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# Lessons in Détente or know thy host: The immunomodulatory gene products of myxoma virus

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The poxvirus, myxoma virus, encodes within its genome at least eleven different proteins that compromise, skew, or disable the innate and adaptive responses of its hosts. In the laboratory rabbit, *Oryctolagus cuniculus*, these effects result in myxomatosis, a fatal condition characterized by skin lesions and systemic immunosuppression. Interestingly, while myxoma infection also causes skin lesions in its natural host and in natural populations of *O. cuniculus* in Australia where this novel host and the virus have co-evolved, the condition of myxomatosis does not ensue and infection is not fatal. In this review I discuss the biochemical properties of the characterized immunomodulatory proteins of myxoma virus, and their pathogenic effects in laboratory rabbits. Disruption of any one myxoma immunomodulatory gene diminishes the severity of the infection without compromising infectivity. Thus, the characterized immunomodulatory genes appear not to be required for a productive infection *in vivo*. The differences in the severity of their effects in laboratory-bred versus wild *O. cuniculus* suggest that the outcome of myxoma infection is a consequence of the interplay between the viral immunomodulatory gene products and the cells and molecules of the host immune system.

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## 1. Introduction

In the past several years it has become clear that most viruses encode gene products that are essential for productive replication in immunocompetent hosts (Bugert and Darai 2000; McFadden and Murphy 2000). Some of these immunomodulatory viral gene products skew the type of immune response elicited, resulting in the evasion of host immune responses (at least temporarily), with or without concomitant pathogenesis. This is especially evident in the poxviruses, large DNA viruses that replicate relatively autonomously in the cytoplasm of infected cells (Cudmore *et al* 1997; Grosenbach and Hurby 1998; Sodeik and Krijnse-Locker 2002). One of the best studied

models of host-poxviral interactions in infection and disease is the case of myxoma virus infection of the laboratory rabbit, *Oryctolagus cuniculus*.

Myxoma virus infection in its natural host, *Sylvilogus brasiliensis*, is relatively benign. By contrast, in the European rabbit, *O. cuniculus*, which is commonly used in the laboratory, myxoma virus causes myxomatosis, a usually fatal disease characterized by fulminating lesions and immunosuppression (Fenner and Ratcliffe 1965). The severity of the disease in laboratory-bred *O. cuniculus* is almost certainly due to lack of opportunity in this setting for virus and host to adapt to each other. In contrast, in Australia, where myxoma virus was introduced some fifty years ago to control wild populations of *O. cuniculus*

**Keywords.** Gene products; myxoma virus; *Oryctolagus cuniculus*; poxvirus; skin lesions

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Abbreviations used: AIF, Apoptosis-inducing factor; CTL, cytotoxic T lymphocyte; EGF, epidermal growth factor; EMP2, epithelial membrane protein-2; ER, endoplasmic reticulum; ICE, interleukin-1 $\beta$ -converting enzyme; IFN- $\alpha/\beta$ , interferon- $\alpha/\beta$ ; MGF, myxoma growth factor; ORF, open reading frame; PTP1B, protein tyrosine phosphatase-1B, SFGF, Shope fibroma growth factor.

lus, myxoma virus does not cause myxomatosis (Fenner and Ratcliffe 1965). Moreover, myxoma virus isolates from wild Australian *O. cuniculus* do not cause myxomatosis in laboratory-raised *O. cuniculus* (Best *et al* 2000; Best and Kerr 2000), indicating that in Australia avirulence has been selected through co-evolution of host and virus (reviewed in Zúñiga 2002). A second important point to bear in mind when considering the pathology of myxoma infection in laboratory rabbits is that skin lesions at the site of infection are characteristic of myxoma infection both in wild *O. cuniculus* in Australia and in its South American host, *S. brasiliensis*. These primary skin lesions are the source of infectious virus, and in the wild they persist over winter months and provide infectious virus for mosquito vectors that emerge in the spring (Best *et al* 2000; Best and Kerr 2000). However, secondary lesions, arising when virus is transmitted from primary lesions to distal sites, are uniquely characteristic of myxomatosis. Hence, virulence of myxoma in laboratory-bred *O. cuniculus* results from a failure to restrict myxoma virus to primary sites of infection. With these different consequences of myxoma infection of *S. brasiliensis* and *O. cuniculus* in mind, I discuss in this review the identified immunomodulatory gene products of myxoma virus, their biochemical properties, and their effects in cells grown in culture and in laboratory-reared *O. cuniculus*. Several of the immunomodulatory gene products of myxoma virus have homologues in other poxviruses. However, myxoma is the only poxvirus for which there has been a systematic study of the effects of viral gene products both *in vitro* and *in vivo*. For this reason, this review will focus on the gene products of myxoma virus, with brief mention of other poxviral orthologues for which information is available.

## 2. Myxoma virus infection and the inflammatory response at cutaneous sites

Replication of myxoma virus occurs initially in class II MHC<sup>+</sup> cells having a dendritic morphology and then in T lymphocytes (figure 1) in the paracortical region of the draining lymph node (Best *et al* 2000). The receptor(s) by which myxoma virus gains entry into cells are not identified. However, NIH 3T3 cells transfected with genes encoding human CCR1, CCR5, or CXCR4 chemokine receptors are fully permissive for myxoma virus infection (Lalani *et al* 1999b). Moreover, adsorption of myxoma virus to NIH 3T3 cells transfected with the CD4 and CCR5 genes is rapidly followed by tyrosine phosphorylation of CCR5 protein, the association of CCR5 with p56<sup>lck</sup> and Jaks, and subsequent Jak phosphorylation. These effects are observed even with UV-inactivated myxoma virus (Masters *et al* 2001).

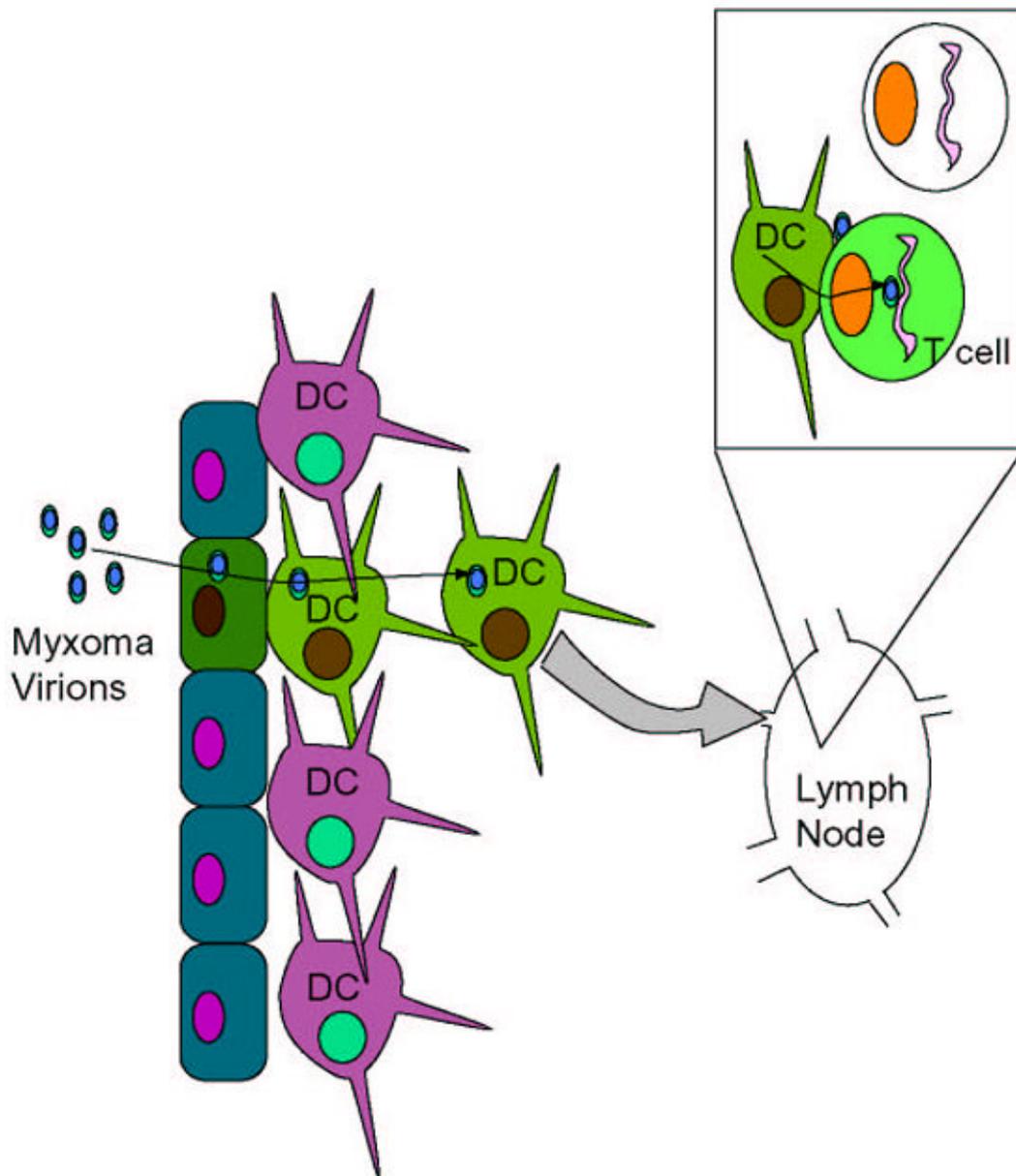
Chemokines (chemotactic cytokines) are small proteins that selectively control the activation, chemotaxis, and adhesion of many types of leukocytes (Yoshie *et al* 2001). Different sets of chemokine receptors are expressed by various leukocytes, endothelial cells, and a variety of non-hematopoietic cells. Hence, leukocyte trafficking, angiogenesis, and inflammation all are regulated by chemokines (Mahalingam and Karupiah 2000; Murdoch and Finn 2000; Yoshie *et al* 2001). The exact rabbit chemokine receptors that facilitate myxoma infection and their mechanisms of action *in vivo* are yet to be identified, but the use of chemokine receptors in myxoma infection may enable the virus to influence the type of immune and inflammatory responses that ensue. For example, *in vivo* studies on virulent and natural isolates of attenuated myxoma virus showed that predominantly granulocytes infiltrate the sites of infection in rabbits that succumb to myxomatosis, while chiefly macrophages and monocytes occur in lesions of rabbits that resolve infection (Best *et al* 2000). Macrophages, but not granulocytes, can engulf apoptotic cells and senescent neutrophils at sites of inflammation (Cox *et al* 1995; Ren and Savill 1995). Ingestion of senescent neutrophils protects tissues from leakage of noxious products and enhances antigen presentation to T-cells by macrophages, thus enabling eventual elimination of virally infected cells.

## 3. Apoptosis: a hallmark of myxoma infection

Most mammalian cell types respond rapidly to viral infection by secreting the antiviral cytokines interferon  $\alpha/\beta$  (IFN- $\alpha/\beta$ ), which then activate multiple antiviral pathways (reviewed in Barber 2001). Apoptosis of infected cells is an early and important mechanism for limiting viral infections. Lymphocyte apoptosis is a cardinal feature of myxoma infection, regardless of the development of myxomatosis (Best *et al* 2000). Within 24 h post-infection, myxoma virus is found in the draining lymph node, and by 4 days post-infection marked apoptosis is evident in the paracortex and germinal centers (Best *et al* 2000; Best and Kerr 2000). Interestingly, the apoptotic cells do not contain detectable virus antigen, but rather are adjacent to virally infected cells (Best *et al* 2000), suggesting that virally infected cells induce apoptosis of neighbouring cells. However, as discussed later, virally infected cells are protected from apoptosis by at least four viral proteins.

## 4. Immunomodulatory gene products of myxoma virus and their effects in laboratory-bred *O. cuniculus*

In the past several years there has been considerable progress in the identification and characterization of myxoma



**Figure 1.** Initial steps of myxoma virus infection *in vivo*. Replication of myxoma virus is initially in class II MHC<sup>+</sup> cells of the skin having a dendritic morphology (labelled DC). Subsequently myxoma virus replicates in T lymphocytes (labelled T-cell) in the paracortical region of the draining lymph node. The transmission of myxoma virus from a dendritic cell to a T-cells is shown in the enlarged segment of the lymph node. In laboratory rabbits these T lymphocytes are believed to disseminate virus to distal sites, thereby leading to secondary lesions and the development of myxomatosis. Myxoma virions are blue. Virally infected cells are green.

virus immunomodulatory molecules. To date, eleven myxoma virus gene products have been shown to have effects on the host immune system (table 1). These include M-T1, a chemokine binding protein, M-T2, a soluble homologue of the TNF receptor, M-T7, a soluble IFN- $\gamma$  receptor homologue which also binds chemokine

receptors, myxoma growth factor (MGF), a member of the epidermal growth factor (EGF) family, M-T4, M-T5, and M11L, all of which modulate apoptosis of host cells, three serpins (SERP1, SERP2, and SERP3), and MV-LAP, a protein which down-regulates cell-surface class I MHC. Six of these viral genes, the *M-T1*, *M-T2*, *M-T4*,

*M-T5*, *M-T7*, and *SERP1* genes, are diploid because they map within the 11.5 kb terminal inverted repeat in the myxoma virus genome (Cameron *et al* 1999). McFadden and his colleagues have noted that the myxoma genome contains other sequences bearing homology to known immunomodulatory proteins (Barrett *et al* 2001). These will not be discussed here.

### 5. Myxoma gene products that interfere with cytokine or chemokine function: M-T1

M-T1 is a secreted 43 kDa glycoprotein which is produced continuously during the course of myxoma infection. M-T1 binds with high affinity to a broad range of CC-chemokines and inhibits their chemoattractant activities *in vitro* (Graham *et al* 1997; Lalani *et al* 1998). The M-T1 protein is not required for a productive infection in laboratory rabbits. The localized cellular infiltrate in primary infection sites was heightened during the first few days of infection in rabbits infected with M-T1 knock-out (M-T1<sup>-</sup>) myxoma virus, as compared to lesions of rabbits infected with unaltered myxoma virus (Lalani *et al* 1999a). The cellular infiltrate at sites of infection by the M-T1<sup>-</sup> myxoma virus consisted chiefly of monocytes and macrophages. Five of six rabbits infected with the M-T1<sup>-</sup> myxoma virus succumbed to myxomatosis. The sixth rabbit began to resolve infection within 12 days and completely recovered. While binding of M-T1 protein chemokines is believed to block leukocyte activation, there is considerable redundancy amongst chemokines, and the chemoki-

nes that attract granulocytes and those that attract macrophages and monocytes in the rabbit are yet to be identified. Hence, the role of the M-T1 protein in myxomatosis remains unclear.

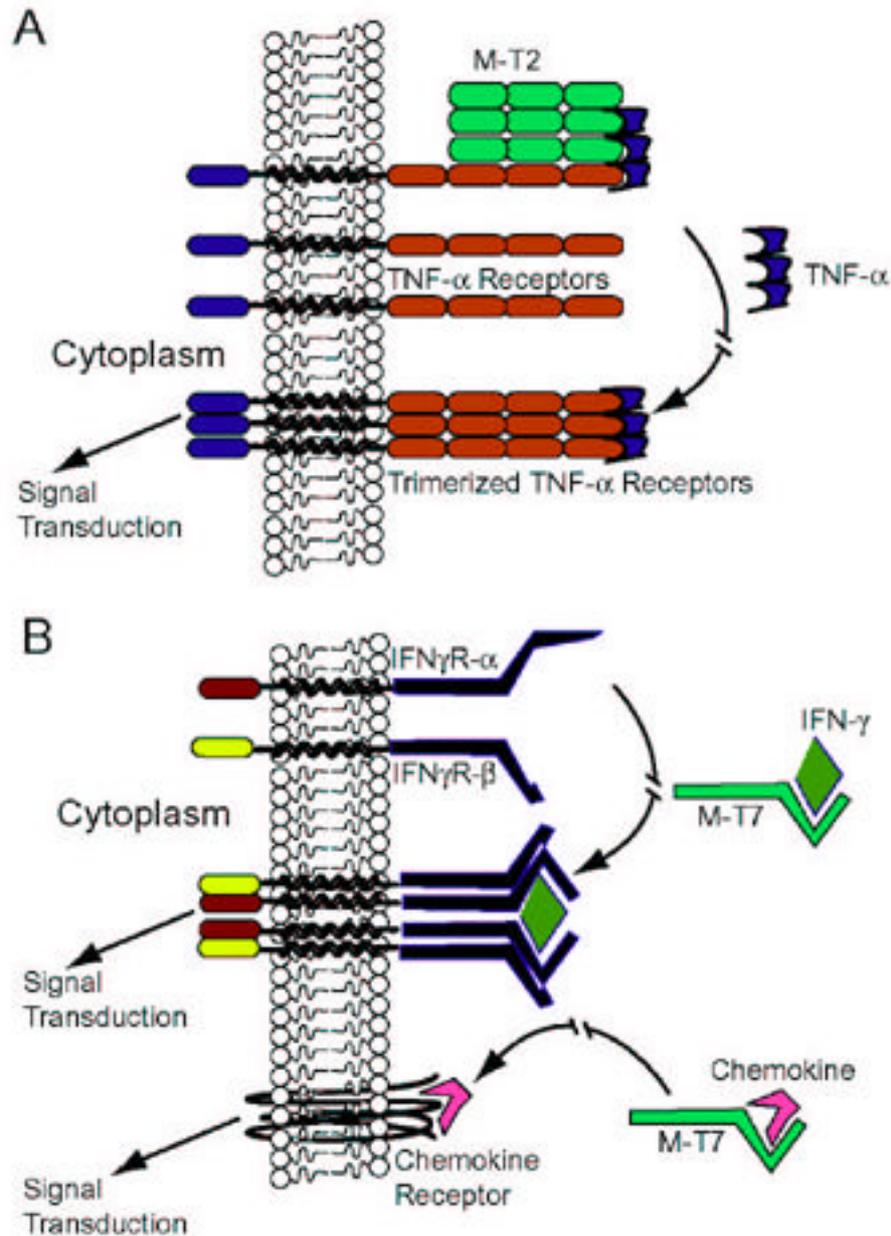
### 6. MT-2: a soluble TNF receptor

The 52–56 kDa M-T2 protein is an early product of myxoma virus-infected cells. M-T2 has significant sequence similarity to the N-terminal ligand-binding domains of cellular TNF receptors, and contains three N-terminal cysteine-rich domains which are required for TNF- $\alpha$  binding. M-T2 is species-specific in that it binds rabbit TNF- $\alpha$  but not human or murine TNF- $\alpha$  (Upton *et al* 1991). Both monomeric and dimeric forms of M-T2 are secreted by virally infected cells and have similar affinity for rabbit TNF- $\alpha$  ( $K_d = 170$  and  $195$  pmol, respectively). The dimer form is a more potent inhibitor of TNF- $\alpha$ , presumably because it more effectively inhibits receptor oligomerization (Schreiber *et al* 1996), thereby abolishing signal transduction through the cellular TNF receptors (figure 2).

Cellular TNF- $\alpha$  exhibits diverse regulatory activities (Baud and Karin 2001). Its potent pro-inflammatory activity regulates innate immunity. Moreover, TNF- $\alpha$  is involved in the regulation of cell differentiation, proliferation and apoptosis. Rabbit RL-5 T lymphocyte cells infected with an M-T2 knock-out myxoma virus undergo apoptosis, resulting in an aborted infection (Macen *et al* 1996). These results suggest that the binding of TNF- $\alpha$

**Table 1.** Myxoma immunomodulatory gene products.

Gene name	Copy No.	Proposed gene product function and/or properties	Required for infection <i>in vivo</i>
M-T1	2	C-C chemokine binding protein; inhibits chemokine chemoattractant properties <i>in vitro</i>	No
M-T2	2	Soluble TNF- $\alpha$ receptor; binds rabbit TNF- $\alpha$	No
M-T7	2	Soluble IFN- $\gamma$ receptor; also binds C-C and C-X-C chemokines <i>in vitro</i>	No
MGF	1	Member of EGF family; epithelial cell mitogen	No
M-T4	2	ER-resident anti-apoptotic protein; also appears to retard class I MHC egress from ER	No, but required for replication in T lymphocytes <i>in vivo</i>
M-T5	2	Ankyrin repeat-containing protein; required for progression to late gene expression in RL-5 cells in culture	No, but required for replication <i>in vivo</i>
M11L	1	Mitochondria-resident protein; prevents mitochondrial permeability transition and subsequent induction of apoptosis cascade	No, but required for replication in monocytes <i>ex vivo</i> in absence of mitogen
SERP1	2	Serine protease inhibitor; inhibits plasmin urokinase, tissue plasminogen activator, and C1s complement protein <i>in vitro</i>	No
SERP2	1	Serine protease inhibitor; substrates unknown	No
SERP3	1	Putative serine protease inhibitor; substrates unknown	No
MV-LAP	1	ER-resident protein with zinc finger motif; promotes loss of cell-surface class I MHC, perhaps by preventing recycling to cell surface	No



**Figure 2.** Abrogation of signal transduction via soluble TNF receptors, IFN- $\gamma$  receptors, and chemokine receptors of myxoma virus. **(A)** Cellular TNF- $\alpha$  receptors trimerize upon the binding of TNF- $\alpha$ . Trimerization is essential for activation of the downstream signal transduction cascade mediated by cytosolic proteins, which associate with the TNF receptor cytoplasmic domains. One consequence of signalling through the trimerized TNF- $\alpha$  receptors is induction of apoptosis via the death domains of the receptor chains. Myxoma virus M-T2 (coloured green) binds TNF- $\alpha$  via its three N-terminal cysteine-rich domains, thereby abolishing signal transduction through cellular TNF receptors. One effect of M-T2 is the prevention of T lymphocyte apoptosis. **(B)** The IFN- $\gamma$  receptor  $\alpha$  and  $\beta$  chains associate upon binding of IFN- $\gamma$ . Because IFN- $\gamma$  (green diamond) also is a dimer, ligand binding results in the formation of an IFN- $\gamma$  tetramer consisting of two  $\alpha$  chains and two  $\beta$  chains. Oligomerization of the IFN- $\gamma$  receptor initiates cell activation via signal transduction through the Jak-Stat pathway (not shown). The myxoma M-T7 protein binds to IFN- $\gamma$ , and blocks its biological activity, presumably by serving as a competitive inhibitor of the cellular IFN- $\gamma$  receptor. M-T7 also binds to cellular chemokines (coloured pink) and prevents their binding to chemokine receptors, which are transmembrane proteins with seven membrane spanning regions. As discussed in the text, signalling through chemokine receptors leads to leukocyte activation, chemotaxis, and adhesion.

by M-T2 blocks TNF- $\alpha$ -mediated apoptosis of infected T-cells. Curiously, a truncated M-T2 protein consisting of only the first two cysteine-rich domains also prevents apoptosis of infected cells, even though it is predicted to be retained intracellularly (Schreiber *et al* 1997). It has been suggested that truncated M-T2 associates with intracellular domains of TNF receptors (Schreiber *et al* 1997). It is perhaps more likely that the truncated M-T2 associates with TNF receptors during their biosynthesis in the ER, thereby preventing the transport of TNF receptors to the cell-surface. For example, when synthesized by CD4<sup>+</sup> cells, HIV-1 gp120 associates with CD4 in the endoplasmic reticulum (ER) and blocks its transport to the cell surface (Buonocore and Rose 1990; Crise *et al* 1990).

M-T2 is not required for productive infection in laboratory-bred *O. cuniculus*. However, disease is markedly attenuated, and primary and secondary lesions are less extensive than those in rabbits infected with the parental Lausanne strain (Upton *et al* 1991). Five of eight rabbits infected with M-T2<sup>-</sup> myxoma virus recovered completely and were immune to subsequent infection with the parental Lausanne myxoma virus (Upton *et al* 1991). In this regard it is interesting to note that a myxoma virus strain isolated from the field in Australia which is attenuated in virulence has been found to bear mutations within and 5' to the M-T2 gene (Saint *et al* 2001).

How do the *in vivo* findings correlate with M-T2's inhibition of apoptosis of T-cell lines in culture? As noted above, TNF- $\alpha$  is a potent pro-inflammatory cytokine that has diverse effects on macrophages and neutrophils. For example, phagocytosis of apoptotic neutrophils by macrophages is potentiated by TNF- $\alpha$  (Ren and Savill 1995). Hence, M-T2's function as a virulence factor *in vivo* is unlikely to be due solely to inhibition of apoptosis. A detailed histological examination of infection sites and a study of cytokine profiles in rabbits infected with the M-T2<sup>-</sup> virus may provide insights into the role of the cellular infiltrate in the pathology of myxomatosis.

### 7. M-T7 protein: a soluble IFN- $\gamma$ receptor and chemokine receptor-binding protein

M-T7 is a secreted, viral homologue of cellular IFN- $\gamma$  receptor and inhibits the biological activity of rabbit IFN- $\gamma$  (Upton *et al* 1992). Curiously, M-T7 also binds a wide range of C-C, and C-X-C chemokines from various species through its heparin-binding domain and prevents chemokine binding to proteoglycan on endothelial cell surfaces (Lalani *et al* 1997). These properties suggest that *in vivo* M-T7 may impair leukocyte trafficking. Consistent with this proposal, a vigorous cellular reactivity was observed in secondary lymphoid organs of laboratory

rabbits infected with M-T7<sup>-</sup> myxoma virus, and not in rabbits infected with the parental virus (Mossman *et al* 1996b). Additionally, substantial numbers of lymphocytes in germinal centers of lymph nodes and spleens of rabbits infected with M-T7<sup>-</sup> myxoma virus appear to be activated (Mossman *et al* 1996b). Collectively, these observations suggest that M-T7 disrupts lymphocyte activation in secondary lymphoid organs by sequestering IFN- $\gamma$  or blocking its activity (figure 2). Indeed, M-T7, while not required for productive infection of laboratory rabbits, is required for myxomatosis. It would be interesting to know if the IFN- $\gamma$  and chemokine-binding activities of M-T7 can be abolished independently. If so, then *in vivo* studies with myxoma virus bearing mutant M-T7 that binds only IFN- $\gamma$ -binding activity or only chemokine binding activity would determine the relative importance of these functions for virulence in laboratory rabbits.

## 8. Myxoma gene products with known effects on T lymphocytes

### 8.1 M-T4

The M-T4 gene product of myxoma virus localizes to the ER of infected cells. Infection of the RL-5 T lymphocyte cell line with the M-T4<sup>-</sup> myxoma virus caused apoptosis and hence an aborted infection (Barry *et al* 1997). M-T4 may inhibit apoptosis by interfering with the function of BAP31 (figure 3 and Hnatiuk *et al* 1999), an ER-resident integral membrane protein that forms a complex with Bcl-2/Bcl-XL and procaspase-8 (Ng *et al* 1997). Cleavage of BAP31 at sites in its cytosolic domain by caspases generates a BAP31 fragment that remains in the membrane and which, when expressed ectopically, is a potent inducer of apoptosis (Ng and Shore 1998; Nguyen *et al* 2000). Additionally, BAP31, in which the caspase cleavage site has been altered to render it resistant to caspase cleavage inhibits Fas-mediated apoptotic membrane fragmentation and mitochondrial release of cytochrome *c* (Nguyen *et al* 2000).

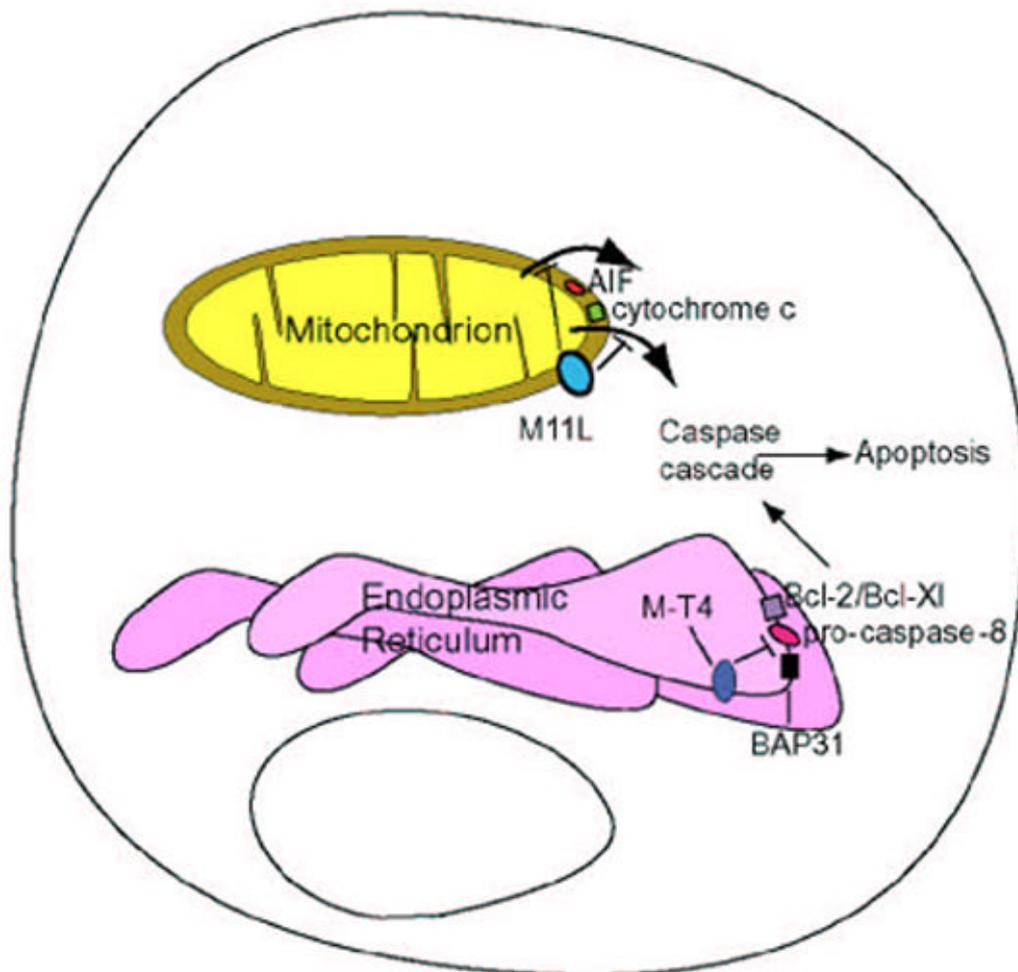
M-T4 has an ER-retention motif (RDEL). However, M-T4 lacking this motif nevertheless localized to the ER (Hnatiuk *et al* 1999). The RDEL<sup>-</sup> M-T4 was weakly anti-apoptotic in fibroblasts infected with myxoma virus bearing the RDEL<sup>-</sup> M-T4 gene (Hnatiuk *et al* 1999). It is unclear why removal of the RDEL motif diminishes M-T4's anti-apoptotic effect. One interesting possibility is that the RDEL motif may be essential for the localization of M-T4 in a microdomain of the ER enriched for pro-apoptotic molecules or for its association with such molecules.

Pulse chase studies in cells infected with unaltered myxoma virus or with the M-T4<sup>-</sup> variant showed that M-

T4 retards class I MHC egress from the ER (Zúñiga 2001). This effect also is observed in fibroblast cell lines and thus is not a T-cell-specific property (Zúñiga 2001). Co-immunoprecipitation studies indicate that M-T4 binds to class I MHC molecules (Zúñiga 2001). These results are intriguing, because in addition to having a role in regulating apoptosis, BAP31 appears to regulate egress of class I MHC molecules and other proteins from the ER (Annaert *et al* 1997; Spiliotis *et al* 2000). BAP31 co-immunoprecipitates with class I MHC molecules and has been suggested to recruit newly assembled proteins into

transport vesicles (Spiliotis *et al* 2000, figure 4). It will be interesting to determine if M-T4 association with class I MHC prevents class I MHC binding to BAP31 or its recruitment into cargo vesicles leaving the ER (figure 4).

M-T4 is a virulence factor that is required for myxomatosis but not for viral infectivity. The lesions on laboratory rabbits infected with M-T4<sup>-</sup> myxoma virus began to heal within 9 days, and the infected rabbits recovered fully within 10–21 days post-infection (Barry *et al* 1997; Hnatiuk *et al* 1999). Perhaps myxomatosis does not develop in rabbits infected with M-T4<sup>-</sup> myxoma virus because



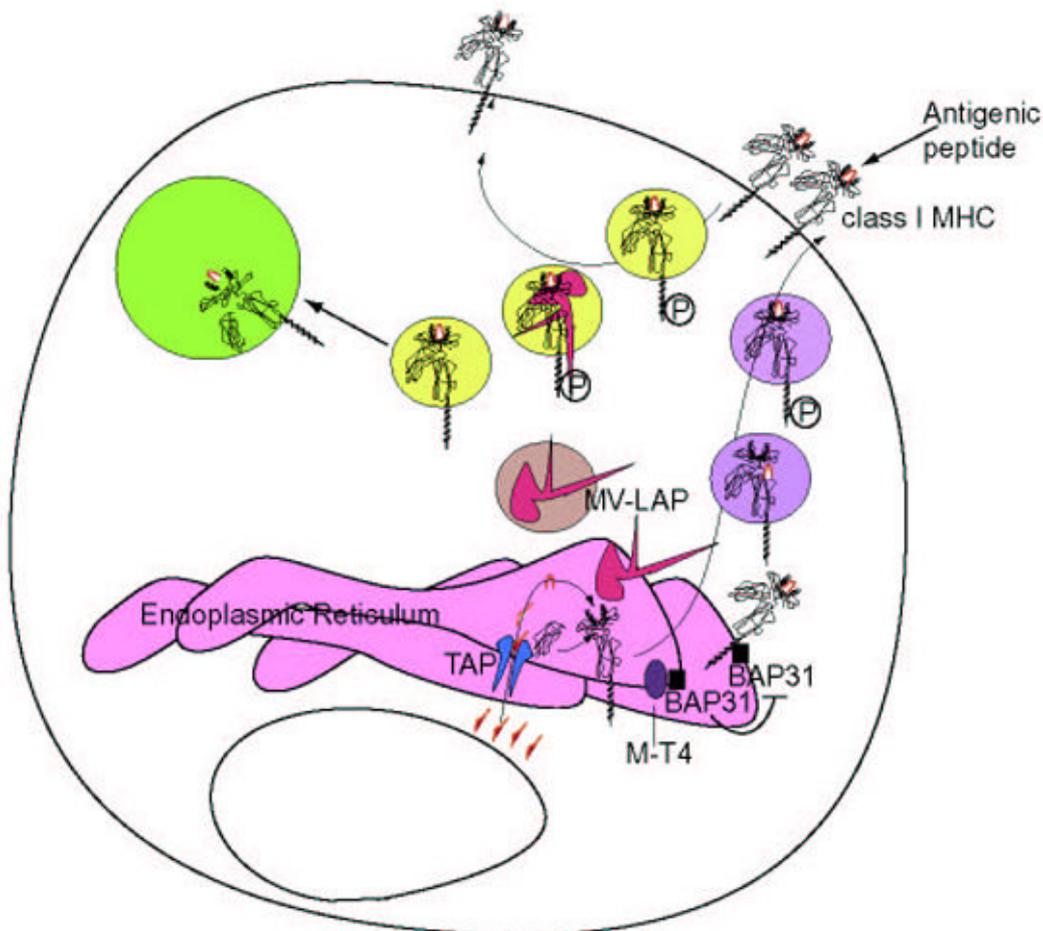
**Figure 3.** Proposed anti-apoptotic roles of myxoma M11L and M-T4 immunomodulatory proteins. The myxoma virus M11L protein is targeted to mitochondrial membranes where it prevents the mitochondrial membrane from undergoing the permeability transition, an obligatory step for release of cytochrome *c* and apoptosis-inducing factor (AIF). This blocks progression through the apoptotic cascade. Another intracellular apoptotic cascade involves protein interactions at the endoplasmic reticulum (ER) membrane. Here the ER-resident protein, BAP31, forms a complex with Bcl-2/Bcl-XL and pro-caspase 8. Cleavage of BAP31 at a caspase recognition motif generates a fragment of BAP31 that remains resident in the ER membrane and induces apoptosis. In myxoma virus-infected T-cells the ER-resident viral M-T4 protein is targeted to the ER membrane where it is proposed to inhibit this apoptotic cascade, perhaps by interfering with the assembly of the pro-apoptotic complex and subsequent cleavage of BAP31.

in the absence of M-T4, infected T-lymphocytes apoptose and hence are unavailable to carry myxoma virus to distal lymph nodes. However, as noted above, M-T4 inhibits class I MHC transport. Myxoma virus also causes the loss of CD4 from the surfaces of the RL-5 T-cell line grown in culture (Barry *et al* 1995). If these effects prevail *in vivo*, then T-cell-mediated immunity may be diminished in rabbits infected with M-T4<sup>+</sup> myxoma virus. Laboratory rabbits infected with myxoma virus bearing a RDEL-M-T4 exhibited an exaggerated inflammatory response at secondary sites of infection (Hnatiuk *et al*

1999). However, the infiltrating inflammatory cells were not identified. More detailed studies with wild type and mutant M-T4 may elucidate the functions of M-T4 and the functional significance of the RDEL motif *in vivo*.

## 8.2 M-T5

M-T5 is a 483 amino acid-long ankyrin repeat-containing protein (Mossman *et al* 1996a). The ankyrin repeat motif mediates protein-protein interactions and is found in a



**Figure 4.** Proposed mechanisms for down-regulation of cell-surface class I MHC by myxoma viral proteins M-T4 and MV-LAP. M-T4 protein associates with endoplasmic reticulum (ER)-resident class I MHC molecules and prevents their egress from the ER. M-T4 may also bind to BAP31, which has been proposed to facilitate egress of certain proteins from the ER. Individually or collectively, these interactions may abrogate the recruitment of class I MHC molecules into cargo vesicles that carry newly synthesized proteins from the ER to the Golgi. Retained class I MHC molecules are degraded by proteasomes (not shown). The myxoma virus MV-LAP protein also localizes to the ER, but its effects appear to be on the cell-surface population of class I MHC molecules. MV-LAP promotes the loss of cell-surface class I MHC. In the model proposed here MV-LAP intercepts endocytosed class I MHC in a 'dephosphorylation compartment' (coloured yellow) where it mediates dephosphorylation of phosphoserines in the class I MHC cytoplasmic tail. Dephosphorylated class I MHC may then be targeted to the lysosome (coloured green) where it is degraded. Continued endocytosis of class I MHC from the cell surface in the absence of recycling back to the plasma membrane is proposed to lead to a loss of cell-surface class I MHC. Another model for MV-LAP function, not illustrated here, invokes its action on cellular proteins that regulate cell-surface levels of class I MHC, as discussed in the text.

diverse array of protein families, including transcription factors, cytoskeletal proteins, proteins which regulate development, and toxins (Andrade *et al* 2001). In RL-5 T-cells infected with M-T5<sup>-</sup> myxoma virus viral replication is terminated prior to late gene expression. It remains unclear why M-T5 is required for productive infection of T-cells since it is not required for productive infection *in vivo*. However, M-T5 is required for myxomatosis (Mossman *et al* 1996a). One interpretation of all of these results is that M-T5 is required for productive infection of T lymphocytes in draining lymph nodes and that virally infected T lymphocytes transport the virus to distal sites at which secondary lesions normally occur during myxomatosis.

### 9. The M11L and MGF (M10L) virulence gene products

MGF is a secreted glycoprotein, having sequence homology to members of the EGF family of proteins. TGF- $\alpha$ , Shope fibroma growth factor (SFGF), and the vaccinia growth factor all can replace MGF in the induction of myxomatosis in rabbits, despite the fact that MGF has less than 40% sequence homology with TGF- $\alpha$  and 80% sequence identity with SFGF (Opgenorth *et al* 1993). The target cells for mitogenic stimulation by MGF, SFGF, and TGF- $\alpha$  are identical, including the epithelial cell layers overlying myxoma tumours in the conjunctiva and respiratory tract (Opgenorth *et al* 1993). MGF is not required for viral replication in unstimulated or mitogen stimulated splenic cultures nor for productive infection *in vivo* (Opgenorth *et al* 1992). Only 25% of the rabbits infected with MGF<sup>-</sup> myxoma virus became moribund. The remaining rabbits recovered completely and were immune to subsequent challenge with virulent myxoma virus (Opgenorth *et al* 1992).

The M11L virulence factor was discovered by McFadden and his colleagues in their studies of MGF. The carboxyl terminus of an upstream open reading frame (ORF), designated M11L, which is predicted to encode a 166-residue polypeptide, partially overlaps with the MGF ORF. The M11L protein is targeted to mitochondria by a short carboxyl terminal motif (Everett *et al* 2000). This subcellular localization of M11L is interesting because mitochondria play a central role in the commitment phase of apoptosis. Caspases are not activated until mitochondrial proteins, particularly cytochrome *c* and apoptosis-inducing factor (AIF), are released into the cytosol (see figure 3; Kroemer and Reed 2000; Boya *et al* 2001). Evidence for an anti-apoptotic role of M11L comes from studies showing that M11L blocked staurosporine-induced apoptosis of cultured cells (Everett *et al* 2000) by preventing the mitochondrial membrane from under-

going the permeability transition that is requisite for release of cytochrome *c* and AIF (Everett and McFadden 2001). Localization of M11L to mitochondria was required for this activity. Furthermore, at 12 h post-infection three times as many rabbit peripheral blood monocytes infected with M11L<sup>-</sup> myxoma virus *ex vivo* underwent apoptosis, as compared to the number of apoptotic monocytes in cultures infected with unaltered myxoma virus (Everett *et al* 2000).

In rabbits infected with M11L<sup>-</sup> virus primary and secondary lesions regressed within 17–21 days, and all infected rabbits recovered completely by day 40 post-infection and were immune to subsequent challenge with virulent myxoma virus. Significantly, lesions in rabbits infected with the M11L<sup>-</sup> virus had a robust inflammatory cell infiltrate (Opgenorth *et al* 1992). Of perhaps some relevance to these results is the observation that M11L<sup>-</sup> virus only replicated in mitogen-stimulated spleen cells and with a ten-fold lower viral yield (Opgenorth *et al* 1992). This observation is puzzling in view of the fact that mitogen activation up-regulates the expression of pro-apoptotic proteins such as Fas (Yoshino *et al* 1994). Identification of the molecular partners of M11L may resolve this apparent paradox.

Rabbits infected with the double mutant MGF<sup>-</sup> M11L<sup>-</sup> myxoma virus did not develop myxomatosis. Indeed, only primary lesions developed and infected rabbits completely recovered by day 14 post-infection and were immune to subsequent challenge with virulent myxoma virus (Opgenorth *et al* 1992). Infections with the M11L<sup>-</sup> and MGF<sup>-</sup> M11L<sup>-</sup> viruses were observed to be largely similar, suggesting that the M11L gene, but not the MGF gene, is required for myxoma virus replication in lymphocytes *in vivo*, and hence for dissemination to secondary sites.

### 10. Myxoma virus-encoded serine protease inhibitors

#### 10.1 SERP1

Serpins are irreversible inhibitors of serine proteases that regulate myriad physiological processes, including apoptosis, inflammation, angiogenesis, complement activation, fibrinolysis, and coagulation (Potempa *et al* 1994). The 55 kDa myxoma virus serpin 1 (SERP1) is a secreted glycoprotein (Upton *et al* 1990). *In vitro* it inhibits plasmin, urokinase, tissue plasminogen activator, and the C1s complement protein, which initiates the classical pathway of complement (Lomas *et al* 1993). These inhibitory effects are interesting in light of the roles of these serine proteases in immunity. Plasma proteinases are chemotactic for inflammatory cells *in vivo*. The comple-

ment system enhances the inflammatory response and promotes lysis of virally infected cells (Frank and Fries 1991). Histological analysis of tissues from rabbits infected with SERP1<sup>-</sup> myxoma virus revealed a significant infiltration of mononuclear cells and a more pronounced inflammatory response than in the rabbits infected with parental SERP1<sup>+</sup> myxoma virus (Macen *et al* 1993). Nevertheless, the tissue damage was much less severe in rabbits infected with SERP1<sup>-</sup> myxoma virus. In fact, 60% of laboratory rabbits infected with SERP1<sup>-</sup> myxoma virus began to recover by 14 days post-infection, and upon recovery were resistant to subsequent myxoma infection. These results are consistent with other studies showing that clearance of infections correlates with monocyte infiltration into lesions (Best *et al* 2000).

### 10.2 SERP2

Upon its discovery, the SERP2 protein was considered to be the functional equivalent of the CrmA protein of cowpox virus (Petit *et al* 1996), which inhibits the interleukin-1 $\beta$ -converting enzyme (ICE, also known as caspase-1). However, several observations are inconsistent with SERP2 being a CrmA orthologue. Firstly, SERP2 and CrmA share only 35% sequence identity. Secondly, myxoma virus SERP2 is a weak inhibitor of granzyme B *in vitro*, having a  $K_i$  of 420 nM as compared to a  $K_i$  of 100 nM for CrmA, and SERP2 also is a weak inhibitor of ICE *in vitro* having a  $K_i$  of 80 nM as compared to 4 pM for CrmA (Turner *et al* 1999). Thirdly, unlike CrmA, SERP2 does not form a stable complex with ICE. Finally, unlike CrmA, SERP2 cannot block apoptosis in cowpox virus-infected cells (Turner *et al* 1999).

SERP2 is not required for myxoma virus replication in culture, but it is a virulence factor (Messud-Petit *et al* 1998). Histological analysis of tissues from SERP2<sup>-</sup> myxoma virus-infected rabbits revealed a robust inflammatory response (Messud-Petit *et al* 1998). Lymph node lymphocytes from these rabbits were apoptotic (Messud-Petit *et al* 1998). Since apoptosis is a hallmark of unaltered myxoma virus, this finding is not informative. Further, studies are necessary to determine if SERP2 protects lymphocyte from apoptosis, thereby enabling them to disseminate virus to secondary sites.

### 10.3 SERP3

A third serpin-like myxoma virus gene product, SERP3, has a serpin motif that lacks several sequences common to most cellular and viral serpins (Guerin *et al* 2001). However, molecular modeling studies indicate that SERP3 has a signature serpin fold. SERP3 is not required for viral replication *in vitro* nor *in vivo*. SERP3 is a viru-

lence factor in that dissemination of SERP3<sup>-</sup> myxoma virus in infected rabbits is limited, secondary lesions do not arise, and disease is attenuated (Guerin *et al* 2001). Further, biochemical analysis is necessary to determine the physiological substrates of SERP3. Nevertheless, the *in vivo* data certainly suggest that it does not share physiological substrates with either SERP1 or SERP2.

## 11. MV-LAP

Myxoma virus infection induces a profound loss of cell-surface class I MHC molecules (Boshkov *et al* 1992), apparently by multiple mechanisms (Zúñiga *et al* 1999a,b; Zúñiga 2001). As discussed above, class I MHC egress from the ER is retarded, possibly via an interaction with M-T4 (Zúñiga *et al* 1999b; Zúñiga 2001). In addition to this and other previously described mechanisms for which viral gene products have not been identified (Zúñiga *et al* 1999b; Zúñiga 2001), myxoma virus down-regulates cell-surface class I MHC apparently by interfering with its normal cycling patterns (Zúñiga *et al* 1999a). Normally cell-surface class I MHC molecules are endocytosed (Capps *et al* 1989, 2000) and recycled back to the cell surface, traversing an acidic compartment during their journey (Capps *et al* 2000). However, in myxoma-infected cells endocytosed class I MHC molecules are not recycled to the cell-surface, but rather are degraded in endolysosomal compartments (Zúñiga *et al* 1999a). A recently identified myxoma gene product, called MV-LAP (myxoma virus leukemia-associated protein), which has been shown to abrogate cytotoxic T lymphocyte (CTL)-mediated lysis of myxoma virus-infected cells *in vitro* (Guerin *et al* 2002), may be responsible for this effect.

MV-LAP has an atypical zinc finger motif (the 'LAP domain') and two hydrophobic stretches that may serve as membrane spanning regions (Guerin *et al* 2002). MV-LAP lacks a canonical ER-retention motif, yet is localized to ER. The LAP motif is also found in the K5 protein of human herpesvirus-8 (Kaposi's sarcoma associated herpesvirus or KSHV), which also localizes to the ER (Paulson *et al* 2001). MV-LAP and the KSHV K5 protein have been suggested to down-regulate cell-surface class I MHC by promoting its endocytosis (Guerin *et al* 2002; Paulson *et al* 2001). Another possible interpretation of these data is that these viral proteins do not promote class I MHC endocytosis, but rather interfere with its recycling back to the cell surface (figure 4). There is precedent for such a mechanism. Protein tyrosine phosphatase-1B (PTP1B) resides on the cytoplasmic side of the ER membrane and yet dephosphorylates receptor tyrosine kinases internalized from the plasma membrane (Haj *et al* 2002). Fluorescence resonance energy transfer (FRET) meas-

urements and confocal microscopic studies indicate that receptor tyrosine kinases transiently associate with PTP1B in an intracellular compartment (Haj *et al* 2002). These observations led to the suggestion that during their trafficking to lysosomes or their re-exocytosis to the cell surface, receptor tyrosine kinases cycle through a 'dephosphorylation compartment' where they encounter PTP1B (Haj *et al* 2002). Phosphorylation and dephosphorylation of serine residues on the class I MHC cytoplasmic tail accompany class I MHC recycling (Capps and Zúñiga 2000). Since K5 protein of KSHV inhibits phosphorylation of the class I MHC cytoplasmic tail (Paulson *et al* 2001), it is possible that MV-LAP (or K5 in KSHV-infected cells) acts similarly to PTP1B. In this model MV-LAP (or K5 in KSHV-infected cells) encounters endocytosed class I MHC in an intracellular compartment where it dephosphorylates it (see figure 4). Dephosphorylation of class I MHC may prevent its recycling back to the cell surface and instead may target it to endolysosomal compartments for degradation (see figure 4).

An alternative possibility is that MV-LAP-mediated loss of cell-surface class I MHC is due to its effects on cellular proteins required for class I MHC recycling. For example, recent studies have identified epithelial membrane protein-2 (EMP2), a tetraspan transmembrane protein, as a regulator of cell-surface levels of class I MHC (Forbes *et al* 2002). Ectopic over-expression of EMP2 increases the cell surface display of class I MHC and increases target cell susceptibility to lysis by CTLs (Forbes *et al* 2002). It is thus possible that MV-LAP abrogates cell-surface class I MHC display by blocking EMP2-mediated recruitment of endocytosed class I MHC into recycling transport vesicles. Regardless of its mechanism of action, MV-LAP must act on class I MHC endocytosis and/or recycling and not on the deposition of newly synthesized class I MHC at the plasma membrane, because biosynthetic studies have shown that in myxoma infected cells class I MHC that exits the ER is transported to the cell surface at the same rate as in uninfected cells (Zúñiga *et al* 1999a).

MV-LAP is not required for infectivity, but virulence and immunosuppression are diminished in rabbits infected with MV-LAP<sup>-</sup> myxoma virus (Guerin *et al* 2002) consistent with its role in evasion of immunosurveillance by CTLs.

### 12. The role of virulence genes in myxoma infection

A noteworthy point that is evident from the above discussion is that the loss of even a single virulence factor attenuates virulence of myxoma virus. In keeping with this conclusion, restriction fragment length polymor-

phism (RFLP) analysis of 37 Australian field isolates of infectious myxoma virus (Saint *et al* 2001) revealed only subtle genetic changes in the myxoma genome in spite of the rapid loss of virulence after myxoma virus was introduced into Australia (Fenner and Ratcliffe 1965). One might have expected more mutation of the immunomodulatory genes, since none of the eleven identified thus far is required for a productive infection in laboratory rabbits (table 1). On the other hand, in a natural setting the skin lesions are the source of infectious virus for insect vectors and must persist over winter months for the virus to be transmitted the following spring (Fenner and Ratcliffe 1965). As discussed above, infection with myxoma virus in which the virulence genes were disrupted individually led to complete healing of the lesions after a few months in several cases. Since mutations in virulence genes that allow more rapid healing of lesions would be selected against, virulence genes that are required for lesion persistence are expected to be maintained in natural isolates of myxoma virus.

### 13. Future directions

As discussed earlier in this review, an immediate effect of myxoma infection is activation of signal transduction cascades in the host cell. It is important to determine the ultimate effects of viral modulation of or interference with signal transduction events. Does myxomatosis result from altered trafficking of neutrophils, macrophages, and lymphocytes in rabbits infected with virulent myxoma virus? Are these trafficking patterns not perturbed in rabbits infected with avirulent myxoma virus? It is also important to study the progress and resolution of myxoma infection in disease-resistant *O. cuniculus* rabbits to elucidate the role of myxoma immunomodulatory proteins in a more natural situation. Only then can we appreciate the true function of immunomodulatory proteins in myxoma virus' relationship with its host and understand the mechanisms by which myxoma virus subverts the host immune system without compromising its ability to be maintained in wild populations from year to year.

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