Vaccinia virus protein A40 is an immunomodulator

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Declaration

The work detailed herein is the work of the candidate except where clearly indicated.

The in vivo animal injections were undertaken by Dr. Ron A-J Chen.

The leukocyte infiltration flow cytometry analysis and cytotoxicity assay on extracted cells detailed herein were undertaken in a collaborative manner with Dr. Nathalie Jacobs.

The polyclonal anti-A40 antibody was generated by Harlan Sera-Lab (Leicestershire, England).

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Susan A. Jarmin
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Abstract

_Vaccinia virus_ (VACV) strain Western Reserve gene _A40R_ encodes a type II membrane glycoprotein with a C-type lectin-like domain at the C terminus. The A40 protein is not incorporated into virions, is nonessential for virus replication in cell culture and does not affect virus virulence in a murine intranasal model of infection. However, A40 does affect the outcome of infection in an intradermal infection model in which the virus lacking gene _A40R_ produced smaller lesions and alterations in the host immune response.

A40 has amino acid similarity to C-type lectins, such as NKG2A and DC-SIGN. This observation together with its location on the infected cell surface and its ability to bind to the surface of cells of the immune system is consistent with A40 functioning as an immunomodulator. It is possible that A40 might function by mimicking native host lectins or by modulating recognition of VACV-infected cells by cells of the immune system.

To investigate the mechanism by which A40 affects the outcome of infection _in vivo_, a cloning and experimental strategy was devised to search for its ligand(s) and try to determine its structure in collaboration with protein crystallographers. To achieve these goals, the recombinant A40 protein has been produced in _E. coli_, mammalian cells, and from insect cells infected with recombinant baculoviruses. Bacterially expressed recombinant A40 was used to generate an antibody specific to A40 and this was then purified, characterised and used to further the characterisation of A40. The potential for A40 to interact with a ligand on the surface of another cell was investigated by a cell binding assay using recombinant A40 protein. This protein was produced in a mammalian system and was found to bind to the surface of immune cells but not to epithelial cells. In cytotoxicity assays, the absence or over-expression of A40 was found to modulate the ability of NK cells to kill VACV infected cells.
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**Abbreviations**

α- antibody against

$A_x$ absorbance at x nm

aa amino acid(s)

Ab antibody or antibodies

ADCC Antibody-dependent cellular cytotoxicity

AICL activation-induced C-type lectin

AIDS acquired immunodeficiency syndrome

Amp Ampicillin

APAF adaptor molecule protease activating factor

APC allophycocyanin

APC antigen presenting cell

AraC cytosine arabinoside

ASFV African swine fever virus

ATI A-type inclusion body

ASFV *African swine fever virus*

β beta

BAC bacterial artificial chromosome

BAP Biotin acceptor protein

Bcl B-cell lymphoma

BH Bcl-2 homology

$β_{2m}$ $β_2$-microglobulin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>β-ME</td>
<td>beta mercaptoethanol</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BR</td>
<td>Brighton Red</td>
</tr>
<tr>
<td>BS&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Bis (Sulfosuccinimidyl) suberate</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CAV</td>
<td>cell associated virus- IMV, IEV and CEV</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-motif ligand</td>
</tr>
<tr>
<td>CCP</td>
<td>complement control proteins</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation antigen</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CEV</td>
<td>cell-associated enveloped virus</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>Ci</td>
<td>curies</td>
</tr>
<tr>
<td>CLECT</td>
<td>C-type lectin-like domain</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin-related protein</td>
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<tr>
<td>CMC</td>
<td>carboxymethylcellulose</td>
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<td>CMLV</td>
<td><em>Camelpox virus</em></td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>COP</td>
<td>Copenhagen</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<td>CPXV</td>
<td><em>Cowpox virus</em></td>
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<tr>
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<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>CT</td>
<td>cytoplasmic tail</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>C-type lectin-like domain</td>
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<td><em>Chorioallantois virus Ankara</em></td>
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<td>δ</td>
<td>delta</td>
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<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of IFN regulatory factors</td>
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<tr>
<td>DAP</td>
<td>DNAX-activating protein (of molecular mass 10 or 12 kDa)</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-diamin-2-phenyllin-dol-dihydrochloride</td>
</tr>
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<td>dATP</td>
<td>(deoxy) adenosine 5’-triphosphate</td>
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<td>DC</td>
<td>dendritic cell</td>
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<td>DC-SIGN(R)</td>
<td>DC-specific ICAM-3-grabbing non-integrin (receptor)</td>
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<td>DCIR</td>
<td>DC Immunoreceptor</td>
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<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>deionised distilled water</td>
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<td>DED</td>
<td>death effector domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleic triphosphate</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>EBV</td>
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<td><em>Eco</em> gpt</td>
<td><em>E. coli</em> guanine phosphoribosyltransferase gene</td>
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<td><em>Ectromelia</em> virus</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EEV</td>
<td>extracellular enveloped virus</td>
</tr>
<tr>
<td>EGF(R)</td>
<td>epidermal growth factor (receptor)</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (β-aminoethyl ether)-N,N,N’N’-tetraacetic acid</td>
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<td>eIF</td>
<td>eukaryotic initiator factor</td>
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<td>Endoglycosidase H</td>
</tr>
<tr>
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<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>endoplasmic reticulum-Golgi intermediate compartment</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal regulated kinase</td>
</tr>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<td>EYFP</td>
<td>enhanced yellow fluorescent protein</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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</table>
FBS  foetal bovine serum
Fc   fragment, crystallisable
FC   flow cytometry
FITC fluorescein isothiocyanate
FWPV Fowlpox virus
γ    gamma
g    gram(s)
GAF  IFN-γ-activated factor
GAGs glycosaminoglycans
GalN D-galactosamine sequence
GAS  IFN-γ-activated
GFP  green fluorescent protein
GlcA D-glucouronic acid
GlcN D-glucosamine
GlcNAc N-Acetylglucosamine
GM-CSF granulocyte-macrophage colony stimulating factor
GMEM Glasgow minimum essential medium
GTP  guanosine 5'-triphosphate
h    hour(s)
HA   haemagglutinin
HCMV Human cytomegalovirus
HEPES N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid)
HIV  human immunodeficiency virus
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<tr>
<th>Abbreviation</th>
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<td>human leukocyte antigen</td>
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<td>h.p.i.</td>
<td>hours post infection</td>
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<td>human papillomavirus</td>
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<td>horse radish peroxidase</td>
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<td><em>Horsepox virus</em></td>
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<td>inclusion body</td>
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<td>intermediate compartment</td>
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<td>inhibitory concentration 50</td>
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<td>ICAM</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<td>IκB kinase</td>
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<td>interleukin</td>
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<td>ILB</td>
<td>insect lysis buffer</td>
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<td>immunoglobulin-like transcript</td>
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<td>ISRE</td>
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<td>Immunoreceptor tyrosine-based inhibition motif</td>
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<td>inverted terminal repeat</td>
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<td>kanamycin</td>
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<td>kilobase pairs</td>
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<td>kDa</td>
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<td>killer cell lectin-like receptor</td>
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<td>LLT</td>
<td>lectin-like transcript</td>
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<td>LMA</td>
<td>low melting agarose</td>
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<td>lipoprotein receptor</td>
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<td>lipopolysaccharide</td>
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<td>M</td>
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<td>µ/mg</td>
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<td>µ/ml</td>
<td>micro/milli litre</td>
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<tr>
<td>µ/mM</td>
<td>micro/milli molar</td>
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<td>mAb</td>
<td>monoclonal antibody</td>
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<td>MAC</td>
<td>membrane attack complex</td>
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<td>MAFA</td>
<td>mast cell function-associated antigen</td>
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<td>MAL</td>
<td>MyD88 adaptor like</td>
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<td>mitogen-activated protein kinase</td>
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<td>MBL-associated serine proteases</td>
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<td>MBL</td>
<td>mannan-binding lectin</td>
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<td>MCP</td>
<td>macrophage chemotactic protein</td>
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<td>MCS</td>
<td>multiple cloning site</td>
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</table>
MDA  melanoma differentiation-associated gene
MHC  major histocompatibility complex
MIC  MHC class-I-chain related protein
min  minute(s)
MIP  macrophage inflammatory protein
MMR  macrophage mannose receptor
MOI  multiplicity of infection
MOPS  3-(N-morpholino) propane sulphonic acid
MPA  mycophenolic acid
MPXV  *Monkeypox virus*
(m)RNA  (messenger) ribonucleic acid
MT  microtubules
MTOC  microtubule organizing centre
Mult  mouse ULBP-like transcript
MVA  modified vaccinia Ankara
MW  molecular weight
MXPV  *Monkeypox virus*
MyD88  myeloid differentiation factor 88
N-/C- terminus  amino/carboxy terminus
NE  neutrophils elastase
NEAA  non-essential amino acids
ND<sub>50</sub>  neutralisation dose 50
NF-κB  nuclear factor kappa B
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<td>NKR</td>
<td>NK receptor</td>
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<td>nano molar</td>
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<td>nitric oxide</td>
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<td>nonidet P-40</td>
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<td>New York City Board of Health</td>
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<td>oligoadenylate synthetase</td>
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<td>OD</td>
<td>optical density</td>
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<td>OEP</td>
<td>overlapping extension PCR</td>
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<td>OMCP</td>
<td>Orthopoxvirus MHC class-I-like protein</td>
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<td>OPV</td>
<td>Orthopoxvirus</td>
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<td>ORF</td>
<td>open reading frame</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PBSF</td>
<td>phosphate buffered saline containing 10% FBS</td>
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<td>PCR</td>
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<td>pDC</td>
<td>plasmacytoid dendritic cells</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>PEG</td>
<td>polyethylene glycerol</td>
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<td>PEI</td>
<td>polyethylenimine</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<td>p.f.u</td>
<td>plaque forming unit(s)</td>
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<td>post infection</td>
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<td>phosphatidylinositol 3-kinase</td>
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<td>proteinase K</td>
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<td>PKR</td>
<td>protein kinase RNA-dependent</td>
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<td>PLC-γ</td>
<td>phospholipase C-γ</td>
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<td>polymorphonuclear neutrophilic leukocytes</td>
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<td>PNGase F</td>
<td>Peptide: N-Glycosidase F</td>
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<td>regulated on activation normal T cell expressed and secreted</td>
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<td>rabbit</td>
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<td>Rev</td>
<td>revertant</td>
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<td>retinoic acid-inducible gene</td>
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<td>RK-13</td>
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<td>ribonuclease</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute (medium) 1640</td>
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<td>RPXV</td>
<td><em>Rabbitpox virus</em></td>
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<td>RT</td>
<td>room temperature</td>
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<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<td>s</td>
<td>second(s)</td>
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<td>SAP</td>
<td>shrimp alkaline phosphatase</td>
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<td>SCR</td>
<td>short consensus repeat</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEL</td>
<td>synthetic early/late promoter</td>
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<td>SEM</td>
<td>standard error of mean</td>
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<td>SeMet</td>
<td>selenomethionine</td>
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<td>SH2</td>
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<td>SH2-domain-containing protein</td>
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<td>s6Le&lt;sup&gt;x&lt;/sup&gt;</td>
<td>sialyl 6-sulpho Lewis X</td>
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<td>salt optimized broth with carbon</td>
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<td>SPI</td>
<td>serine protease inhibitor</td>
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<td>ss</td>
<td>single-stranded</td>
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<td>STAT</td>
<td>signal transducers and activators of transcription</td>
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<td>STING</td>
<td>stimulator of interferon genes</td>
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<td>spleen tyrosine kinase</td>
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<td>TAA</td>
<td>tumour-associated antigens</td>
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<td>TNF-associated kinase</td>
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<td>Terrific broth</td>
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<td>TBK</td>
<td>TANK-binding kinase</td>
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<td>TBS</td>
<td>tris buffered saline</td>
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<td>trichloroacetic acid</td>
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<td>T cell receptor</td>
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<td>N,N,N’,'N’-Tetramethylethylenediamine</td>
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<td>transcription factor</td>
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<td>transmembrane domain</td>
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<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>TNF receptor-associated factor</td>
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<td>unique long</td>
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<td>ultraviolet</td>
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<td><em>Variola virus</em></td>
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<td>vaccinia early transcription factor</td>
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<td>VGF</td>
<td>virus growth factor</td>
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<td>VACV immunoglobulin</td>
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<td>v/v</td>
<td>volume/volume</td>
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<td>weight/volume</td>
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<td>zeta</td>
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<td>ZAP-70</td>
<td>zeta-chain-associated protein kinase</td>
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Chapter 1. Introduction

1.1 Introduction to poxviruses and their historical significance

The Poxviridae are a family of complex enveloped viruses with double-stranded (ds) DNA genomes, which replicate entirely in the cytoplasm of infected cells (Moss, 2007). Vaccinia virus (VACV) is the most extensively studied member of the Poxviridae, which comprise 2 subfamilies, the Chordopoxvirinae and the Entomopoxvirinae, which infect chordates and insects respectively. The Chordopoxvirinae is divided into 8 genera (Table 1.1) and VACV is the prototype species of the Orthopoxvirus (OPV) genus, whose members include Variola virus (VARV) (Moss, 2007), the causative agent of smallpox.

Table 1.1 Poxvirus classifications.
Prototypical viruses are underlined and viruses with sequenced genomes are in bold.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
<th>Member viruses</th>
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<tbody>
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<td>Chordopoxvirinae</td>
<td>Avipoxvirus</td>
<td>fowlpox, canarypox, turkeypox</td>
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<tr>
<td></td>
<td>Capripoxvirus</td>
<td>goatpox, sheeppox, lumpy skin disease</td>
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<td>Lepripoxvirus</td>
<td>myxoma, rabbit fibroma</td>
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<td>Molluscipoxvirus</td>
<td>molluscum contagiosum</td>
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<tr>
<td>Orthopoxvirus</td>
<td></td>
<td>camelpox, cowpox, ectromelia, monkeypox, taterapox, vaccinia, variola</td>
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<td>Parapoxvirus</td>
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<td>swinepox</td>
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<td>Yatapoxvirus</td>
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<td>yaba monkey tumour, tanapox, yaba-like disease</td>
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<td>Entomopoxvirinae</td>
<td>Alphaentomopoxvirus</td>
<td>melontha melontha</td>
</tr>
<tr>
<td></td>
<td>Betaentomopoxvirus</td>
<td>amsacta moorei, melanoplus sanguinipes</td>
</tr>
<tr>
<td></td>
<td>Gammaentomopoxvirus</td>
<td>chironomus luridus</td>
</tr>
<tr>
<td>Unclassified poxviruses</td>
<td></td>
<td>crocodilepox, deerpox</td>
</tr>
</tbody>
</table>

In 1798, Edward Jenner exploited the immunological cross-reactivity between OPVs and started the process of vaccination (Jenner, 1798; Baxby, 1979; Baxby, 1996). Smallpox is the only human disease to have been eradicated through human
intervention. By the 16th century, smallpox was endemic in Europe and through European colonisation spread to other parts of the world. It was a greatly feared disease with up to 40% mortality and those who survived were usually left with deep-pitted scars (Mack, 1972; Fenner, 1993). The last known natural case occurred in 1977 in Somalia and the World Health Organisation (WHO) certified the disease as eradicated in 1980 (Fenner et al., 1988). Eradication was achieved through vaccination but the precise mechanisms of protection remain largely unknown.

The most commonly used VACV strains in the WHO eradication program were Lister and Elstree in Europe, New York City Board of Health (NYCBH), Dryvax and Wyeth in the United States, EM-63 in Russia and Temple of Heaven (Tian Tan) in China (Fenner, 1977). Of the many VACV strains isolated, VACV strains Copenhagen (Cop) (Goebel et al., 1990), Western Reserve ((WR), a derivative of NYCBH strain adapted by serial brain passage in mice) and International Health Division (IHD)-J (Parker, 1941) are the most commonly utilised laboratory strains. VACV has a broad host range although its origin and natural host remain unknown (Baxby, 1977).

Despite the eradication of smallpox more than 3 decades ago VACV continues to be a focus of research because its many interactions with the host cell and immune system have provided an insight into virology, cell biology and immunology. It was developed as an expression vector in 1982 (Mackett et al., 1982; Panicali & Paoletti, 1982) and became widely used as a recombinant expression vector for laboratory research and vaccine development (Moss et al., 1983; Paoletti et al., 1984). As an expression vector it has several useful properties, such as the relative ease of constructing recombinant VACV, a large capacity for exogenous DNA (Smith & Moss, 1983), relatively high levels of expression and a wide range of cells that can be targeted (Panicali & Paoletti, 1982; Moss et al., 1983; Mackett & Smith, 1986). Recombinant VACVs expressing genes from other pathogens, such as influenza virus (Panicali et al., 1983; Smith & Moss, 1983) or hepatitis B virus (Smith et al., 1983), have induced in vaccinated animals immune responses against the foreign antigen expressed by the virus making VACV a potential vaccine vector. Attenuated strains of VACV, such as modified virus Ankara (MVA) (derived from chorioallantois virus Ankara (CVA) (Meisinger-Henschel et al., 2007)), are being developed with the aim of producing vaccines that induce protection but which are safer, causing fewer side-effects and complications.
1.2 Genome structure of OPVs

VACV has a linear, AT-rich (67%) dsDNA genome (Goebel et al., 1990; Moss, 2001) which is approximately 190 kilobase pairs (kbp), although this varies depending on the strain. For instance, MVA is 165 kbp, (Antoine et al., 1998) and Duke is 199 kbp (Li et al., 2006). The two DNA strands are covalently linked at the termini by incompletely base-paired hairpin loops into one continuous molecule (Baroudy et al., 1982). The VACV strain Copenhagen genome contains about 200 open reading frames (ORFs) (Goebel et al., 1990; Johnson et al., 1993) and no introns have been identified.

Bioinformatic analyses of poxvirus genomes that have been sequenced revealed approximately 90 proteins are conserved in all chordopoxviruses and about half of these are present in all poxviruses (Upton et al., 2003; Gubser et al., 2004). The conserved genes are usually essential for replication and virion assembly and cluster in a central region of the genome, whilst genes that are non-essential and involved in immunomodulation or host range are usually located in the more variable terminal regions (Fig. 1.1).

At either end of the genome there are inverted terminal repeats (ITR) that consist of identical but oppositely orientated sequences. The ITRs of different VACV strains and OPVs vary in length (Gubser & Smith, 2002) due to various deletions, tandem repeat sequences and transpositions (Wittek & Moss, 1980). A highly conserved sequence is located near the terminal hairpin loops and is essential for the resolution of concatemeric DNA intermediates formed during DNA replication (DeLange & McFadden, 1987; Merchlinsky & Moss, 1989; Merchlinsky, 1990).

![Fig. 1.1 Diagrammatic representation of an OPV genome](image-url)
The first sequence of a VACV genome was determined for the Copenhagen strain (COP) (Goebel et al., 1990) and with its publication, a genetic nomenclature was adopted in which the VACV ORFs were named according to their relative position (left to right) in the 16 fragments produced upon digestion of the VACV-COP genome with HindIII restriction endonuclease. The fragments were labelled A to P, with A being the largest fragment and P the smallest and the transcriptional orientation of each ORF was denoted by an L or R. Exceptions to this rule included ORFs in fragment C, which are located in the highly variable left end and which are numbered right to left. More recently, ORFs in the sequenced poxviruses have been numbered continuously along the genome, left to right, but the original nomenclature established for VACV Copenhagen remains a useful nomenclature for poxvirologists.

1.3 Virion structure

VACV virions are large (360 x 270 x 250 nm), brick-shaped particles (Hollinshead et al., 1999; Cyrklaff et al., 2005) that are visible by light microscopy and lack the symmetry seen with other viruses such as helical and icosahedral capsids. VACV produces three distinct types of virion: intracellular mature virus (IMV), intracellular enveloped virus (IEV) and extracellular virions that include both the cell-associated enveloped virus (CEV) and the extracellular enveloped virus (EEV). These different virions have different structures, locations, abundance and roles in the virus life-cycle. Some authors also refer to these virions as mature virus (MV) for IMV, wrapped virus (WV) for IEV and extracellular virus (EV) for both CEV and EEV (Moss, 2006).

All the virions are composed of a viral core surrounded by an IMV membrane. The core contains the genome associated with numerous viral structural proteins and enzymes required for early transcription (Munyon et al., 1967). The core is surrounded by the core wall, with a biconcave structure formed by separation of the core wall from the IMV membrane (Condit et al., 2006). The bilateral concavities are filled by amorphous masses termed ‘lateral bodies’ (Dubochet et al., 1994). IEV is formed by the wrapping of IMV with a double membrane derived from early endosomes or trans-Golgi network (Tooze et al., 1993; Schmelz et al., 1994). IEV are transported on microtubules to the cell surface where its outer membrane fuses with the plasma
membrane to form CEV on the cell surface (Blasco & Moss, 1992) or EEV that is released from the cell. IMV represents the majority of progeny but remains inside the infected cell until cytolysis and is important for long-term stability in the environment and transmission between hosts. In contrast, EEV is important for in vivo dissemination of the virus (Payne, 1980).

1.4 VACV life cycle

An overview of the VACV lifecycle is shown in Fig. 1.2 and can be divided into several steps: virus binding, entry, uncoating, gene expression, DNA replication, virion assembly and virion release.

1.4.1 Virus binding

The binding of VACV to permissive cells is the primary step in establishing infection. Poxviruses are notable for their omission from a summary of the known cellular receptors of viruses (Sieczkarski & Whittaker, 2005). VACV can infect many diverse cell types, suggesting that either the viral receptor is ubiquitously expressed on cells or that the virus can bind to many different receptors (Moss, 2001). Several studies have shown that cell surface glycosaminoglycans (GAGs) can bind to distinct virus proteins but these interactions are not essential for VACV entry (Carter et al., 2005). Several other types of molecules including cholesterol (Chung et al., 2005) and laminins (Chiu et al., 2007) may affect VACV binding and its subsequent entry.

1.4.2 Virus entry

Due to the morphological differences between the two infectious forms of VACV, the IEV and EEV enter cells by different mechanisms (Vanderplasschen et al., 1998a). Studies on IMV entry suggest there are several possible routes. IMV binds to cells and the IMV membrane fuses with the plasma membrane releasing the core into the cytoplasm (Carter et al., 2005). Fusion and entry may also take place within endosomes at low pH (Townsley & Moss, 2007) and a recent study supports a role for macropinocytosis (Mercer & Helenius, 2008) as an alternative pathway in the entry of
IMV. IMV fusion is mediated by a large fusion complex consisting of at least 9 essential viral proteins (Senkevich et al., 2004; Brown et al., 2006).

The entry of EEV is complicated by the presence of an additional outer membrane, for simple fusion of the outer EEV membrane with the plasma membrane would release an IMV into the cell, which would still require removal of the IMV lipid envelope to obtain access of the core to the cytosol. Two models of EEV entry have been proposed and their relative importance may depend on the cell type being studied. In a process termed ligand-dependent non-fusogenic dissolution (Law et al., 2006), the EEV can interact with GAGs on the plasma membrane causing disruption of the outer membrane and exposure of the IMV to the cell surface. The IMV can then fuse to plasma membrane and release the core into the cytoplasm, leaving the outer membrane as a shroud over the fusing virion. In an alternative method, EEV is endocytosed by the cell and the EEV membrane is disrupted by acidification within an endosome, exposing the IMV. The IMV is then able to fuse with the endosomal membrane to release the core into the cytoplasm (Ichihashi, 1996; Vanderplasschen et al., 1998a).

1.4.3 Gene Expression

Following entry into the cytoplasm, the viral core moves on microtubules (MT) deeper into the cell (Carter et al., 2003) and initiates a highly regulated temporal cascade of transcription and genome replication, all of which occur in the cytoplasm (Broyles, 2003). VACV genes are transcribed in a strictly controlled order that can be defined by four temporal gene classes: immediate-early, early, intermediate and late (Assarsson et al., 2008) and viral messenger RNAs (m RNAs) are capped and polyadenylated like eukaryotic mRNAs (Kates & Beeson, 1970; Wei & Moss, 1975).

1.4.3.1 Immediate-early genes

The core contains all the necessary enzymes for early transcription associated with the genome (Golini & Kates, 1985; Rohrmann & Moss, 1985) including a DNA-dependent RNA polymerase (Kates & McAuslan, 1967; Munyon et al., 1967), methylation capping enzyme, poly-A polymerase and various transcription factors (TF) (Yuen et al., 1987; Broyles et al., 1988). Recent genome-wide analysis of mRNA
expression from VACV WR revealed a set of 35 genes exhibiting immediate-early expression (Assarsson et al., 2008). This class of genes is characterised by expression within 0.5 h post infection (h.p.i.) and maximum expression was reached by 2 h.p.i before declining. Immediate early genes include those involved in virulence, immune evasion and DNA replication.

1.4.3.2 Early genes

Early genes make up the largest class of ORFs, with 73 genes (as classified by Assarsson et al., 2008). Expression is detectable within 1 h.p.i. and reaches maximum levels at 2 h.p.i. The early genes include proteins involved in transcription, DNA replication, immune evasion proteins and host cell modulators (Broyles, 2003). Early VACV promoters are approximately 15-bp long and lie at positions -28 bp to -13 bp upstream of the RNA start site. These promoters have the consensus sequence, AAAAAATGAAAAAAA/TA, separated by a T-rich spacer sequence, (-12 to -2) from a 7-bp region (-1 to +6) within which initiation occurs, usually at a purine (Davison & Moss, 1989a). Transcription of early genes terminates 30 – 50 bp downstream of a TTTTTNT signal (N represents any nucleotide), on the non-template strand of the DNA (Yuen & Moss, 1987; Piacente et al., 2008).

As yet there is no functional or genetic evidence for the subdivision of early and immediate-early genes. Intermediate-early and early transcription occurs within the core and the viral mRNA is extruded through pores in its surface and when the core is disassembled early transcription ceases. The uncoating of the viral genome from the core requires the proteolysis of some viral proteins and hydrolysis of the virion phospholipid (Zaslavsky, 1985) and is dependent on the novel synthesis of at least one early viral protein (Pedersen et al., 2000).

1.4.3.3 Intermediate genes

Once DNA replication has started, intermediate transcription is initiated and requires several virus-encoded proteins including virus intermediate transcription factors (VITFs). (Sanz & Moss, 1999; Katsafanas & Moss, 2004). Intermediate genes encode proteins including late TFs (Keck et al., 1990; Zhang et al., 1992; Baldick &
Moss, 1993; Broyles, 2003). Intermediate transcripts are detected after 1.5 h.p.i. and peak at 2 h.p.i before declining. Intermediate promoters contain two elements, a 14 bp A/T rich upstream element separated by 10 or 11 bp from a TAAA initiator sequence, giving an overall core element with the sequence $(A/T)_{12}N_{12}TAA(T/A)GG$ (Baldick & Moss, 1993).

For both intermediate and late genes, a 5’ untranslated poly(A) leader sequence of about 35 bases is created by slippage of the VACV RNA polymerase on the AAA sequence of the transcription initiation motif (Schwer et al., 1987). During the intermediate and late phases of infection, the mRNAs are heterogeneous in length and do not have a defined 3’ end (Mahr & Roberts, 1984; Baldick & Moss, 1993) due to a failure to terminate at the early termination sequences, which are no longer recognised by the RNA polymerase.

1.4.3.4 Late genes

Late gene transcription is detected after 2 h.p.i and synthesis continues throughout the replication cycle (Baldick & Moss, 1993). Transcription begins once the necessary transcription factors and newly synthesised RNA polymerase are available (Broyles, 2003). Late gene products include the majority of structural proteins, the enzymes and TFs required for early transcription that become assembled into the virion, as well as some virulence factors that enhance viral immune evasion (Broyles, 2003; Assarsson et al., 2008). In addition, late gene products are responsible for the resolution of the DNA concatemers into single genomes that are packaged into new virions.

The late gene promoter is also bipartite, with the overall core sequence $(A/T)_{6}N_{9}TAAAT$, consisting of an upstream A/T-rich core region of around 20 bp and a conserved TAAAT element within which transcription initiates (Davison & Moss, 1989b). This is often followed by a G with the resultant ATG acting as the translation start codon.
1.4.4 DNA replication

DNA replication occurs after early gene expression, within the cytoplasm at discrete sites termed viral factories. Individual infectious particles have the ability to initiate a separate factory and thereby, the number of factories in a cell is proportionate to the multiplicity of infection (MOI) (Moss, 2001). Several VACV proteins are required for synthesis of DNA precursors and for DNA replication. These include a DNA polymerase (Challberg & Englund, 1979; Earl et al., 1986), DNA helicases (Koonin & Senkevich, 1992), DNA ligase (Kerr & Smith, 1989), and enzymes for the synthesis of new nucleotides including ribonucleotide reductase (Slabaugh et al., 1988), thymidine kinase (Weir et al., 1982), thymidylate kinase (Smith et al., 1989a) and enzymes involved in proofreading such as deoxyuridine triphosphate (Broyles, 1993).

Replication starts approximately 1-2 h.p.i., with the introduction of a nick into one of the DNA strands near the terminal hairpin. The nick exposes a free 3’OH group that acts as a primer for the initiation of replication (Moyer & Graves, 1981) and allows DNA polymerase to copy the hairpin and elongate to the end of the genome. The newly synthesised strand is self complementary and folds back on itself, allowing the DNA polymerase to continue along the genome and round the opposite hairpin so forming concatemers. These are resolved by specific nucleases (Shuman & Moss, 1987; DeLange, 1989; Garcia & Moss, 2001; Eckert et al., 2005) into monomers after late gene expression.

1.4.5 Virus assembly, maturation & release

Virion assembly also occurs in the viral factories and involves a series of complex stages. The viral DNA and core proteins (Cassetti et al., 1998) are surrounded by membrane crescents which gradually extend into spherical immature virions (IV). These structures are then converted to a brick-shaped IMV particle by maturation via proteolytic cleavage of some capsid proteins (Moss & Rosenblum, 1973; Yang, 2007), loss of the scaffold protein D13 and condensation of the nucleoprotein core into the IMV particles.

The origin and number of the crescent membranes surrounding the IMV have been protractedly disputed since the mid 1990s. In the early 1960s Dales and co-workers proposed that the membrane is a single lipid bilayer that is not associated with
cellular organelles. This theory has been supported by a large amount of EM data (Hollinshead et al., 1999; Heuser, 2005). A second proposal (Sodeik et al., 1993) suggested that the membrane was two tightly opposed membranes that originated from the immediate compartment between the endoplasmic reticulum (ER) and the Golgi. The one membrane model theory is now accepted in the field and is consistent with the membrane fusion mechanism for IMV entry (Carter et al., 2005). The debate as to whether the virion membrane is derived from de novo biogenesis (Dales & Mosbach, 1968) or derived from pre-existing intracellular membranes (Sodeik et al., 1993; Sodeik & Krijnse-Locker, 2002) still rages (reviewed in Roberts & Smith, 2008).

The majority of IMV are released from the infected cell during lysis but a proportion undergoes further morphogenesis to form EEV. This occurs predominantly in early stages of infection, as wrapping membranes can become depleted later in infection. IMV are transported out of virus factories on MTs (Sanderson et al., 2000; Ward, 2005) to near the microtubule organising centre (MTOC) where they become wrapped in a double-layer of membrane derived from the endosomes (Tooze et al., 1993) or trans Golgi network (TGN) (Schmelz et al., 1994) to form IEV. The IEV are transported to the cell surface on MTs, utilising a kinesin-dependent mechanism (Hollinshead et al., 2001; Rietdorf et al., 2001; Ward & Moss, 2001), where the outer membrane fuses with the plasma membrane to form CEV that remain on the cell surface (Blasco & Moss, 1992) or is released from the cell as EEV (Smith et al., 2002a). Actin nucleation, below the plasma membrane, induced by CEV propels the virion away from the infected cell and into surrounding cells or the extracellular space (reviewed in Smith & Law, 2004).

1.5 Effect of VACV on host cells

VACV infection induces early changes in cell morphology, adhesion properties and metabolism and eventually results in death of the infected cells. These changes are collectively termed the cytopathic effect (CPE). Effects include the inhibition of host protein synthesis (Bablanian et al., 1981), alterations to the extracellular matrix of cells leading to Ca^{2+}-independent adhesion (Sanderson & Smith, 1998), cell rounding in a reversible and microtubule-dependent manner (Schepis et al., 2006) and virus-induced cell motility (Sanderson et al., 1998).
Modified from Roberts & Smith, 2008
Fig. 1.2 Overview of the VACV lifecycle
A cartoon diagram illustrates the VACV lifecycle. Extracellular enveloped virions (EEV, 1) and intracellular mature virions (IMV, 2) bind to and enter the cell, releasing the core into the cytoplasm. The core is translocated (3) by microtubules (MT) deeper into the cell to a perinuclear site. Production of early mRNAs (4) leads to uncoating of the core, release of the genome (5) and following replication of the viral genome (6), the production of intermediate (7) and late mRNAs (8). These are translated into structural proteins and early gene transcription machinery (9). Within the viral factories, a single genome interacts with the early transcription machinery plus structural proteins and these are assembled within membrane crescents to form immature virions (IV, 10). The IV undergoes nucleoprotein condensation and proteolytic cleavages of structural proteins to form IMV (11). Many IMV build up in the cell and are released upon cell lysis (12). However, a proportion undergo further morphogenesis. The IMV are transported on MT to sites of wrapping (13) by the trans golgi network (TGN) or early endosomes. IMV particles are wrapped by a double membrane (14) to form intracellular enveloped virions (IEV, 15). IEV are transported to the cell surface on MT (16). The outer IEV membrane fuses with the plasma membrane (17) to form cell-associated enveloped virions (CEV) on the cell surface. Actin polymerisation beneath the CEV forms actin tails (18), driving CEV towards neighbouring cells or releasing EEV that can infect either neighbouring or distant cells.
1.6 Use of VACV in medical research

The ability to generate recombinant VACV that express foreign genes (Mackett et al., 1982; Panicali & Paoletti, 1982) or delete VACV genes (Earl et al., 2001) has numerous applications in research. Recombinant VACVs are being used as a gene delivery agent (Guo & Bartlett, 2004), a vaccine vector for delivery of exogenous antigens for presentation to the immune system (Sutter & Staib, 2003; Gherardi & Esteban, 2005) or an oncotherapeutic agent (Shen & Nemunaitis, 2005). The use of VACV in these ways has been enabled by advances in the understanding of VACV’s interaction with its host.

Recombinant VACV are generated by the construction of a plasmid containing the gene of interest under the control of a VACV promoter, flanked by VACV sequences that determine the site of insertion into the virus genome. Homologous recombination between the plasmid and the virus genome gives rise to recombinant VACV. The inclusion of selective markers such as β-galactosidase (Chakrabarti et al., 1985), fluorescent proteins such as green fluorescent protein (GFP) (Dominguez et al., 1998), or drug-selective proteins such as *Escherichia coli* (E. coli) guanine phosphoribosyltransferase (Eco gpt) (Boyle & Coupar, 1988) can be used to positively select for the recombinant viruses (Carroll & Moss, 1997). Alternative methods include *in vitro* ligation of a foreign gene into the virus genome (Merchlinsky & Moss, 1992), the rescue of infectious virus using the bacterial artificial chromosome (BAC) system with a non-replicating helper virus such as fowlpox virus (Domi & Moss, 2002; Cottingham et al., 2008) and the use of lepripoxviruses to aid recombination (Yao & Evans, 2003).

As well as being able to study the function of individual genes such as *C12L* (Symons et al., 2002a) or *NIL* (Bartlett et al., 2002) by null mutants for non-essential genes, techniques for inducible gene expression allow essential genes to be studied and can provide a system for replication control (Rodriguez & Smith, 1990; Ward et al., 1995).

1.6.1 VACV-based vaccines

After the success of VACV in the vaccination against and elimination of smallpox, research has turned to its use as a gene delivery system for heterologous
antigens. VACV has been used in a variety of clinical trials as vaccines for treatment of infectious agents such as rabies (Wiktor et al., 1984; Pastoret et al., 1988; Brochier et al., 1991) in which a recombinant VACV-rabies vaccine was developed for oral vaccination of foxes against rabies. Field trials proved safe and efficacious and eradicated rabies in areas of Europe where it was used.

Apprehensions about the safety profile of VACV are being addressed by the generation of attenuated vectors, focusing especially on MVA. MVA has lost the ability to replicate in most mammalian cells, is apathogenic even in immunodeficient animals and is safe in humans considered at risk from conventional VACV vaccines (Mayr & Danner, 1978; Blanchard et al., 1998; Sutter & Staib, 2003; Harrer et al., 2005). In animal models, MVA vaccines have been shown to be immunogenic and protect against various infectious agents including influenza virus (Breathnach et al., 2004; Veits et al., 2008), flaviviruses (Men et al., 2000; Nam et al., 2002), plasmodium parasites (Schneider et al., 1998; Moorothy et al., 2001) and simian immunodeficiency virus (Smith et al., 2004; Van Rompay et al., 2005; Martinon et al., 2008), either alone or as part of a heterologous prime-boost protocol.

Clinical trials in humans have confirmed that MVA is safe in immunocompromised patients and that MVA-based prime-boost strategies can induce T cell responses against human immunodeficiency virus (HIV) (Mwau et al., 2004; Goonetilleke et al., 2006) or tuberculosis specific (Hawkridge et al., 2008) antigen. Whilst these trials are encouraging, the need to improve the vectors has been recognised due to the failure of large scale phase II trials using MVA-malaria vaccines (Bejon et al., 2007a; Bejon et al., 2007b). Several strategies are being studied including the removal of immunomodulatory proteins from MVA (Staib et al., 2005) or the co-expression of stimulatory molecules (Abaitua et al., 2006) as well as refining the choice of antigen to help induce a more potent immune response.

1.6.2 VACV-based oncotherapy

VACV has been used in the treatment of cancer in three ways: as a vector for the delivery of anti-cancer genes to tumour cells, a vector for tumour-associated antigens and co-stimulatory molecules and as an oncolytic agent to selectively lyse tumour cells. The aim of the first two strategies is to stimulate immune responses that
target and eliminate active tumour cells. A recombinant VACV expressing the tumour suppressor p53 was generated using the Lister strain as a backbone (Timiryasova et al., 1999; Fodor et al., 2005) and showed in vivo, the synergistic effect of combining an anti-tumour antigen with the co-expression of cytokines and co-stimulatory molecules such as interleukin (IL)-2 and IL-12 (Chen et al., 2000) to inhibit tumour growth. Recombinant VACV expressing specific tumour antigens have also been developed. Immune responses are often repressed by tumour-induced immune anergy, and VACV-based vectors expressing tumour-associated antigens (TAAs) are designed to re-stimulate the immune response to these cells. Some TAAs are expressed at low levels in normal tissues but over-expressed in a particular cancer including carcinoembryonic antigen (CEA) (Greiner et al., 2002; Schmitz et al., 2002), prostate-specific antigen (PSA) (DiPaola et al., 2006) and mucin 1 (Scholl et al., 2000). Other vaccines target TAAs that are involved in carcinogenic viral lifecycles such as MVA-E2, where antibodies to human papillomavirus (HPV) E2 target macrophages to destroy tumour cells (Rosales et al., 2000).

VACV itself is capable of directly lysing tumour cells in which it replicates. Oncolytic therapeutic strategies exploit the naturally high level of VACV replication in tumour cells or alternatively VACV may be modified to target it preferentially to tumour cells (McCart et al., 2001; Mullen & Tanabe, 2002; Thorne et al., 2005).

1.7 Innate immune response to VACV infection

At the onset of infection, VACV is confronted by the innate and subsequent adaptive immune response that contains and eventually eliminates the viral infection. In response to this threat, VACV has evolved numerous ways to modulate the immune response and its immune evasion mechanisms have become the subject of intense study in recent years.

In animal models of VACV infection, infection occurs through exposure at the subcutaneous layer of the skin or through mucus membranes in the airways and the first host immune response is mediated by innate leukocytes and non-specific mechanisms of microbial recognition. VACV induces, and thereby needs to modulate, innate immune mechanisms including the complement system, macrophages, natural killer (NK) cells, γδ T cells and the antiviral state induced by interferons (IFNs).
1.7.1 Complement

The complement system is an important effector component of both the adaptive and innate response. The complement system is made up of at least 30 plasma and cell surface proteins and includes 3 activation pathways. The main function of these pathways is to mark targets permanently for destruction, to recruit other proteins and cells that facilitate the target’s destruction and in the case of some bacteria and viruses to participate directly in the destructive process by osmotic lysis. Many of the proteins of the activation pathways are proteinases and activation occurs in a cascade by proteolytic activation of one zymogen that then activates the next zymogen in the pathway. Antigen-antibody complexes provide the activating signal for the classical pathway of complement activation. Sequential activation of complement components C1, C4 and C2 produces the key enzyme C3 convertase which acts to cleave and activate C3. C3 cleavage results in a small C3a fragment, a potent anaphylatoxin that induces mast cell degradation, promotes inflammation and recruits phagocytotic cells, and the larger C3b fragment that covalently attaches to the activating antigen, marking it for destruction. C3b acts as a site for activation and assembly of the complement membrane attack complex (MAC), a self-assembling pore-forming complex formed from plasma membrane proteins C5, C6, C7, C8 and C9 that kills targets by osmotic lysis. C3b also acts as an opsonin, enhancing phagocytosis by binding complement receptors on neutrophils and macrophages.

The second pathway, the alternative pathway, is activated without antibody (Ab) by microbial structures that neutralise inhibitors of spontaneous complement activation via C3bBb, the alternative pathway convertase. This leads to the deposition of C3b and the activation of MAC. The mannan-binding lectin (MBL) pathway utilises the MBL protein, a serum protein of the collectin family that is structurally and functionally similar to C1q of the classical pathway and binds to mannose-containing carbohydrates on microbial surfaces. This interaction activates MBL-associated serine proteases (MASP) 1 and 2 that activate C4 and thereby the remainder of the pathway. Recent studies have implicated a role for extrinsic proteases and coagulating molecules in the initiation of complement, expanding its role in the innate immune response (Atkinson & Frank, 2006; Markiewski et al., 2007). Activation of the complement system can lead to the lysis of virions, the neutralisation of virions through
opsonisation, membrane lysis of infected cells, increased phagocytosis and chemotaxis of neutrophils.

VACV encodes a 35 kDa secreted protein, related to the family of complement control proteins (CCP), called vaccinia complement control protein (VCP). It is most closely related to human C4b-binding protein (C4BP), contains multiple short consensus repeats (SCRs) and interacts with cells bearing C4b to prevent binding to the complement receptor 1 (Kotwal & Moss, 1988b). VCP also inhibits complement activation by both the classical and alternative pathways (Isaacs et al., 1992a). There have been three other VACV proteins proposed as complement modulates: B5 has amino acid (aa) similarity to factor H, a regulator of the alternative pathway, and two proteins from VACV strain rabbitpox (RPXV) that show significant aa similarity to complement proteins. RPXV B20 is similar to the complement component C5 whilst RPXV M2 shows similarity to C4a and C3 (Bloom et al., 1991).

VACV also resists complement-induced neutralisation by the incorporation of cellular modulators, cluster of differentiation (CD)55 and CD59 proteins, into the outer membrane of EEV (Vanderplasschen et al., 1998b). Host cells express these regulatory proteins to down-regulate complement activation and prevent unwanted damage to host tissues. CD55 inhibits the formation and accelerates the decay of C3-convertases involved in the classical and alternative pathways whilst CD59 prevents the formation of MAC.

1.8 Innate immune cells

Phagocytic cells engulf pathogens and use intracellular vacuoles to focus toxic effector molecules such as nitric oxide (NO) and degenerative enzymes in an effort to destroy the organism. The major phagocytic cells are neutrophils, monocytes and macrophages and are crucial initiators and regulators of innate and adaptive immune responses.

1.8.1 Neutrophils

Neutrophilic polymorphonuclear leukocytes (PMN) mediate their role in host defence after their rapid recruitment to sites of infection via chemotaxis, by the
phagocytosis of pathogens, secretion of the contents of their vesicles and production of reactive oxygen intermediates. Activated neutrophils also interact with dendritic cells (DC) to induce DC maturation (van Gisbergen et al., 2005). In this way, neutrophils may contribute indirectly to triggering adaptive responses via T cells. Activated neutrophils release neutrophil elastase (NE, also called Elastase 2), a powerful serine protease that along with its degenerative properties can induce inflammation (Korkmaz et al., 2008) through the modulation of cytokines (Bank & Ansorge, 2001) and up-regulation of gene expression by the activation of IL-1R/Toll-like receptor (TLR) pathways. NE up-regulates IL-8 via TLR-4 (Walsh et al., 2001; Wiedow & Meyer-Hoffert, 2005), and thereafter IL-8 attracts and activates neutrophils in a positive feedback loop.

Neutrophils can phagocytose radiolabelled VACV in vitro (West et al., 1987). In the intradermal (i.d.) infection model of VACV infection, granulocytes were seen to increase slightly by day 2 p.i and continue to increase to day 10 p.i. when their numbers began to decline (Jacobs et al., 2006). VACV also down-regulates the NE signalling cascade by interfering with the TLR signalling pathways (discussed in section 1.9.1) instigated by NE (Carroll et al., 2005).

1.8.2 Macrophages

Macrophages have important functions as antigen presenting cells (APC) for the activation of NK and T cells, phagocytosis of pathogens and immunomodulation by the secretion chemokines, cytokines and components of the complement system (Fujiwara & Kobayashi, 2005). They also have an important role in cross-presentation of antigen to activated T cells (Ramirez & Sigal, 2002). Macrophages are a major cell population in most tissues in the body and their numbers increase further during inflammation, wounding and malignancy. In tissues with close contact to the environment, such as the skin, approximately 50% of murine leukocytes are macrophages (Dupasquier et al., 2004) whilst they represent 95% of resident phagocytic cells in the respiratory tract and lungs (Curtis & Kaltreider, 1989).

Resident macrophages constitute a major subpopulation of leukocytes in mouse dermal tissue and early after VACV infection (day 4), increase in number (Jacobs et al., 2006). Their importance in the control of VACV infection is illustrated by several
studies. When macrophages are depleted in a murine intranasal (i.n.) model of VACV infection, the overall severity of infection increased, with reduced clearance of VACV and a more pronounced weight loss (Luker et al., 2005; Rivera et al., 2007). VACV replication is abortive in activated macrophages (McLaren et al., 1976; Buchmeier et al., 1979; Natuk & Holowczak, 1985). In freshly isolated murine peritoneal macrophages, VACV infection induces nitric oxide synthase expression (Karupiah & Harris, 1995) and is important in control of VACV and ectromelia virus (ECTV) infection, inhibiting DNA replication and late protein synthesis (Karupiah et al., 1998). Whilst in the murine macrophage cell line RAW 246.7, VACV inhibited NO production (Bellows et al., 2003). VACV infection of macrophages results in apoptosis via a mechanism that is dependent on early gene expression and is NO-independent (Humlova et al., 2002).

### 1.8.3 Natural killer cells

NK cells are large granular lymphocytes that contribute to the early immune response to virus infection either by lysis of infected cells or by the release of antiviral cytokines in particular IFN-γ (Biron & Brossay, 2001; Mercer et al., 2005; Vivier et al., 2008). During inflammation, viral infection and tumour growth, NK cells are rapidly recruited from the blood into injured tissues. They are activated by IFNs and macrophage-derived cytokines (IL-2 and tumour necrosis factor (TNF)-α) and may recognise infected cells either directly, through identification of alteration in expression of major histocompatibility complex (MHC), or via Fc receptors on bound Abs, leading to Ab-dependent cellular cytotoxicity (ADCC).

A balance between activating and inhibitory receptors from cellular interactions and soluble mediators control the cytolytic activity of NK cells, with their activation being stringently controlled by inhibitory receptors that act as a ‘fail-safe’ to avoid inadvertent stimulation and consequential harm to normal cells. The susceptibility of target cells to natural killing is inversely related to their expression of MHC class I molecules. NK cells survey cells for down-regulation or over-expression of MHC class I or ‘missing-self’ (Fig. 1.3), with activating receptors dampening or regulating signals depending on the target cell encountered.
1.8.3.1 NK cell receptors

Most activating and inhibitory NK cell receptors are encoded by genes in two genomic regions, the NK-gene complex (NKC, (Yokoyama et al., 1991; Yokoyama & Plougastel, 2003)) and the leukocyte-receptor complex (LRC (Wende et al., 1999)). While the receptors in the LRC belong to the immunoglobulin (Ig) superfamily, the NKC-encoded molecules encode lectin-like molecules.

The human killer Ig-like receptors (KIR), are a family of type I membrane proteins which possess two (2D) or three (3D) Ig domains, with either a long (L) or short (S) cytoplasmic domain. Each member interacts with a group of closely related human leukocyte antigen (HLA) class I molecules that are determined by the aa belonging to the C-terminal portion of the α1 helix of the MHC class I molecule (Boyington & Sun, 2002). KIRs with long cytoplasmic tails are inhibitory and contain immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences, while those with short tails activate cytotoxicity via interactions with immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor molecules by a charged aa residue in the transmembrane region (TM).

A second group of receptors known as immunoglobulin-like transcript (ILT) are also members of the Ig superfamily and are categorised into three groups: those with ITIM motifs, those with short cytoplasmic domains and a charged aa residue in the TM, and a member that contains no transmembrane segment (Borges et al., 1997). These receptors are expressed in monocytes, macrophages, DCs, B, T and NK cells (Colonna et al., 1999). ILT-2 is expressed on NK cells and is targeted by the human cytomegalovirus (HMCV) protein UL18, a MHC class I homolog (Cosman et al., 1997; Wilkinson et al., 2008).

Members of the NKC lectin-like family of NK receptors are dimeric type II transmembrane glycoproteins, with each chain comprising an extracellular C-type lectin-like domain (CTLD, (Drickamer, 1999)) connected by a stalk region of 25-75 residues to transmembrane and cytoplasmic domains. These receptors include the murine Ly49 receptors (Wilhelm et al., 2002), the natural killer cell receptor protein 1 (NKR-P1) receptors (Giorda et al., 1990), the human and murine CD94/NKG2 family of receptors (Boyington et al., 1999) and CD69 (Llera et al., 2001). The Ly49 family (Karlhofer et al., 1992; Wilhelm et al., 2002) comprise receptors with a single CTLD
and are expressed as disulphide-bonded homodimers. Ly49A, C and G2 contain cytoplasmic ITIMs and function as MHC class-I-specific inhibitory receptors (Matsumoto et al., 1998), although Ly49G2 has some peptide specificity (Brennan et al., 1996). Ly49D and Ly49H function as activating receptors, with a transmembrane arginine residue for association with the adaptor protein DNAX-activating protein of molecular mass 12 kDa (DAP12). Activating Ly49 receptors regulate lymphocyte function-associated antigen 1 (LFA-1) intercellular adhesion molecule 1 (ICAM-1) dependent adhesion between cytolytic cells and their target cells (Osman et al., 2007), and promote the delivery of cytotoxic granules towards target cells (Bryceson et al., 2005).

The NKG2 family, like Ly49 and NKP-P1 families, contains activating (NKG2C, -E and –H) and inhibitory members (NKG2A and –B) (Plougastel et al., 1996). NKG2 molecules must dimerise with the invariant CD94 molecule for expression at the cell surface and signalling functions (Lazetic et al., 1996). Human CD94/NKG2A and CD94/NKG2C recognise the non-classical MHC class I molecule HLA-E (Braud et al., 1998), while the murine CD94/NKG2A, -C and –E recognise the HLA-E related molecule Qa-1 (Vance et al., 1998). HLA-E and Qa-1 are similar to MHC class I molecules but bind and present leader peptides of classical MHC class I molecules and non-classical HLA-G. Thereby, NK cells can monitor individual HLA allele changes by KIRs but also the global expression of class I molecules and HLA-E itself by CD94/NKG2 heterodimers. CD94/NKG2 receptors are expressed on most NK cells, γδ T cells and a subset of effector/memory CD8+ γδ T cells

The activating receptor NKG2D is distinct from other NKG2 molecules as it is expressed as a disulphide-bonded homodimer by NK, NKT cells, γδ T cells, CD8+ T cells and macrophages (Bauer et al., 1999). There is also only limited sequence similarity between NKG2D and other NKG2 molecules (28% aa identity for the lectin-like domain) whereas other NKG2 molecules are closely related to each other (~ 70% aa identity). In humans, the NKG2D ligands are MHC class-I-chain related protein (MIC) A, MICB, and the unique long (UL)16-binding protein (ULBP) family (Bauer et al., 1999; Cosman et al., 2001). The murine ligands are the minor histocompatibility molecule H-60 (Diefenbach et al., 2000), members of the retinoic acid early transcript (Rae) 1 family (Cerwenka et al., 2000) and mouse ULBP-like transcript 1 (Mult1, (Carayannopoulos et al., 2002; Diefenbach et al., 2003)). The NKG2D ligands are type
I transmembrane proteins related to MHC class I molecules but do not associate with β2-microglobulin (β2m), nor do they bind peptides. The NKG2D receptor thereby recognises self proteins that are inducible (Groh et al., 1996; Li et al., 2001) and up-regulated on the surface of most tumours and many infected cells. Signalling pathways associated with infection, such as TLR signalling (see Section 1.9.1), lead to the up-regulation of NKG2D ligands on macrophages (Hamerman et al., 2004).

The NKR-P1 family (Giorda et al., 1990; Mesci et al., 2006) includes NK1.1 (NKR-P1C, (Ryan et al., 1992)), a serological marker of NK cells from C57Bl/6 mice. NKR-P1C acts as an activating receptor, with a charged residue at the TM and interacts with FcεRIγ. Only NKR-P1A (Lanier et al., 1994) has been identified in humans, and is found on only a subset of NK cells whilst murine NKR-P1C is expressed by all NK cells and a subset of T cells (NKT cells). Originally NKR-P1 receptors were thought to recognise carbohydrates (Bezouska et al., 1994) but physiological ligands were shown to comprise a group of C-type lectin-like proteins designated C-type lectin-related protein (Clr) (or Clec2 or Ocil (Plougastel et al., 2001; Zhou et al., 2001; Iizuka et al., 2003; Carlyle et al., 2004)). Although they lack at least one of the conserved cysteines thought to form disulphide bridges in CTLDs, Clr-b can recognise certain GAGs in a Ca2+-independent manner (Plougastel et al., 2001). Only two ligands for the murine NKR-P1 receptors have been demonstrated: Clr-b is a ligand for the inhibitory NKR-P1B and D receptors (Carlyle et al., 2004) and is expressed on a broad range of cells, whereas Clr-g is a ligand for the activating receptor NKR-P1F (Iizuka et al., 2003) and is expressed preferentially in activated NK cells.

Other molecules encoded by the NKC have been identified. These include the killer cell lectin-like receptor subfamily F1 (KLRF1, or NKp80, (Roda-Navarro et al., 2000; Vitale et al., 2001)) and a human NKC-encoded lectin-like molecule that contains cytoplasmic tyrosine residues that are not typical of ITIM or ITAM and has no charged TM residues. The ligand of KLRF1 is another lectin-like receptor, human activation-induced C-type lectin (AICL, (Hamann et al., 1997; Welte et al., 2006)). KLRG1 (also known as mast cell function-associated antigen (MAFA)) was identified on rat mast-cells (Guthmann et al., 1995). Human and murine orthologues are expressed on NK and T cell subsets but not mast cells (Blaser et al., 1998; Corral et al., 2000) and bind cadherin-E, -N and –R to inhibit lysis of cells expressing the cadherins (Ito et al., 2006). Human lectin-like transcript 1 (LLT1, (Boles et al., 1999)), AICL and

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mouse Clr form another family, related to CD69 and are among the earliest cell-surface molecules induced during lymphocyte activation.

Each NK cell usually expresses only a selection of these receptors, so the NK cell population has a diverse repertoire of different MHC class I specificities and other ligands including DNA-damage or stress-induced ligands (Valiante et al., 1997; Kubota et al., 1999; Raulet et al., 2001). NK cells can also recognise target cells indirectly through CD16, a receptor specific for the Fc region of IgG, thereby activating ADCC towards these Ab-bound cells.

1.1.1.1 Signalling from inhibitory and activating NK receptors

Inhibitory receptors mediate their effect through ITIM sequences (V/IxYxxL/V) in their cytoplasmic domains. When phosphorylated, ITIMs recruit and activate Src homology 2 (SH2)-domain-containing protein tyrosine phosphatases (SHP)-1, SHP2 (Campbell et al., 1996; Olcese et al., 1996; Yusa & Campbell, 2003) or SH2-containing inositol polyphosphate 5-phosphatase (SHIP), depending on the receptor analysed. The phosphorylation of SHP-1 or SHP-2 results in a decrease in phosphorylation of numerous intracellular proteins involved in activatory signal transduction, see below (Valiante et al., 1996; Palmieri et al., 1999; Stebbins et al., 2003)).

Activating receptors lack an intracellular ITIM and instead have a charged residue in their TM domain that allows their non-covalent association with signalling adaptor molecules containing an intracytoplasmic ITAM. The ITAM is defined by the prototype sequence Yxx(L/I)_{n} Yxx(L/I) (Reth, 1989). NK cells express three ITAM-containing adaptor proteins FceRIγ (Hibbs et al., 1989), CD3ζ (Anderson et al., 1989), and DAP12 (Lanier et al., 1998). The phosphorylation of the tyrosine residue recruits protein tyrosine kinases including spleen tyrosine kinase (Syk) and zeta-chain-associated protein kinase 70 kDa (ZAP-70). Their recruitment and phosphorylation initiate several kinase cascades and the release of intracellular calcium to trigger the cytolytic cascade and cytokine release (McVicar et al., 1998). This response is coupled to a series of downstream regulators including phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3K), extracellular-signal regulated kinase (ERKs), the Vav family guanine nucleotide exchange factor (Galandrini et al., 1999) and Rho-Rac.
guanosine 5'-triphosphate (GTP)-binding proteins and their effectors, reviewed in (Colucci et al., 2002). The mouse NKR-P1C associates with the ITAM-containing adaptor protein FcεRIγ (Arase et al., 1997) while the human NKR-P1A receptor does not contain ITIM sequences nor does it contain a charged residue in the TM (Lanier et al., 1994). The human NKR-P1A receptor modulates NK cell function via the activation of acid sphinomyelinase and N-acyl-sphingosine (ceramide) generation, which can induce apoptosis, proliferation, and differentiation in cells (Pozo et al., 2006).

In most cell lines NKG2D pairs with DAP10, which lacks an ITAM, and instead contains the tyrosine-based motif YxxM that binds to the p85 subunit of PI3K (Wu et al., 1999). In murine NK cells, NKG2D associates with DAP12 (Diefenbach et al., 2002; Gilfillan et al., 2002) and DAP12 is necessary for NKG2D-induced cytokine production (Diefenbach et al., 2002). This is due to mice having two isoforms of NKG2D, that are generated by alternative splicing and differ in the presence (NKG2D-L) or absence (NKG2D-S) of 13 aa at the N terminus in the cytoplasmic domain (Diefenbach et al., 2002; Gilfillan et al., 2002). NKG2D activation can also activate Janus activated kinase (JAK) 2, signal transducers and activators of transcription (STAT) 5, ERK 1/2 and mitogen-activated protein kinase (MAPK) 1/2 (Sutherland et al., 2002).

1.8.3.2 NK cell activity in OPV infection

Several observations suggest that NK cells play a role in defence against VACV infection. In mice, NK cells are recruited to the site of infection in mice as early as 6 h.p.i. (Natuk & Welsh, 1987; Prlic et al., 2005). In the i.d. model of VACV infection, NK cells represented only <1% of the lymphocyte population in mock-infected ears, but increase after infection up to day 7 p.i. in infected ears (Jacobs et al., 2006). Depletion of NK cells renders mice more susceptible to VACV infection (Bukowski et al., 1983; Karupiah et al., 1991). In athymic nude mice, which lack functional T cells, NK activity was 3-fold higher than in control animals, perhaps to compensate for reduced CD8+ T cell activity (Karupiah et al., 1990). Following infection by ECTV, NK cells are necessary for initial recovery (Jacoby et al., 1989; Fang & Sigal, 2005).
In humans, VACV provokes an increase in the susceptibility of cells to NK cell-directed lysis *ex vivo* (Brooks *et al.*, 2006; Chisholm & Reyburn, 2006). Direct action of type I IFN on NK cells, but not on DCs, is required for NK activation in response to VACV infection *in vitro* and *in vivo*, and this activation of NK cells can lead to efficient VACV clearance (Martinez *et al.*, 2008), although in the context of VACV infection *in vivo*, DCs participate in the direct activation of NK cells (Raulet, 2004). As infection progresses, there is a reduction in cytolytic capacity of NK cells, indicating that NK cells are subject to infection themselves (Brutkiewicz *et al.*, 1992; Kirwan *et al.*, 2006), although it could be that down-regulation of their activity occurs by mechanisms other than direct infection. Some groups have seen sufficient down-regulation of MHC class I molecules (Kirwan *et al.*, 2006) or non-classical MHC class Ib molecules on the cell surface of infected cells to prevent KIR-mediated or NKG2A protection of the target cell respectively and NK cells exhibit increased lysis of VACV-infected targets *in vitro* (Baraz *et al.*, 1999).

Although deletion of the VACV genes *NIL* (Jacobs *et al.*, 2008) or *F3L* (Froggatt *et al.*, 2007) altered the NK cell responses in the i.n. model, VACV encoded proteins that directly prevent NK-mediated destruction of target cells, such as those encoded by human and murine cytomegalovirus (Krmpotic *et al.*, 2002; Dunn *et al.*, 2003; Lodoen & Lanier, 2005) have not been reported.

In Cowpox virus (CPXV) and Monkeypox virus (MPXV), the orthopoxvirus MHC class I-like protein (OMCP) is secreted during infection and is a competitive antagonist of NKG2D, thereby inhibiting NKG2D-dependent NK killing of infected cells (Campbell *et al.*, 2007). It has been hypothesised that OPVs will encode a modulator of NK function only if they modulate MHC expression excessively, as is seen with CPXV (Byun *et al.*, 2007; Dasgupta *et al.*, 2007) and some herpes viruses (Ziegler *et al.*, 1997; Fruh *et al.*, 2002; Trgovcich *et al.*, 2006) but not VACV. MPXV does not down-regulate MHC class I or II *in vitro* but has been shown to inhibit T cell responses by a mechanism that requires cell-to-cell contact and is MHC class I and II processing/presentation independent (Hammarlund *et al.*, 2008).

As well as modulating expression of host proteins including MHC class I molecules (van der Wal *et al.*, 2002; Barel *et al.*, 2003) and NKG2D ligands (Beck & Barrell, 1988; Dunn *et al.*, 2003), some CMV encode decoy ligands to immune cell
receptors (Lodoen & Lanier, 2005). Whilst MHC class I homologs (Arase et al., 2002; Smith et al., 2002b) inhibit T and NK cell function in an MHC-dependent manner, rat CMV (RCMV) encodes the C-type lectin-like protein RCTL that acts as a decoy ligand for the inhibitory NK receptor NKR-P1B (Voigt et al., 2001; Voigt et al., 2007) in an MHC-independent manner. During RCMV infection, expression of NKR-P1B’s cellular ligand, Clr-b, is rapidly down-regulated. RCTL subverts NK killing of infected cells by counteracting the loss of Clr-b.

1.8.4 NKT cells

NKT cells are a subset of T lymphocytes that express NK markers (Bendelac, 1995). The T cell receptor (TCR) consists of an invariant T cell α chain paired with one of the three β chains and NKT cells are regarded as non-specific innate cells. NKT cells recognise CD1d, a non-polymorphic MHC class I-like ligand which binds self- and foreign lipids and glycolipids. Upon activation, NKT cells are able to produce large quantities of IFN-γ, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF) and multiple other cytokines and chemokines (such as IL-2 and TNF-α) within minutes of activation (Ota et al., 2005). Both activatory and inhibitory NK receptors (Section 1.8.3.1) are expressed by subsets of T cells, including NKG2D, NKR-P1, KIRs, Ly49 and CD94/NKG2 receptors (Raulet, 2003; Vivier & Anfossi, 2004).

In the i.d. model of VACV infection, subsets of T cells expressing the NK cell marker NK1.1 were very low (<1%) in uninfected tissue and did not increase after infection, whilst a subset expressing the marker DX5 (CD49b) emerged p.i. and underwent >400-fold expansion in cell number. At day 9 p.i. 67% were DX5+CD3+CD4+ and 28% were CD8+. After day 10 p.i. the percentage and number of these cells had decreased corresponding with the observed decrease in lesion size (Jacobs et al., 2006).

1.8.5 Importance of C-type lectins in immune response

C-type lectins are a group of Ca²⁺-dependent (C-type) carbohydrate-binding (lectin) proteins that contain a compact domain that mediates carbohydrate binding called the carbohydrate recognition domain (CRD). Comparison of different proteins
CRDs revealed conserved residues that are characteristic of the domain (Drickamer, 1988; Drickamer, 1989). As the number of proteins identified with these motifs has grown it became apparent that not all proteins with CRDs can bind carbohydrates or Ca\(^{2+}\) and the term C-type lectin-like domain (CTLDs, (Drickamer, 1999)) was coined to describe such domains. The superfamily of proteins containing CTLDs is a large group of proteins with diverse functions. The classification of the family members is based on the overall domain architecture of the CTLD-containing proteins reviewed in (Drickamer & Fadden, 2002; Zelensky & Gready, 2005). The CTLD fold is a double-loop structure (loop-in-loop), stabilised by two conserved disulphide bridges located at the bases of the loops formed by four cysteines (C1-C4). The second loop, (long loop region) is structurally and evolutionary flexible and is involved in Ca\(^{2+}\)-dependent binding. These domains bind the hydroxyl groups of monosaccharides in complex with a Ca\(^{2+}\) ion, which is coordinated by a highly conserved Asp-Glu-Cys (EDC) motif. CTLDs can be divided into two groups depending on the presence (canonical) or absence (compact) of a long loop region. CTLDs that are not related to the classical fold by sequence, have been identified, such as the link-protein type module of CD44 (Kohda et al., 1996) and the angiogenesis inhibitor protein endostatin (Hohenester et al., 1998). CRDs generally have a low affinity for their ligands but this is compensated by having multiple CRDs in a single peptide or oligomerisation of multiple single CRDs (Bouyain et al., 2001; Cambi & Figdor, 2005).

Whilst many CTLDs selectively bind carbohydrates, others bind proteins (NK receptors, (see Section 1.8.3.1), reviewed in (Natarajan et al., 2002)), lipids ((Sano et al., 1998; Shi et al., 2001), inorganic compounds (such as calcium carbonate (Geider et al., 1996)) and ice (Ewart et al., 1992).

The NK cell receptor group of CTLD-containing proteins (Group V lectins), as described in section 1.8.3.1 are all type II TM proteins containing divergent CTLDs. Group V not only contains the NK cell receptors but also structurally-related proteins such as the lectin-like oxidized low-density lipoprotein receptor (LOX-1), which plays an important role in leukocyte extravasation upon inflammatory stimuli and in scavenger function (Hayashida et al., 2002; Honjo et al., 2003) and Dectin-1. Dectin-1 lacks cysteines in its stalk region, and therefore probably does not dimerise and has an ITAM in its cytoplasmic tail. Unusually, Dectin-1 recognises carbohydrates (\(\beta\)-glucan,
(Weis et al., 1998; Brown et al., 2003)) but lacks many of the conserved residues in the CRD indicative of carbohydrate binding in other lectins (Drickamer, 1999).

Another important family of C-type lectins are the selectins (E-, L- and P-selectin), which mediate adhesion and homing of leukocytes by the recognition of carbohydrates sialyl-Lewis X (sLe^X) and sialyl 6-sulpho Lewis X (s6Le^X), (Erbe et al., 1992; McEver et al., 1995; Nelson et al., 1995; Bouyain et al., 2001). In a similar way, the macrophage mannose receptor (MMR) is multifunctional, acting as a scavenger receptor via carbohydrate binding (Leteux et al., 2000) and in phagocytosis of microbial pathogens (Weis et al., 1998) and under certain conditions, can mediate lymphocyte binding via L-selectin (Irjala et al., 2001). Another family of CTLD proteins involved in trafficking of leukocytes is the type II receptors which include DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), DC-SIGN receptor (DC-SIGNR) and DC immunoreceptor (DCIR). All the type II receptors contain one Ca^{2+}-dependent CRDs. The interaction between DC-SIGN and ICAM-2 regulates the chemotaxis of DCs across resting and activated endothelium (Geijtenbeek et al., 2000a), mediates DC-T cell interactions (Geijtenbeek et al., 2000c; van Kooyk & Geijtenbeek, 2002) and acts as an antigen uptake receptor on DCs (Engering et al., 2002). The latter process is exploited by HIV (Geijtenbeek et al., 2000b; Geijtenbeek et al., 2000c). The CRD of DC-SIGN is separated from the TM region by a neck region, consisting of seven complete and one incomplete tandem repeat sequences. This neck region is required for oligomerisation (Mitchell et al., 2001) and ligand binding (Bernhard et al., 2004).

The collectins are a group of soluble C-type lectins that can neutralise a broad range of bacterial and fungal pathogens through aggregation or complement activation via the MBL pathway. Collectins can also induce the production of pro-inflammatory molecules, such as cytokines, and reactive oxygen species in phagocytes by interacting with other cell surface receptors or by scavenging of bacterial molecules, for example lipopolysaccharide (LPS).

Utilising a diverse superfamily of proteins, the host can distinguish self and non-self as well as drive cell trafficking to specific areas, as and when different cell populations are required. C-type lectins have been described in bacteria and viruses. Bacterial CTLD-containing proteins include toxins such as pertussis toxin (Stein et al., 1994) whilst viral CTLD proteins include structural envelope proteins and have been
found in many viruses including VACV (Duncan & Smith, 1992; Wilcock et al., 1999), fowlpox virus (Afonso et al., 2000), African swine fever virus (ASFV, (Neilan et al., 1999; Galindo et al., 2000)), Epstein-Barr virus (EBV, (Mullen et al., 2002)) and alcelaphine herpes virus (Enser et al., 1997). Whilst bacterial CTLD proteins were assigned to the CTLD superfamily on the basis of structural similarity only, viral CTLD proteins contain a canonical CTLD and show similarity to mammalian CTLDs.
Fig. 1.3 Representation of NK cell-target interactions that can activate NK function
Left panels depict the signalling components of the NK cell as a balance of contact-mediated inhibitory (polygons) and stimulatory (circles) signals. Innate cytokines (stars and sliding weight) can alter the equilibrium. Right panels schematise interactions between the two cell types. (a) A resting NK cell in contact with a normal uninfected target is inhibited, as inhibitory signals constitutively outweigh activating signals. Infected cells often down-regulate inhibitory MHC class I molecules (b) and up-regulate ligands for activating receptors (c). They also induce ‘innate’ cytokines that bias NK-cell ligand recognition and signalling in favour of triggering (d). Adapted from (Carayannopoulos & Yokoyama, 2004)
Fig. 1.4 Natural killer cell receptors and their signalling pathways

(A) Representation of the types of receptors and their domains present on human and mouse NK cells (Vivier & Anfossi, 2004) (B) Signal transmission from NK cell receptors. (i) The recruitment and activation of ITIM-containing adaptors such as SHP-1, by inhibitory receptors leads to the dephosphorylation of proteins whose phosphorylation is necessary for conveying activating signals. (ii) Activating receptor complexes on NK cells associate with the ITAM-bearing adaptor molecule DAP12 through charged residues in their transmembrane regions. The membrane adaptor protein DAP10 does not have an ITAM in its cytoplasmic region but has an YxxM motif that is a potential Src homology 2 (SH2) binding domain. Activation of NK cells through ligation of these complexes leads to recruitment and activation of SH2 domain-containing protein tyrosine kinases, such as Syk or ZAP-70, except for NKG2D which recruits PI3-kinase. Adapted from (Borrego et al., 2002)
1.8.6 DCs

DCs are APCs that play an essential role in bridging innate and adaptive responses to infection by activating naïve CD4$^+$ and CD8$^+$ T cells. Immature DCs, such as Langerhans cells (LC), reside underneath the epithelia of peripheral tissues where they are ideally located to acquire antigens of invading pathogens. Pathogen structures such as LPS and endogenous signals from CD40-CD40L interactions and TNF-α production by NK cells mediate DC maturation. Mature DCs leave the peripheral tissues and migrate to lymph nodes to present MHC-bound antigen to T cells which in turn initiates T cells responses. A subset of DCs termed plasmacytoid DCs (pDCs) are responsible for the production of high levels of type I IFN in some viral infections that activate NK cells (Andoniou et al., 2005) and inducing an antiviral state in cells bearing IFN receptors (discussed in Section 1.9.3.1).

CD11c$^+$MHC II$^+$ APC cells (dermal DCs and LCs) increase only slightly post-VACV infection and their number are comparable to the number seen in situ prior to infection (Jacobs et al., 2006) when they make up approximately 10% of the cell population in the mouse dermis (Dupasquier et al., 2004). Direct visualisation of sectioned lymph nodes by confocal microscopy showed direct presentation of VACV antigen to prime CD8$^+$ T cells by infected DCs (Norbury et al., 2002). Depletion of DCs in mice abrogates the production of IFN-γ by T cells following MVA infection, suggesting DC-mediated antigen presentation is required for the induction of T cell-mediated responses in vivo (Liu et al., 2008). Whilst MVA induces moderate activation of DCs (Drillien et al., 2004), VACV WR or VACV Cop inhibits maturation of immature DCs (Engelmayer et al., 1999; Drillien et al., 2000; Drillien et al., 2004) inducing delayed apoptosis ex vivo and infection of DCs by both strains is abortive (Drillien et al., 2004). Mature DCs seem to be more resistant to VACV-induced cell death and can induce naïve CD8$^+$ T cell responses (Yates & Alexander-Miller, 2007). VACV infection inhibits their migration without altering surface expression of chemokine receptors or impairing cellular locomotion (Humrich et al., 2007). In vivo, VACV infection induces DC maturation but impaired DC antigen-presentation function by reducing MHC class II expression (Yao et al., 2007). Despite this modulation of DC function by VACV, uninfected bystander DCs may induce viral antigen-specific cytotoxic T lymphocytes (CTLs) by cross-presentation rather than by direct antigen presentation (Larsson et al., 2001; Ramirez & Sigal, 2002; Liu et al., 2008).
1.8.7 T cell receptor γδ cells

TCR γδ cells are a minor proportion of lymphocytes (<10%) in peripheral blood but are abundant in the epidermis (Asarnow et al., 1988) and are the first line of defence that is crucial for the control of viral infection (Bukowski et al., 1994; Ninomiya et al., 2000) including infection with VACV. In contrast to αβ T cells, γδ T cells can target antigens directly, not just via MHC presentation (Kaufmann, 1996).

In the i.d. model of VACV infection, γδ T cells were the major populations of lymphocytes up to day 4 p.i. with their number increasing up to this point but with no change relative to the percentage of the total leukocyte population until after day 4 when their relative percentage began to decrease (Jacobs et al., 2006). In mice lacking γδ T cells, virus titres were significantly higher and these mice had increased mortality compared to normal mice (Welsh et al., 1997; Selin et al., 2001) attributed to enhanced VACV replication early after infection. Upon infection by VACV, there was a 4-fold increase in γδ T cells and 12% of the γδ T cells were producing IFN-γ compared to 1% in uninfected mice (Selin et al., 2001). In a VACV-challenge study on vaccinated patients, an expansion of VACV-specific γδ T cells was seen, indicating that a memory effector γδ T response was induced (Abate et al., 2005; Agrati et al., 2006).

1.9 Pattern recognition receptors (PRRs)

The recognition of microbial ‘non-self’ lies in the ability of the host to recognise conserved products of microbial metabolism that are unique to the microbial organism and not produced by the host, termed pathogen-associated molecular patterns (PAMPs) and these include nucleic acid, protein or lipid structures. These PAMPs are recognised by receptors called pattern recognition receptors (PRRs). PRRs can be secreted, membrane bound or soluble. Secreted PRRs bind to microbial cells and flag them for destruction by the complement system or phagocytosis, whilst membrane bound and soluble receptors activate signalling pathways that induce anti-microbial responses upon PAMP recognition. The importance of TLRs in the detection of viral infection and activation of antiviral effector mechanisms is highlighted by the ways that viruses have developed to modulate these signalling pathways.
**1.9.1 Toll-like receptors (TLRs)**

TLRs are a family of PRRs expressed particularly on macrophages and DCs but also on neutrophils, eosinophils and many epithelial cells. TLRs are present in the plasma membrane or endosomal membrane. There are at least 10 human TLRs and 12 murine TLRs. Each TLR is a transmembrane receptor that contains a cytoplasmic Toll/IL-1 receptor (TIR) domain and an extracellular leucine-rich repeating (LRR) domain and recognises a unique subset of PAMPs (Takeda & Akira, 2007). Upon activation, the TIR domain recruits TIR domain-containing adaptors, myeloid differentiation factor 88 (MyD88) for TLR1, 2, 4-9 and TIR domain-containing adaptor inducing IFN-β (TRIF) for TLR 3 and 4. Both pathways lead to the activation of transcription factors such as nuclear factor – kappa –light chain-enhancer of B cells (NF-κB) and IFN-regulatory factors (IRFs). These regulate the expression of genes that encode IFNs and pro-inflammatory cytokines (Fig. 1.5). TLR 1, 2, 5 and 6 are mostly expressed on the plasma membrane. TLR4 is expressed on the plasma membrane and early endosomes and TLR3, 7, 8 and 9 are restricted to late endocytic components (reviewed in (Blander, 2007).

1.9.1.1 **TLR 2**

TLR2 recognises a variety of components from microbial pathogens and have been shown to detect Gram-positive bacteria via lipopeptides. Infection of TLR2−/− mice with VACV showed that the VACV-stimulated secretion of pro-inflammatory cytokines by DCs is dependent on the TLR2/MyD88 pathway and this pathway is required for activation of innate immunity to control of VACV in vivo (Zhu et al., 2007). This pathway is also important in the development of effector and memory formation in VACV infection in vivo by survival of activated T cells (Quigley et al., 2009).

1.9.1.2 **TLR3**

TLR3 recognises dsRNA and thereby is expected to be a key component in host recognition of viruses. DNA viruses as well as RNA viruses can produce dsRNA due to hybridisation of complementary transcripts. In an i.n. model of VACV infection TLR3−/− mice were unexpectedly more resistant to infection than wild-type (WT) with less
weight loss and reduced morbidity and mortality (Hutchens et al., 2008a). These findings were attributed to the role of TLR3 in the recruitment of leukocytes to the site of infection and its role in the induction of cytokines including IL-6 and TNF-α, which may regulate the adverse affects of VACV in vivo.

1.9.1.3 TLR4

The role of TLR4 in recognition of LPS, a major component of Gram-negative bacteria has been well characterised. TLR4 can also recognise components of the extracellular matrix produced in response to tissue damage. As well as MyD88-dependent signalling, TLR4 can activate TRIF-dependent signalling and has been reported to limit replication of a number of viruses (Kurt-Jones et al., 2000; Georgel et al., 2007). TLR4 was shown to mediate protection against VACV in an i.n. infection when viral titre was compared in C3H/HeJ mice (with a TLR4 mutation) and WT mice (Hutchens et al., 2008b).

1.9.1.4 TLR7, 8 and 9

TLRs7, 8 and 9 recognise single stranded (ss)RNA, nucleotide analogues and ss CpG-DNA, respectively, within endosomal compartments. The role of TLR9 in VACV infection was demonstrated using TLR9-deficient mice and highlighted the importance of TLR9 in DC recognition of poxviruses. MVA and ultraviolet (UV)-inactivated CVA activated DCs in normal mice whilst infectious CVA inhibited TLR9 recognition. MVA was less efficient at inducing maturation of TLR9-deficient DCs, suggesting MVA has lost some of the immune inhibitory components and this recognition utilises a TLR9-dependent component (Samuelsson et al., 2008).

TLR7 and TLR8 have also been postulated to play a role in the innate response to VACV infection (Miller et al., 2008) via the recognition of non-host nucleic acids by macrophages and DCs (Bauer et al., 2008), but these responses have not been fully characterised.
1.9.1.5 Modulation of TLR signalling by VACV

The intracellular VACV protein A52 inhibits the activation of NF-κB by multiple TLRs via its association with interleukin 1 receptor associated kinase 2 (IRAK2) and TNF receptor-associated factor 6 (TRAF6) (Harte et al., 2003). This association displaces TNF-associated kinase (TAK)-1 binding protein (TAB1) from the TRAF6 complex and MyD88 adaptor-like (MAL) from the IRAK2 complex. A46 targets host TIR adaptors such as MyD88 and TRIF (Stack et al., 2005) and thereby inhibits the translocation of NF-κB into the nucleus. A46 also interacts with two other TIR adaptor proteins MAL and TRIF-related adaptor molecule (TRAM) and thereby down-regulates TRIF-dependent pathways that induce IRF3 activation leading to expression of IFN-β.

The roles of A46 and A52 are not redundant as demonstrated by the attenuated phenotype resulting from deletion of either gene (Harte et al., 2003; Stack et al., 2005). Furthermore, both proteins are needed to efficiently shut down TLR3 signalling, whilst only A46 is required for the inhibition of TLR-induced MAPK activation (Stack et al., 2005).

NF-κB modulates the expression of many genes that are critical for the innate and adaptive immune response to infection and can be stimulated by multiple pathways including TLRs and by cytokines such as IL-1 and TNF-α (reviewed in (Perkins, 2007)). The importance of this molecule in controlling virus infection is exemplified by the number of VACV proteins that modulate its function or signalling pathways leading to its activation.

VACV protein B14 is an intracellular virulence factor made early during infection (Chen et al., 2006). B14 interacts with the inhibitor of kappa B (IκB) kinase (IKK) complex via IKK-β, thereby inhibiting the activity of the IKK complex and preventing NF-κB activation from multiple signalling pathways (Chen et al., 2008).

M2 inhibits the phosphorylation of ERK2 and subsequent NF-κB activation (Gedey et al., 2006). Unlike the aforementioned modulators of NF-κB, M2 is located exclusively at the ER and this localisation is necessary for its function (Hinthong et al., 2008). It has been postulated that M2 could inhibit the ER-localised protein, stimulator of interferon genes (STING, (Ishikawa & Barber, 2008)), which is involved in NF-κB...
and IRF3 activation and the subsequent induction of IFN, although this hypothesis has yet to be investigated (Barber, 2008).

K1L is a VACV protein that prevents the degradation of IκBα (Shisler & Jin, 2004) and thereby the translocation of NF-κB into the nucleus. It also has a role in VACV host cell restriction and allows replication in rabbit cell lines (Bradley & Terajima, 2005). The significance of its NF-κB-regulatory function in this host restriction and the mechanism by which it inhibits IκBα degradation is not well defined.

VACV K7 is a cytosolic protein that inhibits induction of IFN-β (Schroder et al., 2008) by interacting with the RNA helicase protein DEAD-box protein 3 (DDX3) (Kalverda et al., 2009) preventing TANK-binding kinase (TBK) 1 and IKKe from activating IRF3.

Fig. 1.5. Representation of TLR signalling and VACV modulation of these pathways
Adapted from (Akira & Takeda, 2004).
1.9.2 Soluble PRRs

The cytosol is also equipped with PRRs capable of detecting viral infections.

1.9.2.1 DNA sensing molecules

The DNA-dependent activator of IFN regulatory factors (DAI) protein binds cytosolic Z-DNA (Takaoka et al., 2007). Upon binding, the protein undergoes homodimerisation and phosphorylation and recruits TRAF-associated NF-κB activator (TANK)-binding kinase (TBK)1 and IRF3 and induces type I IFN expression (Wang et al., 2008). Wang et al., also demonstrated the presence of additional cytosolic DNA-sensing molecules, by suppressing the DAI protein, but these remain unidentified.

1.9.2.2 RNA sensing molecules

The RNA helicases retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) mediate recognition of viral RNA (Asahina et al., 2008; Takeuchi & Akira, 2009). Both proteins contain two N-terminal cysteine-aspartic acid protease (caspase) activation and recruitment domains (CARDs) and a C-terminal DexD/H box RNA helicase domain. Whilst RIG-1 recognises the 5'-triphosphate end of RNA and MDA-5 senses dsRNA both proteins associate with the adaptor protein IFN-β promoter stimulator 1 (IPS-1) via its CARD (Kawai et al., 2005). IPS-1 then associates with TRAF3, which recruits TBK1 and IKKε leading to phosphorylation of IRF-3 and IRF-7 leading to type I IFN production.

The dsRNA-dependent PKR contains two dsRNA binding domains and a serine-threonine kinase domain that phosphorylates eukaryotic initiator factor (eIF) 2, leading to an arrest in translation of both cellular and viral mRNAs (Garcia et al., 2007). PKR also functions downstream of TLR3 signalling to amplify the signal by interaction with TBK1. PKR can transmit these signals to eIF2 but also to the STAT protein family, IRF1, c-Jun N-terminal kinases (JNKs), p38 MAPK and NF-κB (Deb et al., 2001; Takada et al., 2007) and can also induce apoptosis (Lee & Esteban, 1994).

The dsRNA-dependent enzyme 2’5’-oligoadenylate synthetase (2’5’-OAS) converts adenosine triphosphate (ATP) to 2’5’-linked oligoadenylates with a 2’5’phosphodiesterase bond instead of 3’5’ (Hovanessian, 1991). These molecules
activate the endoribonuclease RNase L, which cleaves mRNA and rRNA, thereby leading to inhibition of protein synthesis.

The VACV E3 protein contains an N-terminal domain with similarity to the Z-DNA binding domain of DAI (Kim et al., 2003). This domain contributes to virus virulence in a murine intracranial model (Brandt et al., 2005), can inhibit apoptosis and gene transactivation (Kwon & Rich, 2005), but its involvement in the DAI pathway remains unconfirmed. The effect of E3 on the RNA-independent induction of IFN-α via the phosphorylation of IRF3 and IRF7 (Smith et al., 2001) could be explained by a down-regulation of the DAI pathway.

E3 also has a role in the modulation of dsRNA sensing by its C-terminal domain that binds and sequesters dsRNA (Rice & Kerr, 1984; Chang & Jacobs, 1993; Beattie et al., 1995). E3 inhibits the activity of PKR (Chang et al., 1992) and OAS (Rivas et al., 1998), thereby inhibiting the IFN antiviral response to VACV.

The role of the VACV K3 protein overlaps with E3 but accomplishes the inhibition of translation by a different mechanism. K3 has aa similarity to a domain of eIF2α and acts as a pseudo-substrate for PKR thereby preventing the phosphorylation of eIF2 (Beattie et al., 1991; Davies et al., 1992; Davies et al., 1993; Kawagishi-Kobayashi et al., 1997). Both K3 and E3 influence VACV host range. The importance of E3 and K3 varies depending on the cell type and level of different viral nucleic acid sensors (Shors et al., 1997; Langland & Jacobs, 2002).

![Diagram](Fig. 1.6 Modulation of soluble PRR signalling by VACV infection)
Representation of soluble PRRs involved in the innate immune response to VACV infection and the targets of VACV immune evasion proteins E3 and K3.
1.9.3 Soluble mediators of immune response

1.9.3.1 Interferon (IFNs)

IFN plays a crucial role in host defence against virus infection and can induce direct antiviral effects, growth-inhibitory effects, as well as promote adaptive immune responses such as promoting T helper cell type 1 (Th1) responses (Muller et al., 1994). IFN was discovered more than 50 years ago as an agent that inhibited the replication of influenza A virus (Isaacs & Lindenmann, 1957). The IFNs now refer to a family of structurally-related cytokines and three classes of IFN (IFN I, II and III) as well as IFN-like cytokines have been identified.

Type I IFNs (Pestka et al., 2004; Langer, 2007) include IFN-α, β, κ (LaFleur et al., 2001), ε, ω, τ (Roberts et al., 1999), δ (Lefevre et al., 1998) and ξ (limitin) (Oritani et al., 2000) and bind a common receptor complex composed of two subunits, IFNAR1 and IFNAR2 (Abramovich et al., 1994). IFNs α and β are the best characterised and are induced by PRRs and their downstream signalling cascades. IFNs are among the earliest host molecules up-regulated upon viral infection. IFN-β induction leads to up-regulation of IFN-α. The binding of type I IFNs to IFNAR can induce the induction of more than 300 IFN-stimulated genes (ISGs). Many of the gene products encode PRRs that detect viral molecules and modulate signalling pathways as well as TFs that form an amplification loop resulting in the generation of an anti-viral state in cells. Some ISGs encode proteins with direct anti-viral activity that catalyse cytoskeletal remodelling, induce apoptosis, induce shut-down of protein synthesis (including RNaseL and PKR), stimulate the expression of MHC class I molecules on the surface of the cell and induce more pro-inflammatory cytokines.

In contrast to type I IFNs, there is only one type II IFN, IFN-γ: IFN-γ does not have marked structural similarity to type I IFNs and binds a different dimeric receptor comprising IFNGR1 and IFNGR2. IFN-γ has direct anti-viral activity but is also associated with the adaptive immune response because it is secreted by T cells and promotes Th1 adaptive responses. IFN-γ is also secreted by macrophages and NK cells and leads to the activation of further inflammatory cells. Many ISGs are regulated by both type I and II IFNs, whereas others are selectively regulated by distinct IFNs.

Type III IFNs (IFN-λ1-3) were discovered more recently (Kotenko et al., 2003) and have antiviral properties. Type III IFNs signal through a receptor composed of
IFNLR1 and IL-10Rβ. They can induce similar outcomes to type I IFNs but are less widely expressed than type I IFNs although they are abundant in dermal tissues.

1.1.1.1.2 Classical IFN signalling pathway

Exposure of cells to viruses or dsRNAs induces the production of IFN-α, IFN-β and IFN-ω through the activation of PRRs especially the TLRs, cytosolic dsRNA and DNA sensors as discussed above. Each of the IFN receptor subunits interact with a member of the JAK family (Darnell et al., 1994). The IFNAR1 subunit associates with tyrosine kinase (TYK) 2 whereas IFNAR2 is associated with JAK1, whilst for the type II IFN receptor, IFNGR1 and IFNGR2 associate with JAK1 and JAK2, respectively. For all three types of IFNs, the initial step in signalling is the dimerisation and autophosphorylation of the associated JAKs, which in turn phosphorylate members of the STAT family, including STAT1 and STAT2 (Ihle & Kerr, 1995; Darnell, 1997). Tyrosine phosphorylation of the STATs leads to homo or heterodimerisation and the formation of transcriptional activator complexes. These complexes translocate to the nucleus where they bind to specific DNA sequences. An important transcriptional complex that is induced by type I IFNs is ISG factor 3 (ISGF3), comprised of a heterodimer of phosphorylated STAT1, STAT2 and IRF9. ISGF3 binds to specific elements known as IFN-stimulated response elements (ISREs), (Kessler et al., 1990) that are present in certain ISGs. The IFN-γ-activated factor (GAF) is a homodimer of STAT1, which binds to IFN-γ-activated sequence (GAS) (Decker et al., 1991; Lew et al., 1991). Of the hundreds of known ISGs, some have only ISREs or only GAS elements in their promoters, whereas others have both.

Although IFN-γ signals through a distinct receptor, constitutive sub-threshold IFN-alpha/beta signalling is critical for eliciting strong responses to IFN-γ (Takaoka et al., 2000) and IL-6 (Mitani et al., 2001). Type I IFNs also influence the adaptive immune response via the activation of T cells. T cells lacking IFNAR1 cannot respond efficiently to antigen stimulation and the CXCR3 chemokine system is regulated by IFN-α/β in CD8+ T cells, and it is critical for efficient cell activation (Ogasawara et al., 2002).
Type III IFNs are induced upon viral infection and mediate anti-viral activity through the induction of ISGs such as OAS, PKR and MxA leading to the formation of ISGF3 (Onoguchi et al., 2007).

Although the JAK/STAT pathway is the primary component in IFN-induced signal transduction, additional signal transducers support IFN signalling via STAT-dependent and STAT-independent ways. Examples of these components are ERK2, which is required for STAT-dependent effects of IFN-β (David et al., 1995) and SHP-2 which becomes phosphorylated upon IFN-α/β stimulation (You et al., 1999). The p38 MAPK pathway also functions in the induction of type I IFN-mediated signals in a variety of cell lines (Goh et al., 1999; Uddin et al., 1999; Uddin et al., 2000).

1.1.1.3 VACV modulation of IFN signalling

Several lines of evidence indicate that IFNs play a crucial role in controlling VACV infection. An early study showed that rabbit IFN protected rabbits from an intradermal VACV infection if administered before inoculation (Isaacs & Westwood, 1959). More recently, infection of transgenic mice lacking IFNRs or downstream signalling components showed enhanced sensitivity to OPV infection (Xu et al., 2008). In macaques, a rapid up-regulation of genes involved in the type I and II IFN response to VARV infection was demonstrated (Rubins et al., 2004).

As discussed above, VACV proteins E3 and K3 block the functions of IFN-induced proteins such as PKR and OAS. In addition, there are several other VACV proteins that modulate IFN pathways by acting as IFN decoy receptors or blocking IFN signalling. The VACV WR B18 protein is a secreted type I IFN receptor, which binds IFN-α and IFN-β (Colamonici et al., 1995; Symons et al., 1995). B18 binds to human and rabbit IFN-α with high affinity, while it has a reduced affinity for bovine and rat IFN-α. Its affinity for murine IFN-β is low (nM range) but loss of the B18R gene (v∆B18R) still attenuates the phenotype of VACV infection in the i.n. model (Symons et al., 1995).

B8 is a soluble IFN-γ receptor from VACV WR and has aa sequence similarity to the IFN-γ binding domain of cellular IFN-γ receptor. B8 is expressed early in infection and binds to IFN-γ, thereby preventing the IFN-γ-IFN-γR interaction (Upton
et al., 1992; Alcamì & Smith, 1995). B8 has broad species specificity for IFN-γ and can inhibit human, bovine, equine, ovine and rat IFN-γ but not murine (Alcamì & Smith, 1995; Mossman et al., 1995a; Symons et al., 2002b) whilst the myxoma virus M-T7 binds and inhibits only rabbit IFNγ (Mossman et al., 1995b).

VACV also modulates IFN functions by blocking IFN signalling at the level of STAT phosphorylation. The H1 phosphatase, termed VH1, (Liu et al., 1995), blocks IFN-γ-stimulated presentation of antigen in VACV-infected cells by dephosphorylating the STATs (Najarro et al., 2001). H1 can inhibit the phosphorylation of STAT1 and STAT2 in response to either type I or type II IFNs and VACV can also block STAT1-independent IFN-induced gene expression, suggesting that VACV encodes other proteins that block additional IFN-mediated signalling pathways (Mann et al., 2008).

Fig. 1.7 Type I and II IFN signalling pathways and their modulation by VACV
1.9.3.2 Other cytokines

IL-18, first known as the IFN-γ-inducing factor, mediates NK and T-cell activation via the induction of IFN-γ production in NK and T cells. IL-18 acts with IL-12 to promote Th1-mediated responses. IL-18 is produced by activated macrophages as an inactive precursor that is processed by caspase 1, IL-1β-converting enzyme (ICE), and the mature form is secreted from cells and binds IL-18R. Humans and mice express soluble proteins that can bind to IL-18 called IL-18BP that regulates IL-18 activity. VACV expresses a related protein called C12 that binds IL-18 and reduces NK and T cell cytotoxic activity, IFN-γ production and NO activity (Calderara et al., 2001; Symons et al., 2002a; Reading & Smith, 2003b). The importance of this pathway is highlighted by VACV modulating it in several ways. As well as targeting IL-18 itself, VACV A46 and A52 (discussed in Section 1.9.1.5) inhibit signalling from the IL-18R and encode an inhibitor of caspase 1, VACV B13(Kettle et al., 1995; Kettle et al., 1997). B13 (or serpin protease inhibitor (SPI)-2), is an intracellular protein, expressed early in infection and inhibits caspase 1. B13 also prevents maturation of IL-1β and IL-18 and has a role in apoptosis (discussed in Section 1.10).

VACV also modulates the IL-1β signalling pathway by secreting a soluble IL-1β receptor encoded by gene B15R in the VACV strain WR (Smith & Chan, 1991; Alcami & Smith, 1992; Spriggs et al., 1992a). B15 binds to IL-1β to inhibit febrile response during systemic infection. In the MVA background, the absence of B15 enhanced CD8+ T cell memory responses (Staib et al., 2005).

VACV also encodes a protein, C16 (Fahy et al., 2008), with a short peptide sequence that was identified in the IL-1 receptor antagonist (IL-1Ra) (Kluczyk et al., 2002; Kluczyk et al., 2004). IL-1ra is secreted from cells binds to the IL-1R but does not induce signal transduction, thereby acting as an antagonist. However, the C16 protein is intracellular and so must have a different function.

Several orthopoxviruses also encode modulators of TNF, such as soluble or cell surface TNFRs. Although most VACV strains do not express TNFRs, 3 strains, Lister, USSR and Evans, were identified that did and each expressed more than 1 TNFR (Alcami et al., 1999; Reading et al., 2002). VACV also expresses intracellular inhibitors of TNF mediated signalling, such as B14 (see section 1.9.1.5).
Chemokines are small (8- to 13-kDa) heparin-binding proteins that are grouped according to shared structural characteristics. These include the presence of cysteines in conserved locations that are essential to forming a characteristic tertiary fold, termed a ‘Greek key’, and are classified into C, CC, CXC or CX3C subfamilies. Chemokines and chemokine receptors play a critical role in the host defence against viruses by mobilising leukocytes to sites of infection, injury and inflammation. Chemokines mediate their actions via seven-transmembrane G-protein-coupled receptors expressed by different subsets of leukocytes. The cellular distribution of different receptors determines the leukocyte subset that predominates in the inflammatory response. In general, CC chemokines attract macrophages and T cell populations and CXC chemokines attract neutrophils, while lymphotactin (XCL1) attracts T and NK cells. Soluble fractalkine (CX3CL1, the only CX3C chemokine) attracts monocytes, T cell subsets and NK cells. Large DNA viruses, such as poxviruses and herpes viruses have evolved several strategies for exploiting or subverting chemokine networks.

VACV expresses a secreted protein that binds CC chemokines with high affinity and is termed the viral chemokine inhibitor (vCCI) or viral CC chemokine binding protein (vCKBP) (Alcami et al., 1998; Beck et al., 2001; Seet et al., 2001; Reading et al., 2003b). The VACV vCCI binds RANTES (regulated on activation normal T cell expressed and secreted) a CC-motif ligand (CCL5) chemokine (Alcami et al., 1998), macrophage-inflammatory protein 1α (MIP-1α, CCL3), macrophage chemotactic protein 1 (MCP-1, CCL2) and eotaxin (CCL11) and thereby blocks the recruitment of inflammatory cells to the lungs of VACV-infected mice (Graham et al., 1997; Reading et al., 2003b).

The VACV A41 protein is a secreted glycoprotein that shares sequence similarity with vCCI (Ng et al., 2001) and blocks the binding of some CC chemokines to GAGs (Bahar et al., 2008). Although it has relatively low affinity for a subset of CC chemokines and consequently does not block the interaction of chemokines with their receptors, it can still function in vivo by blocking the establishment of a chemokine concentration gradient on endothelial cells and thereby inhibiting leukocyte chemotaxis. The deletion of the A41L gene from VACV reduces virulence, but induces stronger CD8+ T cell response and confers better protection against subsequent challenge (Clark et al., 2006).
1.9.3.4 Vaccinia growth factor

VACV growth factor (VGF) (Brown et al., 1985; Twardzik et al., 1985), is related to the epithelial growth factor (EGF) family of cytokines that stimulate epithelial cells to divide. By mimicking EGF, VGF induces proliferation of uninfected cells surrounding the site of infection, so that as the virus spreads it encounters cells with high metabolic activity that support virus replication. The importance of EGF for virus virulence was demonstrated by the attenuated phenotype of the deletion mutant (Buller et al., 1988).

1.10 Apoptosis

Apoptosis or programmed cell death plays an essential role in development, tissue homeostasis and the elimination of pathogen-infected cells. In response, viruses have evolved several mechanisms to block apoptosis and allow completion of the replication cycle before cell death. Apoptosis can be induced by extracellular stimuli, such as TNF or Fas-binding, or by intracellular stimuli that cause DNA damage, and mitochondrial dysfunction or signal microbial invasion. Central to apoptosis are the caspases and B-cell lymphoma 2 (Bcl-2) proteins. The caspase family comprise aspartic proteases, which are present as inactive pro-enzymes that are activated in a proteolytic cascade and contain an N-terminal death domain (DD), either a CARD (caspases 1, 2, 4, 5, 9 and 11) or a death effector domain (DED, caspases 8 and 10). They can be activated by extracellular stimulation such as Fas ligand that binds to Fas. Binding induces trimerisation of the receptor and binding of adaptor proteins such as Fas-associated protein with death domain (FADD) via DDs. The adaptor proteins also contain a DED that recruits and activates caspase 8. This initiating step induces a cascade of proteolytic caspase cleavage that leads to the eventual disassembly of cellular structures and dys-regulation of homeostatic cellular activity.

Alternatively, apoptotic stimuli can be sensed intracellularly by the mitochondrion, ER or Golgi complex. These signals converge the mitochondrion, and result in the loss of membrane potential and the release of pro-apoptotic proteins such as apoptosis inducing factor, second mitochondria-derived activator of caspases (SMAC), endonuclease G and cytochrome c. Cytochrome c initiates caspase 9
recruitment and activation via its interaction with the adaptor molecule protease activating factor 1 (APAF-1).

The mitochondrial ‘checkpoint’ of apoptosis is regulated by pro- and anti-apoptotic members of the Bcl-2 family. These proteins are characterised by up to four domains termed Bcl-2 homology (BH) domains and can function as homo- or heterodimers to regulate apoptosis. The relative levels of pro- and anti-apoptotic members within a cell modulate the balance between apoptosis and cell survival.

VACV modulates apoptosis in several ways including interference with caspases, interference with Bcl-2 proteins and alterations in Ca\(^{2+}\) homeostasis, reviewed in (Taylor & Barry, 2006). For instance, VACV strain WR protein B13 is a serpin that inhibits caspase 1 activity (Kotwal & Moss, 1988a; Smith et al., 1989b) and seems to inhibit cell death during VACV infection (Dobbelstein & Shenk, 1996; Kettle et al., 1997). VACV encodes a second serpin, B22 (also called SPI-1, (Kotwal & Moss, 1988a; Smith et al., 1989b; Kettle et al., 1995), which acts as a pseudo-substrate for target proteases and thereby modulates apoptotic signalling. Deletion of B22R from RPXV restricts host range and in the cells that become non-permissive, the restriction is due to the induction of apoptosis (Brooks et al., 1995). B22 was shown to interact with cathepsin G, a serine proteinase, via serpin motifs and thereby modulate apoptosis. In an intraperitoneal model, lack of B13 or B22 induced stronger T helper and cytotoxic responses (Legrand et al., 2004).

The VACV proteins F1 and N1 are Bcl-2-like proteins that inhibit the activity of pro-apoptotic BCl-2signalling (Cuconati & White, 2002). F1 localises to the mitochondrion, via a single transmembrane domain (Stewart et al., 2005), where it inhibits the release of cytochrome \(c\) and thereby regulates the initiation of the apoptotic cascade (Wasilenko et al., 2003; Taylor et al., 2006). This inhibition may be due to the direct interaction of F1 with the BH3 domains of the Bcl-2 proteins (Fischer et al., 2006). VACV N1 is a virulence factor in the i.d., i.n. and intracranial models of infection (Bartlett et al., 2002; Abrahams et al., 2005; Jacobs et al., 2008). Structurally N1 is a Bcl-2-like protein (Aoyagi et al., 2007; Cooray et al., 2007) and interacts with pro-apoptotic Bcl-2 proteins to protect infected cells against staurosporine-induced apoptosis (Cooray et al., 2007). The structure of F1 also showed a Bcl-2 protein with an open surface groove for binding BH3 peptides.
1.11 Adaptive immune responses to VACV infection

1.11.1 Humoral responses

The importance of an Ab response to VACV infection is shown by the many ways in which Ab binding can help clear the infection. Anti-VACV Ab can bind directly to VACV particles, causing aggregation and inhibiting virus binding and entry to cells. Ab-binding to virions can also initiate complement-mediated lysis or opsonisation. Ab can also bind to infected cells, leading to ADCC.

The relative contributions of humoral and cellular immunity to VACV infection were assessed in mice that were B-cell deficient or CD4+ T cell depleted that were unable to clear a VACV infection (Xu et al., 2004). The Ab response is primarily CD4+ T cell-dependent (Xu et al., 2004) and is predominantly of an IgG2a isotype (Fogg et al., 2004). IgM appears by 7 days p.i. and IgG by day 14 (Spriggs et al., 1992b) and Ab levels peak around 6 weeks p.i. (Wyatt et al., 2004; Meseda et al., 2005) and can be maintained for more than 3 months (Coulibaly et al., 2005; Meseda et al., 2005) in mouse models.

Vaccination studies in humans demonstrate a strong Ab response is elicited after primary vaccination. Abs are not detected before day 10 post vaccination (McCarthy et al., 1958; McClain et al., 1997) but are apparent by day 13/14 (Greenberg et al., 2005) and Ab levels continue to rise until approximately day 28 (Frey et al., 2003; Greenberg et al., 2005). Re-vaccination elicits a more rapid Ab response, with Ab levels peaking approximately day 14, considerably earlier than after primary immunisation and re-vaccination also boosts previous Ab levels (McCarthy et al., 1958; Frey et al., 2003; Greenberg et al., 2005).

Ab levels decrease slowly to a steady level but are long-lived, being maintained up to 50 years post-vaccination (Fenner et al., 1988; Hammarlund et al., 2003; Putz et al., 2005). VACV-specific memory B cells are also generated after smallpox vaccination (Crotty et al., 2003). At 4 weeks to 6 months post-vaccination, 1% of circulating IgG+ memory B cells were VACV-specific and a moderate correlation was seen between memory B cells and serum Abs, indicating that multiple factors may be involved in Ab maintenance (Slifka et al., 1998; Crotty et al., 2003).
1.11.1.1 Important immunogens in the humoral response

In animal and human studies, approximately 10 immunodominant antigens have been identified by immunoblotting (Wilton et al., 1986; Cheliapov et al., 1988; Demkowicz et al., 1992; Jones-Trower et al., 2005) and proteome arrays (Davies et al., 2005a; Duke-Cohan et al., 2009). In general the major targets are core proteins such as A4, E3 and A10, IMV surface antigens such as H3, D8, D13, and A27 and EEV-specific antigens such as A56, B5, A33 and F13. There was some variation in strength of targeting to a particular antigen between species. The targets for neutralising Ab activity are the IMV and EEV surface antigens. IMV targets include H3 (Chertov et al., 1991; Demkowicz et al., 1992; Lin et al., 2000; Davies et al., 2005b), D8 (Hsiao et al., 1999; Sakhatskyy et al., 2006), A27 (Rodriguez et al., 1985; Hooper et al., 2003; Hooper et al., 2004), L1 (Fogg et al., 2004; Wyatt et al., 2004), A17 and F9 whilst B5 is the only EEV target for neutralising Abs in the absence of complement (Putz et al., 2006). However, anti-A33 Ab can neutralise EEV in the presence of complement (Fogg et al., 2004).

EEV antigens A33 and A56 are not targets for neutralising Abs without complement but A33-specific Abs can inhibit comet tail formation (Galmiche et al., 1999; Lustig et al., 2004) and A33 is important for Ab-resistant direct cell-to-cell spread (Law et al., 2002). In the presence of complement, A33-specific Abs induce EEV membrane lysis, exposing the IMV which can be neutralised (Lustig et al., 2004). A56 is the viral haemagglutinin (HA) protein but A56-specific Abs have not been shown to neutralise EEV (Law & Smith, 2001; Putz et al., 2006).

1.11.2 Cellular response

There have been many studies of cellular immunity to VACV infection, utilising proliferation assays, cytotoxic killing assays and detection of IFN-γ and other cytokines.

1.11.2.1 T cell response

In mice, both CD4+ and CD8+ T cell responses are elicited following VACV infection (Xu et al., 2004). Kinetic analysis of infection shows that VACV induces a
potent primary CD8⁺ T cell response as well as long-term memory in vivo. As early as day 5 p.i., a IFN-γ positive CD8⁺ T cell response was detected and peaked at day 7, with 30% of CD8⁺ T cells in the spleen being VACV-specific. There was also a strong CTL response by day 7 (Harrington et al., 2002). Despite the large CD8⁺ response, in CD8⁺-depleted or null mice, virus was cleared at similar rates to the control, suggesting that the activated CD8⁺ T cells were not essential for virus clearance following acute infection in the intraperitoneal model (Xu et al., 2004) and subcutaneous model (Spriggs et al., 1992b) of infection. However, in the absence of CD4⁺ T cell response, a partial function of CD8⁺ T cells was demonstrated (Xu et al., 2004).

The CD4⁺ T cell response displayed similar kinetics to that of CD8⁺ T cells but at lower frequencies (Harrington et al., 2002). The response peaked at day 7 p.i., with 3% of the CD4⁺ T cell being VACV-specific. When compared to CD8⁺ T cells, a higher percentage of CD4⁺ T cells produced IL-2, demonstrating an increased proliferative response compared to the CD8⁺ T cell compartment. This is consistent with CD4⁺ T cells as T helper cells. Although the response declined by approximately 90% by day 30, it was maintained for over 300 days (Harrington et al., 2002).

Within the i.d model, the lymphocyte populations from the adaptive immune response was observed later p.i. with CD4⁺ T cells, increasing in percentage even up to day 17 p.i. despite most lesions having resolved by this time-point. In contrast, the percentage of CD8⁺ T cells started to decrease after day 7 p.i. and the absolute number for both subsets started to decrease after day 10 p.i. The CTL activity against EL4 cells infected with VACV WR at 10⁴ plaque forming units (p.f.u) was detected by day 7 p.i. (Jacobs et al., 2006).

The influence of the mouse strain, on the immune responses is highlighted by the resistance of C57BL/6 mice to VACV (and ECTV) compared to BALB/c mice. BALB/c mice are more susceptible and exhibit a type II polarised (IL-4) cytokine response. In contrast, C57BL/6 mice are more resistant and exhibit a type I polarised (IFN-γ, IL-2, TNF) cytokine response. Depletion of CD8⁺ T cells in a type II bias environment results in greater susceptibility (Belyakov et al., 2003). The Th1 skewed response in C57BL/6 mice indirectly indicates an important role in innate immunity. Recombinant VACV expressing IFN-γ (Kohonen-Corish et al., 1990) or IL-2 (Ramshaw et al., 1987) were less virulent whereas IL-4 expressing recombinant VACV
(Sharma et al., 1996) was more virulent and expression of IL-2 activates NK cells and CD4+ T cells.

Overall, these data from murine studies indicate that Abs, CD4+ T cells and CD8+ T cells are not essential for survival but all make valuable contributions. Whilst Abs contribute to the prevention of infection, in the absence of Abs, T cells are necessary and sufficient for survival. In a more virulent OPV infection, such as ECTV, both humoral and cellular responses become necessary for recovery and survival (Chaudhri et al., 2004).

In humans, strong CD4+ and CD8+ T cell responses are elicited following smallpox vaccination. In general, CD8+ T cell responses are of a greater magnitude (2- to 4-fold higher) after immunisation (Amara et al., 2004; Rock et al., 2006) but CD4+ T cell responses are maintained for a greater length of time at higher frequencies (Hammarlund et al., 2003; Amara et al., 2004; Rock et al., 2006). VACV-specific CD4+ T cells were detected in the majority (82-100%) of individuals vaccinated up to 75 years previously unlike CD8+ responses (Hammarlund et al., 2003; Amara et al., 2004). The importance of CD4+ T cells was highlighted by the outcome of vaccination in HIV positive individuals. In the USA, after a review of approximately 300 HIV-infected military personnel, the smallpox vaccine is not recommended for individuals with a CD4+ T cell count below 200 cells/mm3 (Bartlett, 2003). In one case, an HIV positive vaccinee developed progressive vaccinia which was treated successfully with VACV immunoglobulin (VIG) (Redfield et al., 1987).

1.1.1.4 T cell epitopes

The investigation of not only the global T cell response to VACV infection, but also the specific T cell epitopes has been important, especially for future vaccine development. Approximately 50 murine MHC-I-restricted (Mathew et al., 2005; Tscharke et al., 2005; Moutaftsi et al., 2006; Tscharke et al., 2006) and human HLA-A/B-restricted VACV epitopes (Drexler et al., 2003; Terajima et al., 2003; Oseroff et al., 2005; Pasquetto et al., 2005) have been identified. The 49 HLA-restricted human epitopes are from 35 different proteins, spanning almost 20% of predicted ORFs and are recognised by PBMCs from vaccinated humans (Oseroff et al., 2005). Overall, the proteins involved are usually greater than 100 aa in size and approximately 50% are
made early during infection and 50% late during infection. Furthermore, there was no
obvious bias towards a particular function or location of the protein containing the
epitope.

In mice, analysis of MHC-II-restricted epitopes showed that the pattern of
antigens recognised by CD4⁺ T cells differs substantially from the pattern of antigens
recognised by CD8⁺ T cells (Moutaftsi et al., 2007). In humans, 122 VACV proteins
elicted CD4⁺ T cell responses (Jing et al., 2008) and the proteins recognised most
frequently tended to be virion structural proteins, with higher molecular weights and to
be expressed late during infection.

1.12 Vaccination

Apart from trying to determine important immune responses for prevention and
elimination of an OPV infection, many studies have also investigated what factors are
important for protection from a secondary infection. In general, protection conferred by
licensed vaccines such as measles/mumps/rubella, hepatitis B or yellow fever vaccines
is due to the production of neutralising or binding Abs (Burton, 2002; Zinkernagel &
Hengartner, 2006) that can neutralise a pathogen immediately. In contrast, T cell
activation can take several days to develop and consequently, are more involved in
clearance and recovery. This is also the case for protection against OPV. In humans, the
correlates of protection remain uncertain but animal studies have helped elucidate
possible mechanisms.

Primary vaccination in humans, by i.d. inoculation with replication-competent
VACV strains results in local viral replication followed by complete virus elimination.
Vaccination is via scarification of the epidermal layers of the upper deltoid region of
the arm. VACV replicates in the basal layer of the skin where a papule appears at the
site of vaccination within 3 – 5 days and develops into a pustular vesicle, following a
vigorous inflammatory and adaptive infiltrate, which reaches maximum size within 8 –
10 days. A scab then forms, which separates after 17 -21 days, leaving a vaccination
scar (Fenner, 1989). Aside from the primary site lesion, most vaccinees experienced a
mild fever and localised discomfort but local responses could include erythema, pain
and swelling following the release of cytokines and chemokines from necrotic and
infected cells (Fenner, 1989; Frey et al., 2002; Orr et al., 2004; Artenstein et al., 2005;
Greenberg et al., 2005). Systemic responses included fever, fatigue, malaise, headaches, nausea, diarrhoea and vomiting (Frey et al., 2002; Orr et al., 2004).

On rare occasions, severe complications were observed after vaccination. These were commonly associated with virus escape from the site of vaccination and included accidental infection of the eye, eczema vaccinatum, generalised vaccinia, postvaccinial encephalitis, progressive vaccinia and myopericarditis (Kempe, 1960; Neff et al., 1967a; Neff et al., 1967b; Lane et al., 1969; Bray & Wright, 2003). Accidental infection of a secondary site such as the eye were more common in children, occurring at a rate of 25-529 cases per million. Eczema vaccinatum (Bacic, 2007) was observed in 10-40 cases per million, in people with skin disorders as a result of mechanical and blood borne viral dissemination. The outcome ranged from benign to fatal and human VIG was often used to treat such complications (Kempe, 1960). VIG was also used to treat generalised vaccinia, a condition where additional lesions form at secondary sites about 6-9 days after vaccination following blood-borne dissemination. Generalised vaccinia was observed in 23-241 cases per million (Vellozzi et al., 2005). Postvaccinial encephalopathy was more severe and often fatal, but had a lower incidence rate, occurring in 3-12 vaccinees per million (Kempe, 1960). The most severe complication of vaccination was progressive vaccinia (Bray & Wright, 2003), which occurred at a rate of 1 per million and resulted from an inability to control virus replication due to T cell deficiencies. The condition resulted in the expansion of the primary site and formation of additional lesions. Depending on the immune status of the vaccinee, the condition could be treated with several doses of VIG. Myopericarditis was only recognised as a complication following more recent military vaccine trials in the USA (Chen & Lane, 2003).

Protection induced by vaccination is shared between Ab and cellular immunity (Belyakov et al., 2003). In murine studies, B cell-deficient mice when immunised with either Wyeth vaccine or MVA were protected against challenge with VACV WR 28 days later were protected (Wyatt et al., 2004; Xu et al., 2004), indicating that Abs are not essential for VACV immunity. However, Abs are sufficient for protection, as shown by passive transfer of sera from immunised animals (Boulter et al., 1961; Law et al., 2005). Likewise, T cells are not strictly necessary for protection. Depletion of CD8+ T cells prior to challenge with VACV WR had no effect compared to control animals (Belyakov et al., 2003) nor did challenge in β2m− mice (Wyatt et al., 2004). In
contrast, CD4⁺-deficient mice (MHC II−) were not protected from challenge (Wyatt et al., 2004). Following adoptive transfer of CD4⁺ and/or CD8⁺ T cells from MVA-immunised mice, naïve mice were not protected upon challenge with VACV WR (Wyatt et al., 2004). In a separate study, adoptive transfer of WR-activated memory CD8⁺ T cells resulted in a 100-fold reduction of virus in the ovaries (Xu et al., 2004).

The precise duration of protection afforded by vaccination is still unclear. Whilst Jenner originally claimed protection was life-long, the WHO estimated the duration of protection to be around 5-10 years (Henderson, 2003). In Europe between 1950 and 1971, only 2%, 6% and 7% respective fatality rates were observed in individuals vaccinated 0-10, 11-20 and > 20 years previously and who subsequently became infected or exposed to VARV (Mack, 1972). Approximately 30% of today’s population are contraindicated to the current smallpox vaccine, including individuals with eczema, HIV infection, those receiving immunotherapy and pregnant women, emphasizing a need for new and safer vaccines. To this end, the immune response to VACV infection and vaccination is crucial.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Complement modulation</strong></td>
<td></td>
</tr>
<tr>
<td>C3/VCP</td>
<td>Binds C3 and C4b; inhibits the classic and alternative pathways of complement activation (Isaacs et al., 1992b)</td>
</tr>
<tr>
<td><strong>PRR signal modulation</strong></td>
<td></td>
</tr>
<tr>
<td>A46</td>
<td>Targets IRAK2 and TRAF6 (Bowie et al., 2000; Stack et al., 2005)</td>
</tr>
<tr>
<td>A52</td>
<td>Targets TIR adaptor proteins (Bowie et al., 2000; Harte et al., 2003)</td>
</tr>
<tr>
<td>B14</td>
<td>NF-κB inhibitor; down-regulates IKK by targeting IKK-β (Chen et al., 2008)</td>
</tr>
<tr>
<td>K1</td>
<td>NF-κB inhibitor; suppresses IκB-α degradation. Host range gene (Shisler &amp; Jin, 2004; Bradley &amp; Terajima, 2005)</td>
</tr>
<tr>
<td>K7</td>
<td>Interacts with TBK1 and IKKε (Schroder et al., 2008)</td>
</tr>
<tr>
<td>M2</td>
<td>NF-κB inhibitor (Hinthong et al., 2008)</td>
</tr>
<tr>
<td><strong>IFN modulation</strong></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>Binds to dsRNA, preventing PKR and OAS activation (Langland &amp; Jacobs, 2002; Kwon &amp; Rich, 2005)</td>
</tr>
<tr>
<td>K3</td>
<td>Pseudo-substrate of PKR (Davies et al., 1992)</td>
</tr>
<tr>
<td>B8</td>
<td>Binds to and inhibits IFN-γ (Alcamí &amp; Smith, 1995; Symons et al., 2002b)</td>
</tr>
<tr>
<td>B18</td>
<td>Binds to and inhibits IFN-α/β (Colamonici et al., 1995; Symons et al., 1995)</td>
</tr>
<tr>
<td>H1</td>
<td>IFN signalling inhibitor; dephosphorylates STAT1 (Najarro et al., 2001; Mann et al., 2008)</td>
</tr>
<tr>
<td><strong>Cytokine &amp; chemokine modulation</strong></td>
<td></td>
</tr>
<tr>
<td>A41</td>
<td>Chemokine binding protein (Clark et al., 2006; Bahar et al., 2008)</td>
</tr>
<tr>
<td>B15</td>
<td>Binds to and inhibits IL-1β (Alcamí &amp; Smith, 1992; Spriggs et al., 1992a; Alcamí &amp; Smith, 1996)</td>
</tr>
<tr>
<td>C12</td>
<td>IL-18 binding protein; inhibits IL-18 induced IFN-γ production (Symons et al., 2002a; Reading &amp; Smith, 2003b)</td>
</tr>
<tr>
<td>C16</td>
<td>Intracellular IL-1Ra-like protein; (Fahy, 2008)</td>
</tr>
<tr>
<td>vCKBP/CCI</td>
<td>CC chemokine binding protein (Graham et al., 1997; Lalani et al., 1997; Alcamí et al., 1998)</td>
</tr>
<tr>
<td>CrmE</td>
<td>Binds to and inhibits activity of TNF-α (Reading et al., 2002)</td>
</tr>
<tr>
<td><strong>Apoptosis modulation</strong></td>
<td></td>
</tr>
<tr>
<td>B13</td>
<td>Serpin; inhibits caspase 1 and thereby i) maturation of IL-1β and IL-18 and ii) TNF or Fas-induced apoptosis (Blake et al., 1995; Kettle et al., 1997)</td>
</tr>
<tr>
<td>B22</td>
<td>Serpin (Blake et al., 1995)</td>
</tr>
<tr>
<td>F1</td>
<td>Interferes with cytochrome c release from mitochondria and apoptosis (Taylor et al., 2006)</td>
</tr>
<tr>
<td>N1</td>
<td>Inhibits apoptosis (Cooray et al., 2007)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>A39</td>
<td>Semaphorin glycoprotein (Gardner et al., 2001)</td>
</tr>
<tr>
<td>A40</td>
<td>Type II membrane glycoprotein that affects the murine i.d. (Tscharke &amp; Smith, 1999; Wilcock et al., 1999)</td>
</tr>
<tr>
<td>A44</td>
<td>3-β-hydroxysteroid dehydrogenase; synthesises steroid hormones that inhibit inflammatory response (Moore &amp; Smith, 1992; Reading et al., 2003a)</td>
</tr>
<tr>
<td>A55</td>
<td>Kelch-like protein that affects the murine i.d. (Beard et al., 2006)</td>
</tr>
<tr>
<td>C2</td>
<td>Kelch-like protein that affects the murine i.d. (Pires de Miranda et al., 2003)</td>
</tr>
<tr>
<td>F3</td>
<td>Kelch-like protein that affects the murine i.d. (Froggatt et al., 2007)</td>
</tr>
</tbody>
</table>
1.13 A40

VACV WR gene A40R encodes a type II membrane glycoprotein with a C-type lectin-like domain at the C terminus. The A40 protein is expressed early in infection, is nonessential for viral replication and is not incorporated into virions (Wilcock et al., 1999). VACV proteins required to provide precursors for macromolecular synthesis or having roles in evasion of host defences are often not required for viral replication in tissue culture cells. Wilcock et al. (1999) also reported that A40 partitioned to the membrane fraction when infected cells were treated with Triton X-114. Four proteins (18, 28, 35 and 38 kDa) were identified in lysates from VACV-infected cells but were absent from deletion mutant-infected cells by immunoblotting using an A40 specific Ab (Wilcock et al., 1999). In the presence of tunicamycin, only the 18 kDa protein was found indicating A40 contains N-linked carbohydrate. Furthermore, in preparations omitting β-mercaptoethanol, one additional band at 70-76 kDa was identified and may represent homodimers of the glycosylated A40.

Wilcock et al. (1999) found the highest FASTA score (198) with a non-poxvirus protein was against the human natural killer cell G2-A protein (accession no. P26715). The predicted CRD domain of A40 contains 5/14 invariant and 17/32 conserved residues of the CRD consensus sequence (Duncan & Smith, 1992; Wilcock et al., 1999). However, it lacks one of the cysteine residues predicted to form intradomain disulphide bonds and residues equivalent to loop 2 and β sheet 5. Some lectins lacking several conserved residues and containing a deletion in the loop regions still may bind carbohydrate. The putative interaction between A40 and carbohydrates remains to be investigated further.

In contrast to the conclusions of Wilcock et al., (1999), Palacios et al., (2005) reported that A40 was modified by sumoylation (SUMO-1) rather than glycosylation and was an intracellular protein that was not associated with membranes (Palacios et al., 2005). A number of proteins, mainly located in the nucleus, are sumoylated (Yeh et al., 2000; Muller et al., 2001; Seeler & Dejean, 2003). Sumoylation is a pathway similar to ubiquitination that modifies proteins reversibly and post-translationally and can alter a protein’s intracellular location, activity, stability and interactions. Palacios et al., (2005) proposed that sumoylation of A40 prevents A40 forming polymers and a small amount of unsumoylated A40 could aid bringing ER membranes together before
they fuse to generate an ER envelope that is proposed to surround the virus factory (Sodeik & Krijnse-Locker, 2002). These differences were proposed to be due to a lack of specificity of the Ab used in the study by Wilcock et al., (1999). However, controls were provided and the Ab reacted with proteins in cells infected with WT and revertant viruses but not a virus mutant lacking the A40R gene or mock infected cells (Wilcock et al., 1999). In comparison, Palacios et al., 2005 did not show controls for the specificity of the peptide Ab and no inhibitor of glycosylation was used to support claims that the protein lacks N-linked glycans. If the peptide Ab used by Palacios et al., is binding A40, the presence of A40 in the ER may reflect its normal transport through the host cell’s secretory pathway.

A recombinant VACV lacking the A40R gene (vΔA40R) was constructed and was found to replicate normally in cell culture (Wilcock et al., 1999). However, compared to WT and revertant (Rev) controls it was attenuated in a murine i.d. infection model (Tscharke et al., 2002) and induced a 30% reduction in lesion size compared to WT and Rev virus (Tscharke et al., 2002). This was observed in Balb/c and C57B1 mice.

The low but significant aa similarity of A40 to C-type lectins such as NKG2A and DC-SIGN, its location on the infected cell and the attenuation in the murine i.d. model of infection, suggest that A40 may function as an immunomodulator by mimicking native host lectins or modulating recognition of VACV-infected cells by cells of the immune system and consequently, interrupting host responses to the viral infection.

1.14 Project Aims

The project aims are to:

1) identify the ligand(s) with which A40 interacts.

2) understand the mechanism by which A40 modulates the host immune response.

To identify cell surface binding partners for A40, recombinant A40 proteins were produced and used in cell binding assays, with binding being assessed by flow
cytometry. Recombinant protein was produced in bacterial, mammalian and baculovirus systems.

Due to the poor affinity of the present anti-A40 Abs, a new A40 Ab was produced and used to re-investigate the localisation, biochemical properties and to try to characterise the interacting partner(s) of A40.

To determine the structure of A40, in collaboration with Dave Stuart’s group at Oxford, recombinant A40 was produced, purified and used in crystallisation trials.
Chapter 2. Materials & Methods

2.1 Chemicals, reagents and rotors

All chemicals were obtained from VWR, UK, or Sigma-Aldrich Co., UK, unless otherwise stated. All centrifugation steps were performed in an Allegra 6R benchtop centrifuge with GH-3.8A rotor unless otherwise stated.

2.2 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Specificity</th>
<th>Dilution (immunoblot)</th>
<th>Dilution (IF)</th>
<th>Reference or supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAb A40</td>
<td>Rabbit</td>
<td>VACV A40</td>
<td>1:1000</td>
<td>1:400</td>
<td>-</td>
</tr>
<tr>
<td>mAb BAP (LX035-9)</td>
<td>Mouse</td>
<td>Biotin Acceptor Protein tag</td>
<td>1:1000</td>
<td>1:400</td>
<td>Law M (Smith lab)</td>
</tr>
<tr>
<td>mAb HA</td>
<td>Mouse</td>
<td>HA-tag</td>
<td>1:1000</td>
<td>-</td>
<td>Covance</td>
</tr>
<tr>
<td>Tetra-His</td>
<td>Mouse</td>
<td>His-tag</td>
<td>1:1000</td>
<td>-</td>
<td>Qiagen</td>
</tr>
<tr>
<td>mAb MBP</td>
<td>Mouse</td>
<td>MBP-Tag</td>
<td>1:5000</td>
<td>-</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>mAb α-tubulin</td>
<td>Mouse</td>
<td>α-tubulin</td>
<td>1:5000</td>
<td>-</td>
<td>Upstate</td>
</tr>
<tr>
<td>mAb 19C2</td>
<td>Rat</td>
<td>VACV SCR2 of B5</td>
<td>1:500</td>
<td>-</td>
<td>(Schmelz et al., 1994)</td>
</tr>
<tr>
<td>mAb AB 1.1</td>
<td>Mouse</td>
<td>VACV D8</td>
<td>1:2000</td>
<td>-</td>
<td>(Parkinson &amp; Smith, 1994)</td>
</tr>
<tr>
<td>RAb B14</td>
<td>Rabbit</td>
<td>VACV B14</td>
<td>1:2000</td>
<td>-</td>
<td>(Chen et al., 2006)</td>
</tr>
<tr>
<td>RAb K7</td>
<td>Rabbit</td>
<td>VACV K7</td>
<td>1:2000</td>
<td>-</td>
<td>Lucas S (Smith lab)</td>
</tr>
<tr>
<td>mAb 15B6</td>
<td>Rat</td>
<td>VACV F13</td>
<td>1:1000</td>
<td>-</td>
<td>(Schmelz et al., 1994)</td>
</tr>
<tr>
<td>α-Mouse IgG HRP</td>
<td>Goat</td>
<td>Mouse</td>
<td>1:1000</td>
<td>-</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>α-Rabbit IgG HRP</td>
<td>Goat</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>-</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>α-Rat IgG HRP</td>
<td>Goat</td>
<td>Rat</td>
<td>1:2000</td>
<td>-</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>α-Mouse Alexa488</td>
<td>Donkey</td>
<td>Mouse</td>
<td>-</td>
<td>1:400</td>
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</tr>
<tr>
<td>α-Rabbit Alexa 488</td>
<td>Donkey</td>
<td>Rabbit</td>
<td>-</td>
<td>1:400</td>
<td>Sigma-Aldrich</td>
</tr>
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</table>
### Table 2.2. Conditions for usage of Ab in FACS analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Reference or supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220 TC</td>
<td>Rat</td>
<td>Mouse B220</td>
<td>1:100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD14 FITC</td>
<td>Mouse</td>
<td>Human CD14</td>
<td>1:100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD3 FITC</td>
<td>Rat</td>
<td>Mouse CD3</td>
<td>1:50</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD3 APC</td>
<td>Rat</td>
<td>Mouse CD3</td>
<td>1:100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD3 FITC</td>
<td>Mouse</td>
<td>Human CD3</td>
<td>1:100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD4 APC</td>
<td>Rat</td>
<td>Mouse CD4</td>
<td>1:100</td>
<td>BD Biosciences</td>
</tr>
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2.3 Cell culture and transfection techniques

2.3.1 Cell lines

RK_{13} (rabbit kidney, ATCC CL-37) cells, TK 143 (thymidine kinase negative human osteosarcoma) and BS-C-1 (African green monkey kidney, ATCC CL-26) cells were used for growth and titration of VACV. COS-7 (African green monkey kidney fibroblasts, ATCC CL-1651) cells were used for the production of recombinant proteins using mammalian expression systems. NK92MI (human natural killer cells, ATCC CRL-2408) cells, Jurkat E6.1 (human acute T cell leukaemia cells, ATCC TIB-152) cells, RAW (mouse spleen lymphoma cells, ATCC TIB-50) cells, HEK-293T (human epithelial cells ATCC CRL-11268) cells, DG75 (human B lymphocytes ATCC CRL-2625) cells, AK31 (human B lymphocytes) cells, HeLa (human negroid cervix epitheloid carcinoma, ECACC 93021013) cells and B3Z (human T cell hybridoma) cells were used for protein binding studies. K562 suspension cells (human lymphoma, ATCC CCL-243) were used in \textit{in vitro} cytotoxicity assays whilst Yac-1 (mouse lymphoma (induced by moloney murine leukaemia virus) cells, ATCC TIB-160) cells and EL4 (mouse (C57BL/6) lymphoma, ATCC TIB-39) cells were used as targets for NK and CTL cytotoxicity assays respectively. Sf9 (\textit{Spodoptera frugiperda} derived cells (Novagen)) were used for production and growth of recombinant baculoviruses. Mouse hybridoma cells (LX035-9) were used to produce an anti-BAP mAb. AK31 and DG75 cells were kindly provided by Professor Paul Farrell and B3Z cells were kindly donated by Dr. Keith Gould.

2.3.2 Maintenance of cell stocks

All cells were maintained in medium supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin (Gibco BRL) unless stated. BS-C-1, TK 143, HEK-293T, K562 and EL4 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL) supplemented with 10% heat-treated (1 h, 56 °C) foetal bovine serum (FBS, Gibco BRL) and 2 mM L-glutamine (Gibco BRL) referred to here as DMEM/10. COS-7 cells were maintained in DMEM/10 then adapted to DMEM supplemented with 2% FBS for protein production (DMEM/2). Jurkat E6.1 cells and B3Z cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco BRL) supplemented with 10% FBS (RPMI/10). DG75, AK31 and YAC-1 cells
were maintained in RPMI 1640 supplemented with 10% superior serum (SS, BioWhittaker (RPMI/SS)). NK92MI cells were maintained in alpha – modified Eagle’s medium (α-MEM, Gibco BRL) supplemented with 12% FBS, 0.1 mM β- mercaptoethanol, 0.2 mM inositol, 0.02 mM folic acid, (α-MEM complete). RK13, RAW and HeLa cells were maintained in minimum essential medium (MEM, Gibco BRL) supplemented with 10% FBS, 1% non-essential aa, and 2 mM L-glutamine (MEM/10). The LX035-9 hybridoma cell line was maintained in RMPI 1640, supplemented with 5% ultra low IgG foetal bovine serum (Invitrogen), 1% non-essential aa, 0.2% peptone, 0.25% D-glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol (Integra medium, IM). The Sf9 cells were maintained in SF900 II SFM (SF900) (Gibco BRL) with 5% FBS (SF900/5). All cells except Sf9s were incubated in a humidified incubator (Heraeus) with 5% CO₂ at 37 °C. The Sf9 cells were incubated as monolayer cultures in a humidified incubator (Hera Cell, Heraeus) at 28 °C and as suspension cultures in a shaking incubator (Orbital Incubator S150, Stuart Scientific) at 28 °C. Cell culture and virus work were performed in a laminar flow hood (Microflow Biological Safety Level II) under biological safety level II conditions.

**2.3.3 Subculture of cells**

For adherent cells, the supernatant was removed and cells were detached with 0.5 mM EDTA/trypsin (Gibco BRL) at 37 °C. Trypsinisation was stopped by adding medium containing 10% serum and any cell clumps were disaggregated by pipetting gently several times. Cells were split as necessary. For suspension cells, the cells were collected by centrifugation at 1,000 x g for 5 min at 4 °C, resuspended in growth medium and seeded at the appropriate density.

**2.3.4 Cryopreservation of cells**

Cells were harvested as described as above, and resuspended in freezing medium (90% FBS, 10% DMSO) at 1 x 10⁷ cells/ml. The cells were aliquoted into Cryo tubes (Nunc) and frozen slowly (-1 °C per h) then transferred to liquid nitrogen (CellBank).
2.3.5 Revival of frozen cells

Cells were thawed quickly in a 37 °C water bath and then added to 10 ml pre-warmed medium in a sterile 50 ml Falcon tube. The cells were collected by centrifugation and resuspended in growth medium and transferred to a T25 flask (CellStar).

2.3.6 Histological staining of extracted cell populations

Cells extracted from mouse tissues were cyto spun onto Superfrost slides (VWR) by loading ~ 2x 10^4 cells into the sample chamber of a disposable cytofunnel case. The cells were spun onto the slide for 10 min at 800 rpm. The slide was carefully removed from the cytofunnel and the cells air dried for 30 min before being fixed and stained using the Diff-Quik kit (Themoshandon) according to the manufacturer’s instructions. Briefly, the slides were dipped slowly into the Diff-Quik fixative solution then solution 1 (xanthene) followed by solution 2 (thiazine) for 30 s each and finally washed in distilled water three times. The stained slides were air dried before mounting with DePex solution under coverslips and samples were examined with and 4 representative fields were counted to check the percentage purity of the extracted cells.

2.3.7 Transfection of COS-7 cells

Liposome-mediated transfection was employed for the transfection of COS-7 cells (FuGene6 transfection reagent Roche). Cells were seeded at 50-70% confluency the day before transfection in DMEM/2%. The culture medium was replaced with pre-warmed medium (1 ml for 6-well plates and 20 ml for a T175 flask). For transfection in 6-well plates, 4 μl FuGene6 reagent (Roche) or 1.5μl polyethylenimine (PEI, Park Scientific Ltd) was added to 100 μl serum-free DMEM, then 1 μg plasmid DNA was added and the mixture was incubated at room temperature (RT) for 20 min and added to the cells. After 24 h the medium was replaced with DMEM/SF and 72 h later the supernatant was harvested and cell debris was removed.
2.3.8 *Enrichment of cell populations*

Peripheral blood mononuclear cells (PBMCs) were revived in RMPI with 10% human serum (heat inactivated) and human IL-2 (100 ng/ml) and seeded in a T75 tissue flask overnight. The lymphocyte enriched population (non-adherent) was harvested by carefully removing the supernatant and centrifugation at 1,000 x g for 5 min. The monocyte enriched (adherent) population was also harvested. The cell monolayer was gently washed with PBS and this was removed and replaced with PBS/0.5 mM EDTA and the cells incubated at 37 °C for 5 min. The flask was gently tapped and the cells harvested by centrifugation at 1,000 x g for 5 min. The cells in each fraction were enumerated with trypan blue exclusion and a haemocytometer and re-suspended in binding buffer for cell binding assays or FACS buffer for antigen staining.

2.4  *Growth of vaccinia viruses*

2.4.1 *VACV strains and mutants*

VACV strain WR (Smith *et al*., 1991), the VACV mutants vΔA40, vA40HA (Wilcock *et al*., 1999) and vB14HA (Chen *et al*., 2006) and the recombinant VACV WR vT7lacIO (Ward *et al*., 1995) were all described previously.

2.4.2 *Maintenance of virus stocks*

Viruses were kept at -70 °C as master stocks. These were used to seed sub-master, which in turn were used to seed working stocks in RK13 cells. The master stocks were sonicated 3 times for 10 s at 20% power output (Sonicator 3000, Sonimix), diluted and used to infect a T175 cm² flask of RK13 cells at 0.01 p.f.u/cell in DMEM/2.5% for 90 min at 37 °C. The infected cells were incubated at 37 °C in DMEM/10% until complete CPE developed (usually 2 -3 days). The cells were harvested into the medium by a few vigorous taps of the flasks and were collected by centrifugation at 2000 rpm for 10 min. The sub-master stock was resuspended in 2 ml DMEM/SF medium, sonicated, aliquoted and the virus titre assessed (section 2.4.3). This sub-master stock was used to infect 10 x T175 cm² flasks of RK13 cells at 0.01 p.f.u/cell as described for the sub-master stocks. After 2 days, the infected cells were harvested and the pellets were resuspended in chilled 10 mM Tris pH 9.0 (Tris 9.0
buffer) and left on ice for 15 min for the cells to swell. The cells were lysed on ice using a tight fitting dounce homogeniser (25 strokes) and the cell debris was removed by centrifugation at 2000 x g for 10 min at 4 °C. The pellet was resuspended in 10 ml Tris 9.0 buffer, re-dounced and centrifuged again. The supernatants were pooled and sonicated briefly before being loaded onto 18 ml 36% (w/v) sucrose solution in Tris pH 9.0 buffer in Beckman Ultra-Clear tubes and centrifuged at 13,500 rpm for 80 min at 4 °C using a Beckman L8M ultracentrifuge and SW28 rotor. The supernatant was removed by aspiration and the pellet was resuspended in 1 ml Tris 9.0 buffer, sonicated and stored at -70 °C. All viruses were titrated by plaque assay on BS-C-1 cells.

2.4.3 Virus titration

The infectivity of the virus was determined by plaque assay. Sonicated virus stocks were diluted 10 or 100-fold through a dilution series in DMEM 2.5% in triplicate. One ml of the dilutions was used to infect 6-well plates of confluent BS-C-1 cells. The plates were incubated at 37 °C and rocked gently every 30 min for 1.5 h. The inocula were aspirated and the cells were overlaid with 1.5 ml DMEM (2.5% FBS) containing 1.5% carboxymethylcellulose (CMC). After 48 or 72 h, the overlay was aspirated, the cells were rinsed in PBS and the cells were stained for 2 h with 0.01% (w/v) crystal violet in 15% (v/v) ethanol. Cells were rinsed by submersion in water and the plaques were counted.

2.5 Recombinant vaccinia virus preparation

2.5.1 Infection/transfection

A T25 flask of confluent CV-1 cells were infected at 0.05 p.f.u/ml with the vT7LacOI virus (Section 5.5). After 90 min incubation at 37 °C, cells were washed twice with DMEM/SF and then 2 ml DMEM/SF was added to each flask. One μg plasmid DNA in 50 μl DMEM/SF and 2 μl Lipofectamine 2000 in 50 μl DMEM/SF were incubated at RT for 15 min then combined and incubated for a further 20 min. This mixture was then added to the flask and cells were incubated for 4 h at 37 °C. The DMEM/DNA/Lipofectamine 2000 mix was replaced with DMEM/2.5% and cells were
incubated for two days at 37 °C. Cells were harvested by scraping, collected by centrifugation at 2000 x g for 5 min and resuspended in 500 μl DMEM.

2.5.2 Plaque purification, selection and analysis of recombinant viruses

VACV recombinants expressing the *E. coli* guanine (-xanthene) phosphoribosyl transferase (*Ecogpt*) gene may be selected in the presence of mycophenolic acid (MPA) as described previously (Boyle & Coupar, 1988). MPA blocks VACV replication in most cell lines due to its inhibition of inosine monophosphate dehydrogenase in the *de novo* pathway of purine synthesis. If *Ecogpt* is expressed from a VACV promoter such as the P7.5 early/late promoter, the inhibition in purine synthesis is overcome if xanthine and hypoxanthanine, substrates for the purine salvage pathway, are added.

Confluent monolayers of BS-C-1 cells in 6-well plates were incubated for 4 h with DMEM containing 50 μg/ml MPA, 250 μg/ml xanthine and 15 μg/ml hypoxanthanine (HX). Hypoxanthanine stocks were prepared as 10 mg/ml stocks in 0.1 M NaOH, so an equal volume of 0.1 M HCl was added to the medium. The medium containing MPA and HX was removed and cells were infected as described above (section 2.4.2) with 10-fold serial dilutions of the infection/transfection inoculum. Infected cells were overlaid with medium containing 1% low-melting point agarose (Sigma) in 2.5% FBS DMEM containing MPA, hypoxanthine and xanthine at the concentrations above. The agar was allowed to set and plaques were incubated at 37 °C for 2 days. Monolayers were then stained by the addition of 2 ml semi-solid overlay containing 33 mg/L neutral red solution (Sigma). After 1-4 h plaques were visualised as areas which have not taken up the red stain. These plaques were picked as a plug of agarose using a 200 μl blunt-ended micropipette tip and were transferred to a 1.5 ml microfuge tube containing 500 μl 2.5% FBS medium.

Plaque solutions were frozen and thawed twice to break open cells and then sonicated for 20 sec. A fraction of each plaque isolate (50 μl) was used to infect one well of a 96-well plate of confluent RK13 cells. After 90 min at 37 °C, 150 μl of MEM/10% FBS was added to each well and the cells were incubated for 2 days before proteinase K digestion (section 2.7.11) and PCR analysis (section 2.7.12).
Recombinant viruses were plaque purified three times in BS-C-1 cells and 100 μl of the final plaque isolate was used to infect a T25 flask of BS-C-1 cells. After three days, the cells were harvested by scraping, collected by centrifugation and resuspended in 1 ml DMEM. This was freeze-thawed three times and stored at -70 °C as a master stock. This master stock was used to infect a T175 flask of RK13 cells to create a submaster stock which in turn was used to create a working stock (Section 2.4.2).

2.6 Recombinant baculovirus production

2.6.1 Direct plaquing transfection

For each transfection mix, Sf9 cells were diluted to ~ 5 x 10^5 cells/ml in prewarmed medium and were seeded at 2 ml per well of a 6 well-plate plus 5 ml in a T25 flask. The plates were gently rocked to ensure an even monolayer and left to attach at 28 °C for at least 20 min.

The transfection mix was prepared in a sterile 5 ml polystyrene bijou. One hundred ng BacVector-1000 Triple Cut Virus DNA was mixed with 500 ng transfer plasmid in a total volume of 25 μl with BacVector Insect Cell Medium (Novagen). In a separate bijou, 5 μl Insect GeneJuice Transfection Reagent (Novagen) was mixed with 20 μl nuclease free water. This was added immediately to the plasmid/DNA/medium mix and swirled to mix and incubated at RT for 15 min.

During this incubation, the Sf9 cells were washed twice in SF900 medium, removing the second wash just prior to adding the transfection mix to the cells. After the incubation was completed, 0.45 ml BacVector Medium was added to the DNA/Insect GeneJuice mix and vortexed gently to become a 1/10 dilution. Then 0.4 ml BacVector Medium was added to to fresh tubes and using a fresh sterile tip for each dilution, 0.1 ml from the 1/10 was added to the first tube (1/50) and this mix was vortexed gently, then 0.1ml of this mix was added to the second tube (1/250).

The second wash medium was removed from the cells and 0.1 ml of 1/10, 1/50 and the 1/250 dilution were added to the freshly drained wells in duplicate by slowly pipetting the dilution onto the centre of the monolayer. The plates were incubated for 1 h at RT with rocking every 20 min. The residual 0.3 ml of the 1/10 dilution were added to the T25 flask as a back up to the direct plaquing transfection and incubated for 1 h at
RT. During this incubation, the agarose overlay was prepared from 3% BacPlaque melted agarose stock diluted 1:2 with pre-warmed SF900 II SFM/5%.

After the incubation, 2 ml agarose overlay was added to each well by pipetting slowly down the side of the well and allowed to solidify for 20 min at RT, after which 1 ml SF900 II SFM/5% was added gently to the centre of each well. The plates were incubated at 28 °C for 4 days and monitored for plaque formation. 6 ml SF900/5% was added to the T25 and this was incubated for 4 days at 28 °C.

2.6.2 Plaque staining and isolation

After incubation for 4 days, the transfections were monitored by staining plaques in the monolayer with neutral red staining. The liquid overlay was removed from the agarose and replaced with 1 ml diluted neutral red (0.33% [w/v] neutral red stock solution diluted 1:13 with sterile PBS just before use). The plates were incubated for 2 h at 28 °C, the staining solution was removed and the plates were incubated at RT for at least 3 h or overnight.

For each transfection, several well-isolated, independent plaques were picked and eluted into 1 ml SF900 in a sterile screw-cap tube and the mixture was incubated overnight at RT to allow the virus to diffuse from the agarose. Each plaque pick was purified by replaquing onto freshly seeded Sf9 cells (Section 2.6.1) in duplicate.

2.6.3 Preparation of high titre virus stocks

A seed stock was prepared from a well isolated plaque. For each virus stock, a T25 flask was seeded using 2.5 x 10⁶ cells obtained from an exponentially growing suspension culture of Sf9 cells. The flasks were incubated at RT for 20 min to allow the cells to attach, the medium was removed and 0.3 ml of virus suspension was added to the cells. The cells were incubated at RT for 1 h and the liquid rocked across the cells twice and 5 ml SF-900/5% medium was added to the flask and the infected cells were incubated at 28 °C until CPE was observed (3-5 days). The medium was then harvested as a seed stock of virus. This was then used to inoculate a T75 flask of SF9 cells seeded with 2 x 10⁷ cells from an exponentially growing suspension culture. The seeding medium was removed from the flask and 0.5 ml seed stock was added to the cells
carefully and incubated for 1 h at RT. The inoculum was rocked across the cells twice during the incubation. To each flask, 10 ml of SF-900/5% was then added and incubated at 28 °C until CPE was observed (3-5 days). The medium was harvested into sterile 15 ml Falcon tubes and centrifuged (1000 x g for 10 min) to remove cell debris. The supernatant was removed to a fresh sterile tube and constituted the master stock of virus. As a backup, 0.9 ml of the master stock was added to 100 μl FBS and stored at −70 °C while the rest was stored at 4 °C. The master stock was titrated on freshly seeded Sf9 cells in duplicate and the plaques stained with neutral red solution. The master stock was then used to seed a high titre stock. Exponentially growing Sf9 cells at ~ 2 x 10^6 cells/ml in a suspension culture were infected at 0.01 p.f.u/cell. The culture was incubated at 28 ºC with shaking for 5 days and harvested by centrifugation at 2,000 rpm for 15 min at 4 ºC. The supernatant (high titre stock) was aliquoted into foil covered tubes and stored at 4 ºC. This stock was titred and then used to infect Sf9 cells to produce recombinant protein (sections 2.8.10 & 2.8.11).

2.7 Manipulation of DNA & cloning techniques

2.7.1 Quantification of DNA

The concentration of DNA was determined by measuring UV absorbance at 260 nm (1 OD_{260}=50 μg/ml dsDNA) using a NanoDrop ND-1000 spectrophotometer. The purity of the nucleic acids was assessed by the OD_{260/280} ratio. Pure DNA has a value between 1.8 and 2.0.

2.7.2 Restriction enzyme digestion of DNA

Restriction digests were performed with 5 U of enzyme per μg DNA for 2 h under conditions recommended by supplier (Promega, Roche or New England Biolab). For SfiI (New England Biolabs, NEB) restriction was performed at 10 U of enzyme per μg DNA for 20 h at 50 °C. The products were purified by extraction from an agarose gel (Sections 2.7.13 & 2.7.14).
2.7.3 Dephosphorylation of 5’ends of DNA fragments

Dephosphorylation was performed at 1 U of shrimp alkaline phosphatase (SAP, Amersham Biosciences) per µg DNA for 1 h at 37 °C under conditions recommended by supplier. The reaction was terminated by heating at 65 °C for 15 min.

2.7.4 DNA ligation

The molar ratio of insert to vector was 3:1 for sticky-end ligation. The reaction was performed in 30 µl containing the insert and vector DNA (50 ng), 1 x buffer (66 mM Tris-HCl, 5 mM MgCl₂, 5 mM DTT, 1 mM ATP, pH 7.5) and 1 U T4 DNA ligase (Roche) and the reaction mix was incubated at 16 °C for 20 h.

2.7.5 Plasmids

Five DNA plasmid vectors, pSEL (Ahmad, 2003), pSecTag2C (Invitrogen), pET-28a (Novagen), pVOTE 1 (Ward et al., 1995), and pBac-2cp (Novagen) were used. pSecTag2C was used for the construction of mammalian expression clones of truncated A40 protein. pSEL was used to express full-length A40 protein tagged with BAP in VACV-infected mammalian cells. pET-28a was utilised to create truncated proteins expressed in E. coli whilst pBac-2cp was used to create recombinant baculoviruses containing truncated A40 protein tagged with His and BAP expressed from insect cells. pVOTE 1 was used to create recombinant VACV expressing full-length A40 protein tagged with BAP and FLAG, and truncated A40 protein tagged with BAP under an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible system.

2.7.6 Transformation of bacterial cells

Transformation competent cells were thawed slowly on ice. Plasmid DNA (50 ng) or ligation products (5 µl, described above) was added to the cells and left on ice for 30 min. The cells were heat-shocked at 42 °C for 2 min and cooled on ice for 10 min. Two hundred µl of SOC (Invitrogen) was added and the transformation mixture was incubated at 37 °C for 1 h with shaking. One hundred µl of the transformation mixture was spread on agar plates containing 100 µg/ml ampicillin (Amp⁺) or 50 µg/ml kanamycin (Kan⁺) and the plate was inverted and incubated overnight.
2.7.7 Bacterial glycerol stocks

A single bacterial colony on an agar plate was inoculated into 5 ml Luria-Bertani broth (LB) and incubated at 37 °C overnight with shaking. A 500 µl aliquot of the culture was mixed with an equal volume of glycerol solution (65% glycerol [v/v], 10 mM Tris-HCl pH 8.0, 100 mM MgSO₄) and stored at -80 °C. When required, the frozen glycerol stock was scraped using a sterile loop, streaked onto an agar plate to generate discrete colonies, and the plate was incubated overnight.

2.7.8 Preparation of plasmid DNA: miniprep

This protocol was used to prepare good quality plasmid DNA for general DNA manipulations such as restriction enzyme digestion, sub-cloning and DNA sequencing. A commercial plasmid purification system, QIAprep Spin Miniprep Kit (Qiagen), was used following the supplier’s instructions. Plasmids were eluted in double distilled water (ddH₂O) and stored at -20 °C.

2.7.9 Preparation of plasmid DNA: Maxiprep

This protocol was used to prepare up to 500 µg of high purity plasmid DNA for cell transfection using a commercial plasmid purification system, HiSpeed Plasmid Maxi Kit (Qiagen) following the supplier’s instructions. The plasmid DNA was eluted in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and stored at -20°C.

2.7.10 DNA sequencing

DNA samples (200 ng of template dsDNA) were mixed with 32 pmol of oligonucleotide primer in a reaction volume of 10 µl. Samples were submitted to the MRC Sequencing Service, Hammersmith Campus, Imperial College London. The results were analysed using BioEdit software (Hall T.A) and the NCBI website (http://www.ncbi.nlm.nih.gov/).
2.7.11 Preparation of viral template DNA for PCR analysis

Confluent BS-C-1 cells were infected with 50 μl of plaque isolate medium (Section 2.5.2) for 48 h in a 96-well plate in the presence of selection drugs. The medium was removed and the cells washed in PBS once. Proteinase K solution [50μL (50 μg/ml Proteinase K (Fluka), 10x Mg free PCR buffer (Promega)] was added to each well and the plate was freeze - thawed once. The contents of each well was transferred into 0.2 ml eppendorf tubes and incubated at 56°C for 20 min, followed by 85 °C for 10 min to inactivate the proteinase K.

For analysing recombinant baculoviruses, the virus was first lysed then treated with proteinase K. The high titre stock virus (section 2.6.3, > 1 x 10^8 p.f.u/ml,) was added to 89 μl Baculo lysis buffer (10 mM Tris, pH 8.3, 100 μg/ml gelatine, 0.45% Triton X-100, 0.45% Tween-20, 50 mM KCl) and then 1 μl proteinase K (10.8 mg/ml) was added. The samples were incubated for 1 h at 60°C after which, the proteinase K was heat inactivated at 95 °C for 10 min and the samples were cooled on ice. A PCR was performed to determine the presence/absence of the required gene within the virus using 3 μl of template per PCR.

2.7.12 Polymerase chain reaction (PCR)

PCR was used to amplify specific DNA sequences for cloning and to screen bacterial clones. For cloning, Platinum Taq DNA High Fidelity (HiFi Taq) (Invitrogen) was used whereas GoTaq (Roche) was used for screening. As an overall strategy, a standard PCR in a 50 μl reaction volume contained 10 to 100 ng of template DNA, oligonucleotide primers (50 pmol each), 200 μM dNTPs, 1 x PCR buffer (HiFi Taq: 60 mM Tris-SO_4 [pH 8.9], 18 mM ammonium sulphate; GoTaq: 2 mM MgCl_2 [HiFi Taq] and 2 U of thermal polymerase. PCR conditions used were (i) initial denaturation at 94 °C for 2 min, (ii) 30 cycles of 94 °C for 30 s, annealing for 30 s, extension at 72 °C (HiFi Taq), 74 °C (GoTaq) for 2 min. The extension time for DNA sequences was approximately 1 min/kb. The PCR products were analysed by agarose gel electrophoresis and where necessary purified from the excised fragment of agarose (section 3.5.12 and 3.5.13).
2.7.13 Resolution of DNA by agarose gel electrophoresis

DNA fragments were resolved by agarose gel electrophoresis. Samples were mixed with 1/5 vol of 6 x loading buffer (30% glycerol [v/v], 0.25% bromophenol blue, 0.25% xylene cyanol FF) before loading into an agarose gel. The concentration of agarose in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) was selected according to the size of DNA fragments to be resolved (0.7% agarose for DNA fragment sizes between 800 bp and 12 kb, 1% for 500 bp to 10 kb, 1.5% for 200 bp to 3 kb and 2% for 50 bp to 2 kb). Molten agarose was supplemented with either ethidium bromide (10 μg/ml) or more recently SYBR Safe DNA gel stain (Invitrogen) and allowed to set. Electrophoresis was performed with 1 x TAE buffer in a minigel electrophoresis unit (BioRad) at 80 volts for 30 min. A 1 kb DNA marker (Hyperladder V, Bioline) or TrackIt 50 (Invitrogen) was electrophoresed alongside the samples. The DNA fragments were visualised with a UV transilluminator (UVP) and photographed using a gel-documentation imager (Bio-Rad Chemi Doc).

2.7.14 Purification of DNA by gel extraction

Resolved DNA fragments in agarose gel were excised by a scalpel blade under a long wave UV lamp (302 nm). The DNA was extracted from the agarose gel using a Qiaquick Gel Extraction Kit (Qiagen) following the manufacturer’s instructions.
Table 2.3 Oligonucleotides used for cloning & sequencing in this study
Restriction sites are underlined. Start and stop codons are bold. Biotin acceptor peptide (BAP), HA, and FLAG sequences are italic. Nucleotides inserted for correcting ORF are bracketed. FP, forward primer; RP, reverse primer

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Table 2.3 continued. Oligonucleotides used for cloning & sequencing in this study

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2.8 Protein expression, purification and analysis

2.8.1 Production and purification of anti-BAP monoclonal antibody

The mouse hybridoma cell line LX035-9 was expanded to 1 L, containing ~ 1 x 10^7 cells. The cells were collected by centrifugation at 2,000 x g for 10 min and the supernatant was harvested. The cells were resuspended in 1 L of RPMI /SF and incubated with rotation for 7 days until maximum levels of mAb had been produced. The supernatant was centrifuged (1000 x g, 10 min) to remove cell debris, filtered through a 0.45 μM filter and applied to a protein G HiTrap column (GE Healthcare) using a peristaltic pump. The column was washed with PBS and the bound IgG was eluted with 100 mM glycine pH 2.8 on an AKTA purifier (Amersham Biosciences). The fractions were assessed for IgG content by SDS-PAGE and coomassie blue staining.

2.8.2 Production of anti-BAP columns

The IgG containing fractions were pooled, concentrated by using a Centriprep-10 protein concentrator (Ambicon) and washed twice in Ab buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% sodium azide). The IgG was coupled to cyanogen bromide (CNBr)-activated Sepharose beads (Pharmacia) following the manufacture’s protocol. The beads were poured into Econo-Columns (BioRad) and washed with BAP affinity column buffer (25 mM Tris, 140 mM NaCl, 0.02% sodium azide [v/v], pH 7.4). The columns were stored at 4 °C until needed.

2.8.3 Bacterial expression systems

2.8.3.1 Bacterial protein expression for generation of anti-A40 antibody

Bacterial plasmids containing truncated A40 (pET-28a-HisA40ΔTM or pET-28a-HisA40ΔTMΔSt, Section 2.7.5.) were expressed in E. coli by transformation into the E. coli strain Rosetta Gami 2 (Novagen), a strain that has trxB/gor mutations for disulphide bond formation and improved protein folding in vivo.

A single bacterial colony was used to inoculate 4 ml of LB containing 50 μg/ml kanamycin (Kan) at 37 °C with shaking. This culture was then used to inoculate 2 L LB
(plus antibiotic) and was incubated at 37 °C with shaking until reaching an OD$_{595}$ reading of 0.6. IPTG was added to a final concentration of 1 mM and the incubation was continued for 3 h. The cells were harvested by centrifugation at 3,000 rpm for 20 min at 4 °C.

2.8.3.2 **Bacterial protein expression for crystallisation trials**

*E. coli* strain Rosetta Gami 2 (DE3) was transformed with bacterial plasmids containing truncated A40 (pET-28a-HisA40ΔTM or pET-28a-HisA40ΔTMΔSt, section 2.7.5.). A single bacterial colony was used to inoculate 10 ml of LB broth containing 50μg/ml Kan and the culture was incubated at 37 °C with shaking. After overnight incubation, the culture was used to inoculate 2 L LB with Kan with shaking until reaching an OD$_{595}$ reading of 0.6. IPTG was added to a final concentration of 1 mM and the incubation was continued at 37 °C for 3 h with shaking. The cells were harvested by centrifugation at 3,000 rpm for 20 min at 4 °C.

2.8.3.3 **Bacterial protein expression with Selenomethionine labelling for crystallisation studies**

*E. coli* strain Rosetta Gami 2 (DE3) was transformed with bacterial plasmids containing truncated A40 as above. A single bacterial colony was used to inoculate 200 ml of Selenomethionine (SeMet) base (190 ml SeMet O/N broth (AthenaES), 0.8 ml L-methionine (10 mg/ml), 10 ml nutrient mix) containing 50 μg/ml Kan at 37 °C with shaking O/N. The culture was centrifuged at 3,000 rpm for 10 min and the cells were washed in 50 ml PBS, resuspended in 10 ml PBS and re-centrifuged as above and then resuspended in 8 ml PBS. This was added to 2 L of SeMet labelling broth (1.9 L SeMet base, 100 ml glucose free nutrient base, 8 ml selenomethionine (Merck chemicals). The bacteria were incubated with shaking at 37 °C until reaching an OD$_{595}$ of 0.6. IPTG was added to a final concentration of 1 mM and the incubation was continued at 16 °C for 16 h with shaking. The cells were harvested by centrifugation at 3,000 rpm for 30 min at 4 °C.
2.8.3.4  Small scale protein extraction to assess solubility of expressed recombinant A40

*E. coli* strains B834 (Invitrogen), BL21 (Invitrogen) or Rosetta Gami 2 were transformed with either pET-28aHisA40ΔTM or pET-28aHisA40ΔTMΔSt. A single colony of the strains was inoculated into 1 ml LB containing 50 μg/ml Kan and incubated at 37 °C with shaking. This culture was used to inoculate 100 ml LB with Kan and shaken at 37 °C until reaching an OD$_{595}$ of 0.6. This culture was split into 5 ml cultures and incubated at either 16, 30 or 37 °C for various times with either 0.1, 0.5 or 1 mM IPTG or induced by the Autoinduction system (Novagen). Before harvesting, the final OD$_{560}$ of each culture was measured. Cell pellets from the induced 5 ml bacterial culture were frozen and thawed once. The cells were resuspended in 200 μl lysis buffer (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 2% [v/v] Triton X-100) per 1.0 OD$_{560}$ of measured culture, vortexed rigorously and sonicated on ice for 15 s at 20% power output with 30 s incubation between each sonication for a total of 2 min. The cell lysates were centrifuged at 15,000 rpm for 15 min to separate soluble and insoluble proteins. The supernatants (soluble proteins) were collected and the insoluble proteins were suspended in an equal volume of lysis buffer. An equal volume of 2 x SDS buffer was added to the soluble and insoluble fractions and the samples were boiled at 95 °C for 7 min. The three fractions were analysed by SDS-PAGE (section 2.8.13).

2.8.3.5  Purification of insoluble His-tagged proteins

Two litre cultures of induced cells were harvested and resuspended in 20 ml PBS. The cells were sonicated ten times on ice for 30 s at 20% power output with 30 s incubation between each sonication. The soluble fraction was harvested by centrifugation at 15,000 x g for 30 min and the supernatant was harvested. The fractions were analysed by SDS-PAGE and coomassie staining.

The pellet was suspended in inclusion body wash buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.5% Triton X-100,) and re-collected by centrifugation at 15,000 x g for 30 min. This wash step was repeated 4 times. The pellet was washed once in inclusion body wash buffer lacking Triton X-100 and the pellet was resuspended in denaturing buffer (100 mM Tris, 6 M guanidine, 2 mM DTT) and incubated at 4 °C overnight with
rotation. The lysate was then harvested by centrifugation at 15,000 x g for 30 min and the supernatant was decanted.

Protein refolding was performed using rapid dilution of denatured protein into refold buffer (50 mM Tris pH 8.0, 1 M L-arginine monohydrochloride, 3.7 mM cysteamine, 6.5 mM cystamine, 1 protease inhibitor complete cocktail tablet (Roche) per 50 ml) drop-wise with stirring at 4 °C and left to refold overnight. The refolded protein was concentrated to ~ 25 ml and dialysed against His binding buffer (0.5 M NaCl, 20 mM Tris; pH 8.0).

The refolded protein was applied to an equilibrated HisTrap HP column (GE Healthcare) and the column was washed in His binding buffer. The bound proteins were eluted with a 50 mM to 500 mM imidazole gradient. Fractions were tested for the presence of recombinant protein by SDS-PAGE followed by coomassie blue staining or immunoblotting for the His tag. Fractions containing the recombinant protein were combined and concentrated using a Centricon YM-10 to 2 ml. This was applied to a Superdex 75 10/300 column (GE Healthcare) in 0.5 ml aliquots and eluted subsequently in PBS. Fractions were assessed for protein by SDS-PAGE, coomassie blue staining or immunoblotting. Fractions containing the desired recombinant protein were combined.

2.8.4 Transient transfection of COS-7 cells

FuGENE 6 Transfection Reagent (Roche) was used for transfection according to the manufacturer’s instruction. Briefly, cells were seeded at 30-50% confluency. After 16-18 h, the culture medium was replaced by pre-warmed complete medium (1 ml per well for a 6-well plate, 20 ml for a T175 flask). For 6-well plates, 4 μl of liposome was added to 100 μl serum-free DMEM for 5 min, and then 1 μg plasmid DNA was added into the mixture. The reagents were mixed gently, incubated for 20 min at RT and added onto cells.
2.8.5 *Mammalian expression system and in vitro biotinylation of BAP-fusion proteins with BirA enzyme*

The procedure for biotinylating BAP-fusion proteins was adopted from (Brown *et al.*, 1998). COS-7 cells were transfected with the psBAPA40ΔTM or psBAPA40ΔTMSt plasmids as described above. After transfection and overnight incubation, the supernatant was replaced by serum-free DMEM. After 3 days incubation, the supernatant containing the secreted recombinant protein was collected, centrifuged for 5 min at 2000 rpm at 4 °C and filtered to remove cell debris. The supernatant was then concentrated 20-fold by centrifugation using a Centriprep-10 protein concentrator (Ambicon). The concentrated proteins were diluted with 10 mM Tris pH 8.0 to the original volume and again concentrated 20-fold as above.

The concentrated recombinant BAP fusion proteins were purified over anti-BAP affinity column (section 2.8.1). The concentrated supernatant was loaded onto the column, washed with 15 ml washing buffer (25 mM Tris; 140 mM NaCl; 0.02% azide; pH 7.4) and bound proteins were eluted by addition of 15 ml elution buffer (100 mM glycine pH 2.8). The eluant was concentrated 5-fold. Enzymatic biotinylation was performed using 1 μg (5000 U) of BirA enzyme (Avidity) overnight at RT as recommended by the manufacturer. Uncoupled biotin was removed by dialysis against PBS.

2.8.6 *Concentration of proteins in the supernatant from transfected COS-7 cells by precipitation with trichloroacetic acid*

One ml of harvested supernatant was mixed with 0.1 ml of trichloroacetic acid (TCA) and the samples were vortexed and left overnight at 4 °C. Samples were centrifuged for 15 min at 13,000 x g at 4 °C and the supernatant was discarded. Pellets were washed twice in ice-cold acetone, vortexed and then centrifuged for 5 min at 13,000 x g at 4 °C. Pellets were air-dried, re-dissolved in 100 µl 2 x SDS protein loading buffer (100 mM Tris-HCl pH 6.5; 4% SDS; 10% glycerol; 0.2% bromophenol blue; 100 mM DTT) and boiled at 95 °C for 6 min.
2.8.7 Treatment of transfected COS-7 with tunicamycin

Cells were transfected as described above (section 2.8.4) with recombinant plasmids psBAPA40ΔTM, psBAPA40ΔTMΔSt, psHAA40ΔTM or psHAA40ΔTMΔSt in 6-well plates. The day after transfection, the medium was changed and the cells were incubated in the presence or absence of 1 μg/ml of tunicamycin 3 days. The supernatant was harvested, centrifuged at 2000 rpm to remove cell debris and then concentrated by TCA precipitation (section 2.8.6). Pellets were resuspended in 2 x loading buffer. Cells were harvested in lysis buffer 1 (LB1, 50 mM Tris HCl pH 7.4; 140 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% [v/v] Triton X-100; mini complete Protease tablet) for 30 min at 4 °C and then 1/6 vol of 6 x SDS protein loading buffer (300 mM Tris HCl pH 6.5; 12% SDS; 30% glycerol; 0.6% bromophenol blue; 100 mM DTT) was added and the samples were boiled at 95 °C for 6 min. Cell lysate and supernatant were analysed by immunoblotting with anti-HA or anti-BAP mAb.

2.8.8 Treatment of proteins with endoglycosidases PNGase F and Endo H

Proteins were incubated with 1 x denaturing buffer at 95°C for 10 min then the samples were made up to 20 μl with 1 x Reaction buffer (G7 for PNGase F, G5 for Endo H), 1% Nonidet P40 (NP-40) and 2 μl endoglycosidase. The samples were incubated at 37 °C for 6 h before 4 μl 6 x SDS loading buffer was added, the samples boiled at 95 °C for 5 min and analysed by SDS-PAGE (section 2.8.13) and Coomassie blue (section 2.8.14) staining and by immunoblotting (section 2.8.15). RNase B was used as a control protein for deglycosylation.

2.8.9 Protein expression from inducible recombinant vaccinia viruses

Confluent monolayers of BS-C-1 cells were infected with recombinant VACVs at 5 p.f.u./cell in DMEM/2.5%. After 90 min incubation at 37 °C, the inoculum was removed and replaced with DMEM with 190 mM NaCl and 1 mM IPTG. Cells were incubated for 24 h at 37 °C and then the supernatant was harvested and centrifuged at 2,000 rpm for 10 min to remove cell debris. The supernatant was concentrated to 5 ml (per flask) using centrifugal concentrators with a molecular cut-off of 10 kDa (Centriprep YM-10). Virus infectivity was destroyed by the addition of psoralen and treatment with long-wave UV light for 10 min.
The concentrated supernatant was then dialysed against 20 mM sodium phosphate buffer pH 7.0 and then applied to a Resource S column that had been pre-equilibrated with 3 column volumes of 20 mM sodium phosphate buffer. Protein was eluted over a gradient of 0 – 1 M NaCl in 1 ml fractions. Twenty μl aliquots of each fraction were analysed by SDS-PAGE (section 2.8.13) and Coomassie blue (section 2.8.14) staining and by immunoblotting (section 2.8.15).

2.8.10 Trial expression of protein from insect cells

A 50 ml shaking culture of Sf9 cells were seeded at 5 x 10⁵ cells/ml and allowed to grow at 28 °C to 2 x 10⁶ cells/ml (2 days). The culture was infected at 5 p.f.u./cell and 5 ml samples were collected at various times thereafter. Infected cells were collected by centrifugation and frozen at -20 °C. When needed the cells were lysed in 1 ml insect lysis buffer (ILB, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors) on ice for 45 min with occasional vortexing. The lysate was centrifuged for 30 min at 3000 rpm at 4 ºC and the supernatant was harvested in a 1.5 ml screw-cap tube. Two hundred μl 6 x SDS protein loading buffer was added to the lysate and the samples were analysed by SDS-PAGE (section 2.8.13) and Coomassie blue (section 2.8.14) staining and by immunoblotting (section 2.8.15).

2.8.11 Large scale expression of protein from insect cells

Four 500 ml shaking cultures were seeded with Sf9 cells in log phase at 5 × 10⁵ cells/ml and grown at 28 °C in an orbital shaker (150 rpm) to a density of 2 × 10⁶ cells/ml. The culture was infected with recombinant baculovirus (section 2.5) at 5 p.f.u./cell and incubated with shaking at 150 rpm at 28ºC. Cells were harvested at the time of maximal expression of the target protein (as determined previously in section 2.8.10 above). Cells were collected by centrifugation for 30 min at 3000 rpm at 4 ºC, the supernatant was removed and the cells were lysed in ILB (section 2.8.10) on ice for 45 min with occasional vortexing. The lysate was centrifuged for 30 min at 3000 rpm at 4 ºC and the supernatant was harvested for protein purification by application to a His column. The column was washed in HisTrap binding buffer and the protein was eluted with a 50 mM to 500 mM imidazole gradient. Fractions were tested for the presence of
recombinant protein by SDS-PAGE followed by coomassie blue staining or immunoblotting for the His tag.

2.8.12 Measurement of protein content

Samples were diluted in distilled water and mixed individually with Dye Reagent Concentrate (Bio-Rad) according to manufacturer’s instructions. After adding the dye, the absorbance was measured at 595 nm and compared to a standard curve using either BSA or bovine gamma globulin.

2.8.13 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved by SDS-PAGE using the Tris/glycine discontinuous buffer system and a vertical electrophoresis unit Mini-PROTEAN III apparatus (Bio-Rad). For reducing gels, the samples were denatured by heating at 95 °C for 5 min in 1 x Laemmli loading buffer with 10 mM β-mercaptoethanol (β-ME). The samples were electrophoresed into the stacking gel (125 mM Tris, pH 6.8, 5% acrylamide, 0.1% ammonium persulphate and 0.1% N,N,N′,N′-tetramethylethylenediamine, TEMED) at 60 V and resolved by the separating gel (375 mM Tris, pH 8.8, 10 - 15% acrylamide, 0.1% SDS, 0.1% ammonium persulphate and 0.04% TEMED v/v) at 180 V for 45 min in protein running buffer (25 mM Tris, pH8.3, 250 mM glycine, 0.1% SDS). After electrophoresis, the gels were processed for coomassie blue staining or immunoblotting.

2.8.14 Coomassie blue staining

Polyacrylamide gels were stained in coomassie blue stain solution (10% [v/v] acetic acid, 45% [v/v] methanol, 0.25% [w/v] coomassie brilliant blue (BDH)) for 1 h at RT, and then destained in destain buffer (10% [v/v] acetic acid, 45% [v/v] methanol). The destain buffer was exchanged until a suitable background was obtained. The gels were dried between 2 sheets of pre-wet cellophane membrane (Bio-Rad) in a hot-air gel dryer (GelAir drying system, Bio-Rad).
2.8.15 Immunoblot analysis of proteins

Polyacrylamide gels were equilibrated for 30 to 60 min in transfer buffer (190 mM glycine, 25mM Tris, 0.1% SDS, 20% methanol) then transferred to Hyperbond ECL nitrocellulose membranes (GE Healthcare) at 18 V for 30 min. After transfer, the membranes were rinsed once in PBS and then blocked (5% milk, PBS) for 1 h at RT. The primary Ab (Table 2.1) was added in blocking solution and incubated overnight at 4 °C. The membrane was washed 3 times in 0.1% Tween/PBS for 10 min, incubated with secondary Ab in blocking solution for 1 h at RT, washed 3 times in 0.1% Tween/PBS for 10 min and exposed with ECL Plus Western blotting detection reagent (GE Healthcare).

2.8.16 Cell binding assay using microspheres coated with recombinant proteins

In a standard binding assay, 5 µl SPHERO™ Avidin - coated fluorescent particles (0.4-0.6 µm, 0.1% w/v, Spherotech Inc) were washed in binding buffer (10 mM HEPES, 140 mM NaCl, 10 mM CaCl$_2$, 10 mM MgCl$_2$, pH 7, 1% BSA). Saturating amounts of biotinylated recombinant protein (50 ng/µl beads) were incubated in 100 µl volume on ice for 1.5 h with occasional agitation. The beads were collected by centrifugation at 13,000 x g for 5 min, the supernatant was removed and the beads were washed with 500 µl binding buffer and recentrifuged. The beads were resuspended in 100 µl binding buffer and sonicated by a cup-horn sonicator (20% power output, 3 x 20 s).

Cells (2 x 10$^5$) were added to beads in a 100 µl volume in binding buffer and incubated for 1 h on ice with occasional agitation. Samples were transferred to 5 ml Falcon tubes and topped up with 500 µl binding buffer and analysed by flow cytometry (FC, Calibre Becton Dickinson). The binding of the fluorescent beads was analysed (excitation 488 and emission at 538) in channel FL1 (FITC channel) with an argon laser. Compensation for non-specific binding was performed using a cell only sample after gating on forward and side scatter profiles.
2.8.17 Co-immunoprecipitation of proteins using microspheres coated with recombinant proteins

SPHERO™ Avidin coated fluorescent particles (25 µl, 0.4-0.6 µm, 0.1% w/v, Spherotech Inc) were washed in binding buffer and prepared as above (section 3.6.13). Cells (2 x 10^6) were lysed in 100 µl LB1 on ice for 30 min with agitation every 10 min. The lysate was centrifuged for 15 min at 15,000 x g to remove cell debris and the supernatant was collected.

The clarified lysate was added to the sonicated beads overnight at 4 °C with rotation. The cells were washed 4 times in LB1. The mixture was centrifuged at 3,000 x g for 1 min, the supernatant was removed and fresh buffer was added. The samples were rotated for 15 min at 4 °C and then recentrifuged as above. The final bead pellet was resuspended in 50 µl 2 x SDS loading buffer, boiled for 5 min at 95 °C and spun for 30 s. Samples were analysed by SDS-PAGE and coomassie blue staining.

2.8.18 Immunoprecipitation of proteins using Protein G beads

Cells (2 x10^7) were lysed in LB1 on ice for 30 min, agitating the mix every 10 min by vortexing. The lysate was centrifuged for 15 min at 15,000 x g to remove cell debris and the supernatant was collected. Lysates were pre-cleared against 30 µl Protein-G Sepharose beads for 2 h on a rotor wheel at 4 °C. In parallel, 40 µl Protein G Sepharose beads per sample were washed in LB1 and spun for 1 min at 3000 x g. The supernatant was removed and the beads were incubated with a dilution 1:500 anti-BAP Ab for 2 h at 4 °C with rotation. The cell lysate:bead mix was centrifuged at 10000 x g and the supernatants of the cell lysates were harvested as pre-cleared cell lysate. The bead-Ab mix was centrifuged at 3000 x g for 3 min and the pellet was resuspended in the pre-cleared lysate and incubated for 4 h at 4 °C with rotation. The bead pellet was harvested by centrifugation at 3000 x g for 3 min and washed in LB1 four times. The beads were then resuspended in 2 x SDS loading buffer, centrifuged briefly and heated to 95 °C for 5 min. The beads were then centrifuged prior to analyzing the supernatants by SDS-PAGE and immunoblotting.
2.8.19 Membrane protein extraction using ProteoExtract Native Membrane Protein Extraction (M-PEK) kit

A T175 flask of TK143 cells was infected with vA40HA at 5 p.f.u/cell in DMEM/2.5% for 1 h at 37 °C or mock-infected, after which the cells were washed carefully in PBS and incubated in DMEM/10% for 8 h at 37 °C. The cells were washed in ice-cold PBS and membrane proteins were extracted using the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem) following the manufacturer’s protocol. Each fraction was concentrated 10 x using a Centriprep Centrifugal YM-3 Filter. Each of the concentrated fractions were analysed by SDS-PAGE and immunoblotting (sections 2.8.13 & 2.8.15).

2.8.20 Immunofluorescent analysis of the cellular localisation of A40

Cells used for fluorescent and confocal microscopy were grown on 22-mm sterilised glass coverslips (borosilicate glass, BDH) in 6-well plates. For infections, cells were seeded ~ 80% confluency overnight and infected with 10 p.f.u./cell. At various time-points, the cells were chilled on ice for 5 min in preparation for staining. For transfection/infections using pSEL, the cells were seeded at ~ 50% onto the coverslips overnight. The cells were transfected as described in section 2.8.4, and after 4 h the medium was changed and the cells infected with 1 p.f.u./cell. At various time-points the cells were chilled on ice for 5 min prior to staining.

For surface staining, the cells were washed once in ice-cold PBS and incubated with primary Ab diluted in DMEM/5% on ice for 1 h. Cells were washed three times in ice-cold PBS and then fixed in ice-cold 4% paraformaldehyde (PFA, diluted in 0.5 M HEPES pH 7.4) for 10 min on ice. The supernatant was removed and replaced with 8% PFA and incubated for 20 min at RT. Cells were washed three times (in PBS at RT), before being quenched for 10 min with 50 mM NH4Cl at RT. The cells were washed three times in 5% FBS/PBS then incubated for 30 min with Alexa488-conjugated secondary Ab (diluted in FBS/PBS) for 30 min at RT. The coverslips were washed three times in PBS followed by one wash in ddH2O. Then they were mounted in Mowiol-4’, 6-diamidino-2-phenylindole (DAPI) mounting medium.

For intracellular staining, the cells were washed three times in ice-cold PBS, before being fixed with 4% PFA for 10 min on ice then with 8% PFA and quenched as
described above. After another three washes (in PBS at RT), cells were permeabilised and blocked in 0.2% (w/v) saponin/ 5% FBS in PBS for 1 h. The cells were then incubated for 1 h at RT with primary Ab diluted in the same blocking mixture. The cells were washed, incubated with secondary antibody and prepared for mounting as described for surface staining. Samples were examined with a Zeiss LSM5 Pascal confocal microscope using Zeiss LSM software and images were reconstructed and processed using Confocal Assistant and Adobe Photoshop software.

2.9 Animal work

2.9.1 Virulence Assays in murine models

2.9.1.1 Intradermal infection model

Groups of female C57Bl/6 mice, between 6 and 8 weeks old, were anaesthetised and inoculated with sucrose-purified VACV in 10 μl of PBS into each ear pinna (Tscharke et al., 2002). Mice were examined daily and the diameter of lesions at the inoculation site was measured using a micrometer. Titres of inocula were confirmed by plaque assay.

2.9.1.2 Vaccination and challenge model

Groups of female Balb/c mice, between 6 and 8 weeks old were anaesthetised and inoculated with sucrose-purified VACV in 10 μl of PBS in one ear pinna as above. Ears were monitored over the subsequent 7 days to establish “take”. Twenty-eight days later, mice were anaesthetised and inoculated i.n. with a lethal dose (1x 10^7 p.f.u) of VACV WR in 20 μl PBS. Mice were weighed daily before and after infection. Signs of illness were also scored as above. Titres of inocula on both vaccination and challenge days were confirmed by plaque assay.

2.9.2 Extraction of cells from infected ear tissues

Ear lobes from infected C57Bl/6 mice were collected, rinsed in 70% ethanol and allowed to dry. The central and dorsal dermal sheets were separated using fine forceps and transferred immediately to RPMI-10 containing 1 mg/ml collagenase
(Sigma-Aldrich, crude type XI) and 0.004% DNase I (Sigma-Aldrich) and incubated for 75 min at 37 °C. The cells were passed through 0.45 μm cell strainers and centrifuged at 800 x g for 5 min. Cells were resuspended in erythrocyte lysis buffer for 3 min before RPMI-10 was added and cells were centrifuged as before. Cells were enumerated using a haemocytometer and trypan blue exclusion.

2.9.3 Virus analysis from infected tissues.

The titres of virus in tissues extracted as above were determined by plaque assay on duplicate monolayers of BSC-1 cells.

2.9.4 Analysis of infiltrating cells at the site of infection

Live cells were counted, washed with flow assisted cell sorting (FACS) buffer (0.1% BSA, 0.1% NaN₃ in PBS), blocked with FACS blocking buffer containing 10% normal rat serum, and a 1:500 anti-CD16/CD32 Fc γ receptor in FACS buffer for 30 min at 4 °C. Cells were stained with appropriate combinations of FITC-, phycoerythrin (PE)-, allophycocyanin (APC) - or tricolour (TC)-labelled antibodies (Table 2.2) in 10 μl FACS block solution per 2 x 10⁵ cells. These were grouped into anti-CD3, anti-CD8 and anti-CD4 for T lymphocytes, anti-NK1.1 for NK cells, anti-TCRγδ for TCR γδ cells, anti-Ly6G on neutrophils and anti-F480 on macrophages. Isotype controls for each fluorophore were obtained from BD biosciences as described (Jacobs et al., 2006), as well as single stains for each Ab to allow compensation between the different fluorophores to be judged accurately. These solutions were incubated with the cells for 30 min at 4 °C in the dark. Cells were washed three times in FACS buffer. The presence of cell-surface markers was determined on a FACSCalibur flow cytometer with CellQUEST software (BD Biosciences). A lymphocyte gate was used to analyse data from at least 50000 events.

2.9.5 Preparation of splenocytes

Spleens were placed into a Petri dish and 3 ml of RPMI containing 2.5% FBS was added. The end of a 5 ml syringe was used to mash each spleen until all visible clumps of cells had dispersed. Cells were then strained through a cell strainer (Falcon)
into fresh 10 ml centrifuge tubes and the volume of medium in each tube was made up to 10 ml. Cells were centrifuged (800 x g, 8 min) and the supernatant was aspirated. Red blood cells were depleted by a 5 min incubation with 2 ml lysis buffer (10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA pH 8.0) followed by the addition of 8 ml RPMI containing 2.5% FBS and another centrifugation (800 x g, 8 min). Cells were resuspended in 10 ml of RPMI containing 2.5% FBS and were counted using a Neubauer Haemocytometer with 20% trypan blue to visualise live cells.

Splenocytes were separated into enriched populations for cell binding assays as described for PBMCs in section 2.3.8.

2.9.6 Preparation of mouse resident peritoneal macrophages

C57Bl/6 mice (4-6 week old) were euthanized and the abdomen were sterilised with 70% alcohol. A 30-cc syringe was attached to 19-G needle and was filled with harvest medium (RPMI/SF). With the bevelled end of needle facing up, the needle was inserted through the peritoneal wall and 10 ml harvest medium was injected into each mouse. Using the same syringe and needle, the needle was inserted bevelled end down into peritoneum and raised slightly to cause tenting of peritoneal wall and the peritoneal fluid was withdrawn slowly. A single 30-cc syringe was used to inject and collect fluid from three mice.

The pooled peritoneal fluid was transferred to a 50-ml polypropylene centrifuge tube on ice and the enumerated using a haemocytometer and trypan blue exclusion and 0.2 ml was differentially stained (section 2.3.6). The peritoneal lavage fluid was centrifuged for 10 min at 800 x g at 4 °C, the supernatant discarded and the cell pellet resuspended in RPMI/10%.

2.9.7 Isolation of mouse bone marrow cells

C57Bl/6 mice (4-6 week old) were euthanized and using aseptic technique, the skin was peeled from the top of each hind leg and down over the foot. The foot was cut off with the skin and discarded. The hind legs were cut off and placed in a dish containing sterile PBS. Excess muscle was removed from the legs by holding end of
bone with forceps and using sterile scissors to push the muscle downward away from forceps and the leg bones were severed between the joints.

A 25-cc syringe was attached to a 26-G needle and filled with sterile PBS. The needle was inserted into the bone marrow cavity of the femur or tibia and the cavity was flushed with 5 ml PBS, or until bone cavity appears white. The wash medium was allowed to collect in a sterile 50-ml conical centrifuge tube on ice and the cells were either used to prepare bone marrow derived macrophages or neutrophils (see below).

2.9.8 Preparation of bone marrow derived macrophages

The cells collected in section 2.9.7 were centrifuged for 10 min at 1600 rpm at RT and the supernatant was discarded. The cell pellet was resuspended in 5 ml serum-free Eagle’s MEM (EMEM). Lymphocyte separation medium (5ml) was placed in a 15-ml conical tube and overlaid with 5 ml of the bone marrow suspension. The separation medium was centrifuged for 20 min at 500 x g at RT with the brake off. The cells were gently removed at the interface with a Pasteur pipette by placing the pipette tip at the interface and slowly moving it over the interface while slowly drawing cells into the pipette. The cells were pooled and centrifuged for 10 min at 500 x g at 4 °C, resuspended in 10 ml EMEM/10 % FCS (EMEM/10) and cell numbers were counted. The remaining cells were centrifuged again for 10 min at 500 x g at 4 °C and resuspended in EMEM/10 to a final concentration of 5 x 10^6 cells/ml. Cells were aliquoted at ~ 1–3 x 10^7 cells per 25-cm² tissue culture flasks containing 10 ml EMEM/10 and IL-2 at 10 ng/ml and cells were incubated for 24 h at 37 °C. The non-adherent cells were then transferred to a 75-cm² tissue culture flask and 10 ml EMEM/10/IL-3 was added. The cells were incubated for a further 4 days after which a further 10 ml EMEM/10/IL-3 was added and the cells were incubated for another 3 days.

The mature macrophages were then harvested by removing and discarding the culture supernatant. The remaining adherent cells were washed with 15 ml PBS. The PBS wash was discarded and 5 ml PBS/EDTA was added to the flask and incubated for 5 min at 37 °C. The cells were loosened by banging the side of flask and EMEM/10 was added to the flask and the detached cells were collected by centrifuging the cells for 10 min at 500 x g at 4 °C. The cells were resuspended in 5 ml of EMEM/10 and
using a haemocytometer and trypan blue exclusion and 2 x 10^4 cells were taken for differential staining (section 2.3.6).

2.9.9 Preparation of mouse bone marrow derived neutrophils

The cells collected in Section 2.9.7 were resuspended and cell clumps were disaggregated with a 19G needle and the cells collected by centrifugation at 112 x g for 6 min at RT. The cell pellet was resuspended in 2 ml of DMEM/SF.

A Percoll gradient was made fresh each time using isotonic Percoll by adding one part 10 x PBS to nine parts Percoll. Percoll solutions of three different Percoll concentrations (52%, 64% and 72% [v/v] Percoll in PBS) were made and 2 ml of each concentration was added to a 15 ml centrifuge tube, starting with 72% on the bottom, then 64% and finally 52%. The 2 ml cell solution was layered onto the Percoll gradient and centrifuged for 30 min at 1060 x g at RT with the brakes off. The mature neutrophils were collected from between the 64% and the 72% layer using a Pasteur pipette and were washed twice in DMEM. Cells were enumerated using a haemocytometer and trypan blue exclusion and the purity was checked with differential staining (section 2.3.6).

2.9.10 Cytotoxicity assay

2.9.10.1 NK cytotoxicity assays using NK cells extracted from infected ears

Yac-1 target cells were centrifuged and resuspended at 10^6 cells/ml. Cells were labelled with Na_2^{51}CrO_4 (2 MBq in 1 x 10^6 cells) for 1 h at 37 °C and agitated every 15 min. After labelling, cells were washed three times in RPMI/10 and resuspended at 1 x 10^5 cells/ml. Labelled target cells were then aliquoted into the wells of v-bottomed 96-well microtitre plates. The proportion of NK cells was assessed by flow cytometry so that the ratio of NK cells to labelled targets was standardised in each sample. In a separate plate, 100 µl doubling dilutions of effector cells were made, starting at a concentration of 10^6 cells per well [to give a final effector:target (E:T) ratio of 100:1]. Samples were diluted in triplicate and then transferred into the corresponding wells of the plate containing the target cells (final volume of 200 µl). Minimum lysis was
determined by incubating labelled targets in medium alone. For maximal lysis, an aliquot of each target population was incubated with RPMI containing 1% Triton X-100. The plates were centrifuged at 1390 r.p.m. for 1 min and incubated at 37 ºC. After 4 h, plates were re-centrifuged and 50 µl supernatant from each well was transferred to the corresponding region on a Lumaplate-96 (Packard Instrument Company, Inc.) and counted. The percentage of specific $^{51}$Cr release was calculated as specific lysis = [(experimental release - spontaneous release)/(total detergent release – spontaneous release)] x100. The spontaneous-release values were always <5% of total lysis.

Statistical analysis. Student’s t-test (two-tailed) was used to test the significance of the results (P<0.05).

2.9.10.2  
**CTL cytotoxicity assay using CTL cells extracted from infected ears**

EL-4 target cells were mock-infected or were infected with VACV WR at 10 p.f.u./cell for 1 h at 37 ºC in 250 µl RPMI in the presence of Na$_2^{51}$CrO$_4$. These cells were washed twice and counted. Serial dilutions of effector cells were incubated in duplicate cultures with either mock-infected or VACV-infected target cells in 96-well v-bottomed plates and incubated as described above for NK cytotoxicity assays.

2.9.10.3  
**NK cytotoxicity assay using NK92MI cell line**

Human NK92MI cells were mock-infected or infected at 1 p.f.u./cell for 16 h before labelling with $^{51}$Cr (section 2.9.10.1) and incubation with K562 targets cells mock-infected or infected at 1 p.f.u./ml for 16 h as described above. vTAPA40 induction was performed in DMEM/10 with 2 mM IPTG and 190 mM NaCl.

2.10  
**Production and purification of anti-A40 serum**

2.10.1  
**Production of rabbit anti-A40 polyclonal serum**

Affinity-purified recombinant A40 protein was sent to Harlan Sera-Lab (Leicestershire, England) to generate a polyclonal rabbit serum. Pre-bleed serum samples were taken from two rabbits prior to immunisation and their serum was tested
for reactivity against VACV-infected cell lysates. Purified protein (100–200 µg with an estimated purity of 90%) was injected into New Zealand White rabbits with Freund’s complete adjuvant full adjuvant to produce anti-A40 serum. Boosters of protein with Freund’s incomplete adjuvant were injected at 14, 28, 42, 56 and 70 days after the primary immunisation. Test-bleeds were taken in 10 ml aliquots at 35, 49 and 63 days after the primary immunisation, and the terminal bleeds were taken 77 days after the initial immunisation.

2.10.2 Purification of antiserum by pre-absorption on infected cells

To remove some of the non-specific binding of the antibody, the α-A40 (171) polyclonal antiserum was absorbed on RK13 cells that had been infected for 12 h with 5 p.f.u/cell of vΔA40. Five T175 cm² flasks of infected cells were scraped into the medium and collected by centrifugation at 2,000 rpm for 5 min at 4 °C. The cells were washed three times in ice-cold PBS and centrifuged between each wash. The washed cells were resuspended in 1ml PBS and were fixed by the addition of 1 ml 8% PFA and incubated on ice for 10 min. The fixed cells were recovered by centrifugation at 2000 rpm for 5 min at 4 °C, the supernatant was removed and the cell pellet was resuspended in 2 ml 8% PFA and incubated on ice for 50 min. The fixed cells were washed twice in 100 mM glycine in PBS followed by centrifugation at 2000 rpm for 5 min at 4 °C. The washed cells were permeabilised in 5ml 0.1% Triton X-100 in PBS for 5 min at RT. The permeabilised cells were resuspended in PBS and collected by centrifugation at 2000 rpm for 5 min at 4 °C. These permeabilised cells were then blocked with 10% FBS in PBS for 1 h at RT. The FBS was removed from the cells by centrifugation for 5 min at 2000 rpm at 4 °C. The pelleted cell material was resuspended in 2 ml α-A40 antiserum and incubated at 4 ºC for 12-14 h with rotation. Cells were removed by centrifugation at 15,000 rpm for 15 min at 4 ºC (Heraeus Biofuge 15R). The supernatant was transferred to a fresh tube, sodium azide was added to a final concentration of 0.02% (w/v) to inhibit microbial growth and the pre-absorbed antiserum was aliquotted and stored at -20 ºC.
2.10.3 Purification of IgG from antiserum

The IgG fraction of the antiserum was purified in an attempt to improve specificity and sensitivity of the antibody. The pre-absorbed α-A40 (171) antibody (section 2.10.2) was purified on a 1 ml Protein A Sepharose HiTrap affinity column (GE Healthcare) that was connected to an AKTApurifier 900 (GE Healthcare) for automated use. The column was equilibrated with 10 column volumes (CV) of 20 mM sodium phosphate buffer pH 7.0. The pre-absorbed α-A40 antibody was diluted 1:1 in 20 mM sodium phosphate buffer and gradually introduced onto the column using a syringe fitted to the luer adaptor. The column was washed with 10 CV of 20 mM sodium phosphate pH 7.0 to remove unbound material and the bound IgG was eluted with 100 mM citric acid pH 3.0 in 1ml fractions into tubes containing 100 μl 1.5M Tris-HCl pH 8.8 to neutralise the acidity of the elution buffer and to preserve the activity of the acid-labile IgG. The IgG elution profile was monitored electronically by measuring absorbance at 280 nm and the fractions were assessed for IgG content by SDS-PAGE and coomassie staining. The IgG containing fractions were pooled, concentrated by using a Centriprep-10 protein concentrator (Ambicon) and dialysed against Ab buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% sodium azide) using ‘Slide-A-Lyser’ dialysis cassettes (Pierce) according to the manufacturer’s instructions. This preparation of antibody is referred to as α-A40 IgG.
Chapter 3. Characterisation of the A40 protein from the VACV strain WR

3.1 Introduction

Two laboratories have characterised the A40 protein previously (Duncan, 1992; Wilcock et al., 1999; Tscharke et al., 2002; Palacios et al., 2005) but these two groups have generated conflicting data. This project began by re-assessing the basic properties of the A40 protein from VACV WR and then sought to understand the function of A40 by investigating its contribution \textit{in vivo} and by seeking to identify its binding partners.

3.2 Previous characterisation

3.2.1 VACV WR A40 characterisation by the Smith lab

The VACV strain WR A40R open reading frame (ORF) (YP_233047.1) is 480 bp long and is located in the right-hand region of the VACV genome (Smith et al., 1991). Computational analysis predicted that A40R encodes an 18,152 Dalton protein of 159 aa with a pI of 8.4. The protein was predicted to have a type II membrane topology with a hydrophobic N terminus, which may act as a TM signal and anchor sequence. The TM domain is followed by a short alpha-helical region and finally a C-type lectin-like domain (Duncan, 1992; Duncan & Smith, 1992) that would be exposed on the outside of the cell or the lumen of an intracellular vesicle. Three potential sites for N-linked glycosylation and three for O-linked glycosylation were identified.

An experimental characterisation of the A40 protein was undertaken by Wilcock \textit{et al.} (1999). A rabbit polyclonal Ab (anti-A40R1) was raised against A40 aa 77-159 (corresponding to most of the C-type lectin-like domain) that had been expressed in \textit{E. coli}. This Ab was used to identify the \textit{A40R} gene products in infected cells (by immunoblotting) as four proteins (18, 28, 35 and 38 kDa) that were present in lysates from VACV-infected cells but were absent from cells infected by a deletion mutant lacking the \textit{A40R} gene (Wilcock \textit{et al.}, 1999). In the presence of tunicamycin, an inhibitor of N glycosylation, only the 18-kDa protein was found indicating that A40 contains N-linked carbohydrate. Furthermore, in cell lysates prepared without β-
mercaptoethanol, one additional band at 70–76 kDa was identified and it was suggested that this might represent homodimers of the glycosylated A40.

Analysis of transcriptional regulatory signals indicated that A40 is expressed early in infection, with the presence of the sequence (A$$^3$$TGA$$^2$$T) at the 5’ end of the ORF, which closely resembles the early promoter consensus (A$$^3$$TGA$$^2$$S) but no late transcriptional initiation consensus at its 5’ end. Northern blotting and S1 nuclease protection analysis of RNA extracted from VACV-infected cells revealed an early transcript of ~ 600 nucleotides and an early initiation site 12 bases upstream of the ORF but no late initiation site was identified (Wilcock et al., 1999). Analysis of proteins made at different times during infection and in the presence of the inhibitor of DNA replication, cytosine arabinoside (AraC), when only early genes are able to be transcribed, indicated that the A40 proteins were made early during infection, consistent with the promoter region of the A40R gene. The isolation of the virus deletion mutant (vΔA40) showed that the A40R gene was non-essential for virus replication, which is consistent with the ORF not being conserved in all OPVs and its loss did not affect either virus plaque size or replication kinetics (Wilcock et al., 1999). The A40 protein, was not incorporated into virions, unlike the A34 C-type lectin-like protein that is in the EEV outer membrane (Duncan & Smith, 1992).

Wilcock et al. (1999) also reported that the A40 protein partitioned to the membrane fraction when infected cells were treated with Triton X-114. In vitro transcription analysis of the A40R gene and translation of the mRNA in vitro in the presence or absence of microsomes, confirmed that the A40 protein was glycosylated and that it was membrane associated with its lectin-like domain present within the lumen of the vesicle (type II membrane topology).

Bioinformatic analysis showed that the highest FASTA score (198) with a non-poxxvirus protein was against the human natural killer cell G2-A protein (accession no. P26715). The predicted CRD domain of A40 contains 5/14 invariant and 17/32 conserved residues of the CRD consensus sequence (Duncan & Smith, 1992; Wilcock et al., 1999). However, it lacks one of the cysteine residues predicted to form intradomain disulphide bonds and residues equivalent to loop 2 and β sheet 5. Some C-type lectins lacking several conserved residues and containing a deletion in the loop regions still may bind carbohydrate.
An investigation into the virulence of the virus deletion mutant compared to WT and Rev controls showed that the deletion mutant remained virulent in an intranasal infection model (Wilcock et al., 1999), whilst a subsequent study showed that in an intradermal model the deletion mutant induced smaller lesions that were cleared faster compared to the control viruses (Tscharke et al., 2002).

3.2.2 Opposing data by Palacios et al.

In contrast to the conclusions of Wilcox et al., (1999), Palacios et al., (2005) reported that A40 was modified by sumoylation rather than glycosylation and was an intracellular protein that was not associated with membranes (Palacios et al., 2005). To reach these conclusions, they raised a peptide antibody against 71 -86 aa of the A40 protein which they tested against cells infected with VACV strain WR. They did not use a deletion mutant to control for the specificity of the serum and indeed suggested that the A40R gene was essential because they had been unable to isolate a deletion mutant. This suggestion ignored the facts i) a well characterised deletion mutant had already been reported and ii) some other orthopoxviruses, such as VARV have the A40R ORF disrupted into small fragments by mutation (Aguado et al., 1992).

A number of proteins, mainly located in the nucleus, are sumoylated (Yeh et al., 2000; Muller et al., 2001; Seeler & Dejean, 2003). Sumoylation is a pathway similar to ubiquitination that modifies proteins reversibly and post-translationally and can alter a protein’s intracellular location, activity, stability and interactions. Palacios et al., (2005) proposed that sumoylation of A40 prevented it forming polymers and a small amount of unsumoylated A40 could aid bringing ER membranes together before they fuse to generate an ER-derived envelope that is proposed to surround the virus factory (Sodeik & Krijnse-Locker, 2002).

The differences between the conclusions of Palacios et al., (2005) and those reported by Wilcock et al., (1999) were proposed by Palacios et al., (2005) to be due to a lack of specificity of the Ab used by Wilcock et al. However, controls had been provided and the Ab reacted with proteins in cells infected with WT and Rev viruses but not a virus mutant lacking the A40R gene (Wilcock et al., 1999). In comparison, Palacios et al., 2005 did not show controls for the specificity of the peptide Ab. Palacios et al., also claimed that the A40 protein was not glycosylated, but showed no
data supporting this claim. If the peptide Ab used by Palacios et al., is binding A40, the presence of A40 in the ER may reflect its normal transport through the host cell’s secretory pathway.

![Fig. 3.1. VACV strain WR A40 protein.](image)

(A) Amino acid sequence of A40 protein. Conserved CRD amino acids are underlined. Predicted N-glycosylation sites are in blue and underlined. (B) Kyte & Doolittle hydropathy plot of A40. (C) Schematic of A40’s predicted structure.
Proteins, identified using BLASTP and the PBR centre, were aligned using GeneDoc. When several strains have identical sequences, only one name is listed in the alignment and the remaining proteins in this group are listed below. VACV-WR (VACV WR protein A40, YP-233047), VACV-Lister (VACV strain Lister protein 211, VBR_C_gene_id 46242), CXPV-GER (cowpox virus strain Germany 91-3 protein C-type lectin-like type-II membrane protein, ABD97512), VACV_CXPV_BR (cowpox virus strain Brighton Red protein 177, NP_851590), CXPV_GRI-90 (cowpox virus strain GRI-90 protein A42, CAD90709), HSPV_MNR-76 (horsepox virus strain MNR-76 protein 162, ABH08271), VACV_MVA (VACV strain modified vaccinia virus Ankara protein 152, AAB96533), VACV_Copenhagen (VACV strain Copenhagen protein A40, AAA48171), VACV_Acam2000 (VACV strain Acambis 2000 protein 176, AAR18008), VACV_3737 (VACV strain 3737 protein 159, ABD57694), VARV_Garcia (Variola minor virus strain Garcia-1966 protein A48), VARV_India (Variola virus strain India 1967 protein A42). 1) Proteins with identical sequences to VACV-Lister. VACV-CVA (VACV strain CVA protein 173, CAM58337), VACV-TianTan (VACV Tian Tan protein 51R, AA089444), RXPV_Utrecht (rabbitpox virus strain Utrecht protein 148, AAS49861), VACV_LC16m8 (VACV strain LC16m8 protein m8221, AAW23600), VACV_LC16m0 (VACV strain LC16m0 protein m0221, AAW23882), VACV_Lister (VACV Strain Lister 107 protein 157, ABD52656). 2) Proteins with identical sequences to VACV-MVA. VACV_MVA1721 (VACV strain MVA 1721 protein 154, VBRC_genome), (VACV strain Acambis 3000 Modified Virus Ankaara protein 154, AAT10550). 3) Proteins with identical sequences to VACV-3737. VACV_Acambis 3 (VACV strain Acambis 3 protein 176, AAQ93262), VACV_Duke (VACV strain Duke protein 173, ABD98635). Shading shows conserved amino acid identities. Black = 100 %, dark grey = 80 %, light grey = 60 % of aligned sequences have the same amino acid at that position. Consensus sequence shown below aligned sequences.
Table 3.1 Amino acid identity of proteins compared to VACV WR A40

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<tr>
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<td>Mus musculus</td>
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<td>16</td>
<td>NP_081248</td>
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<td>Homo sapiens</td>
<td>8</td>
<td>17</td>
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<tr>
<td>Viral proteins:</td>
<td></td>
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<tr>
<td>RCMV RCTL</td>
<td>Rat cytomegalovirus</td>
<td>25</td>
<td>28</td>
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</tr>
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<td>Vaccinia virus</td>
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<td>13</td>
<td>YP_233039</td>
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<td>FWPV strain HP1-438 Munich</td>
<td>9</td>
<td>12</td>
<td>CAE52549</td>
</tr>
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</table>
Fig. 3.3 Amino acid alignment of CRD domain of A40 with distal poxvirus proteins and non-viral proteins

Residues 56-159 of VACV WR A40 were aligned with related proteins from other poxviruses and non-viral proteins (identified by BLASTP). The alignment was generated using GeneDoc. VACVWRA40 (VACV strain WR protein A40), VACVWRA34 (VACV strain WR protein A34, YP_233039), Clr-b (Rat Clr-b, Q0H8B9), RCTL (RCMV RCTL protein, AAG40235), CD94 (human protein CD94, AAH28009), NKG2A (human protein NKG2A, NP_998823), KLRG1 (human protein KLRG1, AAH12621), LSIGN (human protein LSIGN (CD209L), NP_999841), DCSIGN (human protein DCSIGN (CD209), AAH32591), CD23 (human protein CD23, AAH62591), DpV83 (Mule deer poxvirus strain W-848-83 protein 144, YP_227519), fp9008 (FPV strain HP1-438 protein 008, CAE52554), fp9003 (FPV strain HP1-438 protein 003, CAE52549). Shading shows conserved amino acid identities. Black = 100%, grey = 60% conserved. The consensus sequence is shown below aligned sequences.
3.3 Bioinformatic analysis of VACV WR A40

3.3.1 Amino acid sequence alignment with VACV WR A40 compared to:

3.3.1.1 Other orthopoxvirus proteins

Proteins from cells and poxviruses that are related to A40 (YP_233047.1) were found by a comparison of the A40 aa sequence (Fig. 3.1 A) with the European Molecular Biology Laboratory (EMBL) database using BLASTP 2.2.19+ (Altschul et al., 1997) and the Poxvirus bioinformatics resource (PBR) centre (www.poxvirus.org) (Fig. 3.2 and Fig. 3.3).

The full-length A40 protein is found in 2 out of the 7 species of orthopoxviruses (VACV and CPXV) and is conserved in many VACV strains including CVA, Tian Tan (TA51R), Lister (Lister 221) and its derivative LC16m8 (211R) (Morikawa & Ueda, 1993), and RPXV Utrecht (RPXV148). In other VACV strains there are three different types of modification to the sequence of the A40 protein; i) single aa substitutions, ii) internal changes in length and iii) C-terminal frameshift mutations near the C-terminus.

Horsepox virus (HSPV) strain MNR-76 protein 162 (HSPV162) (Tulman et al., 2006) and CXPV strain GRI-90 protein A42 both share 94.5% identity to WR A40 but have an insertion of 6 aa at position 145, and 4 independent substitutions each. However, they each contain the same C-terminal residues as A40 WR. CXPV strain Brighton Red protein 177 (CPXV177) has 8 aa changes compared with A40 WR including a single insertion at residue 41, but overall keeps the same C-terminal sequence as WR.

In VACV strain Copenhagen, a frameshift mutation replaced the C-terminal 22 residues present in other VACV strains with 31 unrelated residues. VACV strains Acambis 2000 (protein 176), 3737 (protein 159), Duke (protein 173) and Acambis 3 (protein 176) have the Copenhagen C terminal modification except for one aa substitution compared to Copenhagen, as well as 4 independent aa substitutions relative to WR. VACV MVA protein 152 and VACV strain MVA I721 protein 154 are identical to A40 WR from aa residue 1 to 154, but the last five residues are replaced with 14 unrelated residues (Fig. 3.2).

The VACV International Health Department (IHD)-J strain does not express A40 (Duncan & Smith, 1992; Wilcock et al., 1999) and in all sequenced VARV strains,
such as Harvey 1947, India 1967 and Bangladesh 1975 the gene is disrupted. This is in contrast to VACV protein A34, another a C-type lectin-like protein, which is highly conserved between the viruses listed above, and is incorporated into the EEV (Duncan & Smith, 1992). This alignment suggests that the WR strain sequence is the predominant sequence and this was why the WR strain sequence was chosen to be studied.

The A34 protein from VACV WR has 19% aa identity to A40. A40 and A34 have the same type II membrane topology with a C-type lectin-like domain in the C-terminal region.

3.3.1.2 FWPV genes

When A40 was compared to proteins from other chordopoxviruses such as avipoxviruses, several proteins are found in both fowlpox virus (FWPV) strains Iowa and Munich. For instance, FPV253 and FPV008, from FWPV Munich have 10% identity to A40 over the whole protein, rising to 13% if only the CRD is compared (Table 3.1 and Fig. 3.3).

3.3.1.3 RMCV RCTL

The RCMV RCTL protein has previously been aligned with VACV A40 Cop (Voigt et al., 2001). When aligned to VACV A40 WR, there is 26% aa identity and 38% aa similarity along the whole of the proteins. When only the CRD domain is considered, RCMV RCTL and VACV A40 WR share 28% aa identity (Table 3.1 and Fig. 3.3).

3.3.1.4 Non-viral proteins

While OPV A40 proteins are very closely related, non-viral protein matches are very distant (~ 20% aa identity) and are various C-type lectin-like proteins. The closest match along the whole protein is with the rat Clr-b protein then human CD94 protein (Fig. 3.3 and Table 3.1), and the highest identity within the CRD domain (aa 40 – 159) are with the rat Clr-b, the NKG2 proteins, CD94 and murine and human DC SIGN. Clr-
b is a ligand of the NKR-P1B inhibitory receptor. CD94 is expressed as disulphide-linked heterodimer with members of NKG2 family, including NKG2A, which transduces inhibitory signals and NKG2C which transduces activatory signals. The extracellular portion of CD94 has been shown by X-ray crystallography to have a unique variation on the classical C-type lectin fold. The CRD is altered significantly and the calcium binding site appears non-functional (Borrego et al., 2002).

3.3.2 A40 is a glycoprotein with type II membrane topology

In agreement with previous bioinformatic analysis of A40R, the WR 165 ORF of 480 bp is predicted to encode a type II membrane protein (https://www.expasy.ch/tools). A hydrophobicity plot predicts that A40 is likely to be a membrane-integrated protein and has no putative cleavage sites (http://www.cbs.dtu.dk/services/SignalP/). A short seven aa tail at the N terminus resides in the cytoplasm, followed by ~ twenty hydrophobic residues comprising a transmembrane spanning region (8 – 29, coloured red, Fig. 3.1C). Adjacent to this region ~ twenty residues (30-55, green, Fig. 3.1C) are predicted to form an alpha helical region designated the ‘stalk’. Residues 56 – 159 are predicted to encode a carbohydrate recognition domain (CRD) or C-type lectin-like domain (CLECT). Within this CRD there are three predicted N-linked glycosylation sites (aa 121-123, 132-134 & 136-138, http://www.cbs.dtu.dk/services/NetNGlyc/).

Several motif prediction programmes (http://ca.expasy.org/tools/) did not predict any likely conserved motifs such as phosphorylation sites within the small cytoplasmic tail that would point to a way in which A40 could function.

The analysis of A40 protein sequence by several secondary structure prediction programmes (Protein Homology/analogY Recognition Engine (PHYRE), a profile – profile matching algorithm, Fugue, an aa-dependent algorithm and GenTHREADER, a protein fold recognition program) suggested A40 may have a similar tertiary structure, within the CRD, to a snake coagglutinin beta chain from the Habu snake (Trimeresurus flavoviridis), part of the heterodimeric coagulation factors IX/X-binding protein (IX/X-BP) (Mizuno et al., 1997) and CD69 (Natarajan et al., 2000). Although the aa sequence showed a low percentage identity (14%) it is possible that there is reasonable structural
similarity. The newly resolved X-ray crystal structures of such CRD domains could provide a basis to model A40 upon.

3.4 Expression of A40 in VACV-infected cells

Although the data provided by Wilcock et al., 1999 are more convincing than those from Palacios et al., 2005, at the outset of this thesis it was important to confirm the properties and location of the protein before designing and undertaking an experimental strategy to seek A40 binding partners and understand the mechanism of action. To do this, I started by preparing a new and improved anti-A40 polyclonal antiserum, as the one described by Wilcock et al., (1999) did not work well in immunofluorescence. The new Ab was raised against aa 30 – 159 of WR A40 expressed in E. coli (section 5.3. & 2.10). This was used in immunoblot analysis to determine when the protein is expressed, its size, localisation and expression in a range of OPVs.

3.4.1 VACV WR A40 is expressed early in infected cells

To investigate the time of expression of A40, BS-C-1 cells were infected with a WT VACV strain WR called vWt (Wilcock et al., 1999) in the presence or absence of cytosine arabinoside (AraC), an inhibitor of DNA replication and therefore intermediate and late protein expression. Infected cells were harvested at various times p.i. and cell lysates were immunoblotted with anti-A40 Ab. The cell lysates were also blotted for a late VACV protein, D8, to confirm that these infections and time-points are characteristic of published analysis (Fig. 3.4 A). A40 was detected 2 h after infection, increased up to 8 h, and was still present at 24 h. Multiple bands ranging from ~25 – 50 kDa were detected at all time-points from virus infected cells and absent from mock infected cells. In the presence of AraC, A40 was still produced, supporting the previous conclusion that A40 is an early protein. In contrast, D8 was detected only after 6 h, increasing at 8 h and is still present at 24 h. In the presence of AraC, no D8 protein is detected, showing that the inhibition of late protein expression was complete. This agrees with expression data from Wilcock et al., (1999) and also kinetic analysis for the temporal transcription profile of VACV WR published by Assarsson et al., (2008).
Fig. 3.4 Characterisation of A40 in VACV-infected cells

(A) Time course of A40 expression. BS-C-1 cells were infected with WT at 5 p.f.u./cell in the presence or absence of 1 μg/ml AraC. Cells were harvested at 0, 2, 4, 6, 8, 12 and 24 h p.i and expression of A40 was analysed in lysates by SDS-PAGE (15% gel) followed by immunoblotting with the α-A40 Ab, 171 or D8.

(B) Post-translational modification of A40 (i) BS-C-1 cells were infected with vA40HA or vB14HA (Chen et al., 2006) at 1 p.f.u./cell for 24 h in the presence/absence of tunicamycin (1 μg/ml). Cells were lysed and lysates were analysed by SDS-PAGE (15% gel) followed by immunoblotting with the HA mAb.

(ii) BSC-1 cells were infected with WT at 5 p.f.u./ml for 24 h. Cells were lysed and lysates were treated with/out the endoglycosidase PNGase F for 16 h at 37 °C then analysed by SDS-PAGE (15% gel) followed by immunoblotting with the Ab 171.

(C) Oligomerisation of A40. BS-C-1 cells were infected with WT at 5 p.f.u./cell for 24 h. Cells were lysed and lysates were resuspended in 2x Laemmli buffer with or without β-mercaptoethanol then analysed SDS-PAGE (15% gel) followed by immunoblotting with the Ab 171. Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) (A40 171) or HRP-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) (D8 or HA) both diluted 1:2000 and ECL reagents (Amersham). Positions of molecular mass markers are shown on the left with sizes in kDa.
Fig. 3.5 Localisation of A40HA within VACV-infected cells

(A) TK-143 cells were infected at 1 p.f.u./cell for 18 h. Proteins were extracted using the M-PEK kit (Pierce) and were analysed by SDS-PAGE and immunoblotted for (i) HA & (ii) B5 (mAb 19C2) and K7. M and S represent membrane; and soluble fractions respectively. Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) (A40), HRP-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) (B5) or HRP-conjugated goat anti-rat IgG antibody (Sigma-Aldrich) diluted 1:2000 and ECL reagents (Amersham). Positions of molecular mass markers are shown on the left with sizes in kDa. (B) BS-C-1 cells were transfected with either pSEL (mock), pSEL(N)BAPA40 or pSEL(C)A40BAP (Section 5.4.4) and incubated overnight. Cells were then infected with vDelA40 at 1 p.f.u./cell for 6 h. For surface staining, cells were incubated with an anti-BAP Ab at a 1:400 dilution for 1 h on ice. The cell were then fixed and incubated with an anti-mouse Alexa488-conjugated secondary Ab. For internal staining, the cells were fixed in PFA, permeabilised in saponin (0.2 % w/v) for 1 h and then incubated with an anti-BAP Ab followed by an anti-mouse Alexa488-conjugated secondary Ab.
3.4.2 VACV WR A40 is post-translationally modified by glycosylation

BS-C-1 cells were infected with vA40HA (with or without tunicamycin and cell extracts were analysed by immunoblotting (Fig. 3.4Bi). In the absence of tunicamycin, several proteins were detected ranging from ~24 to ~ 40 kDa corresponding with data from (Wilcock et al., 1999). Furthermore, in the presence of tunicamycin, these proteins were replaced by a single protein of ~18 kDa confirming the higher molecular weight forms are glycosylated. VACV B14 was used as a control. B14 is a 15 kDa protein that is not N-glycosylated and therefore, no shift in mobility is seen.

As an alternative method to demonstrate that A40 is glycosylated, VACV WR cell lysates were treated with endoglycosidases. Peptide: N-Glycosidase F ((PNGase F), cleaves between the innermost N-Acetylglucosamine (GlcNAc) and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins). Endoglycosidase H ((Endo H) cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins). These lysates were then analysed by immunoblotting (Fig. 3.4Bii). In the absence of PNGase F treatment, several protein bands were detected ranging from ~ 25 to ~38 kDa, again corresponding to data from Wilcock et al. With PNGase F treatment, these bands are replaced by a single band ~ 18 kDa.

These BS-C-1 cell lysates were also used to determine if A40 forms oligomers. The lysates were dissolved in Laemmli buffer in the presence or absence of β-ME and then immunoblotted with the α-A40 Ab (Fig. 3.4C). In the absence of β-ME several higher bands are seen ranging from ~ 50 – 75 kDa which are not present when the samples are treated with β-ME, suggesting that A40 forms oligomers within an infected cell.

3.4.3 VACV WR A40 localises to the infected cell surface membrane

The subcellular localisation of A40 was investigated by immunoblotting and immunofluorescence. Cells were either mock-infected or infected with vA40HA and proteins were extracted using the ProteoExtract Native Membrane Protein Extraction Kit (Section 2.8.19) and analysed by immunoblotting (Fig. 3.5A). A40HA was detected only in the membrane fraction but not in the soluble fraction. As a control the samples
were also blotted for B5, a known VACV membrane protein and K7 as a cytosolic protein (Fig. 3.5Aii). As expected, B5 was only found in the membrane fraction, whereas K7 was detected only within the cytosol.

To assess the localisation of A40 within an infected cell by an independent method, A40 was tagged with either a C- or N-terminal BAP tag (Section 5.2 & 5.4.4). These genes were inserted downstream of the early/late promoter in vector pSEL (Ahmad, 2003). HeLa cells were transfected with either plasmid, incubated overnight and then infected with vΔA40 (Wilcock et al., 1999) for 8 h (section 2.8.20). Cells were either stained live on ice (left panel, Fig. 3.5B) or fixed, permeabilised and then stained (right panel, Fig. 3.5B).

In the live stained cells, BAP-tagged A40 was detected with the C-terminally tagged A40 but not the N-terminally tagged protein and no protein was detected in the mock transfected/infected cells (left panel, Fig. 3.5B). In the permeabilised cells (right panel, Fig. 3.5B), BAP-tagged A40 was detected in both the cells transfected with N- and C-terminally tagged A40 but not in the mock-transfected cells. In this system, A40 is localised to the surface membrane of the infected cells and because the C-terminally tagged A40 was detected without membrane permeabilisation (left panel), these data show that A40 is a type II membrane protein with the C-terminal domain exposed outside the cell.

3.4.4 A40 is expressed by several VACV strains

Whilst the bioinformatic analysis (Fig. 3.1 - 3 and section 3.3.1) indicated that A40 orthologues were found in 2 of the 7 different sequenced OPV species, a comparison by immunoblotting for expression of A40 confirmed that A40 is expressed by different VACV strains, some of which have been sequenced, and whose similarity to WR A40 was discussed above.

RK13 cells were infected with 17 different VACV strains and CPXV strain BR and the A40 deletion mutant vΔA40. The cell lysates were analysed by immunoblotting for A40, F13 (a late viral protein (35 kDa) to check for viral infection) and tubulin (to check for consistent loading).
As previously described, WR A40 is present as multiple bands ~ 25-38 kDa and these are glycosylated. In Fig. 3.6, the A40 Ab detected similar multiple bands from lysates of cells infected with VACV strains Lister, Tian Tan and RPXV (as expected from their sequences). Two of the sequenced CPXV strains (CPXV BR and Germany 91-3 (Ger)), have a single aa insertion at position 39 (Fig. 3.2) which may alter the protein, but for CPXV BR, a band is seen ~ 37 kDa whilst for elephantpox-2 (EL-2), the A40 Ab did not detect anything above the non-specific bands present in all the lanes. VACV Copenhagen A40 has a predicted molecular mass of 19.3 kDa but is detected faintly with the A40 Ab with bands present ~ 28 kDa and 35 kDa. As mentioned in section 3.3.1.1, the sequence of the VACV Copenhagen A40 protein varies from VACV WR A40 in the last 22 residues. The replacement of these residues with 31 unrelated residues accounts for the difference in size and may explain why the α-A40 Ab is not as efficient at detecting VACV Copenhagen compared to VACV WR A40. No A40 protein was detected in lysates of cells infected with VACV MVA suggesting either the Ab cannot detect this variant of A40 or that the substitution of the terminal five aa with 14 unrelated aa relative to WR makes the MVA protein unstable.

The A40 protein from VACV strain buffalopox was detected strongly with the same size bands as WR, but with a stronger signal at each size. For VACV strains Evans, Patwadangar and USSR the multiple band pattern of WR A40 is seen. In contrast, VACV Dairen, King’s Institute, Tashkent and Wyeth do not express detectable A40 detected by this anti-A40 Ab. These VACV strains have not been sequenced, and so it is probable that the A40 protein in Evans, Patwadangar and USSR are very similar to VACV WR by sequence, like Lister and Tian Tan. VACV strains Dairen, King’s Institute and Tashkent may have insertion and/or mutations as seen with the different strains of CPXV, VACV Copenhagen and MVA.

3.5 Summary

3.5.1 Reaffirmation of A40 characteristics

The expression and localisation of A40 has been confirmed independently as a glycoprotein that is expressed early in infection and is present on the cell surface with type II membrane topology.
As predicted by their known sequences, VACV strains Lister, Tian Tan and rabbitpox express A40 and A40 is also expressed by VACV strains buffalopox, Evans, Patwadangar and USSR.

3.5.2 Possible protein-protein interaction searches

The localisation, topology and predicted domain structure of A40, will influence its function and are important when considering strategies to identify its interacting partners. Fig. 3.7 summarises four possible ways in which A40 could associate with ligands. The first is that A40 could interact with a molecule(s) expressed on the surface membrane of immune cells (Fig. 3.7A). This association would most likely be mediated by the predicted C-terminal CRD domain of A40.

A second possibility is that A40 could interact with a protein expressed at the surface of the VACV-infected cell (Fig. 3.7B). Through this interaction A40 could disrupt or alter the interacting partner’s function. This interaction could be mediated by the C-terminal CRD domain, the cytoplasmic tail or the interceding stalk region and transmembrane domain, or a combination of all of them.

A third possibility is that A40 could interact in the extracellular matrix with a soluble protein that has been secreted by the host cells (Fig. 3.7C). Lastly, A40 could interact with an intracellular protein expressed with the infected cell via the A40 short N-terminal region (Fig. 3.7D). Unlike proteins such as NKG2, A40 has no predicted protein-protein interaction domains such as ITIM or ITAM, and the cytoplasmic sequence is also very short (7 amino residues) suggesting that this possibility is less likely than the others.

In consideration of these possible associations, a cloning and experimental strategy was devised to explore each of these options and clarify the function of A40 and its proposed role in modulating the host immune system during infection.
Fig. 3.6 Expression of the A40 protein by VACV strains
RK13 cells were infected at 5 p.f.u./cell for 24 h. The cells were washed in PBS then lysed in LB1 on ice (section 2.8.7) and analysed by SDS-PAGE (15% gel) and immunoblotting with the anti-α-tubulin mouse Ab (1:5000), the anti-F13 mouse Ab (1:1000) or the anti-A40 rabbit (1:1000). Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich) (A40) or HRP-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) (tubulin and F13) both diluted 1:2000 and ECL reagents (Amersham). Positions of molecular mass markers are shown on the left with sizes in kDa.
1) **Cell – cell interaction:**

![Diagram of cell-cell interaction](image)

**Summary:**
- **VACV infected cell**: A40 extracellular domains interact with membrane protein(s) (ligand A) on another cell. This neighbouring cell might be part of the immune system.

2) **Protein – protein between A40 and another membrane protein on the same cell:**

![Diagram of protein-protein interaction](image)

**Summary:**
- **A40 could interact with other membrane proteins (ligand B)** on the same cell. This could occur via any of the A40 domains.

3) **Interaction with a soluble protein:**

![Diagram of protein-soluble interaction](image)

**Summary:**
- **A40 could interact with soluble protein(s) (ligand C)** within the extracellular matrix. This interaction could be mediated through the A40 extracellular domain.

4) **Interaction of A40 with an intracellular protein:**

![Diagram of protein-intracellular interaction](image)

**Summary:**
- **A40 could interact with intracellular protein(s) (ligand D)** expressed in the VACV-infected cell.

---

**Fig. 3.7 Summary of possible A40 interactions**

1) **Cell – cell interaction.** The A40 extracellular domains may interact with membrane protein(s) (ligand A) on another cell. This neighbouring cell might be part of the immune system.

2) **A40 could interact with other membrane proteins (ligand B) on the same cell.** This could occur via any of the A40 domains.

3) **A40 could interact with soluble protein(s) (ligand C) within the extracellular matrix.** This interaction could be mediated through the A40 extracellular domain.

4) **A40 could interact with intracellular protein(s) (ligand D) expressed in the VACV-infected cell.**
Chapter 4. A40 affects the outcome of infection in the murine intradermal infection model

4.1 Introduction

Although deletion of A40 has no effect on *in vitro* growth properties of the VACV (Wilcock *et al.*, 1999) there are many examples of VACV proteins (particularly immunomodulatory proteins) that do not alter virus replication in tissue culture, but have a significant effect on the outcome of *in vivo* infections. Examples include secreted proteins such as the VACV vIL-1βR (encoded by the WR gene *B15R*) (Alcamí & Smith, 1992; Spriggs *et al.*, 1992a), as well as intracellular proteins such as N1 (Bartlett *et al.*, 2002; Cooray *et al.*, 2007) all of which down-regulate the functioning of a component of the host immune system.

Two mouse models have been used to study the contribution of specific genes in VACV virulence in our laboratory; the intranasal (i.n.) model (Turner, 1967) and the intradermal (i.d.) model (Tscharke & Smith, 1999; Tscharke *et al.*, 2002). In the i.n. model, there is virus replication in the lungs, dissemination to other organs including the brain and induction of a systemic infection the outcome of which is dependent on the virus dose administered (Williamson *et al.*, 1990). In severe infections, death is attributed to pneumonia since a marked consolidation of the lungs can be observed (Turner, 1967; Taylor *et al.*, 1991; Law *et al.*, 2005). The severity of infection in this model is measured by monitoring weight loss and grading signs of illness (Alcamí & Smith, 1992). Mice are sacrificed if they reach humane endpoints defined by weight loss and signs of severe disease to comply with animal welfare legislation.

In contrast, the i.d. model mimics a dermal vaccination (Tscharke *et al.*, 2002) causing a highly localised infection with a local skin lesion in the infected ear and little if any virus spread to other sites (Tscharke & Smith, 1999). In the i.d. model, virulence is measured by the size of the lesion. Virus titres present at various times p.i. can be determined by tissue extraction and plaque assay, and the inflammatory infiltration can be determined by cell extraction and flow cytometry (Jacobs *et al.*, 2006). Within our lab, both models are used to assess the virulence of different VACV strains and VACV mutants engineered to lack specific genes. A specific deletion mutant may show a
phenotype in one model but not the other, both models, or neither model (Tscharke et al., 2002). Also gene deletion may either increase or decrease virulence.

A comparison of the recombinant VACV strain WR vΔA40, in which the A40R gene is disrupted and partially deleted, with the wild-type (WT) parent virus and a revertant (Rev), in which A40R is restored to its natural locus within the deletion mutant, showed that the removal of A40 from VACV does not produce a phenotype in the i.n. model (Wilcock et al., 1999) but does in the i.d. model (Tscharke et al., 2002). In the i.d. model, vΔA40R induced a 30% reduction in lesion size compared to WT and Rev viruses in both Balb/c and C57B1/6 mice (Tscharke et al., 2002). This would suggest that A40 interacts with one or more cellular ligands within the dermis and in doing so, modulates the way in which VACV interacts with its host. Within the i.d. model, it is possible to characterise the leukocytes infiltrating into the infected lesion and this was done with WT VACV WR previously (Jacobs et al., 2006). Here the consequence of deleting A40 on leukocyte infiltration was assessed at different times after infection.

4.2 Intradermal infection with vΔA40

4.2.1 Effect of A40 on the infiltration of leukocytes into VACV-infected lesions

To examine if loss of A40 affected virus virulence in the i.d. model, C57Bl/6 mice were infected i.d. (section 2.9.1) as described previously (Tscharke & Smith, 1999; Tscharke et al., 2002). The titre of each virus inoculum used for infection was checked by titration of diluted virus samples on BSC-1 cells and the infectious doses administered were titrated and found to be within 0.2 p.f.u./ml for all the different viruses. The migratory cells in the infected ears were collected by collagenase-based extraction (Jacobs et al., 2006) and section 2.9.2) and analysed by flow cytometry (section 2.9.4) at days 2, 4, 7, 10 and 17 p.i.

To begin with, the total number of cells recruited to the ear dermis of mice infected with the three different viruses was assessed by trypan blue staining and enumeration using a haemocytometer (Fig. 4.1). At day 2 p.i., the total number of cells per ear was higher in the ears of mice infected with the virus lacking A40 but this difference was not significant. The number of cells in vΔA40 infected ears was
significantly higher at days 4 and 7 p.i., but this significance was lost at day 10 p.i. compared to the cell numbers recruited by WT and Rev infections. At day 17 p.i., the number of cells within vΔA40 infected ears was significantly less than the cell numbers within WT and Rev infected ears.

Fig. 4.1 Leukocyte infiltration in the intradermal mouse model
Mice (n=4) were infected in both ears with 1 x 10^4 p.f.u. of vΔA40 and control viruses and at the indicated times p.i. cells that migrated into each ear of each infected mouse were pooled. The total number of viable cells was determined by trypan blue exclusion. Data are expressed as means +/- SD of cell counts. P-values were calculated using the Student T-test, ¥ = 0.064, *= 0.05, $ = 0.02, ** = 0.01, *** = 0.005.
The type of leukocytes infiltrating the site of infection was investigated further by flow cytometry. The number of CD3⁺ T lymphocytes was significantly greater at days 2 and 7 post-infection (p.i.), equivalent at day 10 p.i. and significantly less at day 17 p.i. with vΔA40 compared to WT and Rev (Fig. 4.2A.). The infiltration of F4/80⁺ macrophages after infection with vΔA40 was equivalent at day 2 p.i. but increased to be significantly greater than controls at day 4 p.i. (Fig. 4.2B). Thereafter, the numbers were equivalent for all three viruses.

The T lymphocyte population was examined further by looking at the separate CD4⁺ and CD8⁺ subpopulations and TCRγδ⁺ cells. The number of CD8⁺ T cells was equivalent between the mice infected with the different viruses at days 2, 4 and 10 p.i. However, infection by vΔA40 induced a greater infiltration at day 7 p.i., and a smaller infiltration at day 17 p.i. compared to controls (Fig. 4.2C). For CD4⁺ T cells (Fig. 4.2D), there is a significantly higher number at day 2 and day 7 p.i. with no difference between the three viruses at day 10 p.i and then significantly less cells at day 17 p.i. The TCRγδ T cells in vΔA40-infected mice were significantly higher in number than the controls at day 2 p.i. with a trend to be higher at day 7 p.i. but at this time-point this difference was not statistically significant. At day 10 p.i. there was a significantly higher number after infection with vΔA40 but this was not apparent at day 17 p.i. (Fig. 4.2E)

There was no significant difference in either NK1.1⁺ NK cells (Fig. 4.2F) or Ly6G positive granulocytes (Fig. 4.2G) after infection with the different viruses. Although there were no significant changes, there was a trend for more NK cells at day 7 p.i. in the absence of A40.

The expression of MHC class II on cells within the lesions was also analysed and a significantly higher number of cells expressing MHC class II was seen at days 2 and 7 p.i. and a significantly lower expression seen at day 17 p.i. (Fig. 4.2H) relative to WT and Rev cell numbers.
Fig. 4.2 Analysis of infiltrating cells in the intradermal infection model
Mice (n=5) were infected in both ears with 1 x 10^4 p.f.u. of vΔA40 and control viruses and at the indicated times p.i. cells that migrated into each ear of each infected mouse were pooled. The total number of viable cells was determined by trypan blue exclusion. Data are expressed as means +/- SD of cells counts. In this cell population the (A) CD3+ cells, (B) macrophages, (C) CD8+ T cells, (D) CD4+ T cells, (E) TCRγδ T cells, (F) NK cells, (G) granulocytes and (H) MHC class II+ cells were identified by appropriate surface markers as described in Section 2.9.4. The means +/-SD of data from 4 infected mice were analysed by cell staining and flow cytometry at the days indicated. P-values were calculated using the Student T-test, $p = 0.064$, $* = 0.05$, $\$ = 0.02$, ** = 0.01, *** = 0.005.
4.2.2 Natural killer activity of extracted Natural killer cells

Given that the number of NK cells was increased slightly from the deletion mutant infected dermal tissue, although this difference was not statistically significant, the NK cells from infected ears (days 4–7 p.i.) were assessed for their NK killing activity by chromium release assays using Yac-1 target cells (Section 2.9.10.1). The release of $^{51}$Cr was assessed and the percentage of cytotoxicity calculated (Fig. 4.3).

There was no significant difference in NK killing activity by NK cells from ears infected with the different viruses at any of time-points assessed.

**Fig. 4.3 Natural killer cell activity**
Chromium release assay of cells isolated from infected ears against NK-sensitive Yac-1 cells. Mice (n=3) were infected in both ears with $1 \times 10^3$ p.f.u. of vΔA40 and control viruses and at the indicated times p.i. cells that migrated into each ear of each infected mouse were pooled. The total number of viable cells was determined by trypan blue exclusion. The ratio of effector:target cells was calculated with the absolute number of NK cells as determined by FACS. Data shown are means ± SEM of two experiments. Test was measured in triplicate.
4.3 Cytotoxic T-lymphocyte activity of extracted T lymphocytes

The cytolytic T cell activity of extracted T lymphocytes was also assessed (Section 2.9.10.2). The targets in these $^{51}$Cr release assays were EL4 cells (specific for T cells from C57Bl/6 mice). If the effector cells were incubated with mock-infected EL4 cells (dashed lines), there was no significant difference between the viruses at day 6 or 7 p.i. (Fig. 4.4).

If the EL4 cells were infected prior to incubation with the effector cells (solid lines) all three viruses increased cytolytic of the extracted cells but no difference was seen between the three viruses (Fig. 4.4).

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**Fig. 4.4** Cytolytic T lymphocyte activity of extracted T lymphocytes from infected dermal samples
Chromium release assay of cells isolated from infected ears against cytotoxic T cell-sensitive EL4 cells. Mice ($n=3$) were infected in both ears with $1 \times 10^4$ p.f.u. of vΔA40 and control viruses and at the indicated times p.i. cells that migrated into each ear of each infected mouse were pooled. The total number of viable cells was determined by trypan blue exclusion. Mock EL4 targets (- - -), EL4 infected targets (+). Ratio of Effector:Target cells was calculated with the absolute number of CD8 T cells as determined by FACS. Data shown are means ± SEM of two experiments. Test was measured in triplicate.
4.4 Immunisation challenge study

The ability of the vΔA40 virus to protect mice against a lethal challenge with WT virus was assessed relative to the control viruses (Fig. 4.5). Mice were infected with $10^4$ p.f.u./ml virus i.d. and assessed for ‘take’ of the virus for 7 days. After 28 days, the mice were challenged i.n. with a lethal dose of WT virus ($10^7$ p.f.u.). The weight loss of the challenged mice was assessed every day post-challenge. By day 3 p.i., all the challenged mice had lost ~15% of their body weight. By day 4, the mice inoculated with vΔA40 showed significantly less weight loss compared to the control mice, with the weight loss levelling off whilst the control mice still lost weight at day 4 compared with day 3. By day 5, all the challenged mice began to gain weight and the mice inoculated with vΔA40 still had a significant difference in weight at day 7 p.i. compared to the WT and Rev mice.

**Fig. 4.5 Vaccination and challenge model**
All mice were vaccinated i.d. with $10^4$ p.f.u. of the indicated virus. Mice were challenged i.n. 28 days p.i. with $1 \times 10^7$ p.f.u. of WT virus and weights were measured daily. The data are presented as the indicated mean +/- SEM. P-values were calculated using the Student T-test.
4.5  The effect of A40 on natural killer activity in vitro

4.5.1 Effect of infection of NK cells on NK killing activity

The role of A40 in NK recognition of infected cells was also assessed in vitro (section 2.9.10.3). Human NK92MI cells were infected prior to incubation with $^{51}$Cr-labelled WR infected K562 target cells. Infection of NK cells with all three viruses increased their cytotoxic activity relative to mock infected NK cells but no significant difference in cytotoxicity between the vΔA40 virus infected NK cells and those infected with the control viruses was observed (Fig. 4.6A).

The infection of the NK92MI cells was assessed by immunoblotting for A40 and tubulin (Fig. 4.6Ci).

4.5.2 Effect of infection of target cells on NK killing activity

If the K562 cells were infected prior to $^{51}$Cr labelling (Section 2.9.10.3) and incubation with uninfected NK cells, the absence of A40 from the infected target cells seems to increase cytotoxicity relative to the controls (Fig. 4.6Bi). At E:T ratios 1:6 and 1:12, the cytotoxicity of NK cells to target cells infected with virus lacking A40 is significantly increased compared to cytotoxicity to target cells infected with Wt or Rev virus.

Conversely, if A40 was over-expressed from an IPTG inducible virus (vTAPA40) there is less cytotoxicity to these target cells relative to the control infected cells (Fig. 4.6Biiv). At the lowest (1:3) and highest (1:100) E:T ratios, the cytotoxicity of NK cells to target cells over-expressing A40 were significantly lower compared to cytotoxicity towards target cells infected with Wt and Rev viruses. When the E:T ratios are increased, the Wt and ΔA40 lines converge whilst vTAPA40 diverges from the controls suggesting what as the amount of A40 increases, its inhibitory effect on the cytotoxicity of the effector NK cells increases. All the cytotoxicity assays for this section were performed at the same time but have been separated into two graphs for ease of assessment.

The expression of A40 was assessed by SDS-PAGE and immunoblotting for A40 and tubulin (Fig. 4.6Cii).
Fig. 4.6 Natural killer activity in vitro
Chromium release assay of NK92MI cells against NK-sensitive K562 cells. (A) NK92MI cells were infected with vΔA40 and control viruses at 1 p.f.u./cell 16 h prior to incubation with labelled target cells. (B) (i) K562 cells were infected with vΔA40 and control viruses at 1 p.f.u./cell, 1 for 16 h prior to labelling and incubation with uninfected effector cells. (ii) K562 cells were infected with vTAPA40 and control viruses at 1 p.f.u./cell and induced for 16 h prior to labelling and incubation with uninfected effector cells. The total number of viable cells was determined by trypan blue exclusion. Ratio of effector: target cells was calculated with the absolute number of NK cells. Data shown are means ± SEM of two experiments. Test was measured in triplicate. (C) (i) NK and (ii) K562 cells were analysed for infection by SDS-PAGE (15%) and immunoblotting for A40 and tubulin as a loading control. Positions of molecular mass markers are shown on the left with sizes in kDa. P-values were calculated using the Student T-test, ¥ = 0.064, *= 0.05, $ = 0.02, ** = 0.01, *** = 0.005.
4.6 Summary

The deletion of A40 from the VACV WR genome causes attenuation of leukocyte infiltration to the site of VACV infection in the murine i.d. model. The absence of A40 from an infection results in an increase in the number of cells recruited to the site of infection and this difference is seen from day 4 p.i. A difference in cell number is also seen at day 17 p.i. correlating with the earlier clearance of A40 from an infected animal and the smaller lesion size of vΔA40 previously observed (Tscharke et al., 2002).

These changes were analysed and an increase in macrophages, CD3+ T cells and TCRγδ T cells was seen in mice infected with vΔA40 compared to the control viruses whilst no significant difference was seen in NK cell infiltration. When the CD3+ T cell populations were analysed, a significant increase in CD4+ T cells was seen at day 2 p.i. followed by an increase in CD8+ T cells at day 7 p.i.

The increase in MHC class II+ cells correlates with the increase in macrophage cell number. It may also represent an increase in other types of APCs which were not present in a significant enough number to be analysed with the total cell population, which could increase in the vΔA40 infected ears relative to WT and Rev, but their contribution to the immune response may be masked by the relative abundance of the other leukocyte cell populations.

The cytotoxic killing activity of NK and CTL T cells from infected ears was not affected by the absence of A40 at the times assessed.

The ability of the vΔA40 virus was to protect mice against a lethal challenge of WT virus was better than mice vaccinated with WT and Rev viruses.

In vitro, infection of NK cells prior to chromium release assays increased the cytotoxicity of human NK92MI cells but the absence of A40 does not affect this killing. This increase in ability to kill target cells does not correlate with observations by Chisholm et al., (2006) that infection of NK cells by VACV inhibits their ability to sense target cells. The increase in cytotoxicity could be due to VACV activating the NK cells or due to the NK cells co-infecting the target cells during the incubation period.
When the target cells were infected prior to incubation with NK92MI cells, there is an increase in cytotoxicity with all three viruses relative to the cytotoxicity towards the mock target cells. When A40 is absent from infected target cells, there is an increase in NK killing activity but there is no significant difference relative to the cytotoxicity directed towards WT and Rev infected target cells. If A40 is over-expressed in target cells, this seems to provide some protection from NK killing as the percentage cytotoxicity to these cells is reduced relative to the percentage cytotoxicity of target cells infected with WT or Rev. Collectively, these data indicate that A40 may regulate NK recognition/killing of infected cells to the advantage of the virus.
Chapter 5. Protein production & characterisation

5.1 Introduction

Having fully established the localisation of WR A40 within the cell and its characteristics such as time of expression in infection and how it is post-translationally modified in mammalian cells, a series of protein mutants were designed to help elucidate A40’s role in viral infection and to search for its potential binding partners.

5.2 Cloning strategy

Six DNA plasmid vectors, pSecTag2C (Invitrogen), pSEL (Vasta et al., 2004), pCI (Promega), pET-28a (Novagen), pVOTE.1 (Ward et al., 1995) and pBac-2cp (Novagen) were used to generate recombinant protein.

Two truncated versions of A40 were developed. In A40ΔTM, the short N-terminal region (aa 1-6) and transmembrane region (aa 7-29) of A40 were removed to aid expression in bacteria and to enable fusion with a signal peptide for secretion of soluble protein from mammalian cells. In A40ΔTMΔSt, in addition to the two regions mentioned above, the hypothesised “stalk” region (aa 30-49) between the transmembrane region and C-type lectin-like domain was also removed. This was to determine whether this region influenced the ability of A40 to bind either to itself or to any possible interacting partners.

pET-28a was utilised to create 2 different versions of A40 in bacteria. Both lacked the transmembrane domain and one also lacked the stalk region. In each case A40 was fused with an N-terminal His6 tag and utilised the natural A40 termination codon. This bacterial expression vector had the extracellular domains (Fig. 5.1 and Fig. 5.2B, C). The A40 protein produced from E. coli was used for binding studies and also for X-ray crystallisation trials.

pSecTag2C was used for expression in mammalian cells of truncated A40 fused with a biotin acceptor protein (BAP) (PNSGSLHHILDAQKMWVNH) (Schatz, 1993) at the N terminus (Fig. 5.1 and Fig. 5.2D & E). The BAP peptide enables the recombinant proteins to be labelled in vitro at the lysine residue within the fusion
protein with biotin using the bacterial enzyme BirA (Schatz, 1993; O'Callaghan C et al., 1999). Furthermore, the BAP can be fused at either the N or C terminus allowing maintenance of the correct orientation of the recombinant proteins for binding studies.

The domains of A40 and BAP were amplified by PCR from pSAD3 (Fig. 5.3A) (Duncan, 1992) and pEMR-Bio (Law, 2001) respectively (Fig. 5.3B). The A40 and BAP sequences were fused together by splicing by overlap extension (Horton et al., 1989) using the PCR products from the first reaction as templates (Fig.5.3C) and were inserted into the expression vector pSecTag2C. BAP-A40ΔTM, and BAP-A40ΔTMΔSt, were digested with restriction enzymes (RE) HindIII and BamHI and inserted into the multiple cloning site (MCS) of pSecTag2C to generate the recombinant clones psBAP-A40ΔTM and psBAP-A40ΔTMΔSt. The fusion products were inserted between the human CMV (HCMV) immediate early promoter (Boshart et al., 1985) and bovine growth hormone polyadenylation sequence for high levels of protein expression in eukaryotic cells (Goodwin & Rottman, 1992). All the fusion products were inserted in frame and downstream of the Igκ-chain leader sequence allowing secretion of the recombinant proteins. BAP-tagged products relied on the translation start site within the vector.

pSEL was used to express BAP-tagged (Fig. 5.1 and Fig. 5.2F and G) full-length A40 in VACV-infected cells. The BAP was fused to either the N or C terminus. pSEL was generated by insertion of the VACV early/late promoter (Davison & Moss, 1990) into the pBluescript SK (+) plasmid (Stratagene) (Ahmad, 2003). The same cloning strategy for pSecTag was used to insert full-length A40 into the vector under the control of the VACV early/late promoter (E/L). BAP-A40 and A40-BAP were digested with restriction enzymes BamHI and HindIII to insert into the MCS of pSEL. Clones pSEL(N)BAPA40 and pSEL(C)A40BAP were generated using this method. These plasmids were used to study the localisation of A40 (section 3.4.3) and seek its interacting partners (section 6.2).

pCI was used to express BAP-tagged full-length A40 (Fig. 5.1 and Fig. 5.2F and G) in mammalian cells. The constructs from pSEL were re-cloned into pCI under the control of the CMV enhancer/promoter.

The pVOTE1 plasmid was used to construct recombinant VACVs derived from vT7lacOI (Ward et al., 1995) that expressed A40 in an IPTG-inducible manner (section
5.5). BAPA40ΔTM and BAPA40ΔTMΔSt from the pSecTag2 vectors including their Igκ secretion sequence (METDTLLLWVLWWWPGSTGDAAQPARRAVRL) were amplified using the primers (N)IgκBAPA40_F & (N)IgκBAPA40_R. The PCR products were digested with NcoI and SaI and ligated into pVOTE.1. The resulting plasmids pVOTE-BAP40ΔTM & pVOTE-BAPA40ΔTMΔSt were used to produce recombinant viruses that produce secreted truncated A40 with an N terminal BAP tag upon addition of IPTG. A full-length A40 tagged with both a FLAG and a BAP tag at the N terminus was also produced. The dual tagged A40 was named TAPA40. The FLAG tag was incorporated into the forward primer (N)FLAG_BAP_F and fused to the BAPA40 from pSEL by overlapping extension to produce (N)FLAGBAPA40. This was then digested with NcoI and SalI and cloned into pVOTE1. This plasmid (pVOTE-TAPA40) was then used to construct a recombinant VACV (Section 2.5.).

pBac-2cp was used to generate recombinant baculoviruses expressing proteins in insect cells. This protein was designed as an alternative source of protein for cell binding studies and to study if post-translation modifications, such as glycosylation, are important for binding. BAP-A40ΔTM and BAP-A40ΔTMΔSt were re-amplified using the alleles within pSecTag2, digested with the restriction enzymes BamHI and HindIII and inserted into pBac-2cp in frame downstream of the His tag sequence. Flanking baculovirus sequences are present to permit the generation of recombinant baculoviruses. In this way, the clones pBacHisBAPA40ΔTM and pBacHisBAPA40ΔTMΔSt were constructed and used to regenerate recombinant baculoviruses expressing HisBAPA40ΔTM (Fig. 5.1 and Fig.5.2 I) and HisBAPΔTMΔSt (J).

For all vectors, the clones were verified by restriction digestions and DNA sequencing. The calculated molecular mass of the recombinant proteins are displayed in Table 5.1.
Table 5.1 Predicted properties of the recombinant proteins expressed in mammalian, bacterial and viral expression systems

<table>
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<th>Protein</th>
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<td>20.97</td>
<td>8.94</td>
</tr>
</tbody>
</table>
**Fig. 5.1 Amino acid sequences of A40 and recombinant A40 proteins**

A40, BAP-A40, A40-BAP, FLAG-BAP-A40 BAP-A40ΔTM, BAP-A40ΔTMΔSt, His-A40ΔTM, His-A40ΔTMΔSt, His-BAP-A40ΔTM & His-BAP-A40ΔTMΔSt. The transmembrane region is italicised. Asparagine (N) - linked glycosylation sites are underlined and bold. BAP, His or FLAG tag sequences are underlined.

**A40**: 159 aa

1. MNKHKTDAYG YACCVICGLI VGIIFTATLL KVVERKLVHT PSIDTIKDA YIREDCPTDW
61. ISYNKCIHL STDRTWEEG RNACKALNPN SDLIKIETPN ELSFLRSIRR GYWGESEI
121. NOTTPYNFIA KNATKNGT KK RKYICSTTN TFKLHSCYTI

**His-A40ΔTM**: 164 aa

1. MGSSHHHHHH SSGLVPRGSH MASMTGGGQQM GRGSLKVVER KLVHTPSIDK TIKDAYIRED
61. CPTDWISEYN CIIHLSTDRT WEERGRNACK ALNPSDRLIK IETPNELSFL RSIRRGYWVG
121. ESEILNOTTP YNFIA KNATKNGT KK RKYICSTTN TFKLHSCYTI

**His-A40ΔTMΔSt**: 144 aa

1. MGSSHHHHHH SSGLVPRGSH MASMTGGGQQM GRGSAIRED CPTDWISEYN CIIHLSTDRT
61. TWEERGRNACK ALNPSDRLIK IETPNELSFL RSIRRGYWVG ESEIL NOTTP YNFIA KNATKNGT
121. NOTTKKKRYIC STTNTFKLHS CYTI

**BAP-A40**: 150 aa

1. MGSSHHHHHH SSGLVPRGSH MANMTG GQQM GRS GLKYIREDCPTDW
61. ISYNNKCIHL STDRKTWEEG RNACKALNPN SDLIKIETPN ELSFLRSIRR GYWGESEI
121. KNATKNGT KK RKYICSTTN TFKLHSCYTI

**BAP-A40ΔTM**: 150 aa

1. MGSSHHHHHH SSGLVPRGSH AWMTG GQQM GRS GLKYIREDCPTDW
61. ISYNNKCIHL STDRKTWEEG RNACKALNPN SDLIKIETPN ELSFLRSIRR GYWGESEI
121. KNATKNGT KK RKYICSTTN TFKLHSCYTI

**BAP-A40ΔTMΔSt**: 130 aa

1. MGSSHHHHHH SSGLVPRGSH AWMTG GQQM GRS GLKYIREDCPTDW
61. ISYNNKCIHL STDRKTWEEG RNACKALNPN SDLIKIETPN ELSFLRSIRR GYWGESEI
121. KNATKNGT KK RKYICSTTN TFKLHSCYTI

**BAP**: 180 aa

1. PNPSGLS HHH Ild AQKimvWNHR LKVVERKLVT TPSIDTIKDA YIREDCP TD WISYSNNKCIH
61. LSTDRTWEEG RNACKALNPN SDLIKIETPN ELSFLRSIRR GYWGESEI
121. NOTTPYFIA KNATKNGT KK RKYICSTTN TFKLHSCYTI

**FLAG-BAP-A40**: 189 aa

1. MDYKKDDDKS GPNSGLLHILI DQAQK MVWNHR RNKHK TDAYG YACC VICGLI VGIIFTATLL KVVERKLVHT PSIDTIKDA YIREDCPTDW
61. ISYNKCIHL STDRTWEEG RNACKALNPN SDLIKIETPN ELSFLRSIRR GYWGESEI
121. NOTTPYFIA KNATKNGT KK RKYICSTTN TFKLHSCYTI

**His-BAP-A40ΔTM**: 206 aa

1. MMVHLLLHLLG SAGLVPRGSG KETAAKFER QHMDASAGGG DDDDKSPGF SESKGLDPNSG
61. SLNHILDAQRKD WNVNRKLVVVDKTVHPSGD ERTCPDMS QHMDASAGGG DDDDKSPGF SESKGLDPNSG
121. RKTWEERGNA ENC丰富多彩 INIETPNELS FLRSIRRGYW IGESEIL NOTTPYFIA KNATKNGT KK RKYICSTTN TFKLHSCYTI

**His-BAP-A40ΔTMΔSt**: 186 aa

1. MMVHLLLHLLG SAGLVPRGSG KETAAKFER QHMDASAGGG DDDDKSPGF SESKGLDPNSG
51. SLNHILDAQRKD WNVNRKLVVVDKTVHPSGD ERTCPDMS QHMDASPSEI DDDDKSPGF SESKGLDPNSG
121. RKTWEERGNA ENC丰富多彩 INIETPNELS FLRSIRRGYW IGESEIL NOTTPYFIA KNATKNGT KK RKYICSTTN TFKLHSCYTI

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Fig. 5.2 Schematic of A40 recombinant proteins

(A) Full length A40. (B) Truncated A40 with the transmembrane region removed and a His tag fused to the N terminus. (C) Truncated A40 with the transmembrane region and stalk region removed and a His tag fused to the N terminus. (D) Truncated A40 with the transmembrane region removed (ΔTM) and a BAP tag fused to the N terminus. (E) Truncated A40 with the transmembrane region and stalk region removed (ΔTMΔSt) and a BAP tag fused to the N terminus. (F) Full-length A40 with a BAP tag fused to the N terminus. (G) Full-length A40 with a FLAG & a BAP tag fused to the N terminus. (H) Full-length A40 with FLAG and BAP tags fused to the C terminus. (I) Truncated A40 with the transmembrane region removed and a His tag and a BAP tag fused to the N terminus. (J) Truncated A40 with the transmembrane region and stalk region removed and a His tag and a BAP tag fused to the N terminus.
Fig. 5.3 Generation of recombinant BAP fusion proteins by overlapping extension PCR (OEP)

(A) The complete A40R ORF or the region encoding the extracellular domains of A40 were amplified from pSAD3 by PCR. BAP sequences were linked at the 5’-end of the forward primers (N)BioA40R_R, (N)BioA40R_Rt, pSEL(N)BioA40R_F and the reverse primer pSEL(C)A40RBio_R. (B) BAP sequences were amplified from pEMR-Bio plasmid DNA by PCR using primers (N)Biotin_F, (N)BioA40R_R and (N)BioA40R_Rt for the fusion with A40RΔTM and A40RΔTMΔSt respectively and pSEL(N)Biotin_F and pSEL(N)BAPA40R_R for the fusion with A40R. In the case of A40RΔTM, A40RΔTMΔSt & (N)A40R sequences were linked with the BAP sequence in the 5’-end of the reverse primers (N)BioA40R_R, (N)BioA40R_Rt and pSEL(N)BioA40R_R respectively. The (C)A40R sequence was linked with the BAP sequence in the 5’-end of the forward primer pSEL(C)A40RBio_F. (C) OEP. The PCR products from (A) & (B) were purified and used as templates for the generation of BAP-fusion sequences by OEP. As overlapping sequences were introduced into the ends of the PCR products, the PCR fragments can be joined together by OEP to form BAP-A40ΔTM, BAP-A40ΔTMΔSt, BAPA40 and A40BAP. “ATG” and “STOP” represent the translation initiation and stop codons, respectively.
5.3 Production of recombinant His–tagged A40 from E.coli

5.3.1 Generation of rabbit anti-A40 polyclonal serum

To produce an antibody to A40, recombinant truncated A40 was expressed from pET-28a in E. coli. Small scale cultures were used to determine conditions giving the most soluble protein (Table 5.2). In all conditions tested the recombinant HisA40ΔTM and HisA40ΔTMΔSt produced were completely insoluble. Rosetta gami 2 (DE3) pLys cells induced with 1 mM IPTG for 3 h were chosen as these conditions gave the best level of expression and scaled up to 1 L cultures (Fig. 5.4). This recombinant protein was harvested, denatured and purified by on-column refolding on a HisTrap column (section 2.8.3.1). Protein derived from denatured inclusion bodies (IB) was applied to a HisTrap column pre-equilibrated with denaturing His-binding buffer containing 8 M Urea. The column was washed in binding buffer and the His-tagged protein refolded using buffer exchange with a linear gradient, gradually reducing the concentration of urea to zero (Fig. 5.5A). The protein was eluted in 500 mM imidazole (Fig. 5.5B) and the fractions were assessed for HisA40ΔTM by SDS-PAGE and coomassie staining (Fig. 5.5C). A band was present in the elution fractions 19 -22 at the predicted size of HisA40ΔTM (~18 kDa, Table 5.1). These fractions were pooled together, concentrated to 0.5 ml and applied to a pre-equilibrated Superdex 75 column (Fig. 5.6A) and the eluted fractions were analysed by SDS-PAGE, coomassie staining (Fig. 5.6B) and immunoblotting (Fig. 5.6 C) for the His tag. Fractions 17 - 22 were pooled and the concentration determined (using the NanoDrop) to be 1.2 mg/ml. This purified protein was sent to Harlan to inoculate two rabbits per recombinant protein (section 2.10.1).

5.3.2 Purification of antiserum by pre-absorption on infected cells

The final bleeds were tested against recombinant A40 to analyse the antiserum. When tested by immunoblotting against cell lysate from VACV infected cells, the antiserum showed specific bands characteristic of A40 and these were absent from cells infected with the vΔA40 virus and from mock-infected cells. However, non-specific bands were seen in all of lysates (Fig. 5.7Bi).

To remove these bands, anti-A40 antiserum was absorbed against vΔA40 cell lysates. Five flasks of confluent RK13 cells were infected with vΔA40 at 5 p.f.u/cell for
18 h. The cells were washed, harvested and fixed in PFA (section 2.10.2). The cells were then permeabilised and incubated with 1 ml of antiserum overnight at 4 ºC. The unbound antiserum was collected, centrifuged at 15,000 rpm for 15 min at 4 ºC (Heraeus Biofuge 15R) to remove cell debris.

To purify the antibody further, the IgG fraction was isolated by affinity chromatography on a HiTrap Protein G column connected to the Akta (section 2.10.3). The elution of the antibody was monitored by absorbance at 280 nm and the pH of the eluant was neutralised by pre- aliquoting 1 M Tris pH 8.8 in the collection tubes. As seen in Fig. 5.7A most of the bound protein eluted in fractions 2 & 3. These fractions were combined together, dialysed against 2x antibody buffer (section 2.10.3), diluted 1:1 with sterile glycerol, aliquoted and stored at 4 ºC for immediate use and at -20 ºC for long term storage.

The purified IgG was compared with the original and pre-cleared antiserum by using comparable dilutions in immunoblots to detect the A40 protein from virus-infected cell lysates (Fig. 5.7B). The purified antibody gave a much better signal and lowered the background significantly (Fig. 5.7Bii) compared to the original antiserum (Fig. 5.7Bi). This purified antibody was subsequently used for immunoblotting and immunofluorescence to analyse the A40 protein.
Fig. 5.4 Production of recombinant His-tagged A40 proteins in bacterial cells

HisA40ΔTM and HisA40ΔTMΔSt were transformed into Rosetta Gami 2 (DE3) pLys and scaled up to 1 L of LB medium with 50 μg/ml kanamycin. Expression was induced for 3 h with 1 mM IPTG. The cells were harvested and lysed by sonication in lysis buffer on ice (section 3.6.4). A small aliquot was taken (Total) and 1/5 vol of 6 x SDS loading buffer was added. The lysate was centrifuged at 13,000 x g for 30 min and separated into soluble protein fraction (supernatant) and insoluble fraction (pellet). The insoluble fraction was resuspended in an equal volume of lysis buffer. Each fraction was analysed for the presence of His-tagged A40 protein by SDS-PAGE (15% gel). (A) Coomassie staining of small-scale production from pET-28a recombinant plasmids. (B) Immunoblot for (N) terminal His tag. (T) Total, (S) Soluble, (I) Insoluble. Positions of molecular mass markers are shown on the left with sizes in kDa.
HisA40ΔTM was produced in *E. coli*, the insoluble protein fraction was harvested and the inclusion bodies were washed and then denatured (section 2.8.3.5). The denatured protein was applied to a HisTrap column. (A) Binding, washing and on-column refolding of HisA40ΔTM. The column was washed in buffer containing 8 M urea, then the protein was refolded using buffer exchange with a linear gradient of 8–0 M urea. (B) Elution of HisA40ΔTM using a step gradient of imidazole. The refolded protein was washed with 5 mM imidazole, then 300 mM imidazole and finally was eluted in 500 mM imidazole. (C) Fractions were tested for HisA40ΔTM by SDS-PAGE and coomassie staining. Positions of molecular mass markers are shown on the left with sizes in kDa.

**Fig. 5.5 Purification of recombinant His-tagged protein from *E. coli* using a HisTrap column**
Fig. 5.6. Purification of HisA40ΔTM by gel filtration.
HisA40ΔTM from on-column refolding fractions 18-24 were pooled and concentrated to 0.5 ml and applied to a Superdex 75 column pre-equilibrated in PBS. (A) Chromatogram of HisA40ΔTM elution from Superdex in PBS. Eluted fraction 18 – 25 were analysed for presence and purity of HisA40ΔTM by SDS-PAGE and (B) coomassie staining or (C) immunoblotting for His tag. Positions of molecular mass markers are shown on the left with sizes in kDa.
Fig. 5.7 Purification of the anti-A40 antiserum and isolation of IgG fraction

Anti-serum raised against HisA40ATM was pre-cleared against vΔA40-infected cells to remove cell non-A40 background bands (section 2.10.2). The pre-cleared serum was diluted 1:2 in PBS and applied to a pre-equilibrated Protein A column (A). The column was washed in PBS and the IgG eluted with 100 mM Citric acid pH 3. The fractions were neutralised with 1.5 M Tris pH 8.8 and fractions 2 and 3 pooled and dialysed against 2 x Ab buffer. (B) BS-C-1 cells were mock-infected or infected with either WT ΔA40 or Rev virus at 15 p.f.u./ml for 16 h. Cells were lysed in LB1 (Section 2.8.7) and the proteins separated by SDS-PAGE and analysed by immunoblotting with (i) anti-A40 Rb 171 anti-serum, (ii) purified IgG fraction of pre-cleared anti-A40 (α-A40 IgG). Positions of molecular mass markers are shown on the left with sizes in kDa.
5.3.3 Crystallisation trials

To improve the chances of obtaining material suitable for crystallisation trials, the conditions of expression of recombinant A40 expressed from pET-28a in *E. coli* were varied to try to improve the protein solubility. Three different strains of *E. coli* commonly used within our lab were tried. In addition, the temperature, IPTG concentration and length of induction and the presence of 1% glucose within the induction medium were all tested.

In all the conditions tested, the test cultures were harvested, the final OD$_{595}$ tested and the pellets were resuspended in lysis buffer with added protease inhibitors, sonicated and the solubility of expression with each set of conditions analysed by SDS-PAGE (section 2.8.3.4.). Whilst all the conditions tested did not increase the amount of soluble A40 protein, the Rosetta Gami 2 cells gave the highest expression overall and it was decided that the best way to obtain recombinant A40 was to harvest the insoluble protein within cleaned inclusion bodies and denature, refold and affinity purify this material (section 2.8.3.2).

A two L culture of RG2 cells transformed with pET28a-HisA40ΔTM and induced for 3 h with 1 mM IPTG at 37 °C with shaking was harvested by centrifugation and the pellet analysed for expression. To process the cells, the pellet was resuspended in PBS containing protease inhibitors on ice. After the samples were sonicated, they were centrifuged and the soluble fraction removed. The insoluble pellet was then resuspended in inclusion body wash buffer and the inclusion bodies were washed four times in this buffer to remove as much contaminating protein as possible. This IB preparation was then denatured using 8 M guanidine overnight. The denatured samples were harvested by centrifugation to remove any remaining insoluble material and transferred to a fresh tube. The protein concentration was determined and the denatured proteins were diluted into refold buffer with stirring. This refolded material was then concentrated and dialysed against His binding buffer to remove the L-arginine within the refold buffer. This is needed within the refold buffer to stabilise the protein as it refolds, but is detrimental to the HisTrap column. As the refolded protein was concentrated, some material did aggregate and this probably represented either misfolded protein or refolded protein aggregating as it became more concentrated.
The concentrated, dialysed material was applied to a pre-equilibrated HisTrap column, washed in His binding buffer and then eluted in a gradient of imidazole. The elution was assessed by absorbance at 280 nm (Fig. 5.8) and the fractions were assessed by SDS-PAGE followed by Coomassie staining (Fig. 5.8B).

The fractions containing His-tagged A40 were combined, re-concentrated and dialysed against PBS and applied to a pre-equilibrated gel filtration column. The elution profile of the A40 protein was again assessed by absorbance at 280 nm (Fig. 5.9A) and fractions were assessed by SDS-PAGE and coomassie staining (Fig. 5.9B). Samples of the fractions were prepared for SDS-PAGE in SDS loading buffer with or without β-ME. In the fractions containing HisA40ΔTM, in the absence of β-ME the protein runs as a doublet ~ 38 kDa whilst in the presence of β-ME, HisA40ΔTM is present as a band ~ 18 kDa.

The pooled purified protein was further assessed by SDS-PAGE and immunoblotting for the His-tag (Fig. 5.10A). His-tagged N1 (Cooray et al., 2007) was used as a control and runs at ~ 15 kDa as predicted, whilst HisA40ΔTM is present ~ 18 kDa. The samples were also assessed in a native gel, and immunoblotted for His (Fig. 5.10B) or A40 (Fig. 5.10C). N1 (detected by the His tag) runs lower than A40.

Fractions 50-58 from the gel filtration column were combined and concentrated to 0.8 mg/ml and sent to our collaborators at the Division of Structural Biology, Oxford for crystallisation trials. Of the different conditions trialled for A40, crystals were obtained when diffused against 0.1 M HEPES pH 7.5, 0.5 M lithium acetate, 18% w/v PEG 4000 (Fig. 5.11A) or 0.1 M MES pH 6.0, 0.4 M potassium acetate, 18% PEG 4000 using free-interface diffusion at RT and these were taken to the ESRF synchrotron in Geneva. The diffraction pattern obtained from these crystals was only to 8 Å (Fig. 5.11B). The protein was also analysed by dynamic light scattering (DSL) and dye-binding shift (thermofluor) experiments to assess the stability and that the protein is folded properly (data not shown). The protein was monomeric by DSL and shown to be natively folded by the refolding experiments. The protein was not made more stable by the addition of Ca^{2+} to the thermofluor experiments. When assessed by MALDI mass spectrometry, HisA40ΔTM was shown to have a mass of 19.1 kDa, which is 500 Da more than predicted but when digested with trypsin and sequenced, the protein was shown to be A40. The reason for this small difference remains unknown.
Fig. 5.8. Affinity purification of refolded HisA40ΔTM
(A) Refolded and concentrated HisA40 from *E. coli* inclusion bodies was applied to a pre-equilibrated HisTrap column. The column was washed in native His binding buffer and His tagged A40ΔTM was eluted using a linear gradient of 20 – 500 mM imidazole. (B) Fractions were tested for HisA40ΔTM by SDS-PAGE and coomassie staining. Positions of molecular mass markers are shown on the left with sizes in kDa.
Fig. 5.9. Purification of HisA40ΔTM by gel filtration.
Refolded HisA40ΔTM fractions from HisTrap column were applied to a Superdex 75 10/300 column for gel filtration. Half ml aliquots were applied to the column and were eluted in PBS. The fractions (0.5 ml) were analysed for the presence of His-tagged recombinant protein by SDS-PAGE. (A) Separation of HisA40ΔTM using gel filtration. (B) Coomassie staining of eluted fractions prepared with or without β-mercaptoethanol in the SDS loading buffer. Positions of molecular mass markers are shown on the left with sizes in kDa.
Fig. 5.10. Analysis of refolded HisA40ΔTM for crystallization trials
Fractions 48-58 from gel filtration were pooled and analysed by SDS-PAGE and on native gels to assess oligomeric properties of purified HisA40ΔTM. HisA40ΔTM and purified N1 protein were analysed by (A) SDS-PAGE (15 %) and (B) native gel (10 %). The recombinant proteins were detected by immunoblotting with α-His Ab. (C) The native membrane was stripped and re-blotted using α-A40 IgG. Positions of molecular mass markers are shown on the left with sizes in kDa for (A).
Fig. 5.11 Crystallisation of HisA40ΔTM and diffraction pattern obtained.

Purified HisA40ΔTM was sent to Prof. D Stuart’s group, Division of Structural Biology, Oxford for crystallisation trials. The purified protein was dialysed in TBS and concentrated to 3.4 mg/ml and used to set up 96-well microfluidics crystallisation experiments. (A) Crystals were obtained from two conditions. HisA40ΔTM in one condition (0.1 M HEPES pH 7.5, 0.5 M lithium acetate, 18% w/v polyethylene glycol 4000) is shown. (B) Diffraction pattern obtained from crystals. Resolution obtained was 8 Å.
Table 5.2 Conditions used for expressing recombinant His tagged A40 in *E. coli*.

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5.3.4 Selenomethionine labelling of recombinant A40

A40 shares only around 20% aa identity with proteins that have had their crystal structure determined, and therefore it was likely that selenomethionine-labelled A40 crystal would be necessary to overcome the phase problem and obtain a high resolution crystal structure. The *E. coli* strain B834 are specifically adapted for in selenomethionine labelling studies because they are methionine auxotrophs. However in these cells, no detectable HisA40 was seen in any of the conditions used. Rosetta cells have been used as an alternative source of selenomethionine labelled protein by using the biosynthesis poisoning pathway method in conjunction with selenomethionine containing culture medium (Graham *et al.*, 2007). However the conditions used to induce unlabelled HisA40ΔTM in RG2 cells did not produce any detectable protein, so the conditions were varied again and some insoluble protein was detected when the culture was induced at ~ OD$_{560}$ 0.6 with 1 mM IPTG and left 32 h.

A trial affinity purification using Ni NTA beads using guanidine to denature the IBs and binding the denatured insoluble material to the beads in denaturing conditions with urea showed that the denatured protein could be bound to the nickel beads.

A trial purification of the selenomethionine labelled refolded protein from IBs was unsuccessful as most of the protein aggregated with the concentration step before application to the HisTrap column. Whether the protein produced is labelled, correctly folded or could be successfully purified was beyond the time limits of this project and will have to be continued in any future project.
Fig. 5.12. Expression & characterisation of recombinant A40 protein in mammalian cells
(A) COS-7 cells were transfected with pSecTag2, psBAPA40ΔTM or psBAPA40ΔTMΔSt. After overnight incubation, the medium was replaced and cells were incubated in absence (-) or presence (+) of tunicamycin (1 μg/ml). Four days after transfection, the cells and supernatant were harvested and the samples were analysed by SDS-PAGE and immunoblotting for presence of BAP tag using α-BAP Ab.
(B) RNase B (control protein) (i) or purified BAP-tagged A40 proteins (ii & iii) were treated with either endoglycosidase PNGase F (P), Endo H (E) or mock treated (-) for 6 h @ 37 °C and analysed by SDS-PAGE (15%) and coomassie staining or immunoblotting for BAP tag using α-BAP Ab. Positions of molecular mass markers are shown on the left with sizes in kDa.
5.4 Production of recombinant fusion proteins using a mammalian expression system

To generate soluble glycosylated recombinant protein for cell binding studies, truncated versions of A40 were expressed by transient transfection of COS-7 cells with plasmids psBAP-A40ΔTM or psBAP-A40ΔTMΔSt (section 2.3.7.). Small-scale transfections were performed in 6-well plates. The tissue culture supernatants were collected 3 days after transfection and concentrated by TCA precipitation (Section 2.8.6).

To characterise the fusion proteins produced, psBAP-A40ΔTM and psBAP-A40ΔTMΔSt were transfected into 6-well plates of COS-7 cells, and incubated with or without tunicamycin to inhibit the addition of N-linked glycans. The supernatant was harvested and the cells were lysed (section 2.8.7). Both samples were analysed by immunoblotting against their fusion tag. In psBAP-A40ΔTM transfected samples, in the absence of tunicamycin, several proteins of apparent molecular mass ranging from ~30 - 45 kDa were seen in the supernatant (Fig. 5.12A). Similarly, for psBAP-A40ΔTMΔSt proteins of ~ 27 - 42 kDa (Fig. 5.12A) were observed in the supernatant fraction. No bands were observed in the cell lysates for either form of A40, showing that the great majority of protein is secreted. However, in the presence of tunicamycin, a single band of ~ 17 and 15 kDa for BAP-A40ΔTM and BAP-A40ΔTMΔSt, respectively, was present only in the cell extracts and not in the culture medium, indicating that glycosylation is needed for protein secretion (Fig. 5.12A). These smaller proteins are the expected size of BAP-A40ΔTM and BAP-A40ΔTMΔSt (Fig. 5.12A).

The presence of multiple bands in the supernatants of the transfected cells and the observation that tunicamycin reduced the size of these bands to a single intracellular protein, indicates that the proteins are both secreted and glycosylated and suitable for purification under non-denaturing conditions.

Once expression had been verified, the production of A40 was scaled up to 5 x T175 flasks. The supernatants were collected, concentrated and buffer-exchanged at day 3 post-transfection. This concentrated supernatant was purified by application to a BAP affinity column and eluted at pH 2.8 (section 2.8.5). The α-BAP columns were generated by coupling α-BAP mAb, grown in culture and purified on a protein G column, to CNBr-activated Sepharose beads and the coupled beads were poured in
Eco-columns. The column fractions were analysed by SDS-PAGE and immunoblotting against mAb BAP (Fig. 5.13). Fractions 2 - 5 were pooled and concentrated and the recombinant BAP-fused proteins were biotinylated with BirA enzyme (Fig. 5.14). After dialysis against PBS, the concentration of each protein was calculated by spectrometry at OD\textsubscript{280} and each protein’s coextinction coefficient (http://www.expasy.ch/tools/protparam.html). The yields of proteins were 1.5 µg/10\textsuperscript{7} cells of BAP-A40\textDelta TM and 3 µg/10\textsuperscript{7} cells of BAP-A40\textDelta TM\Delta St.

To verify that the recombinant BAP-A40 proteins are modified by glycosylation, the purified BAP-A40\textDelta TM and BAP-A40\textDelta TM\Delta St were treated with either PNGase F or Endo H. RNase B is a high mannose glycoprotein and was used as a positive control for the endoglycosidases. RNase B has a single N-linked glycosylation site, which makes it ideal for SDS-PAGE gel shift assays.

When RNase B was treated with PNGase F or Endo H, a reduction in size was seen (Fig. 5.12B). Similarly, when the BAP-tagged A40 proteins were treated with PNGase, the multiple higher bands seen in the mock treated proteins are replaced with a single band ~ 15 kDa for BAPA40\textDelta TM\Delta St and ~ 17 kDa for BAPA40\textDelta TM representing the unmodified protein. When the proteins were treated with Endo H, this shift was not seen suggesting that the N-linked glycans have not been cleaved indicating that the N-glycosylation on A40 is not of a high mannose form.
Fig. 5.13 Purification of secreted BAP-tagged A40 proteins from COS-7 cells
Five T175 flasks of COS-7 cells were transfected with psBAP-A40ΔTM or psBAP-A40ΔTMΔSt. Four days after transfection the supernatant was harvested, concentrated using a Centricon YM-10 to 10 ml, re-diluted in Tris buffer and re-concentrated. This concentrated supernatant was applied to a pre-equilibrated BAP affinity column. The column was washed in PBS until absorbance returned to base level. Bound BAP-tagged proteins were eluted in 100 nM glycine, pH 2.8. To each eluted fraction a 1/10 volume 1 M Tris pH 8.0 was added. Each fraction was analysed for the presence of BAP-tagged protein by SDS-PAGE and immunoblotting using mAb BAP with mAb BAP run as a control. M, Marker, St, Starting material, FT, Flow through, W, Wash, E, Elution, Ab, BAP mAb control (A) BAP-A40ΔTM recombinant protein (B) BAP-A40ΔTMΔSt recombinant protein. Positions of molecular mass markers are shown on the left with sizes in kDa.
Biotinylation of purified BAP-tagged proteins

Purified BAP-tagged proteins were biotinylated using BirA and dialysed against PBS. Samples were analysed for successful biotinylation by SDS-PAGE (15%) and immunoblotting using extravidin Ab. Positions of molecular mass markers are shown on the left with sizes in kDa.

5.4.1 Generation of stable cell lines expressing secreted recombinant A40

To reduce the need to repeatedly transfect COS-7 cells to generate A40 protein, stably transfected cell lines expressing the two secreted forms of truncated BAP-tagged A40 were produced.

The pSecTag2 plasmid includes a zeocin resistance gene that can be used to select for cells that harbour the required plasmid. A killing curve was performed on COS-7 cells to determine the concentration of zeocin at which non-transfected cells would die off but stably transfected cells would be allowed to grow and form colonies.

Cells were seeded overnight and then transfected with either of the two plasmids expressing the BAP-tagged A40 protein. The cells were left for 3 days in DMEM/2% to reach confluency and the supernatant was harvested and analysed by immunoblotting for A40 protein to check that the transfection has been successful. The cells were then seeded at various ratios to give low confluency and the medium changed to include 0.2% zeocin. The cells were left to expand and the medium changed every 2-3 days for 3 weeks. At this point, the plates containing the mock transfected samples were devoid of cells, whilst, in contrast, both psBAPA40ΔTM and psBAPA40ΔTMΔSt-transfected cell plates had distinct colonies.
Several of these colonies were picked for each transfection and used to seed a T25 flask and this in turn was expanded to a T175 flask in the presence of zeocin. Once the cells had expanded enough, they were tested for the level of A40 protein expression by immunoblotting (section 2.8.15, Fig. 5.15) and others were harvested for cryopreservation (section 2.3.4).

Cell lines from both transfections expressed secreted A40 with the characteristic multiple bands (Fig. 5.15). These cell lines will aid future studies of A40, in the same way that previous BAP-tagged VACV protein expressing cell lines have aided the study of the Ab response to VACV vaccination (Law et al., 2005; Putz et al., 2006).

Fig. 5.15 Generation of stable cell lines expressing secreted recombinant truncated A40
COS-7 cells adapted to 2% serum were transfected with either (A) psBAPA40ΔTM.8 or (B) psBAPA40ΔTMΔSt.1 and left for 3 days. The cells were split and selective pressure for stable transfection applied (0.2% zeocin [v/v] in DMEM/2%). After 3 weeks, individual colonies were picked and expanded. To test for expression of the secreted recombinant proteins, individual cell lines were seeded in a well of a 6-well plate and left for 3 days in 1 ml of serum free medium. The supernatant was harvested, cell debris removed by centrifugation and proteins in the resultant medium TCA precipitated and analysed by SDS-PAGE (15% gel), and immunoblotting with α-BAP mAb. Positions of molecular mass markers are shown on the left with sizes in kDa. M = mock.
5.4.2 Expression of BAP-tagged control proteins from COS-7 cells

To validate the cell binding assay data (section 6.2) three BAP-tagged protein constructs were obtained from Prof. Neil Barclay, Oxford. These were rat CD4 (domains 3 & 4, pEF-BIOS-rCD4d3d4), rat CD200 (pEF-BIOS-CD200) or mouse SIRPα (pEF-BIOS-mSIRPα) with an N-terminal BAP tag and have been used previously to study the interactions of CD200 (Hoek et al., 2000; Hatherley & Barclay, 2004; Hatherley et al., 2005) and SIRPα (Vernon-Wilson et al., 2000; Barclay et al., 2002) with other immune proteins and cell types.

Plasmids encoding these three proteins were transfected into COS-7 cells in the same way as the A40 constructs (sections 2.3.7, 2.8.4 & 1.1). Three days after transfection, the supernatant from a small-scale transfection was analysed for the presence of the BAP-tagged proteins by TCA precipitation, SDS-PAGE and immunoblotting with the anti-BAP mAb. Transfection of COS-7 cells with pEF-BIOS-rCD4d3d4 resulted in a secreted BAP-tagged protein of ~25 kDa, whilst pEF-BIOS-CD200 resulted in a secreted protein of ~45 kDa and pEF-BIOS-mSIRPα expressed a secreted protein ~ 50 kDa. Therefore, larger amounts of protein were produced and purified by anti-BAP affinity columns. The purified proteins were then biotinylated and dialysed as described for the BAP-tagged A40 proteins (sections 2.8.5 & 1.1).

The concentrations of the proteins were assessed using a Nanodrop spectrometer (section 2.8.12) and the proteins were then aliquoted and frozen until needed.

![Fig. 5.16 Expression of control BAP-tagged proteins from COS-7 cells](image)

COS-7 cells were transfected with either pEF-BIOS-rCD4d3d4, pEF-BIOS-CD200 or pEF-BIOS-mSIRPα. After 3 days the supernatants were harvested and 1ml was tested for the presence of BAP-tagged protein by TCA precipitation of proteins, followed by SDS-PAGE (10% gel) and immunoblotting with α-BAP mAb. Positions of molecular mass markers are shown on the left with sizes in kDa.
5.4.3 Transient transfection of mammalian cells with pCI expressing A40

To study the effect of A40 in the absence of any other VACV proteins, full-length A40R ORF with either a BAP or HA tag at the N or C terminus were generated using OEP and cloned into the pCI vector under the HCMV immediate-early enhancer/promoter region. The resulting plasmids pCI-(N)HAA40, pCI(C)A40HA, pCI-(N)BAPA40 (Fig. 5.1F and Fig. 5.2F) and pCI-(C)A40BAP (Fig. 5.1G and Fig 5.2G) were transfected into several cell types to test for expression, using pCI(C)B14HA (Chen et al., 2006) as a positive control. After 48 h the supernatant was removed, the cells were washed in ice-cold PBS and lysed with ice-cold LB1 buffer. After removing the nuclear debris, the lysates were analysed by SDS-PAGE and immunoblotting with the anti-HA mAb and the anti-A40 Ab (Fig. 5.17).

Using the HA Ab, the HA tagged B14 protein is seen ~ 15 kDa but neither of the HA-tagged A40 constructs are detected (Fig. 5.17). When using the A40 Ab, only the N-terminally BAP-tagged A40 is detected (Fig. 5.17). Due to the poor expression of A40 from these vectors, further analysis using these plasmids was not pursued.

5.4.4 Expression of BAP tagged A40 from pSEL

At the same time as cloning the full-length BAP-tagged A40 alleles into pCI (section 5.4.3), the PCR products generated by OEP were also cloned into the pSEL plasmid under the VACV early/late promoter. The resultant plasmids pSEL-(N)BAPA40 and pSEL(C)BAPA40 were transfected into HeLa cells and after 4 h the cells were infected with vΔA40 at 1 p.f.u./cell and incubated for 16 h. The cells were harvested and lysed in LB1 and the clarified lysates were analysed by SDS-PAGE and immunoblotted for the presence BAP-tagged recombinant A40 (Fig. 5.18). Both plasmids expressed A40 within the cell that was detected by the anti-A40 Ab with bands ~ 26 – 38 kDa and no bands were detected with either Ab in the mock lanes where the cells were transfected with pCI and infected with vΔA40.

These plasmids were then used to transfect HeLa cells seeded on coverslips that had been infected with vΔA40R. These cells were analysed by immunofluorescent staining to study the localization of A40 within an infected cell as described in section 3.4.3 and Fig. 3.5.
HeLa cells were transfected with pCI, pCI-(N)BAPA40, pCI-(C)A40 or pCI(C)B14HA. Cells were harvested 48 h after transfection and analysed by SDS-PAGE (15%) and immunoblotting with α-A40, α-HA Ab (A) and α-tubulin Ab as a loading control. Positions of molecular mass markers are shown on the left with sizes in kDa.

HeLa cells were transfected with pSEL, pSEL-(N)BAPA40 or pSEL-(C)A40. Cells were infected with vΔA40 at 1 p.f.u./ml for 24 h. Cells were harvested and analysed for the presence of A40 by SDS-PAGE (15%) and immunoblotting with α-A40 and D8 as virus infection control and α-Tubulin Ab as a loading control. Positions of molecular mass markers are shown on the left with sizes in kDa.
Fig. 5.19 The VOTE system

(A) Regulation of A40 expression in the presence (blue) and absence (red) of inducer (IPTG). The lac repressor is transcribed under the control of the VACV early/late (P_EL) promoter. In the absence of inducer, the repressor binds to the lac O site (LO), preventing transcription of the T7 RNA polymerase (T7 gene I) and also to the modified lac O (SLO) preventing transcription of the target gene should leaky synthesis of the T7 RNA polymerase occur. When IPTG is added, the repressor dissociates from the operator and transcription of the T7 RNA polymerase ORF occurs. After translation T7 RNA polymerase binds to the T7 promoter and transcribes the target gene. Transcription is terminated at the transcriptional terminators (TT) and the stability and translation of A40 transcripts is improved by the encephalomyocarditis virus internal ribosome entry site (IRES).

(B) i) The pVOTE plasmid. HA (A56R) fragments for insertion into the VACV genome and position of the MCS. Illustration adapted from (Ward et al., 1995). ii) A40R inserted at the HA locus. Primers for PCR amplification to validate that recombinant A40 has recombined into the HA locus are shown.
5.5 The VOTE system

The VOTE system (Vaccinia-lac Operon-T7-Encephalomyocarditis virus upstream translational enhancer element) (Ward et al., 1995) is constructed using vT7lacOI, a VACV WR virus with the lac I and T7 gene 1 inserted into the tk locus as shown in Fig. 5.19A. The virus contains an intact HA gene, into which the target gene and control elements are inserted using the pVOTE plasmid (Fig. 5.19Bi). The pVOTE plasmid contains the Ecogpt selectable marker so that viruses containing the pVOTE DNA can be selected in the presence of MPA. Recombination occurs between the fragments of the HA gene in pVOTE and the HA gene in the virus. This inserts the gene of interest between the HA_L and HA_R fragments of the VACV genome.

The two pVOTE plasmids available (Ward et al., 1995) pVOTE.1 and pVOTE.2 differ in the restriction endonuclease site for the insertion of the 5’ end of the target gene (NcoI in pVOTE.1 and NdeI in pVOTE.2). Although each plasmid contains 7 restriction endonuclease sites at which the 3’ end of the target gene can be inserted, the position of the 5’ end of the target gene cannot be varied, as this affects translational efficiency of T7 RNA polymerase transcripts. Since A40R does not contain either of the 5’ restriction endonuclease sites, either plasmid was suitable and pVOTE.1 was selected.

5.5.1 Expression of recombinant A40 from VOTEA40 viruses

5.5.1.1 Secreted BAP-tagged truncated A40 viruses

DNA encoding truncated A40 tagged with BAP was cloned from psBAPA40ΔTM & psBAPA40ΔTMΔSt including the Igκ leader sequence into pVOTE.1 plasmid and the resultant plasmids (pVOTE-BAPA40ΔTM & pVOTE-BAPA40ΔTMΔSt) were transfected into RK13 cells that had been infected with the vT7lacOI virus at 0.05 p.f.u./cell. The virus population, obtained two days later were then plaque purified on BS-C-1 cells three times in the presence of MPA to select recombinant viruses. The presence of the inserted gene was checked by PCR (section 2.7.11 - 2.7.12 & Table 2.3). The vT7lacOI virus contains an A40R gene at its natural locus in addition to that being inserted within the A56R gene locus. Therefore it is possible for the transfected plasmid to recombine within the genome at this point.
instead of at the HA locus. To check that integration had occurred at the A56R gene, the HA locus was amplified using a primer specific to the A56R gene (A56R_R) and a primer for A40R (A40_R). A PCR product of the expected size was seen for the newly isolated VOTE-BAPA40ΔTM and VOTE-BAPA40ΔTMΔSt viruses. A positive isolate for each virus was then used to seed a master, submaster and working stock.

These viruses were then used to infect cells to check for expression of the required protein. Different parameters were checked to maximise protein expression. These included various cell types (RK13, BS-C-1 and HEK 293T cells), different m.o.i. (0.1 – 10), differing NaCl concentration in the culture medium (150-250 mM) and different concentrations of IPTG (0.1 – 5 mM). Cells were infected for 90 min in medium containing 2.5% FBS. The medium was changed to induction medium (serum free) containing varying concentrations of IPTG and NaCl and harvested after 16 h. The medium was harvested, cell debris were removed by centrifugation and proteins in the clarified supernatant were concentrated by TCA precipitation overnight (section 2.8.6). Proteins were dissolved and analysed by SDS-PAGE and immunoblotting for the presence of BAP-tagged A40. It was found that an infection at 5 p.f.u/ml in HEK 293T cells for 16 h in medium containing 190 mM NaCl and 2 mM IPTG gave the best production of secreted BAP-tagged A40. These conditions were used to express truncated A40 for the use in cell binding studies (section 6.2).

For large-scale A40 expression, 5 x T175 flasks were infected per virus and the secreted proteins were harvested at 16 h p.i., concentrated approximately 10-fold to 5 ml and buffered against 10 mM Tris pH 8.0. The concentrated supernatant was treated with psoralen and then placed under long-wave UV to inactivate virus infectivity. To purify the secreted protein, the psoralen/UV treated supernatant was purified using anti-BAP affinity columns and biotinylated as described for the COS-7 expressed proteins. The concentration of the proteins was calculated using the Nanodrop and each protein’s coextinction coefficient. Yields were approximately 6.6 µg/10⁷ cells for vBAPA40ΔTM and 9.8 µg/10⁷ cells for vBAP-A40ΔTMΔSt.
5.5.1.2 Full-length A40 from VOTEA40 virus

Using the plasmid pVOTE-TAPA40, the virus vVOTE-TAPA40 was generated in the same way as described above for VOTE-BAPΔTM and VOTE-BAPA40ΔTMΔSt. The presence of the inserted A40R gene was checked by PCR and a product of the expected size was obtained.

The same range of conditions was used to test the expression of this full-length recombinant A40 within cells. Instead of harvesting the supernatant, infected cells were harvested 16 h after induction with LB1 (section 2.8.7) and analysed by SDS-PAGE and immunoblotting for BAP-tagged A40 within the cell lysates (Fig. 5.20C). This showed that VOTE-TAPA40 expressed BAP-tagged A40 within infected cells as multiple bands (~ 30- 40 kDa) are detected with both the α-A40 Ab (and α-BAP mAb, data not shown).
Fig. 5.20 Generation of recombinant VACVs, expressing recombinant A40 proteins inducibly

(A) Primers A56_R and A40R_R gave a PCR product of approximately 1.6 kbp from viruses where pVOTE-BAPA40ΔTM had recombined into the HA locus. A PCR product of 1.5 kbp was obtained from viruses where pVOTE-BAPA40ΔTMΔSt had recombined successfully and a PCR product of 2.2 kbp was obtained from viruses generated using pVOTE-TAPA40.

(B) Anti-A40 immunoblot of supernatants taken 24 h p.i. from uninfected cells, VOTE-BAPA40ΔTM or VOTE-BAPA40ΔTMΔSt infected cells with or without 2 mM IPTG.

(C) Cells were either mock infected or infected with VOTE-TAPA40 at 5 p.f.u./cell and incubated for 24 h with or without 2 mM IPTG added to the medium. Cell were harvested and lysed in LB1 and analysed for the presence of TAPA40 by SDS-PAGE (15%) and immunoblotting with anti-A40 Ab. Positions of molecular mass markers are shown on the left with sizes in kDa.
5.6 Generation of recombinant baculoviruses expressing A40

To generate an alternative source of unglycosylated, recombinant A40 protein, recombinant baculoviruses were constructed using pBac-2cp. The truncated BAP-tagged A40R alleles from pSecTag2 were inserted into pBac-2cp inframe with the N-terminal His tag. The plasmids pBacHisBAPA40ΔTM and pBacHisBAPA40ΔTM were used to co-transfect Sf9 cells (section 2.6) along with BacVector Triple Cut Virus DNA to generate bvHisBAPA40ΔTM and bvHisBAPA40ΔTMΔSt. The recombinant viruses were then plaque purified on Sf9 cells twice and the presence of the inserted gene was checked by PCR using viral DNA from infected cells as template (section 2.7.11 and Fig. 5.21A). A positive isolate for each virus was used to seed master stocks and these were used to generate high titre stocks from shaking cultures of infected Sf9 cells.

The recombinant baculoviruses were then checked for expression of BAP-tagged A40. Shaking cultures of Sf9 cells were either infected with bvHisBAPA40ΔTM or bvHisBAPA40ΔTMΔSt at 5 p.f.u./cell and samples were removed from the shaking culture at several time-points p.i. The cells were harvested by centrifugation, the supernatant was removed and the pellets were freeze/thawed before being lysed in ILB (section 2.8.10). The lysate was analysed for the expression of His-tagged recombinant A40 using SDS-PAGE and immunoblotting using an α-His Ab (Fig. 5.21B).

A single band at the predicted size of 23 kDa was detected using the α-His Ab on lysates from bvHisBAPA40ΔTM infected Sf9 cells (Fig. 5.21Bi) and a single band at ~20 kDa is seen in bvHisBAPA40ΔTMΔSt infected cells (Fig. 5.21Bii). For both infections, the proteins are expressed from 24 h p.i., and are detectable at 72 h p.i. but seem to be degraded thereafter (Fig. 5.21B).

Having verified that the recombinant viruses express the truncated A40 proteins, a large scale shaking culture was infected with either of the viruses at 5 p.f.u./cell and the cells harvested 72 h p.i. The pellets were lysed and the recombinant A40 applied to a HisTrap column for affinity purification. For both proteins, the HisTrap was not successful and no protein was eluted with imidazole and the recombinant A40 may have precipitated out of solution as the cell lysate supernatant was applied to the column. Due to time constraints, this method of producing recombinant A40 was not further pursued.
Fig. 5.21 Generation of recombinant baculoviruses expressing His-tagged truncated proteins

(A) Primers (N)BiBcin_F and A40R_R gave a PCR product of approximately 450 bp in viruses where pBacHisBAPA40ΔTM had combined with Baculo DNA to produce bacHisA40ΔTM. A PCR product of 390 bp was obtained in viruses where pBacHisBAPA40ΔTMΔSt had recombined successfully.

(B) Sf9 cells were infected with (i) bacHisBAPA40ΔTM or (ii) bacHisBAPA40ΔTMΔSt at 5 p.f.u./ml and samples were taken every 24 h. The cells were lysed and analysed for the presence of His-tagged protein by SDS-PAGE and immunoblotting using α-His Ab. Positions of molecular mass markers are shown on the left with sizes in kDa.
5.7 Summary

Recombinant A40 has been expressed from several systems in an attempt to produce sufficient quantities of protein to aid in the search for a ligand for A40 and help analyse how A40 acts within an infected cell and ultimately its role in an infected host system.

A40 expressed from *E. coli* was used to i) generate a new anti-A40 Ab to help characterise A40 and ii) crystallisation trials in Oxford for structure determination. Whilst A40 was successfully crystallised using this protein, to increase the resolution and solve the structure, a selenomethionine labelled form of the protein will need to be produced and crystallised.

Secreted recombinant forms of A40 were produced in mammalian cells which were glycosylated and similar to A40 expressed by VACV. This protein was then used in cell binding assays (Chapter 6) to find an interacting partner for A40. Stable cell lines expressing these BAP-tagged A40 proteins were produced. BAP-tagged control proteins were also produced and purified to help validate the results of the cell binding assay.

As an alternative source of glycosylated recombinant A40, recombinant VACV were generated that express the secreted forms of BAP-tagged A40 under an IPTG-inducible promoter. These proteins were purified and used in conjunction with the proteins produced from pSecTag to validate cell binding results. A VACV-expressing a full-length FLAG-BAP-tagged A40 virus was also generated and characterised.

Recombinant baculoviruses were also generated expressing His-BAP tagged truncated A40, which did express the recombinant proteins but due to time constraints their purification was not followed up.

These recombinant proteins are a valuable source of reagents for use in further characterising the role of A40 in infection.
Chapter 6. Identifying potential binding partners of A40

6.1 Introduction

To search for potential binding partners of A40, soluble A40 was added to a variety of different cells and binding was measured by flow cytometry.

The recombinant A40 protein expressed from mammalian cells by either pSecTag or the IPTG-inducible VACVs, was characterised and found to be similar to the native A40 made during virus infection. In both cases A40 was modified by N-linked glycosylation and was either transported to the cell surface or secreted (if the N-terminal transmembrane domain was absent). The two different proteins BAP-A40ΔTM and BAP-A40ΔTMΔSt were added to different types of cells. Both proteins were included to assess the importance of the alpha helical region or ‘stalk’ region in the interactions of A40 with potential ligands.

Previously, a similar binding assay has been used to assess the binding of VACV proteins B5, A33, A56 and A34 (Law, 2001) and for other low-affinity binding molecules such as CD48 (Brown et al., 1998). The recombinant proteins were labelled with BirA at a highly specific residue in the BAP sequence. As the interaction between biotin and avidin has a very high affinity (\(K_d = 10^{-14}\) M) and avidin is tetrameric, this system provides a rapid method for multimerising proteins to increase the affinity in binding studies whilst keeping the orientation of the protein with the C-type lectin-like domain exposed externally.

The biotinylated forms of A40 were coupled to fluorescent polystyrene beads covered with avidin and the binding of these proteins to various cells was investigated by fluorescent activated cell sorting (FACS). In addition to the two forms of recombinant A40, three biotinylated proteins were used as controls. There were biotinylated CD4d3d4 (recombinant IgG domains 3 and 4 of rat CD4, (Brown et al., 1998)), mouse CD200 (CD4d3d4-mCD200) and mouse SIRPα (CD4d3d4-mSIRPα) and all contained the BAP sequence preceding the CD4d3d4 domain and were kindly provided by Dr N Barclay (University of Oxford).

The conditions used by M Law (Section 2.8.16.) were selected to trial A40 binding. The binding was performed in binding buffer with 1% BSA, pH 7 and
incubated at 4 °C to try to reduce non-specific phagocytosis of the protein-coated beads. The amount of each recombinant protein was determined by Nanospectrometry and 250 ng of recombinant protein, diluted in binding buffer was added to the washed beaded per assay. The biotinylated-A40 protein coated beads were incubated with the target cells for 1 h before binding was assessed by FACS.

### 6.2 Binding of recombinant A40 proteins to cells

The fluorescent polystyrene beads covered with either form of A40 did not bind to 293T cells (Fig. 6.1A), BS-C-1 cells (Fig. 6.1B) or HeLa cells (Fig. 6.1C). In contrast, binding to RAW 264.7 cells (Fig. 6.3A) B3Z cells (Fig. 6.3B), SP3 cells (Fig. 6.3C), AK31 cells (Fig. 6.2C) and NK92MI cells (Fig. 6.4A) was observed. The same molar amount of the recombinant proteins was used in each assay (250 ng), suggesting that BAPA40ΔTM binds to NK92MI cells at the highest level, followed by B3Z cells, SP3 cells and RAW 264.7 cells (Fig. 6.9). When the binding of BAP-fusion protein coated beads to different cell types was expressed as a percentage of the difference in mean fluorescence between the control (cells and beads only) and BAP-fusion coated beads, there was an increase in mean fluorescence (Fig. 6.9) for Jurkat E6.1 cells (4.81% & 3.7%), RAW cells (BAP-A40ΔTM 63.87% & BAPA40ΔTMASt 62.72%), AK31 cells (4.77% & 100.92%), SP3 cells (49.66% & 67.70%), B3Z cells (34.23% & 75.16%) and NK92MI cells (143.30% & 74.42%). For 293T cells, BS-C-1 cells and HeLa cells there was on average a slight decrease (0.02% & -1.14%), (-3.05% & -0.38%), and (-5.03% & -12.86%) respectively. This suggested a non-specific binding of the avidin-coated beads to these cells in the absence of A40. This non-specific binding was eliminated when the beads were incubated with the BAP-fusion recombinant proteins prior to incubation with cells. This non-specific binding seems to be especially strong on incubation with BS-C-1 cells. For DG75 cells there was a slight increase for BAPA40ΔTM (2.87%) but not for BAPA40ΔTMASt (-1.6%) (Fig. 6.2B). Overall, recombinant A40 binds to NK cells, macrophages and some T cell lines but does not bind to epithelial cells.

To investigate the nature of the putative surface molecule interacting with A40, NK92MI cells were treated with trypsin to remove surface proteins. The cells were harvested in the same way as untreated NK92MI cells except for being incubated in
0.05% Trypsin/EDTA for 10 min at RT before washing once in FBS containing medium and twice in PBS (-Mg\(^{2+}\), -Ca\(^{2+}\)). This treatment inhibited binding of both the recombinant A40 proteins to NK92MI cells (Fig. 6.4B, 29.62% & 7.85%). Therefore, the ligand(s) bound by A40 were either proteins or were molecules bound to proteins.

Having found some cell lines that recombinant A40 could bind to, the binding to primary cells from human PBMCs and mouse bone marrow derived cells and splenocytes was analysed by the same assay. Human PBMCs were harvested from volunteers’ blood samples by Lymphoprep separation and cryo-preserved until required. Once revived, the cells were incubated with human IL-2 overnight and the adherent cells allowed to attach to the culture flask. The non-adherent cells were harvested in the culture medium and analysed separately to the adherent cells (Fig. 6.5). The recombinant A40 proteins did not bind to the non-adherent cells (Fig. 6.5A, BAPA40ΔTM (4.80%) and BAPA40ΔTMΔSt (8.12%)) which represent the lymphocyte populations of PBMCs (Fig. 6.8 A) but did bind to the adherent cell population (Fig. 6.5B) with an increase in mean fluorescence upon incubation with BAPA40ΔTM of 266.63% and of 317.85% for BAPA40ΔTMΔSt. The adherent cell portion of PBMCs is enriched in monocytes, macrophages and IL-2 induced adherent natural killer cells (Fig. 6.5B). The recombinant A40 protein binds to one or more of these cells in the PBMCs but unfortunately, exactly which cell type(s) were binding A40 was not successfully determined in the time available.

When primary mouse cells were analysed, peritoneal macrophages (Fig. 6.6A) gave an increase in fluorescence with BAPA40ΔTM (49.52%) and slightly less with BAPA40ΔTMΔSt (22.83%) whilst with mouse splenocytes there was no strong binding with either the adherent cell population (Fig. 6.6C, 9.88% & 4.01%) or non-adherent cells (Fig. 6.6B, 4.81% & 5.79%).

When the difference in average increase in mean fluorescence for each cell type was analysed, there was no significant difference between the binding capacity of BAPA40ΔTM and BAPA40ΔTMΔSt except for AK31 cells where BAPA40ΔTMΔSt bound to the cells whilst BAP40ΔTM does not (P = 0.007). With B3Z cells, both forms of A40 bound but binding was increased significantly with the form lacking the stalk (P=0.001). Conversely, for NK92MI and the mouse peritoneal macrophages, binding
was statistically significantly increased when A40 has the stalk (P = 0.005 & 0.03, respectively).

To verify that the binding seen with A40 and the cell types was specific, BAP-tagged control proteins were expressed and purified in the same way as the recombinant A40 proteins. Examples of the binding seen with different cell types are shown in Fig. 6.7. As expected CD4d3d4, which is a negative control, did not bind to any of the cell types tested with A40, suggesting that the increase seen in the binding assays with recombinant A40 is specific. CD200, which binds to its ligand CD200R on macrophages from many tissues, shows higher binding to RAW 264.7 cells than seen with the A40 proteins (Fig. 6.7B). This higher affinity for RAW 264.7 cells is also seen with SIRPα, which binds to CD47 on all cells.
Fig. 6.1 Does recombinant biotinylated BAPA40ΔTM or BAPA40ΔTMΔSt bind to epithelial cells?
The biotinylated recombinant A40 proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before being adding cells (2 x 10^5 cells per 5 µl beads). After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data were analysed using Cellquest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells and beads control. Chromatographs are representative of one average binding assay. The assays were repeated at least 3 times. (A) HEK 293T cells, (B) BS-C-1 cells and (C) HeLa cells.
The biotinylated recombinant A40 proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before being added to cells (2 x 10^5 cells per 5 µl beads). After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data were analysed using Cellquest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells and beads control. (A) Jurkat E6.1 T cells, (B) DG75 B cells and (C) AK31 B cells.
Fig. 6.3 Binding of recombinant biotinylated A40 proteins onto immune cells

The biotinylated recombinant A40 proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before being added to cells (2 x 10^5 cells per 5 µl beads). After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data were analysed using Cellquest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells and beads control. (A) Raw 264.7 cells, (B) B3Z hybridoma T cells and (C) SP3 T cells.
Fig. 6.4 Binding of recombinant biotinylated A40 proteins onto NK92MI cells
The biotinylated recombinant A40 proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before being added to cells (2 x 10^5 cells per 5 µl beads). For trypsin treated cells (B), NK92MI cells were treated with 0.5% Trypsin for 5 min, washed in PBS/EDTA and re-suspended in binding buffer prior to incubation with sonicated protein coated beads. After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data was analysed using CellQuest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells and beads control.
The biotinylated recombinant A40 proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before adding cells (5 x 10⁵ cells per 5 µl beads). PBMCs were split into monocyte enriched (Adherent) and lymphocyte enriched (non-adherent) populations by incubation in tissue culture flasks overnight (section 2.3.8). After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data were analysed using CellQuest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells and beads control. 

(A) Non-adherent cell population of human PBMCs, (B) adherent cell population of human PBMCs.
Fig. 6.6 Binding of recombinant biotinylated A40 proteins onto mouse macrophages and splenocytes

The biotinylated recombinant A40 proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before adding cells (5 x 10^5 cells per 5 µl beads). Splenocytes were split into monocyte-enriched (adherent) and lymphocyte enriched (non-adherent) populations by incubation in tissue culture flasks overnight (sections 2.3.8 & 2.9.5) prior to incubation with protein coated beads. After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data were analysed using CellQuest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells & beads control. (A) Mouse peritoneal macrophages, (B) non-adherent cell population of mouse splenocytes, (C) adherent cell population of mouse splenocytes.
Fig. 6.7 Binding of recombinant biotinylated control proteins to validate cell binding data

The biotinylated recombinant A40 proteins or control BAP-tagged proteins were coupled to avidin-coated yellow fluorescent polystyrene beads at a ratio of 50 ng per µl of beads. The beads were washed, re-suspended in binding buffer and sonicated before adding cells (5 x 10^5 cells per 5 µl beads). Human PBMCs were split into monocyte enriched (adherent) and lymphocyte enriched (non-adherent) populations by incubation in tissue culture flasks overnight (sections 2.3.9 & 2.9.5) prior to incubation with protein coated beads. After incubation, the samples were analysed by FACS on a FACS-Calibre (Becton Dickinson, BD). The binding of the fluorescent beads to cells was examined by the filter sets (excitation at 488 nm and emission at 538 nm) and the data were analysed using CellQuest software (BD) and WinMDI. The background fluorescence of the cells without any beads (cells only) is shown as well as cells and beads control. (A) HEK 293T cells, (B) mouse RAW 264.7 macrophage cells,
Fig. 6.8 Cell populations in enriched fractions of human peripheral blood mononuclear cells
Human PBMCs were revived from frozen overnight, in medium containing IL-2. The cells were harvested in adherent and non-adherent fractions (section 2.9.5), re-suspended in FACS buffer and stained for characteristic antigen markers to determine which cell types are within each fraction (i) CD3, (ii) CD20, (iii) CD56, (iv) CD14. (A) non-adherent PBMCs, (B) adherent PBMCs.
Fig. 6.9 Cell binding assessed by increase in mean fluorescence
Increase in mean fluorescence upon incubation of cells pre-coated with either BAPA40ΔTM or BAPA40ΔTMΔSt. n=3 independent experiments. These data are presented as the indicated mean +/- SEM. P MØ = peritoneal macrophage cells, PBMC N = non-adherent PBMCs, PBMC A = adherent PBMCs, Spleen N = non-adherent splenocytes, spleen A = adherent splenocytes. Clear bars = cell binding to BAPA40ΔTM, filled bars = cell binding to BAPA40ΔTMΔSt.
6.3 Immunoprecipitations of A40

To be able to identify a ligand for A40, the ability to precipitate A40 from either infected or transfected cells needed to be demonstrated. BS-C-1 cells were infected with vTAPA40 and induced to express A40 with IPTG for 16 h. The cells were washed and then harvested in lysis buffer. The lysate was divided in half and incubated with either α-A40 Ab pre-bound to protein A sepharose beads or α-BAP Ab bound to protein G sepharose beads. The beads were washed and then re-suspended in SDS loading buffer and any protein precipitated by the Ab-bound beads was analysed by SDS-PAGE and immunoblotting for the presence of A40 protein.

In the lysate incubated with α-A40 Ab, no A40 protein was detected even with overnight exposure of the membrane (Fig. 6.10A) and this might be due to the fact that this polyclonal Ab was raised against antigen derived from expression in *E. coli*. However, using the α-BAP mAb, BAP-tagged A40 was detected in the immunoprecipitated lysate (Fig. 6.10B).

6.3.1 Co-immunoprecipitations using vTAPA40

Having been able to immunoprecipitate A40 from infected cells using an α-BAP Ab, this method was used in co-immunoprecipitations using cells or membrane preparations from cells that bound recombinant A40 in the cell binding assays (section 6.2) including NK92MI, RAW 246.7 and human PBMCs. The co-immunoprecipitations were also performed with cross-linking reagents such as bis (Sulfosuccinimidyl) suberate (BS₃) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) but due to time constraints, this section of work was unable to be completed.
Fig. 6.10 Immunoprecipitation of A40

BS-C-1 cells were infected at 1 p.f.u./ml of vTAPA40 and induced with 2 mM IPTG for 16 h. The cells were harvested (Section 2.8.18) and the lysates were incubated with (A) α-A40 Ab:protein A beads or (B) α-BAP mAb:protein G beads. The immunoprecipitated proteins were analysed by SDS-PAGE (12% gel) followed by immunoblotting with α-A40 Ab. Positions of molecular mass markers are shown on the left with sizes in kDa.
6.4 Summary

As a first step towards understanding the mechanism by which A40 affects the infiltration of leukocytes to the site of infection, the binding of biotinylated A40 to a variety of cell types was investigated. Recombinant A40 was found not to bind to the epithelial cells tested but did bind to various immune cells including NK cells, macrophages and T cells.

In view of these data, binding of A40 to primary human PBMCs was investigated and it was found that A40 bound preferentially to monocyte enriched populations compared to lymphocyte-enriched populations. BAP-tagged A40 protein produced by transient expression in uninfected mammalian cells and also from recombinant-VACV infected cells gave comparable results. Both forms of recombinant A40 (with or without the stalk) behave in a similar manner in the binding assays (with minor exceptions).
Chapter 7. Discussion & Future work

7.1 Introduction

After the eradication of smallpox, research with VACV continued particularly in two areas. The first focused on increasing our understanding of VACV interactions with the host cell and immune system. Not only has this brought advancement in our understanding of how VACV subverts numerous pathways for its own benefit, but it has also increased our understanding of the host immune system and cell signalling pathways. The second area of research with VACV focuses on the development of vaccine vectors. The large size of the VACV genome, the ease of its genetic manipulation and the broad host cell range, as well as the improved safety profile have maintained VACV at the forefront of vaccine research in many disease models.

The two areas converge when considering the numerous VACV proteins that interfere with the host immune response. Identifying these proteins and characterising how they function not only allow better understanding of viral-host interactions, but also enables improvement of VACV immunogenicity. VACV immunomodulatory proteins characterised hitherto can be loosely divided into two groups. VACV proteins that are secreted from the infected cells and bind to inflammatory cytokines, chemokines or IFNs include B18, B8, B15, C12 and CrmE. These bind IFN-α/β, IFN-γ, IL-1β, IL-18 and TNF-α respectively, and thereby prevent the activation of the inflammatory cascades initiated by these cytokines, and impede the host immune response to infection. The second group act within the cell upon intracellular signalling pathways that orchestrate anti-viral responses. This group include A46, A52, B14, K1, K7, and M2 which inhibit the intracellular TLR/IL-1 receptor pathway and NF-κB activation, while F1 and N1 inhibit pathways leading to apoptosis.

While these proteins have been identified and their functions in infection defined, at least in part, there are many VACV proteins that are still ill-defined. At the outset of this project, VACV WR A40 was among these. It was noted that A40 bears sequence similarity to C-type lectins and had a type II membrane topology. Characterisation of VACV WR A40R (Wilcock et al., 1999) showed that the gene was transcribed early during infection and the gene product was glycosylated and present on the surface of infected cell but not incorporated into virions. While the absence of the
A40 protein did not affect the outcome of VACV infection in the mouse i.n. model (Wilcock et al., 1999), it resulted in attenuation in the i.d. model (Tscharke et al., 2002). This suggested A40 might act as an immunomodulator. Unlike the two groups of VACV immunomodulators described above, A40 is present on the cell surface and so might act via altering cell-to-cell contacts. In contrast to the work of Wilcock et al., (1999) another group reported that the A40 protein was modified by sumoylation, allowing it to localise to the viral factories where it was postulated to have a role in the fusion of ER cisternae to enclose the VACV replication sites.

The goals of this project were to re-define the properties of the VACV WR A40 protein including its temporal expression, localisation and post-translational modifications, and to understand where, when and how A40 might exert its function. The mechanisms by which A40 affects the host immune response were investigated focusing on its role in the in vivo intradermal model of infection. To understand the mechanism of action of A40, binding partners for A40 were sought by the production of recombinant A40 that was tagged with a biotin acceptor peptide.

### 7.2 VACV WR A40R gene and protein product

VACV WR A40R is located in the right-hand region of the virus genome, a region rich in immunomodulatory proteins. For instance, gene A41L encodes a CC chemokine binding protein (Ng et al., 2001; Clark et al., 2006; Bahar et al., 2008), gene B8R encodes an IFN-γ receptor (Alcami & Smith, 1995) and gene B15R encodes an IL-1R (Smith & Chan, 1991; Alcami & Smith, 1992; Spriggs et al., 1992a). The A40R ORF is 480 bp long and is predicted to encode a 159 aa protein with a mass of 18.1 kDa. Bioinformatic analysis showed A40 WR is identical to proteins encoded by many strains of VACV including Lister and RPXV Utrecht. In other VACV strains or orthopoxviruses there are closely related proteins that have variations near the C–terminus and some additional amino acid substitutions. For instance, the VACV Copenhagen A40 protein shares 80 % aa identity with VACV WR but differs in the last 22 aa where these residues are replaced with 31 distinct residues due to a frameshift mutation. Whilst VACV A40 WR differs in aa sequence from these strains, the A40 from WR represents the majority species and so justifies why this strain was studied rather than Copenhagen. The Ab raised against the VACV strain WR A40 protein
expressed in bacteria, detected the A40 protein in cells infected by all the VACV strains tested that encode an identical A 40 protein. In addition, this Ab detected slightly different A40 proteins made by other VACV strains including Copenhagen. The ability of this Ab to detect VACV Copenhagen A40 shows that the A40 variants made by VACV strains WR and Copenhagen are stable. Although VACV WR & Wyeth are both derived from NYCBH, the Wyeth strain does not express A40 recognised by this polyclonal Ab. The reasons for this are unclear, but possible explanations could be a promoter or frameshift mutation.

The characteristics of the A40 protein defined by Wilcock et al., (1999) were verified and A40 was shown to be expressed early in infection appearing within 2 h p.i. This is logical for its role in modulating the innate immune response to infection. Its expression prior to viral DNA replication would allow it to interact with the host immune system before late viral proteins are expressed and enable it to either prevent or delay the host response to infection. The A40R gene product is heavily glycosylated with the unglycosylated 18.1 kDa protein representing only a small fraction of the A40 protein produced during infection. Proteins ranging from ~25 – 40 kDa are the major forms produced during infection and these were shown to be glycosylated because treatment of infected cells with tunicamycin, or digestion of A40 protein with the endoglycosidase PNGase F, caused reduction in A40 size. The use of PNGase F to verify the post-translational glycosylation was necessary because tunicamycin can induce ER stress and consequently the production of unmodified A40 might have been due to disruption of the host cell translation and modification machinery. In addition to being glycosylated, analysis of A40 by native PAGE and SDS-PAGE in the absence of β-ME, indicated that A40 can form oligomers, predominantly dimers. The oligomerisation of the glycosylated A40 may allow to A40 to bind to its ligand with higher affinity as exemplified by C-type lectins. The stalk region of C-type lectins, such as DC-SIGN, is important for oligomerisation and this enhances Ag binding (Feinberg et al., 2005). In DC-SIGN, repeats of 23 amino acids form an extended neck region. The regions nearest the CRD stabilise the dimer while the regions near the N terminus are needed to stabilise a tetramer. A40 only contains 20 amino acids in the possible stalk region. This region may be able to stabilise oligomers by forming a coiled-coil and thereby increase the affinity for binding.
While most characterised VACV immunomodulators are either secreted or intracellular, the localisation of A40 at the cell surface with type II topology suggests it modulates the immune response from this location. The type II topology means that the CLECT domain is exposed on the cell surface in a position to interact with other host cells.

The expression and purification of recombinant A40 from *E. coli* enabled the production of another Ab that was very valuable for this project. The new Ab recognised denatured A40 in immunoblots but also native A40 by immunofluorescence and thus allowed the study of A40 localisation within infected or transfected cells. The antibody was raised against the C-type lectin domain and therefore its recognition of A40 on live (non-permeabilised) cells confirmed the type II membrane topology of A40. This knowledge was essential in focusing the project towards binding studies using recombinant A40, rather than focusing on A40 with the infected cells. The recombinant A40 produced in *E. coli* was also used in crystallisation studies. The microfluidic trials produced crystals which diffracted to 8 Å. This was a good step forward, considering the protein was denatured from inclusion bodies and then refolded but needs to be improved. The use of selenomethionine to label the recombinant protein was tried but the yields were too low to be efficient by this mechanism and so the structure of A40 remains unsolved.

### 7.3 Role of A40 in the intradermal immune response

Although the sequence of A40 from VACV strains WR and Copenhagen differ, the fact that deletion of the *A40R* gene from VACV strain WR resulted in virus attenuation and alteration in the immune response to infection, indicated that the WR A40 protein is functional. It was interesting that vΔA40 was attenuated in the i.d. but not i.n. infection model (Wilcock *et al.*, 1999; Tscharke *et al.*, 2002). In this regardA40 is not alone in influencing infection in one model but not another (Tscharke *et al.*, 2002). The comparison of the response to infection by wild type or deletion mutant can be used to dissect the role of a particular protein even if a difference is seen in only one model. A40 may have a measurable effect on the i.d. but not i.n. model because of the difference in cell populations within the two areas. In the i.n. model, the outcome of infection can be measured by studying the weight change and infiltration of leukocytes.
into the lungs or spleen. Within both the i.n. and i.d. model, T lymphocytes are recruited to the sites of infection by day 7 p.i and αβ-TCR+ T cells predominate over γδTCR+ T cells. In contrast to leukocyte recruitment in the i.d. model, neutrophils comprise <10% of the cells recruited to the lungs in an i.n. infection (Reading & Smith, 2003a; Jacobs et al., 2006).

In the i.d model the loss of A40 caused an increase in the recruitment of macrophages and T cells early in infection. The increase in γδTCR cells in the absence of A40 in the i.d. model indicates this could be true as γδTCR cells are a large subpopulation with the skin. The local increase in MHC class II+ cells following i.d. infection with vΔA40R compared to WT virus would attract more CD4+ T cells and these would produce cytokines that help attract CD8+ T cells and other leukocytes to target the infected cell and clear the host of VACV. This correlates with the observed slight increase in CD4+ T cells at day 2 p.i., which is followed at day 4 p.i. by an increase in CD8+ T cells.

The increase in macrophages seen in the absence of A40 allows the immune system to detect the infection earlier and this detection would allow APCs to return to the lymph nodes and interact with T cells, thereby increasing the response to the infected area. The macrophages would also help with the clearance of infected cells. Activation of tissue macrophages would result in secretion of IL-12 and TNF-α. IL-12 induces differentiation of naive T cells to Th1. This promotes the production and release of IFNγ, which in turn promotes IL-12. This feedback loop maintains the activation and infiltration of further macrophage and NK cells. The Th1 response is important in clearing the virus. Collectively, these observations might suggest that A40 is skewing the immune response towards a Th2 biased response.

The recruitment of leukocytes to the skin depends on cytokine and chemokines especially CCL2 & CCL3 (MCP-1 & MIP-1α) which recruit macrophages and T cells. When VACV strain WR gene B14R was deleted, there was also an increase in macrophage and T cell numbers in the infected tissue after i.d. infection (Chen et al., 2008). This was explained by the ability of B14 to inhibit activation of IkB kinase and thereby the downstream pathway leading to NF-κB activation. NF-κB activation is needed for the production of many pro-inflammatory cytokines that are needed for leukocyte recruitment. While A40 could not function in this way because it is
expressed on the cell surface, it might interact with either macrophages or NK cells and thereby modulate the activation of these cells. In turn this would affect the production of cytokines and the consequent recruitment of more cells.

Although there was no significant difference in NK cell number when A40 was absent, A40 could still influence NK function by affecting the activation state of the infiltrating NK cells. Whilst no difference was seen in the cytolytic activity of NK or CTL cells extracted from infected ears, there may be a difference at earlier time-points that was not seen due to the low numbers of cells present. These cells may have been too few to give reproducible data within a cytotoxicity assay. In vitro, it was observed that infection of NK cells prior to incubation with uninfected target cells activated the NK cells and increased their cytotoxicity. This does not correlate with data from Kirwan et al., 2006 and these authors concluded that VACV infection of NK cells inhibited their ability to kill target cells. A plausible explanation is that the extent to which the NK cells are infected determines whether they are activated or rendered non-functional. A higher m.o.i., or infection for longer duration, would tend to render the cells non-functional, whereas a lower m.o.i., or infection for a shorter time might merely activate the cells. The in vitro cytotoxicity assays demonstrated that A40 was produced in infected NK cells. This could be of advantage to the virus by allowing infected NK cells to further modulate the immune response even if viable virus progeny are not produced from these cells. The effect of A40 in vivo on cytotoxicity could be masked by the function of another, yet unidentified, VACV protein or the effect may not be significant at the particular time-points or p.f.u. used in these assays.

The in vitro cytotoxicity assays using the vΔA40 and vTAPA40 to infect target cells prior to incubation with NK cells, showed that the presence of A40 affects the ability of NK cells to lyse infected cells. VACV infection in the absence of A40 slightly increased the ability of NK cells to recognise and kill infected cells. Conversely, in an infection where A40 was over-expressed, the ability of NK cells to kill these infected cells was diminished. This correlates with the ability of recombinant A40 to bind to NK cells. In vivo, the ability of NK cells to recognise infected tissue and lyse target cells as well as the activation and the resultant recruitment of further leukocyte populations is important in the clearance of infection. Whilst this ability to modulate NK killing was not seen in the extracted cells, this may be due to the time-points analysed and the number of cells able to be extracted. Even if A40 does not
affect the number of NK cells recruited to the site of infection, an ability to modulate the activation of the NK cells that are recruited would be advantageous to VACV. By inhibiting NK activation and consequential release of cytokines, A40 would help mask the presence of VACV within the infected tissue, for at least a while, allowing the virus to replicate unhindered by the response mounted by the immune system.

As outlined in section 1.8.3.2, different OPVs affect NK cell function in different ways and A40 could have a novel function not yet seen in these viruses. Whilst MPXV does not affect MHC class I expression greatly, it does encode a protein that down-regulates T cell recognition, needs direct cell-cell contact and is MHC I & II independent (Hammarlund et al., 2008). In contrast, the CPXV protein CPV203 retains MHC class I molecules in the ER, thereby modulating the recognition of infected cells through presented viral peptide surveillance (Byun et al., 2007). Other genes of viruses such as the RCMV encode a PKR-P homologue thereby modulating NK recognition (Voigt et al., 2007). RCMV infection shuts off the expression of Clr-b, leaving infected cells sensitive to NK killing. By acting as a decoy ligand, the RCMV protein RCTL subverts the immune system’s MHC-independent ‘self-nonself’ surveillance. Could A40 act in a similar fashion to the RCMV protein RCTL which protects infected cells from NK killing by it interaction with NKR-P1B?

The modification of VACV strains to generate a better vaccine against smallpox and also to allow VACV to be used as a vector to express foreign antigens for vaccination against other diseases such as HIV or malaria is at the forefront in the study of VACV proteins and their interactions with the host. The consequence of the removal of A40R from VACV WR on virus immunogenicity was analysed by challenging animals that had been immunised with WT, deletion or revertant viruses 28 days previously with VACV WR i.n. This showed that animals immunised with vΔA40 lost less weight upon challenge and so were better protected. This indicates that the removal of A40 would beneficial for vaccine development. Many studies focus on the use of VACV strain MVA, due to its loss of many immunomodulators and its good safety profile, even in immunocompromised individuals. MVA still contains the A40R gene, but the protein has a different C-terminal sequence to VACV WR and this could affect function. However, the effect of this difference remains to be characterised. It remains possible that deletion of A40 from MVA might improve immunogenicity.
The potential for A40 to interact with a ligand on the surface of another cell was investigated by a cell binding assay using recombinant A40 protein fused to BAP. This protein was produced in a mammalian system, allowing it to be glycosylated in the same way that it is during VACV infection. The biotinylation is highly specific and produces a ratio of one biotin molecule per recombinant protein molecule. This allows the molarity of the recombinant proteins to be determined using a known biotinylated standard. As the interaction between biotin and its ligand avidin is specific and high affinity ($K_d = 10^{-14}$), and avidin and its derivatives are tetrameric, this provides a method for multimerising proteins and increasing the avidity for binding studies (Hanke et al., 1999). The biotinylated A40 proteins were bound to avidin-coated fluorescent beads and screened against different cell types. This screen revealed that both recombinant forms of A40 (with or without stalk region between the membrane and CRD) were able to bind some cell types but not others. Epithelial cells such as HeLa, HEK 293T and BS-C-1 cells did not bind A40, but some NK, B and T cells (NK92MI, RAW 246.7, AK31 and B3Z cells) did. Generally, the binding of A40 containing the stalk region was higher than binding of A40 lacking this domain. When this study was expanded to include human PBMCs, recombinant A40 bound preferentially to populations enriched in monocytes. This is consistent with the binding seen to RAW 246.7 cells. CD8$^+$ T cells express NK cell associated molecules like Ly49 and NK1.1 upon activation and this might explain why A40 bound to certain T cells types in culture but not others. The ability of A40 to bind to immune cells and not epithelial cells indicates its function involves the modulation of the immune response not the surrounding epithelial cells or infected cell in which it was produced.

This binding to immune cells suggests that VACV A40 WR belongs to the expanding group of VACV immunomodulators that are not secreted and not involved in intracellular signalling and have been identified in other viruses such as the MHC class I mimics of CMV. In VACV, the group could be said to include VACV B18 and CrmE. While both proteins are present in a soluble form, they also have a transmembrane form which either block IFN-α transmembrane signalling (Colamonici et al., 1995; Alcami et al., 2000) or sequester TNF to prevent TNF-mediated killing (Saraiva & Alcami, 2001; Reading et al., 2002).

The nature of the molecule(s) bound by A40 remain unknown but this (these) includes a protein component because trypsin treatment prevented A40 binding. While
it is possible that the CRD of A40 is binding to carbohydrate attached to a protein backbone, and the trypsin treatment removed this backbone, it seems unlikely that A40 interaction with cells is by carbohydrate alone. This is because all cells have carbohydrate on their surface, yet A40 bound to some cells and not others. A40 lacks the final conserved cysteine found in classical C-type lectins and that is required for carbohydrate binding (Drickamer, 1989). This might suggest that A40 is not a carbohydrate binding lectin. However, other lectins such as Clr lack a conserved cysteine and yet recognise certain GAGs in a Ca\(^{2+}\) independent manner (Plougastel et al., 2001; Carlyle et al., 2004).

### 7.4 Future work

The limitations of time meant that while the characterisation of A40 was advanced in this study, many questions are still unanswered. Using the reagents generated in this project, the study of A40 can be continued and the function of A40 in an immune response investigated further.

Expansion and refinement of the co-immunoprecipitation studies using membrane preparations from positive-binding cell types is a priority for this study.

The analysis of leukocyte infiltration could be expanded to investigate the activation state of the cells recruited in the presence and absence of VACV WR A40. Focusing on early time-points p.i., the recruitment and binding of A40 to other APCs, including DCs, could be investigated. In vivo, the analysis of leukocyte recruitment by flow cytometry could be focused on days 0-4 and on particular subsets of cells. In particular, cells which were relatively rare in the previous samples analysed, which would include but are not limited, to DCs. In vitro, the cell-binding assay could be expanded to include DCs cultured from mouse samples in collaboration with Dr Nathalie Jacob, University of Liege. Focusing on these cell types in i.d. murine samples may overcome the limiting sensitivity of the assay and the rarity of these cells in the previously performed wider screen.

To investigate in greater detail the host response to infection in the presence or absence of A40, the levels of cytokine expression could be measured by RT-PCR using RNA extracted from infected tissue, especially early after infection (day 0 -7). An
increase in pro-inflammatory cytokines might be anticipated in vΔA40 infected tissues that would recruit the increased number of macrophages and T cells to the site of infection. Primers have already been designed to encompass a variety of cytokines and chemokines thought to be important in the recruitment of leukocytes and the response to virus infections. For instance, transforming growth factor (TGF)-β is a chemoattractant for macrophages and it would be interesting to see if levels are elevated in after infection with vΔA40 compared to WT and Rev. Similarly, it would be interesting to investigate if IL-8 was increased after infection by vΔA40. IL-8 attracts neutrophils to sites of infection just before macrophages, which then produce TNF & IL-12.

So far the consequences of immunization with a virus lacking A40 have been investigated focusing on early time points (up to day 10 p.i.) by looking at the cellular response to infection and late time points (28 day p.i.) by evaluating the protection this immunisation provides. But there has been no measurement of antibody responses or the memory T cell response to VACV antigens. These parameters need investigation, for example by measurement of Ab levels and IFN-γ levels in vaccinated mice samples by ELISA.

Using the His-BAP-tagged A40 produced from the baculoviruses generated in this project, the question of whether the glycosylation of A40 affects its binding affinity or range could be investigated. Alternatively, binding studies with de-glycosylated A40 proteins produced in mammalian cells could be performed. To perform these tests efficiently, larger quantities of the mammalian protein should be produced and purified from the stable cell lines grown in multi-flask culture systems. This was not preformed due to time limitations and would only be prioritised after identification of A40’s ligand(s).

In summary, the most important task remaining is the identification of ligands for A40 on the surface of specific cells of the immune system. The identification of these ligands is key to understanding the mechanism of action of A40. The identification of such ligands might improve our understanding of the immune system and as precedent for this, the study of the HCMV UL18 protein and its ligands resulted in the discovery of the leukocyte immunoglobulin-like receptor (LIR)-1 (Cosman et al., 1997).
Chapter 8. References


mediated antitumor gene therapy with p53, IL-2, and IL-12 in a glioma model. 
*Cancer Gene Ther* 7, 1437-47.


Cosman, D., Mullberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M. & Chalupny, N. J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14, 123-33.


Duncan, S. A. (1992) Analysis of three vaccinia virus genes, one of which is essential for plaque formation. Sir William Dunn School of Pathology, Pages.


Hinthong, O., Jin, X. L. & Shisler, J. L. (2008). Characterization of wild-type and mutant vaccinia virus M2L proteins' abilities to localize to the endoplasmic


Leteux, C., Chai, W., Loveless, R. W., Yuen, C. T., Uhlín-Hanssen, L., Combarrous, Y., Jankovic, M., Maric, S. C., Misulovin, Z., Nussenzweig, M. C. & Feizi, T. (2000). The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and B and
sulfated oligosaccharides of blood group Lewis(a) and Lewis(x) types in addition to the sulfated N-glycans of lutropin. *J Exp Med* **191**, 1117-26.


