Investigating a role of ASPP2 in the cellular response to infection

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Masters of Science (by Research) in Clinical Medicine

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DECLARATION

All the work presented in this thesis is the result of my own work and does not constitute part of any other thesis unless indicated otherwise. The work here described was performed while I was a graduate student at the Ludwig Institute for Cancer Research, under the supervision of Prof. Xin Lu.
ACKNOWLEDGEMENTS

First of all, gratitude must be expressed towards my supervisor, Prof. Xin Lu for allowing me to undertake research in her lab and for helping with the acquisition of reagents from several EBV labs. I am indebted to the past and present members of the Lu lab, as well as those from the Ludwig Institute in general. Many, many thanks to Dr. Ludo Buti, who mentored me during the past year; to the rest of the Lu lab for their wonderful support and advice; to Dr. Sarah De Val, who has offered personal advice and helped me with thesis revisions; to Prof. Rob Goldin, who has provided me with precious human biopsy samples and his expertise; to Indi who patiently processed them; and finally to Mark Shipman who provided technical help for microscopy and image analysis.

I would also like to thank my EBV collaborators from the University of Birmingham and Imperial College, Dr. Christopher Dawson and Prof. Paul J Farrell, who provided us with the NPC cell lines and EBV plasmids. I must also express my gratitude to collaborators from the Jenner Institute, Dr. Elena Stylianou and Prof. Helen McShane, as well as Dr. Gopi Suthendra from LICR, who helped me in the BCG/Mtb infection studies. Last but not least, thanks to Sir Anthony Epstein, Yvonne Barr and Bert Achong for the discovery of EBV in 1964 (and to Robert Koch who discovered Mtb 130 years ago)! Happy 50th EBV anniversary!
Intracellular pathogens such as Epstein-Barr virus (EBV) and *Mycobacterium tuberculosis* (*Mtb*), the causative agents of nasopharyngeal cancer (NPC) and tuberculosis (TB), respectively, can establish lifelong persistent infection in their respective host cells and have a profound effect on the dynamics of the host microenvironment. Studies have explored the molecular components and mechanisms that are involved in complex host-pathogen interactions that lead to infection and cancer. In particular, the transcription factor (TF) p53 plays a critical role in protecting the host against cancer and infectious agents by inducing cell cycle arrest and apoptosis. Dysregulation of p53 pathways contributes to the pathogenesis of cancer and inflammatory diseases. Here, it is investigated whether regulators of p53, such as p63 and ASPP2, can link infection, immune activation and their involvement in NPC and TB. Both p63 and ASPP2 are determinants of epithelial integrity and cell apoptosis. In addition, ASPP2 has been shown to interact with members of the p53 family and the pro-inflammatory TF NF-kB. Notably, an inverse relationship is shown between the expression levels of p63 and EBV infection *in vitro*. Moreover, EBV-infected NPC cells exhibit features of epithelial-to-mesenchymal transition (EMT), and a lack of p63 may play a role in NPC progression by improving tumour motility and invasion. Interestingly, human NPC biopsies exhibit nuclear p63, which contradicts the *in vitro* data outlined in this report. It is postulated that the histology analyses in this study are more likely to accurately reflect the intrinsic environment of tumourigenesis, which are in agreement with the published literature. Induction of ASPP2 *in vivo* is shown in NPC, as well as BCG- and *Mtb*-infected lung biopsies. Furthermore, examination of these samples revealed aberrant accumulation of immune cells at the lesion site. Administration of inflammatory agonists, such as lipopolysaccharide (LPS), triggered up-regulation of ASPP2 in macrophage-like THP-1 cells. This differential expression indicates that ASPP2 may
be involved in the immunological eradication of cancerous or granulomatous lesions. Understanding the interplay between regulators of p53 and the anti-inflammatory responses will shed light on the complexity of the host’s immune surveillance system.
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<td>$\Delta N$</td>
<td>delta N</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAM</td>
<td>alternatively activated macrophages</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Acryl/Bis</td>
<td>acrylamide/bis acrylamide</td>
</tr>
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<td>AG</td>
<td>arabinogalactan</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
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<tr>
<td>AJC</td>
<td>apical-junctional complex</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cells</td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>AraLAM</td>
<td>arabinofuranosyl-terminated lipoarabinomannan</td>
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<td>ASPP</td>
<td>apoptosis-stimulating protein of p53</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BARTs</td>
<td>BamHI A rightward transcripts</td>
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<td>BCG</td>
<td>Bacillus Calmette–Guérin</td>
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<td>Bcl</td>
<td>B cell lymphoma</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<td>BL</td>
<td>Burkitt’s lymphoma</td>
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<td>bp</td>
<td>base pair</td>
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<td>BRCA1</td>
<td>breast cancer 1</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>c.f.u.</td>
<td>colony-forming units</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>calcium</td>
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<td>CagA</td>
<td>cytotoxin-associated gene A</td>
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<td>CAM</td>
<td>classically activated macrophages</td>
</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate</td>
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<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
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<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
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<tr>
<td>chr</td>
<td>chromosome</td>
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<tr>
<td>CK</td>
<td>cytokeratin</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
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<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<td>CpG</td>
<td>cytosine phosphate guanosine</td>
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<tr>
<td>CREB</td>
<td>cyclic AMP response element-binding</td>
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<td>CSF1</td>
<td>colony-stimulating factor 1</td>
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<tr>
<td>Ct</td>
<td>threshold cycle</td>
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<td>CTAR</td>
<td>carboxyl terminal activation region</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte</td>
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<td>CTLA-4</td>
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<td>CWF</td>
<td>cell wall fraction</td>
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<td>CWS</td>
<td>cell wall skeleton</td>
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<tr>
<td>CXCL</td>
<td>chemokine (C-X-C motif) ligand</td>
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<tr>
<td>d.p.i.</td>
<td>days post infection</td>
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<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole dihydrochloride hydrate</td>
</tr>
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<td>DBD</td>
<td>DNA-binding domain</td>
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DC  dendritic cell
DC-SIGN  dendritic cell-specific ICAM-3 grabbing non-integrin
DD  death domain
DED  death effector domain
DISC  death-inducing signalling complex
DMEM  Dulbecco's Modified Eagle's medium
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
DRAM  damage-regulated autophagy modulator
DS  dyad symmetry
DTT  dithiothreitol
E. coli  Escherichia coli
EA  early antigen
EBER  EBV-encoded RNA
EBNA  Epstein-Barr nuclear antigen
EBV  Epstein-Barr virus
EBVaGC  Epstein-Barr virus-associated gastric cancer
ECL  enhanced chemoluminescence
EDTA  Ethylenediaminetetraacetic acid
EEA-1  early endosome antigen 1
EGFR  epidermal growth factor receptor
EMT  epithelial-to-mesenchymal transition
EREG  epiregulin
FACS  fluorescence activated cell sorting
FADD  Fas-associated via death domain
FasL  Fas ligand
FBS  foetal bovine serum
FR  family of proteins
FSG  fish skin gelatin
G418  genticin sulfate
G-CSF  granulocyte-colony stimulating factor
GDI  GDP dissociation inhibitor
GOF  gain-of-function
gp  glycoprotein
GPI  glycosylphosphatidylinositol
GTP  guanosine-5’-triphosphate
H&E  haematoxylin & eosin
H. pylori  Helicobacter pylori
HBV  hepatitis B virus
HCC  hepatocellular carcinoma
HCl  hydrogen chloride
HCV  hepatitis C virus
HHV-4  human herpesvirus 4
HIV  human immunodeficiency virus
HL  Hodgkin's lymphoma
HLA  human leukocyte antigen
HPV  human papilloma virus
HRP  horse-radish peroxidase
HTLV-1  human T-lymphotropic virus 1
IARC  International Agency for Research on Cancer
IE  immediate-early
IF  immunofluorescence
IFN  interferon
Ig  immunoglobulin
IHC immunohistochemistry
IL interleukin
IM infectious mononucleosis
iNOS inducible nitric oxide synthase
IRAK interleukin-1 receptor-associated kinase
IRF iron regulatory factor
IRF interferon regulatory factor
ISH in situ hybridisation
IκB inhibitor of NF-κB
JNK c-Jun N-terminal kinase
kb kilo base pair
kDa kilo dalton
KSHV Kaposi's sarcoma-associated herpesvirus
L litre
LAM lipoarabinomannan
LB Luria-broth
LCL lymphoblastoid cell line
LM lipomannan
LMP latent membrane protein
LOH loss of heterozygosity
LPS lipopolysaccharide
LxA4 lipoxin A4
M molar
M. avium Mycobacterium avium
M. leprae Mycobacterium leposy
M. marinum Mycobacterium marinum
mAb monoclonal antibody
mAGP mycolyl-arabinogalactan-peptidoglycan
ManLAM mannose-capped lipoarabinomannan
MAPK mitogen-activated protein kinase
Mcl myeloid cell leukaemia
MCP1 monocyte chemotactic protein
mdm2 murine double minute 2
MDR multi-drug resistant
MFI mean immunofluorescence intensity
MGC multi-nucleated giant cell
MHC major histocompatibility complex
miR micro ribonucleic acid
MLN mesenteric lymph nodes
MMP-9 matrix metalloproteinase 9
mRNA messenger ribonucleic acid
Mt Mycobacterium tuberculosis
myD88 myeloid differentiation primary response protein 88
n = number equal
NaCl sodium chloride
NaOH sodium hydroxide
NF-κB nuclear factor-kappa B
NICD Notch intracellular domain
NK natural killer
NO nitric oxide
NOD nucleotide-binding oligomerisation domain
NP-40 Nonidet P-40
NPC nasopharyngeal carcinoma
OD oligomerisation domain
OHL oral hairy leukoplakia
<table>
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<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>USP7</td>
<td>ubiquitin-specific-processing protease 7</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>VCA</td>
<td>viral capsid antigen</td>
</tr>
<tr>
<td>VPS</td>
<td>vacuolar protein sorting</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>XDR</td>
<td>extensively drug-resistant</td>
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<td>YAP</td>
<td>Yes-associated protein</td>
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<td>ZEB1</td>
<td>zinc finger E-box binding homeobox 1</td>
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CHAPTER I: INTRODUCTION

1.1 Infection and cancer: a general overview

1.1.1 The hallmarks of cancer

In order for cancer cells to thrive and exhibit a competitive advantage over normal/benign cells, they must acquire the following capabilities: (i) a sustained proliferative potential, (ii) self-sufficiency in growth signalling, (iii) resistance to death by preventing apoptosis, (iv) achievement of replicative immortality, (v) the ability to induce metastasis, and (vi) angiogenesis (Hanahan and Weinberg, 2000). Both tumour suppressor genes (TSGs) such as TP53, BRCA1, Rb and PTEN, and oncogenes such as Ras, myc, EGFR and PDGFR, are frequently mutated in most cancers (Vogelstein and Kinzler, 2004). Inactivation of TSGs is required to override cell cycle checkpoints and therefore facilitates insensitivity to anti-growth signals, while constitutive oncogenic signalling facilitates self-sufficiency with regard to mitogenic signals (Ashworth et al., 2011). Tumourigenesis is a progressive, multistep process and an accumulation of genomic instability is observable over time.

Recently, additional hallmarks of cancer have emerged. These include tumour-promoting inflammation and the evasion of immune detection, and are supported in part by the observation of higher tumour incidence rates among the immunocompromised (Hanahan and Weinberg, 2011). Infiltration of immune cells such as macrophages, B-lymphocytes, natural killer (NK) cells and T-lymphocytes have been observed at the site of tumours in hepatic, lung and oesophageal carcinomas (Gooden et al., 2011, Wang et al., 2014a). It was initially thought that the
body mounts an anti-tumour response by activating its innate and adaptive immunity in an attempt to obliterate these abnormal malignant cells. However, it is now hypothesised that these tumour-infiltrating immune cells may actually contribute to tumour progression by promoting metastasis and inflammatory angiogenesis (Whiteside, 2008). Secretion of growth factors and pro-inflammatory cytokines, and the recruitment of a variety of cell types (including myeloid cells, endothelial cells, fibroblasts and macrophages), stimulates neovascularisation and favours a pro-tumour microenvironment (Dinarello, 2006). The dual roles of the host immune system in cancer progression have led to the concept of “cancer immunoediting” (Kim et al., 2007). “Immunoediting” is envisaged to occur in 3 phases: elimination, equilibrium and escape. The intact host immune surveillance system acts as a defence mechanism to detect and destroy neoplastic cells. At equilibrium, some tumour cells become resistant as they develop evasion strategies, while the non-resistant tumour is selectively eliminated. These resistant tumour cells overcome immune checkpoints by inducing immunological tolerance, which involves active dampening of cytotoxic T-lymphocyte activity, recruiting immunosuppressive T-regulatory (T_{reg}) cells and producing immunosuppressive cytokines (i.e. TGFβ, IL-10) (Facciabene et al., 2012). Several cancer immunotherapies, such as autologous tumour-specific cell therapies and anti-CTLA-4 and PD-1 antibodies, aim to harness the patient’s own immune system to target tumour-specific epitopes, overcome peripheral tolerance and induce immunological memory to avoid the recurrence of cancer (Kyi and Postow, 2014).

### 1.1.2 Infection-attributable cancers

Infectious agents (virus, bacteria and parasitic infections) are attributed to approximately 20% of malignancies globally (Vineis and Wild, 2014). Pathogens...
including human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus (EBV), human T-lymphotropic virus type I (HTLV-1), Kaposi sarcoma-associated herpesvirus (KSHV) and the bacterium *Helicobacter pylori* have been listed as cancer-causing agents (IARC, 2012). These carcinogens can promote tumourigenesis by actively driving the transformation of normal cells to neoplastic cells through the expression of viral oncogenes, or by stimulating chronic inflammation that causes genetic and epigenetic alterations.

1.2 Epstein-Barr virus (EBV)

1.2.1 EBV

EBV, also known as human herpesvirus 4 (HHV-4), is a very successful pathogen as it infects about 90% of the human population and persists asymptotically for life. Discovered by Anthony Epstein, Yvonne Barr and Bert Achong using electron microscopy in 1964, it is the first γ-herpesvirus to have been discovered within a Burkitt’s lymphoma (BL) cell line (Epstein et al., 1964). Primary EBV infection usually happens during childhood and infected individuals become long-term carriers of the virus (Faulkner et al., 2000). However, if contraction of EBV is delayed until adolescence, which occurs predominantly in developed countries, patients may develop infectious mononucleosis (IM) which is also known as glandular fever or the “kissing disease” (Henle et al., 1968).

EBV exhibits a very narrow host tropism as it infects only B-lymphocytes and epithelial cells. It gains access to B-lymphocytes via attachment of the viral envelope glycoprotein gp350/220 to the host cell surface receptor CD21 (also known as
complement receptor 2 (CR2). Blockade of this ligand-receptor interaction by a soluble gp350/220, a deletion mutant gp350/220 or a gp350/220-specific EBV-neutralising antibody can prevent absorption of the virus to the B-lymphocytes (Tanner et al., 1988). This suggests that this may be the predominant or sole virus entry method. The subsequent trigger for internalisation via endocytosis involves the binding of a second glycoprotein, gp42, to human leukocyte antigen (HLA) class II molecules on B-lymphocytes. It has been suggested that EBV gains cell entry into CD21-negative epithelial cells by the fusion of its glycoprotein gH/gL complex to host integrins αvβ6 and αvβ8 (Chesnokova et al., 2009). The efficiency of “cell-free” EBV infection in vitro is variable and inefficient. To facilitate its infection of epithelial cells, an artificial system has been devised by co-culturing a monolayer of epithelial cells with recombinant EBV-producing lymphoblastoid cell lines (Imai et al., 1998). EBV is transferred from a neighbouring B-lymphocyte to epithelial cells via viral glycoproteins gp85 and gp110, following the formation of an intercellular synapse (Shannon-Lowe et al., 2006). This cell-to-cell infection method has a higher infection efficiency (1,000 to 10,000-fold) compared to cell-free EBV infection.

EBV, like other viruses in the herpes family, displays a biphasic life cycle consisting of a lytic and a latent phase. During the lytic replication cycle, the virus produces large numbers of infectious virus progenies, which are shed into the saliva and transmitted to naïve hosts through skin-to-skin contact. The transcription of two immediate-early (IE) genes, BZLF1 and BRLF1, which encode the transcription factors Zta/ZEBRA and Rta, are activated upon induction of the lytic cycle (Rooney et al., 1989). These transactivators act synergistically to initiate the lytic cascade by activating promoters of downstream viral early and late genes, as well as cellular genes. Among the 80 immunogenic viral proteins expressed by the virus are DNA replication factors and structural proteins, such as capsid antigens, which are required for linear EBV genome replication and packaging of the virion (Steven et al.,
Epstein-Barr nuclear antigen 1 (EBNA-1) transcription is also initiated from an early lytic promoter BamHI F promoter (Fp) during the lytic phase and thousands of copies of viral genomes per cell are replicated during the viral productive cycle (Lear et al., 1992). To date, the mechanism that enables the switching from latency to the lytic cycle has yet to be elucidated.

After the initial lytic infection, EBV establishes latency in B-lymphocytes by expressing a limited set of latent products: six nuclear antigens, Epstein-Barr nuclear antigens (EBNAs) - EBNA-1-6; and three membrane proteins, latent membrane proteins (LMPs) - LMP-1, LMP-2A and LMP-2B. The EBV genome persists as circular episomal double-stranded DNA during quiescence and is propagated with the host replication machinery (FIGURE 1.1). It replicates only once and maintains a low copy number to evade the immune response. Transcription of the restricted set of latent EBV viral antigens is initiated from one of the three distinct BamHI promoters: Cp, Wp and Qp, depending on the type of latency programme (TABLE 1).

Type III latency gene expression, where the full spectrum of latent gene products are transcribed, was first recognised in lymphoblastoid cell lines (LCL) (Klein, 1989). These infected cells adopt the promoter of the Wp/Cp locus to establish immortalisation. The Wp promoter is only utilised early in infection and is subsequently repressed by methylation, at which point the Cp promoter is activated. This shift of promoter usage occurs in a mutually exclusive manner (Woisetschlaeger et al., 1990). In other forms of latency, transcription is initiated from the Qp promoter and the Wp/Cp promoter is silenced. Only EBNA-1 is transcribed in the type I latency displayed in Burkitt’s lymphoma and EBV-associated gastric carcinoma (EBVaGC), while EBNA-1 as well as LMP-1, LMP-2A and LMP-2B are produced in type II latency, exhibited by Hodgkin’s lymphoma and nasopharyngeal carcinoma (NPC). These are summarised in TABLE 1. Two small non-polyadenylated coding RNAs,
including EBV-encoded RNAs (EBER1 and EBER2) and BamHI A rightward transcripts (BARTs), can be detected in all forms of viral latency, though their exact role is yet to be determined. Due to its ubiquitous expression, molecular examination of EBER by in situ hybridisation (ISH) has been the gold standard to detect EBV in patient’s biopsies. Other detection methods include PCR of EBV DNA, and the determination of the presence of IgA or IgE antibodies against virus early (EA) and capsid antigen (VCA) in the serum.
FIGURE 1.1 - Schematic diagram showing the structure of the EBV DNA episome. The EBV genome persists in a circular, double-stranded form, which is not integrated into the host genome during latency. Different latency programmes utilise different BamHI promoters. The non-coding EBV-encoded RNAs (EBER1 and EBER2) and BamHI A rightward transcripts (BARTs) coding for BARF0 are abundantly expressed in all viral latency programmes. In type III latency, B-lymphocytes express all latency products EBNA-1, EBNA-2, EBNA-3A-3C, EBNA-LP, LMP-1, LMP-2A and LMP-2B. In latency II, EBNA-1 and all LMP antigens are expressed, whereas only EBNA-1 is expressed in type I latency. EBNA-1 is transcriptionally initiated from the Wp or Cp promoter (green arrow) in the latent III programme, while it is transcribed from the Qp promoter (pink arrow) in latency types I and II. The origin of replication (oriP) (orange) contains numerous binding sites for EBNA-1 for genome replication and maintenance. The larger green arrows represent the direction and the location transcriptional start sites of the latent proteins. Adapted from Young and Rickinson, 2004.
<table>
<thead>
<tr>
<th>Latency programme</th>
<th>EBV antigens expressed</th>
<th>EBV-associated diseases</th>
<th>Transcriptional promoter usage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td>EBNA-1 only</td>
<td>Burkitt’s lymphoma (BL), gastric carcinoma (EBVaGC)</td>
<td>Qp</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td>EBNA-1, LMP-1, LMP-2A</td>
<td>Hodgkin’s lymphoma (HL), nasopharyngeal carcinoma (NPC), peripheral T/NK-lymphoma</td>
<td>Qp</td>
</tr>
<tr>
<td><strong>Type III</strong></td>
<td>All latency proteins expressed – EBNA-1, LMP-1, LMP-2A, LMP-2B, EBNA-2-6</td>
<td>Lymphoproliferative diseases <em>i.e.</em> AIDS-associated lymphoma and post-transplant lymphoproliferative disorder (PTLD)</td>
<td>Wp or Cp</td>
</tr>
</tbody>
</table>

**TABLE 1** - EBV gene expression in all forms of latency programmes.
1.2.2 EBV-associated malignancies

According to an IARC (International Agency for Research on Cancer) report published in 2012, EBV is classified as a Group 1 human carcinogen due to its causal association with several malignancies (IARC, 2012). Chronic infection with EBV is known to cause BL (which is endemic in Africa and common in children), Hodgkin’s disease (HD), oral hairy leukoplakia (OHL), and NPC. High EBV antibody titer is also detected in immunocompromised individuals and could cause post-transplant lymphoproliferative disorders (PTLD) and HIV/AIDS-associated lymphoma. There is limited evidence that EBV can contribute to gastric cancer, as EBV has been detected in 10% of gastric carcinomas and has a distinct lymphoepithelioma histopathology (Imai et al., 1994). EBV-associated T- and NK-cell lymphoproliferative lymphomas is also reported in East Asia, although these are relatively rare (Kimura et al., 2001).

1.2.3 Nasopharyngeal carcinoma (NPC)

NPC is a head and neck cancer of the nasopharyngeal surface epithelium that is endemic in southern Asia but rarely found in western countries. Both genetic and environmental factors are known to facilitate NPC’s pathogenesis. Epidemiological studies have shown a correlation of exposure to dietary salted preservatives, such as \(N\)-nitroso compounds (regularly consumed in Southern Asia), with increased risk of NPC. Furthermore, the presence of EBV in the form of DNA, non-coding RNA and oncogenic proteins has been detected in all undifferentiated NPC biopsies, strongly suggesting that EBV may be the causative agent. This is further supported by other findings concluding that pre-invasive dysplastic cells carry monoclonal EBV viral
genomes, implying that EBV infection is a pre-requisite of NPC malignancy and progression (Pathmanathan et al., 1995) (FIGURE 1.2).

FIGURE 1.2 - Schematic representation of the molecular mechanism of nasopharyngeal tumourigenesis. Early transition to pre-malignant tissue from normal mucosal epithelium is due to loss of heterozygosity (LOH) on chromosomes 3p and 9p, which results in the inactivation of tumour suppressors such as CDK inhibitor CDKN2A and pro-apoptotic Ras-binding protein RASSF1A. High frequencies of chr 3p deletion in the Southern Chinese population, and the consumption of carcinogens such as volatile nitrosamine present in preserved fish, increases the risk of developing NPC. EBV infection is a necessary precursor to transformation of high-grade lesions as EBV is detected in most NPC biopsies. The induction of EBV oncoproteins such as LMP-1 and LMP-2A confers survival and growth advantages. Further genetic mutations and epigenetic alterations contribute to NPC progression and invasion. Adapted from Young and Rickinson, 2004.

The World Health Organisation (WHO) has proposed three main histological classifications of NPC: type I squamous cell carcinoma (SCC); type II keratinising, undifferentiated carcinoma; and type III non-keratinising carcinoma. Undifferentiated nasopharyngeal tumour biopsies often show dense infiltration of lymphoid infiltrates in the stroma, together with islands of malignant cells containing vesicular nuclei and scattered mitotic figures. This NPC histopathology was coined “lymphoepithelioma-
like carcinoma" by Regaud and Reverchon in 1921. As EBV is a human-specific virus with little or no capacity to infect other species (hence the absence of a robust small animal model), the investigation of EBV pathogenesis is very challenging. Also, the EBV genome is lost in culture during the propagation of epithelial carcinoma cell lines isolated from patient’s biopsies. Nevertheless, a Japanese group has developed a method to re-infect NPC cells with recombinant EBV isolates using the cell-to-cell infection technique (Imai et al., 1998). This serves as a common in vitro model to study EBV.

1.2.4 EBV proteins

**EBNA-1**

EBNA-1 is essential for viral genome replication and the mitotic segregation of EBV episomes during cell division by tethering itself to host chromosomes (Lupton and Levine, 1985). It therefore plays an important role in the maintenance of the EBV genome in infected cells, especially when the EBV episomes persist in a low copy number. EBNA-1 binds to multiple sites within the origin of replication (oriP) of the EBV genome, which contains two cis-acting elements: dyad symmetry (DS) and family of repeats (FR) (Reisman et al., 1985). The core domain of EBNA-1 is homologous to the structure of the E2 transactivator protein in bovine papillomavirus and hence shares a similar DNA binding mechanism and function.
EBNA-1 is also a transcriptional regulator that modulates both viral and host gene expression. Differential gene regulation is necessary for cell transformation, provides a selective advantage for neoplastic cells and exerts anti-apoptotic effects. It has been observed that EBNA-1-expressing carcinoma cell lines display an increase in signal transducers and activators of transcription 1 (STAT1) expression, particularly the transcriptionally active STAT1α p91 form (Wood et al., 2007). However, the role of STAT1 in epithelial tumourigenesis is not clear. In contrast, EBNA-1’s antagonisation of TGFβ-induced βig-h3 gene transcription was thought to promote the invasiveness of carcinoma. EBNA-1 regulates its own expression by binding to two lower affinity binding sites at position +10 of the Qp promoter. This allows the maintenance of latency I and II by restricting the expression of other latent proteins, such as EBNA-2-6, while inducing the expression of LMP-1 and LMP-2A (Yoshioka et al., 2008). To confer resistance to apoptosis, EBNA-1 binds to USP7, an ubiquitin-specific protease that deubiquitinates p53 (FIGURE 1.3). The affinity between EBNA-1 and USP7 is greater than that of the USP7-p53 complex, resulting in the destruction of p53 (Saridakis et al., 2005). It also induces the expression of survivin, an inhibitor of apoptosis proteins (Lu et al., 2011).
LMP-1

LMP-1 acts as a viral equivalent of a constitutively active ligand-independent B-lymphocyte CD40 receptor, which is a member of the tumour necrosis factor (TNF) receptor family. It is a 356 amino acid (aa) long protein that contains a short amino-terminal cytoplasmic region, six membrane-spanning domains and a 200 aa long C-terminal cytoplasmic region (FIGURE 1.4). LMP-1 is oncogenic as its overexpression alone is sufficient to transform both rodent NIH3T3 and Rat-1 fibroblasts. It is also essential for B-lymphocyte immortalisation upon EBV infection (Wang et al., 1985). Two functional domains that are present at the C-terminal tail of LMP-1, carboxyl terminal activation regions CTAR1 and CTAR2, recruit TRAFs (TNF receptor associated factors) to confer downstream oncogenic signalling pathways, such as phosphoinositide 3-kinase (PI3K) and nuclear factor-kappa B (NF-κB). Both CTAR1 and CTAR2 can activate NF-κB signalling, although the CTAR2 region is known to be the predominant contributor (~70%), which results in tumour survival and proliferation (Huen et al., 1995). It is also thought that recruitment of the adapter protein TRADD (TNF receptor-associated death domain) to CTAR2 allows LMP-1 to mask its pro-apoptotic effects (Schneider et al., 2008). The combination of such abnormal signalling induced by LMP-1 leads to phenotypic changes in cell growth, survival and migration.
FIGURE 1.4 - LMP-1 has multiple membrane-spanning domains and self-aggregates in the lipid raft to mimic activated CD40 monomers. The cytoplasmic tail of LMP-1 contains CTAR1 and CTAR2 domains that recruit proteins involved in cell survival, proliferation and migration signalling pathways. Activation of STAT3 results in the up-regulation of anti-apoptotic effectors, such as Bcl-3. Also, c-Jun N-terminal kinase (JNK), usually activated under environmental stress, induces downstream AP-1 transcriptional activity and regulates cell apoptosis and proliferation. It is known that aberrant NF-κB signalling is required for B-lymphocyte immortalisation.

LMP-2A

LMP-2A is a 12 transmembrane protein that mimics the activated B cell receptor (BCR). Two of the eight tyrosine residues (Y74 and Y85) present at the amino-terminus of LMP-2A form an immunoreceptor tyrosine-based activation motif (ITAM), which interacts with the SH2 domains of Syk tyrosine kinase (Fruehling and Longnecker, 1997). Competitive binding to Syk (which is also a substrate for the cellular BCR) and its subsequent phosphorylation blocks BCR signalling and
prevents EBV lytic-activation (Miller et al., 1995). Although LMP-2A is not required for the immortalisation of B-lymphocytes, there is now increasing evidence that LMP-2A plays a role in EBV tumourigenesis by enabling cell survival through activation of the PI3K/Akt pathway (Longnecker, 2000, Portis and Longnecker, 2004). Studies have also shown that the promoter of LMP-2A can be regulated by Notch (Anderson and Longnecker, 2008, Anderson and Longnecker, 2009). The promoter of LMP-2A consists of two consensus RBPJκ (recognition signal binding protein Jκ) sites, which can interact with several transcriptional activators, including Notch intracellular domain (NICD) and EBNA-2. Using luciferase transcriptional assays, LMP-2A has been reported to control its expression through auto-regulation of its promoter in the absence of EBNA-2 in epithelial cells.

1.3 *Mycobacterium tuberculosis* (*Mtb*)

### 1.3.1 Tuberculosis – a global burden

*Mycobacterium tuberculosis* (*Mtb*) is a very successful pathogen that infects a third of the world’s population. It was discovered by Robert Koch on 24th March 1882, almost 130 years ago, as the causative agent of tuberculosis (TB). TB is currently the second leading infectious cause of death worldwide. 90% of those who are infected with the bacteria are able to contain the infection by mounting an effective immune response and are therefore asymptomatic carriers. The other 10%, however, progress rapidly to develop the clinical disease, which is referred to as primary TB. It has been reported that 5-10% of asymptomatic carriers will develop active TB in their lifetime (Andrews et al., 2012). Reactivation risk increases in the immunocompromised, especially in HIV-positive individuals, due to viral perturbations.
of the protective host immunity. Individuals that are infected with \textit{Mtb} can be tested by the Mantoux tuberculin skin hypersensitivity test (TST). However, TST cannot differentiate between latent infection and active TB, nor can it be used to predict the risk of progression or reactivation.

According to the WHO global health report, TB accounted for 8.6 million new cases and 1.3 million deaths in 2012, with HIV-associated TB mortality being approximately 13\% (WHO, 2012). In addition, the misuse and non-adherence of antibiotics has led to the escalation of acquired resistant strains of \textit{Mtb}, resulting in outbreaks of multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). The first line of defence antibiotics, isoniazid and rifampicin, are ineffective towards both these strains of TB. XDR strains have also developed resistance to second line antibiotics, fluoroquinolones. In 1993, the WHO declared TB as a global health emergency. There are urgent programmes, such as the Stop TB strategy and Stop TB Partnership's Global Plan, that aim to reduce TB-associated morbidity and mortality by 50\% compared to figures in 1990.

TB is transmitted by the respiratory route via the inhalation of air droplets containing the \textit{Mtb} bacillus. In 2012, over 53\% of TB incidence cases reported in the UK were pulmonary, where \textit{Mtb} causes extensive damage to the lungs by actively forming necrotic regions known as caseous granuloma (Pedrazzoli \textit{et al.}, 2013). Through dissemination of the bacteria to other organs via the lymph nodes, a process known as “lymphohematogenous seeding”, TB can also manifest itself in the spleen, liver, central nervous system (CNS), bone, and pericardium. Systemic symptoms of TB include coughing, haemoptysis, fever, malaise, night sweats and weight loss.
1.3.2 *Mtb* and its virulence factor

The causative agent of tuberculosis is a GC-rich, rod-shaped, Gram-positive mycobacteria. It belongs to the genus *Mycobacterium*, which is comprised of over 130 species, including the *Mycobacterium bovis* Bacille Calmette-Guérin (BCG), *

*M. avium*, *M. marinum* and *M. leprae* (the causative agent of leprosy) (Rogall *et al.*, 1990). The non-pathogenic BCG is currently used as a live, attenuated vaccine and is effective against TB in newborns and children, but is inconsistent in efficacy (0 - 80%) against adult pulmonary TB (Colditz *et al.*, 1994, Mangtani *et al.*, 2014). Nevertheless, following an observation made in 1928, in which patients with lesions of active tuberculosis were less likely to develop bladder cancer, BCG is also being used to treat bladder cancer (Pearl, 1928). An effective anti-tumour response is mounted by BCG: (i) attachment and internalisation of BCG by the urothelial cells; (ii) secretion of cytokines; (iii) inhibition of cancer cell proliferation and (iv) induction of cell cycle arrest (Redelman-Sidi *et al.*, 2014).

*Mtb* persists in phagosomes within host antigen-presenting cells (APCs) in a latent or dormant state. It has an unusual cell wall architecture, formed by an outer envelope and a cell membrane (*FIGURE 1.5*). The heteropolysaccharide mycolyl-arabinogalactan-peptidoglycan (mAGP) complex of the cell wall skeleton (CWS) forms the backbone of the envelope and is mainly responsible for the maintenance of cell shape and size. It also serves as a platform, allowing the assembly of signalling complexes, and connects extracellular cues to the activation of downstream intracellular effectors. The mAGP complex is an effective adjuvant and is often used in combination with anti-mycobacterial treatments and vaccines as, together, they are highly immunogenic. The cell membrane is asymmetrical and contains various proteins, lipids and lipidglycans. These include lipomannan (LM), lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM) (*FIGURE 1.6*). Lipoproteins may
become soluble and can be shed during cell wall disruptions and activate immunity, such as toll-like receptor (TLR) signalling in APCs. The cell envelope is a major determinant of virulence for \textit{Mtb} as its components are able to mediate host immunosuppression and confer the mycobacterium resistance to therapeutics.

\textbf{FIGURE 1.5 – The components of, and their physical organisation within, the mycobacterial cell envelope.} The asymmetric cell wall architecture consists of two segments: an inner plasma membrane and an upper hydrophobic leaflet with myolic acids covalently linked to arabinogalactan (AG). AG is bound to the muramyl residues of the peptidoglycan. The AGP complex forms the insoluble cell wall core of \textit{Mtb}. Many lipid species are synthesised and anchored to the cell envelope, each with a different function in their interactions with the host and the microenvironment.
FIGURE 1.6 - Biogenesis and structure of lipid moieties present within the mycobacterial cell wall. (A) Lipoproteins are produced as pre-prolipoproteins and undergo additional post-translational processing by three lipid enzymes to yield the mature lipoprotein. Lgt catalyses the addition of a diacylglycerol residue to the cysteine moiety. The pre-prolipoprotein also contains an amino-terminal signal sequence, which is subsequently cleaved by LspA. Finally, Lnt mediates the N-acylation of the cysteine residue of the diacylglycerol prolipoprotein to form a triacylated lipoprotein that sits in the hydrophobic plasma membrane. (B) Glycoconjugates such as phosphatidylinositol mannosides (PIMs) are non-covalently linked to the cell wall through their GPI anchor. PIM forms the precursor for LM and LAM. Mannose residues can be added to form ManLAM and further acylation can also occur. These glycolipids are highly virulent and immunogenic.
Mtb has evolved several mechanisms for its continued survival in host cells and evasion of immune surveillance, such as: (i) perturbation of phagocytosis by altering trafficking pathways and inducing phagosome maturation arrest; (ii) inhibition of the production of anti-mycobacterial products, such as nitric oxide (NO), reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI); (iii) inhibition of apoptotic cell death; (iv) induction of the anti-inflammatory response by stimulating the release of the immunosuppressive cytokine IL-10; and (v) inhibition of antigen presentation.

The mycobacterial genome encodes large numbers of lipid enzymes that are anchored within the cell envelope, and each plays a different role in bacterial persistence. In particular, the glycolipid LAM can inhibit antigen presentation of APCs to CD4+ T-lymphocytes. It has been shown that the mannose-capped version of LAM ligates to the dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) receptor expressed on the dendritic cells (DCs) to inhibit Mtb internalisation, prevent DC maturation and modulate cytokines production (Geijtenbeek et al., 2003). In addition, prolonged TLR stimulation by other mycobacterial lipoproteins such as LpqH, LpRG, LpRA and Psts1 can lead to the inhibition of MHC class II antigen presentation. This can result in diminished antigen presentation and an inability of the inflammatory cells to prime naïve T-lymphocytes (Pecora et al., 2006).

LAM attaches and incorporates itself onto the macrophage cell membrane through the acyl chains of its glycosylphosphatidylinositol (GPI) anchor and alters its lipid raft composition, which consists of signalling complexes enriched with various components such as small GTPases, Src domain-containing tyrosine kinases, adapter proteins and cytosolic signal transducers. Lipid rafts form the cell surface platform for downstream host immune signalling transduction pathways; hence LAM is able to modulate the inflammatory response. Paradoxically, it induces the secretion
of pro-inflammatory cytokines such as TNFα, IL-1α and IL-6, though it also inhibits T-lymphocyte proliferative responses (Ilfangumaran et al., 1995). Excess inflammation has deleterious effects on the host but is advantageous for Mtb. It is believed that a pro-inflammatory response favours granulomatous reactions at the inflamed site.

Various bacterial constituents such as ManLAM, SecA2 and nucleoside diphosphate kinase (Ndk) are able to prevent acidification and maturation from early endosome (Rab5-positive, ~ pH 6.4) to late endosome (Rab7-positive, ~ pH 4.8), therefore inhibiting phagosomal fusion with lysosomes (Fratti et al., 2003, Sullivan et al., 2012, Sun et al., 2010). ManLAM activates the stress-induced p38-MAPK, which leads to the phosphorylation of GDP dissociation inhibitor (GDI). Phosphorylated GDI complexes with the Rab5 GTPase and locks it in its inactive GDP form. This reduces the amount of Rab5 in the early endosomes and consequently inhibits its recruitment of early endosome antigen 1 (EEA-1), which is essential for phagolysosomal fusion (Cavalli et al., 2001). The lysosome contains lysosomal hydrolases that degrade and kill viable bacilli that are present in the vacuoles. Likewise, ManLAM can also avoid apoptosis by inducing the phosphorylation of the pro-apoptotic protein Bad at S136 via the PI3K pathway (Maiti et al., 2001). Normally, Bad binds to the pro-survival proteins Bcl-2 and Bcl-Xl, forms a heterodimer with them, prevents their activation and promotes cytochrome c release, triggering apoptotic cell death. Phosphorylated Bad is sequestered in the cytoplasm and is therefore unable to prevent apoptosis.
1.3.3 Innate recognition - pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs)

Cells continuously survey the cellular microenvironment for the presence of pathogens, a process known as innate immune sensing, to enable them to mount a robust first-line defence against any infection. In order for the host to identify self from non-self, it evolves a system of PRRs, which act as a molecular mechanism for pathogen sensing. These receptors are usually present on the surface of patrolling monocytes and tissue-resident APCs, such as DCs and macrophages, and recognise and bind to motifs expressed on the pathogens, which are known as PAMPs. PAMPs are highly conserved and expressed exclusively by the infectious agents. Thus, they are recognised as “foreign” by the host immune system. In addition, PAMPs are indispensable for physiological functions. Genetic mutations of PAMPs can affect microbial virulence and survival so they are rarely mutated. These pathogen-specific features include viral nucleic acids (DNA, RNA) and bacterial products (flagellin, lipopolysaccharide (LPS), glycolipids, lipoproteins, peptidoglycans and zymosan). Activation of PRRs by PAMPs generates protective immune responses and mediates cross-talk between innate and adaptive immunity.

PRRs facilitate the uptake of pathogens into host phagosomes. These include TLRs, DC-SIGN, complement receptors, mannose receptors, scavenger receptors and cytosolic nucleotide-binding oligomerisation domain (NOD)-like receptors. TLRs are type I transmembrane receptors that stay anchored on the plasma membrane or the endosomal membrane. There are at least 10 known TLRs. Normally, adapter proteins are recruited to form a multi-protein signalling complex, which leads to the activation of downstream expression of genes encoding key effectors that modulate immunity (FIGURE 1.7). For example, LPS, an endotoxin secreted only by the
Gram-negative bacteria such as *Escherichia coli (E. coli)*, is a prototypic activator of TLR4 and its co-receptors CD14 and MD-2.

Several mycobacterial components are potent agonists for the TLR receptors, especially TLR2 (Sutcliffe and Harrington, 2004). These include myolic acids, lipoproteins and glycolipids expressed within the cell wall architecture (FIGURE 1.5). They can induce distinct host cellular responses, and it is reported that ManLAM is known to be more pathogenic compared to the non-capped version (AraLAM). TLRs usually exist as homo- or heterodimers. In particular, TLR2 associates with TLR1 or TLR6, along with the co-receptor CD36 (Hoebe *et al.*, 2005). TLR2 receptor dimerisation can influence ligand specificity. For example, TLR2-TLR6 heterodimers bind to diacylated lipoproteins while TLR2-TLR1 heterodimers bind to triacylated lipoproteins. On the other hand, the intracellular TLR9 binds to unmethylated CpG (cytosine phosphate guanosine) motifs in bacterial DNA. Following its recognition in the endosomal compartment, bacterial proteins are processed by intravacuolar proteolysis into peptides and presented in the context of MHC class I to CD8+ T-lymphocytes. *Mtb* can activate both TLR2 and TLR4 receptors and it has been demonstrated that transfection of Chinese hamster ovary (CHO) cells with TLR2 and TLR4 receptors confers sensitivity to *Mtb* (Means *et al.*, 1999).

Following recognition of *Mtb* by PRR receptors, several adapter proteins, such as myeloid differentiation primary response protein 88 (myD88) and toll-interleukin-1 receptor domain-containing adaptor protein (TIRAP), are recruited, leading to the activation of the NF-κB signalling pathway. It is clearly established that myD88 is necessary for TLR2 and IL-1 receptor signalling. In addition, the TLR-myD88 complex can function as a death receptor, as myD88 possesses a death domain at its N-terminus and is therefore able to interact with the adapter protein Fas-associated via death domain (FADD) (Aliprantis *et al.*, 1999). FADD further binds...
to pro-caspase 8 through its death effector domains (DEDs), forming a death-inducing signalling complex (DISC). DISC initiates a cascade of activation of downstream caspases that mediates apoptotic effector functions. This activation of apoptosis promotes the host’s defence and is crucial for the control of Mtb infection.

In agreement, studies have shown that upon LPS stimulation, macrophages as well as endothelial cells undergo myD88- or FADD-dependent apoptotic signal relay (Ruckdeschel et al., 2002, Choi et al., 1998).

NF-κB signalling is pivotal for the regulation of innate and adaptive immunity and is responsible for the stimulation of pro-inflammatory cytokines. The NF-κB family is comprised of five members: p65 (RelA), RelB, cytoplasmic Rel, p50 and p52. The NF-κB complex is formed by the homo- or heterodimerisation of two of the family members. Its function is tightly regulated by the inhibitory protein IκB and its inactive form is located in the cytoplasm. In the canonical pathway, NF-κB is liberated from its inhibitory complex upon the phosphorylation and subsequent proteolysis of IκB, which enables its translocation into the nucleus and the activation of its target gene sets (FIGURE 1.7). Genes that are transcriptionally activated by NF-κB include the pro-inflammatory cytokines and chemokines TNFα, IL-2, IL-6, IL-8, IL-12β and the neutrophil-responsive granulocyte-colony stimulating factor (G-CSF). The expression of these cell adhesion molecules and chemoattractants allows the recruitment and activation of circulating monocytes, macrophages, lymphocytes and neutrophils to the site of infection. Inducible nitric oxide synthase (iNOS) transcription is also induced through TLR signalling in murine macrophages and mediates the production of NO, which has direct bactericidal activity (Brightbill et al., 1999). Another investigation has shown that the inhibition of hTLR2 receptors in peripheral blood lymphocytes results in the attenuation of anti-microbial ROS production (Aliprantis et al., 1999).
FIGURE 1.7 - TLR receptors are type I membrane proteins that are either expressed on the surface of APC or intracellularly. They usually exist in homo- or heterodimers, act as “pathogen sensors”, and are able to discriminate against different classes of infectious agents. Interaction of specific components of pathogens, such as the cell wall, membrane, flagella or nucleic acids, with TLR triggers a conformational change in the cytoplasmic tail of the receptor. The “activated” receptor in turn recruits adaptor proteins IRAKs and TRAFs through specific protein interaction domains and assembles a signalling complex that leads to the activation of downstream NF-κB, MAK kinases and interferon regulatory factor (IRF) signalling pathways. Activated DNA-binding TFs, including CREB, AP-1, NF-κB and IRFs, translocate into the nucleus, binding to promoters of specific gene signatures that participate in the regulation of immunity.
1.3.4 Recruitment of inflammatory cells and granuloma formation

As mentioned above, *Mtb* is transmitted by air droplets and it is, therefore, not surprising that it inhabits the lungs and causes pulmonary TB. During the acute phase of infection, the bronchoalveolar space of the lung becomes a "battlefield" for the bacterium *Mtb* and the host immune system. To combat the pathogen and contain the infection, the resident APCs, including alveolar macrophages and DCs, engulf the pathogen via receptor-mediated phagocytosis and launch innate immune signalling. Robust inflammatory responses are induced locally by the production of an array of pro-inflammatory cytokines and chemokines, such as TNFα, interferon-γ (IFN-γ), IL-12, CCL2 and CXCL10, as well as the extravasation of myriad immunologic components to activate mycobactericidal and mycobacteriostatic functions, such as NO and its metabolites (Russell, 2007). These inflammatory cytokines, especially TNFα, regulate chemotaxis and the accumulation of inflammatory mediators (neutrophils, T-lymphocytes, B-lymphocytes and macrophages) at the infected site. It has been reported that TNFα-deficient mice have increased susceptibility to *Mtb* infection and are unable to limit bacteria growth, and these mice show dramatically reduced survival rates compared to wild type (WT) controls (Flynn *et al.*, 1995). The inflammatory response is further potentiated by the elaboration of cytokines and the cross-priming of the Th1 response by DCs (adaptive immunity). Preceding studies have shown elevated expression of iNOS at sites of infection in the lungs post 20 days of infection (Jung *et al.*, 2002). iNOS is required for the catalysis of L-arginine to NO, which has microbicidal activity. However, the immune response mounted against *Mtb* is only partially effective, as *Mtb* has evolved several evasive mechanisms. For instance, the engulfed bacteria are able to prevent being disposed of by preventing phagolysosomal maturation and therefore reside in vacuoles in macrophages.
Mtb can be visualised using the Ziehl-Neelsen staining method by microscopy, due to the ability of its thick, waxy, hydrophobic cell wall to retain dyes. As a result of long term persistence of the bacteria and infiltration of successive waves of effector immune cells, the inflammation becomes chronic and progressive. This phenomenon is also known as delayed-type hypersensitivity, caused by an overstimulation of inflammatory mediators. At this stage, a “tubercle” or “granuloma” is formed de novo. Granuloma is a signature of tuberculosis and is formed by cellular infiltration of the immune cells including macrophages, DCs, T-lymphocytes, B-lymphocytes, and neutrophils, as well as fibroblasts (FIGURE 1.8). These infected or activated macrophages adopt an epithelial-like morphology and are hence known as epithelioid cells. Some can fuse with each other to form multi-nucleated giant cells (MGCs). These granulomas can be further classified by their histopathological profiles (Barry et al., 2009). The most common type found in TB is caseous granuloma, which is comprised of a caseous (“cheese-like”), necrotic core. This central region is hypoxic and lipid-rich (which comes from the cell wall of Mtb), and contains large numbers of the bacteria as well as dead host cells. The caseous centre is surrounded by epithelioid macrophages, foam cells, giant cells, followed by a cuff of T- and B-lymphocytes, and is further bound by fibroblasts and a layer of collagen deposits. Non-necrotising granuloma is another type of granuloma, often seen in the mouse model of tuberculosis, where it lacks the central necrotic core and caseation. It is comprised mainly of macrophages surrounded by lymphocytes. Mtb is usually found intracytoplasmically in the macrophages. Fibrotic or calcified granulomas are sometimes seen in latent TB and are mostly populated by fibroblasts with very few inflammatory cells, and can be encapsulated by mineral aggregates.

Formation of granuloma is a double-edged sword in that it benefits both the host and the bacteria. From the host-centric perspective, the initiation of granuloma formation by macrophages is a means of protective immune response to contain Mtb infection
at the infection site by restricting bacterial spreading. Higher morbidity is seen in individuals who fail to develop granuloma as they are unable to prevent rapid widespread dissemination of the bacteria (Flynn et al., 1995). However, paradoxically, recent evidence has suggested that the establishment and persistence of granuloma accelerates the progression of TB by creating a favourable niche for *Mtb* amplification and transmission (Davis and Ramakrishnan, 2009). The advantage of living within the host’s phagosomes is that the pathogen is able to evade immune surveillance. How the bacteria are able to retain, survive and proliferate in the macrophages is still unclear. Likewise, they can enter the dormant or latent state for an extended period of time until reactivation at a later time point. Granuloma can also act as a defence against anti-TB drug penetration. The breakdown of granulomas by inflammatory mediators, assisted by the induction of matrix metalloproteinase 9 (MMP-9), allows bacterial dissemination and transmission into the airways (Volkman et al., 2010). Therefore, the striking a balance between the activation and suppression of the host’s immunity is important in TB pathogenesis, especially in latent infection.

Infected macrophages can disseminate to a different anatomical site to establish secondary granulomas and cause extrapulmonary TB. Phagocytes (DCs and macrophages) are used as vehicles to colonise other parts of the body and emigrate to lung-draining lymph nodes by haematogenous spreading. The homing of DCs to the lymphatic system is regulated by the CCR7-CCL19 chemokine axis. The CCR7 receptor, which is expressed on the cell surface of DCs, is attracted towards the chemotactic gradients of CCL19 or CCL21 generated by the mesenteric lymph nodes (MLN). IL-12p40 cytokine is associated with macrophage accumulation within the inflamed lungs and can stimulate the migration of infected DCs to the lymph nodes. IL-12p40-deficient mice are defective in DC migration to the lymph nodes and therefore compromise DCs’ ability to initiate T-lymphocyte expansion (Khader et al.,
Hence, the rate-limiting steps of the initiation and development of adaptive immunity are the homing of the activated APCs to the lung-draining lymph nodes and cross-presentation of the mycobacterial antigen (Wolf et al., 2008). These APCs briefly interact with naïve T-lymphocytes in the lymph nodes and form immunological synapses, which results in T-lymphocyte activation, proliferation and cytokine secretion. Primed T effector cells are then trafficked to the lungs to encounter the pathogen and attempt to resolve the infection. Insufficient APC activation or delayed recruitment of T-lymphocytes can lead to a failure to arrest TB progression and bacterial persistence. Despite the onset of adaptive immunity, complete eradication of Mtb is difficult to achieve. In mouse experimental models of TB, bacterial burden slowly accumulates and stays at a stationary level post-20 days of infection, resulting in the eventual death of the animal (North et al., 1999).

Several experimental models have been used to study the mechanism of TB pathogenesis or test the efficacy of anti-bacterial treatment strategies. The use of murine models is most common and has been invaluable due to its cost-effectiveness. However, the mouse model for TB resembles acute primary TB infection and fails to develop well-structured typical caseous necrotic granulomas. There is high bacterial burden throughout the course of infection as Mtb clearance is generally poor in mice, which eventually results in the accumulation of viable bacilli, excess inflammation and the death of the animal. Two latent infection models were further developed in mice: Cornell/drug-induced and the low dose/chronic model. In the Cornell mouse model, sterilised mice were challenged with 1-3 x 10⁶ Mtb followed by 12 weeks of antibiotic treatment with isoniazid and pyrazinamide. There is low bacterial burden (paucibacillary) during the early stage until reactivation following the withdrawal of antibiotics for 90 days, or the introduction of immunosuppressive drugs (McCune et al., 1966). In comparison, the low dose model involves challenging mice with low doses of Mtb aerogenically; spontaneous
reactivation occurs at least 18 months later. This murine model may be preferred as it is analogous to natural latent infection in humans. On the other hand, guinea pigs and rabbits are more susceptible to TB infection and, therefore, produce robust immune responses against *Mtb* and can form caseous necrotic granulomas. The use of non-human primates such as monkeys and rhesus macaques may represent a repertoire identical to that in human TB, as a full spectrum of granulomas can be isolated from both primary and reactivated TB. However, the limitations of using non-human primates include outbred genetic variation and the cost of conducting these experiments.

**FIGURE 1.8 - A typical *Mtb* granuloma structure.** It is usually composed of several immune cell types, primarily monocytes/macrophages, DCs, B- and T-lymphocytes, neutrophils and natural killer (NK) cells. The macrophages can be differentiated by subtypes such as “classically-activated” macrophages (CAM) or “alternatively-activated” macrophages (AAM). Depending on the strain of *Mtb*, the bacteria-containing macrophages can undergo either apoptosis or necrosis. Monocytes and macrophages can also fuse to form a multi-nucleated giant cell (MGC) or differentiate into epithelioid or foam cells. These immune cells play a vital role in innate immune responses against *Mtb*.
1.3.5 Immune effectors

Macrophages

Tissue macrophages and DCs differentiate from circulating peripheral blood monocytes following cytokine stimulation. Depending on their functionality and phenotypic markers, they can be categorised into two major macrophage subsets: M1, classically activated macrophages (CAM); or M2, alternatively activated macrophages (AAM). IFNγ, LPS and G-CSF are strong M1-polarising factors, and the M1 subset is characterised by its secretion of pro-inflammatory cytokines (IL-1, IL-12, IFN-γ, TNFα) and high expression of iNOS. On the other hand, the M2 subset is characterised by the secretion of anti-inflammatory cytokines IL-4 and IL-13, and high arginase I and mannose receptor expression. During the acute infection phase, macrophages are polarised into the M1 subset and play a protective role in the control of TB pathogenesis. iNOS-positive macrophages are abundant in Mtb-infected tissues containing granulomas, and are essential for the generation of respiratory bursts (Facchetti et al., 1999). M1 macrophages promote autophagy, phagosome maturation and acidification, and the release of anti-microbial peptides to eradicate intracellular mycobacteria. Activation of either the M1 or M2 macrophage subset can affect lymphocytic outcome: for example M1-induced IL-12 encourages differentiation into CD4+ Th1 cells. M1 macrophages trigger respiratory bursts and the release of RNI and ROI, which have potent bactericidal activity. However, these metabolites can damage neighbouring bystander (uninfected) cells. Hence, in later stages of Mtb infection, M2-polarised macrophages are generated as a negative feedback mechanism to antagonise and resolve the exuberant inflammatory response generated by Th1-mediated immunity. Excess inflammation has detrimental effects, such as tissue damage, and could accelerate TB pathogenesis.
Thus, homeostatic reprogramming can limit the magnitude and duration of macrophage inflammation. It is also speculated that M2 macrophages may be induced by \textit{Mtb} to promote tolerance and circumvent the host’s defence mechanisms (Benoit \textit{et al.}, 2008). M2 macrophages are associated with the secretion of immunosuppressive IL-4 and IL-10, decreased phagocytosis and macrophage autophagy.

Destruction of the pathogen can be achieved by either direct cytotoxicity to the infected cells or clearing of the infected or bystander cells by programmed cell death (apoptosis). Apoptotic vesicles which contain engulfed \textit{Mtb} bacilli undergo a process known as “efferocytosis”, as a host defence mechanism that limits the mycobacterial spread and survival without inducing inflammation. Apoptosis is not only limited to macrophages, as it has been demonstrated that \textit{Mtb} infection can also induce T-lymphocyte apoptosis. A previous study showed that CD4+ T-lymphocytes fail to undergo apoptosis in the absence of IFN\textsubscript{γ}, leading to a huge bacterial burden and the sequestration of activated T-lymphocytes in the spleen and central nervous system of experimental mice (Dalton \textit{et al.}, 2000). Nevertheless, \textit{Mtb} is able to exhibit its virulence by modulating the balance between apoptosis and necrosis, consequently leading to mycobacterial spread. Enhanced necrosis was reported in hypervirulent \textit{Mtb} strains such as H37Rv, as opposed to stimulation of apoptosis in attenuated or avirulent mutants such as BCG and H37Ra (Keane \textit{et al.}, 2000). Virulent H37Rv increases the expression of anti-inflammatory mediator lipoxin A4 (LxA\textsubscript{4}), which inhibits the production of cyclooxygenase-2 (COX2)-derived prostaglandin E2 (PGE\textsubscript{2}). PGE\textsubscript{2} protects against pathogen-induced plasma membrane microdisruptions and mitochondrial damage. Cells infected with the avirulent strains exhibit hallmarks of apoptosis, for example DNA fragmentation, nuclear condensation, plasma membrane blebbing and caspase cascade activation. On the other hand, cells infected with virulent \textit{Mtb} suffer from membrane lesions and...
these membranes remain unrepaired (Divangahi et al., 2009). Hence, LxA4 is anti-apoptotic and pro-necrotic. Rupturing of the plasma membrane during necrosis results in the release of viable mycobacteria into the intracellular space, which favours bacterial dissemination, subsequent reinfection and promotes excess inflammation and tissue damage. In addition, Mtb is capable of up-regulating host anti-apoptotic proteins such as Mcl-1 and Bcl-XL, as well as mycobacterial anti-apoptotic factors nuoG and secA2 (Sly et al., 2003, Velmurugan et al., 2007). nuoG encodes a subunit of the type I NADH dehydrogenase complex, and it has been reported that its deletion results in a marked increase in apoptosis and a reduction in Mtb virulence.

**T-lymphocytes**

T helper cells are essential components of the adaptive immune system and are necessary to contain Mtb infection. Failure to activate or delayed onset of T-lymphocyte-mediated immunity has deleterious consequences and can lead to accelerated TB pathogenesis. This is apparent in immunocompetent HIV-TB co-infected individuals. HIV patients suffer from progressive reduction of CD4+ T-lymphocytes, which are required to curtail Mtb replication, thus increasing their risk of TB reactivation (Kwan and Ernst, 2011). In HIV-TB co-infected patients, granulomas may fail to form or it may contain fewer numbers of lymphocytes, and infected organs are usually populated with disorganised aggregates of macrophages.

Histopathological analyses have revealed the presence of antigen-specific T-lymphocytes around clusters of macrophages within TB granulomas. In the context of TB progression, the protective immunity favours dominant Th1 polarisation, and CD4+ T-lymphocytes represent the major T-lymphocyte subset (Nunes-Alves et al.,
Antigen-mediated stimulation of naïve T-lymphocytes occurs in lymphoid organs, followed by clonal expansion, differentiation and trafficking of primed T-effector cells to the lung. As mentioned earlier, IFNγ, along with TNFα and IL-12, contributes to mycobacterial resistance and clearance. Secretion of these cytokines by CD4+ T-lymphocytes allows the recruitment of a second wave of innate inflammatory cells, including monocytes and granulocytes, amplifying immune signal strength.

CD8+ T-lymphocytes, sometimes known as cytotoxic T-lymphocytes (CTLs), have a distinctive role in protective immunity against Mtb during primary and latent infection. CTLs can mediate the directed killing of infected APCs via the perforin and granulysin exocytosis cytotoxicity pathway, which results in cell lysis and apoptosis. Activated CTLs also contain Fas ligands (FasL) which are able to deliver a “lethal hit” by binding to the Fas receptors present on target cells and inducing apoptosis, consequently reducing bacterial viability (Oddo et al., 1998). However, the relative contribution from CD8+ T-lymphocytes in orchestrating anti-mycobacterial immunity has been controversial. It is believed that pro-inflammatory IFNγ production (mostly by CD4+ T-lymphocytes) is the major protective mechanism compared with the cytotoxic killing exerted by CTLs. Previous reports show that CD4+ T-lymphocyte-deficient mice are more susceptible to mycobacteria and succumb to death more rapidly (~50 days) compared to CD8+ T-lymphocyte-deficient mice (>150 days) (Mogues et al., 2001). This correlates with the exponential growth of tubercle bacilli in the lungs of mice devoid of CD4+ T-lymphocytes, while mycobacterial burden is only 1 log higher in the absence of CD8+ T-lymphocyte immunity compared with WT controls. Hence CD8+ T-lymphocytes cannot fully compensate for a lack of CD4+ T-lymphocytes.

Mtb has evolved several ways to dampen an exuberant immune response, one of
which is the induction of the immunosuppressive cytokine IL-10 and its production contributes to chronic TB infection. IL-10, as well as TGFβ, is secreted by tolerant or regulatory T-lymphocytes, such as Foxp3+ (CD4+ CD25+). IL-10 is able to: (i) deactivate macrophage function, (ii) reduce production of RNI and ROI, (iii) reduce internalisation, processing and presentation of \textit{Mtb} antigens, (iv) inhibit migration of DCs to the neighbouring lymph nodes, and (v) inhibit Th1 polarisation and cytokine production. Abrogation of IL-10 signalling using an anti-IL-10R antibody allows the stabilisation or reduction of bacterial counts, induction of IFNγ and an increase in T-lymphocytes numbers in the lungs (Beamer et al., 2008).

1.4 The tumour suppressor p53 and its family members

1.4.1 The p53 family

It is well-documented that the tumour suppressor p53 is the most frequently mutated gene in human cancers. For example, cancer genome studies have shown that 96% of ovarian cancers contain \textit{TP53} genetic alterations (Olivier et al., 2002). Gain-of-function (GOF) mutation of p53 by single amino acid substitution is common, and may block WT p53 function by binding directly to DNA or sequestering other transcription co-factors (Dittmer et al., 1993). p53 is a DNA damage checkpoint protein and is activated upon DNA damage to halt the progression of cell cycle until the DNA is repaired. If the damage is irreparable, downstream p53 pathways can suppress proliferation and trigger apoptosis. Hence p53 can modulate a range of effector genes including pro-apoptotic genes such as Bax and PIG3, alongside cell cycle arrest or regulation genes such as \textit{mdm2} and \textit{p21waf}. p53-mediated apoptosis can be further potentiated or inhibited by members of the apoptotic-stimulating
protein of p53 (ASPP) family. A previous investigation has demonstrated that mutation of p53 in NPC is extremely rare, however, overexpression of its stable inactive form has been reported in NPC patients (Sheu et al., 1995).

Several lines of evidence have shown that there is extensive interplay between p53 and inflammation. During inflammatory conditions, p53 is able to transactivate genes that modulate the levels of ROS following genotoxic insult by nitrogen or oxygen species (de Moraes et al., 2007). For example, under low stress conditions, p53 elicits an anti-oxidant response to defend against oxidative damage, carries out cell repair work and induces senescence. If the damage is severe, however, p53 induces the expression of pro-oxidant effectors to stimulate the production of ROS and activate the downstream apoptotic machinery, in order to eliminate damaged or dysregulated cells. In general, the p53 and NF-κB pathways can regulate each other by reciprocal negative feedback to modulate a balance between cell survival, apoptosis and inflammation. NF-κB is a master regulator of the immune system as it directly regulates the gene transcription of pro-inflammatory cytokines. Hence, it is not surprising that tumour cells promote the transcription of NF-κB gene programmes while abrogating p53-mediated apoptosis. In addition, the expression of several components of the immune system is subject to p53 regulation. These include colony-stimulating factor 1 (CSF1), monocyte chemotactic protein (MCP1), interferon regulatory factor 9 (IRF9) and TLRs (Menendez et al., 2013).
FIGURE 1.9 - The genomic structure of p53 and p63. The blue boxes represent transactivating domains (TAD), the red boxes represent the DNA-binding domains (DBD), the orange boxes represent the oligomerisation domain (OD) and the purple boxes represent the sterile alpha motif (SAM). Several isoforms are generated due to alternative splicing (indicated by alternative lines at the C-terminal end) and different transcription initiation sites represented by P1 or P2.

1.4.2 p63

p63 and p73 belong to the p53 family of proteins. They share high homology with p53 as well as exhibiting similar functions to interacting proteins, including the ASPPs (FIGURE 1.10). p63 plays a fundamental role in regulating epithelial stratification. Although p63-knockout mice are viable, they quickly die of dehydration after birth due to a lack of skin and limbs (Mills et al., 1999, Yang et al., 1999a). Like p53, p63 stimulates a diverse network of signalling pathways and can bind to at least 5,800 gene target sites (Yang et al., 2006).
Alternative p63 transcriptional initiation sites exist that generate mRNAs for either the full-length transactivating isoform (TAp63) or the truncated dominant-negative ΔNp63 isoform (FIGURE 1.9). Alternative splicing at the carboxyl-terminal leads to the expression of six gene variants: TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β and ΔNp63γ (Murray-Zmijewski et al., 2006). Differential localisation of the two isoforms has been observed in physiological tissues (Nylander et al., 2002). ΔNp63 is the most abundant isoform in the skin and is confined to the highly proliferative basal cell population located in the stratum basale. On the other hand, TAp63 is expressed predominantly in the female germline, though it has been shown to be detected at low levels in the epidermis (Suh et al., 2006). Up-regulation of ΔNp63 expression is observed in epithelial cancers, in particular SCC and oesophageal carcinoma (Tordella et al., 2013, Hu et al., 2002, Crook et al., 2000). In contrast, decreased ΔNp63 expression is associated with bladder and prostate cancer progression and poor clinical outcome (Urist et al., 2002, Tucci et al., 2012). Several studies have reported strong p63 nuclear staining in NPC biopsies. However, its role in NPC progression has yet to be elucidated (Guo et al., 2006, Crook et al., 2000). Mutations of the p63 gene are rare in nasopharyngeal malignancies and Guo et al. have reported that increased expression of p63 is due to an increase in protein stability. p63 plays a role in maintaining the “stemness” and proliferative ability in epithelial stem cells, as well as inducing cell death by transactivating p53-responsive genes (Bergholz and Xiao, 2012). It is well-established that the ability of p63 to transactivate p53 target genes is mediated by the TAp63 isoform, while ΔNp63 is required for cell proliferation and stem cell maintenance. Nevertheless, it has been shown that ΔNp63 can compete for DNA binding sites of the p53 promoters and block TAp53 or TAp63 function in a dominant-negative manner (Yang et al., 1998). Therefore, it is widely believed that the ΔNp63 isoform plays a major role in carcinogenesis.
1.5 The ASPP family of proteins

1.5.1 The ASPP family

ASPP stands for both apoptosis stimulating protein of p53, and ankyrin-repeat, SH3-domain and proline-rich-region-containing protein. The ASPP family consists of ASPP1, ASPP2 and iASPP and they all bind to p53, p63 and p73 to stimulate or inhibit p53-mediated apoptosis (Samuels-Lev et al., 2001). ASPP1 and ASPP2 specifically potentiate p53-induced pro-apoptotic function, while oncogenic iASPP inhibits p53’s apoptotic ability. The ASPPs modulate the apoptosis function through their C-terminal domains, which contain the signature motifs: ankyrin repeats, SH3 and proline-rich domains (FIGURE 1.10). Low patient survival rates have been linked with an overexpression of iASPP (Cao et al., 2013).

FIGURE 1.10 - Structure of ASPP family proteins. ASPP1, ASPP2 and iASPP share structural homology at their C-terminal ends, which consists of ankyrin repeats, a SH3 domain and a proline-rich region. ASPP1 and ASPP2 also have an α-helical structure at their N-termini, which interacts with other proteins, and are pro-apoptotic. iASPP, on the other hand, is anti-apoptotic.
ASPP2 is a haploinsufficient tumour suppressor and binds to members of the p53 family to transactivate pro-apoptotic genes such as Bax, Puma and PIG3. A study performed in our group observed the increase of frequency of “spontaneous” tumours in ASPP2 heterozygous mice as compared to WT mice (enhanced in ASPP2+/− background) (Vives et al., 2006). This indicates that ASPP2 has a protective role in tumourigenesis. Down-regulation of ASPP1 and ASPP2 via epigenetic promoter hypermethylation has been observed in tumour biopsies including hepatitis B-positive hepatocellular carcinoma (HCC) (Zhao et al., 2010). The aberrant inactivation of such a tumour suppressor gene triggers malignant progression and metastasis. A previous study has identified that ASPP2 acts as a suppressor of SCC by interacting with the inflammatory mediator NF-κB p65 to down-regulate p63, which is elevated in SCC (Tordella et al., 2013). Moreover, ASPP2 can form an apical-junctional complex (AJC) with Par-3 and ZO-1 with its N-terminus to maintain the polarity of the neural cells during CNS development (Sottocornola et al., 2010). Through the formation of a ternary complex with β-catenin and E-cadherin, ASPP2 is able to reverse epithelial-to-mesenchymal transition (EMT) and inhibit tumour metastasis by preventing the transactivation of EMT transcription factor ZEB1 (Wang et al., 2014b).

In addition, ASPP2 is indispensable for maintenance of the homeostasis of the intestinal epithelium. Loss of cell polarity results in intestinal epithelial disorganisation in ASPP2-deficient mice (Koch, 2013). Furthermore, these mice exhibit increased susceptibility to chronic inflammation. Other amino-terminal binding partners include YAP and H. pylori virulence factor CagA (Vigneron et al., 2010, Buti et al., 2011). Another study by our group has shown that ASPP2 is directly induced following microbial LPS treatment of murine macrophages through TLR4 activation of STAT1 signalling, suggesting that, in addition to its tumour suppressor role, ASPP2 can also regulate the inflammatory responses (Turnquist et al., 2014).
1.6 Aims & objectives

The aim of this thesis is to address the links that may exist between the members of the ASPP family, particularly ASPP2, and the host defence against pathogenic infections such as EBV and Mtb.

p53, the “guardian of the genome”, is often targeted in tumourigenesis and abrogation of p53’s effector functions can promote tumour progression. One of the most conserved functions of p53 is to induce apoptotic cell death in response to multiple insults. In recent years, there has been increasing evidence regarding p53’s involvement in inflammation and cancer. Also, the full implications of pathogen-stimulated apoptosis remain elusive. Thus, it is of interest to study the involvement of regulators of p53, such as p63 and ASPP2, in nasopharyngeal carcinogenesis and the immunopathogenesis of tuberculosis.

*Nasopharyngeal carcinogenesis*

Persistent EBV infection is aetiologically associated with undifferentiated NPC. Notably, p63 overexpression is detected in NPC and its contribution to NPC pathogenesis is not well defined. The ASPPs function as regulators of p53 and p63, thus, the aim of this study is to investigate the role of the ASPPs and p53/p63 in NPC development and address the following issues:

- To characterise the host’s cellular gene expression of p53 and the ASPP family in NPC cell lines and biopsies.
- To understand the roles of altered proteins’ expression and their downstream signalling pathways in promoting NPC tumourigenesis.
• To understand the interactions between NPC and its microenvironment \textit{in vivo}.

\textit{Immunopathogenesis of tuberculosis}

Previous studies carried out in our lab have demonstrated a novel role of ASPP2 as a gatekeeper to inflammation. STAT1-induced ASPP2 transcription is triggered by LPS-TLR4 stimulation in macrophages, and ASPP2-deficient mice show enhanced colonic and neuroinflammation. Therefore, it is of interest to further elucidate the participation of ASPP2 in inflammatory signalling for the control of tuberculosis:

• To characterise the immune response (formation of granuloma) and growth kinetics of BCG \textit{in vivo}.
• To understand the cellular signalling pathways central to the activation of immune cells following BCG and \textit{Mtb} infection \textit{in vivo}.
• To understand the effects of various mycobacterial virulence factors on the protein expression of the ASPPs and pro-inflammatory mediators, using an \textit{in vitro} monocyte/macrophage cell model.
CHAPTER II: MATERIALS & METHODS

2.1 Materials

2.1.1 Animals

All animals were bred and maintained at the Wellcome Trust Centre for Human Genetics, Oxford, and were handled by Dr. Gopinath Sutendra from the Ludwig Institute according to Institutional Animal Care and Use Committee approved guidelines. All procedures were carried out following the guidelines of the UK Home Office Animal Scientific Procedures Act 1986 (PIL: 30/10391). All mice used in the present study were WT female BALB/c mice (7 weeks old) and were obtained from the Jackson’s Laboratory.

2.1.2 Reagents

All chemicals, unless otherwise stated, were obtained from Sigma (MO, USA) or BDH Chemicals (UK). All autoradiography films (Hyperfilm) and ECL (enhanced chemoluminescence) reagents were purchased from Amersham Pharmacia Biotech (UK). Nitrocellulose membranes were purchased from Whatman (Germany). All tissue culture dishes and flasks were obtained from Geiner (UK). GenElute HP plasmid maxiprep kits were purchased from Sigma (MO, USA), and RNeasy midi kits from Qiagen (UK).
Ammonium persulfate (APS)

10% (w/v) stock solution was prepared in water and stored at -20°C in single-use aliquots.

Ampicillin stock

0.5g of the antibiotic was dissolved in 10ml sterile distilled water, creating a 50mg/ml solution. This was stored at -20°C in single-use aliquots.

Blocking solution

5% fat-free milk (Marvel, UK) or 5% (w/v) bovine serum albumin (BSA) (Sigma, USA) was prepared in 1x TBS-T.

Cell wall fraction (CWF) of Mtb

1mg of cell wall fraction of Mtb, strain H37Rv (BEI resources), was reconstituted in sterile-filtered PBS and stored at -80°C until use.

Citric acid

21g of citric acid was dissolved in 1L of distilled water.

Citric buffer

10x (0.1M) citric buffer was prepared by dissolving 29.4g sodium citrate in 1L distilled water. The pH was adjusted to 6.0 with citric acid. This 10x stock solution was sterile-filtered and stored at 4°C for up to a year. For use, the 10x solution was diluted in distilled water to make a 1x working solution.
**Complete™ protein inhibitor cocktail**

One Complete™ protein inhibitor (Boehringer Mannheim, UK) tablet was dissolved in 2ml of sterilised distilled water as a 20x stock solution which was stable at -20°C for 12 weeks.

**DAPI staining solution**

1000x stock solution of 10mg/ml was prepared by dissolving 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) in water and kept at -20°C.

**EDTA solution**

A 0.5M C$_{10}$H$_{14}$N$_2$O$_8$Na$_2$.2H$_2$O (EDTA) stock solution was made by dissolving 186g of EDTA in 700ml distilled water. The pH was adjusted to pH 8.0 with NaOH, and the volume made up to 1L with distilled water.

**Freezing medium**

- 50% (v/v) FBS
- 45% (v/v) complete DMEM or RPMI medium
- 5% (v/v) DMSO

Made fresh every time.

**G418 genticin sulfate**

A 1000x (400mg/ml) stock solution was prepared by dissolving 400mg G418 (Calbiochem) in 1ml distilled water, sterile-filtered and stored at -20°C. For use, 1000x stock was diluted in complete DMEM or RPMI medium to make a 1x working solution.
**LB (Luria-broth) agar plates**

LB-agar powder was dissolved as recommended by the manufacturer’s instructions and autoclaved. The solution was then cooled to 55°C before the addition of 100µg/ml ampicillin. 20ml of LB-agar was then poured into 10cm² dishes, allowed to set and plates stored at 4°C.

**LB medium**

LB powder was dissolved as recommended by the manufacturer’s instructions, autoclaved and stored at room temperature (RT).

**Lipoarabinomannan (LAM)**

500µg of lyophilised purified LAM from *Mycobacterium tuberculosis*, strain H37Rv (BEI Resources), was reconstituted in 1ml of sterile-filtered water and stored at -80°C until use.

**Lipopolysaccharide (LPS)**

LPS-EK (LPS from *E. coli* K12) Ultrapure (Invivogen) stock solution was prepared according to the manufacturer’s instructions and stored at -20°C.

**Lipotransfection reagents**

Lipotransfections were performed with Lipofectamine®2000 (Invitrogen) following the manufacturer’s instructions or as otherwise stated.

**Paraformaldehyde solution (4%)**

4% (w/v) solution was prepared by first dissolving paraformaldehyde in water in the fume-hood. 10x PBS was added until the final concentration of PBS was 1x. The pH
was adjusted to 7.0 with 1M HCl and the solution stored in single-use aliquots at -20°C.

**Phorbol 12-myristate 13-acetate (PMA)**

1mg of PMA (Sigma) was dissolved in 1ml of DMSO. Stock solution was further diluted in complete RPMI medium to achieve a 100ng/ml working concentration.

**Phosphatase inhibitor cocktail**

1 PhosSTOP phosphatase inhibitor cocktail (Roche) was dissolved in 10ml cold RIPA lysis buffer before use.

**Phosphate-buffered saline (PBS)**

12.5Mm NaCl

1Mm sodium dihydrogen phosphate, NaH$_2$PO$_4$

1.6Mm disodium dihydrogen phosphate, Na$_2$HPO$_4$

The pH was adjusted to 7.0 and autoclaved. The solution was prepared by the Ludwig Institute washroom team.

**Ponceau S staining solution (10 X)**

5% (v/v) acetic acid

2% (v/v) Ponceau S (sodium salt)

30% (w/v) trichloroacetic acid CCl$_3$.COOH

30% (w/v) 5-sulfosalicyclic acid C$_7$H$_6$O$_6$S.2H$_2$O (Sigma, MO, USA)

The solution was dissolved in water to a 1x dilution before use.
Primers used in PCR

All primers were synthesised by Operon, Germany. The lyophilised primers were dissolved in sterile water to a stock concentration of 100mM.

Protein molecular weight markers

The pre-stained protein markers were purchased from New England Biolabs (UK) and were used as a size standard for SDS-polyacrylamide gel electrophoresis.

RIPA (radioimmunoprecipitation assay) lysis buffer

150mM NaCl
1% (v/v) NP-40
0.1% (w/v) SDS
50mM Tris base

RIPA buffer was stored at RT. Complete protease inhibitor cocktail (1:20) was added to the buffer. Depending on the experiment, phosphatase inhibitor cocktail was also added.

Ribonuclease A (RNase A)

50mg of ribonuclease A (Sigma) was dissolved in 1ml of 10mM Tris-HCl, pH 7.5, 15mM NaCl to make a 10mg/ml stock solution which was stored in single-use aliquots at -20°C. Boiling to remove DNase was not recommended by the manufacturer.

RNeasy Mini Kit

The kit for purification of total RNA from cells and tissues was purchased from QIAGEN and kept at RT.
**SDS Solution**

A 10% (w/v) solution of sodium dodecyl sulphate (SDS) was dissolved in water and stored at RT.

**5x SDS-PAGE loading dye**

- 250nM Tris-HCl
- 10% (w/v) SDS
- 50% (v/v) glycerol
- 12.5% (v/v) β-mercaptoethanol
- 0.5% (w/v) bromophenol blue

**10X SDS-PAGE Running Buffer**

- 720g glycine
- 150g Tris base
- 50g SDS

5L of running buffer were prepared using distilled water.

**10X SDS-PAGE Transfer Buffer**

- 725g glycine
- 145g Tris

5L of transfer buffer were prepared using distilled water. 20% ethanol was added to 1X SDS-PAGE transfer buffer before use.
SuperScript® First Strand Synthesis System

The kit for cDNA production from RNA was purchased from Invitrogen and stored at -20°C.

TO-PRO

The nuclear dye was purchased as a liquid from Invitrogen and stored at -20°C in aliquots.

10X Tris buffered saline and Tween-20 (TBS-T)

121g Tris base
36.53g NaCl
250ml Tween-20

The pH was adjusted to 7.6 with HCl and a total volume of 5L prepared.

Triton-X-100

Triton-X-100 was purchased from Sigma Aldrich and stored at RT.

Urea buffer

8M urea
1M thiourea
0.5% (w/v) CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate
50mM DTT (dithiothreitol)
24mM spermine
Nanopure water (Type I) generated from the milliQ water system was used for all procedures.
2.1.3 SDS-polyacrylamide gels

All plates were cleaned, dried and assembled in the casting trays (Biorad). The acrylamide content of the gels varied between 6%-12% depending on the size of the protein of interest. The acrylamide gels were overlaid with 70% isopropanol solution and left to polymerise. After polymerisation, the isopropanol was washed away with water and a 4% stacking gel was set with the appropriate number and size of wells.

<table>
<thead>
<tr>
<th>Acryl/Bis</th>
<th>2ml</th>
<th>2.7ml</th>
<th>3.3ml</th>
<th>4.0ml</th>
<th>1.3ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5M Tris-HCl pH 8.8</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>--</td>
</tr>
<tr>
<td>1.0M Tris-HCl pH 6.8</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
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<tr>
<td>10% APS</td>
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<td>100μl</td>
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<td>50μl</td>
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<tr>
<td>TEMED</td>
<td>10μl</td>
<td>8μl</td>
<td>5μl</td>
<td>5μl</td>
<td>10μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.3ml</td>
<td>4.6ml</td>
<td>4.0ml</td>
<td>3.3ml</td>
<td>6.1ml</td>
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<tr>
<td>Total volume</td>
<td>10ml</td>
<td>10ml</td>
<td>10ml</td>
<td>10ml</td>
<td>10ml</td>
</tr>
</tbody>
</table>

**TABLE 2 – Preparation of SDS-polyacrylamide gels.** All resolving and stacking gels were prepared using 30% acrylamide/bis acrylamide (Acryl/Bis) 29:1 (NBL, UK or BioRad, UK). Values given are per 10ml of gel required. Abbreviations: APS, ammonium persulfate; TEMED, \textit{N,N',N''-}tetramethylethylenediamine; Tris, Tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulphate.
### 2.1.4 Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Name</th>
<th>Host/ type</th>
<th>Source (catalogue #)</th>
<th>Working conditions</th>
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<tbody>
<tr>
<td>ASPP1</td>
<td>Lx54.2</td>
<td>mouse mAb</td>
<td>ascites</td>
<td>1:1000 1:100</td>
</tr>
<tr>
<td>human ASPP2</td>
<td>Dx54.10</td>
<td>mouse mAb</td>
<td>ascites</td>
<td>1:1000 1:100</td>
</tr>
<tr>
<td>mouse ASPP2</td>
<td>S32</td>
<td>rabbit pAb</td>
<td>serum</td>
<td>1:1000 1:200</td>
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<tr>
<td>β-actin-HRP</td>
<td>-</td>
<td>mouse mAb</td>
<td>Abcam (ab20272)</td>
<td>1:5000 -</td>
</tr>
<tr>
<td>CD3</td>
<td>-</td>
<td>rabbit pAb</td>
<td>Abcam (ab5690)</td>
<td>- 1:100</td>
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<tr>
<td>CD3</td>
<td>CD3-12</td>
<td>rat mAb</td>
<td>AbD Serotec (MCA1477)</td>
<td>- 1:100</td>
</tr>
<tr>
<td>CD11b</td>
<td>MI/70</td>
<td>rat mAb</td>
<td>Abcam (ab8878)</td>
<td>- 1:100</td>
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<tr>
<td>CD14</td>
<td>M305</td>
<td>rabbit pAb</td>
<td>Santa Cruz (sc-9150)</td>
<td>- 1:100</td>
</tr>
<tr>
<td>CD45R/B220</td>
<td>RA3-6B2</td>
<td>rat mAb</td>
<td>BD (554879)</td>
<td>- 1:100</td>
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<tr>
<td>CD68</td>
<td>ED1</td>
<td>mouse mAb</td>
<td>Abcam (ab31630)</td>
<td>- 1:100</td>
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<tr>
<td>cleaved caspase-3 (D175)</td>
<td>5A1E</td>
<td>rabbit mAb</td>
<td>Cell Signalling (9664)</td>
<td>- 1:100</td>
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<tr>
<td>GAPDH-HRP</td>
<td>-</td>
<td>rabbit pAb</td>
<td>Abcam (ab9385)</td>
<td>1:5000 -</td>
</tr>
<tr>
<td>Antibody</td>
<td>Source</td>
<td>Dilution</td>
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<td></td>
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<tr>
<td>----------</td>
<td>--------</td>
<td>----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA-1 R4</td>
<td>rabbit pAb</td>
<td>1:500-1000</td>
<td></td>
<td></td>
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<tr>
<td>EBNA-1 0211</td>
<td>mouse mAb</td>
<td>Abcam (ab20777) 1:100</td>
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<td></td>
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<tr>
<td>E-cadherin 24E10</td>
<td>rabbit mAb</td>
<td>Cell Signalling (3195) 1:1000 1:200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4/80 M300</td>
<td>rabbit pAb</td>
<td>Santa Cruz (sc-25830) - 1:100</td>
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</tr>
<tr>
<td>F4/80 C7</td>
<td>mouse mAb</td>
<td>Santa Cruz (sc-377009) - 1:100</td>
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<td></td>
</tr>
<tr>
<td>iASPP Lx49.3</td>
<td>mouse mAb</td>
<td>ascites 1:3000 1:300</td>
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<td></td>
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<tr>
<td>LMP-1 CS.1-4</td>
<td>mouse mAb</td>
<td>Dako (M0897) 1:1000 1:50</td>
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<tr>
<td>LMP-2A 14B7</td>
<td>rat mAb</td>
<td>Abcam (ab59026) 1:1000 1:100</td>
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<tr>
<td>macrophage (MAC) MAC387</td>
<td>mouse mAb</td>
<td>Abcam (ab22506) - 1:100</td>
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<tr>
<td>N-cadherin -</td>
<td>mouse mAb</td>
<td>BD (610920) 1:1000 1:100</td>
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<tr>
<td>NF-κB p52/p100 -</td>
<td>rabbit pAb</td>
<td>Abcam (ab31432) - 1:100</td>
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<td></td>
</tr>
<tr>
<td>NF-κB p65 -</td>
<td>rabbit pAb</td>
<td>Santa Cruz (sc-109) 1:500</td>
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<tr>
<td>NF-κB p-p65 (S536) -</td>
<td>rabbit pAb</td>
<td>Cell Signalling (3031) 1:1000</td>
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<tr>
<td>NOS2 (iNOS) C11</td>
<td>mouse mAb</td>
<td>Santa Cruz (sc-7271) - 1:100</td>
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</tr>
<tr>
<td>Notch-1 C-20</td>
<td>goat pAb</td>
<td>Santa Cruz (sc-6014) - 1:200</td>
<td></td>
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<tr>
<td>pan-cytokeratin AE1/AE3</td>
<td>mouse mAb</td>
<td>Dako (M3515) - 1:100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Antibody</td>
<td>Type</td>
<td>Dilution</td>
<td>Dilution</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>p53</td>
<td>DO-1</td>
<td>mouse mAb</td>
<td>1:2500</td>
<td>1:500</td>
</tr>
<tr>
<td>mouse p53</td>
<td>CM5</td>
<td>rabbit pAb</td>
<td>Leica Biosystems (NCL-p53-CM5)</td>
<td>-</td>
</tr>
<tr>
<td>p63</td>
<td>4A4</td>
<td>mouse mAb</td>
<td>Santa Cruz (sc-8341)</td>
<td>1:1000</td>
</tr>
<tr>
<td>p63</td>
<td>H137</td>
<td>rabbit pAb</td>
<td>Santa Cruz (sc-8343)</td>
<td>1:1000</td>
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<tr>
<td>p63</td>
<td>-</td>
<td>rabbit pAb</td>
<td>Abcam (ab53039)</td>
<td>-</td>
</tr>
<tr>
<td>TAp63</td>
<td>-</td>
<td>rabbit pAb</td>
<td>BioLegend (poly6189)</td>
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</tr>
<tr>
<td>△Np63</td>
<td>-</td>
<td>rabbit pAb</td>
<td>BioLegend (poly6190)</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-STAT1 (S727)</td>
<td>EPR3146</td>
<td>rabbit mAb</td>
<td>Abcam (ab109461)</td>
<td>-</td>
</tr>
<tr>
<td>p-STAT1 (Y701)</td>
<td>58D6</td>
<td>rabbit mAb</td>
<td>Cell Signalling (9167)</td>
<td>1:1000</td>
</tr>
<tr>
<td>vimentin</td>
<td>D21H3</td>
<td>rabbit mAb</td>
<td>Cell Signalling (5741)</td>
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</tr>
<tr>
<td>ZEB1</td>
<td>H102</td>
<td>rabbit pAb</td>
<td>Santa Cruz (sc-25388)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

**TABLE 3 - List of primary antibodies.**

Abbreviations – Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody; IHC, immunohistochemistry; IF, immunofluorescence; WB, western blot.
<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Application</th>
<th>Working conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse IgG</td>
<td>Dako</td>
<td>WB</td>
<td>1:2000</td>
</tr>
<tr>
<td>rabbit IgG</td>
<td>Dako</td>
<td>WB</td>
<td>1:2000</td>
</tr>
<tr>
<td>Alexa-Fluor® 488 F(ab’)₂ goat-anti-mouse IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa-Fluor® 488 F(ab’)₂ goat-anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa-Fluor® 488 F(ab’)₂ goat-anti-rat IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa-Fluor® 546 F(ab’)₂ goat-anti-mouse IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa-Fluor® 546 F(ab’)₂ goat-anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
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<tr>
<td>Alexa-Fluor® 568 F(ab’)₂ goat-anti-rat IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa-Fluor® 647 F(ab’)₂ donkey-anti-mouse IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
<tr>
<td>Alexa-Fluor® 647 F(ab’)₂ goat-anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>IF/ IHC</td>
<td>1:400</td>
</tr>
</tbody>
</table>

**TABLE 4 – List of secondary antibodies.**

Abbreviations – HRP, horseradish peroxidase; IHC, immunohistochemistry; IF, immunofluorescence; WB, western blot.
### 2.1.5 Cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Tissue/ type</th>
<th>Culture Medium*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>human lung carcinoma</td>
<td>DMEM</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>A549 rEBV</td>
<td>human lung carcinoma, stably infected with</td>
<td>DMEM</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td></td>
<td>recombinant Akata's strain of EBV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C666.1</td>
<td>EBV-positive human undifferentiated NPC</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>CNE1</td>
<td>EBV-negative human immortalised NPC</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>CNE1 rEBV</td>
<td>human immortalised NPC, stably infected with</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td></td>
<td>recombinant Akata's strain of EBV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNE2</td>
<td>EBV-negative human immortalised NPC</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>CNE2 rEBV</td>
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<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
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<tr>
<td></td>
<td>recombinant Akata's strain of EBV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1299</td>
<td>p53-null human lung carcinoma</td>
<td>DMEM</td>
<td>ATCC</td>
</tr>
<tr>
<td>HNE1</td>
<td>EBV-negative human immortalised NPC</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>HNE1 rEBV</td>
<td>human immortalised NPC, stably infected with</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Cell Line</td>
<td>Description</td>
<td>Medium</td>
<td>Supplier</td>
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<tr>
<td>-----------</td>
<td>-------------</td>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td>SUNE1</td>
<td>EBV-negative human immortalised NPC</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>SUNE1 rEBV</td>
<td>human immortalised NPC, stably infected with recombinant Akata’s strain of EBV</td>
<td>RPMI</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>THP-1</td>
<td>human monocytic cell line</td>
<td>RPMI</td>
<td>ATCC</td>
</tr>
<tr>
<td>TW04</td>
<td>EBV-negative human immortalised NPC</td>
<td>DMEM</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>TW04 rEBV</td>
<td>human immortalised NPC, stably infected with recombinant Akata’s strain of EBV</td>
<td>DMEM</td>
<td>Prof. C. Dawson (University of Birmingham)</td>
</tr>
<tr>
<td>U2OS</td>
<td>human osteosarcoma</td>
<td>DMEM</td>
<td>ATCC</td>
</tr>
</tbody>
</table>

**TABLE 5 – List of established cell lines.**

Abbreviations – ATCC, American Type Culture Collection; DMEM, Dulbecco’s Modified Eagle’s Medium; EBV, Epstein-Barr virus; rEBV, recombinant EBV; RPMI, Roswell Park Memorial Institute-1640. *All culture medium were supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Pen/Strep) and 1% L-glutamine. Cell lines were stored in liquid nitrogen until required.

The stable EBV-infected cell lines were kind gifts from Prof. C. Dawson from the University of Birmingham and were generated by transducing the cells with WT rEBV (Akata’s strain) carrying a neomycin-resistance gene and selected using G418 at a concentration of 400µg/ml.
### 2.1.6 Plasmids

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Plasmid</th>
<th>Control</th>
<th>Source</th>
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<td>EBNA-1</td>
<td>pSG5-EBNA-1</td>
<td>pSG5</td>
<td>Prof. P. Farrell (Imperial College)</td>
</tr>
<tr>
<td>LMP-1</td>
<td>pSG5-LMP-1</td>
<td>pSG5</td>
<td>Prof. P. Farrell (Imperial College)</td>
</tr>
<tr>
<td>LMP-2A</td>
<td>pSG5-LMP-2A</td>
<td>pSG5</td>
<td>Prof. P. Farrell (Imperial College)</td>
</tr>
</tbody>
</table>

**TABLE 6 – List of plasmids.**

All plasmids were received from other laboratories and sent to Source Bioscience for sequencing to confirm their identity.
2.2 Methods

2.2.1 Tissue culture

Basic media

DMEM was purchased from Lonza (Belgium), RPMI from Gibco-BRL (UK) and stored at 4°C.

Media supplements

FBS was purchased from PAA Laboratories and tested for its ability to support the growth of various cell lines. It was heat-inactivated for 30 minutes at 55°C and stored at -20°C in 50ml aliquots. L-Glutamine was purchased from Gibco-BRL at a 200mM concentration, stored at -20°C and used at a final concentration of 2mM. Pen/Strep was purchased from Gibco-BRL at 10,000units/ml and stored at -20°C. A final concentration of 200units/ml was used.

Maintenance of cell lines

All cell lines listed in TABLE 5 were cultured in complete culture medium (DMEM or RPMI) supplemented with 10% FBS, 1% Pen/Strep and 1% L-glutamine, in dishes or flasks maintained in a Heraeus incubator at 37°C, in the presence of 10% CO₂. Medium was changed every 2-3 days depending on the cell line. Upon reaching 75 - 90% confluence, cells were washed once with 1x PBS and incubated with 7ml of pre-warmed Trypsin-EDTA 0.05% (Gibco-BRL) at 37°C until the cells dissociated.
from the flasks or dishes. A cell scraper was used if the incubation time with trypsin exceeded 10 minutes. Trypsin was inhibited by the addition of the appropriate volume of fresh growth medium and cells were pelleted by centrifugation at 1000rpm for 5 minutes at RT and the supernatant removed. Cell pellets were resuspended in 10ml of complete medium and counted using an automated cell counter (Bio-Rad). This culture was seeded onto fresh flasks or dishes at the desired density.

G418 was added to stably infected NPC cell lines to maintain the expression of EBV genes at a concentration of 400µg/ml.

All cell lines adhered readily to the plastic surface with the exception of THP-1 and C666.1.

**Cryopreservation of cell lines**

Cells were grown to about 80-90% confluence and collected by trypsinisation (as described above). The cell pellet was resuspended in the appropriate amount of freezing medium and 1ml aliquots were transferred into cryovials (Nunc). The vials were then labelled and cooled at a rate of 1°C per minute in a Nalgen Cryo 1°C freezing container and placed in a -80°C freezer (New Brunswick Scientific) for at least 24hrs, before being transferred to a liquid nitrogen tank for long term storage.

For the recovery of frozen stocks, vials were placed in a 37°C water bath for 2-3 minutes. To remove the freezing media, 5ml of pre-warmed growth medium was added before centrifugation for 5 minutes at 1,000rpm. The cell suspension was transferred to a 10cm² dish or T25 flask with the appropriate pre-warmed fresh growth medium and kept in a 37°C incubator overnight for recovery.
2.2.2 DNA and RNA techniques

Bacterial strains and culture

Chemically competent *E. coli* Alpha-Select™ silver efficiency (Bioline) was used as a host for plasmid DNA. Bacteria were cultured in LB media containing 100μg/ml ampicillin at 37°C.

Transformation

Silver efficiency competent cells were thawed on ice. The desired plasmid DNA (100ng, ~1μl) was added to 50μl of the competent cells. The mixture was kept on ice for 30 minutes. Heat shock was applied to the bacteria by first placing the vial in a 42°C water bath for 45 seconds, and then on ice for another 2 minutes. 100μl of SOC (super optimal broth containing glucose) medium (Invitrogen) was added to the cells, and they were shaken at 37°C for 1 hour before plating on agar plates containing the appropriate antibiotic. Plates were incubated at 37°C overnight to allow colony formation and colony selection the following day.

Large scale preparation of plasmid DNA (maxi-prep)

A single bacterial colony was picked and inoculated in 300ml of LB medium containing the appropriate antibiotic. The bacterial suspension was left shaking at 37°C, overnight. The cells were centrifuged at 6,000xg for 15 minutes at 4°C (Beckman Coulter J-26XP, rotor F10BCI-6x500y). The plasmid DNA extraction was carried out according to the GenElute HP plasmid Maxi-prep Kit manufacturer’s
protocol. The cDNA concentration was measured by OD260 using a Nanodrop spectrophotometer (Thermo Scientific).

RNA extraction and reverse transcription

RNA was extracted from cells according to the Qiagen RNeasy mini kit protocol, including treatment with DNase I (Qiagen) to eliminate possible genomic DNA contamination. Up to 5μg of total RNA was used to generate cDNA with the SuperScript® First Stand Synthesis System (Invitrogen) following the manufacturer’s protocol.

Real-time quantitative PCR (rt-qPCR)

Real-time quantitative PCR was performed on the 7500 real time PCR system (Applied Biosystem) using the QuantiTect SYBR Green PCR kit (Qiagen). Each reaction was performed in duplicate using 1μl of cDNA in a final volume of 25μl (TABLE 7). An initial denaturation step of 15 minutes at 95°C was included to activate the HotStarTaq DNA polymerase. Reactions were run for 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Quantitect SYBR Green PCR Mastermix</td>
<td>12.5</td>
</tr>
<tr>
<td>Forward &amp; Reverse primer mix</td>
<td>2.5</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>9</td>
</tr>
<tr>
<td>cDNA</td>
<td>1</td>
</tr>
</tbody>
</table>

TABLE 7 - Mastermix for rt-qPCR.
For each experiment, the threshold cycles (Ct) were automatically determined by the Applied Biosystems 7500 software. Previously published primers were used for all of the genes analysed (sequences reported in TABLE 8). The expression level of each target gene was analysed based on the ΔΔCt method, with GAPDH as an internal control.

The fold change in mRNA levels for each rt-qPCR sample was calculated using the equation: Fold change = $2^{\Delta\Delta CT}$, where $\Delta\Delta CT = [(C_t \text{ gene of interest} - \text{average } C_t \text{ GAPDH}) \text{ EBV-infected cells} - (C_t \text{ gene of interest} - \text{average } C_t \text{ GAPDH}) \text{ parental cells}]$. 
<table>
<thead>
<tr>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAp63</td>
<td>GCCCATTGACTTGAACCTT</td>
<td>Eurofins</td>
</tr>
<tr>
<td>GGGTGGCTGCAATCCTG</td>
<td>GTCTGGCGGAGGGAAT</td>
<td>Eurofins</td>
</tr>
<tr>
<td>EBNA-1</td>
<td>GGGTGGCTGGAAGACAT</td>
<td>Eurofins</td>
</tr>
<tr>
<td>GAPDH</td>
<td>-</td>
<td>Qiagen (QT00079247)</td>
</tr>
</tbody>
</table>

TABLE 8 – List of primers.
2.2.3 Protein manipulation

Sample preparation

Cells grown in monolayers were washed once with 1x PBS and lysed in RIPA or urea lysis buffer (1ml per 10cm² dish). The cells were scraped with a sterile disposable cell scraper (Greiner), transferred to labelled eppendorf tubes and left on ice for 30 minutes. The mixture was cleared by centrifugation at 13,200rpm at 4°C for 20 minutes. The resulting lysate was removed to a fresh eppendorf tube and the cell debris discarded.

Mouse tissue was previously frozen at -80°C and crushed into fine powder using a pestle and mortar. The powder was mixed with RIPA lysis buffer and incubated on ice for 30 minutes to encourage the powder to dissolve. The lysate was then sonicated 3 times for 10 seconds and clarified by centrifugation at 13200rpm, 4°C for 20 minutes. Centrifugations were repeated until no pellet was obtained. The resulting supernatant (soluble fraction) was collected in a fresh eppendorf tube. The cell pellet was incubated with urea lysis buffer and processed as described above to obtain the insoluble fraction.

Protein concentration determination

The protein concentration of cell and tissue extracts was determined using the BioRad (Bradford) protein assay reagent system. In a 96-well plate, 1μl of cell lysate was dissolved in 200μl of 1x BioRad assay reagent. In the same plate, known concentrations of BSA (Sigma) were used to establish a standard curve concentration/absorbance. The plate was read in the spectrophotometer (Anthus
Labtech Instrument, using Magellan software) at 595nm, and the concentration of the cell lysate was obtained by extrapolating the BSA standard curve.

**SDS-polyacrylamide gel electrophoresis (PAGE)**

Known concentrations of protein were mixed with appropriate volumes of 5x SDS-PAGE loading dye and boiled at 80 - 90°C for 5 minutes. The resolving gels were made with 6 - 12% of polyacrylamide (TABLE 2), depending on the size of protein of interest. Equal amounts of protein were loaded in the wells of the stacking gel. The gels were resolved at 130V for 90 minutes in 1x running buffer using a Bio-Rad running system. Pre-stained protein markers, broad range (7 - 175kDa) (New England Biolabs) were used as molecular weight standards.

**Immunoblotting**

After the samples were separated through the gel, the gel was transferred to a wet transfer unit containing 1x SDS-PAGE transfer buffer. The proteins were electrophoretically transferred onto nitrocellulose membrane (GE healthcare) for either 3 hours at a constant voltage of 80V, or overnight at 30V, using the Mighty Small II transfer electrophoresis system (Amersham). The membrane was then stained with Ponceau S solution to determine the success of the transfer of proteins and equal loading of the lanes. The membrane was subsequently washed with distilled water and blocked in 5% dried skimmed milk in TBS-T for 30-60 minutes at RT. Primary antibody was added at the recommended concentrations (TABLE 3), and diluted in TBS-T plus 5% milk overnight at 4°C. After three washes in distilled water, the secondary HRP-labelled antibody was added for 1 hour at RT (1:2000).
The membrane was washed another three times with distilled water and once with TBS-T for 10 minutes. Signals were visualised by enzymatic ECL detection (GE Healthcare) using X-ray films (Fujifilm).

### 2.2.4 Cell-based assays

#### Cell transfection

Lipofectamine™2000 (Invitrogen) was used as a DNA-lipid carrier according to the manufacturer's protocol.

#### Immunofluorescence (IF)

Cells were grown on coverslips in 24-well sterile plates (Corning) to 70-90% confluence. Once experimental procedures were completed, cells were fixed in 4% paraformaldehyde for 15 minutes and washed twice with 1x PBS. Subsequently, the cells were permeabilised in 0.2% Triton-X 100 in PBS for 10 minutes, followed by two PBS washes before blocking in blocking buffer (0.2% fish skin gelatin (FSG) in PBS) for an hour. Primary antibodies were diluted in blocking buffer and incubated with the cells for an hour. Three PBS washes were performed to remove unbound primary antibodies before incubation with a nuclear marker, DAPI (1:1000) or TO-PRO (1:1000), and the appropriate fluorescent secondary antibody (1:400) (Invitrogen) for 30 minutes. This step was followed by another 3 washes with PBS, after which the coverslips were mounted using Fluoromount (Southern Biotech) and the images collected using a Zeiss LSM 710 microscope.
Stimulation of THP-1 cells with bacterial constituents

Prior to stimulation with TLR agonists, 1.5 – 2.0 x 10^6 THP-1 cells were seeded to each well (6-well plates) and induced to differentiate into macrophages for 72 hours by the addition of 100ng/ml PMA to the complete medium. The cells were briefly washed with 1x PBS and rested for 24 hours in PMA-free complete medium before incubation with LPS (1, 5, 10, 25, 50 and 100 ng/ml), LAM (1, 5, 10, 25, 50 and 100 ng/ml) or CWF (1, 5, 10, 25, 50 and 100 µg/ml) in complete medium for 5 hours. Cell lysates were obtained using urea buffer as described previously.

2.2.5 Mouse work

BCG infection

15 female BALB/c mice of 7 weeks of age were challenged with 5 x 10^5 colony forming units (c.f.u.) of *M. bovis* BCG Pasteur strain via the intranasal route. Three animals were sacrificed at specific intervals after infection: 1, 7, 14, 21 and 28 days. A WT (non-infected) mouse was included at each time point for comparison. The lungs, spleens and livers of these animals were removed. Half of each organ was used for c.f.u. determination while the other half was used for histology and protein analysis. The BCG infection was performed by Dr. Elena Stylianou from the Jenner Institute and the animals were handled by Dr. Gopinath Sutendra.
Determination of bacterial load

Mouse lungs and spleens were homogenised and plated onto nutrient 7H11 agar plates using serial dilutions to determine the viable bacterial cell count. The number of c.f.u. was counted following incubations of these agar plates in a 37°C incubator for 4 weeks. This experimental procedure was performed by Dr. Elena Stylianou.

Histological techniques

Mouse lungs, spleens and livers were removed and washed in PBS before fixation in formalin for 48 - 72 hours. The organs were then dehydrated in an ethanol series, cleared in histoclear and embedded in paraffin wax.

Human NPC tissue and control biopsies were obtained from the Imperial College Healthcare NHS Trust Tissue Bank, in collaboration with Prof. Rob Goldin, a histopathologist at St. Mary’s Hospital, Imperial College, London. Sections were cut at 4µm thickness and either stained for haematoxylin and eosin (H&E), or processed for immunostaining.

H&E staining

Tissue sections were de-waxed in histoclear, rehydrated in an ethanol series, immersed in Harris haematoxylin (3 minutes), differentiated with acid alcohol (5 dips), blued in Scott Water (30 seconds) and then immersed in eosin (5 minutes) with washes in water between the steps. Sections were then dehydrated and permanently mounted (Vectamount – Vector Labs, CA, USA).
Immunohistochemistry (IHC)

Samples were cleared with histoclear and rehydrated in an ethanol series before blocking with 3% hydrogen peroxide in methanol for 10 minutes. The slides were subjected to heat-mediated antigen retrieval in pre-boiled citrate buffer (pH 6.0) for 10 minutes and left to cool to RT (~20 minutes) on the bench. The sections were washed once with PBS, blocked in Image-iT® FX Signal Enhancer (Invitrogen) for 30 minutes, then incubated overnight at 4°C with primary antibody diluted in antibody diluent (Dako). Slides were washed in PBS 3 times for 10 minutes, before being incubated with biotinylated secondary antibody diluted in antibody diluent (1:250) for 30 minutes at RT. To remove unbound antibody, the slides were washed in PBS 3 times for 5 minutes. Sections were incubated in an avidin/biotin-based peroxidase complex solution (Vector), for 15 minutes at RT, and washed in PBS 3 times for 5 minutes. Subsequently, sections were incubated in a DAB (3,3’-diaminobenzidine) peroxidase substrate solution (Vector) for up to 10 minutes at RT. Slides were then rinsed in water and counterstained by immersion in haematoxylin for 15 seconds. Finally, the slides were dehydrated through increasing graded alcohols and immersed in histoclear for 5 minutes, before being mounted in ProLong® Gold Antifade Reagent (Invitrogen) to preserve staining.

Tissue immunofluorescence on paraffin sections

Slides were deparaffinised and hydrated through graded alcohols to water. Slides were subjected to heat-mediated antigen retrieval in pre-boiled citric acid buffer for 10 minutes and left in the buffer until cold enough to handle (~20 minutes). The sections were briefly washed with PBS and blocked in Image-iT® FX Signal Enhancer. Subsequently, the slides were incubated in primary antibody diluted in
antibody diluent overnight at 4°C. The day after, slides were washed in PBS 3 times for 10 minutes. Next, fluorophore-conjugated secondary antibodies (1:400) diluted in antibody diluent were applied to all sections for an hour at RT, followed by three 5 minutes washes in PBS. Finally, the slides were incubated with DAPI (1:1000) for 10 minutes, washed in distilled water and mounted in ProLong® Gold Antifade Reagent. Stained sections were kept at 4°C, and protected from light until they were visualised using a Zeiss LSM 710 microscope.

2.2.6  Data analysis

**Computer images**

All autoradiographs were scanned using an Epson perfection 1660 Photo scanner and Adobe Photoshop CS6 software. Images were manipulated only as a whole for size, brightness and contrast. No signal was modified in relation to the whole image. ImageJ® software was used to quantify gel bands from western blots.

**Statistics**

All statistical analysis for histological characterisation was conducted using three different mice (n = 3). Differences were considered significant at a value of p≤0.05. The two-tailed student’s t-test was used to calculate the statistical significance between two measurements (GraphPad Prism® Software, version6, San Diego, CA, USA).
CHAPTER III: NASOPHARYNGEAL CARCINOGENESIS

To investigate the role of the ASPPs and p53/p63 in NPC development, we determine whether EBV infection or the expression of EBV proteins has an effect on the expression levels of ASPP and p53 family proteins. Therefore, we screened for p53 and ASPP family proteins in EBV-infected cell lines and in NPC biopsies.

3.1 Validation of recombinant EBV protein expression

In order to check for the specificity of the EBV antigen antibodies, the osteosarcoma U2OS cell line was seeded in 10cm² plates with coverslips and transiently transfected with pSG5 plasmid carrying one of the viral latent proteins EBNA-1, LMP-1 or LMP-2A. Cells were allowed to grow for another 24 hours before harvesting. The molecular weights of these proteins were assessed by immunoblotting, while their intracellular localisation was analysed using immunofluorescence staining. The molecular weights of EBNA-1, LMP-1 and LMP-2A were approximately 80kDa, 52kDa and 46kDa, respectively (FIGURE 3.1). A parental NPC cell line and its EBV-infected counterpart were also checked for endogenous EBNA-1 expression. As shown in FIGURE 3.1, endogenous EBNA-1 expression was detected in the EBV-infected HNE1 cell line. However, its expression was relatively low compared to an EBV-infected lymphoblastoid cell line, PD-LCL, which expresses the full spectrum of latency genes, and could only be detected with increased exposure time. Also, it was observed that earlier passages of EBV-infected
NPC lines had higher endogenous EBNA-1 expression compared to later passages (data not shown).

As expected, EBNA-1 was found to be localised in the nucleus upon overexpression, while LMP-1 and LMP-2A were stained in the cytoplasm or the peri-nuclear region (FIGURE 3.1D). EBNA-1 expression was not detected in EBV-infected cell lines using IF, due to its low copy number. The punctuate staining of EBNA-1 in the C666.1 cell line, which is derived from a Cantonese NPC patient, further confirmed its cellular localisation (data not shown). The C666.1 cell line retains its EBV genome in long-term cultures and hence serves as a good in vitro model with which to study EBV.
FIGURE 3.1 - Overexpression of EBV latent antigens in U2OS cells. (A) Immunoblotting of EBNA-1 in untransfected, EBNA-1-transfected, HNE1 parental and EBV-infected HNE1 cells. (B, C) Western blots confirming the exogenous expression of LMP-1 and LMP-2A in U2OS cells. An EBV-transformed B lymphoblastoid cell line, PD-LCL, which is known to express endogenous EBNA-1, LMP-1 and LMP-2A is used as positive control. β-actin protein level is used as a loading control. (D) Immunofluorescence staining of EBNA-1, LMP-1 and LMP-2A in U2OS-transfected cells. DAPI is used as a counterstain whereas omitted primary antibody is used as a negative control (data not shown).

3.2 In vitro characterisation of p53 and ASPP family proteins in parental NPC and its EBV-infected counterparts

The alteration by EBV of the gene expression of cancer-related pathways by EBV in NPC tumours, such as cell proliferation, anti-apoptosis and metastasis, has been reported in several studies (Chou et al., 2008). This leads to genomic instability and contributes to NPC pathogenesis. EBV viral machinery has been shown to modulate the expression of several target genes that regulate apoptotic cell death, such as Puma, Bim, Survivin and Tomm22 (Choy et al., 2008, Marquitz et al., 2011, Yip et al., 2006). As p53 works in concert with the ASPP family members to mediate the cell’s apoptotic response, particularly with ASPP1 and ASPP2 to specifically potentiate p53’s transactivation of the promoters of pro-apoptotic genes such as Bax and Puma, we sought to examine the expression of p53, p63 (a p53-related protein), ASPP1, ASPP2 and iASPP in vitro during the course of EBV infection (Samuels-Lev et al., 2001, Bergamaschi et al., 2003).

Five pairs of parental NPC lines and their EBV-infected counterparts (CNE1, CNE2, HNE1, SUNE1 and TW04) were screened to evaluate the expression levels of p53 and the ASPP family members to identify if there were distinct patterns of up- or
down-regulation, by western blotting. Due to loss of the EBV genome from cells isolated from NPC patients following prolonged culture, these EBV-negative NPC lines were re-infected with neomycin-resistant recombinant EBV (Akata’s strain which only expresses EBNA-1) and selected using 400μg/μl G418 (henceforth referred to as EBV-infected cells).

FIGURE 3.2 - Comparison of p53/ASPP family protein levels between parental and EBV-infected cell lines. Western blot analysis of ASPP1 (Lx54.2), ASPP2 (Dx54.10), iASPP (Lx49.3), p53 (DO-1), p63 (4A4) and NF-κB p65 (sc-109) protein patterns in different NPC parental cell lines and their EBV-infected counterparts. β-actin signal is used as a loading control.

As shown in FIGURE 3.2, we observed no significant changes in the expression levels of ASPP1, ASPP2, iASPP and p53. The lack of a specific expression pattern was consistent between pairs of cell lines (n = 3). Interestingly, down-regulation of p63 protein expression was observed in EBV-infected cell lines compared to their
parental counterparts. Since the important role of p63 in epithelial cell proliferation is well-documented, the effect of different seeding densities in p63 protein expression levels was investigated. NPC cells were plated at low (3.75 x 10^5 cells per 10 cm^2 plate), medium (7.5 x 10^5 cells) and high densities (11.25 x 10^5 cells), and allowed to grow for 48 hours before harvesting in urea buffer (FIGURE 3.3). p63 levels were consistently down-regulated after EBV infection in NPC cell lines and an adenocarcinomic alveolar cell line, A549, despite variation in seeding densities. Its cellular localisation was determined using IF staining.
FIGURE 3.3 - EBV infection in NPC lines associates with reduced expression of p63.

Western blot analysis demonstrates p63 reduction in EBV-infected cell lines regardless of seeding densities. EBNA-1 expression is used to confirm EBV infection in CNE2. Anti-pan p63 monoclonal antibody (4A4) that detects both the TA\(p63\) and ΔN\(p63\) isoforms is used and β-actin is used as a loading control.

Strong nuclear p63 expression was detected in the parental NPC lines, but greatly diminished following EBV infection (FIGURE 3.4). Mean immunofluorescence intensity (MFI) was determined for NPC cells in response to EBV infection, and quantitative analysis showed a significant decrease of p63 MFI in EBV-infected cell lines compared to its non-infected counterparts (\(p<0.0001\) vs parental NPC cells, student's unpaired two-tailed \(t\)-test, \(n > 128\)). This observation is in accordance with the western blot data (FIGURES 3.2 & 3.3). Also, it is noted that both parental and EBV-infected NPC lines expressed heterogeneous nuclear p63. Some cells had higher p63 intensity within the same culture conditions. At present, the molecular mechanisms involving p63 in NPC pathogenesis are unclear. A previous study carried out in our lab has demonstrated that ASPP2 is able to suppress p63 expression through RelA/p65-mediated repression (Tordella et al., 2013).

Transfection of ASPP2 in the SCC cell line O40 led to an accumulation of nuclear p65, and down-regulation of p63 in a mutually exclusive pattern. Therefore, it was speculated that EBV-induced p63 repression was due to downstream NF-κB activation. Nevertheless, there was no significant change in total p65 protein expression levels following EBV infection (FIGURE 3.2). As such, the expression levels of activated or phosphorylated NF-κB should be investigated, and its cellular localisation determined in both parental and EBV-infected cell lines.

There are two major classes of p63 isoform, TA\(p63\) and ΔN\(p63\), and they play opposite roles in cancer pathogenesis. TA\(p63\) is pro-apoptotic and can transactivate p53 responsive promoters, while ΔN\(p63\) is known to act in a dominant-negative
manner and binds to WT p53 to inhibit its function. It is not clear which isoforms of p63 were down-regulated, as the 4A4 antibody used to detect p63 expression in the above experiments could recognise both TAp63 and ΔNp63 isoforms. Hence, specific TAp63 and ΔNp63 antibodies were used to differentiate the expression levels of different p63 isoforms in CNE2 and EBV-infected CNE2 cells.
FIGURE 3.4 - Nuclear p63 expression is decreased in EBV-infected NPC cell lines compared to their parental counterparts. NPC cells are allowed to grow for 48 hours before they are fixed and stained for p63 (red). The figure shows representative images from confocal analysis of a single experiment. DAPI is used as counterstain. There is a significant decrease in the total p63 fluorescence intensity in EBV-infected cells. IF intensity is determined using ImageJ software and statistical analysis is carried out using GraphPad 6. \( p < 0.0001 \) vs parental cells, student's two-tailed t-tests, \( n > 128 \).

Down-regulation of the ΔNp63 isoform was observed in EBV-infected CNE2 cell lines, and there was no difference for TAp63 expression (FIGURE 3.5). Protein levels were evaluated using densitometry, but these results do not yet have statistical significance.

FIGURE 3.5 - EBV-infection modulates ΔNp63 protein expression levels in CNE2 cells. (A) Protein expression levels of TAp63 and ΔNp63 are comparable between parental CNE2 cells and EBV-infected CNE2 cells at different seeding densities. β-actin level is used as a loading control. (C) Scanned band intensities are quantified by ImageJ densitometry analysis. \( n = 1 \)
Next, rt-qPCR was performed on total mRNA to examine p63 transcript levels in parental and EBV-infected NPC lines using isoform-specific primers. rt-qPCR analysis revealed that there were relatively lower levels of ΔNp63α in EBV-infected NPC cell lines compared to non-infected ones. In CNE2 cells, EBV infection had no significant effect on the transcript level of the TA-specific isoform. Elevated TAp63α transcript levels were observed in EBV-infected CNE1, HNE1 and SUNE1 cells when compared with their parental counterparts only if they were seeded at a high density, but these results also require confirmation.

**FIGURE 3.6 – rt-qPCR of TAp63α and ΔNp63α in non-infected and EBV-infected NPC lines.** Graphs summarising the relative mRNA levels of TA-specific and the ΔN isoform of p63 in each NPC cell line, compared to their EBV-infected counterparts at various seeding densities (low density (red), medium density (blue) and high density (green)) \((N = 1; n = 2)\).
In summary, the *in vitro* data including immunoblotting, immunofluorescence and rt-qPCR, are in agreement with one another. EBV infection suppresses p63 expression, particularly that of the ΔNp63 isoform. These results, however, contradict the published literature, which has demonstrated high expression levels of ΔNp63 in NPC in comparison with normal cells/tissue (Crook *et al.*, 2000, Yip and Tsao, 2008).

3.3 High levels of tumour-infiltrating lymphocytes and p63+ and ASPP2+ cells in NPC tumour biopsies

Human undifferentiated NPC tissue biopsies were obtained from the Imperial College Healthcare NHS Trust Tissue Bank to study the role of p63 and its regulator ASPP2 in NPC progression. H&E staining showed a large fraction of poorly-differentiated tumour masses present as nests and lobules. Scattered inflammatory lymphocytes were also present (*FIGURE 3.7*). This biopsy would be classified under the type III subgroup of NPC, according to the WHO guidelines. As expected, NPC tumours were highly heterogeneous in terms of cell types, as almost half of the tumour mass was made up of infiltrating immune cells. Also, there was a degree of invasion of the carcinoma into the surrounding desmoplastic stroma. Next, the expression of the EBV antigen, EBNA-1, was detected in these biopsies to determine whether these NPCs were EBV-associated.
FIGURE 3.7 - Morphological analyses of undifferentiated NPC biopsies. (A) H&E stainings show local infiltration of lymphocytes and plasma cells at the tumour site. (B) NPC is composed of large epithelioid and spindled cells with indistinct borders. (C) Irregular small nests of pleomorphic tumour cells surrounded by dense inflammatory infiltrates composed of lymphocytes, plasma cells and eosinophils. (D) Closer view of a representative area of NPC. 20x magnification for A, B, C; 40x magnification for D. (n = 3)

Methods of EBV detection, such as examination of the presence of EBV DNA or EBER by ISH, are commonly used in the literature. However, EBNA-1 is indispensable for viral genome replication and is maintained in infected cells. It has been previously demonstrated that EBNA-1 is consistently detected in EBV-associated tumour biopsies, while LMP protein expression varies (Young et al., 1988). Consequently, the demonstration of EBNA-1 expression (protein or transcript levels) was deemed sufficient to indicate EBV-positive status in EBV-infected cell lines or a given tumour in this study.
IHC examination for EBNA-1 expression was performed, and it was demonstrated that the NPC tumours were indeed EBV-positive (FIGURE 3.8A, B). EBNA-1 was localised in the nucleus (FIGURE 3.8B) and absent in non-cancerous nasopharyngeal tissue (FIGURE 3.8C, D). It was also observed that EBNA-1 intensity in NPC was variable from one cell to another, suggesting variations in EBV gene expression. Previously, p63 heterogeneity had been demonstrated within the NPC cell population in vitro. Therefore, p63 expression was examined in these biopsies to confirm this and better our understanding of NPC tumour heterogeneity and the degree of epithelial differentiation.
NPC biopsies revealed marked expression of nuclear p63 (FIGURE 3.9A, B), whereas normal nasopharyngeal mucosa tissue displayed weak cytoplasmic p63 immunostaining (FIGURE 3.9C, D). This finding was consistent with numerous studies but did not agree with the previously obtained, in vitro results described above (Guo et al., 2006, Crook et al., 2000).

The role of the ASPP family in NPC has not been previously investigated. Hence its in vivo expression in NPC was characterised. High levels of nuclear ASPP2 were detected within NPC tumours (FIGURE 3.10A, B), while its expression was absent in
normal nasopharyngeal epithelium (**FIGURE 3.10C**). It has been demonstrated that ASPP2 interacts with p53/p63 proteins to stimulate the transcription of pro-apoptotic *Bax* and *Puma* genes. Increased ASPP2 accumulation may suggest the up-regulation of p53- or p63-mediated apoptosis in NPC. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay or co-staining with cleaved caspase 3 should, therefore, be performed to assess apoptotic activity in NPC biopsies. Note that ASPP2 was expressed in the nuclei of cells of smaller size, therefore, it may be expressed by the tumour-infiltrating lymphocytes (TILs) rather than the malignant carcinoma. Hence, further analysis that involves co-staining with lymphocyte or cytokeratin markers is required.

**FIGURE 3.10 - Immunohistochemical analysis of NPC tumour samples for ASPP2.** (A,B) Immunophenotypes of ASPP2 in NPC biopsies obtained using the Dx54.10 antibody. Haemotoxylin is used as a counterstain. (C) Negative expression of ASPP2 in non-malignant tissue. (D) Negative controls (primary antibodies omitted) are used to check the specificity of
the ASPP2 antibody. 20x magnification for A, B, C and E; 40x magnification for D and E. ($n = 1$)

3.4 EBV infection promotes an EMT phenotype in NPC lines

Having demonstrated that p63 is down-regulated upon EBV infection in NPC cell lines (FIGURES 3.3 - 3.6), it was speculated that the loss of p63 in these infected cells could be due to a loss of the epithelial phenotype and the adoption of a mesenchymal morphology. This phenotypic switching phenomenon is termed EMT and is common in tumours (Moreno-Bueno et al., 2008). The advantages of acquiring a mesenchymal phenotype include enhanced cell motility and invasion, which allows tumour dissemination and metastasis. Higher magnification of phase contrast photomicrographs showed that CNE2 acquired a fibroblastoid-like morphology following EBV infection (FIGURE 3.11A, lower panel). The EBV-infected cultures were heterogeneous and consisted of spindle-shaped cells of different size, large flat cells and small round cells. Also, they were disorganised and largely overlapping (loss of contact inhibition) compared to non-infected counterparts.

Although the function of Notch in EMT is unclear, it has been reported that Notch targets or ligands such as Hey1 and Jagged1 could be up-regulated upon TGFβ-induced EMT expression (Zavadil et al., 2004). Uninfected and EBV-infected CNE2 cells were immunostained for Notch1, which is the receptor for Notch ligands, to check for its cell localisation. Notch1 expression was found localised at the cytoplasmic compartment and membranes of both CNE2 and EBV-infected CNE2 cells. Nevertheless, endogenous Notch was expressed at the cell membrane after EBV infection, while it was localised at the perinuclear region in parental CNE2 cells. Also, EBV-infected cells were more polarised with protruding membranes, which is a feature of cell migration (FIGURE 3.11B). Other cell lines had less remarkable
differences in their morphology after EBV infection (data not shown). Several reports in the literature have cited EMT-related events in epithelial cells after EBV infection. For example, EBV has been shown to transform gastric epithelial cells *in vitro* (Nishikawa *et al.*, 1999) and EBV-negative NPC cells (Teramoto *et al.*, 2000).

To confirm further that these EBV-infected cells were undergoing EMT, EMT markers were screened for in these NPC lines using WB and IF methods. During EMT, cells start to lose the expression of proteins that maintain cell polarity and adhesion between cells, such as E-cadherin, β-catenin, claudin and occludin, to increase their motility (Thiery, 2002). In parallel, mesenchymal markers such as vimentin, fibronectin, and EMT inducers such as Snail, Twist and ZEB1, are up-regulated. In the cell lines studied here, a down-regulation of E-cadherin and an up-regulation of mesenchymal markers N-cadherin and vimentin was noted in EBV-infected CNE2 and SUNE1 cells (FIGURE 3.12). There were no significant differences in epithelial or mesenchymal markers in infected CNE1, HNE1 and TW04 cells assayed by immunoblotting. E-cadherin expression in CNE2 cells at the cell membrane was lost after infection, while an increase in vimentin expression in the cytoplasm was observed (FIGURE 3.12B, C). This *in vivo* data further supports the hypothesis. FIGURE 3.13 showed negative expression of E-cadherin in NPC tumours while it was strongly expressed at the membrane and cell-to-cell junctions in non-malignant nasopharyngeal epithelial cells in the same biopsy sections. The transcription factor ZEB1, which participates in developmental EMT, was also expressed in the nuclei of NPC tumours (FIGURE 3.14A, B).
FIGURE 3.11 - Induction of EMT by EBV infection. CNE2 cells are infected with EBV and allowed to grow on coverslips for two days before morphological analysis (A) or immunostaining with Notch (B). $(n = 1)$
FIGURE 3.12 - EBV promotes EMT by down-regulating epithelial markers and inducing the expression of mesenchymal markers. (A) Parental NPC and their EBV-infected counterparts are screened for E-cadherin, N-cadherin and vimentin expression. β-actin is used as a loading control for protein determination. (B) E-cadherin expression at the cell junctions is lost after EBV infection in CNE2 cells. (C) The intensity of the IF of vimentin is increased in CNE2 and SUNE1 cells following EBV infection. Nuclei are counterstained with DAPI. (n = 1)

FIGURE 3.13 - Representative photomicrograph showing E-cadherin expression in NPC samples. Loss of E-cadherin expression is noted in NPC tumours (A and B). In contrast, the non-malignant section in the NPC biopsy displays diffuse E-cadherin expression (C and D). (n = 3)
FIGURE 3.14 - Representative photomicrograph showing ZEB1 expression in NPC biopsies. Strong nuclear ZEB1 expression is detected in NPC tumours (A and B). In contrast, ZEB1 expression is absent in non-cancerous nasopharyngeal tissue (C and D). \((n = 1)\)
3.5 Discussion

Viruses account for more than 10% of tumour incidence, thus there is a need to understand viral-mediated oncogenesis, especially with regard to how normal cellular signalling pathways are perturbed (Schiller and Lowy, 2014). EBV infects most individuals but only causes NPC in specific populations, mostly Asians in Southern China, Hong Kong, Taiwan, Malaysia and Singapore. It is speculated that the aetiological factors in multistep NPC pathogenesis include genetic predisposition, dietary environmental exposure to salted food and EBV infection.

NPC is a malignancy of epithelial origin and it is well known that p63, a member of the p53 family, plays an important role in epithelial differentiation and stratification. This prompted investigation of what role p63 plays in NPC pathogenesis. It has been suggested that p63 or ΔNp63 can be used as a diagnostic or prognostic marker for NPC, as accumulation of nuclear p63 expression in NPC tissue biopsies has been observed (Guo et al., 2006, Crook et al., 2000). Indeed, heterogeneous p63 expression was detected in this study in the nuclei of malignant NPC tumours (FIGURE 3.9). Conversely, in vitro studies demonstrated an inverse relationship between the expression of p63 and EBV infection. Specifically, ΔNp63 expression was shown to be down-regulated after EBV infection. The mechanism behind the lack of p63 expression is yet to be identified, although there are several possibilities. These include p63 protein degradation or promoter hypermethylation, which would result in low or no gene transcription. Similarly to p53, mutation of the p63 gene is rare in undifferentiated NPC, therefore, it is unlikely that mutations abrogate p63 transcription. Despite the fact that several studies have been performed, very little is known about the precise functional significance of p63 in NPC, its regulation and downstream pathological consequences. Our hypothesis is that p63 or ΔNp63
behaves as a metastasis inhibitor, and that its loss of expression facilitates NPC development by increasing cell motility and invasion. Hence, this phenomenon is associated with more aggressive, metastatic cells, while non-invasive primary NPC tumours retain p63 expression. Comparison of type I NPC or low-grade NPC with aggressive advanced NPC (or distant metastases) may shed light on the molecular circuits linking p63 and the progression of NPC.

FIGURE 3.15 - Physiological role of p63 in the morphogenesis and maintenance of epithelial tissue. (A) ΔNp63 accumulates at the basal layer of the stratified epidermis, is involved in the self-renewal of epithelial stem cells and favours cell proliferation. In contrast, committed epithelial progenitors located at the suprabasal layers weakly express TAp63. (B) The TAp63 isoform can co-operate with p53 to transactivate downstream pro-apoptotic genes and is, hence, a regulator of cell death and the cell cycle. ΔNp63 acts in a dominant-negative manner to inhibit TAp63 or p53-mediated apoptosis by blocking the DNA binding site at the target promoter, or by sequestering TAp63 or p53.

In our hypothesis, the decreased expression of p63 in EBV-infected cell lines is associated with the onset of EMT. During EMT, epithelial cells lose their epithelial properties by reducing their cell polarity while up-regulating their mesenchymal gene expression signatures, to facilitate cell migration and invasion. Our experiments
showed that altered cellular morphology and the expression of EMT markers, N-cadherin and vimentin, occurred in NPC cell lines, particularly CNE2 and SUNE1 cells (FIGURE 3.12). Further analysis of the EMT markers by rt-qPCR would further confirm the western blot and immunofluorescence data. A recent study has demonstrated that ΔNp63-mediated miR205 induction blocks EMT by repressing the transcriptional activity of ZEB1/2, a master regulator of EMT (Tran et al., 2013). Furthermore, Fang et al. have reported the induction of an invasive phenotype in NPC cell lines upon chemical EBV reactivation (Fang et al., 2012). To further test this hypothesis, EMT markers’ expression should be analysed following p63 knockdown by siRNA in parental NPC cells.

Previous findings reported that EBV latent antigens such as LMP-1 and LMP-2A can regulate the NF-κB pathway, which can initiate the tumour-promoting machinery by elevating the gene transcription of genes involved in cell proliferation, blocking apoptosis and cell invasion. NF-κB is usually present in the cytoplasm in an inactive form. Phosphorylation of NF-κB, for example at S276 on p65, leads to the translocation of the NF-κB dimers into the nucleus, where they activate various transcriptional programmes (Arun et al., 2009). A recent study in our lab has shown crosstalk between NF-κB and p63 pathways (Tordella et al., 2013), demonstrating that p63 levels are suppressed by ASPP2-mediated p65 activation. Intriguingly, the immunoblots in this study showed no significant change of p65 levels after EBV infection (FIGURE 3.2). However, it would be of interest to compare expression levels of activated or phosphorylated p65, as well as the cellular localisation of p65, in EBV-infected NPC cell lines and biopsies.

The observation of the presence of the EBV latent antigen EBNA-1 in undifferentiated NPC tumour biopsies confirmed a significant association of NPC with EBV (FIGURE 3.8). NPC tumours were highly heterogeneous in terms of cell types
within the tumour mass and latent EBV gene expression. Nearly half of the tumour density could be attributed to cellular lymphoid infiltration, and there is increasing evidence that these inflammatory cells play a role in the facilitation of NPC progression. Heterogeneity in the microenvironment adds to the complexity of cancer development. There may be potential interplay between apoptotic factors, immune sensing and the dysregulation of host cellular signalling pathways. ASPP2 was detected to be nuclear in NPC biopsies (FIGURE 3.10), while it was retained in the cytoplasm in vitro (data not shown). As previously mentioned, NPC tumours are comprised of various cell types, including immune cells. Therefore, there is a need to address which cells within the tumour, either the infiltrating lymphocytes or malignant cells, are expressing high levels of nuclear ASPP2. Co-staining of p53/p63 and ASPP family proteins with epithelial or immune markers would elucidate the different subpopulations within NPC tumours.

Epithelial cell lines established from EBV-positive NPCs inevitably lose their EBV genomes by exocytosis on serial passaging (Chen et al., 1999). In the experiments described here, this obstacle was overcome by infecting recombinant EBV in these cell lines. However, the recombinant system that was utilised was the BL-derived Akata’s strain, a type I EBV strain. EBV isolated from the Chinese NPC patients exhibit genetic divergence to the Western strains and are more epithelial-tropic. The Akata’s strain was selected due to its ability to produce a high titer of EBV production. Infection of the cell lines using the type II NPC-derived EBV strains such as GD1, GD2, HKNPC1 and M81 may be more reflective of NPC tumours (Kwok et al., 2012, Tsai et al., 2013).
EBV down-regulates p63 expression levels, particularly the ΔNp63 isoform, as well as the epithelial marker E-cadherin in NPC cells. On the other hand, mesenchymal markers such as N-cadherin and vimentin are up-regulated. EBV can induce an EMT-like phenotype via p63-mediated suppression. The expression of the tumour suppressor ASPP2 is induced in NPC biopsies and may play a role in NPC pathogenesis through immune regulation or the stimulation of apoptosis.
CHAPTER IV: IMMUNOPATHOGENESIS OF TUBERCULOSIS

4.1 Distribution and kinetics of mycobacterial accumulation following intranasal administration of BCG in BALB/c mice

From previous work in our lab, it has been shown that LPS-TLR4 stimulation can induce ASPP2 signalling in a STAT1-dependent pathway (Turnquist et al., 2014). ASPP2 mRNA transcript levels are up-regulated in macrophages of patients suffering from inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and tuberculosis. The importance of ASPP2 as a gatekeeper to inflammation is supported by the fact that ASPP2 is targeted and its pro-apoptotic function disrupted by CagA, a secreted toxin of the bacterium *H. pylori* (Buti et al., 2011). ASPP2 contributes to the host’s defence against pathogens by inducing apoptosis through the activation of STAT1-dependent signalling in response to LPS and IFNγ. Through collaboration with the Jenner Institute, University of Oxford, lung biopsies were obtained from mice that were infected with virulent *Mtb*, and a mouse BCG infection study was set up to investigate whether activation of ASPP2 could be observed upon inflammatory stimuli.

First, the infection kinetics of intranasal BCG vaccination in BALB/c mice was examined and the morphological changes of their lungs investigated in order to understand the cellular basis of lung immunity against bacterial pathogens.
FIGURE 4.1 – Bacterial load in lungs and spleens of BALB/c mice after intranasal administration of BCG. Mice are sacrificed at specific time points, their organs excised, homogenised and plated in serial dilutions on 7H11 agar plates for 4 weeks. Enumeration of bacterial counts in each organ is carried out and each colony represents a "colony-forming unit" (c.f.u.). Data are expressed as median and error for two to three mice per time point (1, 7, 14, 21 and 28 d.p.i.).
FIGURE 4.1 shows the pulmonic and splenic bacterial burden changes during the infection period of 28 days. Only half of each of these organs: lung, spleen and liver, was collected for the enumeration of bacterial load as the other halves was used for immunoblotting and immunohistochemistry (IHC). Therefore, the figures reported represent the number of colony-forming units (c.f.u.) from half of each of these organs. With the assumption that viable BCG was distributed equally within the organ (for example, the right lung received the same amount of BCG as the left lung), the bacterial burden can be multiplied by a factor of 2 to obtain the total bacterial count.

The mice showed high bacillary numbers in the lungs at early time points ($4 \times 10^5$ c.f.u.) (FIGURE 4.1, upper panel). An average of 16% of viable bacilli was deposited in the lungs from the total dose given. The bacterial load in the lungs peaked 7 days after BCG challenge (between $6$ and $7 \times 10^5$ c.f.u.) and remained high at about $4 \times 10^5$ c.f.u. throughout the course of infection (14 - 21 days post infection (d.p.i.)). Pulmonary clearance did not happen until 28 days after infection, at which point the number of bacteria dropped slightly to $1.5 \times 10^5$ c.f.u. It is believed that the half-life of bacteria retention in the lungs is dependent on the effector mechanisms of the recruited inflammatory mediators. The pattern of infection in the spleen differed from that in the lungs. Bacterial load was extremely low ($30 - 40$ c.f.u.) during the early days of infection but increased steadily over time. Peak bacterial growth in the spleen was evident by day 21 p.i. (between $2$ and $3 \times 10^3$ c.f.u.) and the bacillary numbers stabilised from day 21 to 28. Note that the lungs displayed significantly higher bacterial load (at least 2 log higher) compared with the spleen. Mycobacterial burden in the murine liver was below detection limits, perhaps due to absence of bacteria in this organ, and was therefore not included in the analysis.
4.2 BCG-infected mice develop pulmonary granulomas

Next, the progression of granuloma formation in these organs was assessed over time. Granulomatous reaction is a characteristic feature of *Mtb* infection and is mediated by the ordered recruitment of inflammatory mediators. It is initiated by the immune cells involved in the first line of the host’s defence (innate immunity), such as monocytes, macrophages and DCs. It is also believed that granuloma formation is the cost of immunopathological damage, which in turn causes fibrosis in the affected areas. As shown in FIGURE 4.2, the mouse experimental model was responsive to mycobacterial infections in comparison with non-infected controls, and pulmonic granulomatous lesions were observed over the course of BCG infection.
FIGURE 4.2 – Serial lung histopathological changes in BCG-challenged BALB/c mice.

(A) Representative H&E staining shows mononuclear inflammatory infiltration, tissue fibrosis and the formation of “tubercles” or granulomas in the lungs. The mice are infected with $5 \times 10^6$ c.f.u. of BCG Pasteur strain via intranasal route and sacrificed at the indicated time points ($n = 3$ for each experimental group). Non-infected mice are used as controls ($n = 5$).

(B) 40x magnification of the granulomatous lesion from the 28 d.p.i. figure panel.

Lung sections from groups of mice infected with BCG for at least 14 days showed extensive granuloma formation, with sizes increasing over the period following infection. The microarchitecture of these granulomas was formed by an accumulation of cells with low nucleo-cytoplasmic ratios, surrounded by clusters of mononuclear cells with high nucleo-cytoplasmic ratios (FIGURE 4.2A). On the basis of their morphological features, cells with larger cytoplasms and less condensed nuclei are often considered as macrophages, and the smaller cells as lymphocytes. This was further confirmed by a histopathologist, and by using IHC markers of immune cells such as CD3 for T-lymphocytes, CD45R/B220 for B-lymphocytes and F4/80 or iNOS for macrophages (FIGURES 4.4 & 4.5A). Compared with day 14, the granulomas formed after day 21 had denser lymphocytic infiltrates. Note that the granulomas were formed in close proximity to respiratory bronchioles and alveoli. Normal, healthy
mouse lungs had large distal air spaces between the alveolar walls, which gave the lungs a spongy appearance. In mice infected with BCG, infiltration of immune cells led to the formation of granulomatous lesions within decreased lung parenchyma volume and consolidation of the air cavities, which in turn contributed to interstitial scarring of the lungs. Lung fibrosis can lead to the progressive destruction of tissue, loss of respiratory function and eventual death of the animal (Marquis et al., 2008). Granulomas formed in the experimental system described here did not exhibit a typical prominent lipid-rich, necrotic caseum core and extensive mineral deposition and, therefore, did not truly replicate the human TB condition. Also, this mouse model of infection resembled acute infection, and not latent or reactivated TB. Scattered foci of granulomatous inflammation varied in size and the number of granulomas formed at each time point was further examined on the basis of macrophage infiltration (FIGURE 4.5).
FIGURE 4.3 - Representative liver histological sections from control and BCG-infected BALB/c mice stained with H&E. (A) Representative H&E staining shows no or rare inflammatory inflammation in the liver of the BCG-infected mice. The mice are infected with 5 x 10^6 c.f.u. of BCG Pasteur strain via intranasal route and sacrificed at the indicated time points. (B) Small foci of inflammatory cell infiltrates are detected in two hepatic specimens at day 21 and 28 (40x magnification). These results are representative of three mice per group, except for non-infected controls (n = 5).

On the other hand, there was no evidence of microscopic granuloma formation in the spleen following H&E histological examination. There were only small numbers of mononuclear cells present in the livers of two infected mice, despite 15 animals being challenged with BCG (FIGURE 4.3B). Histological examination of the liver showed tiny clumps of lymphocytic infiltrates surrounded by liver parenchyma. Accumulation of epithelioid macrophages was not seen in these infiltrates. No necrotic areas were observed and there were no marked differences in the histological appearance of the liver between the majority of the BCG-challenged mice and non-infected controls. The lack of granulomatous lesions was in agreement with an earlier finding that viable bacilli could not be detected in the hepatic tissues. Since granulomas were generally absent in the liver (FIGURE 4.3) and spleen (data not shown), further investigations were focused on pulmonary biopsies.
4.3 Cell-mediated immune response elicited in both BCG and *Mtb*-infected mice

In general, mice showed higher susceptibility and infectivity to more virulent strains of *Mtb* infection compared to *M. bovis* BCG. Lung specimens from *Mtb*-infected mice were shown earlier to have an exacerbated granulomatous response, with extensive areas of parenchymal infiltration (FIGURE 4.4). These positive control sections contained huge fibrotic areas populated with a mixture of innate and adaptive inflammatory mediators. To gain insight into the granuloma-infiltrating cells that culminated in their progression and establishment, *Mtb*-infected lung sections were stained for various immune recognition molecules. A mixture of CD45R/B220+ B-lymphocytes and CD3+ T-lymphocytes was detected in granulomatous areas after IHC examination. Both markers were highly expressed in the cytoplasm by cells with a high nucleo-cytoplasmic ratio (FIGURE 4.4A). Recruitment of inflammatory cells that contribute to the formation of granulomas was strictly localised in the pulmonary vessels and bronchi. Negative control studies with the primary antibody omitted showed no positive staining. Thus, the evidence presented here suggests that *Mtb* infection induces B and T-lymphocyte recruitment, proliferation and activation to promote inflammation at the infected site.
FIGURE 4.4 – Inflammatory mediators enriched at granulomatous lesions in the lung parenchyma of mice infected with *Mtb* for 4 weeks. (A) Top panel confirms no staining in the negative control (primary antibody omitted). Middle panel indicates the presence of B- and T-lymphocytes throughout the lesions, as shown by anti-CD45R/B220 and anti-CD3 stainings. The bottom panel depicts enlarged images of areas indicated by the middle panels in white boxes. (B) Comparison of the expression of macrophage phenotypic markers on pulmonary granulomatous regions.

As previously reported in the literature, macrophages and DCs are one of the first cell types of the innate immune response to be recruited to the inflamed site following BCG or *Mtb* infection. Thus, the infiltration of macrophages in lung granulomas was examined. Several markers are widely used to identify macrophage populations, hence, a list of antibodies was screened against paraffin-embedded sections from mice infected with *Mtb* for 28 days: CD11b, CD68, iNOS, MAC and F4/80. CD11b belongs to the integrin family and contributes to various adhesion and migration interactions of leucocytes, including monocytes, macrophages, neutrophils, NK cells and granulocytes. CD68 encodes an 110kD transmembrane glycoprotein involved in phagocytic activity and is generally expressed by monocytes and macrophages. iNOS plays a role in the conversion of L-arginase to NO, which is responsible for the stimulation of respiratory burst and M1 macrophage activation. The MAC antibody detects L1/calprotectin antigen expressed intra-cytoplasmically within granulocytes, monocytes and tissue macrophages. Finally, F4/80 is the most commonly used antibody to stain for murine macrophages, as it is able to identify a variety of macrophage subsets, including brain microglial cells, liver Kupffer cells, splenic red pulp cells, bone marrow macrophages and lymph-node medullary macrophages.

It was observed that CD11b expression was absent, while CD68 showed weak staining in positive controls (FIGURE 4.4B). The rabbit-raised F4/80 antibody showed diffuse moderate staining and there were significant expression levels of
iNOS and MAC in cells with high cytoplasmic/nuclei ratios within the granulomas. The expression of these markers (CD68, iNOS, MAC and F4/80) was found exclusively in the cytoplasm of the cells. For the purpose of double IF staining with mouse ASPP2 (using the S32 antibody which was raised in rabbits), a F4/80 antibody raised in rats was obtained. However, this antibody showed negative staining for macrophages in positive control tissue (**FIGURE 4.4B**). Due to the inconsistency of the two different F4/80 antibodies, it was concluded that both iNOS and MAC antibodies (both raised in mouse) were suitable for double IF labelling with ASPP2.

4.4 BCG-infected macrophages express iNOS within TB lung lesions

It has been reported that the development of adaptive immunity occurs following the priming of mycobacteria-specific antigens to T-lymphocytes at the lung-draining lymph nodes. The expression phase of adaptive immunity in the murine experimental model used in this study seemed to occur after 14 days in response to BCG challenge. The presence of a subpopulation of CD3+ T-lymphocytes was observed at day 14 p.i. (**FIGURE 4.5A**). T-lymphocyte levels were extremely low or absent between days 1 and 7, and in non-infected controls. Following their activation in the MLN, the T-lymphocytes extravasate into the lungs to encounter the pathogen. A delay of at least 14 days was observed before the adaptive immune effector took effect. In mice challenged with the virulent *Mtb* H37Rv, CD4+ T-lymphocytes slowly accumulated but only peaked at around day 20, after which the number remained constant (Wolf *et al.*, 2008). It has been previously demonstrated that prior BCG vaccination in mice leads to an accelerated recruitment of T-lymphocytes in the
lungs, and significantly lower mycobacterial burden (Connor et al., 2010). Note the mutually exclusive staining pattern between CD3 and iNOS in the lung granulomas (FIGURE 4.5A). This indicated that two distinct populations of T-lymphocytes and macrophages existed within the granulomatous lesions. An increase of M1 macrophage population in the lungs was observed over the course of infection. The inflammatory infiltrate was predominantly populated by iNOS+ cells, suggesting polarisation of macrophages to the M1 phenotype, which was thought to occur in a pro-inflammatory conditioning microenvironment. However, quantification of cell numbers or phenotypic analysis by FACS is necessary to draw conclusive interpretations for these observations. To score the number of granulomas from each tissue specimen, iNOS was used as a marker to characterise the macrophage subpopulation. FIGURE 4.5B shows that there was no demonstrable granuloma in lung sections from non-infected controls and BCG-infected mice from days 1 - 7. The size and number of pulmonary granulomas increased up to day 28. This correlated with disease progression and severity. The preliminary results showed that the experimental murine BALB/c model was sensitive to stimulation by BCG.
FIGURE 4.5 – **Induction of granulomatous lesions after BCG infection.** (A) IF analysis of paraffin-embedded murine pulmonary sections from BCG-infected mice on days 1, 14 and 28 p.i. showing the distribution of immune cells. There is a moderate amount of iNOS+ macrophages (red) surrounded by CD3+ T-lymphocytes (green) on day 28 p.i. compared with day 14 p.i. Nuclear counterstain is DAPI (blue). (B) The number of pulmonary granulomas per tissue is enumerated by counting clusters of macrophages, as depicted in (A – 28 d.p.i.). Progressive fibrosis is observed over time. Data shown (median with range) are representative of \( n = 3 \).

4.5 Induction of p53 and ASPP2 in cells expressing iNOS within TB granulomas

Recent data have indicated that ASPP2, the gatekeeper of inflammation and cancer, is induced in macrophages and brain cells upon LPS stimulation (Turnquist et al., 2014). Thus, the role of ASPP2 on the effector mechanism of \( Mtb \)-elicited responses in the lungs of BCG-challenged mice was investigated. As shown in **FIGURE 4.6**, upper panel, ASPP2 was highly expressed in the lungs, especially in the cytoplasm of cells with low nucleo-cytoplasmic ratios, though a limited amount of cells expressed nuclear ASPP2. This observation raised the question of whether ASPP2-expressing cells are macrophages or other cell types. By staining an adjacent tissue section, a prominent accumulation of F4/80+ cells (macrophages) was demonstrated in the same region of ASPP2+ cells within the granuloma. Hence, it was speculated that macrophages recruited to the granulomatous lesions up-regulate ASPP2 expression. Induction of ASPP2 at the inflamed site can lead to apoptosis, an effective host defence strategy for bacterial clearance. To further support this hypothesis, double IF staining was performed for ASPP2 and iNOS (M1 macrophage marker). Preceding studies have shown that, during acute TB infection, monocyte-derived macrophages become classically activated and up-regulate iNOS expression (Benoit et al., 2008, Quiding-Jarbrink et al., 2010).
FIGURE 4.6 – Induction of the pro-apoptotic protein ASPP2 (upper panel) and macrophage marker F4/80 expression (lower panel) in murine lungs after BCG treatment for 28 days. Right panels depict enlarged images of areas indicated by the left panels in yellow boxes. Microscopic magnifications are 10x, 20x and 40x.
FIGURE 4.7 – iNOS+ macrophages within pulmonary granulomas in mice challenged with BCG for 4 weeks co-express the pro-apoptotic markers ASPP2 and p53, while CD3+ cells do not. Co-staining of iNOS with ASPP2 (A, B) and p53 (C) shows co-localisation of iNOS (green) with both markers. Enlargements of areas near the yellow arrow are shown in (B). Triple staining of CD3 and iNOS/ASPP2/p53 shows expression in distinct cell populations (depicted in the yellow circle in A). It is not possible to perform double staining between p53 and ASPP2 because both antibodies were raised in the same animal species (rabbit). DAPI (grey) is used to visualise the nuclei.

The expression levels of ASPP2 and p53 were compared in lungs collected from BCG-infected mice with positive Mtb-infected murine lung specimens, both at 4 weeks p.i. This study previously demonstrated that iNOS-expressing macrophages were interspersed with T-lymphocytes. As shown in FIGURE 4.7, the data suggested that ASPP2 and p53 co-localised in iNOS-expressing cells and not CD3+ cells. The induction of these two pro-apoptotic proteins in Mtb-infected mice was reminiscent of that observed in BCG-infected mice (FIGURE 4.8). Note that ASPP2 and p53 staining revealed a distinctive irregular punctate/granular staining pattern that coincided with the cytoplasmic distribution of iNOS. This staining pattern of ASPP2 and p53 was consistent in both BCG- and Mtb-infected tissue biopsies. Expression of p53 and ASPP2 was generally low or absent in non-infected mice and infected mice day 1 - 7 p.i. In addition, there was a lack of iASPP expression in these infected biopsies (data not shown). In summary, ASPP2 and p53 expression was mainly detectable in granulomatous regions with macrophages following mycobacterial stimulation. Thus, these findings strongly suggest that ASPP2 and p53 participate in the host’s defence against invading microbial pathogens.
FIGURE 4.8 – ASPP2 and p53 are up-regulated in macrophages within the pulmonary granulomas of mice infected with *Mtb* for 4 weeks. Regions expressing iNOS (red) are always positive for ASPP2 (green) (A) and p53 (green) (B). DAPI (blue) is used to visualise the nuclei. Higher magnifications are shown in the right panels.
4.6 STAT1 nuclear translocation in iNOS+ cells in *Mtb*-infected lung specimens

Next, the upstream signal transduction pathway or molecular mechanism that leads to the activation of p53/ASPP2 was investigated. As reported in a recent publication, LPS stimulation induces STAT1-dependent ASPP2 expression in macrophages *in vitro* (Turnquist *et al.*, 2014). STAT1 is an interesting target to study in these infected tissue samples as it can be activated and is a downstream effector of IFN-γ signalling. In addition, STAT1 directly augments ASPP2 signalling. It has been established that IFN-γ has a massive impact on macrophage functionality and can modulate T-lymphocyte-mediated apoptosis. In this study, differential phosphorylated STAT1 (p-STAT1) cellular localisation was observed in different inflammatory cell clusters (FIGURE 4.9B). In general, p-STAT1 was highly expressed in *Mtb*-infected pulmonary specimens. Macrophages displayed nuclear localisation of the serine-p-STAT1. In higher cell density regions, which were populated by B- or T-lymphocytes, STAT1 was retained cytoplasmically. Co-localisation of p-STAT1 with the nuclear marker DAPI was absent in B- and T-lymphocyte-rich areas.
FIGURE 4.9 – Macrophage clusters within the granulomas reveal marked expression of nuclear phosphorylated STAT1. Paraffin-embedded lung sections from mice infected with Mtb for 28 days are stained with anti-iNOS and anti-p-STAT1 antibodies. p-STAT1 expression (green) co-localises with the nuclear marker in iNOS+ macrophages (red) (shown with white arrows). As shown in (B, lower panel) (orange arrows), p-STAT1 is rarely detected in the nuclear compartment of other cell types. DAPI (blue) is used to visualise the nuclei. (B) depicts enlarged images of areas indicated in (A) in white and orange boxes. Macrophage-rich areas are shown in white boxes, while lymphocyte-rich areas are shown in orange boxes.

4.7 BCG infection up-regulates iNOS expression in the lungs, but not that of ASPP2, as revealed by total protein western blot analysis

Subsequently, the effects of BCG infection were examined on the regulation of ASPP2 and iNOS protein levels by western analysis. In the BCG experimental model, no significant change in expression levels of ASPP2 were detected by western blotting throughout the course of infection (FIGURE 4.10). Although induction of ASPP2 was observed in the cytoplasm of macrophages in the granuloma (FIGURE 4.6), ASPP2 was generally highly expressed in the lungs. Hence, it was postulated that ASPP2 induction within the granuloma would be hard to detect if whole lung homogenates were used. iNOS levels were more abundant in the lungs of mice challenged with BCG post 21- 28 p.i., suggesting the presence of macrophages, or a polarisation of macrophages towards the M1 subset in response to mycobacterial stimulation. Increased expression of iNOS correlated with previous IHC findings and its contribution to both protective innate immunity and immunopathological damage in the form of granuloma formation.
FIGURE 4.10 – Western blot analysis of pulmonary ASPP2 and iNOS expression in BCG-infected mice. Whole lung lysates from BCG-infected mice are prepared using RIPA buffer. These are resolved by SDS-PAGE electrophoresis and membranes immunoblotted using ASPP2 (S32) and iNOS antibodies. β-actin is used as loading control.
4.8 Effects of bacterial virulence factors on the expression levels of pro-inflammatory and apoptotic mediators

Previous reports in the literature have shown the importance of macrophages in mediating innate defence mechanisms against mycobacterial infections. Thus, the feasibility of using human monocytic THP-1 cells as an in vitro model, with which to study the effects of mycobacterial cell wall components on the expression of the proteins of interest, was tested. It has been reported that the recognition of mycobacterial constituents is primarily mediated by TLR2, though other TLRs, such as TLR4 and TLR9, are thought to play a non-redundant role in *Mtb* sensing. TLR2 signalling utilises similar downstream effectors, such as the adapter protein myD88, to TLR4 signalling, which results in the activation and nuclear translocation of the pro-inflammatory transcription factor NF-κB. The mycobacterial cell wall and its associated structures play a crucial role in host-pathogenic interactions, and can mediate the immunoregulatory functions of macrophages and lymphocytes. A preceding study has demonstrated that ManLAM can inhibit apoptosis via Ca\(^{2+}\)-dependent signalling (Rojas *et al.*, 2000). It was therefore of interest to examine whether certain components from the *Mtb* cell wall were able to affect the activity of apoptotic regulators, such as ASPP2 and iASPP, in macrophages.

Two different mycobacterial virulence factors were utilised: fractions from the CWS and lipoarabinomannan (LAM), a major lipoglycan in the cell wall. LPS was used as a positive control in macrophage activation. Previous work in our lab has shown that ASPP2 is up-regulated upon LPS stimulation in both human THP-1 and murine RAW24.7 cells (Turnquist *et al.*, 2014). In this study, a range of concentrations was used and, as negative control, cells were incubated with PBS in complete RPMI (the bacterial immunostimulatory components were constituted in PBS).
The gram-negative LPS induced a vigorous pro-inflammatory response, characterised by an up-regulation of the phosphorylated NF-κB p65 subunit and phosphorylated STAT1 in a dose-dependent manner (FIGURE 4.11A). Following LPS stimulation, endogenous ASPP2 expression was shown to be up-regulated and peaked at 5ng/ml. However, ASPP2 expression reverted to background levels at higher concentrations (25 – 100ng/ml). As a negative control, iASPP expression remained unchanged with increasing amounts of LPS. This confirmed the specificity of induction of ASPP2, and not iASPP, by LPS.
FIGURE 4.11 – Effects of bacterial antigens on the expression of pro-inflammatory effectors and apoptotic regulators in activated THP-1 cells. Human THP-1 cells are activated in 100ng/ml PMA for 72 hours before stimulation them with different concentrations of various bacterial constituents: LPS (A), CWS (B) and LAM (C). Equal amounts of cell lysates are analysed by western blotting using the antibodies indicated. β-actin is used as loading control (n = 1).
The two different mycobacterial cell wall components (LAM and CWS) appeared to have different stimulatory capacities. Pro-inflammatory NF-κB was up-regulated following CWS stimulation, while phosphorylated STAT1 levels did not change significantly. LAM was able to activate both NF-κB and STAT1 but was not able to potentiate STAT1 signalling at higher concentrations (FIGURE 4.11C). ASPP2 protein levels peaked at 5 to 25ng/ml CWS or LAM, while its expression levels were low at higher concentrations. This may indicate that mycobacterial constituents are able to inhibit apoptosis by inhibiting ASPP2’s activity via an NF-κB- and STAT1-independent pathway. iASPP expression was weak in activated THP-1 cells and was not up-regulated following LAM stimulation. On the other hand, CWS was able to induce the expression of iASPP at lower concentrations. However, this experiment needs to be repeated for conclusions to be drawn on the effects of bacterial antigens on the expression of ASPP family proteins.
Manipulation of the death modality of infected APCs is a common strategy by which intracellular pathogens such as *Mtb*, *Listeria monocytogenes* and *Yersinia pestis* evade the host’s immune system. Therefore, in this study, the role of the ASPPs, (primarily ASPP2) was explored in inflammatory signalling in a cellular context, and in vivo following mycobacterial challenge.

In the experimental BALB/c mice study, it was demonstrated that BCG infection resembles acute TB infection, and an early inflammatory response was mounted with recruitment of a mixture of lymphocytes and macrophages in the lungs. This indicated that the mice were sensitive to BCG infection and a granulomatous response was observed at day 14 p.i. (FIGURE 4.5). This was supported by the presence of large numbers of bacteria in the lungs during early infection time points (FIGURE 4.1). The current animal model failed to mimic some aspects of human TB. Human granuloma is composed of a central caseous necrotic core with a "cheese-like" appearance, populated with *Mtb*-containing macrophages. The granulomas formed in our study did not possess a lipid-rich caseous core. The strain of the mice used could have impacted on the TB’s pathogenesis and affected the expression levels of the protein of interest, p53/ASPP2. Mice of resistant strains, for example C57BL/6 and BALB/c, are more able to control TB infection and generally live twice as long as more susceptible strains such as DBA/2, C3H and 129/SvJ.

Macrophage and T-lymphocyte infiltration correlated with the progression of the pulmonary granulomas. Reduction of bacillary numbers was observed in the lungs at day 28 p.i. There are several possible explanations for bacterial clearance: cytotoxic killing, autophagy, apoptosis or dissemination of BCG to the spleen or other organs.
The data outlined here suggest that small amounts of *Mtb* were indeed trafficked to the spleen. However, the low bacterial count was unable to cause granulomatous reactions in the spleen and liver. Notably, inhibition of mycobacterial growth in the lungs as depicted by a drop in bacillary numbers at day 28 p.i., coincided with the presence or activation of CD3+ T-lymphocytes in the lungs at day 21 p.i. This may indicate T-lymphocyte-mediated killing of the mycobacteria and could be further investigated by measuring the expression levels of cytotoxic mediators such as perforin or granulysin.

IHC examination showed up-regulation of pro-apoptotic p53 and ASPP2 within the lung granulomas, and their co-localisation with the pro-inflammatory M1 macrophage marker (*FIGURES 4.7 & 4.8*). This suggests that inflammatory stimuli could activate apoptosis via a p53/ASPP2-dependent signalling pathway. Interestingly, in this study, p53 and ASPP2 induction was detected in the lungs of mice infected with a virulent *Mtb* strain, suggesting similar activation of p53/ASPP2 in various mycobacterial strains.

It would, therefore, be of interest to compare bacteria growth rates and granuloma formation in p53- or ASPP2-deficient mice infected with either the attenuated or virulent strains. The strain and number of bacteria (initial challenge and further doubling), as well as the ability of host inflammatory mediators to restrict spread of infection, especially macrophages, will determine TB pathogenesis in these infected mice. Future studies should also address the time kinetics of the induction of both innate and adaptive immunity, and this could be achieved by measuring the infiltration of macrophages and lymphocytes by flow-activated cell sorting (FACS), which is more quantifiable compared with histological analysis. It is crucial to understand what role ASPP2 plays in the mycobacterial infection context. ASPP2 is known for regulating apoptotic cell death, and it is highly likely that it mediates
apoptosis in mycobacteria-infected macrophages in an attempt to resolve the infection. This could be investigated by detecting activated caspase 3 in tissue biopsies. Caspase 3 is a downstream effector of both intrinsic and extrinsic death signalling pathways and executes the apoptosis process. Likewise, the TUNEL assay is suitable for the detection of apoptosis.

Although the total levels of ASPP2 appeared to be relatively similar in non-infected and BCG-infected mice in western blot analysis (FIGURE 4.10), the possibility remains that ASPP2 levels are up-regulated specifically in the cytoplasm of recruited macrophages within granulomas. Hence, a method needs to be identified that can isolate granulomas from other pulmonary cells. It may be possible to isolate granulomas by FACS sorting of inflammatory mediators, since they populate the granulomatous lesions. Prior cell permeabilisation should be performed before staining for intracellular markers such as ASPP2 and p53.

In this study, first steps have been made to dissect the molecular mechanisms linking pathogen-associated inflammation and ASPP2 induction. STAT1 is one of the cellular signalling mechanisms that mediate ASPP2 activation. Here, nuclear localisation of phosphorylated STAT1 was observed within the macrophage cluster, not within the lymphocyte cluster (FIGURE 4.9). This finding suggests a role for STAT1 activation in host-mediated immunity. Activation of STAT1 and NF-κB was also assessed in mycobacterial-stimulated macrophages in vitro. In agreement with the published literature, LPS stimulation of PMA-treated THP-1 cells resulted in the prominent activation of STAT1 and NF-κB (FIGURE 4.11). Striking differences in phosphorylated STAT1 and NF-κB levels were not observed, although ASPP2 was seemingly up-regulated after incubation with low concentrations of LAM and CWS. The results obtained in this preliminary experiment, however, were not conclusive and therefore should be repeated. Furthermore, it may be useful to generate TLR2,
TLR4 or NF-κB reporter cells to test the immunostimulatory activity of the mycobacterial components. In addition, induction of pro-inflammatory cytokines can be detected by rt-qPCR. Finally, the link between ASPP2 and STAT1/NF-κB needs to be further investigated in order to have a better understanding of the immune effector signalling pathways and their role in containing mycobacterial infection.
As stated in the 'aims & objectives' section, this study sought to increase our understanding of the role of ASPP2 in pathogenic infection, particularly in EBV and Mtb, which are aetiological agents of NPC and tuberculosis respectively. Pathogenesis of these infectious diseases is dictated by a complex and dynamic balance between the host defence system (both innate and adaptive immunity) and microbial virulence. Effective clearance of the pathogen requires recognition of the foreign antigens and rapid recruitment of immune regulators. Paradoxically, exuberant inflammatory responses can lead to the construction of a hospitable microenvironment for the hijacked or transformed cells, through the secretion of growth-stimulating factors by inflammatory mediators. It is becoming clearer that inflammation is one of the acquired hallmark capabilities required for neoplastic progression (Hanahan and Weinberg, 2011). Note that about 20% of cancers have an infectious aetiology.

On the other hand, most of the common types of human cancer are attributable to mutations of the tumour suppressor p53. Additionally, numerous reports have shown its direct involvement in the regulation of the immune response. It has been reported that ASPP2 can also bind to NF-κB p65, a transcription factor that regulates pro-inflammatory gene expression (Yang et al., 1999b). Previous work conducted in our lab has provided some evidence that ASPP2 is intimately linked to inflammation (Buti et al., 2011, Koch, 2013, Turnquist et al., 2014).

This thesis is divided into two chapters, each corresponding to a pathogenic disease. Here, the major findings are summarised: (i) expression of p63 in nasopharyngeal carcinoma cells is reduced after EBV infection; (ii) nuclear p63 and ASPP2 are expressed in NPC tissue; (iii) prominent infiltration of inflammatory mediators is
evident within the NPC tumour; (iv) a granulomatous response is induced by BCG and *Mtb* at 21 d.p.i.; (v) expression of p53, ASPP2 and nuclear p-STAT1 is induced in iNOS+ cells within mycobacterial granulomas; and (vi) activation of NF-κB in PMA-treated THP-1 cells occurs following stimulation with the bacterial ligands LPS, LAM and CWS.

Several studies have shown an association between pulmonary TB and lung malignancies, and there are also case reports of the co-existence of TB and cancer (Yu *et al.*, 2011, Vento and Lanzafame, 2011). A study has demonstrated that interstitial inflammatory macrophages isolated from *Mtb*-infected mice can actively induce DNA damage and the secretion of epiregulin (EREG), an EGFR ligand, which can increase the mitotic activity of tumour cells (Nalbandian *et al.*, 2009). Almost all (95%) lung cancers are of epithelial origin. Chronic inflammation is generally caused by dysregulation of inflammatory mediators and can accelerate the progression of cancer. Pro-inflammatory cytokines secreted by immune cells can induce lung epithelial cells to activate NF-κB signalling, which has pro-tumourigenic effects, such as inducing the expression of anti-apoptotic mediators and propagating inflammation (Grivennikov *et al.*, 2010). The tumour suppressor p53 is able to recruit co-activators, such as ASPP1 and ASPP2, to induce its pro-apoptotic activity and antagonise NF-κB-mediated pro-inflammatory signalling. Therefore, defects in apoptotic signalling pathways can result in uncontrolled lymphocyte proliferation (Lee *et al.*, 2000).

Both NF-κB and STAT1 are important transcriptional regulators in immune signalling, and can be activated through common receptors such as TLR4. A preceding study has suggested synergistic interactions between NF-κB and STAT1 in enhancing the gene transcription of IRF1 and CXCL10 (Ohmori *et al.*, 1997). In addition, p53 is known to interact and regulate these two TFs (Murphy *et al.*, 2011, Townsend *et al.*, 2004). It is highly likely that NF-κB and STAT1 utilise common signalling response
elements to carry out their functions in immune surveillance and tumour suppression. In this study, the interplay between the NF-κB and STAT1 signalling pathways in NPC and TB has yet to be elucidated.

In conclusion, our model outlined here suggests that ASPP2 is an important regulatory factor in mediating pathogenic-specific responses (**FIGURE 5.1**). Nevertheless, the underlying molecular links between the different elements of ASPP2 activation require further investigation.

**FIGURE 5.1** – A proposed schematic model illustrating the possible mechanisms by which ASPP2 may regulate inflammation in pathogenic infections. EBV antigens such as LMP-1 are known to activate the NF-κB pathway to promote tumour cell survival and allow the transcription of pro-inflammatory cytokine and chemokine genes. This can lead to exuberant inflammation and provides a hospitable microenvironment for the pathogen. ASPP2 can bind and regulate NF-κB p65 as well as p63. It has been demonstrated that ASPP2 is able to repress p63 expression via NF-κB-mediated inhibition. Several reports have suggested a role for p63 as a metastasis inhibitor. LPS-TLR4 stimulation can trigger STAT1 activation, resulting in the translocation of phosphorylated STAT1 into the nucleus to mediate the transcription of ASPP2. As a p53 co-activator, ASPP2 is able to induce apoptotic cell death. ASPP2 is also able to regulate autophagy in a p53-independent manner. Several components of the mycobacterial cell wall, as well as EBV-encoded dUTPase and gp350, have been shown to activate TLR2. ASPP2 has been shown in this study to be activated following stimulation with mycobacterial CWS and LAM at low concentrations.
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