The Role of the Ectromelia Virus Protein p28 in Apoptosis and Viral Pathogenesis

A thesis submitted for the degree of Doctor of Philosophy at the Australian National University

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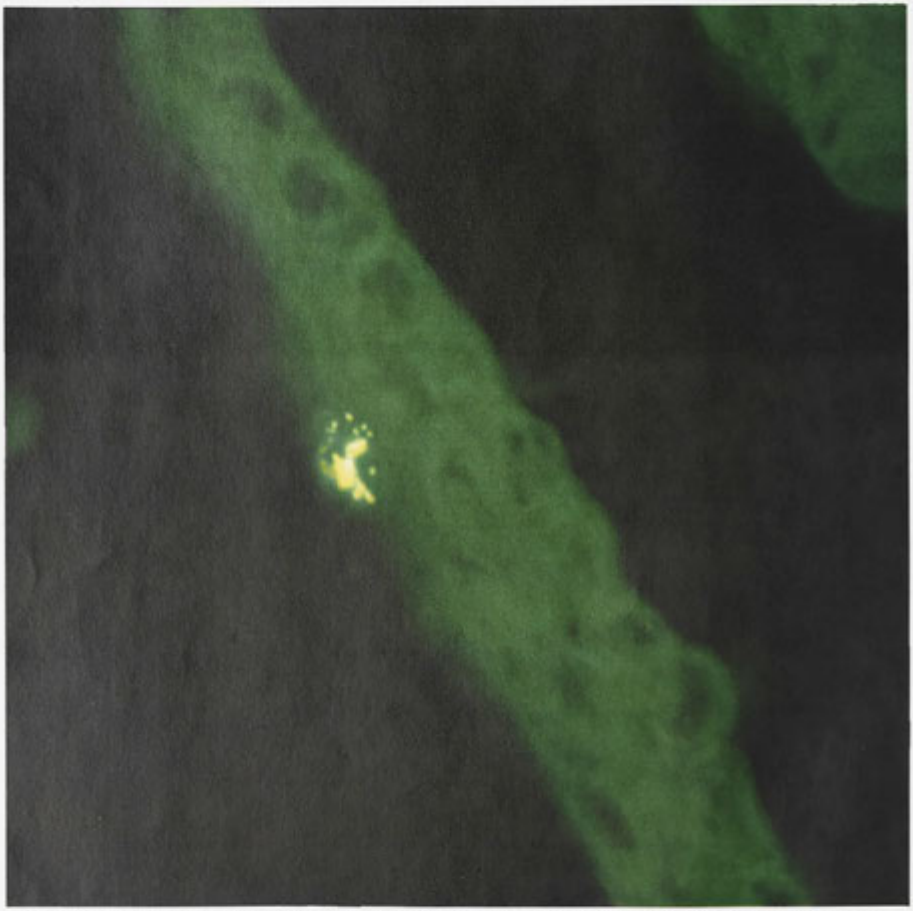
Tables and Figures

p30, paragraph 2, line 5:	for "growth of EV in ANCR mice" substitute "growth of EV in the highly EV-susceptible ANCR mouse strain"
p45, paragraph 2, line 8:	for "designed by SC and Deborah Maguire" substitute "designed by Simone Cuff and Deborah Maguire"
p69, paragraph 3, line 5:	for "which important" substitute "which are important"



Apoptotic cell, situated between bile duct and venule. TUNEL stain: 240x magnification.

Statement

All work described in this thesis is my own work except as outlined below.

The original EVΔp28 virus was created by Tatiana Senkevich
Cl2DMP liposomes were generously provided by Nico van Rooijen
XT-3-11, XT-22 and GL113 antibodies were provided by Janet Ruby, from clones provided by J. Abrams

Simone Cuff

Dedication

For Lois Gould, an unrecognised national treasure.

Acknowledgments

In this tumultuous PhD there have been many people who have helped (or, in some cases, looked in with bemused interest) along the way. In particular, it has been my privilege to work with, and under the watchful eye of, Janet Ruby, who has taught me as much about persistence and critical thinking as about science. Thanks seems just a little inadequate for the support and tireless effort that Janet has put into this work and the development of her students. May she be blessed with a thousand grants and a Nature paper a year!

I'd also like to thank those of JCSMR who helped answer my often obscure yet urgent questions at the drop of a hat: Guna Karupiah, Ian Ramshaw and Alistair Ramsay were especially helpful in furnishing me with answers, mice, equipment and advice. Veronica Ross has been wonderful in helping negotiate the twists and turns of the non-science part of a PhD, and put in time and effort beyond the call of duty when the unexpected has happened. I don't care how much she is paid, she deserves a pay rise. Thanks also to the people of the Graduate School who have taken the thought and time to humanise the administration associated with a PhD.

At Melbourne, I would like to thank Bronwyn Kenshole, Danielle Smith and Steve Turner (with occasional incursions by Naomi Barratt and Sara Hassan) for creating a lively and exciting work environment. Also, from the Zoology department, David Paul and Bruce Abaloz, for teaching me things about microscopes that I didn't know I had to know in time that they didn't really have. John Hill and Stephen Cody generously provided time, disk space, and technical expertise in the use of the confocal microscope, and Paul Waring and Odilia Wijburg patiently taught me how to traverse the uncharted expanse of the murine liver.

From Vet Science, I'd like to thank Jeff Gill, Ian Walker, Craig Cunningham and Liz Washington, for providing me with microscopes, advice (not all of it sought), time, energy, computers, political discourse, sarcastic wit, alcohol and emergency reagents. Jeff and Craig deserve special thanks for their editing. Thanks guys! If you ever slip into sanity, the world will lose a great intellectual resource. Ross Cahill and Wayne

Kimpton also deserve a large vote of thanks for their generosity in allowing me to all but camp in their lab and dominate the computers with little explanation during the writing of this thesis.

To Mum and Gerry, Dad and Vronnie and of course the ever-amazing Maria: thankyou for your unfailing support and understanding - you'll never realise how important that has been. Thanks too to my brother and sisters for bringing the Twilight Zone to life whenever normality threatened.

Finally, my heartfelt thanks to Jason Twohig, whose love, intellectual integrity, scientific curiosity, and ability to make up songs about small fluffy white dogs have been inspiring throughout this PhD.

~

With that I finish thanking the humans. I would also like to put a little note in here to the mice, whom, it seems, get the short end of the stick in science. A lot of mice were used in this study. Without their contribution, none of this work would have been done, and all of the information on p28 would have been at a molecular level; nice, but of unknown physiological significance. To all the mice I used: I tried to do the experiments as rigorously as possible without wasting any of your lives, and I appreciated every single mouse that contributed to this data. I am sure that we are all very relieved that your fellows are now safe for at least another 6 months.

~

Publications & Abstracts

Publications

Cuff SM and Ruby J (1996) "Evasion of apoptosis by DNA viruses" *Immunol Cell Biol* **74** (6):527-37

Cuff SM, Hill J, van Rooijen N, Senkevich T, Buller RML, and Ruby J "An early and distinct apoptotic response to virus infection in vivo is mediated by tumour necrosis factor" *manuscript in preparation*

Cuff SM and Ruby J "Ectromelia virus differentially affects the host response dependent on host strain and the virulence factor p28" *manuscript in preparation*

Proceedings of meetings

Cuff SM and Ruby J (1996) "Apoptosis in viral infections" 8th Annual Conference of the Australasian Society for HIV Medicine, Sydney, NSW.

Cuff SM and Ruby J (1996) "Regulation of apoptosis by host and viral factors in response to infection with ectromelia virus" 26th Annual Scientific Meeting of the Australasian Society for Immunology, Adelaide, SA.

Ruby J, Senkevich T, Buller RML and **Cuff SM** (1997) "A common signalling pathway for apoptosis mediated by CD40 and the p75 TNF receptor" 25th Keystone Symposia, Tamarron, Colorado

Cuff SM and Ruby J (1998) "Tumor necrosis factor (TNF)-dependent responses during poxvirus infection" 7th International TNF Conference, Hyannis Massachusetts

Cuff SM and Ruby J (1998) "Tumor necrosis factor (TNF)-dependent responses during poxvirus infection" 12th International Poxvirus Symposium.

Cuff SM and Ruby J (1998) "An early apoptotic response to virus infection in vivo is mediated by tumour necrosis factor" 28th Annual Scientific Meeting of the Australasian Society for Immunology, Melbourne, Vic.

Cuff SM and Ruby J (1999) "Apoptosis in ectromelia virus infection is specifically blocked by p28" International Union of Microbiological Societies Meeting, Sydney, NSW

List of Abbreviations

129	129Sv mouse strain
AIF	Apoptosis inducing factor
ATP	Adenine triphosphate
B6	C57BL/6 mouse strain
B6/129	C57BL/6 x 129/Sv mouse strain
β2m	β-2 microglobulin
Cl2MDP	Dichloromethylene diphosphonate
cDNA	DNA reverse transcribed from a cellular RNA template
cFLIP	Cellular FLICE-inhibitory protein
CMI	cell mediated immunity
crm	Cytokine response modifier
CTL	Cytotoxic T lymphocyte
d	day/s
ddH_2O	Double-distilled water (milli-Q filtered)
DC	dendritic cell
DD	death domain
DED	death effector domain
DHFR	dihydrofolate reductase
DISC	death inducing signalling complex
DLN	draining lymph node
DMEM	Dulbecco's modified essential media
DNA	deoxyribonucleic acid
DR	death receptor
EDTA	ethylenediaminetetra-acetic acid
ERK	extracellular signal regulated protein kinase

EV	ectromelia virus
EVΔp28	ectromelia virus p28 mutant
FADD	Fas associated death domain (protein)
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
fp	footpad
g	grams
gpt	xanthine-guanine phosphoribosyltransferase
h	hour/s
HCMV	human cytomegalovirus
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
H & E	haemotoxylin and eosin histological stain
HPV	human papillomavirus
HSV	herpes simplex virus
ICE	interleukin 1 converting enzyme
IFN	interferon
I-κB	inhibitor of κb
IL	interleukin
i.p.	intraperitoneal
ITR	inverted terminal repeat
i.v.	intravenous
KIR	natural killer inhibitory receptor
l	litres
LC	Langerhans' cell
LIR	leukocyte inhibitory receptor
LN	lymph node
M	moles
m_	milli_(litres, grams, moles)

μ_	micro_(litres, grams, moles)
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MEM	minimum essential media
MHC	major histocompatability complex
moi	multiplicity of infection
mRNA	messenger RNA
MTD	mean time to death (days)
MTT	thiazolyl blue
muTNF	murine TNF
nm	nanometres
NBF	neutral buffered formalin
neo	neomycin (cassette)
NF-κB	nuclear factor κB
NIK	NF-κB inducing kinase
NK	natural killer (cell)
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
p.i.	post-infection
PLN	popliteal lymph node
POL	polymerase
PS	phosphatidylserine
RING	really interesting new gene
rmp	resistance to mousepox (gene/locus)
RNA	ribonucleic acid
RT	reverse transcriptase

RT-PCR	reverse transcriptase polymerase chain reaction
s	second/s
s.c.	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SM	sphingomyelin
SPI-2	serpin-2
TAP	transporters associated with antigen processing
TcR	T cell receptor
TE	10mM Tris.Cl, 1mM EDTA pH 8.0
TF	transcription factor
TKO	triple knockout (CD40-/-p55-/-p75-/-)
TNF	tumour necrosis factor
TNFR	TNF receptor
Tnfrsf	TNF receptor superfamily
TRADD	TNF receptor associated death domain (protein)
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	terminal dUTP nick end labelling
UL	unique long
US	unique short
UTP	uracil triphosphate
UV	ultraviolet (light)
VV	vaccinia virus
VV-CD40L	vaccinia virus encoding CD40L
VV-TNF	vaccinia virus encoding TNF
wt	wild type

Glossary

Acute	Occurring between 1 and 3d post-inoculation. See also peracute phase of infection
Apoptosis	Specific, morphologically defined form of cell death controlled by intracellular checkpoints. Sometimes referred to as 'cell suicide'.
Attenuation	(of virus) Reduced ability of a virus to replicate *in vivo* (of disease) Reduced pathogenesis of disease
Cachexia	Weight loss as a consequence of disease
EV Moscow strain	Strain of ectromelia virus isolated by Dr. V. D. Soloviev from wild mouse populations. Highly virulent and pathogenic strain of EV.
EV-resistant	Mouse strains in which EV Moscow infection is rarely fatal at normal infectious and experimental doses ($<10^5$pfu)
EV-susceptible	Mouse strains in which EV Moscow infection is usually fatal at normal infectious and experimental doses ($<10^5$pfu)
Hepatomegaly	Enlargement of the liver
Kupffer cells	Resident macrophages of the liver.
Langerhans' cells	Resident dendritic cells of the skin
Morbidity	Symptoms of disease
Mousepox	Disease caused by ectromelia virus
Necrotic (cell)	Cell undergoing uncontrolled cell death. Includes cell death due to trauma and some toxins.

Necrotic (organ)	Organ in which cell death has resulted in breakdown of organ architecture. May be on a small scale, ie groups of several necrotic cells, or larger scale in which both necrotic and apoptotic cells may be evident.
Pathogenesis	Symptoms of disease *in vivo*
Pathogenicity	Ability of an infecting organism to induce pathological changes in the host
Peracute	Occurring within 24h of inoculation. See also acute phase of infection
Plaque	Lesion of liver or spleen macroscopically visible by pale or dark mottling. Microscopically often associated with necrosis.
Pocks	Distinctive cutaneous lesions associated with infection by poxviruses and parapoxviruses. Usually inflamed, leukocytic lesions from which virus may be isolated. Pocks may resolve during infection, leaving hairless scars.
Replication factor	Factor, normally a viral protein, required within the infected cell for replication of the virus particles. See also virulence factor
Splenomegaly	Enlargement of the spleen
Virulence	Ability of a pathogen to replicate *in vivo*. See also pathogenicity.
Virulence factor	Viral protein contributing to viral replication *in vivo* but not required for replication *in vitro*. See also replication factor.

Table of Contents

Abstract

Abstract

Apoptosis is involved in aspects of viral infection from the anti-viral response to virus replication and pathogenesis. This thesis examines the effect of a single antiapoptotic factor on apoptosis and pathogenesis in a natural virus infection of mice. The p28 gene of ectromelia virus (EV) is required for virus replication in A strain but not B6 mice. A p28 mutant EV (EVΔp28) has reduced growth in A strain macrophages, leading to the hypothesis that p28 is a strain specific replication factor. Recently, it has also been discovered that p28 also enables EV to prevent apoptosis induced through CD40 or the p75 TNF receptor in some cell lines. Thus the hypotheses that p28 enhances virulence as a strain specific replication factor, and that p28 is an inhibitor of apoptosis in vivo, were investigated. The effect of p28 on EV virulence and pathogenesis was found to form a spectrum, ranging from necessity for virulence in EV-susceptible mouse strains to no effect on virulence in EV-resistant strains. Unexpectedly, this was independent of both the presence of macrophages and levels of apoptosis during the critical period of infection (d3-9 p.i.). However, expression of p28 was associated with reduced levels of apoptosis in response to EV infection specifically in B6 mice from d3-d9 p.i. Interestingly, the effect of p28 on apoptosis in B6 mice was readily detectable by only 3d p.i., yet was dependent on the presence of CD8+ T cells corresponding with data in other mouse strains suggesting interactions between p28 and cellular antiviral responses. These data suggested that the role of p28 during *in vivo* infection was not directly attributable to a role as a strain-specific replication factor, or preventing apoptosis during d3-9 p.i.

A role for apoptosis early in infection in the priming of the immune response has been suggested by a number of authors. Based on the apoptotic profile in B6 mice, it was hypothesised that p28 may increase EV virulence through preventing apoptosis prior to d3 p.i. It was discovered that the apoptotic response to acute EV infection consists of two distinct, differentially regulated phases. In addition to the response from d3-9 p.i., an additional increase in apoptosis was observed in the livers and ovaries of infected mice within 6h of infection, despite the fact that virus was not yet detectable in these organs. The response was TNF-dependent and only occurred in mice infected with EVΔp28, suggesting that it may be blocked by p28. Apoptosis was dependent on the

presence of the p55 TNF receptor, and required macrophages at the site of inoculation. These observations suggest that TNF synthesis may occur as a very early response to infection, resulting in rapid and transient apoptosis at distal sites. The targeting of the response by EV further suggests that it may be an important component of the host response.

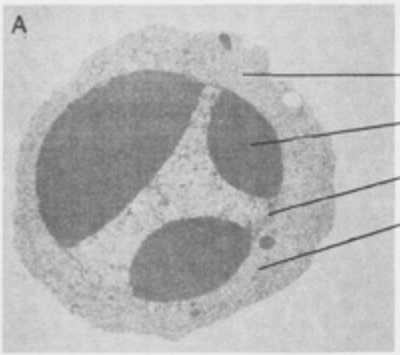
The final question addressed was whether p28 affected apoptosis later in the response through interaction with p75 or CD40 as described *in vitro*. EV and EVΔp28 infection were compared in mice lacking p75, or both p55 and p75 TNF receptors, and in mice lacking two interacting cytokines/cytokine receptors important to the antiviral response: IL-6 and the receptor for IFNγ. All cytokine and cytokine receptor deficient mice were more susceptible to EV than wt mice, but remained resistant to EVΔp28. Closer examination of TNF receptor deficient mice showed that both EV and EVΔp28 mutant viruses replicated to similar levels and induced similar levels of hepatic necrosis, however EVΔp28 stimulated lower levels of leukocyte infiltrates independent of any observable effects on apoptosis. This suggested that effects of p28 from d3-9 p.i. had both TNF-dependent and independent roles. Finally, EV and EVΔp28 infection were compared in CD40-/- mice on EV-resistant and EV-susceptible genetic backgrounds. Results suggested an interaction of p28 with CD40 was important to virulence in the susceptible but not the resistant strain.

The data are consistent with p28 having a role in manipulation of macrophage and TNF function, and suggest that these are likely to be related, especially early in infection. p28 also increased virulence through non-TNF and macrophage dependent pathways. These data also have intriguing implications for TNF and apoptosis during virus infection. The presence of a biphasic response to EV infection implies that systemic sequelae of infection may occur much more rapidly than previously thought. Additionally, the findings in CD40-/- mice suggest that the roles of receptors may differ in importance to the antiviral response in different *in vivo* contexts. By implication, viral factors affecting CD40 may also vary in effect dependent on host strain.

Chapter 1

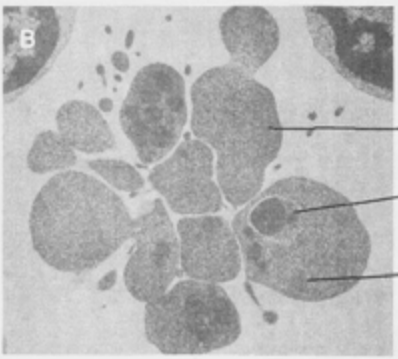
General Introduction

Figure 1.1 Morphological Characteristics of Apoptosis



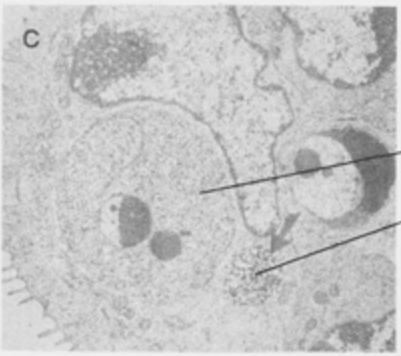
Stage I. Condensation

- Cytoplasmic shrinkage
- Chromatin condensation at nuclear margin
- Nuclear membrane intact
- Organelle morphology preserved



Stage II. Zeiosis

- Membrane bound apoptotic bodies
- Nuclear contents maintained within nuclear membrane
- Organelles intact



Stage III. Uptake of Apoptotic Bodies

- Apoptotic bodies recognised and phagocytosed by surrounding cells
- Contents of apoptotic bodies degraded

Electron micrographs reproduced from "Pathways for Cytolysis" ed. G.M.Griffin and J. Tschopp

1.1 Apoptosis in Virus Infections

Apoptotic cell death is widely recognised as being important both in immune function and in viral pathogenesis. Viral survival strategies reflect this dichotomy: while a high level of apoptosis is a hallmark of some virulent infections (Griebel *et al.*, 1990; Noteborn *et al.*, 1994; Zhang *et al.*, 1996), many viruses encode anti-apoptotic genes, implying that this process of cell death is an important component of the anti-viral response.

There are many potential antiviral roles for apoptosis. The death of host cells prior to the completion of virus replication is likely to be an effective antiviral measure *in vivo*, as shown by studies using Sendai virus and encephalomyocarditis virus (Itoh *et al.*, 1998; Schwarz *et al.*, 1998). Viruses which stimulated rapid apoptosis were found to have reduced viral progeny *in vitro*, correlating with reduced virulence and increased host survival *in vivo*. A second function of apoptosis may be to prime the immune response *in vivo*. Studies by Albert (1997), Bellone (1998) and Inaba *et al.* (1998) have demonstrated that antigens acquired by phagocytic APCs from apoptotic bodies can be presented on class I MHC and are capable of stimulating Ag-specific CTL activity. This suggests that apoptosis may be an elegant mechanism to prime the immune response against intracellular pathogens, yet remain immunologically silent when used to dispose of senescent cells during normal physiology.

Apoptosis may also have proviral roles. Potential roles for apoptosis in enhancing virus growth could include the spreading of virus within apoptotic bodies, or suppression of the immune response. The former is suggested by electron micrographs in which apoptotic debris from ectromelia virus-infected cells can be observed to be phagocytosed before forming new viral factories and subsequent virus particles (Matsumoto 1958). An example of apoptosis in suppression of the T cell response to infection is the measles virus. Infection with measles virus leads to a period of immunosuppression (Griffin and Bellini, 1996) associated with T cell cycle arrest

(Engelking *et al.*, 1999) and enhanced DC and T cell apoptosis (Fugier-Vivier *et al.*, 1997; Okada *et al.*, 2000). This suggests that virus is likely to exploit apoptosis as an effective mechanism of suppressing the T cell response.

1.2 Definition of Apoptosis

Like many physiological processes, cell death does not have a single mechanism or pathway. A range of morphological and biochemical characteristics are seen in cell death, encompassing but not exclusive to those defining classical apoptosis and necrosis.

Classical apoptosis is defined by morphological criteria (Kerr *et al.*, 1972; Fig. 1.1). Early cytoplasmic changes lead to the cell detaching from neighbouring cells and losing specialised surface elements such as microvilli and gap junctions. Concurrently, the nucleus shows characteristic chromatin condensation as the nucleoskeleton becomes compromised. This results in chromatin coalescing to form distinctive electron-dense crescents adjacent to the nuclear membrane. Zeiosis – an active bubbling of the cytoplasmic membrane – and blebbing of the nucleus is then noticeable, although the organelles remain intact and the nucleus and cytoplasm remain membrane bound. As the cytoskeleton is broken down, the cytoplasm and nucleus begin to bleb into apoptotic bodies containing compartmentalised nuclear and cytoplasmic material, including intact organelles. The apoptotic bodies are rapidly phagocytosed by the surrounding cells and degraded within lysosomes. The process can be surprisingly rapid, with the entire process from detachment to degradation taking as little as 20 minutes.

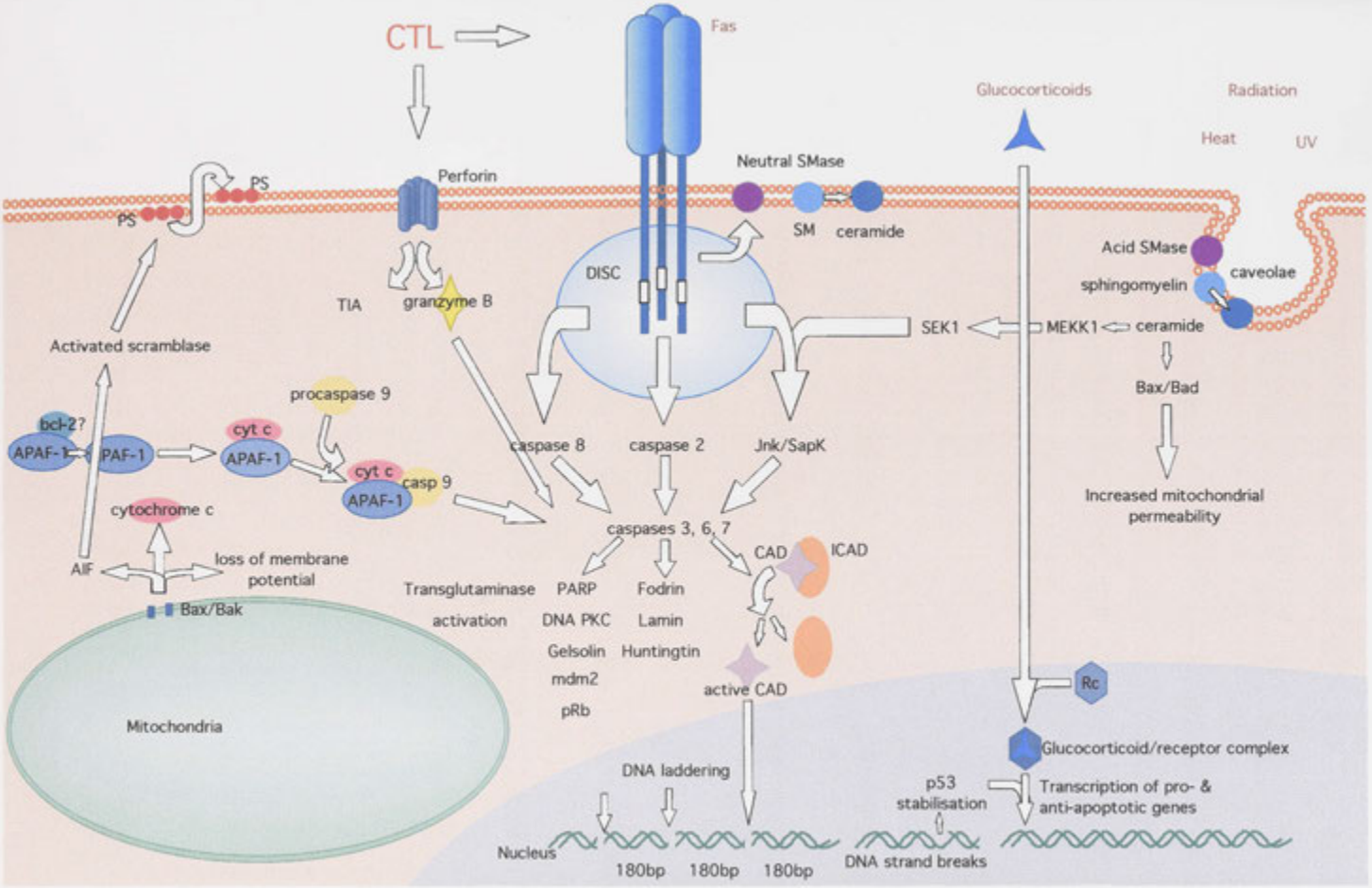
The morphological changes seen in apoptosis are accompanied by distinctive, tightly regulated biochemical cascades. Mitochondria in particular play an essential role in apoptosis. The membrane permeability transition of mitochondria, characterised by a loss of membrane potential (Zoratti and Szabo, 1995) and release of apoptosis-inducing factor (AIF) from the intermembrane space (Susin *et al.*, 1999) is pivotal in apoptosis,

and is thought to be the point at which cell death becomes irreversible. This is usually accompanied by the release of cytochrome c and formation of the pro-apoptotic "apoptosome" complex (see Fig. 1.2; Susin *et al.*, 1999). As the process of apoptosis continues, multiple proteases, particularly the caspase family, are activated. Caspases are important in the apoptotic response as a mechanism of amplifying the initial apoptotic signal, and as effector proteases (for a recent review see Rathmell and Thompson, 1999). The 14 caspases currently known can be defined as initiator or effector caspases, with some overlap in actual function. Initiator caspases can be defined by their long prodomain, usually a protein-protein interaction domain (Slee *et al.*, 1999). On receipt of an apoptotic signal, aggregation of inactive initiator caspases is thought to trigger self-cleavage into active caspases. In addition to cleaving apoptotic targets such as plectin (Stegh *et al.*, 2000) the initiator caspases amplify the original signal by further activation of effector caspases. These are important effectors of apoptosis, playing roles in dismantling the cytoskeleton through cleaving of globular actin and lamins (Rao *et al.*, 1996), and activating further enzymes in the process of apoptosis, such as caspase activated DNase (CAD; Enari *et al.*, 1998). The release of CAD from its inhibitor (ICAD) results in DNA being cleaved in the regions between histones. This leads to the 180bp ladder often used as a biochemical marker of apoptosis (Wyllie, 1980). The translocation of phosphatidylserine from the inner leaf of the cytoplasmic membrane to the outer surface of the cell is also an early event characteristic of apoptosis, which later allows the recognition and phagocytosis of apoptotic bodies by surrounding cells (Fadok *et al.*, 1992; Fadok *et al.*, 2000).

The progression of a cell through apoptosis contrasts starkly with the process of necrosis. Necrosis occurs as a result of an uncontrolled change to the cell or its environment, such as some cytotoxic agents, change of extracellular osmolarity, or a breach of the cytoplasmic membrane. Normally, this results in the cytoplasm and

Figure 1.2 Pathways to apoptosis

Apoptosis initiated by a diverse range of stimuli converge on common pathways: caspase activation, mitochondrial events, and nuclear events. The cytotoxic T cell response can cause lytic death through insertion of perforin as shown. It may have additional roles in disruption of cytoplasmic calcium levels, and regulation of granzyme function (see text for details).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Radiation
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Perforin
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SM
ceramide
Acid SMase
caveolae
sphingomyelin
DISC
TIA
granzyme B
SEK1
MEKK1
ceramide
Activated scramblase
Bax/Bad
procaspase 9
bcl-2?
APAF-1
PAF-1
cyt c
APAF-1
caspase 8
caspase 2
Jnk/SapK
Increased mitochondrial permeability
cyt c
casp 9
APAF-1
cytochrome c
caspases 3, 6, 7
CAD
ICAD
AIF
loss of membrane potential
Transglutaminase activation
PARP
DNA PKC
Gelsolin
mdm2
pRb
Fodrin
Lamin
Huntingtin
Bax/Bak
active CAD
Rc
Mitochondria
Glucocorticoid/receptor complex
DNA laddering
p53 stabilisation
Transcription of pro- & anti-apoptotic genes
Nucleus
180bp
180bp
180bp
DNA strand breaks

organelles of the cell swelling and bursting, releasing cytoplasmic contents into the extracellular milieu. The uncontrolled nature of necrosis is a counterpoint to the tightly regulated mechanism of apoptosis both mechanistically and in the immunological response to death. After apoptosis, the cellular contents are sequestered into surrounding cells, limiting inflammation, but potentially stimulating cell-mediated immunity through presentation of antigen (Albert *et al.*, 1997; Bellone *et al.*, 1998; Inaba *et al.*, 1998). In contrast, necrosis stimulates non-specific inflammation. Interestingly, apoptotic bodies not taken up by surrounding cells undergo secondary necrosis, resulting in the same inflammatory response as after conventional necrosis (Kerr *et al.*, 1972).

1.3 Triggers for Apoptosis During Viral Infection

Apoptosis can be triggered at many stages during viral infection by intracellular or extracellular stimulation. The importance of apoptosis in virus infection is supported by the fantastic array of viral anti-apoptotic strategies which exist (Table 1.1). These include latency, mimicry of host anti-apoptotic proteins, degradation of host pro-apoptotic proteins, sequestration of triggers for apoptosis, and the blocking of signalling pathways by inactive pseudosubstrates.

Three apoptotic stimuli of importance during viral infection – detection of intracellular abnormalities, cytotoxic granules and cytokine receptor mediated apoptosis - and corresponding mechanisms of viral evasion of apoptosis are shown here as an illustration of the interplay of host and virus in manipulating apoptosis.

Table 1.1 Mechanisms of viral inhibition of apoptosis

Pathway	Mechanism	Gene	Virus	Reference
Cell-mediated cytotoxicity				
Recognition of infected cells: The MHC	Blocking MHC transport through Golgi	E19 US3 m152	Adenovirus HCMV MCMV	(Andersson *et al.*, 1985) (Jones *et al.*, 1996) (Ziegler *et al.*, 1997)
	Blocking TAP function	ICP47 US6	HSV HCMV	(Hill *et al.*, 1995) (Ahn *et al.*, 1997)
Recognition of infected cells by NK cells	MHC class I mimics	UL18 m144	HCMV MCMV	(Reyburn *et al.*, 1997) (Kubota *et al.*, 1999)
Granule-mediated apoptosis	Pseudosubstrate of granzyme B	CrmA	Cowpox	(Quan *et al.*, 1995)
Receptor-mediated cytotoxicity				
TNF-mediated apoptosis	TNF receptor homologues	CrmB, C Crm D T2	Cowpox EV Myxomavirus	(Alcami *et al.*, 1999) (Alcami *et al.*, 1999) (Macen *et al.*, 1996)
	cIAP homologues	p35 A224L	Baculovirus ASFV	(Clem and Miller, 1993) (Chacon *et al.*, 1995)
	Blocking of caspase 8 binding through DED	E8 K13 BORFE2 MC160L	EHV-2 HHV-8 BHV-4 Molluscum contagiosum	(Wang *et al.*, 1997a) (Wang *et al.*, 1997a) (Wang *et al.*, 1997a) (Hu *et al.*, 1997) (Bertin *et al.*, 1997)
Cell-Cycle Progression and Apoptosis				
The p53 pathway	Inhibition of p53 transcriptional activity	T antigen	SV40	McCarthy et al. (1994)
	P53 degradation	E6	HPV-16, -18	(Scheffner *et al.*, 1990) (Werness *et al.*, 1990)
Bcl-2 family members	Bcl-2 homologue	E1B-19K A179L	Adenovirus ASFV	(Rao *et al.*, 1992) (Brun *et al.*, 1996)

1.3.1 Intracellular Triggers - Inappropriate Cell Cycling

One of the important roles of apoptosis during normal physiology is the destruction of defective cells prior to cell replication. Cellular abnormalities and cell cycle perturbation are detected by a number of semi-redundant systems within the cell, some of which stimulate cell cycle (comprehensively reviewed in Kohn, 1999). Arrest of the cell cycle allows repair of cell damage prior to S phase. On repair, the stimulus for cell cycle arrest is removed and the cell can resume normal cycling. Apoptosis may be induced in situations in which cells are unable to remove the stimulus for cell cycle arrest, or in cells in which cell cycle arrest and progression are concurrently stimulated. Virally-induced apoptosis may be stimulated through either route. Examples include cell cycle arrest and apoptosis on cellular detection of viral dsRNA (Balachandran *et al.*, 2000; Kaufman, 1999; Kibler *et al.*, 1997; Lee and Esteban, 1994; Takizawa *et al.*, 1996) and the stimulation of inappropriate cell cycling in non-cycling cell types by viral infection (Barry and McFadden, 1998; Fotedar *et al.*, 1996; Moran, 1993; Op De Beeck and Caillet-Fauquet, 1997). The latter is particularly important for viral infections such as adenoviruses and papillomaviruses which stimulate partial cell cycle progression in quiescent cell populations as part of the viral replication cycle.

1.3.1.1 Stimulation of apoptosis through the p53 pathway

Many of the cellular mechanisms for detection of DNA or RNA abnormalities cause cell cycle arrest by the stabilisation of the nucleoprotein p53. During normal cell cycling, p53 has a half-life of around 20 min (Olson *et al.*, 1993). Degradation of p53 is enhanced by binding to mdm-2, which is also a negative regulator of p53-mediated transcription (Fuchs *et al.*, 1998; Honda *et al.*, 1997; Momand *et al.*, 1992; Tao and Levine, 1999). The presence of DNA damage (Shieh *et al.*, 1997) or dsRNA (Cuddihy *et al.*, 1999) leads to post-translational modification of p53 (phosphorylation and acetylation), preventing mdm-2 binding (Shieh *et al.*, 1997; Shieh *et al.*, 1999) and p53 degradation. p53 then forms tetramers, which are active as transcription factors (TF;

Sakaguchi *et al.*, 1997). These directly upregulate cell cycle arrest genes such as pRb and $p21^{waf1}$ (Shiio *et al.*, 1992; El Deiry *et al.*, 1993), feedback inhibitors of the p53 pathway (mdm-2: (Perry *et al.*, 1993) and TFs such as c-fos and c-jun (Ginsberg *et al.*, 1991), while downregulating cell cycle progression genes, such as the E2F family. Thus increased levels of p53 can induce cell cycle arrest.

Accumulation of excess p53 is also associated with apoptosis induction. This is at least partially attributable to the upregulation of bax transcription by p53 and concomitant decrease in the transcription of bcl-2 (Miyashita et al., 1994). While further molecular mechanisms dictating whether stabilisation of p28 induces cell cycle arrest or apoptosis are still uncertain (Matlashewski 1999), the concurrent accumulation of p53, promoting cell cycle arrest, and cell cycle promoting factors such as the E2F family of transcription factors are a strong indicator of apoptosis induction (Kowalik et al., 1995; Shan and Lee, 1994; Wu and Levine, 1994). During cell cycle arrest and repair, apoptosis through the simultaneous stimulation of cell cycle arrest and progression is prevented by the p53-mediated upregulation of pRb. pRb, and the related proteins p107 and p130, bind to E2F and other cell cycle promoting transcription factors to downregulate transcription from a variety of promoters associated with cell cycle progression including DHFR (Blake and Azizkhan, 1989), POLα (Pearson et al., 1991), cyclin A (Schulze et al., 1995), cyclin D (Sala et al., 1994) and cyclin E (Duronio and O'Farrell, 1995).

The importance of the p53 pathway for apoptosis induction during infection with some viruses can be assessed by the finding that nuclear DNA viruses including the papillomaviruses and adenoviruses have evolved multiple pathways with which to block the p53/pRb pathway through convergent evolution.

1.3.1.2 Degradation of host proteins

The interaction of human papillomavirus 16 and 18 (HPV-16 and HPV-18) with the p53 system illustrates the effectiveness of degradation of host pro-apoptotic proteins as a viral anti-apoptotic strategy. HPV-16 and 18 are tumorigenic viruses estimated to be associated with 93% of cervical cancers (Walboomers *et al.*, 1999). The virus infects the quiescent cervical epithelium and exploits two early genes –E6 and E7- in order to stimulate cell cycle progression without inducing apoptosis. The E7 protein physically complexes pRb, preventing downregulation of E2F and so stimulating cell cycle progression. The loss of pRb concurrently leads to stabilisation and overall increased p53 levels. Thus E7 expression in isolation stimulates a rapid apoptotic response (Jones *et al.*, 1997). However, E7 expression in concert with the HPV protein E6 results in maintenance of the cell cycle (Howes *et al.*, 1994; Pan and Griep, 1994). E6 is an early protein which concurrently binds p53 and E6AP, a ubiquitin ligase (Scheffner *et al.*, 1993). This directly flags p53 for degradation, thus very effectively preventing p53-mediated apoptosis.

1.3.2 Cell-Mediated Apoptosis

1.3.2.1 The cytotoxic granules of CTLs and NK cells

Cytotoxic T lymphocytes (CTLs) and natural killer cells (NK cells) provide complementary cell mediated responses during virus infection. CTL-mediated apoptosis is stimulated after the recognition of the MHC class I of the target cell or the cognate T cell receptor (TCR) of the CTL, facilitated by integrins (reviewed in Garcia *et al.*, 1999). NK cells recognise cells through NK cell receptors, which can be either stimulatory or inhibitory (reviewed in Lanier, 1998). Each cell type then has the capacity to induce apoptosis through two pathways: cytotoxic granules and the TNF receptor superfamily.

Cytotoxic granules contain a number of effector molecules, including perforin, granzymes (notably granzymes A and B), and TIA-1. During CTL-mediated cytotoxicity, a tight junction forms between the effector and target cells and cytotoxic granules are delivered to the target cell (Yannelli *et al.*, 1986). The components of the granules each stimulate complementary aspects of apoptosis.

Perforin is a C9-like glycoprotein which aggregates into transmembrane pores in a calcium-dependent manner (Krahenbuhl and Tschopp, 1991). Perforin is of importance for the induction of cytotoxicity (Kagi *et al.*, 1994), and the antiviral activity of CTL and NK cells (Mullbacher *et al.*, 1999a), however the mechanism is a source of debate. Since single channel electrophysical readings have not detected any closing events for the pores (Peters *et al.*, 1990), it would be expected that perforin pore formation would lead to cell lysis and necrotic death. In some cell types perforin-dependent lysis can be induced independent of other granule constituents (Persechini *et al.*, 1990), however CTLs more commonly induce target cell apoptosis. It is likely that perforin contributes to apoptosis through interaction with granzymes, since apoptotic changes to target cells in response to granzymes require perforin (Hayes *et al.*, 1989). Direct observation of granzyme colocalisation with and without perforin suggests that the role may be in delivery of granzymes to the nucleus of the target cell (Jans *et al.*, 1998).

Granzymes are serine proteases with esterase activity and various cleavage site specificities. They are expressed not only in CTLs and NK cells, but also in non-cytolytic T cells and bone-marrow derived mast cells (Jenne and Tschopp, 1988). Most granzyme activity of CTL and NK cell granules is mediated by granzymes A and B.

Granzyme B is the granzyme most closely associated with apoptosis. It has a similar enzymatic activity to the caspase family, and is capable of inducing the apoptotic cascade by cleaving and so activating the effector caspase, caspase 3 (Darmon *et al.*, 1995; Quan *et al.*, 1996; van de Craen *et al.*, 1997). Interestingly, activation of caspases is necessary for the induction of nuclear changes associated with apoptosis after

exposure to granzyme B (Sarin *et al.*, 1998), but apoptotic changes in the cytoplasm induced by the granzyme do not require caspase activity (Atkinson *et al.*, 1998; Heibein *et al.*, 1999). This may be because granzyme B is capable of cleaving some downstream caspase substrates directly (Andrade *et al.*, 1998; Barry *et al.*, 2000; Thomas *et al.*, 2000).

Granzyme A is a tryptase exclusively expressed in CTLs. Granzyme A can induce nuclear apoptotic morphology (Shi *et al.*, 1992) and is synergistic with granzyme B (Nakajima *et al.*, 1995). While the CTLs of granzyme A-deficient mice are functionally indistinguishable from those of wt mice, possibly due to redundancy with granzyme K (Wilharm *et al.*, 1999), the mice have increased susceptibility to some viral infections (Mullbacher *et al.*, 1996), suggesting an important role in the anti-viral response.

Cytotoxic granules also stimulate apoptosis through TIA-1 and TIA-R. These closely related RNA-binding proteins are capable of inducing DNA fragmentation in mammalian cells (Taupin *et al.*, 1995; Tian *et al.*, 1991) during CTL-mediated apoptosis and specific phases of foetal development (Lowin *et al.*, 1996). The link between the binding of the RNA and the DNA degradation is not yet known.

Each of the above proteins is capable of inducing apoptosis independently, however they are also synergistic (Nakajima *et al.*, 1995). Together with the induction of apoptosis through Fas (CTLs: Lowin *et al.*, 1994) this provides a partially redundant, and highly effective, system for the detection and apoptosis of virally infected cells.

1.3.2.2 Blocking of host protein transport

The evasion of CTL responses is particularly important in latent or long-term virus infections. As a consequence, viruses such as the herpes viruses down-regulate MHC class I by inhibiting MHC class I complex formation at several stages of assembly and transport, including the loading of the MHC class I with peptide. During assembly, peptides derived from degradation pathways active in the cytosol must be transported

into the lumen of the endoplasmic reticulum in order to bind and stabilise the immature MHC class I complexes. The translocation occurs through the transporter associated with antigen processing (TAP), in an ATP-dependent process (Lankat and Tampe, 1999). The herpes viruses herpes simplex virus (HSV) (Hill *et al.*, 1995; Tomazin *et al.*, 1996) and human cytomegalovirus (HCMV) (Ahn *et al.*, 1997; Hengel *et al.*, 1997) stably bind the TAP heterodimer, preventing transport of the peptides through the TAP, and thus trap the nascent MHC class I complexes in the ER. In the absence of peptide, these are degraded, and levels of MHC class I presented by the cell are reduced. In addition, ICP47 may destabilise the TAP heterodimer structure, further inhibiting function (Lacaille and Androlewicz 1998). Interestingly, the strategy of TAP inhibition appears to have evolved independently in each virus strain, as ICP47 and US6 are not homologous, and bind in separate domains of the TAP complex.

1.3.2.3 Mimicry

Mimicry of host proteins is an extremely common mechanism for the prevention of apoptosis of infected cells. Mimics or homologues exist of many host proteins, including cytokine receptors (Barry and McFadden, 1997), cell cycle regulators (Lee and Reddy 1999) and antiapoptotic genes (Subramanian *et al.*, 1995). A number of viruses also use homologues of host proteins to prevent CTL or NK cell mediated detection and apoptosis. The human cytomegalovirus (HCMV) MHC class I homologue UL18 is an MHC class I homologue expressed at the cell surface complexed with β2-microglobulin, and capable of binding peptides in the peptide cleft (Browne *et al.*, 1990; Chapman and Bjorkman, 1998; Fahnestock *et al.*, 1995). Expression of UL18 enables the virus to avoid NK mediated cell death despite MHC class I downregulation. UL18 is hypothesised to protect against NK cell mediated death by binding the leukocyte immunoglobulin-like receptor-1 (LIR-1), a glycoprotein closely related to the natural killer inhibitory receptors (KIR), with an affinity >1000 fold higher than the affinity for class I MHC (Chapman *et al.*, 1999). This is consistent with studies in which UL18

transfection of cell lines leads to resistance to killing by IL-2 activated NK cells in virus-free systems (Reyburn *et al.*, 1997).

1.3.3 Receptor-Ligand Interactions

1.3.3.1 The TNF receptor superfamily

The TNF receptor superfamily is defined by the presence of distinctive cysteine-rich pseudorepeats in the extracellular portion of the protein. More than 20 superfamily members have been identified, including OPG, OX40, CD40, CD30, Fas, TNFR 1 (p55), TNFR 2 (p75) and the death receptors (DR1-5). Most members are type I membrane proteins, with the exceptions being two soluble TNF receptor homologues encoded by poxviruses (Smith *et al.*, 1991) and soluble TNF receptor variants. The intracellular domains show no intrinsic enzymatic activity, but instead contain protein-protein interaction domains. Members of the family can be separated into subgroups on the basis of their intracellular domains. In particular, subgroups are defined by the presence or absence of death domains (DDs), or TRAF domains (Fig. 1.3). The presence of these domains allows interactions with other DD or TRAF proteins respectively.

TNF receptor superfamily members have a diverse range of functions, with activities in CD4+ T cell activity (Lane 2000), activation of NK cells (Martin-Fotecha *et al.*, 1999), B cell function (Renshaw *et al.*, 1994), osteoclast function (Wong *et al.*, 1999), increasing vascular permeability (Goldblum *et al.*, 1988; Horvath *et al.*, 1988), stimulation of chemokine production (Tessier *et al.*, 1997), and activation of macrophage functions (Conkling *et al.*, 1988), as well as induction of apoptosis. TNF-mediated apoptosis is most closely associated with stimulation of the death domain receptors: prototypically, p55 (CD120a, TNFR1, Tnfrsf1a) and Fas (CD95, APO-1) (Itoh *et al.*, 1991; Wong and Goeddel, 1994). However, it has also been noted recently

that apoptosis can be signalled via non death domain receptors such as p75 (CD120b, TNFR2, Tnfrsf1b) and CD40 (Hess and Engelmann, 1996; Weiss *et al.*, 1997).

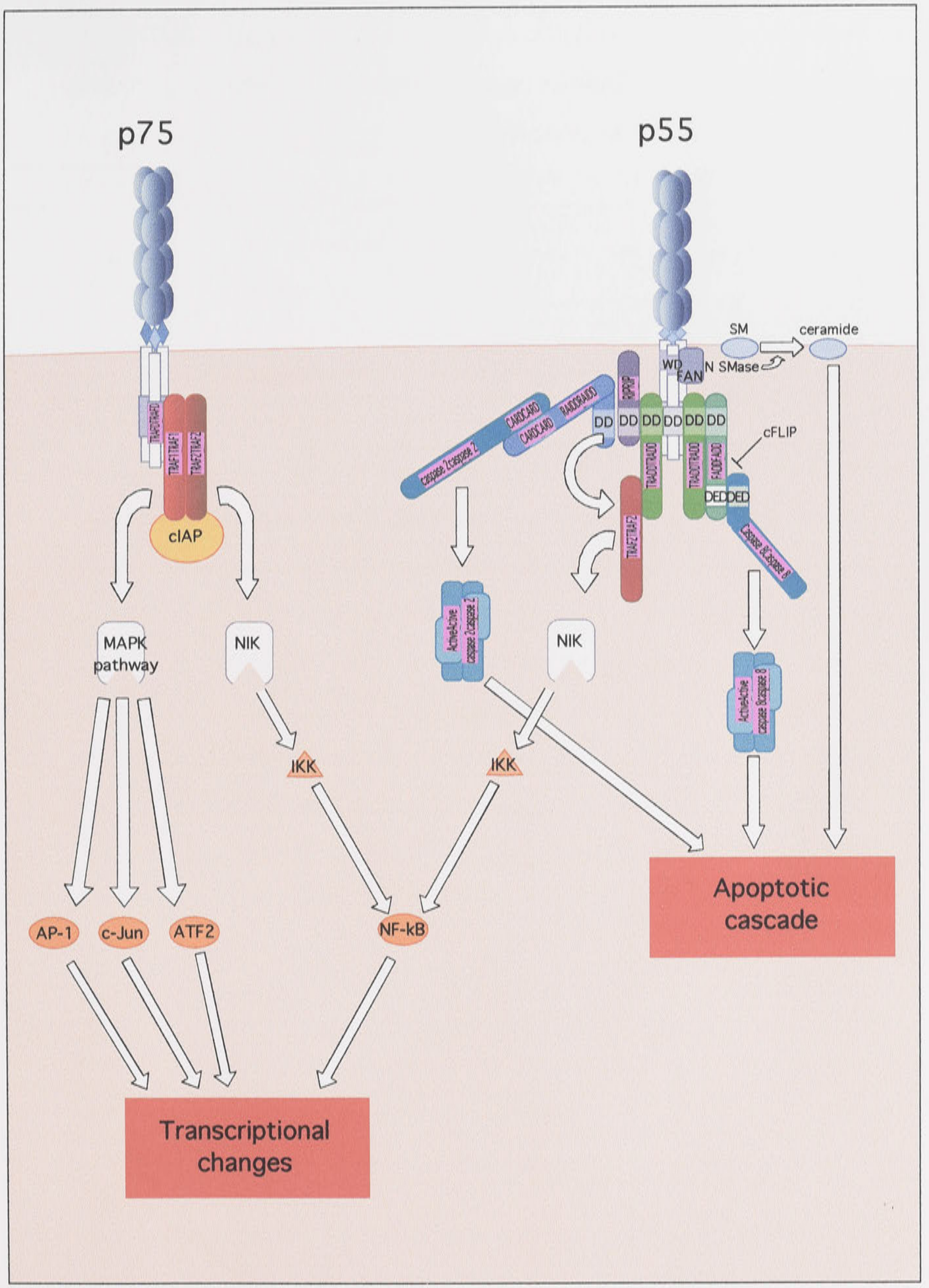
1.3.3.2 DD and non-DD stimulated apoptosis

The "death receptors" are defined by the presence of a cytoplasmic death domain (DD). The DD is a protein docking site, which serves as a seeding point for the complexing of the death-inducing signalling complex (DISC) (Eberstadt *et al.*, 1997). The DISC of the different DD receptors differ in exact components, but remain the same in principle. The DD of p55 recruits TRADD, a cytoplasmic DD protein which further binds FADD to the complex (Hsu *et al.*, 1996). FADD contains death effector domains (DEDs), also protein docking sites, which are necessary for the binding of pro-caspase 8 (FLICE) (Muzio *et al.*, 1996). The aggregation of caspase 8 pro-enzymes in the DISC appears to trigger self-cleavage, resulting in activation and release of the enzyme. Once activated, caspase 8 is in turn capable of activating effector caspases such as caspase 3 and thus stimulating the apoptotic cascade (Srinivasula *et al.*, 1996; Fig. 1.3). Caspase 8 also participates directly in apoptosis through the cleavage of the anti-apoptotic bcl-2 family member BID (Li *et al.*, 1998; Luo *et al.*, 1998) and cytoskeletal components (Stegh *et al.*, 2000). Overexpression studies have also shown that p55 ligation can activate caspase 2 through the cytoplasmic DD protein RAIDD (Duan and Dixit, 1997), although given the lack of p55 signalled cell death in caspase 8 null mice (Varfolomeev *et al.*, 1998), the relevance of this *in vivo* is questionable.

In addition to apoptosis, TRADD binding to the DD may also stimulate transcriptional changes signalled by p55. When overexpressed, TRADD can heterodimerise with TRAF2 (Hsu *et al.*, 1996), a signalling molecule more commonly associated with the signalling of transcriptional changes through the p75 TNF receptor. The dimer binds and allows the activation of NIK (NF-κB inducing kinase) (Malinin *et al.*, 1997;

Figure 1.3 Pathways of TNF receptor superfamily signalling

Adapted from (Darnay *et al.*, 1997) and (Wallach, 1999).


p75
p55
TRADD
TRAF1
TRAF2
cIAP
SM
ceramide
N SMase
WD
FAN
DD
CARD
caspase 2
RAIDD
cFLIP
DED
caspase 8
Active caspase 2
Active caspase 8
MAPK pathway
NIK
IKK
AP-1
c-Jun
ATF2
NF-kB
Apoptotic cascade
Transcriptional changes

Pomerantz and Baltimore, 1999) and degradation of the inhibitor of NF-κB (I-κB; Song *et al.*, 1997), allowing NF-κB activity.

p55 is also capable of transducing signals through other protein-protein interaction domains. In particular, FAN, an adaptor protein required for neutral sphingomyelinase activation by this receptor, binds to a stretch of nine residues upstream of the DD (Adam-Klages *et al.*, 1996). Activation of neutral sphingomyelinase leads to activation of the ERK pathway, ultimately activating NF-κB and resulting in transcriptional consequences of p55 TNF receptor binding (Adam *et al.*, 1996).

Non-death domain receptors include CD27, CD30, CD40 and the p75 TNF receptor. These receptors have a wide variety of functions which are predominantly effected through transcriptional changes. In addition, CD40 and p75 are capable of enhancing apoptosis when coexpressed with p55, and may stimulate apoptosis individually (Declercq *et al.*, 1998; Grell *et al.*, 1999; Weiss *et al.*, 1997), despite the lack of a DD. The primary signalling molecules for non-DD receptors are the TNF receptor associated factors (TRAFs). TRAFs do not appear to have enzymic activity. Instead, TRAFs appear to be primarily adaptor proteins, similar to TRADD and FADD.

The binding of TRAFs by non-DD TNF receptors triggers formation of hetero- or homodimers capable of activating the NIK pathway (Malinin *et al.*, 1997). In addition, the TRAFs also activate the MAPK pathway. This upregulates transcription factors including AP1, cJun, ATF2 and Elk1 (Reinhard *et al.*, 1997; Song *et al.*, 1997), resulting in further changes to transcriptional patterns and mRNA stability. TRAFs may also be transported to the nucleus, and directly enhance transcription through their amino domain (Min *et al.*, 1998).

There are currently two hypotheses as to the mechanism of p75 and CD40 enhancing apoptosis through p55 which are supported by strong experimental evidence and which are not mutually exclusive. The first, supported by Scatchard analysis of TNF receptor

binding assays, is that p75 has a higher on rate and lower off rate than the p55 receptor (Tartaglia *et al.*, 1993a). This would increase the concentration of free TNF near the surface of the cell and thus increase the ligand available for binding to the lower affinity p55 TNF receptor (Grell *et al.*, 1995). A second explanation is that binding of the p75 receptor leads to upregulation of TNF mRNA in the cell. On translation, the TNF is hypothesised to remain cell associated, leading to an increase in autocrine and paracrine signalling. This has been seen to occur in cell lines *in vitro* (Grell *et al.*, 1999; Vercammen *et al.*, 1995). A similar observation has been made with CD40-mediated cell death (Grell *et al.*, 1999; Hess and Engelmann, 1996) and in some cell types may extend to upregulation of other DD-stimulating ligands (Afford *et al.*, 1999). This may also be enhanced or mediated by interaction of shared signalling pathways (Declercq *et al.*, 1998).

1.3.3.3 Preventing enzymatic activity

The poxvirus strategies for preventing signalling through the TNF receptors include TNF receptor homologues which effectively sequester sTNF and prevention of apoptotic signalling through serpins (Barry and McFadden 1997; Turner *et al.*, 1999). The poxvirus serpin crmA/SPI-2 is an effective anti-apoptotic protein, active as a caspase pseudosubstrate (Quan *et al.*, 1995). The protein binds to the active site of the enzymes in a similar manner to the true substrates, however is not cleaved, forming instead a stable inactive complex (Ekert *et al.*, 1999). CrmA has been found to bind strongly to caspase 1 and caspase 8 *in vitro*, with weaker binding to granzyme B and caspase 6 also evident (Quan *et al.*, 1995; Zhou *et al.*, 1997). This is consistent with studies in which crmA as been expressed in transfection systems and found to block death induced by overexpression of the initiator caspase, caspase 1 (Miura *et al.*, 1995), or by Fas/p55 activation (Tewari and Dixit, 1995), but not overexpression of effector caspases such as caspase 3 (Srinivasula *et al.*, 1996). A low affinity for granzyme B may also have functional consequences in reducing cell death due to CTL activity

(Macen *et al.*, 1996; Tewari *et al.*, 1995a). However, given the much higher affinity for caspase 8, it seems likely that the resistance to CTL-lysis observed in several studies is due to resistance to Fas-mediated death.

Whether the function of crmA *in vivo* is to prevent Fas-mediated cell death, to reduce inflammation, or both is not yet certain. Initial studies described crmA in an anti-inflammatory role (Pickup *et al.*, 1986), in which the ability to block the processing of IL-1β by caspase 1 was paramount (Ray *et al.*, 1992; Thompson *et al.*, 1993). However, given the importance of Fas and the CTL response in resolution of viral infections, it would be expected that this too would play an important role. This has not yet been demonstrated in *in vivo* infection. This could be due to the route of infection or the presence of multiple genes blocking the TNF/Fas and IL-1β pathways (Spriggs et al., 1992; Hu et al., 1994) in poxviruses. The influence of crmA on *in vivo* apoptosis is also yet to be described.

1.3.4 Summary

The balance between pathogenesis and virus clearance involves sophisticated host-virus interactions. Apoptotic cell death is an important cellular process manipulated by an array of genes from both the virus and the host. Some of the more direct methods by which apoptosis is induced and prevented have been noted above, but it is likely that more subtle strategies will be discovered as we continue to unravel virus-host interactions. Indeed it is likely that many have already been discovered but not yet identified as having apoptotic consequences.

Given the importance of apoptosis in virus infection and the temporal nature of many of the apoptotic stimuli, it is surprising how little has been published on the kinetics of the apoptotic response during infection, or the effect of anti-apoptotic factors on kinetics of infection. Thus in the current study, apoptosis is studied during acute infection with a virulent pathogen. As a model, the mouse pathogen ectromelia virus has been chosen.

Ectromelia virus (EV), the causative agent of mousepox, has several advantages as a model system. Firstly, it is a well-defined infection. Extensive work by Fenner, Mims, Roberts and coworkers between 1930 and 1965 resulted in a solid body of knowledge on the pathology within EV infected individuals and the spread of infection through groups. In addition, the immunology of EV has been comprehensively studied as a model of a generalised infection by Blanden, Karupiah and coworkers from 1970 to the present time. The genetics of resistance to EV have also been closely examined by Buller, Brownstein and coworkers. Thus EV infection, and the host response to EV infection, have been well characterised.

EV also has an advantage as a model in that the natural host is the mouse. The virus is thus highly adapted to a host which is well defined, and in which inbred strains of known EV-susceptibility are available. Further, manipulation of the immunological environment is aided by the availability of gene knockout and genetically manipulated mice.

Finally, EV encodes an intriguing anti-apoptotic gene which our laboratory has shown to prevent cell death through the non-DD TNF superfamily receptors p75 and CD40. This provides an excellent opportunity to examine the effect of an antiapoptotic gene on apoptosis and virus virulence *in vivo*.

1.4 Ectromelia Virus

1.4.1 Ectromelia Virus and Mousepox

Ectromelia virus (EV) is an orthopoxvirus closely related to vaccinia virus, cowpox and the smallpox agent variola virus. The EV genome consists of a 210kb linear dsDNA with terminal hairpin structures (Esposito and Knight, 1985). Sequences necessary for replication are located internally, and a large number of genes dispensible for *in vitro* growth are present in the inverted terminal repeats (ITRs) of greater than 6.5kb

(Esposito and Knight, 1985; Kotwal and Moss, 1988; Perkus *et al.*, 1991). Among the genes present in the ITRs are a large number of immunomodulatory genes, dispensable for growth *in vitro*, but necessary for virulence *in vivo*.

EV is the causative agent of mousepox, which can be manifested as a rapidly fatal, acute disease, or a chronic form which may be resolved by the host response to infection. At the initial stages of infection EV predominantly infects cells of the macrophage/monocyte system, with virus later being spread to parenchymal cells (Mims, 1959b; Roberts, 1962). Gene expression is rapid, with newly synthesised antigen evident within 1h of infection, and viral inclusion bodies evident in the cytoplasm within 6h of infection. These mark the formation of 'viral factories' and productive EV replication (Marchal 1930, Cairns 1960). The infection can lead to a range of outcomes in inbred mouse strains. These range from a rapid lethal infection in highly susceptible strains such as Balb/c, D2 and A/J to an inapparent infection followed by virus clearance in 129Sv mice (Fenner 1948; Wallace *et al.*, 1985; Jacoby *et al.*, 1989; Brownstein *et al.*, 1992; Brownstein and Gras, 1995). Interestingly, the lethality of infection is not linked to virus replication within the mice, as mouse strains both susceptible and resistant to lethal EV infection may support high levels of virus replication (Wallace *et al.*, 1985). Host resistance is multigenic, and associated with a strong cell-mediated response. Mice in which cell mediated immunity is specifically compromised, for example athymic (Allen *et al.*, 1981; Subrahmanyan and Mims, 1967) or NK cell depleted mice (Karupiah *et al.*,1996) are highly susceptible to EV mediated death. Several resistance to mousepox genes (rmp-1 to 4) have been identified (Brownstein *et al.*, 1992; Brownstein and Gras, 1995; Wallace *et al.*, 1985). Rmp-1, initially identified in A/J ($rmp\text{-}1^s$) and B6 ($rmp\text{-}1^r$) mice, appears to be a non-redundant resistance gene (Wallace *et al.*, 1985), as rmp-4 may be (Brownstein and Gras, 1995), whereas some overlap in the activities of rmp-2 and rmp-3 has been noted (Brownstein *et al.*, 1992). Interestingly, rmp-1 is located extremely close to the NK gene complex,

and may influence the susceptibility of mice to EV-mediated lethality through effects on the NK cell response (Brownstein *et al.*, 1992).

Outcome of infection is also dependent on the strain of virus used. EV has been isolated from wild virus populations on multiple occasions, from Europe, Asia, the United Kingdom and North America. The majority of current studies are performed with two highly virulent strains: NIH-79 and Moscow. The major isolate used in NIH studies, and the studies performed by Buller and colleagues is NIH-79, isolated in 1979 from an outbreak in the NIH laboratories (Allen *et al.*, 1981). NIH-79 is similar in virulence to the highly virulent and lethal Moscow strain. Moscow strain was used in the defining experiments of Andrewes & Elford (Andrewes and Elford, 1947), Fenner (Fenner, 1947; Fenner, 1947a; Fenner, 1948) and Blanden (Blanden, 1970; Blanden, 1971; Blanden, 1971a). All experiments in this thesis use the Moscow strain of virus.

1.4.2 Kinetics of EV Infection

Infection with EV results in acute disease over the first 9 days of infection. Virulent strains may induce significant damage to the liver and spleen during the early stages of disease which can cause death in susceptible mouse strains (Fenner 1949). Survivors of the initial stages of infection will usually have a strong subsequent humoral response to virus which is an effective barrier to reinfection (Fenner, 1949a). The kinetics of the infection are illustrated in Fig. 1.4.

EV normally infects mice first as a very small inoculum through cracks or abrasions in the skin surface, where it is quickly taken up by phagocytes, in particular macrophages (Mims, 1959b). Rapid virus replication, cell to cell spread and cell migration leads to seeding of the local draining lymph node within 24h (Roberts, 1962), where the virus rapidly replicates to high levels and is released into blood and lymph. The resultant viraemia can seed the liver and spleen, the targets of infection, as well as other organs of the body (lungs, intestine, reproductive tract). Within 3d post infection (p.i.) virus

Figure 1.4 Progression of mousepox

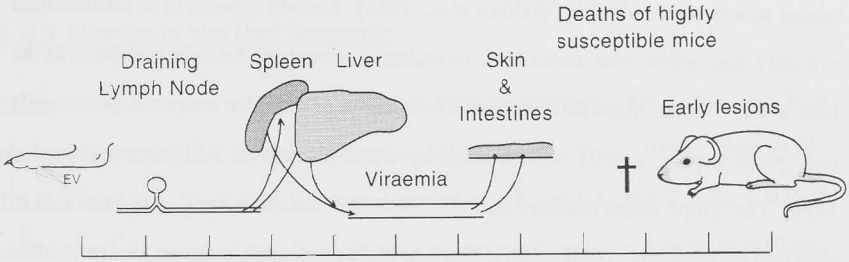
A. Spread of virus during the acute phase of infection after cutaneous or subcutaneous inoculation with ectromelia virus, analogous to natural infection. The positions of early external lesions appearing around d10 to d11 p.i. are marked as shaded areas.

B. Kinetics of the cellular response to ectromelia virus infection.

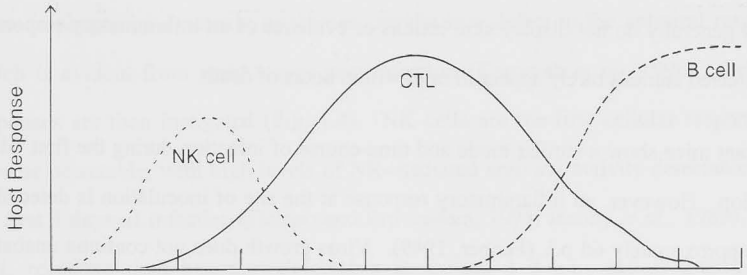
C. Detection of virus at the site of infection (footpad), in the blood and in a target of infection (spleen) during ectromelia virus infection.

Fig. 1A and 1C adapted from Fenner et al. 1949. Fig. 1B adapted from Gardner et al. (1974), and G. Karupiah (pers. comm.).

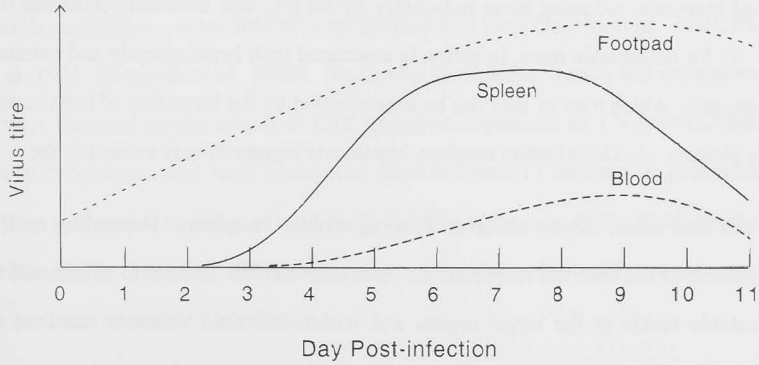
A.


Deaths of highly susceptible mice
Draining Lymph Node
Spleen
Liver
Skin & Intestines
Early lesions
EV
Viraemia
†

B.


Host Response
NK cell
CTL
B cell

C.


Virus titre
Footpad
Spleen
Blood
0 1 2 3 4 5 6 7 8 9 10 11
Day Post-infection

can be titrated from the target organs of infection. The virus replicates quickly in these sites, with up to 10^7pfu per gram isolated from either organ by d6 p.i. in susceptible mouse strains such as Balb/c (Wallace *et al.*, 1985). Growth appears to be uncontrolled in highly susceptible mice, resulting in coagulative necrosis in the liver, and death of the host within 7-9d of infection. Internal examination of the mice reveals hepatomegaly and splenomegaly, usually accompanied by white plaques on both organs (Marchal 1930). The lymph nodes draining the site of infection are often swollen and the area of infection oedematous. Additionally, death in highly susceptible mouse strains is often accompanied by intestinal haemorrhage, which can be microscopically associated with distinctive EV inclusion bodies (Greenwood *et al.*, 1936). Highly susceptible mouse strains generally do not display skin lesions or evidence of an inflammatory response, and infected animals rarely appear ill until within hours of death.

Resistant mice show a similar mode and time-course of infection during the first 6d of infection. However, an inflammatory response at the site of inoculation is detectable from approximately 6d p.i. (Fenner, 1949). Virus growth does not continue unabated until the death of the animal as seen in susceptible mice. Instead, virus titres peak in the liver and spleen at around 6d p.i.. The mice then appear to be able to mount an effective antiviral response, reducing titres noticeably by 9d p.i., and eventually clearing the virus. As for susceptible mice, infection is associated with hepatomegaly and extreme splenomegaly, which may or may not be accompanied by the formation of hepatic and splenic plaques. As the infection resolves, hepatocyte regeneration is evident in the liver, and scar tissue can be observed forming within the spleen. Depending on the effectiveness of the antiviral response, the virus may take 15-28 days to be reduced to undetectable levels in the target organs and within this time viraemia can lead to seeding of the skin and periphery. Hence mice which do not quickly clear the virus are likely to sustain conjunctivitis and virally-induced skin lesions, particularly around the eyes, ears, nose and base of the tail. The mice also suffer from rapid, severe

inflammation at the site of infection, which may result in gangrene and the limb amputation (ectromelia) for which the virus was originally named (Marchal, 1930).

1.4.3 Kinetics of the Host Response

Elements of both the innate and adaptive immune responses are critical for host resistance to lethal EV infection (Blanden, 1971; Jacoby *et al.*, 1989; Tsuru *et al.*, 1983). Infection of resistant B6 mice depleted of NK cells, CD4+ T cells, CD8+ T cells, B cells or macrophages have shown that these cell types contribute to differing stages of the anti-EV response. Cytokines and macrophage function are important in the response to EV from very early times post-infection (see Section 1.4.4). Depletion of macrophages by cytotoxic liposomes results in a defect in the antiviral response which is evident from early stages post-infection (Karupiah *et al.*, 1996). Cellular responses are then instigated (Fig. 1.4). NK cells are the first cellular response to become detectable, with high levels of NK-mediated antiviral activity detectable over the first 4 days of infection (Delano and Brownstein, 1995; Jacoby *et al.*, 1989). The CTL response then becomes detectable at around 3 d p.i., and is maximal at approximately 6 d p.i. (Blanden, 1970; Blanden, 1971; Gardner *et al.*, 1974). Both of these responses are central to the clearance of EV, with loss of either NK cells or CD8+ T cells resulting in severe defects in the clearance of virus (Jacoby *et al.*, 1989; Blanden et al. 1971; Karupiah *et al.*, 1996). Studies by Mullbacher, Simon and colleagues have further focussed on the effects of CTL granule components on EV growth, clearance and pathogenesis, and have found that mice deficient in perforin or granzymes are highly susceptible to EV-mediated death (Mullbacher *et al.*, 1996; Mullbacher *et al.*, 1999a; Mullbacher *et al.*, 1999), supporting an important role for CTL granule-mediated apoptosis in control of EV infection.

Subsequently, from day 8 onwards the antibody response becomes detectable (Blanden, 1970), with IgG strongly induced. The humoral response is not effective in resolution

of infection (Blanden, 1971), but is effective at protecting against recurrent infection (Fenner, 1949; Roberts, 1962).

1.4.4 EV and TNF

The important role of cytokines in host control of poxvirus infection (Ramshaw *et al.*, 1997) and EV infection in particular has been highlighted by the infection of cytokine or cytokine-deficient mice. Notably, mice deficient in Th1-type cytokines (Ramshaw *et al.*, 1997), TNF receptors (Ruby *et al.*, 1997) or the IFNγ receptor (Karupiah *et al.*, 1990) result in significantly increased susceptibility to lethal EV infection. Further, resistance to EV is significantly impaired by loss of either the p55 or p75 TNF receptors, with the loss of p75 resulting in a shorter mean time to death than loss of both receptors (Ruby *et al.*, 1997). Thus both TNF receptors are important for the antiviral response to EV infection.

TNF has multiple antiviral roles, such as increasing vascular permeability (Goldblum *et al.*, 1988; Horvath *et al.*, 1988), stimulating chemokine production (Tessier *et al.*, 1997), and activation of macrophage functions (Conkling *et al.*, 1988), in addition to direct stimulation of apoptosis as described in Section 1.3.3.2. The importance of TNF in EV infection is evident in the multiple mechanisms utilised by the virus to prevent EV signalling. These include soluble decoy TNF receptors and pseudosubstrates of intracellular signalling proteins (Loparev *et al.*, 1998; Turner *et al.*, 2000).

Decoy receptors may be important for blocking not only TNF-mediated signalling and cell death in the infected cell, but also for reducing levels of TNF in the extracellular fluid, thus preventing further immune consequences of TNF expression. Poxviruses encode up to 3 decoy TNF receptors, designated crmB, C and D in cowpox. Of these, crm B is the most widely expressed, with crm C described as an intact ORF only in cowpox (Alcami *et al.*, 1999; Smith, 1996), and crmD described in cowpox and ectromelia (Loparev *et al.*, 1998). The sole functional EV decoy receptor, crmD, has

sequence similarity to the ligand-binding domain of the p75 TNF receptor but lacks the transmembrane or cytoplasmic domains (Howard *et al.*, 1991; Smith *et al.*, 1991; Upton *et al.*, 1991). *In vitro* studies have found that crmD can bind TNF *in vitro*, however its role in *in vivo* inflammation is not yet known (Alcami *et al.*, 1999; Loparev *et al.*, 1998; Smith, 1996).

In addition to decoy receptors, EV also encodes a crmA homologue, SPI-2 (Turner *et al.*, 2000). SPI-2 efficiently prevents TNF and Fas-mediated apoptosis *in vitro*, and is functionally indistinguishable from cowpox crmA in expression studies (Turner *et al.*, 2000); see Section 1.3.3.3).

Data from work by Janet Ruby has also suggested that the early EV gene, p28, may further interfere in TNF-mediated responses (J.Ruby, unpublished data).

1.4.5 p28

p28 is a 241 aa protein consisting of two domains, a carboxy zinc RING finger and an amino domain (Fig. 1.5). It is not a clear homologue of any proteins yet implicated in cell signalling or antiviral function. An important role in virus infection is supported by the presence of highly conserved homologues in pathogenic poxviruses, such as cowpox, variola viruses India 1967 and Bangladesh 1975 and Shope fibroma virus (Upton *et al.*, 1994). In contrast, extensively passaged viruses such as vaccinia Copenhagen and vaccinia WR contain either truncated or non-existent p28 ORFs. A role for p28 in regulation of the antiviral response is suggested by the requirement for p28 for *in vivo* virulence, despite having no detectable effect on viral replication in most cell lines *in vitro* (Senkevich *et al.*, 1994).

p28 is expressed from an early promoter, with its unstable mRNA present from 2 hours post-infection and continuing to be produced for the remainder of the infection independent of viral replication (Senkevich *et al.*, 1995). The vast majority of p28 protein appears to be maintained in the viral factories, with the possibility of some small

amounts being present in the cellular nucleus (Upton *et al.*, 1994). The compartmentalisation of p28 is abrogated by complete, but not partial, removal of the RING finger. The RING finger appears to be required for localisation of the protein into the viral factories (Senkevich *et al.*, 1995); the carboxy domain has not yet been found to have a defined structure or function.

p28 has proved refractory to expression independent of virus, and so all studies published have examined the roles of the protein through comparison of wt EV and a p28 disruption mutant virus (EVΔp28) created by Tania Senkevich and colleagues (Senkevich *et al.*, 1994). Initial papers describing p28 showed that the gene was required for growth of EV in ANCR mice, correlating with reduced EVΔp28 replication in *ex vivo* peritoneal macrophages from the same strain (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995). EVΔp28 was also attenuated in SCID mice (Senkevich *et al.*, 1995), however, both EV and EVΔp28 grew to similar titres in B6 mice (Senkevich *et al.*, 1995), suggesting that the effect of p28 expression on EV virulence may differ dependent on either the genotype of the host or effectiveness of the host response. The p28 Zn RING finger motif is held in common with the TRAF family (Section 1.3.3.2), and led to the hypothesis that p28 may influence virus replication or host cell viability through interaction with TRAF signalling pathways (J. Ruby, pers. comm.). A role in manipulating TNF receptor signalling was confirmed when EV but not EVΔp28 was found to prevent death of the L929 mouse fibroblast line signalled through CD40 or the p75 TNF receptor (J. Ruby, pers. comm.). This has since been found to be concomitant with preventing the upregulation of TNF mRNA after CD40 or p75 TNF receptor ligation. The effect appears to be specific, as housekeeper genes and IL-1 mRNA are not affected (Turner *et al.*, submitted). An additional role for p28 in preventing apoptosis is implied by the work of Brick and colleagues, who found that EV but not the EV p28 mutant virus could prevent UV-mediated apoptosis of virally infected cells (Brick *et al.*, 2000). This could reflect an activity of p28 in preventing virally mediated

A

MEFDPAKINTSSIDHVTILQYIDEPNDIRLPVCIIRNINNITYFINITKINPDLANQFR
AWKKRIAGRDYMTNLSRDTGIQQSKLTETIRNCQKNRNIYGLYIHYNLVINVVIDWITD
VIVQSILRGLVNWYIANNTYNPNTPNSTTISELDIIKILDKYEDVYRVSKEKECGICYE
VVYSKRLENDRYFGLLDSCNHIFCITCINIWHRTRRETGASDNCPICRTRFRNITMSKF
YKLVN

B

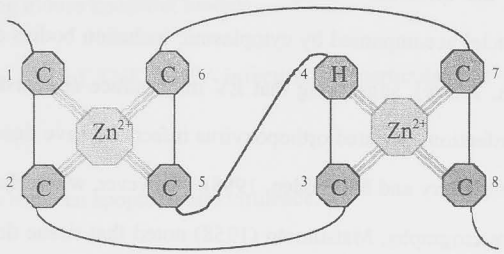


Fig.1.5 The p28 protein.

A. The sequence of the EV protein p28, (Senkevich et al., 1994 ; D. Smith, unpublished data).

B. The predicted topology of the zinc RING finger, comprising 8 cysteine/histidine residues binding 2 Zn^{2+} ions. The RING is likely to be a scaffolding motif, to enable effective folding of the active domains of the protein .

apoptosis, as described for the Shope fibroma virus p28 homologue, N1R (Brick *et al.*, 1998).

Thus p28 was known to be an effective antiapoptotic gene when expressed in a viral context *in vitro*. However, the role of p28 *in vivo*, and apoptosis manipulation by EV using p28, was not known.

1.4.6 EV and Apoptosis

Given the high susceptibility of mice lacking CTL-mediated (Mullbacher *et al.*, 1996; Mullbacher *et al.*, 1999a; Mullbacher *et al.*, 1999), or TNF-mediated (Ruby *et al.*, 1997) apoptosis to lethal EV infection, it is very likely that apoptosis plays an important role in host control of EV infection. Currently, there is little data examining apoptosis directly during the infection. Early microscopy studies revealed the presence of pyknotic cell nuclei accompanied by cytoplasmic inclusion bodies during infection of the liver (Mims, 1959b), suggesting that EV may induce apoptosis of infected cells during *in vivo* infection. Related orthopoxvirus infections have described apoptosis as an antiviral event (Barry and McFadden, 1998). However, when charting EV infection using electron micrographs, Matsumoto (1958) noted that tissue debris from infected cells was phagocytosed by macrophages. Approximately 5h later, the engulfed tissue debris was intermixed with inclusion bodies, which produced EV particles 6h p.i. This suggests that apoptosis may enable infection of macrophages through the phagocytosis of apoptotic bodies, and that apoptosis may enhance EV spread in phagocytic cell types *in vivo* in addition to a role in virus clearance.

These data suggest that apoptosis has multiple functions during *in vivo* EV infection, which may be either pro- or anti-viral. Further, it is possible that apoptosis fulfills differing roles at various stages of the infection. This would imply that viral factors which manipulate apoptosis, including p28, may also differ in effect and role at differing times post infection.

1.4.7 Aims of the Project

In the studies described in this thesis, the kinetics of apoptosis are explored using EV infection of mice as a model system. The EVΔp28 virus is also compared to EV in order to examine the contribution of an apoptosis-blocking virus protein to apoptosis, and how this affects virulence and pathogenesis. Finally, the contribution of TNF to apoptosis and infection progression in EV infection is examined using two approaches – the EVΔp28 virus, and mice lacking TNF receptors.

Thus the aims of this study are

- to describe apoptosis in the context of a natural virus infection
- to determine the contribution of the p28 gene to EV virulence in a variety of mouse strains, including mouse knockout models
- to examine the role of TNF in EV infection, in particular it's contribution to apoptosis
- to explore links between apoptosis and virulence.

Chapter 2

Materials and Methods

2.1 Cell Culture

BS-C-1 cells (African green monkey kidney: ATCC CCL-26) were cultured in MEM supplemented with 5% FCS, 10mM HEPES pH7.4, 2mM L-glutamine, 10µg/ml penicillin and 10µg/ml streptomycin (MEM5). Primary chick epithelial cells were prepared from 13 day old chicken embryos by Allison Condie and cultured in MEM containing 10% FCS and otherwise supplemented as above. WEHI 164 cells (ATCC CRL-1751) were cultured in DMEM supplemented with 10% FCS, (DMEM10) and otherwise supplemented as above (MEM5).

2.2 Viruses

The p28 disruption mutant EV (EVΔp28) was created by Tania Senkevich and is a gpt disruption mutant of ectromelia virus (EV), derived from the replacement of the RING finger of p28 with the bacterial enzyme, xanthine-guanine phosphoribosyltransferase (gpt) (Senkevich *et al.*, 1994). The p28 mutant virus used is functionally indistinguishable from two independently derived p28 mutant viruses and can be reverted to wt phenotype by re-introducing the original segment of p28 (Senkevich *et al.*, 1994).

Stocks of Moscow strain EV and the Moscow strain-derived EVΔp28 were prepared as crude lysates of infected primary chick epithelial cells or BS-C-1 African green monkey kidney cells. The presence of gpt in EVΔp28 allowed periodic testing of virus stock purity by growth in gpt selection medium (MEM5 + 25 µg/ml mycophenolic acid, 250 mg/ml xanthine and 15mg/ml hypoxanthine) (Falkner and Moss, 1988). At the same time as harvest of virus stocks, crude lysates of identical uninfected cells were prepared, aliquoted and stored for use in mock-infection (Section 2.5). All viruses and control lysates were stored in small aliquots at –70°C until use.

2.3 Mice

p55-/- (Rothe *et al.*, 1993), p75-/- (Peschon *et al.*, 1998) and p55-/-p75-/- mice (Peschon *et al.*, 1998) were disruption mutants bred on a hybrid C57BL/6 (B6) x 129/Sv (129) strain background. CD40-/- mice (Kawabe *et al.*, 1994) were also disruption mutants in a B6 x 129 background. Control mice for these strains were B6 x 129 (B6/129)F2. CD40-/- were bred onto a Balb/c background from the above CD40-/- by Paul Foster. B6.β2m-/- (Zijlstra *et al.*, 1989) on a B6 background, IFNγR-/- (Huang *et al.*, 1993), IL-6-/- (Kopf *et al.*, 1994) on a 129 background, and control 129 mice were kindly provided by Alistair Ramsay, JCSMR, ANU. For some studies, B6, Swiss Nude and A/J mice were used. Mice were bred in specific pathogen free facilities at the Animal Breeding Establishment, John Curtin School of Medical Research, Australian National University or at the Department of Microbiology and Immunology, University of Melbourne. Genotype of cytokine receptor null mice was confirmed by PCR (Table 2.1) except for IL-6-/- mice, which were provided directly by Alistair Ramsay. TKO mice were CD40-/-p55-/-p75-/- bred from the above knockout populations by S.C. and Jo McLintock at the Animal Breeding Establishment, JCSMR, ANU. Wt control mice for TKO mice were bred from the same B6/129 F1 crosses used to generate the TKO mice.

2.4 Mouse Genotyping

DNA was extracted from tail tips incubated in 250µl digestion buffer (final concentration: 100mM NaCl, 10mM Tris.Cl pH8.0, 25mM EDTA pH8.0, 0.5% SDS, 100µg/ml proteinase K) for 16h at 56˚C while shaking. The digested tissue was made up to a volume of 400µl with double distilled water. After two phenol-chloroform extractions and a single ethanol precipitation, 1µl of the extracted DNA was analysed in a 25µl PCR reaction using the primers shown in Table 2.1. The PCR conditions comprised an initial 3 min incubation at 94˚C, followed by 35 cycles of 94˚C for 30s, the appropriate annealing temperature (Table 2.1) for 45s, then 72˚C for 90s. A final

extension time of 10 min at 72°C was included, followed by storage at 4°C until analysis.

2.5 *In Vivo* Infection

8-12 week old sex-matched mice were inoculated with 5 x 10^3 pfu of either EV or EVΔp28 diluted in 20µl PBS into the right hind footpad. Control mice were mock-infected with matched uninfected tissue culture prepared at the same time as the virus stocks (Section 2.2), and diluted as for virus. Tissues were harvested at the specified times and fixed in 10% neutral buffered formalin for later apoptotic cell counts, frozen at -20°C for titration, or snap frozen in OCT embedding agent by exposure to liquid nitrogen. For morbidity/mortality studies, mice were infected as above and observed daily. For some experiments, this included daily measurement of mouse weight, and footpad thickness as taken vertically from the pad to the top of the foot. This was compared to weight changes and footpad thickness in mock-infected index mice of the same genotype. All mice in morbidity/mortality studies were autopsied on death.

Surviving mice were sacrificed at 25d p.i. and autopsied, except for Balb/c and Balb/c-CD40-/- mice which were sacrificed at 21d p.i. for humane reasons. Liver and spleen of all mice surviving until the end of the experiment were frozen at –20°C for titration to determine whether virus had been cleared, and facial lesions and/or eye secretions were swabbed for presence of infectious virus.

2.6 *In Vivo* Neutralisation of TNF

To neutralise TNF, mice were given 100µl XT-3-11 anti-TNF mAb ascites i.p. 24h before infection and then at 1, 3, and 5 days p.i. For the shorter time-course, 100µl XT-22 anti-TNF mAb ascites was given i.v. 24h pre-infection. At this dose, both mAbs were equally effective in blocking TNF-dependent production of reactive nitrogen intermediates during virus infection (J. Ruby, personal communication). Control mice

Table 2.1 Primers for genotyping of cytokine deficient mouse strains

Target	Primer	Sequence	Annealing Temperature	Ref
CD40	CD40UpG	5'-GGC AGT AAG ACG ATG TGA CAA CAG A-3'	64°C	A
CD40	CD40Holo	5'-GAG ATG AGA AGG AAG AAT GGG AAA AC-3'	64°C	A
CD40 (inserted neo cassette)	CD40low2T	5'-TAT TGG CTG CAG GGT CGC TCG GTG TT-3'	64°C	A
p55	p60-B	5'-GGA TTG TCA CGG TGC CGT TGA AG-3'	64°C	B
p55	p60-E	5'-TGA CAA GGA CAC GGT GTG TGG C-3'	64°C	B
p55	p60-spe	5'-TGC TGA TGG GGA TAC ATC CAT C-3'	64°C	B
p55/p75 (inserted neo cassette)	pgk5'-66	5'-CCG GTG GAT GTG GAA TGT GTG-3'	64°C	B
p75	p80-i	5'-AAC GGG CCA GAC CTC GGG T-3'	64°C	B
p75	p80-Kas	5'-AGA GCT CCA GGC ACA AGG GC-3'	64°C	B
IFNγR	IFNγR sense	5'-GAT CCT ACA TAC GAA ACA TAC GGT C-3'	60°C	C
IFNγR	IFNγR antisense	5'-GTC ATC ATG GAA AGG AGG GAT ACA G-3'	60°C	C
IFNγR (inserted neo cassette)	Neo	5'-CCT GCG TGC AAT CCA TCT TG-3'	60°C	C

A: (Peschon *et al.*, 1998) B: (Kawabe *et al.*, 1994) C: (Huang *et al.*, 1993)

were pre-treated with an isotype control mAb ascites preparation (GL113, anti-β galactosidase). All 3 clones were kindly provided by J. Abrams, DNAX.

2.7 Sublethal-Irradiation

Groups of 4 animals were irradiated in a rotating container exposed to 650 rad from a ^{60}Cobalt source (Dept. of Chemistry, University of Melbourne). Mice were infected with 5 x 10^3 pfu EV or EVΔp28 into the right hind footpad 24h post-irradiation and observed daily up to 25d p.i.. One group of 2-4 control mice of each genotype were also irradiated and mock-infected for observation up to 26d post-irradiation.

2.8 Macrophage Depletion

To deplete macrophages from the site of infection, age and sex-matched groups of mice were injected with liposomes containing dichloromethylene diphosphonate (Cl2MDP) 2 days before infection, as described in Table 2.2. Liposomes were prepared with phosphatidylcholine and cholesterol as described by van Rooijen and Sanders (1994) (van Rooijen and Sanders, 1994), stored at 4°C and used within 2 weeks. Control (mock-depleted) mice were injected with PBS in an identical manner to the liposomes 2 days before infection (van Rooijen and Sanders, 1994). This was considered a more appropriate control than PBS-containing liposomes, which may concurrently activate macrophages and block normal phagocytic functions (N. van Rooijen, pers. comm.). Cl2MDP was a gift of Boehringer Mannheim GmbH, Mannheim, Germany. Depletion was confirmed by examination of sections for acid phosphatase-positive cells. Briefly, cryosections of livers, spleens or LN were fixed in dry acetone for 10 minutes at room temperature. The cryosections were then allowed to dry completely before being overlaid with a barbital-based acid phosphatase buffer with napthyl AS-BI phosphate as substrate. To create the buffer/substrate mix, 4 solutions were made: (a) barbital buffer (145mM sodium acetate, 145mM sodium barbital in ddH_2O); (b) substrate solution (10mg/ml napthyl AS BI phosphate in dimethylformamide); (c) pararosaniline solution

(40mg/ml pararosaniline-HCl dissolved over heat in 2N HCl, then cooled and filtered); and (d) sodium nitrate solution (40mg/ml sodium nitrate in ddH_2O). Solutions A and B were mixed in ddH_2O (5ml:1ml:12ml respectively) to form solution 1, which was then mixed with solution 2 (800µl solution C mixed with 800µl solution D) before adjusting to pH 5.0 (Kraal *et al.*, 1987). Negative control slides were overlaid with buffer in which substrate solution B was replaced with dimethylformamide. Slides were reacted for 30 min (liver) or 60 min (spleen, LN) at 37°C then washed well in 3 changes of PBS. Sections were then counterstained in Harris' haematoxylin before dehydration and mounting in DPX.

Table 2.2 Depletion of macrophages by injection of Cl2MDP liposomes.

Liposome Injection Site		Macrophage Depletion Site				Ref.
Footpad	i.v.	Footpad	Draining LN	Liver	Spleen	
50µl	-	Y	Y	N	N	(Delemarre *et al.* 1990)
-	150µl	N	N	Y	Y	(van Rooijen *et al.* 1994)
50µl	150µl	Y	Y	Y	Y	Fig. 4.9, this thesis

2.9 Analysis of Serum TNF

Serum TNF was measured according to the bioassay of Espevik and Nissen-Meyer (1986) (Espevik and Nissen-Meyer, 1986). Briefly, experimental sera or murine rTNF in control serum were serially diluted 1:1 across a 96 well microtitre plate in DMEM10. 5×10^4 WEHI 164 cells were added in an equal volume of DMEM10 containing 4µg/ml actinomycin D and incubated for 24h (37°C, 5% CO_2). Cell viability was then ascertained with thiazolyl blue (MTT).

2.10 In Situ Apoptosis Detection

Apoptosis was visualised by TUNEL staining, using a method adapted from Ansari *et al.* (1993) (Ansari *et al.*, 1993) by G.Gobe (University of Queensland) and S.C. Samples were fixed in 10% neutral buffered formalin then set in paraffin. Subsequently, 7-8µm tissue sections were dewaxed and rehydrated. After 15min digestion with 0.05% pepsin at 37°C, sections were permeabilised with 0.1% Tween20/0.1%Triton X100 for 2min at 4°C and rinsed thoroughly with PBS. Sections were then incubated with terminal deoxytransferase (Boehringer-Mannheim, Mannheim, Germany) reaction mix as per manufacturer's instructions for 1h in the dark at 37°C, using FITC-12-UTP as the labelling nucleotide. The reaction was stopped by the addition of 50µl 0.05M EDTA and sections rinsed thoroughly with PBS before mounting in AquaMount for immediate reading, or ProLong for delayed reading. As a positive control, sections of murine ovaries from uninfected mice were included in each batch. These routinely contain large numbers of TUNEL-positive cells in atretic follicles (see Fig. 4.7A). To quantitate apoptotic cells, greater than 10 random 40x fields were assessed by confocal laser scanning microscopy using a Biorad MRC 1000 (Bio-Rad Microscience, Herts, UK) equipped with a 100mW argon ion laser (Ion Laser Technologies, Salt Lake City, UT, USA) which was fitted to a Nikon Eclipse TE300 inverted microscope (Nikon Pty. Ltd., Japan). The excitation wavelength was 488 nm, with emission monitored at wavelengths greater than 515 nm. The resultant images were analysed using the Bio-Rad COMOS analysis software (Bio-Rad Microscience). Apoptotic cells were discriminated as TUNEL-positive cells with apoptotic morphology, as necrotic cells can also be labelled during TUNEL (Grasl-Kraupp *et al.*, 1995). Counting of apoptotic cells was not possible at late times post-infection in some mouse strains, since fragments from individual cells were not distinguishable from those of other cells in areas containing high levels of cell death. Thus the percentage area of the total field which was TUNEL-positive was calculated and translated into absolute cell numbers by calculating the actual area apoptotic and dividing by the

average area of comparable nuclei in that sample. It should be noted that, while this method was tested and found accurate in livers in which apoptotic cells were readily quantifiable, it did not distinguish between TUNEL-positive apoptotic and necrotic cells. This is noted in text where applicable.

2.11 Assessment of Hepatic Morphology

Samples from the right lobe of the liver were collected from each mouse, and immediately fixed in 10% neutral buffered formalin (10% NBF). After greater than 24h fixation, samples were set in paraffin, then sectioned into 6-7μm thicknesses onto Histogrip-coated slides. Sections were then stained with haematoxylin and eosin, using Harris haematoxylin and Eosin-Y. Each section was initially examined first at low power (4x) to determine overall damage, localisation of necrotic and lymphocytic foci, and architectural changes to the liver. Necrotic and lymphocytic foci were then quantitated in a minimum of 10 high power (10x) fields of view by counting both the number of foci and number of nucleated cells per focus. Lymphocytic infiltration not causing foci was semi-quantitated by estimating the ratio of lymphocytes not in foci to the number of hepatocytes present. Sections were also examined at high power (10-40 x magnification) for the presence of ballooning degeneration, kupffer cell activation, vacuolisation and other symptoms of infection or damage. Two sections were assessed per sample, with all sections assessed blind. Photography was performed on an Olympus BX50 light microscope with Olympus PM30 exposure control unit, using Fujichrome Velvia colour slide film. Equipment and advice were kindly provided by David Paul and Bruce Abaloz, Department of Zoology, University of Melbourne.

2.12 Virus Titration

Virus titration was carried out as described by Karupiah *et al.* (1993) (Karupiah *et al.*, 1993). Livers, spleens, lungs and popliteal lymph nodes draining the site of infection were removed from infected mice and homogenised (PRO Scientific Inc., USA) in 1ml

ice-cold PBS. 100µl homogenate was incubated with an equal volume of 1mg/ml trypsin (30min, 37°C) to release the virus and diluted in MEM5 to a final volume of 1ml. Ten-fold dilutions of the trypsinised homogenate in PBS were titrated onto BS-C-1 cells and overlaid with MEM5 supplemented with 1% low viscosity or 0.6% high viscosity carboxymethyl-cellulose. After 4 days incubation at 35°C (5% CO2) monolayers were stained with 0.2% crystal violet in 20% ethanol, and plaques counted.

2.13 Detection of Virus by PCR

DNA was extracted from organs homogenised as above. 100µl of organ suspension was incubated with 100µl double strength digestion buffer (final concentration: 100mM NaCl, 10mM Tris.Cl pH8.0, 25mM EDTA pH8.0, 0.5% SDS, 100µg/ml proteinase K) for 16h at 56°C while shaking. The digested tissue was made up to a volume of 400µl with double distilled water. After two phenol-chloroform extractions and a single ethanol precipitation, 1µl of the extracted DNA was analysed for the presence of viral DNA in a 25µl PCR reaction using the primers p28F and p28R as shown in Table 2.3. Primers and PCR protocol designed by S.C and Deborah Maguire (JCSMR, ANU). The PCR conditions comprised an initial 3 min incubation at 94°C, followed by 35 cycles of 94°C for 30s, 55°C for 45s and 72°C for 90s. A final extension time of 10 min at 72°C was included, followed by storage at 4°C until analysis. To control for variations in DNA extraction efficiency, a PCR for genomic CD40 was performed using the primers CD40UpG and CD40Holo as described in Section 2.4.

Table 2.3 Primers for the detection of viral and genomic DNA.

Target	Primer	Sequence	Annealing Temperature
p28	p28F	5' –GGATATGGAATTCGATCCTGCC- 3'	55°C
p28	p28R	5'–TTATTAGTTAACTAGCTTATAGAACTTGCTC-3'	55°C
CD40	CD40UpG	5'-GGC AGT AAG ACG ATG TGA CAA CAG A-3'	64°C
CD40	CD40Holo	5'-GAG ATG AGA AGG AAG AAT GGG AAA AC-3'	64°C

2.14 Purification of Viral DNA

Viral DNA was prepared from stocks by gently pelleting 750µl stocks (5000 x g, 4°C, 15min) and resuspending in 500µl prechilled TE buffer (10mM Tris.HCl, 1mM EDTA, pH7.4). 500µl prechilled 2x VV DNA extraction buffer was added (final concentrations: 10mM Tris.HCl pH7.6, 50mM β-mercaptoethanol, 25mM NaCl, 10mM EDTA, 1% sarcosyl, 1.5M sucrose) and mixed gently by inversion. DNase-free RNase and proteinase K were added to 100µg/ml and after gentle mixing, incubated at 37°C overnight. Samples were gently phenol/chloroform extracted 3 times by slowly rotating the sample with phenol/chloroform for 15 min at ambient temperature then separating layers (20 000 x g, 4°C, 10 min). This was followed by 2 chloroform extractions performed in the same manner. The resultant aqueous layer was dialysed against 4 changes of TE then quantitated by spectrophotometric analysis (UV 1601).

2.15 Statistical Analysis

Data was analysed using Minitab 10.5 (Minitab Inc., PA). Statistical comparisons were made using the two-tailed Student t-test. For small or non-normally distributed samples, a ranked test (the Wilcoxin U-test) was also used to confirm statistical difference. Figures display the results of Student's t-test. Groups in which testing occurred solely by Student t-test contained greater than 5 readings or individuals. Error bars indicate SD unless otherwise stated.

2.16 Reagents and Suppliers

Actinomycin D	Sigma Chem. Co., MO, USA
Agarose	Progen Industries, Qld, Australia
Aquamount	BDH, UK
β-mercaptoethanol	Sigma Chem. Co., MO, USA
Carboxymethylcellulose	Sigma Chem. Co., MO, USA
Chloroform	Research Organics, OH USA
Cholesterol	Sigma Chem. Co., MO, USA
Cl2MDP	Boerhinger Mannheim, GmbH, Mannheim, Germany
Dimethylformamide	Sigma Chem. Co., MO, USA
DMEM	Media Production Unit, University of Melbourne
DNase-free RNase	Promega, WI, USA
dNTPs	Promega, WI, USA
DPX	BDH, UK
EDTA	Sigma Chem. Co., MO, USA
Eosin Y	BDH, UK
Ethanol	BDH, UK
FCS	CSL Biosciences, Vic, Australia
Haematoxylin	BDH, UK
HEPES	Sigma Chem. Co., MO, USA
Hypoxanthine	Sigma Chem. Co., MO, USA
L-glutamine	Gibco BRL, NY USA
MEM	Media Production Unit, University of Melbourne
Mycophenolic acid	Sigma Chem. Co., MO, USA
Napthyl AS BI phosphate	Sigma Chem. Co., MO, USA
10% NBF	Ajax Finechem, NSW, Australia

OCT embedding agent	Sakura Finetechnical Co., Tokyo, Japan
Pararosanaline	Sigma Chem. Co., MO, USA
PBS	Media Production Unit, University of Melbourne
PCR buffer	Boerhinger Mannheim, GmbH, Mannheim, Germany
Penicillin	CSL Biosciences, Vic, Australia
Pepsin	Sigma Chem. Co., MO, USA
Phenol (pH 7.5)	Research Organics, OH, USA
Phosphatidylcholine	Lipoid GmbH, Ludwigshafen, Germany
Primers	Bresatec, SA, Australia
ProLong	Molecular Probes, OR, USA
Proteinase K	Sigma Chem. Co., MO, USA
rTNF (murine)	Genentech, CA, USA
Sarcosyl	Sigma Chem. Co., MO, USA
SDS	Progen Industries, Qld, Australia
Sodium acetate	Ajax Finechem, NSW, Australia
Sodium barbital	BDH, UK
Sodium chloride	Ajax Finechem, NSW, Australia
Sodium nitrate	Ajax Finechem, NSW, Australia
Sodium nitrite	Ajax Finechem, NSW, Australia
Streptomycin	CSL Biosciences
Sucrose	Ajax Finechem, NSW, Australia
Taq DNA polymerase	Boerhinger Mannheim, GmbH, Mannheim, Germany
Terminal deoxytransferase	Boerhinger Mannheim, GmbH, Mannheim, Germany
Thiozolyl blue	Sigma Chem. Co., MO, USA
Tris.Cl	ICN, OH, USA

Triton-X100	Sigma Chem. Co., MO, USA
Trypsin	Sigma Chem. Co., MO, USA
Tween20	Sigma Chem. Co., MO, USA
Xanthine	Sigma Chem. Co., MO, USA

Chapter 3.

Ectromelia Virus Differentially Affects the Host Apoptotic Response Dependent on Host Strain and the Virulence Factor p28

3.1 Introduction

The poxvirus ectromelia virus (EV) is a virulent mouse pathogen, closely related to the human pathogen smallpox (Buller and Palumbo, 1991; Moss, 1996). Interestingly, EV is not equally pathogenic in all mouse strains. In highly EV-susceptible mouse strains such as D2, ANCR, A/J and Balb/c, EV has been found to cause a rapid lethal infection, whereas EV-resistant strains such as B6, and 129 appear to respond to infection with an effective antiviral response (Delano and Brownstein, 1995; Karupiah, 1998; Niemialtowski *et al.*, 1994; O'Neill and Brenan, 1987). The basis for resistance is multigenic in most strains (Brownstein *et al.*, 1992; Brownstein and Gras, 1995), although studies on B6 and A/J mice have found that resistance segregates with the Rmp-1 (resistance to mousepox-1) gene (Delano and Brownstein, 1995; Wallace *et al.*, 1985), which is closely linked to NK cell activity (Delano and Brownstein, 1995). The response to infection of resistant mice is associated with a predominantly Th1-type cytokine profile and, consequently, a strong cell-mediated immune response (CMI) (Blanden, 1970; Gardner *et al.*, 1974; Jacoby *et al.*, 1989). In contrast, the response to EV infection of susceptible mice is associated with a Th2-like response and ineffective or delayed NK and CTL responses (Karupiah, 1998; Mohan *et al.*, 1997; O'Neill and Brenan, 1987; Schell, 1960). It would be reasonable to hypothesise then that the apoptotic response may vary between susceptible and resistant mouse strains, and that any manipulation of apoptosis by the virus may also vary in effectiveness, dependent on the immune bias of the host.

The EV virulence factor p28 is capable of manipulating apoptosis *in vitro*, but the effects of expression on apoptosis *in vivo* are not known. *In vivo* studies on the role of p28 have been limited to a comparison of virus growth and host death in 2 immunocompetent (ANCR and B6) and 2 immunocompromised (SCID and outbred athymic) mouse strains (Senkevich *et al.*, 1994; Senkevich *et al.*, 1995). It was found that p28 was required for virulence in the highly EV-susceptible A strain, SCID and athymic mice. For these three strains, EV replicated to significantly higher levels than EVΔp28. This result may have indicated that p28 was active as a virulence factor only in the absence of an effective antiviral response;

alternatively, it may have indicated that p28 was of importance in susceptible mice or uniquely ineffective as a virulence factor in B6 mice. Further, it was not evident whether p28 enhances virulence through manipulation of apoptosis (Brick *et al.* 2000, Turner *et al.* submitted), effects on virus growth in macrophages (Senkevich *et al.*, 1995), or consequent effects of p28 expression on host antiviral responses.

Thus in the following experiments the effects of the EV virulence factor p28 on the virulence and pathogenesis of EV are explored, focussing particularly on the effects of p28 expression in host strains of differing susceptibility to EV, and the effects of p28 on the apoptotic response to infection. In addition to better illuminating the role of p28 *in vivo*, this study also aims to map the hepatic apoptotic response occurring during a virulent viral infection.

3.2 Results

3.2.1 The effect of p28 on EV pathogenesis is dependent on host strain

Previous studies in immunocompetent mice have described p28 as a virulence factor in the highly EV-susceptible A strain mice but not EV-resistant B6 mice (Senkevich *et al.* 1995). This led to the hypothesis that p28 might be a strain-specific replication factor. However, it was also possible that p28 was required for EV virulence in susceptible but not resistant mice. To test the effects of host strain and EV-susceptibility on p28 function, the mortality and morbidity of infection with EV or a p28 mutant EV (EVΔp28) was examined in two strains of highly resistant mice (B6 and 129), two strains of highly susceptible mice (A/J and Balb/c) and congenitally athymic Swiss Nudes (Table 3.1). B6x129 F2 mice (B6/129) were also examined in preparation for further experiments (see Chapters 4 and 5). Mice were infected with 5×10^3 pfu EV or EVΔp28 s.c. into the right hind footpad then observed at 24h intervals for 25d p.i. This regimen mimics the natural route of infection (Fenner, 1949; Roberts, 1962). Mortality is shown in Table 3.1. Identically infected mice were also sacrificed 9d p.i. to enable comparison of pathology between mouse strains (see also Section 3.2.4).

All mice were examined daily for symptoms of illness and discomfort (weight loss, lethargy, coat ruffling) as well as symptoms specific to mousepox (presence of pocks, particularly on the face and extremities). Mousepox stimulates a strong cellular immune response (Blanden, 1970), which was monitored indirectly through footpad swelling (Fenner, 1949). Intestinal infection, particularly in EV-susceptible mice, has been reported by several authors (Fenner, 1949; O'Neill and Brenan, 1983). Thus, diarrhoea was also noted as an indicator of intestinal infection. Autopsies performed on mice sacrificed at d9 p.i. examined pathological changes in the target organs of infection (liver and spleen), intestines and the popliteal lymph node draining the site of infection (draining lymph node; DLN). Splenomegaly was measured as an indicator of the host cellular response. In addition, the state of other internal organs not commonly associated with mousepox pathogenesis was examined (lungs, heart, thymus, stomach, kidneys, reproductive tract) for

grossly observable signs of infection. These organs did not typically show overt signs of infection. There was occasional pathology within individuals, however this is likely to reflect differences between individuals rather than groups. In consequence, symptoms in these organs have been omitted for clarity unless displayed by 2 or more mice within the group. Data regarding viral titres in organs of these mice is also not presented, as virus growth is explored more fully later in this chapter (Section 3.2.2). The effects of infection with EV or EVΔp28 on morbidity are summarised in Table 3.2.

Differences between EV and EVΔp28 infection were marked in susceptible mice. A/J mice were highly susceptible to lethal infection with EV, succumbing 8-9d post-inoculation (Table 3.1). Little morbidity, including footpad swelling, was observable, although 3 of 5 mice had minor diarrhoea in the 12h before death. Death was accompanied by extensive viral lesions in the liver, spleen and intestine, associated with intestinal haemorrhage in 3 of 5 mice (Table 3.2). Fatty change of the liver, indicating hepatic stress, was also detected in 3 of 5 mice, and evidence of oedema and tissue breakdown in the reproductive tract was seen in 2 of 5 mice. In contrast, A/J mice were highly resistant to EVΔp28. This was most clearly shown by the lack of mortality in EVΔp28 infected mice (Table 3.1). Morbidity observed was also markedly different, and remained restricted to minor footpad swelling, evident from approximately 9-17d p.i. EVΔp28 infected mice sacrificed at 9d p.i. for comparison to lethally infected mice revealed hepatomegaly and splenomegaly greater than that detected in EV-infected mice, however, these were relatively low in comparison to other mouse strains (Table 3.2). At termination of the experiment at d25 p.i., the p28 mutant virus was no longer detectable in these mice in either the liver or spleen (data not shown). These data confirmed that p28 was necessary for pathogenic EV infection of A/J mice.

Balb/c mice also proved to be highly susceptible to EV-induced mortality and resistant to EVΔp28, demonstrating that resistance to EVΔp28 was not specific to A/J mice (Table 3.1). Progression of EV infection in Balb/c mice was similar to that in the A/J strain. Balb/c mice infected with EV had slightly higher levels of inflammation at the site of

infection than A/J mice (Table 3.2), however 3 of 5 mice did not display further signs of discomfort until shortly before death. This discomfort resulted in distress necessitating euthanasia at 8d p.i. in 2 of the 5 mice infected. All mice additionally had conjunctivitis, with 3 of 5 mice suffering further facial lesions (Table 3.2). Post-mortem examination showed that internal pathology was very similar to that seen in A/J mice. Extensive viral lesions were present in the liver, spleen and intestines, leading to intestinal haemorrhage in 1 of 5 mice. Ovaries and uterus were also inflamed in 4 of 5 mice.

As seen in A/J mice, Balb/c were highly resistant to EVΔp28, however morbidity was noticeably greater. Morbidity was present from d9 p.i., as indicated by weight loss, small eye lesions and footpad swelling (Table 3.2). At d15 p.i. symptoms appeared to resolve, and by d25 p.i. mice showed no evidence of disease. Autopsies at d25 p.i. revealed minor residual hepatomegaly and splenomegaly well below that of EV-infected mice (Table 3.2). It thus appeared that p28 was a virulence factor in at least two strains of EV-susceptible mice. It also appeared that p28 affected virulence to a different extent between A/J and Balb/c strains.

The effect of p28 on EV infection in the highly susceptible but immunocompetent A/J and Balb/c mice was next compared to Swiss nude mice, which are congenitally athymic (Pantelouris, 1968; Green, 1981). The subsequent lack of T cells results in high susceptibility to EV infection (Buller *et al.*, 1987). All Swiss nude mice infected with EV suffered rapid, lethal infection (Table 3.1) with minimal morbidity and no detectable swelling at the site of infection (Table 3.2). Lethal infection was associated with severe intestinal lesions in 4 of 5 mice, and uniformly severe splenic and hepatic lesions similar to those seen in the susceptible A/J and Balb/c strains. In contrast to the immunocompent susceptible mice, Swiss nude mice were also highly susceptible to EVΔp28, with 100% of mice succumbing to lethal infection (Table 3.1). The MTD of EVΔp28-infected mice was significantly extended compared to EV infection ($P<0.05$), however this is unlikely to be biologically significant in view of the similarity of the morbidity (Table 3.2), hepatic indicators of damage (Table 3.4) and virus titres (Fig. 3.2B) associated with EVΔp28

infection compared to those observed in EV infection. Prior to death, morbidity appeared very similar to that in response to EV infection, with post-mortem examination revealing almost identical internal pathology (Table 3.2). Both EV and EVΔp28-infected mice were found to have small, dark spleens and pale, mottled livers, consistent with the abundant viral lesions and low level cellular infiltration found histologically (see also Fig. 3.3). Hence p28 expression was not necessary for EV virulence in Swiss nude mice.

p28 expression had a less obvious effect on EV pathogenesis in EV-resistant mouse strains. All EV-resistant mouse strains survived infection with EV or EVΔp28 (Table 3.1) with minimal morbidity (Table 3.2). While mice did not show typical signs of disease such as pocks or weight loss, all resistant mice showed extreme footpad swelling which was not seen in susceptible mice. Examination of internal pathology at d9 p.i. further showed that footpad swelling correlated closely with splenomegaly for EV and EVΔp28 infections. While internal pathology was low overall, some hepatomegaly and evidence of fatty liver was seen in all 3 resistant mouse strains, particularly B6. More striking was the presence of damage in the infected footpad and the lymph node draining the site of infection. This resulted in lymph node destruction in all EV-infected B6 mice, 3 of 5 EVΔp28 infected B6 mice, 3 of 5 EV infected B6/129 mice, and 2 of 5 EV infected 129 mice.

As described in EV-susceptible mice, the effect of p28 expression on EV pathogenesis was not equal between all EV-resistant mouse strains. In addition to differential LN destruction (Table 3.2), this was most evident through comparison of splenomegaly and footpad swelling. Both were greater in response to EV than EVΔp28 infection in 129 and B6/129 mice. This contrasted with B6 mice, in which p28 expression did not appear to affect either parameter (Table 3.2).

These data showed that p28 expression increased EV pathogenesis in both susceptible (A/J and Balb/c) and resistant (B6/129 and 129) mouse strains. The requirement for p28 for EV virulence was not equal in all mouse strains. This could be clearly observed in susceptible mice, in which mutation of p28 led to responses ranging from dramatic attenuation of the virus and almost no attendant morbidity (A/J), to external lesions which were then resolved

(Balb/c), or lethal infection indistinguishable from that of wt EV (Swiss nude). EV virulence appeared less affected by the presence of p28 during infection of EV-resistant mouse strains, however this was at least partially due to the low level of morbidity evident on EV infection. In these mice, expression of p28 led to detectable differences in the host response to infection, as indicated by increased footpad swelling and splenomegaly (129), or LN destruction with increased footpad swelling and splenomegaly (B6/129), although again, p28 did not affect symptoms of infection in all mice (B6).

These data had broadly addressed whether p28 expression affected the outcome of disease in a number of mouse strains. It was now examined whether the effect of p28 on viral pathogenesis was related to a requirement for p28 for EV replication as suggested by Senkevich and colleagues (1995), or in preventing virus clearance. To do this, the kinetics of virus replication were ascertained in resistant and susceptible mice.

3.2.2 The effect of p28 on EV replication is dependent on host strain

To determine the kinetics of EV and EVΔp28 replication, Swiss nude, A/J, B6, B6/129 and 129 mice were infected with 5 x 10^3 pfu of either EV or EVΔp28 via the footpad as previously and sacrificed from d3 to d9 p.i. Balb/c mice were not used, as suitably matched mice were not available at the time of the experiments. This time-course encompasses the first detection of virus in target organs at d3 (Fenner, 1949), the peak virus titres occurring at d6-7 p.i. (Fenner, 1949), and initiation of virus clearance by CMI (Blanden 1971, Gardner 1974). Virus replication was examined in the target organs of infection (liver and spleen) and the popliteal lymph node draining the site of infection (draining lymph node: DLN) (Fig. 3.1). The DLN is the first site seeded by virus after footpad infection and is an indicator of virus replication at the site of inoculation (Fenner 1949a). The footpad itself was not titrated as the original inoculum would interfere with the titres obtained.

Swiss nude mice did not show any effect of p28 expression on EV replication or clearance in any of the organs tested. EV and EVΔp28 proved highly and equally virulent, with levels of both viruses increasing in the livers of mice until host death. Levels of both

viruses were significantly greater than those seen in any other mouse strain tested (Fig. 3.1; $P<0.01$) and were indistinguishable at each timepoint ($P>0.40$). Hence in Swiss nude mice p28 expression did not affect virus replication or clearance. This was consistent with earlier observations of equal morbidity and mortality.

In contrast, EV clearly required p28 for effective replication in A/J mice. High levels of EV replication occurred in all organs tested from d3 p.i. In contrast, severe attenuation of EVΔp28 was evident in the liver and spleen of infected animals, with virus remaining below detectable levels in the liver. EVΔp28 was also attenuated in the DLN, in which EVΔp28 remained at levels at least 1 log less than the wt virus. This was particularly notable as, despite apparent titres of up to $10^{6.8 \pm 0.4}$ pfu/g, the small mass of DLN (<12mg) means that absolute EVΔp28 levels actually decreased over the period of the infection in the DLN of A/J mice to levels below those injected by d9 p.i., the only mice in which this occurred. Thus p28 was required for virus growth in A/J mice.

The inflammatory responses of the EV-resistant mouse strains to infection with EV or EVΔp28 had suggested that p28 affected virulence in 129 and B6/129, but not B6 mice. However, infection of B6, 129 and B6/129 mice with EV and EVΔp28 led to similar virus replication kinetics in each case. All three strains supported equal growth of EV and EVΔp28 in the DLN. Levels of virus replication were approximately equal in the spleen, however in the liver EVΔp28 titres reached maximum levels approximately 1 log below those of EV at d6 p.i. (B6 $P<0.05$; B6/129 $P<0.01$, 129 $P<0.01$) before the onset of virus clearance, observable at d9 p.i. Virus clearance appeared more effective in 129 and B6/129 mice than B6 mice. This finding was in keeping with the higher observed resistance of these mice to EV-mediated morbidity.

These data showed that EVΔp28 replication was compromised very early in infection of the highly susceptible A/J mice. The effect was unique amongst the mouse strains tested and consistent with the marked lack of pathogenicity in A/J mice. Differences in the responses of EV-resistant mice to p28 expression appeared generally related to levels of viral

replication, since the three resistant mouse strains showed slight but consistent attenuation of EVΔp28, and minor effects of p28 on morbidity.

Two potentially related mechanisms proposed in the literature were consistent with the host strain-specific effects of p28 expression on virus replication. The first, proposed by Senkevich and colleagues, was that p28 was required for EV replication in macrophages from some strains of mice. This was consistent with a requirement for p28 for the growth of EV in *ex vivo* macrophages derived from A strain mice (Senkevich *et al.*, 1995), and the important role of macrophages in the establishment of EV infection (Mims, 1959b; Roberts, 1962). A second mechanism was proposed by Brick and colleagues, and Turner and coworkers (Brick *et al.* 2000; Turner *et al.* submitted). These two studies found that p28 was required for EV to block some forms of apoptosis, which may prevent effective virus replication. Apoptosis may directly compromise virus replication through the death of the infected cell before virus replication is complete (Everett *et al.*, 2000; Itoh *et al.*, 1998; Roulston *et al.*, 1999; Smith *et al.*, 1997), and may additionally promote the antiviral response through priming of the immune response (Albert *et al.*, 1997; Bellone *et al.*, 1998). These proposed mechanisms for p28 activity were not mutually exclusive, as it was possible that apoptosis early after EVΔp28 infection of *ex vivo* A strain macrophages may restrict replication of the p28 mutant virus. Indeed, preliminary experiments have suggested that this is the case (S.C., data not shown). Thus the next experiment examined whether the effect of p28 on EV virulence required the presence of macrophages. To do this, EV and EVΔp28 virulence were tested in mice depleted of macrophages with cytotoxic liposomes.

3.2.3 Macrophages contribute to p28-mediated virulence in A/J mice

Growth of EV in macrophages is critical at several points in infection. Macrophages are amongst the first cell types infected during dermal and subdermal EV infection (Roberts 1962). Additionally, infection of the resident macrophages of the liver (Kupffer cells) is an important and rate-limiting step in the progression of virus infection (Mims 1959). Hence,

activity of p28 as a macrophage-specific replication factor would be consistent with, and sufficient for, the extreme attenuation of EVΔp28 observed in A/J mice. To test the role of p28 and macrophages in EV infection of A/J mice, the virulence of EV and EVΔp28 were compared in mice depleted of macrophages using Cl2MDP liposomes injected into the footpad and i.v. 48h prior to infection (Section 2.8). This protocol effectively depletes macrophages from the liver, spleen, footpad and popliteal lymph (Karupiah *et al.*, 1996; van Rooijen *et al.*, 1989), see also Fig. 4.9). Control mice were mock-depleted with PBS as described in Section 2.8. After liposome or PBS treatment, mice were infected with 5×10^3 pfu of EV or EVΔp28 in the right hind footpad as previously, then observed at 24h intervals for 25d. B6 mice were also tested as controls in which EV and EVΔp28 grew to equal levels and caused similar mortality and morbidity.

The response of mock-depleted A/J mice to infection with EV or EVΔp28 was indistinguishable from that of untreated mice (Section 3.2.1). EV infection resulted in rapid lethal infection with minimal morbidity, whereas EVΔp28 infection resulted in minor morbidity followed by recovery from infection (Tables 3.1, 3.3). Infection of liposome-treated A/J mice with EV led also to rapid lethality, identical to that of PBS-treated EV infected mice. However, liposome-treated A/J mice showed strikingly increased susceptibility to EVΔp28, as illustrated by the increase in mortality from 0% to 80% of infected mice. This indicated that EVΔp28 attenuation was largely reversed by macrophage depletion. However, the p28 mutant virus remained attenuated in comparison to EV as indicated by lower mortality rates and extended MTD ($P<0.01$). Unexpectedly, post-mortem examination did not show overt lesions to the liver or spleen, although all mice showed DLN destruction.

Mock-depleted B6 mice responded to infection as per untreated mice, with all mice surviving infection with both viruses (Table 3.3). Swelling at the site of infection was

severe, and indistinguishable between the wt and p28 mutant viruses. Macrophage depletion of B6 mice led to a significant increase in susceptibility to both EV and EVΔp28. Footpad swelling and splenomegaly were very reduced in comparison to non-treated mice, suggesting compromise of the inflammatory response to infection in macrophage-depleted mice. This is in agreement with the low splenomegaly observed, and previous reports of immune dysfunction in macrophage depleted mice (Karupiah *et al.*, 1996; van Rooijen and Sanders, 1994). Death ensued rapidly, with all mice succumbing to infection at 8d p.i. (Karupiah *et al.*, 1996; Table 3.3). Post-mortem examination revealed very small spleens and the presence of viral plaques on the margins of the liver. It was additionally found that mortality, morbidity and internal pathology did not differ between EV and EVΔp28 infection of liposome-treated B6 mice.

The results of macrophage depletion in A/J and B6 mice confirmed the critical role of macrophages in resistance to EV infection (Karupiah *et al.*, 1996). The presence or absence of p28 did not affect lethality in macrophage-deficient B6 mice. A/J mice depleted of macrophages were more susceptible to EVΔp28 than control mice, suggesting that a major antiviral mechanism targeted by p28 is macrophage-dependent. Despite this, EVΔp28 was still less virulent than EV, with 4 of 5 macrophage-depleted mice surviving for an extended period in comparison to equivalent mice infected with EV, and 1 mouse surviving for greater than 25d p.i. Virus was not detectable in the liver, spleen or LN of this mouse (data not shown), thus the mice were still capable of clearing the infection. This observation suggests that p28 was still required for virulence in macrophage-depleted A/J, and so also inhibits mechanisms which are independent of macrophages.

A role for p28 in virulence through the inhibition of apoptosis has also been implied by previous work (Brick *et al.*, 2000, Turner *et al.*, submitted). The effect of p28 expression on apoptosis during EV infection was next examined.

3.2.4 P28 manipulates apoptosis in B6 mice but not in other strains

To determine whether p28 expression affected apoptosis during EV infection, apoptotic cells were quantitated in the livers of B6, B6/129, 129, A/J or Swiss Nude mice. Liver was chosen, as hepatic damage is thought to be the cause of death in EV infection (Fenner, 1949). Liver samples collected from the mice described in Section 3.2.2 were fixed in 10% NBF and sectioned for detection of apoptosis *in situ*. This has the advantage of allowing the sites and context of apoptosis to be examined in addition to quantitative analysis. Apoptosis was visualised using TUNEL staining, which exploits the ability of terminal deoxytransferase to attach fluorescein-conjugated uracil triphosphate (FITC-12-UTP) to breaks in the DNA strands. The apoptotic cells can then be visualised and quantitated (Section 2.10). Since this staining technique can also stain necrotic cells (Grasl-Kraupp *et al.*, 1995), the morphology of the labelled cells was also examined.

For histological assessment, sections collected in serial with those used for TUNEL staining were stained with H&E. Necrosis, lymphocytic infiltration and lymphocytic foci were then semi-quantitated for comparison to apoptosis (Table 3.4). Swiss nude, A/J, B6 and 129 strains were chosen as representing a variety of responses in susceptible and resistant mice to EV and EVΔp28 infection. B6/129 mice were also subsequently examined (Chapter 5).

The apoptotic response to EV infection and the sensitivity of the response to p28 expression differed between mouse strains independent of the effect of p28 on EV virulence. Swiss nude mice responded to both EV and EVΔp28 infection with high levels of apoptosis at d6 and d9 p.i. (Fig. 3.2). Consistent with viral replication, levels of apoptosis were not significantly different between EV and EVΔp28 infected mice d6 p.i. The apoptotic cells were predominantly present as individual cells associated with areas of necrosis and comparable to those later detected in CTL-deficient mice (Fig. 3.7C). Increased apoptosis was concomitant with increases in both virus titres (Fig. 3.2) and necrosis (Fig. 3.3B, C, Table 3.4), suggesting that these were related in Swiss nude mice. In addition, high levels of vacuolisation (Fig. 3.3B) were detected, which increased over the

first 6d of infection. Quantitation of necrosis showed a non-significant trend towards reduced necrosis in EVΔp28-infected mice at d9 p.i. (P=0.09) This was not detectable by direct observation.

In contrast, apoptosis did not obviously correlate with virus levels in A/J mice. EV replicated to levels significantly higher in the livers of A/J than Swiss nude mice by d6 p.i. (P<0.01; Fig. 3.2), while EVΔp28 did not reach detectable levels. However, A/J mice displayed low levels of apoptosis in response to both virus infections (Table 3.4, Fig. 3.4). Interestingly, hepatic necrosis was present at similar levels in both EV and EVΔp28 infection despite EVΔp28 virus titres being below the limit of detection in the liver (Fig. 3.2). Necrosis was not, however, qualitatively equivalent. Hepatic necrosis in response to EV consisted of areas of hepatic damage with poorly defined margins, similar in appearance to those observed in Swiss nude mice (Fig. 3.3C, 3.4B). In contrast, necrotic areas in A/J mice infected with EVΔp28 were discrete and localised. Further, necroses in EVΔp28-infected A/J mice were associated with inflammatory foci (Fig. 3.4C). This was not seen in EV infection, suggesting that p28 expression inhibited inflammation or leukocyte chemotaxis in the livers of these mice.

The highly EV-resistant 129 mouse strain also responded to EV infection with a low level of apoptosis which was not sensitive to p28 expression (Fig. 3.2), and did not display hepatic necrosis at any point in infection (Table 3.4). EV-resistant mice displayed increased inflammatory cell infiltration into the liver and higher numbers of lymphocytic foci (Table 3.4), consistent with the capacity of the mice to resolve the infection. These observations suggested that a vigorous apoptotic response was not required for an effective antiviral response. Further, the patterns of apoptosis observed in resistant mice differed from those seen in susceptible A/J mice. Whereas apoptotic cells in highly EV-sensitive mice were closely associated with necroses, apoptotic cells in EV-resistant mice were scattered throughout the parenchyma. These may or may not have been associated with foci of inflammatory cells, as the foci were difficult to discern during fluorescence

microscopy (see Figs. 4.2E, 4.2F, 5.9). The pattern of apoptosis observed in 129 and B6/129 mice was shared by EV-infected B6 mice (Fig. 3.7B, 5.9).

The apoptotic response of B6 mice was uniquely sensitive to regulation by p28 expression. B6 mice displayed low levels of apoptosis in response to EV infection, as observed in other resistant strains. However, infection with EVΔp28 led to an increase in apoptosis evident within 3 days of infection which was significantly increased over EV-induced levels throughout the infection ($P<0.05$; Fig. 3.2). Levels of apoptosis in EVΔp28 infected mice were approximately 3 times higher than apoptosis in EV-infected animals from d3 p.i., reducing towards wt levels as the infection began to resolve. For both EV and EVΔp28 infection, apoptosis appeared qualitatively similar; apoptotic cells appeared to be predominantly scattered hepatocytes, with some apoptosis present in the sinusoids and venules. Figs. 4.2E and 4.2F illustrate the most common patterns of apoptosis observed in B6 mice.

Within B6 mice, an accelerated infiltration of lymphocytes and increase in lymphocytic foci was seen in EVΔp28-infected animals across the early stages of infection compared to that in EV infection (Table 3.4). While it is tempting to speculate that this could be the cause of increased apoptosis in EVΔp28-infected B6 mice, it should be noted that increased apoptosis was present before the increase in lymphocyte infiltration and during the time at which both EV and EVΔp28 infected mice had similar numbers of foci. These findings suggest that both inflammation dependent and independent mechanisms activate apoptosis.

This data revealed a complex relationship between the apoptotic response and the antiviral response. In immunocompromised mice (Swiss nude), virus growth was uncontrolled and levels of apoptosis appeared to parallel virus levels. However, in immunocompetent mice, generally low levels of apoptosis were observed, whether or not the mice mounted an effective antiviral response (eg. 129 vs. A/J). In B6 mice however, the apoptotic response appeared to be strongly inhibited by p28 at all timepoints examined.

3.2.5 p28 activity requires both radiation sensitive and radiation resistant elements

In the previous experiments it was noted that EVΔp28 but not EV infection induced the formation of lymphocytic foci in A/J mice, suggesting that p28 may be active in A/J mice by preventing an efficient immune response. EVΔp28 also stimulated accelerated formation of inflammatory cell foci in B6 mice, however, this appeared to have a lesser effect on virulence. In order to test whether a functional immune response was required for attenuation of EVΔp28, A/J and B6 mice were sublethally irradiated before infection with EV or EVΔp28. Sublethal irradiation limits the immune response by causing the death of proliferating cells, including cells responding to antigen (Johnson *et al.*, 1995; Lin *et al.*, 1996) and downregulates some aspects of macrophage function (Anderson and Warner, 1976). Groups of 4 mice were exposed to 650 rad from a ^{60}Co source and infected 24h later with 5 x 10^3 pfu EV or EVΔp28 per mouse as previously. Mice were then examined at 24h intervals for morbidity and mortality. As controls, groups of 3 mice were irradiated and mock-infected, and 4 mice of each genotype were left non-irradiated before infection.

Irradiated mice showed a significant reduction swelling at the site of inoculation and lack of splenomegaly, as seen in macrophage depleted mice (Section 3.2.3). The MTD of irradiated A/J mice infected with EV was equivalent to that of non-irradiated mice, suggesting that irradiation did not increase the sensitivity of the mouse strain to EV-mediated lethality (Table 3.5). Both irradiated and non-irradiated mice displayed similar internal pathology, with grainy, slightly fatty livers. Irradiated mice additionally displayed reduced spleen size in comparison to non-irradiated control mice. Death was associated with significant intestinal damage in 3 of 4 mice in both irradiated mice and non-irradiated mice.

EVΔp28 infection of irradiated A/J mice also resulted in 100% mortality, however MTD was around 18d p.i., substantially greater than that of EV infected mice (Table 3.5; $P<0.01$). Post-mortem examination revealed strikingly different pathology to that seen in EV infected A/J mice. The livers of irradiated A/J mice infected with EVΔp28 were

relatively normal, with some fatty change. No intestinal damage was observed, however spleens were highly enlarged and fibrous. In comparison, non-irradiated mice did not appear to have significant internal pathology, although some splenomegaly was noted.

In contrast, sublethally-irradiated B6 mice were equally susceptible to EV and EVΔp28, with a mean time to death (MTD) of around 10d p.i. for both viruses (Table 3.5). Unlike non-irradiated control mice, irradiated B6 displayed little swelling in the infected footpad. In addition, irradiated mice infected with EV or EVΔp28 possessed extremely small spleens and soft, fatty livers with a grainy texture. Samples were not taken for titration, or histological comparison with susceptible or non-irradiated mice or TUNEL staining since mice had been dead for between 15 min and 16h, during which time variable tissue and virus degradation would have already started to take place. However, morbidity was identical in response to both viruses, suggesting that virulence was also identical. The observation that p28 does not affect virulence in irradiated B6 mice may indicate that p28 manipulates immune-mediated response(s). This is consistent with the regulation of apoptosis by p28 during d3-9 p.i. in this mouse strain. However, it is also possible that the impairment of the host response is to such an extent that p28 is no longer necessary for high EV virulence.

These data showed that irradiation increased susceptibility of A/J mice to EVΔp28 but did not completely reverse attenuation of the virus. Comparison of MTD between irradiated mice shows that survival time was significantly extended in EVΔp28 infected A/J mice compared to those infected with EV ($P<0.01$), or B6 mice infected with either virus ($P<0.01$), revealing a radioresistant aspect of A/J resistance to EVΔp28. Since irradiation prevents the proliferative response to antigen, the continuing resistance of A/J mice to EVΔp28 also implies that p28 has further activities in these mice than the manipulation of the cellular immune response implied in Fig. 3.4. Further, the unique pathology observed in these mice suggests that p28 may be required for localisation of the virus to organs during EV infection, particularly the liver and intestine. Alternatively, p28 may normally function to increase EV virulence in some organs, notably the liver and intestine, leading to

the rapid death observed during infection of A/J mice with the wt virus. In the absence of an immune response, EVΔp28 may be able to replicate to pathogenic levels in other organs such as the lungs. It is not known how this may relate to previous data describing roles for p28 in apoptosis (Brick *et al.*, 2000, Turner *et al.*, submitted), virus growth in macrophages (Senkevich et al. 1995), or changes to hepatic pathology (Section 3.2.1). Given the focus of the current work on apoptosis in infection rather than the basis for EVΔp28 attenuation in A/J mice, this data was noted but not explored further.

In contrast to the startling effect of p28 expression in sublethally-irradiated A/J mice, p28 expression did not affect lethality of infection in similarly irradiated B6 mice. Sublethal irradiation of B6 mice led to high susceptibility to both EV and EVΔp28. No difference could be seen between the two virus infections, a finding in common only with Swiss nude mice. The finding suggests that p28 does not play a role in virulence in irradiated B6 mice. Further, the result, in combination with the differences in apoptosis between EV and EVΔp28 infected B6 (Fig. 3.6) suggests that p28 may be active in B6 mice through manipulation of immune-mediated apoptosis.

3.2.6 The effect of p28 expression on apoptosis is abrogated in MHC class I-deficient mice

Effective NK and CTL responses are critical for resistance to EV. Depletion of either cell type results in susceptibility to EV-mediated lethality (Blanden, 1970; Buller *et al.*, 1987; Jacoby *et al.*, 1989; Karupiah *et al.*, 1996; O'Neill and Brenan, 1987). The two cytotoxic cell types have overlapping kinetics of activation during EV infection. NK cells, which important during virus infections as cytolytic cells and sources of antiviral cytokines (Biron *et al.*, 1999), are predominantly active prior to 4d p.i. The CTL response, mediated by CD8+ T cells is then highly active from 3-10d p.i. (Gardner *et al.*, 1974; Karupiah *et al.*, 1996). It had been noted in Section 3.2.4 that the presence of p28 reduced hepatic apoptosis in B6 mice during the period 3-9d p.i., ie during the period of maximum CTL activity. Thus, it was possible that the difference in levels of hepatic apoptosis between EV and EVΔp28 infected B6 mice was dependent on CD8+ T cells.

To investigate whether CD8+ CTLs contributed to the differences seen in the apoptotic response to EV and EVΔp28 infection in B6 mice, apoptosis and virus growth were examined in B6 and B6.β2m-/- mice. B6.β2m-/- mice are selectively CD8+ CTL deficient due to extremely low levels of class I MHC expression (Zijlstra *et al.*, 1990). In the absence of positive selection, CD8+ T cells cannot mature to provide a CTL response (Zijlstra *et al.*, 1990). Interestingly, NK cell function remains largely intact (Tay *et al.*, 1995). B6 and B6.β2m-/- mice were infected with a low dose (5×10^3 pfu) of either EV or EVΔp28 via the footpad as previously, and organs harvested from 3-9d p.i. for virus titration and apoptotic staining.

As shown in an earlier study (Karupiah *et al.*, 1996), B6.β2m-/- mice are highly susceptible to the lethal effects of EV infection. All B6.β2m-/- mice infected with EV died within 17d of infection, whereas control mice uniformly survived the infection (Table 3.6). Similarly, B6.β2m-/- mice did not survive infection with EVΔp28, and no differences were observed between the MTD of the two infections. Titration of EV and EVΔp28 showed that replication of both viruses was increased in B6.β2m-/- mice, compared to B6 mice. The increase in virus titres was evident from d3 p.i. on infection with EVΔp28 ($P<0.05$) and from d6 p.i. on infection with EV ($P<0.05$; Fig. 3.6). Virus clearance was also significantly compromised, with levels of hepatic EV and EVΔp28 present in B6.β2m-/- mice at d9 p.i. comparable to those in B6 mice at the peak of infection (d6 p.i.; Fig. 3.6). Equivalent patterns of virus growth and clearance were observed in the spleen and DLN (data not shown). Thus, neither virus replication or clearance was affected by p28 expression in B6.β2m-/- mice.

TUNEL staining of hepatic sections showed that apoptosis in B6 mice was similar to that observed previously (Fig. 3.2). While both EV and EVΔp28 infected mice displayed some hepatic apoptosis, the levels of apoptosis were at significantly higher levels in mice infected with EVΔp28 compared to those infected with EV within 3d p.i.($P<0.01$; Fig. 3.6). Enhanced levels of apoptosis were maintained in EVΔp28 infected mice at 6d p.i., before decreasing at d9 p.i. to levels similar to those found in infection with the wt virus. In

contrast, B6.β2m-/- mice responded to both EV and EVΔp28 with low levels of apoptosis at 3d p.i. which increased during the course of the infection. The magnitude of the difference in apoptosis levels between EV and EVΔp28-infected mice was strikingly decreased in B6.β2m-/- mice compared to B6, although it remained significant at d6 p.i. ($P<0.05$) and became significant at d9 p.i. ($P<0.01$; Fig. 3.6). This reflected the similarity of the kinetics of apoptosis in B6.β2m-/- mice to those observed in Swiss nude mice (Fig. 3.2) rather than the parent B6 strain (Figs. 3.2 and 3.6).

A qualitative examination of apoptosis in B6 and B6.β2m-/- mice showed that the differences in levels of apoptosis detected were mirrored by differences in the pathogenesis of EV and EVΔp28. As previously, B6 mice displayed predominantly scattered apoptosis in response to both EV and EVΔp28 (Fig. 3.7B), with some apoptosis associated with foci of leukocytes, as illustrated in Fig. 4.2F. This was consistent from d3-9 p.i., and qualitatively indistinguishable between EV and EVΔp28 infections. Inflammation of the livers as indicated by increased numbers of leukocytes, increased inflammatory foci and some distension of sinusoids was also indistinguishable between the two virus infections (see Fig. 4.2E, F). B6.β2m-/- mice displayed very different patterns of apoptosis, associated with differences in hepatic necrosis and leukocyte infiltration. Like B6 mice, B6.β2m-/- mice had a significant infiltration of inflammatory cells evident from d3 p.i. which appeared to form foci throughout the hepatic tissue. However, this was associated with extensive necrosis not observed in B6 mice (Fig. 3.7C). Apoptosis in B6.β2m-/- mice was similarly strongly associated with necrosis, in contrast to the scattered apoptotic cells of B6 mice.

Hence the difference in levels of apoptosis between EV and EVΔp28 infection in B6 mice was dependent on a functional CD8+ T cell response.

3.2 Discussion

The work presented in this chapter focussed on two questions (1) the role of p28 in the pathogenesis of EV and (2) the role of apoptosis in the host response to infection.

The data showed that p28 contributes to EV virulence to varying degrees in a number of mouse strains, with A/J (p28 absolutely required for EV virulence) and B6 (p28 not required for EV virulence) forming the extreme phenotypes. The effect of p28 expression appeared greater in highly EV-susceptible mouse strains and lesser in mice highly resistant to EV-mediated mortality.

There are a number of mechanisms by which p28 may be a more effective virulence factor in EV-susceptible than EV-resistant mice. Mouse strains highly susceptible to EV are more likely to have a Th2-type cytokine bias and humoral immune response, whereas resistance to EV-mediated mortality is associated with a Th1 response and correspondingly strong cell mediated immunity (Karupiah, 1998). In Balb/c mice, the Th2 bias has been linked with possible defects in cytokine production (Alleva *et al.*, 1998) and/or the response to cytokines (Shibuya *et al.*, 1998). For example, Balb/c CD4+ T cells are capable of producing Th1 type cytokines such as IFNγ after concurrent treatment with IL-12, IL-1 and TNF. In comparison, CD4+ T cells from EV-resistant B6 require only IL-12 treatment (Shibuya *et al.*, 1998). It is known that p28 interrupts the upregulation of TNF mRNA after p75 or CD40 activation (Turner *et al.*, submitted). It is possible that this also occurs *in vivo*. Thus by preventing upregulation of TNF or other cytokines, p28 may prevent a Th2 to Th1 switch in EV-susceptible mice which is not necessary for an effective cell-mediated antiviral response in Th1 biased resistant mice. In A/J mice it is likely that there are further mechanisms very early in the response, as indicated by the extremely low levels of EVΔp28 detectable by d3 p.i. in the liver and spleen. An alternative, and possibly complementary, possibility is that the innate apoptotic response of EV-infected cells to infection is sufficient

to clear the majority of infecting virus in the absence of p28 in the susceptible mouse strains tested. Since this would occur before the timepoints tested, the response would be unlikely to be observed in the liver from 3d p.i. To detect whether this is occurring, TUNEL staining in the infected footpad and local draining lymph node would be required at earlier timepoints. This may be enhanced by a reduced ability to replicate in macrophages (Senkevich *et al.*, 1994). A third, related mechanism may be that early host-induced apoptosis in EVΔp28 infection could serve to prime the immune response through rapid antigen presentation (Albert *et al.*, 1997; Bellone *et al.*, 1998). This might be expected to be of greater importance in susceptible mice than in mice which are independently capable of an effective cell-mediated anti-viral response such as B6 or 129.

A role for p28 in manipulating the immune response is supported by the reduction of EV-EVΔp28 differences in irradiated mice compared to non-irradiated controls, equal virulence of EV and EVΔp28 in congenitally athymic Swiss nude mice, accumulation of inflammatory foci in response to EVΔp28 but not EV in A/J mice, and by suppression of apoptosis by EV but not EVΔp28 in B6 mice. It is also possible that the reduced impact of p28 in EV infection of macrophage-depleted mice may reflect the role of p28 in manipulation of cellular immunity. This is suggested by the significant reductions in CD4+ and CD8+ T cell activity reported in macrophage-depleted mice (Karupiah *et al.*, 1996). In this regard it is interesting to note that the effect of macrophage depletion on EV and EVΔp28-mediated mortality was indistinguishable from that of sublethal irradiation, in which T cell function, and some aspects of macrophage function such as production of inflammatory cytokines, are compromised (Anderson and Warner, 1976)

Earlier studies showed that EVΔp28 was uniformly attenuated in SCID or outbred athymic mice (Senkevich *et al.*, 1995). In contrast, data in this chapter shows EVΔp28 virulence to be only marginally reduced in the congenitally athymic mouse strain, Swiss nude. While it is possible that compensatory mechanisms such as NK cells are masking the effect of p28

mutation, both EV and EVΔp28 grow to high levels in the Swiss nude mice, suggesting that compensating mechanisms are not effective against either virus. It is possible that the difference in EVΔp28 attenuation between this study and the previous study could be a result of differing genetic backgrounds of the athymic mice An analogous finding in this study is that EV and EVΔp28 virulence is equal in sublethally irradiated B6, yet EVΔp28 is attenuated in similarly irradiated A/J mice.

A key question addressed in this chapter was whether p28 was required for virulence as a strain and cell-type specific replication factor in the macrophages of A strain mice (Senkevich *et al.* 1995). This hypothesis was consistent with previous studies in which it has been shown that macrophages are amongst the first cell types to be infected (Mims 1959, Roberts 1962) and productive infection of macrophages is required for hepatic infection (Mims 1959). However, data in this chapter suggests that this hypothesis is unlikely to explain the role of p28, since EVΔp28 virulence in A/J mice was not restored by depletion of macrophages, indicating that p28 had further functions in virulence.

Thus, the data support the presence of macrophage-independent aspects of p28 activity in EV virulence. However, they do not preclude a role for p28 in manipulating other aspects of macrophage activity that may be of importance in the anti-viral response, for example, production of inflammatory cytokines and APC function (Gordon *et al.*, 1992). This is explored further in Chapter 4.

In addition to the role of p28 in infection, this study also focussed on the inter-relationships between apoptosis, virus growth and pathogenesis. Apoptosis has previously been associated with pathogenesis in a number of viral infections (Baize *et al.*, 1999; Lewis *et al.*, 1996; Oberhaus *et al.*, 1997; Roulston *et al.*, 1999). In this study, it was found that apoptosis was only associated with observable EV pathogenesis in immunocompromised mice (Swiss nude and B6.β2m-/-). In contrast, both susceptible and resistant

immunocompetent mouse strains had apoptotic responses unrelated to viral growth or necrosis. There are a number of conclusions that can be drawn. It can be seen from the low levels of apoptosis and high titres of virus at d6 p.i. in most strains tested that apoptosis is very unlikely to be required for EV replication. Similarly, the resistance of B6 and 129 mice to EV infection does not appear to be dependent on a quantitatively large apoptotic response, since levels of apoptosis are very similar to those found in susceptible A/J mice. This is confirmed by the apoptotic response of B6 mice to EVΔp28 infection. While infection with the p28 mutant resulted in significantly higher levels of apoptosis, this was associated with only a relatively minor acceleration of lymphocytic focus formation, which did not affect virulence or the outcome of infection. The lack of correlation between levels of apoptosis and resistance is interesting, given that the major cell types capable of inducing apoptosis during the acute phase of infection - the NK cells and CD8+ CTL- are essential for recovery from EV (Karupiah *et al.*, 1996). In particular B6 mice lacking perforin, and so unable to induce NK or CTL granule-mediated apoptosis, become susceptible to lethal infection (Mullbacher *et al.*, 1999b). In contrast, removal of CD4+ cells, which are of primary importance in cytokine production rather than in induction of cytotoxicity, results in chronic infection and an inability to clear virus (Karupiah *et al.*, 1996). This suggests an important role for the cytotoxic response in control of EV not detectable by *in situ* apoptosis detection. Alternatively, apoptosis early in infection may not be observable in this study because of the organs and timepoints chosen for investigation. For example, it is possible that p28 is influencing apoptosis in the footpad and DLN before the virus reaches the liver. Indeed, it can be seen throughout this chapter that events determining virus replication are likely to be occurring prior to 3d p.i. This can particularly be seen in A/J and B6 mice. In A/J mice, in which EVΔp28 was highly attenuated, virus replication was reduced by d3 p.i. In B6 mice, in which EVΔp28 was not attenuated, a difference in hepatic apoptosis was clearly evident by d3 p.i. The latter was

puzzling, as virus replication and leukocyte infiltration into the liver (including NK cells) was low at this stage. The effect of p28 on the apoptotic response to EV infection prior to d3 p.i. was thus examined more closely in B6 mice.

3.4 Tables and Figures

Table 3.1 Mortality in response to infection with EV or EVΔp28

Mouse Strain	Infection				Mice per group
	EV*		EVΔp28*		
	Mortality (%)	MTD (days)‡	Mortality (%)	MTD (days)	
A/J	100	9.0 ± 0.0	0	-	6
Balb/c	100	8.6 ± 0.5[a]	0	-	5
Swiss Nude	100	7.2 ± 0.3	100	9.0 ± 0.7	5
B6	0	-	0	-	5
129	0	-	0	-	5
B6/129	0	-	0	-	5

*Sex-matched 8-12 week old mice of each genotype were infected with 5x 10^3 pfu of either EV or EVΔp28 in the right hind footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality.

‡MTD, mean time to death ± SD. MTD analysed for statistically significant differences with the Student's t-test. MTD found to be significantly different between EV and EVΔp28 infected Swiss nude mice ($P<0.05$).

a. 2 of 5 EV infected Balb/c sacrificed at 8d p.i. for humane reasons.

Table 3.2 Indicators of morbidity in EV infection of various mouse strains

Symptom	Mouse strain and infection[a]											
	A/J		Balb/c		Swiss nude		129		B6/129		B6	
	EV*	EVΔp28	EV*	EVΔp28	EV*	EVΔp28	EV	EVΔp28	EV	EVΔp28	EV	EVΔp28
Weight loss[b]	-	-	-	+	+	+	-	-	-	-	-	-
Facial lesions[c]	-	-	+++	+	-	-	-	-	-	-	-	-
Intestinal lesions[d]	++	-	++	+/-	++++	++++	-	-	-	-	-	-
Hepatic lesions[e]	+++	-	+++	+/-	+++	+++	-	-	-	-	+/-	+/-
Splenic lesions[f]	+++	-	+++	+	+++	+++	-	-	-	-	+/-	+/-
LN destruction[g]	-	-	-	-	-	-	-	-	+	-	+	+
Hepatomegaly[h]	-	+	+	+	+	+	-	-	+	+	+	+
Splenomegaly[i]	+	++	++	+/-	+	++	++	+	++++	+++	++++	++++
Footpad swelling[j]	-	+	+/-	+	-	-	+++	++	+++	++	+++++	+++++

a. Groups of 5 female mice were infected with 5 x 10^5 pfu FV or EVΔp28 and observed for 25d p.i. Observations quoted in this table were taken at 9d p.i. or at time of death if MTD < 9d. Groups for which readings were taken at time of death are indicated by asterisks.

b. Weight loss was measured by daily weighing of mice before and after infection up to 9d p.i. Weight loss occurred from d6 p.i., and was not observed to reverse during the time measured. Scored at 9d p.i. Scores: Weight maintained at 100-95% uninfected weight (-); reduced to 90-95% uninfected weight (+); reduced to 85-90% uninfected weight (++)

c. Facial lesion scores: No lesions (-); conjunctivitis (+); conjunctivitis with visible pocks (++); extensive visible pocks (+++)

d. All intestinal lesions detected were severe. Score indicates number of mice in group with lesions, from 0 (-) to 5 mice (+++++)

e. Hepatic lesions were grossly visible as pale or dark mottling of liver, primarily at the outer edge of lobules and associated with fatty change. Individual plaques were observable in severe damage. Present in no mice -; low levels present in less than half of the experimental group +/-; low levels present in more than half of the experimental group +; mottling or plaques more prevalent ++; severe hepatic damage +++

f. Splenic lesions were grossly visible as pale or dark mottling, often associated with either softening of the organ or fibrous changes. Scoring as for hepatic lesions.

g. LN destruction scored as either popliteal LN draining the site of infection destroyed (+) or present (-) at 9d p.i.

h. Hepatomegaly: score indicates virus infected liver mass as a percentage of mock-infected liver mass. 0-100%: - ; 100-150%: +

i. Splenomegaly: score indicates virus infected spleen mass as a percentage of mock-infected spleen mass. 0-100%: -; 101-150% +, 151-200% ++, 201 – 300% +++, 301-400% +++, >401% +++++

j. Footpad swelling measured using clinical scores. No swelling -; just detectable +, bones of foot obscured ++; swollen to 3mm thickness +++; very swollen below hock ++++; swelling including hock +++++

Table 3.3 Effects of Cl2MDP treatment on resistance of mice to infection with EV and EVΔp28.

Infection *					
Mouse strain and Treatment	EV		EVΔp28		Mice per group
	Mortality (%)	MTD (days)‡	Mortality (%)	MTD (days)	
A/J					
+ PBS	100	9.8 ± 0.8	0	-	5
+ Liposomes	100	8.8 ± 0.8	80	15.0 ± 5.0	5
B6					
+ PBS	0	-	0	-	5
+ Liposomes	100	8.0 ± 0.0	100	8.0 ± 0.0	5

*Female mice of each strain were treated with 150μl Cl2MDP liposomes i.v. and 50μl liposomes s.c. into the right hand hind footpad. 48h later they were infected with $5x\ 10^3$ pfu of either EV or EVΔp28 in the same footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality.

‡MTD, mean time to death ± SD. MTD was not statistically different between EV-infected A/J mice treated with PBS or liposomes (P=0.10), liposome-treated A/J mice infected with EV or EVΔp28 (P=0.09), or liposome treated B6 mice infected with EV or EVΔp28 using the Student's t-test.

Table 3.4 Hepatic changes associated with infection.

Infection	EV				EVΔp28			
Mouse Strain	Swiss Nude	A/J	B6	129	Swiss Nude	A/J	B6	129
Day post-infection	Necrotic foci[a]							
0	0 ± 0	20.38 ± 61.15	0 ± 0	0 ± 0	0 ± 0	20.38 ± 61.15	0 ± 0	0 ± 0
3	20.38 ± 40.76	61.15 ± 326.11	0 ± 0	0 ± 0	0 ± 0	20.38 ± 407.64	0 ± 0	0 ± 0
6	481427±30166	8947.8 ± 6196.2	101.91 ± 101.91	0 ± 0	40968.2±8805.1	3444.6 ± 978.34	0 ± 0	0 ± 0
9	†[d]	†[d]	8947.8 ± 6196.2	0 ± 0	206268±115975	1732.5 ± 815.29	40.76 ± 40.76	0 ± 0
	Inflammatory cell foci[b]							
0	0 ± 0	0.01 ± 0.01	0.21 ± 0.21	0.10 ± 0.14	0 ± 0	0.01 ± 0.01	0.21 ± 0.21	0.10 ± 0.14
3	0 ± 0	6.48 ± 4.75	1.30 ± 0.82	5.69 ± 3.34	0 ± 0	3.24 ± 2.13	5.84 ± 2.46	4.38 ± 2.65
6	0 ± 0	0 ± 0	5.35 ± 1.15	22.31 ± 6.41	0 ± 0	7.68 ± 3.58	20.60 ± 2.85	18.35 ± 6.24
9	†	†	120.86 ± 39.55	51.26 ± 10.24	0 ± 0	24.65 ± 5.36	103.20 ± 27.46	44.21 ± 11.88
	Inflammatory cell infiltration[c]							
0	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
3	+/-	+/-	+	+/-	+/-	+/-	+	+
6	+/-	++	+	++	+/-	+	++	++
9	†	†	++	+++	+/-	+/-	++	+++

a. Levels of necrosis were semi-quantitated by counting necrotic foci in at least 10 fields of view per section from 2 sections per sample. Size of each necrosis was measured using a micrometer, and the total area per field of view was calculated. All samples were assessed blind. Data represents μm^2 necrotic tissue per field of view ± SD

b. Levels of inflammatory cell foci were semi-quantitated by observing foci in at least 10 fields of view per section from 2 sections per sample. The number of cells per focus and the total number of foci per field of view were counted. Cells per focus x number of foci were calculated to give the number of cells within foci in each field of view. All samples were assessed blind. Data represent cells within foci per field of view ± SD

c. Inflammatory cell infiltration was estimated by comparing the ratio of lymphocytes to hepatocytes present in at least 10 fields of view per section from 2 sections per sample. Lymphocytes present within foci were excluded from the ratio. All samples were assessed blind.

d. † = not assessed due to death of mice.

Table 3.5 Effects of sublethal irradiation on resistance of mice to infection with EV and EVΔp28.

Mouse strain and Treatment	Infection *				Mice per group
	EV		EVΔp28		
	Mortality (%)	MTD (days)‡	Mortality (%)	MTD (days)	
A/J					
Non-irradiated	100	9.0 ± 0.0	0	-	5
Irradiated	100	9.0 ± 0.0	100	18.0 ± 0.0[a]	5
B6					
Non-irradiated	0	-	0	-	5
Irradiated	100	10.5 ± 0.5	100	10.0 ± 0.0	5

*Male mice of each strain were irradiated with 650 rads from a ^{60}Co source 24h prior to infection. A further group of 3 mice of each genotype was irradiated and mock-infected to ensure that irradiation was sublethal and so not contributing directly to mortality of infection. No mortality was seen in this group (data not shown).

‡MTD, mean time to death ± SD. MTD analysed for statistically significant differences with the Student's t-test.

a. MTD found to be significantly different between EV and EVΔp28 infected mice ($P<0.01$).

Table 3.6 Mortality in B6 and B6.β2m-/- mice in response to infection with EV or EVΔp28

Mouse	EV*		EVΔp28		Mice per
Strain	Mortality (%)	MTD (days)‡	Mortality (%)	MTD (days)	group
B6	0	-	0	-	6
B6.β2m$^{-/-}$	100	17.3 ± 0.6	100	17.8 ± 0.3	10

*Sex-matched mice of each genotype were infected with 5x 10^3 pfu of either EV or EVΔp28 in the right hind footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality.

‡MTD, mean time to death ± SD. MTD not significantly different in EV and EVΔp28-infected B6.β2m-/- mice by Student's t-test (P<0.05).

Figure 3.1 EV and EVΔp28 replication in the acute phase of infection

Groups of 5 female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. Liver, spleen and the popliteal lymph node draining the infected footpad (DLN) were titrated as described in Karupiah et al. (1993). Titres and levels of apoptosis were analysed for statistical significance with the Student's t-test (*P<0.05, **P<0.01). Data is representative of two experiments. Limit of detection for titration: $10^{1.7}$ pfu/g. Titres and apoptotic counts are not available for EV-infected Swiss nude and A/J mice at 9d p.i. due to death of the mice (see Table 3.1). Data represents virus titre ± SD.

na: not applicable. DLN were often destroyed during the process of infection and so could not be titrated in B6 mice, or in 129 mice infected with EV at d9 p.i..

nd: not determined.

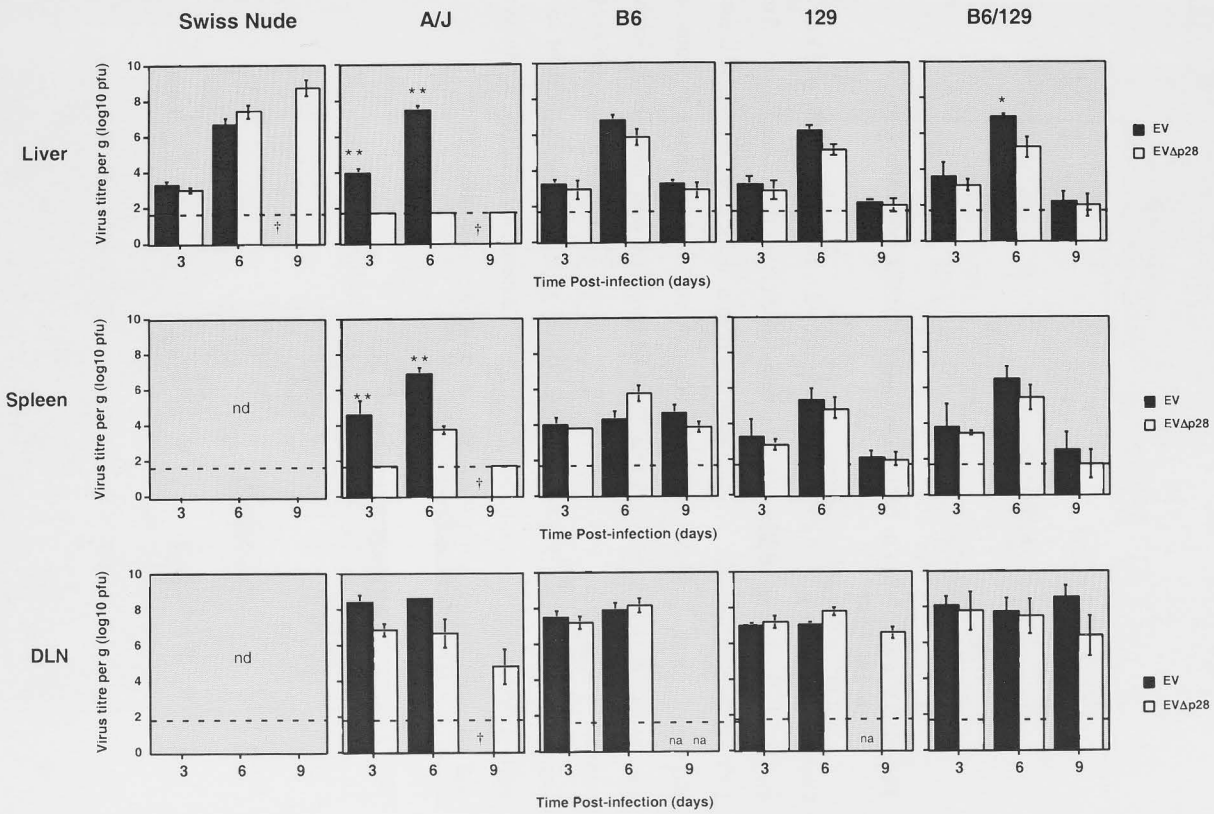

Swiss Nude
A/J
B6
129
B6/129
Liver
Spleen
DLN
Virus titre per g (log10 pfu)
Time Post-infection (days)
EV
EVΔp28
nd
na
†
*
**

Figure 3.2 Apoptosis in response to EV and EVΔp28 infection

Groups of 5 female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. Liver samples were fixed in 10% NBF for sectioning and staining for apoptotic cells using the TUNEL method (Section 2.10; A). Hepatic virus titres from figure 3.1 are also shown for comparison with apoptosis (B). Virus titres and levels of apoptosis were analysed for statistical significance with the Student's t-test (*P<0.05, **P<0.01). Data is representative of two experiments for A/J, B6, Swiss nude and B6/129 mice, and one experiment for 129 mice. Limit of detection for titration: $10^{1.7}$ pfu/g. Data are not available for Swiss nude and A/J mice at 9d p.i. with EV due to death of the mice (see table 3.1).

Error bars represent SD of collated data.

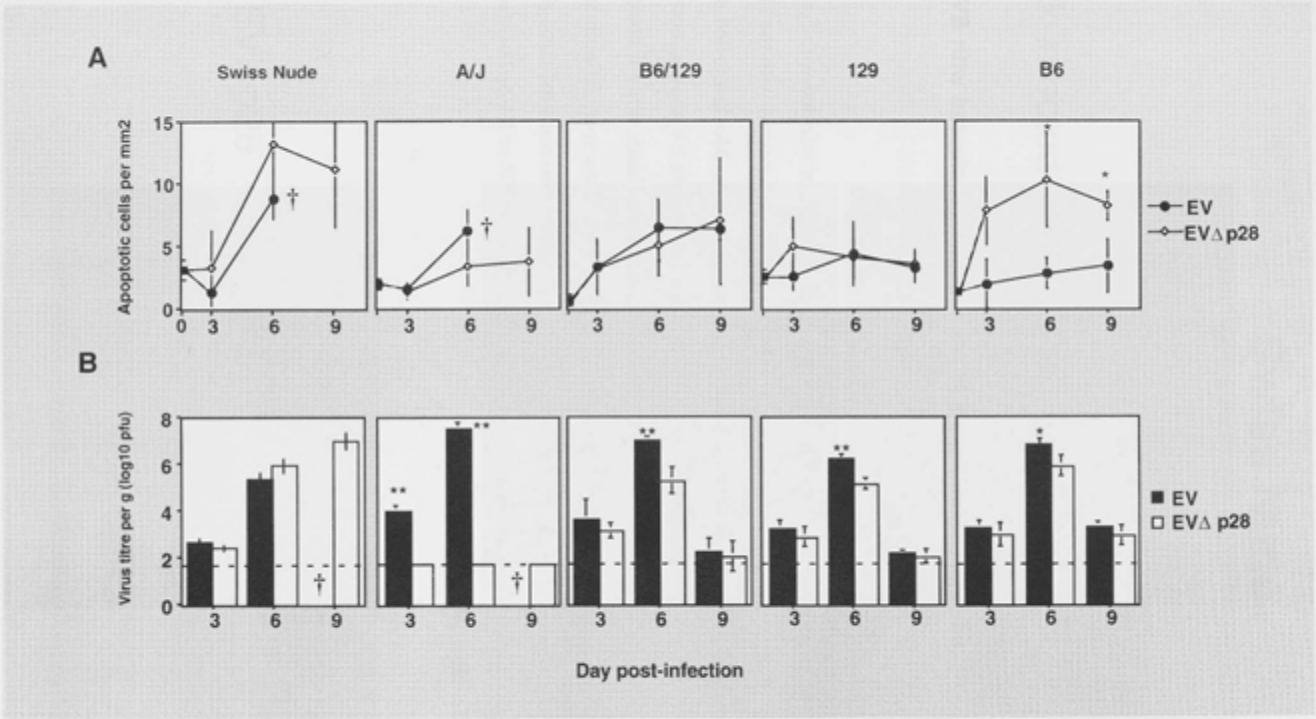

A
Swiss Nude
A/J
B6/129
129
B6
Apoptotic cells per mm2
15
10
5
0
0 3 6 9
EV
EVΔp28
B
Virus titre per g (log10 pfu)
8
6
4
2
0
3 6 9
EV
EVΔ p28
Day post-infection

Figure 3.3 Hepatic morphology of Swiss nude mice infected with EV or EVΔp28

Livers were harvested 6d p.i. from 5 female mice mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained with the H&E as per materials and methods. Representative sections are shown. Mock-infected mice displayed healthy liver morphology (A). Mice infected for 6d with EV showed necrotic changes and severe vacuolisation of cells throughout the tissue (black arrows; compare to portal inflammation in A/J and B6 mice, Figs. 3.4, 3.5). However, inflammatory changes were minimal (B). In EVΔp28 infected mice, necrosis also occurred throughout the tissue (C). No inflammatory infiltrates were seen in either EV or EVΔp28-infected Swiss nude mice, although ballooning necrosis was prominent (white arrows). Magnification: 20x.

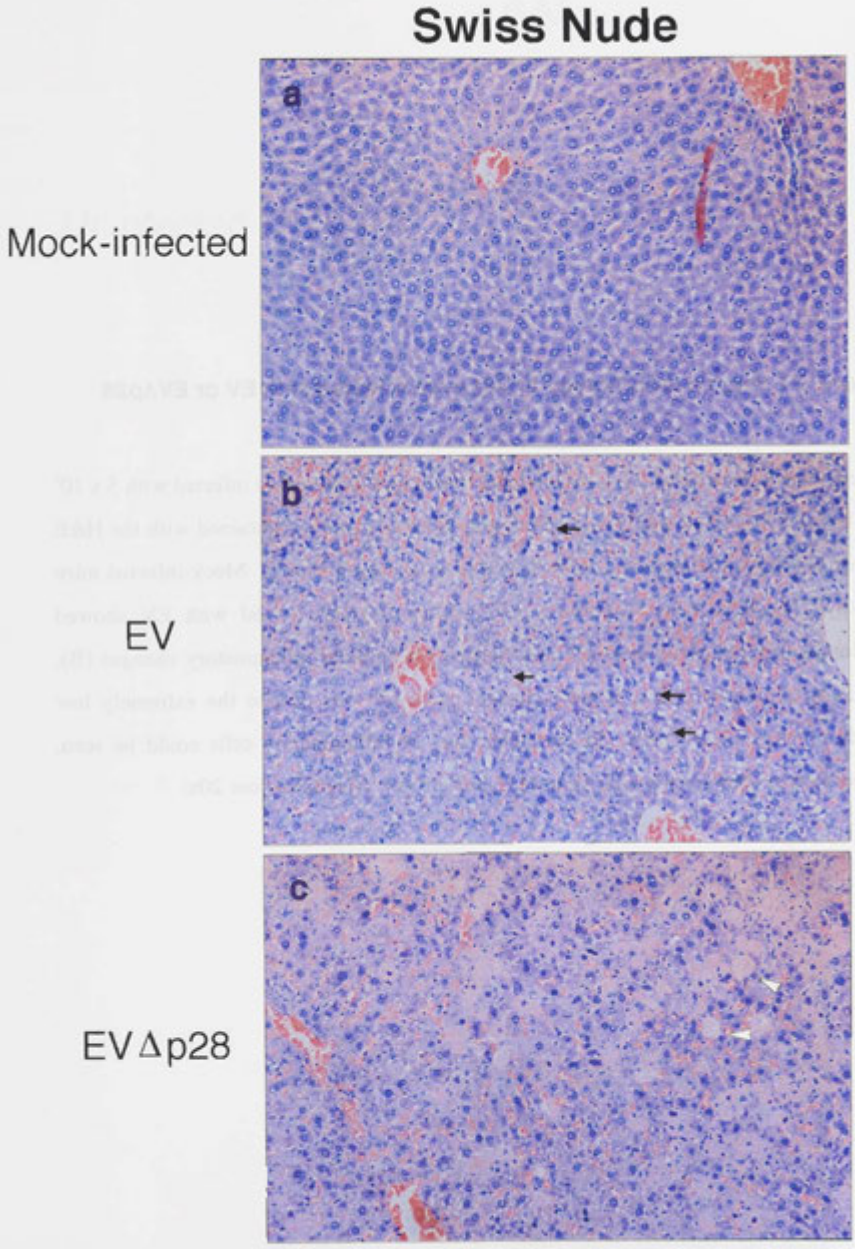

Swiss Nude
Mock-infected
EV
EVΔp28
a
b
c

Figure 3.4 Hepatic morphology of A/J mice infected with EV or EVΔp28

Livers were harvested 6d p.i. from 5 female mice mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained with the H&E as per materials and methods. Representative sections are shown. Mock-infected mice displayed healthy liver morphology (A). Mice infected for 6d with EV showed predominantly periportal necrosis, associated with minimal inflammatory changes (B). Necrotic cells resembled those seen in Swiss Nude mice infected with EVΔp28 (white arrows; see also Fig. 3.3). In EVΔp28 infected A/J mice, hepatic changes were obvious despite the extremely low levels of virus present (C). In particular, foci of inflammatory cells could be seen. These were predominantly near venules. Magnification: 20x.

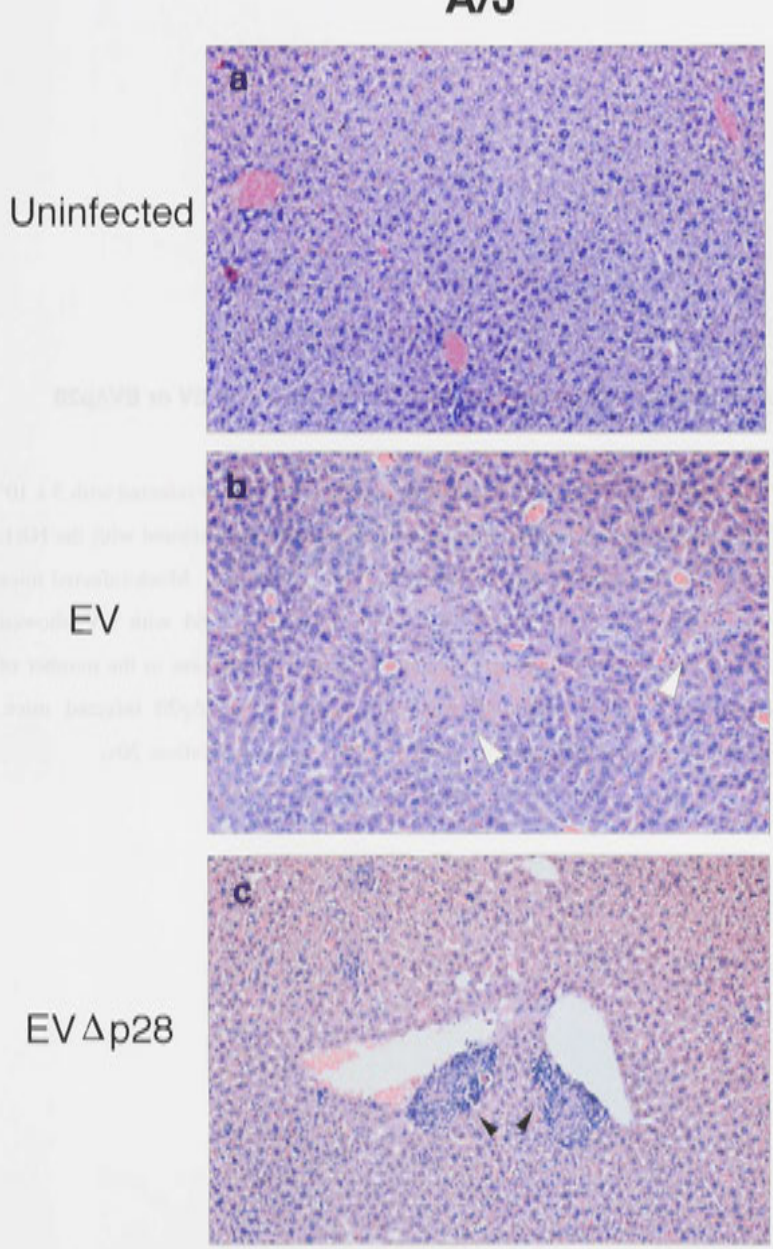
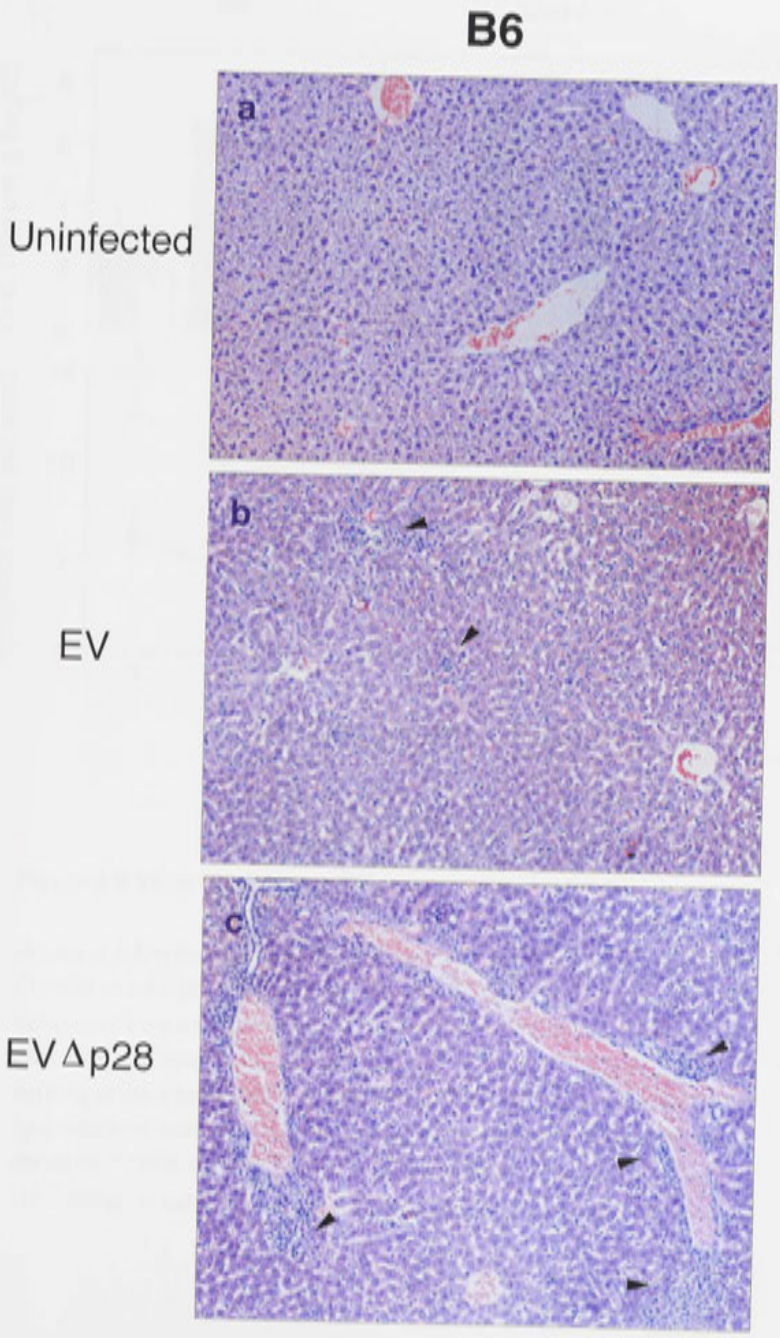

A/J
a
Uninfected
b
EV
c
EV Δp28

Figure 3.5 Hepatic morphology of B6 mice infected with EV or EVΔp28

Livers were harvested 6d p.i. from 5 female mice mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained with the H&E as per materials and methods. Representative sections are shown. Mock-infected mice displayed healthy liver morphology (A). Mice infected for 6d with EV showed inflammatory changes including distended sinusoids and an increase in the number of inflammatory cells (B). Pathology was very similar in EVΔp28 infected mice. Inflammatory infiltrates highlighted by black arrows (B, C). Magnification: 20x (A, B); 40x (C).


B6
a
Uninfected
b
EV
c
EVΔp28

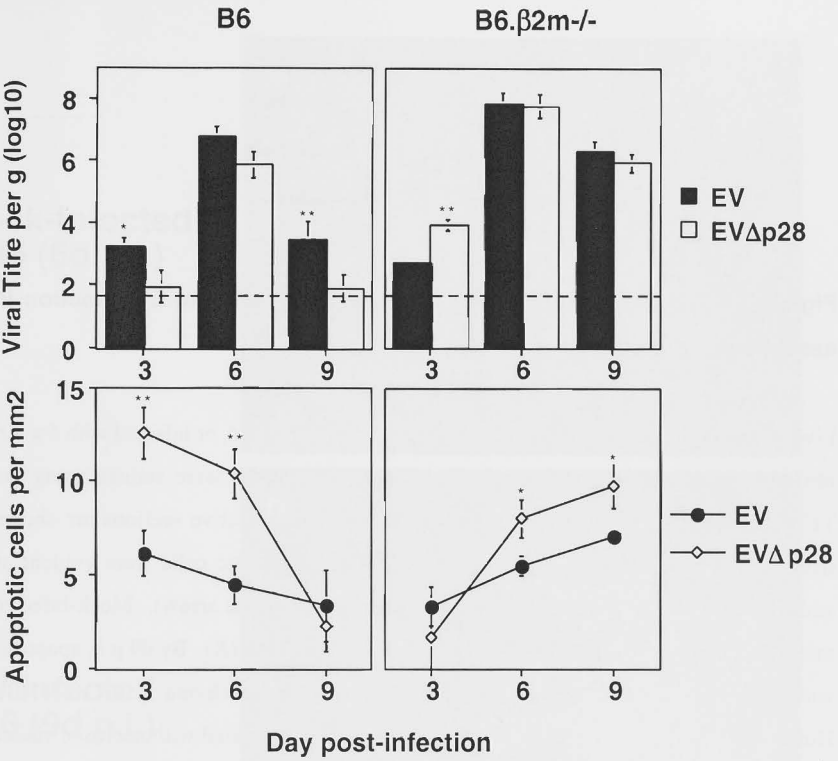


Figure 3.6 Virus growth and apoptosis in B6 and B6.β2m-/- mice

Groups of 5 female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. Samples of liver were harvested for virus titration as described by Karupiah *et al.* (1993) or snap-frozen in liquid nitrogen for cryosectioning and TUNEL staining as described in Sections 2.5 and 2.10 . Virus titres and levels of apoptosis were analysed for statistical significance using the Student's t-test (*P<0.05, **P<0.01). The limit of detection for virus titration was 50 pfu/g ($10^{1.7}$ pfu/g), as indicated by the dashed line. Error bars represent SD.

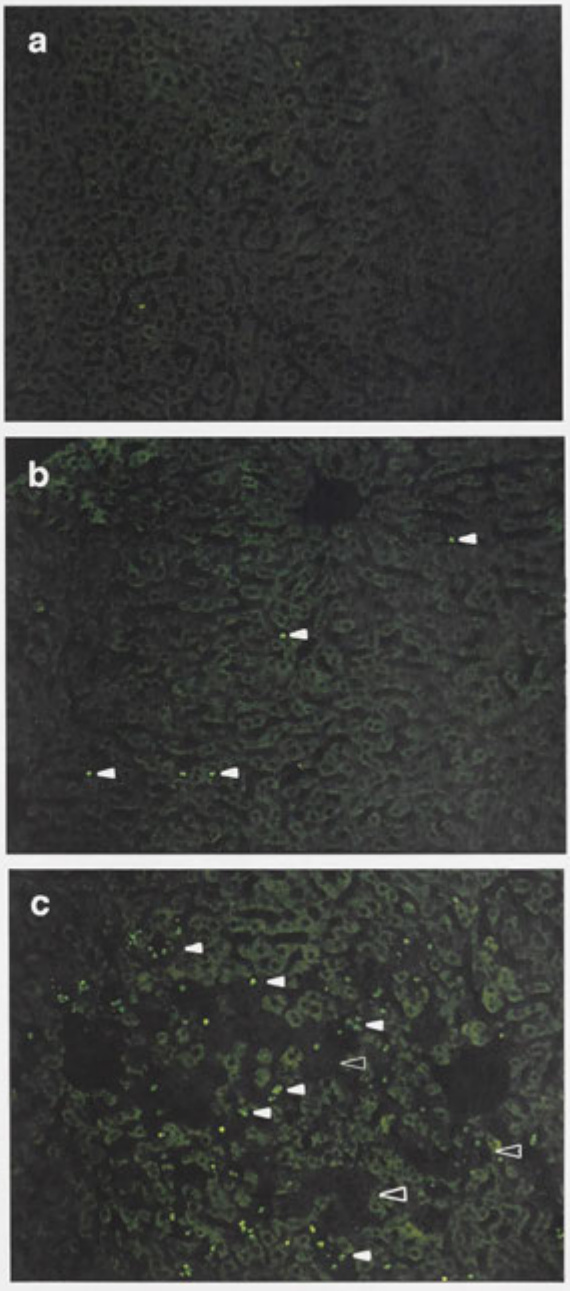
Figure 3.7 The apoptotic response of B6.β2m-/- mice to EV infection is associated with high levels of necrosis

Livers were harvested 6d p.i. from 5 female mice mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained using the TUNEL procedure as per materials and methods. Representative sections are shown from EV-infected samples examined for Fig. 3.6. Apoptotic cells were evident as strongly FITC-positive cells with apoptotic morphology (white arrow). Mock-infected mice displayed low to undetectable levels of hepatic apoptosis (A). By d9 p.i., apoptosis was present as scattered cells throughout the hepatic parenchyma in B6 mice (B). However, in B6.β2m-/- mice apoptosis was commonly associated with necrosis, visible in TUNEL stained sections as large areas of reduced background staining (black arrows). Pathology and the pattern of apoptosis was indistinguishable between EV and EVΔp28 infected mice. Magnification: 20x.

Mock-infected
B6 (6d p.i.)

EV infected
B6 (9d p.i.)

EV infected
B6.β2m-/- (9d p.i.)



Chapter 4

A Novel Apoptotic Response to EV Infection is Suppressed By Expression of p28 *In Vivo*

4.1 Introduction

There is now a large body of evidence supporting the importance of early events in the anti-viral response and outcome of infection. Studies in which key elements of the early host response have been ablated have shown that compromise of the early response is often associated with a reduced ability of the organism to clear virus and successfully recover from infection (Huang *et al.*, 1993; Karupiah *et al.*, 1996). The early response includes production of cytokines such as TNF and IFNγ (Grieder *et al.*, 1997; Ruzek *et al.*, 1997), and may include rapid apoptosis of infected cells (Itoh *et al.*, 1998). Notably, the response has previously been described as a local event (Grieder *et al.*, 1997) which appears to play roles in preventing spread of virus (Itoh *et al.*, 1998) and in the initiation of inflammation (Orange *et al.*, 1997). Infection with a number of complex DNA viruses results in cytokine upregulation within hours of inoculation (Grieder *et al.*, 1997; Ruzek *et al.*, 1997). Similar responses, such as cytokine upregulation, are closely associated with apoptosis and pathology later in infection (Orange *et al.*, 1997).

An important role for TNF early in infection is suggested by its rapid production after virus infection (Grieder *et al.*, 1997), the rapid attenuation of vaccinia virus constructs encoding TNF (Sambhi *et al.*, 1991), and the aberrant inflammatory responses to EV seen in TNF receptor deficient mice (Chapter 5). This corresponds with the *in vitro* finding that TNF is anti-viral within 24h in a variety of infections (Mestan *et al.*, 1986; Wong and Goeddel, 1986; Wong *et al.*, 1992). TNF is also one of the primary factors identified in the host response against EV (Ruby *et al.*, 1997 ; Chapter 5).

It has also been found that the virus gene p28 is necessary for EV to block transcriptional upregulation of TNF *in vitro*, thus preventing TNF-mediated apoptosis in some cell types (Turner *et al.* submitted). The data described in Chapter 3 shows that p28 is important for the virulence of EV in a variety of mouse strains. However, no

consistent effect on apoptotic profiles was observed from d3 to d9 p.i. Given the potential importance of early events in the oucome of infection, it was possible that p28 and/or apoptosis played an important role in EV infection prior to the timepoints chosen.

Thus, in this chapter apoptosis in EV infection and the effects of p28 are examined prior to d3 p.i.

4.2 Results

4.2.1 Two distinct phases of apoptosis occur on infection of mice with p28-mutant but not wild-type ectromelia virus.

During the experiments described in Chapter 3, it was noted that B6 mice displayed increased hepatic apoptosis within 3d of infection, despite low levels of detectable virus and inflammatory cell infiltration (Table 3.2, Fig. 3.2). The data suggested that p28 affected apoptosis prior to d3 p.i. To investigate this hypothesis, B6 mice were infected with 5×10^5 pfu of either EV or EVΔp28 via the footpad as described in Section 2.5, and sacrificed at intervals from 6h to 9d p.i. (Fig. 4.1A, B). The extension of the time course to 9d p.i. allowed direct comparison of apoptosis before d3 p.i. with that already described. The liver, a target of infection, was harvested, sectioned and stained, either using the TUNEL technique in order to visualise apoptotic cells *in situ*, or haematoxylin and eosin (H&E) for general histological examination (Figs. 4.1A, 4.2). Samples were also collected for virus titration (Fig. 4.1B).

Apoptosis was very low in mock-infected mice (Fig. 4.1A). At 24h p.i., both EV and EVΔp28 infected mice displayed a marked increase in hepatic apoptosis. Apoptosis from d3-9 p.i.was similar to that described in Chapter 3 (Fig. 4.1A). Apoptotic cells were predominantly scattered cells within the sinusoids and occasional parenchymal cells. As virus replication, necrosis and the inflammatory response increased through d6 p.i., foci of apoptotic hepatocytes in association with infiltrating lymphocytes or necroses could be observed (Fig. 4.2G) which were also observable at d9 p.i. This response was common to both EV and EVΔp28-infected mice.

In addition, examination of apoptosis before d1 p.i. revealed an apoptotic response to EVΔp28 infection at only 6h p.i. The apoptosis was not observed in EV infection, implying that the response was blocked by the presence of p28 (Figs. 4.1A, 4.2A, B, C, D). The apoptotic cells were distributed randomly through the hepatic parenchyma,

predominantly in the sinusoids, suggesting that the apoptosing cells might be kupffer cells or migrating leukocytes. Further, the apoptotic response was transient, with apoptosis no longer detectable 12h after infection. This apoptotic response was distinctive in that it occurred prior to the detection of infectious virus in the liver (Fig. 4.1B) and was not associated with inflammatory changes seen later in infection such as sinusoid distension (Fig. 4.2E), necrosis (Fig. 4.2F) or foci of inflammatory cells. In contrast, EV-infected mice did not display increased hepatic apoptosis prior to 24h p.i. (Figs. 4.1A, 4.2C).

Titration of livers showed that virus growth was very similar to that described in Chapter 3. Virus first became detectable at d3 p.i. for both EV and EVΔp28. Virus titres then peaked at d6 p.i., followed by a decrease in titres, detectable at d9 p.i. The trend was the same for both viruses, with EVΔp28 titres being significantly lower than EV titres at d3 and d9 p.i. in 2 of 3 experiments ($P<0.05$; Fig. 4.1, and data not shown). Previous studies of apoptosis in viral infection have associated apoptosis with the presence of viral antigen (Lieber *et al.*, 1997). However, titration of the livers had shown that infectious virus was not detectable before d3 p.i. (Fig 4.1B). The possibility existed that during footpad infection a very small amount of virus, below the limit of detection by titration, had entered the bloodstream and thus reached the liver. To investigate the possibility that virus was present in the liver 6h p.i., EV-specific PCR was performed on DNA extracted from the same livers used for the apoptotic studies (Fig. 4.3). It was found that viral DNA could be detected 24h p.i. in both EV and EVΔp28 infected mice, correlating with the marked increase in apoptosis seen at 1d p.i. in both infections (Fig. 4.1A). However, viral DNA could not be detected 6h or 12h p.i. Hence, infection with EVΔp28 but not EV elicited a transient apoptotic response in the liver 6h p.i. in B6 mice. At this very early stage of the infection, no virus could be detected in the liver by titration or PCR.

4.2.2 The early phase of virus-induced apoptosis is dependent on TNF

The finding that EV but not EVΔp28 inhibited CD40 and TNF mediated cell death *in vitro* (Turner *et al.*, submitted) suggested that the apoptosis observed in mice infected with EVΔp28 could be dependent on one of these factors. Both are important in the anti-EV response (Ruby *et al.*, 1995; Ruby *et al.*, 1997), with TNF implicated in the later stages of other viral infections as a cause of hepatic immunopathology (Orange *et al.*, 1997). CD40 was first investigated to determine whether it was necessary for the early phase of apoptosis. CD40-/- and wt B6/129 mice were infected as previously with EV or EVΔp28 and livers harvested for TUNEL staining at 6h and 12h p.i.. To control for the effects of stress and tissue disturbance during inoculation, livers were also harvested from mock-infected mice 6h p.i. as described in Section 2.5. It was found that neither wt (B6/129) nor CD40-/- mice responded to mock-infection with an increase in apoptosis (Fig. 4.4). CD40-/- mice experienced an increase in apoptosis at 6h p.i. in response to EVΔp28 but not EV, as was seen in wt mice (Fig. 4.4). Hence CD40 was not necessary for the early phase of apoptosis to occur.

To investigate whether TNF was required for the early apoptotic response to EVΔp28 infection, apoptosis was also examined in mice in which TNF was depleted. B6 mice were treated with neutralising anti-TNF mAb or an isotype control 24h before infection with EVΔp28, then at 2d intervals thereafter. This antibody and dose has previously been shown to efficiently deplete TNF from the serum (J. Ruby, pers comm), and was found in this experiment to deplete serum TNF 6d p.i. (data not shown) as tested by biological assay (Section 2.9). Livers were harvested 6h or 6d p.i., and apoptotic cells stained using TUNEL. Serum was also collected and tested for the presence of TNF by biological assay.

Apoptotic cells were readily detected in livers from control mAb-treated mice infected with EVΔp28 6h p.i. (Fig. 4.5A). In contrast, apoptosis in equivalent EVΔp28-infected mice treated with anti-TNF mAb remained at background levels. Thus, treatment with

anti-TNF mAb prevented the early phase of apoptosis. A comparison of apoptosis in anti-TNF treated mice with control treated mice at 6d p.i., revealed a trend towards higher apoptosis in the anti-TNF treated subjects although not to a statistically significant extent (P=0.15; Fig. 4.5A). Titration revealed that this corresponded with a similar trend in hepatic virus titres of anti-TNF mAb-treated mice at 6d p.i. (P=0.14; Fig. 4.5B), suggesting that the trend towards increased apoptosis may be due to virus-mediated damage.

It is noteworthy that while TNF was demonstrably depleted at d6 p.i, TNF was below detectable levels in the serum of all mice tested at 6h p.i. (limit of detection 1.2 pg/ml; data not shown).

Thus, the early phase of apoptosis was dependent on the presence of TNF but not CD40.

4.2.3 The early phase of apoptosis is dependent on the p55 TNF receptor

Treatment with anti-TNF mAb demonstrated the apparent dependence of the early apoptotic response on TNF. TNF is capable of signalling through two receptors – p55 and p75 (see Section 1.3.3.1) – as well as potentially mediating signalling events through its cytoplasmic domain (Hribar *et al.*, 1999; Watts *et al.*, 1999). To investigate the contributions of the alternative p55 and p75 TNF receptors to the early apoptotic response, hepatic apoptosis was monitored in wt B6/129 mice and mice lacking either p55, p75, or both TNF receptors (Peschon *et al.*, 1998). Mice were mock-infected with tissue culture lysate or infected with 5 x 10^3 pfu of either EV or EVΔp28 in the hind footpad as previously and liver samples harvested at 6h p.i. for TUNEL staining (Fig. 4.6). As previously, EVΔp28 but not EV infection significantly increased apoptosis above background levels in the livers of wt B6/129 mice at 6h p.i. (P<0.01; Fig. 4.4, 4.6). p75-/- mice also displayed a large increase in apoptosis 6h after infection with EVΔp28, which was not significantly different to the apoptotic response of B6/129 mice to EVΔp28. p75-/- mice were found to further display elevated apoptosis after EV

infection. While not statistically significant (P=0.18, Fig. 4.6), the trend was maintained in duplicate experiments.

Mock-infected and uninfected p55-deficient mice were found to consistently display higher apoptosis than equivalent p75-/- or B6/129 mice. Levels of apoptosis did not increase at 6h p.i. with either EV or EVΔp28 infection, suggesting that the p55 TNF receptor is necessary for the early phase of apoptosis. This was further supported by the lack of an apoptotic response in p55-/-p75-/- mice. Mice lacking both TNF receptors showed extremely low levels of apoptosis which were invariant between EV, EVΔp28 and mock-infection 6h p.i. The pattern of apoptosis in all infected and mock-infected p55-/-p75-/- mice examined 6h p.i. appeared to also reflect the pattern of apoptosis seen in uninfected mice.

Thus p55 was necessary for the early apoptotic response to occur. The response was not dependent on p75, however a role for p75 in p28 activity was implied by the trend towards increased apoptosis in EV-infected p75-/- mice 6h p.i.

4.2.4 The early phase of apoptosis is not exclusive to the liver

The liver is investigated throughout this thesis as the target organ of EV infection in which virally-induced damage is thought to lead to mortality (Fenner 1949). However, it is also an important filter for the body's blood supply and as such is sensitive to systemic changes (Arias, 1988). Hence, it was possible that the changes seen in hepatic apoptosis at 6h p.i. were systemic rather than specific to the liver. Thus apoptosis was also determined in the ovaries, a site of low level EV infection (Karupiah *et al.*, 1993). Apoptosis was not quantitated over the entire ovary since apoptosis occurs continuously in healthy, uninfected ovaries during normal follicular atresia (Guo *et al.*, 1994; Kaipia and Hsueh, 1997; Fig. 4.7A). However, levels of apoptosis are extremely low or undetectable in ovarian stroma of healthy mice (Fig. 4.7A, B), and increased during EV infection (Fig. 4.8). Thus B6/129, p75-/- or p55-/-p75-/- mice were mock-infected, or infected with EV or EVΔp28 via the footpad as previously and ovaries collected at 6h or

6d p.i. for TUNEL staining. Apoptotic cells were then counted in the stroma of each ovary.

The ovaries of mock-infected wt mice were indistinguishable from those of uninfected mice (Figs. 4.7, 4.8A, D, Table 4.1). Upon infection with EV, apoptosis in the ovarian stroma increased in B6/129 and p75-/- mice (Fig. 4.8B), although the ovaries did not appear otherwise damaged. EVΔp28 infection resulted in further increases in stromal apoptosis in B6/129 and p75-/- mice ($P<0.05$; Table 4.1, Fig 4.8A, C). In contrast, p55-/- mice showed no statistically significant increase in apoptosis ($P=0.64$; Table 4.1). Interestingly, these mice exhibited a wide variation in apoptosis between individuals infected with EV or EVΔp28 not seen in other mouse strains. The variation was consistent in two experiments, and consistent with unusually high hepatic apoptosis (Fig. 4.6). p55-/-p75-/- mice responded to EVΔp28 infection with low levels of apoptosis at 6h p.i. While the increase in apoptosis in EVΔp28-infected p55-/-p75-/- mice over that in EV-infected or mock-infected mice remained significant ($P<0.05$), it should be noted that apoptosis stimulated 6h p.i. by EVΔp28 infection of p55-/-p75-/- mice was below levels seen during EV infection of all other mouse strains tested ($P<0.01$).

A comparison of apoptosis levels at d6 p.i. showed that EV infection uniformly stimulated higher levels of apoptosis in the ovarian stroma than EVΔp28 independent of host genotype ($P<0.02$; Table 4.1). Apoptosis at this point was much more prolific than that observed at 6h p.i. ($P<0.01$; Table 4.1, Fig. 4.8E, F). However, levels of apoptosis were not significantly different between wt and TNF receptor knockout mice. This suggested that TNF did not play a role in the ovarian response to EV or EVΔp28 infection during apoptosis 6d p.i. Thus, this data showed that the early apoptotic response occurred in the ovaries as well as in the liver. Further, the apoptotic response in the ovaries was dependent on the p55 TNF receptor.

4.2.5 p28 does not block the early phase of apoptosis in IFNγR-/- mice

TNF *in vivo* is part of a complex web of cytokine interactions. Hence, in addition to TNF receptor knockout mice, apoptosis was examined in mice lacking IFNγR and IL-6, which are known to play a role in the effective host response to EV (Karupiah *et al.*, 1993; Ramshaw *et al.*, 1997). IFNγ is particularly important in antiviral responses (Karupiah *et al.*, 1993) and can enhance TNF production as well as synergising with TNF-mediated anti-viral activity (Beutler *et al.*, 1986; Davignon *et al.*, 1996; Ohmori *et al.*, 1997; Schijns *et al.*, 1991). IL-6 is induced by TNF during hepatic damage and can prevent TNF-mediated hepatic damage under some circumstances (Mizuhara *et al.*, 1994). Mortality studies have shown that IL-6 deficient mice have an increased susceptibility to EV-mediated lethality (Ramshaw *et al.*, 1997). To determine whether the contribution of TNF to the early phase of apoptosis was specific, or due to the less effective antiviral response in TNF receptor deficient mice, mice lacking IL-6 or the receptor for IFNγ were infected with a small amount of EV or EVΔp28 as previously and apoptosis determined in the liver (IL-6-/-) or ovaries (IFNγR-/-). Apoptosis studies were also to include the livers of IFNγR-/- mice, however fixation of the livers was incomplete, resulting in the livers being unusable for TUNEL staining. As IL-6 and IFNγR-/- mice were on a different background to the TNF receptor deficient mice, the background strain 129Sv (129) was also infected and hepatic and ovary samples examined for apoptosis.

Apoptosis in 129 mice 6h p.i. showed the same trends as had been seen in B6 and B6/129 mice. Apoptosis in response to EV 6h p.i. was very low; indeed, levels of hepatic apoptosis stimulated by EV infection were lower than those stimulated by mock-infection (Table 4.3). Apoptosis increased in response to EVΔp28 in both the ovary (Table 4.2) and liver (Table 4.3) in comparison to EV- or mock-infection.

Apoptosis in the ovaries of IFNγR-/- mice was equally induced by EV and EVΔp28 infection (Table 4.2). Apoptosis increased to very high levels within 6h of infection in

response to either virus. Levels of apoptosis were 5 to 10 fold higher than those detected in other strains of mice (Table 4.1). Similarly, at d6 p.i. levels of apoptosis were elevated in response to both viruses, and not significantly different between EV and EVΔp28. Thus p28 did not prevent early apoptosis in IFNγR-/- mice.

Apoptosis in IL-6-/- mice was very similar to that described in the wt 129 strain, and was indistinguishable microscopically. At 6h p.i., EV-infected mice displayed slightly lower levels of apoptosis in the liver than mock-infected mice. Apoptosis increased 6h p.i. with EVΔp28 to levels similar to those described in 129 mice. Hence, IL-6 did not play a detectable role in the apoptotic response to infection 6h p.i.

4.2.6 The early phase of apoptosis is mouse strain dependent

It could be seen from a comparison of apoptosis in B6, B6/129 and 129 mice at 6h p.i. that the early phase of apoptosis was present in EV-resistant mouse strains (Figs. 4.1, 4.4, Table 4.1). However, it was found in Chapter 3 that not all mouse strains responded equally to p28 expression. To investigate the effect of host strain on the early phase of apoptosis, EV-resistant and EV-susceptible mouse strains were mock-infected or infected in the right hind footpad with 5×10^3 pfu of either EV or EVΔp28 as previously, and livers harvested at 6h or 6d p.i. for TUNEL staining. In addition to B6, B6/129 and 129 mice, apoptosis was measured in the livers of EV-susceptible Swiss nude and A/J mice.

EV infection did not stimulate apoptosis greater than mock-infection at 6h p.i., in any mouse strain (Table 4.3). In contrast, EVΔp28 stimulated a host-strain dependent apoptotic response. The apoptotic response to EVΔp28 was highly significant in B6 and B6/129 mice ($P<0.01$). EVΔp28 stimulated lower levels of apoptosis 6h p.i. in 129 mice compared to B6 mice ($P<0.01$), however apoptosis remained significantly higher than that in response to EV ($P<0.05$). In contrast, the apoptotic response to infection of A/J and Swiss Nude mice to EVΔp28 infection did not increase to levels greater than

those seen on mock-infection or EV-infection. Further, a comparison of the early phase of apoptosis (6h p.i.) with the later phase (6d p.i.) showed that the two phases were not observably interdependent. Hence, the early phase of the apoptotic response was dependent on host strain. This appeared to correlate with susceptibility or resistance to EV-mediated lethality. A comparison of apoptosis in resistant mice 6h p.i. with EVΔp28 further shows that the apoptotic response is most significantly increased in the B6 mice. Interestingly, apoptosis in the B6 x 129 hybrid was intermediate between the parent strains and was not significantly different from either, suggesting that the early apoptotic response is heritable and codominant.

4.2.7 The early phase of apoptosis is macrophage dependent

Macrophages have been implicated in the attenuation of EVΔp28 *in vitro* (Senkevich *et al.*, 1995) although their role *in vivo* is more ambiguous (Chapter 3). As a highly phagocytic cell type, macrophages are amongst the first cell types to be infected during dermal (Roberts, 1962) or intravenous (Mims, 1959b) infection. Replication of EV in macrophages is critical for hepatic infection (Mims, 1959b). Macrophages are also important APCs and sources of cytokines. In particular, macrophages are known to produce inflammatory cytokines including TNF on infection *in vitro* and *in vivo* (Biron, 1994; Goldfeld and Maniatis, 1989). To test whether macrophages in the liver or at the site of infection were required for the early apoptotic response in the liver, Cl2MDP-liposomes were used to deplete macrophages from distinct tissue compartments (Kurimoto *et al.*, 1994; van Rooijen and Sanders, 1994). Footpad inoculation with liposomes was used to deplete macrophages from the footpad and medulla and subcapsular sinus of the draining lymph node. This did not include the Langerhans cells of the skin, tissue dendritic cells, or dendritic cells and macrophages of the cortex and germinal centres of the lymph nodes (Delemarre *et al.*, 1990; Kurimoto *et al.*, 1994). Intravenous inoculation was then used to deplete macrophages from the liver and red pulp of the spleen (van Rooijen and Sanders, 1994). Macrophage depletion was monitored by acid phosphatase staining of livers and DLNs from liposome-treated and

control-treated animals using acid phosphatase staining (Section 2.8, Table 2.2; Burnstone 1959, (Kraal *et al.*, 1987). 48h post-inoculation with liposomes, livers and popliteal LN were harvested from treated mice and snap-frozen in liquid nitrogen. Cryosections were then acid-phosphatase stained. No changes in Kupffer cell numbers were found after liposome treatment of the footpad, despite almost complete macrophage depletion in the popliteal lymph node (Fig. 4.9B). In contrast, mice in which Cl2MDP-liposomes were inoculated i.v. were depleted of Kupffer cells from the liver without affecting macrophage numbers in the popliteal lymph node (Fig. 4.9B).

To determine the role of macrophages in the footpad and liver in the early phase of apoptosis, Cl2MDP-liposomes were injected i.v. or into the footpads of B6 mice 2 days prior to footpad infection with 5 x 10^1 pfu EVΔp28. Livers were harvested 6h p.i. and stained using TUNEL for quantification of apoptosis. Levels of apoptosis in PBS-treated mice infected with EV were similar to those in previous control treated mice (Fig. 4.5, 4.9A). The lower levels than those seen in untreated mice (Fig. 4.1) suggests that i.v. treatment may slightly depress the apoptotic response independent of the effects of liposomes or virus. Macrophage depletion from the footpad, accompanied by PBS injection i.v., resulted in a decrease in the number of apoptotic cells in the livers of the infected mice, compared to control-treated animals ($P<0.05$; Fig. 4.9A). In contrast, i.v. inoculation of Cl2MDP-liposomes caused increased levels of TUNEL-positive cells in the livers. This effect was possibly due to residual apoptotic macrophages 48h after i.v. injection of liposomes, or the inefficient clearance of apoptotic debris from macrophage-depleted livers (Falasca *et al.*, 1996; Shi *et al.*, 1998; Shi *et al.*, 1996). These data indicated that the early apoptotic response observed in the liver was dependent on macrophages at the site of infection. While no contribution towards the apoptotic response by Kupffer cells was detected, this may have been complicated by ongoing apoptosis of hepatic phagocytes after i.v. liposome treatment.

4.2.8 Inhibition of the early phase of apoptosis does not influence virus replication

The results presented indicate that p28 was required to block the early phase of apoptosis and that it was also a virulence factor (Chapter 3). To determine whether the effect of p28 on virulence was linked to the early phase of apoptosis, it was necessary to manipulate apoptosis 6h p.i. independent of the later effects of p28 expression (d3-9 p.i.; Fig. 4.1A). Hence, groups of 5 B6 mice were treated with anti-TNF or a control antibody 24h prior to infection, in order to prevent the early phase of apoptosis. Mice were then infected in both hind footpads with a high dose of EVΔp28 (5×10^6 pfu per footpad) and sacrificed at 6h p.i. for assessment of apoptosis, or 1d or 2d p.i. for viral titration.

Assessment of apoptosis showed that apoptosis was increased in response to EVΔp28 to levels similar to those seen in infections with smaller doses of virus (7.34 ± 1.68 apoptotic cells per mm^2 liver; Table 4.3). Anti-TNF treatment led to reduction of peracute apoptosis to a lesser extent than observed previously, which was not signifcant at 6h p.i. (4.75 ± 1.76 apoptotic cells per mm^2 liver; P=0.059). Virus titration from the draining popliteal lymph nodes, livers or lungs showed no difference between the amount of virus recovered from anti-TNF mAb treated mice compared to control mAb treated animals d1 p.i. (Fig. 4.10). While anti-TNF mAb treatment tended to be associated with an increase in splenic virus titres, levels of virus were not significantly higher than those in control mAb-treated animals (P=0.053; Student's t-test). Similarly, the apparent decrease in lung titres on anti-TNF treatment was not significant, as only 3 of 5 mice treated with the control mAb yielded virus from this organ. By day 2 there was no difference in virus levels between anti-TNF mAb and control mAb treated animals in any of the organs tested. Hence, this preliminary experiment did not demonstrate an effect of the early apoptotic response on virus growth.

4.3 Discussion

This chapter demonstrates that EV stimulates a biphasic apoptotic response. Apoptosis was observed in the livers of mice 6h after infection with low doses of EVΔp28 but not wt EV in the footpad. The apoptosis was transient and no longer observable 12h after infection. A second phase of apoptosis was then observed in the liver from 1 to 9d p.i., which coincided with the detection of virus in the liver and was then maintained after the onset of virus clearance at 9d p.i. The data suggest that different pathways are involved in the regulation of the two phases of apoptosis since p28 gene expression clearly suppressed the early phase of apoptosis, but had a variable effect on the later response in different strains of mice. Thus two distinct, differentially regulated phases are present in the apoptotic response to EV infection.

Apoptosis has previously been demonstrated within hours of infection at the site of virus replication *in vivo* (Lieber *et al.*, 1997). Viral antigen and apoptosis are often closely associated (Lewis *et al.*, 1996), however histological colocalisation studies suggest that within an infected tissue apoptosis may be triggered in uninfected cells (Heise and Virgin, 1995; Oberhaus *et al.*, 1997). The current study demonstrates that a rapid apoptotic response may also occur in an organ remote from the site of infection. We found that viral DNA first became demonstrable in the liver 24h p.i., slightly earlier than previous authors (Fenner, 1949; Mims, 1959b). The lack of detectable virus in the liver prior to 24h p.i. suggests that the hepatic apoptosis may be due to a systemic response initiated by local events in the footpad. An apoptotic response was observed in the ovaries which mirrored that in the liver, also supporting a systemic response to infection. Rapid systemic sequelae of local events have previously been suggested by the work of Grieder and colleagues (Grieder *et al.*, 1997) in which cytokine mRNA was measured in various organs in response to footpad infection with the alphavirus, Venezuelan equine encephalitis. IFNγ, TNF and IL-6 mRNA in the local draining lymph node was upregulated within 6h p.i. to levels equal to or greater than those found

at 24h p.i. Further, changes in cytokine mRNA levels in non-draining lymph nodes and spleens of infected animals were found within 24h p.i. This supports the concept of a systemic response during the very early stages of viral infection, potentially triggered by events at the site of infection.

The early phase of apoptosis did not appear to depend on CD40. Two lines of evidence indicated that the apoptotic response was instead critically dependent on TNF. Firstly, neutralisation of TNF using specific mAb significantly reduced levels of apoptosis 6h after infection. Secondly, no early apoptotic response to infection with EVΔp28 was observed in mice lacking the p55 TNF receptor, with or without the presence of p75. A rapid, short-lived increase in TNF levels has been noted during a similar timeframe in other viral infections (Grieder *et al.*, 1997; Lieber *et al.*, 1997) although these studies have not examined apoptosis. Hence, the hypothesis that the peracute apoptotic response to EVΔp28 infection is mediated by a transient increase in TNF levels is supported directly by the data in this chapter and indirectly by work in similar models of infection apoptosis noted in EV infection (Grieder *et al.*, 1997; Lieber *et al.*, 1997). While the reason for the transience of the response has not been investigated in this study, it is possible that the downregulation of TNF may occur rapidly at a transcriptional level as has been found *in vitro* (Sinha *et al.*, 1998). It is also known that release of cellular and poxviral soluble TNFRs is capable of blocking TNF activity (Loparev *et al.*, 1998), suggesting further potential mechanisms for downregulation of the response.

In vitro studies in our laboratory suggest a mechanism by which EV might be blocking the early phase of apoptosis. At the initiation of these studies it was known only that p28 was required for EV to block *in vitro* cell death stimulated by CD40 or p75. It is now known that one pathway by which these two receptors cause apoptosis is the upregulation of TNF mRNA. The TNF produced can cause autocrine or juxtacrine stimulation of the p55 TNF receptor, resulting in cell death (Grell *et al.*, 1999). A recent study by Steve Turner and Janet Ruby has shown that prevention of p75 or

CD40-mediated cell death by EV *in vitro* is associated with reduced upregulation of TNF mRNA by either of these receptors (Turner *et al.* submitted). In contrast, infection with the p28 mutant virus does not change the levels of TNF mRNA produced by infected cells. It is possible that a similar mechanism is responsible for the early phase of apoptosis and its suppression by EV *in vivo*. This is suggested by the observation that EVΔp28-infected mice lacking the p75 TNF receptor have reduced hepatic apoptosis at 6h p.i. compared to wt mice, and is further supported by the finding that the p55 TNF receptor is required for the apoptotic response to occur. However, semi-quantitative RT-PCR of TNF mRNA *in vivo* has found significant variation in TNF mRNA levels between individuals, suggesting that there may be further mediators of apoptosis (S.C. and S. Turner, data not shown).

The finding that EV was less effective at preventing apoptosis in p75-/- mice suggests that p28 may require p75 for efficient anti-apoptotic activity. If the activity of p28 in blocking peracute apoptosis is through the prevention of TNF mRNA upregulation through the non-death domain TNF receptors as hypothesised, then it is interesting that ablation of the p75 TNF receptor only partially blocks the apoptotic response. This suggests that EV may also block death through other p55-dependent mechanisms. This may reflect an activity of p28 on the stability of TNF mRNA independent of the stimulus for upregulation. An alternative, but less likely, explanation is that p28 prevents apoptosis by interfering in an unknown manner with the cytoplasmic signalling cascade after p55 TNF receptor ligation. p75 may then enhance apoptosis through cooperation of signalling cascades from the two receptors (Weiss *et al.*, 1997). However, while a role for p28 in interference with the p55 signalling cascade may be consistent with the data presented in this chapter, there is currently no evidence for this *in vitro*.

Involvement of IFNγ in peracute apoptosis was implied by the extremely high levels of apoptosis seen in ovaries of both EV and EVΔp28-infected IFNγR-/- mice 6h p.i. A role for IFNγR in p28-mediated suppression of the apoptotic response was unexpected,

given that IFNγ has been found to increase apoptosis in ovarian tissue when combined with Fas and/or TNF (Jo *et al.*, 1995; Porter *et al.*, 2000; Quirk *et al.*, 1997; Quirk *et al.*, 1998). However, there are a number of mechanisms by which the absence of IFNγR might have increased levels of apoptosis. These include a role in the activity of p28 at the site of infection, or an unrelated role at the site at which apoptosis was measured. With reference to the former, IFNγ has been found to be produced in the local draining lymph node within 6h of footpad infection with other viruses (Grieder *et al.*, 1997), concurrent with the production of TNF in the same infection. The presence of potential IFNγ-activated sequences (GAS) in the promoter of p28 suggests that IFNγ may enhance p28 mRNA transcription (SC, data not shown). This would be consistent with the presence of an apoptotic response at 6h p.i. in EV infected as well as EVΔp28-infected mice. Alternatively, IFNγR deficiency may specifically affect ovarian apoptosis during EV infection. This is suggested by the observation that IFNγ is produced in preovulatory follicles during normal ovarian function (Grasso *et al.*, 1994). It would be expected that functions of IFNγ in the follicles would be disrupted in IFNγR-/- mice. Given that IFNγ is capable of affecting non-immune functions such as hormone production (Pate, 1995), it is possible that the differences seen in apoptosis between the wt and IFNγR-/- mice may be unrelated to expression of p28 by EV, and may instead be due to an unexpected response of these mice to virus infection. Although no adverse effects of the IFNγR-/- mutation on the ovary have been reported in the literature, the unusually high levels of apoptosis at both 6h and 6d p.i. suggest that some cell types in the ovaries of these mice may be sensitised to apoptosis.

A key finding of this study has been the requirement for macrophages at the site of infection for the early phase of apoptosis to occur. Macrophages are both responsive to and producers of TNF (Gordon *et al.*, 1992) and are central to successful innate antiviral responses and the generation of an effective acquired immune response (Fearon and Locksley, 1996). Depletion of macrophages before EV infection results in a severe defect in the host antiviral response evident within 24h (Karupiah *et al.*, 1996; Tsuru *et*

al., 1983), supporting a role in viral control early in infection. Since macrophages are among the first cells infected after exposure to EV (Roberts, 1962) and are also potent producers of TNF (Gordon *et al.*, 1992), a logical proposal is that early expression of p28 in infected cells in the footpad inhibits local TNF production. As a result, the hypothetical systemic TNF response and its effects, such as apoptosis at distal sites, are also abrogated. Alternatively, other macrophage-derived factors may lead to the production of TNF within the liver in virus-infected mice, or TNF-producing cells may migrate to the liver.

In a preliminary experiment presented in this chapter, no antiviral role for the early apoptosis or the early TNF response was demonstrated as indicated by levels of virus detected early in infection. It is possible that no difference was seen because the large dose of EVΔp28 used rendered the assay insensitive. This is suggested by the decrease in the effect of antiTNF mAb treatment on apoptosis at 6h p.i. (Fig. 4.5, Section 4.2.8). It is also possible that the effects of the early response may not be linked to viral replication. For instance, it has been suggested that some poxviral virulence factors act to regulate inflammation rather than directly affecting host factors which control virus replication (McFadden *et al.*, 1995).

The data presented in this study indicate that EV gene p28 is required for EV to suppress a rapid macrophage- and TNF-dependent apoptotic response to virus infection. The function of the apoptotic response is not yet known, however, there are a number of potential roles. The first relates to a protective function, for example preventing further hepatic damage later in infection. This has been suggested by studies in which it has been shown that TNF-mediated hepatic damage can be followed within hours by an increase in levels of IL-6 which appears to have a negative effect on further TNF production and TNF-mediated hepatic necrosis (Mizuhara *et al.*, 1994). Alternative hypotheses suggest that the apoptosis may be secondary to the TNF response. TNF has previously been found to be important in multiple inflammatory events, including chemotaxis of lymphocytes and monocytes (Dixit *et al.*, 1990; Green *et al.*, 1998). In a

mouse model, the onset of experimental autoimmune encephalomyelitis was delayed in TNF-deficient mice. The delay correlated with defective migration of inflammatory leukocytes into the central nervous system parenchyma (Korner *et al.*, 1997). Hence, a transient increase in TNF may prime the immune response, resulting in a more effective antiviral response and more efficient virus clearance.

Thus these studies had found that TNF was required for a transient apoptotic response that could be blocked by the presence of p28. This led to a further question: did p28 manipulate TNF-mediated apoptosis – or other TNF mediated events – later in infection?

4.4 Tables and Figures

Table 4.1 Apoptosis in ovaries of TNF receptor knockout mice infected with EV or EVΔp28.

Mouse Strain	6h p.i.	6h p.i.		6d p.i.	
	Mock-infected[a]	EV[a]	EVΔp28[a]	EV[a]	EVΔp28[a]
B6/129	1.25 ± 0.95	15.25 ± 3.55[b]	64.52 ± 12.11[b,c]	105.63 ± 18.81[b,d]	13.9 ± 2.84[b]
p75-/-	3.30 ± 1.51	17.67 ± 3.74[b]	57.38 ± 17.95[b,c]	129.61 ± 27.32[b,d]	45.31 ± 16.25[b]
p55-/-	4.26 ± 2.53	11.00 ± 10.56	10.25 ± 8.05	nd	nd
p55-/-p75-/-	1.20 ± 0.98	1.20 ± 0.66	6.70 ± 2.01[b,c]	138.24 ± 21.83[b,d]	36.92 ± 12.17[b]

a. Groups of 5 female 9-14 week old mice were infected with 5 x 10^3 pfu EV or EVΔp28, or mock-infected with tissue culture lysate diluted as for the infectious virus. Ovaries were harvested 6h or 6d later and fixed in 10% NBF for TUNEL staining. Apoptosis was measured by counting apoptotic cells in the stroma of the ovary (Fig. 4.7) and adjusting to the number of apoptotic cells per mm^2 of stroma. Minimum of two sections per ovary counted for both ovaries of each mouse. All sections were assessed blind. Experiment indicative of 2 (p75-/-, p55-/-, p55-/-p75-/-) or 3 (B6/129) replicates. P55-/- mice harvested in groups of 3 mice. Data represents the number of apoptotic cells present per mm^2 of ovarian stroma.

b. Apoptosis in response to virus infection significantly greater than that in response to mock-infection at 6h p.i. ($P<0.05$)

c. Apoptosis in response to EVΔp28 significantly greater than that in response to EV ($P<0.05$)

d. Apoptosis in response to EV significantly greater than that in response to EVΔp28 ($P<0.05$)

nd: Not determined

Table 4.2 The early phase of apoptosis is not blocked by p28 in IFNγR-/- mice

	6h p.i.	6h p.i.		6d p.i.	
Mouse Strain	Mock-infected[a]	EV[a]	EVΔp28[a]	EV[a]	EVΔp28[a]
129	9.21 ± 2.36	12.89 ± 5.46	23.78 ± 3.16[b]	28.64 ± 8.64	26.54 ± 12.61
IFNγR-/-[d]	11.65 ± 2.36	116.17 ± 27.27	198.5 ± 14.44[c]	165.29 ± 31.08	248.62 ± 41.25

a. Groups of 5 female 10-13 week old mice were infected with 5 x 10^3 pfu EV or EVΔp28, or mock-infected with tissue culture lysate diluted as for the infectious virus. Ovaries were harvested 6h or 6d later and fixed in 10% NBF for TUNEL staining. Apoptosis was measured by counting apoptotic cells in the stroma of the ovary (see Fig. 4.7) and adjusting to the number of apoptotic cells per mm^2 of stroma. Minimum of two sections per ovary counted for both ovaries of each mouse. All sections were assessed blind. Data represents the number of apoptotic cells present per mm^2 of ovarian stroma.

b. Apoptosis in response to EVΔp28 significantly greater than that in response to EV ($P<0.05$)

c. Apoptosis in response to EVΔp28 significantly greater than that in response to EV ($P<0.01$)

d. Apoptosis in IFNγR-/- mice infected with EV or EVΔp28 greater than equivalent 129 mice infected with the same virus at both timepoints ($P<0.001$)

Table 4.3 Hepatic apoptosis during the early and late phases of apoptosis in mice infected with EV or EVΔp28.

Mouse Strain	6h p.i.	6h p.i.		6d p.i.	
	Mock-infected[a]	EV[a]	EVΔp28[a]	EV[a]	EVΔp28[a]
A/J[b]	2.02 ± 0.14	1.75 ± 0.63	1.82 ± 0.87	4.03 ± 1.36	3.33 ± 1.53
Swiss nude[b]	2.48 ± 0.60	1.41 ± 0.80	3.13 ± 1.18	7.94 ± 2.86	8.35 ± 1.68
B6[b]	1.28 ± 0.03	0.98 ± 0.02	6.14 ± 0.96[c]	3.26 ± 0.65	7.83 ± 1.02[c]
B6/129[b]	0.21 ± 0.14	0.58 ± 0.29	5.92 ± 1.75[c]	5.22 ± 1.30	5.08 ± 1.17
129	2.17 ± 0.41	0.59 ± 0.16[e]	3.28 ± 0.33[d]	4.41 ± 2.55	4.10 ± 0.53
129.IL-6-/-	1.53 ± 0.20	0.64 ± 0.43[e]	2.68 ± 0.42[d]	nd	nd

a. Groups of 4 to 5 female mice between 8 and 14 weeks old were infected with 5 x 10^5 pfu EV or EVΔp28, or mock-infected with tissue culture lysate diluted as for the infectious virus. Livers were harvested 6h or 6d later and fixed in 10% NBF for TUNEL staining. Staining was performed in 6 mixed batches, with 3 B6 samples repeatedly assayed to control for batch to batch variation. Apoptosis measured by counting apoptotic cells as described in materials and methods. IL-6-/- mice concurrently assayed for comparison to 129 mice. Levels of apoptosis compared statistically using Student's t-test. Data represents the number of apoptotic cells detected per mm^2 hepatic tissue.

b. Representative of 2 to 3 experiments. Experimental replicates limited in 129 and 129.IL-6-/- mice by mouse availability.

c. Apoptosis in response to EVΔp28 significantly greater than that in response to EV ($P<0.01$)

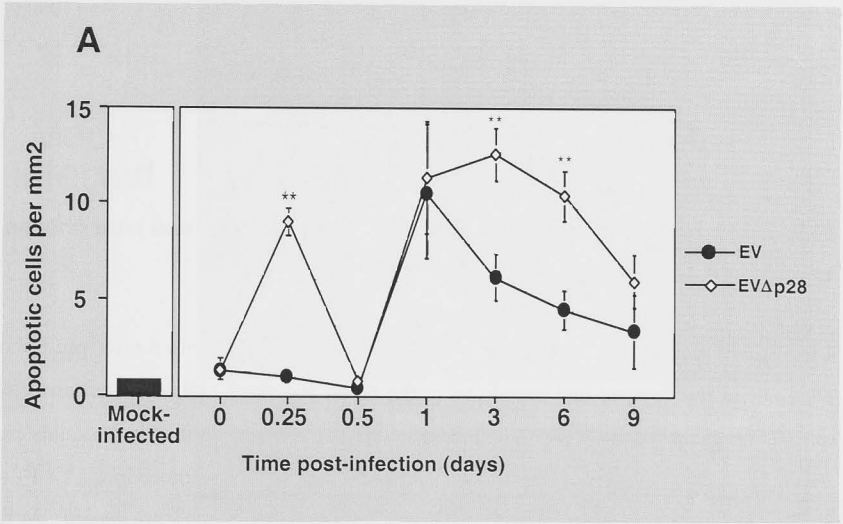
d. Apoptosis in response to EVΔp28 significantly greater than that in response to EV ($P<0.05$)

e. Apoptosis in response to EV significantly less than in response to mock-infection ($P<0.05$)

nd. Not determined

Figure 4.1 B6 mice display a biphasic apoptotic response to EVΔp28 infection

Groups of 5 8-16 week old female B6 mice were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. (A) At least two sections per liver were stained for apoptotic cells using the TUNEL procedure described in materials and methods, and apoptosis quantitated. Data represent mean TUNEL-positive cell numbers ± SD. Data was analysed for statistical significance using Student's t-test (*P<0.05, **P<0.01). The number of TUNEL-positive cells was found to be significantly higher in EVΔp28 infected mice than EV-infected mice in the early phase of apoptosis (before 12h p.i.) and at d3 and d6 p.i. (B) The early phase of apoptosis occurred prior to detection of virus in the liver. Both EV (filled bars) and EVΔp28 (open bars) became detectable by titration in the liver at d3 p.i. Liver samples were harvested at the indicated time points and titrated as described in Section 2.11. The limit of detection of the plaque-forming assay was 100 pfu and samples below this level were assigned a value of 50 (1.7 $\log_{10}$) pfu (broken line). Data represent mean virus titres from 5 mice ± SD and are representative of 3 experiments.


A
Apoptotic cells per mm2
15
10
5
0
**
**
**
Mock-infected
0
0.25
0.5
1
3
6
9
Time post-infection (days)
EV
EVΔp28

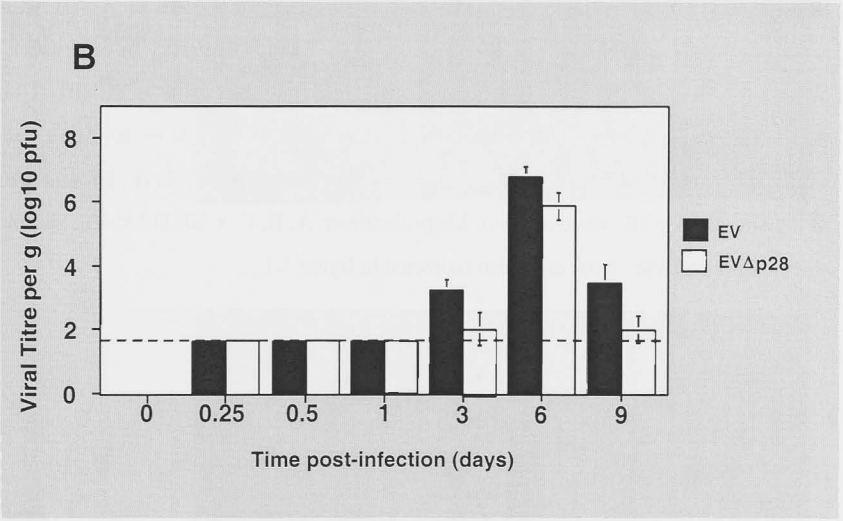

B
Viral Titre per g (log10 pfu)
8
6
4
2
0
0
0.25
0.5
1
3
6
9
Time post-infection (days)
EV
EVΔp28

Figure 4.2 Hepatic morphology differs between the early and later phases of apoptosis.

Livers were harvested from groups of 5 female B6 mice infected with 5 x 10^3 pfu EV or EVΔp28 in the footpad and sacrificed at the times indicated below. Mock-infected animals were inoculated with diluted tissue culture lysate as described in materials and methods. Formalin-fixed sections were stained with the TUNEL procedure (A - F) or H&E (G, H, I) for histological examination. Representative sections are shown. At 6h p.i., no observable changes were present in livers of mock-infected mice (A, G). Infection with EVΔp28 led to the presence of scattered apoptotic cells 6h p.i. (B, white arrows) in the absence of overt changes to hepatic architecture (H). In contrast, EV infection at 6h was not associated with any hepatic changes observable in TUNEL (C) or H&E (I) stained sections. In contrast, apoptosis 6d p.i. with EV or EVΔp28 was associated with inflammatory changes such as distension of sinusoids (E, F), and areas of hepatic damage (F, white arrows). Magnifications: A, C x 20; B, D-I x 40. Samples shown were collected from mice also indicated in Fig. 5.1.

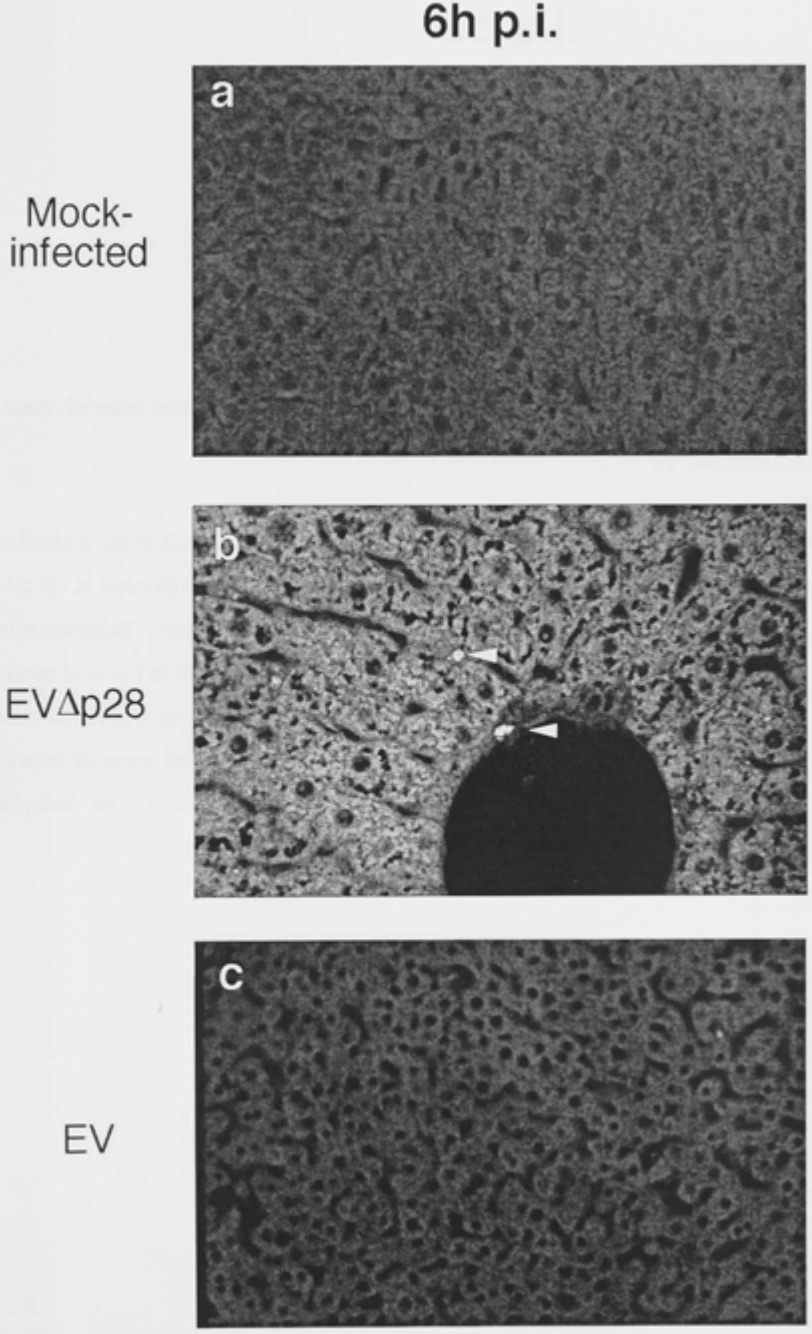

6h p.i.
a
Mock-
infected
b
EVΔp28
c
EV

Figure 4.2 Hepatic morphology differs between the early and later phases of apoptosis. (D – F)

Groups of 5 female B6 mice infected with 5 x 10^3 pfu EV or EVΔp28 in the footpad or inoculated with diluted tissue culture lysate. Livers were then harvested at 6d p.i.. Formalin-fixed liver sections were stained with the TUNEL procedure. Representative sections are shown. At 6d p.i., no observable changes were present in livers of mock-infected mice (D). Apoptosis 6d p.i. with EV or EVΔp28 was associated with inflammatory changes such as distension of sinusoids (E, F), and areas of hepatic damage (F) Apoptotic cells indicated by white arrows. Magnification: x 40. Samples shown were collected from mice also indicated in Fig. 4.1.

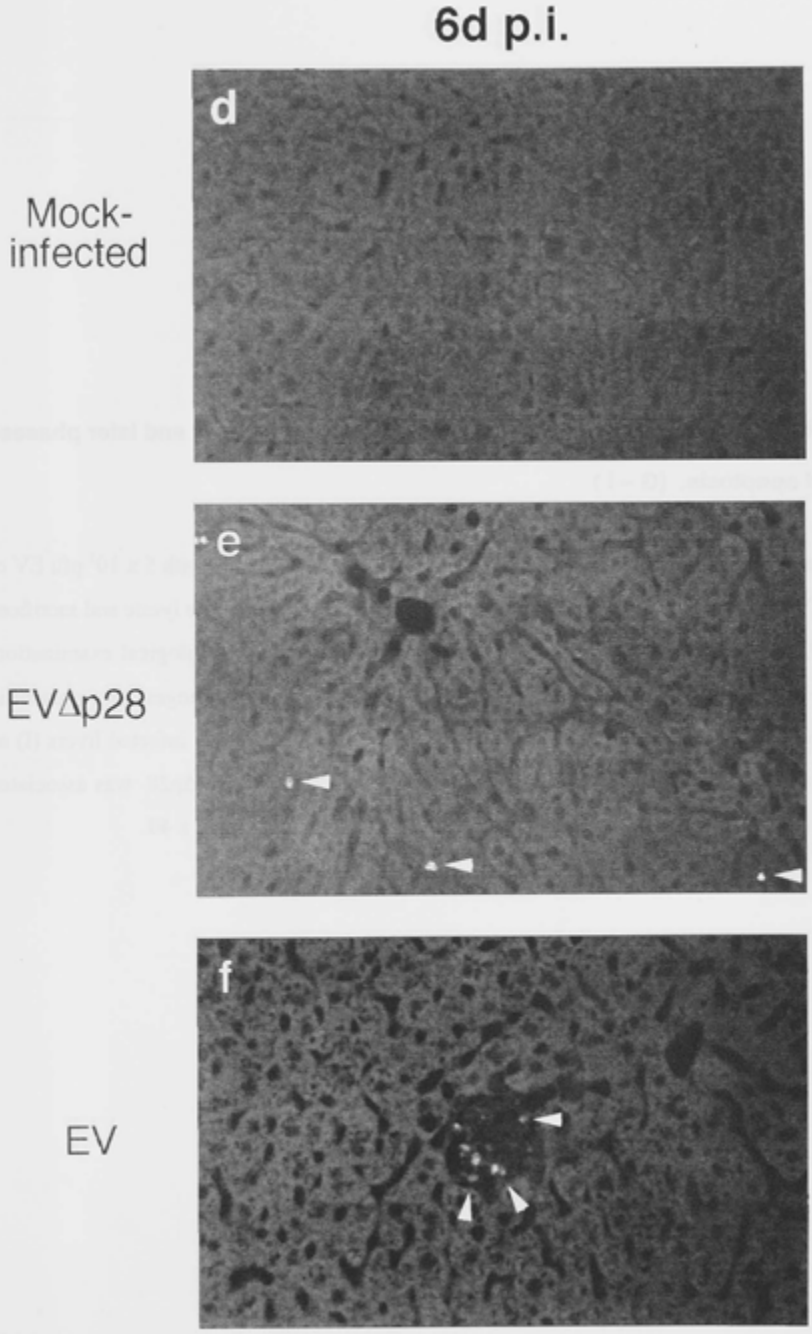
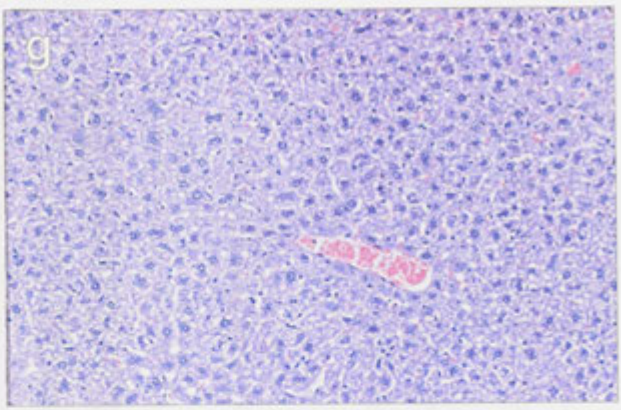

6d p.i.
d
Mock-
infected
e
EVΔp28
f
EV

Figure 4.2 Hepatic morphology differs between the early and later phases of apoptosis. (G – I)

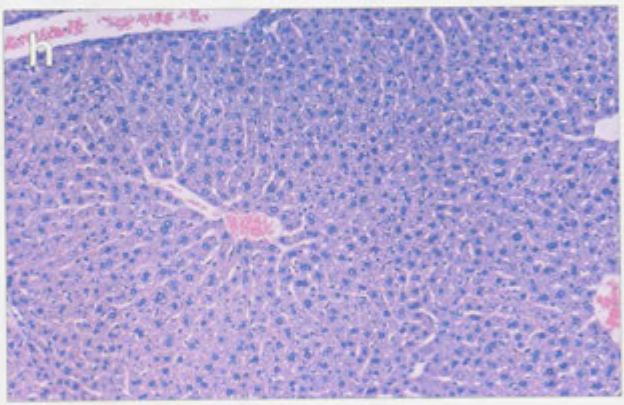
Livers were harvested from groups of 5 female B6 mice infected with 5 x 10^3 pfu EV or EVΔp28 in the footpad, or mock-infected with diluted tissue culture lysate and sacrificed 6h p.i. Formalin-fixed sections were stained with H&E for histological examination. Representative sections are shown. At 6h p.i., no observable changes were present in livers of mock-infected mice (G), EVΔp28 infected livers, or EV infected livers (I) as stained with H&E. In contrast, apoptosis 6d p.i. with EV or EVΔp28 was associated with a inflammatory changes, as shown in Fig. 3.5. Magnification: x 40.

6h p.i.

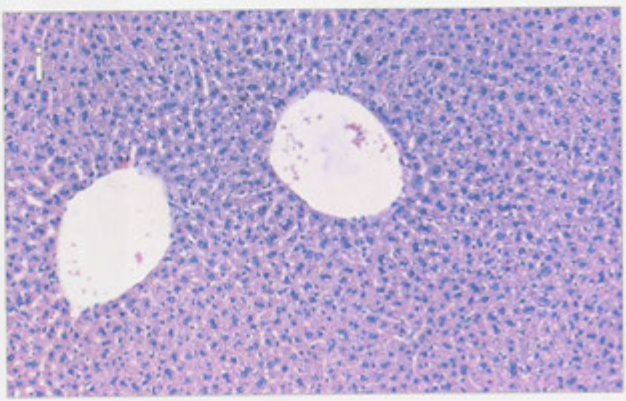
Mock-infected



EVΔp28



EV



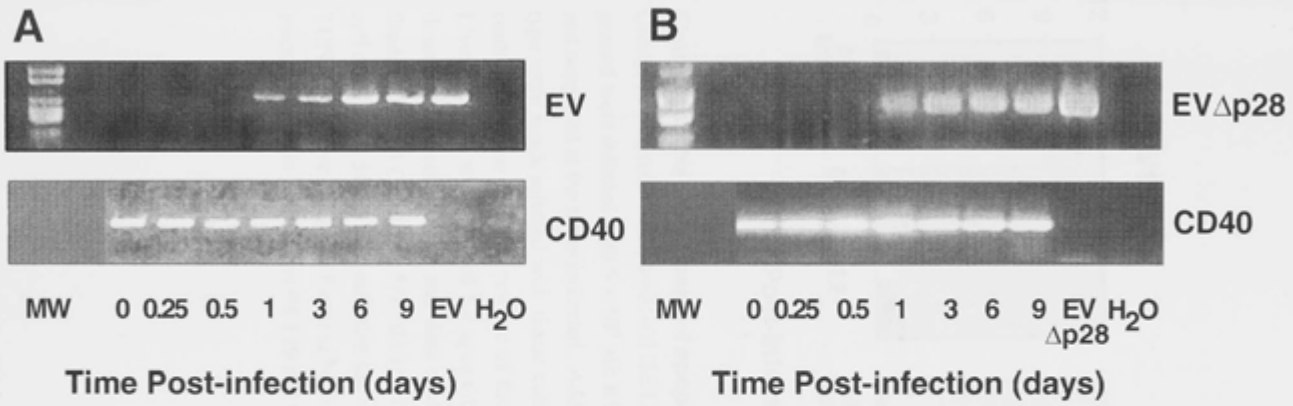


Figure 4.3 Detection of virus in the livers of infected mice by PCR.

10-12 week old female B6 mice were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad as previously and livers harvested at the times indicated. DNA was extracted from the livers of 3 mice per timepoint as described in Section 2.13 before amplification of virus DNA with the primers p28F and p28R (Table 2.3). Both (A) EV and (B) EVΔp28 DNA were detected. Genomic CD40 was also amplified as a control for DNA concentration (Kawabe *et al.* 1994). Samples from single mice representative of each timepoint are shown.

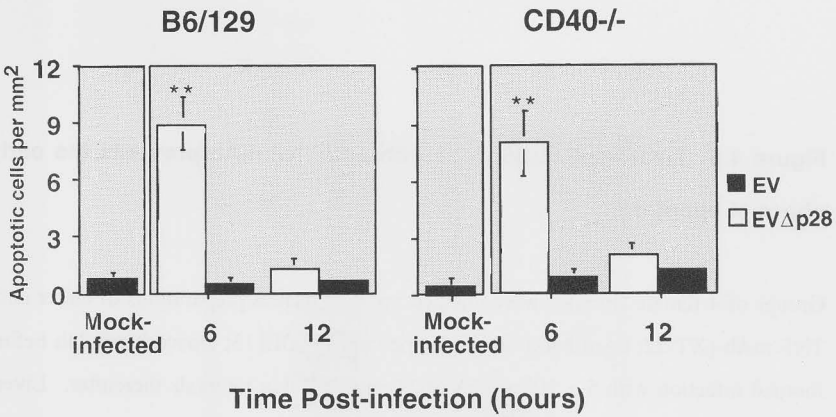
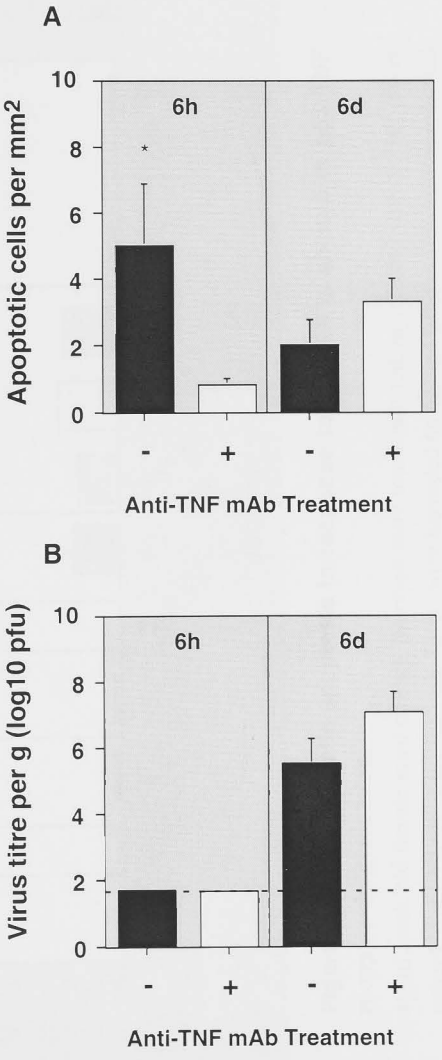


Figure 4.4 The early phase of apoptosis is present in CD40-/- mice
Groups of 5 female 10-14 week old B6/129 or CD40-/- mice on a B6/129 background were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. Additionally, 5 control mice of each genotype were mock-infected with tissue culture lysate and sacrificed at 6h p.i. to control for stress and the presence of tissue culture material in the innoculum. Liver sections were stained for apoptotic cells using the TUNEL procedure described in Section 2.10, and data analysed for statistical significance using Student's t-test (*P<0.05, **P<0.01). Data represent mean TUNEL-positive cell numbers ± SD. Mock-infection led to negligible apoptosis. The number of TUNEL-positive cells was found to be significantly higher in EVΔp28 infected mice at 6h p.i. in both the wt B6/129 and CD40-/- mice (P<0.01).

Figure 4.5 Treatment of B6 mice with anti-TNF mAb prevents the early phase of apoptosis.

Groups of 4 female B6 mice were injected i.p. with ascites preparations of either anti-TNF mAb (XT-22; open bars) or an isotype control (GL113; closed bars) 24h before footpad infection with 5 x 10^3 pfu EVΔp28, and at 2 day intervals thereafter. Livers harvested at 6h or 6d p.i. were formalin-fixed and sections stained for apoptotic cells using the TUNEL method. Samples of each liver were also frozen at -20°C for titration. Apoptotic cells were counted and analysed as Section 2.10. Data shown represent mean number of TUNEL-positive cells ± SD. (A) Anti-TNF mAb-treated mice were found to have significantly lower levels of apoptosis than control mice 6h p.i. (Student's t-test; $*P<0.05$) but not 6d p.i. (B) Anti-TNF treated mice showed an increase in virus titre although it was not statistically significant. This correlated with a similar increase in apoptosis. Samples titrated as described in Section 2.12. The limit of detection of the plaque-forming assay was 100 pfu and samples below this level were assigned a value of 50 (1.7 $\log_{10}$) pfu (broken line). Data represent mean virus titres from 4 mice ± SD.


A
Apoptotic cells per mm2
10
8
6
4
2
0
6h
6d
*
-
+
-
+
Anti-TNF mAb Treatment
B
Virus titre per g (log10 pfu)
10
8
6
4
2
0
6h
6d
-
+
-
+
Anti-TNF mAb Treatment

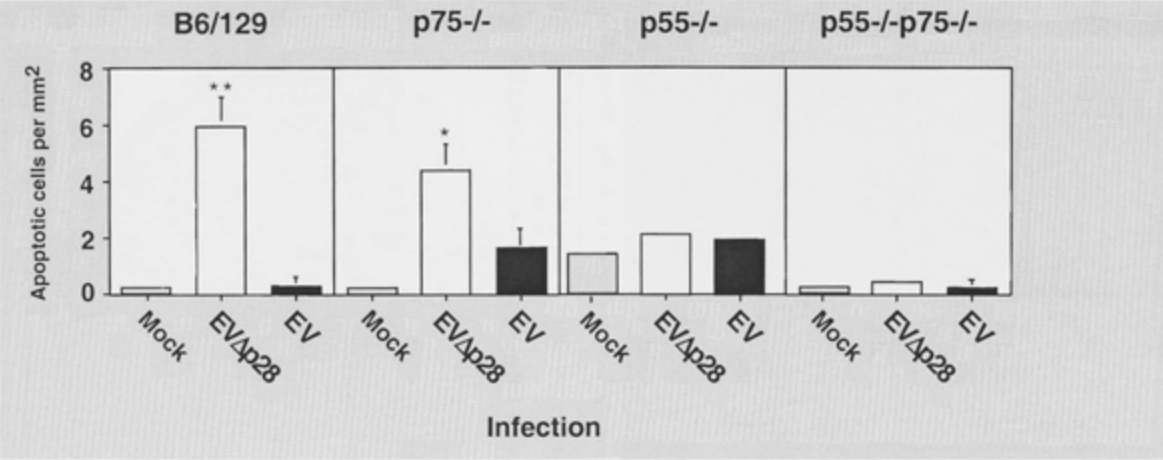


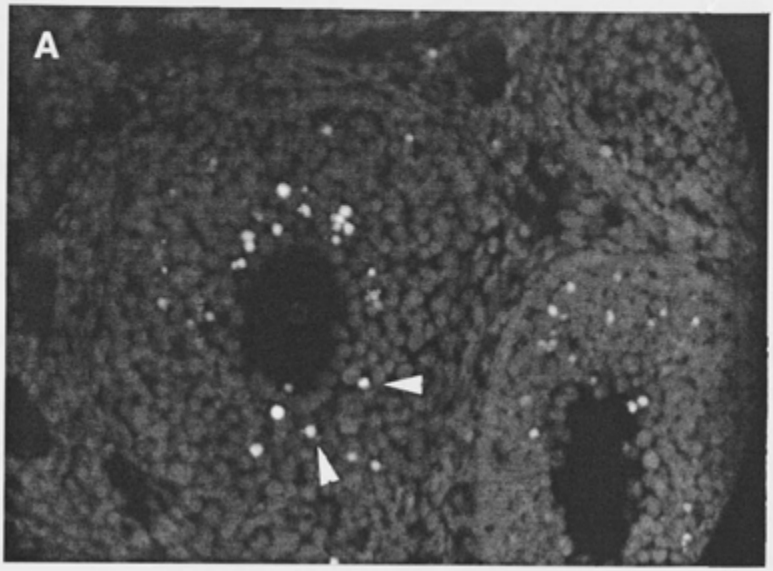
Figure 4.6 Early hepatic apoptosis in response to EVΔp28 is ablated in p55 TNF receptor-deficient mice.

10-16 week old female mice of each genotype were mock-infected, or infected with 5x 10^3 pfu of either EV or EVΔp28 in the footpad. Livers were harvested 6h p.i. and fixed in 10% NBF at indicated time-points. Sections were stained for apoptotic cells using the TUNEL procedure as described in the materials and methods. Data indicates mean number of TUNEL-positive cells ± SEM for 2 to 3 readings per sample, with samples collected from 5 individual mice. Levels of apoptosis were significantly increased in EVΔp28-infected wt (**P<0.01) and p75-/- (*P<0.05) mice compared to EV-infected mice by Student's t-test.

Figure 4.7 Apoptosis occurs during follicular development in the ovaries

Apoptosis occurs in both infected and uninfected ovaries during the normal development of follicles (A B; white arrows). Shown are three follicles from the ovary of an uninfected mouse. During development of the follicle, granulosa cells initially proliferate to form a thick layer of cells around the ovum (see also Fig. 4.8B and D). This is followed by the formation of the thecal layer around the developing follicle, and the apoptosis of granulosa cells to form the antrum (shown in A, B). More advanced antrum formation can be seen in the secondary follicles in Fig. 4.8F, which also display a well developed thecal layer. Apoptosis is not seen in the theca surrounding the follicles or in stromal cells.

Ovary shown was harvested from a healthy 12 week old B6/129 mouse, fixed in 10% NBF and TUNEL stained as per materials and methods. Magnification 40x.


A

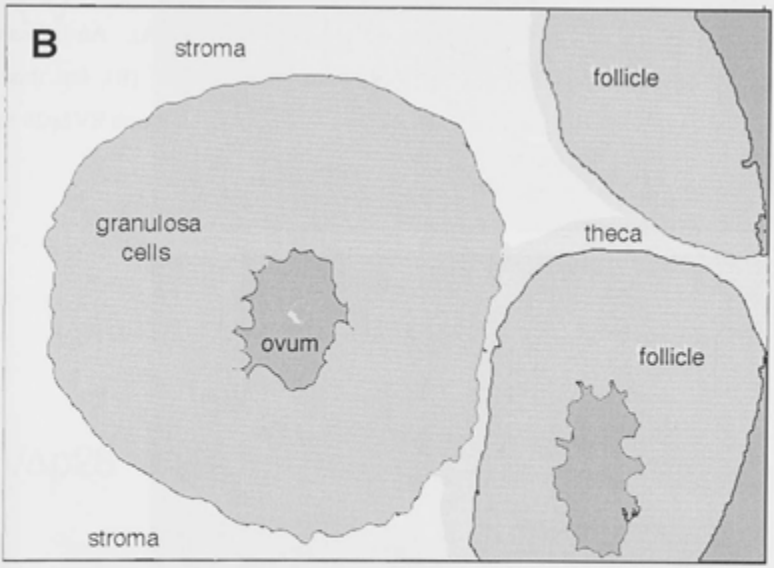

B
stroma
follicle
granulosa cells
theca
ovum
follicle
stroma

Figure 4.8 (A-C) The early phase of apoptosis occurs in the ovaries and is distinct from the later phase of apoptosis

Ovaries were harvested from groups of 5 female mice infected with 5 x 10^3 pfu EV or EVΔp28 in the footpad and sacrificed at 6h or 6d p.i. (see also Table 4.1). Formalin-fixed sections were stained with the TUNEL procedure as per materials and methods. Representative sections from p75-/- mice are shown. Apoptosis occurs in both infected and uninfected ovaries during normal development of follicles (A). An increase in apoptosis was seen in the stroma of EV infected mice 6h p.i. (B), but this was significantly less than the apoptotic response noted in mice infected with EVΔp28 for 6h p.i. (C). Magnification: 40x

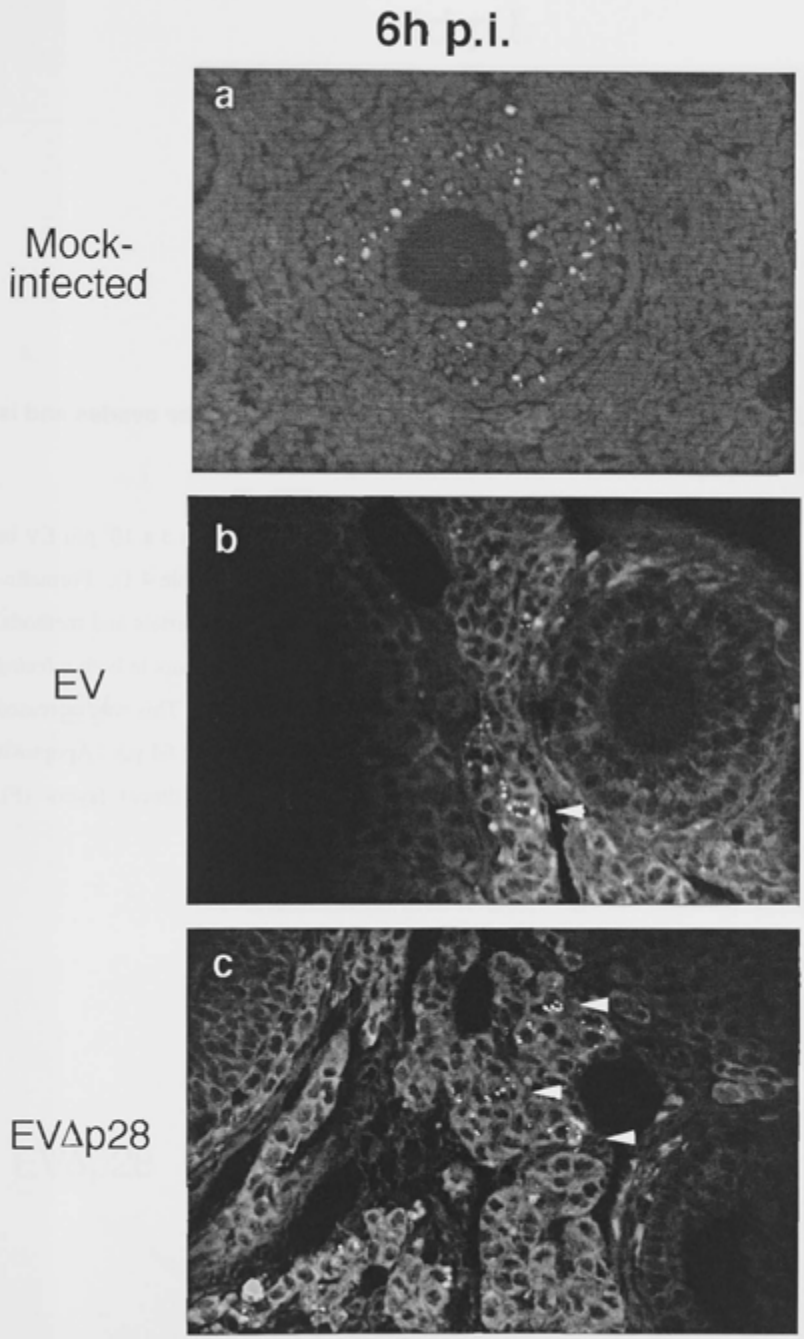

6h p.i.
a
Mock-
infected
b
EV
c
EVΔp28

Figure 4.8 (D-F) The early phase of apoptosis occurs in the ovaries and is distinct from the later phase of apoptosis

Ovaries were harvested from groups of 5 female mice infected with 5 x 10^3 pfu EV or EVΔp28 in the footpad and sacrificed at 6h or 6d p.i. (see also Table 4.1). Formalin-fixed sections were stained with the TUNEL procedure as per materials and methods. Representative sections from p75-/- mice are shown. Apoptosis occurs in both infected and uninfected ovaries during normal development of follicles (D). This was increased in the stroma of mice infected with either EV (E) or EVΔp28 (F) 6d p.i. Apoptosis occurred in all sections of the stroma examined, including thecal layers (F). Magnification: D, F: 40x; E 16x

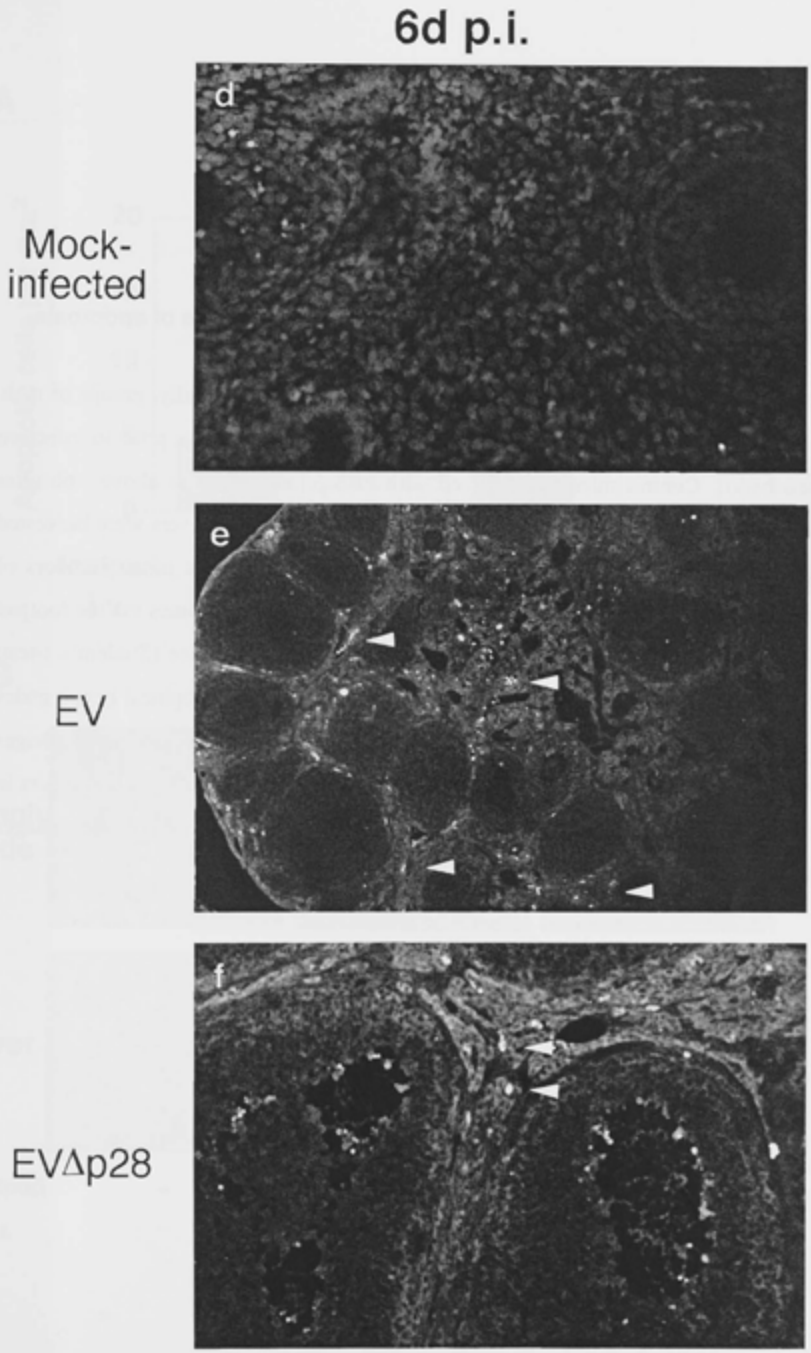
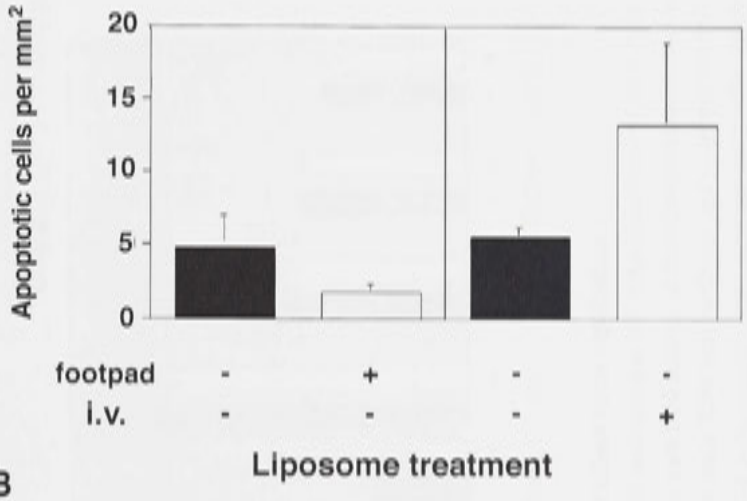

6d p.i.
d
Mock-
infected
e
EV
f
EVΔp28

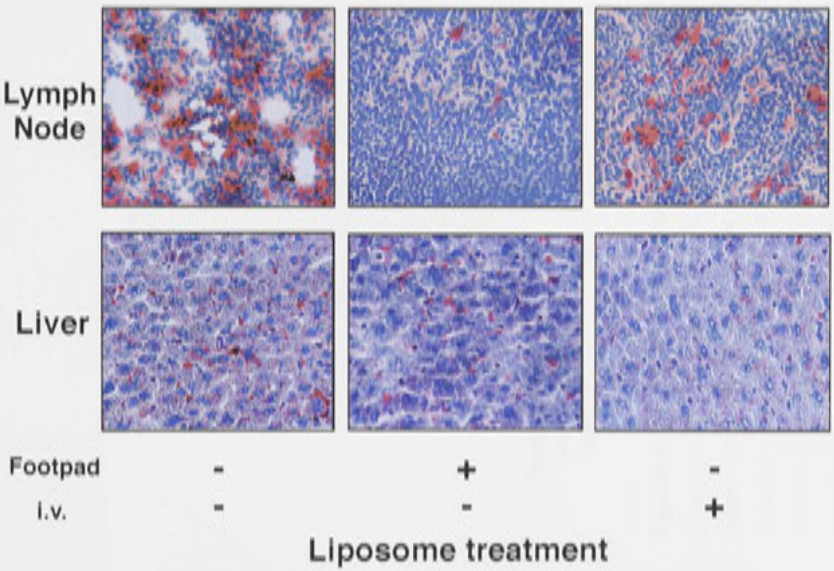
Figure 4.9 Macrophage depletion prevents the early phase of apoptosis.

(A) To deplete macrophages from the site of infection or systemically, groups of male B6 mice received Cl2MDP liposomes via the routes shown 2 days prior to infection (open bars). Control mice were treated with PBS (closed bars) as above. 6h after subsequent infection with 5 x 10^3 pfu of EVΔp28 in the footpad, livers were harvested and formalin fixed for TUNEL staining. Data shown represent mean numbers of apoptotic cells from 5 mice ± SD. Treatment with Cl2MDP-liposomes via the footpad significantly reduced levels of apoptosis, compared to control mice (Student's t-test; $P<0.05$). (B) Mice were liposome-treated as above and livers and popliteal lymph nodes were harvested for acid phosphatase staining. In footpad-inoculated mice, macrophages were depleted from the draining lymph node without affecting Kupffer cell numbers in the liver. In contrast, systemic liposome treatment caused the depletion of macrophages from the liver but not the lymph node. Magnification: 20x.

A


Apoptotic cells per mm2
20
15
10
5
0
footpad - + - -
i.v. - - - +
Liposome treatment

B


Lymph Node
Liver
Footpad - + -
i.v. - - +
Liposome treatment

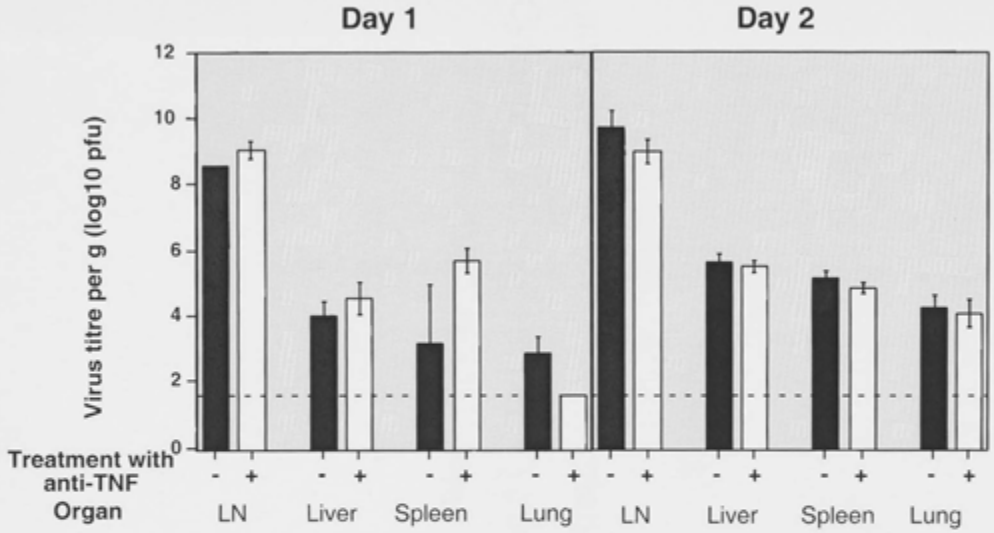


Figure 4.10 The early apoptotic response does not affect virus replication.

Groups of 5, 8-12 week old male B6 mice were injected i.v. with anti-muTNF mAb (open bars) or an isotype control mAb (closed bars) 24h before infection with $5x10^6$ pfu EVΔp28 in each hind footpad, as described in materials and methods. Days 1 or 2 p.i. lung, liver, spleen and draining popliteal lymph node (LN) were collected and virus was titrated. Differences between the virus titres of control mAb-treated and anti-TNF mAb-treated organs were not statistically significant at either day ($P>0.05$, Student's t-test). The limit of detection of the plaque-forming assay was 100 pfu and samples below this level were assigned a value of 50 (1.7 log10) pfu (broken line). Data represent mean virus titres from 5 mice ± SD.

Chapter 5

p28 Contributes to EV Virulence Through Both Cytokine-Dependent and Cytokine-Independent Mechanisms

5.1 Introduction

Experiments described in previous chapters presented an intriguing mix of possible aspects of EV infection affected by p28. Initial experiments found p28 to be an important virulence factor in some strains of mice (Chapter 3). In addition, p28 appeared to regulate a TNF-dependent apoptotic response early in infection (Chapter 4). in B6 mice, there was also evidence that p28 could regulate apoptosis later in infection (Chapter 3). However, it was not known whether the interaction of p28 with TNF receptors or CD40, as shown *in vitro*, was a significant factor in the response to virus later in infection.

Important roles for both CD40 and TNF in antiviral defence have been demonstrated for a number of virus infections (Grell *et al.*, 1998). CD40 is required for the upregulation of the humoral immune response (Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994), macrophage activation (Stout *et al.*, 1996), APC function of B cells, macrophages and dendritic cells (Grewal and Flavell, 1998), and subsequent T cell activation (Clarke, 2000; Grewal *et al.*, 1995). TNF influences multiple aspects of the antiviral response, including upregulation of IFNγ and chemokines (Dixit *et al.*, 1990; Lange *et al.*, 1995; Tessier *et al.*, 1997), apoptosis (Lazdins *et al.*, 1997), leukocyte chemotaxis (Green *et al.*, 1998), and inflammatory changes such as increasing vascular and cellular permeability (Hribar *et al.*, 1999). These activities of TNF are mediated through the two TNF receptors, p55 (TNF receptor 1) and p75 (TNF receptor 2). Most activities have been attributed to the p55 TNF receptor rather than the p75 receptor, correlating with the finding that p55 is required for aspects of TNF antiviral activity (Wong and Goeddel, 1986). However, there is emerging evidence that p75 also plays an important role in the anti-viral response. This has been highlighted by infection of p55-/- and p75-/- mice with EV. While p55-/- mice were more susceptible to EV than

wild-type mice, p75-/- mice were significantly more susceptible again, resulting in a lethal infection (Ruby *et al.*, 1997).

In this study, the hypothesis that p28 may contribute to EV virulence *in vivo* through p75 or CD40 is addressed, with emphasis on the period between 3 and 9d p.i. In addition, two other cytokines important in control of EV infection are examined for comparison: IL-6 and IFNγ. Mice deficient in either of these cytokines due to gene knockout or neutralisation with antibodies are significantly more susceptible to lethal EV infection than the parent mouse strain (Karupiah *et al.* 1993, Ramshaw *et al.* 1997). It has also been shown that recombinant vaccinia viruses constitutively expressing either cytokine are highly attenuated *in vivo* (Ramshaw *et al.*, 1997). Thus this chapter examines the contributions of cytokines or their receptors to EVΔp28 attenuation *in vivo* with particular reference to TNF and the impact of p28-mediated blocking of TNF activity.

5.2 Results

5.2.1 TNF receptor knockout mice are resistant to EVΔp28 infection but not wt EV

To determine whether TNF-dependent antiviral mechanisms were regulated by p28, p75-/-, p55-/-p75-/- and wt B6/129 mice were infected with 5 x 10^3 pfu of either EV or EVΔp28 s.c. into the footpad and examined daily for morbidity and mortality up to 25d p.i. This involved monitoring footpad swelling as an indicator of inflammation at the site of infection (Fenner, 1949), the appearance of pocks; and symptoms of general illness including weight loss, lethargy,and coat ruffling. Additionally, at death or termination of the experiment, internal organs were examined for evidence of pathology.

As described in Chapter 3, wt B6/129 mice were resistant to both EV and EVΔp28 (Table 5.1). B6/129 mice displayed high levels of foot swelling in response to EV, which were diminished in response to EVΔp28 (Fig. 5.1), consistent with the strong inflammatory response of these mice to EV infection (Chapter 3). At no stage did the mice show classical signs of succumbing to mousepox such as lethargy, coat ruffling, hunched appearance, weight loss (Fig. 5.2), or the appearance of pox (Fenner, 1949). Indeed, all wt mice recovered from infection with either virus. In contrast, p28 significantly affected mortality in p75-/- and p55-/-p75-/- mice. 60% of p75-/- mice infected with EV succumbed to disease, with a mean time to death of 11.6d p.i. (Table 5.1). p75-/- mice infected with EV displayed reduced foot swelling compared to EV-infected B6/129 mice ($P<0.01$; Fig. 5.1). No further signs of disease were seen in EV-infected p75-/- mice until 24h before death, at which point some but not all mice became hunched and lethargic. All p75-/- mice infected with EVΔp28 survived infection (Table 5.1). As observed in B6/129 mice, EVΔp28 infection was associated

with lower footpad swelling than EV. Thus the p75 TNF receptor was not required for p28 activity as a pathogenic factor.

p55-/-p75-/- mice displayed higher morbidity in response to both EV and EVΔp28 than p75-/- mice. Cutaneous or mucosal lesions were detected in 5 of 6 mice infected with EV, and discomfort and coat ruffling were present from days 8-21 of infection. Despite the increased morbidity, rates of mortality were equal to those found in p75-/- mice, and mean time to death was significantly extended ($P<0.01$; Table 5.1). Unexpectedly, inflammation in the footpad was also increased in p55-/-p75-/- mice infected with EV compared to p75-/- mice ($P<0.05$; Fig. 5.1). Morbidity in p55-/-p75-/- mice infected with EVΔp28 was restricted to coat ruffling , with all mice surviving infection. Thus, p28 was a significant factor in EV pathogenesis in both p75 and p55p75 TNF receptor-deficient mice.

These data supported previous work describing roles for p75 and p55 in resistance of mice to EV infection (Ruby *et al.*, 1997). The experiments also showed that EV stimulated higher levels of morbidity and lethal infection than EVΔp28 in both TNF receptor deficient mouse strains, indicating that p28 contributed to pathogenesis independent of TNF. To determine whether p28 was contributing to virus replication, or preventing virus clearance through the TNF receptors, the kinetics of virus replication were examined in the wt and TNF receptor deficient mice.

5.2.2 Kinetics of virus replication in TNF receptor deficient mice.

In order to determine the kinetics of EV and EVΔp28 replication in wt and TNF receptor deficient mice, groups of 5 p75-/-, p55-/-p75-/- and wt B6/129 mice were infected with either EV or EVΔp28 as previously, and sacrificed at 3d intervals over d3-9 p.i. Virus titres were obtained from the liver, spleen and popliteal LN draining the site of inoculation, as described in Chapter 3.

Hepatic titres of EV and EVΔp28 were similar in the livers of B6/129, p75-/- and p55-/-p75-/- mice (Fig. 5.3). Maximum hepatic virus titres were obtained at d6 p.i., with EVΔp28 titres approximately 1 log lower than those seen in comparable EV infected mice. By d9 p.i., wt and p28 mutant virus titres were indistinguishable in all three mouse strains, despite the fact that EV, unlike EVΔp28, caused fatal disease in 2 of the 3 strains. At this time both viruses were at uniformly higher titres in the livers of p75-/- and p55-/-p75-/- than B6/129 mice, suggesting that virus clearance from the liver is compromised in the absence of p75 but not further compromised by the removal of p55. Further, the similar titres of EV and EVΔp28 present at 9d p.i. in all strains tested show that virus clearance from the liver was not affected by p28 expression.

Levels of EV in the spleen were sensitive to the presence of both p28 and the TNF receptors. The p75 TNF receptor was clearly required for EV clearance, as virus titres were significantly higher in p75-/- mice than wt mice 9d p.i. ($P<0.05$) and showed a strong trend towards higher levels in p55-/-p75-/- mice 9d p.i. In addition, p28 was required for efficient virus replication in the spleens of p55-/-p75-/- mice (Fig. 5.3). Interestingly, this correlated with unusual splenomegaly in p55-/-p75-/- mice (Fig. 5.4). In B6/129 and p75-/- mice, splenomegaly in response to EVΔp28 was significantly less than that in response to EV ($P<0.01$), however in p55-/-p75-/- mice splenomegaly was significantly enhanced ($P<0.01$) in response to both viruses. Further, splenomegaly was indistinguishable between EV and EVΔp28 in the p55-/-p75-/- mice by d9 p.i. It is possible that this reflects an effect of p28 expression that is dependent on the p55 TNF receptor. Alternatively, this could reflect the unusual immunological environment of these mice. The migration of follicular dendritic cells to the spleen and lymph nodes is compromised in p55-/-p75-/- mice, resulting in a lack of B cell follicles (Kawabe *et al.*, 1994). A CD40 or TNF-dependent role for p28 in B cell expansion, or a TNF-dependent role in T cell expansion would also be consistent with this result.

Virus titres were also determined from the DLN, as an indicator of virus replication in the footpad. Both EV and EVΔp28 were detectable at high levels within the DLN of B6/129 and p75-/- mice within 3d of infection, and remained at high levels at all timepoints (Fig. 5.3). By d9 p.i. EV replication and the associated host response resulted in destruction of the lymph node of p75-/- mice (Fig. 5.3). Replication of EV in the DLN of p55-/-p75-/- mice was similar to that in p75-/- mice, however replication of EVΔp28 was at lower levels early in infection, reaching levels comparable to those of wt virus only at d9 p.i. (Fig. 5.3). This did not delay detection of virus in the livers of infected mice, since both EV and EVΔp28 were present in the liver at similar levels at d3 p.i.

These data confirmed that the TNF receptors, particularly p75, had roles in clearance of both EV and EVΔp28 from the target organs of infection (Ruby *et al.*, 1997). p28 expression mildly enhanced EV replication independent of TNF, however, interaction with TNF receptors did not detectably affect virus clearance. These data show that p28 did not affect EV replication or clearance within the first 9d p.i. through interactions with TNF receptor signalling

Interestingly, hepatic replication of EVΔp28 was only reduced by approximately 1 log compared to EV. The reduced replication of EVΔp28 did not appear to be sufficient to account for its reduced lethality. It was possible that the role of p28 was predominantly in manipulation of the host response. TNF is capable of inducing hepatic necrosis (Lehmann *et al.*, 1987) and is induced by viral infection (Grieder *et al.*, 1997; Orange *et al.*, 1997; Ruzek *et al.*, 1997). To investigate whether p28 affected these parameters through TNF, necrosis and the inflammatory response within EV and EVΔp28 infected livers were next examined.

5.2.3 Hepatic damage is greater in response to infection with wt virus than EVΔp28.

To examine whether p28 affected hepatic pathology through the TNF receptors, NBF-fixed liver samples from the mice described in the previous section were stained with haematoxylin and eosin (H&E) as described in Section 2.11. Each liver was then assessed histologically for the presence of necrotic foci and inflammatory infiltrates. Typical results are shown in Table 5.2.

B6/129 mice responded rapidly to infection with either virus, as shown by infiltration with inflammatory cells and formation of multiple foci from d3 p.i. with EV or EVΔp28 (Table 5.2). Levels of cellular infiltration increased from d3 to d9 p.i. and were accompanied by Kupffer cell hypertrophy and mild inflammation. Necrosis was noticeably absent from infected livers (Figs. 5.5, 5.6, 5.7). The changes appeared similar in EVΔp28 infection, although quantitation of foci revealed reduced numbers of foci (Table 5.2, Fig. 5.7). Neither necrosis nor inflammatory changes were seen in mock-infected animals (Fig. 5.5).

p75-/- and B6/129 mice shared similar responses to infection as measured by quantitation of inflammatory foci (Table 5.2). However, while the control of infection in B6/129 mice was associated with minimal hepatic damage, infection of p75-/- mice with either EV or EVΔp28 resulted in necrotic changes observable from d6-9 p.i. (Table 5.2). Hepatic necrosis in p75-/- mice did not differ quantitatively between viruses, however the response to EV infection was accompanied by the appearance of distinctive necrotic foci, often discrete and separated from foci of inflammatory cells (Fig. 5.6C, D). These were not seen in p75-/- mice infected with EVΔp28 (Fig. 5.7C, D), or B6/129 or p55-/-p75-/- mice infected with either virus (Fig. 5.6, 5.7). The distinctive foci were accompanied by indicators of hepatic damage such as degeneration of hepatocytes, increased blood in the sinusoids and increased infiltration by inflammatory cells. Since virus titres were not appreciably higher in EV-infected mice than EVΔp28

infected mice, it is unlikely that the foci were due to overwhelming levels of virus replication. It is possible that the foci may be due to a protective role for p75 against p55-mediated damage, as has been suggested by Horn and colleagues (Horn *et al.*, 2000).

p55-/-p75-/- mice displayed a large number of smaller inflammatory foci in comparison to the B6/129 and p75-/- mice. Necrotic foci were present in EV and EVΔp28 infected mice at approximately the same levels as those in p75-/- mice but were associated with inflammatory foci in both EV and EVΔp28 infection, contrasting with the lack of necrosis in B6/129 and the distinctive necroses seen in p75-/- mice (Figs. 5.6, 5.7). By d9 p.i., necrosis was accompanied by significant damage to the infected livers (Fig. 5.7E). Inflammatory foci showed a strong trend towards increased numbers in EV infected mice compared to EVΔp28 infected mice, although this was not significant.

These data show that both TNF receptors are necessary for maximal inflammatory cell infiltration, inflammatory focus formation and prevention of necrosis during EV infection, independent of p28 expression. p55 also appeared to have a further role in the hepatic response to virus which was manipulated by p28, as indicated by the presence of the distinctive necrotic foci in p75-/- mice. The finding that the foci were not present in similarly infected p55-/-p75-/- mice suggests that they may require p55 activity.

It has been found that, in addition to blocking TNF-mediated apoptosis (Turner *et al.*, submitted), p28 and its homologues are also capable of blocking other forms of apoptosis *in vitro* (Brick *et al.*, 2000; Brick *et al.*, 1998). Given the important role of apoptosis in the anti-viral response (Clem and Miller, 1993; Itoh *et al.*, 1998; Turner and Moyer, 1998), in hepatic damage (Lawson *et al.*, 1998; Lieber *et al.*, 1997) and in the potential priming of the immune response (Inaba *et al.*, 1998; Rosen *et al.*, 1995), it was possible that p28 also influenced pathogenesis in these mice through manipulation of non-TNF mediated apoptosis. Hence the effect of p28 expression on apoptosis was examined in the livers of wt and TNF receptor-deficient mice.

5.2.4 Apoptosis in EV-infected TNF receptor deficient mice is differentially responsive to p28 expression

The effects of p28 interactions with TNF signalling on hepatic apoptosis were examined using *in situ* TUNEL staining on liver samples from the mice described in Section 5.2.3. Apoptosis was measured as the number of TUNEL-positive cells showing apoptotic morphology at 3d intervals from d3 to d9 p.i., and quantified as described in Section 2.10. Typical staining patterns are shown in Figs. 5.9 - 5.11.

Apoptosis, paradoxically, appeared more responsive to p28 expression in the absence of TNF receptors. Apoptosis in B6/129 mice was not sensitive to p28 expression, showing comparable levels and patterns of apoptosis across the course of infection with either virus (Figs. 5.8, 5.9). As described in Chapter 3, both viral infections stimulated predominantly scattered apoptosis throughout the infection, from d3 p.i. At d6 and d9 p.i., apoptosis was also present in the proximity of leukocyte clusters (Fig. 5.9B, C). Mock-infected B6/129 mice displayed negligible levels of apoptosis (Fig. 5.9A).

Apoptosis in p75-/- mice was at similar levels to B6/129 in mock-infected (Fig. 5.10A) and EV or EVΔp28 infected mice 3d p.i. (Fig 5.8). At d6 p.i., apoptosis showed a trend towards increasing levels in response to infection with either virus, which continued to increase slightly at d9 p.i. in response to EV but not EVΔp28 (Fig. 5.8). The increase in apoptosis in p75-/- mice infected with EV was associated with distinctive apoptosis patterns from d6 p.i. (Fig. 5.10B). Apoptotic cells were clustered near or within small areas of necrosis resembling that illustrated in Fig. 5.6C. Apoptotic cells in EVΔp28-infected p75-/- mice were instead predominantly scattered as seen in B6/129 mice, with a smaller percentage of apoptosis associated with necroses (Fig. 5.10C). It is interesting that despite the very different patterns of apoptosis, the absolute number of cells undergoing apoptosis was not significantly greater in EV infected than EVΔp28 infected mice.

In contrast, apoptosis in p55-/-p75-/- mice was highly sensitive to the presence of p28. Mice exhibited similar levels of apoptosis in response to EVΔp28 to those seen in B6/129 and p75-/- mice (Fig. 5.8). However, the number of TUNEL-positive cells seen in response to wt EV was significantly increased from d3 p.i., reaching very high levels by d9 p.i. ($P<0.01$; Fig. 5.8). This was predominantly individual apoptosing hepatocytes, although both necrosis-associated and single cell apoptosis were evident. By d9 p.i., a larger percentage of TUNEL-positive cells in EV-infected p55-/-p75-/- mice was present as undigested apoptotic bodies than that seen in B6/129 or p75-/- mice (Fig. 5.11B). It is likely that much of the apparent increase in apoptosis was attributable to necrotic cells and secondary necrosis of undigested apoptotic bodies, as late in infection the volume of apoptotic cells and associated debris made the distinction between apoptosis and necrosis extremely difficult (Fig. 5.11B). This contrasted with the apoptosis seen in response to EVΔp28, which more closely resembled the response observed in p75-/- mice (Figs. 5.10C, 5.11C).

Thus, unlike MCMV and adenovirus infections (Hayder *et al.*, 1999; Orange *et al.*, 1997), TNF did not appear to contribute to apoptosis during EV infection from d3-9 p.i. in B6/129 mice. p28 also did not appear to affect the apoptotic response in these mice. However, p28 expression did lead to increased numbers of TUNEL-positive cells in the livers of p55-/-p75-/- mice. This was unrelated to hepatic virus titres, since growth of EV was not significantly greater in the livers of p55-/-p75-/- mice than p75-/- or B6/129 mice (Fig. 5.3). Further, at d9 p.i. equal titres of EV and EVΔp28 were recovered from the livers of p55-/-p75-/- mice (Fig. 5.3). It also appears unrelated to cellular infiltration (Table 5.2). Indeed, the histology suggests that high levels of TUNEL-positive cells are seen because of a lack of uptake of apoptotic particles, and subsequent necrosis.

Given that the presence of p28 affected hepatic necrosis (Fig. 5.6, 5.7) and apoptosis (Fig. 5.8) without affecting the number of inflammatory foci (Table 5.2), it was possible

that p28 might be affecting virulence through reducing the effectiveness of inflammatory cells. To address this possibility EV and EVΔp28 infection was examined in sublethally irradiated mice. These have previously been shown to have limited immune responses due to an inability of immune cells to proliferate on antigenic or other stimulation (Johnson *et al.*, 1995; Lin *et al.*, 1996).

5.2.5 p28 affects virulence through radiation-insensitive elements

In order to better define the elements affected by p28 expression, wt and TNF receptor deficient mice were sublethally irradiated before infection (Section 2.7). Groups of 4 mice were infected 24h later with 5 x 10^3 pfu EV or EVΔp28 per mouse as previously, and examined at 24h intervals for morbidity and mortality. As controls, 3 mice of each genotype were irradiated and mock-infected, and groups of 3-4 sex and age-matched mice of each genotype left non-irradiated before infection.

Mortality rates in non-irradiated control mice were equal to those demonstrated previously (Tables 5.1 and 5.3). B6/129, p75-/- and p55-/-p75-/- mice were uniformly resistant to EVΔp28-mediated lethality. B6/129 mice also proved resistant to EV-mediated lethality, whereas both p75-/- and p55-/-p75-/- mice suffered 60% mortality in response to EV infection. MTD were again significantly longer in p55-/-p75-/- mice (16.5 ± 1.2 days) than p75-/- mice (10.8 ± 0.8 days: $P<0.01$). Irradiation resulted in both B6/129 and TNF receptor knockout mice becoming highly susceptible to EV-mediated mortality (Table 5.3). Strikingly, irradiation rendered wt B6/129 and TNF receptor deficient mice equally susceptible to EV mediated lethality, as indicated by MTD (Table 5.3). Irradiated B6/129 and TNF receptor knockout mice were also highly susceptible to EVΔp28. However, in comparison to EV infection, mortality in EVΔp28-infected mice was uniformly delayed by 4 to 5 days. From these data, two conclusions can be drawn. Firstly, the equal susceptibility of B6/129, p75-/- and p55-/-p75-/- mice to EV after irradiation suggests that TNF requires radiation-sensitive elements for anti-

EV activity. Secondly, p28 enhances virulence through both radiation sensitive, and TNF-independent, radiation-insensitive elements.

The study thus far had examined p28 activity only in the light of its potential interactions with TNF, and had found that it had predominantly TNF-independent activity. There were two possibilities not yet explored. The first was that p28 may be acting through CD40 *in vivo*, consistent with the *in vitro* finding that p28 can block cell death and the upregulation of TNF mRNA through CD40 (Turner *et al.*, submitted). The second possibility was that *in vivo* interactions noted between p28 and the TNF receptors more generally reflected the compromise of the immune response due to the removal of cytokine receptors rather than the TNF receptors specifically. Both of these possibilities were now addressed.

5.2.6 Effects of CD40 deficiency differ between mouse strains

In addition to preventing TNF mediated cell death through p75, p28 has been shown to block CD40-mediated cell death (Turner *et al.*, submitted). In order to test whether the effects of p28 expression were negated in the absence of CD40, CD40-/- mice on the same B6/129 background as the TNF receptor knockout mice were infected. In addition, it was possible that the effect of CD40 deficiency may differ in EV-susceptible and EV-resistant mice, as has been implied for TNF (Shibuya *et al.*, 1998). Thus, the importance of CD40 in the attenuation of EVΔp28 was also investigated by infecting mice lacking CD40 on the EV-susceptible Balb/c background.

As previously, mice were infected with 5 x 10^3 pfu of either EV or EVΔp28 via the footpad and examined daily for 25d p.i. Additionally, at death or termination of the experiment, internal organs were examined for evidence of pathology. Results are shown in Table 5.4 and Fig. 5.12.

B6/129 mice were resistant to EV and EVΔp28 as previously (Chapter 3, Table 5.4). Mice displayed footpad swelling from 8-18d p.i. in response to both viruses, which was

greater in response to EV than EVΔp28, but otherwise showed minimal morbidity in response to infection (Chapter 3, Table 5.4, Fig. 5.1, 5.2 and 5.12A, B). All mice survived infection and examination at d25 p.i. revealed minor residual splenomegaly in otherwise healthy mice. EVΔp28 appeared to be attenuated in B6/129 mice, as indicated by reduced footpad swelling (Fig 5.12A).

CD40 knockouts on the resistant B6/129 background were found to be more susceptible to EV-mediated lethality than wt B6/129 mice (Table 5.4). Increased mortality was associated with slight, but consistent, weight loss in all mice (data not shown) and enhanced swelling at the site of infection (Fig 5.12A). Attenuation of EVΔp28 was also observed in CD40-/- mice, as indicated by reduced lethality of infection (Table 5.4) and footpad swelling (Fig. 5.12A). Hence mutation of CD40 did not restore EVΔp28 virulence, suggesting that CD40 is not required for the activity of p28 as a virulence factor in B6/129 mice.

A comparison of EV and EVΔp28 infection in Balb/c and Balb/c-CD40-/- mice showed that p28 was also required for EV virulence in mice on a Balb/c background. Balb/c mice were found to rapidly succumb to EV infection, but no mortality was seen in response to EVΔp28 (Table 5.4). Instead, Balb/c mice infected with EVΔp28 displayed delayed onset of morbidity by 2 to 3d in comparison to EV infected mice, and symptoms appeared to resolve by d17 p.i. Mice were fully recovered from the infection at termination of the experiment at 25d p.i. (Fig. 5.12C, D; see also Section 3.2.1). Post-mortem examination revealed that the difference in mortality was reflected in marked differences in internal pathology. EV infected Balb/c uniformly displayed hepatic, splenic and intestinal lesions, associated with extreme splenomegaly and highly enlarged Peyer's patches. An EVΔp28-infected Balb/c mouse sacrificed at d8 p.i. for comparison with EV infection displayed potential intestinal damage and discoloured spleen and liver but no splenomegaly or enlargement of Peyer's patches. Further, all

observable symptoms of EVΔp28 infection were resolved at the termination of the experiment (25d p.i.).

Balb/c-CD40-/- mice infected with EV displayed greater morbidity and more rapid onset of disease than similarly infected wt Balb/c mice. EV infected Balb/c-CD40-/- were hunched and lethargic 1d before morbidity was detectable in similarly infected Balb/c mice. This was accompanied by discharge from the eyes in all mice, but no visible pocks. Post-mortem examination revealed severe hepatic damage, as indicated by hepatomegaly and fatty, grainy appearance. All mice also displayed intestinal haemorrhage and severe tissue damage to the uterus and ovaries. Enlarged Peyer's patches and splenomegaly were not observed in EV-infected Balb/c-CD40-/- mice.

An increase in susceptibility of Balb/c-CD40-/- mice could also be seen in response to EVΔp28 infection. Balb/c-CD40-/- mice infected with EVΔp28 suffered severe ongoing morbidity from d10 p.i. which did not begin resolution until around d17 p.i. This included facial lesions, accelerated footpad swelling, lethargy and ruffled fur (Fig. 5.12C, D). Sacrifice of an EVΔp28-infected Balb/c-CD40-/- mouse at d8 p.i. for comparison with EV-infected mice revealed minimal hepatic damage, as indicated by slight hepatomegaly. No eye lesions were visible, and intestines appeared to be intact, with slightly enlarged Peyer's patches. Strikingly, this was associated with extreme splenomegaly, and a lack of visible splenic lesions compared to similarly infected Balb/c mice. Since CD40-/- mice lack an effective B cell response (Kawabe *et al.*, 1994), the splenomegaly suggests that in Balb/c-CD40-/- mice may have strong T cell proliferation in response to EVΔp28, which does not occur in response to EV. This is deserving of further investigation. The data may also have suggested that EVΔp28 was similarly attenuated in Balb/c-CD40-/- mice as in Balb/c. However, pathogenic changes in Balb/c-CD40-/- mice at d8 p.i. were likely to underestimate the pathogenicity of EVΔp28 in these mice. EVΔp28-infected Balb/c-CD40-/- mice did not consistently display signs of infection until d7 p.i. Some discomfort was discernible at d9 p.i. By

d11 p.i., all mice displayed maximal footpad swelling, and signs of significant general discomfort such as hunching and coat ruffling. This was accompanied by lethargy, weight loss and swollen bellies by d12 p.i., which was associated with poorly formed faeces (2 of 5 mice) or diarrhoea (3 of 5 mice) by d13 p.i. Symptoms did not begin to subside until d17 p.i. Mice sacrificed at d25 p.i. showed hepatomegaly and splenomegaly, but no other remaining symptoms of infection. Thus while EVΔp28 infection of Balb/c-CD40-/- mice led to only 20% mortality, the absolute level of mortality seen is likely to understate EVΔp28 virulence.

These limited experiments support the data of Ruby and colleagues (Ruby *et al.*, 1995) describing an important role for CD40 in the anti-viral response. The data further suggest that interactions between CD40 signalling pathways and p28 may not be of importance in EV virulence in B6/129 mice, but play a greater role in virulence in Balb/c mice.

5.2.7 IFNγR and IL-6 deficient mice are resistant to EVΔp28 infection

Many cytokines other than TNF have been demonstrated as important in the control of EV infection (Karupiah, 1998; Karupiah *et al.*, 1993; Ramshaw *et al.*, 1997). It was possible that differences in the effects of p28 expression observed between wt and TNF receptor deficient mice were due to the compromise of the immune response in the TNF receptor knockouts rather than TNF receptor deficiency specifically. Hence, EV and EVΔp28 infection of TNF receptor deficient mice was compared with infection of mice lacking other cytokines or cytokine receptors important in the resolution of EV infection. IFNγ and IL-6 are known from previous studies to be required for resistance to EV infection (Karupiah *et al.*, 1993; Ramshaw *et al.*, 1997; van den Broek *et al.*, 1995). It should be noted for these experiments that while the TNF receptor knockouts were on a B6/129 background, the IL-6 and IFNγR-/- mice were on the more EV-resistant 129 background (for details of 129 mice, see Chapter 3).

IL-6-/-, IFNγR-/- and 129 mice were infected with 5 x 10^3 pfu of either EV or EVΔp28 via the footpad and examined over the course of 25 days. The background 129 mice proved highly resistant to EV and EVΔp28 (Table 5.5). No mortality was observed in response to either virus, and morbidity in response to both viruses was also minimal. Footpad swelling was observed at low levels and appeared to be slightly less in response to EVΔp28 than EV. No other symptoms of infection were observed, and on sacrifice at d25 p.i., virus was not detectable in the liver or spleen by titration (data not shown).

IL-6-/- mice displayed increased mortality in response to EV, resulting in 50% mortality at 14 ± 2d p.i. (Table 5.5). No increase in mortality was seen in EVΔp28 infected mice. However, morbidity was more similar than is suggested by mortality rates. IL-6-/- mice responded to both infections with reduced splenomegaly and increased footswelling compared to that seen in 129 mice. Both aspects were slightly reduced in EVΔp28 compared to EV infection, as seen in the background strain. EVΔp28 infected mice recovered from infection after displaying foot swelling until approximately d17 p.i. EV-infected mice also suffered foot swelling until around d19 p.i. Further gross signs of infection were not evident until 24h before death, at which point they became hunched with ruffled fur. Post-mortem examination revealed macroscopically observable viral plaques on the liver and spleen, but little splenomegaly. Plaques were not present at sacrifice of the remaining surviving mice (d25 p.i.) or in an EVΔp28-infected IL-6-/- mouse sacrificed at d13 p.i. for comparison with lethal EV infections. Thus the loss of IL-6 resulted in higher susceptibility to both viruses. This did not appear to be affected by the presence of p28 to a greater extent in the IL-6-/- mice than in the wt strain, suggesting that IL-6 and p28 are unlikely to interact.

IFNγR-/- mice were also susceptible to both EV and EVΔp28 induced morbidity, and highly susceptible to lethal EV infection. EV-infected IFNγR-/- displayed diarrhoea and multiple pocks in the vicinity of mucous membranes from 7d p.i. Mice became

hunched and lethargic within 9 days of infection, followed rapidly by death. Post-mortems revealed severe intestinal lesions and reduced spleen and liver size. Liver pathology was associated with fatty change and necrosis in 4 of 6 livers examined, and fibrosis in 1 of 6 livers. EVΔp28 infection of IFNγR-/- mice also resulted in pocks on the face and mucous membranes, appearing within 24h of those on EV-infected mice. Mice became lethargic and 6 of 7 mice suffered intestinal damage as indicated by diarrhoea from d11-12 p.i. An EVΔp28-infected mouse sacrificed for comparison with EV-infected mice at d12 showed hepatomegaly, and intestinal lesions similar to those seen in EV infected mice. Symptoms subsided in the following 36h, and all mice survived infection, although external lesions and footpad swelling were maintained. Mice were sacrificed for humane reasons at d21 p.i. At sacrifice, EVΔp28-infected mice displayed residual hepatomegaly and splenomegaly, with no other discernible internal injury. However, pocks were still present and infectious on the face, urogenital openings and at the site of infection (data not shown). Hence the morbidity seen in IFNγR-/- mice was consistent with a non-specific increase in susceptibility to virus infection. These data suggested that the activity of p28 as a virulence factor did not require IFNγR or IL-6.

5.3 Discussion

In this chapter, potential roles of p28 in EV virulence were investigated through the infection of cytokine- or cytokine receptor-deficient mice. It was shown previously that EV but not EVΔp28 can block apoptosis activated by CD40 and p75 *in vitro* (Turner *et al.* submitted). Data presented in this chapter demonstrates that p28 also targets CD40 and p75 independent events *in vivo*. This was most clearly illustrated in mortality studies of B6/129-derived mice. It was expected that if the role of p28 *in vivo* was to block activity through CD40 or p75, then in their absence EVΔp28 virulence would be restored. However, CD40-/-, p75-/- and p55-/-p75-/- mice on a B6/129 background suffered lethal infection in response to EV and recovered fully from EVΔp28 infection. This demonstrated that either p28 affected mortality through pathways additional to those observed *in vitro*, or that p28 must block both TNF and CD40-mediated signalling pathways for EV virulence. The latter is suggested by the presence of intracellular signalling molecules common to both CD40 and p75 (Grell *et al.*, 1999; Rao *et al.*, 1995; Rothe *et al.*, 1995). CD40-/-p75-/- and CD40-/-p55-/-p75-/- mice have been bred which may help to answer this question (SC and Jo McLintock, JCSMR Animal Services). However, the mice appear to have further defects, as indicated by low litter sizes (knockout litter size: 3.0 ± 1.4 pups, control litter size: 5.1 ± 1.6 pups) and unproductive matings (2 of 5 knockout breeding pairs productive, 5 of 5 control breeding pairs productive). While this suggests potentially interesting roles for these receptors in reproduction and/or development, sufficient mice for investigation of p28 activity have not yet been produced.

Previous studies suggested that the attenuation of EVΔp28-mediated mousepox in some mouse strains was due to compromised virus replication (Senkevich *et al.*, 1995). Data in this chapter, like that in Chapter 3, does not support a role for p28 as a replication factor *in vivo*. While quantitation of virus in B6/129 and TNF receptor deficient mice

showed maximum titres of EVΔp28 to be approximately 1 log lower than those of wt EV in B6/129 and TNF receptor deficient mice, the reduced lethality of EVΔp28 infection was unlikely to be due to the difference in virus titres since (1) levels of virus rose to similar levels in B6/129 mice which survived and TNF receptor KO mice which did not, and (2) by d9 p.i., titres of both EV and EVΔp28 were significantly reduced and indistinguishable from one another in all strains tested. This suggested that EVΔp28 replication was not sufficiently compromised to account for the attenuation of disease. Thus, it is likely that the role of p28 in virulence was in virus-host interactions.

An initial hypothesis tested in this chapter was that p28 may block TNF-mediated apoptosis through p75 during the period d3-9 p.i. It was not possible to detemine whether this in fact occurred, as low levels of apoptosis were observed in wt mice in response to both EV and the p28 mutant virus. However, broader TNF-dependent activities were also examined. Unexpectedly, the data suggests that the contribution of p28 to virulence is predominantly TNF-independent. A subtle interaction between p28 and effects of the p55 TNF receptor was suggested by the unusual necroses observed in p75-/- mice, but not wt or p55-/-p75-/- mice in response to EV. There are a number of possible explanations. It can be seen from the data in Table 5.1 and Figure 5.3 that p75-/- mice were as susceptible or more susceptible to EV than p55-/-p75-/- mice. Thus it is possible that the necroses simply reflect the higher susceptibility of the mice, although if this is the case then it is interesting that they do not resemble necroses in any of the EV-susceptible mouse strains examined. Alternatively, a protective role for p75 against p55-mediated damage to the liver is suggested by comparison of necroses from EVΔp28-infected wt, p75-/- and p55-/-p75-/- mice. This has previously been demonstrated to occur on treatment with the hepatotoxin dimethylnitrosamine (Horn *et al.*, 2000), and is consistent with the higher susceptibility of p75-/- mice than p55-/-p75-/-mice. A third alternative is that p28 may interrupt chemotaxis stimulated by TNF by preventing chemokine upregulation (Hornung *et al.*, 2000; Tessier *et al.*, 1997), although this has so far been described as a p75 dependent event (Wang *et al.*, 1997).

A role for p28 in preventing TNF-mediated chemotaxis was also suggested by comparing splenomegaly and infiltration of the liver by leukocytes in B6/129 and p75-/- mice with p55-/-p75-/- mice. B6/129 and p75-/- mice displayed significantly greater splenomegaly in response to EV than EVΔp28 by d9 p.i. ($P<0.05$) In contrast, there was no difference between splenomegaly induced by EV or EVΔp28 in p55-/-p75-/- mice, suggesting that p28 may enhance splenomegaly through a TNF-dependent mechanism. The high splenomegaly may have been due to abnormally high cell proliferation in the spleen, however this is unlikely, since the CD4 and CD8 responses to antigen in these mice have previously been described as normal (Peschon *et al.*, 1998), and levels of leukocytes found in the liver did not increase; indeed, levels of leukocytes infiltrating the liver were lower in p55-/-p75-/- mice than in either B6/129 or p75-/- mice. An alternative proposal is that the splenomegaly observed may reflect a role for TNF in chemotaxis of T cells from the spleen to sites of infection. A role for TNF in lymphocyte chemotaxis through endothelial monolayers has been shown by de Jong and colleagues (de Jong *et al.*, 1996), and further roles for TNF in regulation of chemokines and chemokine receptor expression have been described both *in vitro* (Hornung *et al.*, 2000) and *in vivo* (Tessier *et al.*, 1997). The data from the current study suggests that the chemotaxis of lymphocytes from the spleen requires the presence of the p55 TNF receptor, and is inhibited by the presence of p28. This finding may indicate an important aspect of p28 function, which could be investigated further by examining TNF-stimulated upregulation of chemokine mRNA or protein from a variety of cell types infected with EV or EVΔp28. The further finding that p28 expression increased hepatic apoptosis in p55-/-p75-/- mice and was required for EV growth in the spleen and LN of these mice suggests that p28 had additional roles in EV infection, independent of interactions with TNF.

A TNF independent component of p28 activity is also supported by the attenuation of EVΔp28 in p55-/-p75-/-mice, and the attenuation of the p28 mutant virus in the irradiated mice independent of TNF expression. This may indicate that p28 interacts

with other pathways; for example, other signalling pathways in which TRAFs are utilised (see Section 1.3.3.2). In particular, a role for p28 in CD40 activity is supported by the comparison of EV and EVΔp28 infection in Balb/c and Balb/c.CD40-/- mice (see below).

It is interesting to contrast the effects of p28 expression on infection in TNF receptor knockout mice with those in mice lacking other cytokines. Comparison of EV and EVΔp28 lethality in IFNγR-/- and IL-6-/- mice revealed that EVΔp28 also induced reduced pathogenesis in these mice. However, despite differences in lethality between EV and EVΔp28 infection, differences in pathogenesis appeared to be minor in comparison to those observed in CD40-/-, TNF receptor deficient, Balb/c or A/J mice. This was consistent in 129, IFNγR-/- and IL-6-/- mice, suggesting that the small difference in pathogenesis is not specific to these cytokines, but instead reflects the influence of the 129 background. Collation of data with that obtained at 6h p.i. shows that IL-6 had no observable impact on the effect of p28 expression or ablation at any time within the first 9d p.i. In contrast, IFNγR appears to play a role in enabling p28 activity early in infection (Chapter 4). IFNγR may play a similar role later in infection, as shown by the similarly high morbidity in EV and EVΔp28 infected IFNγR-/- mice, but is not absolutely required for p28 activity later in infection, as shown by the difference in mortality between EV and EVΔp28-infected IFNγR-/- mice. On current data, it is impossible to dissect out the contribution of background strain from the effect of IFNγR mutation. This data is also interesting because the severely curtailed IFNγ responses of A/J and Balb/c mice are likely to play a role in their extreme sensitivity to some viral infections, including EV (Mohan *et al.*, 1997; Schindler *et al.*, 1982). However, while IFNγR-/- mice were highly susceptible to EV, an increase in susceptibility to EVΔp28 was also evident which was not seen in A/J or Balb/c mice, suggesting further that IFNγ is not involved in the attenuation of EVΔp28 in either of these mouse strains.

A comparison of CD40-deficient mice on B6/129 and Balb/c backgrounds also strikingly illustrated the effects of background strain, and suggested that the contribution of p28-receptor interactions may differ in different host strains. The B6/129 strain is a cross of two EV-resistant strains and is itself highly resistant to EV-induced death. CD40-/- mice on the B6/129 background showed increased susceptibility to EV, as seen in TNF receptor deficient mice. The increased morbidity and further reduced MTD reflects the important role of CD40 in the anti-viral response prior to the antibody response (Ruby *et al.*, 1995). These mice also showed increased morbidity in response to EVΔp28 and an accelerated inflammatory response at the site of infection, followed by resolution of the infection. In contrast, Balb/c mice are sensitive to lethal EV infection and resistant to EVΔp28. Mutation of CD40 on a Balb/c background resulted in severe pathology in response to both viruses. A comparison of autopsy results suggested that pathology in response to EVΔp28 was only slightly milder than that of EV. Hence CD40 was more important to p28 activity in the susceptible Balb/c mice than the resistant B6/129. The finding that EVΔp28 stimulates splenic expansion in Balb/c.CD40-/- mice but not Balb/c further suggests that in these mice p28 may play a role in immune suppression. The effect of p28 expression and potential interactions with CD40 signalling in EV-susceptible mice would be a fruitful area to examine further. CD40 is of importance in many stages of the immune response, including APC priming and function, and CD4 activity (reviewed in Grewal and Flavell, 1998) which may be affected by p28 activity and cause the noted low level of EVΔp28 virulence early in infection.

The limited results from these experiments suggest that CD40 may be more important in the antiviral response of the susceptible Balb/c strain than the resistant B6/129 strain. Similar experiments have been planned to examine TNF in susceptible mouse strains, using Balb/c.TNF-/- mice. At present, TNF-/- mice have been crossed onto a Balb/c background, but have not yet been sufficiently backcrossed for experimental work.

This data also revealed information on the role and mechanism of TNF in EV infection. Results of irradiation experiments showed that TNF requires radiation-sensitive elements for anti-EV activity. All irradiated mice suffered lethal infection with both viruses, with no difference seen between MTD of B6/129 mice and the TNF receptor knockouts. This is in apparent conflict with previous studies describing direct antiviral activity of the cytokine *in vitro* (Wong and Goeddel, 1986) and *in vivo* (Ruby *et al.*, 1997). In particular, a vaccinia virus construct encoding TNF has been found to be attenuated in both irradiated and non-irradiated mice (Lidbury *et al.*, 1995). It is possible that the differences between the current and previous studies reflect insufficient induction of TNF in irradiated mice, and potential differences between EV and vaccinia virus. Another possibility is suggested by a study by Elkon and colleagues (Elkon *et al.*, 1997). During infection of wt and SCID mice with recombinant adenovirus, it was found that TNF was an effective antiviral agent against hepatic infection only in wt mice (Elkon *et al.*, 1997). It is thus possible that, unlike the case in VV infection, TNF is not directly anti-viral in EV infection. This data suggests that TNF may require an effective T cell response in order to be effectively anti-viral in resistant mice.

Thus in this chapter it has been found that p28 activity has some TNF dependent activity, but predominantly enhances virulence through TNF-independent mechanisms. These differ between mouse strains: in particular, the activity of p28 was found to be significantly increased in susceptible Balb/c mice lacking CD40 compared to wt mice, yet CD40 mutation had relatively little effect on virulence in EV-resistant B6/129. This suggests that (1) p28 has significant TNF- and CD40-independent activity; (2) the role of p28 in EV virulence may differ dependent on the strain of the host.

5.4 Tables and Figures

Table 5.1 Effects of TNF receptor mutation on resistance of mice to infection with EV and EVΔp28.

Infection*					
Mouse Strain	EV		EVΔp28		Mice per group
	Mortality (%)	MTD ‡ (days)	Mortality (%)	MTD ‡ (days)	
B6/129	0	-	0	-	10
p75-/-	60	11.6 ± 0.9	0	-	10
p55-/-p75-/-	50	18.0 ± 0.0	0	-	6

*8-12 week old sex-matched mice of each genotype were infected with 5 x 10^3 pfu of either EV or EVΔp28 in the right hind footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality.

‡MTD, mean time to death ± SD. MTD significantly greater in p55-/-p75-/- mice infected with EV than p75-/- mice ($P<0.01$) as tested by Student's t-test

Table 5.2 Hepatic changes associated with EV or EVΔp28 infection of TNF receptor deficient mice.

Infection	EV			EVΔp28		
Mouse Strain	B6/129	P75-/-	P55-/-p75-/-	B6/129	P75-/-	P55-/-p75-/-
Day post-infection	Necrotic foci[a]					
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	0 ± 0	2975.4 ± 3220.0	1324.7 ± 1467.4	0 ± 0	1732.3 ± 3892.6	183.4 ± 4259.4
9	0 ± 0	468.7 ± 835.6	14938.5 ± 7214.5	0 ± 0	1793.4 ± 1467.4	6582.7±6134.4
	Inflammatory cell foci[b]					
0	0.21 ± 0.47	0.15 ± 0.33	0 ± 0	0.21 ± 0.47	0.15 ± 0.33	0 ± 0
3	4.34 ± 4.34	0.55 ± 0.92	0.18 ± 0.17	3.09 ± 1.04	0.16 ± 0.23	0.08 ± 0.17
6	16.29 ± 3.11	12.52 ± 12.24	24.33 ± 10.57	11.61 ± 3.24	2.57 ± 0.71	9.07 ± 5.39
9	61.26 ± 14.73	66.81 ± 17.71	46.83 ± 13.00	48.62 ± 9.83	35.53 ± 56.00	24.07 + 15.10
	Inflammatory cell infiltration[c]					
0	+/-	+/-	+/-	+/-	+/-	+/-
3	+/-	+/-	+	+	+/-	+
6	++	++	+	++	+	+
9	+++	+	++	+++	++	+

a. Levels of necrosis were semi-quantitated by counting necrotic foci in at least 10 fields of view per section from 2 sections per sample. The size of each necrosis was measured using a micrometer, and the total area of necrosis per field of view calculated. All samples were assessed blind. Data represents μm^2 necrotic tissue per field of view ± SD

b. Levels of inflammatory cell foci were semi-quantitated by observing foci in at least 10 fields of view per section from 2 sections per sample. The number of cells per focus and the total number of foci per field of view were counted. Cells per focus x number of foci were calculated to give the number of cells within foci in each field of view. All samples were assessed blind. Data represent cells within foci per field of view ± SD

c. Inflammatory cell infiltration was estimated by comparing the ratio of lymphocytes to hepatocytes present in at least 10 fields of view per section from 2 sections per sample. Lymphocytes present within foci were excluded from the ratio. All samples were assessed blind.

Table 5.3 Effect of sublethal irradiation on mortality in response to EV or EVΔp28 infection

Mouse strain	Infection*			
	EV		EVΔp28	
	Mortality (%)	MTD (days) ‡	Mortality (%)	MTD (days) ‡
Non-irradiated				
B6/129	0	-	0	-
p75-/-	60	10.8 ± 0.8	0	-
p55-/-p75-/-	60	16.5 ± 1.2	0	-
Irradiated[a]				
B6/129	100	10.8 ± 0.2	100	15.3 ± 1.8[b]
p75-/-	100	10.0 ± 0.0	100	14.0 ± 0.0[c]
p55-/-p75-/-	100	10.5 ± 0.5	100	14.0 ± 0.5[b]

*Groups of 4 8-12 week old sex-matched mice of each genotype were infected with 5 x 10^3 pfu of either EV or EVΔp28 in the right hind footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality. MTD analysed for significant difference using the Student's t-test

a: Mice were irradiated with 650 rads from a ^{60}Co source 24h prior to infection. Control mice were not irradiated. A further group of mice of each genotype was irradiated and mock-infected as described in Section 2.7 to ensure that irradiation was not lethal. No mortality was seen in this group (data not shown).

b: MTD significantly different between EV and EVΔp28 infected mice of the same genotype ($P<0.05$)

c: MTD significantly different between EV and EVΔp28 infected mice of the same genotype ($P<0.01$)

‡MTD, mean time to death ± SD.

Table 5.4 Effects of CD40 mutation on resistance of B6/129 and Balb/c mice to infection with EV and EVΔp28.

Infection					
Mouse Strain	EV		EVΔp28		Mice per group
	Mortality (%)	MTD (days)	Mortality (%)	MTD (days)	
B6/129	0	-	0	-	5
CD40-/-	66	8.9 ± 1.3	0	-	6
Balb/c	100	8.6 ± 0.5	0	-	5
Balb/c.CD40-/-	100	8.0 ± 0.0	20	21.0 ± 0.0	5

*8-12 week old sex-matched mice of each genotype were infected with 5x 10^3 pfu of either EV or EVΔp28 in the right hind footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality.

‡MTD, mean time to death ± SD.

MTD between EV infected CD40-/-, Balb/c and Balb/c.CD40-/- mice not significantly different as tested by Student's t-test. MTD of Balb/c.CD40-/- infected with EVΔp28 not compared to EV-infected mice statistically, as only one mouse was lethally infected with EVΔp28.

Table 5.5 Effects of IFNγR or IL-6 mutation on resistance of mice to infection with EV and EVΔp28.

Infection*					
Mouse Strain	EV		EVΔp28		Mice per group
	Mortality (%)	MTD ‡ (days)	Mortality (%)	MTD ‡ (days)	
129	0	-	0	-	5
129. IL6-/-	50	14.0 ± 2.0	0	-	6
129.IFNγR-/-	100	11.4 ± 1.1	0	-	7

*8-12 week old sex-matched mice of each genotype were infected with 5x 10^3 pfu of either EV or EVΔp28 in the right hind footpad and observed for 25 days at 24h intervals for signs of morbidity and mortality.

‡MTD, mean time to death ± SD.

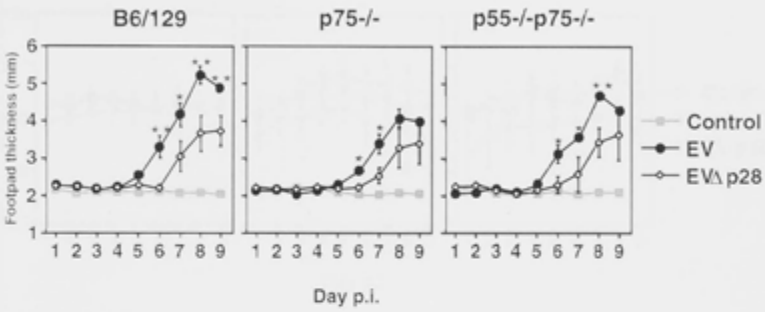


Figure 5.1 Swelling at the site of infection in response to EV or EVΔp28 inoculation

Groups of 5, 10-14 week old female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad. Control measurements were made from 5 mice mock-infected with tissue culture lysate as described in Section 2.5. Footpad thickness was measured from the pad to the top of the foot at 24h intervals. Data represent the mean of 5 measurements ± SD. Statistical significance analysed with the Student's t-test. EV infections resulted in significantly greater footpad swelling than EVΔp28 in B6/129, p75-/- and p55-/-p75-/- mice (*P<0.05, **P<0.01). p75-/- and p55-/-p75-/- mice showed significantly reduced footpad swelling in response to EV from d6 p.i., in comparison to B6/129 (P<0.01) mice. Swelling was reduced more in EV-infected p75-/- than EV-infected p55-/-p75-/- mice (P<0.05). All other comparisons at single time-points were not significant (P>0.05).

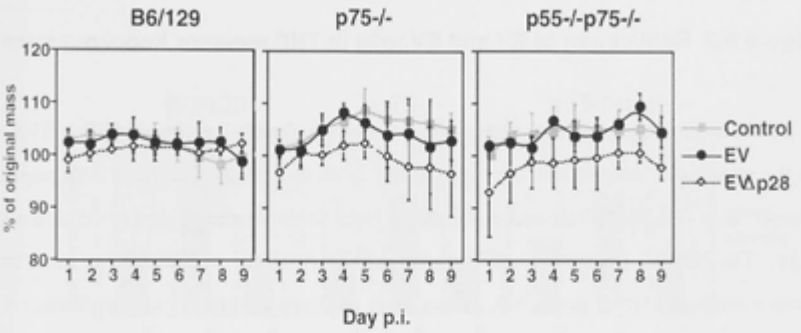


Figure 5.2 Weight changes in response to EV or EVΔp28 infection

Groups of 5, 10-14 week old female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and mice weighed at 24h intervals. Control measurements were made from 5 mice mock-infected with tissue culture lysate as described in materials and methods. Data represent the mean of the 5 measurements ± SD. No significant difference was found in mouse weights from d1-9 p.i. (Student's t-test, $P>0.05$).

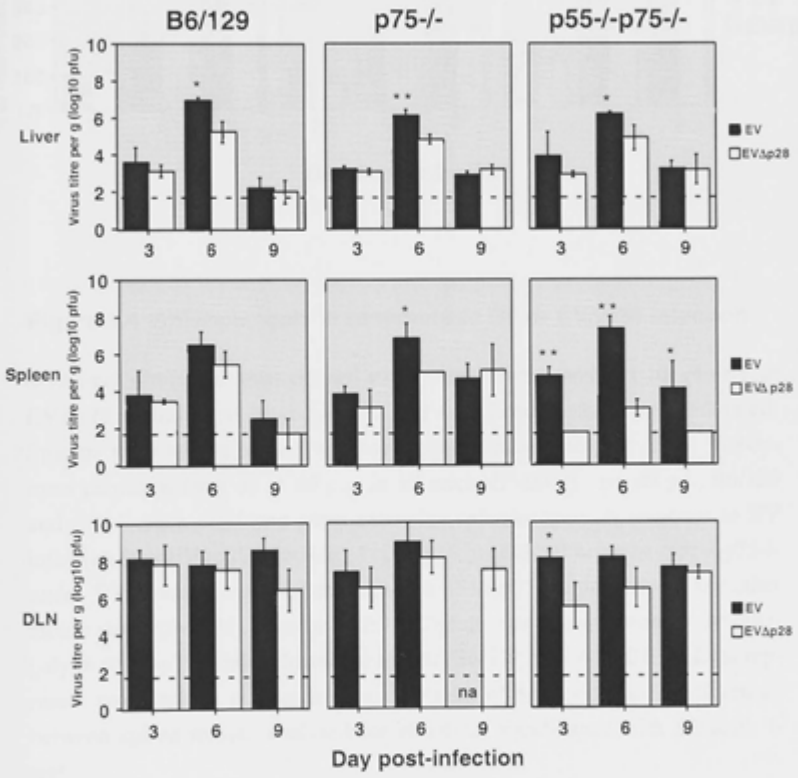
Figure 5.3 Replication of EV and EVΔp28 in TNF receptor knockout mice

8-12 week old female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. The right hind popliteal lymph node (DLN), spleen and a sample of liver were titrated as described in Section 2.12. The limit of detection is $10^{1.7}$ pfu (broken line). Data represent mean virus titres from a minimum of 5 mice ± SD. Titres were analysed for statistical significance with Student's t-test. Hepatic EV titres were statistically different from EVΔp28 titres at d6 p.i. in B6/129 ($P<0.05$), p75-/- ($P<0.01$) and p55-/-p75-/- mice ($P<0.05$). At all timepoints, EV titres were significantly greater than EVΔp28 in p55-/-p75-/- spleen ($P<0.01$). Additionally, EVΔp28 replication in p55-/-p75-/- spleen was significantly less than growth in B6/129 on d3 ($P<0.01$) and d6 p.i. ($P<0.01$) and p75-/- mice on d3 ($P<0.05$), d6 p.i. ($P<0.01$), and d9 p.i. ($P<0.01$). Replication of EV was significantly greater than that of EVΔp28 at d6 p.i. in p75-/- mice ($P<0.01$). EV titres were significantly greater than EVΔp28 titres in DLN of p55-/-p75-/- mice at d3 p.i. ($P<0.05$). All other comparisons at single time-points not significant ($P>0.05$). No titre available for EV-infected p75-/- DLN due to the destruction of the lymph node by infection

na: not applicable.

* EV and EVΔp28 titres significantly different ($P<0.05$)

** EV and EVΔp28 titres significantly different ($P<0.01$)


B6/129
p75-/-
p55-/-p75-/-
Liver
Spleen
DLN
Virus titre per g (log10 pfu)
EV
EVΔp28
na
Day post-infection

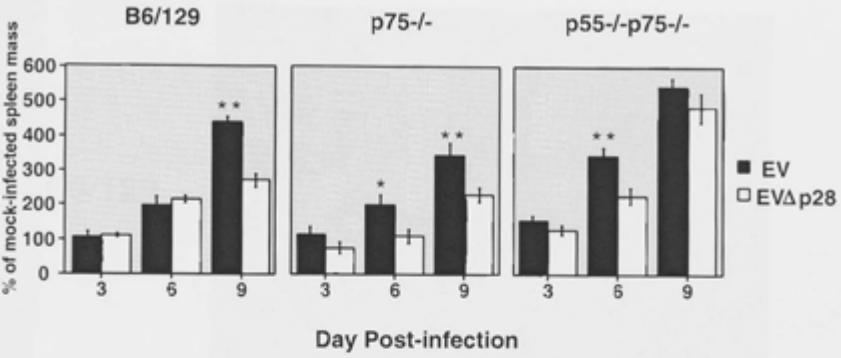


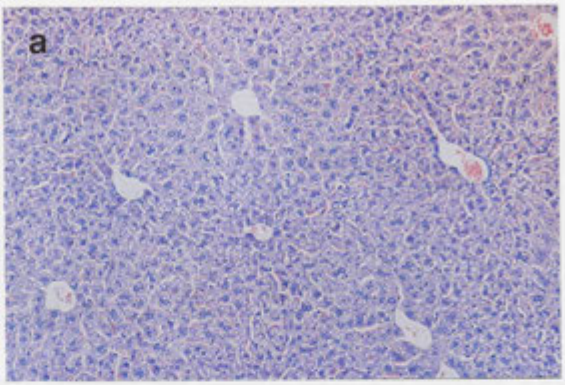
Figure 5.4 Splenomegaly in response to EV or EVΔp28 infection

8-12 week old female mice of each strain were infected with 5 x 10^3 pfu EV or EVΔp28 in the right hind footpad and sacrificed at the times indicated. Spleens were harvested and weighed before titration (see Fig. 5.3). Spleen mass increased from d3 to d9 p.i.. in all mice (P<0.01). By d9 p.i., B6/129 and p75-/- mice displayed more extensive splenomegaly in response to EV infection than EVΔp28 infection (P<0.01). This did not occur in p55-/-p75-/- mice. EV caused greater splenomegaly in p55-/-p75-/- mice than in any other mouse strain (P<0.05). Similarly, EVΔp28 infection caused greater splenomegaly in p55-/-p75-/- mice than other strains (B6/129, p75-/- P<0.01). Data represent mean spleen masses from a minimum of 5 mice ± SD. Differences between spleen masses analysed for statistical significance with Student's t-test.

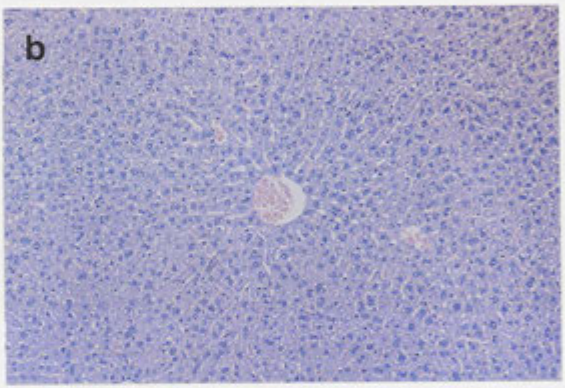
Figure 5.5 Normal hepatic morphology in TNF receptor deficient mice

5 female mice of each genotype between 10 and 16 weeks old were mock-infected with tissue culture lysate and livers harvested 6d p.i.. Formalin-fixed sections were stained with H&E as per materials and methods. Representative sections are shown from B6/129 (A), p75-/- (B) and p55-/-p75-/- mice (C) mice. In each case, mock-infection did not lead to observable pathology. Magnification: 20x.

B6/129



p75-/-



p55-/-p75-/-

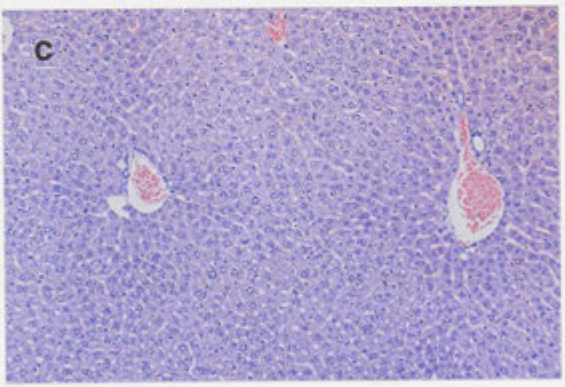
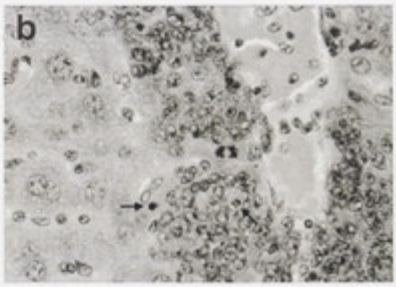


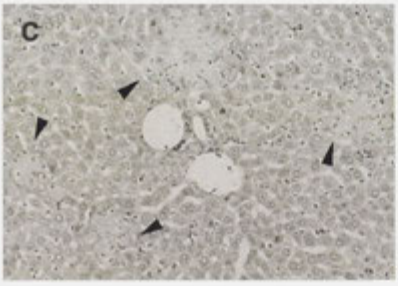
Figure 5.6 Necrotic foci in EV-infected TNF receptor deficient mice

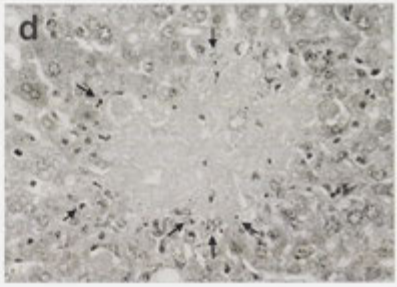
5 10-16 week old female mice of each genotype were infected with EV as previously and livers harvested 9d p.i.. Formalin-fixed sections were stained with H&E as per materials and methods. Representative sections are shown. B6/129 (A, B) and p55-/- p75-/- mice (E, F) displayed necrosis associated with foci of inflammatory cells. However, infection of p75-/- mice with EV led to the presence of necroses associated with little or no inflammatory infiltrate (C, D). Apoptosis was evident in the vicinity of necroses from all three mouse strains, but appeared more prevalent in those seen in EV-infected p75-/- mice (B, D, F). Magnification: A, C, E : 20x, B, D, F : 64x.

B6/129



p75-/-





p55-/-p75-/-

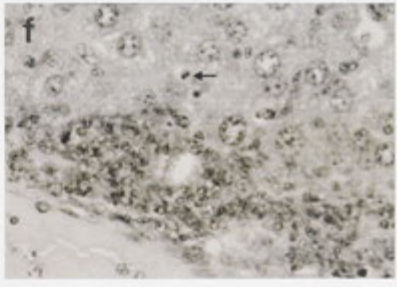
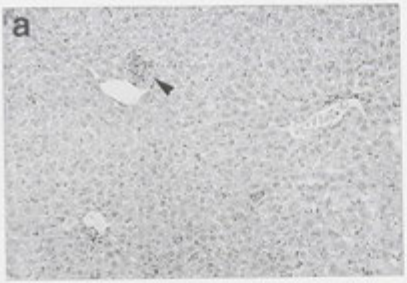
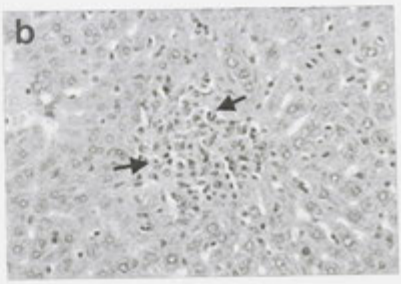


Figure 5.7 Necrotic foci in EVΔp28-infected TNF receptor deficient mice

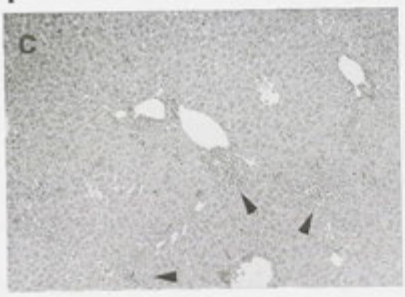
5 10-16 week old female mice of each genotype were infected with EVΔp28 as previously and livers harvested 9d p.i.. Formalin-fixed sections were stained with H&E as per materials and methods. Representative sections are shown. B6/129 (A, B) p75-/- (C,D) and p55-/-p75-/- mice (E, F) displayed necrosis associated with foci of inflammatory cells. Magnification: A, C, E : 20x, B, D, F : 64x.

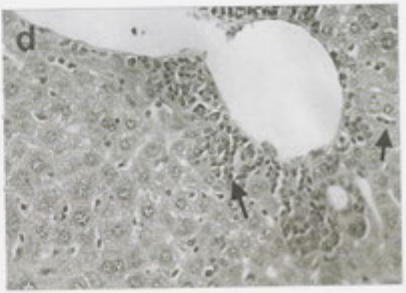
B6/129



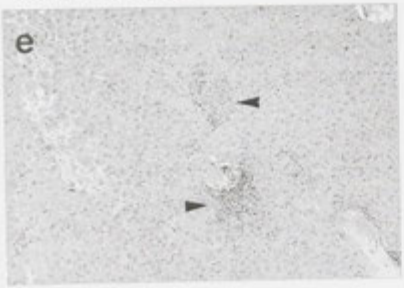


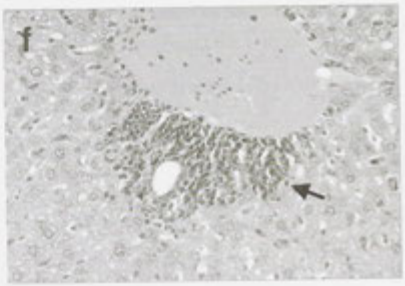
p75-/-





p55-/-p75-/-





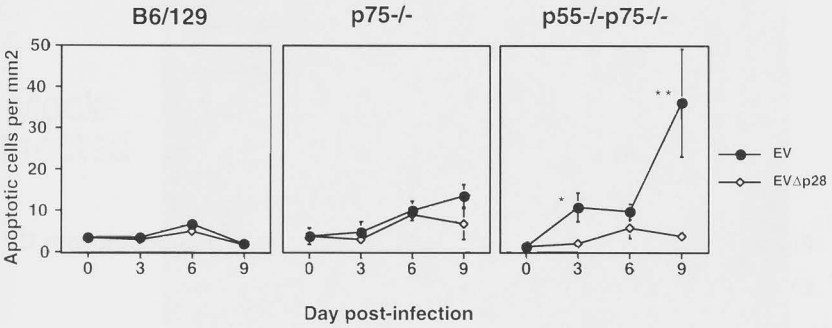


Figure 5.8 Hepatic apoptosis in EV or EVΔp28-infected TNF receptor deficient mice

8-10 week old female mice of each genotype were infected with 5x 10^3 pfu of either EV or EVΔp28 in the footpad and livers harvested at indicated time-points as described in Section 2.5. Sections were stained for apoptotic cells using the TUNEL procedure. Data indicates mean number of TUNEL-positive cells ± SD for 5 to 7 individual mice. Levels of apoptosis were significantly increased in p55-/-p75-/- mice infected with EV at d3 p.i. (* $P<0.05$) and d9 (** $P<0.01$) as tested by Student's t-test.

Figure 5.9 The apoptotic response of B6/129 mice to infection with EV or EVΔp28

Livers were harvested 6d p.i. from 5 10-16 week old female mice mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained with the TUNEL procedure as per materials and methods. Representative sections are shown. Apoptotic cells were evident as strongly FITC-positive cells with apoptotic morphology (white arrow). Mock-infected B6/129 mice displayed low to undetectable levels of hepatic apoptosis (A). Apoptosis became more evident during the infection and by d6 p.i., could be detected as individual apoptotic cells and apoptotic cells in contact with inflammatory infiltrates. This occurred equally in both EV (B) and EVΔp28-infected mice (C). Magnification: (A, B) 40x, (C) 60x.

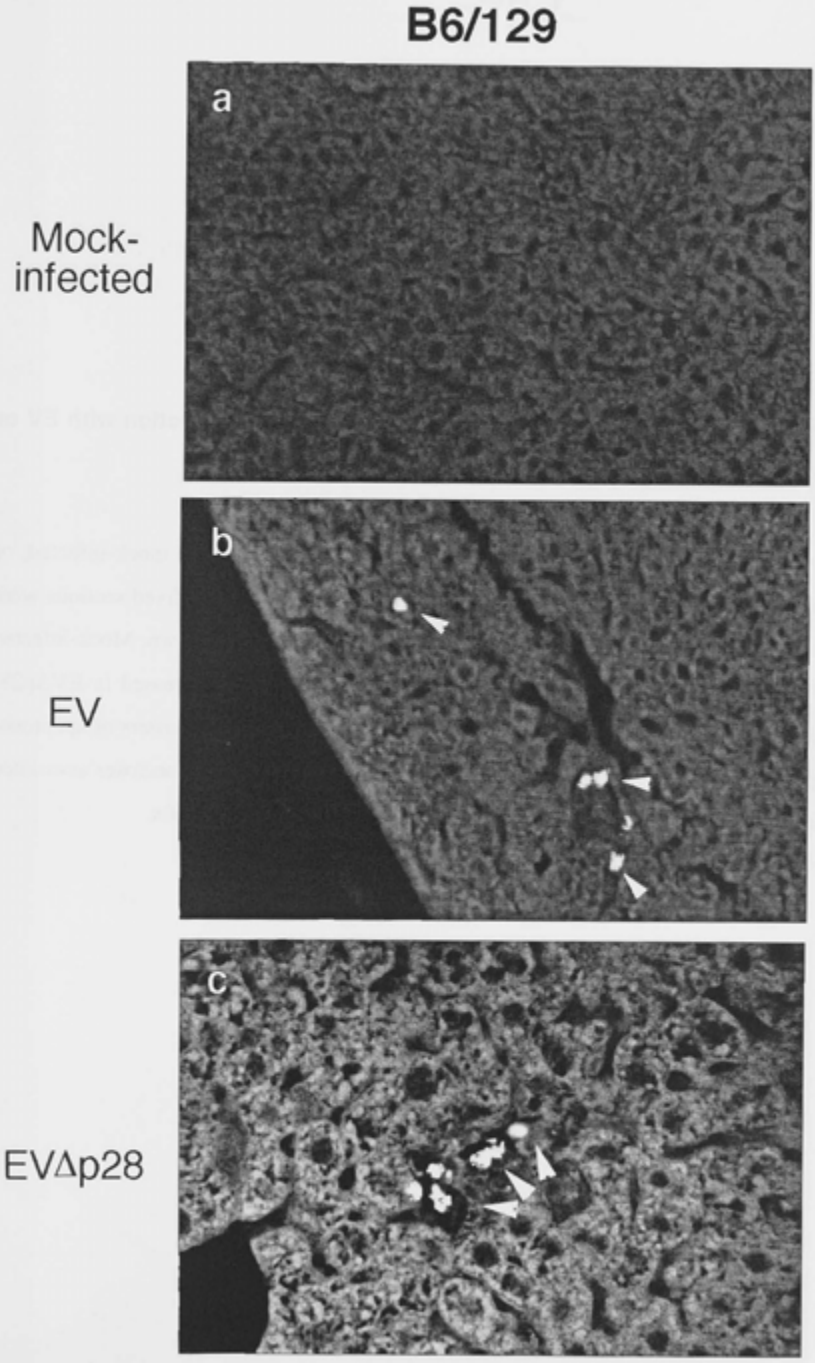

B6/129
a
Mock-
infected
b
EV
c
EVΔp28

Figure 5.10 The apoptotic response of p75-/- mice to infection with EV or EVΔp28

Livers were harvested 6d p.i. from 5 10-16 week old female mice mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained with the TUNEL procedure. Representative sections are shown. Mock-infected p75-/- mice suffered negligible levels of apoptosis (A). This increased in EVΔp28-infected mice, in the form of scattered apoptotic cells (C) or small clusters of apoptosis. Apoptosis was marginally higher in EV-infected mice (B), in which necroses associated with significant apoptosis appeared more prevalent. Magnification: 60x.

p75-/-

Mock-infected

EV

EVΔp28

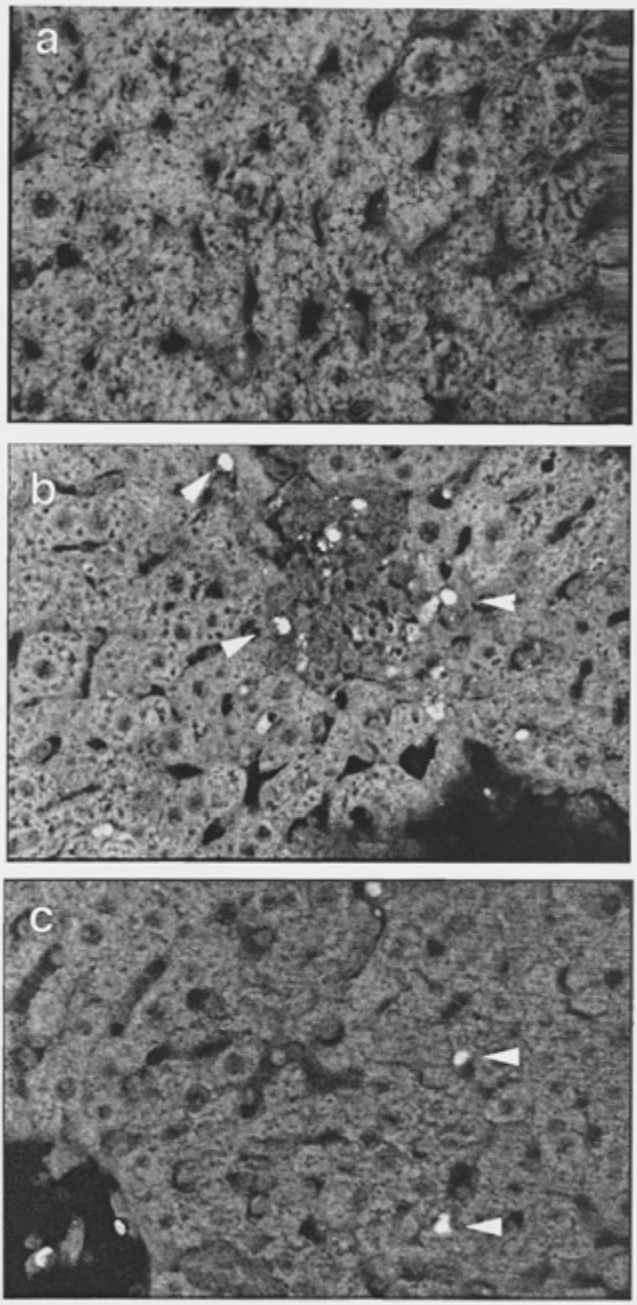
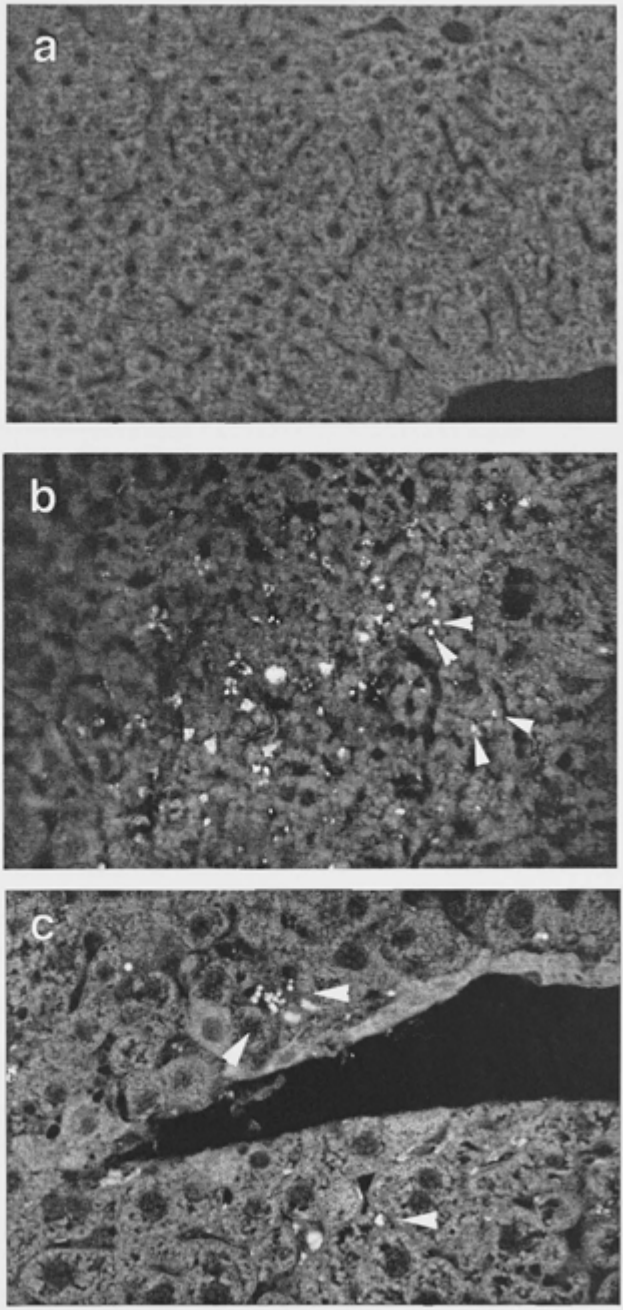


Figure 5.11 The apoptotic response of p55-/-p75-/- mice to infection with EV or EVΔp28

Livers were harvested 6d p.i. from 5 female mice of each genotype mock-infected, or infected with 5 x 10^3 pfu EV or EVΔp28 as previously. Formalin-fixed sections were stained with the TUNEL procedure as described in Section 2.10. Representative sections are shown. While mock-infected p55-/-p75-/- mice had low levels of hepatic apoptosis (A), apoptosis was highly increased in EV infection, mainly as single apoptosing cells (B). This was also increased in EVΔp28 infected mice (C) although to a much lower level. See also Fig. 4.8. High counts of apoptosis in EV-infected-p55-/-p75-/- mice are likely to be due in part to the presence of apoptotic dust in these livers (white arrow). Magnification: A, 40; B, C, 60x.

p55-/-p75-/-



Mock-infected

EV

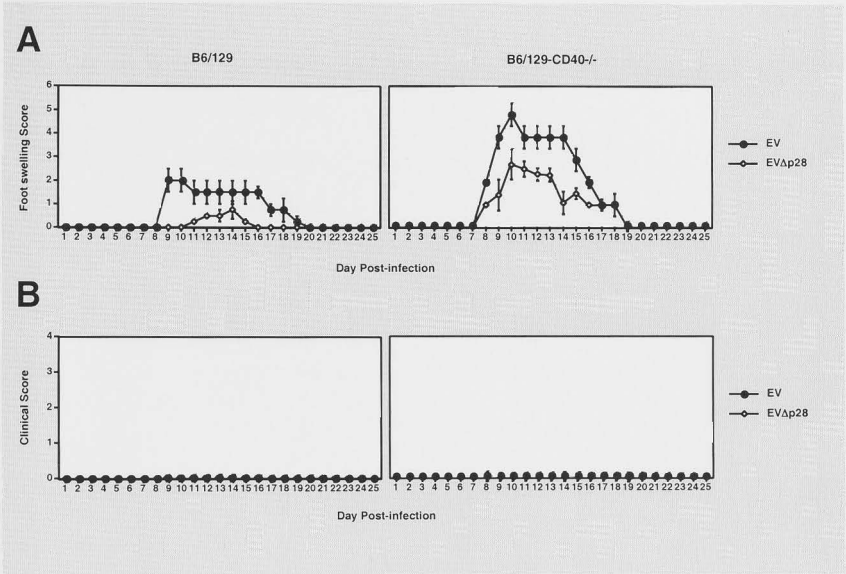
EVΔp28

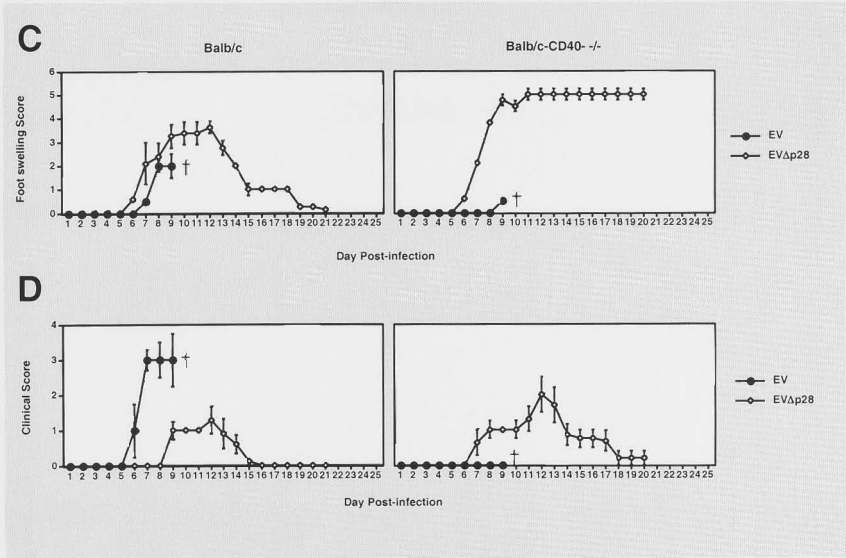
Figure 5.12 Morbidity of CD40 deficient mice in response to infection with EV or EVΔp28

Groups of 5 female 8-10 week old CD40-/- mice on a B6/129 (A, B) or Balb/c (C, D) background were infected with 5 x 10^3 pfu EV or EVΔp28 as previously, and morbidity examined at 24h intervals for up to 25d p.i.

(A, C) Swelling at the site of infection was assessed visually and scored according to the following scale: 0 – healthy footpad, 1- slight swelling of footpad, 2- bones of foot obscured by swelling, 3- feet approximately 3mm in diameter, restricted to foot below hock, 4- extremely swollen, restricted to foot below hock, 5- extremely swollen including hock.

(B, D) Clinical score based on presence of facial lesions, lethargy, and coat ruffling. Lesions scored 1 to 3 (1-lesions just detectable, 3- confluent lesions across face and muzzle), and added to lethargy (0-not present, 0.5-present) and coat ruffling (0-not present, 0.5- present).


A
B6/129
B6/129-CD40-/-
Foot swelling Score
EV
EVΔp28
Day Post-infection
B
Clinical Score
EV
EVΔp28
Day Post-infection


C
Balb/c
Balb/c-CD40- -/-
Foot swelling Score
EV
EVΔp28
Day Post-infection
D
Clinical Score
EV
EVΔp28
Day Post-infection

Chapter 6

Discussion

The experiments described in this thesis were designed to address effects of the expression of the virulence factor p28 on apoptosis and pathogenesis during the course of a natural poxvirus infection, ectromelia virus. During these studies, insights have been gleaned into the roles of TNF and apoptosis in viral infection, as well as the role that p28 plays in the virulence of EV.

6.1 Apoptosis in EV infection

An important aim of this study was to examine the kinetics of the apoptotic response during a virulent virus infection, using EV infection of mice as a model of a coevolved host-virus system. This allowed the frequency of tissue apoptosis to be correlated with virus growth, histologically detectable events such as tissue inflammation, and selected physiological parameters such as host morbidity and mortality. Surprisingly, *in situ* staining of the livers of EVΔp28 infected mice showed that EVΔp28 infection leads to two distinct, differentially regulated phases of apoptosis. These appeared independent of one another. The early phase of apoptosis was a transient apoptotic response, detectable in the liver and ovaries 6h after footpad infection of mice with EVΔp28 but not wt EV. The induction of the early apoptosis required macrophages local to the site of infection and was clearly TNF dependent. A later apoptotic phase was also evident in infections with either wt or mutant virus from 3 to 9d pi, coinciding with elevated rates of viral replication.

While the early phase of apoptosis was blocked by the presence of p28, the sensitivity of the later stage to regulation by p28 depended on host strain. These data imply that the two bursts of apoptotic activity may be mediated by different pathways and associated regulatory elements. Alternatively, if the activity of p28 itself can be regulated by other proteins (either virus or host) during infection, it can be equally hypothesised that the activity of p28 itself was differentially altered at different stages of the infection. This supposes that p28 expression by virus occurs at sufficient and

equivalent levels during both phases of apoptosis to mediate the inhibition of apoptosis. Indeed, the more highly progressed the infection, the more likely that the tissue milieu contains host and viral proteins involved in modifying existent apoptotic signalling pathways.

The work described in Chapter 4, together with previous *in vitro* findings, suggests that the early phase of apoptosis in the liver is stimulated by rapid cytokine release from an unidentified source after infection. Interestingly, increases in the production of TNF, IFNγ, and IL-12 mRNA have been described within 6h at the site of infection in response to several other viruses, including adenovirus (Lieber *et al.*, 1997) and the alphavirus Venezualan equine encephalitis (Grieder *et al.*, 1997). Additionally, similar increases have been described in response to infection with intracellular bacteria such as *Listeria monocytogenes* (Ehlers *et al.*, 1992; Golovliov *et al.*, 1995; Iizawa *et al.*, 1992). In these studies, changes in cytokine mRNA levels were found at the earliest timepoint tested (30min: Iizawa *et al.* 1992; 3h p.i.: Lieber *et al.* 1997). These data suggest that the induction of the apoptotic response may be a general response to pathogens. It is likely that the route of infection and the pathogen additionally affect the early cytokine and apoptotic response (Orange *et al.*, 1997; Ruzek *et al.*, 1997; Ruzek *et al.*, 1999; Sprecher and Becker, 1992).

Perhaps one of the most intriguing discoveries during these studies was the demonstration that within hours of footpad infection, apoptosis was rapidly signalled to distal organs. This study is the first to show rapid systemic consequences of local events on virus infection. The increase in apoptosis is just one of many possible consequences of a transient increase in TNF. The many roles of TNF in the antiviral response (see Section 6.2.1) suggest that investigation of further possible consequences such as the upregulation of other inflammatory cytokines at remote sites would allow a greater insight into the immediate response to infection and the mechanisms by which the host response to infection is initially shaped.

These experiments only briefly examined the possib le functions of an early apoptotic response. The early phase of apoptosis did not correspond with the later apoptotic response, observable changes in leukocyte responses, or changes to pathology indicative of a more effective anti-viral response. However, if apoptosis in the liver is concomitant with apoptosis at the site of infection, two interesting functions that immediately present themselves are apoptosis as an effective antiviral response at the site of infection, and apoptosis of infected cells allowing rapid uptake of antigen through phagocytosis of apoptotic bodies by APCs. The former would be consistent with the low level of virus growth in A/J mice (Senkevich *et al.*, 1994, Chapter 3) and the observation that *ex vivo* macrophages from this strain support low levels of virus replication (Senkevich *et al.*, 1995). An anti-apoptotic role for poxviral cytokine-modifying proteins has previously been described in myxomavirus, in which the T2 gene product has separate extracellular anti-TNF activity, and intracellular anti-apoptotic activity (Schreiber *et al.*, 1997; Sedger and McFadden, 1996). The second possibility is suggested by studies in which it has been found that APCs are capable of presenting antigen obtained from the engulfment of apoptotic bodies (Albert *et al.*, 1997; Bellone *et al.*, 1998). Thus an early apoptotic response may allow very rapid presentation of viral antigen, and enhance the speed of the T cell response to infection.

The later phase of apoptosis is differentially regulated to the early stage, as indicated by the insensitivity of the later phase of apoptosis to lack of TNF (Chapter 5), and to p28 in all strains except B6 (Chapter 3). The low levels of apoptosis and high levels of virus in most strains during the later stage of infection suggests that a large hepatic apoptotic response is not required for EV replication. In addition, the low level of observable apoptosis during the clearance of EV from highly resistant mice show that high levels of apoptosis are also not required for EV clearance. At first glance, this appears to be in contradiction to previous findings, in which an important role for apoptosis in the clearance of EV – particularly apoptosis mediated by the immune system (Mullbacher *et al.*, 1999a) - has been implied. However, there are at least three alternative

interpretations of the data: (1) The apoptotic response may be of importance as a low level, targeted response. This interpretation is supported by work in which sections from the livers of LCMV-infected mice have been stained for perforin, CD8, NK1.1 and virus, showing a surprisingly low number of perforin positive cells in the organ (Muller *et al.*, 1989; Young *et al.*, 1989) consistent with the low levels of apoptosis detected in this thesis (Chapters 3 and 5). (2) The number of cells undergoing apoptosis is potentially larger than that observed. The number of cells labelled by TUNEL is only a proportion of the cells undergoing apoptosis. The process of apoptosis is rapid, with TUNEL and the necessary apoptotic phenotype only visible for a part of the total time in which cells are apoptotic. The rapid nature of apoptosis and uptake of apoptotic bodies further suggests that there may be substantial apoptosis occurring, with only a small proportion of the cells visibly apoptotic at any point in time. (3) Although the liver is a target of infection it is likely to be of interest to examine apoptosis in other organs; notably, the spleen and intestine. The latter is suggested by a number of highly susceptible mouse strains in which death was commonly associated with intestinal haemorrhage (Fenner *et al.* 1989; Chapter 3). This site potentially offers a useful future direction for studying apoptosis in EV infection.

6.2 The EV protein, p28

At the time these studies commenced, activity of p28 was known only from two closely related papers. In these, p28 was described as a strain-specific virulence factor required for growth of EV in macrophages from ANCR mice and for EV virulence in ANCR mice *in vivo* (Senkevich *et al.*, 1994, 1995). A third paper on the rabbitpox p28 homologue N1R described the gene as being capable of preventing vaccinia virus (VV)-mediated cell death in some cell lines (Brick *et al.*, 1998), a finding which has now been expanded to include UV-mediated cell death for both N1R and p28 (Brick *et al.*, 2000). Additionally, our laboratory had found that EV but not EVΔp28 could prevent TNF-mediated cell death in a mouse fibroblast line *in vitro* (J. Ruby, pers. comm.). Further

explorations of p28 activity were hampered by an inability to express functional p28 independent of virus (J. Ruby, D. Segal, S.C. [data not shown], and D. Smith) or detect p28 using previously prepared (Senkevich *et al.* 1994) or newly designed and created antibodies (S.C., data not shown). Indeed, despite the testing of multiple strategies for the production and detection of p28, production remains limited to insoluble protein in cell free or bacterial systems (D. Smith and J. Ruby, pers. comm.; Senkevich *et al.*, 1994), and p28 remains detectable only through RT-PCR detection of mRNA or biological activity.

The mechanism by which EV but not EVΔp28 may prevent TNF-mediated apoptosis has now started to be defined. Cell types such as the murine fibroblast line L929 are sensitive to cell death through CD40 and the p75 TNF receptor. In response to stimulation of either receptor, the cells upregulate TNF mRNA and subsequently apoptose (Turner *et al.* submitted). Apoptosis can be blocked by preventing signalling through the p55 TNF receptor pathway, consistent with the model of p75-mediated cell death in which p75 stimulation leads to transcriptional upregulation of TNF and subsequent autocrine stimulation of the p55 TNF receptor (Grell *et al.* 1999). On infection with EV, stimulation of p75 no longer results in an increase in TNF mRNA, and the cells become refractory to p75 TNF receptor-mediated cell death (Turner *et al.* submitted). In contrast, EVΔp28 infection does not prevent either TNF mRNA upregulation or cell death. The effect of p28 appears to be specific to TNF mRNA, as other cytokine and housekeeper mRNAs examined were not affected. However, it should be noted that chemokine mRNA was not examined (see Sections 6.2.2, 6.2.3).

6.2.1 p28 and TNF interaction

A fundamental question addressed in this thesis was whether p28 activity *in vivo* was equivalent to its activity *in vitro*. No direct evidence was found for p28 preventing TNF-mediated apoptosis of infected cells *in vivo*, suggesting instead that p28 may downregulate other consequences of TNF-mediated signalling. Data presented in

Chapter 4 show that p28 prevented TNF-dependent apoptosis *in vivo* at 6h p.i. However, it is extremely unlikely that this reflected directly antiapoptotic activity of p28, since the apoptosis occurred remote from the site of infection and was in greater quantities than would occur if only virally infected cells were undergoing apoptosis. Given the *in vitro* data, it is hypothesised that p28 prevents the early phase of apoptosis through impeding upregulation of TNF at the site of infection. The hypothesis implies the interruption of other TNF-mediated activities, including leukocyte activation, and leukocyte and APC chemotaxis (Dixit *et al.*, 1990; Green *et al.*, 1998) (see also Section 6.2.2). It is interesting to speculate what effects this may have on infection in the footpad. Both p75 and CD40 are involved in many stages of the antiviral response, including events from initial injection of the virus to infection of the LN (see Fig. 1.4). In particular, p75 has been shown to be of importance in the migration of dendritic cells from the site of infection to the lymph node (Wang *et al.*, 1997). CD40 is important in the subsequent maturation of the DCs and the provision of help to T cells within the lymph node (Grewal and Flavell, 1996). It is possible that p28 may interfere in either of these pathways. EV initially infects phagocytic cell types such as Langerhans cells and macrophages (Roberts, 1962). These, and keratinocytes, are major sources of TNF on cutaneous or dermal viral infection (Sprecher and Becker, 1992) or application of irritants (Wang *et al.*, 1997). TNF is then capable of stimulating chemotaxis of leukocytes towards the site of infection (Dixit *et al.*, 1990; Green *et al.*, 1998), as well as the maturation of DCs and their migration to the local LN (Cumberbatch *et al.*, 1994; Grewal and Flavell, 1996). By preventing any of these stages of chemotaxis, and concomitant cell maturation, it is possible that p28 may influence aspects of the earliest antiviral responses.

To test the hypothesis that p28 interrupts TNF production early in infection, semiquantitative cytokine mRNA assays could be performed in tissue from the footpad, popliteal LN, contralateral LN, liver and spleen of B6 mice, which displayed the strongest apoptotic response to infection to be clearly prevented by p28 expression.

Preliminary experiments to examine this have already been performed, although data so far has been equivocal (SC; data not shown).

No evidence was found for p28 interacting with TNF-dependent apoptosis later in the infection. This was possibly linked to the differential triggers for apoptosis active later in infection as compared to those active in the early phase (see Section 6.1).

6.2.2 CD40 and potential p28 activity

A second pathway through which EV p28 prevented transcription *in vitro* was that of CD40. CD40 has multiple activities in the antiviral response. In addition to being required for B cell activation (Calderhead *et al.*, 2000; Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994), CD40 is capable of activating NK cells (Carbone *et al.*, 1997; Martin-Fontecha *et al.*, 1999), and is also necessary for effective CD8+ T cell memory initiation (Borrow *et al.*, 1996; Borrow *et al.*, 1998), and APC function, particularly the production of IL-12 from DCs (Mosca *et al.*, 2000; Schulz *et al.*, 2000). CD40 and CD40L deficient mice have been found to have compromised immune responses as shown by a lack of B cell follicles (Calderhead *et al.*, 2000; Kawabe *et al.*, 1994; Renshaw *et al.*, 1994; Xu *et al.*, 1994) and ineffective antiviral responses to some but not all viral infections (Borrow *et al.*, 1996; Ruby *et al.*, 1995; Whitmire *et al.*, 1996).

Data in this thesis suggests that the interaction of p28 with CD40-mediated activities may be more important for EV virulence in EV-susceptible than EV-resistant mouse strains. This may reflect the multiple roles for CD40 in the antiviral response, and in particular the role of CD40 in upregulation of IL-12 during virus infection. IL-12 appears to be more important in the generation of effective cell-mediated immunity in Th2-biased mouse strains such as Balb/c than Th1-biased strains such as B6. This is illustrated by the requirement of Balb/c mice for IL-12 in order to generate an effective Th1-type antiviral response (Galbiati *et al.*, 2000). In contrast, B6 mice were found to generate Th-1 type T cells in both the presence and absence of IL-12. Thus one

mechanism by which p28 may be affecting EV-mediated mortality may be by manipulation of CD40, resulting in an ineffective Th2-type T cell response and subsequent death of the host.

Although effects of p28 on CD40 activity were most clearly evident in susceptible mice, it is possible that p28 also has effects on resistant mice which have not been assayed in the current system. Experiments in this thesis concentrated on events during the first 9 days of infection, and overall mortality of infection. CD40 function is also required for effective $CD8^+$ CTL memory and effective B cell responses (Borrow *et al.*, 1996; Whitmire *et al.*, 1996). Thus it is possible that p28 may play a role in resistant mice in the downregulation of long-term protection against future EV infection. Indeed, given that many wild populations of mice carry EV without overt pathogenesis (Fenner, 1996), it is possible that the long term effects of p28 expression may be of more importance in propagation of EV outside of laboratory conditions than the acute effects noted in the highly susceptible mouse strains.

6.2.3 p28 and immune function

Several pieces of evidence suggest interaction between p28 and elements of the immune system, which may or may not be linked with p28-TNF interactions. Firstly, liver histology in selected mouse strains (A/J and p75-/- mice; Chapters 3 and 5) showed different patterns of leukocyte infiltration and necrosis in response to EV or EVΔp28. In particular, EV-infected A/J mice displayed an unexpectedly disorganised, low level hepatic inflammatory infiltrate despite the high levels of virus present. In contrast, EVΔp28 infection led to the presence of inflammatory infiltrates and definite foci, as described in EV-resistant mouse strains. Secondly, several experiments showed a reduced impact of p28 expression in T cell deficient mice. In Chapter 3, p28 was shown to be a virulence factor in two immunocompetent EV-susceptible mouse strains (A/J and Balb/c) but not in the T cell deficient Swiss nude strain. The difference in the activity of p28 as a virulence factor may have been due to either the lack of T cells, or

the background genotype of Swiss nude mice. It was not possible to differentiate between these possibilities, as no background strain for Swiss nudes is available. However, quantitation of apoptosis in B6 and B6.β2m-/- mice (Chapter 3) allowed comparison of EV and EVΔp28 infection in wt or CD8+ T cell null mice. While EVΔp28 induced high levels of apoptosis in B6 mice, EV induced reduced levels of apoptosis. The difference between levels of apoptosis induced by EV or EVΔp28 infection was abrogated in mice lacking CD8+ T cells, suggesting that the effect of p28 on apoptosis later in infection may require a functional CD8 response.

It is possible that the interaction between p28 and cellular immunity may reflect interruption of TNF- or CD40-mediated events by p28. Examination of the inflammatory infiltrates in infected mice shows that in some mouse strains (p75-/- and A/J) significant differences occur between EV and EVΔp28-stimulated leukocyte infiltration into the liver. A role for TNF in NK cell recruitment to the liver has been previously described (Pilaro *et al.*, 1994). Additionally, TNF can stimulate production of a number of chemokines, including Mig, RANTES, MIP-1α, MIP-2 and JE (Ohmori *et al.*, 1997; Tessier *et al.*, 1997) and through these can also stimulate chemotaxis of other cell types (Tessier *et al.*, 1997). Thus it is very likely that p28 manipulation of cellular chemotaxis through TNF plays a role in the contribution of p28 to EV virulence.

An interaction of p28 with cellular immunity distinct from TNF is implied by the pathology of the p55-/-p75-/- mice in response to EV or EVΔp28 (Chapter 5). In p55-/-p75-/- mice, splenomegaly in response to EVΔp28 was similar to that induced by EV, the only mice in which this occurred. It was also associated with reduced splenic EVΔp28 replication. This finding may additionally indicate a cell-type dependent role for p28 in the absence of TNF. It is possible that this reflects a similar cell type-specific effect of p28 expression to that previously described in the macrophages of A/J mice (Senkevich et al. 1995). These data show that p28 has roles in manipulation of immune

function outside of TNF manipulation, and strongly suggests an effect on virulence through regulation of a TNF-independent mechanism.

A possibility that p28 may regulate signalling through other TNF family members was not tested in these experiments. This could occur through preventing TRAF-pathway stimulated transcription similar to the pathways used by CD40 and p75, or through preventing upregulation of the mRNA after CD40 or p75 activation. For example, OX40 is known to be important in development of an efficient immune response (Lane, 2000). In particular, the ligand for OX40 is upregulated in splenic DCs by CD40 ligation during the DC-CD4+ T cell interaction which leads to CD4+ T cell activation (Lane, 2000; Walker *et al.*, 2000). It is possible that p28 activity may interrupt upregulation of OX40L in a similar manner to that observed for TNF. While no definitive evidence for EV-mediated interruption of CD4+ T cell function has yet been found, the possibility cannot be discounted on the weight of current evidence.

6.2.4 p28 in wt mice

The experiments described in this thesis found that the aspects of virulence and pathogenesis affected by p28 expression varied between host strains (Chapter 3). The variation in strain-specific responses to p28 expression formed a spectrum, with A/J and B6 mice forming the extreme phenotypes. In A/J mice expression of p28 was required for production of high virus titres. In addition, a role for p28 in immune suppression was implied by the increased splenomegaly and numbers of hepatic inflammatory infiltrates during the later stages of EVΔp28 infection. In contrast, in B6 mice the apoptotic response to infection was sensitive to p28 expression although no significant effect was detected in virus virulence, pathogenesis or the inflammatory response. The more resistant 129 mice experienced only a minor effect of p28 expression on inflammation and pathogenesis which could be entirely accounted for by the slightly reduced level of EVΔp28 replication in comparison to EV.

Thus p28 activity resulted in different effects on EV virulence dependent on host strain. Interestingly, this result correlates with previous studies in which the importance of TNF and its receptors in the anti-viral or anti-pathogen response has been shown to differ between mouse strains. The differences can be clearly observed in a comparison of Balb/c mice with B6. Primary B6 CD4+ T cells require dendritic cells or splenocytes plus IL-12 for induction of a Th1 phenotype from naive cells *in vitro*, whereas those isolated from Balb/c mice additionally require concurrent treatment with IL-1α and TNF (Shibuya *et al.*, 1998). These data suggest that p28 interference with TNF levels early in infection could efficiently prevent a Th1-like response in Balb/c mice while having a minimal effect on the response of resistant mouse strains such as B6 or 129. Further, since Balb/c but not B6 IFNγ levels were also dependent on the provision of IL-1α and TNF (Shibuya *et al.*, 1998), it is possible that a similar mechanism could prevent effective anti-viral responses before the induction of a T cell response. This would be interesting to test through comparison of T cell responses in EV and EVΔp28 infected susceptible mouse strains.

This work underlines the need to examine the effects of virulence factor expression in a number of mouse strains. These differences in susceptibility of mouse strains to various infections have been widely recognised in other infections and often used to investigate the attributes that lead to resistance to particular virus infections. It is interesting that they are rarely utilised in investigation of virus attributes. Given that wild host populations consist of individuals with differing genotypes, it is possible that by addressing the effects of virus genes on pathogenesis in a number of mouse strains we may form a clearer and more accurate picture of how these genes may be of importance to pathogenesis outside of the laboratory.

6.2.5 Summary

The data in this thesis has concentrated on the possible *in vivo* ramifications of p28 activities which have been defined *in vitro*. In particular, data has examined regulation of TNF – related functions by p28. It is hypothesised that regulation of TNF-mediated transcriptional activity as described *in vitro* may result in some of the differences seen in immune responses to EV and EVΔp28. Interruption of TNF mediated cell death, also described *in vitro*, was not noted during the majority of the response, however it is possible that this plays a role at the site of infection. To investigate the contribution of apoptosis at this site, further investigation of the apoptotic response in the footpad or draining popliteal LN would be required.

Interruption of other forms of cell death, eg. virally induced and UV induced apoptosis have been shown by previous authors (Brick *et al.*, 1998, Brick *et al.*, 2000). The prevention of apoptosis in these systems did not occur through p75, since the experiments used HeLa cells which do not express the p75 TNF receptor. The experiments in this thesis confirm that p28 enhances EV virulence through TNF-independent mechanism(s), however, no evidence has been found implicating apoptosis independent of TNF.

In addition to the examination of p28 activity *in vivo*, this study has also mapped the hepatic response to EV in a number of mouse strains. Examination of apoptosis during the first 9d of virus infection has revealed a complex, multifactorial interaction between virus and host. Cytokines were found to be necessary for apoptosis early in infection, and may have directly triggered the apoptotic response at the site remote from infection. Later in the infection, apoptosis was present at unexpectedly low levels unless high levels of necrosis were present, suggesting that low levels of apoptosis as detected by TUNEL were sufficient for virus clearance.

These data have shown that p28 can have multiple effects *in vivo* which are not entirely accounted for by our current knowledge of p28 activity. In addition, examination of

apoptosis during the first 9d of virus infection has revealed a complex, multifactorial interaction between virus and host. Cytokines were found to be necessary for apoptosis early in infection, and may have directly triggered the apoptotic response at the site remote from infection. Later in the infection, apoptosis was present at unexpectedly low levels unless high levels of necrosis were present, suggesting that low levels of apoptosis as detected by TUNEL were sufficient for virus clearance.

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