficking network whereby invaginated clathrin-coated pits allow access of membrane and membrane-bound proteins into the cell, which can be transferred through early endosomes to the sorting multi-vesicular body, or sorting endosome, and from here they are targeted for recycling or degradation in the lysosome. Specific endosomal compartments involved in these various processes can be defined by molecular analysis of their membrane proteins (Deneka, Neeft et al. 2003).

1.9.1 Endocytosis, clathrin and its effectors

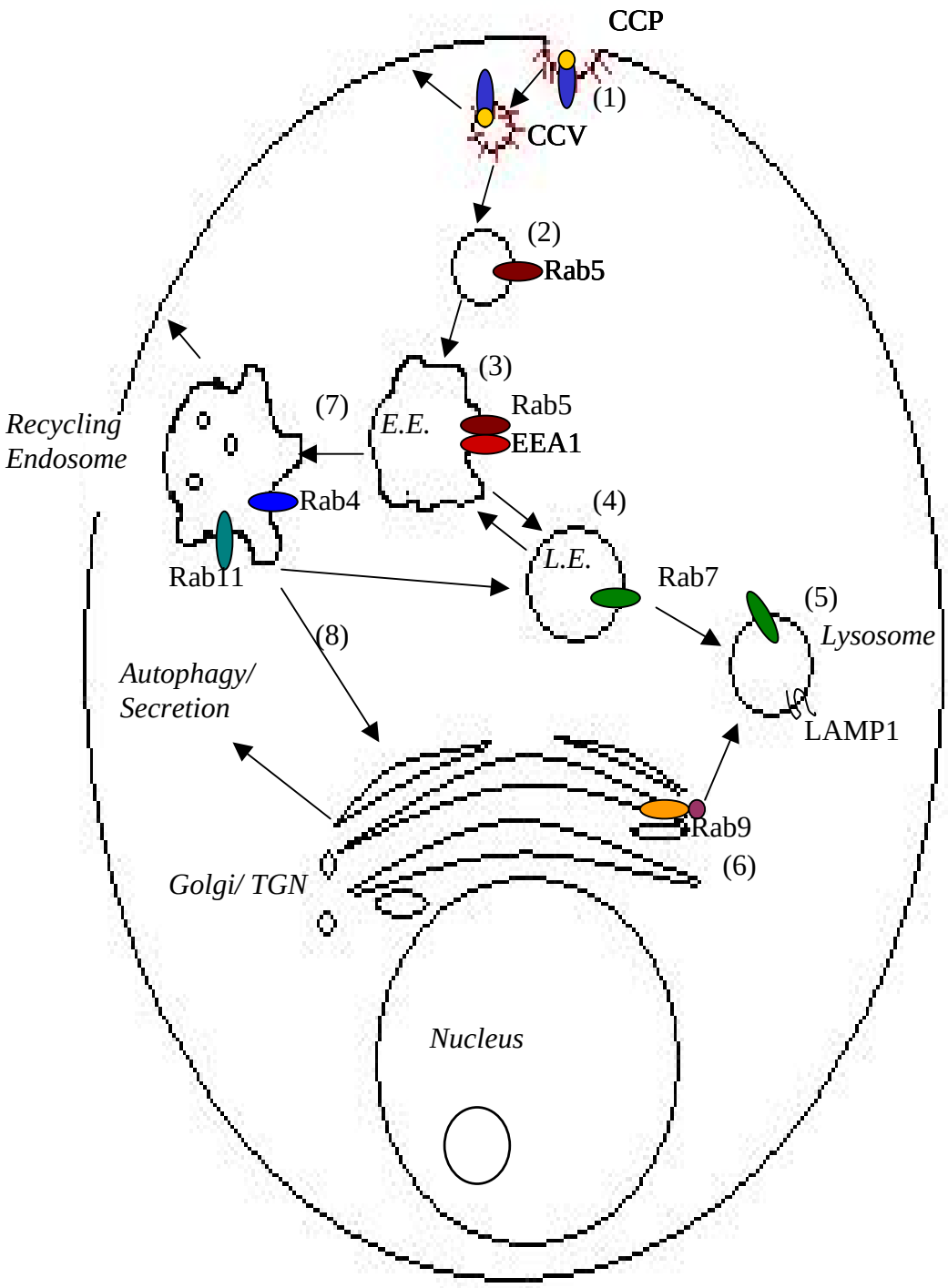
Endocytosis is a process by which cells uptake exogenous proteins from their immediate environment and in doing so internalises membrane and membrane-bound proteins. Much of the current research focuses on clathrin-dependent endocytosis (CDE), although clathrin-independent mechanisms have also been identified (CIE) (Donaldson, Porat-Shliom et al. 2009). CDE is a mechanism that involves complex protein-protein and protein-lipid interactions. These mechanisms include those dependent on caveolin-coated structures, which is linked to proteins localising to cholesterol-rich membrane regions, in addition to

Figure 1.3 Endosomal-Lysosomal Trafficking

Schematic of endosomal to lysosomal intracellular trafficking network by which receptors are endocytosed, sorted and targeted for recycling or degradation in the lysosome. (1) Endocytosis is controlled in a clathrin dependent manner and results in internalisation of membrane and membrane bound receptors and proteins first in clathrin coated pits (CCP) and secondly in clathrin coated vesicles (CCV). These vesicles are then carried through the cell and interact with Rab5 to form Rab5 early vesicles (2), Rab5 is a small GTPase that controls both early and late stages of endosome and vesicle intracellular traffic. Upon recruitment of its effector proteins (GEFs) (3) including EEA1, Rabex-5 and Rabaptin-5, this activated Rab5 and facilitates vesicle to endosome fusion and homotypic early endosome (EE) fusion.

The contents of the vesicle are passed to the EE and here through the interaction of GEFs, Rab5 and membrane bound phospholipids trafficking to the late endosome (LE) is effected (4). EE to LE switching is facilitating by changes in the membrane architecture of the compartments, including loss of Rab5 and the presence of Rab7. EE conversion events to LE conversion events occur more often than the reverse and from the LE compartmental contents are passed to the acidic lysosome for receptor:ligand detachment and degradation of content.(5) The lysosomal pH is maintained by presence of the v-ATPase holoenzyme and degradation is mediated by the presence of acid hydrolases including Cathespins. LAMP1 is a membrane bound lysosomal protein that is used a marker of this compartment and contributes to the highly carbohydrate component of the membrane via glycosylation events. (6) Rab9 is responsible for shuttling, MPRs to the lysosome from the Trans Golgi Network

(TGN), which are bound to acid hydrolases. (7) Endocytosed content can also be recycled back to the membrane via the multi-vesicular body (MVB)/sorting endosome, Rab4 and Rab11 are localised here, Rab4 controlling short recycling, not via the TGN and Rab11 long recycling, through the GA (golgi apparatus) (8). Authophagy, exosome formation and synthesis and secretion of protein are controlled by the TGN/GA and are initiated here. This Figure is adapted from a variety of sources, (Woodman 2000; Zerial and McBride 2001; Rodriguea-Boulan, Kreitzer et al. 2005)


CCP
(1)
CCV
(2)
Rab5
(3)
Rab5
EEA1
E.E.
(7)
Recycling
Endosome
Rab4
(4)
L.E.
Rab11
Rab7
(5)
Lysosome
LAMP1
(8)
Autophagy/
Secretion
Rab9
(6)
Golgi/ TGN
Nucleus

phagocytosis and macropinocytosis (Mousavi, Malerod et al. 2004) which are both dependent on cellular cortical actin filaments. Other cascades of CIE have been identified and deal with a unique set of membrane components, regulatory factors and cargo (Brodsky 1988). The primary focus of this thesis pertains to CDE. A facet that was not investigated during this research, but which is of interest in light of the localisation of the LMP2A and LMP2B proteins to cholesterol-enriched lipid rafts, is the contribution of caveolin-driven endocytosis on the levels of cell surface receptor. It is therefore likely that this pathway could be modulated in the presence of LMP2 expression.

It has been shown that clathrin-mediated endocytosis occurs in three main stages (Brodsky 1988). First, clathrin is assembled into polygonal lattices on the plasma membrane, which also involved re-organisation and complex formation of a variety of membrane-bound proteins and intracellular cytosolic effectors. These complexes concentrate receptors to distinct regions of the membranes, which increases the efficiency of sorting endocytosed vesicles by endosomes later on. Uncoated internalisation is not as efficient and is in no way specific, similar to the mode of entry of viruses and toxins. Accumulation of these clathrin complexes precedes formation of clathrin-coated pits and invaginations that internalise portions of the plasma membrane and allow infiltration of extra cellular fluid into these invaginations. Finally, vesicles are formed through clipping or pinching off of the invaginated membrane, and the subsequent loss of the clathrin coat (Mousavi, Malerod et al. 2004). These free vesicles are then targeted to the endosomal trafficking networks and sorted depending on the lipid and protein component of the internalised membrane. Many factors are involved in the regulation of this process, with two of the best characterised components being the adaptor protein 2 (AP2) and Dynamin proteins (Shih, Gallusser et al. 1995). AP2 is a protein composed of four subunits and is thought to localise and facilitate the accumulation of clathrin at the plasma membrane, and in so doing, can contribute to lattice formation. Sorting of plasma membrane

proteins by APs into clathrin-coated pits or depressions leads to the formation of internalised clathrin-coated vesicles (Brodsky 1988; Hansen, Sandvig et al. 1993; Shih, Gallusser et al. 1995; Wucherpfennig, Wilsch-Brauninger et al. 2008). Dynamin has an N-terminal domain responsible for the hydrolysis of GTP: its three forms are differentially expressed and their functions appear to overlap. It is thought that this GTPase activity mediates, either directly or indirectly, closing of the neck of the invaginated pit and subsequent vesicle fission. More recently it has been shown that dynamin may recruit actin filaments, and in doing so, can further facilitate vesicle scission (Wucherpfennig, Wilsch-Brauninger et al. 2008).

In the case of receptor-mediated endocytosis, ligand binding to the receptors results in their activation and subsequent signal transduction. The receptor complex is endocytosed and internalised in a clathrin-dependent manner, and upon uncoating of the internalised vesicle, they are exposed to intracellular compartments, which serve to acidify the vesicle and its contents thus segregating the ligand from the receptor (Bananis, Murray et al. 2000). These then separate and enrich two separate populations of vesicles, one is of which is targeted for degradation and has a high ligand content, whilst the other is recycled to the membrane and contains a higher proportion of receptor. For this process to occur it is necessary for pre-segregated vesicles to bind to, and move along, microtubules to undergo fission (Bananis, Murray et al. 2000). Early endocytic vesicles have the capacity to undergo fission and receptor-ligand segregation is dependent on binding to microtubules, whilst their motility is dependent on ATP. Vesicles that undergo binding with kinesin have been shown to contain unsegregated receptor-ligand complexes. These vesicles are then capable of fusing with compartments of the early endosome trafficking network in a Rab5 dependent manner, (Woodman 2000). From here the fate of internalised molecules is decided, whether targeted for lysosomal degradation or recycled to the plasma membrane, as in the case of receptors whose signalling cascade has been initiated in earlier compartments upon interaction with signalling intermediate adaptor

molecules or activating ligands. This trafficking network is a small fraction of a much larger extensive intracellular dynamic membrane process, which can include the trans-golgi network (TGN), the endoplasmic reticulum, exosome secretion and autophagy (Woodman 2000; Zerial and McBride 2001).

The transport of cargo between endocytosed vesicles and cellular compartments, or between compartments, involves the movement and subsequent tethering of the vesicle to its destination membrane, and finally fusion of the two membranes. Soluble N-ethylmaleimide sensitive fusion protein receptors (SNAREs) are integral membrane proteins that are localised to compartments throughout the trafficking network, and have been shown to be responsible for this fusion between the phospholipid bilayers of the target membrane and the incoming vesicle (Deneka, Neeft et al. 2003). SNAREs, are tightly regulated, and although expressed on a wide variety of organelles, are only active at specific membrane sites. These proteins are fundamental to vesicular transport, and have been shown to interact directly with another family of proteins, the Rab GTPases. Through these interactions it is thought that the Rab proteins regulate activation of SNARE machinery and provide cues as to the specific timing and location of fusion events. A significant level of cross talk exists between the SNARE fusion machinery and the Rab GTPases (Raiborg and Stenmark 2009). Specific examples of this include the interaction of EEA1 and Rabenosyn5 (effectors of Rab5 activity) with SNAREs, syntaxin 6, 7 and 13, and the interaction between Rabenosyn5 and the Sec1 family member, VPS45 (Christoforidis, McBride et al. 1999; Raiborg and Stenmark 2009). These interactions are integral to full endosomal trafficking but a detailed discussion of their mechanics is beyond the scope of this investigation. Experimental data in this thesis has focused on the Rab proteins and the effect of their impaired expression on trafficking networks as a result of LMP2A/2B expression.

1.9.2 The early endosome: Rab 5

Rab 5 is one of the best characterised members of the Rab family of proteins, which constitute the largest family of monomeric small GTPases that function at specific sites of the endosome/lysosome pathway (Woodman 2000). In the early endocytic pathway Rab5 regulates vesicle and early endosome fusion, as well as homotypic endosome-to-endosome fusion. Rab5 also acts as a chaperone to endocytosed vesicles, transporting them to the early endosome and facilitating the tethering of vesicles to endosomal membranes. These processes are mediated by a positive feedback loop between Rab5 and its guanine exchange factor (GEF), Rabex-5 (Horiuchi, Lippe et al. 1997). Following membrane recruitment of Rab5, Rabex-5 activates Rab5 GTPase activity, allowing recruitment of its effector Rabapatin-5. This facilitates enhanced Rabex-5 activity and the subsequent enrichment of Rab5 levels at the membrane. Rab5 can also interact with PI3-K to form phosphatidylinositol-3-phosphate (PtdIns-3-P) (Deneka, Neeft et al. 2003). The co-localisation of Rab5 with newly formed PtdIns-3-P enables binding of the Rab5 effector proteins, EEA1 and Rabenosyn5, which function to stabilise the classical early endosome and promote its fusion with vesicles and other endosomal membranes (Simonsen, Lippe et al. 1998). EEA1, a classical marker of early endosomes, is a coiled protein that contains two Rab5 binding domains. It is thought to contribute to fusion by bridging two Rab5-positive membranes through these domains, and two FYVE Zn finger domains, which can interact specifically with PtdIns-3-P (Christoforidis, McBride et al. 1999). Rabenosyn5 is recruited in a similar FYVE-dependent manner and is involved in the docking and fusion of endosomal membranes, an example of the complex level of interactions occurring between lipids and proteins at the membrane, especially given that the enrichment of PtdIns-3-P on the membrane could instigate binding of additional effectors in a Rab5-independent manner (Barnekow, Thyrock et al. 2009).

The role of Rab5 and its importance in both the early and late stages of the network is highlighted in studies where a GTP-deficient Rab5 was expressed in normal rat kidney (NRK) cells (Hirota, Kuronita et al. 2007). This study uncovered the existence of 3 distinct classes or types of endosomes. Firstly, formation of type I endosomes occurs: these can undergo homotypic endosome fusion and also fusion with carrier vesicles from the TGN and endocytosed vesicles (homotypic fusion still occurs due to the endogenous pool of Rab5 remaining in the cell, and as this is depleted, the rate of homotypic fusion is diminished). This is followed by the formation of type II endosomes, which are morphologically distinct from "classical early endosomes". They are giant endosomes that display all the characteristics of the type I endosomes and late endosomes, yet appear to be a hybrid between these and intermediary cargo vesicles. Finally type III clustered endosomes form via the vesiculation of the type II giants, allowing reformation of late endosomes and lysosomes (Hirota, Kuronita et al. 2007). When cells expressing this defective form of Rab5 are treated with wortmannin, a specific inhibitor of PI3-K activity, the rate of vesiculation of the type II giants is accelerated, which is perhaps unsurprising given the role of PtdIns-3-P in mediating the function of Rab5 and its effectors as described above. Early endosomes can fuse with late endosomes: an event that is promoted by, and dependent on, active forms of Rab5. The effect of expression of defective Rab5 on lysosomal formation as described below is indicative of a role for Rab5 in the late stages of endosomal trafficking, in tandem with its role in early endosome fusion, as described above (Hirota, Kuronita et al. 2007).

Cells display distinct subpopulations of early endosomes, which are dynamic but do not necessarily intermix: early endosomes containing Rab5 only; Rab5- and Rab4-carrying endosomes; and finally Rab4- and Rab11-recycling endosomes (McCaffrey, Bielli et al. 2001; Stenmark and Olkkonen 2001). Rab4 can bind to Rab5 effectors and vice versa, indicative of the fluid nature of the Rab-controlled membrane network and the cross reactivity of their

machinery. Rab4 and Rab11 are the GTPases responsible for sorting membranes either for recycling or degradation, and are localised within recycling endosomes and the multi-vesicular body (MVB) (McCaffrey, Bielli et al. 2001). Rab4 is responsible for short loop recycling and Rab11 is responsible for long loop recycling (that which passes through the TGN) (Zerial and McBride 2001). Another Rab family member, Rab6, has been shown to have a role in microtubule development through interaction with Rabkinesin6, which has roles in vesicle motility and cytokinesis. Development of such intracellular structures enables endosomes and endosome-like compartments to traffic through the cell (Bananis, Murray et al. 2000). Plus end-directed kinesin binds Rab6 via KIF16B, which is recruited by PtdIns3, implicating Rab6 further in the process of compartment movement (Barnekow, Thyrock et al. 2009).

1.9.3 Early to late fusion: Rab7

The endosome can fuse with additional intracellular compartments and it has been shown that loss of Rab5 from an intracellular compartment can induce Rab7 positivity (Rink, Ghigo et al. 2005). Rab7 is a marker of the late endosome and the lysosome. The numbers of Rab5-positive endosomes increases over time, with the loss of EEA1 from these compartments being observed as they become Rab7 positive. Loss of EEA1 enhances loss of Rab5 through destabilisation of Rab5 effector complexes (Rink, Ghigo et al. 2005). Inactivation of RabGAP-5 results in the swelling of endosomes and a reduction in lysosomal conversion and trafficking (Hirota, Kuronita et al. 2007). Rab5 also regulates the attachment to, and movement of, early endosomes along microtubules, dynamically fluctuating on individual early endosomes. Via repetitive fusion events, degradative cargo becomes highly enriched in progressively fewer and larger compartments (Woodman 2000). These are then removed from the cell centre via loss of Rab5 and acquisition of Rab7, a process requiring the class c VPS/HOPS complex (a Rab7 GEF) (Bucci, Thomsen et al. 2000). This is the most striking example of membrane identity

remodelling via exchange of Rab machinery, and is another example of SNARE regulation by the Rab domains on organelles that are not static, but rather merge and separate in a dynamic manner, changing the properties of their binding compartments (Markgraf, Peplowska et al. 2007). This cyclical nature of Rab5 machinery means that endosomes are either stable for minutes or lose identity rapidly in conversion events. Early and late systems are thus separated; yet remain connected through Rab5-mediated control of these events.

Rab7 is implicated in early endosome to late endosome trafficking. Expression of an activated Rab7 resulted in increased levels of perinuclear aggregates, as does overexpression of wild-type Rab7 (Bucci, Thomsen et al. 2000). Inactive dominant negative mutants showed the reverse, and localised to the cytosol, whilst lysosomal content also became increasingly dispersed. Rab7 is therefore essential for the maintenance of the perinuclear lysosomal compartments. Once structures begin to acquire Rab7 and lose Rab5, direction of movement of these late endocytic structures toward the perinuclear aggregate or lysosomes occurs, likely recruiting dynesin/kinesin to serve as tracks for this movement. Rab7b has been shown to negatively regulate TLR4 signalling through investigation of its role in LPS-induced production of TNFα, IL-6, nitrous oxide (NO) and IFNβ (Wang, Chen et al. 2007); and has also been shown to potentiate activation of MAPK, NFκB and IRF3 signalling through TLR4 (Wang, Chen et al. 2007). Lack of Rab7b has been shown to increase IRF3 and NFκB activity and concomitantly increase the levels of their effector cytokines. This silencing of Rab7b also led to the upregulation of TLR4 expression, indicative of a possible role for Rab7b in TLR4 degradation (Wang, Chen et al. 2007).

1.9.4 The lysosome

The lysosome is an acidified intracellular compartment responsible for the digestion of membranes and membrane-bound proteins by acid hydrolases, such as cathepsin D (Eskelinen,

Tanaka et al. 2003). These products undergo trafficking to the lysosome by phagocytosis, autophagy, and also by the process of endocytosis and endosomal sorting described above. Structurally, the membrane of the lysosome is densely packed with carbohydrate and is spanned by a number of proteins including the highly glycosylated proteins, lysosome associated membrane protein 1 (LAMP1) and LAMP2; the tetraspannin, CD63; and Niemann Pick C1 protein (NPC1) (Eskelinen, Tanaka et al. 2003). Acidity is maintained through the action of the V-ATPase holoenzyme (Bucci, Thomsen et al. 2000). Lysosomes are conspicuous by their lack of mannose-6-phosphate receptors (MPRs), which bind lysosomal enzymes from the TGN and transport them to late endosomes in a Rab9-dependent manner. Rab9 is localised to late endosomes and the TGN and has been shown to control traffic between both compartments, specifically transporting newly synthesised MPRs bound to cathepsins via the Rab9 effector, TIP47 (Dodeller, Gottar et al. 2008). Due to the acidic conditions of the compartment, the cathepsins become activated; the ligands dissociate from their cognate receptors and are released into the late endosome. These lysosomal hydrolysis events facilitate both receptor recycling and degradation of receptors and ligands (Forgac 2007).

1.9.5 Ubiquitination as a sorting signal

While poly-ubiquitination is mainly responsible for directing proteins to the proteasome for degradation, additional forms of post-translational modification exist that require ubiquitin binding. Mono- or multi-ubiquitination has been shown to be largely involved in the stability and activity of a variety of proteins, and has also been linked to the maintenance and control of interactions that specify subcellular localisation of certain classes of membrane-bound receptors (Piper and Luzio 2007). In yeast, it has been shown that mono-ubiquitination is sufficient to induce internalisation of receptors and this hypothesis has been broadened to include the endocytosis and secretory networks of mammalian cells. The fate of TGFβ

receptors, for example, can be controlled by the extent of ubiquitination of the cytosolic domains of the receptor. When associated with Smad7/Smurf-2 complexes, the TGFβ receptor is endocytosed in a caveolin-dependent manner and targeted for proteaosomal degradation; however, when associated with Smad anchor proteins, it is internalised through clathrin-mediated endocytosis and signalling is activated (Piper and Luzio 2007). Ubiquitin also has a role in the sorting of internalised proteins in the multi-vesicular body (MVB) or multi-vesicular endosome (MVE), whereby non-ubquitinated proteins are targeted for recycling to the plasma membrane and those ubiquitinated are cycled to late endosomes, and eventually the lysosome for degradation by acid hydrolases. LPS is endocytosed through receptor-mediated endocytosis, which is both clathrin- and dynamin-dependent. TLR4 is then ubiquitinated and targeted for recycling or degradation. Inhibition of endocytosis and sorting leads to increases in LPS-induced signalling (Wang, Chen et al. 2007). Trafficking of these receptor complexes is therefore essential for signal termination and optimal LPS-associated antigen presentation. Other receptors are internalised and activated in different ways. For example, the EGF receptor is internalised within minutes after ligand-binding by clathrin-mediated endocytosis (Keegan, Sheflin et al. 2000; Repetto, Yoon et al. 2007), whereas IL-2 receptor complexes are taken up in a raft-dependent manner that is reliant on caveolin.

1.9.6 Viral proteins and cell traffic.

1.9.6.1 The E5 protein of Papillomavirus

The HPV-encoded E5 protein is a small hydrophobic protein that is expressed in virus infected keratinocytes. E5 has been implicated in interfering with a wide range of normal cell functions including signal transduction, receptor turnover and compartment pH regulation (Thomsen, Deurs et al. 2000; Ashby, Meagher et al. 2001). E5 is localised to the endoplasmic reticulum and can induce the anchorage-independent growth of immortalised fibroblasts but

not primary keratinocytes (Disbrow, Hanover et al. 2005). BPV E5 has been shown to interact with the V-ATPase complex, resulting in alkylation of the golgi which is partially responsible for MHC class I retention. Interacting with both V-ATPase and MHC in complexes, E5 retains newly assembled MHC complexes in the golgi apparatus (GA) by preventing GA acidification. By physical interaction these complexes are shunted into the lysosome for degradation (Disbrow, Hanover et al. 2005). HPV16 E5 transiently alters endosomal, but not GA, pH. It is possible that E5 sequesters V-ATPase regulators in the ER, thus inhibiting the function of the complex. Interaction of E5 and V-ATPase may be lost due to the removal of the bound protein during "turning" of the ATP-driven proton pump explaining the transient nature of this dysregulation (Ashby, Meagher et al. 2001; Marchetti, Ashrafi et al. 2006).

1.9.6.2 The K3 and K5 proteins of KSHV

Viral disruption of antigen presentation by MHC links both intracellular trafficking and immune responses. In the context of KSHV, the K3 and K5 proteins have been shown to downregulate MHC class I molecules from the cell surface (Hewitt, Duncan et al. 2002). These proteins remove HLA molecules from the surfaces of cells via rapid endocytosis of the molecules: K5 is responsible for downregulating HLA A and B, but has a weaker effect on HLA class C molecules; K3 downregulates cell surface expression of all HLA types (Ishido, Wang et al. 2000). K3 and K5 have also been shown to target IFNγR1 for increased endocytosis by ubiquitinating the receptor, thereby contributing to the viral mechanisms of immune evasion (Li, Means et al. 2006). Increased endocytosis is also responsible for decreased levels of B-72 and ICAM-1 (proteins involved in T-cell stimulation) in BJAB cells expressing K5 (Coscoy and Ganem 2001). These processes and additional immunomodulatory effects of KSHV are reviewed elsewhere (Means, Ishido et al. 2002; Lehner, Hoer et al. 2005; Rahim-Rezaee, Cunningham et al. 2006).

1.9.6.3 The Nef protein of HIV

The HIV protein, Nef, internalises CD4, which functions to bind to the viral envelope glycoprotein, GP120, and permit viral entry. CD4 binds to the N-terminus of Nef, and increased internalisation of this molecule has three secondary effects: combating immune surveillance; preventing superinfection; yet allowing trafficking of ENV proteins. This occurs in a clathrin-dependent manner and it has been demonstrated that Nef expression induces pit formation, resulting in the accumulation of endosomes, and that the stability of CD4 is sensitive to lysosomal inhibitors (Foti, Mangasarian et al. 1997; Mangasarian, Foti et al. 1997; Sanfridson, Hester et al. 1997). Nef binding protein 1 (NBP1) can bind to the C-terminus of Nef and encodes a component of the V-ATPase holoenzyme. The V-ATPase complex can bind to AP2, which is complexed with clathrin at the cell surface, thus facilitating the increase of clathrin density, and consequently endocytosis (Lu, Yu et al. 1998). Nef has also been shown to promote actin filament remodelling to further disrupt intracellular signalling and T-cell immune responses (Haller, Rauch et al. 2007).

These are examples by which viral proteins can interact with and disrupt intracellular trafficking mechanisms to evade detection by the immune system. Other examples include the role of the Tip protein of Herpes virus saimiri (HVS) in downregulating CD4 and T-cell receptor (TCR) from the cell surface (Cho, Kingston et al. 2005), an effect that is dependent on its localisation in lipid rafts, is important for this, which is significant given LMP2A/2B's similar intracellular location.

1.10 Aims and Objectives

The aim of this body of research was to elucidate the exact mechanisms by which, LMP2A and LMP2B contribute to EBV-associated epithelial malignancy through maintenance of viral latency by immune evasion. To perform such analysis comparisons were made between panels of epithelial cell-lines, CNE-2, H103 and H157, stably expressing LMP2A, LMP2B or a Neomycin control. Firstly comprehensive investigations were undertaken to characterise the effect of viral protein expression on the TLR signalling network, the first mediator of the host response to viral infection. This was performed at a variety of levels from basal and activated receptor expression to activation and read-out of signalling responses, including Ap-1 and NFκB activity and IFNβ production extended across nine of the TLRs. These analyses involved using standard techniques such as immunoblotting, immunofluorescent staining and luciferase assay. Secondly this study aimed to identify the exact effect of expression of the LMP2 proteins on endosomal-lysosomal trafficking network, identifying a putative mechanism for the reported modulation of type I interferon responses. Utilising both standard and live-cell confocal imaging allowed a comprehensive overview of intracellular trafficking to be performed in the cell-lines described above. Finally generation of attenuated forms of the LMP2 proteins through sequential deletion of the transmembrane domains was undertaken to highlight the importance of these protein regions in mediation of these effects and to ascertain, which of these loops is necessary for internal membrane localisation.

CHAPTER 2

Materials and Methods

2.1 Tissue Culture

2.1.1 Tissue culture media and solutions

2.1.1.1 Media

RPMI 1640: Gibco®, liquid in 500ml sterile bottles, supplemented with L-glutamine (0.2M) and stored at 4°C.

DMEM-F12,1:1: Gibco®, liquid in 500ml sterile bottles, supplemented with L-glutamine (0.2M) high glucose, phenol red and sodium pyruvate, pH 7.0 and stored at 4˚C.

Opti-MEM® : Gibco®, liquid in 500ml sterile bottles with a reduced amount of phenol red.

Freezing media: 70% normal growth media, 20% FCS and 10% Dimethyl sulfoxide (DMSO) (Sigma Aldrich).

2.1.1.2 Other solutions and buffers

Foetal Calf Serum (FCS): Gibco®, liquid in sterile 500ml bottles (mycoplasma/virus tested), stored in 25ml aliquots at -20 °C.

Antibiotics: Sigma-Aldrich, Penicillin/streptomycin solution containing 10,000 units/ml penicillin and 10 mg/ml streptomycin in 0.9% sodium chloride. Sterilised by filtration and used at 5ml per 500ml media.

Phosphate- Buffered Saline (PBS): Oxoid, supplied as tablets, containing NaCl 0.8g, KCL 0.02g, Na_2HPO_4 0.115g, KH_2PO_4 0.02g. 100 tablets dissolved in 10 litres of SDW. aliquoted into 500ml bottles and sterilised by autoclaving at 15psi and 120 °C for 20 minutes.

Trypsin: Gibco®, Trypsin-EDTA (0.25% Trypsin with EDTA) 1X solution 100ml sterile bottles, Porcine parvovirus and mycoplasma treated. Solution contains 2.5 g/L of Trypsin (1:250), 0.38 g/L of EDTA and phenol red. Stored at -20 °C, thawed and heated to 37°C prior to use and stored at 4°C thereafter.

Genticin (G418): Sigma-Aldrich, 5g of G418 powder dissolved in 100ml SDW for a stock solution of 50mg/ml. Sterilised by filtration and stored in 5ml aliquots at –20°C.

Fibronectin: Sigma-Aldrich, pre-sterilised human plasma 0.1% (solution) derived fibronectin at a concentration of 1mg/ml, stock solutions are stored at 4°C.

Hydrocortisone: Sigma Aldrich, 100ng hydrocortisone sodium-succinate powder dissolved in 10ml SDW. 10ml of absolute ethanol was added to give a stock solution of 5mg/ml in 50% ethanol, which was subsequently filtered using a 0.2μm filter and stored at 4ºC.

2.1.2 Cell lines

The *CNE-2* cell line is an immortalised nasopharyngeal carcinoma. *CNE-2 Neo* has been retrovirally transduced to express a neomycin resistance cassette and is used as a control, *CNE-2 WT rEBV* is derived from parental *CNE-2s* that are stably infected with the recombinant Akata strain of EBV. *CNE-2 LMP2A-rEBV* is derived from parental *CNE-2s* that are stably infected with a recombinant Akata strain of EBV that carries a deletion for the EBV gene LMP2A. *CNE-2 LMP2A* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2A. *CNE-2 LMP2B* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2B. Medium: RPMI-1640 supplemented with 5% FCS and antibiotics.

The *Ad/Ah* cell line is an immortalised human adenocarcinoma derived from the nasopharynx. *Ad/Ah Neo* has been retrovirally transduced to express a neomycin resistance cassette and is used as a control, *Ad/Ah WT rEBV* is derived from parental *Ad/Ahs* that are stably infected with the recombinant Akata strain of EBV. *Ad/Ah LMP2A-rEBV* is derived from parental *Ad/Ahs* that are stably infected with a recombinant Akata strain of EBV that carries a deletion for the EBV gene LMP2A. *Ad/Ah LMP2A* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2A.

Ad/Ah LMP2B has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2B. Medium: RPMI-1640 supplemented with 5% FCS and antibiotics.

The *H103* cell line is an immortalised human squamous oral carcinoma, *H103 Neo* has been retrovirally transduced to express a neomycin resistance cassette and is used as a control. *H103 LMP2A* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2A. *H103 LMP2B* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2B. Medium: DMEM-F12 supplemented with 10% FCS, hydrocortisone (0.4mg/ml) and antibiotics.

The *H157* cell line is another immortalised human squamous oral carcinoma, *H157 Neo* has been retrovirally transduced to express a neomycin resistance cassette and is used as a control. *H157 LMP2A* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2A. *H157 LMP2B* has been retrovirally transduced to express a neomycin resistance cassette and the SV40 promoter driven EBV gene LMP2B. Medium: DMEM-F12 supplemented with 10% FCS, hydrocortisone (0.4mg/ml) and antibiotics.

The *C666-1* cell line is an EBV positive cell line derived from undifferentiated nasopharyngeal carcinoma (NPC). C666-1 is a subclone of the parental cell line, C666, derived from a xenograft of Southern Chinese origin. Medium: RPMI-1640 supplemented with 10% FCS and antibiotics.

2.1.3 Maintenance of cell lines

All cell lines were grown and maintained in culture in incubators at 37°C and 5% $C0_2$. Cells were grown on various treated plastic vessels (Iwaki, Corning, MatTek) depending on cell

number required for experimentation. All cell lines adhered readily to the plastic surface with the exception of the *C666-1*line, which required the flasks to be pre-coated in fibronectin at a concentration of 10µg/ml. Maintenance of stock cultures was performed in 75cm^2 or 150cm^2 tissue culture treated flasks and passaged twice a week. Once cell stocks had reached approximately 90-95% confluency, medium was removed and the cells washed gently with 5mls of PBS, this was subsequently removed and 5ml of trypsin added. Flasks were then incubated at 37°C until cells detached. Deactivation of the trypsin was performed by the addition of 10ml of normal growth medium. Cells were pelleted by centrifugation at 1500rpm for 5 minutes and the supernatant removed. Cell pellets were then resuspended in 10ml of growth medium and counted using a haemocytometer, depending on the experimental requirement cells were plated at varying densities onto various types of plastics. Stocks were typically maintained by adding 2ml of cell suspension into 13ml of normal growth Medium in fresh flasks. G418 was added to maintain expression of EBV genes at a concentration of 400µg/ml.

2.1.4 Cryopreservation of cell lines

Cells were trypsinised and recovered as above. Cells were frozen at a usual density of 1 x 10^6 in 1ml of freezing medium in sterile freezing ampoules (Nunc) and transferred to a cryopreservation box (Mr Frosty, Nalgene). This was then placed into -80^{o}C freezer and the cells cooled by 1^{o}C per minute. Ampoules were left overnight at -80^{o}C and for long-term storage transferred to the vapour phase of nitrogen (-140^{o}C). Recovery of frozen stocks was performed by thawing ampoules in a 37°C water bath and transferring the cell suspension slowly, in a drop-wise manner, into 5-10ml of warmed growth medium. Cells were allowed to recover for 10mins at 37°C before seeding in the normal manner.

2.2 DNA Transfection into mammalian cells

2.2.1 Lipofectamine and Plus Reagent transfection

Lipofectamine (Invitrogen) is a lipid-based system of successfully delivering DNA plasmids into cultured eukaryotic cells. Its efficiency is greatly increased by first pre-complexing the DNA using Plus reagent (Invitrogen), optimisation of manufacturers instructions was carried out by altering DNA concentrations, cell numbers and reagent volumes. For transfection onto a six-well plate (Iwaki), the following amounts were deemed to be the most effective (Table 2.2.1.1)

Table 2.2.1.1: Optimum volumes for DNA transfection of cells in culture.

DNA amount (µg)	Plus reagent (µl)	Dilution volume (µl)	Lipofectamine Reagent (µl)	Dilution Volume (µl)	Transfection Volume (µl)
0.5-2	6	100	4	100	800

For a typical transfection using a 6-well plate, cells of interest were seeded at a density of 2.5 x 10^5 and allowed to adhere to the surface by incubation overnight. The media was then removed and the dish washed with PBS followed by two washes with serum free Opti-Mem®. The DNA to be transfected was mixed with the appropriate volume of Opti-Mem® medium and 6µl Plus reagent per reaction added. This was incubated at room temperature for 15 minutes, following which, 4µl of Lipofectamine diluted in Opti-Mem® was added and allowed to mix for an additional 15 minutes. During the second incubation 800µl of serum free Opti-Mem® was added to each well of a 6-well plate. The appropriate volume of transfection mixture was then added in a drop-wise manner onto the six-well plate and the plate placed into the incubator for 3hours. Following this the reactions were supplemented with 2ml of 10% FCS-Opti-Mem® per well, this was returned to the incubator overnight. The following day the medium was removed and either 2ml of normal growth medium added for 24hrs prior to harvesting or additional transfections/treatments were performed again using serum free Opti-

Mem®. In order to assess transfection efficiency a plasmid expressing green fluorescent protein (p-eGFP) was used and a percentage of positivity determined by fluorescent microscopy.

*Table 2.2.1.2:*List of plasmid DNA vectors

Gene of Interest	Plasmid	Control
LMP2A	pSG5-LMP2A	pSG5
LMP2B	pSG5-LMP2B	pSG5
LMP2A-HA	pLXSN-LMP2A-HA	pLXSN-Neo
LMP2B-HA	pLXSN-LMP2B-HA	pLXSN-Neo
GFP	pLenti-6-GFP	pLenti6
LMP2A	pLenti-6-LMP2A	pLenti6
LMP2B	pLenti-6-LMP2B	pLenti6
LMP2-L1	pLenti-6-L1del	pLenti6
LMP2-L3	pLenti-6-L3del	pLenti6
LMP2-L4	pLenti-6-L4del	pLenti6
LMP2-L5	pLenti-6-L5del	pLenti6
LMP2-L6	pLenti-6-L6del	pLenti6
EBERS	pBSAII-EBER	pBSAII

2.3 Tissue culture treatment of cells

2.3.1 Stimulation of signalling responses

In order to stimulate signalling responses of certain classes of immunological receptors, purified versions of their ligands are commercially available. Cells of interest were seeded at the required density depending on the experimental design and allowed to adhere overnight in the incubator at 37°C and 5% C02. Once adhered, the media was removed and the dishes washed with PBS followed by serum free Opti-Mem®. This removes any residual FCS, which could interfere with signalling responses. Cells were then incubated with Opti-Mem® containing the required concentration of treatment (Table 2.3.1.1) and incubated for a variety

of timepoints prior to harvesting and analysis. For treatment of cells with Poly (I:C) a synthetic double stranded RNA molecule (Invivogen), Lipofectamine 2000(Invitrogen) was used. This is a similar reagent to Lipofectamine (described in section 2.2.1) but does not require Plus reagent. It is specifically designed for the transfection of RNA molecules. Briefly, cells of interest were seeded at a density of 2.5 x 10^5 and allowed to adhere overnight at 37°C. Following this the cells were washed with PBS and serum free Opti-Mem®, the RNA to be transfected was diluted in Opti-Mem® and incubated for 30mins at room temperature. This was then added in a drop-wise manner to the 6-well plate and the reactions returned to the incubator. As Liopfectamine 2000 was only used to transfect P(I:C) it was not deemed necessary to supplement the media after 3hours with 10% FCS.

Table 2.3.1.1: Cell treatments and stimulations

Signalling Pathway	**Treatment**	**Concentration**	**Timepoints**
TLR1	Pam3CSK4	0.5Mμg/ml	6 and 9 hours
TLR2	HKLM	10^8 cells/ml	6 and 9 hours
TLR3	Poly (I:C)	5μg/ml	6 and 9 hours
TLR4	LPS	200ng/ml	6 and 9 hours
TLR5	Flagellin	50ng/ml	6 and 9 hours
TLR6	FSL-1	400ng/ml	6 and 9 hours
TLR7	Imiquimod	2.5μg/ml	24 and 48 hours
TLR8	ssRNA	1μg/ml	6 and 9 hours
TLR9	CpG DNA	1μM	6 and 9 hours

2.3.2 Dual luciferase reporter assay (Promega)

The dual luciferase reporter assay system (Promega) is a method by which, the luciferase activities of both firefly (*Phontinus pyralis)* and Renilla (*Renilla reinformis)* can be quantified from the same sample effectively and efficiently. The firefly luciferase signal expressed by the promoter of interest is measured through quantitation of the luminescent signal emitted upon

addition of luciferase reagent II (LARII). This signal is quenched by the addition of the second, Stop and Glo', reagent, which allows the constitutively active Renilla luciferase signal to then be measured. Cells were transfected with the reporter plasmid to be analysed, consisting of the promoter of the gene of interest cloned upstream of the firefly luciferase coding DNA sequence, or an appropriate negative control vector, in the manner described in section 2.2.1. In tandem with transfection with the reported construct the Renilla (pRL-TK) was co-transfected into all cell samples, including negative controls. For transfection into a 6-well plate, 0.5µg of each of the vectors were used, (see Table 2.3.2.1 for list of reporter plasmids and their control counterparts). Further stimulation of the reporter by receptor ligands was performed depending on experimental design as described in section 2.3.1.

*Table 2.3.2.1.:*List of Luciferase reporter constructs and their control counterparts

Reporter	**Control Counterpart**
pIFNβ-Luc	pTAL-luc
p-κBconA-Luc	pGL2-basic
pAP1-Luc	pGL3-basic

The samples were harvested at the appropriate time point by removing all of the media and washing the wells gently with PBS. All PBS was removed on-ice and 500µl of 1 x Passive Lysis Buffer (Promega) per well of a 6-well plate was added. Samples were subjected to two cycles of freeze and homogenised by repetitive pipetting. 20µl of each sample was added to the wells of a 96-well microtitre plate in triplicate. 50µl of LARII reagent was added to each well, mixed gently and the firefly luminescence read using a Victor luminometer. 50µl of Stop and Glo' reagent was then added to each sample, mixed and the Renilla values measured. Relative activity was calculated by normalising the firefly values to their Renilla counterparts. Standardisation was performed according to experimental design, usually using the control

vector as a baseline or the level of firefly activity prior to stimulation. Histograms of these data were constructed using Excel™ and statistical significance assigned using an F-test followed by the relevant student's T-test.

2.4 Immunofluorescent staining

2.4.1 Solutions and Buffers

4% Paraformaldehyde (PFA)

20g PFA powder was dissolved by stirring overnight at 42°C in 500ml SDW. Once in solution, 5 Dulbecco A tablets were added, the pH adjusted to 7.4 and the solution covered in foil, to protect from light.

20% HINGS

Heat inactivated normal goat serum, diluted to 20% (v/v) in PBS.

Triton-X-100

Triton-X-100 detergent, diluted to 0.5% (w/v) in PBS.

1, 4 Diazabicyclo (2,2,2) octane triethylene diamine (DABCO)

90ml of glycerol was added to 10ml PBS containing 2.5g of DABCO powder, the pH adjusted to 8.6 and the solution covered in foil, to protect from light.

2.4.2 Immunofluorescent Staining

12-well microdot Teflon coated slides (Henley) were seeded with cells of interest at a density of $2x10^4$. Cell samples were supplemented with normal growth media up to a total volume of 50-75µl per well. Slides were placed into $10cm^2$ dishes and grown overnight at 37°C and 5% C02. Media was then removed and slides washed in PBS for 2 minutes at room temperature. Extra PBS was removed using blotting paper and the cells fixed according to the primary antibody. For PFA fixation slides were incubated with 4% PFA for 10 minutes, washed twice

for 5 minutes in PBS and permeabilised in 0.5% Triton-X for 10 minutes. Two, 5 minute washes were repeated and all excess PBS removed using blotting paper. Other methods of fixation included methanol, acetone and methanol/acetone. In these cases, slides were incubated in of the ice-cold solution for 8-10 minutes, removed and washed (2 x 5 minutes) in PBS. Once dry, cells were blocked in 50µl/well of 20% HINGS/PBS for one hour in a moist box. Primary antibodies were diluted in 20% HINGS/PBS (Table 2.4.2.1) and 50µl added to each well of interest, adding 20% HINGS/PBS with no antibody to negative controls. Upon incubation for 1 hour in a moist box, slides were then washed (2 x 5 minutes) in PBS and excess PBS removed using blotting paper. Secondary antibodies, one of either, Alexa 594, Alexa 546, Alexa 488 or Alexa 364 (Molecular Probes) (Table 2.4.2.2.) were diluted 1:1000 in 20%HINGS/PBS and 50µl added in a species specific manner depending on primary antibody. Slides were washed, dried and coverslips mounted using DABCO.

Co-staining for co-localisation experiments were performed in a similar manner, with primary antibodies added and incubated together and various labelled secondaries used depending on the species of the primary. For fixed staining of intracellular lysosomes, cells were seeded onto microdot slides and grown overnight at 37°C. Wells were then treated with serum free Gibco® Opti-Mem® media containing 150nM LysoTracker™ Dye (Invitrogen) for 45mins-2hours, fixed and mounted as above. All slides were analysed using fluorescent and/or confocal laser microscopy.

Table 2.4.2.2: Primary Antibodies used for Immunofluorescent staining

Primary antibody	**Species**	**Dilution**	**Supplier/ Reference**
A1- VTPase	Rabbit	1:500	Sanata Cruz (sc-28801)
CD63	Mouse	1:25	Gift from R.Sadej
EEA1	Mouse	1:50	AbCam (ab 18175)
Ductin	Rabbit	1:50	Chemicon (AB5469)
Dynamin	Mouse	1:1000	BS Transduction
HA	Rat	1:1000	Roche (3f10)
HA(Y112)	Rabbit	1:1000	Santa Cruz (sc-805)
IRF1	Rabbit	1:100	Santa Cruz (sc-497)
IRF2	Rabbit	1:100	Santa Cruz (sc-498)
IRF3	Rabbit	1:100	Santa Cruz (sc-9082)
IRF7	Rabbit	1:100	Santa Cruz (sc-9083)
LAMP1	Rabbit	1:200	Abcam (ab 24170)
LMP2	Human Sera	1:50	Gift from C. Dawson
MyD88	Rabbit	1:300	Imgenex IMG 5519
Rab5	Rabbit	1:100	Santa cruz (sc-28570)
Rab5	Mouse	1:50	Santa cruz (sc-46692)
Rab7	Rabbit	1:100	Santa cruz (sc-10767)
SOCS2	Rabbit	1:100	Santa Cruz (sc-9022)
SOCS3	Rabbit	1:200	Santa Cruz (sc-9023)
SOCS6	Rabbit	1:200	Santa Cruz (sc-5608)
TLR3	Mouse	1:500	Imgenex
TLR3	Goat	1:50	Santa Cruz (sc8691)
TLR4	Mouse	1:500	Imgenex
TLR4	Rabbit	1:50	Santa Cruz (sc-10741)
TLR7	Rabbit	1:75	Santa Cruz (sc-30004)
TLR9	Mouse	1:100	Serotec CD289

Table 2.4.2.2: Secondary Antibodies used for Immunofluorescent staining

Secondary antibody	**Dilution**	**Supplier**
Alexa-Fluor 350 Goat Anti-Rabbit	1:1000	Molecular Probes
Alexa-Fluor 350 Goat Anti-Mouse	1:1000	Molecular Probes
Alexa-Fluor 350 Goat Anti-Human	1:1000	Molecular Probes
Alexa-Fluor 488 Goat Anti-Rabbit	1:1000	Molecular Probes
Alexa-Fluor 488 Goat Anti-Mouse	1:1000	Molecular Probes
Alexa-Fluor 488 Goat Anti-Human	1:1000	Molecular Probes
Alexa-Fluor 546 Goat Anti-Rabbit	1:1000	Molecular Probes
Alexa-Fluor 546 Goat Anti-Mouse	1:1000	Molecular Probes
Alexa-Fluor 546 Goat Anti-Human	1:1000	Molecular Probes
Alexa-Fluor 594 Goat Anti-Rabbit	1:1000	Molecular Probes
Alexa-Fluor 594 Goat Anti-Mouse	1:1000	Molecular Probes
Alexa-Fluor 594 Goat Anti-Human	1:1000	Molecular Probes

2.4.3 Live cell imaging

Cells of interest were seeded at a density of $2.5x10^4$ cells, onto a well of a 24-well uncoated, 0.13mm, 13mm diameter glass bottomed plate available from MatTek™ and grown overnight in serum free Gibco® Opti-Mem®. Cells were then incubated with 150nM LysoTracker™ Dye (Invitrogen) for 2hours. Post incubation, lysosomes were visualised with a Zeiss Axiovision LSM 510Meta confocal microscope with the stage and chamber set at temperature of 37°C. Acidification of lysosomes was performed in a similar manner as above with the media replaced with Gibco® Opti-Mem® containing 1μM Yellow/Blue DND-160 (PDMPO) LysoSensor™ (Invitrogen) and placed into the microscope chamber immediately. Time-lapse

imaging was used to scan the cells capturing a number of images of the same cells over varying incubation timepoints.

2.4.4 Translocation Assays

Visualising the nuclear translocation of proteins was performed by seeding the cells of interest onto microdot slides. Cells were grown overnight in normal growth media, the media was then replaced with either serum free Gibco® Opti-Mem® containing the appropriate concentration of chemical treatment or media alone. Slides were incubated for a variety of timepoints, and following incubation were fixed and incubated with primary and secondary antibodies as above. Slides were analysed using confocal laser microscopy and translocation assessed by the comparing the subcellular localisation of proteins with and without chemical treatment.

Table 2.4.4.1: Antibodies and treatment used in Translocation Assay

Antibody	Species	Dilution	Supplier/ Reference	Treatment
IRF3	Rabbit	1:500	Santa Cruz (sc-9082)	Poly(I:C) 6 hours
P65	Rabbit	1:50	Santa Cruz (sc-372)	Poly(I:C) 6 hours

2.5 Immunoblotting

2.5.1 Solutions and buffers

Tris buffered saline-Tween (TBS-T)

24.2g Tris and 80g NaCl, dissolved in 10L SDW and pH adjusted to 7.6. 10ml of Polyoxyethylene sorbitan monolaurate (Tween-20) added and the solution mixed thoroughly.

Resolving gel buffer

1.5M Tris (pH 8.8) and 0.4% (w/v) SDS dissolved in SDW and stored at room temperature.

Stacking gel buffer

0.25M Tris (pH 6.8) and 0.2% (w/v) SDS dissolved in SDW and stored at room temperature.

Running buffer

0.25M Tris, 1.92M glycine and 1% (w/v) SDS dissolved in SDW, and pH adjusted to 8.8.

Transfer buffer

0.25M Tris and 1.92M glycine dissolved in SDW and 20% (v/v) methanol added.

5% Milk blocking buffer

5% (w/v) powdered milk dissolved in TBS-T, stored at 4°C.

5% Bovine serum albumen (BSA)

5% (w/v) powdered BSA dissolved in TBS-T, stored at 4°C.

RIPA Lysis buffer

50mM Tris (pH 7.4), 150mM NaCl, 1mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, dissolved in SDW. Protease inhibitors: 20µg/ml leupeptin (Sigma L8511), 20µg/ml aprotinin (Sigma L1153) and 20µg/ml pepstatin (Sigma P5318) and 0.5mM sodium orthovanadate (Sigma, S6508), 0.5mM sodium fluoride (Sigma S7920) and 1mM PMSF (Sigma P7626) added prior to use and this complete lysis buffer stored short term at -20°C.

3X Lamelli SDS Sample Buffer

187.5 mM Tris-HCL pH 6.8, 6% w/v SDS, 30% Glycerol and 0.03% w/v bromophenol blue mixed and stored at -20 ^{o}C.

Stripping Buffer

100mM Tris-HCl pH 6.8, 2% (w/v) SDS, 50mM 2-β-mercaptoethanol.

2.5.2 Protein extraction

Whole cell lysates of adherent cells were prepared from the cells of interest by removing all growth media and washing dishes with ice-cold PBS, on ice. All PBS was removed and 200-500µl of complete RIPA lysis buffer (ice-cold) was added. Cells were mechanically lysed using a cell scraper (Starstedt) and the lysate transferred to a prechilled labelled 1.5 ml

eppendorf Suspension cell cultures were pelleted by centrifugation, resuspended in ice-cold PBS, spun again, all PBS removed and complete lysis buffer added. All lysates were then sonicated for 10 seconds. Protein concentrations were determined using the Biorad protein assay kit (Biorad). Briefly, 5µl of diluted (50% in SDW) lysate was aliquoted in triplicate to wells of a 96-well plate, 25µl of Reagent A and 200µl of Reagent B were added. The plate was shaken gently for 5 minutes and the absorbance read at a wavelength of 620nm. A standard curve was constructed using known BSA standards and the concentrations of samples extrapolated from this. A minimum of 30µg of whole cell lysate was diluted with 3X Lamelli SDS sample buffer and boiled at 95°C for 5 minutes and returned to ice prior to loading.

2.5.3 SDS-polyacrylamide gel electrophoresis, (SDS-PAGE)

SDS-polyacrylamide gels were cast using the mini-protean 3 electrophoresis system (Biorad). Varying percentages of acrylamide gels were prepared according to the molecular weight of the protein under analysis, typically containing 30% (w/v) acrylamide, 0.8% (v/v) N, N-bis acrylamide solution (National Diagnostics, UK) to a final concentration of 7.5-20%, 25% (v/v) resolving buffer, and 0.06% (v/v) TEMED. Addition of 20% APS (ammonium persulphate) to a final concentration of 0.1% enabled gel polymerisation. 100% ethanol was gently added to remove any air bubbles and removed using SDW upon gel setting. Stacking gels were prepared in a similar manner irrespective of the concentration of resolving gel, containing, acrylamide/ bis-acrylamide solution to a final percentage of 5% (v/v), 50% (v/v) stacking buffer, 0.1% (v/v) TEMED, and 0.1% APS. Combs were inserted into the stacking gel and once set wells were washed with SDW. The tank was filled with running buffer, appropriate volumes of samples added, alongside a molecular weight protein marker (Spectra Broad Range Pre-stained Protein Ladder, 10-260kDa Fermentas) and run at 135V and for 1.5-2 hours.

2.5.4 Immunoblotting

Upon electrophoresis resolved protein samples were transferred to a nitrocellulose membrane, (Pall-Life Sciences 66-485) by removing gels from the plates and placing into the Mini-transblot kit (Biorad). The gels along with the membrane were sandwiched between two layers of 3MM filter paper (Whatman), soaked in transfer buffer and run at 90V for 90 minutes on ice. Once transferred, membranes were added to blocking buffer (milk or BSA depending on primary antibody) and incubated with agitation for approximately 1 hour. The membrane was then transferred to 10mls of blocking buffer containing the primary antibody (Table 2.5.3.1.) and incubated with agitation overnight at 4°C or 3 hours at room temperature. Following primary incubation membranes were washed (3 x 5 minutes) with wash buffer and added to blocking buffer containing the appropriate secondary (HRP-conjugated, see Table 2.5.3.2.) and incubated for approximately 1 hour with agitation. Membranes were then washed (3 x 5minutes) with wash buffer and immunoreactive bands detected using ECL (Enhanced chemiluminescence) (Amersham International, EZ-ECL Biological Industries) and visualised using autoradiography on hyperfilm (Amersham International) and developed on a Kodak X-OMAT-1000 processor. Re-probing of membranes with additional primary antibodies was performed by incubating the membrane in heated stripping buffer, 50-55°C, for 1 hour in a sealed container at room temperature. The membrane was then washed (4 x 5 minutes) in wash buffer, re-blocked and probed as above.

Table 2.5.4.1: Primary Antibodies used for Immunoblotting

Antibody	**Species**	**Dilution**	**Company**
A1- VTPase	Rabbit	1:500	Sanata Cruz (sc-28801)
β-actin (AC-15)	Mouse	1: 20000	Sigma (A5441)
CD63	Mouse	1:50	Gift from R.Sadej
Ductin	Rabbit	1:300	Chemicon (AB5469)
HA	Rat	1:1000	Roche (3f10)
HA(Y112)	Rabbit	1:1000	Santa Cruz (sc-805)
IFNαR1	Rabbit	1:500	Santa Cruz (sc-845)
IFNαR1	Mouse	1:500	Santa Cruz (sc-7391)
IFNγR	Mouse	1:500	Santa Cruz (sc-12755)
IκBα	Rabbit	1:500	CST (9242)
IRF1	Rabbit	1:500	Santa Cruz (sc-497)
IRF1	Rabbit	1:1000	CST (4699)
IRF2	Rabbit	1:500	Santa Cruz (sc-498)
IRF3	Rabbit	1:500	Santa Cruz (sc-9082)
IRF7	Rabbit	1:500	Santa Cruz (sc-9083)
Jak1	Rabbit	1:500	Santa Cruz (sc-7288)
Jak1	Mouse	1:500	Santa Cruz (sc-1677)
P65	Rabbit	1:50	Santa Cruz (sc-372)
P-p65	Rabbit	1:1000	CST (3031)
p-IκBα	Rabbit	1:1000	CST (2859)
p-IRF3	Mouse	1:750	CST (4961)
Rab5	Rabbit	1:500	Santa cruz (sc-28570)
Rab5	Mouse	1:500	Santa cruz (sc-46692)
Rab7	Rabbit	1:500	Santa cruz (sc-10767)
SOCS2	Rabbit	1:500	Santa Cruz (sc-9022)
SOCS3	Rabbit	1:500	Santa Cruz (sc-9023)
SP-1	Rabbit	1:100	Santa Cruz (sc-59)

Table 2.5.3.2: Secondary Antibodies used for Immunoblotting

Secondary antibody	**Dilution**	**Supplier**
Anti-Rabbit-HRP	1:1000	DAKO
Anti-Mouse-HRP	1:1000	DAKO
Anti-Rat-HRP	1:1000	DAKO
Anti-Human protein A	1:8000	Zymed

2.6 Flow cytometry analysis (Fluorescence-activated cell sorting FACS)

2.6.1 Solutions and buffers

1% PFA (Paraformaldehye) 1g PFA powder was dissolved by stirring overnight at 42^{o}C in 100ml SDW. Once in solution, 1 Dulbecco A tablet was added, the pH adjusted to 7.4 and the solution covered in foil, to protect from light.

0.1% Saponin 0.1g of Saponin detergent dissolved in 100ml SDW and adjusted to a pH of 7.4.

2.6.2 FACs analysis

Cell to be labelled were seeded in triplicate on 10cm^2 dishes at a density of 1 x 10^6 and grown until approximately 80% confluent. All media was then removed and the cells rinsed in PBS and then twice in EDTA. In order to prevent loss of cell surface receptors, cells were detached from the plastic in the absence of trypsin using fresh EDTA and incubated at 37°C until cells loosened. Cell suspensions were pelleted at 1500rpm for 5 minutes, all EDTA removed and resuspended in 10ml of normal media for 30mins at 37°C. Cells were then counted and plated into the wells of a pre-chilled 96-well V plate at a density of 5 x 10^5 cells per well. Plates were centrifuged at 2000rpm for 2 minutes at 4°C and supernatants discarded. Samples were then washed three times in 1%FCS/PBS, pelleting the cells and discarding the supernatants as above. Cell pellets were resuspended in 1% FCS/PBS containing the appropriate concentration

of primary antibody (Table 2.6.2.1.) to a total volume of 50µl and incubated for 1.5hours on ice, in the dark. After incubation samples were washed in 1% FCS/PBS three times, as above, and incubated on ice for 1.5 hours with 1% FSC/PBS containing the relevant Alexa 488 conjugated secondary antibody (Table 2.6.2.2.) to a total volume of 50µl. Plates were wrapped in foil to protect from the light. Post incubation the cells were washed three times in 1% FCS/PBS and supernatants discarded, following the final wash the cells were resuspended in 50µl 1%FCS/PBS and added to FACS analysis tubes (Starstedt) containing 1ml 1% PFA. Samples were wrapped in foil and stored at 4°C until read on a flow cytometer.

Table 2.6.2.1: Primary Antibodies used for FACS analysis

Antibody	**Species**	**Dilution**	**Company**
TLR3	Mouse	1:1000	Serotec CD283
TLR4	Mouse	1:1000	Serotec CD284
TLR7	Rabbit	1:1000	Santa Cruz (sc-30004)
TLR9	Mouse	1:1000	Serotec CD289

Table 2.6.2.2: Secondary Antibodies used for FACS analysis

Secondary Antibody	**Dilution**	**Company**
Alexa-Fluor 488 Goat Anti-Rabbit	1:2000	Molecular Probes
Alexa-Fluor 488 Goat Anti-Mouse	1:2000	Molecular Probes
Alexa-Fluor 488 Goat Anti-Human	1:2000	Molecular Probes

FACS analysis was also performed on permeabilised cells to compare the levels of receptors on the surface of cells compared with those bound to internal membranes. This was performed in a similar manner as above with the addition of 0.1% Saponin detergent at the primary and

secondary incubation steps. Washing and fixing were performed as normal. Data analysis of the output of a FACS analysis was performed using the WinMidi™ program, measuring only viable cells and standardising against negative internal controls.

2.7 Immunohistochemistry

2.7.1 Solutions and buffers.

EDTA buffer: 100ml EDTA-Tween buffer diluted in 900ml SDW.

Hydrogen Peroxide: Sigma, provided as a 30% Hydrogen peroxide solution in water and diluted to 0.3% in SDW.

2.7.2 Agitated Low Temperature Epitope Retrieval Immunostaining Technique

Paraffin embedded sections to be stained were cut using a micro-tome, transferred onto slides and heated to 60°C. Slides were then transferred to Xylene in a fume hood for 5 minutes, followed by IMS for 5 minutes. Slides were then washed in water to remove all traces of IMS, before placing into a 0.3% hydrogen peroxide solution for 15 minutes. Following this, slides were transferred into EDTA buffer pre-heated to 65°C for 16hours / overnight, with constant heat and stirring. The following day the buffer was cooled by placing under a running tap, the slides removed, covered with a cover plate and mounted onto a Sequenzer™ rack. Slides were then washed for five minutes with TBS, no Tween (pH 7.6). TBS containing the primary antibody at the required concentration (Table 2.7.2.1) at a total volume of 100µl was added and incubated for 1 hour at room temperature. Sections were washed in TBS-Tween for five minutes, following which; 2 drops of Chemate Envision Secondary (Molecular probes) were added and incubated for 30 minutes. Following another 5 minute wash with TBS-Tween sections were places onto a staining rack and kept moist by addition of TBS. Epitopes were then visualised by adding chromagen (DAB) using a Pasteur pipette and incubating for 5

minutes. Slides were then washed in SDW and counterstained in haematoxylin for 10-30 seconds. Excess haematoxylin was removed by rinsing with warm water and the sections incubated in IMS for 5mins. Slides were then immersed in Xylene for 2 minutes dried and coverslips mounted using DPX (TBS Biosciences). Stained sections were visualised and photograph using a phase microscope.

Table 2.7.2.1: Antibodies used for IHC staining.

Antibody	Species	Dilution	Company
TLR3	Mouse	1:25	Imgenex 417-A
TLR4	Mouse	1:25	Imgenex 579-A

2.8 Electrophoretic mobility shift assay (EMSA)

2.8.1 Solutions and buffers

1% TBE Buffer: 10.8g Tris, 4.5g orthoboric acid and 0.74g EDTA in 1 litre SDW, 10mls in 990mls of SDW and sterilised by autoclave.

0.5% TBE Buffer: As above but diluted to 0.5% in SDW and autoclaved.

10X binding buffer: 100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5.

2.8.2 Nuclear and cytosolic protein extractions

Cells of interest were grown on 10cm^2 dishes until 80-90% confluent. Normal growth media was replaced with serum free media alone or supplemented with a chemical treatment (see section 2.3.1), removal of serum ensured that in control samples only a basal level of transcription factor binding would be observed. Lysis of cells was performed using the NE-PER nuclear and cytosolic extraction reagents (Pierce Biotechnology). Once cells had reached the required harvesting timepoint, all media was removed and the dishes washed gently with ice-cold PBS, on ice. All PBS was removed from the dish and cells lysed by addition of 100µl CERI reagent, on ice. Lysates were scraped, collected and transferred into pre-chilled, labelled

1.5ml eppendorf tubes. Samples were vortexed for 15 seconds and incubated for 10mins on ice. 11µl of CERII was then added to each sample, vortexed and incubated for on ice for an additional minute. Lysates were then centrifuged for 5 minutes at 13000rpm and 4°C, the pellet contained the nuclei and the supernatant, which was removed and transferred to a clean eppendorf, the cytosolic fraction. The nuclear pellet was resuspended in 50µl of NERI on ice and vortexed for 15 seconds every 10 minutes for 40 minutes. Samples were then centrifuged as before and the supernatant containing the nuclear fraction removed and transferred to clean pre-chilled 1.5ml eppendorf. Protein concentration was measured as described previously (section 2.5.2.) and samples stored at –80°C. To ensure efficient separation of cell fractions, immunoblotting was performed on both extracts using antibodies specific to the cytoplasmic and nuclear markers, β-tubulin and Sp-1 respectively (data not shown), expression of each only detectable in the relevant extract.

2.8.3 Preparation and annealing of probes

EMSAs were performed using commercially available (MWG) end-labelled IR-Dye-700 probes designed for specific DNA binding sites (See Table 2.8.3.1. for probes used). The oligonucleotides were diluted with DEPC treated water to 100pmole and for annealing 5µl of each of the sense and anti-sense complimentary probes were mixed with 90µl of Restriction buffer B (Roche). This was incubated at 95°C for 10 minutes, the heat block switched off and the sample allowed to cool overnight. The annealed probe was then aliquoted (20µl), covered in foil (to protect from light) and stored at –20°C until further use.

Table 2.8.3.1: IRD-700 end labelled probes used for EMSA assays.

Probe	**Sequence**
PRDI FWD	5'-GGGAGAAAGTGAAAGTG-3'
PRDI REV	3'-CACTTTCACTTTCTCCC-5'
PRD11 FWD	5'-GGGAAATTCCGGGAAATTCC-3'
PRD11 REV	3'- GGAATTTCCCGGAATTTCCC-5'
NFKB FWD	5' - AGTTGAGGGGACTTTCCCAGGC -3'
NFKB REV	3' - TCAACTCCCCTGAAAGGGTCCG -5'

2.8.4 Preparation of native-PAGE gels

6% Native-PAGE gels were prepared as outlined in Table 2.8.4.1, these were poured into Novex cassettes (Invitrogen) and stored at 4°C. Prior to sample loading the gels were placed into Novex cassette tanks (Invitrogen) and filled with 0.5% TBE buffer, combs removed and gels pre-run at 70V for 30 minutes.

*Table 2.8.4.1:*Native-PAGE gel preparation for approx 8 10-well gels.

Volume	**Reagent**
13.3ml	30% Acrylamide (0.8%N,N bis acrylamide)
22.7ml	SDW
44ml	TBE Buffer
450µl	20% APS
45µl	TEMED

2.8.5 DNA: Protein binding reactions

EMSA binding reactions were set up as outlined in Table 2.8.5.1, the value 'x' denoting the amount of sample to be loaded for a 5µg total. Once mixed reactions were incubated in the dark at room temperature for 30 minutes, following this 4µl of 5X Loading Buffer (Odyssey®) was added to each sample and mixed by gentle tapping. Total sample volumes were loaded onto the pre-cast gels and run at 100V for 1-1.5 hours or until the dye front reached the bottom of the gel. Visualisation and densitiometric analysis was performed on the Odyssey® infrared imaging system (Li-cor).

Table 2.8.5.1: EMSA binding reaction

Volume	Reagent
Xµl	Nuclear Extract (5µg)
(14-x)µl	SDW
1µl	Annealed IRD-700 labelled Probe
1µl	Poly (dI:dC) 1µg/µl
1µl	25 mm DTT/ 2.5% Tween-20
2µl	10X Binding Buffer

2.9 Molecular biology

2.9.1 RNA extraction from cells in culture

RNA extraction was performed using the Trizol reagent (Invitrogen) and a modified form of the manufacturers protocol. Prior to addition of Trizol cells of interest were grown to 80-85% confluency, all media removed and dishes washed with PBS. Upon removal of all PBS, cells were lysed by addition of 500µl per well of a 6-well plate of Trizol. Lysis was aided by repetitive pipetting and the lysate collected and transferred to a sterile 15ml screw cap polypropelene tube (Starstedt) and shaken vigorously for 2 minutes. 100µl of chloroform was

added to each sample, the tubes shaken for 2 minutes and centrifuged for 30 minutes at 3000rpm and 4°C. The upper phase was carefully removed and transferred to a fresh, labelled 1.5ml eppendorf tube. An equal volume of 100% isopropanol was then added, solution mixed by inversion and incubated at room temperature for 10 minutes. Samples were then centrifuged for a further 30 minutes at 3000rpm and 4°C and supernatant removed. 75% ethanol was then added and the pellets washed overnight at –20°C. The following day, samples were centrifuged for 10 minutes at 3000rpm and 4°C, ethanol removed and pellets allowed to air-dry for 5-10 minutes. Pellets were resuspended in 200µl DEPC (diethylpyrocarbonate) treated water (Sigma Aldrich). Samples were then incubated for 10 minutes at 55°C and stored at –80°C. Concentration of the RNA was measured at absorbances of 260nm and 280nm using a Nanodrop ND-1000 spectrophotometer (Labtech International).

2.9.2 cDNA synthesis

Production of cDNA was performed using Random primers (Promega) and SuperScript III® reverse transcriptase, the reaction master mix was prepared on ice and is outlined in Table 2.9.2.1.

Table 2.9.2.1: cDNA synthesis reaction mix.

Volume	**Reagent**
4µl	5X Buffer (Invitrogen)
1µl	0.1M DTT (Invitrogen)
1µl	Random Primers
1µl	dNTPs (10mM) (Invitrogen)
1µl	RNaseOUT ribonuclease Inhibitor (40U/µl) (Invitrogen)
1µl	Superscript III Reverse Transcriptase (200U/µl) (Invitrogen)

1µg of RNA was diluted up to a total volume of 11µl in DEPC water and denatured at 65°C for 5 minutes in a thermocycler. Following this 9µl of the reaction master mix detailed above was added and the samples returned to the thermocycler and the following program run: 25^{o}C for 5 minutes, 50^{o}C for 1hour, 70^{o}C for 15 minutes, with a final hold at 4^{o}C. Samples were then further diluted with 80µl of DEPC, aliquoted and stored at -20°C until further use.

2.9.3 Reverse transcriptase - polymerase chain reaction (RT-PCR)

RT-PCR was performed using cDNA synthesised as described in section 2.9.2 and using GoTaq green polymerase mastermix (Promega). Reaction mixtures were made according to Table 2.9.3.1, and these were run on a thermocycler following the program in Table 2.9.3.2, where 'x' denotes the primer set specific annealing temperature.

Table 2.9.3.1: RT-PCR reaction master mix

Test Volumes	Control Volumes	Components
25µl	25µl	GoTaq Green
1µl (100pmol)	1µl (100pmol)	5' Primer
1µl (100pmol)	1µl (100pmol)	3' Primer
18µl	23µl	DEPC treated H_2O
5µl (50ng)	0µl	cDNA

Table 2.9.3.2: Typical RT-PCR thermocycler program

Number of Cycles	Temperature	Duration
1	95^{o}C	1min
25-35	94^{o}C	45 sec
	x^{o}C	45 sec
	72^{o}C	1 min
1	72^{o}C	10 min
	4^{o}C	Hold

Primer sets were specifically designed to avoid self-annealing, formation of hairpins and according to the following rules; GC content of 40-60%, high 5' GC content, high 3' AT content and a 3' GC content less than 40%. The primers were also designed to span intron/exon boundaries to ensure mRNA amplification. Table 2.9.3.3. shows a list of primer sets used, their annealing temperatures and cycle numbers. GAPDH primers were used as an internal control to ensure equal levels of cDNA in each sample.

Table 2.9.3.3: List of RT-PCR primer sets.

Gene	**Sequence**
LMP2A	Forward: ATGGGGTCCCTAGAAATGGT Reverse: TTATACAGTGTTGCGATATGGG
LMP2B	Forward: ATGAATCCAGTATGCCTGCCT Reverse: TTATACAGTGTTGCGATATGGG
IFNβ	Forward: CTGGCTGGAATGAGACTA Reverse:GCAGAATCCTCCCATAATA
IRAK1	Forward: CGGGCAATTCAGTTTCTACAT Reverse:CCTCCTCAGCCTCCTCTT
IRAK4	Forward: CCAGCTGACTCCACTACTAA Reverse: GGCCCTTTCAACAGTCTCAA
MAP3K7 (TAK1)	Forward: CGACCACCATGATAAAA Reverse: CCAGTGTAAGATAAGCCAT
MYD88	Forward: GCGGGCATCACCACACTT Reverse: GCGAGTCCAGAACCAAGATT
TIRAP (Mal)	Forward: AGTAGTCGCTGGAGCAAA Reverse: CTTACAACGCATGACAGCTT
TRAF3	Forward: CCGTGGAGGACAAGTACAA Reverse: CATGTGGCTTCCCGGTATT
TRAF6	Forward: GCCCAGGCTGTTCATAGTT Reverse: GATTGTGGGTCGCTGGAAA

2.9.4 Agarose gel electrophoresis

2.9.4.1 Solutions and Buffers

TBE buffer: 10.8g Tris, 4.5g orthoboric acid and 0.74g EDTA dissolved in 1L SDW.

2.9.4.2 Gel electrophoresis

PCR and RT-PCR products were analysed and separated via horizontal gel electrophoresis. Depending on the expected size of the PCR product varying percentages of agarose gels were prepared (0.5-2% w/v) by dissolving the appropriate weight of agarose powder (Eurogentec) in TBE buffer. The solution was then heated and upon sufficient cooling 0.5µg/ml of Ethidium Bromide (Thermo Scientific, 0.625 mg/ml) was added to allow visualisation of DNA. The gel was then poured into a gel tray of appropriate size, to a thickness of approximately 7mm and allowed to cool until fully set. The combs were then removed and the gel submerged in TBE buffer in an electophoresis tank, samples loaded (usually 25µl of RT-PCR products) alongside 500ng of a 100bp molecular weight marker (GeneRuler™ 100bp Plus, Fermentas). Electrophoresis was performed at 1-5V/cm, depending on the expected size of the fragment and the percentage of the gel. Following electrophoresis the DNA was visualised and photographed by ultraviolet illumination with a Syngene Bioimagine GeneFlash.

2.9.5 Real time quantitative PCR (RT-QPCR)

RT-QPCR for target genes was carried out on cDNA generated as described in section 2.9.2, using commercially available TaqMan® primer and probe sets (Applied Biosciences) (Table 2.9.5.1). Cellular target primer and probes sets were FAM labelled and the internal baseline control used for data normalisation was a VIC labelled huGAPDH primer/probe set. Primers were designed to span intron-exon boundaries to ensure mRNA amplification. Reactions were set-up according to Table 2.9.5.2, loaded in triplicate onto an optical 96-well plates (Applied Biosystems) and run on an ABI 7500 Fast real-time PCR machine.

Table 2.9.5.1: Q-PCR primer probe sets from ABI Biosystems.

Gene	Catalogue Number
TLR3	Hs01551078_m1
TLR4	Hs00152939_m1
IFNβ	Hs00277188_s1
STAT1	Hs01014002_m1
ISGF3/IRF9	Hs00959316_g1
IRF1	Hs00971960_m1
MXA1	Hs00895598_m1
MXA2	Hs01550808_m1
OAS1	Hs00973637_m1
OAS2	Hs00942643_m1

Table 2.9.5.2: Q-PCR reaction mix.

Volume	Components
10µl	2 x ABI Q-PCR Master Mix
0.5µl	VIC huGAPDH primer/probe
1µl	FAM Target primer/probe
7.5µl	DEPC treated H_2O
5µl (50ng)	cDNA sample

Cycle threshold or Ct values are defined as the cycle number where the fluorescent signal exceeds that of the background, an inverse relationship existing between the level of target DNA in a sample and the cycle number. Values for the dCt (for experiments performed in triplicate) were obtained by normalising the test Ct values against the GAPDH Ct values in the multiplexed reactions. The mean dCt was used to calculate the ddCt by normalisation using sample controls, the inverse relationship between cycle number and level of DNA in the cell was resolved by applying the formula 2^-ddCt.

2.10 Cytokine Array

In order to assess the cytokines secreted from a variety of cell lines upon various treatments a cytokine array was performed (R & D Systems). The Human Cytokine Array Panel A Array Kit was used according to manufacturers protocols. The principle of the assay is to incubate supernatant from growing cells with a nitrocellulose membrane spotted in duplicate with selected capture antibodies. Cytokines within the supernatant will be bound onto the membrane by their cognate antibodies and following this can be visualised using Strepdavidin-HRP (Horseradish peroxidase) and chemiluminescent detection. The observed signal is directly proportional to the level of cytokine bound to the membrane and internal controls allow for normalisation within and between samples so that comparisons can be made between cytokine production in a number of cell lines for a number of cell treatments. Briefly, following stimulation with a variety of chemical treatments (see section 2.3.1, Table 2.3.1.1) the growth medium, 1.5ml per well of a 6-well plate, was removed and transferred to a labelled eppendorf. RNA was extracted from the cells for validation and optimisation purposes as described in section 2.9.1. Prior to sample incubation the membrane was blocked for 1 hour in Array Buffer 4 on a rocking platform. While blocking, 15µl of Cytokine Array Panel A Detection Antibody Cocktail to each sample and incubated at room temperature for 1 hour. As the samples were growth medium from cell culture it was not necessary to dilute them further. Once blocked Array Buffer 4 was removed and the sample/antibody mixtures added to each membrane, this was incubated overnight at 4°C on a rocking platform. Following this the membranes were removed from the supernatant mix and washed (3 x 10 minutes) with the supplied Wash Buffer. The Strep-HRP antibody was diluted in Array Buffer 5, added to each of the membranes and incubated for 30 minutes at room temperature. Membranes were washed as above. Once washed ECL and autoradiography was used to visualise the membranes. Densitiometric analysis was performed using a Biorad© densitometry scanner.

CHAPTER 3

Generation of a panel of LMP2 loop mutants in a lentiviral expression system.

3.1 Introduction

To further investigate the effects of stable LMP2A and LMP2B expression in epithelial cells and to ascribe a specific function to particular protein domains, a panel of LMP2 loop mutants were generated. As described previously the LMP2 proteins comprise 12 transmembrane spanning domains and, in the case of LMP2A, an amino-terminal cytosolic signalling domain. Although most of the effects attributed to LMP2A expression in epithelial cells are thought to result from the disruption of normal cellular signalling by this N-terminal domain, the effect of LMP2B expression alone on cell signalling pathways and on growth and differentiation cannot be explained by such a hypothesis (Allen, Young et al. 2005; Shah, Stewart et al. 2009). To examine the role, if any, of the transmembrane domains on the intracellular localisation and biological properties of LMP2A/2B, the loops of the LMP2A/2B protein were serially deleted. It is known that the transmembrane domains anchor the protein to internal phospholipid membranes (Longnecker, Merchant et al. 2000) and it is likely that this localisation can alter intracellular trafficking which in turn can attenuate and, in some cases, prime signalling responses. The use of a lentiviral vector expression system for this cloning strategy provided a means by which stable cell lines could be readily generated by viral transduction. These viruses could also be used to transduce primary keratinocytes, allowing an investigation into the contribution of the LMP2 proteins in early stages of cell transformation. As the transmembrane spanning domains of LMP2A and LMP2B are identical, recombinant lentiviral vectors expressing defined LMP2 membrane loops were engineered from a plasmid containing an LMP2B cDNA.

3.2 Generation of recombinant pCR8/GW/TOPO transfer plasmids containing LMP2 membrane deletion mutants.

3.2.1 PCR of LMP2 mutants.

LMP2 loop mutant constructs were derived from the pMSCV/LMP2B-HA plasmid using oligonucleotide primers targeting sequences within the LMP2B cDNA such that the transmembrane domains would be sequentially deleted. *Figure 3.1,* shows a schematic of the binding sites for these primers and the expected gene and protein products. They include primers to delete loop 1 (LMP2-L1-Del), loops 1, 2 and 3 (LMP2-L3-Del), loops 1-4 (LMP2-L4-Del), loops 1-5 (LMP2-L5-Del) and loops 1-6 (LMP2-L6-Del). The 3' primer was designed to target sequences within the plasmid downstream of the STOP codon so that PCR amplification would include the coding sequence of the HA tag. *Table 3.2.1.1* lists the sequences of each of the primers used and their respective annealing temperatures. The PCR reactions were set-up using High Fidelity DNA Polymerase according to *Table 3.2.2.2*, and run on a thermocycler according to the program in *Table 3.2.2.3.* The optimum annealing temperatures for each primer set were used.

Table 3.2.3.1: PCR amplification of LMP2 loop mutant reactions

Primer	**Sequence, 5'-3'**	**Annealing temp**
LMP2-L1 Del Fwd	GGCTCGAGATGTCATCTTGCCGCTGC	62.4˚C
LMP2-L3 Del Fwd	GCCTCGAGATGACTCTTGGTGCAG	62.4˚C
LMP2-L4 Del Fwd	GGCCTGAGATGCTGGCACGACTGTTCC	62.4˚C
LMP2-L5 Del Fwd	GGCTTCGAGATGCTTGCTATCCTGACCG	60.2˚C
LMP2-L6 Del Fwd	GGCCTCGAGATGTGCCGCTACTGCT	62.4˚C
Rev Primer	GGGGATCCCCAGACTGCCTTGGGAAAA	N/a

Figure 3.1.Schematic representation of the cloning procedure for generation of LMP2 loop deleted mutants.

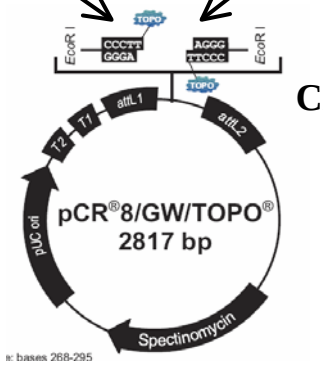
The full-length sequence of LMP2B is shown with each transmembrane loop denoted by a differently coloured sequence. LMP2-L1 Del mutant is comprised of deletion of the first section of the gene (green), LMP2-L3 Del the first three loop domains (green, light blue, navy blue), LMP2-L4 Del the first four loop domains (green, light blue, navy blue and magenta), LMP2-L5 Del the first five loop domains (green, light blue, navy blue, magenta and green) and finally the LMP2-L6 Del, the majority of the gene with only the C terminal domain remaining (black). 5' PCR primers for each of these sequences bind immediately upstream of the deletion with 3' primer binding downstream of the HA tag (not denoted in schematic). Upon amplification by PCR the gene fragments were ligated into the pCR8/GW/TOPO™ vector at the site shown. Validation of correct insertion was performed using diagnostic digestion and sequencing. Following this the recombinant pCR8 vectors were recombined with the promoter of choice, pENTR™5'-UbCp, and the final pLenti6/R2R4/V5-DEST destination vector. The generated lentiviral vectors were then validated, expression of the mutant proteins assessed, virus produced and transduction of cell lines performed. Plasmid maps were obtained from the Invitrogen website.

LMP2B

```
1    ATGAATCCAG TATGCCTGCC TGTAATTGTT GCGCCCTACC TCTTTTGGCT GGCGGCTATT GCCGCCTCGT GTTTCACGGC
81   CTCAGTTAGT ACCGTTGTGA CCGCCACCGG CTTGGCCCTC TCACTTCTAC TCTTGGCAGC AGTGGCCAGC TCATATGCCG
161  CTGCACAAAG GAAACTGCTG ACACCGGTGA CAGTGCTTAC TGCGGTTGTC ACTTTCTTTG CAATTTGCCT AACATGGAGG
241  ATTGAGGACC CACCTTTTAA TTCTCTTCTG TTTGCATTGC TGGCCGCAGC TGGCGGACTA CAAGGCATTT ACGTTCTGGT
321  GATGCTTGTG CTCCTGATAC TAGCGTACAG AAGGAGATGG CGCCGTTTGA CTGTTTGTGG CGGCATCATG TTTTTGGCAT
401  GTGTACTTGT CCTCATCGTC GACGCTGTTT TGCAGCTGAG TCCCCTCCTT GGAGCTGTAA CTGTGGTTTC CATGACGCTG
481  CTGCTACTGG CTTTCGTCCT CTGGCTCTCT TCGCCAGGGG GCCTAGGTAC TCTTGGTGCA GCCCTTTTAA CATTGGCAGC
561  AGCTCTGGCA CTGCTAGCGT CACTGATTTT GGGCACACTT AACTTGACTA CAATGTTCCT TCTCATGCTC CTATGGACAC
641  TTGTGGTTCT CCTGATTTGC TCTTCGTGCT CTTCATGTCC ACTGAGCAAG ATCCTTCTGG CACGACTGTT CCTATATGCT
721  CTCGCACTCT TGTTGCTAGC CTCCGCGCTA ATCGCTGGTG GCAGTATTTT GCAAACAAAC TTCAAGAGTT TAAGCAGCAC
801  TGAATTTATA CCCAATTTGT TCTGCATGTT ATTACTGATT GTCGCTGGCA TACTCTTCAT TCTTGCTATC CTGACCGAAT
881  GGGGCAGTGG AAATAGAACA TACGGTCCAG TTTTTATGTG CCTCGGTGGC CTGCTCACCA TGGTAGCCGG CGCTGTGTGG
961  CTGACGGTGA TGTCTAACAC GCTTTTGTCT GCCTGGATTC TTACAGCAGG ATTCCTGATT TTCCTCATTG GCTTTGCCCT
1041 CTTTGGGGTC ATTAGATGCT GCCGCTACTG CTGCTACTAC TGCCTTACAC TGGAAAGTGA GGAGCGCCCA CCGACCCCAT
1121 ATCGCAACAC TGTATAA
```

Sequence of Loop Mutants

Amplification and Ligation



Cloning Destination Vector

Transformation and Validation

Homologous Recombination

Transformation and Validation

Virus Production and Transduction

Table 3.2.3.2: PCR amplification of LMP2 loop mutant reactions

Volume	**Reagent**
1µl	Plasmid Template DNA (10ng)
1µl	Forward Primer
1µl	Reverse Primer
5µl	10 X Reaction Buffer
1µl	DNTPs
0.35µl	High Fidelity DNA Polymerase
40.65	DEPC Water

Table 3.2.3.3: PCR thermocycler program

Number of Cycles	**Temperature**	**Duration**
1	96^{o}C	1min
25	96^{o}C	10 sec
	50^{o}C	5 sec
	60^{o}C	4 min
Hold	4^{o}C	Hold

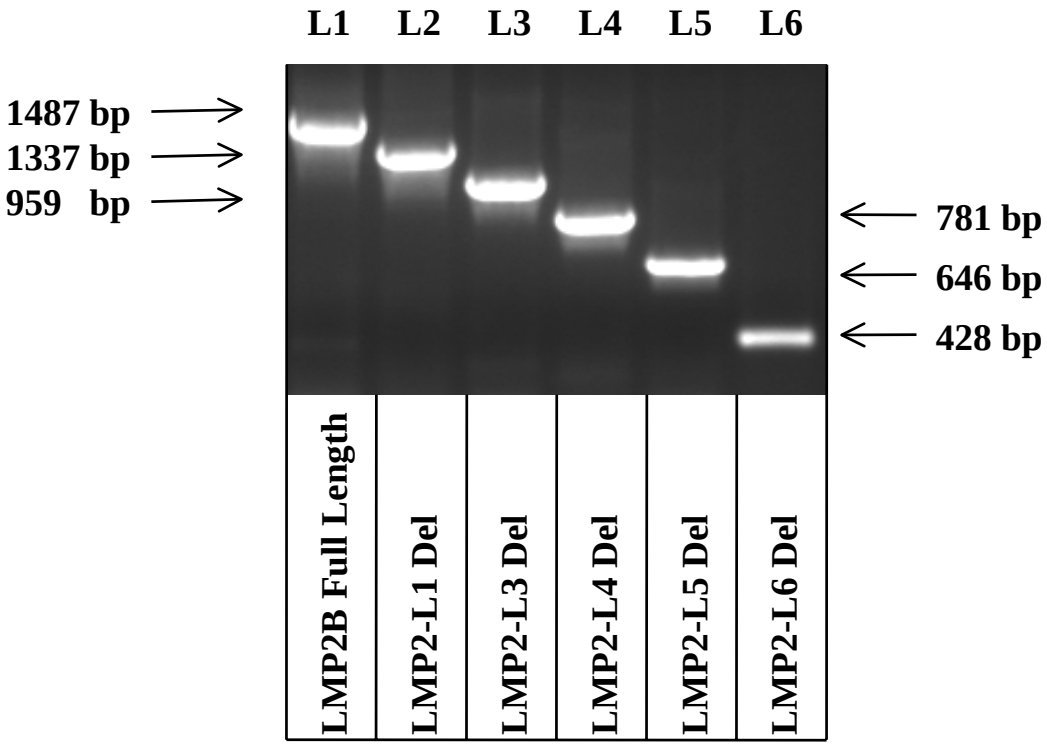
Gel-loading buffer was added to the PCR reactions and they were run on an agarose gel, and photographed, *Figure 3.2* shows each of the discrete bands and the difference in size depending on the extent of the deletion.

3.2.2 Gel extraction of PCR products

Once resolved by agarose gel electrophoresis, PCR products were compared with the DNA ladder to ensure their correct molecular weight. Each fragment was then isolated and purified for further cloning using a gel extraction kit from Qiagen (Qiagen). The products of interest were excised from the gel using a clean sterile scalpel and transferred into clean, labelled 1.5ml

Figure 3.2. PCR amplification of LMP2 gene fragments.

Amplification of the required gene fragments was performed by PCR using the template pMSCV/LMP2B/HA plasmid, 5' primers designed to delete required loop domains, a 3' primer encompassing the C-terminal HA tag and High fidelity DNA polymerase. Following this gel electrophoresis was carried out and the resulting gel visualised using EtBr and photographed. Lane 1 (L1) shows the control full length LMP2B band and lanes 2-6 (L2-L6) the expected smaller loop deleted fragments. Fragment sizes were identified through comparison with DNA ladders (Norgen) and are denoted to the left and right of the photograph.


L1
L2
L3
L4
L5
L6
1487 bp
1337 bp
959 bp
781 bp
646 bp
428 bp
LMP2B Full Length
LMP2-L1 Del
LMP2-L3 Del
LMP2-L4 Del
LMP2-L5 Del
LMP2-L6 Del

expenders. Gel slices were weighed and appropriate volumes of buffer QG were added (100µl per 100mg), this was heated to 55˚C for 15 minutes to dissolve polymerised agarose. The mixture was added onto a DNA binding column and centrifuged to remove dissolved agarose, while retaining required DNA in the column, at 13000rpm for 1 minute. Flow-through was discarded, 750µl of wash buffer WE added and the column centrifuged as above. Wash buffer was discarded and the now empty column centrifuged as above to ensure complete removal of buffer traces. The inner sleeve of the binding column was removed and placed into a clean pre-labelled eppendorf, 40µl of DEPC treated water added to the centre of the filter and the samples centrifuged again. Flow through containing purified DNA was stored at –20˚C and its concentration subsequently measured using a Nano-drop spectrophotometer.

3.2.3. Ligation of PCR-amplified products into pCR8/GW/TOPO® vector

The gel-isolated PCR products were ligated into the destination vector, pCR8/GW/TOPO®, supplied by Invitrogen in the TA cloning kit. Reactions were set-up according to manufacturers protocol for chemically competent *E. coli* cells. As the PCR products varied in length the reaction mix was incubated for 15minutes at room temperature to ensure correct ligation of the larger fragments, e.g. LMP2-L1 Del mutant. The reaction mixture is outlined below, Table 3.2.3.1. Reactions were cooled to 4˚C and if necessary stored at –20˚C.

Table 3.2.3.1: pCR8/GW/TOPO® vector ligation mix.

Volume	**Reagent**
2µl	Freshly Prepared PCR product
1µl	Salt Solution
2µl	DEPC Water
1µl	TOPO® Vector

3.2.4 Transformation of One Shot ® TOP10 *E. coli.*

pCR8/GW/TOPO® vectors containing various LMP2B loop deletion mutants were used to transform the TOPO-10 *E. coli* cells supplied with the cloning reaction kit, by heat-shocking the plasmid DNA into the bacterial cells. 2µl of each ligation reaction was mixed with one vial of bacterial cells (30µl) and incubated on ice for 30 minutes. Following this, samples were placed into a 42˚C water bath for 30 seconds and immediately returned to the ice. 250µl of pre-warmed SOC medium was added to each culture and the samples incubated with horizontal shaking (200rpm) at 37˚C for 1 hour. 75µl of each culture was then plated onto LB-Agar plates containing 100mg/ml spectinomycin and grown overnight in a 37˚C incubator. As a positive control 10pg of PUC-19 plasmid was transformed in a similar manner, but under ampicillin selection, a non-transformed bacterial culture was used as a negative control.

3.2.5. Analysis of Transformants.

After overnight incubation, agar plates were screened for bacterial colony growth. For each of the five loop deleted mutants, 4 colonies were picked using a sterile loop and grown overnight in 3ml of LB nutrient broth containing 100mg/ml spectinomycin at 37˚C with shaking at 220rpm.

3.2.5.1 Miniprep

Bacterial cultures were removed from the incubator and 1.5ml removed and transferred to a clean eppendorf, cells were pelleted by centrifugation at 13000rpm for 10 minutes and the supernatant discarded. Plasmid DNA was then extracted using the Qiagen DNA Miniprep Kit. (Qiagen). The cell pellet was first resuspended in 100µl PB buffer and then lysed by addition of 250µl of buffer PL, mixed by inversion and incubated on ice for 15 minutes. Cellular lysis was neutralised by the addition of 350µl Buffer NB and the solutions mixed by inversion.

Samples were then transferred into the supplied DNA binding columns and centrifuged at 13000rpm at 4˚C for 2 minutes. Flow-through was discarded, 750µl wash buffer carefully added and each sample centrifuged as above. Complete removal of wash buffer from the DNA binding column was ensured by a second centrifugation upon emptying the waste. The inner sleeve of each column was then transferred into a clean labelled 1.5ml eppendorf and 20µl DEPC water added to the centre of the binding column. This was incubated for 2 minutes at room temperature, centrifuged as before, a further 20µl of DEPC water added and re-centrifuged. The staggered addition of DEPC water ensured the highest yield of plasmid DNA possible was obtained. Concentrations were measured using a Nano-drop spectrophotometer and samples stored at –20˚C.

3.2.5.2 Diagnostic digests

To ensure that the pCR8/GW/TOPO® vectors contained the desired PCR-amplified fragments in the correct orientation, a series of diagnostic restriction digests was performed. These involved incubating the plasmid of interest with a restriction endonuclease and its required salt buffer and resolving the samples on an agarose. *Figure 3.3*, shows representative results of these digests and depicts the samples that were deemed to have digested correctly. As shown in *Figure 3.3 B*, although some colonies grew and plasmid DNA was successfully extracted, for all of the LMP2 Loop deletion mutants, not all contained fragments that were inserted in the correct orientation. Digest reaction mixtures were set-up according to *Table 3.2.5.2.1*, and incubated at room temperature for 16 hours or overnight. Following incubation, 4 X Gel loading buffer was added and samples separated by gel electrophoresis. EcoRI (New England Biosciences) was used to analyse which samples contained an inserted fragment of the correct size and subsequent digestion with Nco1 and Xba1 (New England Biosciences) revealed

fragment orientation. *Table 3.2.5.2.2,* contains a list of the band sizes expected for correctly orientated inserts.

Table 3.2.5.2.1: Restriction Endonuclease reaction mix

Reagents	**Single Digest**	**Double Digest**
Plasmid DNA (0.5µg)	Xµl	Xµl
Enzyme 1	1µl	1µl
Enzyme 2	--------	1µl
10x Buffer	2µl	2µl
DEPC Water	17-Xµl	16-Xµl

Table 3.2.5.2.2: Expected fragment sizes upon gel electrophoresis

Sample	**EcoR1 Sizes**	**Orientation Fragment Sizes**
LMP2-L1 Del	1337bp	433bp, 350bp, linearisied vector
LMP2-L3 Del	959bp	433bp, 350bp, linearisied vector
LMP2-L4 Del	781bp	433bp, 350bp, linearisied vector
LMP2-L5 Del	646bp	433bp, 350bp, linearisied vector
LMP2-L6 Del	428bp	250bp

3.2.5.3 Sequencing

In addition to diagnostic restriction digestion, samples selected from this initial screening were subjected to sequencing to ensure specificity prior to homologous recombination with the lentiviral vector. Chosen samples were sequenced using the ABI Prism 310 (Applied Biosystems) sequencing protocol. Briefly, this involved sequencing the gene sequence of interest with primers specific to sequences on the pCR8 plasmid surrounding the inserted gene. These GW1 and GW2 primers are situated no less than 55 nucleotides from the insertion site

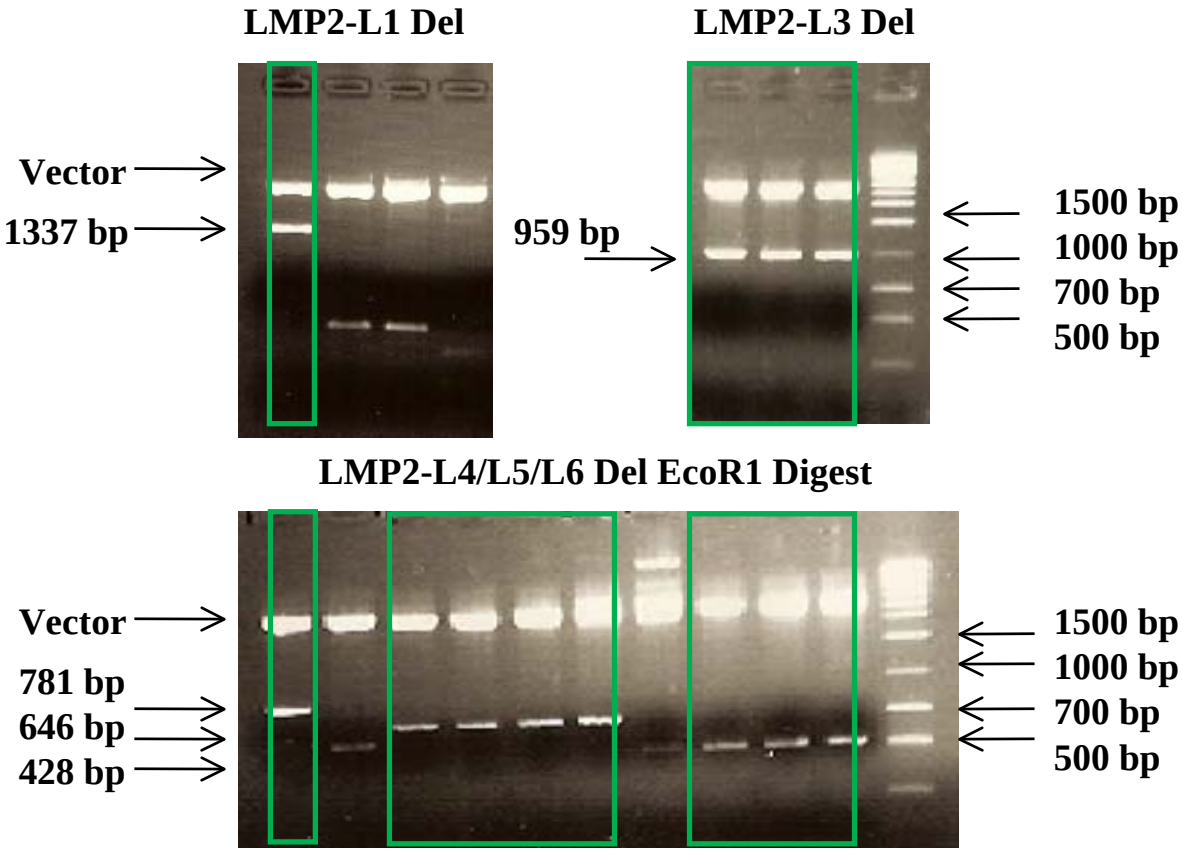
Figure 3.3. Diagnostic digests of pCR8/GW/TOPO™ containing amplified LMP2 gene fragments.

A series of diagnostic digestions were performed to validate insertion of the LMP2 gene fragments into the pCR8/GW/TOPO™ plasmid.

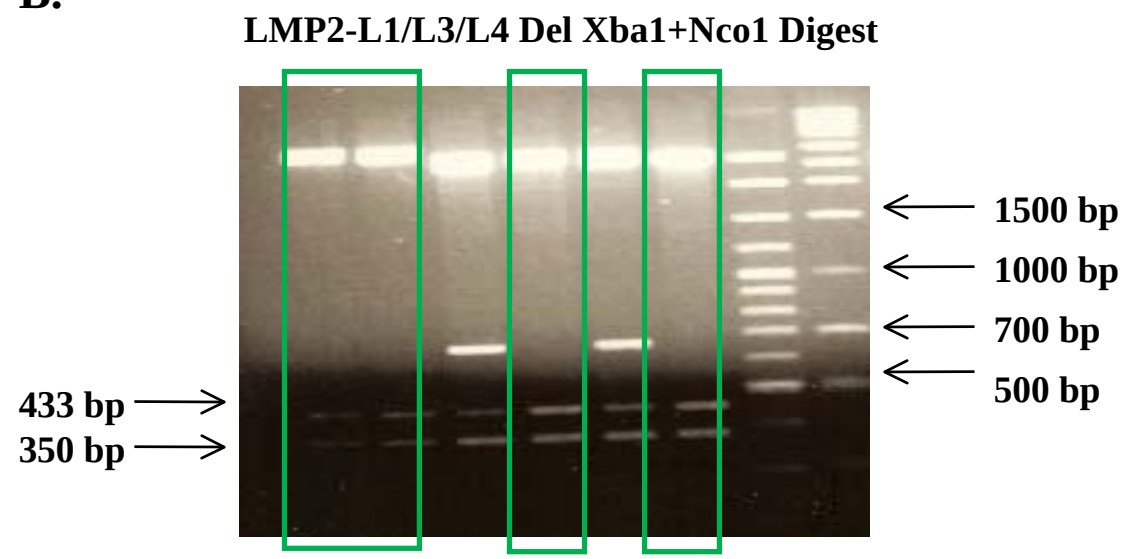
(A) Restriction endonuclease digestion of ligated pCR8/GW/TOPO™ plasmids by EcoR1 was performed to validate insertion of gene fragments of interest. Upon incubation reactions were resolved by gel electrophoresis, visualised and photographed. LMP2-L1 Del panel shows digestion of four prepped LMP2-L1 Del samples, LMP2-L3 Del panel three digested samples and the LMP2-L4/L5L6 panel two LMP2-L4 Del samples, and four of each of the LMP2-L5 Del and LMP2-L6 Del vectors. Correctly digested samples are denoted by the green boxes and the band size measured by comparison against the DNA CloneSizer ladder.

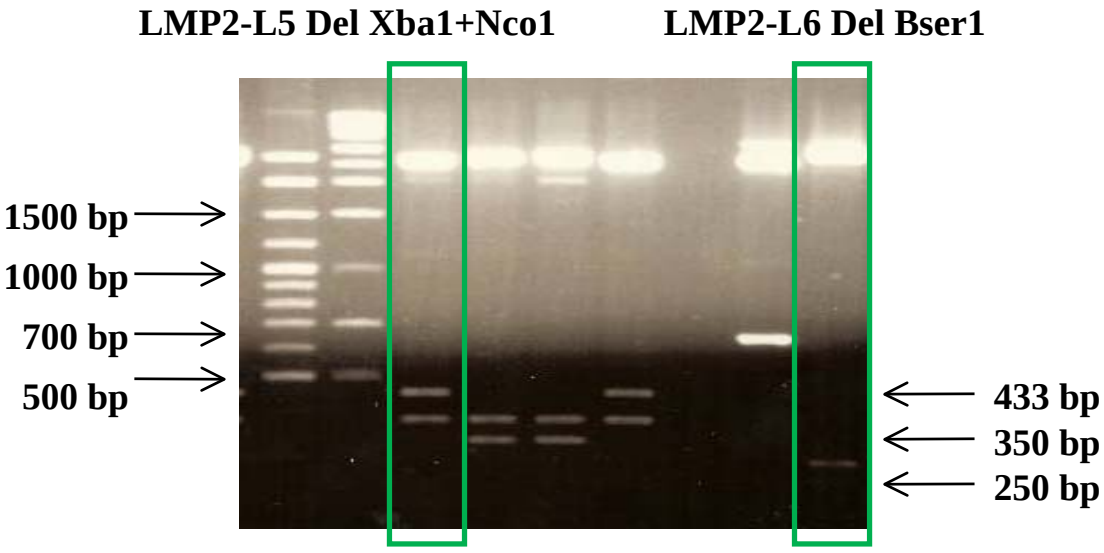
(B) Double Restriction endonuclease digestion of ligated pCR8/GW/TOPO™ plasmids by Xba1 and Nco1 (LMP2-(L1-L5) Del) and a single digest with the Bser1 enzyme (LMP2-L6 Del) were performed to validate the correct orientation of inserted gene fragments of interest. Samples were visualised by gel electrophoresis and photographed. LMP2-L1/L3/L4 Del panel shows digestion of two LMP2-L1-Del, three LMP2-L3 Del and one LMP2-L4 Del samples. LMP2-L5 Del Xba1+Nco1 shows four samples, an empty lane and two LMP2-L6 Del samples digested by BseR1. Green boxes denote correctly digested samples, the expected fragment sizes are shown, for LMP2-(L1-L5) samples these are of equal size whereas the linearised plasmid sizes differ.

A.


LMP2-L1 Del
LMP2-L3 Del
Vector
1337 bp
959 bp
1500 bp
1000 bp
700 bp
500 bp
LMP2-L4/L5/L6 Del EcoR1 Digest
Vector
781 bp
646 bp
428 bp
1500 bp
1000 bp
700 bp
500 bp

B.


LMP2-L1/L3/L4 Del Xba1+Nco1 Digest
1500 bp
1000 bp
700 bp
500 bp
433 bp
350 bp


LMP2-L5 Del Xba1+Nco1
LMP2-L6 Del Bser1
1500 bp
1000 bp
700 bp
500 bp
433 bp
350 bp
250 bp

and fall between the attL1 and attL2 sites on the restriction map in *Figure 3.1*. The PCR reaction mix was set-up for each respective correctly digested plasmid according to *Table 3.2.5.3.1* and run on a thermocycler using the program outlined in *Table 3.2.5.3.2*. Following amplification the resulting extension product was purified by addition of 2µl of 125mM EDTA, followed by addition of 2µl 3M sodium acetate and 50µl of 100% ethanol. Samples were then mixed by inversion, incubated at R.T. for 15 minutes and centrifuged at 2000g for 45mins at 4˚C. The supernatant was then carefully removed and 70µl of 70% ethanol added, this was then centrifuged for 15mins at 1650g, supernatant removed and samples air-dried. 10µl of Hi-Di™ (Applied Biosystems) was added to each reaction and the samples run on the sequencer or stored at 4˚C.

Table 3.2.5.3.1: Sequencing PCR reaction mix

Volume	**Reagents**
Xµl	DNA sample (100ng)
2µl	Ready Reaction Premix (Applied Biosystems)
2µl	Big Dye Sequencing Buffer (Applied Biosystems
1µl (3.2pmol)	Primer GW1 –or- GW2
15-Xµl	DEPC Water

Table 3.2.5.3.2: Sequencing PCR cycle.

Number of Cycles	**Temperature**	**Duration**
1	96^{o}C	1min
25	96^{o}C	10 sec
	50^{o}C	5 sec
	60^{o}C	4 min
Hold	4^{o}C	Hold

The sequences were then analysed using the Chromas software, and the sequences checked against the consensus LMP2 sequence using the online alignment tool BLAST (bl2seq), (NCBI). This ensured that the DNA sequences generated during the cloning process had not acquired mutations. *Figure 3.4.*shows sequencing results from one of the samples as viewed in Chromas and the respective BLAST alignment.

3.3 Recombination

3.3.1. Homologous Recombination

pCR8 plasmids containing correctly sequenced inserts were recombined with the lentiviral destination vector, pLenti6/R4R2/V5-DEST (Invitrogen) and a plasmid containing the Ubiquitin promoter, pENTR™-UbCp. These reactions involved incubating all the component parts of the new recombinant vector, pLenti6 backbone, promoter of choice and the recombinant pCR8/GW/TOPO® plasmids according to *Table 3.3.1.1*.

Table 3.3.1.1: Homologous recombination reaction

Volume	**Reagents**
2µl	Recombinant pCR8
2µl	pENTR™-UbCp
1µl	pLenti6/R4R2/V5-DEST
2µl	Gateway® LR Clonase™ II Enzyme Mix
3µl	DEPC Water

The reactions were incubated at room temperature for 16 hours, following which, 1µl of 2µg/µl Proteinase K Solution (Invitrogen) was added, samples incubated at 37˚C for 10 minutes and stored at 4˚C until transformation.

Figure 3.4. Representative example of the output of the sequencing reaction.

Sequencing, using the ABI Prism 310 protocol, of the pCR8/GW/TOPO™ vectors was used to validate the gene fragment inserted.

(A) Schematic representation of the output of a sequencing reaction following visualisation using the Chromas program. Each peak represents a discrete signal from each base of the analysed sequence, Red: Thymine, Green: Adenine, Blue: Cytosine and Black: Guanine. Depicted here is the sequence of the LMP2-L1 Del fragment, using the GW1 forward primer.

(B) Gene sequences were exported from Chromas in FASTA format and aligned against the full-length LMP2B sequence using BLAST. A sample of the BLAST output is shown where the LMP2-L1 Del sequence above is shown to have 98% identity with the consensus LMP2B sequence. Small errors in sequence were analysed and resolved by matching mismatches in the alignment with overlapping peaks visualised in Chromas.

A.

B.

Query ID	lcl|51901	**Subject ID**	51903
Description	(gi|86261677:59-272, 360-458, 540-788, 871-951, 1026-1196, 1280-1495, 1574-1680) Human herpesvirus 4 complete wild type genome	**Description**	1.1fwd_001_A01 sequence exported from chromatogram file
Molecule type	nucleic acid	**Molecule type**	nucleic acid
Query Length	1137	**Subject Length**	1208
		Program	BLASTN 2.2.21+ ▷Citation

Other reports: ▷Search Summary [Taxonomy reports]

▼ Graphic Summary

Distribution of 1 Blast Hits on the Query Sequence

Mouse over to see the defline, click to show alignments

Color key for alignment scores

<40 | 40-50 | 50-80 | 80-200 | >=200

Query

0 200 400 600 800 1000

▶ Dot Matrix View

▶ Descriptions

▼ Alignments ☐ Select All Get selected sequences NEW

```
>lcl|51903 1.1fwd_001_A01 sequence exported from chromatogram file
Length=1208

 Score = 1735 bits (939),  Expect = 0.0
 Identities = 973/987 (98%), Gaps = 12/987 (1%)
 Strand=Plus/Plus

Query  151   TCATATGCCGCTGCACAAAGGAAACTGCTGACACCGGTGACAGTGCTTACTGCGGTTGTC  210
             ||||||||||  ||    ||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TCATATGCCG-TG--GAAAGGAAACTGCTGACACCGGTGACAGTGCTTACTGCGGTTGTC  117

Query  211   ACTTTCTTTGCAATTTGCCTAACATGGAGGATTGAGGACCCACCTTTTAATTCTCTTCTG  270
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118   ACTTTCTTTGCAATTTGCCTAACATGGAGGATTGAGGACCCACCTTTTAATTCTCTTCTG  177

Query  271   TTTGCATTGCTGGCCGCAGCTGGCGGACTACAAGGCATTTACGTTCTGGTGATGCTTGTG  330
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178   TTTGCATTGCTGGCCGCAGCTGGCGGACTACAAGGCATTTACGTTCTGGTGATGCTTGTG  237

Query  331   CTCCTGATACTAGCGTACAGAAGGAGATGGCGCCGTTTGACTGTTTGTGGCGGCATCATG  390
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

3.3.2. Transformation of ONE SHOT® Stbl3™ *E. coli.*

Transforming the ONE SHOT® Stbl3™ strain of *E. coli* was carried out in a manner similar to that described in section 3.2.4, using one vial of competent cells (30µl) and 2µl of recombination reaction. Post-heat shock, 75µl of culture was plated onto pre-warmed LB-Agar plates containing 100mg/ml ampicillin and grown overnight at 37˚C.

3.3.3. Analysis of Transformants

As above in section 3.2.5, colonies were picked and grown overnight in 3ml of LB broth containing 100mg/ml ampicillin. 1.5ml of these cultures were miniprepped as outlined in section 3.2.5.1, the remaining cultures were stored at 4˚C for Maxi-Prep of cultures deemed to contain successfully recombined plasmids.

3.3.3.1 PCR amplification of genes of interest

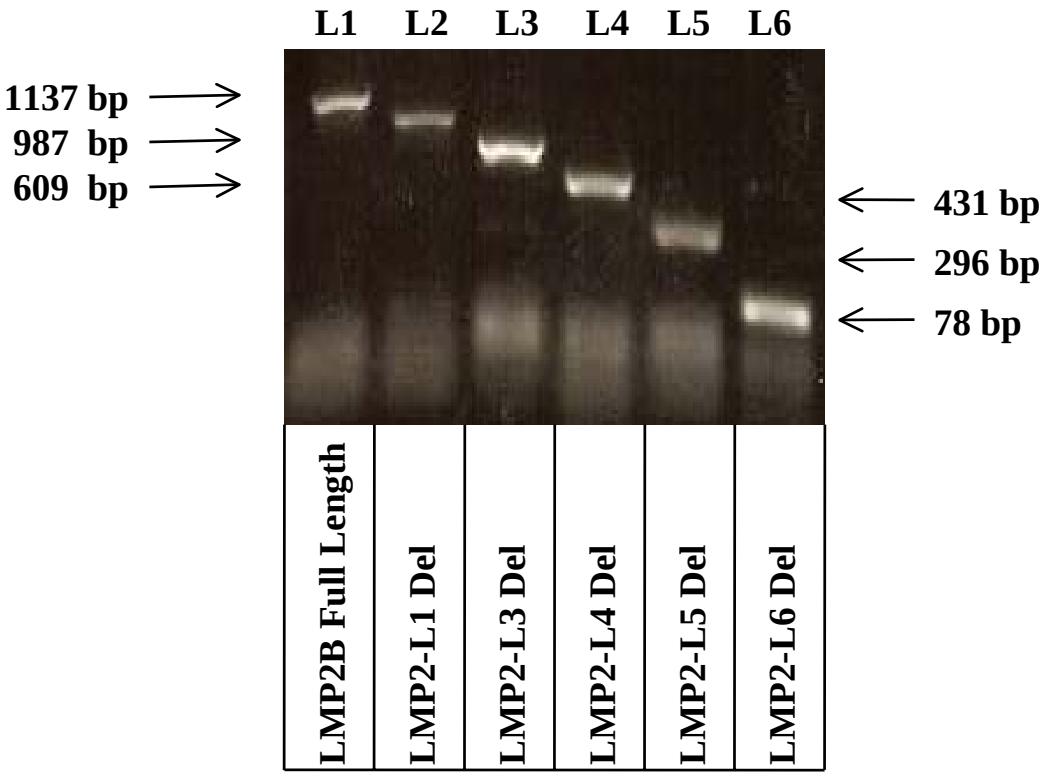
To further validate the insertion of the correct LMP2B loop deletion mutant, the generated plasmids were subjected to PCR using the primers from the initial amplification step of the cloning process. *Figure 3.5,* shows a photograph of PCR products following gel electrophoresis. From this it can be observed that the inserted genes could be amplified from the recombinant pLenti6/R4R2/V5-DEST vectors. Further validation was carried out to show that expression of these genes occurs in the correct manner upon transfection into human epithelial cells.

3.3.4. Maxi prep

The refrigerated cultures corresponding to samples that yielded correctly sized fragments after digestion were used to inoculate 500ml of LB-broth containing 100mg/ml ampicillin, these were then grown overnight at 37˚C with shaking at 220rpm. Using the PureLink™ Hi Pure

Figure 3.5. Validation of recombinant pLenti6/R2R4/V5-DEST by PCR of inserted gene fragments.

PCR was performed on the pLenti6/R2R4/V5-DEST vectors using the 5’ forward primers used in the initial amplification step and a reverse 3’ primer designed to amplify LMP2B without the HA tag., to ensure correct insertion of LMP2 deleted gene fragments. Following amplification samples were resolved by gel electrophoresis, visualised and photographed. Lane 1 shows the expected band for PCR of full-length LMP2B, lanes 2-6, (L2-L6) show the expected smaller loop deleted gene fragments. Band sizes were compared against a DNA CloneSizer ladder and are indicated to the left and right of the photograph.


L1
L2
L3
L4
L5
L6
1137 bp
987 bp
609 bp
431 bp
296 bp
78 bp
LMP2B Full Length
LMP2-L1 Del
LMP2-L3 Del
LMP2-L4 Del
LMP2-L5 Del
LMP2-L6 Del

plasmid DNA purification Kit (Invitrogen, UK) the DNA was extracted and purified according to manufacturers instructions. The bacterial cells were first pelleted using a Sorvall bucket centrifuge at 5000rpm for 10 minutes. Supernatants were carefully decanted off and discarded, 20ml of buffer R3 was added and the pellet resuspended by repetitive pipetting and vortexing. Cells were lysed by addition of 20ml ice-cold L7 lysis buffer, gently mixed by inversion and incubated at R.T. for no longer then 5 minutes. 20ml of buffer N3 was added to each sample to both neutralise the lysis buffer and to precipitate cellular debris and solutions were then centrifuged for 10 minutes at 12000rpm. The DNA binding columns were equilibrating by adding 30ml of buffer EQ1 to each column, this was allowed to drain through by gravity.

Once centrifuged the supernatants were carefully transferred into the now empty columns and allowed to drain by gravity. Columns were then washed with 60ml W8 wash buffer, and once drained the inner sleeve of the column was removed and the DNA eluted by addition of 15ml of E4 elution buffer. 10.5ml of ice-cold 100% isopropanol was added to each collection tube and then centrifuged at 15000rpm for 30mins at 4˚C. Supernatants were discarded, the pellet washed in 5ml 70% ethanol and samples centrifuged at 15000rpm for 10 minutes at 4˚C. All residual ethanol was carefully removed and the pellet allowed to air-dry prior to resuspension with 500µl DEPC treated water. The DNA was transferred to clean 1.5ml eppendorfs and the concentration of each plasmid was then measured using a Nano-drop spectrophotometer. Samples were aliquoted and stored at –20˚C and 1µg/µl for further use.

3.5 Generation of virus

The human kidney epithelial packaging cell line 293FT was used to produce whole virus from the recombinant plasmids generated in the sections above. Cells were seeded into $10cm^2$ at a density of 6×10^6 and grown until dishes were 85-90% confluent. 4µg of each of the packaging plasmids, psPAX2, pMD2G and 4µl of each of the recombinant lentiviral plasmids containing

the LMP2 loop deletion mutants were transfected into each 10cm^2 dish. Following transfection the cells were grown in 5ml of normal growth medium for approximately 72 hours. The media was then collected and centrifuged at 3000rpm for 15 minutes to remove and cellular debris. Supernatants, containing virus were then transferred to a fresh universal tube and stored at –80˚C. A GFP lentivirus was created in tandem with the LMP2 mutants for use as a transduction control. CNE-2 cells to be transduced by whole virus were seeded onto the wells of a 6-well plate at a density of 2.5 x 10^5 and grown at 37˚C until 65-70% confluent. 2µl of polybrene (5mg/ml) (Millipore) was mixed with 1ml of the virus containing supernatant and added to each well of a six-well plate and cells returned to a 37˚C incubator overnight. Polybrene was used to increase the efficiency of viral transduction. 2ml of normal growth media was then added to each well and the cells grown for a further 2-3 days. Viral infection was assessed by visualisation of GFP in those cells transduced with the control virus. Drug selection was effected through the use of the blastocidin resistance cassette within the pLenti6/V5-DEST backbone, this allowed cells stably expressing mutant forms of LMP2B to be generated. Although drug selection was undertaken time constraints prevented generation of stable cell lines and as such validation of expression of the recombinant lentiviruses was performed transiently.

3.5 Validation of recombinant Lentiviral vectors.

3.5.1. Transient expression

CNE-2 cells were seeded into 10cm^2 dishes and grown until 80 % confluent. These were then transfected 2µg of one of the recombinant lentiviral plasmids containing LMP2B loop mutants, full length LMP2A or LMP2B in a lentiviral backbone (provided by Dr K. Shah) or pSG5-LMP2A-HA, pSG5-LMP2B-HA, or a pSG5 control. Cells were grown for 48 hours to allow expression of proteins of interest prior to harvesting for RNA and protein extraction. A panel

of transiently transfected dishes was also trypsinised and cells seeded at a density of 1 x 10^4 onto each well of a 12-well microdot Teflon-coated slide for immunofluorescence staining.

3.5.2 RT-PCR of RNA extracts

Post-transfection, cells were harvested for RNA extraction and isolation (*section 3.5.1*). The RNA was used to synthesise cDNA, which was subjected to RT-PCR for the LMP2 gene inserts, using primers specific to full length LMP2A, LMP2B and the primers used to amplify the LMP2B loop deletion mutants. *Figure 3.6* shows a photograph of a representative RT-PCR reaction following gel electrophoresis. Products of the correct size were observed for each of the cDNA samples analysed.

3.5.3 Immunofluorescence staining for the LMP2B loop deletion mutants

To determine the intracellular localisation of the LMP2B loop deletion mutants, transfected cells were seeded onto microdot Teflon-coated slides (see section 3.5.1) and subjected to immunofluorescent staining with a mAb specific for the HA epitope. Slides were viewed and photographed on a confocal microscope, *Chapter 2, section 2.4.* One representative analysis, shown in *Figure 3.7,* illustrates the pattern of LMP2 staining as green punctate staining. When comparing the localisation of full-length LMP2A and LMP2B proteins (transient transfecton of pSG5-LMP2A-HA/pSG5-LMP2B-HA) with the LMP2B loop deletion mutants, there is a distinct loss of perinuclear intracellular membrane localisation as the loops are sequentially deleted. Thus, whereas LMP2A, LMP2B and the LMP2-L1-del and LMP2-L2-del mutants localise to perinuclear vesicles, the LMP2-L4-Del mutant begins to show loss of signal, with all signal lost in the cases of LMP2-L5-Del and LMP2-L6-Del. It is hypothesised that the loss of membrane anchoring due to the deletion of transmembrane domains results in an unbound and unstable protein that is degraded.

Figure 3.6. RT-PCR of LMP2 mutant gene fragments upon transient expression in CNE-2 parental cells.

CNE-2 parental cells were seeded at a density of 2.5 X 10^5 per well of a 6-well plate and incubated overnight at 37˚C and 5% CO_2 to allow cell adherence. The following day the cells were transiently transfected with the recombinant lenti-viral plasmids, grown for 48 hours to allow expression and RNA extracted and purified. cDNA was synthesised and all samples subjected to RT-PCR for expression of the loop deleted genes. The original 5' forward primers were used in tandem with a 3' reverse primer specific to a site upstream of the STOP codon of LMP2B. Samples were resolved by gel electrophoresis, visualised and photographed. Lanes 1-3 are samples from LMP2-L1 transfected samples, lanes 4-6 LMP2-L3 Del, lanes 7-9 LMP2-L4 Del, lanes 10-12 LMP2-L5 Del and lanes 13-15 LMP2-L6 Del. Band sizes were compared against a DNA CloneSizer ladder. RT-PCR was also performed for GAPDH acting as a control to ensure equal cDNA concentrations across samples.

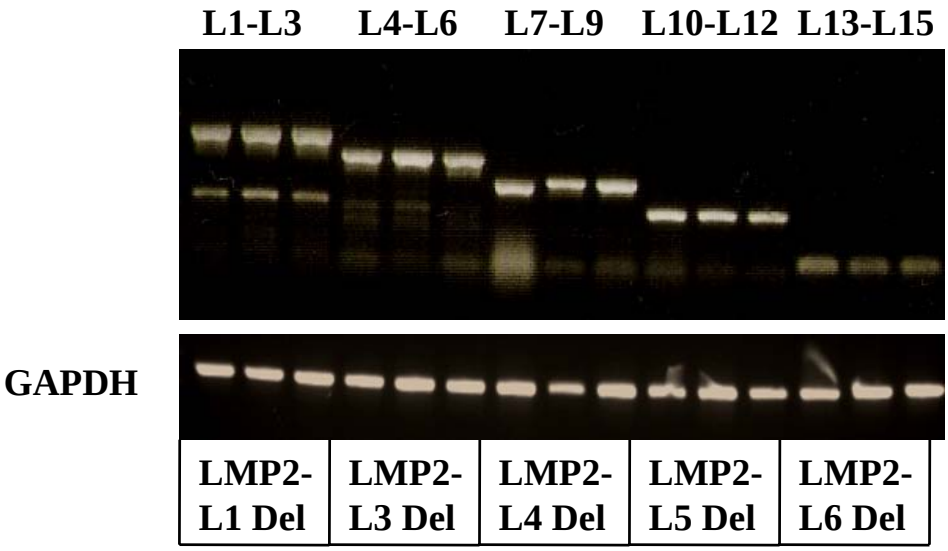
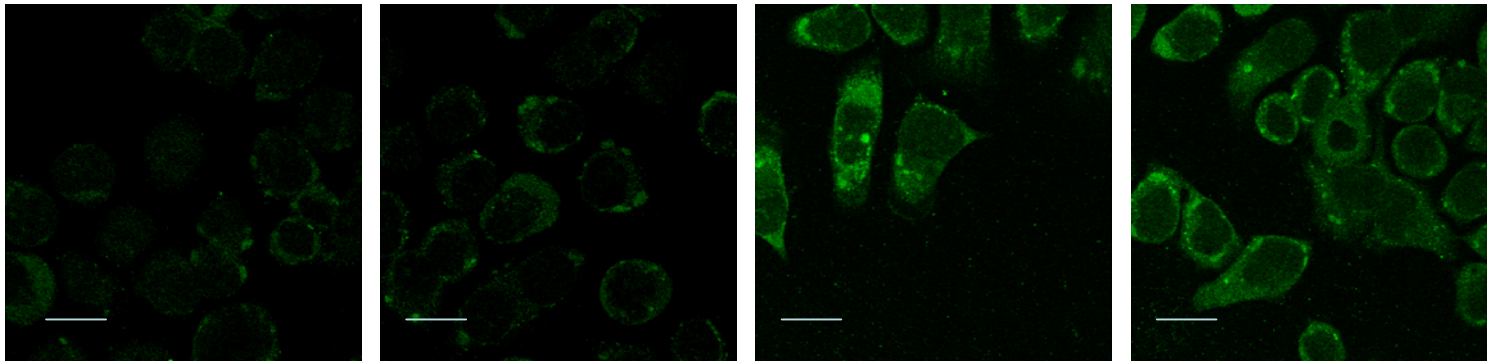

L1-L3
L4-L6
L7-L9
L10-L12
L13-L15
GAPDH
LMP2-L1 Del
LMP2-L3 Del
LMP2-L4 Del
LMP2-L5 Del
LMP2-L6 Del

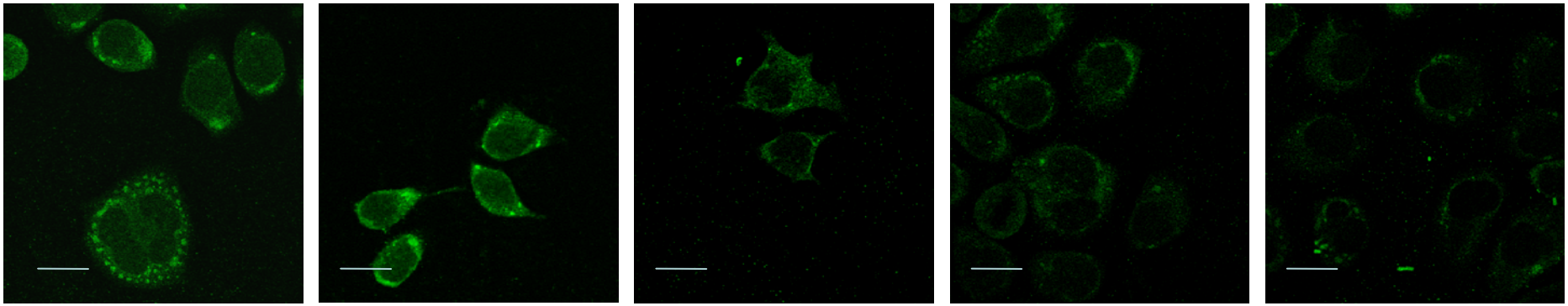
Figure 3.7. Validation of recombinant protein expression by Immunofluorescent staining for HA-tag.

CNE-2 parental cells were seeded at a density of 2.5 X 10^5 per well of a 6-well plate and incubated overnight at 37˚C and 5% CO_2 to allow cell adherence. The following day the cells were transiently transfected with the recombinant lenti-viral plasmids, grown for 48 hours to allow expression and removed from the dishes by addition of trypsin. Cells were then seeded at a density of 2 x 10^4on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day slides were subjected to immunofluorescent staining for the HA-tag. Cells transiently transfected with pSG5/LMP2A/HA and pSG5/LMP2B/HA were used as positive controls while a null transfectant and those transfected with the control pSG5 vector were negative controls.

Bar = 10μm.



Parental PSG5 only LMP2A-HA LMP2B-HA



LMP2-L1 Del LMP2-L3 Del LMP2-L4 Del LMP2-L5 Del LMP2-L6 Del

3.6 Discussion and Future Work

While some small experiments were performed to validate the efficacy of the lentiviral vectors as plasmids, their use in investigative research was limited by time constraints. It was possible however to transiently express the loop deleted forms of the LMP2 protein in three epithelial cell backgrounds and to examine their effect on lysosome numbers, *Chapter 5, Figure 5.9.* This small investigation highlights the benefit of the truncated tagged versions of LMP2 in further investigating and understanding the effect of LMP2 expression on epithelial cells and its contribution to carcinogenesis and tumour progression. In the context of this thesis it would be of particular interest to further investigate the effect of stable expression of the truncated LMP2 proteins on the modulation of endosomal and lysosomal trafficking. In order to do so, panels of cells stably expressing the modified LMP2 proteins would be generated, facilitating a more robust experimental system and one which is readily comparable with those used in the remaining chapters of this thesis.

Using a lentiviral system to create the vectors enables easy and effective infection of additional epithelial cell-lines and even primary keratinocytes with full length and truncated forms of LMP2, upon creation of whole virus. This would allow further examination of the role of the LMP2 protein in early stages of tumourigenesis, as the current cell models use systems that are initially tumourigenic and have subsequently been retro-virally transduced to express the EBV protein, e.g. CNE-2, H103. The use of a Ubiquitin driven promoter ensures expression of the proteins at a high level, while vectors could be readily made with an inducible metallothionein promoter, thus allowing lower levels of expression. Generation of the variant forms of LMP2 was successful and the small investigations carried out utilising them demonstrate how applicable they are in terms of understanding the function of LMP2.

CHAPTER 4

Modulation of innate immune signalling by the LMP2A and LMP2B proteins

4.1 Introduction

LMP2A expression in epithelial cells has been shown to affect a variety of signalling pathways, which include PI3K/Akt, ERK-MAPK and Wnt/β-catenin (Scholle et al., 2000; Morisson et al., 2003; Lu, Lin et al. 2006; Ikeda and Longnecker, 2007; Anderson and Longnecker, 2008a/b). Although LMP2B lacks intrinsic signalling ability, previous work from this laboratory has shown that LMP2B can influence cell adhesion and motility through as yet unidentified mechanisms (Allen et al., 2005). Like LMP2A, LMP2B can modulate signalling from certain classes of immune receptors, particularly those involved in the Type I interferon response (Shah, Stewart et al. 2009). Here, these analyses are expanded to investigate the effect of LMP2A and LMP2B expression on the TLR signalling network. These receptors are responsible for the first wave of the innate immune response and are also shown to modulate adaptive immunity (Goodsell 2001). This is important in terms of both initial viral infection and more specifically in terms of maintenance of viral latency in cells of epithelial origin, where stable latent infection with EBV can predispose cells to growth transformation. In the context of viral latency, evasion of the innate immune response is fundamental to viral persistence (Levy and Garcia-Sastre 2001). Recognition of products of viral replication (RNA and protein) within the infected cell is performed by the pattern recognition receptors (PRRs), of which two classes have been identified. The membrane bound, Toll-like receptors (TLRs) and the cytsolic retinoic acid inducible gene I/ Mda 5 complexes (Chen, Huang et al. 2007).

TLR signalling is initiated through recognition of a PAMP, pathogen associated molecular pattern, these molecules varying from components of the bacterial cell wall to viral DNA or RNA products of replication (Sandor and Buc 2005). Five receptors are responsible for this

process including, TLR3, TLR4, TLR7, TLR8 and TLR9. Activation of the receptor results in phosphorylation of signalling intermediates and adaptor molecules, including, among others, the IRAK proteins, IRAK1 and IRAK4, the TRAF proteins TRAF3 and TRAF6 and adaptor molecules such as TRIF and MyD88. The signalling pathways result in the activation by phosphorylation, nuclear translocation and dimerisation of a variety of transcription factors, including IRF1, IRF3, IRF7 and IRF9; NFκB subunits, specifically p65/RelA; p50 heterodimers; and subunits of the AP1 transcription factor family (Albiger, Dahlberg et al. 2007). Transcriptional activation results in series of positive feedback loops to amplify production of pro-inflammatory cytokines such as IFNβ, which can signal in an autocrine or paracrine manner to mount a type I interferon response (Bowie and Haga 2005). The TLRs focused on here include those known to be involved in regulating IFNβ, whose expression is controlled by an enhancesome complex (Yie, Senger et al. 1999; Panne 2008). Activation of this transcription factor complex is the first step in the innate immune response and is thought to be the gateway for cellular immunological responses to viral infection.

Whilst studies in B-cells show that LMP2A enhances signalling through these receptors (Wang, Nicholas et al. 2006), data presented here suggest that LMP2A, and to a lesser extent, LMP2B, attenuate TLR signalling through mechanisms involving transcriptional repression of the receptors and signalling intermediates. Together with previously published data, these findings indicate the wide scope of LMP2A effects on innate immune responses in epithelial cells.

Modulation of TLR expression by LMP2A results in dampening of signalling responses, due most likely to the disruption of IRF3 activity. Investigations into each of the individual components of the enhancesome revealed that whilst efficient IRF3 activation is attenuated, the

activity of NFκB activity is enhanced, findings which reflect a complexity in LMP2A signalling. Assessing the levels of cytokine production of control cells compared to LMP2A expressing counterparts revealed distinct differences, showing both increases and decreases of levels of secreted cytokines, indicative perhaps of the functional consequences of dampening one signal whilst initiating another. The scope of these findings was expanded to include an analysis of the effect of LMP2A on EBER induced IFNβ activity and the consequences of this in EBV infected cells.

Findings below indicate that the LMP2A and, to a lesser extent LMP2B, dramatically alters the innate immune signalling landscape of epithelial cells. The significance of this in terms of the maintenance of viral latency and tumour progression are discussed as well as an attempt to elucidate the mechanisms underlying these effects.

4.2 LMP2A and LMP2B modulate type I interferon responses

4.2.1 Expression level of type I interferon receptors and tyrosine kinases.

To assess the integrity of the interferon-signalling pathway in CNE2 cells, the expression of various components of the IFNR signalling network were characterised at the mRNA and protein level by RT-PCR and immunoblotting. It has previously been shown that type I interferon responses are modulated by LMP2A and LMP2B in epithelial cells (Shah et al., 2009). To confirm this, immunoblotting was performed for the IFNα/β-receptor-I (IFNAR), the IFNγ-receptor-I (IFNGR) and Tyk2 and Jak1, two tyrosine kinases that associate with these receptors (Garcia-Sastre and Biron 2006). A representative immunoblot, shown in *Figure 4.1*, shows that compared to control cells, expression of both the IFNAR and IFNGR and the JAK and Tyk2 tyrosine kinases are reduced in cells expressing LMP2A and LMP2B, the effect

being more pronounced for LMP2A. Reprobing of the blot with a monoclonal antibody (mAb) to β-actin confirmed equal protein loading.

4.2.2 LMP2 expression modulates expression of IFN signalling intermediates

4.2.2.1 Effect of LMP2 on IRF protein expression

Extending the above findings to regulatory elements of the IFN signalling cascade, immunofluorescence staining and immunoblotting was performed for positive and negative regulators of the IFN signalling pathways. The interferon regulatory factors (IRFs) are a family of proteins whose function is to control signalling from type I interferon receptors and to control immune responses of a variety of additional pathways including Toll-like receptors (TLRs) (Honda and Taniguchi 2006). As transcription factors, the IRF proteins control expression of a variety of genes involved in cytokine production and mounting further type I interferon cascades. IRF1, is involved in control of antibacterial and antiviral innate immunity and regulates expression of a variety of genes including, NOS2 and GBP1 (Ko, Gendron-Fitzpatrick et al. 2002). IRF2 is a negative regulator of innate immunity and has been shown to attenuate the type I interferon response (Honda and Taniguchi 2006). IRF3 is also involved in positive regulation of anti-viral responses and controls expression of a variety of genes including IFNB, IFNA4 and CCL5 (Hiscott 2007). The final IRF examined, IRF7, is involved in antiviral immunity and is inducible by the type I interferon response; its function is closely tied to that of IRF3 and is known to control expression of IFNβ and IFNα and is of most significance in mediating responses from the cytosolic pattern-recognition receptors, e.g. retenoic-acid-inducible gene I (RIG-I) (Gingras, Parganas et al. 2004; Honda, Yanai et al. 2005). Immunofluorescence staining was performed on the CNE-2 cell panel (see *section 2.4,*) using antiserum or mAbs specific for IRF1, IRF2 and IRF3. Representative confocal images are shown in *Figure 4.2*. Under basal conditions, cells expressing LMP2A showed reduced

levels of cytoplasmic IRF1 protein expression compared to control cells, with intermediate levels of expression being observed in LMP2B expressing cells. Unlike IRF1, expression of IRF2 and IRF3 appeared extremely low in control and LMP2B expressing cells whereas they were barely detectable in LMP2A expressing cells.

Immunoblotting using antisera or mAbs specific for IRF1, IRF2, IRF3 and IRF7, (*Figure 4.2 B*), shows a general reduction in IRF protein expression in LMP2A and LMP2B expressing cells compared to control cells, although the reduction was always more pronounced in cells expressing LMP2A. Whilst expression of IRF2, IRF3 and IRF7 was most noticeably reduced in LMP2A expressing cells, lower levels of IRF1 were also observed. In the case of IRF2, IRF3 and IRF7, immunoblotting revealed the expression of the higher molecular weight species, which may represent different phosphorylated forms of IRF2, IRF3 and IRF7. However, in all cases, the abundance of these species was also reduced in LMP2A expressing cells. There are apparent discrepancies between the levels of expression comparing between the immunofluorescent and immunoblotting data, this is attributable to the differences in sensitivity of the antibodies in different applications/protocols. The extent of the modulation in LMP2A expressing cells was surprising given the varying functions of the IRFs throughout the IFN signalling network, especially in the case of IRF2, which has been shown to attenuate type I interferon responses, for reasons which are highlighted later.

4.2.2.2 Effect of LMP2A and LMP2B on SOCS protein expression

While positive regulators and initiator factors of type I IFN responses appeared to be reduced by LMP2A, it was deemed appropriate to examine the effect, if any, on negative regulators of this pathway. The suppressors of cytokine signalling, (SOCS) (Yoshimura, Naka et al. 2007), are a family of proteins that are induced by cytokines, such as IFNγ, and by certain TLRs as

part of a negative feedback loop to attenuate IFN signalling responses (Andrea, Frey et al. 2004). They have been shown to function to control innate immunity through a variety of mechanisms including disruption of JAK-STAT signalling. Immunofluorescence staining was performed on the CNE-2 cell panel (see *section 2.4)* using antiserum or mAbs specific for three of the eight family members. SOCS-2 is a 22kDa protein that is shown to inhibit JAK signalling and interacts with insulin-like growth factor I receptor (IGF-1R) (Dey, Spence et al. 1998; Lee, Moon et al. 2008; Quentmeier, Geffers et al. 2008). SOCS-3 is a 24kDa protein that is also involved in negative regulation of JAK signalling, interacting with JAK2 (Dey, Furlanetto et al. 2000; Yamamoto, Yamaguchi et al. 2003). Finally, SOCS-6 is a 59kDa protein that has been shown to interact with and degrade STAT3 (Hwang, Min et al. 2007). Representative confocal images of immunofluoresence staining for SOCS proteins in the CNE2 panel is shown in *Figure 4.3A.* In contrast to the IRF proteins (*Figure 4.2.A*), expression of SOCS 2, SOCS3 and SOCS6 were elevated in LMP2A expressing cells relative to control and LMP2B expressing cells. Although predominantly cytosolic, a diffuse punctate pattern of staining was also observed in the nuclei of LMP2A expressing cells.

Immunoblotting using antiserum specific for SOCS-3 and SOCS-6 (see *section 2.5.4*) are shown in *Figure 4.3 B.* Mirroring findings from immunofluorescence staining, expression of SOCS-3 was elevated in LMP2A expressing cells compared to either control or LMP2B expressing cells, whilst expression of SOCS 6 was increased in both LMP2A and LMP2B expressing cells. Reprobing of the blot with an antibody to β-actin confirmed equal protein loading. Taken as a whole, these findings demonstrate that LMP2A down-regulates the expression of proteins involved in innate immune responses, by decreasing IFN receptor and associated tyrosine kinase expression, decreasing positive regulators of signalling and augmenting the expression of proteins involved in negatively regulating these pathways.

Figure 4.1: Immunoblotting for type I interferon receptors and related tyrosine kinases across the CNE-2 cell panel.

Protein extracts were isolated from CNE-2 cells expressing either a neomycin resistance cassette, full length LMP2A or full length LMP2B. Samples were then subjected to immunoblotting analysis using antibodies specific for the receptors (A) IFNα/βR1 and (B) IFNγR1 and the tyrosine kinases (C) Tyk2 and (D) Jak1. The panels show representative immunoblots of each of the proteins examined, using β-Actin as a loading control. Expression of IFNα/βR1 is down regulated in LMP2A expressing cells and to a lesser extent in LMP2B expressing cells compared to controls. IFNγR1 expression follows a similar pattern of expression, with the tyrosine kinases showing marked downregulation in both LMP2A and LMP2B expressing cells compared to controls.

CNE-2

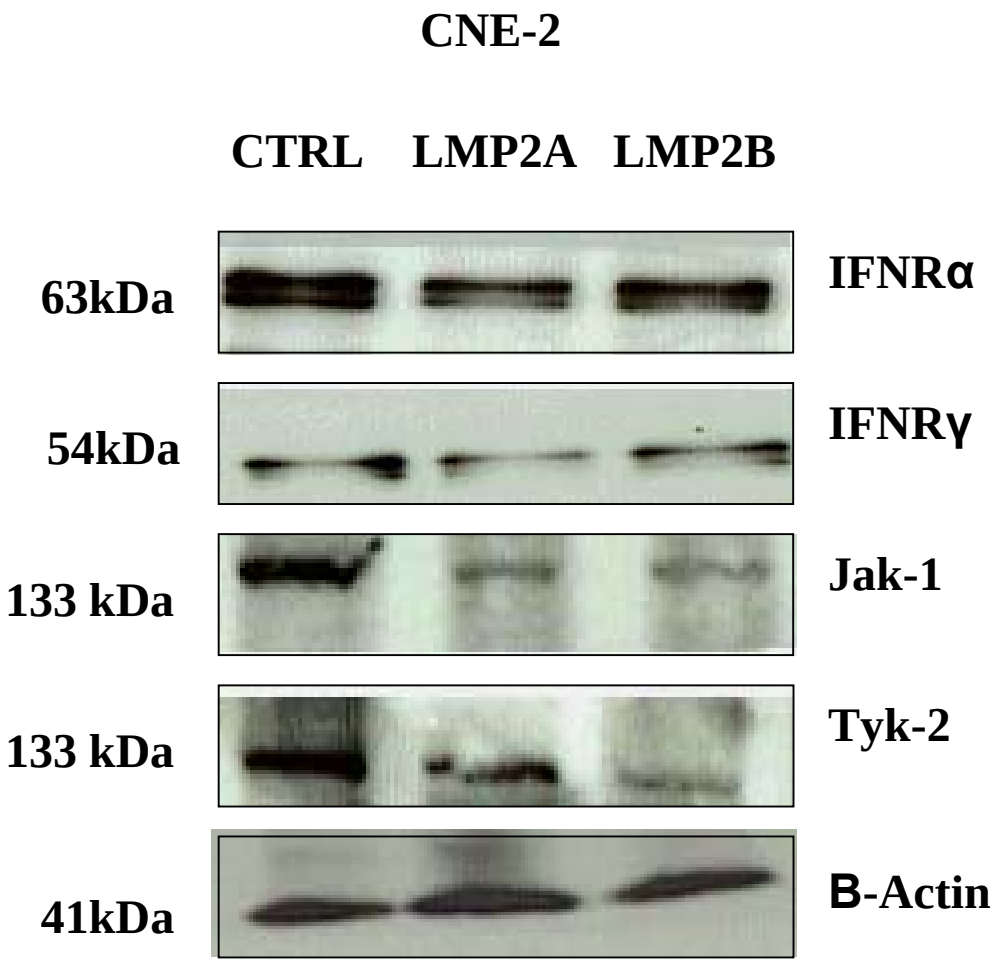

CTRL
LMP2A
LMP2B
63kDa
IFNRα
54kDa
IFNRγ
133 kDa
Jak-1
133 kDa
Tyk-2
41kDa
B-Actin

Figure 4.2: Protein expression of the IRF proteins across the CNE-2 cell panel by immunoblotting and immunofluorescent staining.

(A) CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent staining was performed for members of the IRF family of proteins, known to be regulators of the type I interferon response, including (i) IRF-1, (ii) IRF-2 and (iii) IRF-3. Slides were viewed and photographed using a Zeiss LSM510-meta confocal microscope. Comparing signal levels between control and LMP2A expressing cells, levels of expression appear to be attenuated for all proteins examined, this is most pronounced in the case of IRF-1. LMP2B expressing cells do not show such differences and signals here are comparable with control levels.

(B) Protein extracts were isolated from the CNE-2 cell panel and immunoblotting performed with anti-bodies specific to the following members of the IRF family of proteins, (i) IRF-1, (ii) IRF-2, (iii) IRF-3, and (iv) IRF-7. Representative immunoblots of each analysis are shown with β-Actin used as a loading control. Comparing levels of expression between LMP2 expressing cells and their control counterparts it is clear that the level of all IRFs examined is decreased in the presence of LMP2A and to a lesser extent LMP2B when compared to control levels.

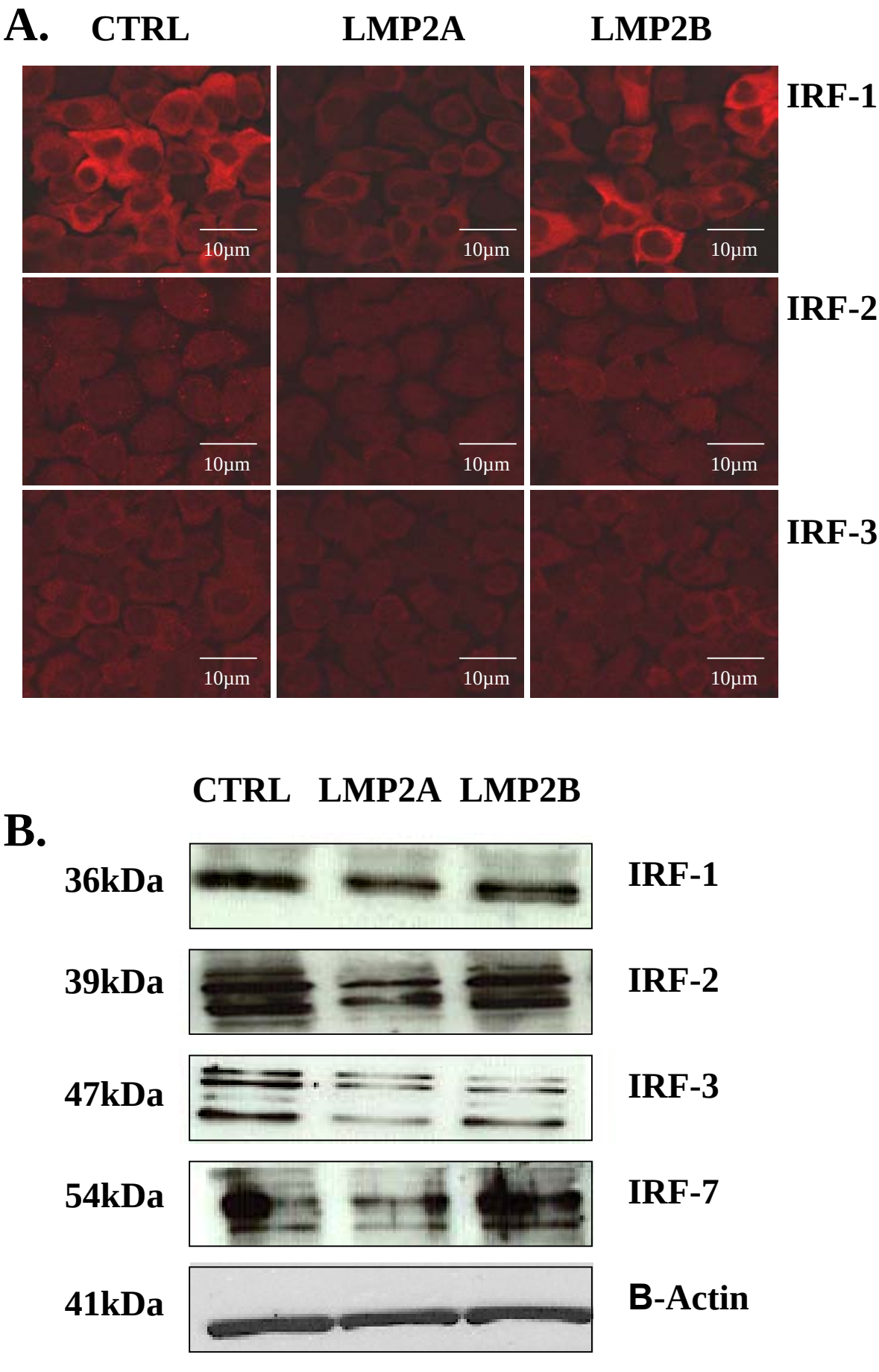
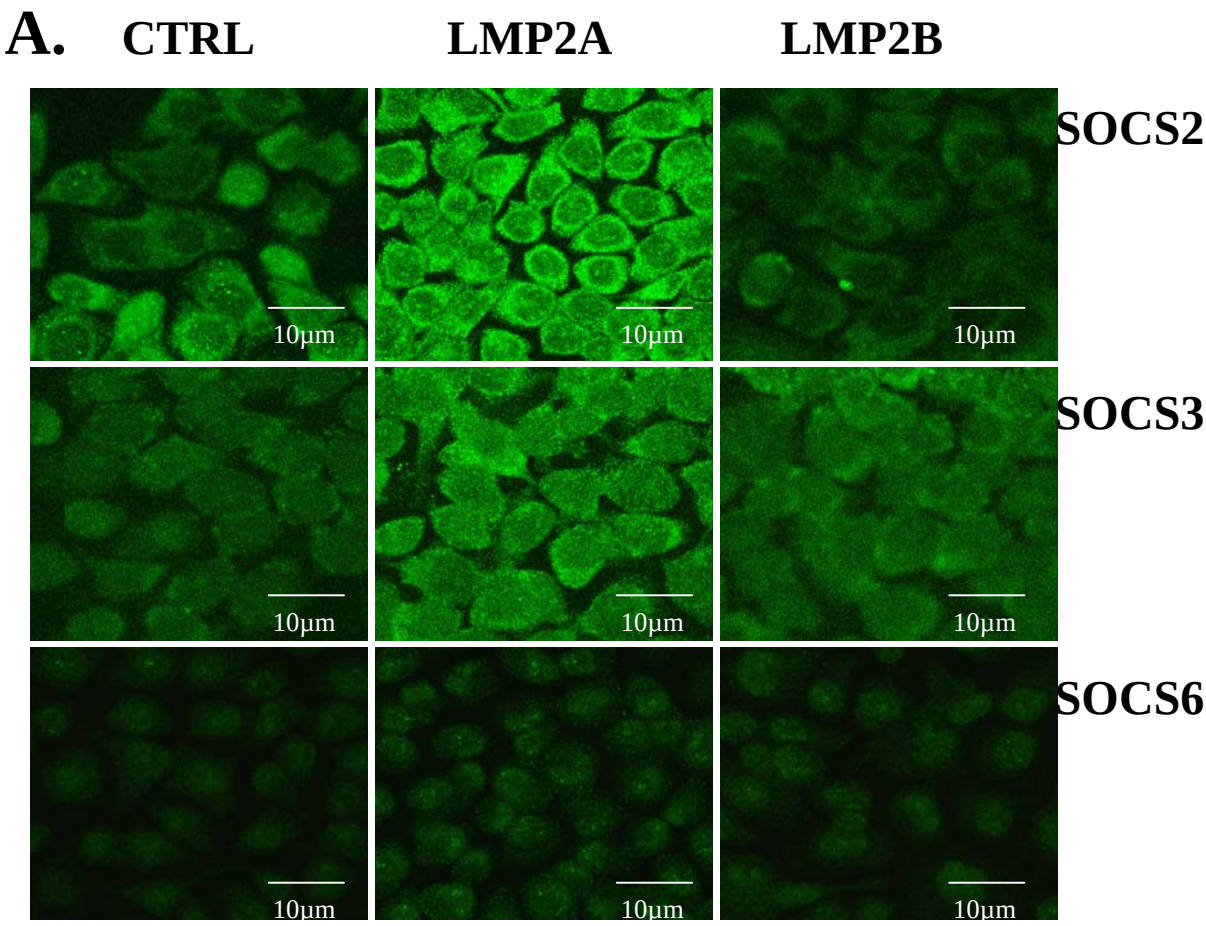

A.
CTRL
LMP2A
LMP2B
IRF-1
IRF-2
IRF-3
10μm
B.
CTRL LMP2A LMP2B
36kDa
39kDa
47kDa
54kDa
41kDa
IRF-1
IRF-2
IRF-3
IRF-7
B-Actin

Figure 4.3: Protein expression of the SOCS proteins across the CNE-2 cell panel by immunoblotting and immunofluorescent staining.

(A) CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent staining was performed for members of the SOCS family of proteins, known to negatively regulate the type I interferon response, including (i) SOCS-2, (ii) SOCS-3 and (iii) SOCS-6. Slides were viewed and photographed using a Zeiss LSM510-meta confocal microscope. Comparing the levels of detected signal between controls and LMP2A and LMP2B cells, it is clear that in the presence of LMP2A expression of each SOCS protein examined is augmented. Small increases can be detected in LMP2B expressing cells compared to their control counterparts for SOCS-3 and SOCS-6 but this is much less significant than the LMP2A induced differences.

(B) Protein samples were isolated from the CNE-2 cell panel and subjected to immunoblotting with antibodies specific for the (i) SOCS-3 and (ii) SOCS-6 proteins. Representative immunoblots of each analysis are shown with β-Actin used as a loading control. Comparing levels of expression between LMP2A expressing cells and their control counterparts, a clear augmentation of expression of both proteins is observable in the presence of LMP2A. LMP2B expression also appears to slightly augment SOCS-3 and SOCS-6 expression compared to control cells but not to the same extent as in LMP2A expressing cells.


A.
CTRL
LMP2A
LMP2B
SOCS2
SOCS3
SOCS6
10μm
B.
CTRL
LMP2A
LMP2B
24kDa
59kDa
41kDa
SOCS3
SOCS6
B-Actin

4.2.2.3 LMP2 modulates the induction of IFN signalling in response to TLR stimulation

To extend these data beyond assessing levels of protein expression, quantitative reverse transcriptase polymerase chain reaction analysis was performed (see *section 2.9.5*), for signalling intermediates (STAT1, IRF9) and downstream effectors (IRF1) involved in type I IFN signalling. The function of IRF-1 is outlined above and it is known to be an integral effector of type I interferon signalling. STAT1 (signal transducer and activator of transcription) is a transcription factor responsible for regulation of interferon-stimulated genes (ISGs), binding to either ISRE or GAS elements in the promoter of these genes. It functions as a phosphorylated trimer when bound to ISRE elements, with either a dimer of itself and IRF-9 or another STAT e.g. STAT2 and IRF-9 (Levy and Garcia-Sastre 2001; Hu, Chen et al. 2007). It binds to GAS elements upon phosphorylation and dimerisation with another of the STAT family, e.g STAT2, STAT3 or itself. IRF-9 is a component of the ISGF3 complex, along with members of the STAT family, it is also known as ISGF3γ or p48 and is integral for signalling from IFNαβ receptors (Levy and Garcia-Sastre 2001).

To examine the impact of LMP2A expression on the transcription of STAT1, IRF1 and IRF9, their expression in response to TLR stimulation was examined. This was achieved by stimulation of cells with polyinosinic-polycytidylic acid (poly(I:C)), a synthetic double-stranded RNA molecule known to activate type I interferon responses by signalling through TLR3 and/or RIG-I/mda-5. Once stimulated, RNA was extracted from the cells (see *section 2.9.1*) and RT-QPCR performed using primer and probe sets specific for the genes of interest.

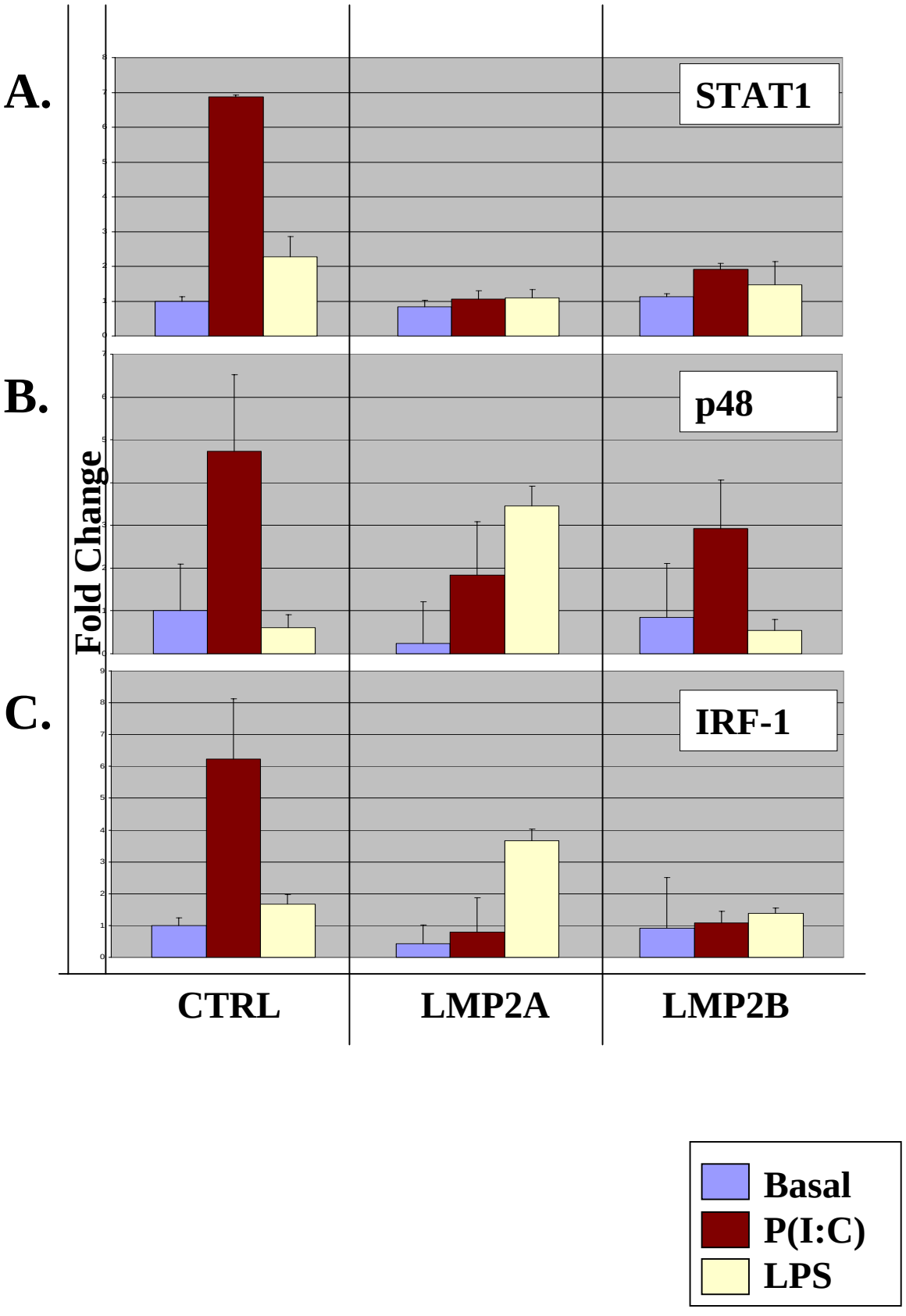
Graphical representations of the expression fold-changes between control, LMP2A and LMP2B expressing cells under basal and stimulated conditions are shown in *Figure 4.4*. Although basal levels are comparable between control, LMP2A and LMP2B expressing cells,

the levels of STAT1 mRNA were induced six fold in control cells in response to poly(I:C) stimulation. In marked contrast, cells expressing LMP2A were almost completely refractory to poly(I:C) stimulation with less than a one fold induction observed upon poly(I:C) stimulation. Similar results were observed for LMP2B, where poly(I:C) stimulation resulted in a one fold induction in STAT1 mRNA expression. This result mirrors those previously published data (Shah, Stewart et al. 2009) demonstrating a dampened type I interferon response in LMP2A and LMP2B expressing cells.

Compared to control and LMP2B expressing cells, the basal levels of IRF9 mRNA expression were lower in LMP2A expressing cells, findings that are in broad agreement with immunofluorescence staining for other IRF family members (*Figures 4.2 A, B).* Poly(I:C) treatment stimulated IRF-9 mRNA expression almost four fold in control cells whilst this induction was reduced to two fold in LMP2A and LMP2B expressing cells. Although the basal levels of IRF1 mRNA expression were lower in LMP2A expressing cells relative to control cells, they were similar between control and LMP2B expressing cells. However, in response to poly(I:C) stimulation, the levels of IRF-1 mRNA were induced five fold in control cells yet barely induced in both LMP2A and LMP2B expressing cells. From these RT-QPCR data it is shown that LMP2A expression modulates basal RNA expression of IRF-9 compared to controls. Upon poly(I:C) stimulation LMP2A appears to attenuate control expression responses for all three genes examined, while LMP2B attenuates expression of STAT-1 IRF-9 and IRF-1 to a lesser extent.

Figure 4.4 RT-QPCR for components of the type I interferon signalling pathway.

CNE-2 cells expressing either a control neomycin resistance cassette, full-length LMP2A or full length LMP2B were stimulated with the artificial agonists for TLR3, 5μg/ml poly(I:C) and TLR4, 200ng/ml LPS. After 6 hours, RNA was extracted from each cell type, pre- and post-treatment. cDNA was subsequentially synthesised and RT-QPCR performed using primer-probe sets specific for each of the following genes, (A) STAT-1, (B) IRF-9 and (C) IRF-1, using probes for the housekeeping gene GAPDH as internal baseline controls. Data was analysed using Microsoft Excel and the fold change differences calculated between cell types and between each cell type before and after stimulation. Histograms are displayed, depicting fold change differences (mean = +/- SD, n=3). STAT-1 expression increases after stimulation with either poly(I:C) or LPS, in control cells compared to basal levels. This increase is attenuated in LMP2A expressing cells and to a lesser extent, LMP2B expressing cells. IRF-9 and IRF-1 expression follow similar patterns but show augmented expression in LMP2A expressing cells upon stimulation with LPS.


A.
STAT1
B.
p48
C.
IRF-1
Fold Change
CTRL
LMP2A
LMP2B
Basal
P(I:C)
LPS

4.2.3 LMP2A and, to a lesser extent LMP2B, attenuates IFNβ expression in response to TLR3 and TLR4 stimulation

While the previous data has focused on the effect of LMP2A and LMP2B on signalling from IFNα/βR1 and IFNγR1 receptors, their effects on the TLR signalling pathway was examined given that TLR activation stimulates the induction of IFNs. To investigate this, the expression of IFNβ was assessed across the CNE-2 cell panel by RT-QPCR (see *section 2.9.5*). Basal levels of expression were compared to those after agonist stimulation of cells with poly(I:C) and LPS to activate TLR3 and TLR4, respectively (see *section 2.3.1.*). A graphical representation of the levels of IFNβ mRNA expression across the CNE2 cell panel, with and without stimulation, is shown in *Figure 4.5*. Basally, both LMP2A and LMP2B expressing cells synthesised lower levels of IFNβ mRNA. However, upon stimulation with either poly(I:C) or LPS, IFNβ mRNA expression was markedly increased twelve fold, for poly(I:C), and five fold, for LPS, in control cells. Comparing this activation across the cell panel it was evident that LMP2A, and to a lesser extent LMP2B, attenuate the ability of TLR3 and TLR4 to stimulate IFNβ expression. Although levels of expression increase upon stimulation the response is dramatically attenuated when compared to the effect of stimulation of control cells, for both agonist treatments. IFNβ expression is a good output to use when assessing TLR3 and TLR4 activity as these receptors are two of the five TLRs shown to be involved in IFNβ production (Colonna 2007).

4.2.4 LMP2A and LMP2B attenuate IFNβ induction of "classical" IFN targets in response to TLR3 and TLR4 stimulation

The above data reveal that IFNβ expression is decreased in LMP2A and LMP2B expressing cells both the basally, and in response to TLR activation. To investigate the effect of this abrogation on IFN signalling, RT-QPCR analysis was performed for four classical interferon

stimulated genes: MXA1, MXA2, OAS1 and OAS2. As in previous analyses, basal levels were compared to those after TLR3 and TLR4 stimulation. Graphical representations of the Q-PCR data obtained are shown in *Figures 4.6A and 4.6B*. From *Figure 4.6.A,* it is evident that TLR3 and TLR4 activation stimulates a 6-fold increase in the expression of MXA-1 and a 40-fold (TLR3) to 6-fold (TLR4) increase in MXA-2, in control cells. Comparing the effect of these stimuli in LMP2A and LMP2B expressing cells with control cells, clear differences are apparent and, although some activation occurs, it is severely attenuated. Similar results were observed using primer and probe sets for OAS1 and OAS2 (*Figure 4.6.B*). Again, the expression of OAS1 and OAS2 are increased in control cells upon TLR3 and TLR4 stimulation, OAS1 15-fold and 6-fold respectively and OAS2, 17-fold (TLR3) with no apparent activation with TLR4. These increases are attenuated in cells expressing LMP2A and, to a lesser extent, LMP2B. Taken as a whole, the above results suggest that LMP2A and, to a lesser extent, LMP2B modulate early events in the type I interferon response by modulating TLR signalling.

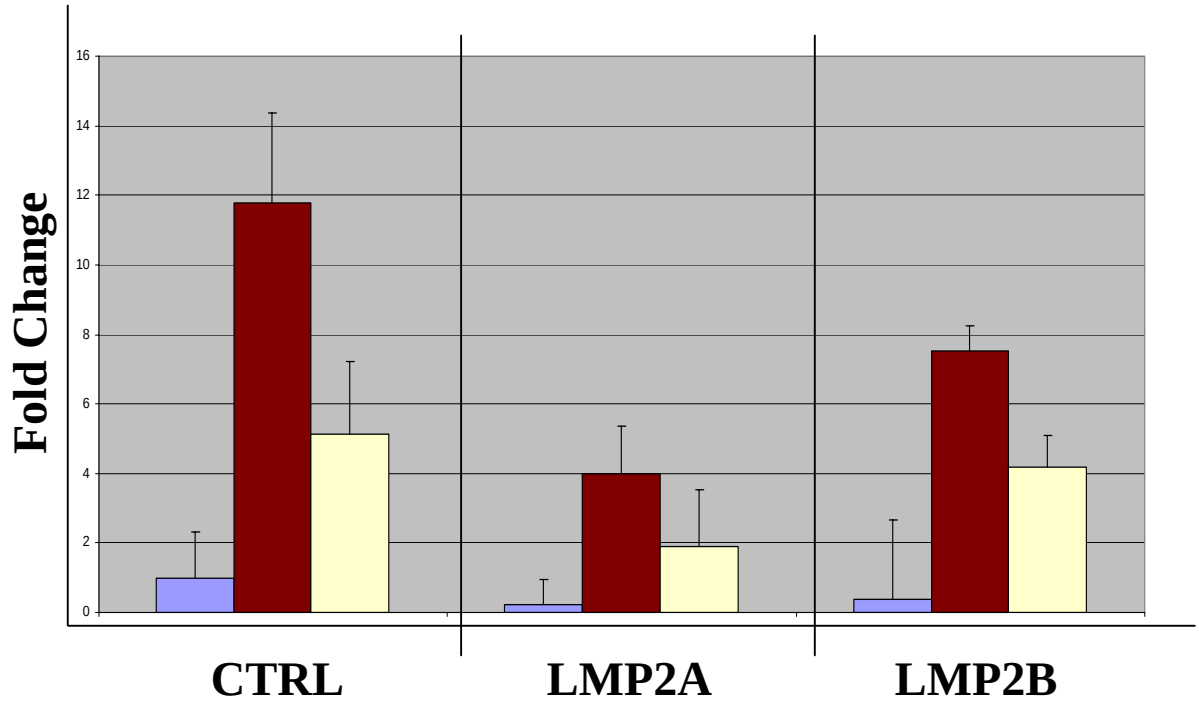
4.3 Expression profiling of TLRs in control, LMP2A and LMP2B expressing cells

4.3.1 LMP2A modulates the expression of TLR3 and TLR4 mRNAs

To ascertain whether attenuation of the TLR-mediated type I IFN response by LMP2A and LMP2B occurred as a result of modulation of the TLR receptors themselves, TLR profiling was performed on the CNE2 panel by RT-QPCR. The TLR signalling network is described in detail in *section 1.7.1.* This study focussed on TLRs that localise on internal membranes of epithelial cells; namely, TLR3, TLR4, TLR7, TLR8, and TLR9 (Nishiya, Kajita et al. 2005) as it was reasoned that LMP2A and LMP2B, which also localise to internal membranes, may somehow modulate their activity.

Figure 4.5 Modulation of IFNβ transcription by LMP2A in CNE-2 cells pre- and post-stimulation with TLR artificial agonists.

CNE-2 cells expressing either a control neomycin resistance cassette, full-length LMP2A or full length LMP2B were stimulated with the artificial agonists for TLR3, 5μg/ml poly(I:C) and TLR4, 200ng/ml LPS. After 6 hours, RNA was extracted from each cell type, pre- and post-treatment and cDNA synthesised. RT-QPCR was performed using a primer-probe set specific for IFNβ and using the housekeeping gene GAPDH as an internal baseline control. Data was analysed using Microsoft Excel and the fold change differences calculated between cell types and between each cell type before and after stimulation. Histograms are shown displaying mean fold change differences (mean = +/- SD, n=3). Basally, small fold changes differences are observable between LMP2A expressing cells and their control and LMP2B expressing counterparts. Upon stimulation with either agonist IFNβ transcription increases in control cells, such augmentation is markedly attenuated in LMP2A expressing cells and to a lesser extent LMP2B expressing cells, for both cell treatments.


Fold Change
16
14
12
10
8
6
4
2
0
CTRL
LMP2A
LMP2B

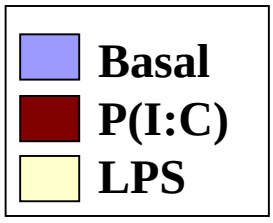
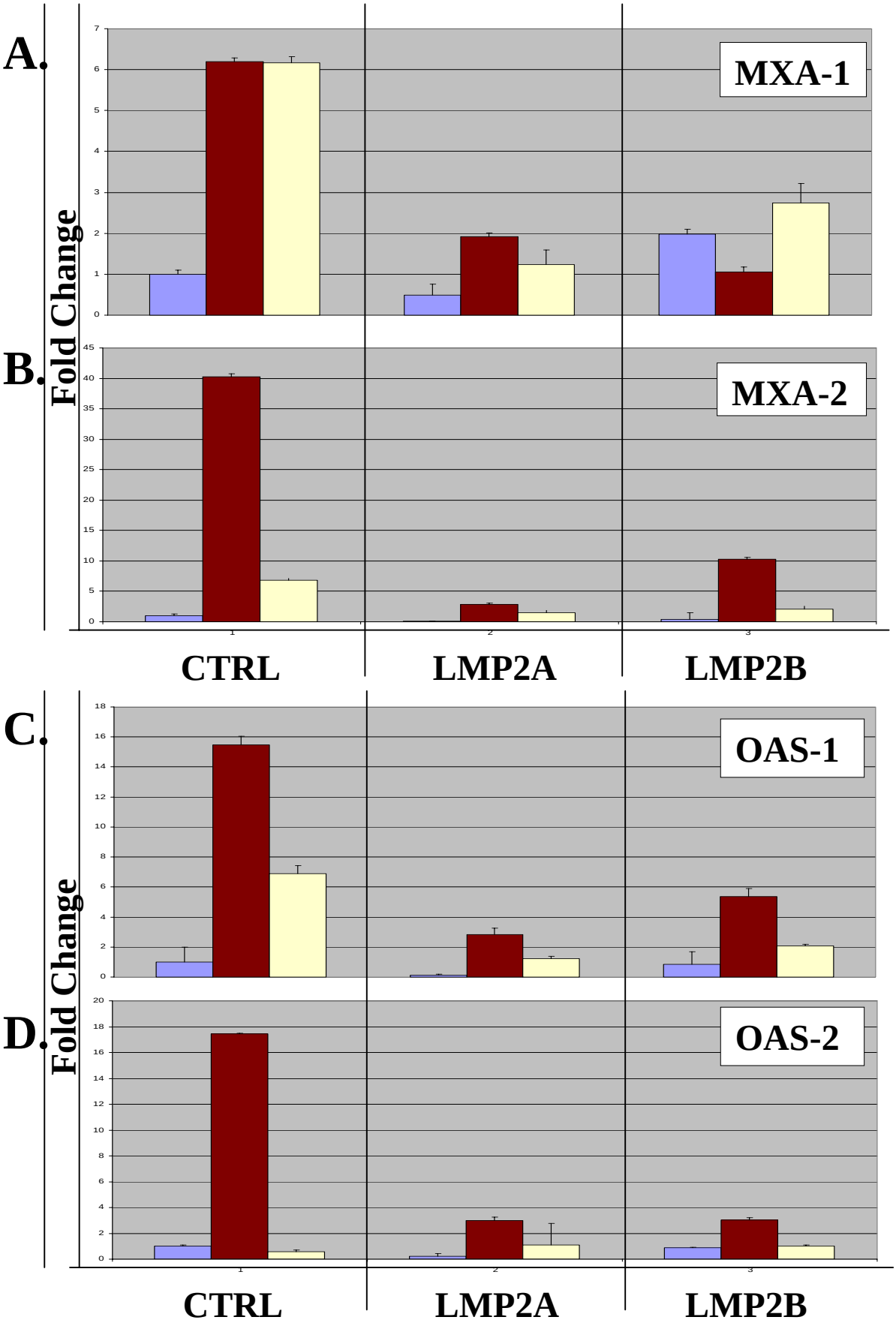

Basal
P(I:C)
LPS

Figure 4.6 Modulation of transcription of targets of IFNβ by LMP2A and LMP2B in CNE-2 cells pre- and post-stimulation with TLR artificial agonists.

CNE-2 cells expressing either a control neomycin resistance cassette, full-length LMP2A or full length LMP2B were stimulated with the artificial agonists for TLR3, 5μg/ml poly(I:C) and TLR4, 200ng/ml LPS. After 6 hours, RNA was extracted from each cell type, pre- and post-treatment, and cDNA synthesised. RT-QPCR was performed using primer-probe sets for classical targets of IFNβ signalling, (A) MXA1, (B) MXA2, (C) OAS1 and (D) OAS2, and the housekeeping gene GAPDH was used as an internal baseline control. Data was analysed using Microsoft Excel and the fold change differences calculated between cell types and between each cell type before and after stimulation. Histograms are shown displaying mean fold change differences (mean = +/- SD, n=3). In the case of MXA1, stimulation with either agonist increases expression levels in control cells. This increase is attenuated in LMP2A expressing cells and to a lesser extent in those expressing LMP2B. MXA2 expression is augmented upon stimulation in control cells for both treatments such increases are however, attenuated in the presence of both LMP2A and LMP2B. OAS1 expression is similarly augmented upon stimulation in control cells, an increase that is attenuated upon LPS stimulation in both LMP2A and LMP2B expressing cells. Upon poly(I:C) stimulation the attenuation is most pronounced in LMP2A expressing cells compared to its LMP2B counterparts. OAS2 expression level is not increased upon LPS treatment of any cell type and while poly(I:C) augments expression in controls, this increase is attenuated in both LMP2A and LMP2B expressing cells


A.
MXA-1
B.
MXA-2
Fold Change
CTRL
LMP2A
LMP2B
C.
OAS-1
D.
OAS-2
Fold Change
CTRL
LMP2A
LMP2B

Primers and probes specific for TLR3 and TLR4 were used in the manner described in *section 2.9.5* to assay the levels of TLR expression in the CNE2 cell panel. A graphical representation of the Q-PCR data is shown in *Figure 4.7.* In addition to assaying the levels of TLR3 and TLR4 basally, the effects of agonist stimulation on their expression was examined to determine whether LMP2A or LMP2B interfere with the positive feedback loops that control TLR expression levels. No significant differences were observed between control and LMP2A or LMP2B expressing cells for either TLR3 or TLR4 expression. Upon stimulation with poly(I:C), TLR3 expression increased dramatically in control cells, whereas LMP2A expressing cells show no increase in expression denoting a signalling network that is markedly attenuated. Similar findings were observed in LMP2B expressing cells but to a lesser extent. In a similar manner, LPS stimulated TLR4 expression in control cells, yet this response was attenuated in LMP2A expressing cells and to a lesser extent in cells expressing LMP2B.

4.3.2 LMP2A and LMP2B modulate the levels of TLR3 and TLR4 protein.

4.3.2.1 Immunofluorescence staining for TLR expression.

Although LMP2A and LMP2B modulated TLR expression at the RNA level, it was important to ascertain whether they also affected the levels of protein expression. Immunofluorescence staining was performed on both the CNE-2 cell panel and another squamous cell carcinoma derived cell line H103, transduced to stably express LMP2A and LMP2B (see *section 2.4*). As described in *section 2.1.2,* the H103 cells were retrovirally transduced to express a full neomycin resistance cassette and either full length HA-tagged LMP2A or LMP2B. These serve to act as a direct counterpart to the CNE-2 cell panel and allow extending hypotheses of signalling modulation across another epithelial cell line.

Immunofluorescence staining was performed on cells grown *in situ*, using monoclonal antibodies specific for TLR3, TLR4, TLR7 and TLR9. Representative confocal images are shown in *Figure 4.8* and *Figure 4.9.* In both cell panels it can be seen that the receptors are expressed throughout the cytoplasm of cells, with some plasma membrane staining observed in the H103 cells. In the case of TLR4, staining was observed at both the plasma membrane and internal endosomal membranes reflecting the dual localisation of this receptor (Sandor and Buc 2005). Comparing expression levels across the cell panels, compared to control cells, expression of all TLRs examined was reduced in cells expressing LMP2A, whilst levels of TLR expression were broadly similar to control cells in cells expressing LMP2B. Overall it is evident that at the protein level, expression of internal TLRs is decreased upon stable expression of LMP2A.

4.3.2.2 Quantitation of TLR expression by FACS

To extend these findings, flow cytometric analysis was performed quantitate the levels of cell surface and cytosolic TLR expression between control, LMP2A and LMP2B expressing cells. This was achieved by performing immunofluorescence staining in the presence or absence of 0.1% Saponin, a detergent that allows access of antibodies to the cell cytoplasm. FACS analysis was performed (see *section 2.6*) and data analysed using the WinMidi program. Graphical representations of the analysis with mean fluorescent intensities are shown in *Figure 4.10*. In the case of TLR3, increased levels of staining were observed in control cells treated with 0.1% saponin, indicating higher levels of internal TLR3. Comparisons within each cell panel revealed reduced expression in LMP2A expressing cells and, to a lesser extent, in cells expressing LMP2B. A similar pattern can be observed across the remaining TLRs examined. TLR4 staining does not have as dramatic a comparison between permeabilised and non-permeabilised cells, indicating its dual localisation at both the plasma membrane and cytosolic

membranes. Mean fluorescent intensity values are also shown which have been normalised against control samples for each analysis.

4.3.3 LMP2A expression reduces the basal levels of TLR signalling components.

Although altered expression of TLRs would have a detrimental effect on cellular responses to TLR agonists, an examination of TLR signalling intermediates was undertaken to determine whether LMP2A and LMP2B altered the expression of TLR signalling components. RT-PCR analysis was performed using oligonucleotide primers specific for IRAK1, IRAK4, MyD88, TIRAP, MAP3K7, TRAF3 and TRAF6. Primers to GAPDH were used as a control to normalise the amounts of input RNA. A representative analysis, shown in *Figure 4.11,* shows that compared to control and LMP2B expressing cells, there is a general reduction in expression of all TLR signalling components in LMP2A expressing cells. These results indicate that not only are levels of receptor expression altered but also that LMP2A affects the expression of components of the signalling pathways themselves. It must be noted however, that endpoint RT-PCR reactions such as these and no quantitative inference can be made from the results of such analyses. It is possible that signalling intermediate expression is modulated through indirect distortion by LMP2A of the feedback loops controlling their expression.

4.3.4 Immunohistochemical analysis of TLR3 and TLR4 expression in normal nasopharyngeal epithelium and NPC biopsies.

Thus far it has been identified that expression at both the RNA level and the protein level of certain classes of TLRs is modulated in epithelial cell lines expressing LMP2A and to a much lesser extent LMP2B. Examining these expression levels in an *in vivo* context allows increased significance to be attributed to these findings in the context of tumourigenesis and disease progression. To this end, immunohistochemical staining was performed in the manner

described in *section 2.7,* using antisera specific for TLR3 and TLR4. Comparisons were made between normal nasopharyngeal epithelium, tonsil epithelium and clinically diagnosed NPC tumour biopsies provided in the form of a tissue array (kindly provided by Dr C.F.Hu, Institute for Cancer Studies). Representative immunohistochemical staining, *Figure 4.12*, shows that TLR3 and TLR4 localise predominantly to the basolateral surface of normal nasopharyngeal epithelium. Although speculative given the number of sections available it is interesting to note that stronger staining was observed in nasopharyngeal epithelium compared to tonsil epithelium. In contrast, the NPC sections showed weaker staining for both TLR3 and TLR4. Unfortunately, due to the lack of a suitable reagent, expression of LMP2A could not be performed. Due to the lack of available biopsy material these data were the result of a single staining experiment, further examination and increased numbers of NPC sections are needed to attach significance to these findings.

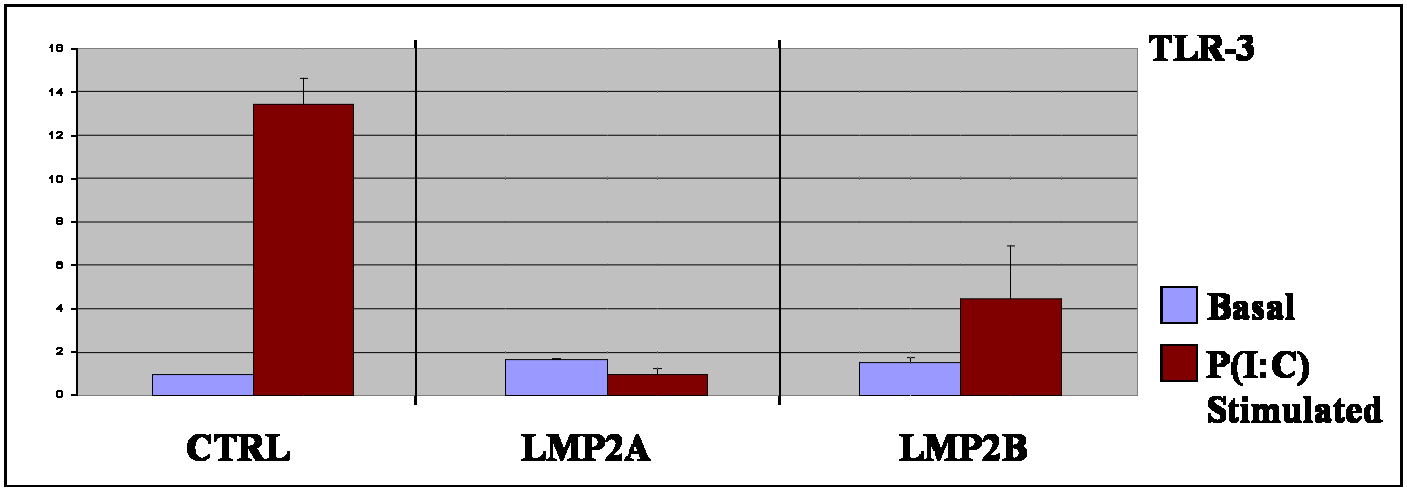
4.4 TLR signalling capacity is modulated in LMP2A expressing cells

As mentioned previously, the signalling pathways of the TLRs examined in the course of this study are responsible for the initiation of innate immunity. Activation of transcription factors that produce IFNβ, NFκB and AP1 subunits is a vital result of the pathway and assessing LMP2's effect on this functionality was the next focus. Nuclear translocation of transcription factors is an integral process in full signalling of the receptors, the transcription factors themselves include, IRF3, IRF7 homo and heterodimers, NFκB heterodimers, specifically containing p65/RelA and the AP1 subunits, Atf-2 and c-Jun. (Panne 2008). Once in the nucleus these factors and others, CBP/p300, form a complex known as the enhancesome, binding of which is fundamental for transcription of IFNβ. In order to assess whether or not LMP2 expression affected formation and functionality of the complex each component part was analysed.

Figure 4.7 Modulation of TLR3 and TLR4 expression by LMP2A in CNE-2 cells.

CNE-2 cells expressing either a control neomycin resistance cassette, full-length LMP2A or full length LMP2B were stimulated with the artificial agonists for TLR3, 5μg/ml poly(I:C) and TLR4, 200ng/ml LPS. After 6 hours, RNA was extracted from each cell type, pre- and post-treatment and cDNA synthesised. RT-QPCR was performed using primer-probe sets specific for (A) TLR3 and (B) TLR4 and using the housekeeping gene GAPDH as an internal baseline control. Data was analysed using Microsoft Excel and the fold change differences calculated between cell types and between each cell type before and after stimulation. Histograms are shown displaying mean fold change differences (mean = +/- SD, n=3). Upon stimulation with either agonist expression of its respective receptor is increased in control cells, this augmentation is abrogated in LMP2A expressing cells and markedly attenuated in LMP2B expressing cells.

A.


TLR-3
Basal
P(I:C) Stimulated
CTRL
LMP2A
LMP2B

B.

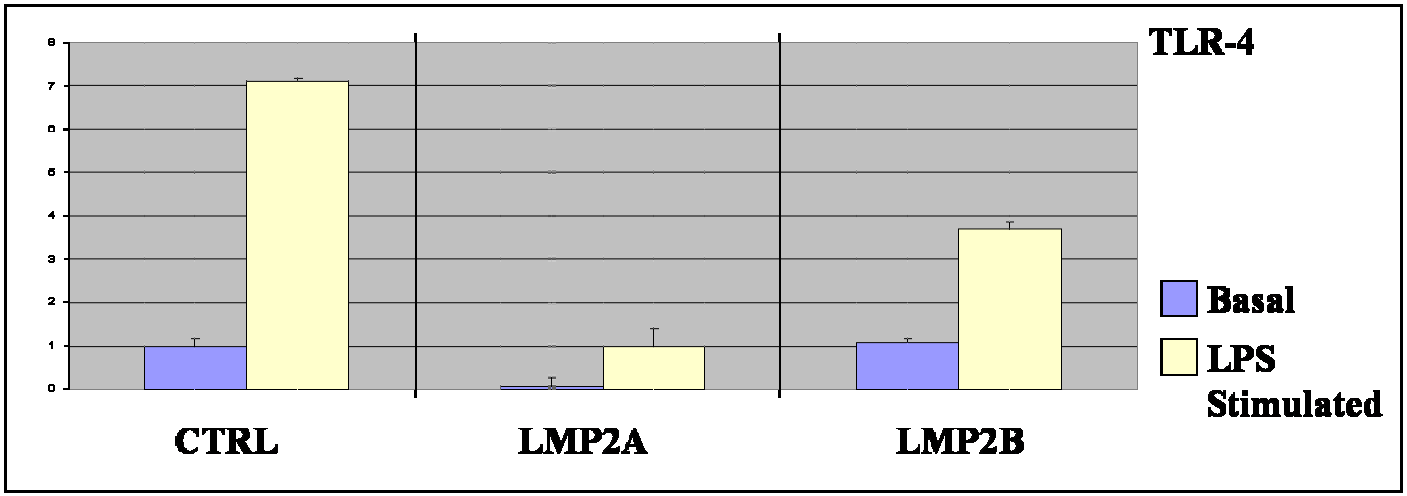

TLR-4
Basal
LPS Stimulated
CTRL
LMP2A
LMP2B

Figure 4.8: Immunofluorescent staining of Toll-like receptors in the CNE-2 cell panel.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent staining was performed for (A) TLR3, (B) TLR4, (C) TLR7, and (D) TLR9. Comparisons between the cell lines reveal that for all TLRs examined expression at the protein level is decreased in cells expressing LMP2A compared to controls and decreased to a lesser extent in LMP2B expressing cells.

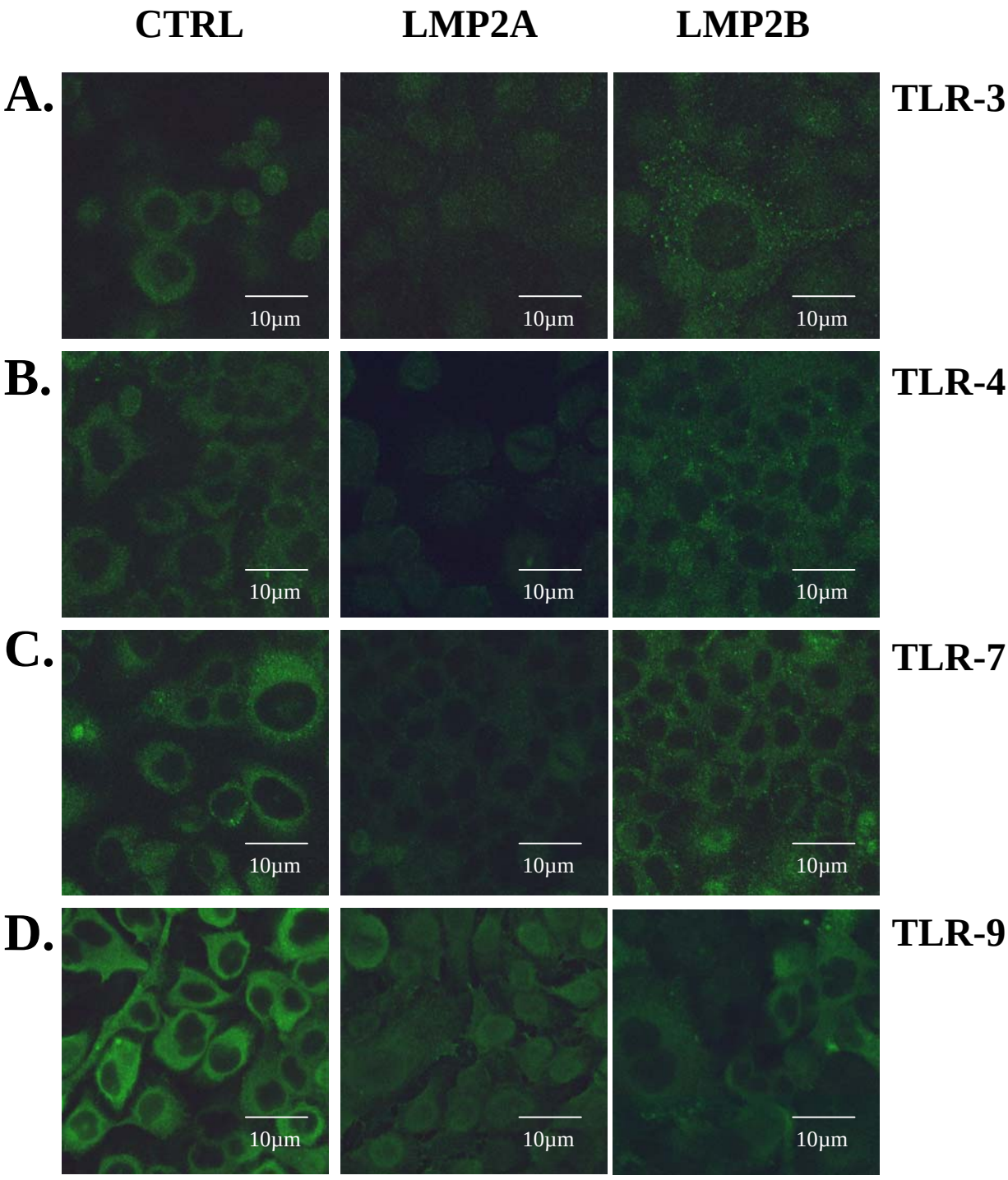

CTRL
LMP2A
LMP2B
A.
TLR-3
B.
TLR-4
C.
TLR-7
D.
TLR-9
10µm

Figure 4.9: Immunofluorescent staining of Toll-like receptors in the H103 cell panel.

H103 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent staining was performed for (A) TLR3, (B) TLR4, (C) TLR7, and (D) TLR9. Comparisons between the cell lines reveal that for all TLRs examined expression at the protein level in decreased in cells expressing LMP2A compared to controls and decreased to a lesser extent in LMP2B expressing cells.

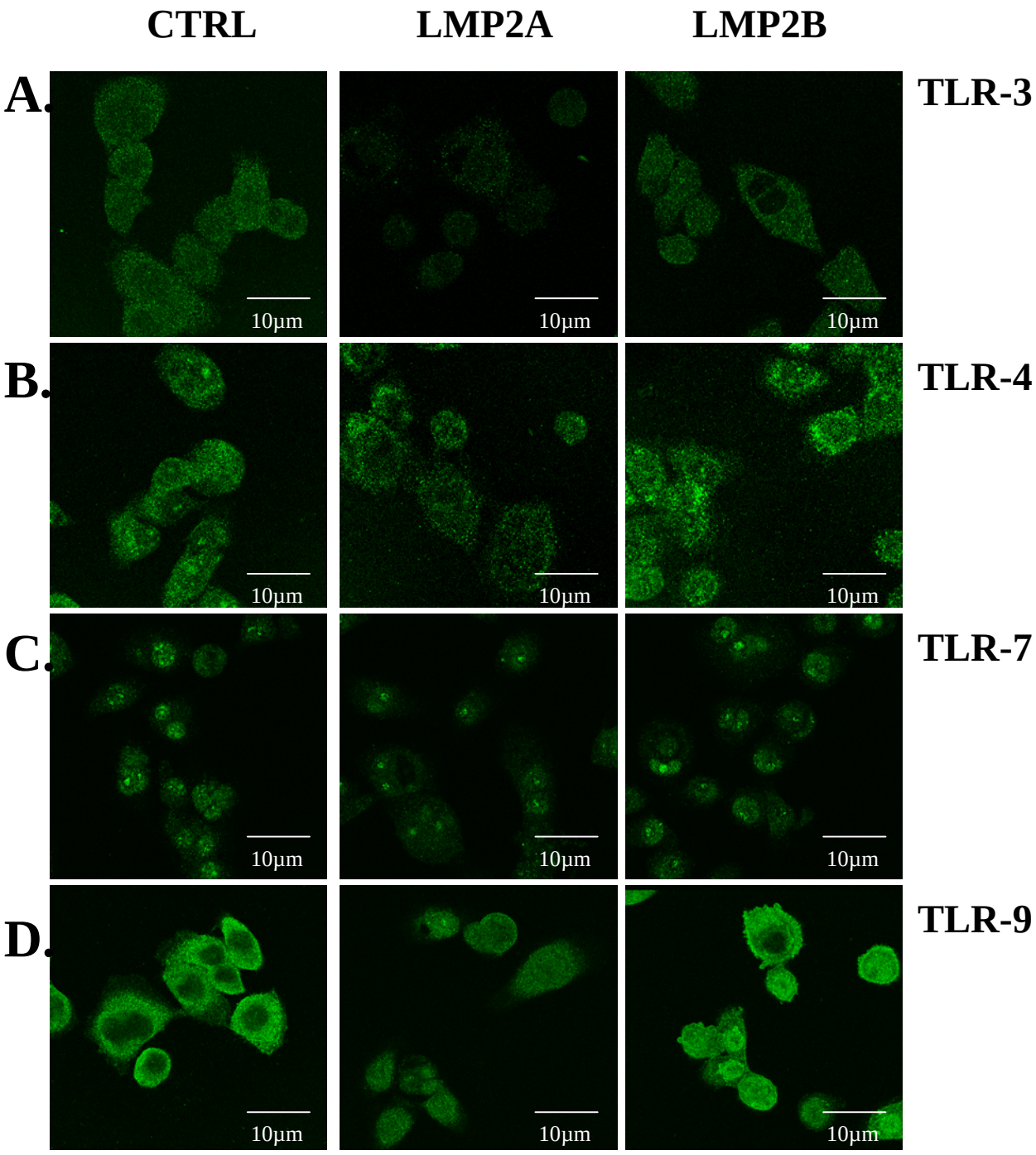

CTRL
LMP2A
LMP2B
A.
TLR-3
B.
TLR-4
C.
TLR-7
D.
TLR-9
10μm

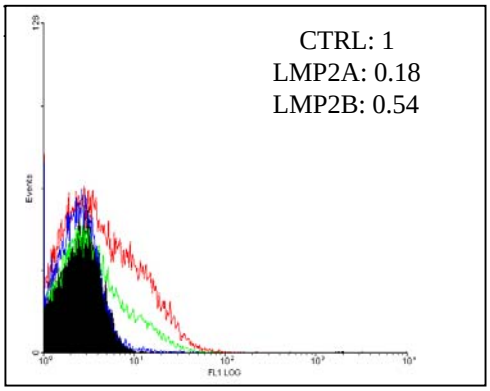
Figure 4.10: FACS analysis of TLR expression in CNE-2 cells pre- and post-permeabilisation.

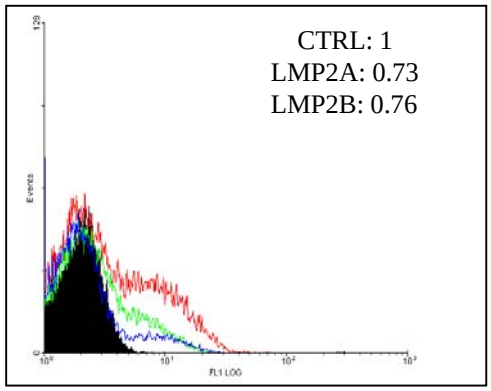
CNE-2 cells expressing either a neomycin resistance cassette, full length LMP2A or full length LMP2B were seeded at a density of 1 x 10^6 onto 10cm^2 dishes and grown to 80% confluence. Following this cells were removed from the surface of the plastic through successive washing and incubation with EDTA. Samples were then probed with antibodies specific to, TLR3, TLR4, TLR7 and TLR9 with and without addition of 0.1% saponin. Cells were fixed in 1% PFA and fluorescence measured using a flow cytometer. Mean fluorescent intensities and graphical representations of the analyses are displayed. Movement of the graph peaks to the right along the X-axis are indicative of an increase of receptor detection on a log-scale. In non-permeabilised samples, across all TLRs examined levels of receptor are decreased in LMP2A expressing cells compared to control and LMP2B counterparts. Upon permeabilisation of cells increased levels of receptor are detectable for all TLRs examined and similarly LMP2A expressing cells show decreased levels of receptor expression compared to their control and LMP2B counterparts. Mean fluorescent intensity (MFI) values are also depicted which have been normalised against the isotype controls and are relative to Neomycin control values.

External

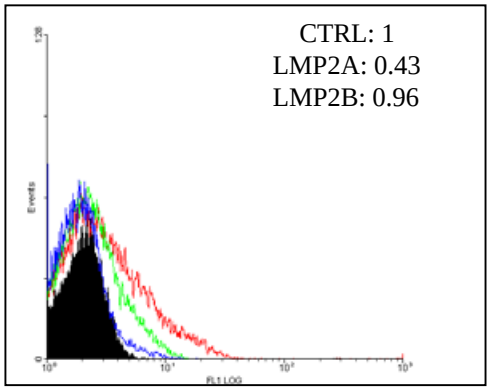
Internal Receptor

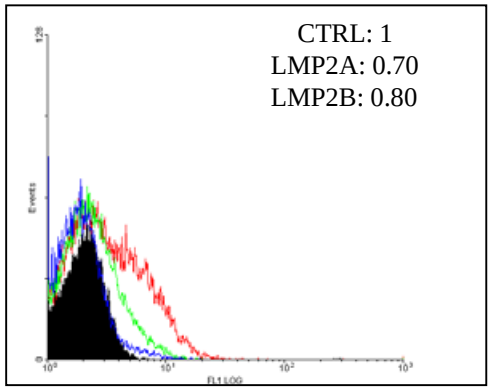
TLR-3



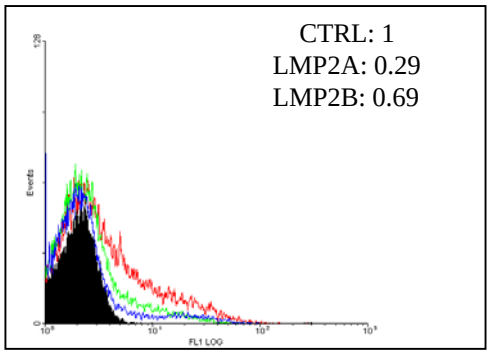


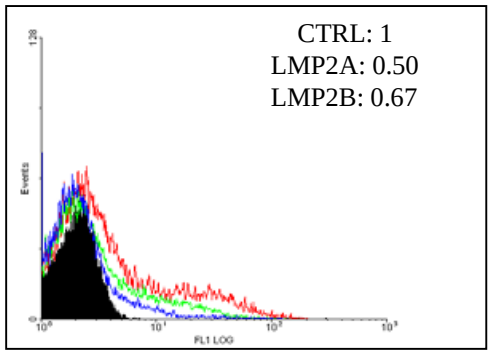
TLR-4



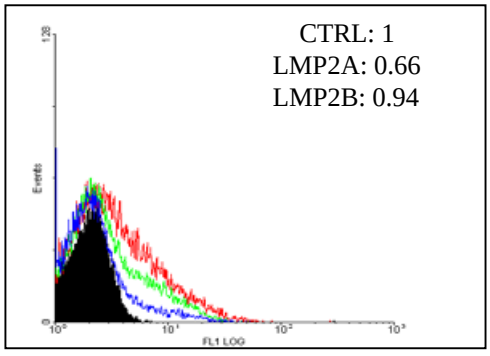


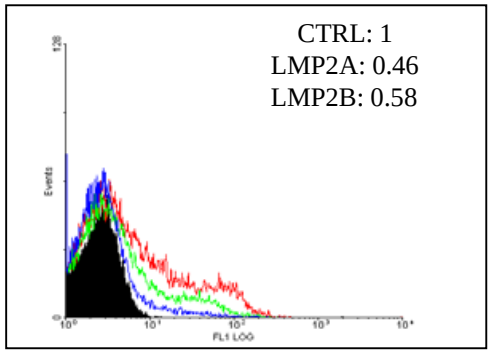
TLR-7





TLR-9





CTRL **LMP2A** **LMP2B**

Figure 4.11: LMP2A modulates expression of intermediates of TLR signalling pathways.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or full length LMP2B were seeded at a density of 1 x 10^6 onto 10cm^2 dishes and allowed to grow to 80% confluence. Following this RNA was extracted and cDNA synthesised. RT-PCR was performed using primers specific to components of TLR signalling pathways including (A) IRAK1, (B) IRAK4, (C) MyD88, (D) TIRAP, (E) MAP3K7, (F) TRAF3, (G) TRAF6 and the housekeeping gene GAPDH was included as an internal control to confirm equal RNA input into the PCR reactions. Reactions were examined using gel electrophoresis and representative photographs are displayed. Comparing levels of expression of each gene examined between control and LMP2A and LMP2B expressing cells, it is observable that in the presence of LMP2A levels all components examined are decreased compared to levels in both controls and LMP2B expressing cells. It must be noted however, that endpoint RT-PCR reactions such as these and no quantitative inference can be made from the results of such analyses.

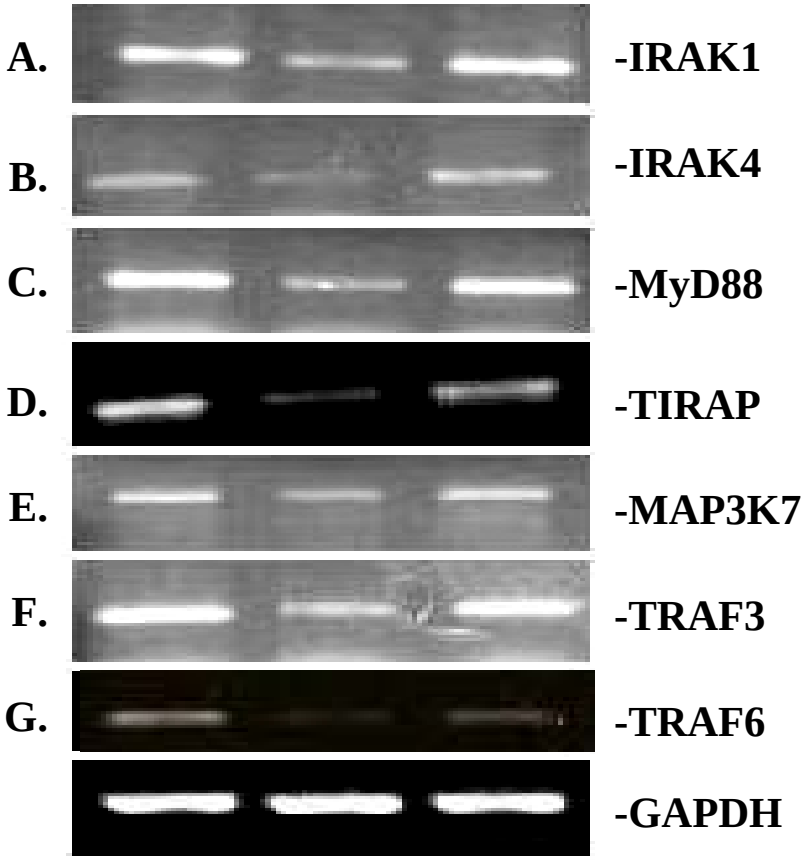
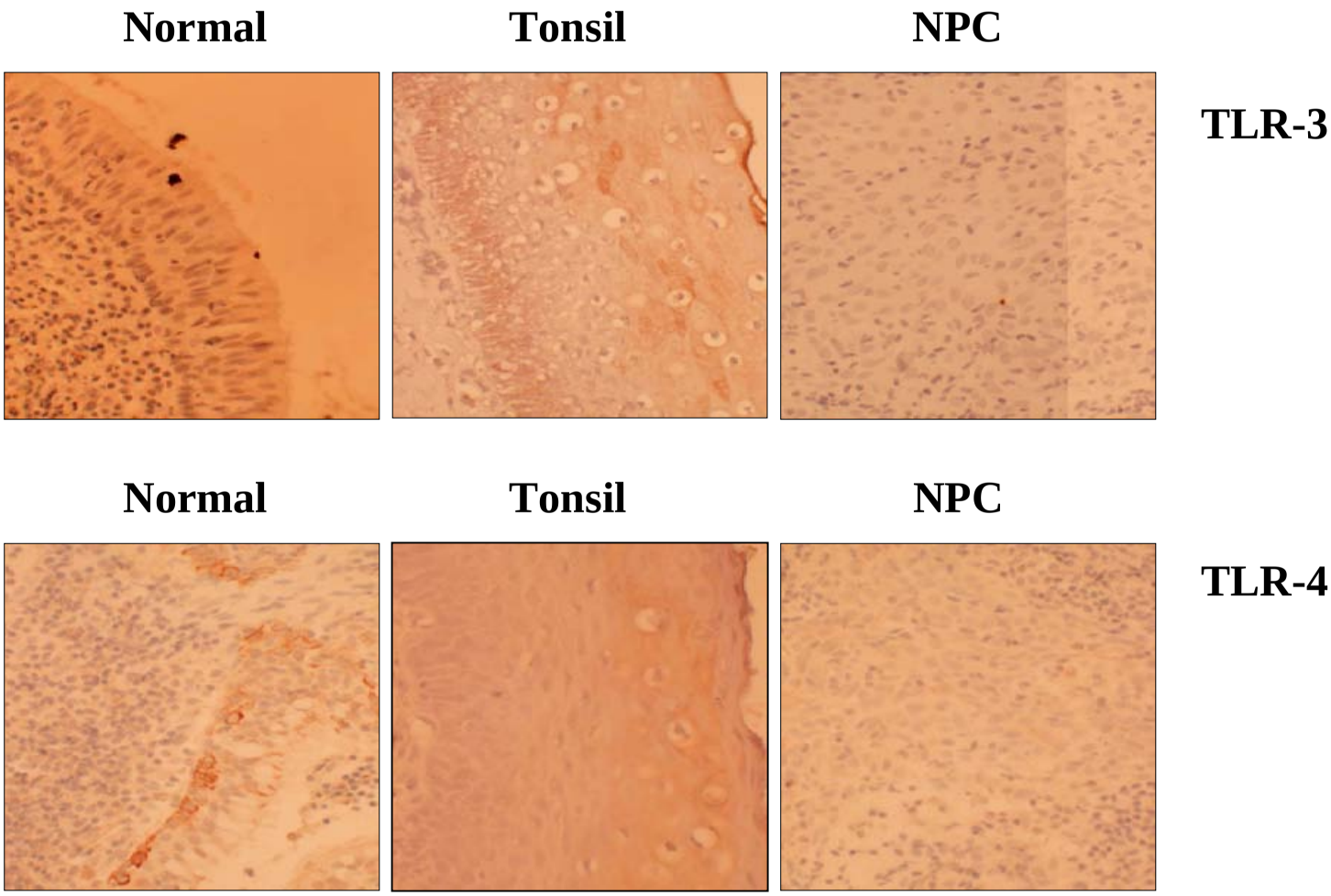

A.
-IRAK1
B.
-IRAK4
C.
-MyD88
D.
-TIRAP
E.
-MAP3K7
F.
-TRAF3
G.
-TRAF6
-GAPDH
CTRL
LMP2A
LMP2B

Figure 4.12: Immunohistochemical staining for TLR3 and TLR4.

Paraffin embedded tissue sections, including normal epithelium, tonsillar epithelium and NPC biopsies were ALTER treated to retrieve the respective antigens and probed using IHC compatible antibodies specific to TLR3 and TLR4. Slides were viewed and photographed using a light phase microscope. Representative images from the analyses are shown. Comparing between the epithelial sections and the tumour itself it is clear that receptor expression is downregulated and that cell morphology is also altered.


Normal
Tonsil
NPC
TLR-3
Normal
Tonsil
NPC
TLR-4

Prior to this, as it has been shown previously, *Figure 4.5,* that IFNβ expression is attenuated in the presence of LMP2A, analysis of the activity of the IFNβ promoter activity as whole was carried out. This determined whether LMP2 expression effected enhancesome formation and thus IFNβ production. To assess this luciferase reporter assays were performed using stimuli for each of the relevant TLRs. This allowed comparison of the basal level of promoter activity compared to that of an activated signalling network while also allowing for comparisons between the cell panels examined.

4.4.1 IFNβ promoter activity is attenuated in presence of LMP2A

4.4.1.1 Effect of LMP2A on TLR3 induced IFNβ activity

Briefly, cells of interest, the CNE-2 and H103 controls and their LMP2A or LMP2B expressing counterparts were transfected with a luciferase reporter plasmid or the relevant control plasmid (see *section 2.3.2*) prior to stimulation with the appropriate agonist. Luciferase assays were then carried out to examine the extent of IFNβ promoter activity at two time points, 6 and 9 hours, using the Dual Luciferase Assay technique (Promega).

A graphical representation of the IFNβ reporter assay of the CNE-2 cell panel after poly(I:C) stimulation is shown in *Figure 4.13.A*. Each assay was performed in biological and technical triplicates and the standard errors calculated. The control samples show, at the basal level, activity of the IFNβ promoter, upon stimulation this activity is, as expected, augmented four-fold, indicating a functioning signalling pathway. This augmentation occurs at both 6 and 9 hours post-incubation with poly(I:C). Comparing this to the LMP2A expressing cells, it can be seen that even basal levels of transcriptional activity are attenuated, mirroring the RT-QPCR data, *Figure 4.5.* Stimulating these cells with poly(I:C) has little effect on the IFNβ promoter, with little or no augmentation observed. LMP2B expressing cells display results matching

those of their control counterparts, with a similar four-fold augmentation upon stimulation and as such this attenuation of TLR3 induced IFNβ transcription is an LMP2A specific effect. *Figure 4.13.B,* shows a graphical representation of the analyses performed on the H103 cell panel, in agreement with the CNE-2 cells, LMP2A expression here is concomitant with attenuated TLR3 signalling. Although the fold differences are of the order of a two-fold difference. The effect of LMP2A is also more pronounced at a basal level in these cells and samples appear almost refractory to poly(I:C) stimulus.

4.4.1.2 Effect of LMP2A on TLR4 induced IFNβ activity

Extending these findings to other TLR members, cells were stimulated with LPS to activate TLR4. A graphical representation of the resulting data in the CNE-2 cell panel is shown in *Figure 4.14.A*. Control and LMP2B expressing cells again show robust activation of IFNβ promoter activity upon LPS stimulation at both the 6-hour and 9-hour time points with approximately two to three fold induction in activity. LMP2A expressing cells show a slight increase in IFNβ promoter activity but compared to controls and LMP2B expressing cells the effect is markedly decreased. *Figure 4.14.B.* shows graphical representation of the same analyses but performed in the H103 cell panel. Comparing null controls to the IFNβ promoter expressing counterparts, basal activity of the promoter is increased and an augmentation occurs upon stimulation with LPS on both LMP2B expressing cells and the neomycin controls. LMP2A expressing cells in line with the CNE-2 result show no such augmentation and similar to that observed upon poly(I:C) stimulation, appear refractory to TLR4 activation by LPS.

4.4.1.3 Effect of LMP2A on TLR7, TLR8 and TLR9 signalling

Figure 4.15.A, depicts the effect of TLR7 stimulation on IFNβ promoter activity analysed in the CNE-2 cell panel. The agonist for TLR7, Imiquimod is a synthetic drug that has been used

previously to treat basal and squamous cell carcinoma. Here it does not appear to activate IFNβ promoter activity in control LMP2A or LMP2B expressing cells. Although the cells express TLR7, *Figures 4.8, 4.9 and 4.10,* agonist treatment does not appear to stimulate IFNβ transcription. The concentrations used were sufficient to mount a TLR7 response, as observed in later sections, but not to induce IFNβ. Although it may appear that LMP2A disrupts the limited activation observed in control and LMP2B samples, comparing the basal attenuation of IFNβ promoter activity between controls and LMP2A expressing cells demonstrates that this is not the case. *Figure 4.15.B.* is a graphical representation of IFNβ promoter activity upon TLR8 stimulation with ssRNA. Mirroring the result observed with poly(I:C) and LPS stimulation, there is an attenuation of signal observed in LMP2A expressing cells when compared with their LMP2B expressing and control counterparts. Promoter activity in LMP2B expressing cells appears to be primed, whereby there is an augmentation of activity upon treatment greater than that seen in the control samples. This could be due to the effect of LMP2B on TLR8 retention in the endosome, which could facilitate super-activation of the receptor complex, or an increased uptake of the ssRNA agonist due to increased endosomal traffic, an effect that many be counteracted in LMP2A expressing cells due to the signalling capacity of its amino-terminal domains, see *chapter 5. Figure 4.15.C,* shows the effect of TLR9, CpG DNA stimulation on IFNβ promoter activity across the CNE-2 cell panel. Here similar to the effects observed after TLR8 stimulation with ssRNA, the augmentation of activity is not as great as in the case of TLR3 and TLR4. However, LMP2A expression attenuates the limited capability of TLR9 agonists to induce IFNβ promoter activity.

The effects of TLR8 and TLR9 stimulation on IFNβ activity here are not as dramatic as with TLR3 and TLR4 stimulation. This can be explained by, the different pathways that the receptors utilise to mount a signalling response. While TLR3 signals MyD88 independently,

and TLR4 can signal through both MyD88 independent and dependent mechanisms, TLRs, 7, 8 and 9, use exclusively MyD88 mediated signalling pathways. Although there is an observable attenuation upon TLR8 and TLR9 stimulation it is likely that LMP2A preferentially exerts its effect on TLR3 signalling given its activation by poly(I:C) and the potential for the EBV encoded EBERs to activate this response.

4.4.1.4 Modulation of TLR1, TLR2, TLR5 and TLR6 signalling by LMP2A

Findings presented thus far have focused on TLR members that localise to internal membranes and the effect that LMP2A has on their expression and signalling. To examine whether these effects were broad i.e. affecting other TLR family members, IFNβ promoter luciferase reporter assays were performed using commercially available agonists specific for TLR1, TLR2, TLR5 and TLR6. A graphical representation of IFNβ promoter activity as assessed by luciferase assay across the CNE-2 cell panel in response to TLR1, TLR2, TLR5 and TLR6 agonist stimulation is shown in *Figures 4.16 A, B, C and D.* In all cases, IFNβ promoter activity is not markedly augmented in CNE2 control cells upon stimulation, indicative of the lack of direct activation of IFNβ responses from these receptors. However, in keeping with observation on TLR3 and TLR4, the basal levels of activity in LMP2A expressing cells is reduced compared to control and LMP2B expressing cells. Augmentation of activity in cells expressing LMP2A and LMP2B upon treatment with any of these four agonists is comparable to levels observed in control cells. Thus these assays clearly demonstrate that, IFNβ production is not associated with activation of signalling from these receptors and that LMP2A or LMP2B do not modulate the little activity that is observable. Analysis of the activity of TLR signalling in terms of IFNβ production revealed an LMP2A specific effect on IFNβ promoter activity.. To examine this in more detail, the effect of LMP2A on components of the IFNβ enhancesome was analysed.

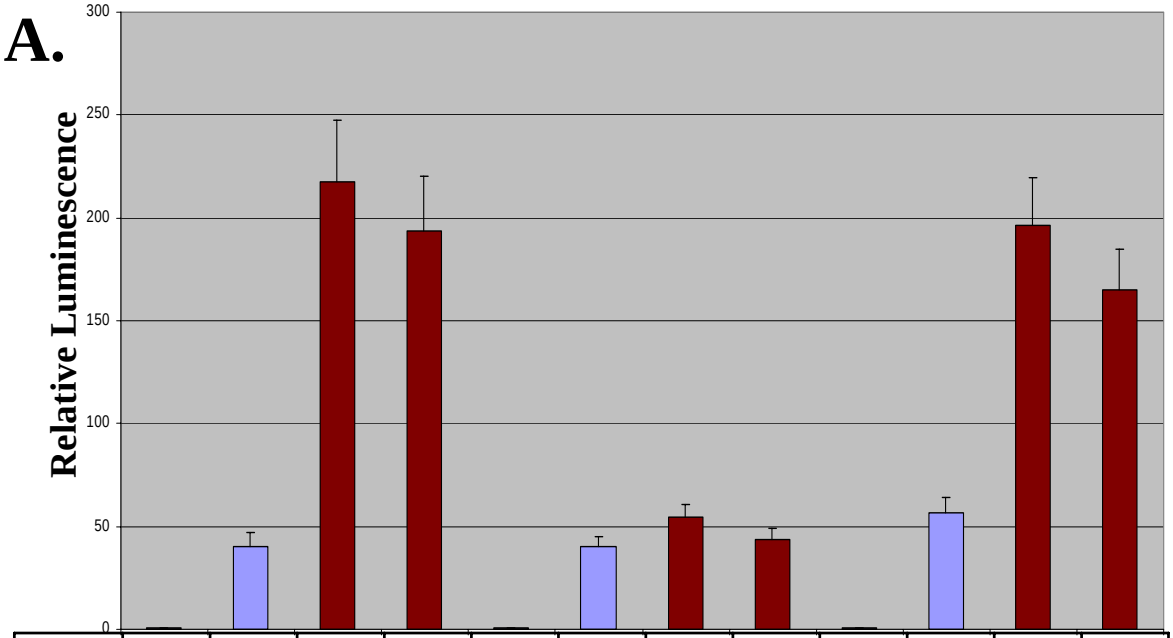
Figure 4.13 Modulation of IFNβ promoter activity by LMP2A expression in epithelial cells upon stimulation of TLR3.

(A) CNE-2 cells and (B) H103 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an IFNβ promoter luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with 5μg/ml poly(I:C) for six and nine hours, harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample set standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the IFNβ promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3).

(A) CNE-2 cells: Comparing basal levels of activity between controls, LMP2A and LMP2B expressing cells, there are discrete differences observable, LMP2A expressing cells showing slightly decreased activity. Upon stimulation of TLR3 by exogenous poly(I:C) control cells show significant increases in IFNβ promoter activity when compared with baseline levels at both time points examined. This augmentation is also seen in LMP2B expressing cells but is abrogated in LMP2A expressing cells, where activity does not markedly increase beyond basal levels.

(B) H103 cells: Comparing basal levels of activity between controls, LMP2A and LMP2B expressing cells, differences are readily observable, LMP2A expressing cells showing slightly decreased activity compared to both LMP2B and control samples. Upon stimulation of TLR3 by exogenous poly(I:C) control cells show significant increases in IFNβ promoter activity when compared with baseline levels at both time points examined. This augmentation is also seen LMP2B expressing cells but is completely abrogated in LMP2A expressing cells, where activity does not markedly increase beyond basal levels and appears almost refractory to stimulation.

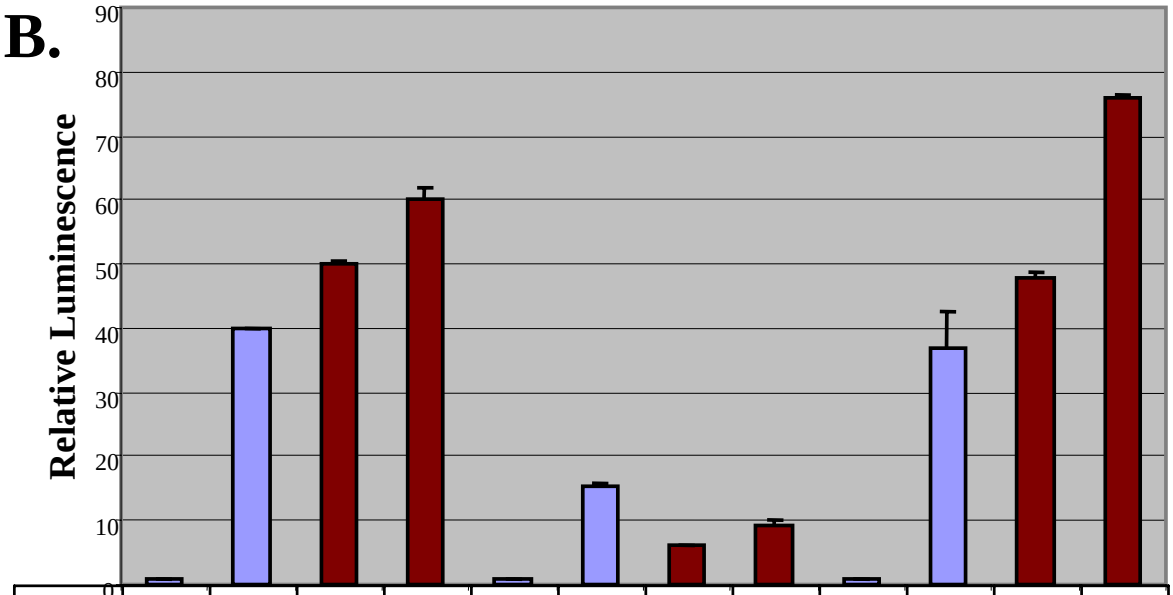
A.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNβ	-	+	+	+	-	+	+	+	-	+	+	+
P(I:C)	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**

CTRL **LMP2A** **LMP2B**

B.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNβ	-	+	+	+	-	+	+	+	-	+	+	+
P(I:C)	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**

CTRL **LMP2A** **LMP2B**

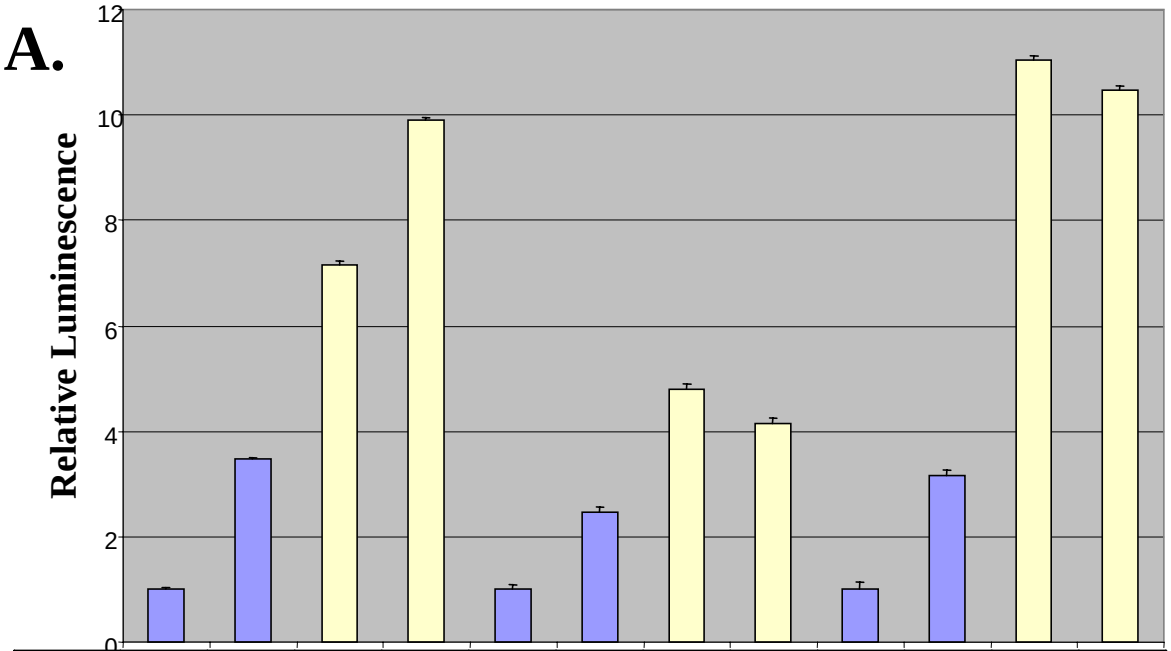
Figure 4.14 Modulation of IFNβ promoter activity by LMP2A expression in epithelial cells upon stimulation of TLR4.

(A) CNE-2 cells and (B) H103 cells, expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an IFNβ promoter luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with 200ng/ml LPS for six and nine hours, harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the IFNβ promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3).

(A) CNE-2 cells: Comparing basal levels of activity between controls, LMP2A and LMP2B expressing cells, there are discrete differences observable, LMP2A expressing cells showing slightly decreased activity. Upon stimulation of TLR4 by exogenous LPS control cells show significant increases in IFNβ promoter activity when compared with baseline levels at both time points examined. This augmentation is also seen LMP2B expressing cells but is attenuated in LMP2A expressing cells, where activity does not markedly increase beyond basal levels.

(B) H103 cells: Comparing basal levels of activity between controls, LMP2A and LMP2B expressing cells, differences are readily observable, LMP2A expressing cells showing decreased activity compared to both LMP2B and control samples. Upon stimulation of TLR4 by exogenous LPS control cells show significant increases in IFNβ promoter activity when compared with baseline levels at both time points examined. This augmentation is also seen LMP2B expressing cells but is completely abrogated in LMP2A expressing cells, where activity does not markedly increase beyond basal levels and appears almost refractory to stimulation.

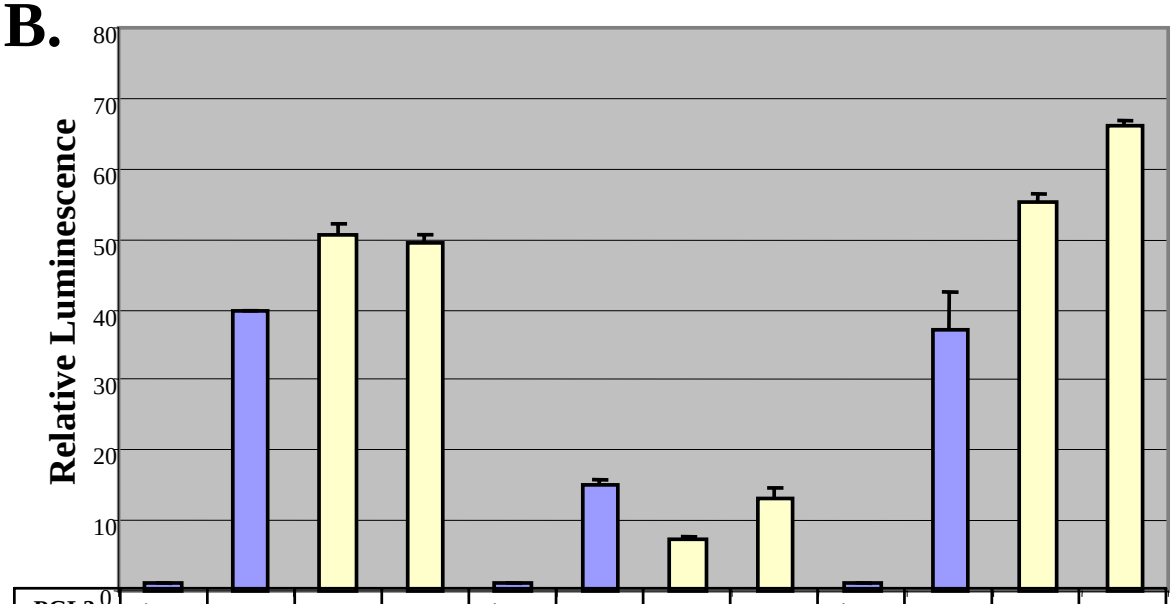
A.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNβ	-	+	+	+	-	+	+	+	-	+	+	+
LPS	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**

CTRL **LMP2A** **LMP2B**

B.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNâ	-	+	+	+	-	+	+	+	-	+	+	+
LPS	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**

CTRL **LMP2A** **LMP2B**

Figure 4.15 Modulation of IFNβ promoter activity by LMP2A expression in CNE-2 cells upon stimulation of TLR7, TLR8 and TLR9.

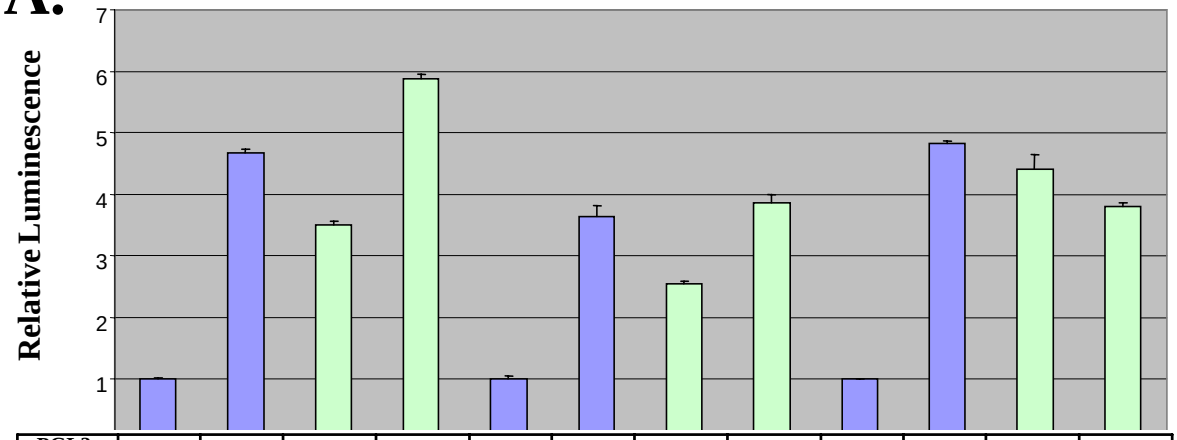
CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an IFNβ promoter luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with (A) 2.5μg/ml Imiquimod for twenty-four and forty-eight hours (TLR7), (B) 1μg/ml ssRNA for six and nine hours (TLR8) or (C) 1μM ODN2006 CpG DNA for six and nine hours (TLR9), harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample set standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the IFNβ promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3).

(A) TLR7: Basal levels of IFNβ promoter activity are attenuated in LMP2A expressing cells compared to control and LMP2b expressing cells. Stimulation with the TLR7 agonist however does not appear to augment this activity in any cell type examined.

(B) TLR8: Discrete differences in promoter activity between control and LMP2A expressing cells are observable. Upon stimulation with ssRNA levels of activity are increased in basal and LMP2B expressing cells, this increase is attenuated in LMP2A expressing cells.

(C) TLR9: In a similar manner to the above result, discrete differences are observable between basal levels of promoter activity between control and LMP2A expressing cells. The augmentation of this activity by CpG DNA in control cells is attenuated in LMP2A expressing cells.

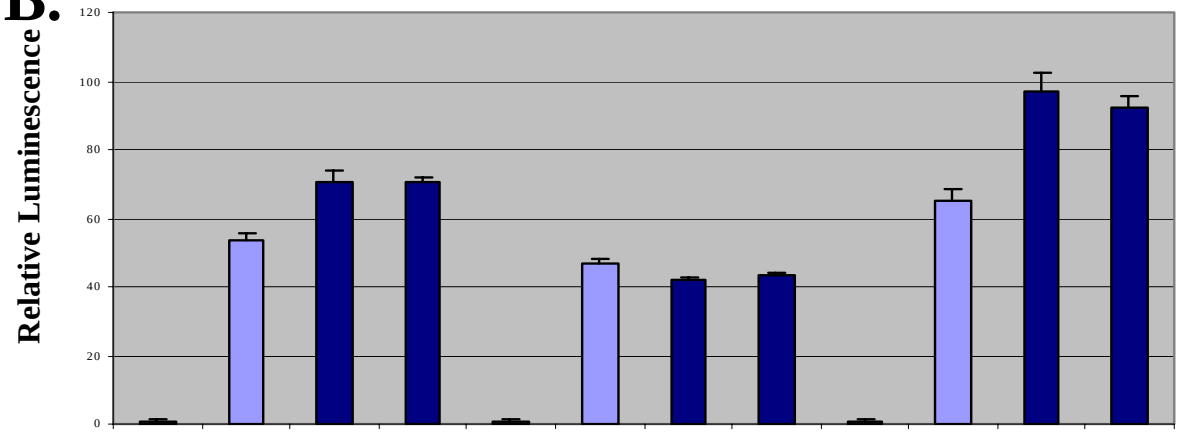
A.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNâ	-	+	+	+	-	+	+	+	-	+	+	+
Imiq	-	-	24hrs	48hrs	-	-	24hrs	48hrs	-	-	24hrs	48hrs

CTRL LMP2A LMP2B

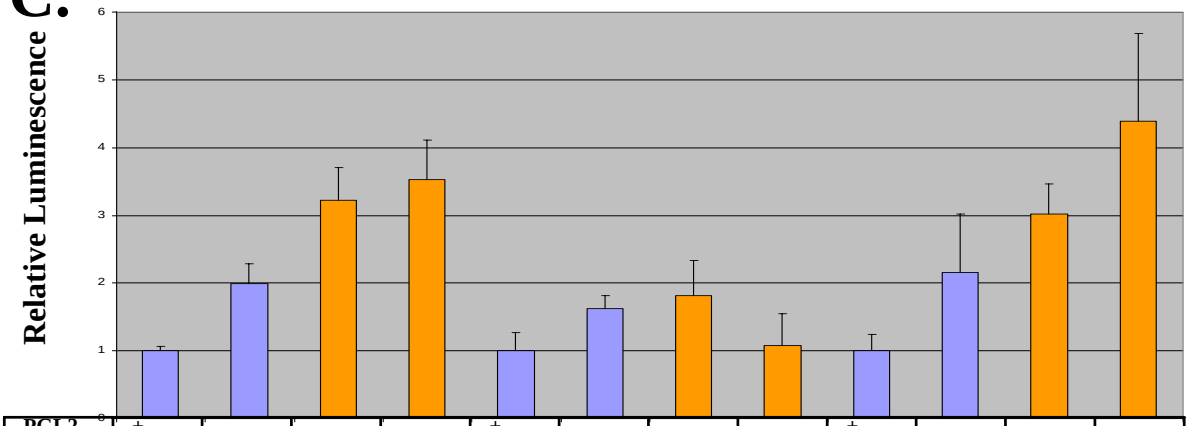
B.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNâ	-	+	+	+	-	+	+	+	-	+	+	+
ssRNA	-	-	6hrs	9hrs	-	-	6hrs	9hrs	-	-	6hrs	9hrs

CTRL LMP2A LMP2B

C.

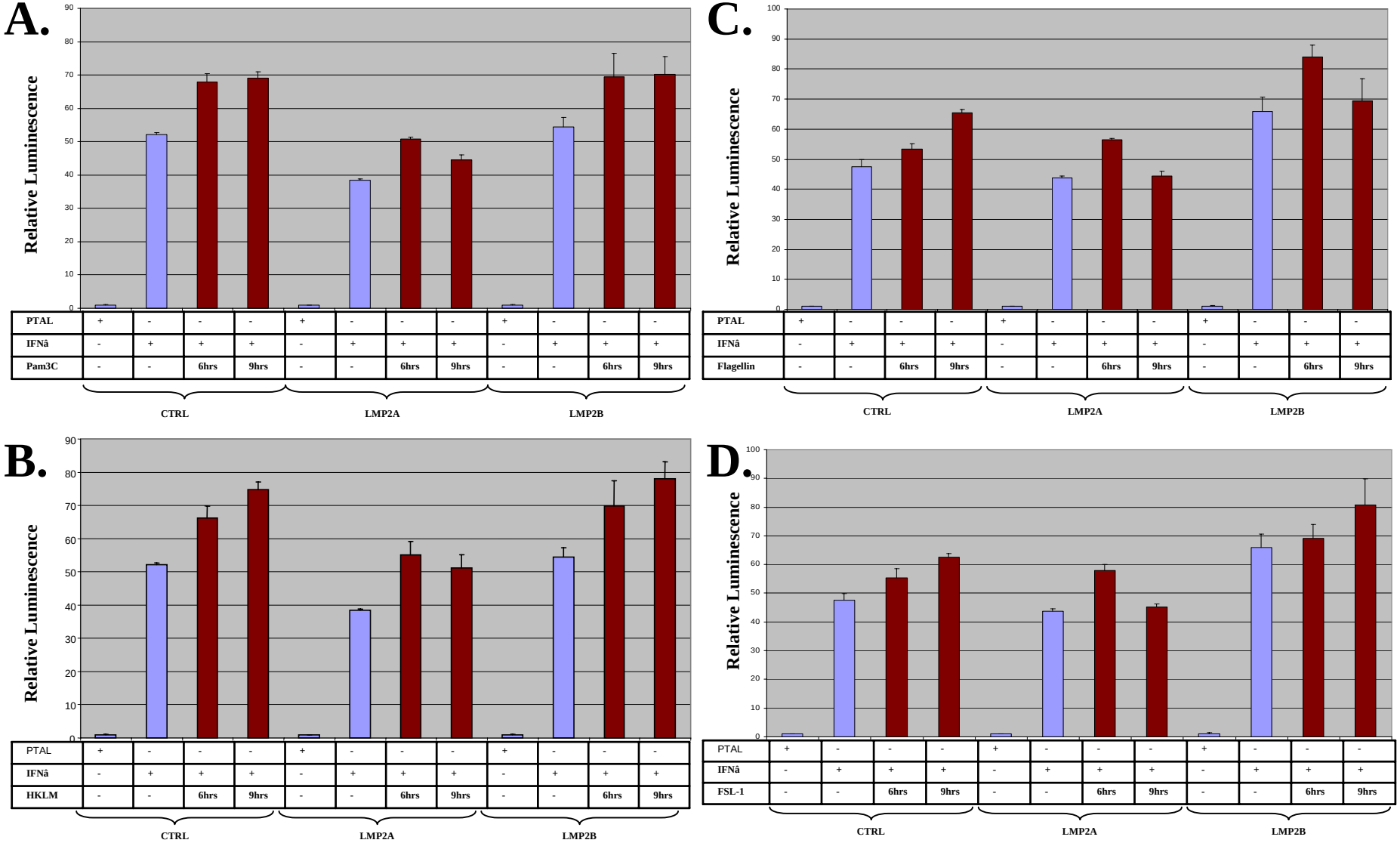


PGL2	+	-	-	-	+	-	-	-	+	-	-	-
IFNâ	-	+	+	+	-	+	+	+	-	+	+	+
CpG	-	-	6hrs	9hrs	-	-	6hrs	9hrs	-	-	6hrs	9hrs

CTRL LMP2A LMP2B

Figure 4.16 Effect of LMP2A expression on IFNβ promoter activity in CNE-2 cells upon stimulation of TLR1, TLR2, TLR5 and TLR6.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an IFNβ promoter luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with (A) 0.5Mμg/ml Pam3CSK4 (B) 10^8 cells/ml HKLM (C) 50ng/ml ST-Flagellin or (D) 400ng/ml FSL-1 for six and nine hours, harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample set standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the IFNβ promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3). For all stimulations on all cell types no significant differences were detected.


A.
Relative Luminescence
PTAL
IFNâ
Pam3C
6hrs
9hrs
CTRL
LMP2A
LMP2B
C.
Relative Luminescence
PTAL
IFNâ
Flagellin
6hrs
9hrs
CTRL
LMP2A
LMP2B
B.
Relative Luminescence
PTAL
IFNâ
HKLM
6hrs
9hrs
CTRL
LMP2A
LMP2B
D.
Relative Luminescence
PTAL
IFNâ
FSL-1
6hrs
9hrs
CTRL
LMP2A
LMP2B

4.4.2 LMP2A expression does not alter AP1 activity in CNE-2 cells

The AP1 transcription factor is composed of homo or hetero dimers of the Jun, Fos and ATF protein families (Malliri, Symons et al. 1998; Ozanne, Spence et al. 2006). It can function to either trans-activate or repress transcriptional activity of a variety of genes involved in cellular pathways including, transformation, cell migration, cell cycle and immune regulation (Vesely, Staber et al.). As outlined in the introduction, AP1 is a key component of the IFNβ enchancesome complex and, in conjunction with NFκB and IRF3, regulates IFNβ transcription. To ascertain whether LMP2A affects AP1 activity, a generic AP1 luciferase reporter plasmid was used to examine whether LMP2A affected TLR-mediated AP1 activation. In a similar manner to the experiments described above, AP1 activity was assessed using the Dual-luciferase reporter kit (Promega). Graphical representations of the data are shown in *Figures 4.17.A, B, C and D*. In response to TLR4, TLR7 and TLR8 agonist stimulation, AP1 reporter activity was increased in control, LMP2A and LMP2B expressing cells equally, with comparable levels of activation being observed between the different TLR agonists. However, a slight attenuation in AP1 activity was observed in LMP2A expressing cells treated with the TLR3 agonist, poly(I:C), *Figure 4.16.A,* where a much weaker response was observed compared to control and LMP2B expressing cells. Overall however, it is determined that AP1 activity is not modulated enough to account for the observable disruption of IFNβ promoter activity, e.g. *Figure 4.13.*

4.4.3 LMP2A attenuates IRF3 activation in response to TLR stimulation

The second component of the complex to be examined in more detail was the transcription factor IRF3. As shown previously, basal protein expression of this transcription factor was downregulated in cells expressing LMP2A compared to their control counterparts, *Figure 4.2A, B.* It is known that IRF3 is more important than IRF7 in the initial early phase of an interferon responses as it is expressed constitutively in all cells, whereas IRF7 is expressed

only at low levels (Peters, Smith et al. 2002), which are augmented by IFNβ, contributing to paracrine and autocrine signalling loops necessary for full type I responses (Hiscott 2007). IRF3:IRF7 heterodimers are involved in the secondary stage of IFNβ production and as the data here are focused on the first wave of the innate response analysis of IRF3 binding to the IFNβ promoter were of primary importance (Dragan, Hargreaves et al. 2007).

4.4.3.1 Nuclear translocation of IRF3 in epithelial cells

To further analyse the effect of LMP2A on IRF3 activity, a translocation assay was performed using immunofluorescence staining, as outlined in *section 2.4.* Briefly, the CNE-2 and H103 cell panels were grown *in situ* prior to TLR3 activation with poly(I:C). After 6 hours, cells were fixed, permeabilised and subjected to immunofluorescence staining with an antiserum specific for IRF3. Representative confocal images of the CNE-2 cell panel and the magnified insets*, Figure 4.18*, show the translocation more clearly, with arrows indicating the nuclei of cells. Comparing basal levels of expression of IRF3 between control, LMP2A expressing cells and those expressing LMP2B, this result mirrors the immunoblotting results, where levels of IRF3 are slightly lower in LMP2A expressing cells. Whereas most, if not all IRF3 is cytoplasmic in unstimulated control cells, upon stimulation with poly(I:C), a robust nuclear translocation is observed in many cells. A similar effect is observed in the LMP2B expressing cells, although the magnitude of the response appeared somewhat reduced. The LMP2A cell panel however shows much less translocation when compared to control and LMP2B expressing counterparts. This indicates a disruption in IRF3 activation by LMP2A, a finding which may account for the attenuation in IFNβ promoter activity described in *section 4.4.1*. To ensure that these findings were not cell type specific, identical experiments were performed in the H103 cell panel. Representative confocal images of immunofluoresence staining for IRF3 are shown in *Figure 4.19*. As with CNE2 cells, control and LMP2B expressing cells expressed similar basal levels of IRF3 with LMP2A having a slightly lower levels of expression. Upon

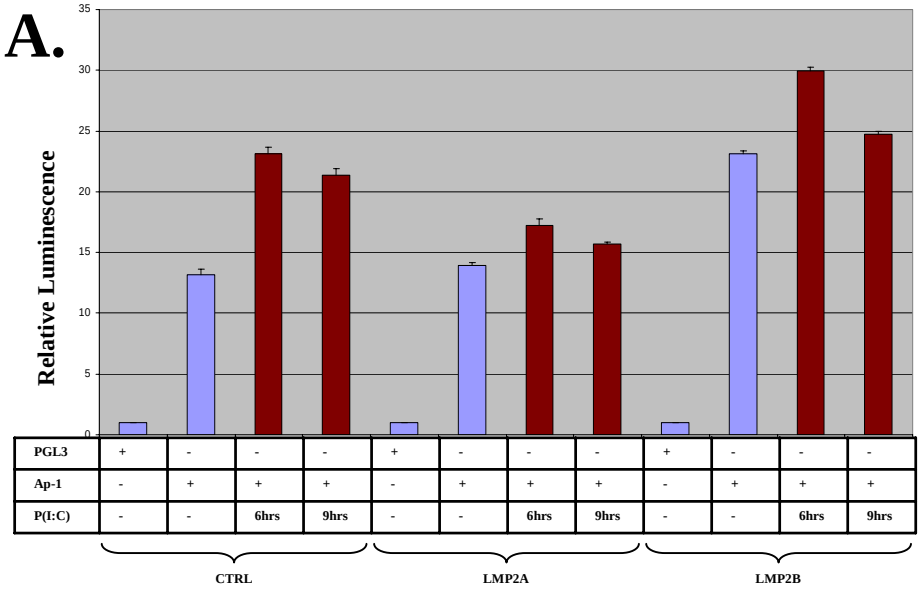
poly(I:C) stimulation, nuclear translocation occurs in control and LMP2B cells but is attenuated in cells expressing LMP2A.

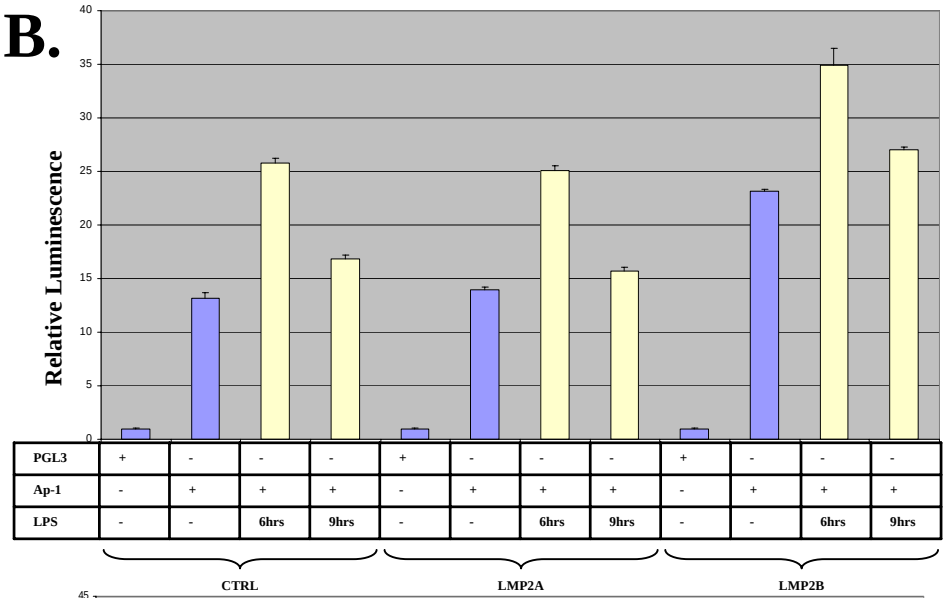
4.4.3.2 IRF3:DNA binding is disrupted by LMP2A

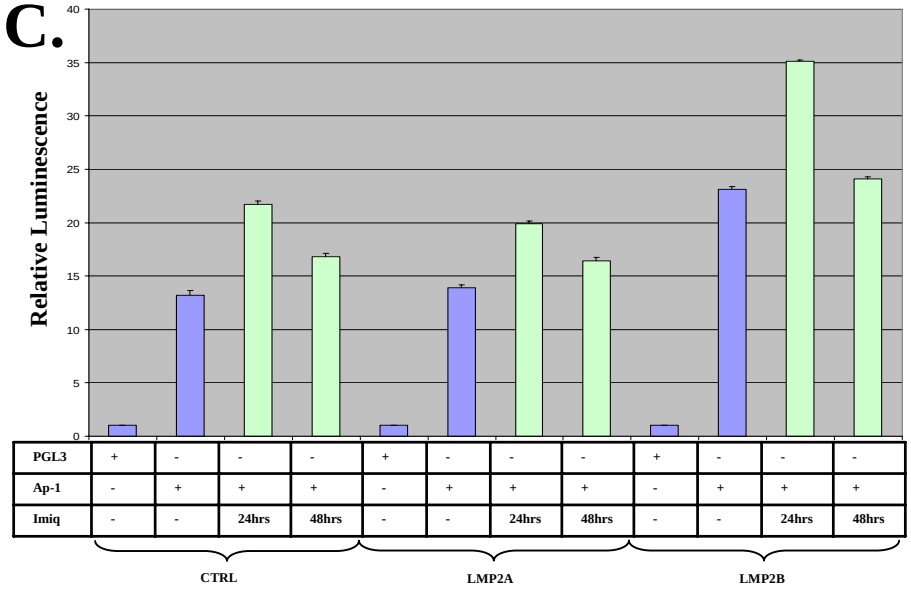
Having demonstrated that LMP2A and., to a lesser extent LMP2B, attenuated IRF3 activation and nuclear translocation, the binding of IRF3 to its target sequences within the IFNβ promoter was explored. IRF3 homodimers are known to bind to two sequences within this region: the PRDI and the PRDIII domains. The NFκB p50/p65 heterodimers bind to the PRDII domain and the AP1 subunits bind to the PRDIV domain (Panne 2008). Full binding of all of the subunits of the enhancesome complex is necessary for its function and interference at one or more of these sites could disrupt transcriptional activity of the promoter, *Chapter 1, section 1.7.6*. To assess this binding, electrophoresis mobility shift assays (EMSA) were performed, as described in *section 2.8.3*. Briefly, nuclear extracts were isolated from the CNE-2 cell panel that had been stimulated with poly(I:C) to activate TLR3. To ensure efficient separation of cell fractions, immunoblotting was performed on both extracts using antibodies specific to the cytoplasmic and nuclear markers, β-tubulin and Sp-1 respectively (data not shown), expression of each only detectable in the relevant extract. Nuclear extracts were then incubated with IRD-700 labelled probes specific to the PRDI binding domain of the IRF3 transcription factor. Binding was measured after scanning of the EMSA gel on a LiCor© infrared laser device. DNA:Protein complexes were resolved and the binding of proteins to the synthetic oligonucleotide PRD domain probe quantitated by measuring relative fluorescence intensities. Densiometric analyses, shown graphically, along with the scanned EMSA gels are shown in *Figure 4.20.* The level of protein binding to the PRDI probe is increased in response to poly(I:C) treatment in control cells. In marked contrast, the level of binding to the PRDI probe observed with nuclear extracts from LMP2A expressing cells is markedly reduced, whereas intermediate levels of binding are observed with extracts from LMP2B expressing cells.

Figure 4.17 Effect of LMP2A expression on AP-1 activity in CNE-2 cells upon stimulation of TLR3, TLR4, TLR7 and TLR8.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an AP-1 luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with (A) 5μg/ml poly(I:C) (TLR3), (B) 200ng/ml LPS (TLR4) for six and nine hours, (C) 2.5μg/ml Imiquimod for twenty-four and forty-eight hours (TLR7) and (D) 1μg/ml ssRNA for six and nine hours (TLR8), harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample set standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the IFNβ promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3). For all stimulations on all cell types no significant differences were detected., although stimulation with agonists did augment AP-1 activity to an equal level across cell types, with ssRNA (TLR8) being the most effective.


A.
Relative Luminescence
0
5
10
15
20
25
30
35
PGL3 + - - - + - - - + - - -
Ap-1 - + + + - + + + - + + +
P(I:C) - - 6hrs 9hrs - - 6hrs 9hrs - - 6hrs 9hrs
CTRL
LMP2A
LMP2B


B.
Relative Luminescence
0
5
10
15
20
25
30
35
40
PGL3 + - - - + - - - + - - -
Ap-1 - + + + - + + + - + + +
LPS - - 6hrs 9hrs - - 6hrs 9hrs - - 6hrs 9hrs
CTRL
LMP2A
LMP2B


C.
Relative Luminescence
0
5
10
15
20
25
30
35
40
PGL3 + - - - + - - - + - - -
Ap-1 - + + + - + + + - + + +
Imiq - - 24hrs 48hrs - - 24hrs 48hrs - - 24hrs 48hrs
CTRL
LMP2A
LMP2B

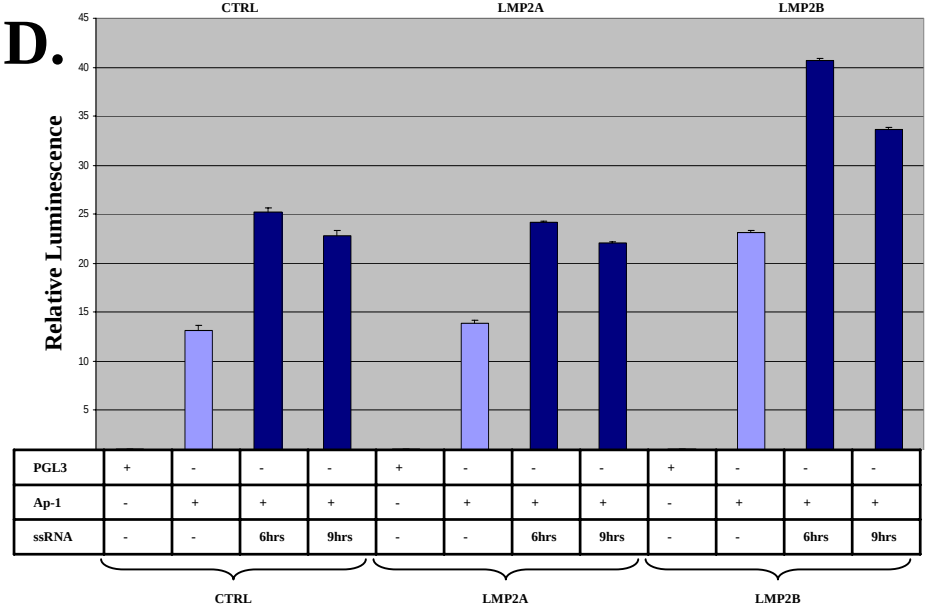

D.
Relative Luminescence
5
10
15
20
25
30
35
40
45
PGL3 + - - - + - - - + - - -
Ap-1 - + + + - + + + - + + +
ssRNA - - 6hrs 9hrs - - 6hrs 9hrs - - 6hrs 9hrs
CTRL
LMP2A
LMP2B

Figure 4.18 Effect of LMP2A expression on IRF-3 translocation in CNE-2 cells upon stimulation of TLR3.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 1 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day samples were stimulated *in situ,* with 5µg/ml poly(I:C) for six hours. Slides were fixed and permeabilised with 4% PFA and 0.5% Triton-X and immunofluorescent staining performed on both stimulated and unstimulated samples using an antibody specific to IRF-3. Samples were viewed and photographed using a Zeiss LSM510-meta confocal microscope and representative images are shown. Levels of IRF-3 protein detected were slightly lower in LMP2A expressing cells when compared to their LMP2B and control counterparts, in line with data from *Figure 4.2(A)(iii).* Upon stimulation with poly(I:C), translocation of IRF-3 from the cytosol to the nucleus is observed in control and LMP2B expressing cells. Levels of translocation in LMP2A expressing cells however, are attenuated when compared with other cell types. Inset images are displayed to show effects at increased magnification with arrows indicating the signal detected with the nuclei of cells.

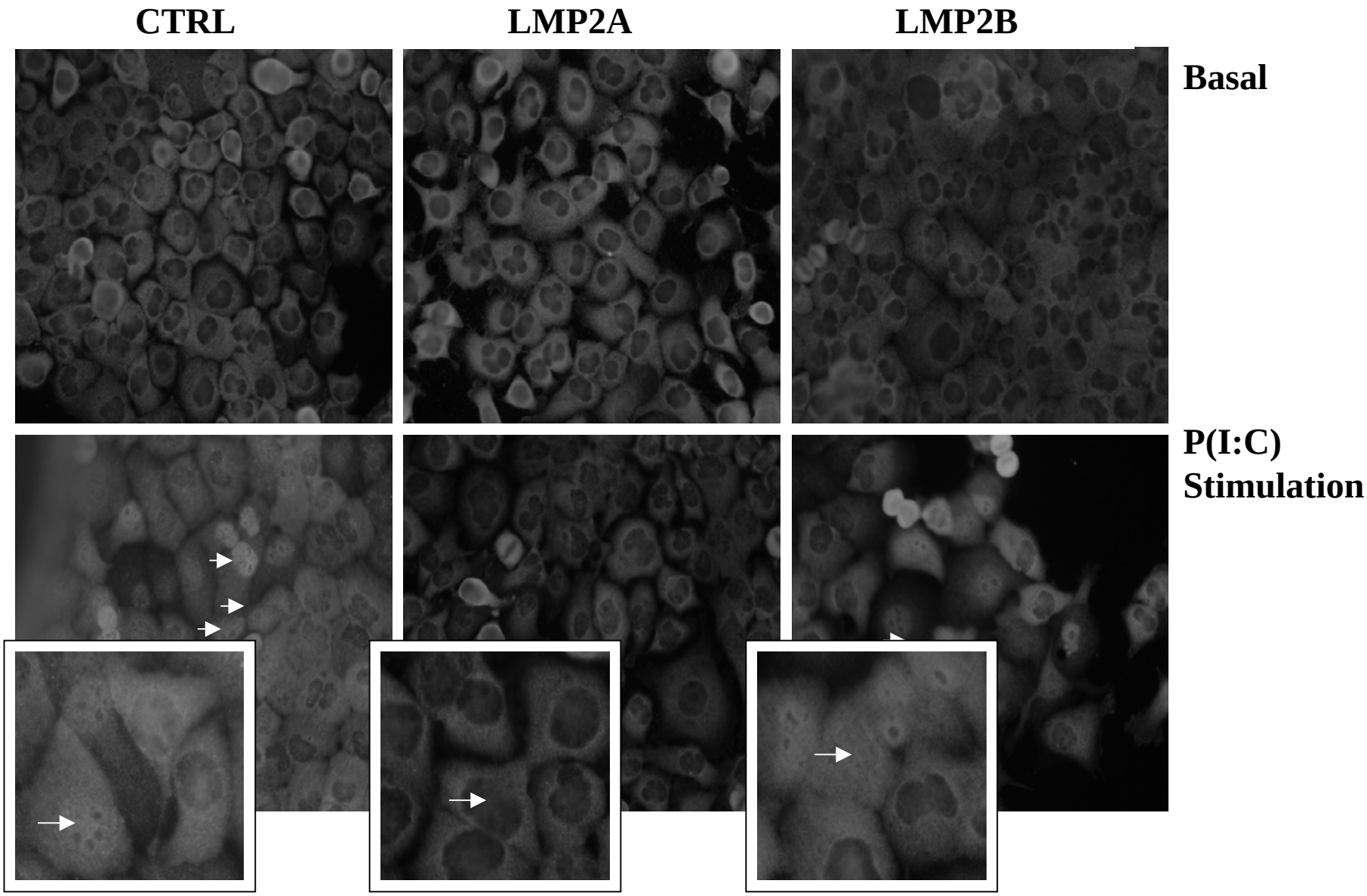

CTRL
LMP2A
LMP2B
Basal
P(I:C)
Stimulation

Figure 4.19 Effect of LMP2A expression on IRF-3 translocation in H103 cells upon stimulation of TLR3.

H103 cells expressing either a control neomycin resistance cassette, full length HA tagged LMP2A or LMP2B were seeded at a density of 1 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day samples were stimulated *in situ,* with 5μg/ml poly(I:C) for six hours. Slides were fixed and permeabilised with 4% PFA and 0.5% Triton-X and immunofluorescent staining performed on both stimulated and unstimulated samples using an antibody specific to IRF-3. Samples were viewed and photographed using a Zeiss LSM510-meta confocal microscope and representative images are shown. Levels of IRF-3 protein detected were slightly lower in LMP2A expressing cells when compared to their LMP2B and control counterparts. Upon stimulation with poly(I:C), translocation of IRF-3 from the cytosol to the nucleus is observed in control and LMP2B expressing cells. Levels of translocation in LMP2A expressing cells however, are attenuated when compared with other cell types. Inset images are displayed to show effects at increased magnification with arrows indicating the signal detected with the nuclei of cells.

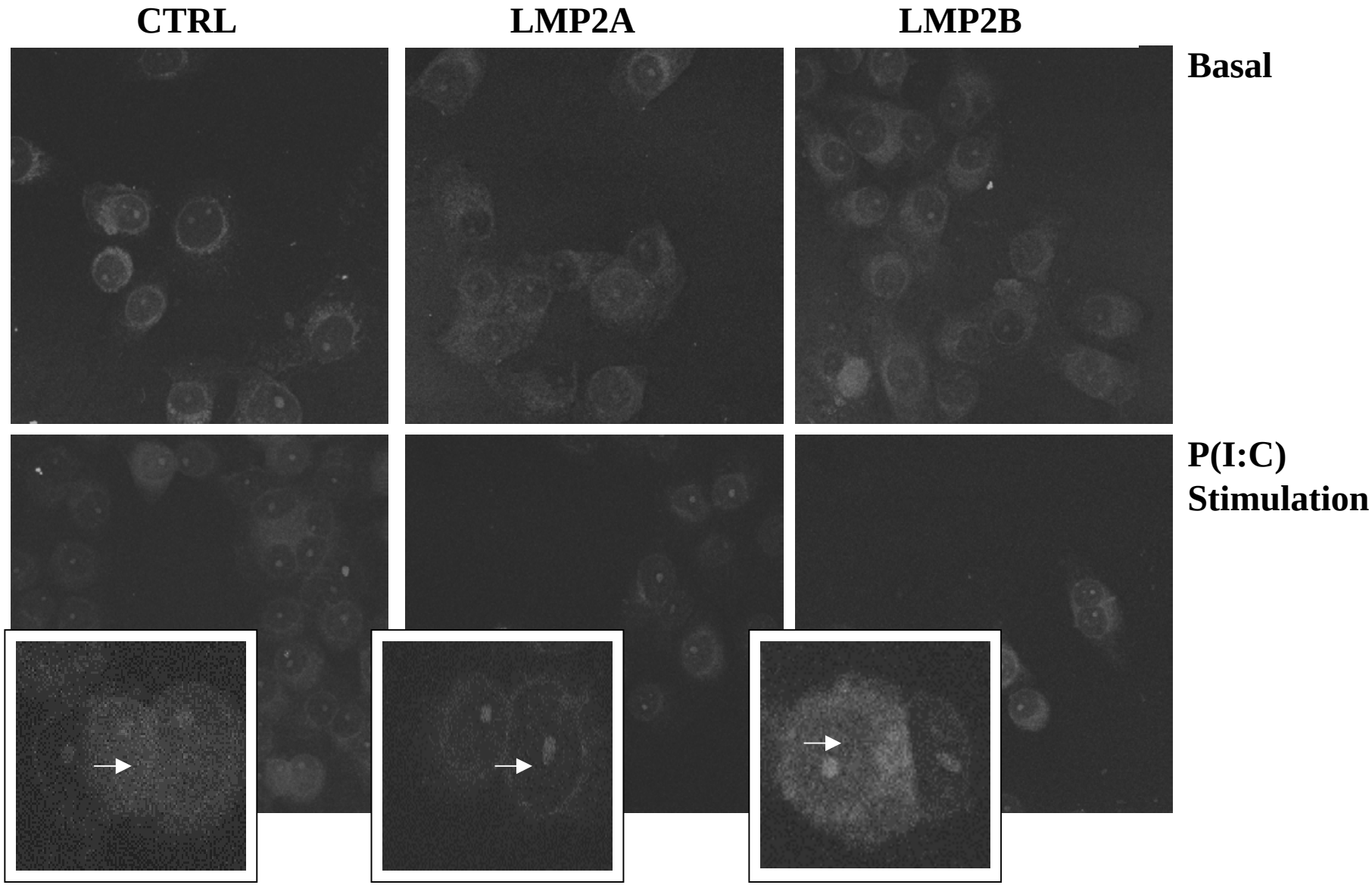
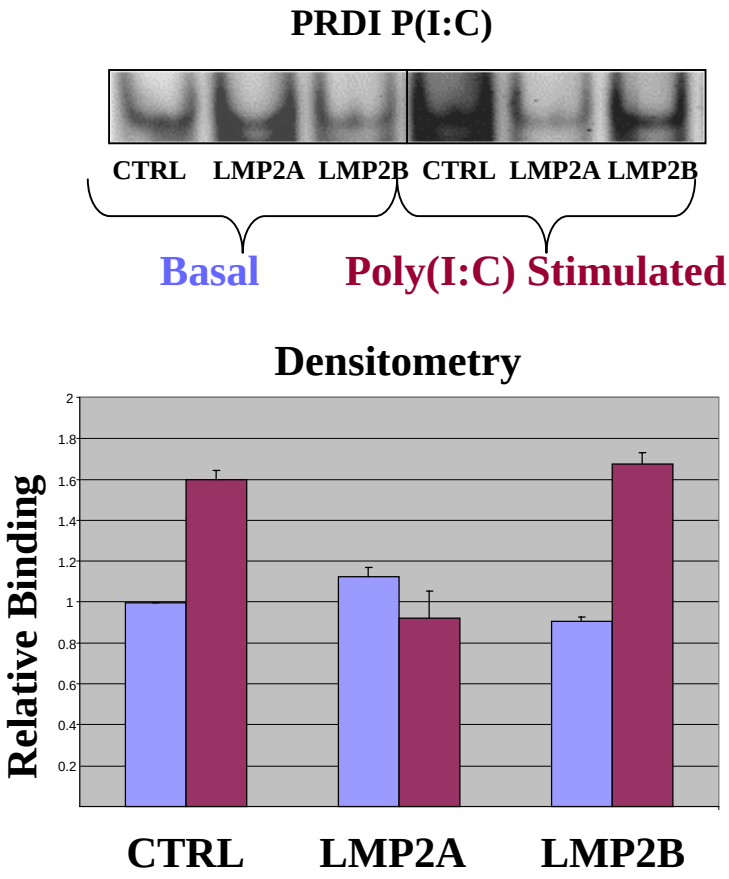

CTRL
LMP2A
LMP2B
Basal
P(I:C)
Stimulation

Figure 4.20 Effect of LMP2A on binding at PRDI domains in CNE-2 cells upon stimulation of TLR3.

CNE-2 cells expressing either a control neomycin resistance cassette, LMP2A or LMP2B were seeded at a density of 1 x 10^6 cells per 10cm^2 dish and allowed to adhere overnight at 37˚C and 5% CO_2**.** The following day samples were stimulated with 5μg/ml poly(I:C) for six hours and nuclear and cytosolic extracts were isolated from control and stimulated samples. The nuclear extracts were then incubated with IRD-700 probes specific to the PRDI domain of the IFNβ promoter. Samples were then separated by gel electrophoresis and analysed using infrared scanning and imaging. Densitometry was also performed and histograms created depicting mean differences in binding affinities between cell types with and without stimulation. Histograms and representative images of the infrared scanning are shown (mean ± SD; n=3). Basally no significant differences are observable between cell types, upon stimulation with poly(I:C) binding is augmented in control and LMP2B expressing cells. This increase is attenuated in LMP2A expressing cells.


PRDI P(I:C)
CTRL LMP2A LMP2B CTRL LMP2A LMP2B
Basal
Poly(I:C) Stimulated
Densitometry
Relative Binding
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
CTRL
LMP2A
LMP2B

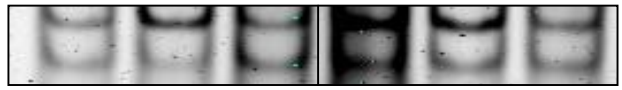
Enhancesome activity at the IFNβ promoter depends also on binding of p50/p65 dimers to the PRDII domain. Identical experiments were performed using a labelled-probe specific to this domain. Graphical representations of the results and the scanned EMSA gels are shown in *Figure 4.21*. Here, comparing the binding intensities of nuclear extracts prepared from unstimulated and poly(I:C) stimulated cells, there is a robust increase in probe binding in control cells. Comparing this to LMP2B expressing cells a small augmentation occurs in stimulated cells, but the level here is more comparable to the attenuated activation state observable in LMP2A expressing cells. These results demonstrate an attenuation of PRDII domain binding in both LMP2A and LMP2B expressing cells upon TLR-3 stimulation, whereas binding at PRDI domains is attenuated only upon LMP2A expression.

These experimental results clearly outline a proposed mechanism for the disruption of TLR3 induced IFNβ promoter activity by attenuating binding of IRF3 transcription factor at the PRDI domain of the promoter. To further investigate this effect it would be necessary to examine the PRDIII domain as this has been reported to be important in both IFNβ and IFNα4 transcription. Although probes were designed and analysed, investigations into the binding affinities in the CNE-2 cell panel were unsuccessful. Data for the PRDII domain revealed that binding here was decreased in both LMP2A and LMP2B expressing cells when compared with controls. Taken together with the effect of LMP2A on binding at the PRDI domain, these results could account for the observed decreases in IFNβ promoter activity described earlier.

Figure 4.21 Effect of LMP2A on binding at PRDII domains in CNE-2 cells upon stimulation of TLR3.

CNE-2 cells expressing either a control neomycin resistance cassette, LMP2A or LMP2B were seeded at a density of 1 x 10^6 cells per 10cm^2 dish and allowed to adhere overnight at 37˚C and 5% CO_2**.** The following day samples were stimulated with 5μg/ml poly(I:C) for six hours and nuclear and cytosolic extracts were isolated from control and stimulated samples. The nuclear extracts were then incubated with IRD-700 probes specific to the PRDII domain of the IFNβ promoter. Samples were then separated by gel electrophoresis and analysed using infrared scanning and imaging. Densitometry was also performed and histograms created depicting mean differences in binding affinities between cell types with and without stimulation. Histograms and representative images of the infrared scanning are shown (mean ± SD; n=3). Basally no significant differences are observable between cell types, upon stimulation with poly(I:C) binding is augmented in control. This increase is attenuated in LMP2A expressing cells and to a lesser extent in cells expressing LMP2B.

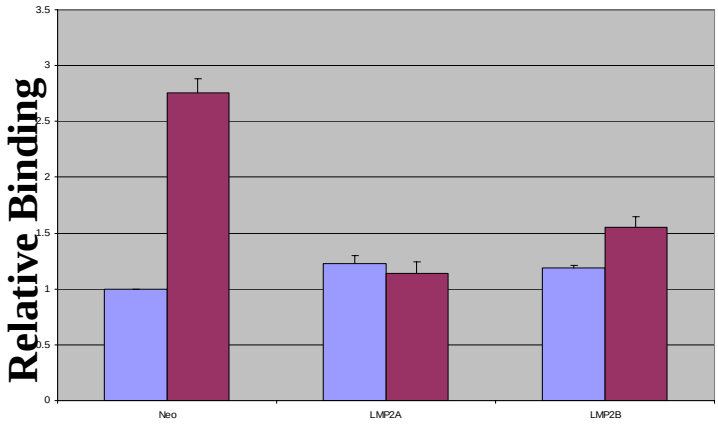
PRDII P(I:C)



CTRL LMP2A LMP2B CTRL LMP2A LMP2B

Basal **Poly(I:C) Stimulated**

Densitometry



CTRL **LMP2A** **LMP2B**

4.4.4 Modulation of NFκB activity by LMP2A

4.4.4.1 Effect of LMP2A on TLR3-induced NFκB activity

The final component of the enhancesome complex to be examined was the NFκB transcription factor. To investigate the impact of LMP2A and LMP2B on NFκB activity, luciferase reporter assays were performed using pConALuc, a plasmid containing tandem consensus NFκB binding motifs from the HIV LTR (Huen et al., 1995). CNE-2 and H103 cell panels were transfected with the NFκB reporter plasmid or the relevant control vector and subsequently stimulated with TLR agonists for 6 and 9 hours. *Figures 4.22A and B,* show graphical representations of the relative levels of basal activity, and the extent of NFκB activation following poly(I:C) stimulation. These results were expected to show little variation in augmentation activity upon stimulation between LMP2A or LMP2B expressing cells and controls, in line with the results for the AP1 promoter analyses. Or if differences in activation levels were observed it was reasoned that LMP2A expressing cells would show decreased NFκB activity, in agreement with lower levels of IFNβ production. Surprisingly however, it is clear from both the CNE-2, *Figure 4.22.A* and the H103, *Figure 4.22.B* cell panels that this was not the case. Although the basal level of NFκB activity were similar between control and LMP2B expressing cells, LMP2A expressing cells displayed elevated levels of NFκB activity, up to a six fold increase in basal NfκB activity, a result that is at odds with the IFNβ data described earlier. Upon stimulation with poly(I:C) little increases in activity are observable across all cell types compared to their basal levels. These results are recapitulated in the H103 cell panel indicating that it is not a cell type specific phenomenon, with LMP2A expressing cells displaying a two to three-fold increase in NFκB activity compared with controls and LMP2B expressing cells.

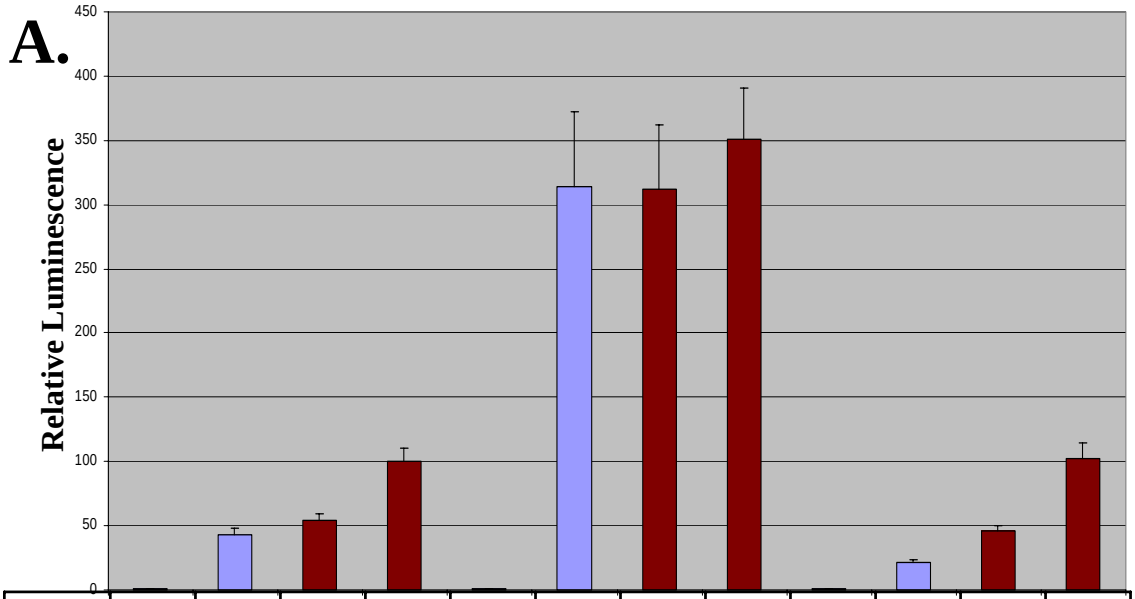
Figure 4.22 Modulation of NFκB activity by LMP2A in epithelial cells upon stimulation of TLR3.

(A) CNE-2 cells and (B) H103 cells, expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an NFκB (3Enh-κB-ConAluc) luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with 5μg/ml poly(I:C) for six and nine hours, harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the NFκB promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=4).

(A) CNE-2 cells: Basal levels of activity of the NFκB reporter are increased in LMP2A compared to both control and LMP2B expressing samples. Upon stimulation with poly(I:C), activity is augmented in control and LMP2B expressing cells at the nine-hour time point compared to basal levels. LMP2A expressing cells do not show increased activity beyond the basal response.

(B) H103 cells: A similar situation to that for CNE-2 cells is observed, whereby activity is augmented in control and LMP2B expressing cells upon stimulation with poly(I:C) and activity in LMP2A expressing cells is not augmented beyond basal responses.

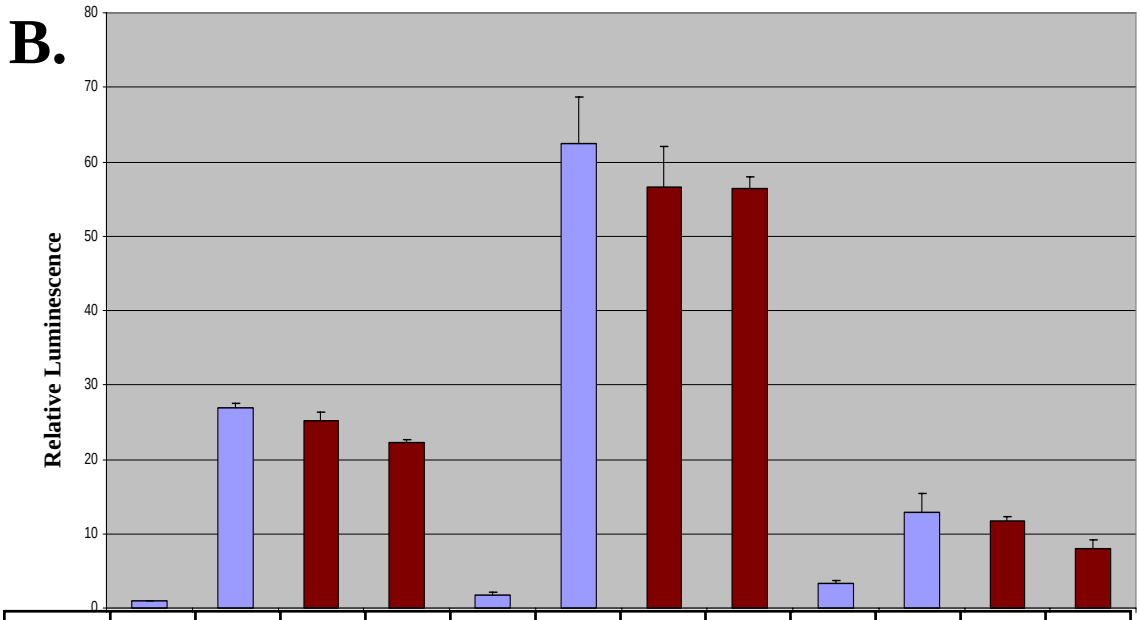
A.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
NFκB	-	+	+	+	-	+	+	+	-	+	+	+
P(I:C)	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**

CTRL **LMP2A** **LMP2B**

B.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
NFκB	-	+	+	+	-	+	+	+	-	+	+	+
P(I:C)	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**	-	-	**6hrs**	**9hrs**

CTRL **LMP2A** **LMP2B**

4.4.4.2 Effect of LMP2A on TLR4-induced NFκB activity

Extending this to examine the effect of other TLR agonists on NFκB signalling was necessary in light of the results from poly(I:C) stimulation. The CNE-2 and H103 cell panels were treated with LPS to activate TLR4. *Figures 4.23.A,B,* show graphical representations of the results for both cell panels. In agreement with the previous investigation, NFκB activity in LMP2A expressing cells appeared higher compared to LMP2B expressing cells and the control counterparts. However, upon stimulation with LPS, NFκB activity in control cells increased considerably, with a similar result observed in LMP2B expressing cells. Surprisingly, LMP2A expressing cells did not show increased NFκB activation and appeared refractory to TLR4 stimulation. A similar pattern of results can be observed in the H103 cell panel, *Figure 4.23.B,* indicating once again that the effects are not cell type specific. Comparing these results with those obtained from investigations of poly(I:C) stimulation demonstrates an intriguing phenomenon. LMP2A expression in epithelial cells appears to augment NFκB signalling at the basal level and upon stimulation with poly(I:C) no increases in activity are observed. But stimulation of TLR4 signalling results in augmentation of signalling in control and LMP2B expressing cells and abrogation of the primed response in LMP2A expressing cells. A rationale for these data may be explained through examining the signalling cascades involved in TLR3 and TLR4 activity. As mentioned previously, *Chapter 1, section 1.7,* TLR3 signalling occurs signals in a MyD88 independent manner, whilst TLR4 signalling can occur in both MyD88 dependent and independent manners. To further determine whether this effect could explain, at least in part, these data, analysis of NFκB signalling in response to TLR members that signal solely through MyD88 was undertaken.

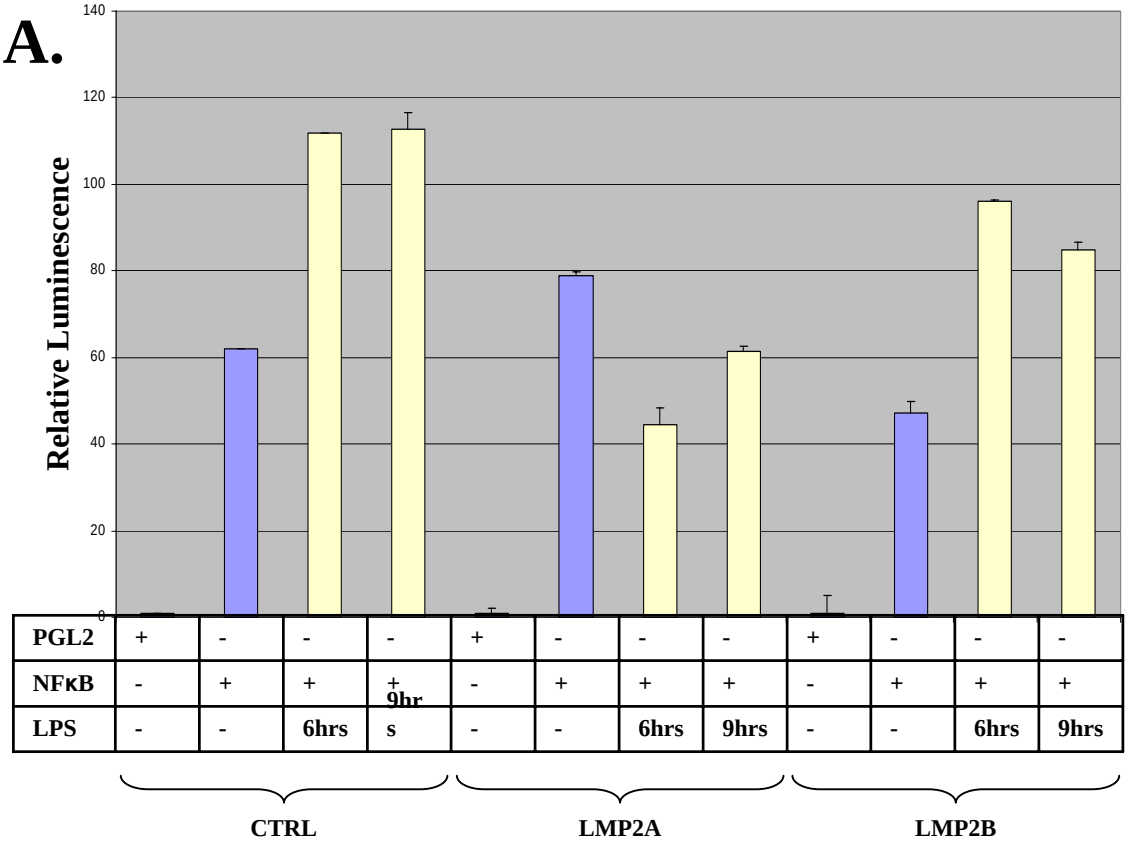
Figure 4.23 Modulation of NFκB activity by LMP2A in epithelial cells upon stimulation of TLR4.

(A) CNE-2 cells and (B) H103 cells, expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an NFκB (3Enh-κB-ConAluc) luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with 200ng/ml LPS for six and nine hours, harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the NFκB promoter and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3).

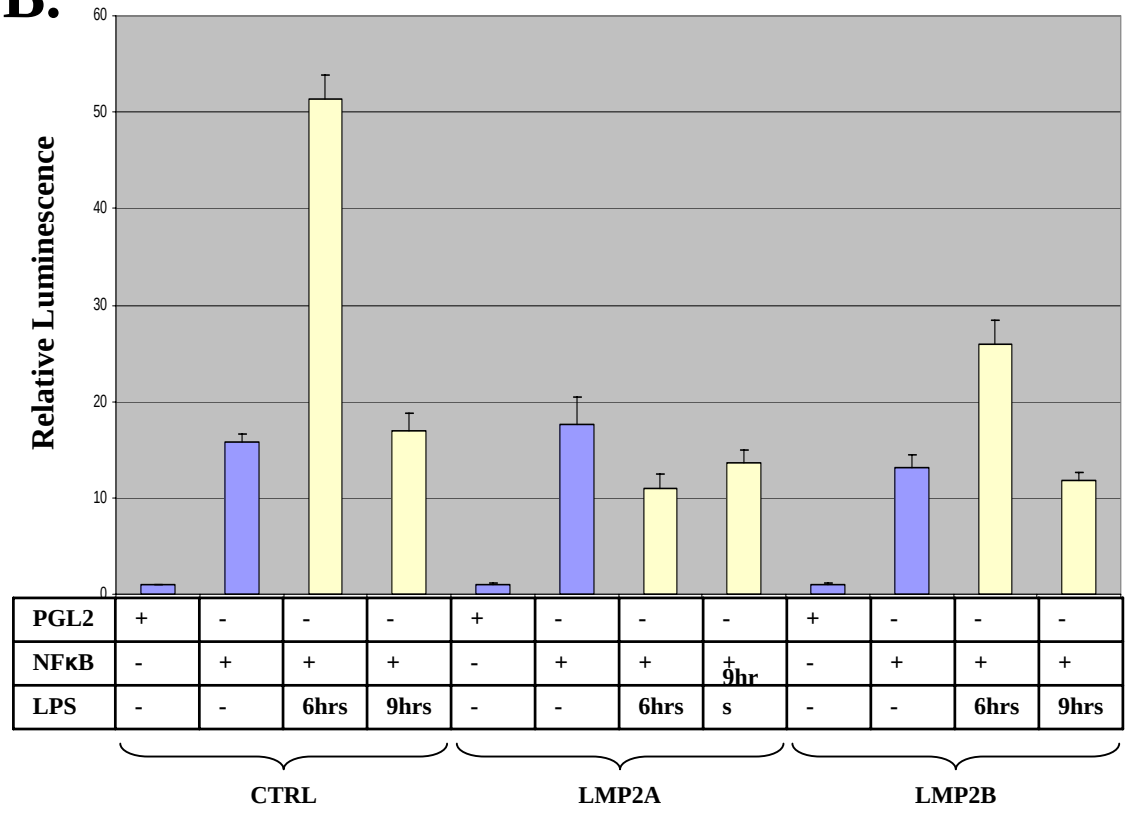
(A) CNE-2 cells: Basal levels of activity of the NFκB reporter are increased in LMP2A compared to both control and LMP2B expressing samples. Upon stimulation with LPS activity is augmented in control and LMP2B expressing cells compared to basal levels. LMP2A expressing cells do not show increased activity and activity was measured as less than basal levels.

(B) H103 cells: Basal levels of activity of the NFκB reporter are increased in LMP2A compared to both control and LMP2B expressing samples. Upon stimulation of TLR4, reporter activity was augmented in control cells at the six-hour time point and to a lesser extent in LMP2B expressing cells. LMP2A expressing cells show no increase in activity post-stimulation with LPS.

A.


Relative Luminescence
140
120
100
80
60
40
20
0
PGL2 + - - - + - - - + - - -
NFκB - + + + - + + + - + + +
LPS - - 6hrs 9hrs - - 6hrs 9hrs - - 6hrs 9hrs
CTRL
LMP2A
LMP2B

B.


Relative Luminescence
60
50
40
30
20
10
0
PGL2 + - - - + - - - + - - -
NFκB - + + + - + + + - + + +
LPS - - 6hrs 9hrs - - 6hrs 9hrs - - 6hrs 9hrs
CTRL
LMP2A
LMP2B

4.4.4.3 LMP2A attenuates TLR7, TLR8 and TLR9-induced NFκB activity.

Analyses performed to measure NFκB activity upon stimulation with TLR7, TLR8 and TLR9 agonists in the CNE-2 cell panel are shown in *Figure 4.24.A, B and C*. Here, basal levels of NFκB activity appear to be augmented in cells expressing LMP2A compared to control and LMP2B expressing cells. In agreement with experiments involving LPS stimulation, significant increases in NFκB activity were observed after stimulation with Imiquimod, ssRNA and CpG DNA in control and LMP2B expressing cells. Taken together, these data with those for the LPS stimulation of epithelial cells, indicate that MyD88 dependent TLR activation of NFκB signalling is attenuated in LMP2A expressing cells. MyD88 independent signalling, as in the case of TLR3 activity appears unaltered in LMP2A expressing cells. Basally however, an increase in NFκB activity is observed and although this is at odds with previously published data from our group (Stewart et al 2005), which demonstrated decreased in NFκB mediated IL-6 activity, these data are deemed important into the analysis of the complex mechanism of LMP2A action. The previous publication dealt with the affect of LMP2A expression in an adeno-carcinoma cell line, Ad/Ah, whereas here the epithelial cell lines examined are the NPC derived, CNE-2 cell line and the squamous cell carcinoma, H103, which are postulated to bear more physiological resemblance and thus significance to NPC research. As mentioned above however, this measurement of NFκB activity was performed using a luciferase reporter construct for one of NFκB's target genes. Many signalling pathways can activate NFκB activity and as such in order to demonstrate that LMP2A expression could modulate p65 activity in a more direct manner, a translocation assay and an EMSA were performed.

4.4.4.4 LMP2A augments poly(I:C) induced p65 translocation

Representative confocal images of a p65 translocation assay are shown in *Figure 4.25*. Briefly, CNE-2 cells, seeded into wells of a teflon-coated microscope slide were serum-starved for 3

hours prior to stimulation with poly(I:C). After 6 hours, cells were fixed, permeabilised and subjected to immunofluorersence staining with a mAb/antiserum specific for p65. Although no significant differences in the basal levels of p65 expression were apparent between control cells and those expressing LMP2A and LMP2B, a robust p65 translocation from the cytosol to the nucleus was observed upon poly(I:C) stimulation. Although this effect was observed in all cell types it was more pronounced in cells expressing LMP2A, findings which suggest that LMP2A primes NFκB responses in response to poly(I:C)/TLR3 activation. Given the basal decrease in expression of TLR3 in LMP2A positive cells, it is difficult to reconcile how priming of NFκB signalling could be mediated through this receptor. However, although TLR3 expression is decreased in LMP2A expressing cells basally it is not absent and there is a priming of NFκB responses without any stimulation. It is therefore hypothesised, that sufficient receptor is expressed to further initiate an already primed response resulting in an increased level of p65 translocation in LMP2A expressing cells compared to their control counterparts. The importance of TRAF proteins to this should also not be discounted and is discussed in detail below.

4.4.4.5 DNA binding of p65 in presence of LMP2A.

To determine whether the increased nuclear translocation of p65 in LMP2A expressing cells treated with poly(I:C) was associated with increased DNA binding, EMSAs were performed using an IRD-700 labelled oligonucleotide NFκB probe containing consensus p65 binding sites. Previously it has been shown protein binding to the PRDII domain in the IFNβ promoter was reduced in cells expressing LMP2A and LMP2B. It is thought that this could be a direct effect of disrupting enhancesome formation through IRF3 binding attenuation at neighbouring sites rather than decreases in NFκB activity, which as shown above appears to be augmented in the presence of LMP2A (see *Figure 4.22*). Briefly, nuclear extracts isolated from basal and

poly(I:C) stimulated cells, along with the relevant controls, were incubated with the IRD-700 labelled NFκB probe, separated by gel electrophoresis and the extent of protein binding measured after scanning of the EMSA gel on a LiCor© infrared laser device. DNA:Protein complexes were resolved and the binding of proteins to the synthetic oligonucleotide NFκB probe quantitated by measuring relative fluorescence intensities.

A scan of the gel and a graphical representation of fluorescence intensities are shown in *Figure 4.26*. Mirroring data obtained from the immunofluorescence staining, the basal levels of binding between control and LMP2A or LMP2B expressing cells do not yield significant differences. Upon poly(I:C) stimulation however, increased p65 binding is observed in cells stably expressing LMP2A compared to both control and LMP2B expressing cells.

It is hypothesised that the augmentation of NFκB activity in LMP2A expressing cells may be due to p65 independent mechanisms of activation. It is thought that this basal augmentation could prime p65 responses, allowing them to become activated beyond control levels upon stimulation of TLR3 signalling with poly(I:C), which could account at least in part for the increased binding and translocation of the p65 protein. Further analysis of the specific alterations in NFκB signalling by LMP2A are necessary to test and confirm this hypothesis, which is beyond the scope of this thesis. Preliminary analysis using the TLR4 agonist LPS, which investigated p65 binding, showed that LMP2A expressing cells show decreased p65 binding post-stimulation compared with controls (data not shown), instigating MyD88 dependent signalling as a mechanism of this attenuated affect and highlighting the differential effects of LMP2A on the various pathways of the TLR signalling network.

Figure 4.24 Modulation of NFκB activity by LMP2A in CNE-2 cells upon stimulation of TLR7, TLR 8 and TLR9.

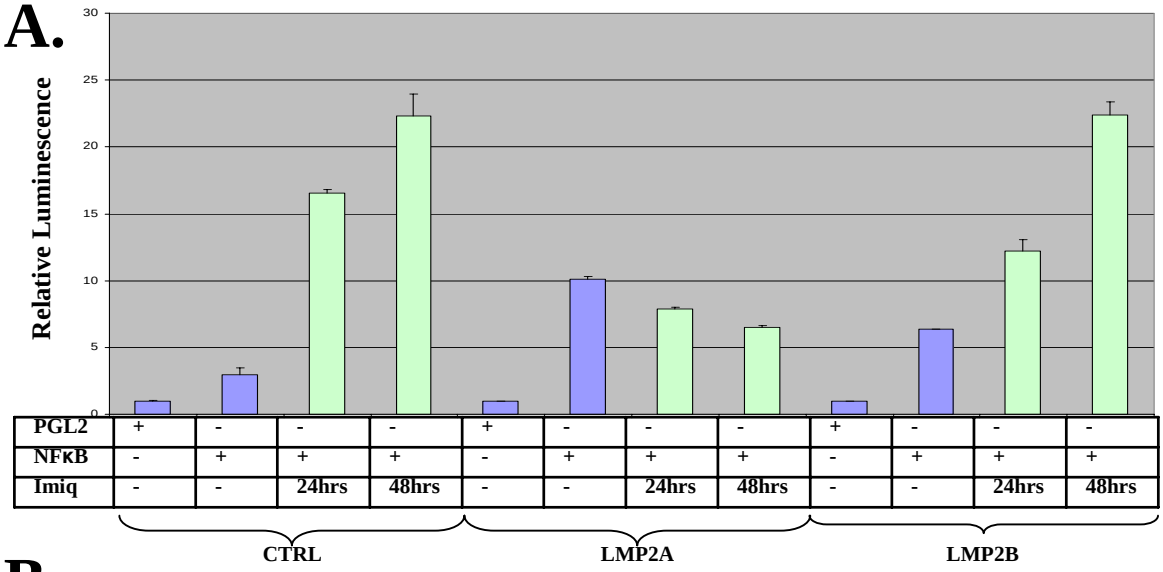
CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with a NFκB luciferase reporter construct (3Enh-κB-ConAluc) and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover for 24 hours and subsequentially stimulated with (A) 2.5μg/ml Imiquimod for twenty-four and forty-eight hours (TLR7), (B) 1μg/ml ssRNA for six and nine hours (TLR8) or (C) 1μM ODN2006 CpG DNA for six and nine hours (TLR9), harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample set standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the NFκB construct and also stimulated cells with the unstimulated basal level of promoter activity. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3).

(A) TLR7: Basally NFκB activity is augmented in LMP2A expressing cells compared with their control and LMP2B expressing counterparts. Post-stimulation with Imiquimod, activity is increased in control and LMP2B expressing cells but these increases are attenuated in cells expressing LMP2A.

(B) TLR8: NFκB activity is increased in control and LMP2B expressing cells upon stimulation with ssRNA, however this augmentation is abrogated in LMP2A expressing cells.

(C) TLR9: Basally NFκB activity is augmented in LMP2A expressing cells compared to control and LMP2B expressing samples. Upon stimulation with ssRNA increased activation occurs after nine hours in control and LMP2B expressing samples but this activation is attenuated in LMP2A expressing cells.

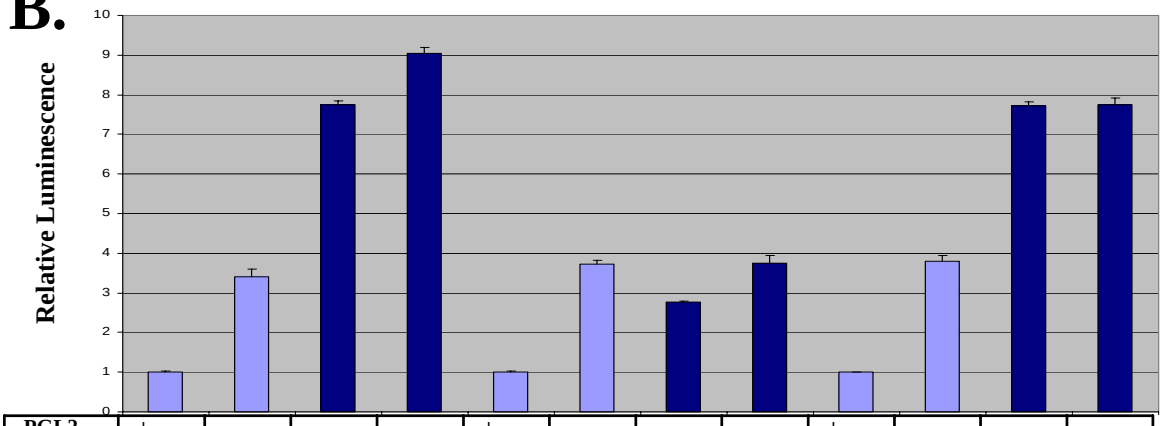
A.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
NFκB	-	+	+	+	-	+	+	+	-	+	+	+
Imiq	-	-	24hrs	48hrs	-	-	24hrs	48hrs	-	-	24hrs	48hrs

CTRL LMP2A LMP2B

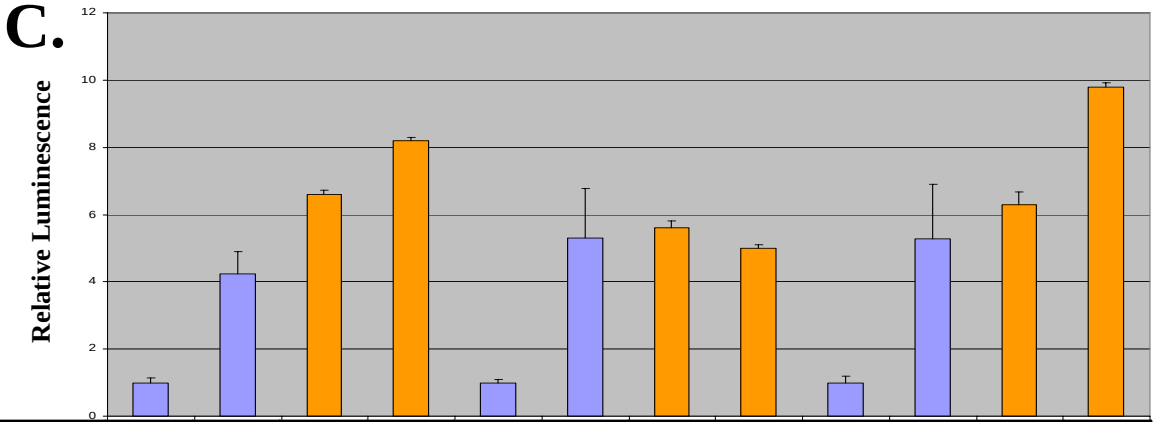
B.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
NFκB	-	+	+	+	-	+	+	+	-	+	+	+
ssRNA	-	-	6hrs	9hrs	-	-	6hrs	9hrs	-	-	6hrs	9hrs

CTRL LMP2A LMP2B

C.



PGL2	+	-	-	-	+	-	-	-	+	-	-	-
NFκB	-	+	+	+	-	+	+	+	-	+	+	+
CpG	-	-	6hrs	9hrs	-	-	6hrs	9hrs	-	-	6hrs	9hrs

CTRL LMP2A LMP2B

Figure 4.25 Effect of LMP2A expression on p65 translocation in CNE-2 cells upon stimulation of TLR3.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 1 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day samples were stimulated *in situ,* with 5µg/ml poly(I:C) for six hours. Slides were fixed and permeabilised with 4% PFA and 0.5% Triton-X and immunofluorescent staining performed on both stimulated and unstimulated samples using an antibody specific to p65. Samples were viewed and photographed using a Zeiss LSM510-meta confocal microscope and representative images are shown. Basal levels of p65 protein detected did not alter greatly across the cell panel. Upon stimulation with poly(I:C) however, translocation of p65 from the cytosol to the nucleus is observed in control and LMP2B expressing cells. The level of this translocation in LMP2A expressing cells however, is markedly augmented when compared with that observed in the other cell samples.

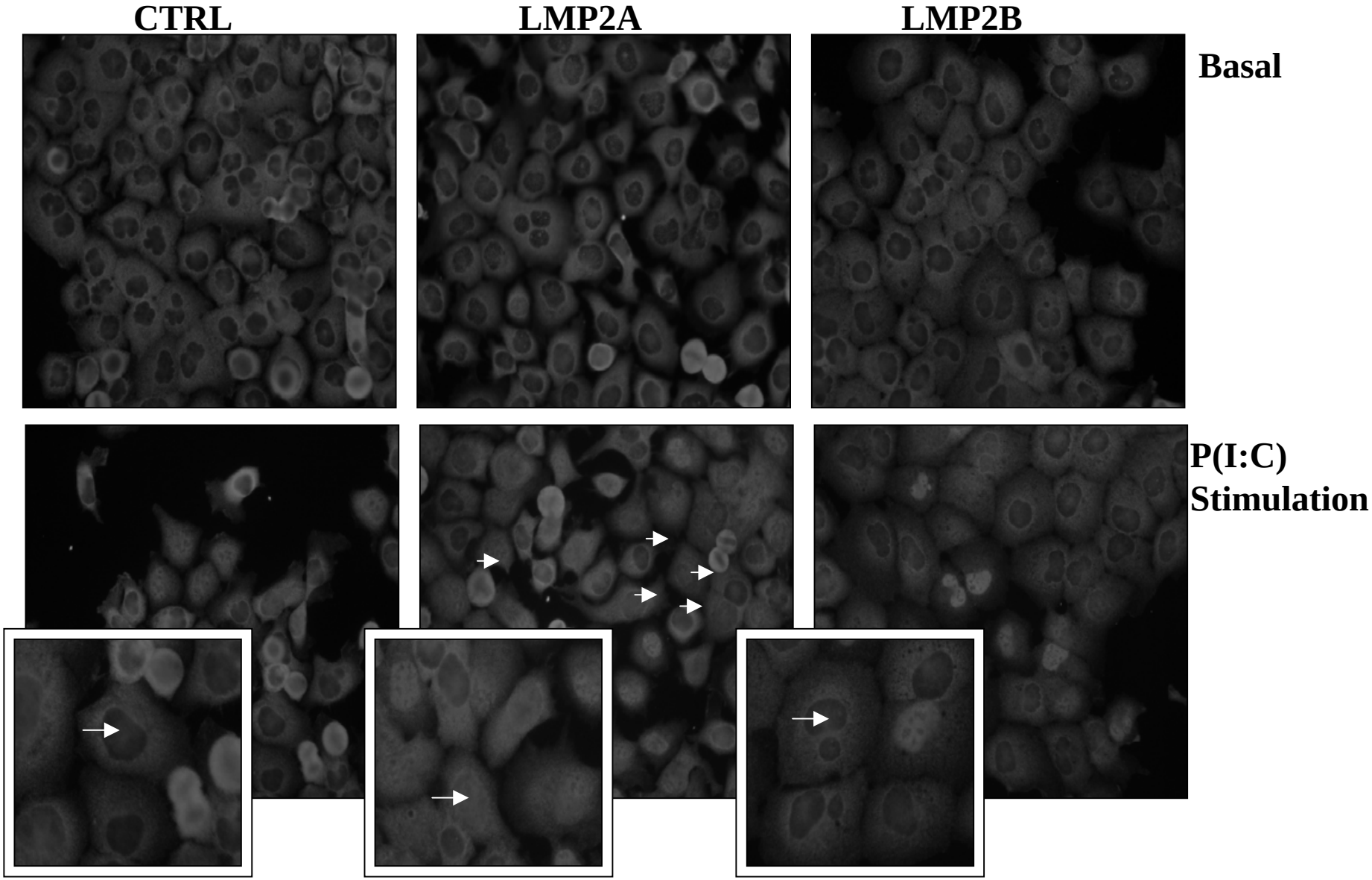
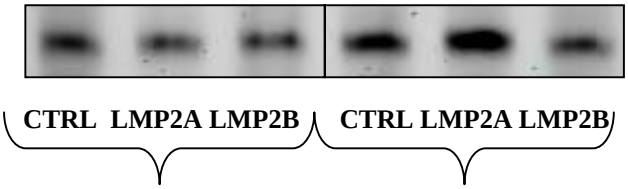

CTRL
LMP2A
LMP2B
Basal
P(I:C)
Stimulation

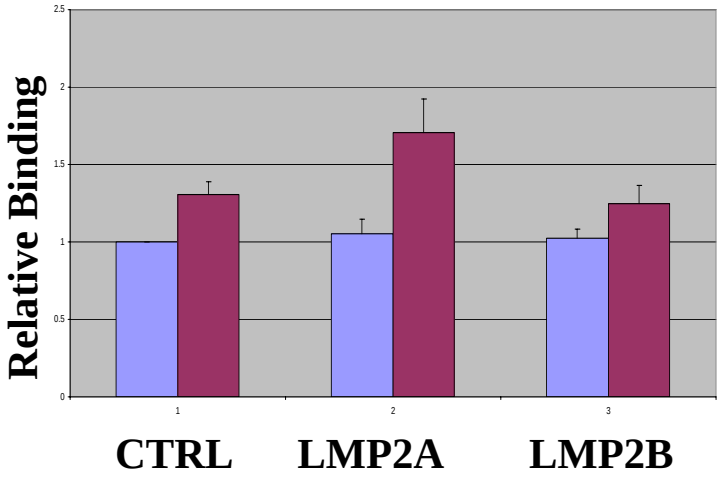
Figure 4.26 Effect of LMP2A on p65 nuclear binding in CNE-2 cells upon stimulation of TLR3.

CNE-2 cells expressing either a control neomycin resistance cassette, LMP2A or LMP2B were seeded at a density of 1 x 10^6 cells per 10cm^2 dish and allowed to adhere overnight at 37˚C and 5% CO_2. The following day samples were stimulated with 5μg/ml poly(I:C) for six hours and nuclear and cytosolic extracts isolated from both control and stimulated samples. The nuclear extracts were then incubated with IRD-700 labelled probes specific to the p65-binding domain of promoters. Samples were then separated by gel electrophoresis and analysed using infrared scanning and imaging. Densitometry was also performed and histograms created depicting mean differences in binding affinities between cell types with and without stimulation. Histograms and representative images of the infrared scanning are shown (mean ± SD; n=3). Basally no significant differences are observable between cell types, upon stimulation with poly(I:C) binding is increased across the cell panel. This increase is augmented in LMP2A expressing cells compared to the effect in control and LMP2B expressing counterparts.

P65 P(I:C)



Densitometry

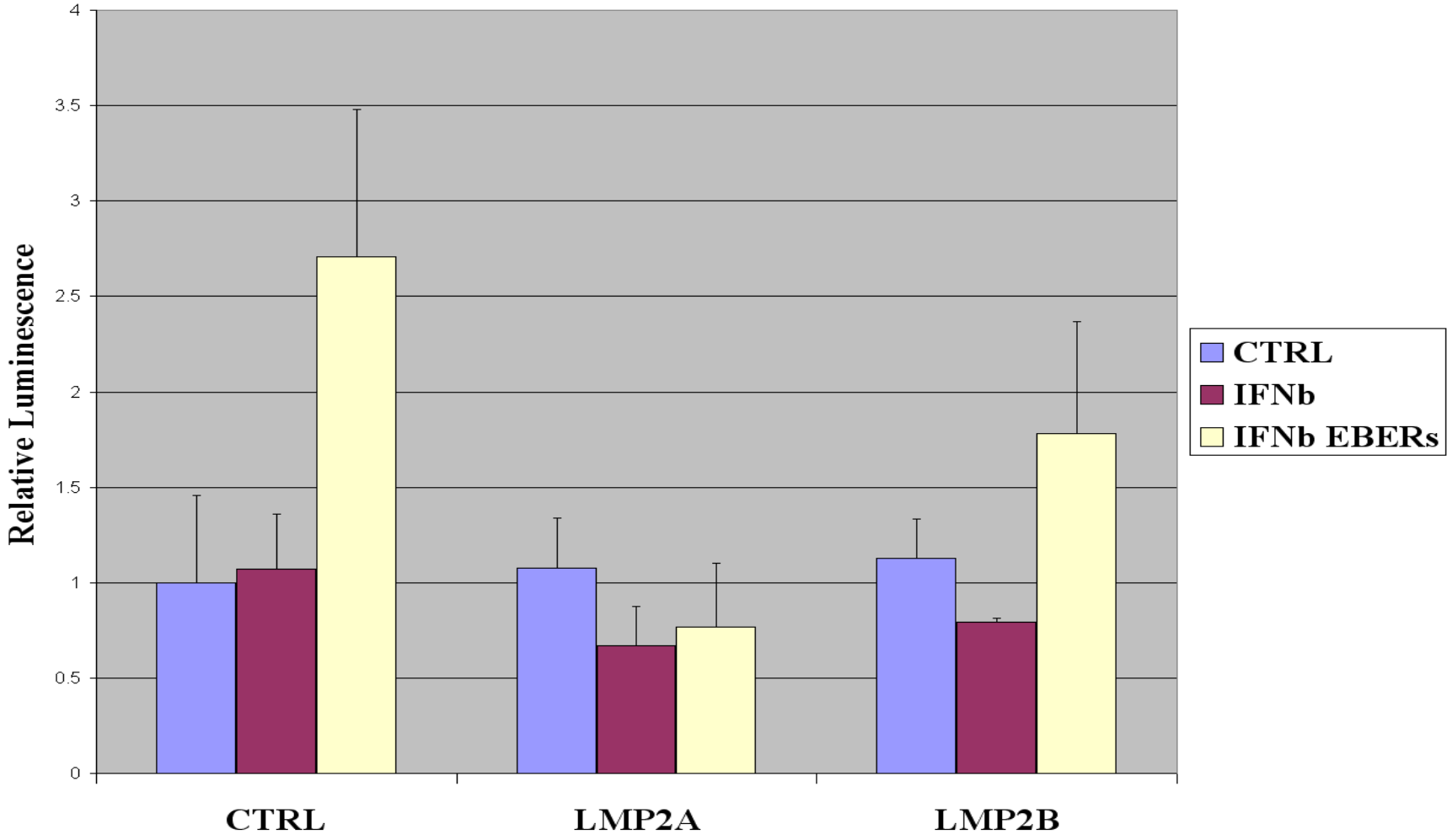


4.5 LMP2A and, to a lesser extent, LMP2B, modulate EBER1/2 induction of IFNβ.

As mentioned previously, in the context of EBV infection, poly(I:C) and ssRNA are the most physiologically relevant TLR agonists given that EBV encodes both dsRNA (i.e. EBER1/2) and ssRNA (BART transcripts) at very high levels in latently infected epithelial cells. Indeed, numerous studies have demonstrated that the EBER RNAs can stimulate IFNβ expression when introduced into epithelial cell lines (Takada, 2001) although it is presently unknown if the BARTs can stimulate TLR8. Although speculative, it is possible that modulation of TLR3 signalling by LMP2A provides a mechanism by which EBV can subvert the "anti-viral" effects of EBER expression on the innate immune signalling network. To further investigate this and to ascertain whether EBER1/2 expression could mount a type I interferon response, IFNβ promoter luciferase reporter assays were performed using plasmids expressing EBERI and EBER2. The CNE-2 panel were transfected with the reporter plasmids, allowed to recover for 16 hours and subsequentially transfected with a plasmid expressing the EBER1/2 RNAs, as described in *section 2.2.1,* and incubated for an additional 24 hours to allow EBER1/2 expression. A graphical representation of the data is shown in *Figure 4.27*. In control cells it is clear that IFNβ promoter activity is activated upon EBER1/2 expression. Comparing this activation with the effect in cells expressing LMP2A or LMP2B, it can be seen that the LMP2B samples that there is significant activation but it is slightly attenuated. LMP2A expressing cells do not however show any activation of IFNβ promoter activity. This demonstrates the ability of LMP2A to limit EBER1/2-induced type I interferon responses and infers physiological and functional relevance to LMP2A's modulation of TLR3 signalling in the context of whole virus infections.

Figure 4.27 Effect of LMP2A expression on EBER induced IFNβ activity in CNE-2 cells.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or LMP2B were seeded at a density of 2.5 x 10^5 cells per well of a six-well dish and allowed to adhere overnight at 5% $C0_2$ and 37°C. The following day cells were transfected with an IFNβ promoter luciferase reporter construct and a plasmid expressing Renilla firefly luciferase or relevant controls. Cells were allowed to recover overnight and subsequentially transfected with a plasmid expressing both EBERI and EBERII or relevant controls and allowed to recover for 24 hours at 5% $C0_2$ and 37°C. The following day samples were harvested and read using a luminometer. Histograms are shown which, display the relative luminescence of each sample set standardised against Renilla firefly signals and comparisons made between control cells transfected with a control vector with those transfected with the IFNβ promoter construct and also EBER expressing cells with those transfected with control plasmids. LMP2A and LMP2B expressing cells are normalised against the activity measured in the control cells with and without stimulation (mean ± SD; n=3). Basally no differences in promoter activity are detected between control cells and those expressing LMP2A or LMP2B. Upon expression of the EBERs IFNβ promoter activity is augmented in control and to lesser extent in LMP2B expressing cells. This increase in activity is attenuated in cells expressing LMP2A.


Relative Luminescence
4
3.5
3
2.5
2
1.5
1
0.5
0
CTRL
LMP2A
LMP2B
CTRL
IFNb
IFNb EBERs

4.6 LMP2A modulates cytokine release upon TLR stimulation

4.6.1 LMP2 attenuates IFNβ production

To further establish the physiological effects of LMP2A and LMP2B on innate immune signalling, cytokine profiling was performed on the CNE2 panel using a commercially available custom cytokine antibody array (Tebu-Bio). This analysis served to explore the modulatory role of LMP2A and LMP2B in TLR-mediated cytokine secretion. The cytokine array was performed as described in *section 2.10*. The CNE2 cell panel was either left untreated or stimulated with the TLR3 and TLR4 agonists, poly(I:C) and LPS for 3, 6, 9 and 12 hours. Cell culture supernatants were removed and applied to small array units containing duplicate embedded antibodies specific for 26 cytokines. Total RNA was extracted from each of the samples and RT-QPCR performed for IFNβ in the manner described in *section 2.9.2.* This identified the ideal time point to perform the array analysis. *Figure 4.28,* shows a graphical representation of the data. Three of the time-points chosen are depicted: 6 hours, 24 hours and finally 72 hours; intermediate time points between these show similar patterns of expression (data not shown). This analysis showed that compared to control and LMP2B, LMP2A attenuates IFNβ production in response to TLR3 and TLR4 agonist stimulation. It was decided, using these data as a guideline, that supernatants from samples stimulated with the agonists for 72 hours would best serve this experiment, given the differences in IFNβ production by RT-QPCR and also allowing for sufficient time for secretion of cytokines.

4.6.2 Cytokine profiling of the CNE-2 cell panel

A representative cytokine antibody array is shown in *Figure 4.29.A*. Comparing the arrays visually, it is apparent that LMP2A expressing cells show modulation of the cytokine profiles compared to control and LMP2B expressing cells, a tenet that is most pronounced upon stimulation with the TLR agonists. The effect of stimulation on cytokine release is also

observed when comparisons are made between control and TLR-stimulated samples within the same cell type. Densitometric analysis was performed by comparing signals from each pair of embedded antibodies with the internal positive controls. This allowed standardisation of each array and also direct comparisons between stimulated and unstimulated samples and between control and LMP2A and LMP2B expressing cells.

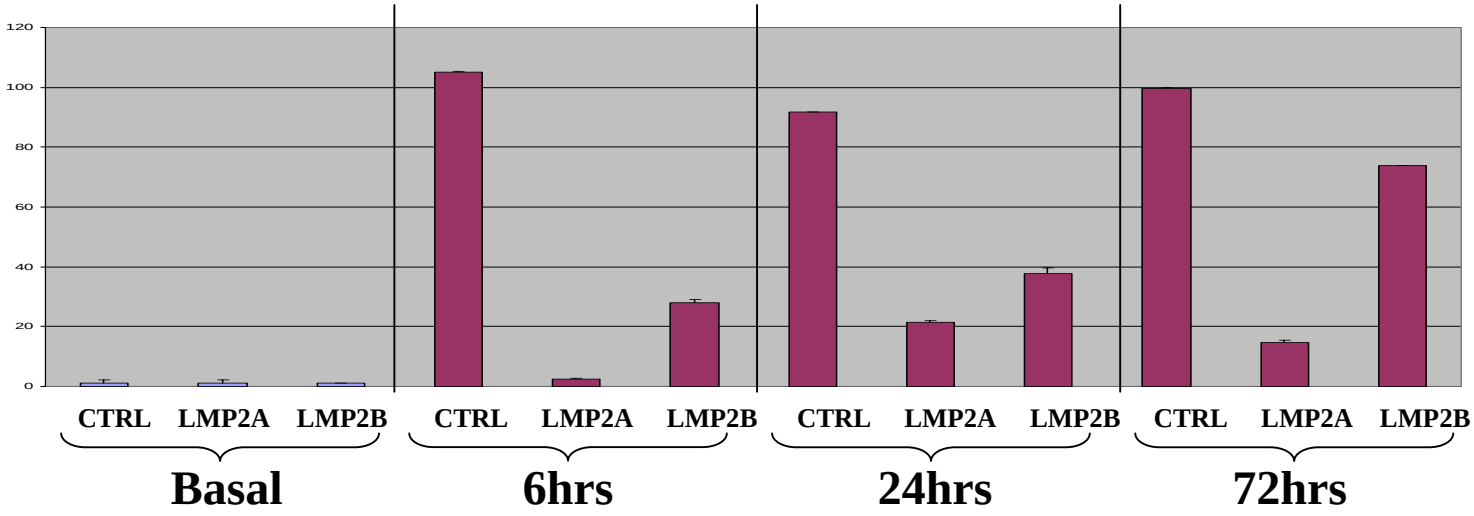
Figure 4.29 B, shows the cytokines whose expression was most modulated in response to TLR stimulation in the CNE2 panel. Table B top, consists of the levels of two of the cytokines examined, levels of which were deemed to be markedly decreased in LMP2A expressing cells upon stimulation with the TLR agonists. Expression of RANTES or CCL5 as mentioned above is controlled by IRF3 and so decreased levels of its secretion add further weight to the argument that LMP2A expression abrogates IRF3 activity. Soluble ICAM-1 is a cytokine involved in pro-inflammatory response and as such decreases in its expression in LMP2A expressing cells compared to controls sits well with the IRF3 and IFNβ signalling data. Table B bottom, shows those cytokines whose expression is increased in LMP2A expressing cells relative to controls upon TLR agonist stimulation. An increase in IL-6 and IL-8 secretion in LMP2A expressing cells is in general agreement with the increases in NFκB activity outlined above. The increases however in the cytokines, IL-1α, IL-1Rα and IP-10 were surprising given the attenuation of the type I interferon responses described previously and confirmed here. Further analysis into these data is necessary to elucidate the exact nature of these alterations. Overall however, it is clearly demonstrated through this assay that the consequences of LMP2A's effect on the TLR signalling is borne out functionally through alteration of the cytokine complement of the cells examined.

Figure 4.28 Modulation of IFNβ transcription by LMP2A in CNE-2 post-stimulation of TLR3 and TLR4.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A or full length LMP2B were stimulated with the artificial agonists for (A) TLR3, 5μg/ml poly(I:C) and (B) TLR4, 200ng/ml LPS, for six hours, nine hours, twelve hours, twenty four hours, thirty six hours, forty eight hours and seventy two hours. RNA was extracted from each cell type, pre- and post- treatment and cDNA synthesised. RT-QPCR was performed using a primer-probe set specific for IFNβ and using the housekeeping gene GAPDH as an internal baseline control. Data was analysed using Microsoft Excel and the fold change differences calculated between cell types and between each cell type before and after stimulation. Histograms are shown displaying mean fold change differences, for control samples and three of the above time points, (mean = +/- SD, n=3). Upon stimulation with either (A) or (B) agonists IFNβ expression is augmented in control cells and to a lesser extent in those expressing LMP2B. Samples expressing LMP2A show attenuated responses for both stimulations, in terms of IFNβ expression.

A.

Poly(I:C) Stimulated



B.

LPS Stimulated

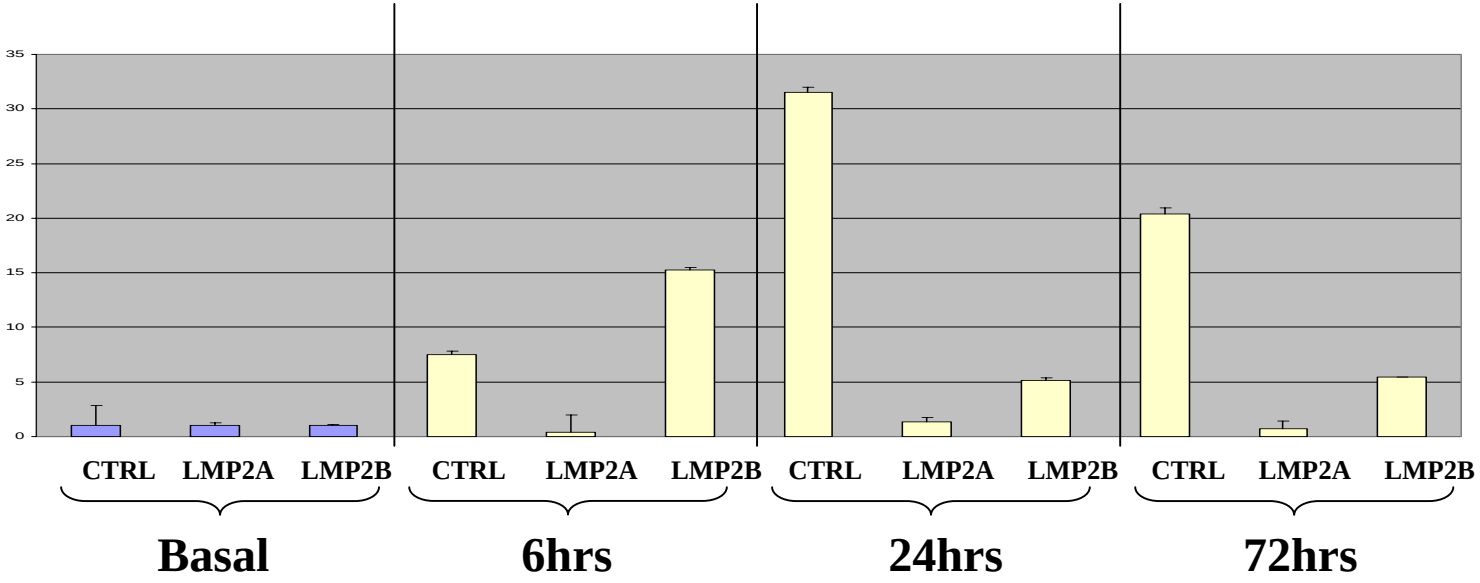


Figure 4.29 Effect of LMP2A expression on cytokine production in CNE-2 cells upon stimulation of TLR3 and TLR4.

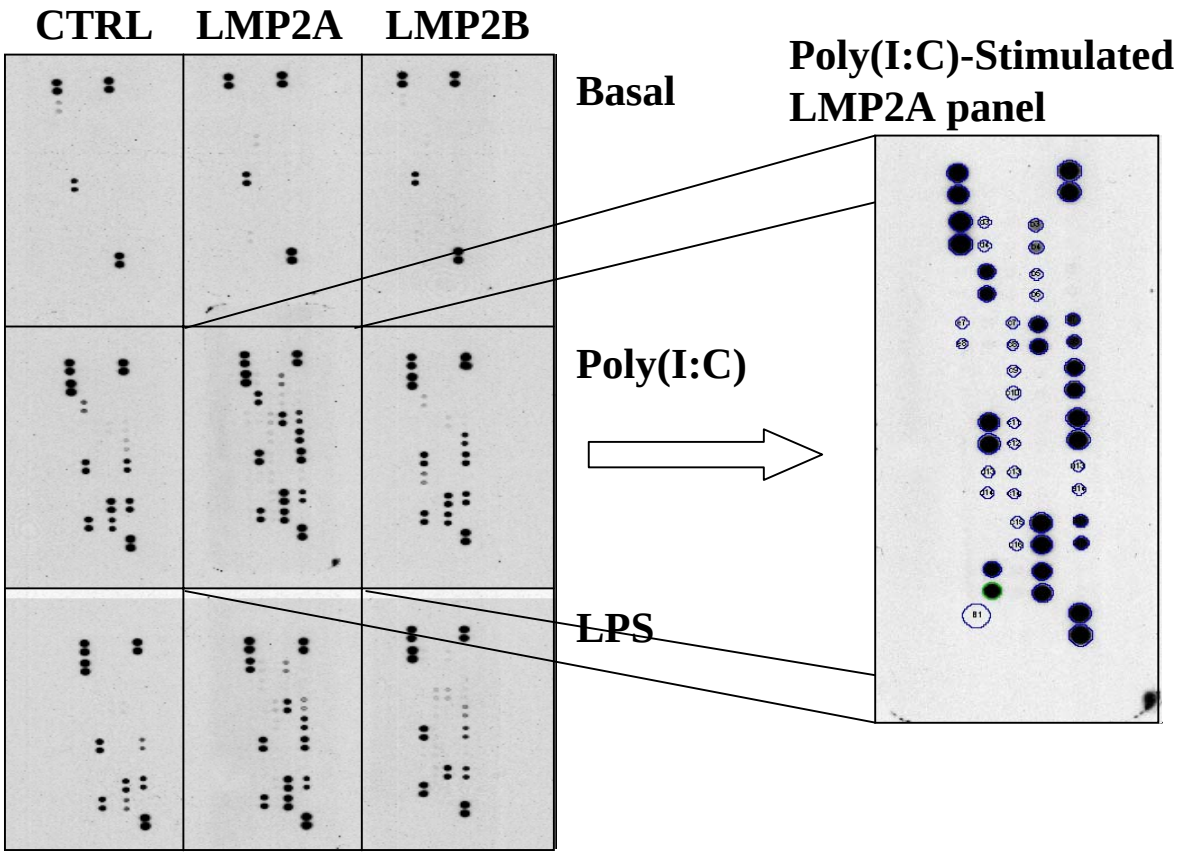
Supernatants were removed from CNE-2 cells expressing either a control neomycin resistance cassette, LMP2A or LMP2B, pre and post-stimulation with 5μg/ml poly(I:C) for 72 hours or 200ng/ml LPS. Samples were incubated with array membranes embedded with antibodies specific to a variety of cytokines, (R & D Systems) washed and signals detected using chemiluminesence.

(A) Scans of the films of the array membranes upon signal detection are shown including an example of the method of densitometric analysis used, performed with a BioRad scanner.

Data analysis was performed using Microsoft Excel, whereby the results of densitometric analysis, i.e. the binding affinities of each of the cytokines was normalised against an internal membrane control for each array. Each binding affinity value was then normalised against the unstimulated Neomycin CTRL value. Stimulated samples for each cell type, CTRL, LMP2A and LMP2B expressing cells were then compared to their respective basal counterparts. Tables were then formed displaying the cytokines with the greatest degree of difference, upon receptor stimulation.

(B) The top section of the Table depicts the cytokines displaying attenuated expression in the presence of LMP2A and/or LMP2B compared to controls, upon stimulation with agonists for TLR3 or TLR4. The bottom section of the Table depicts the cytokines displaying increased production and secretion in the presence of LMP2A and/or LMP2B compared to controls, upon stimulation with TLR3 or TLR4.

A



B

Cytokine		Basal			P(I:C)			LPS	
	CTRL	LMP2A	LMP2B	CTRL	LMP2A	LMP2B	CTRL	LMP2A	LMP2B
RANTES	1.00	1.91	1.33	12.12	6.07	7.84	9.95	3.89	9.57
sICAM-1	1.00	0.91	0.94	6.71	4.14	5.51	4.05	4.02	3.40

Cytokine		Basal			P(I:C)			LPS	
	CTRL	LMP2A	LMP2B	CTRL	LMP2A	LMP2B	CTRL	LMP2A	LMP2B
G-CSF	1.00	0.90	0.92	1.06	4.45	1.03	0.90	1.84	1.17
GM-CSF	1.00	0.91	0.93	1.08	10.78	4.83	0.90	6.30	1.91
GROalpha	1.00	0.92	0.93	6.58	14.16	8.18	2.05	9.06	3.30
IL-1alpha	1.00	0.92	0.93	1.03	2.33	1.03	0.91	1.78	1.17
IL-1ra	1.00	0.90	0.92	1.05	10.60	1.14	0.96	9.41	1.47
IL-6	1.00	0.91	0.95	6.70	9.03	5.32	3.94	6.95	5.46
IL-8	1.00	0.90	0.94	5.67	11.19	6.19	2.01	11.50	2.32
IP-10	1.00	0.90	0.92	2.41	9.02	1.20	0.92	1.07	1.08

4.7 Discussion and Future Work

Data presented in this chapter confirm and extend the original observations of Shah and colleagues (Shah et al., 2009) demonstrating an ability of LMP2A and LMP2B to modulate innate immune response in nasopharyngeal epithelial cells. This study has revealed modulation of IFN receptors, regulatory factors (IRFs/SOCS) and signalling intermediates (Jak/Tyk2) by LMP2A and LMP2B, an effect that renders cells less responsive to IFN. Although LMP2A and LMP2B both modulate IFN receptor signalling, this study has identified an effect on TLR signalling that appears to be specific for LMP2A.

Although the mechanism(s) by which LMP2A modifies TLR signalling was not fully resolved, the lack of responsiveness to TLR agonists is likely linked to reduced expression of the TLRs themselves. RT-PCR, IF and FACS profiling of cells revealed a global reduction in TLR expression in LMP2A expressing cells, an effect that was only marginal for LMP2B. These observations imply that the amino-terminal signalling domain of LMP2A is required for TLR modulation and suggests that the effects of LMP2A on IFNR and TLR signalling may occur through distinct mechanisms. Although LMP2A mutants defective for the ITAM (Y74/85), Lyn kinase (Y112) and ubiquitin-ligase (WWW) domains were in the process of development, time did not permit a thorough investigation into their function. Thus, the domains and/or signalling pathways responsible for LMP2A's effects on TLR expression could not be identified.

Previous studies in B-cells have shown that the amino-terminal domain of LMP2A is required for cell signalling. By sequestering and targeting tyrosine kinases (Lyn and Syk) for ubiquitin-mediated degradation, LMP2A inhibits B-cell receptor signalling whilst providing a tonic signal to promote cell survival (Caldwell, Wilson et al. 1998). Findings presented here

demonstrate that LMP2A also acts to inhibit signalling from certain classes of receptors in epithelial cells, in this case, IFNRs and TLRs. Whether this constitutes the true function of LMP2A in EBV infected epithelial tissue is unknown, although modulation of both IFNR and TLR function may limit epithelial cell responsiveness to the growth inhibitory effects of viral infection and may facilitate viral persistence.

Findings show that LMP2A is clearly able to attenuate IFNβ promoter activity, both basally, and in response to TLR agonist stimulation. This does not appear to involve the disruption of AP1 or NFκB, as LMP2A does not influence their expression or activity of these transcription factors; in the case of the latter, LMP2A appears to prime NFκB responses. However, findings do show that the inhibitory effects on IFNβ promoter activity may be due, in part, to attenuation of IRF3 activation. Although not formally assayed, the lack of IRF3 may prevent the formation of a "complete" enhancesome complex and thus fail to initiate IFNβ transcription. Decreases in IFNβ production also has an effect in terms of cytokine output by decreasing levels of the pro-inflammatory cytokines sICAM-1 and RANTES (CCL5), which are regulated by IRF3. Putting these observations into context, it appears that whilst LMP2A and LMP2B can both limit the actions of IFN, by inhibiting signalling from both the IFNAR and IFNGR, LMP2A can also reduce the extent of TLR signalling, thereby providing a more robust blockade of anti-viral activity.

The differences in extent of signalling attenuation between the TLRs by LMP2A may be partially explained through analysis of their signalling pathways. MyD88-independent pathways are utilised only by TLR3 and TLR4, (Kawai and Akira 2006) with signalling being solely dependent on TRIF, *Chapter 1, section 1.7*. In contrast, TLR7, TLR8 and TLR9 signalling is mediated through MyD88. Preliminary results indicate that the expression of

Myd88 are equal across the CNE-2 cell panel, which is surprising given reduced expression of MyD88 at the mRNA level in LMP2A expressing cells (see *Figure 4.11*). Although the levels of Myd88 protein were not assayed, it is possible, given that the levels of mRNA are reduced, that LMP2A targets components of the TLR3 and TLR4 signalling complex for degradation. Further examination is necessary to fully characterise this apparent disparity and the roles of both MyD88 and TRIF in attenuation of signalling response. It is likely that LMP2A preferentially disrupts TRIF-IRF3-mediated signalling facilitating a robust dampening of responses from the pathways engaged by TLR3 and TLR4. TRIF engages the IRF3 and NFκB transcription factors through the engagement of TRAF3 and TRAF6, (Häcker, Redecke et al. 2006). It is known that differential ubiquitination of TRAF3 can result in its degradation or activating potential for IRF3, (Nakhaei, Mesplede et al. 2009; Tseng, Matusuzawa et al. 2010) and there maybe a role of the E3 ubiquitin ligases OTUB1, OTUB2 which negatively regulative IFN induction in this process, (Li, Zheng et al. 2010). Two additional ubiquitin ligases, which have been shown to regulate the different ubiquitin status of TRAF3, cIAP1/2, could also play a role in LMP2A's modulation of response, (Mao, Li et al. 2010). The disparity in terms of activation of NFκB and attenuation of IRF3 could be explained by these ubiquitination events. Future studies will determine whether LMP2A alters TRAF3/6 expression or activity to impede IRF3 activation yet augment TRAF6-mediated NFkB activation including analysis of the ubiquitin status of TRAF3 in the presence of LMP2A.

Signalling through TLR9 is mediated mainly by IRF7 (Honda and Taniguchi 2006) and, as shown above, some modulation of its signalling capacity is observed by LMP2A. Given this and the previous data on IRF3 activation, it would be interesting to determine the effect on the secondary wave of IFNβ production, mediated through IRF3 and IRF7 heterodimers, and to analyse whether disruption of IRF3 activity is limited to the initial wave of response or if the

secondary wave is also affected. Characterisation of IRF7 activity was beyond the scope of this thesis but a more robust understanding of LMP2A's effect on this transcription factor would yield further understanding into the viral protein's mode of action. It should be noted however that while it is hypothesised that attenuation of IRF3 binding is the main contributing factor to decreases in IFNβ production, IRF7 also binds to PRDI domains and this may also contribute to the observed modulation specifically during secondary waves of response.

The issue of NFκB "priming" was surprising given the extent of the attenuation of TLR-3 and TLR-4 driven IFNβ production by LMP2A. Although overall p65 binding is increased in cells expressing LMP2A, borne out by concomitant increases in NFκB controlled cytokine secretion (e.g. IL-6 and IL-8), p65 binding at the IFNβ promoter PRDII domain is reduced in LMP2A expressing cells. It is hypothesised that the observed decreases in binding at this domain are the result of disruption of enhancesome formation and not decreases in p65/RelA activity. The priming of NFκB activity remains the most surprising and one of the more interesting aspects of the above results. It appears that LMP2A must be acting to both repress the activity of certain TLR signalling cascades whilst initiating or promoting others. Although speculative, this could be due to the recruitment of TLR signalling components by the amino-terminal domains of LMP2A resulting in a constitutive activation of the NFκB. Co-immunoprecipitation experiments and co-localisation studies could elucidate whether this is the case. It is also necessary to further expand these studies to consider the role of the TRAF proteins (TRAF3/6) in these effects. A more in depth analyses of intermediates involved in this pathway and how their function is altered in the presence of LMP2A is necessary to confirm this. Preliminary data suggests that IκBα degradation is increased in LMP2A expressing cells compared to control and LMP2B expressing counterparts stimulated for TLR3 and TLR4 and treated with cycloheximide, an inhibitor of *de novo* protein synthesis (data not shown).

Putting the findings of LMP2A in a physiological context, data presented here shows that EBER1/2 were able to activate IFNβ production and that LMP2A could attenuate this response. In the context of a viral infection this highlights an important mechanism by which EBER1/2 can exert its effects on tumour progression (Wu, Maro et al. 2007) with other viral gene products, such as LMP2A limiting anti-viral responses. It would be interesting to observe the effect of EBER1/2 expression in the presence of LMP2A on NFκB activity. A more robust cell line model is being developed where both the EBERs and LMP2A expression are expressed stably thus allowing more in depth analysis of this effect. It is thought that this would illuminate the exact nature of the interplay of expression of these viral gene products and aid understanding of epithelial cells with latent EBV infection.

Experimentally there are many avenues worth exploring to advance the findings presented here. Key experiments have been performed in another epithelial cell line, H103, to ensure that the data is not cell type specific, and these results match those from the CNE-2 cell panel as shown above. Preliminary EMSAs for the PRDI and PRDII domains in this cell line match the CNE-2 results, indicating that LMP2A expression can also modulate IRF3 binding in this epithelial cell background. As mentioned above, to examine the possibility that LMP2A inhibits the formation of the IFNβ enhancesome, EMSAs should be performed on the PRDIII domain of the IFNβ promoter, as this is reported to be an important additional binding site for IRF3 homodimers and IRF3:IRF7 heterodimers. Analysis of the effect of LMP2A on the CBP/p300 (Yoneyama, Suhara et al. 1998) protein would also illuminate this result more clearly. Throughout these experiments stimulation of TLR3 with poly(I:C) was performed by transfecting the agonist into cells of interest in order to eliminate activation of response from the cytosolic PRRs, RIG-I and Mda5. Examination of IFNβ production upon stimulation of

these receptors would define whether attenuation of IRF3 activity is the sole reason for the LMP2A induced decreases in IFNβ, or if LMP2A is also acting further upstream in this signalling cascade to interfere with signalling, possibly at the level of the TRIF adaptor protein. Such an experiment would also define the role of the localisation of LMP2A on internal membranes in this process.

Overall, data presented here clearly show that LMP2A modulates the expression and function of a broad range of TLRs in epithelial cells. These data account in part for the attenuation of type I responses previously reported (Shah, Stewart et al. 2009) whilst highlighting important differences between LMP2A and LMP2B. Further analysis is required to pinpoint exact mechanisms for the observed modulation and to further understand the total contribution of this to disease progression. One further avenue of exploration involves the effects of LMP2A and LMP2B on LMP1 signalling. LMP1 utilises components of the IL1R/TLR signalling pathway to activate NFκB. LMP2A may inhibit or modulate LMP1 signalling whereas LMP2B may not. Physiological relevance is also attributed to these results in the context of the interplay of viral gene products during EBV latency and also in terms of cytokine secretion into the extra-cellular milieu.

CHAPTER 5

Modulation of the endosomal trafficking network by the LMP2A and LMP2B proteins.

5.1 Introduction

The endosomal/lysosomal network, as described in detail in *Chapter 1, Section 1.9,* is a series of interconnected intracellular vesicles that function to transduce signals from cell surface receptors or membrane-associated proteins. This system controls important aspects of the signalling response, regulating receptor internalisation, receptor signalling, receptor recycling and/or degradation. Many viruses express proteins that high-jack this pathway to modulate signalling, both positively and negatively, from membrane proteins involved in growth factor responses and immune modulation. As part of a strategy to limit host cell responses, many viruses target IFNRs, TLRs or MHC molecules to limit the actions of interferon and thereby facilitate immune evasion. Here, the role of the LMP2A and LMP2B proteins has been investigated in this context and their ability to disrupt endosomal trafficking and receptor turnover examined. Dysregulation of this interconnected vesicular transport system by LMP2A and LMP2B may play an important role in maintaining latent EBV infection of epithelial cells. This function may facilitate host immune recognition of EBV infected cells, thereby inadvertently promoting viral-mediated tumourigensis (Pegtel, Subramanian et al. 2005).

Findings presented here show that LMP2A and LMP2B disrupt normal endosome trafficking in epithelial cells, an effect that may enhance the transport of IFN and TLR receptors from the cell surface to the lysosome for degradation. Previous studies in this laboratory have shown that LMP2A and LMP2B promote degradation of internalised IFN receptors thereby abrogating full IFNR signalling responses (Shah, Stewart et al. 2009). Previous reports have

mainly focused on the function of LMP2A in B-cells and its role in attenuating signalling from the BCR. In the case of epithelial cells, LMP2A attenuates signalling networks that engage NFκB, STAT3 (Stewart, Dawson et al. 2004), whilst stimulating the Wnt/β-Catenin (Morrison, Klingelhutz et al. 2003) and ERK/MAPK signalling pathways (Allen, Young et al. 2005). LMP2A has previously been shown to alter trafficking and antigen processing in B-cells through its ability to block phosphatidylcholine-specific phospholipase D (PLD) activity (Snyder and Pierce 2006), an enzyme that has been implicated in vesicular trafficking (McDermott, Wakelam et al. 2004). These data however focus not on LMP2A-specific effects, which require the amino-terminal signalling domain, but rather on effects that are common to both LMP2A and LMP2B.

Vesicular trafficking encompasses many different pathways and processes, including secretion of newly synthesised protein from the golgi network, extracellular secretion of cytokines and chemokines, autophagy, antigen presentation and processing, protein degradation and receptor signalling. The overall network is vast, dynamic and intricately controlled by a variety of proteins, e.g. the Rab small GTPases and phospholipids. This study has focused on one section of this network, namely the internalisation of receptors into early endosomes and their trafficking from these compartments to the lysosome for degradation.

Briefly, activated receptors are endocytosed, along with additional membrane bound factors in a clathrin/Rab5-mediated manner. These internalised vesicles then fuse with early endosomes and the receptor and endocytosed membrane form part of the internal membrane. Endosomal movement continues and the internalised molecules are transported to Rab7 positive late endosomes and finally to the lysosome, were proteins are degraded by acid hydrolysis. Prior to this, receptor recycling can occur through Rab4 and Rab11 associated endosomes (Zerial and

McBride 2001; Deneka, Neeft et al. 2003; McDermott, Wakelam et al. 2004; Hirota, Kuronita et al. 2007).

To investigate whether LMP2A and LMP2B influence receptor internalisation and turnover through modulation of the endosomal/lysosomal network, the integrity and activity of these networks was examined in more detail. This involved characterising these pathways in the CNE-2, H103 and H157 cell lines by comparing the expression of proteins integral to the correct functioning of the pathway. Increases in the levels of both early and late effectors of the pathway and of markers of early and late endosome/lysosomal compartments indicated that the effects were "global", with both viral proteins affecting all stages of the trafficking network. However, it is important to note that the LMP2 proteins are almost exclusively localised to the trans-golgi network (Lynch, Zimmerman et al. 2002) and, as assessed by immunofluorescence staining, co-localise with EEA1 and Rab5, to early endosomes, but not to acidified late endosomes or lysosomes. The effects of LMP2A and LMP2B on these compartments involve both an increase in the number of late endosomes/lysosomes and an increase in their degradative properties. Whether LMP2 acts to directly increase the levels of late compartments or whether by increasing levels of Rab5 and indeed Rab5 activity, increased Rab5 to Rab7 conversion occurs, thus facilitating membrane changes sufficient for lysosome formation, remains to be determined. Results discussed below allude to a mechanism that involves both increases in these conversion events and also interference by LMP2 at later stages of the trafficking network.

5.2 Profiling components of the endosomal/lysosomal network in LMP2A and LMP2B expressing cells.

5.2.1 Immunoblotting

To investigate whether expression of LMP2A and LMP2B influence the levels of protein components of the endosomal/lysosomal network, immunoblotting was performed on control, LMP2A and LMP2B expressing CNE-2 and H103 cells (as described in *section 2.5)*, using antibodies specific for Rab5, Rab7, CD63 and Ductin (a component of the V-ATPase complex). Results from a representative experiment are shown in *Figure 5.1*. Reprobing of the blots with a mAb to β-actin served as a loading control.

Rab5 is a well-characterised small GTPase that has defined functions in the biogenesis of early endosomes, endocytic vesicles and a role in endosomal trafficking (Woodman 2000; Mousavi, Malerod et al. 2004). Rab7 is another GTPase that is most associated with late endosomes (Bucci, Thomsen et al. 2000), and has been shown to be responsible for deciding the fate of certain classes of receptors, including growth factor receptors (Saxena, Bucci et al. 2005), and those involved in immune surveillance (Wang, Chen et al. 2007). CD63 is a member of the tetraspanin superfamily and is associated with acidified lysosomes (Metzelaar, Wijngaard et al. 1991) and, although this localisation is well defined, its role in endosomal traffic remains to be fully elucidated. It is thought however that it acts in tandem with the Vacuolar ATPase machinery in the process of lysosomal acidification (Forgac 2007), of which the 16kd protein, Ductin is a key component. Results presented in *Figure 5.1*, show that the expression of all proteins analysed is increased in cells expressing LMP2A or LMP2B relative to control cells. The larger more diffuse bands for CD63 are likely to result from protein glycosylation. These increases are indicative of an increased level of intracellular compartments and vesicles, an increase, which is not restricted to a particular stage of the network but rather a global

upregulation of the endosomal – lysosomal pathway. Given their roles in lysosomal acidification, the increased levels of Ductin and CD63 provide further evidence for the hypothesis that expression of LMP2A and LMP2B results in increased intracellular traffic, which may act to turnover and inactivate immune receptors through lysosomal degradation in acidified compartments.

5.2.2 Immunfluoresence staining for endosomal/lysosomal proteins.

To further examine the extent of the increase in endosome/lysosme compartments in LMP2A and LMP2B expressing CNE2 cells, immunofluorescence staining was performed for the early endosome markers Rab5 and EEA1, and a protein associated with the lysosomal membrane, lysosome associated membrane protein 1 (LAMP1). Cells of interest were fixed and permeabilised as described in *section 2.4* and probed with antibodies specific for Rab5 and EEA1. *Figure 5.2* shows representative confocal images for Rab5, EEA1 and LAMP1 after staining with selected mAbs and human serum, SK, used to detect LMP2A and LMP2B. In agreement with the immunoblotting data, levels of Rab5 were increased in cells expressing LMP2A and LMP2B compared to control cells. Levels of the second early endosome marker EEA1, which is associated with Rab5 on the membrane of the early endosome compartment and has been characterised as the first effector of Rab5 GTPase activity (Christoforidis, McBride et al. 1999), were also markedly upregulated. Staining for LAMP1, a highly glycosylated membrane bound protein with one spanning domain and a luminal N-terminal shown to be involved in tumour metastasis (Eskelinen, Tanaka et al. 2003), revealed an upregulation in cells expressing LMP2A and LMP2B. Taken together with findings from immunoblotting, it is likely that LMP2A and LMP2B expression results in the increase in formation of at least two intracellular compartments in epithelial cells.

Figure 5.1 Immunoblotting for proteins associated with endosomal trafficking components.

Protein lysates were extracted from CNE-2 and H103 cells expressing either a Neomycin resistance cassette, full length LMP2A or LMP2B. Immunoblotting was then performed for markers of endosomal traffic using antibodies specific to the early endosome/endocytic vesicle marker Rab5, the late endosome/lysosome marker Rab7, the lysosome-associated protein CD63 and the 16kD V-ATPase component, Ductin. β-actin was used as a loading control. Representative scans of the immunoblots are shown, in which it can clearly be seen that in cells expressing LMP2A or LMP2B, in both cell types, expression of all proteins examined is increased when compared with neomycin controls. The bands for the CD63 are more diffuse compared with the others due to protein glycosylation.

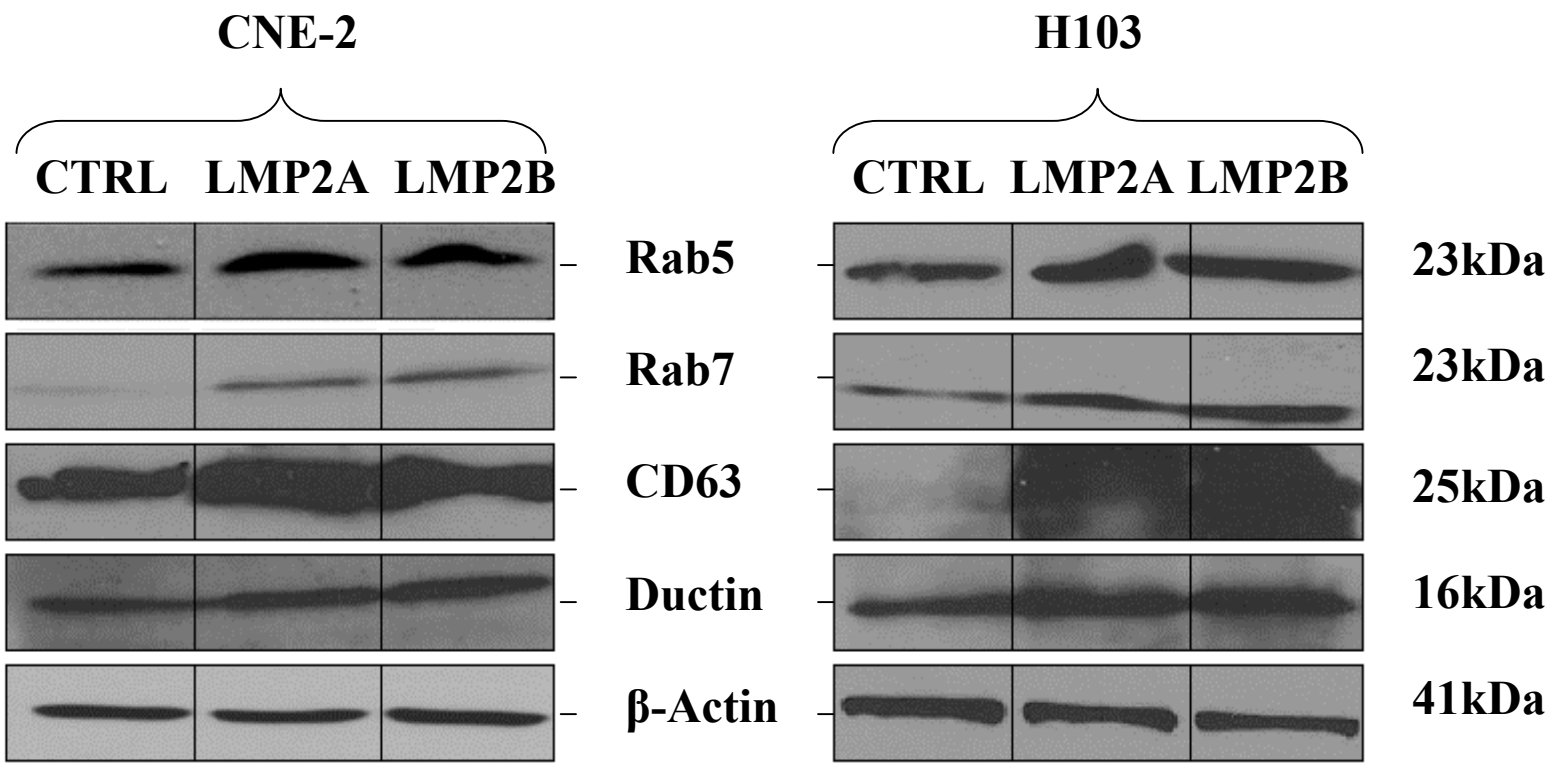
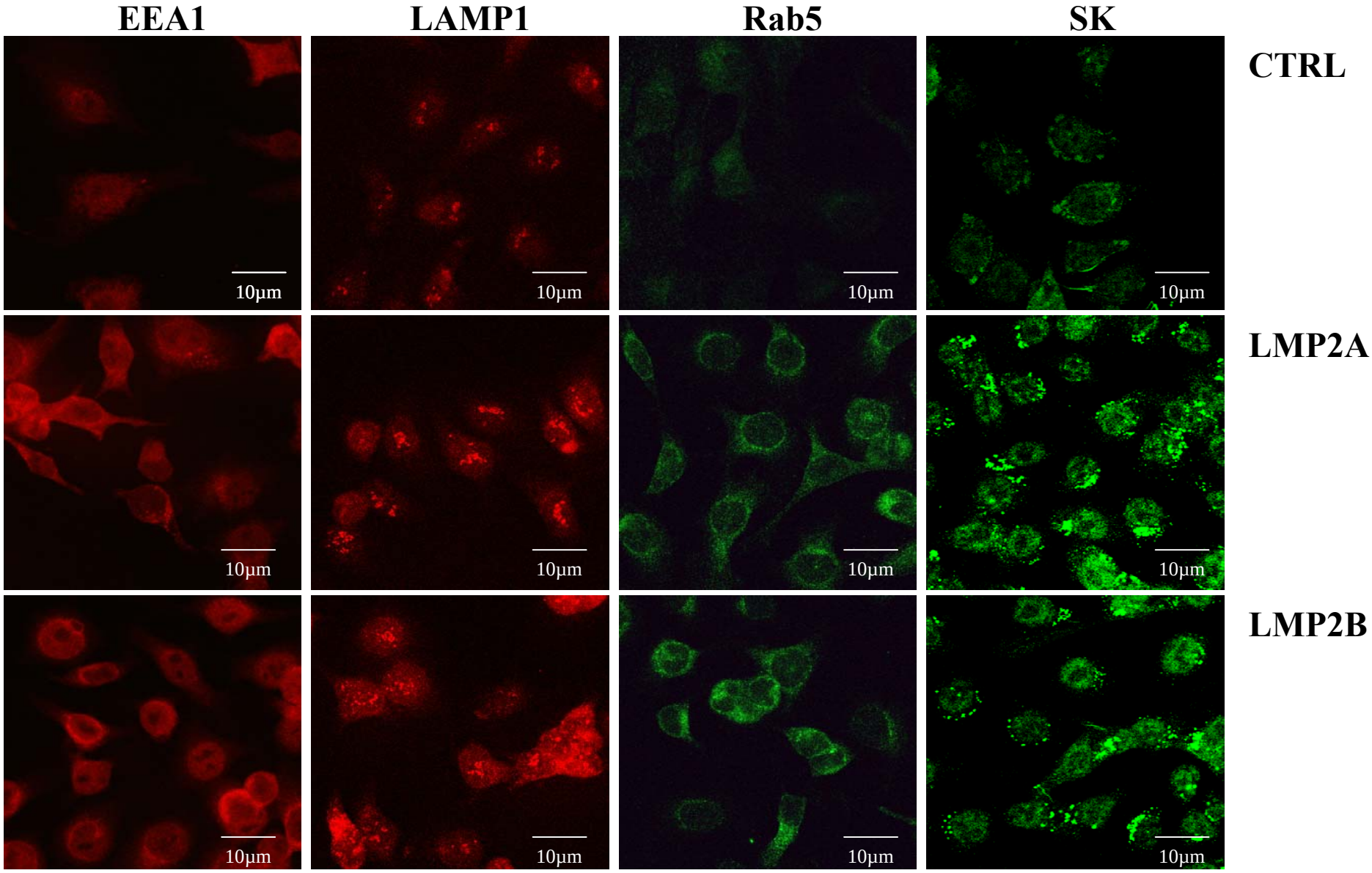

CNE-2
H103
CTRL
LMP2A
LMP2B
CTRL
LMP2A
LMP2B
Rab5
Rab7
CD63
Ductin
β-Actin
23kDa
23kDa
25kDa
16kDa
41kDa

Figure 5.2 Immunofluorescent staining for markers of endosomal compartments.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A of LMP2B were seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent staining was performed for, the early endosome protein, Early Endosome Antigen 1 (EEA1), the early endosome/endocytic vesicle associate Rab protein, Rab5, the lysosome associated protein, Lysosome associated membrane protein 1 (LAMP1), and finally the human serum SK was used to detect LMP2 expression. Representative confocal images are shown of each cell type with each antibody. Comparing the signals observed between control and LMP2 expressing cells, it can be seen that levels of each of the markers examined are increased with LMP2 expression. Differential subcellular location is also observable between each antibody demarcating the varying localisations of each of the markers.

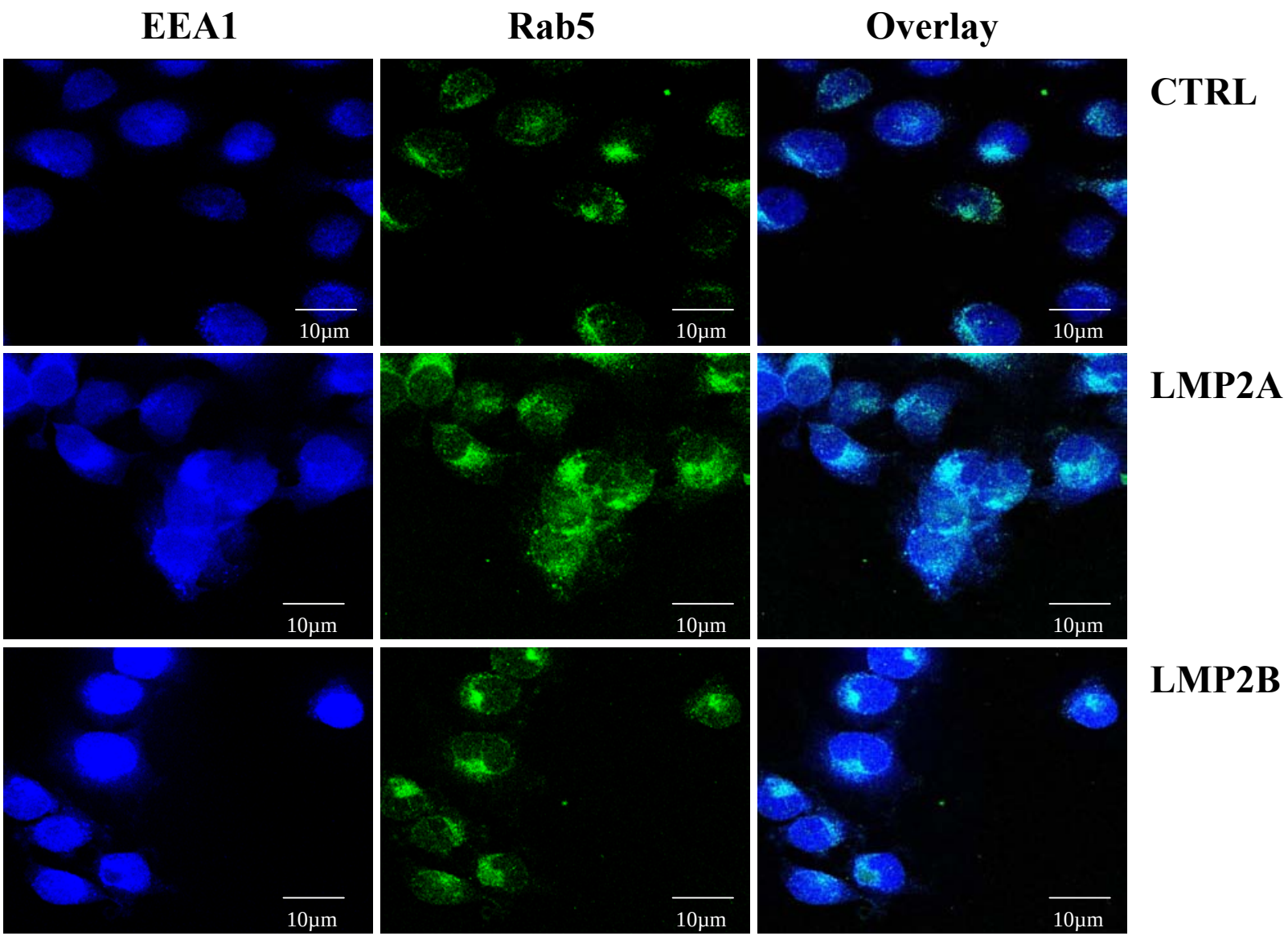

EEA1
LAMP1
Rab5
SK
CTRL
LMP2A
LMP2B
10μm

5.3 Co-localisation of Rab5 and EEA1 in CNE-2 cells

To ascertain whether the increase in Rab5 expression was linked to increases in the numbers of early endosomes or whether expression at additional intracellular locations distinct from EEA1 positive membranes was also altered, co-staining for EEA1 and Rab5 was performed. Rab5 is associated with endocytic vesicles (Mousavi, Malerod et al. 2004), where it has been shown to control the level of clathrin-mediated endocytosis. While EEA1 is not found at these sites, it has been shown to interact with Rab5 in the early endosome and this interaction occurs upon fusion of Rab5 positive vesicles, serving to activate Rab5 GTPase activity (Woodman 2000). Thus co-staining facilitated investigation of the effect of LMP2A and LMP2B expression on the levels of Rab5 positive endocytic vesicles. Control, LMP2A and LMP2B expressing CNE2 cells were subjected to immunofluorescence staining after fixation and permeabilisation using mAbs specific for Rab5 and EEA1, as described in *section 2.4. Figure 5.3* shows confocal images of representative immunofluorescence staining. Comparing LMP2A and LMP2B expressing cells with control cells it is clear that the number of early endosomes, identified by the markers EEA1 and Rab5, is markedly increased in cells expressing LMP2A and LMP2B. As predicted a high degree of co-localisation was observed between EEA1 and Rab5. However, Rab5 expression was also observed in small punctate clusters that are EEA1 negative, this corroborates the published data discussed previously, reporting that Rab5 is also localised to endocytic and trafficking vesicles. Although this increase in endosomal marker expression has been described above, the immunofluorescence staining also indicates an increase in smaller Rab5 vesicles, which have been shown to be responsible in mediating both the early events of endocytosis and later events in endosomal-lysosomal trafficking. Augmentation of endocytosis by Rab5 positive vesicles is likely to account, at least in part, for the reduction of signalling from certain classes of immunoreceptors in LMP2A and LMP2B expressing cells as previously reported (Shah, Stewart et al. 2009).

Figure 5.3 Immunofluorescent co-staining for early endosome associated proteins EEA1 and Rab5 in the CNE-2 cell panel.

CNE-2 cells expressing either a control neomycin resistance cassette, full length LMP2A of LMP2B were seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent co-staining was performed for; the early endosome protein, Early Endosome Antigen 1 (EEA1) and the early endosome/endocytic vesicle associate Rab protein, Rab5. Representative confocal images are shown. Comparing the signals observed between control and LMP2 expressing cells, it can be seen that levels of each of the markers examined are increased with LMP2 expression. In tandem with this, although a high degree of co-localisation is observed between each of the markers, Rab5 expression can also be observed in subcellular locations that are EEA1 negative, indicative of Rab5's association with endocytic vesicles.


EEA1
Rab5
Overlay
CTRL
LMP2A
LMP2B
10μm

5.4 LMP2 expression on internal membranes: co-localisation with early endosomes

When expressed in epithelial cells, LMP2A and LMP2B localise to internal perinuclear membranes, which constitute part of the endosome network (Dawson et al., 2001). To examine if LMP2A and LMP2B localise to the early endosome, which could account for the observed increase in the number of early endosomes, double immunofluorescence staining was performed for the endosome markers (EEA1, Rab5) and LMP2A or LMP2B. As described above CNE-2 cells stably expressing either LMP2A or LMP2B have increased levels of EEA1 and Rab5, as assessed by immunoblotting and immunofluorescence staining, compared to control cells. Although the SK or Bala human sera could have been used to detect LMP2A and LMP2B in the CNE-2 cell panel, these sera may inhibit full and correct binding of other antibodies to epitopes on LMP2-associated proteins. However, the H103 and H157 cell panel have, as previously described, *section 2.1*, have been transduced with recombinant retrovirues to express HA-tagged versions of full length LMP2A or LMP2B. This HA-tagged epitope allows efficient detection of both proteins by immunofluorescence with minimal interference such as might be observed with polyclonal human sera. Cells grown *in situ* were fixed and permeabilised, as described in *section 2.4*, and probed with either antibodies specific for Rab5, EEA1 and the HA-tagged-epitope.

Figures 5.4(A, B), show representative confocal images of double immunofluorescence staining. In agreement with the immunoblotting data in H103 cells, *Figure 5.1*, and mirroring findings from the immunofluoresence staining of the CNE-2 cell panel, *Figure 5.2*, Rab5 expression in H103 and H157 cells expressing LMP2A and LMP2B was markedly increased compared to control cells. HA staining was visible as punctate peri-nuclear aggregates that were also positive for Rab5. However, expression of Rab5 was not limited to LMP2-positive vesicles and was also observed in smaller clusters nearer the plasma membrane, which are

believed to be endocytic vesicles. LMP2A and LMP2B therefore appeared to co-localise with Rab5 on early endosomes but not on vesicles closely localising with the plasma membrane. *Figures 5.5A, B,* show that EEA1 positive vesicles are also markedly up-regulated in cells expressing LMP2A and LMP2B compared with control cells in both the H103 and H157 cell lines. Extensive co-localisation is also observed between EEA1 and both LMP2A and LMP2B.

5.5 LMP2A and LMP2B increase the numbers of lysosomes: live-cell imaging

Although increases in protein levels of Ductin and CD63, *Figure 5.1*, by LMP2A and LMP2B indicate increases in acidification of a proportion of lysosomes, and immunofluorescent staining for LAMP1 revealed significant augmentation of this protein in LMP2A and LMP2B expressing cells, further investigation was necessary to fully elucidate LMP2's effect on the these compartments. LysoTracker™ Red is a commercially available dye that identifies lysosomes in mammalian cells. Incubating living cells with the dye for up to two hours facilitates dye uptake for subsequent detection by fluorescent microscopy. The immunoblotting and immunofluorescent staining described above suggest that in tandem with an increase in numbers of early endosomes there is also an upregulation of acidified lysosomes in LMP2A and LMP2B expressing cells.

To further analyse this, live cell confocal imaging was performed on the CNE-2, H103 and H157 epithelial cell panels, expressing full length LMP2A, LMP2B or control cells expressing a neomycin resistance cassette. Cells were seeded at a density of 2.5×10^4 onto each well of a glass bottomed 24-well microscopy plate, (MatTek©) and allowed to adhere overnight at 37°C and 5% $C0_2$. Serum free medium containing 150nM LysoTracker™ was added to each well incubated for 2 hours at 37°C and subsequently analysed using live cell confocal imaging. Representative images of a typical analysis are shown in *Figure 5.6*. In all cell lines examined

perinuclear aggregates of the dye were observed in control cells after 2 hours incubation. Comparing this with cells expressing LMP2A and LMP2B, it is clear that the number of lysosomal compartments was elevated in the presence of LMP2A and LMP2B. The distribution of lysosomes also appeared to be altered in LMP2A and LMP2B expressing cells, where they are spread throughout the cytosol in comparison to a more clustered localisation at one end of the nuclear compartment in control cells. Live cell imaging, rather than fixed staining allows analysis of samples without influencing the intracellular trafficking of vesicles. This allows for the fluid, dynamic nature of intracellular trafficking to be considered and observations of the same cells can be made over a period of time rather than a single snapshot. This method also ensures that the increased signal observed is not a result of build-up of unbound dye due to increased Rab5-Clathrin endocytosis, as unbound dye would be exocytosed from the cell upon further incubation of cell samples following initial confocal analysis (data not shown).

5.6 LMP2A and LMP2B do not localise to lysosomal compartments

From findings presented in the previous section, it was clear that a proportion of LMP2A and LMP2B localise to EEA1/Rab5 positive early endosomes, whilst also stimulating the numbers of lysosomes. As the intracellular trafficking of endosomal vesicles is a dynamic process, it is possible that LMP2A and LMP2B could migrate to other internal membranes including lysosomal compartments. To assess whether there is a direct interaction between LMP2, the lysosome and components of the V-ATPase machinery, immunofluoresence staining was performed staining for LysoTracker™ and LMP2A and LMP2B, using SK serum and a HA-specific mAb to identify LMP2A and LMP2B in the CNE2 and H103 or H157 cells lines respectively. Cells were seeded at a density of 1 x 10^4 onto each well of a 12-well teflon coated microdot slide and incubated overnight at 37°C and 5% $C0_2$ to allow cellular adherence. The

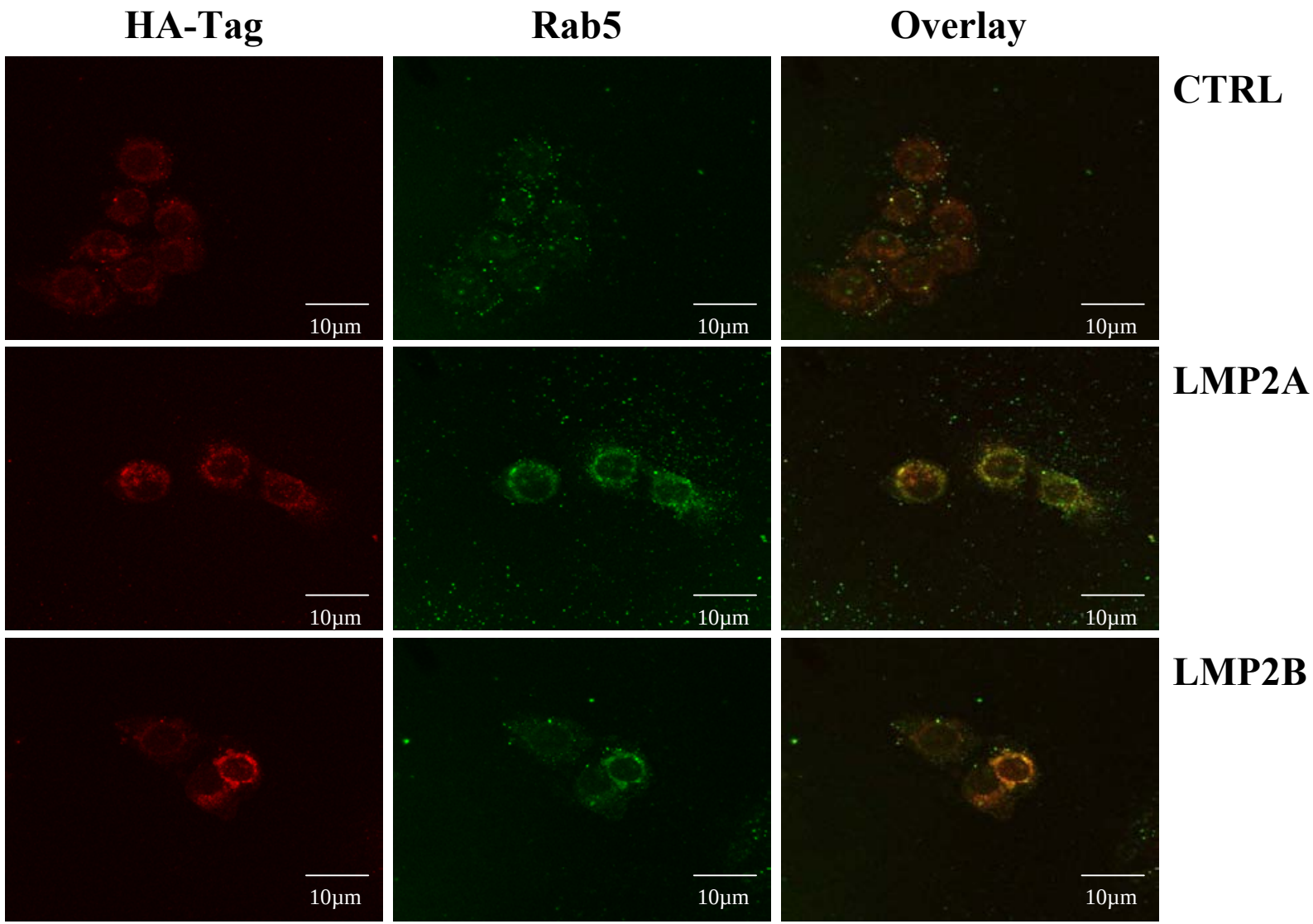
following day the growth media was removed and replaced with serum free media containing 150nM LysoTracker™ and incubated for 2 hours at 37°C. Cells were then fixed and permeabilised, using Acteone/Methanol to prevent disruption of LysoTracker™'s affinity for lysosomal compartments, as described in *section 2.4.* Samples were then probed with a mAb specific for the HA-tag or the SK serum to identify the localisation of LMP2A and LMP2B. Incubating the cells prior to fixation allows the dye to be taken into the cell and minimises signal interference by either addition of primary antibodies or the fixation procedure.

A representative analysis, shown in *Figure 5.7(A),* confirmed an increase in the number of lysosomes in LMP2A and LMP2B expressing cells. However, a significant level of co-localisation between LMP2A and LMP2B and the lysosomes was not observed, indicating that these proteins do not associate with the lysosomal compartment but rather increase their number through interacting with components at an earlier stage in the endosomal-lysosomal network. Essentially identical results were obtained in H103 cells, *Figure 5.7(B)*, and H157 cells, *Figure 5.7(C)*. These representative images show an increase in lysosome numbers and no significant level of colocalisation with LMP2A or LMP2B, as assessed by immunofluoresence staining using the HA-tag as a marker for LMP2A or LMP2B. Taking into consideration the degree of colocalisation between early endosome components and LMP2A and LMP2B, across all cell lines, it is hypothesised that LMP2A and LMP2B localise to endosomal-like compartments and that this interferes with normal trafficking mechanics, disrupting the correct sorting of membranes and their bound proteins to target these for degradation in the lysosome.

Figure 5.4 Immunofluorescent co-staining of Rab5 and LMP2 in the H103 and H157 cell panels.

Cell panels were seeded at a density of 2 x 10^4 on to the wells of 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent co-staining was performed for the early endosome/endocytic vesicle associated Rab protein, Rab5 and LMP2 using an antibody specific for Rab5 and a second antibody specific for the HA-tag of the LMP2 protein. Representative confocal images are shown of, (A) the H103 cell panel and (B) the H157 cell panel. Comparing the signals observed between control and LMP2 expressing cells in both (A) and (B), it can be seen that levels of Rab5 are increased with LMP2 expression in agreement with the images in *Figure 5.2.* Rab5 expression is observed as punctate clusters throughout the cytoplasm with some co-localisation with the LMP2 HA-tag signal.

(A) H103



(B) H157

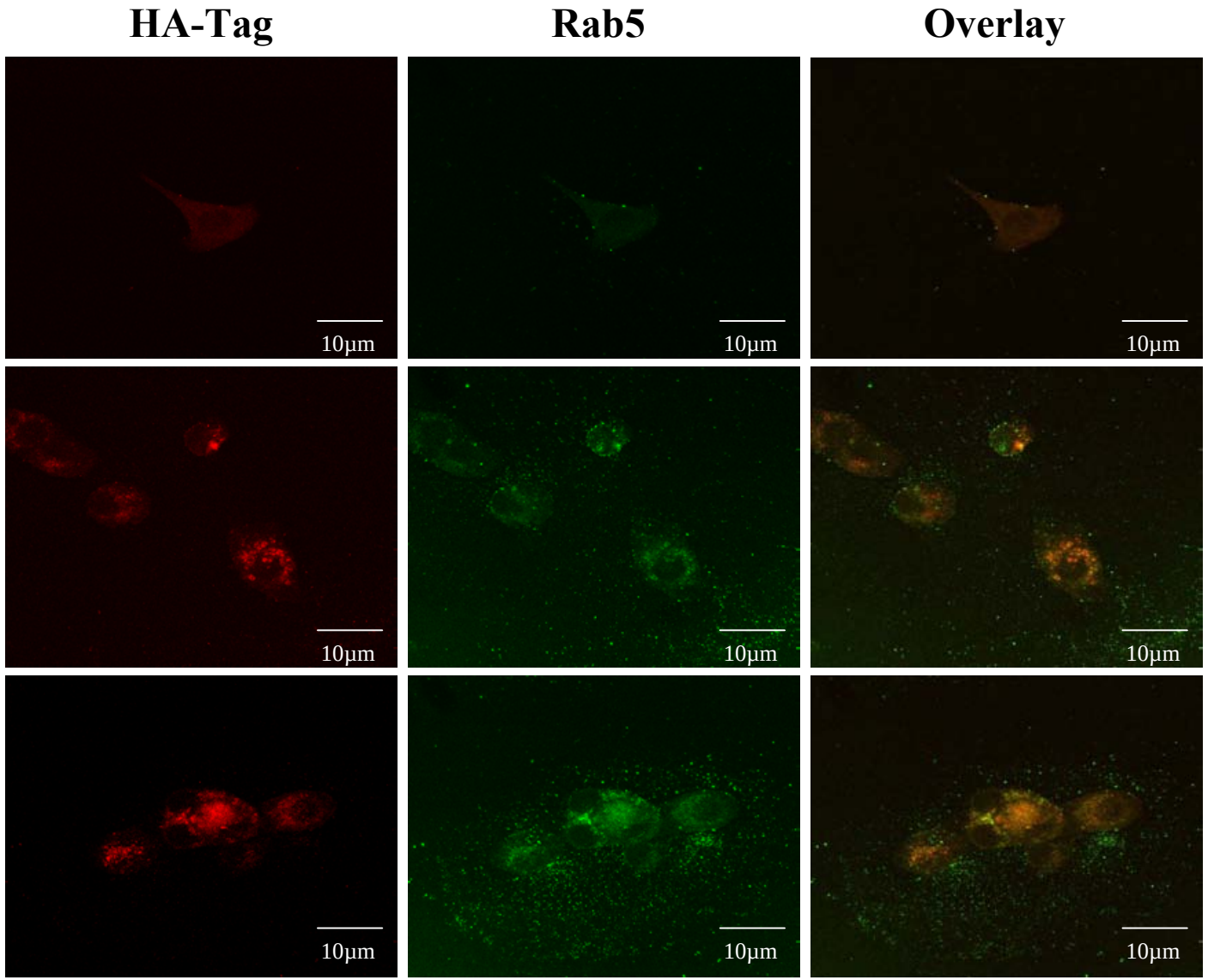
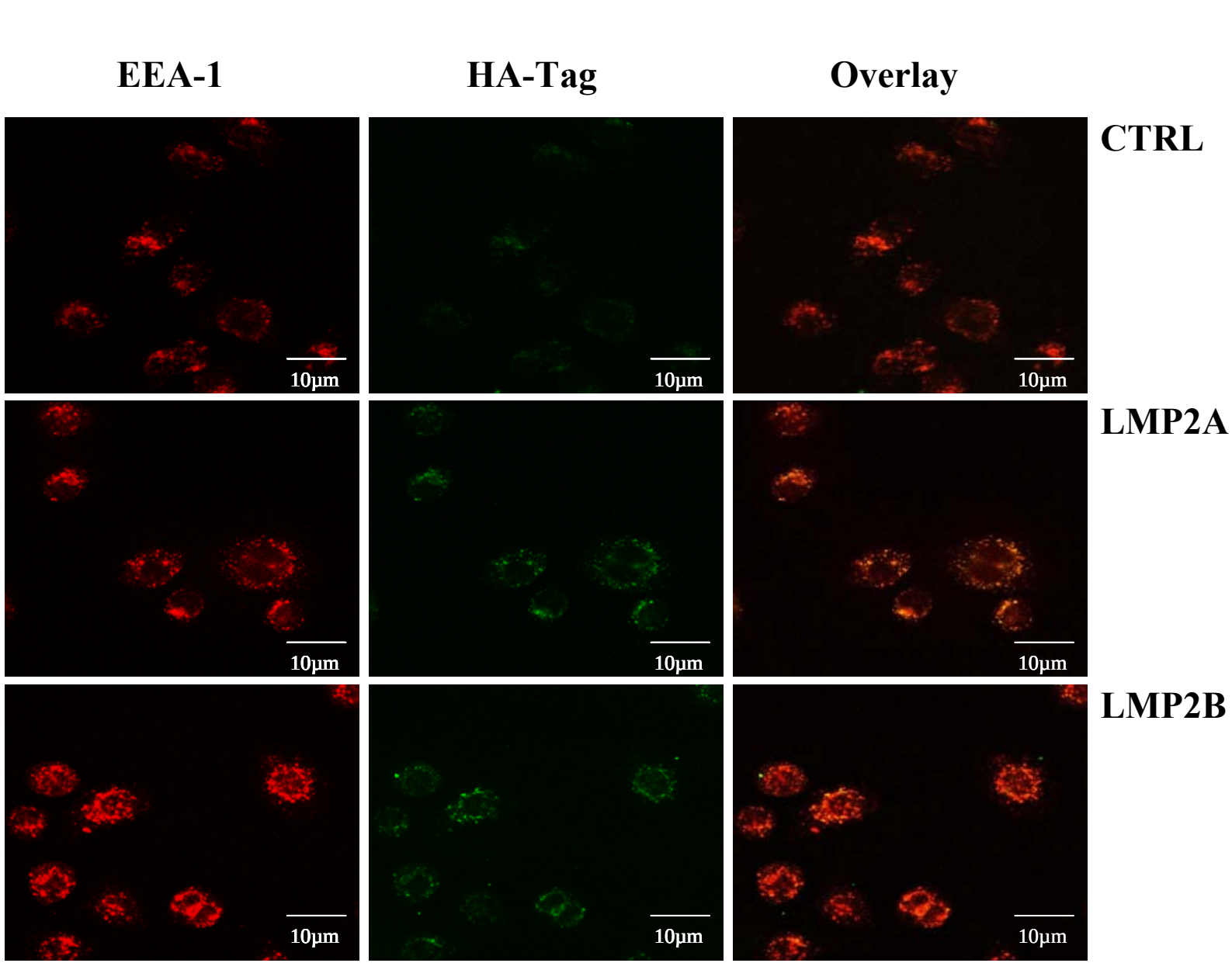


Figure 5.5 Immunofluorescent co-staining of EEA1 and LMP2 in the H103 and H157 cell panels.

Cell panels were seeded at a density of 2 x 10^4 on to the wells of 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. The following day immunofluorescent co-staining was performed for the early endosome associated protein, EEA1 and LMP2 using an antibody specific for EEA1 and a second antibody specific for the HA-tag of the LMP2 protein. Representative confocal images are shown of, (A) the H103 cell panel and (B) the H157 cell panel. Comparing the signals observed between control and LMP2 expressing cells in both (A) and (B), it can be seen that levels of EEA1 are increased with LMP2 expression in agreement with the images in *Figure 5.2*. EEA1 expression has a high degree of co-localisation with the HA-tag, demonstrating expression of LMP2 on the membrane of early endosomes**.**

(A) H103



(B) H157

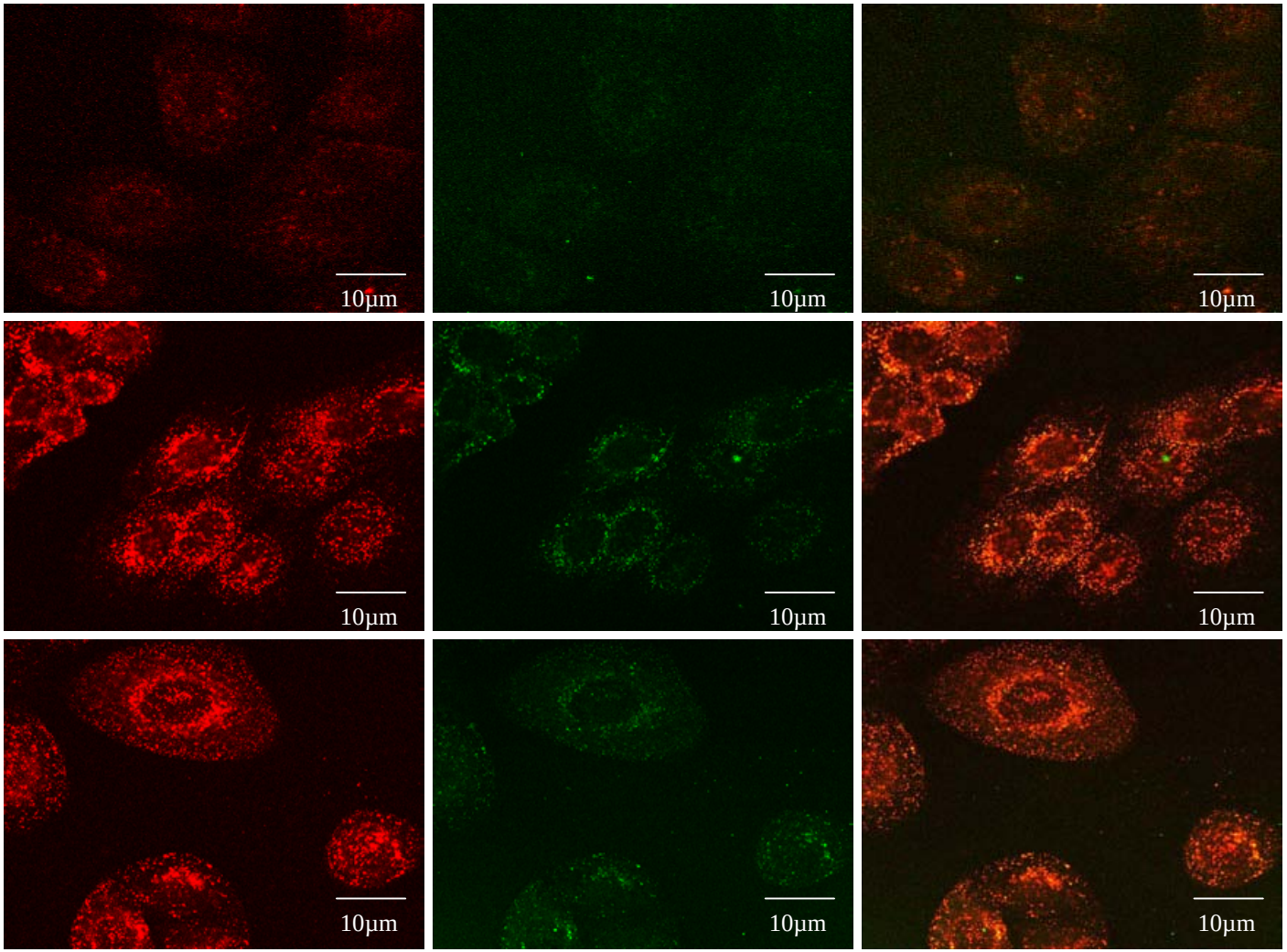


Figure 5.6 Live-cell fluorescent imaging of lysosomes in the CNE-2, H103 and H157 cell panels.

Cells panels were seeded at a density of 2.5 x 10^4 cells, onto wells of a 24-well uncoated, 0.13mm, 13mm diameter glass bottomed plate and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. Upon treatment with LysoTracker™ cells were photographed using live confocal microscopy. Representative images of each cell panel are shown. Across all cell types, comparing between control and LMP2 expressing cells it is observed that the number of lysosomes is increased with LMP2 expression. Distribution of lysosomal compartments is also altered in LMP2 positive cells, where signals are detected throughout the cytoplasm rather than in distinct peri-nuclear clusters.

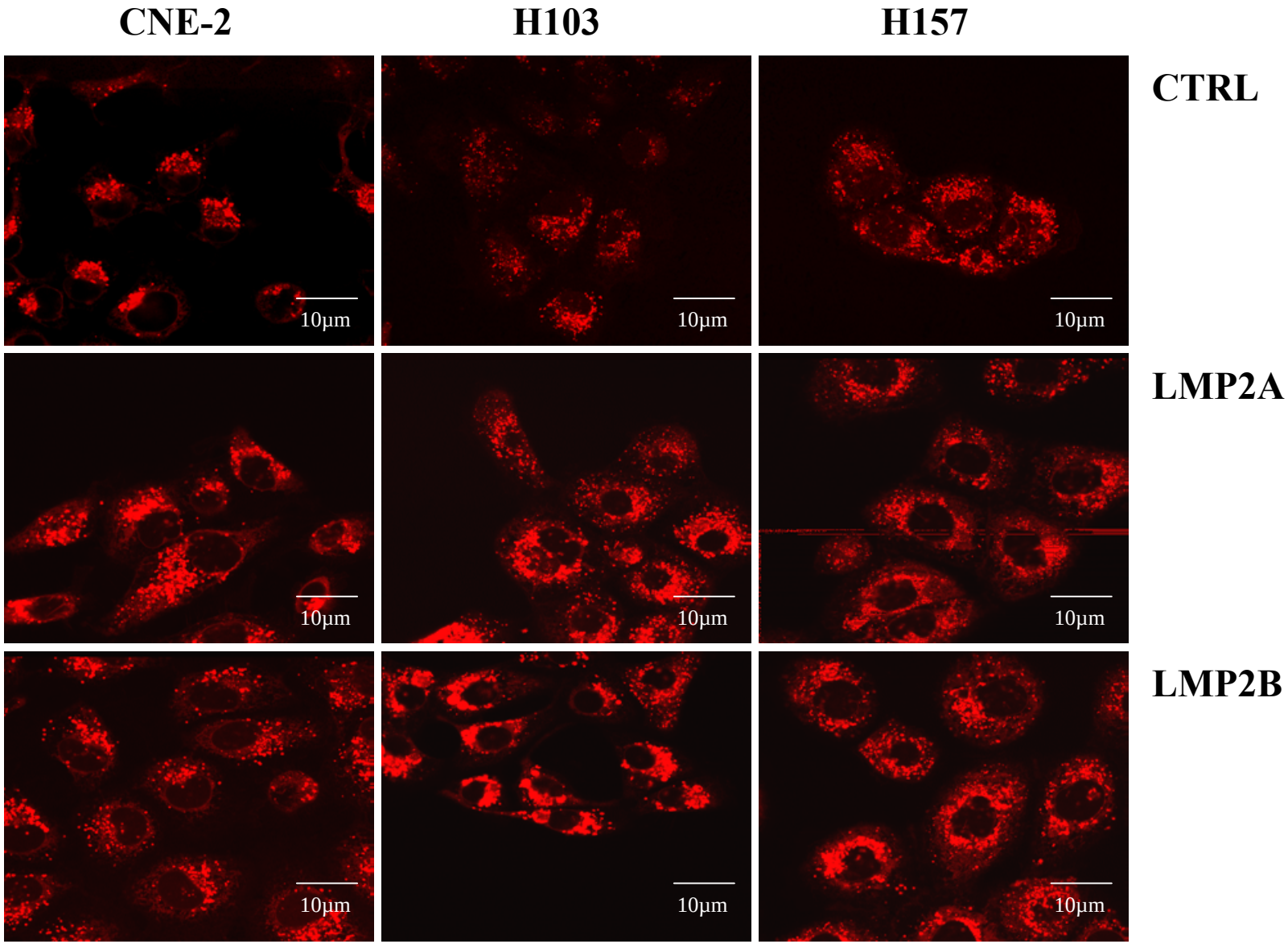
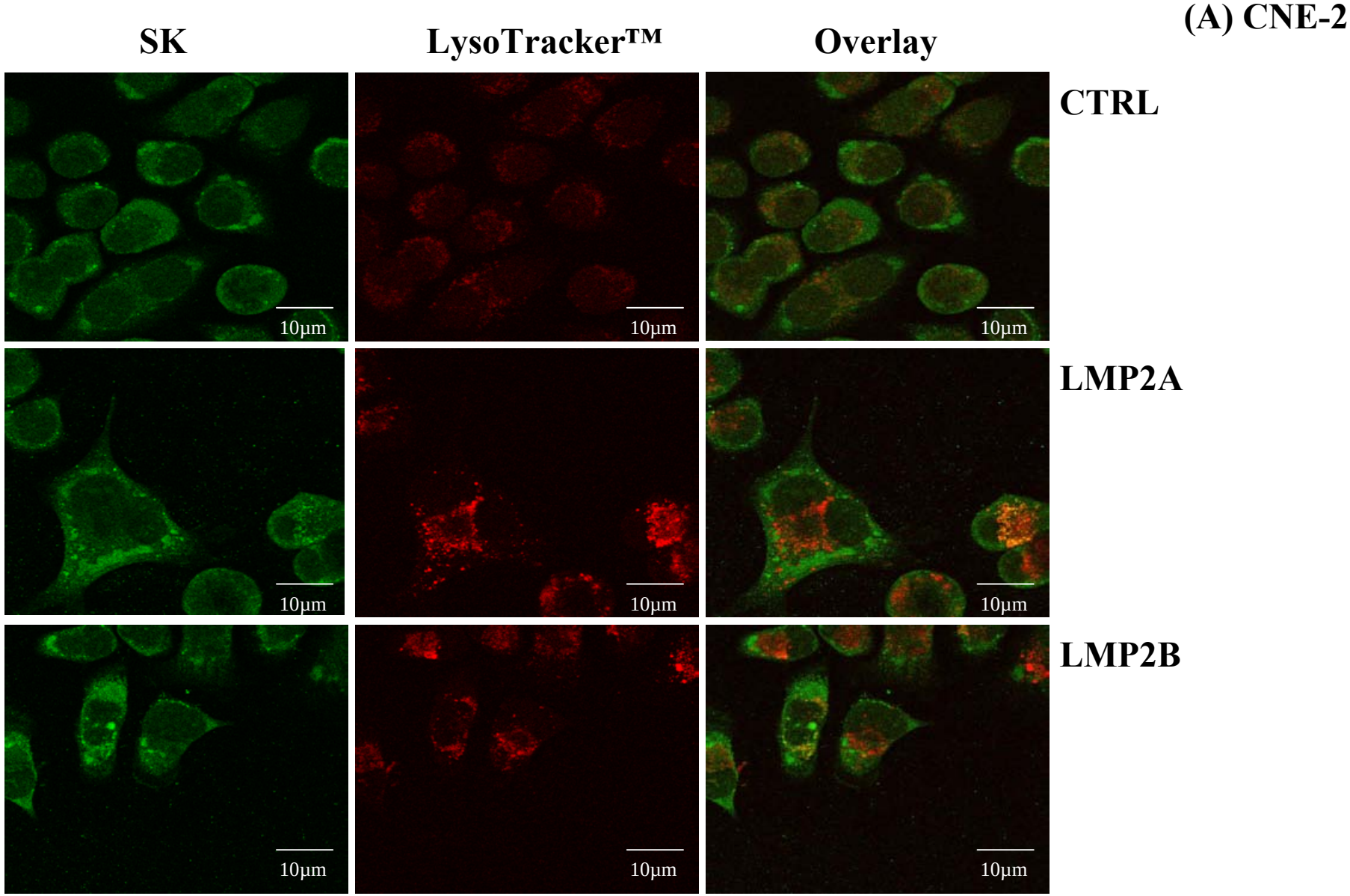
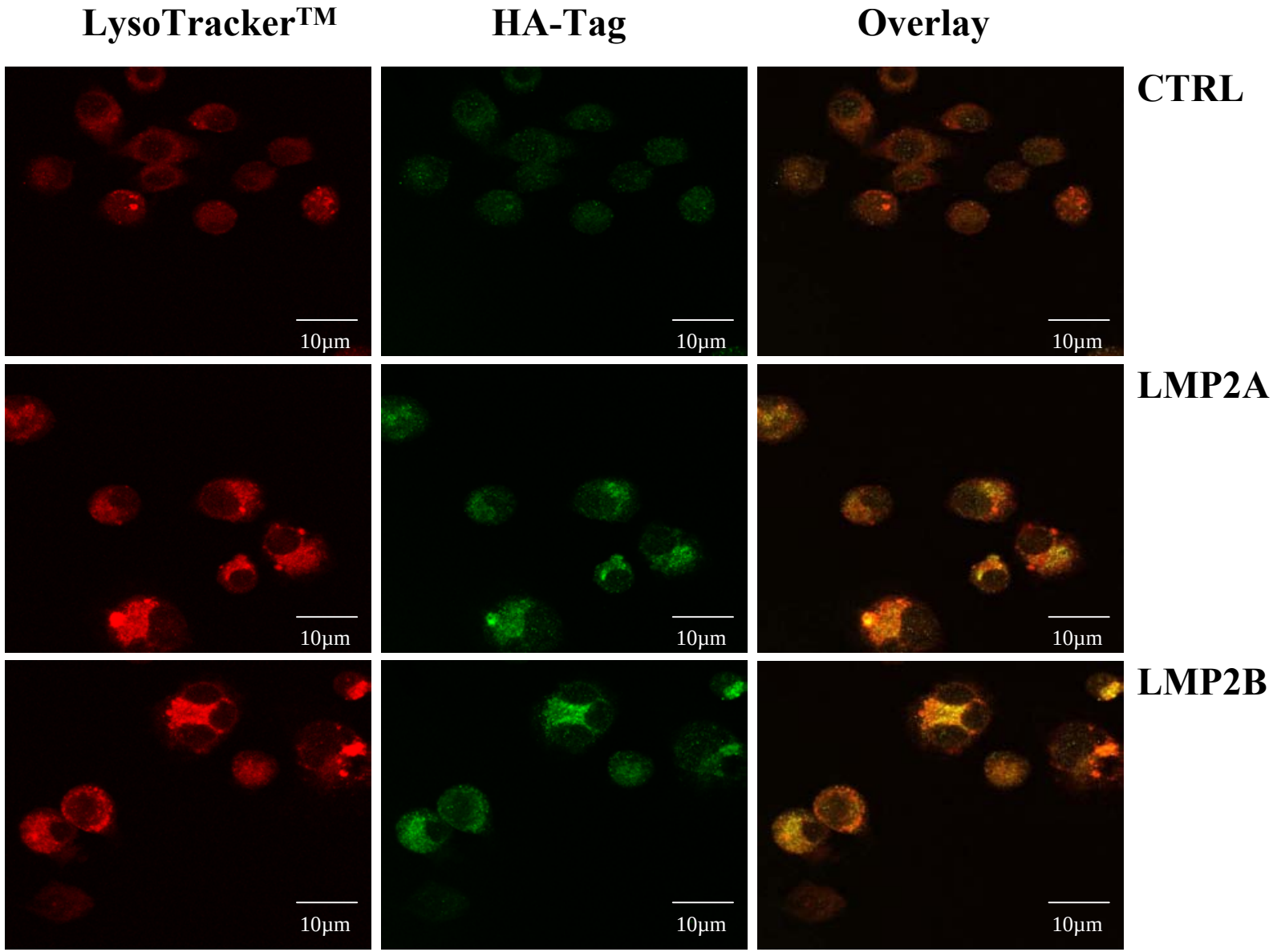

CNE-2
H103
H157
CTRL
LMP2A
LMP2B
10μm
10μm
10μm
10μm
10μm
10μm
10μm
10μm
10μm

Figure 5.7 Immunofluorescent co-staining of lysosomes and LMP2 in the CNE-2, H103 and H157 cell panels.

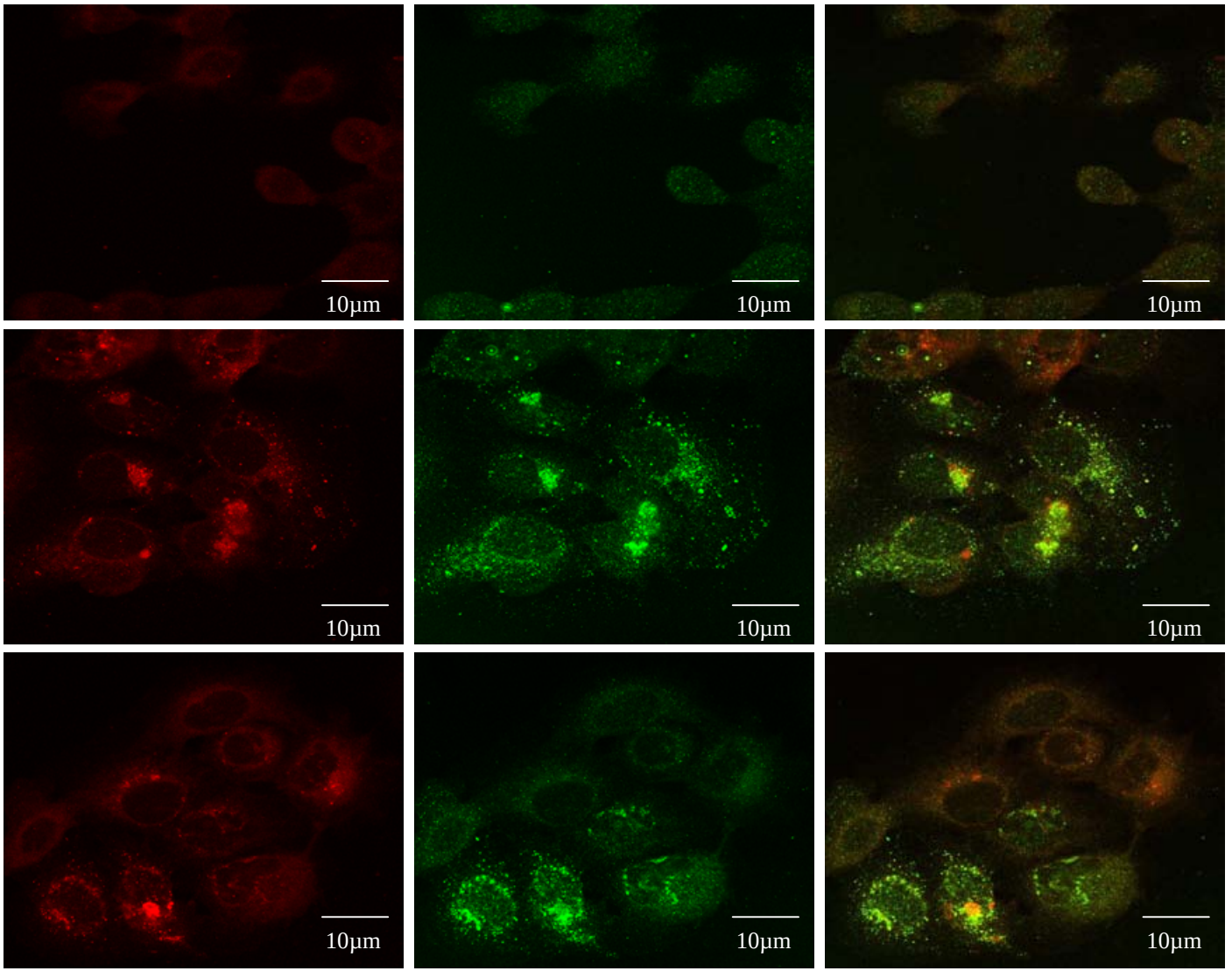
Cell panels, (A) CNE-2, (B) H103 and (C) H157, were seeded at a density of 2 x 10^4 on to the wells of 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. LysoTracker™ treatment was performed prior to fixation and permeabilisation of samples. Immunofluorescent staining was then performed using the SK human serum in (A) the CNE-2 cell panel or an antibody specific for the HA-tag in (B) H103 and (C) H157 cell panels to detect LMP2 expression. Increased numbers of lysosomes are observed in LMP2 expressing cells when compared with controls, across all cell types, in agreement with images from, *Figure 5.6,* and LMP2 expression is detected in the relevant cells. No significant co-localisation is observed between lysosomes and LMP2 indicating that LMP2 is not expressed in the membrane of this compartment.


(A) CNE-2
SK
LysoTracker™
Overlay
CTRL
LMP2A
LMP2B
10μm

(B) H103



(C) H157



5.7 Lysosome acidification and trafficking is altered by LMP2 expression

The above results show that the number of lysosomes is increased in cells expressing LMP2A and LMP2B and, given this, the immunoblotting results described in *section 5.2.1,* showing significant increases in the levels of CD63 and Ductin expression in LMP2A and LMP2B expressing cells is not surprising. To ascribe some functional significance to these observations it was necessary to investigate any effects on endosome acidification to determine whether LMP2A and LMP2B influence the kinetics of endosome acidification. An increase in the number of lysosomes, is suggestive of an intracellular trafficking network that has the potential to degrade receptors and endocytosed membrane proteins more effectively. To address this issue, LysoSensor ™ a commercially available dye (Invitrogen) was used to qualitatively measure the acidity of endosome and lysosomal compartments in LMP2A and LMP2B expressing cells relative to control cells. Unlike Lysotracker™, the signal intensity generated using Lysosensor™ is pH sensitive, with the extent of emission proportional to endosome pH; a decreasing pH results in stronger emission as read by the confocal microscope an viewed as a blue fluorescent signal.

The CNE-2, H103 and H157 cell panels were incubated with LysoTracker™ as described in *section 2.4* and, following confocal microscopic analysis to ensure that the lysosomes had been stained correctly, medium removed and replaced with serum free medium containing 1μM LysoSensor™. Time-capture laser scanning was then used to monitor the movement of the LysoSensor™ through the cell and to measure the signal emitted upon reaching the lysosome. Due to the increased numbers of lysosomes it was expected that the signal observed in LMP2A and LMP2B expressing cells would be greater compared to control cells, which was the case. Representative images from a typical analysis, taken 5 minutes and 15 minutes post incubation with the dye are shown in *Figure 5.8(A, B, C).* Here, an increase in lysosome number is

observed and thus a proportional increase in the occurrence of an acidified signal, what is most interesting to note however, is that the signal observed in control cells compared with LMP2A and LMP2B expressing cells was much stronger indicating an increase in acidification of these lysosomes. LMP2A and LMP2B appear to increase the number of lysosomes within the cell, by a mechanism that is independent of a direct LMP2:lysosome interaction, while also increasing the acidity of these lysosomes, compared with control cells.

The increase in numbers of endosomes observed in response to LMP2A and LMP2B expression would increase the total amounts of internalised endosomal membranes, which require processing, either by degradation or through recycling to the plasma membrane. The concomitant increase in lysosomes could therefore be a result of LMP2 interaction with early endosomes. Further investigation is necessary to fully understand the exact mechanism of this modulation and what effects this has on the signalling capacity of affected cells.

5.8 Effect of truncated forms of LMP2 on lysosome number

The modulation of endosomal trafficking observed in LMP2A and LMP2B expressing cells is a feature common to both proteins. Given the lack of an N terminal signalling domain in the LMP2B protein, the transmembrane domains of the proteins are likely to contain the protein motifs, which dictate these functions. These membrane loops are responsible for the protein's subcellular localisation on internal membranes and it is this tethering to membranes, which is postulated to facilitate LMP2's disruption of endosomal traffic. To further investigate this and to characterise which domains of the protein are necessary for binding, the LMP2-Loop deleted mutants, *chapter 3*, were used. A similar study using mutant forms of the LMP2 protein (Rowe and Tomaszewski-Flick 2007) has shown that the regions preceding the fourth loop are responsible for membrane binding. This would suggest that expression of LMP2 mutant

proteins that express only the latter transmembrane domains of the protein would not display such modulation of intracellular traffic.

CNE-2, H103 and H157 cells were transiently transfected with one of the following plasmids: pSG5, pSG5 LMP2A-HA-tag, pSG5 LMP2B-HA-tag, pLenti6 LMP2-L1Del, pLenti6 LMP2-L3Del, pLenti6 LMP2-L4Del, pLenti6 LMP2-L5Del and pLenti6 LMP2-L6Del. 48 hours post-transfection, cells were seeded onto each well of a 12-well teflon coated microdot slide and, following overnight incubation, treated for 2 hours with serum free media containing 150nM LysoTracker™, as described in *section 2.4.* Expression of LMP2A, LMP2B or mutant LMP2B proteins was visualised after probing with an antibody specific for the HA-tagged epitope and photographed on a Zeiss LSM 510Meta confocal microscope. Representative images of the staining are shown in Figures *5.9(A-F).*

Transient expression of full length, HA-tagged LMP2A and LMP2B for 72 hours resulted in increased numbers of lysosomes compared to cells transfected with the empty vector control plasmid, pSG5, in all cell types. In agreement with the co-staining performed with cells stably expressing LMP2A and LMP2B no significant overlap was observed between LMP2A and LMP2B and the LysoTracker™ positive signals; however, a degree of colocalisation was observed in the transient setting when compared with stable expression. This disparity may occur as a result of over-expression of the protein upon transient transfection and the degree of colocalisation may be diminished upon establishment of more stable expression.

Cells transiently expressing the mutant forms of LMP2 deleted for domains up to and including transmembrane loop one, those expressing LMP2 deleted past transmembrane loop three and to a lesser extent those expressing LMP2 truncated beyond the fourth transmembrane loop, panels

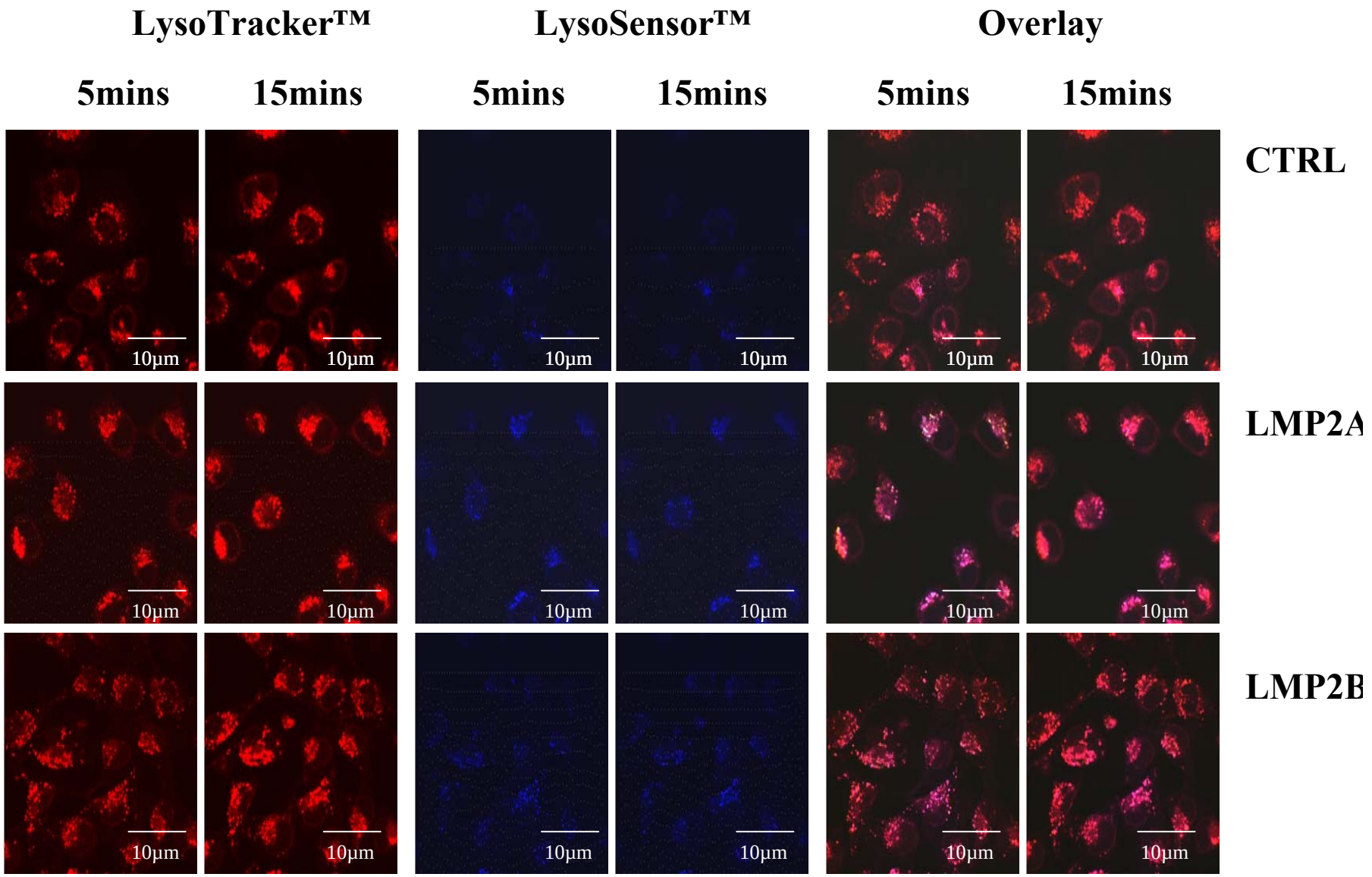
(v), (vi) and (vii) respectively, showed increased numbers of lysosomes when compared to null transfected controls, while maintaining a positive signal for the HA-tag, indicating expression of the indicated LMP2B deletion mutant. The HA-tag appeared to be lost from those cells expressing the two remaining truncated forms of LMP2, Loop5-deleted and Loop-6 deleted, panels (viii) and (ix). This may indicate a loss of membrane association and turnover of these proteins due to instability. The lack of a HA-staining is concomitant with a reduction in the number of lysosomes, indicated by a weaker LysoTracker™ signal, to levels comparable with null transfected controls, (i) and (ii).

Here it is clear that even in a transient system, expression of LMP2A or LMP2B or truncated versions of LMP2, which include at least the fourth transmembrane domain, for 72 hours, is sufficient to augment formation of lysosomal compartments. It must also be noted that this is a novel finding, which for the first time puts forward a direct effect of LMP2B expression that mirrors the effect of LMP2A without acting as an antagonist or rheostat. The data also underpins the importance of the trans-membrane domains of both proteins and reveals this protein structure as a mediator of intracellular signalling.

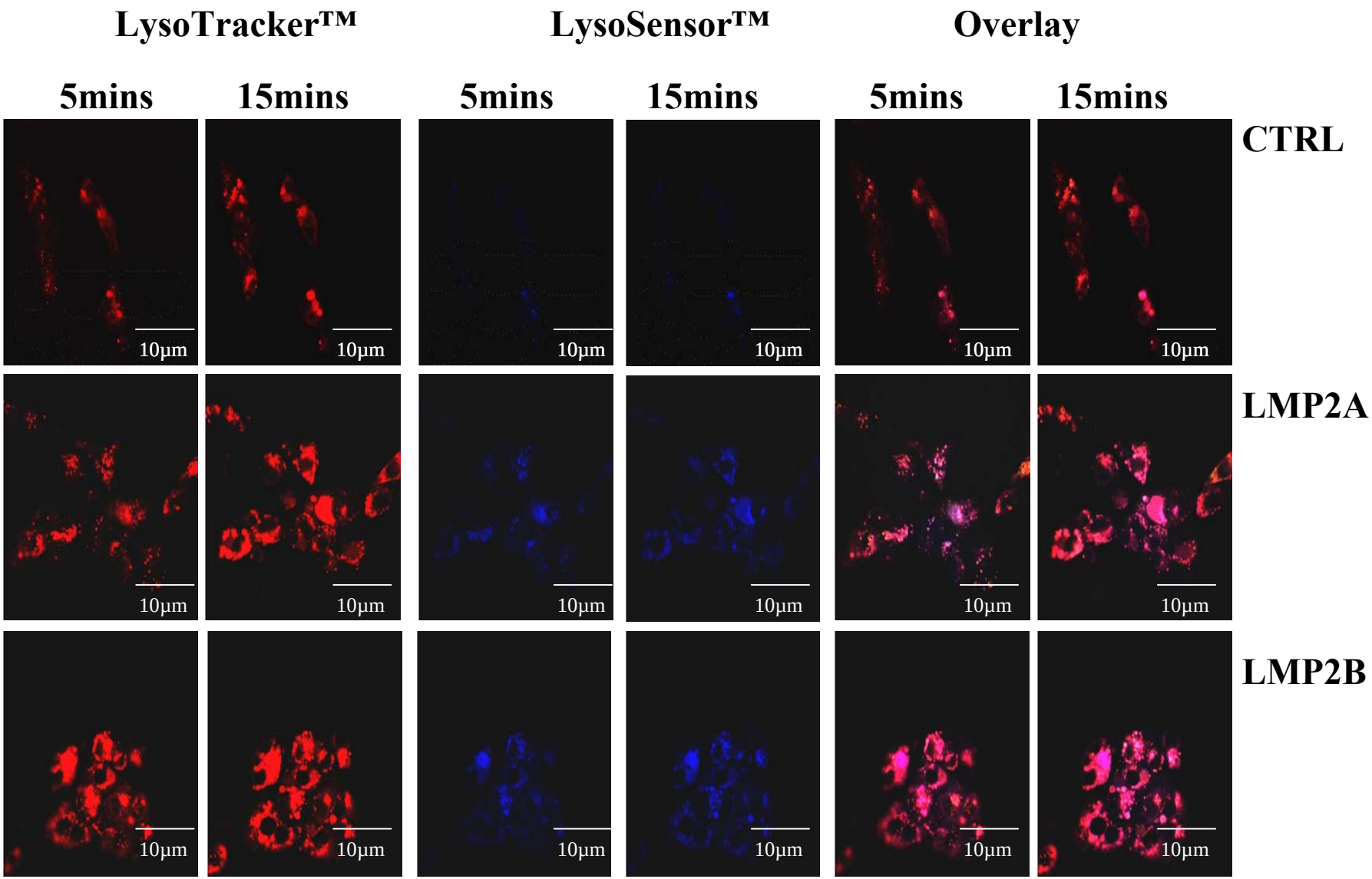
Figure 5.8 Live-cell fluorescent imaging of acidified lysosomes in the CNE-2, H103 and H157 cell panels.

Cells panels, (A) CNE-2, (B) H103 and (C) H157, were seeded at a density of 2.5 x 10^4 cells, onto wells of a 24-well uncoated, 0.13mm, 13mm diameter glass bottomed plate and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. Upon treatment with LysoTracker™ and LysoSensor™ cells were photographed using live time-capture confocal microscopy. Representative images of each cell panel are shown at the 5 minute and 15 minute time-points. Across all cell types, comparing between control and LMP2 expressing cells it is observed that the number of lysosomes is increased in cells expressing LMP2 and in agreement with *Figure 5.6,* distribution of these compartments is also altered. Signals for LysoSensor™, which detects the degree of acidification of these compartments, are also increased in cells expressing LMP2, indicating increased acidity of these compartments. Magnified versions of these images, (D) CNE-2, (E) H103 and (F) H157, demonstrate that the signal strength of LysoSensor™ is increased in individual lysosomes between control and LMP2 expressing cells, indicating that the increased acidification signal is not only a result of increased numbers of lysosomes. Comparing the movement of these dyes through the cell by time-capture between control and LMP2 positive cells, it is demonstrated, albeit simply, that the speed of this traffic is increased in LMP2 expressing cells. Arrows indicate movement of a smaller vesicle to a larger more acidified compartment.

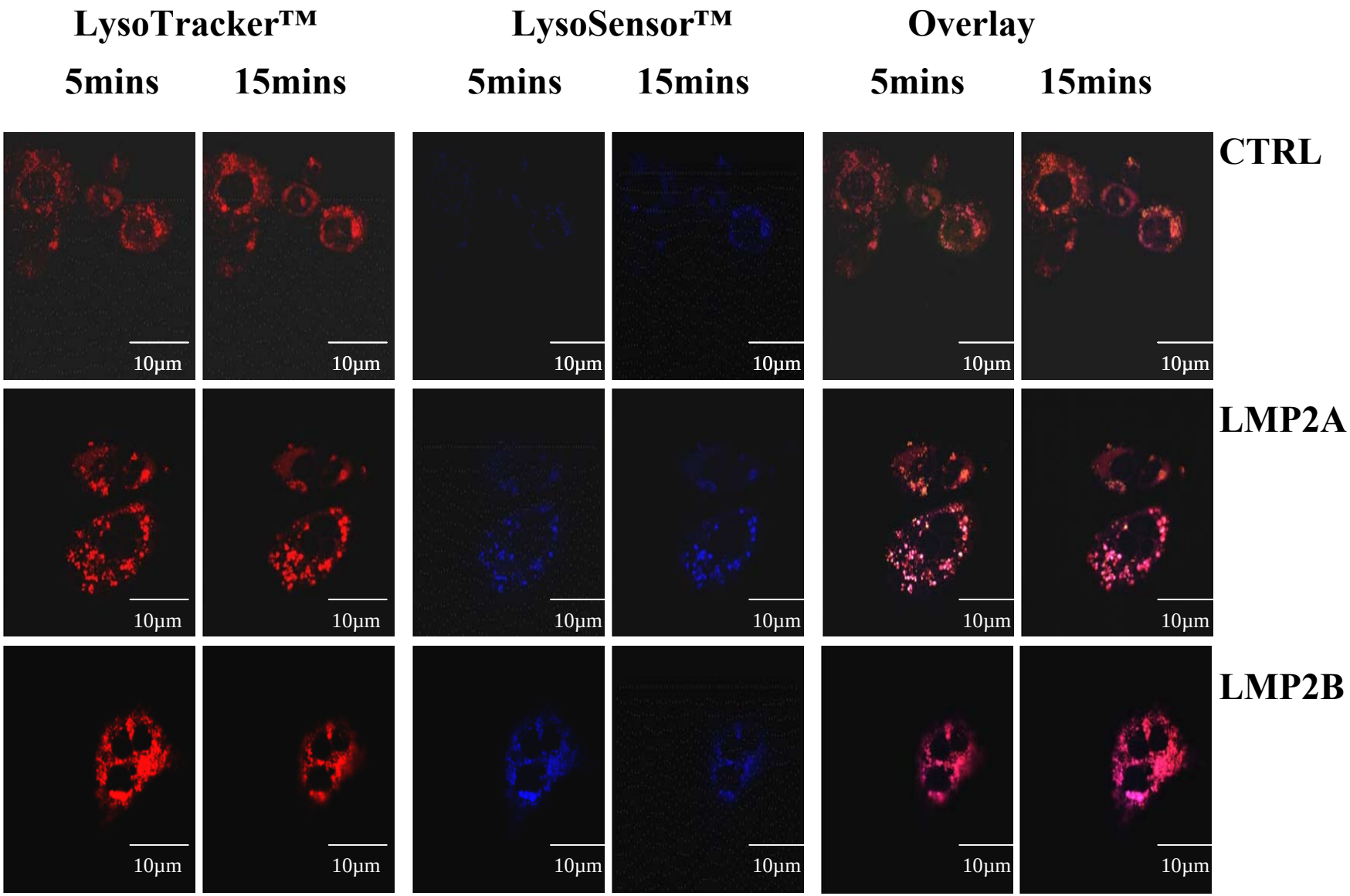
(A) CNE-2


LysoTracker™
LysoSensor™
Overlay
5mins
15mins
5mins
15mins
5mins
15mins
CTRL
LMP2A
LMP2B
10μm

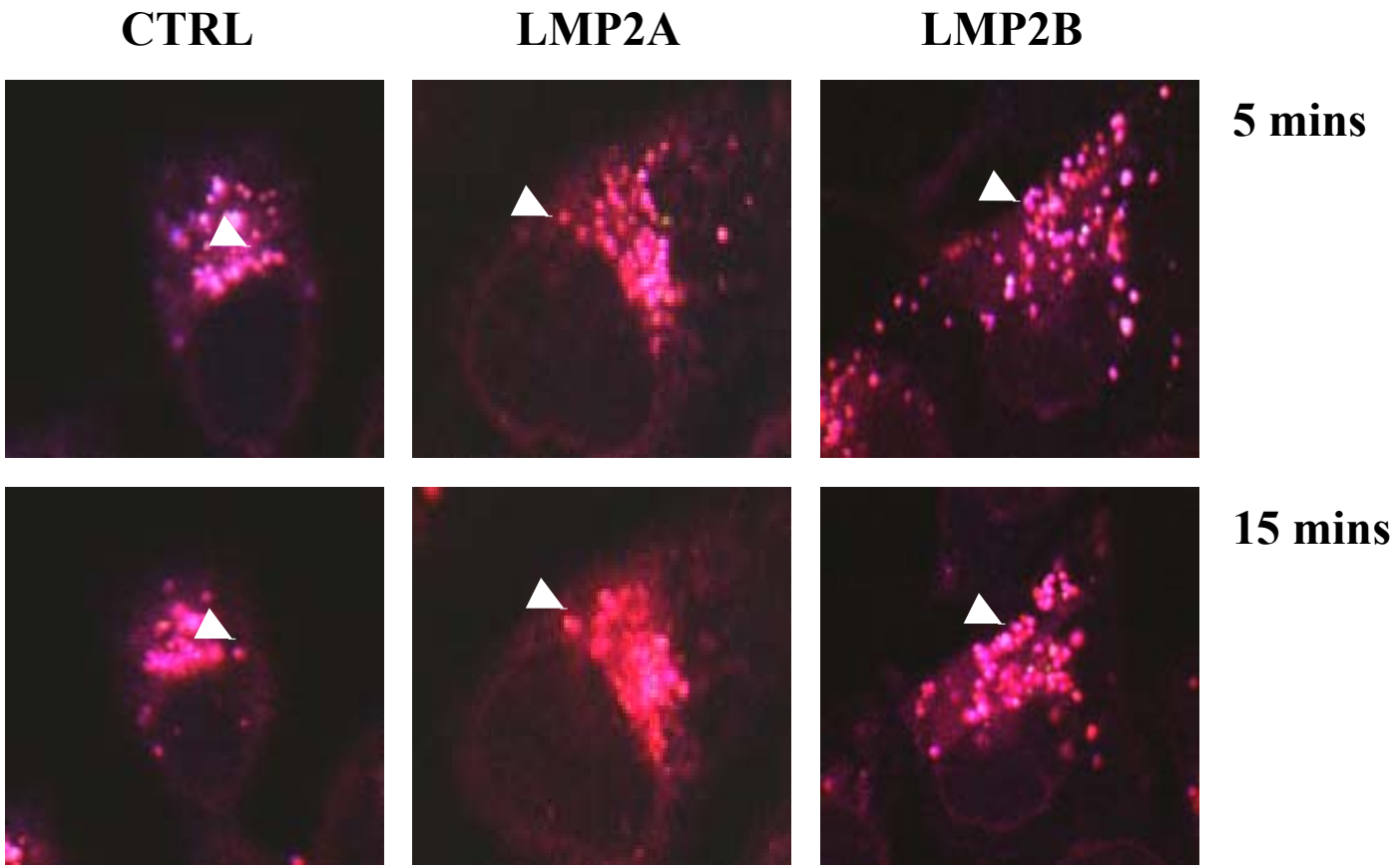
(B) H103


LysoTracker™
LysoSensor™
Overlay
5mins
15mins
5mins
15mins
5mins
15mins
CTRL
LMP2A
LMP2B
10μm

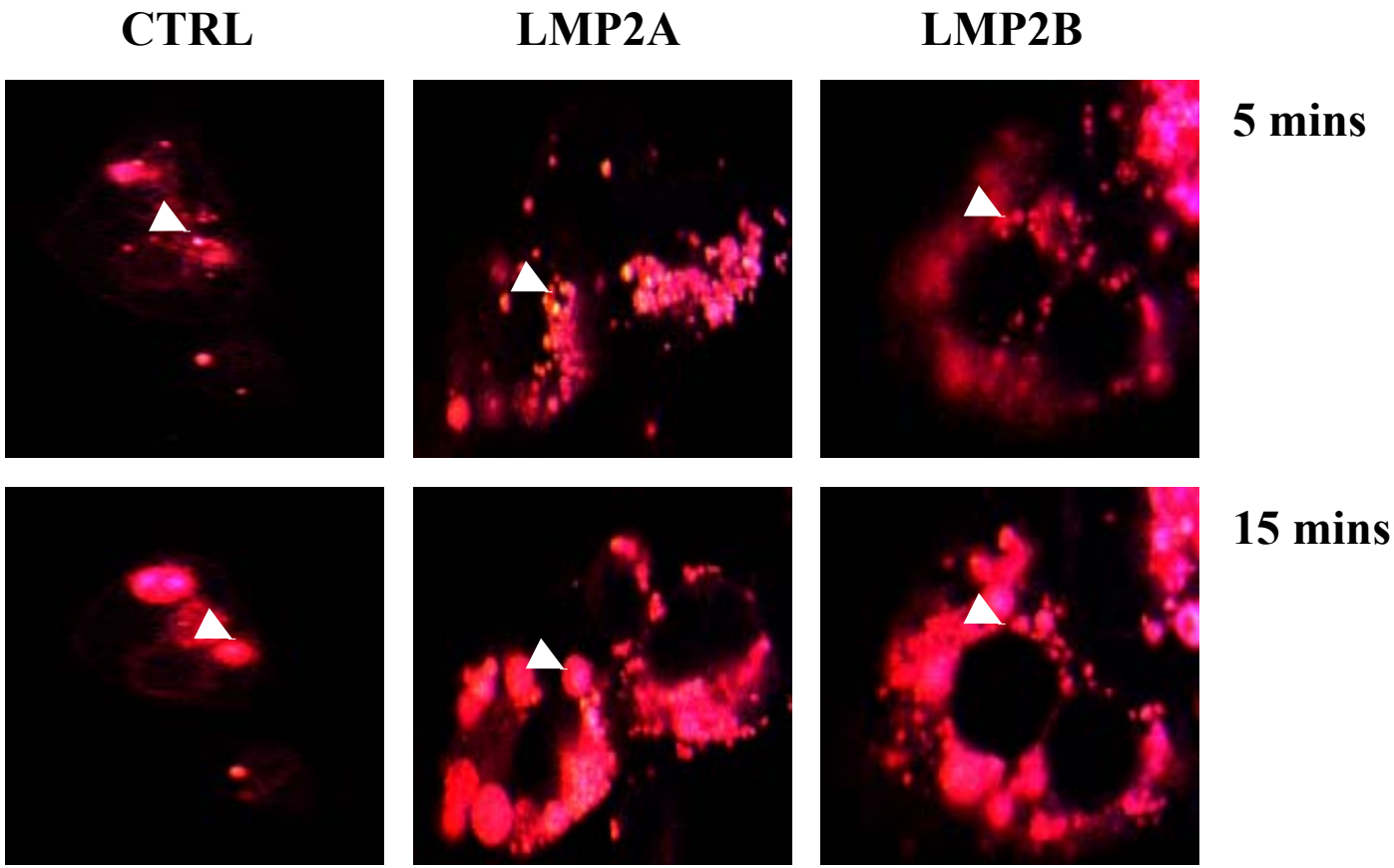
(C) H157


LysoTracker™
LysoSensor™
Overlay
5mins
15mins
5mins
15mins
5mins
15mins
CTRL
LMP2A
LMP2B
10μm

(D) CNE-2



(E) H103



(F) H157

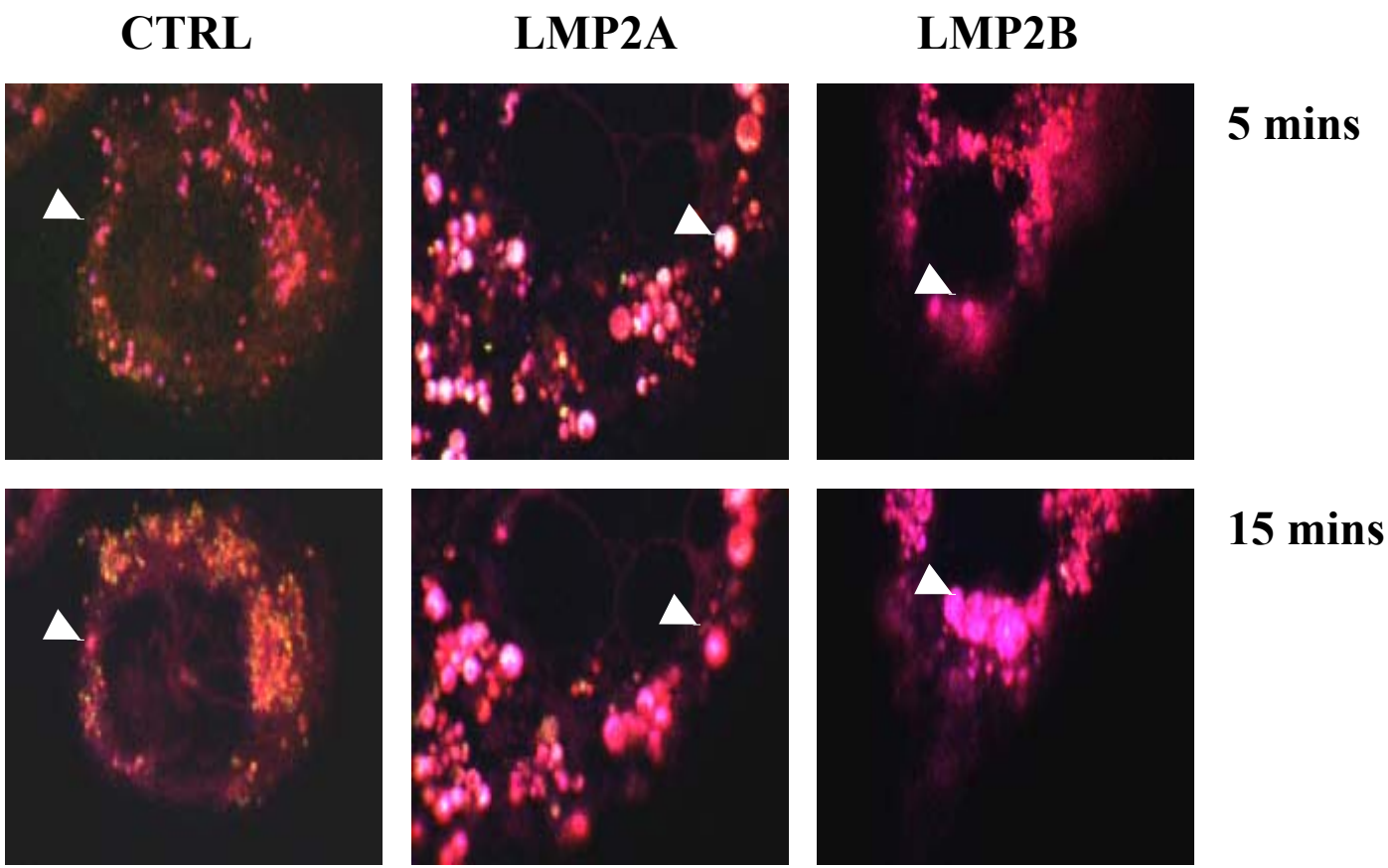
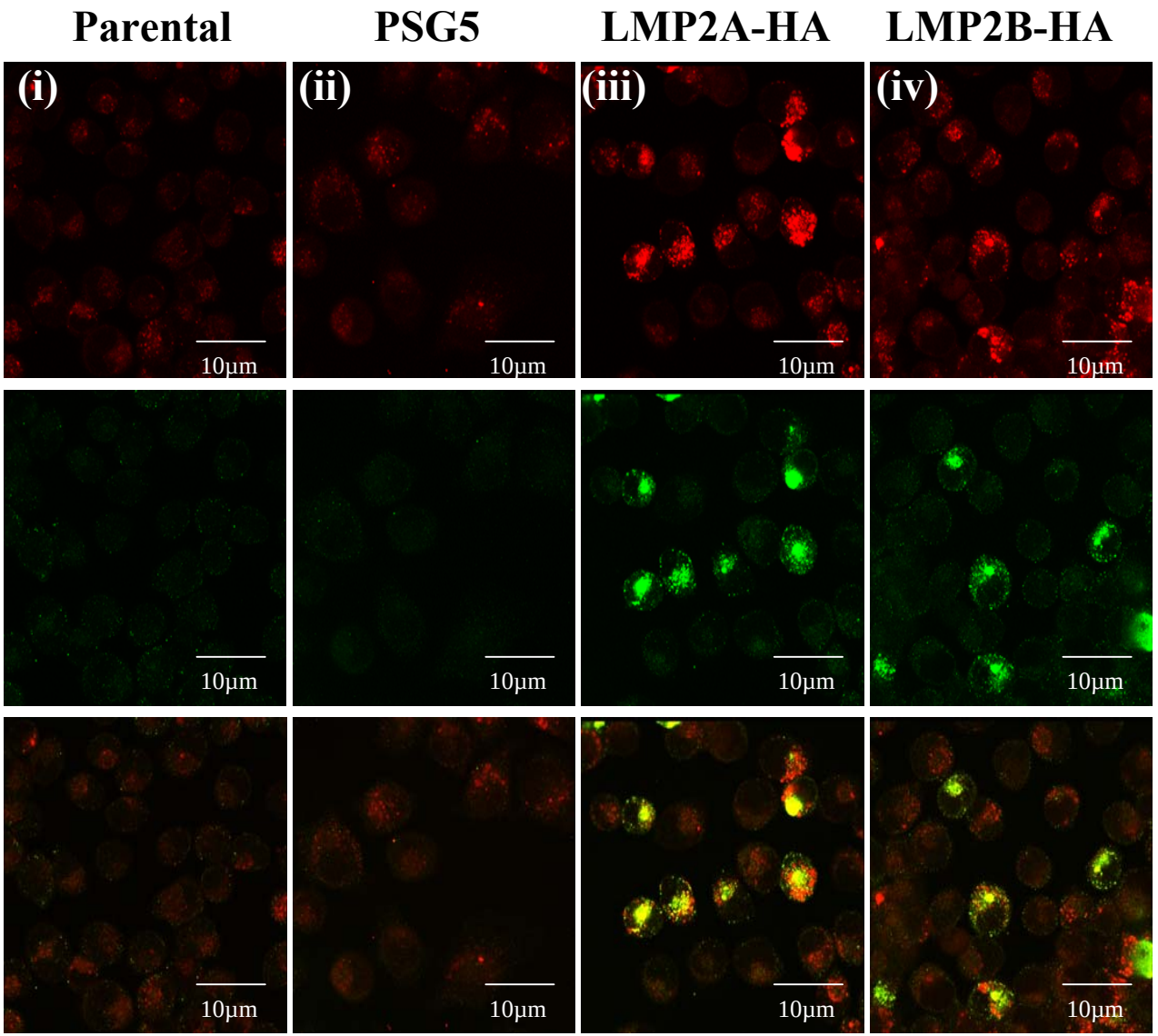
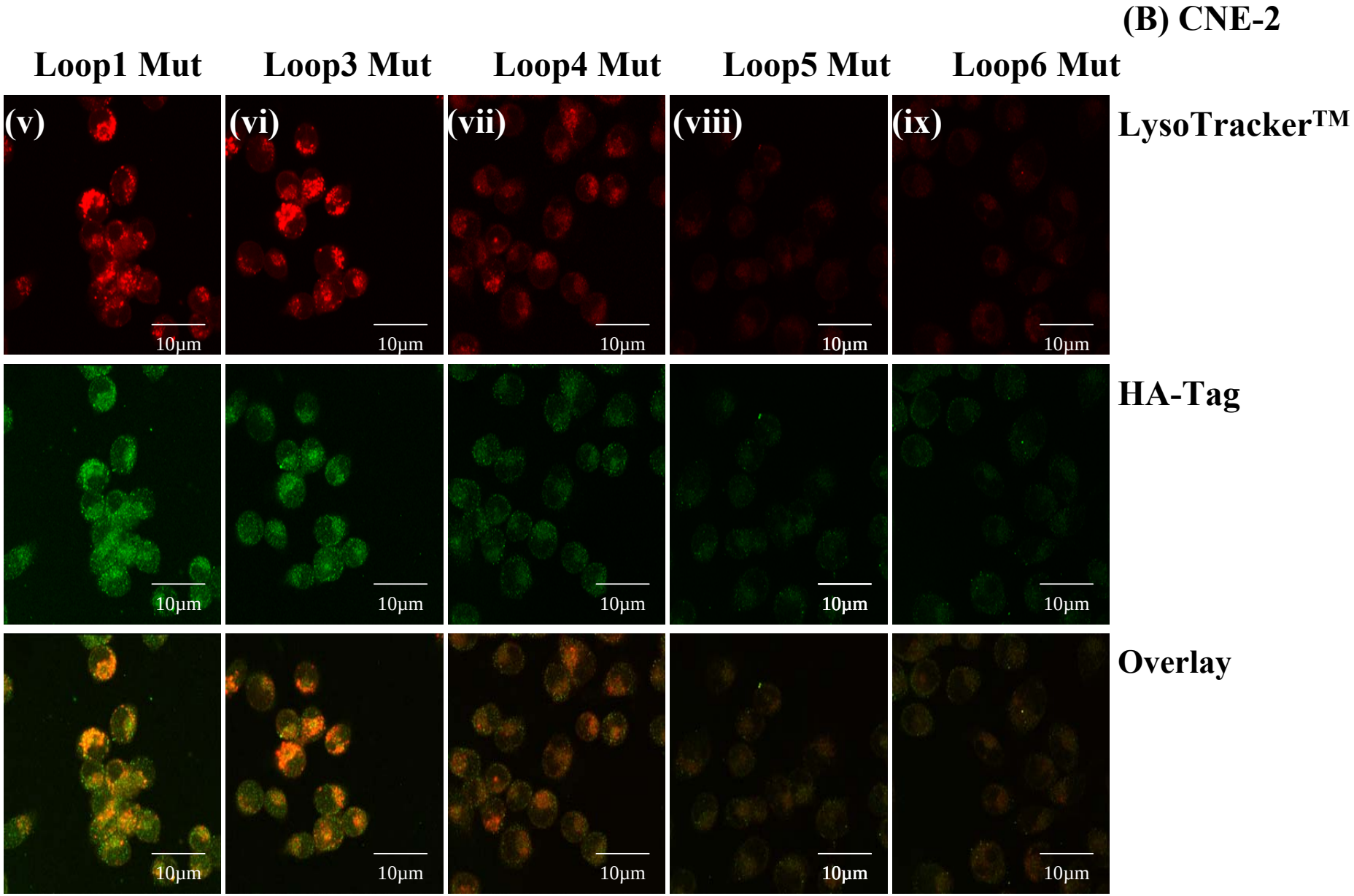


Figure 5.9 Immunofluorescent co-staining for LysoTracker™ and transiently expressed LMP2 variants in CNE-2, H103 and H157 cell panels.

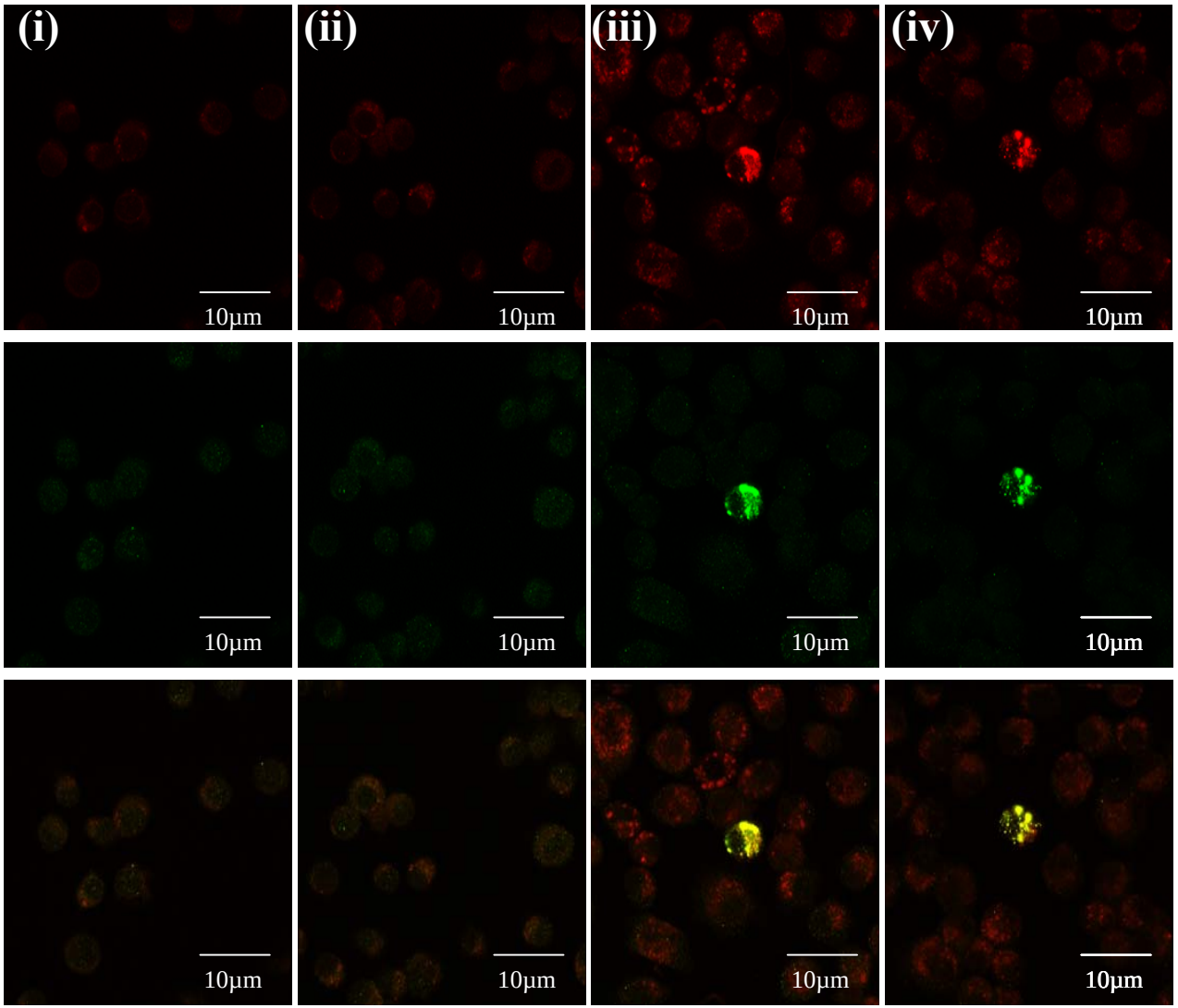
(A, B) CNE-2, (C, D) H103 and (E, F) H157 parental cells were seeded at a density of 2.5 x 10^5 cells per well of a 6-well plate and incubated overnight at 37˚C and 5% CO_2 to allow cell adherence. The following day the cells were transiently transfected with the recombinant lentiviral plasmids or relative controls and grown for 48 hours to allow expression. Transfected cells were then seeded at a density of 2 x 10^4 on to 12-well microdot Teflon coated slides and placed into a 37˚C incubator with 5% CO_2 overnight to allow cell adherence. All samples were treated with LysoTracker™ prior to fixation and permeabilisation. Immunofluorescent staining was performed for LMP2 using an antibody specific for the HA-tag. Representative confocal images are shown. In panels (A), (C) and (E), it is demonstrated that transient expression of LMP2 is sufficient to cause an increase in numbers of lysosomes when compared with null transfected controls. Compared with images shown in *Figure 5.7,* there is greater co-localisation of signals in the transient setting when compared to stably expressed LMP2. Panels (B), (D) and (F) show representative images of the effect of expression of the loop deleted forms of LMP2. Here it is demonstrated that while deletion of the transmembrane loops 1, 1-3 and 1-4 of LMP2, does not impair LMP2's ability to increase lysosomal number. Deletion beyond Loop 5 results in loss of membrane binding and stability of the protein and a concomitant return to lysosome number that is comparable with control levels. This loss of membrane binding is also observed in *Chapter 3, Figure 3.8*

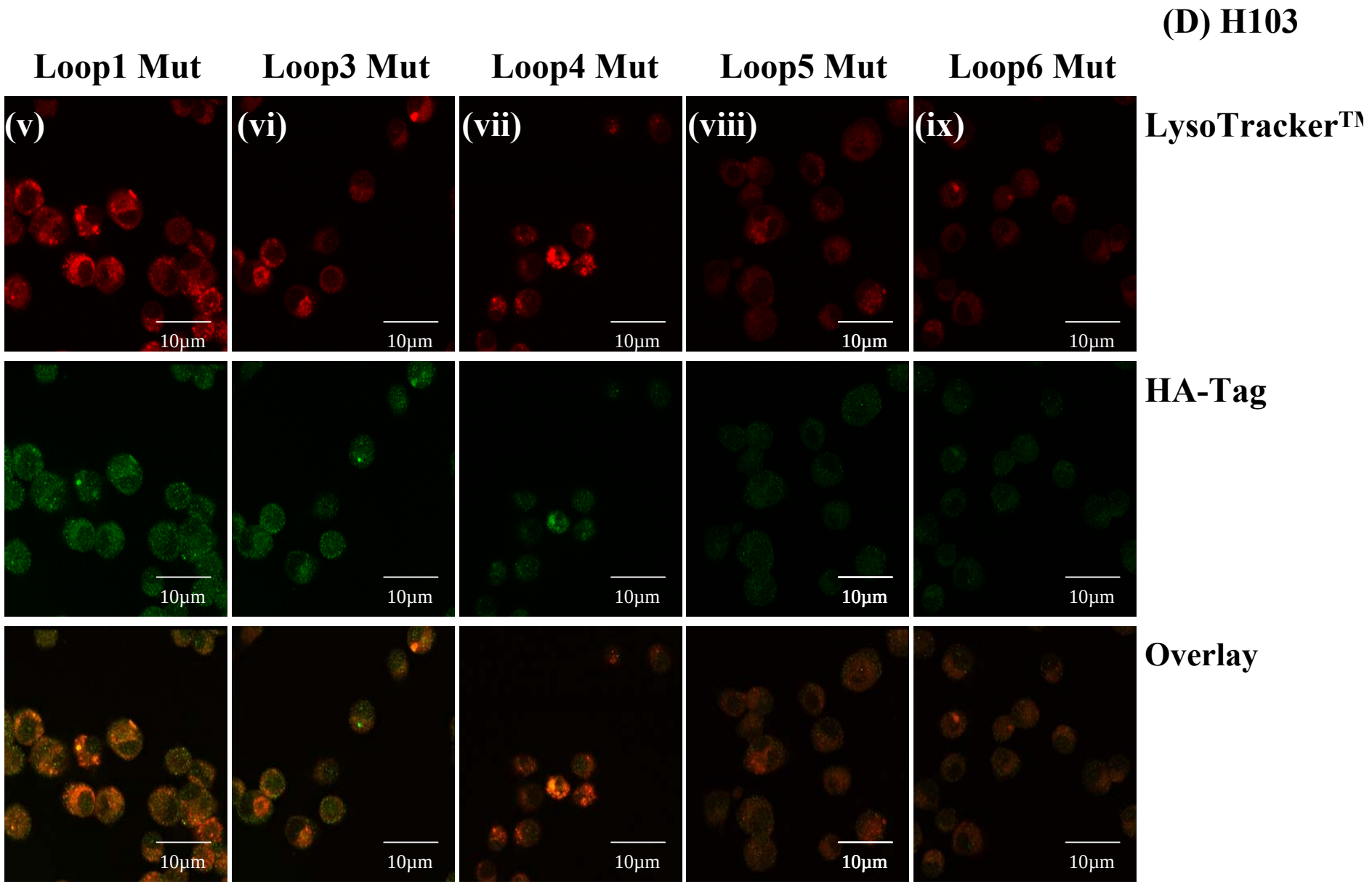
(A) CNE-2


Parental
PSG5
LMP2A-HA
LMP2B-HA
(i)
(ii)
(iii)
(iv)
LysoTracker™
HA-Tag
Overlay
10μm

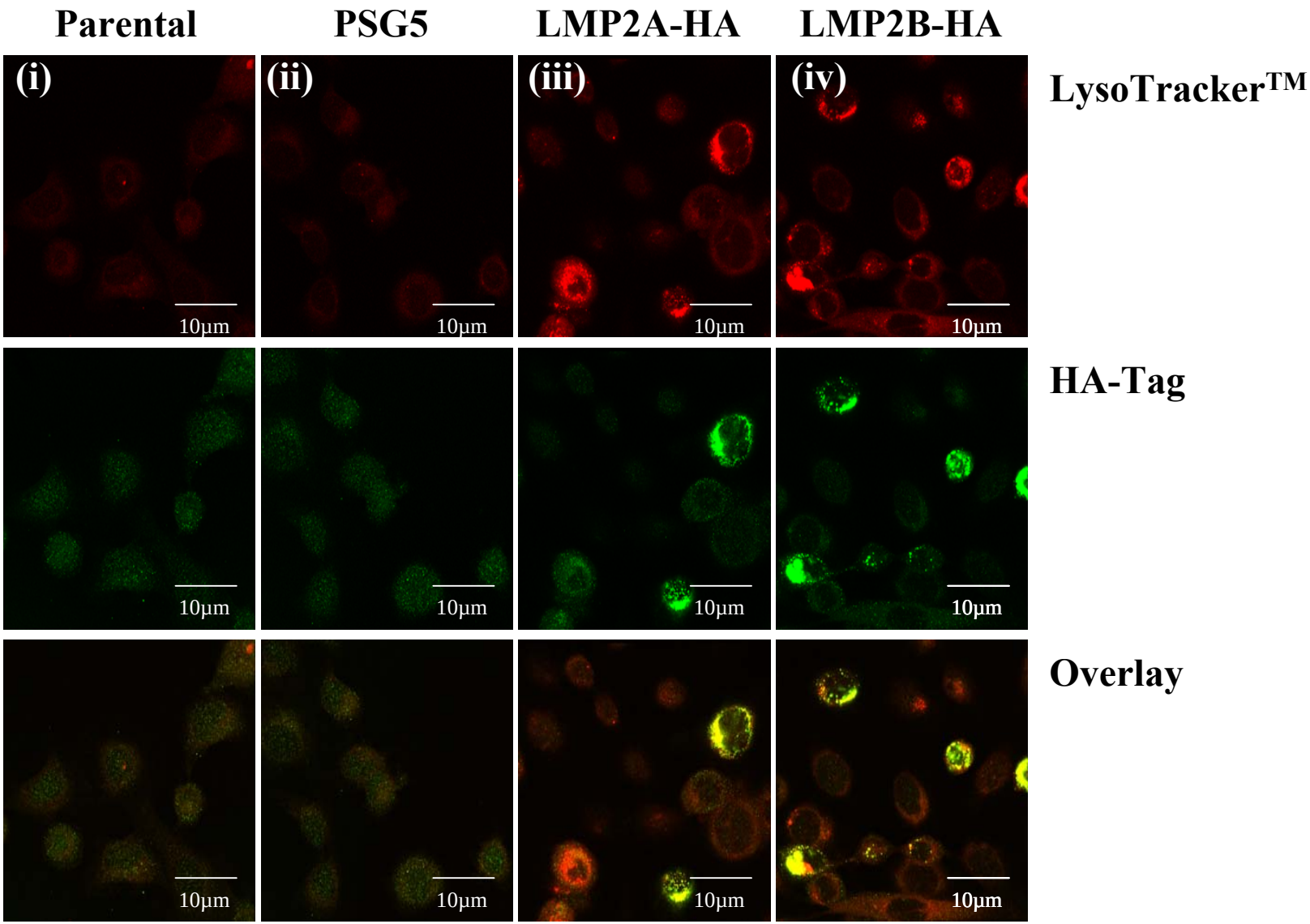

(B) CNE-2
Loop1 Mut
Loop3 Mut
Loop4 Mut
Loop5 Mut
Loop6 Mut
(v)
(vi)
(vii)
(viii)
(ix)
LysoTracker™
HA-Tag
Overlay
10μm

(C) H103

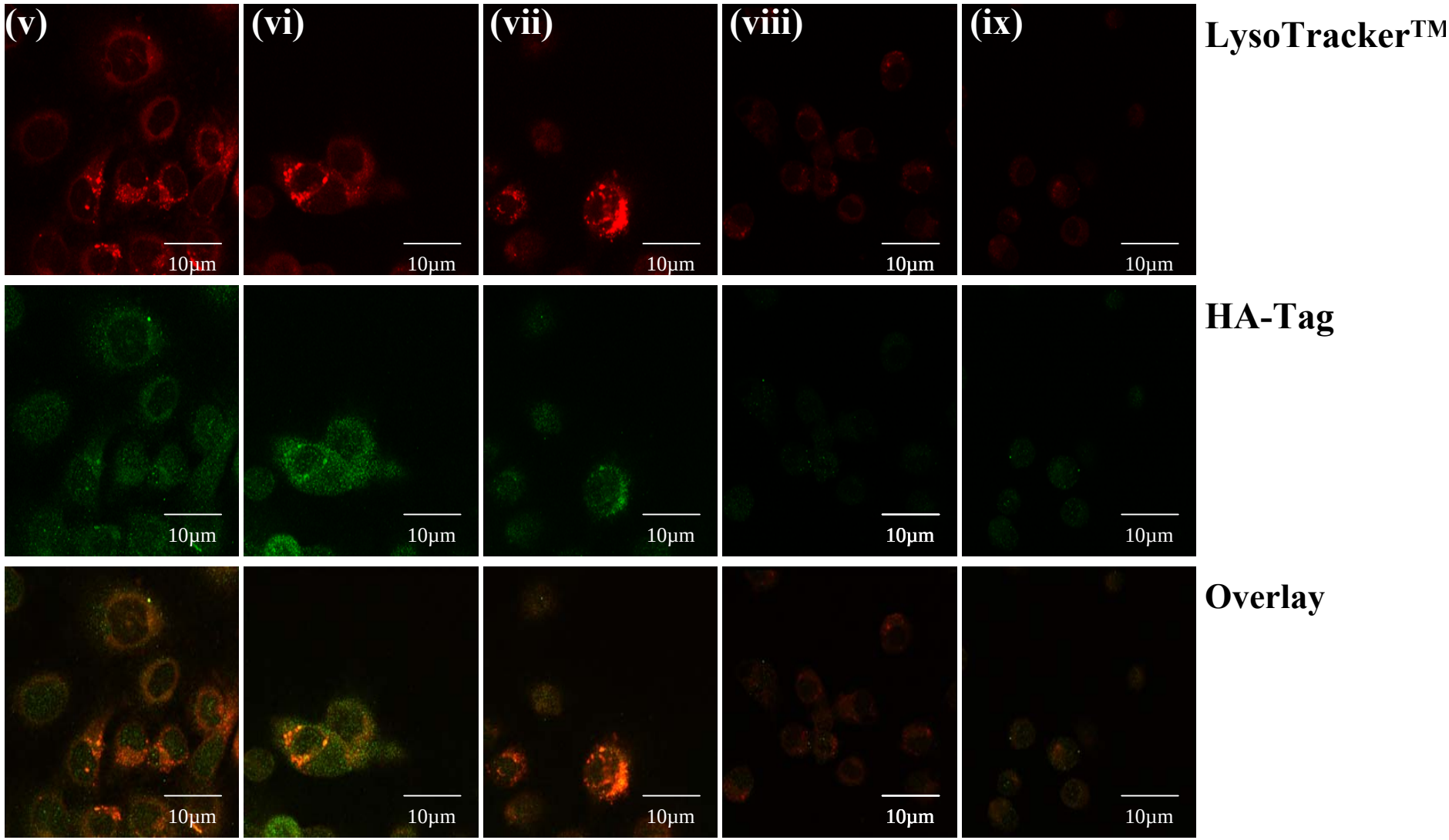

Parental
PSG5
LMP2A-HA
LMP2B-HA
(i)
(ii)
(iii)
(iv)
LysoTracker™
HA-Tag
Overlay
10µm


(D) H103
Loop1 Mut
Loop3 Mut
Loop4 Mut
Loop5 Mut
Loop6 Mut
(v)
(vi)
(vii)
(viii)
(ix)
LysoTracker™
HA-Tag
Overlay
10μm

(E) H157


Parental
PSG5
LMP2A-HA
LMP2B-HA
(i)
(ii)
(iii)
(iv)
LysoTracker™
HA-Tag
Overlay
10µm

(F) H157


Loop1 Mut
Loop3 Mut
Loop4 Mut
Loop5 Mut
Loop6 Mut
(v)
(vi)
(vii)
(viii)
(ix)
LysoTracker™
HA-Tag
Overlay
10μm

5.9 Discussion and future work.

While the novel findings presented here show that LMP2A and LMP2B alter the endosome-lysosome trafficking network in epithelial cells, the mechanism(s) by which this is achieved are unclear, and the physiological consequences of these effects are unknown. However, taken as a more "global" examination, findings presented here show that both LMP2A and LMP2B increase the number of early endosomes and endocytic vesicles, an effect which could facilitate increased endocytosis of plasma membrane-bound material such as receptors or integral membrane proteins. In tandem with this, the numbers of late endosomes and degradative lysosomes and are also increased, perhaps indicating that these viral proteins influence the rate at which receptors or signalling proteins are targeted for degradation. An important finding is the discovery that LMP2A and LMP2B increase the kinetics of endosome acidification, an effect that may have an effect on the signalling properties of receptors and other membrane-associated proteins. Protein studies on the mechanism of Rab5 have shown that EEs can fuse to late endosomes (LE) and acidic compartments (Rink, Ghigo et al. 2005). Concomitant increases in lysosome number given increased levels of Rab5 are therefore not surprising.

Increases in both Rab5 and EEA1 could indicate an increased level of endosomal fusion and thus an increase in merging of early endosomes into their Rab7 positive late endosomal counterparts. This would add weight to the hypothesis that LMP2 expression on internal membranes increases kinetics of traffic in the cell, rather than creating a bottleneck, where the level of one compartment severely outnumbers others. However this explanation cannot account for increases in acidification of individual lysosomal compartments, as assessed by imaging with LysoSensor™. It is postulated that LMP2A and LMP2B expression somehow interferes with functioning of vacuolar ATPases and as such decreases compartment pH, as shown by increases in Ductin expression in LMP2A and LMP2B expressing cells.

Thus far, the functional significance of this dysregulation has not been fully assessed, although it is hypothesised that an increase in the number of acidified compartments or increased kinetics of endosome acidification could prevent cell signalling via increased turnover and degradation of receptors and contribute to a down-regulation of receptor signalling. Initiation of signalling from certain receptors occurs in intra-cellular compartments where adaptor molecules, ligands and receptors form complexes and activate signalling cascades. Disrupting the external membrane architecture of these compartments, distorting the usual cellular ratios of compartment types and decreasing luminal pH would, in these cases, have detrimental effects on signalling networks. How LMP2A and LMP2B regulate this mechanism and the extent of this effect on receptors are as yet unknown. As localisation of the LMP2 proteins to internal membranes is integral to these processes, further analysis of stable expression of truncated forms of LMP2 on compartmental acidification will aid further understanding of the mechanism by which LMP2A and LMP2B exert these effects. It is shown that loss of membrane tethering through serial deletion of LMP2's transmembrane loops alleviates increases in lysosome number and it is expected to also alleviate decreases in pH.

Experimentally, further investigating these data would involve characterising the exact interaction of LMP2 with components of the early endosome cell membrane and assessing its relationship with Rab5 activity. Pulse chase experiments using labelled, radio-labelled or otherwise, versions of receptors would enable more robust proof that internalisation through clathrin-Rab5 mediated endocytosis is increased in LMP2 positive cells. Immuno-precipitation for tagged versions of LMP2 would allow characterisation of interacting intermediates and would determine relationships between LMP2 and, Rabex5, EEA1 and Rabapatin 5, effectors of Rab5 activity. Rab7 interaction with LMP2 would be assessed in a similar manner, to determine if LMP2 facilitates conversion of endosomes, or whether the kiss-and-run

hypothesis of vesicular trafficking enables a Rab7: LMP2 interaction. Tracing the path of internalised receptors by large-scale time-capture confocal microscopy would facilitate determining the postulated degradative fate of these molecules while also ascertaining the effect of LMP2 expression on recycling Rab4/Rab11 positive endosomes. In order to extend these findings it may also be prudent to examine the parallel effects of the E5 protein of HPV. In a similar manner this protein alters the pH of the GA but does not affect V-ATPase activity (Ashby, Meagher et al. 2001; Marchetti, Ashrafi et al. 2006), it is likely that the LMP proteins may act in a similar manner.

Taken together the above data demonstrate a definitive effect of LMP2A and LMP2B on the endosome-lysosome trafficking network; however, this work is very much in its infancy and further more specific investigation is necessary to tease out the exact nature of this modulation.

CHAPTER 6

General Discussion and Future Perspectives

The association of EBV with malignancies of both B-cells and epithelial cells is well documented. Although there is some level of understanding of the role the virus plays in the development and progression of these diseases, many of the effects of viral protein expression in both B-cell and epithelial cells remains to be elucidated. In B-cells, LMP2A acts as a surrogate BCR allowing B-cell maturation and survival, ensuring viral latency whilst preventing lytic reactivation (Swanson-Mungerson, Bultema et al. 2006; Wang, Nicholas et al. 2006). During B-cell infection, the potentiation of a surrogate BCR signal by LMP2A affords the virus a mechanism of persistent latent infection while also engaging with other signalling cascades to promote cell survival and preventing programmed cell death (Caldwell, Wilson et al. 1998). The "non-signalling" LMP2B protein is thought to function as a rheostat, antagonising LMP2A's ability to maintain viral latency (Rovedo and Longnecker 2007; Rechsteiner, Berger et al. 2008; Rechsteiner, Bernasconi et al. 2008). Little, if any, information is available regarding specific signalling capabilities of LMP2B in this cell type. The tenet of LMP2A and LMP2B's contribution to immune evasion in B-cells has been examined, and while their function in this cell type is becoming clearer, their role in epithelial cells is less well defined (Miller, Lee et al. 1994; Wang, Nicholas et al. 2006).

Although LMP2A and LMP2B are expressed in almost all NPC cases, their contribution to this epithelial malignancy remains elusive (Brooks, Yao et al. 1992). It has been shown that expression of the viral protein can alter epithelial cell adhesion and motility (Allen, Young et al. 2005; Lu, Lin et al. 2006), inhibit differentiation, and promote anchorage-independent growth, (Scholle, Bendt et al. 2000)) through engagement of the PI3-kinase/Akt and Wnt/β-catenin signalling pathways (Morrison, Klingelhutz et al. 2003; Fukada and Longnecker 2004; Pang, Lin et al. 2009). In terms of epithelial malignancy the majority of research has

focused on LMP2A and the effect of its amino-terminal signalling domain on cell function. Few, if any studies have addressed the role of LMP2B in epithelial cells.

This thesis can be divided into two distinct but linked areas of research. Findings presented in *Chapter 4* demonstrate an LMP2A-specific effect on Toll-like receptor (TLR) signalling, effects that can be attributed to the amino-terminal signalling domain given the inability of LMP2B to influence signalling from TLR receptors. These analyses were undertaken as part of a study to further explore the effects of LMP2A and LMP2B on attenuation of the type I interferon signalling in epithelial cells (Shah, Stewart et al. 2009). This previous study demonstrated that modulation of the type I IFN signalling response did not require the amino-terminal domain of LMP2A, but rather, required domains common to both the LMP2A and LMP2B proteins. Findings presented in *Chapter 5* have attempted to analyse the effects of LMP2A and LMP2B on intracellular trafficking and modulation of the endosomal/lysosomal network, with a view to exploring the possibility that LMP2A and LMP2B influence the fate of internalised receptors through mechanism(s) involving endosome acidification. Findings presented here suggest that although this may be true for the IFNRs (Shah, Stewart et al. 2009), the effects of LMP2A on TLR signalling must occur through as yet unidentified mechanism(s).

Viral evasion of the host's immune response can be achieved through a number of mechanisms, as outlined in *section 1.8*, many of which involve modulation of the innate immune response and disruption of the induction of cellular interferons. The idea that LMP2A could contribute to such an effect came from the study of epithelial cells infected with a recombinant EBV deleted for LMP2A (Stewart, Dawson et al., 2004). Unlike wild-type infected epithelial cells, cells carrying an LMP2A-deleted form of EBV displayed increased basal expression of NF-κB, STAT3 and IRF7, an effect which resulted in expression of the major viral transforming gene, LMP1 (Stewart, Dawson et al. 2004). In tandem with this expression of LMP2A and to a lesser extent LMP2B revealed that these proteins could modulate IFN signalling directly in the

NPC-derived cell lines, Hone-1 and Ad/AH. The pathways involved in innate immunity that control creation of an anti-viral state are initially mediated by the Toll-like receptors. Analyses of TLR signalling described in *Chapter 4* revealed that cells expressing LMP2A were almost refractory to TLR agonist activation of these responses, whilst LMP2B had little, if any effect on TLR signalling. These findings build upon the previous observations reported by Shah and colleagues and demonstrate that whilst both LMP2A and LMP2B are capable of limiting the actions of interferon, LMP2A alone is capable of modulating TLR signalling responses.

Five TLRs are known to control viral induction of the type I interferon response: TLR 3, 4, 7, 8 and 9; the remaining family members are involved in recognition of bacterial and fungal PAMPs (Kawai and Akira 2006). Findings presented in this study showed that LMP2A did not alter the signalling capabilities of the latter, suggesting that their effects were specific to viral and bacterial-encoded PAMPs. LMP2A did however have a profound effect on expression and signalling from TLR family members that mediate anti-viral immune responses. IFNβ production is a vital component of initialising an immune response to viral infection and its promoter activity was used as a read-out for the activation of these TLRs (Samuel 2001). In all cases, IFNβ promoter activity was attenuated in LMP2A expressing cells compared to their control and LMP2B expressing counterparts in response to TLR agonist stimulation. While indicative of a global dampening of TLR responses, differences in the extent and level of modulation by LMP2A were observed. These variations are attributable to the various signalling pathways and adaptor intermediates utilised by each TLR in the induction of type I interferon expression. The effect of LMP2A was most pronounced in the cases of TLR3 and TLR4, which can both signal via the TRIF-dependent pathway to activate IRF complexes (Honda and Taniguchi 2006). IRF3 in particular is important for signalling from these receptors and findings presented here demonstrate that its activity was attenuated by LMP2A. Although the exact mechanism(s) involved in this attenuation remains to be uncovered, it may likely involve

interference of LMP2A with either dimerisation or phosphorylation events, both of which facilitate activation and nuclear translocation. Examination of the phosphorylation of IRF3 is an important future perspective for continuation of this research and initial data demonstrate that upon stimulation of TLR3, the levels of phosphorylated IRF-3 are reduced in LMP2A expressing cells compared to control and LMP2B expressing counterparts.

Activation of TLR3 signalling was performed using poly (I:C) delivery to endosomes by liposome-mediated delivery, which ensured minimal cross-activation of the IFNβ promoter by signalling through the RLHs. It is thought however, that signalling through these receptors would be modulated in a similar manner given the effect of LMP2A expression on the activity of IRF-3. Analysis of the effect of LMP2A on EBER function demonstrates this. EBERs have been shown to modulate innate immunity via binding to the PKR and preventing activation of the eIF-2a transcription factor, (Nanbo and Takada 2002); however, they can also initiate signalling through RIG-1 activation (Samanta, Iwakiri et al. 2006) and more recently through activation of TLR3 (Iwakiri, Zhou et al. 2009). Transient expression of the EBERs resulted in a robust stimulation of IFNβ promoter activity in control and LMP2B expressing cells, whereas this activation was attenuated in LMP2A expressing cells, demonstrating an ability of LMP2A to dampen EBER induction of IFNβ expression. This is important in the context of virus infection, where the EBER RNAs are always expressed. The interchangeable roles of IRF7 and IRF3 in TLR signalling pathways (Barnes, Richards et al. 2004) was not examined and as such it is shown that modulation of signalling by LMP2A is known to be contained to early signalling events prior to expression of IRF7. IRF7 expression however relies on type I interferon signalling and given that LMP2A modulates IGSF3 formation it is likely that IRF7 mediated TLR signalling would also be attenuated. Whether the activation of IRF7 is directly affected by LMP2A remains a future avenue of research.

NFκB activity was also examined by luciferase activity to further characterise the effect of LMP2A on TLR-mediated activation of the IFNβ promoter. Here it was surprising to see a primed NFκB response given the aforementioned effects on IRF3 activation and IFNβ promoter activity. These effects were most striking in the context of TLR3 stimulation in response to poly(I:C) stimulation, whereas stimulation with the remaining four TLR agonists (TLR 4,7,8,9) showed dampened responses in NFκB activity. Here, LMP2A appeared to augment the primed response. Indeed immunofluorescent staining for p65 pre- and post-stimulation shows that p65 nuclear translocation is increased in the presence of LMP2A. Again this can only be attributed to the route by which these TLRs induce IκBα phosphorylation and degradation, with TLR3 signalling only through TRIF-mediated pathways with the remaining receptors facilitating signal through MyD88 (Sandor and Buc 2005). This hypothesis holds up in the case of TLR4 signalling as this pathway preferentially activated IRF complexes via TRIF in early cascades and utilises its MyD88 adaptor capacity for additional signalling (Shen, Tesar et al. 2008).

Inactivation of IRF3 by LMP2A and hyper-activation of p65 are results that appear to be antagonistic to one another especially given the binding of both factors in the enhancesome complex (Panne 2008). EMSA analyses of two of the four domains of the IFNβ promoter element produced results in agreement with dampening of IFNβ production by LMP2A. IRF3-IRF7 hetero/homo-dimers bind to PRDI while NFκB p65 subunits bind at PRDII. Upon stimulation with poly(I:C), binding of protein complexes to both sites were decreased in cells expressing LMP2A, whereas robust binding was observed in control and LMP2B expressing cells. These findings demonstrate the indispensable nature of IRF subunit binding in controlling this promoter and, although NFκB activity is increased, p65/p50 subunits cannot bind to PRDII domains without the co-operative binding of the remainder of activated IRF dimers. A general consensus sequence for NFκB activity did however demonstrate that binding of these subunits was effective elsewhere.

Physiologically in the context of cytokine release, LMP2A for the most part augments the production and secretion of pro-inflammatory cytokines. Basally this disparity is evident but it becomes much more pronounced upon analysis of cells post-stimulation with the TLR3 and TLR4 agonists, poly (I:C) and LPS. Production of these increased levels of cytokines fits nicely with a situation where NFκB activity is "primed" and becomes hyper-activated in LMP2A expressing cells. Two of the cytokines examined however displayed decreased levels in LMP2A expressing cells. These cytokines, RANTES/CCL5 and sICAM-1, are known to be under the control of IFNβ signalling through the type I response, and as such adds further weight to the ability of LMP2A to limit the induction of IFNβ. Taken as a whole, these findings suggest that LMP2A attenuates the induction of IFNβ-regulated cytokines, whilst augmenting the production of other pro-inflammatory cytokines. The observation that cytokine release is both augmented in some cases and decreased in others removes any ambiguity as to the specificity or efficacy of the experiment and indicates a situation where one signalling cascade is initiated and the other attenuated. The role of the lymphocyte infiltrate of NPC is a topic under current investigation and scrutiny and results such as these indicate that the situation may be more complex than first considered (Niedobitek, Young et al. 1992; Agathanggelou, Niedobitek et al. 1995; Wu, Chien et al. 2005), that the tumour cell may attract the infiltrate to promote an environment that is more permissive to tumourigenesis.

Another aspect of this model of signalling modulation by LMP2A is the role of PI3K signalling. This pathway is engaged by LMP2A, *section 1.5.3.2*, which results in constitutive phosphorylation and activation of Akt. PI3K also plays a role in TLR signalling, in particular mediating the response through TLR3 (Schroder and Bowie 2005). Modulation of this signalling pathway could indirectly prevent IRF3 phosphorylation downstream of TLR3. Interference of TLR signalling by LMP2A would afford the virus a cellular context incapable of mounting or maintaining an "anti-viral" state. The contribution of LMP2A to NFκB signalling observed here

is at odds with previous data showing that the protein decreased the levels of IL-6 secretion, although these findings were based on deletion of LMP2A from the whole virus (Stewart, Dawson et al. 2004). Also experiments were performed in the adenocarcinoma cell line Ad/Ah, while here the cell lines used are either derived from squamous carcinoma of the head and neck, H103, or derived from NPC itself, CNE2. In the context of whole virus infection, the augmentation of LMP1 induced NFκB activity by LMP2A has been reported and the data presented here further implicate LMP2A in this signalling cascade (Dawson, George et al. 2001). Results of analyses using the EBERs reveal that although these RNAs can mount RIG-1 induced IFNβ activity, this is attenuated in the presence of LMP2A, facilitating EBER action without establishment of an innate immune response.

The second area of investigation regarding the effect of viral protein expression on intracellular trafficking was undertaken to bridge the gap between the exclusive signalling modulation of the TLR network by LMP2A and the function of both proteins in controlling signalling through IFNαβR. The endosomal lysosomal network controls the endocytosis, degradation and recycling of signalling receptors, (Bonnerot, Briken et al. 1997; Lorenzo, Ploegh et al. 2001; Boulan, Kreitzer et al. 2008). Distortion of this network could account, in part, for the dampening of signalling allowing the virally infected cells to evade immunosurveillance. Although effects of LMP2A and LMP2B on modulation of the endosome/lysosomal network have been uncovered, the mechanism(s) involved have yet to be elucidated. It was thought that the effect of the viral proteins on endosomal traffic may also play a role in modulation of TLR signalling, given that the receptors investigated, with the exception of TLR4, bind to, signal from and are activated by ligands recognised within the endosome (Colonna 2007). However although the numbers and apparent activities of these compartments are altered, the fact that LMP2B expressing cells displayed no differences in levels of TLR signalling discount this possibility. In

these cells although movement of compartments is augmented it is hypothesised that this does not distort ligand:TLR binding and activation.

Early endosomes are responsible for the endocytosis and sorting of membrane bound receptors in order to facilitate signalling and receptor turnover (Woodman 2000). Characterisation of endosomes has been aided by the identification of markers specific to compartments involved in different stages of vesicular trafficking enabling early and late endosomes to be distinguished (Stenmark and Olkkonen 2001; Barnekow, Thyrock et al. 2009). Rab5 is a marker of the early endosome, immuno-blotting using whole cell lysates showed an increase in expression in the presence of LMP2A and LMP2B. Immunofluoresence staining and confocal imaging revealed that this was due to an increase in the numbers and/or formation of early endosomes. A similar scenario emerged when analysis of Rab7 was undertaken, indicating that late endosome numbers also appeared to be increased. Rapid endocytosis from the membrane of MHC molecules is a mechanism utilised by many viruses in evading immune surveillance (Ishido, Wang et al. 2000), *section 1.9.6*. The K3 and K5 proteins of KSHV, *section 19.6.2,* are known to exert this effect on both MHC molecules and the IFNγ receptor (Li, Means et al. 2006). It is hypothesised that the increased number of early endosomes observed in LMP2A and LMP2B expressing cells may increase the turnover of cell-surface receptors or membrane associated proteins, which may include the IFNRs or TLRs. Pulse-chase experiments using fluorescently-labelled ligands would provide an indication that the level of endocytosis in these cells is increased, thus accounting for increases in the number of membrane-associated early vesicles.

As described in *section 1.9*, binding of Rab GTPase effector proteins is necessary and important for compartmental conversion events. This machinery is adaptable and significant overlap exists between the Rab proteins involved in its regulation (Rink, Ghigo et al. 2005) allowing Rab5 positive endosomes to progress to sorting Rab4 positive endosomes and switch to

Rab7 positive late endosomes (Barnekow, Thyrock et al. 2009). Findings presented here show that LMP2A and LMP2B localise to early endosomal rather than in late endosomal compartments. Increases in expression of EEA1 a Rab5 effector protein (Simonsen, Lippe et al. 1998; Christoforidis, McBride et al. 1999) indicates that Rab5 activity may also be increased which could facilitate compartmental conversion more rapidly, increasing the movement of cargo through the cell. The sorting endosome or MVB is designated by the expression of both Rab4 and Rab11 and future work will focus on this as one possible site for LMP2's interference with the pathway (McCaffrey, Bielli et al. 2001), with a putative role for the Nedd4-like ubiquitin ligase binding domain of LMP2A possibly influencing sorting of proteins by ubiquitination (Longnecker, Merchant et al. 2000; Winberg, Matskova et al. 2000; Piper and Luzio 2007). It is suggested that disruption of activity here could redirect cargo to be degraded rather than recycled, accounting for an attenuation in receptor signalling.

Rab7 is as mentioned a marker for late endosomes but is also expressed on the membranes of lysosomes (Bucci, Thomsen et al. 2000). The lysosome is the degradative vacuole of the cell, the acidic luminal environment coupled with the presence of acid hydrolases facilitate, ligand-receptor de-coupling and degradation of endocytosed particles (Eskelinen, Tanaka et al. 2003). Live cell confocal microscopy demonstrated that lysosomal number was increased in LMP2A and LMP2B expressing cells and that the lysosomes of these cells were also more acidic. The nature of these experiments allowed identification of the lysosome using one dye and the concomitant identification of acidification using another. The dyes are endocytosed and thus follow the pathway through the trafficking network, using time capture it was demonstrated that movement of the dye is markedly faster in LMP2 expressing cells. There are two main theories accounting for this, firstly the overabundance of lysosomes may facilitate uptake of the dye more quickly or given the increases in early endosome number that the shuttling of endocytosed material in LMP2 expressing cells is augmented. Techniques using this

type of live imaging are being optimised to allow comparison of the traffic of labelled receptors between LMP2 positive and negative cells.

Increases in lysosomal acidity may also in part be attributed to self-aggregation of the viral proteins on internal membranes. Interaction of these aggregates with the vacuolar ATPase machinery could account for such increases, or indeed the aggregates themselves could act as porin-like structures. It is shown here that expression of a component of this complex, Ductin, is increased upon LMP2A and LMP2B expression, although this can be attributed to an increase in lysosomal number, rather than an increase in numbers and activity of endogenous vacuolar ATPase complexes. While it is tempting to hypothesise that LMP2 could exert such effects, a significant degree of co-expression was not observed between the viral protein and the lysosomal markers. This could be due to the detection system currently used for LMP2 but it is more likely to be indicative of an indirect LMP2 effect. While initially these analyses were performed to define a mechanism for dampening of innate immune signalling, increased intra-cellular traffic could have far reaching effects on signalling responses controlled by other receptors, such as the EGFR (epidermal growth factor receptor). This receptor initiates signalling via MAPK and Akt to control cell motility and proliferation (Miller and Raab-Traub 1999; Repetto, Yoon et al. 2007), is regulated through tyrosine kinases and is a target or viral oncoproteins including, E5 of papilloma viruses BPV and HPV16, which has been shown to alter trafficking to prevent MHC presentation, (Thomsen, Deurs et al. 2000; Marchetti, Ashrafi et al. 2006). The ability of LMP2A to sequester tyrosine kinases and the ability of LMP2A and LMP2B to alter membrane traffic may play a role in distortion of EGFR signalling, which may promote tumorigenesis (Lu, Lin et al. 2006; Sandilands and Frame 2008).

The necessity of the internal membrane localisation of the LMP2 proteins in order to exert these effects is demonstrated clearly upon generation and expression of the loop deleted mutants of this transmembrane region, *Chapter 3.* Expression of these proteins reversed the

increase in lysosome formation once membrane attachment was lost. Little is known about the activity of the transmembrane domains apart from membrane anchoring, (Tomaszewski-Flick and Rowe 2007). Here it is shown that proteins appear increasingly unstable after deletion of loop 4 and that this unstable unbound protein exerts little or no effect on lysosome formation. Previous published data revealed that these transmembrane domains were necessary for internal membrane anchoring and that the carboxyl-terminus domain was responsible for self-aggregation of the protein, (Rowe and Tomaszewski-Flick 2007). Most of the current research of these proteins focuses exclusively on the role of the signalling domain unique to LMP2A, while investigations into the role of LMP2B in the B-cell context have focused on its apparent ability to modulate these signals (Rovedo and Longnecker 2007; Rechsteiner, Berger et al. 2008; Rechsteiner, Bernasconi et al. 2008). Generation of the loop-deleted mutants of the proteins allows in depth analysis into the role of the LMP2B protein while also highlighting the importance of certain transmembrane domains for the function of LMP2A. Although these studies remain in their infancy it is believed that generation of cell lines stably expressing these truncated proteins will underpin the importance of certain domains within the transmembrane portion of the protein in controlling the localisation of the protein and its ability to induce its effect on endomembrane dynamics. While not presented here, a bioinformatic examination of these domains has revealed putative myristylation consensus sequences, which could account for the binding of LMP2A and LMP2B to lipid rafts on intracellular membranes of epithelial cells. Such a location allows the proteins to alter the endosomal to lysosomal trafficking network, which as demonstrated results in modulation of signal from certain classes of immune receptors. In B-cells, the association of LMP2A with lipid rafts allows this viral protein to act as a surrogate BCR; indeed, recent studies show that this ability is dependent on cholesterol (Ikeda and Longnecker 2007). Similar cholesterol depletion experiments are currently being performed to characterise whether lipid rafts in epithelial cells are the sites of LMP2 action. Interactions

between phospho-lipids and proteins are a fundamental part of intracellular traffic and signal transduction. PI3K pathways have been shown to be involved in Rab5 activation, further implicating LMP2A's modulation of this pathway as a possible mechanism of endosomal distortion (Horiuchi, Lippe et al. 1997; Hirota, Kuronita et al. 2007). The Tip protein of HSV may also illuminate a potential mechanism for this interaction as it also binds to lipid rafts and modulates cell signalling, specifically TCR by interaction with STAT3 (Cho, Kingston et al. 2005). It is known that these lipid rafts serve as a base for creation of "signalosome" complexes and immunological synapses, it is possible that distortion of these raft structures by the presence of the LMP2 proteins could modulate formation and disassociation of receptors, ligand and adapter proteins. It is possible that the signalling effects seen upon LMP2A expression result from the interaction of its amino-terminal domain upon distortion of lipid rafts, possibly explaining the apparent disparity in function of these two viral proteins. Further analyses with these mutants would allow a clearer understanding of the influence binding of these proteins to lipid rafts have on membrane mechanics.

In conclusion, novel functions of the LMP2 viral proteins have been identified highlighting the function of these proteins in promotion of latency and tumourigenesis by innate immune evasion. Signalling through TLRs is modulated upon expression of LMP2A but is not affected by LMP2B, while endosomal trafficking and the putative effect of this on receptor signalling is altered by both transmembrane proteins. Taken together these data demonstrate the unique signalling potential of the amino-terminal signalling domain of LMP2A while also illustrating the result of intracellular membrane binding by the transmembrane regions of both proteins.

References

A, T., H. Yanai, et al. (2005). "Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors." Nature **434**(7030): 243-249.

Agathanggelou, A., G. Niedobitek, et al. (1995). "Expression of Immune Regulatory Molecules in Epstein-Barr Virus-Associated Nasopharyngeal Carcinomas with Prominent Lymphoid Stroma." American Journal of Pathology **147**(4): 1152-1160.

Albiger, B., S. Dahlberg, et al. (2007). "Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors." Journal of Internal Medicine **261**: 511-528.

Alcami, A. and U. H. Koszinowski (2000). "Viral mechanisms of immune evasion." Trends in Microbiology **8**(9): 410-418.

Alexander, W. S. and D. J. Hilton (2004). "The Role of Suppressors of Cytokine Signaling (SOCS) Proteins in Regulation of the Immune Response." Annual Review of Immunology **22**: 503-29.

Alexopoulou, L., A. Czopik Holt, et al. (2001). "Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3." Nature **413**: 732-738.

Allen, M. D., L. S. Young, et al. (2005). "The Epstein-Barr Virus Encoded LMP2A and LMP2B Proteins Promote Epithelial Cell Spreading and Motility." Journal of Virology **79**(3): 1789-1802.

Ambinder, R. (2003). "Posttransplant lymphoproliferative disease: pathogenesis, monitoring, and therapy." Current Oncology **5**(5): 359-363.

Ambinder, R., P. Browning, et al. (1993). "Epstein-Barr virus and childhood Hodgkin's disease in Honduras and the United States." Blood **81**(2): 462-467.

Anagnostopoulos, I., H. Herbst, et al. (1989). "Demonstration of monoclonal EBV genomes in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma by combined Southern blot and in situ hybridization." Blood **74**(2): 810-816.

Anderson, L. and R. Longnecker (2008). "An auto-regulatory loop for EBV LMP2A involves activation of Notch." Virology **371**(2): 257-266.

Anderson, L. and R. Longnecker (2008). "EBV LMP2A provides a surrogate pre-B cell receptor signal through constitutive acitvation of the ERK/MAPK pathway." Journal of General Virology **89**(7): 1563-1568.

Andrea, B., M. Frey, et al. (2004). "Suppressor of Cytokine Signaling (SOCS) Proteins Indirectly Regulate Toll-like Receptor Signaling in Innate Immune Cells." The Journal of Biological Chemistry **279**(52): 54708-54715.

Anwar, N., D. Kingma, et al. (1995). "The investigation of Epstein-Barr viral sequences in 41 cases of Burkitt's lymphoma from Egypt: epidemiologic correlations." Cancer **76**(7): 1245-1252.

Araujo, I., H. Foss, et al. (1999). "Frequent expansion of Epstein-Barr virus (EBV) infected cells in germinal centres of tonsils from an area with a high incidence of EBV-associated lymphoma." Journal of Pathology **187**(3): 326-330.

Arnulf, B., C. Copie-Bergman, et al. (1998). "Nonhepatosplenic gammadelta T-cell lymphoma: a subset of cytotoxic lymphomas with mucosal or skin localization." Blood **91**(5): 1723-1731.

Ashby, A. D. M., L. Meagher, et al. (2001). "E5 transforming proteins of papillomaviruses do not disturb the activity of the vacuolar H+-ATPase." Journal of General Virology **82**: 2353-2362.

Au, W., P. Moore, et al. (1998). "Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes." Journal of Biological Chemistry **273**(44): 29210-29217.

Baer, R., A. Bankier, et al. (1984). "DNA sequence and expression of the B95-8 Epstein-Barr virus genome." Nature **310**(5974): 207-211.

Balkhi, M., K. Fitzgerald, et al. (2010). "IKKalpha negatively regulates IRF-5 function in a MyD88-TRAF6 pathway." Cell Signalling **22**(1): 117-127.

Bananis, E., J. W. Murray, et al. (2000). "Microtubule and Motor-dependent Endocytic Vesicle Sorting In Vitro." The Journal of Cell Biology **151**(1): 179-186.

Barnekow, A., A. Thyrock, et al. (2009). "Rab proteins and their interaction partners." International Review of Cell and Molecular Biology **274**(Chapter 5): 235-274.

Barnes, B., J. Richards, et al. (2004). "Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection." Journal of Biological Chemistry **279**(43): 45194-45207.

Baumforth, K., J. Flavell, et al. (2005). "Induction of autotaxin by the Epstein-Barr virus promotes the growth and survival of Hodgkin lymphoma cells." Blood **106**(6): 2138-2146.

Becker, J., D. Schrama, et al. (2009). "Merkel cell carcinoma." Cellular and molecular life sciences **66**(1): 1-8.

Belpomme, D., P. Irigaray, et al. (2007). "The multitude and diversity of enviromental carcinogens." Enviromental Research **105**(3): 414-429.

Bernard, S., R. Cartwright, et al. (1987). "Hodgkin's disease: case control epidemiological study in Yorkshire." British Journal Cancer **55**(1): 85-90.

Biggin, M., M. Bodescot, et al. (1987). "Epstein-Barr virus gene expression in P3HR1-superinfected Raji cells." Journal of Virology **61**(10): 3120-3132.

Blander, J. M. and R. Medzhitov (2006). "Toll-dependent selection of microbial antigens for presentation by dendritic cells." Nature **440**: 808-812.

Blum, K., G. Lozanski, et al. (2004). "Adult Burkitt leukemia and lymphoma." Blood **104**(10): 3009-3020.

Bonnerot, C., V. Briken, et al. (1997). "Intracellular signaling and endosomal trafficking of immunoreceptors Shared effectors underlying MHC class II-restricted antigen presentation." Immunology Letters **57**: 1-4.

Borza, C. and L. Hutt-Fletcher (2002). "Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus." Nature Medicine **8**(6): 594-599.

Boulan, E., G. Kreitzer, et al. (2008). "Organisation of vesicular trafficking in epithelia." Nature Reviews Molecular Cell Biology **6**: 233-247.

Boutell, C., A. Orr, et al. (2003). "PML Residue Lysine 160 Is Required for the Degradation of PML Induced by Herpes Simplex Virus Type 1 Regulatory Protein ICP0." Journal of Virology **77**(16): 8686-8694.

Bowie, A. G. and I. R. Haga (2005). "The role of Toll-like receptors in the host response to viruses." Molecular Immunology **42**: 859-867.

Brodsky, F. M. (1988). "Living with Clathrin: its role in intracellular membrane traffic." Science **242**: 1396-1402.

Brooks, L., A. Lear, et al. (1993). "Transcripts from the Epstein-Barr virus BamHI A fragment are detectable in all three forms of virus latency." Journal of Virology **67**(6): 3182-3190.

Brooks, L., Q. Yao, et al. (1992). "Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts." Journal of Virology **66**(5): 2689-2697.

Bucci, C., P. Thomsen, et al. (2000). "Rab7: A key to lysosome Biogenesis." Molecular Biology of the Cell **11**: 467-480.

Buettner, M., A. Greiner, et al. (2005). "Evidence of abortive plasma cell differentiation in Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma." Hematological Oncology **23**(3-4): 127-132.

Burkitt, D. (1958). "A sarcoma involving the jaws in African children." British Journal of Surgery **46**(197): 218-223.

Busson, P., C. Keryer, et al. (2004). "EBV-associated nasopharyngeal carcinomas: from epidemiology to virus-targeting strategies." Trends in Microbiology **12**(8): 356-360.

Cai, X., A. Schäfer, et al. (2006). "Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed." Plos Pathogens **2**(3): e23.

Caillaud, A., A. Hovanessian, et al. (2005). "Regulatory serine residues mediate phosphorylation-dependent and phosphorylation-independent activation of interferon regulatory factor 7." Journal of Biological Chemistry **280**: 17671-17677.

Caldwell, R. G., R. C. Brown, et al. (2000). "Epstein-Barr Virus LMP2A-Induced B-Cell Survival in Two Unique Classes of EuLMP2A Transgenic Mice." Journal of Virology **74**(3): 1101-1113.

Caldwell, R. G., J. B. Wilson, et al. (1998). "Epstein-Barr Virus LMP2A Drives B Cell Development and Survival in the Absence of Normal B Cell Receptor Signals." Immunity **9**: 405-411.

Cao, W. and Y. Liu (2007). "Innate immune functions of plasmacytoid dendritic cells." Current Opinions in immunology **19**: 24-30.

Carbone, A., U. Tirelli, et al. (1993). "Human immunodeficiency virus-associated systemic lymphomas may be subdivided into two main groups according to Epstein-Barr viral latent gene expression." Journal of Clinical Oncology **11**(9): 1674-1681.

Chan, A., P. Teo, et al. (2004). "Pathogenesis and treatment of nasopharyngeal carcinoma." Seminars in Oncology **31**(6): 794-801.

Chan, A., P. Teo, et al. (2002). "Nasopharyngeal carcinoma." Annals of Onocology **13**(7): 1007-1015.

Chan, A., K. To, et al. (2000). "High frequency of chromosome 3p deletion in histologically normal nasopharyngeal epithelia from southern Chinese." Cancer Research **60**(19): 5365-5370.

Chan, K. and Y. Lo (2002). "Circulating EBV DNA as a tumor marker for nasopharyngeal carcinoma." Seminars in Cancer Biology **12**(6): 489-496.

Chang, M. (2009). "Cancer prevention by vaccination against hepatitis B." Recent results in cancer research **181**: 85-94.

Chang, M., H. Lee, et al. (2007). "Role and prognostic significance of proapoptotic proteins in Epstein-Barr virus-infected gastric carcinomas." Anticancer Research **27**(2): 785-791.

Chang, Y., C. Tung, et al. (1999). "Requirement for cell-to-cell contact in Epstein-Barr virus infection of nasopharyngeal carcinoma cells and keratinocytes." Journal of Virology **73**(10): 8857-8866.

Chen, F., L. Hu, et al. (1995). "Coupled transcription of Epstein-Barr virus latent membrane protein (LMP)-1 and LMP-2B genes in nasopharyngeal carcinomas." Journal of General Virology **76**(1): 131-138.

Chen, F., C. Liu, et al. (2005). "Epstein-Barr Virus Latent Membrane 2A (LMP2A) Down-Regulates Telomerase Reverse Transcriptase (hTERT) in Epithelial Cell Lines." International Journal of Cancer **113**: 284-289.

Chen, K., J. Huang, et al. (2007). "Toll-like receptors in inflammation, infection and cancer." International Immunopharmacology **7**: 1271-1285.

Chen, S.-Y., J. Lu, et al. (2002). "Epstein-Barr Virus Latent Membrane Protein 2A Regulates c-Jun Protein through Extracellular Signal-Regulated Kinase." Journal of Virology **76**(18): 9556-9561.

Cheung, F. M. F., S. W. Pang, et al. (2004). "Nasopharyngeal intraepithelial lesion: latent Epstein-Barr virus infection with malignant potential." Histopathology **45**: 171-179.

Chevallier-Greco, A., E. Manet, et al. (1986). "Both Epstein-Barr virus (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter." EMBO **5**(12): 3243-3249.

Cho, N.-H., D. Kingston, et al. (2005). "Association of Herpesvirus Saimuri Tip with Lipid Raft Is Essential for Downregulation of T-Cell Receptor and CD4 Coreceptor." Journal of Virology **80**(1): 108-118.

Christoforidis, S., H. M. McBride, et al. (1999). "The Rab5 effector EEA1 is a core component of endosome docking." Nature **397**(6720): 621-625.

Ciuffo, G. (1907). "Imnesto positiv con filtrado di verrucae volgare." Giorn Ital Mal Venereol **48**: 12-17.

Cochet, C., D. Martel-Renoir, et al. (1993). "Expression of the Epstein-Barr virus immediate early gene, BZLF1, in nasopharyngeal carcinoma tumor cells." Virology **197**(1): 358-365.

Cohen, J., F. Wang, et al. (1989). "Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation." PNAS **86**(23): 9558-9562.

Colonna, M. (2007). "TLR pathways and IFN-regulatory factors: To each its own." European Journal of Immunology **37**: 306-309.

Coscoy, L. and D. Ganem (2001). "A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates Tcell costimulation." The Journal of Clinical Investigation **107**(12): 1599-1606.

Dalla-Favera, R., M. Bregni, et al. (1982). "Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells." PNAS **79**(24): 7824-7827.

Damania, B. (2006). "DNA tumor viruses and human cancer." Trends in Microbiology **15**(1): 38-44.

Dambaugh, T., K. Hennessy, et al. (1984). "U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2." PNAS **81**(23): 7632-7636.

Danve, C., G. Decaussin, et al. (2001). "Growth Transformation of Primary Epithelial Cells with a NPC-Derived Epstein-Barr Virus Strain." Virology **288**: 1-13.

Dawson, C., A. Eliopoulos, et al. (1995). "BHRF1, a viral homologue of the Bcl-2 oncogene, disturbs epithelial cell differentiation." Oncogene **10**(1): 69-77.

Dawson, C., L. Laverick, et al. (2008). "Epstein-Barr virus-encoded LMP1 regulates epithelial cell motility and invasion via the ERK-MAPK pathway." Journal of Virology **82**(7): 3654-3664.

Dawson, C. W., J. H. George, et al. (2001). "The Esptein-Barr Virus Encoded Latent Membrane Protein 2A Augments Signaling from Latent Membrane Protein 1." Virology **289**: 192-207.

Dayaram, T. and S. J. Marriott (2008). "Effect of Transforming Viruses on Molecular Mechanisms Associated With Cancer." Journal of Cellular Physiology **216**: 309-314.

De-Thé, G. (1979). "The epidemiology of Burkitt's lymphoma: evidence for a causal association with Epstein-Barr virus." Epidemiological Reviews **1**: 32-54.

de Oliveira, D. E. (2007). "DNA viruses in human cancer: An integrated overview on fundamental mechanisms of viral carcinogenesis." Cancer Letters **247**: 182-196.

Deacon, E., G. Pallesen, et al. (1993). "Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells." Journal of Experimental Medicine **177**(2): 339-349.

Deneka, M., m. Neeft, et al. (2003). "Regulation of Membrane Transport by rab GTPases." Critical Reviews in Biochemistry and Molecular Biology **38**(2): 121-142.

Deng, L., C. Wang, et al. (2000). "Activation of the IkB Kinase Complex by TRAF6 Requires a Dimeric Ubiquitin-Conjugating Enzyme Complex and a Unique Polyubiquitin Chain." Cell **103**: 351-361.

Desgranges, C., H. Wolf, et al. (1975). "Nasopharyngeal carcinoma. X. Presence of epstein-barr genomes in separated epithelial cells of tumours in patients from Singapore, Tunisia and Kenya." International Journal of Cancer **16**(1): 7-15.

Dey, B. R., R. W. Furlanetto, et al. (2000). "Suppressor of Cytokine Signaling (SOCS)-3 Protein Interacts with the Insulin-like Growth Factor-I Receptor." Biochemical and Biophysical Research Communications **278**(1): 38-43.

Dey, B. R., S. L. Spence, et al. (1998). Interaction of Human Suppressor of Cytokine Signaling (SOCS)-2 with the Insulin-like Growth Factor-I Receptor. **273:** 24095-24101.

Dhar, V. and C. Schildkraut (1991). "Role of EBNA-1 in arresting replication forks at the Epstein-Barr virus oriP family of tandem repeats." Molecular Cell Biology **11**(12): 6268-6278.

Diebold, S., T. Kaisho, et al. (2004). "Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA." Science **303**(5663): 1529-1531.

Disbrow, G. L., J. A. Hanover, et al. (2005). "Endoplasmic Reticulum-Localized Human Papillomavirus Type 16 E5 Protein Alters Endosomal pH but Not trans-Golgi pH." Journal of Virology **79**(9): 5839-5846.

Dodeller, F., M. Gottar, et al. (2008). "The Lysosomal Transmembrane Protein 9B Regulates the Activity of Inflammatory Signaling Pathways." The Journal of Biological Chemistry **283**(31): 21487-21494.

Dolan, A., C. Addison, et al. (2006). "The genome of Epstein-Barr virus type 2 strain AG876." Virology **350**(1): 164-170.

Donaldson, J. and D. Williams (2009). "Intracellular assembly and trafficking of MHC class I molecules." Traffic **10**: 1745-1752.

Donaldson, J. G., N. Porat-Shliom, et al. (2009). "Clathrin-independent endocytosis: A unique platform for cell signaling and PM remodeling." Cellular Signalling **21**: 1-9.

Dragan, A. I., V. V. Hargreaves, et al. (2007). "Mechanisms of activation of interferon regulator factor 3: the role of C-terminal domain phosphorylation in IRF-3 dimerization and DNA binding." Nucleic Acid Research.

Dupuis, J., J. Emile, et al. (2006). "Prognostic significance of Epstein-Barr virus in nodal peripheral T-cell lymphoma, unspecified: A Groupe d'Etude des Lymphomes de l'Adulte (GELA) study." Blood **108**(13): 4163-4169.

Eliopoulos, A. and L. Young (2001). "LMP1 structure and signal transduction." Seminars in Cancer Biology **11**(6): 435-444.

Epstein, J., C. Sherlock, et al. (1991). "Hairy leukoplakia-like lesions following bone-marrow transplantation." AIDS **5**(1): 101-102.

Epstein, M., B. Achong, et al. (1964). "VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA." Lancet **1**(7335): 702-703.

Eskelinen, E.-L., Y. Tanaka, et al. (2003). "At the acidic edge: emerging functions for lysosomal membrane proteins." Trends in Cell Biology **13**(3): 137.

Eskelinen, E., Y. Tanaka, et al. (2003). "At the acidic edge: emerging functions for lysosomal membrane proteins." Trends in Cell Biology **13**(3): 137-145.

Evans, A. and N. Mueller (1990). "Viruses and Cancer. Causal associations." Annals of Epidemiology **1**(1): 71-92.

Everett, R. and M. Chelbi-Alix (2007). "PML and PML nuclear bodies: implications in antiviral defence." Biochemie **89**(6-7): 819-830.

Feederle, R., M. Kost, et al. (2000). "The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators." EMBO **19**(12): 3080-3089.

Feng, B., M. Jalbout, et al. (2007). "Dietary risk factors for nasopharyngeal carcinoma in Maghrebian countries." International Journal of Cancer **121**(7): 1550-1555.

Ferry, J. (2006). "Burkitt's lymphoma: clinicopathologic features and differential diagnosis." Oncologist **11**(4): 375-383.

Fingeroth, J., M. Diamond, et al. (1999). "CD21-Dependent infection of an epithelial cell line, 293, by Epstein-Barr virus." Journal of Virology **73**(3): 2115-2125.

Fingeroth, J., J. Weis, et al. (1984). "Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2." Proceedings of the Natural Academy of Sciences **81**(14): 4510-4514.

Fitzgerald, K., E. Palsson-McDermott, et al. (2001). "Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction." Nature **413**(6851): 78-83.

Fixman, E., G. Hayward, et al. (1995). "Replication of Epstein-Barr virus oriLyt: lack of a dedicated virally encoded origin-binding protein and dependence on Zta in cotransfection assays." Journal of Virology **69**(5): 2998-3006.

Flemington, E. and S. Speck (1990). "Autoregulation of Epstein-Barr virus putative lytic switch gene BZLF1." Journal of Virology **64**(3): 1227-1232.

Flemington, E. and S. Speck (1990). "Epstein-Barr virus BZLF1 trans activator induces the promoter of a cellular cognate gene, c-fos." Journal of Virology **64**(9): 4549-4552.

Forgac, M. (2007). "Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology." Nature Reviews Molecular Cell Biology **8**: 917-929.

Foti, M., A. Mangasarian, et al. (1997). "Nef-mediated clathrin coated pit formation." Journal of Cell Biology **139**(1): 37-47.

Frangou, P., M. Buettner, et al. (2005). "Epstein-Barr virus (EBV) infection in epithelial cells in vivo: rare detection of EBV replication in tongue mucosa but not in salivary glands." Journal of Infectious Disease **191**(2): 238-242.

Frappier, L., K. Goldsmith, et al. (1994). "Stabilization of the EBNA1 protein on the Epstein-Barr virus latent origin of DNA replication by a DNA looping mechanism." Journal of Biological Chemistry **269**(2): 1057-1062.

Fruehling, S., S. K. Lee, et al. (1996). "Identification of Latent Membrane Protein 2A (LMP2A) Domains Essential for the LMP2A Dominant-Negative on B-Lymphocyte Surface Immunoglobulin Signal Transduction." Journal of Virology **70**(9): 6216-6226.

Fruehling, S. and R. Longnecker (1997). "The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction." Virology **235**(5): 241-251.

Fukada, M. and R. Longnecker (2004). "Latent Membrane Protein 2A Inhibits Transforming Growth Factor-beta1-Induced Apoptosis through the Phosphatidylinositol 3-kinase/Akt Pathway." Journal of Virology **78**(4): 1697-1705.

Fukada, M. and R. Longnecker (2007). "Epstein-Barr Virus Latent Membrane Protein 2A Mediates Transformation through Constitutive Activation of the Ras/PI3-K/Akt Pathway." Journal of Virology **81**(17): 9299-9306.

Fukayama, M., J. Chong, et al. (2001). "Pathology and molecular pathology of Epstein-Barr virus-associated gastric carcinoma." Current Topics in Microbiology and Immunology **258**: 91-102.

Fukayama, M., R. Hino, et al. (2008). "Epstein-Barr virus and gastric carcinoma: virus-host interactions leading to carcinoma." Cancer Sci **99**(9): 1726-1733.

Galani, E. and C. Christodoulou (2009). "Human papilloma viruses and cancer in the post-vaccine era." Clinical Microbiology and infection **15**(11): 977-981.

Gan, Y., J. Chodosh, et al. (1997). "Epithelial cell polarization is a determinant in the infectious outcome of immunoglobulin A-mediated entry by Epstein-Barr virus." 71 **1**(519-526): 519.

Garcia-Sastre, A. and C. A. Biron (2006). "Type 1 Interferons and the Virus-Host Relationship: A Lesson in Détente." Science **312**: 879-882.

Georgel, P., Z. Jiang, et al. (2007). "Vesicular stomatitis virys glycoprotein G activates a specific antiviral Toll-like receptor dependent pathway." Biochemical and Biophysical Research Communications **336**: 496-506.

Gerle, B., A. Korokani, et al. (2007). "Acetylated histone H3 and H4 mark the upregulated LMP2A promoter of Epstein Barr virus in lymphoid cells." Journal of Virology **81**(23): 13242-13247.

Gingras, S., E. Parganas, et al. (2004). "Re-examination of the Role of Suppressor of Cytokine Signaling 1 (SOCS1) in the Regulation of Toll-like Receptor Signaling
" The Journal of Biological Chemistry **279**(52): 54702-54707.

Goldsmith, D., T. West, et al. (2002). "HLA associations with nasopharyngeal carcinoma in Southern Chinese: a meta-analysis." Clin Otolaryngol Allied Sci **27**(1): 61-67.

Goodbourn, S., L. Didcock, et al. (2000). "Interferons:cell signalling, immune modulation, antiviral responses and virus countermeasures." Journal of General Virology **81**: 2341-2364.

Goodsell, D. S. (2001). "The Molecular Perspective: Interferons." The Oncologist **6**: 374-375.

Gottipati, S., N. L. Rao, et al. (2007). "IRAK1: A critical signaling mediator of innate immunity." Cellular Signalling **20**(2): 269-276.

Grandvaux, N., B. R. tenOever, et al. (2002). "The interferon antiviral response: from viral invasion to evasion." Current Opinion in Infectious Diseases **15**: 259-267.

Gratama, J., M. Oosterveer, et al. (1988). "Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency." PNAS **85**(22): 8693-8696.

Gratama, J., M. Zutter, et al. (1991). "Expression of Epstein-Barr virus-encoded growth-transformation-associated proteins in lymphoproliferations of bone-marrow transplant recipients." International Journal of Cancer **47**(2): 188-192.

Greenspan, D., Y. De Souza, et al. (1990). "Efficacy of desciclovir in the treatment of Epstein-Barr virus infection in oral hairy leukoplakia." Journal of AIDS **3**(6): 571-578.

Greenspan, J., D. Greenspan, et al. (1985). "Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion." New England Journal of Medicine **313**(25): 1564-1571.

Gregory, C., C. Edwards, et al. (1988). "Isolation of a normal B cell subset with a Burkitt-like phenotype and transformation in vitro with Epstein-Barr virus." International Journal of Cancer **42**(2): 213-220.

Guasparri, I., D. Bubman, et al. (2008). "EBV LMP2A affects LMP1-mediated NF-kB signaling and survival of lymphoma cells by regulating TRAF2 expression." Blood **117**(7): 3813-3820.

Gulley, M., D. Pulitzer, et al. (1996). "Epstein-Barr virus infection is an early event in gastric carcinogenesis and is independent of bcl-2 expression and p53 accumulation." Human Pathology **27**(1): 20-27.

Häcker, H., V. Redecke, et al. (2006). "Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6." 439 **7073**(204-207): 204.

Haller, C., S. Rauch, et al. (2007). "HIV-1 Nef employs two distinct mechansism to modulate lck subcellular locaisation and TCR induced actin remodelling." Plos One **11**(e1212): 1-13.

Hammerschmidt, W. and B. Sugden (1988). "Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus." Cell **55**(3): 427-433.

Hammerschmidt, W. and B. Sugden (1989). "Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes." Nature **340**(6232): 393-397.

Hanahan, D. and R. A. Weinberg (2000). "The Hallmarks of Cancer." Cell **7**(1): 57-70.

Hansen, S., K. Sandvig, et al. (1993). "Clathrin and HA2 adaptors: efefcts of potassium depletion, hypertonic medium and cytosol acidification." The Journal of Biological Chemistry **121**: 61-72.

Hansen, T. and M. Bouvier (2009). "MHC class I antigen presentation: learning from viral evasion strategies." Nature Reviews Immunology **9**: 503-513.

Harabuchi, Y., N. Yamanaka, et al. (1990). "Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma." Lancet **335**(8682): 128-130.

Harris, N. (1999). "Hodgkin's disease: classification and differential diagnosis." Modern Pathology **12**(2): 159-175.

Hayden, M. and S. Ghosh (2004). "Signaling to NF-kappaB." Genes and Development **18**(18): 2195-2224.

Hayes, D., A. Brink, et al. (1999). "Expression of Epstein-Barr virus (EBV) transcripts encoding homologues to important human proteins in diverse EBV associated diseases." Molecular Pathology **52**(2): 97-103.

Hayward, S., L. Nogee, et al. (1980). "Organization of repeated regions within the Epstein-Barr virus DNA molecule." Journal of Virology **33**(1): 507-521.

Hemmi, H., T. Kaisho, et al. (2002). "Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway." Nature Immunology **3**(2): 196-200.

Henle, G., W. Henle, et al. (1968). "Relation so Burkitt's tumor-associated herpes-type virus to infectious mononucleosis." PNAS **59**(1): 94-101.

Henle, W. and G. Henle (1969). "The relation between the Epstein-Barr virus and infectious mononucleosis, Burkitt's lymphoma and cancer of the postnasal space." East African Medical Journal **46**(7): 402-406.

Henle, W., G. Henle, et al. (1970). "Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms, and control groups." J Natl Cancer Inst **44**(1): 225-31.

Herbst, H., J. Samol, et al. (1997). "Modulation of interleukin-6 expression in Hodgkin and Reed-Sternberg cells by Epstein-Barr virus." Journal of Pathology **182**(3): 299-306.

Heussinger, N., M. Büttner, et al. (2004). "Expression of the Epstein-Barr virus (EBV)-encoded latent membrane protein 2A (LMP2A) in EBV-associated nasopharyngeal carcinoma." Journal of Pathology **203**(2): 696-699.

Hewitt, E. W., L. Duncan, et al. (2002). "Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation." The EMBO Journal **21**(10): 2418-2429.

Hildesheim, A., L. Anderson, et al. (1997). "CYP2E1 genetic polymorphisms and risk of nasopharyngeal carcinoma in Taiwan." Japan National Cancer Research Institute **89**(16): 1207-1212.

Hill, A. B. (1965). "The Environment and Disease: Association or Causation?" Proceedings of the Royal Society of Medicine **58**(5): 295-300.

Hirota, Y., T. Kuronita, et al. (2007). "A role for Rab5 activity in the biogenesis of endosomal and lysosomal compartments." Biochemical and Biophysical Research Communications **364**: 40-47.

Hiscott, J. (2007). "Triggering the innate antiviral response through IRF-3 activation." The Journal of Biological Chemistry **282**(21): 15325-15329.

Hislop, A., G. Taylor, et al. (2007). "Cellular responses to Viral infection in humans: lessons from EBV." Annual Review of Immunology **25**: 587-617.

Hitt, M., M. Allday, et al. (1989). "EBV gene expression in an NPC-related tumour." EMBO **8**(9): 2639-2651.

Hochberg, D., J. Middeldorp, et al. (2004). "Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo." PNAS **101**(1): 239-244.

Honda, K. and T. Taniguchi (2006). "IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors." Nature Reviews Immunology **6**: 644-658.

Honda, K., H. Yanai, et al. (2005). "IRF-7 is the master regulator of type-I interferon-dependent immune responses." Nature **434**(7034): 772-777.

Horiuchi, H., R. Lippe, et al. (1997). "A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function." Cell **90**: 1149-1159.

Hu, X., J. Chen, et al. (2007). "Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation." Journal of Leukocyte Biology **82**(2): 237-243.

Huang, D., J. Ho, et al. (1974). "Demonstration of Epstein-Barr virus-associated nuclear antigen in nasopharyngeal carcinoma cells from fresh biopsies." International Journal of Cancer **14**(5): 580-588.

Husebye, H., O. Halass, et al. (2006). "Endocytic pathways regulate Toll-like receptor 4 signaling and link innate and adaptive immunity." The EMBO Journal **25**: 683-692.

Hwang, M.-N., C.-H. Min, et al. (2007). "The nuclear localization of SOCS6 requires the N-terminal region and negatively regulates Stat3 protein levels." Biochemical and Biophysical Research Communications **360**(2): 333-338.

Ikeda, M., A. Ikeda, et al. (2000). "The Epstein-Barr Virus Latent Membrane Protein 2A PY Motif Recruits WW Domain-Containing Ubiquitin-Protein Ligases." Virology **268**: 178-191.

Ikeda, M. and R. Longnecker (2007). "Cholesterol is critical for Epstein-Barr virus latent membrane protein 2A trafficking and protein stabiliy
" Virology **360**: 461-468.

Imai, S., J. Nishikawa, et al. (1998). "Cell-to-cell contact as an efficient mode of Epstein-Barr virus infection of diverse human epithelial cells." Journal of Virology **72**(5): 4371-4378.

Irigaray, P., J. A. Newby, et al. (2007). "Lifestyle-related factors and environmental agents causing cancer: An overview." Biomedicine and Pharmacotherapy **61**(10): 640-658.

Isaacs, A. and J. Lindenmann (1957). "Virus interference. I. The interferon." Proceedings of the Royal Society of London Biology **147**: 258-267.

Ishido, S., C. Wang, et al. (2000). "Downregulation of Major Histocompatibility Complex Class I Molecules by Kaposi's Sarcoma-Associated Herpesvirus K3 and K5 Proteins." Journal of Virology **74**(11): 5300-5309.

Iwakiri, D., L. Zhou, et al. (2009). "Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3." Journal of Experimental Medicine **206**(10): 2091-2099.

Iwasaki, A. and R. Medzhitov (2004). "Toll-like receptor control of the adaptive immune response." Nature Immunology **5**: 987-995.

Jarrett, R., A. Gallagher, et al. (1991). "Detection of Epstein-Barr virus genomes in Hodgkin's disease: relation to age." Journal of Clinical Pathology **44**(10): 844-848.

Johansson, B., G. Klein, et al. (1970). "Epstein-Barr virus (EBV)-associated antibody patterns in malignant lymphoma and leukemia. I. Hodgkin's disease." International Journal of Cancer **6**(3): 450-462.

Johnsen, I., T. Nguyen, et al. (2006). "Toll-like receptor 3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling." EMBO **25**(14): 3335-3346

Jones, R., L. Smith, et al. (2003). "Epstein-Barr virus nuclear antigen 1 (EBNA1) induced cytotoxicity in epithelial cells is associated with EBNA1 degradation and processing." Virology **313**(2): 663-676.

Jurk, M., F. Heil, et al. (2002). "Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848." Nature Immunology **3**(6): 499.

Kagan, J. C., T. Su, et al. (2008). "TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon beta." Nature Immunology **9**(4): 361-368.

Kanzler, H., R. Küppers, et al. (1996). "Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells." Journal of Experimental Medicine **184**(4): 1495-1505.

Kawai, T. and S. Akira (2005). "Toll-like receptor downstream signaling." Arthritis Research and Therapy **7**(1): 12-9.

Kawai, T. and S. Akira (2006). "Innate immune recognition of viral infection." Nature Immunology **7**(2): 131-137.

Kawai, T. and S. Akira (2006). "TLR signaling." Cell Death and Differentiation **13**: 816-825.

Kawai, T. and S. Akira (2008). "Toll-like receptor and RIG-1-like receptor signalling." Annals New York Academy of Science **1143**: 1-20.

Kawai, T., S. Sato, et al. (2004). "Interferon alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. ." Nature Immunology **5**: 1061-1068.

Kaye, K., K. Izumi, et al. (1993). "Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation." PNAS **90**(19): 9150-9154.

Keegan, B. P., L. G. Sheflin, et al. (2000). "The Internalization and Endosomal Trafficking of the EGF Receptor in Response to EGF Is Delayed in the waved-2 Mouse Liver." Biochemical and Biophysical Research Communications **267**: 881-886.

Kelly, G., A. Bell, et al. (2002). "Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2." Nature Medicine **8**(10): 1098-1104.

Kennedy, G., J. Komano, et al. (2003). "Epstein-Barr virus provides a survival factor to Burkitt's lymphomas." PNAS **100**(24): 14269-14274.

Kile, B. T. and W. S. Alexander (2001). "The suppressors of cytokine signalling (SOCS)." Cellular and Molecular Life Sciences **58**: 1627-1635.

Kim, T. W., K. Staschke, et al. (2007). "A critical role for IRAK4 kinase activity in Toll-like receptor-mediated innate immunity." The Journal of Experimental Medicine **204**(5): 1025-1036.

Kintner, C. and B. Sugden (1979). "The structure of the termini of the DNA of Epstein-Barr virus." Cell **17**(3): 661-671.

Klein, E., L. Kis, et al. (2007). "Epstein-Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions." Oncogene **26**(9): 443-453.

Klein, G., B. Giovanella, et al. (1974). "Direct evidence for the presence of Epstein-Barr virus DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of the nasopharynx." PNAS **71**(12): 4737-4741.

Klein, G. and E. Klein (1985). "Evolution of tumours and the impact of molecular oncology." Nature **315**(6016): 16-22.

Klumb, C., R. Hassan, et al. (2004). "p53 protein expression does not correlate with EBV status in childhood B non-Hodgkin lymphomas." Pediatric Blood Cancer **43**(2): 115-119.

Knox, P., Q. Li, et al. (1996). "In vitro production of stable Epstein-Barr virus-positive epithelial cell clones which resemble the virus:cell interaction observed in nasopharyngeal carcinoma." Virology **215**(1): 40-50.

Ko, J., A. Gendron-Fitzpatrick, et al. (2002). Susceptibility of IFN Regulatory Factor-1 and IFN Consensus Sequence Binding Protein-Deficient Mice to Brucellosis. **168:** 2433-2440.

Kobayashi, T., G. Takaesu, et al. (2006). "Mal-function of TLRs by SOCS." Nature Immunology **7**(2): 148-155.

Kurt-Jones, E., L. Popova, et al. (2000). "Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus." Nature Immunology **1**: 398-401.

Kusano, S. and N. Raab-Traub (2001). "An Epstein-Barr virus protein interacts with Notch." Journal of Virology **75**(1): 384-395.

Laux, G., F. Dugrillon, et al. (1994). "Identification and characterization of an Epstein-Barr virus nuclear antigen 2-responsive cis element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes." Journal of Virology **68**(11): 6947-6958.

Laux, G., M. Perricaudet, et al. (1988). "A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome." EMBO **7**(3): 769-774.

Lee, K.-H., K.-J. Moon, et al. (2008). "Increased cytoplasmic levels of CIS, SOCS1, SOCS2, or SOCS3 are required for nuclear translocation." FEBS Letters **582**(15): 2319-2324.

Lee, M., M. Diamond, et al. (1999). "Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus." Journal of Virology **73**(4): 2974-2982.

Lehner, P. J., S. Hoer, et al. (2005). "Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases." Immunological Reviews **207**: 112-125.

Lemaitre, B., E. Nicolas, et al. (1996). "The dorsoventral regulatory gene cassette spatzie/Toll/cactus controls the potent antifungal response in *Drosophila* adults. ." Cell **86**: 973-983.

Lenoir, G., M. Vuillaume, et al. (1985). "The use of lymphomatous and lymphoblastoid cell lines in the study of Burkitt's lymphoma." IARC Sci Publication **60**: 309-318.

Leung, S., K. Chau, et al. (1998). "p53 overexpression is different in Epstein-Barr virus-associated and Epstein-Barr virus-negative carcinoma." Histopathology **33**(4): 311-317.

Levitskaya, J., A. Sharipo, et al. (1997). "Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1." PNAS **94**(23): 12616-12621.

Levy, D. E. and A. Garcia-Sastre (2001). "The virus battles: IFN induction of the antiviral state and mechanisms of the viral evasion." Cytokine and Growth Factor Reviews **12**: 143-156.

Li, H., C. Man, et al. (2006). "Molecular and cytogenetic changes involved in the immortalization of nasopharyngeal epithelial cells by telomerase." International Journal of Cancer **119**(7): 1567-1576.

Li, Q., R. Means, et al. (2006). "Downregulation of interferon gamma receptor I by Kaposi's Sarcoma-Associated Herpesvirus K3 and K5." Journal of Virology **10**.

Li, Q., S. M. Turk, et al. (1995). "The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells." J Virol **69**(7): 3987-94.

Li, Q., L. Young, et al. (1992). "Epstein-Barr virus infection and replication in a human epithelial cell system." 1992 **356**(6367): 347-350.

Li, S., Y. Chang, et al. (1996). "Effect of a 10-amino acid deletion on the oncogenic activity of latent membrane protein 1 of Epstein-Barr virus." Oncogene **12**(10): 2129-2135.

Li, S., A. Strelow, et al. (2002). "IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase." PNAS **99**(8): 5567-5572.

Li, S., H. Zheng, et al. (2010). "Regualtion of virus triggered signalling by OTUB1 and OTUB2 mediated deubiquitination of TRAF3 and TRAF6." The journal of Biological chemistry **285**(7): 4291-4297.

Li, X., R. Fasano, et al. (2009). "HLA associations with nasopharyngeal carcinoma." Current opinions in Molecular Medicine **9**(6): 751-765.

Liao, J. B. (2006). "Viruses and Human Cancer." Yale Journal of Biology and Medicine **79**: 115-122.

Lo, K. and D. Huang (2002). "Genetic and epigenetic changes in nasopharyngeal carcinoma." Seminars in Cancer Biology **12**(6): 451-462.

Longnecker, R., M. CL, et al. (1992). "The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential." Journal of Virology **66**(11): 6461-6469.

Longnecker, R., B. Druker, et al. (1991). "An Epstein-Barr Virus Protein Associated with Cell Growth Transformation Interacts with a Tyrosine Kinase." Journal of Virology **65**(7): 3681-3692.

Longnecker, R. and E. Kieff (1990). "A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1." Journal of Virology **64**(5): 2319-2326.

Longnecker, R., M. Merchant, et al. (2000). "WW- and SH3-domain interactions with Epstein-Barr virus LMP2A." Experimental Cell Research **257**(2): 332-340.

Longnecker, R. and T. Portis (2003). "Epstein-Barr virus (EBV) LMP2A alters normal transcriptional regulation following B-cell receptor activation." Virology **318**: 524-533.

Lorenzo, M. E., H. L. Ploegh, et al. (2001). "Viral immune evasion strategies and the underlying cell biology." Immunology **13**: 1-9.

Lu, J., W.-H. Lin, et al. (2006). "Syk Tyrosine Kinase Mediates Epstein-Barr Virus Latent Membrane Protein 2A-induced Cell Migration in Epithelial Cells." The Journal of Biological Chemistry **281**(13): 8806-8814.

Lu, X., H. Yu, et al. (1998). "Interactions between HIV1 Nef and Vacuolar ATPase Facilitate the Internalization of CD4." Immunity **8**: 647-656.

Lung, M., R. Chang, et al. (1990). "Epstein-Barr virus genotypes associated with nasopharyngeal carcinoma in southern China." Virology **177**(1): 44-53.

Lung, M., W. Lam, et al. (1991). "Detection and prevalence of the "f" variant of Epstein-Barr virus in southern China." Virology **185**(1): 67-71.

Luo, B., Y. Wang, et al. (2005). "Expression of Epstein-Barr virus genes in EBV-associated gastric carcinomas." World Journal of Gastroenterology **11**(5): 629-633.

Lupton, S. and A. Levine (1985). "Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells." Molecular and Cellular Biology **5**(10): 2533-2542.

Lynch, D. T., J. S. Zimmerman, et al. (2002). "Epstein-Barr virus latent membrane protein 2B (LMP2B) co-localizes with LMP2A in perinuclear regions in transiently transfected cells " Journal of General Virology **83**: 1023-1035.

M'Faydean, J. and F. Hodbay (1898). "Note on the experimental transmission of warts in the dog." Journal of Comparative Pathology **11**: 341-344.

Malliri, A., M. Symons, et al. (1998). "The Transcription Factor AP-1 Is Required for EGF-Induced Activation of Rho-like GTPases, Cytoskeletal Rearrangements, Motility, and In Vitro Invasion of A431 Cells " The Journal of Cell Biology **143**(4): 1087-1099.

Mangasarian, A., M. Foti, et al. (1997). "The HIV1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane,." Immunity **6**(1): 67-77.

Mao, A.-P., S. Li, et al. (2010). "Virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of IFN-β and cellular antiviral response." Journal of Biological Chemistry: 1-8.

Marchetti, B., G. H. Ashrafi, et al. (2006). "The E5 protein of BPV-4 interacts with the heavy chain of MHC class I and irreversibly retains the MHC complex in the Golgi apparatus." Oncogene **25**: 2254-2263.

Markgraf, D., K. Peplowska, et al. (2007). "Rab cascade and tethering factors in the endomembrane system." FEBS letters **581**: 2125-2130.

Marshall-Clarke, S., J. E. Downes, et al. (2007). "Polyinsinic acid is a ligand for Toll-like receptor 3." The Journal of Biological Chemistry **282**(34): 24759-24766.

Martin, H. J., J. M. Lee, et al. (2007). "Manipulation of the toll-like receptor 7 signaling pathway by Epstein-Barr virus." Journal of Virology **81**(18): 9748-9758.

Masucci, M. G. (2004). "Epstein-Barr virus oncogenesis and the ubiquitin-proteasome system." Oncogene **23**: 2107-2115.

Matsumoto, M., K. Funami, et al. (2003). "Subcellular localization of Toll-like receptor 3 in human dendritic cells." Journal of Immumology **171**(6): 3154-3162.

McCaffrey, M., A. Bielli, et al. (2001). "Rab4 affects both recycling and degradative endosomal trafficking." FEBS letters **495**: 21-30.

McDermott, M., M. J. O. Wakelam, et al. (2004). "Phospholipase D." Biochemistry and cell biology **82**(1): 225-253.

McGeoch, D. and D. Gatherer (2007). "Lineage structures in the genome sequences of three Epstein-Barr virus strains." Virology **359**(1): 1-5.

McGettrick, A. F. and L. A. J. O'Neill (2004). "The expanding family of MyD88-like adaptors in Toll-like receptor signal transduction." Molecular Immunology **41**: 577-582.

McLaughlin-Drubin, M. E. and K. Munger (2008). "Viruses associated with human cancer." Biochimica et Biophysica Acta **1782**: 127-150.

Means, R. E., S. Ishido, et al. (2002). "Multiple endocytic trafficking pathways of MHC class I molecules induced by a Herpesvirus protein.." The EMBO Journal **21**(7): 1638-1649.

Metzelaar, M. J., P. L. Wijngaard, et al. (1991). CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. Journal of Biological Chemistry. **266:** 3239-3245.

Miller, C., J. Lee, et al. (1994). "Epstein-Barr virus protein LMP2A regulates reactivation from latency by negatively regulating tyrosine kinases involved in sIg-mediated signal transduction." Infectious Agents **3**(2-3): 128-136.

Miller, N. and L. Hutt-Fletcher (1992). "Epstein-Barr virus enters B cells and epithelial cells by different routes." Journal of Virology **66**(6): 3409-3414.

Miller, W. E. and N. Raab-Traub (1999). "The EGFR as a target for viral oncoproteins." Trends in Microbiology **7**(11): 453-458.

Mirvish, S. (1995). "Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC." Cancer Letters **93**(1): 17-48.

Mitarnun, W., S. Suwiwat, et al. (2002). "Epstein-Barr virus-associated peripheral T-cell and NK-cell proliferative disease/lymphoma: clinicopathologic, serologic, and molecular analysis." American Journal of Hematology **70**(1): 31-38.

Molesworth, S., C. Lake, et al. (2000). "Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells." Journal of Virology **74**(14): 6324-6332.

Morris, M., C. Dawson, et al. (2008). "Epstein-Barr virus-encoded LMP1 induces a hyperproliferative and inflammatory gene expression programme in cultured keratinocytes." Journal of General Virology **89**(11): 2806-2820.

Morris, M., C. Dawson, et al. (2009). "Role of the Epstein-Barr virus-encoded latent membrane protein-1, LMP1, in the pathogenesis of nasopharyngeal carcinoma." Future Oncology **5**(6): 811-825.

Morrison, J. A., A. J. Klingelhutz, et al. (2003). "Epsteinn-Barr Virus Latent Membrane Protein 2A Activates B-Catenin Signaling in Epithelial Cells." Journal of Virology **77**(22): 12276-12284.

Morrison, J. A. and N. Raab-Traub (2005). "Roles of the ITAM and PY Motifs of Epstein-Barr Virus Latent Membrane Protein 2A in the Inhibition of Epithelial Cell Differentiation and Activation of B-Catenin Signaling." Journal of Virology **79**(4): 2375-2382.

Mousavi, S. A., L. Malerod, et al. (2004). "Clathrin-dependent endocytosis." Journal of Biochemistry **377**: 1-16.

Mueller, N., A. Evans, et al. (1989). "Hodgkin's disease and Epstein-Barr virus. Altered antibody pattern before diagnosis." New England Journal of Medicine **320**(11): 689-695.

Murray, P. (2007). "The JAK-STAT signaling pathway: input and output integration." Journal of Immumology **175**(8): 2623-2629.

Murray, P., G. Niedobitek, et al. (1996). "In situ detection of the Epstein-Barr virus-encoded nuclear antigen 1 in oral hairy leukoplakia and virus-associated carcinomas." Journal of Pathology **178**(1): 44-47.

Murray, P. and L. Young (2002). "The Role of the Epstein-Barr virus in human disease." Frontiers in Bioscience **7**: 519-540.

Murray, P., L. Young, et al. (1992). "Immunohistochemical demonstration of the Epstein-Barr virus-encoded latent membrane protein in paraffin sections of Hodgkin's disease." Journal of Pathology **166**(1): 1-5.

Nakhaei, P., T. Mesplede, et al. (2009). "The E3 ubiquitin ligase Triad 3A negatively regulates the RIG-1/MAVS signalling pathwya by targeting TRAF3 for degradation." Plos Pathogens **5**(11): 1-14.

Nanbo, A. and K. Takada (2002). "The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis." Reviews Medical Virology **12**(5): 321-326.

Nemerow, G., C. Mold, et al. (1987). "Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d." Journal of Virology **61**(5): 1416-1420.

Nemerow, G., R. Wolfert, et al. (1985). "Identification and characterization of the Epstein-Barr virus receptor on human B-lymphocytes and its relationship to the C3d complement receptor (CR2)." Journal of Virology **55**(2): 347-351.

Ni, Y., M. Chang, et al. (2001). "Hepatitis B Virus infection in children and adolescents in a hyperendemic area: 15 years after mass hepatits B vaccination." Annals of Internal Medicine **135**(9): 835-836.

Niedobitek, G., H. Herbst, et al. (1992). "Epstein-Barr virus and carcinomas. Expression of the viral genome in an undifferentiated gastric carcinoma." Diagn Mol Pathology **1**(2): 103-108.

Niedobitek, G., N. Meru, et al. (2001). "Epstein-Barr virus infection and human malignancies." Viruses and Cancer **82**: 149-170.

Niedobitek, G., N. Meru, et al. (2001). "Epstein-Barr virus infection and human malignancies." International Journal of Experimental Pathology **82**(3): 149-170.

Niedobitek, G., D. Mutimer, et al. (1997). "Epstein-Barr virus infection and malignant lymphomas in liver transplant recipients." International Journal of Cancer **73**(4): 514-520.

Niedobitek, G., L. Young, et al. (1992). "Expression of Epstein-Barr virus genes and of lymphocyte activation molecules in undifferentiated nasopharyngeal carcinomas." American Journal of Pathology **140**(4): 879-887.

Nishikura, K., A. ar-Rushdi, et al. (1983). "Differential expression of the normal and of the translocated human c-myc oncogenes in B cells." PNAS **80**(15): 4822-4826.

Nishiya, T., E. Kajita, et al. (2005). "TLR3 and TLR7 Are Targeted to the Same Intracellular Compartments by Distinct Regulatory Elements." The Journal of Biological Chemistry **280**(44): 37107-37117.

Nonkwelo, C., J. Skinner, et al. (1996). "Transcription start sites downstream of the Epstein-Barr virus (EBV) Fp promoter in early-passage Burkitt lymphoma cells define a fourth promoter for expression of the EBV EBNA-1 protein." Journal of Virology **70**(1): 623-627.

O'Neill, L. A. J. and A. G. Bowie (2007). "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling." Nature Reviews Immunology **7**: 353-364.

Ojima, H., T. Fukuda, et al. (1997). "Infrequent overexpression of p53 protein in Epstein-Barr virus-associated gastric carcinomas." Japan Journal of Cancer Research **88**(3): 262-266.

Osato, T. and S. Imai (1996). "Epstein-Barr virus and gastric carcinoma." 71 **4**(175-182): 175.

Ozanne, B. W., H. J. Spence, et al. (2006). "Transcription factors control invasion: AP-1 the first among equals." Oncogene **26**(1): 1-10.

Pagano, J., M. Blaser, et al. (2004). "Infectious agents and cancer: criteria for a causal relation." Seminars in Cancer Biology **14**(6): 453-471.

Pallesen, G., K. Sandvej, et al. (1991). "Activation of Epstein-Barr virus replication in Hodgkin and Reed-Sternberg cells." Blood **78**(5): 1162-1165.

Pang, M., K. Lin, et al. (2009). "The signaling pathways of Epstein-Barr virus-encoded latent membrane protein 2A (LMP2A) in latency and cancer." Cellular and molecular biology letters **14**(2): 222-247.

Panne, D. (2008). "The enhanceosome." Current Opinion in Structural Biology **18**(2): 236-242.

Panousis, C. and D. Rowe (1997). "Epstein-Barr virus latent membrane protein 2 associates with and is a substrate for mitogen-activated protein kinase." Journal of Virology **71**(6): 4752-4760.

Pathmanathan, R., U. Prasad, et al. (1995). "Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia." American Journal of Pathology **146**(6): 1355-1367.

Pattle, S. and P. Farrell (2006). "The role of Epstein-Barr virus in cancer." Expert Opinions in Biological Therapy **6**(11): 1193-1205.

Pegtel, D., J. Middeldorp, et al. (2004). "Epstein-Barr virus infection in ex vivo tonsil epithelial cell cultures of asymptomatic carriers." Journal of Virology **78**(22): 12613-12624.

Pegtel, D. M., A. Subramanian, et al. (2005). "Epstein-Barr-Virus-Encoded LMP2A Induces Primary Epithelial Cell Migration and Invasion: Possible Role in Nasopharyngeal Carcinoma Metastasis." Journal of Virology **79**(24): 15430-15442.

Peters, K. L., H. L. Smith, et al. (2002). "IRF-3-dependent, NFkB- and JNK-independent activation of the 561 and IFN-B genes in response to double-stranded RNA." PNAS **99**(9): 6322-6327.

Piper, R. and J. Luzio (2007). "Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes." Current Opinion in Cell Biology **19**: 459-465.

Pisani, P., F. Bray, et al. (2002). "Estimates of the world-wide prevalence of cancer for 25 sites in the adult population." International Journal of Cancer **97**(1): 72-81.

Platanias, L. (2005). "Mechanisms of type-I- and type-II-interferon-mediated signalling." Nature Reviews Immunology **5**(5): 375-386.

Portis, T., M. Ikeda, et al. (2004). "Epstein-Barr virus LMP2A: regulating cellular ubiquitination processes for maintenance of viral latency?" Trends in Immunology **25**(8): 422-426.

Powles, T., G. Matthews, et al. (2000). "AIDS related systemic non-Hodgkin's lymphoma." Sex Transm Infect **76**(5): 335-341.

Quentmeier, H., R. Geffers, et al. (2008). "SOCS2: inhibitor of JAK2V617F-mediated signal transduction." Leukemia **22**(12): 2169-2175.

Raab-Traub, N. (2002). "Epstein-Barr virus in the pathogenesis of NPC." Seminars in Cancer Biology **12**(6): 431-441.

Raab-Traub, N. and K. Flynn (1986). "The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation." Cell **47**(6): 883-889.

Radkov, S., R. Touitou, et al. (1999). "Epstein-Barr virus nuclear antigen 3C interacts with histone deacetylase to repress transcription." Journal of Virology **73**(7): 5688-5697.

Rahim-Rezaee, S., C. Cunningham, et al. (2006). "Kaposi's sarcoma-associated herpesvirus immune modulation: an overview." Journal of General Virology **87**: 1781-1804.

Raiborg, C. and H. Stenmark (2009). "The ESCRT machinery in endosomal sorting of ubquitylated membrane proteins." Nature **458**: 445-452.

Randall, R. and S. Goodbourn (2008.). "Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures." Journal of General Virology **89**: 1-47.

Rechsteiner, M. P., C. Berger, et al. (2008). "Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection." Journal of Virology **82**(4): 1739-1747.

Rechsteiner, M. P., M. Bernasconi, et al. (2008). "Role of latent membrane protein 2 isoforms in Epstein–Barr virus latency " Trends in Microbiology **16**(11): 520-527.

Reichart, P., A. Langford, et al. (1989). "Oral hairy leukoplakia: observations in 95 cases and review of the literature." Journal of Oral Patol Med **18**(7): 410-415.

Repetto, E., I.-S. Yoon, et al. (2007). "Presenilin 1 Regulates Epidermal Growth Factor Receptor Turnover and Signaling in the Endosomal-Lysosomal Pathway." The Journal of Biological Chemistry **282**(43): 31504-31516.

Resnick, L., J. Herbst, et al. (1988). "Regression of oral hairy leukoplakia after orally administered acyclovir therapy." JAMA **259**(3): 384-388.

Rezk, S. and L. Weiss (2007). "Epstein-Barr virus-associated lymphoproliferative disorders." Human Pathology **38**(9): 1293-1304.

Rickinson, A., M. Rowe, et al. (1984). "T-cell-mediated regression of "spontaneous" and of Epstein-Barr virus-induced B-cell transformation in vitro: studies with cyclosporin A." Cellular Immunology **87**(2): 646-658.

Rickinson, A., L. Young, et al. (1987). "Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells." Journal of Virology **61**(5): 1310-1317.

Rickinson, A. B. a. K., E. (2001). Epstein-Barr virus. Fields Virology. a. P. M. H. D. M. Knipe. Philadelphia, PA, Lippincott, Williams and Wilkins.

Rink, J., E. Ghigo, et al. (2005). "Rab conversion as a mechanism of progression from early to late endosomes." Cell **122**: 735-749.

Robertson, E., J. Lin, et al. (1996). "The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa)." Journal of Virology **70**(5): 3068-3074.

Rochford, R., M. Cannon, et al. (2005). "Endemic Burkitt's lymphoma: a polymicrobial disease?" Nature Reviews Microbiology **3**(2): 182-187.

Rodriguea-Boulan, E., G. Kreitzer, et al. (2005). "Organization of vesicular trafficking in epithelia." Nature Reviews Molecular Cell Biology **6**: 233-247.

Roizman, B. and J. Baines (1991). "The diversity and unity of herpesviridae." Compartative Immunology, Microbiology and Infectious Diseases **14**(2): 63-79.

Rothenfusser, S., N. Goutagny, et al. (2005). "The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I." Journal of Immunology **175**(8): 5260-5268.

Rous, P. (1910). "A TRANSMISSIBLE AVIAN NEOPLASM. (SARCOMA OF THE COMMON FOWL.)." Journal of Experimental Medicine **12**(5): 696-705.

Rousset, R., C. Desbois, et al. (1996). "Effects on NF-κB1/p105 processing of the interaction between the HTLV-1 transactivator Tax and the proteasome." Nature **381**: 328-331.

Rovedo, M. and R. Longnecker (2007). "Epstein-Barr Virus Latent Membrane Protein 2B (LMP2B) Modulates LMP2A Activity." Journal of Virology **81**(1): 84-94.

Rowe, D. T. and M. J. Tomaszewski-Flick (2007). "Minimal protein domain requirements for the intracellular localization and self aggregation of Epstein-Barr Virus Latent Membrane Protein 2." Virus Genes **35**(2): 225-234.

Rowe, M., A. Lear, et al. (1992). "Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes." Journal of Virology **66**(11): 122-131.

Rowe, M., C. Rooney, et al. (1985). "Distinctions between endemic and sporadic forms of Epstein-Barr virus-positive Burkitt's lymphoma." International Journal of Cancer **35**(4): 435-441.

Rowe, M., L. Young, et al. (1989). "Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins." Journal of Virology **63**(3): 1031-1039.

Ruf, I., P. Rhyne, et al. (2000). "Epstein-Barr virus small RNAs potentiate tumorigenicity of Burkitt lymphoma cells independently of an effect on apoptosis." Journal of Virology **74**(21): 10223-10228.

Salamon, D., M. Takacs, et al. (2003). "High-resolution methylation analysis and in vivo protein-DNA binding at the promoter of the viral oncogene LMP2A in B cell lines carrying latent Epstein-Barr virus genomes." Virus Genes **27**(1): 57-66.

Samanta, M., D. Iwakiri, et al. (2006). "EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN." EMBO **25**(18): 4207-4214.

Sample, J., D. Liebowitz, et al. (1989). "Two related Epstein-Barr virus membrane proteins are encoded by separate genes." Journal of Virology **63**(2): 933-937.

Sample, J., L. Young, et al. (1990). "Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes." Journal of Virology **64**(9): 4084-4092.

Samuel, C. E. (2001). "Antiviral Actions of Interferons." Clinical Microbiology Reviews **14**(4): 778-809.

Sandilands, E. and M. C. Frame (2008). "Endosomal trafficking of Src tyrosine kinase." Trends in Cell Biology **18**(7): 322-329.

Sandor, F. and M. Buc (2005). "Toll-like Receptors. II. Distribution and Pathways Involved in TLR Signalling." Folia Biologica (Praha) **51**: 188-197.

Sandvej, K., L. Krenács, et al. (1992). "Epstein-Barr virus latent and replicative gene expression in oral hairy leukoplakia." Histopathology **20**(5): 387-395.

Sanfridson, A., S. Hester, et al. (1997). "Nef proteins encoded by human and simian immunodeficiency viruses induce the accumulation of endosomes and lysosomes in human T cells." PNAS **94**(3): 873-878.

Sarkar, S., K. Peters, et al. (2004). "Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling." Nature Structural Molecular Biology **11**(11): 1060-1067.

Sarkar, S. N., C. P. Elco, et al. (2007). "Two Tyrosine Residues of Toll-like Receptor 3 Trigger Different Steps of NF-kB Activation." The Journal of Biological Chemistry **282**(6): 3423-3427.

Sato, M., H. Suemori, et al. (2000). "Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for IFN-[alpha]/[beta] Gene Induction." Immunity **13**(4): 539-548.

Sato, S., H. Sanjo, et al. (2005). "Essential function for the kinase TAK1 in innate and adaptive immune responses." Nature Immunology **6**: 1087-1095.

Sato, S., M. Sugiyama, et al. (2003). "Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling." Journal of Immumology **171**(8): 4303-4310.

Satoh, T., H. Kato, et al. (2010). "LGP2 is a positive regulator of RIG-I– and MDA5-mediated antiviral responses." PNAS **107**(4): 1512-1527.

Saxena, S., C. Bucci, et al. (2005). "the Small GTPase Rab7 Controls the Endosomal Trafficking and Neuritogenic Signaling of the Nerve Growth Factor Receptor TrkA." The Journal of Neuroscience **25**(47): 10930-10940.

Schaefer, B., J. Strominger, et al. (1997). "Host-cell-determined methylation of specific Epstein-Barr virus promoters regulates the choice between distinct viral latency programs." Molecular and Cellular Biology **17**(1): 364-377.

Scholle, F., K. M. Bendt, et al. (2000). "Epstein-Barr Virus LMP2A Transforms Epithelial Cells, Inhibits Cell Differentiation and Activates Akt." Journal of Virology **74**(22): 10681-10689.

Scholle, F., R. Longnecker, et al. (1999). "Epithelial cell adhesion to extracellular matrix proteins induces tyrosine phosphorylation of the Epstein-Barr virus latent membrane protein 2: a role for C-terminal Src kinase." Journal of Virology **73**(6): 4767-4775.

Scholle, F., R. Longnecker, et al. (2001). "Analysis of the phosphorylation status of Epstein-Barr virus LMP2A in epithelial cells." Virology **291**(2): 208-214.

Schroder, M. and A. G. Bowie (2005). "TLR3 in antiviral immunity: key player or bystander." Trends in Immunology **25**(9): 462-468.

Sen, G. and B. Williams (2003). "A viral on/off switch for Interferon." Science **300**: 1100-1101.

Sen, G. C. and S. N. Sarkar (2005). "Transcriptional signaling by double-stranded RNA: role of TLR3." Cytokine and Growth Factor Reviews **16**: 1-14.

Seo, J., S. Jun, et al. (2010). "Establishment and characterization of gastric carcinoma cell clones expressing LMP2A of Epstein-Barr virus." International Journal of Molecular Medicine **25**(1): 11-6.

Seto, E., T. Ooka, et al. (2008). "Reconstitution of nasopharyngeal carcinoma-type EBV infection induces tumorigenicity." Cancer Research **68**(4): 1030-1036.

Shah, K. M., S. E. Stewart, et al. (2009). "The EBV-encoded latent membrane proteins, LMP2A and LMP2B, limit the actions of interferon by targeting interferon receptors for degradation." Oncogene **28**(44): 3903-3914.

Shanmugaratnam, K. (1978). "Histological typing of nasopharyngeal carcinoma." IARC Sci Publication **20**: 3-12.

Shannon-Lowe, C., B. Neuhierl, et al. (2006). "Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells." PNAS **103**(18): 7065-7070.

Shao, Y., S. Poirier, et al. (1988). "Epstein-Barr virus activation in Raji cells by extracts of preserved food from high risk areas for nasopharyngeal carcinoma." Carcinogenesis **9**(8): 1455-1457.

Shen, H., B. M. Tesar, et al. (2008). "Dual Siganling of MyD88 and TRIF Is Critical for Maximal TLR4-Induced Dendritic Cell Maturation." The Journal of Immunology **181**: 1849-1858.

Sheu, L., A. Chen, et al. (1996). "Enhanced malignant progression of nasopharyngeal carcinoma cells mediated by the expression of Epstein-Barr nuclear antigen 1 in vivo." Journal of Pathology **180**(3): 243-248.

Shih, W., A. Gallusser, et al. (1995). "A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes." The Journal of Biological Chemistry **270**: 31083-31090.

Shimizu, N., H. Yoshiyama, et al. (1996). "Clonal propagation of Epstein-Barr virus (EBV) recombinants in EBV-negative Akata cells." Journal of Virology **70**(10): 7260-7263.

Simonsen, A., R. Lippe, et al. (1998). "EEA1 links PI(3)K function to Rab5 regulation of endosome fusion." Nature **394**: 494-498.

Sinclair, A., M. Jacquemin, et al. (1994). "Reduced signal transduction through glucocorticoid receptor in Burkitt's lymphoma cell lines." Virology **199**(2): 339-353.

Sinclair, A., I. Palmero, et al. (1994). "EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus." EMBO **13**(14): 3321-3328.

Sixbey, J., J. Nedrud, et al. (1984). "Epstein-Barr virus replication in oropharyngeal epithelial cells." The New England Journal of Medicine **310**(19): 1225-1230.

Snyder, M. D. and S. K. Pierce (2006). "A Mutation in Epstein-Barr Virus LMP2A Reveals a Role for Phospholipase D in B-Cell Antigen Receptor Trafficking." Traffic **7**: 993-1006.

Stenmark, H. and V. Olkkonen (2001). "The Rab GTPase family." Genome Biology **2**(5): 1-7.

Stewart, S., C. W. Dawson, et al. (2004). "Epstein-Barr virus encoded LMP2A regulates viral and cellular gene expression by modulation of the NF-kB transcription factor pathway." PNAS **101**(144): 15730-15735.

Sun, Q., L. Sun, et al. (2006). "The specific and essential role of MAVS in antiviral innate immune responses." Immunity **24**(5): 633-642.

Swaminathan, S., B. Tomkinson, et al. (1991). "Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro." PNAS **88**(4): 1546-1550.

Swanson-Mungerson, M., R. Bultema, et al. (2006). "Epstein-Barr Virus LMP2A Enhances B-Cell Responses In Vivo and In Vitro." Journal of Virology **80**(14): 6764-6770.

Swart, R., S. Fruehling, et al. (1999). "Tyrosines 60, 64, and 101 of Epstein-Barr virus LMP2A are not essential for blocking B cell signal transduction." Virology **263**(2): 485-495.

Swart, R., I. Ruf, et al. (2000). "Latent membrane protein 2A-mediated effects on the phosphatidylinositol 3-Kinase/Akt pathway." Journal of Virology **74**(22): 10838-10845.

Takada, K. (2000). "Epstein-Barr virus and gastric carcinoma." Journal of Clinical Pathology: Molecular Pathology **53**: 255-261.

Takada, K. and Y. Ono (1989). "Synchronous and sequential activation of latently infected Epstein-Barr virus genomes." Journal of Virology **63**(1): 445-449.

Takaoka, A., H. Yanai, et al. (2005). "Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors." Nature **434**(7030): 243-249.

Takeda, K., T. Kaisho, et al. (2003). "Toll-like receptors." Annual Review of Immunology **21**: 335-376.

Tang, J., E. Rohwäder, et al. (2007). "Evaluation of Epstein-Barr virus antigen-based immunoassays for serological diagnosis of nasopharyngeal carcinoma." Journal of Clinical Virology **40**(4): 284-288.

Tang, X., J. Gao, et al. (2007). "Acetylation-dependent signal transduction for type I interferon receptor." Cell **161**(1): 93-105.

Tanner, J., J. Weis, et al. (1987). "Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis." Cell **50**(2): 203-213.

Thomas, J., M. Allday, et al. (1991). "Epstein-Barr virus-associated lymphoproliferative disorders in immunocompromised individuals." Advances in Cancer Research **57**: 329-380.

Thomas, J., D. Felix, et al. (1991). "Epstein-Barr virus gene expression and epithelial cell differentiation in oral hairy leukoplakia." American Journal of Pathology **139**(6): 1369-1380.

Thompson, M. and R. Kurzrock (2004). "Epstein-Barr virus and cancer." Clinical Cancer Research **10**(3): 803-821.

Thomsen, P., B. v. Deurs, et al. (2000). "The HPV16 E5 oncogene inhibits endocytic trafficking." Oncogene **19**: 6023-6032.

Thorley-Lawson, D. A. (2001). "Epstein-Barr virus: exploiting the immune system." Nature Reviews Immunology **1**: 75-82.

Tierney, R., N. Steven, et al. (1994). "Epstein-Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state." Journal of Virology **68**(11): 7374-7385.

Tomaszewski-Flick, M. J. and D. T. Rowe (2007). "Minimal protein domain requirements for the intracellular localization and self-aggregation of Epstein-Barr Virus Latent Membrane Protein 2." Virus Genes.

Trümper, L., G. Brady, et al. (1993). "Single-cell analysis of Hodgkin and Reed-Sternberg cells: molecular heterogeneity of gene expression and p53 mutations." Blood **81**(11): 3097-3115.

Tsai, C., C. Lee, et al. (1999). "Additive effect of Sp1 and Sp3 in regulation of the ED-L1E promoter of the EBV LMP 1 gene in human epithelial cells." Virology **261**(2): 288-294.

Tsai, C., S. Liu, et al. (1995). "Identification of a novel promoter located within the Bam HI Q region of the Epstein-Barr virus genome for the EBNA 1 gene." DNA Cell Biology **14**(9): 767-776.

Tseng, P.-h., A. Matusuzawa, et al. (2010). "Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines." Nature Immunology **11**(1): 70-76.

Tsujimura, H., T. Tamura, et al. (2004). "Toll-like 9 signalling activates NF-kappaB through IFN regulatory factor-8/IFN consensus binding protein in dendritic cells." Journal of Immunology **172**: 6820-6827.

Tsurumi, T., M. Fujita, et al. (2005). "Latent and lytic Epstein-Barr virus replication strategies." Review of Medical Virology **15**(1): 3-15.

Uematsu, S. and S. Akira (2007). "Toll-like Receptors and Type I Inteferons." The Journal of Biological Chemistry **282**(21): 15319-15323.

Uzé, G. and D. Monneron (2007). "IL-28 and IL-29: newcomers to the interferon family." Biochimie **89**(6-7): 729-734.

Vesely, P. W., P. B. Staber, et al. "Translational regulation mechanisms of AP-1 proteins." Mutation Research/Reviews in Mutation Research **682**(1): 7-12.

Walling, D., A. Brown, et al. (2003). "Multiple Epstein-Barr virus infections in healthy individuals." Journal of Virology **77**(11): 6546-6550.

Walling, D., C. Flaitz, et al. (2001). "Persistent productive Epstein-Barr virus replication in normal epithelial cells in vivo." Journal of Infectious Disease **184**(12): 1499-1507.

Wang, D., D. Liebowitz, et al. (1985). "An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells." Cell **43**(3pt2): 831-840.

Wang, H., M. W. Nicholas, et al. (2006). "EBV Latent Membrane Protein 2A Induces Autoreactive B Cell Activation and TLR Hypersensitivity." The Journal of Immunology **177**: 2793-2802.

Wang, Q., S. Tsao, et al. (2006). "Anti-apoptotic role of BARF1 in gastric cancer cells." Cancer Letters **238**(1): 90-103.

Wang, R.-P., M. Zhang, et al. (2008). "Differential regulation of IKKa mediated activation of IRF3/7 by NIK" Molecular Immunology **45**: 1926-1934.

Wang, Y., T. Chen, et al. (2007). "Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4." Immunobiology **110**(3): 962.

Wang, Y., T. Chen, et al. (2007). "Lysosome-associated small Rab GTPase Rab7b negatively regulates TLR4 signaling in macrophages by promoting lysosomal degradation of TLR4." Blood **110**(3): 962-971.

Webster-Cyriaque, J., J. Middeldorp, et al. (2000). "Hairy leukoplakia: an unusual combination of transforming and permissive Epstein-Barr virus infections." Journal of Virology **74**(16): 7610-7618.

Webster-Cyriaque, J. and N. Raab-Traub (1998). "Transcription of Epstein-Barr virus latent cycle genes in oral hairy leukoplakia." Virology **248**(1): 53-65.

West, A. P., A. A. Koblansky, et al. (2006). "Recognition and Signaling by Toll-Like Receptors." Annual Review of Cell and Developmental Biology **22**: 409-439.

Wilson, J., J. Bell, et al. (1996). "Expression of Epstein-Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice." EMBO **15**(12): 3117-3126.

Winberg, G., L. Matskova, et al. (2000). "Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases." Molecular and Cellular Biology **20**(22): 8526-8533.

Wong, N., A. Hui, et al. (2003). "Molecular cytogenetic characterization of nasopharyngeal carcinoma cell lines and xenografts by comparative genomic hybridization and spectral karyotyping." Cancer Genetics and Cytogenetics **140**(2): 124-132.

Woodman, P. G. (2000). "Biogenesis of the Sorting Endosome: The Role of Rab5." Traffic **1**: 695-701.

Wu, C.-C., K.-Y. Chien, et al. (2005). "Cancer cell-secreted proteomes as a basis for searching potential tumor markers: Nasopharyngeal carinoma as a model." Proteomics **5**: 3173-3182.

Wu, Y., S. Maro, et al. (2007). "Epstein-Barr virus (EBV)-encoded RNA (EBER2) but not EBER1 plays a critical role in EBV-Induced B-cell growth transformation." Journal of Virology **81**(20): 11236-11245.

Wucherpfennig, T., M. Wilsch-Brauninger, et al. (2008). "Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release." The Journal of Cell Biology **161**(3): 609-624.

Wullaert, A., K. Heyninck, et al. (2006). "Ubiquitin: tool and target for intracellular NF-kappaB inhibitors." Trends in Immunology **27**(11): 533-540.

Wysokenski, D. and J. Yates (1989). "Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus." Journal of Virology **63**(6): 2657-2666.

Yamamoto, K., M. Yamaguchi, et al. (2003). "SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor [beta]2 subunit." Biochemical and Biophysical Research Communications **310**(4): 1188-1193.

Yates, J., N. Warren, et al. (1985). "Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells." Nature **313**(6005): 812-815.

Yie, J., K. Senger, et al. (1999). Mechanism by which the IFN-Î² enhanceosome activates transcription. **96:** 13108-13113.

Yoneyama, M., M. Kikuchi, et al. (2005). "Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity." Journal of Immumology **175**(5): 2851-2858.

Yoneyama, M., M. Kikuchi, et al. (2004). "The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses." Nature Immunology **5**(7): 730-737.

Yoneyama, M., W. Suhara, et al. (1998). "Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300 " The EMBO Journal **17**(4): 1087-1095.

Yoshimura, A., T. Naka, et al. (2007). "SOCS proteins, cytokine signalling and immune regulation." Nature Reviews Immunology **7**: 454-464.

Young, L., C. Alfieri, et al. (1989). "Expression of Epstein-Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease." New England Journal of Medicine **321**(16): 1080-1085.

Young, L., C. Dawson, et al. (1999). "Epstein-Barr virus and apoptosis: viral mimicry of cellular pathways." Biochem Soc Trans **27**(6): 807-812.

Young, L., R. Lau, et al. (1991). "Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia." Journal of Virology **65**(6): 2868-2874.

Young, L. and P. Murray (2003). "Epstein-Barr virus and oncogenesis: from latent genes to tumours." Oncogene **22**(33): 5108-5121.

Young, L. and A. Rickinson (2004). "Epstein-Barr virus: 40 years on." Nature Reviews Cancer **4**(10): 757-768.

Yuen, S., L. Chung, et al. (1994). "In situ detection of Epstein-Barr virus in gastric and colorectal adenocarcinomas." American Journal of Surgical Pathology **18**(11): 1158-1163.

Zalani, S., E. Holley-Guthrie, et al. (1996). "Epstein-Barr viral latency is disrupted by the immediate-early BRLF1 protein through a cell-specific mechanism." PNAS **93**(17): 9194-9199.

Zech, L., U. Haglund, et al. (1976). "Characteristic chromosomal abnormalities in biopsies and lymphoid-cell lines from patients with Burkitt and non-Burkitt lymphomas." International Journal of Cancer **17**(1): 47-56.

Zeng, M., D. Li, et al. (2005). "Genomic sequence analysis of Epstein-Barr virus strain GD1 from a nasopharyngeal carcinoma patient." Journal of Virology **79**(24): 15323-15330.

Zerial, M. and H. McBride (2001). "Rab proteins as membrane organizers." Nature Reviews Molecular Cell Biology **2**: 107-119.

Zhang, J.-G., A. Farley, et al. (1999). "The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation." PNAS **96**: 2071-2076.

Zhao, B. and C. Sample (2000). "Epstein-barr virus nuclear antigen 3C activates the latent membrane protein 1 promoter in the presence of Epstein-Barr virus nuclear antigen 2 through sequences encompassing an spi-1/Spi-B binding site." Journal of Virology **74**(11): 5151-5160.

Zimber-Strobl, U. and L. Strobl (2001). "EBNA2 and Notch signalling in Epstein-Barr virus mediated immortalization of B lymphocytes." Seminars in Cancer Biology **11**(6): 423-434.

Zimber-Strobl, U., L. Strobl, et al. (1994). "Epstein-Barr virus nuclear antigen 2 exerts its transactivating function through interaction with recombination signal binding protein RBP-J kappa, the homologue of Drosophila Suppressor of Hairless." EMBO **13**(20): 4976-4982.

Zimber-Strobl, U., K. Suentzenich, et al. (1991). "Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene." Journal of Virology **65**(1): 415-423.

Zimber, U., H. Adldinger, et al. (1986). "Geographical prevalence of two types of Epstein-Barr virus." Virology **154**(1): 56-66.

Zur Hausen, A., B. van Rees, et al. (2004). "Epstein-Barr virus in gastric carcinomas and gastric stump carcinomas: a late event in gastric carcinogenesis." Journal of Clinical Pathology **57**(5): 487-491.

zur Hausen, H. (1999). "Viruses in human cancers." European Journal of Cancer **35**(14): 1878-85.

zur Hausen, H., H. Schulte-Holthausen, et al. (1970). "EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx." Nature **228**(5276): 1056-1058.