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Technical knockout: understanding poxvirus pathogenesis by selectively deleting viral immunomodulatory genes

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Summary

The study of viral pathogens with genomes as large and complex as poxviruses represents a constant experimental challenge. Advances in recombinant DNA technologies have provided sophisticated methods to produce mutants defective in one or more viral genes, termed knockout (KO) viruses, thereby facilitating research into the impact of specific gene products on viral pathogenesis. Such strategies have rapidly advanced the systematic mining of many poxvirus genomes and enabled researchers to identify and characterize poxvirus genes whose functions represent the culmination of host and pathogen coevolution. Of particular interest are the multiple classes of virus-encoded immunomodulatory proteins that have evolved specifically to allow poxviruses to evade, obstruct or subvert critical elements within the host innate and acquired immune responses. Functional characterization of these viral genes by generating KO viruses and investigating the phenotypic changes that result is an important tool for understanding the molecular mechanisms underlying poxvirus replication and pathogenesis. Moreover, the insights gained have led to new developments in basic and clinical virology, provided a basis for the design of new vaccines and antivirals, and increased the potential application of poxviruses as investigative tools and sources of biotherapeutics for the treatment of human diseases.

Introduction

The poxviruses are a large family of double-stranded DNA viruses that includes members that infect vertebrates (*Chordopoxvirinae*) as well as insects (*Entomopoxvirinae*) (Moss, 2001). Poxviruses are notable among DNA viruses for their large virion size and the ability to replicate within the cytoplasm of infected cells autonomous of the host nuclear machinery. Poxviruses also possess one of the largest viral genomes, ranging in size from 135 kb to 290 kb and encoding as many as 260 open reading frames (ORFs), with termini that form covalently closed hairpin loops. In general, genes that are centrally located in the genome are relatively conserved among poxviruses and have common essential molecular functions, such as replication and virion assembly (Moss, 2001). However, increasing interest has been engendered by the products of the more variable, terminally located genes that have been shown to encode a diverse array of proteins that function in host-range restriction and modulation or inhibition of the host responses to infection.

The sequences of over two dozen poxvirus genomes have been determined and multiple immunomodulatory proteins have been identified and broadly divided by function into a three strategic classes: virostealth proteins, virotransducers and viromimetics (virokines and viroceptors) (Nash *et al.*, 1999). Virostealth encompasses a general strategy in which the visible signals of infection are masked in order to reduce the ability of cell-mediated immune responses to recognize and eliminate infected cells. Virotransducers are viral proteins that act intracellularly to inhibit innate antiviral pathways and the signal transduction cascades that mediate host range. Virokines and viroceptors represent virus-encoded proteins that mimic host cytokines or their receptors, respectively, thereby blocking extracellular communication signals and promoting a protected microenvironment for the virus within immuno-exposed tissues (Fig. 1). Generally, viroceptors, which can be either secreted or localized to the surface of infected cells, are related to cellular receptors and act by competing for ligands that promote antiviral
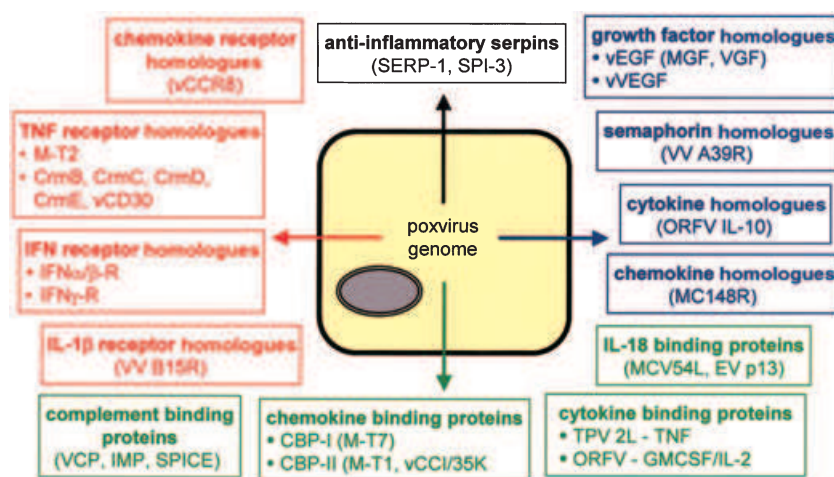
Fig. 1. Virokines and viroceptors produced by poxvirus-infected cells. Indicated are select poxvirus viromimetics representing cytokine receptor homologues (red), cytokine and complement binding proteins (green), viral homologues of immune molecules (blue) and the anti-inflammatory poxviral serpins (black). IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; VCP, vaccinia complement protein; IMP, inflammation modulatory protein; SPICE, smallpox inhibitor of complement enzymes; CBP, chemokine binding protein; vEGF, viral epithelial growth factor; vVEGF, viral vascular endothelial growth factor.

immune or inflammatory processes. In contrast, virokines are generally secreted viral proteins that mimic host molecules, such as cytokines, complement regulators or their inhibitors.

KO poxvirus genes – strategies, considerations and effective analyses

Although the obvious sequence similarity between some poxvirus genes and the cDNA versions of related cellular counterparts provides insight into their function, the evolutionary origins of other ORFs are more obscure (Moss, 2001). Thus, genetic analyses employing recombinant DNA technologies are essential tools for studying the contribution of specific viral genes and proteins to virus–host interactions and viral pathogenesis. Much of what is known about viral pathogenesis can be traced back to discoveries made with knockout (KO) viruses, mutant viruses in which the targeted disruption of a specific viral gene produces phenotypic changes reflective of the normal biological function of its protein product (Coen and Ramig, 1996). The typical method for generating KO mutants of poxviruses (Fig. 2) employs a step-wise approach that harks back to early marker-transfer mutagenesis strategies and closely resembles the ancestral retrotranscription/recombination events by which many immunomodulatory proteins were likely acquired by poxviruses from their vertebrate hosts. The information that can be obtained from KO analyses is not sufficient to definitively assign a particular function to a viral gene product. Rather, the biological activity of purified proteins must be confirmed in relevant *in vitro* assays to provide further insight into how poxviral immunomodulatory proteins interact with their targets. Several recent reviews have provided in depth accounts of the advances made using these techniques for the study of poxviruses and the reader is referred to select examples for more infor-

mation (Moss and Shisler, 2001; Turner and Moyer, 2002; Seet *et al.*, 2003).

The requirement for an animal model for in-depth analyses renders KO strategies ineffective for the study of poxviruses like molluscum contagiousum virus (MCV), an obligate human pathogen for which neither animal models nor tissue culture systems are available (Smith and Skelton, 2002). Consequently, investigation of the properties of poxviral immunomodulatory proteins using KO viruses has largely employed murine models in the study of orthopoxviruses, such as vaccinia virus (VV), and rabbit models in the study of leporipoxviruses, such as myxoma virus (MV). VV exhibits a broad host range that includes several mammalian species, but the natural reservoir for the virus is unknown (Moss, 2001). The route of inoculation also significantly impacts upon disease course and VV infection is achieved under experimental conditions using intranasal, intracerebral, intraperitoneal or intradermal routes. In contrast, MV is an obligate rabbit pathogen that establishes only a localized infection in its natural host species, the *S.* American *Sylvilagus* rabbit, but a lethal disseminated disease (myxomatosis) in European *Oryctolagus* rabbits (Nash *et al.*, 1999). Moreover, MV is transmitted under natural conditions by arthropod vectors and intradermal injection is the most common mechanism for introducing virus. Despite these differences, both VV and MV cause generalized disseminated infections characterized by the formation of a primary lesion at the initial site of inoculation and a viremia that spreads the infection through the host lymphoreticular system to establish internal and external lesions in secondary organs and tissues. As a consequence of their immunosuppressive capacities, infection with VV and MV supports the development of supervening bacterial infections that ultimately lead to the death of host. Thus, targeted disruption of poxviral immunomodulatory genes can impact greatly on disease progression in these models.
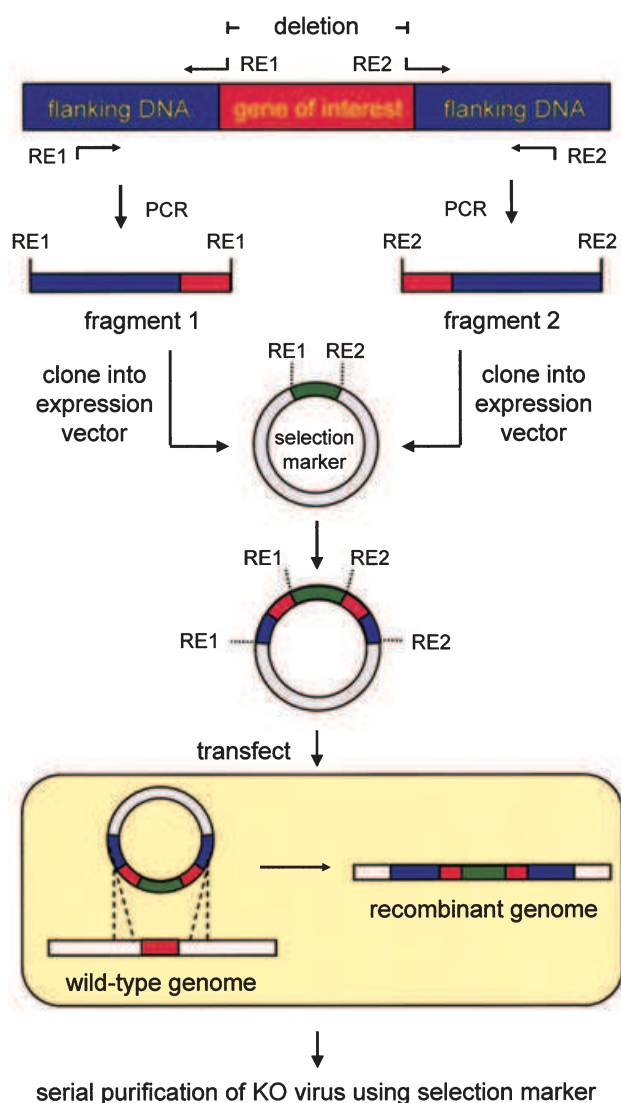
Fig. 2. General strategy for constructing knock-out poxviruses. A fragment of the poxvirus genome containing the gene to be disrupted and flanking sequences of varying lengths is cloned and used as a template for subsequent manipulations. Two subfragments are amplified by PCR using primers designed to selectively delete a portion of the targeted ORF while introducing restriction sites (RE) for subcloning the fragments. Each fragment is cloned in series into a transfer vector containing a selection marker under a poxvirus promoter (green fluorescence protein shown), such that the marker sequences further disrupt the target ORF. The vector encoding the poxvirus sequences is transfected into permissive cells that are infected at low multiplicity with wild-type virus. Within infected-transfected cells, homologous recombination occurs between the transfected sequences and wild-type virus genomes. KO viruses are isolated from wild-type virus and serially purified according to the selection criteria conferred by the selection marker.

Viromimicry – seeing a familiar face

The MV and VV immunomodulatory genes that have been investigated using KO viruses are summarized in Table 1 and addressed in greater detail below with relevant examples from other poxviral species. In a recent minireview,

we focused on the role of virostealth and virotransduction in poxvirus immune evasion (Johnston and McFadden, 2003). Thus, we emphasize here the large body of literature devoted to gene KO analysis of poxviral virokines and viroceptors.

TNF viroceptors

Tumour necrosis factor (TNF) is a potent proinflammatory and proapoptotic cytokine secreted by macrophages and activated T-cells. To inhibit the activities of this cytokine, many poxviruses encode soluble proteins that resemble secreted versions of the extracellular domains of cellular TNFR, termed vTNFRs (Cunnion, 1999). vTNFRs function primarily as molecular scavengers that bind to and sequester TNF, thereby blocking the interaction between the ligand and its native receptor. The T2-like vTNFRs, found in the leporipoxviruses MV and Shope fibroma viruse (SFV), are secreted glycoproteins that bind TNF with high affinity (Sedger and McFadden, 1996). SFV T2 has been reported to bind both TNF-α and TNF-β from several species (Smith *et al.*, 1991), but MV T2 exhibits specificity for rabbit TNF-α (Schreiber *et al.*, 1996). The orthopoxvirus cytokine response modifier (Crm)-like vTNFRs, of which four major classes (CrmB, C, D and E) have been identified, also vary widely in distribution and biological activity (Saraiva and Alcami, 2001; Cunnion, 1999). CrmD is found primarily in poxviruses that lack CrmB and CrmC (Alcami *et al.*, 1999), while functional CrmE homologues has been identified only in cowpoxvirus (CPV) and select VV strains (Reading *et al.*, 2002; Saraiva and Alcami, 2001). Recently, members of a fifth Crm-like vTNFR family closely resembling CD30 have also been identified in CPV and ectromelia virus (EV) (Panus *et al.*, 2002; Saraiva and Alcami, 2001).

KO studies. Despite the importance of TNF in host antiviral responses, naturally arising MV strains deficient in M-T2 have been reported (Saint *et al.*, 2001) and M-T2 KO viruses retain the capacity to infect susceptible hosts. However, rabbit lymphocytes infected *in vitro* with MV disrupted in both copies of the M-T2 gene undergo apoptosis and abortive infection (Macen *et al.*, 1996). Consistent with this finding, the M-T2 KO virus is markedly attenuated *in vivo*, exhibiting decreased lethality and a pathology in which opportunistic bacterial infections are less frequent, primary lesions are smaller and less pronounced and secondary lesions are largely absent (Upton *et al.*, 1991). Both the *in vivo* and *in vitro* observations suggest that deletion of M-T2 impairs virus replication and spread in an immunocompetent host, emphasizing the importance of inhibiting TNF to poxviral pathogenesis. KO analysis of Crm-like vTNFRs is hindered by virus strain-dependent variability in their expression; thus, the roles these proteins play in pathogenesis are more commonly

Table 1. Effects of disrupting select immunomodulatory genes on MV and VV pathogenesis.

Virus[a]	Gene	Function[b]	Relative virulence[c]	Host range[d]
MV	M-T1	CC chemokine inhibitor	++++	N
	M-T2	TNF receptor mimic	++	Y
	M-T4	ER apoptosis	−	Y
	M-T5	ankyrin domain protein	−	Y
	M-T7	Type 2 IFN receptor mimic	+	N
	M10L	growth factor (EGF-like)	+	N
	M11L	mitochondrial apoptosis	−	Y
	M131R	superoxide dismutase	+++++	N
	M150R	NF-κB binding protein	−	N
	M153R	PHD/LAPIN (scrapin)	++	N
	SERP-1	anti-inflammatory serpin	++	N
	SERP2	caspase-inhibiting serpin	+	N
	SERP3	serpin	+++	N
VV	CrmE	TNF-R mimic	+++	N
	VGF	growth factor (EGF-like)	+++	N
	A39R	semaphorin-like	++++	N
	A41L	inflammation regulator	+++	N
	A45R	superoxide dismutase	+++++	N
	A46R	TLR inhibitor	+++	N
	A52R	TLR inhibitor	+++	N
	A53R	TNF-R mimic	+++++	N
	B8R	Type 2 IFN-R mimic	+++	N
	B12R	Ser-Thr kinase	+++++	N
	B15R	IL-1β-R mimic	+++++	N
	B18R	Type 1 IFN-R mimic	+++	N
	C7L	host range protein	N/D	Y
	C12L	IL-18 bp	++	N
	C21L	complement control	+++	N
	E3L	dsRNA binding protein	−	Y
	K1L	host range protein	N/D	Y
	K3L	eIF2-α mimic	+++	Y
	SPI-1	serpin	+++++	Y
	SPI-2	anti-apoptotic serpin	+++++	N
	SPI-3	anti-inflammatory serpin	+++++	N

a. Select immunomodulatory genes from myxoma (MV) and vaccinia (VV) virus for which KO analyses have been performed are shown.
b. TNF, tumour necrosis factor; ER, endoplasmic reticulum; IFN, interferon; TLR, toll-like receptor; IL, interleukin.
c. Virulence exhibited by KO viruses is compared to wild-type virus on a descending scale from fully virulent (+++++) to avirulent (−). N/D, not done.
d. Deleting a gene restricts (Y) or does not affect (N) replication in a permissive host or cell line.

studied by expressing their ORFs in a background that normally lacks them. For example, recombinant VV expressing CPV CrmB, CrmC or CrmE is more virulent in mice than wild-type virus, causing rapid weight loss and mortality (Reading *et al.*, 2002). The contribution of the two vTNFRs encoded by VV (strain USSR), CrmE and A53R, however, has been assessed in both intradermal and intranasal mouse models using KO viruses. Deletion of A53R did not impact on virulence following infection by either route, but loss of CrmE resulted in marked attenuation of the virus when delivered by the intranasal route (Reading *et al.*, 2002). Thus Crm-like vTNFRs likely contribute to pathogenesis, but in a manner that reflects the complex regulation of TNF in the host.

IFN viroceptors

The integral role played by interferons (IFNs) in host antiviral responses is underscored by the fact that all poxviruses employ at least one mechanism to disrupt its activity (Sen, 2001). As with TNF, a common strategy is

to encode soluble viral mimics of both Type I (α/β) and Type II (γ) IFN receptors (IFN-R) that bind to and sequester these cytokines. For example, the B8R genes of both VV and EV encode proteins that bind IFN-γ from several species (Alcami and Smith, 1995), although only the B8R homologue of EV inhibits murine IFN-γ despite the ability of both viruses to infect mice (Smith and Alcami, 2002). Both viruses also encodes a protein (B18R) that closely resembles the IL-1 receptor but actually strongly interacts with Type I IFNs from several species (Smith and Alcami, 2002; Symons *et al.*, 1995). Of note, the VV B18R product has been detected as both a secreted protein and localized to the surface of infected and uninfected cells (Alcami *et al.*, 2000), suggesting that it protects infected cells from the direct action of IFN-α/β and uninfected cells from IFN-induced resistant to infection. MV encodes an IFN-γ receptor homologue, M-T7, whose anti-IFN properties is rabbit-specific (Mossman *et al.*, 1996), but purified M-T7 protein also exhibits the surprising ability to bind to diverse families of human chemokines (Lalani *et al.*, 1997).

KO studies. Deletion of both copies of M-T7 from MV attenuates the virus and leads to elevated inflammatory responses in primary lesions (Mossman *et al.*, 1996). Given the contribution of chemokines to inflammation, however, this finding must be viewed in the context of the reported capacity for M-T7 to bind both chemokines and IFN. Deletion of the VV B8R gene has been reported to either enhance or not affect virulence in mice (Symons *et al.*, 2002a; Verardi *et al.*, 2001), although B8R-deletants are attenuated in other rodent species (Verardi *et al.*, 2001). The increased virulence reported in some mice infected with B8R KO virus is particularly surprising because the protein does not bind mouse IFN, possibly indicating that like M-T7 this protein has multiple activities (Alcami and Smith, 1995). The VV B18R gene product also exhibits low affinity for murine IFN-α/β *in vitro*, but deletion of this gene from VV significantly attenuates virulence in mice resulting in minimal disease symptoms, limited mortality and loss of neuroinvasiveness (Symons *et al.*, 1995).

IL-1β viroceptors

The VV B15R ORF encodes a secreted interleukin (IL)-1β receptor that was shown to bind to both murine (Spriggs *et al.*, 1992) and human (Alcami and Smith, 1992) IL-1β and to block the activity of the former in a functional bioassay (Spriggs *et al.*, 1992). Although deletion of B15R influences VV pathogenesis in mice, the extent of the effect has been shown to vary according to the route of inoculation. Intracranial injection of VV deleted for B15R results in significant attenuation compared to wild-type virus (Spriggs *et al.*, 1992), but B15R KO virus delivered intranasally exhibits lethality comparable to wild-type VV despite the earlier emergence of clinical symptoms (Alcami and Smith, 1992). In fact, B15R-deleted virus delivered intranasally induced greater fevers and was marginally more pathogenic. Thus, the effects of deleting a specific virus gene can vary according to non-genotypic factors such as the inoculation route, likely reflecting regional differences in host immune responses. The pathogenic contribution of the IL-1β receptors encoded by other orthopoxviruses such as CPV and EV has yet to be determined in the context of an *in vivo* infection.

Viral IL-18 binding proteins

Poxviruses also target the IFN pathway indirectly through proteins that scavenge IL-18, a pleiotrophic pro-inflammatory cytokine that induces IFNγ production. Mammalian IL-18 binding protein (IL–18 BP) is a natural antagonist of IL-18 and many poxviruses have been shown or are predicted to encode soluble homologues of this protein (Smith *et al.*, 2000; Xiang and Moss, 1999).

The IL-18BPs encoded by orthopoxviruses that are able to infect rodents, such as VV, EV and CPV, exhibit much greater affinity for murine IL-18 than human (Calderara *et al.*, 2001). Of the three putative IL-18BPs encoded by the human poxvirus MCV (MC51L, 53 L and 54 L), only MC54L appears to bind to IL-18 (Xiang and Moss, 1999). This interaction exhibits high affinity for human and murine IL-18, but its role in pathogenesis can not be assessed in the context of MCV infection for the reasons detailed above.

KO studies. Recombinant murine IL-18 introduced systemically by intravenous injection has been shown to decrease the development of lesions and increased the activity of cytolytic immune cells following VV infection of mice (Tanaka-Kataoka *et al.*, 1999). Deletion of the C12L gene encoding the VV IL–18 BP produces similar attenuation in mice inoculated intranasally (Symons *et al.*, 2002b), further supporting the importance of IL-18 in controlling VV infections. In contrast, disruption of the EV IL–18 BP gene (p13) by insertional mutagenesis has little impact on pathogenic outcome beyond an observed increase in NK cell activity that correlates with moderate increases in the clearance of infected cells (Born *et al.*, 2000). It should be noted that the presence of other intact immunomodulatory factors, such as the EV IFN-γR described above, may have compensated for the loss of IL–18 BP activity.

Viral IL-10

IL-10 is a multifunctional cytokine with both immunostimulatory and immunosuppressive effects. Homologues of IL-10 have been identified in the genomes of *orf* virus (ORFV), Yaba-like disease virus (YLDV) and lumpy skin disease virus (LSDV), but only the ORFV IL-10 has been characterized to date and shown to have biological activity similar to that of ovine IL-10 (Fleming *et al.*, 1997). *In vitro*, ORFV IL-10 promotes thymocyte proliferation, costimulates mast cell growth and suppresses macrophages activation (Imlach *et al.*, 2002), suggesting a role in immune evasion that involves mimicking the suppressive effects of host IL-10 on Th1-mediated responses. More recently, ORFV IL-10 has also been implicated in the impairment of acquired immunity by inhibiting the maturation of and antigen presentation by dendritic cells (Lateef *et al.*, 2003), possibly explaining why ORFV can repeatedly infect the same host. In a sheep model, deletion of ORFV IL-10 was shown to produce elevated levels of IFN-γ in infected tissue compared to wild-type virus (Haig *et al.*, 2002).

Viral growth factors

Many poxviruses encode proteins that resemble mammalian growth factors, most notably the homologues of epi-

dermal growth factor (EGF) detected in members of virtually all poxvirus genera and vascular endothelial growth factor (VEGF) found only in parapoxviruses (Seet *et al.*, 2003). The EGF homologues encoded by VV and MV, termed VGF and MGF, respectively, are secreted proteins produced early in infection that compete with cellular EGF for receptors (EGFR) expressed on epithelial cells overlying sites of infection (Opgenorth *et al.*, 1992; Stroobant *et al.*, 1985). The functional consequences of this interaction include promoting EGFR autophosphorylation and the generation of mitotic responses, as well as reducing EGFR downregulation and degradation to prolong the duration of proliferative signals. The parapoxviruses, ORFV and pseudocowpoxvirus (PCPV), encode biologically active homologues of mammalian VEGF-A that have been shown to stimulate proliferation of vascular endothelial cells and promote vascular permeability (Wise *et al.*, 1999). Moreover, they exhibit a receptor binding profile that is unique among VEGF family members in its apparent specificity for VEGFR-2 and neuropilin-1 (Meyer *et al.*, 1999). Thus, their function in pathogenesis is likely to contribute to the proliferative and highly vascularized nature of parapoxvirus lesions.

KO studies. Deletion of VGF affects VV pathogenesis in both mice and rabbits. For example, KO virus delivered by an intracranial route exhibits decreased neurovirulence in mice, whereas virus introduced intradermally produces less localized cellular proliferation in lesions (Buller *et al.*, 1988). Similarly, infection of rabbits with MV KO virus lacking MGF was found to result in less mortality and milder disease symptoms, including less hyperplastic lesions and fewer secondary bacterial infections (Opgenorth *et al.*, 1992). Disruption of the VEGF-like gene in ORFV results in the loss of the three VEGF activities associated with the parent virus: mitogenesis of vascular endothelial cells, induction of vascular permeability and activation of VEGF receptor 2 (Savory *et al.*, 2000). *In vivo*, loss of viral VEGF does not impact greatly on viral replication, but it does result in lesions characterized by decreased genesis of blood vessels at sites of infection, reduced inflammatory cell influx and abrogation of epidermal cell proliferation (Savory *et al.*, 2000).

Viral semaphorins

Semaphorins represent a family of cellular regulatory proteins implicated in both neuronal development and activation of B and T lymphocytes. Included in this family are several poxviral proteins, most notably the products of the VV and EV A39R genes, which have been shown to possess the defining 500-amino acid 'sema' domain (Comeau *et al.*, 1998). Moreover, these proteins interact with a novel virus-encoded semaphorin protein receptor, termed VESPR, a plexin family cell surface receptor for

which the natural ligand is unknown. Surprisingly, studies into the function of the EV A39R protein have suggested pro-inflammatory properties that manifest as increased recruitment of immune cells to sites of infection because of IL-6 and -8 upregulation (Comeau *et al.*, 1998). This strategy likely favours virus dissemination within the host by attracting immune cells that can be subsequently infected.

KO studies. The VV (strain Western Reserve) A39R gene product is naturally truncated and insertion of full-length A39R from VV (strain Copenhagen) was shown to have only minimal effect on pathogenesis in mice (Gardner *et al.*, 2001). Consistent with its pro-inflammatory potential, moderately increased inflammation leading to larger lesions that were slower to resolve was observed following infection with virus containing A39R (Gardner *et al.*, 2001). However, disease symptoms and viral titres remained unaffected. Similarly, deletion of the intact gene from VV (strain Copenhagen) did not influence pathogenesis, suggesting that poxviral semaphorins may promote, but are not essential to, infection.

Viral anti-inflammatory serpins: SERP-1 and SPI-3

Serpins are a family of serine proteases inhibitors that regulate complex proteinases-dependent pathways involved in such processes as inflammation, apoptosis and tissue remodelling. The MV SERP-1 is the first poxviral serpin shown to be secreted and therefore it is technically a virokine (Upton *et al.*, 1990). The orthopoxvirus SPI-3 is not secreted from RPV-infected cells and shares limited sequence homology with SERP-1, but the presence of a common P1 Arg residue in the active site of the protein confers a similar inhibitory profile *in vitro* (Turner *et al.*, 2000). Both proteins inhibit a range of trypsin-like serine proteinases *in vitro*, including tissue plasminogen activator, urokinase, plasmin, thrombin and factor Xa (Turner *et al.*, 2000; Nash *et al.*, 1998; Lomas *et al.*, 1993), suggesting that the primary function of these serpins is to modulate host inflammatory responses to infection. Despite these similarities, however, SERP-1 and SPI-3 are not functionally interchangeable (Wang *et al.*, 2000). The precise targets and receptors through which SERP-1 acts *in vivo* are not yet defined, although purified SERP-1 has recently been shown to interact with native vascular urokinase-type plasminogen activator receptors to inhibit inflammatory cell responses in a mouse model (Dai *et al.*, 2003).

KO studies. Deleting the SPI-3 gene from the genome of orthopoxviruses such as CPV and VV has limited effect on pathogenesis *in vivo* and KO viruses exhibit only moderate reductions in virulence murine models (Thompson *et al.*, 1993). In contrast, SERP-1 is an important viru-

lence factor for MV in its rabbit host. Deletion of SERP-1 markedly reduces the lethality of MV compared to wild-type virus and produces a pathology characterized by rapid induction of host inflammatory responses, reduced leukocyte infiltration and the development of fewer secondary lesions (Upton *et al.*, 1990). These observations suggest that SERP-1 is important to the dissemination of MV *in vivo* as well as the control of host inflammatory responses to infection.

Manipulation of chemokine function by poxviruses

Chemokines (chemoattractant cytokines) are small, secreted cytokines that contribute to the host efforts to limit virus infections by coordinating the activation and mobilization of leukocytes that mediate inflammatory responses in areas of infection. Consequently, all poxviruses attempt to modulate chemokine activity by encoding chemokine receptor homologues and secreted ligand mimics and chemokine binding proteins (CBPs) (Mahalingam and Karupiah, 2000). Bioinformatic analyses have identified putative G-protein-coupled chemokine receptor (GPCR) homologues and chemokine ligand mimics in the genomes of several poxviruses (Alcami, 2003). With the exception of the product of the MCV MC148R gene, which has been shown to function as a selective antagonist of human CCR8 (Ishikawa-Mochizuki *et al.,* 1999), functional studies on these proteins are limited and their role in pathogenesis *in vivo* remains speculative. Greater insight has been gained into the role of poxvirus CBPs, however. Classified as either Type I (low affinity) or Type II (high affinity), their roles in poxvirus virulence have been examined extensively using KO viruses.

Type I poxviral CBPs

The dual-function IFNγR homologue of MV, M-T7, is the sole Type I poxvirus CBP identified to date. The capacity for M-T7 to inhibit the activity of a broad spectrum of chemokines, in addition to IFN-γ, likely arises from its ability to interact with the heparin binding domains common to many chemokines (Lalani *et al.*, 1997). In doing so, M-T7 has the potential to interfere with generalized chemokine binding to glycosaminoglycans and disrupt the localization of a large number of C, CC and CXC chemokines in tissues. Deletion of the M-T7 gene produces marked attenuation that prevents dissemination of the virus to distal sites of infection (Mossman *et al.*, 1996). Locally, loss of MT-7 function is associated with leukocyte infiltration into the primary dermal sites of viral replication and activation of leukocytes in secondary immune tissues, such as the lymph nodes and spleen (Mossman *et al.*, 1996). Naturally, the question remains as to whether this phenotype reflects loss of binding to chemokines, IFN-γ or both classes of molecules.

Type II poxviral CBPs

Type II CPB, also termed CPB-IIs or vCCIs, have been identified in several poxvirus species and are exemplified by the 35 kDa vCCI encoded by many orthopoxviruses (Smith *et al.*, 1997) and the product of the M-T1 gene of MV (Graham *et al.*, 1997). Despite lacking sequence similarity with known mammalian proteins, type II CBPs target the GPCR binding conserved among many CC chemokines to competitively inhibit their ability to interact with diverse cellular receptors (Seet and McFadden, 2002). Like the high binding affinities exhibited by these proteins, this property likely reflects the unique structure of type II CPBs that distantly resembled the collagen-binding domain of the *Staphylococcus aureus* adhesin molecule (Carfi *et al.*, 1999). The proposed function of poxviral CBPs is based on *in vitro* studies that support the ability of these proteins to bind chemokines and impede leukocyte migration. However, loss of type II CBP activity appears to have limited effect on the virulence *in vivo*. For example, infection of rabbits with MV lacking M-T1 differs from wild-type infections only in the development of heightened localized cellular inflammation in primary lesions, together with a moderate increase in infiltrating monocytes and macrophages early in infection (Lalani *et al.*, 1999). Similarly, the virulence of a rabbitpox (RPV) KO virus lacking the 35 kDa CBP-II gene was shown to differ little from wild-type virus in mice (Martinezpomares *et al.*, 1995). These results are perhaps unsurprising when the considerable redundancy in chemokine function and the combined effects of other poxviral immunomodulators that indirectly impact on chemokine function are considered.

Control of the complement system by poxviruses

The complement system is an integrated network of cell-associated effector proteins and secreted regulatory proteins that participate in the identification and destruction of invading pathogens, as well as the initiation and amplification of inflammatory responses. The VV complement control protein (VCP) typifies poxviral strategies to modulate this system, with genes encoding similar products identified several other orthopoxviruses (Kotwal, 2000). VCP targets both the classical and alternative complement activation pathways by directly and indirectly promoting the decay of the C3 convertase (Sahu *et al.*, 1998; Kotwal *et al.*, 1990). A similar mechanism of action has been demonstrated for the smallpox inhibitor of complement enzymes (SPICE), the VCP homologue of the variola virus (VaV) (Rosengard *et al.*, 2002). Given the virulence of VaV in humans compared to VV, the finding that SPICE inhibits human C3 activity nearly 100-fold more than VCP is of particular interest (Rosengard *et al.*, 2002).

KO studies. Various murine models have been used to

assess the function of the CPV VCP homologue, the inflammation modulatory protein (IMP). Although results varied extensively with host strain and route of inoculation, these studies suggest that modulation of host complement contributes little to poxvirus virulence. For example, the IMP KO virus was not attenuated in BALB/c mice inoculated using either footpad injections (Miller *et al.*, 1997) or a connective tissue air pouch model (Kotwal *et al.*, 1998), exhibiting lethality comparable to the wild-type. However, inflammation and mononuclear cellular infiltration at sites of infection were greater in animals infected with the IMP KO virus compared to wild-type CPV. Because BALB/c mice express only low constitutive levels of C3, mice that were either fully deficient in C3 or expressed high levels of C3 were also studied (Kotwal *et al.*, 1998). Infection with either wild-type or KO virus produced similar disease pathologies in C3-deficient mice, but the differences in inflammatory responses elicited by the viruses was comparable to that observed in BALB/c mice despite greater levels of host C3 expression.

Overview

The examples provided above illustrate the important contribution of immunomodulatory genes in the progression and resolution of poxvirus infections and their ability to impact on host range, virulence and pathogenicity. However, it is difficult to predict the effect of deleting a specific gene because of the redundancy inherent to many poxvirus immune evasion strategies and the capacity for individual immunomodulatory proteins to have multiple functions. Consequently, the phenotype of a KO virus may not necessarily reflect the full extent to which a gene product contributes to pathogenesis. Despite these limitations, certain trends do manifest when KO viruses are compared on the basis of the function of the gene disrupted. As shown in Table 1, deletion of genes whose products regulate host antiviral responses that influence survival at the level of the infected cell, such as apoptosis, more profoundly affect virulence than genes encoding proteins that modulate more global host antiviral responses, such as chemokine and complement networks. Although manipulation of the latter strategies are important to the efficient spread of the virus once an infection has been established, failure to block innate defence mechanisms evolved to remove infected cells prevents the infection from being established at all. Thus, it is not surprising that poxviral proteins that modulate these critical host responses are determinants of host range as well as virulence.

Applications of the information gained from KO viruses

The immunomodulatory strategies of poxviruses are so effective that new avenues of research, collectively known as virotherapeutics, have emerged in the attempt to exploit viral immunomodulatory proteins for the treatment of human diseases (Smith and Kotwal, 2001). For example, several of the poxviral proteins described in this review have been used in animal models to prevent allograft and xenograft transplant rejection, and to inhibit adverse immune responses in models of arterial injury following balloon angioplasty (Dabbagh *et al.*, 2000; DeBruyne *et al.*, 2000; Liu *et al.*, 2000; Lucas *et al.*, 1996; Maksymowych *et al.*, 1996). In many of these applications, viral proteins are used as purified biotherapeutics outside the context of the intact virus and pathogenesis is not a consideration. However, other virotherapies based on live poxviruses that have been modified to be less virulent or to exhibit a specific phenotype are becoming increasingly prevalent. These include the use poxviruses in vaccines (Mayr, 2003) and as therapeutic vectors and oncolytic agents (Kwak *et al.*, 2003; Vanderplasschen and Pastoret, 2003). The rational design of such therapies requires detailed information about how modification of the viral genome impacts on the biology of poxviruses and emphasizes the importance of KO virus analyses. In addition to the therapeutic benefit afforded by the characterization of poxvirus immunomodulators, the study of immune modulation by viruses contributes greatly to our understanding of how the immune system responds to infection and the selective pressures that drive the coevolution of virus and host. Greater understanding of the role individual genes play in poxvirus pathogenesis also has the potential to provide insights into novel targets on which to base antiviral strategies, information of particular relevance given the potential use of smallpox as a bioterrorism agent.

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