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Review

Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity

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Virus infection of mammalian cells is sensed by pattern recognition receptors and leads to an innate immune response that restricts virus replication and induces adaptive immunity. In response, viruses have evolved many countermeasures that enable them to replicate and be transmitted to new hosts, despite the host innate immune response. Poxviruses, such as vaccinia virus (VACV), have large DNA genomes and encode many proteins that are dedicated to host immune evasion. Some of these proteins are secreted from the infected cell, where they bind and neutralize complement factors, interferons, cytokines and chemokines. Other VACV proteins function inside cells to inhibit apoptosis or signalling pathways that lead to the production of interferons and pro-inflammatory cytokines and chemokines. In this review, these VACV immunomodulatory proteins are described and the potential to create more immunogenic VACV strains by manipulation of the gene encoding these proteins is discussed.

Introduction

Vaccinia virus (VACV) is the live vaccine used to immunize against smallpox (Fenner *et al.*, 1988) and, whilst it remains the only vaccine to have eradicated a human disease, its origin and natural host are unknown (Baxby, 1981). VACV is the most intensively studied member of the genus *Orthopoxvirus* of the family *Poxviridae* (Moss, 2007). It is distinct from cowpox virus (CPXV), the virus presumed to have been used by Edward Jenner in 1796 when he introduced vaccination against smallpox. Properties of orthopoxviruses include a dsDNA genome of approximately 200 kb, a cytoplasmic replication cycle, the production of two distinct forms of infectious virus particle called intracellular mature virus (IMV) and extracellular enveloped virus (EEV), virus-encoded enzymes for transcription and DNA replication, and many virulence factors that modulate the innate immune response to infection.

Although smallpox was declared eradicated in 1980, interest in VACV has continued because of the development of VACV as a vector for the expression of foreign genes, and because VACV represents an excellent model for studying virus–host interactions. In the early 1980s, recombinant VACV strains expressing foreign genes were constructed (Mackett *et al.*, 1982; Panicali & Paoletti, 1982) and shown to have potential application as new live vaccines (Panicali *et al.*, 1983; Smith *et al.*, 1983a, b; Paoletti *et al.*, 1984) that could induce both antibody and T-cell responses to the foreign antigen (Bennink *et al.*, 1984). The capacity of VACV for foreign DNA is at least 25 kb (Smith & Moss, 1983) and therefore multiple foreign genes can be expressed simultaneously to create a polyvalent vaccine (Perkus *et al.*, 1985). Apart from the use of recombinant VACVs as live vaccines, these viruses are valuable research tools that have been used, for instance, to identify which virus antigens are recognized by CTLs (Yewdell *et al.*, 1985; Bennink *et al.*, 1986, 1987; McMichael *et al.*, 1986; Osman *et al.*, 1999) and to identify the human immunodeficiency virus (HIV) co-receptor fusin (CXCR4) (Feng *et al.*, 1996). Whilst the concept of reusing VACV as a vaccine for other infectious diseases was attractive, the imperfect safety record associated with its use (Lane *et al.*, 1969) necessitated improvements in safety before its reuse in man. To this end, researchers sought to identify VACV virulence genes and then remove these from the genome to create stably attenuated vaccines. This search and other approaches led to the discovery of the many VACV proteins that inhibit the innate response to infection, the subject of this review. For earlier reviews of this topic, see Smith (1994), Smith *et al.* (1997b), Moss & Shisler (2001), Alcamí (2003) and Seet *et al.* (2003).

The linear dsDNA genome of VACV and other orthopoxviruses may be divided into a conserved central region of about 100 kb that encodes proteins that are mostly

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essential for virus replication, and more variable terminal regions, which encode non-essential factors affecting virulence, host range and immunomodulation and represent about half the virus genes (Gubser *et al.*, 2004). The proteins encoded by these terminal genes have diverse functions, but many target the innate immune system and can be grouped according to which component they target. The function of some of these proteins was inferred from amino acid similarity with cellular proteins of known function. For instance, the VACV complement protein (VCP) (Kotwal & Moss, 1988), the soluble IL-1 receptor (Smith & Chan, 1991; Alcamí & Smith, 1992) and the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme (Moore & Smith, 1992). In other cases, however, the protein function was deduced by experimentation or determination of its three-dimensional structure, comparison with protein structure databases and experimentation. The study of these proteins has established some general principles. First, these immunomodulatory proteins are very numerous and VACV devotes between one-third and one-half of its genetic coding capacity to them. Secondly, VACV has many gene families that indicate duplication and diversification of an ancestral gene (Goebel *et al.*, 1990; Smith *et al.*, 1991). Thirdly, the great majority of these proteins are expressed early during infection to combat the innate immune system rapidly, although a notable exception is the soluble IL-1 receptor (IL-1R), which is expressed late during infection (Alcamí & Smith, 1992). Fourthly, there are several examples where multiple proteins apparently target the same cellular pathway and yet loss of each protein individually alters the virus phenotype *in vivo*, indicating that the proteins have non-redundant functions. Lastly, a protein may have multiple immunomodulatory functions.

This review focuses largely on proteins from VACV rather than poxviruses in general and this is dictated by length restrictions. Thus, we regret it has not been possible to cite all articles on this topic; however, many of the principles learnt by VACV are applicable to these other viruses, and in some cases reference is made to other orthopoxviruses. Different aspects of the innate immune system, and how VACV interferes with these, are described in turn, and the review concludes with a consideration of the influence of virus immunomodulators on virus virulence and immunogenicity, and future prospects for development of therapeutics and vaccines.

Complement

The complement system represents an important host defence that can destroy viruses or virus-infected cells and promote the phagocytosis of virions that have been opsonized by antibody via recognition of the Fc region of bound immunoglobulin. To counteract complement, VACV expresses an abundant, 35 kDa protein called VCP that is secreted from infected cells and contains four short consensus repeats that are characteristic of the family of complement control proteins (Kotwal & Moss, 1988). VCP binds to complement components C3b and C4b and functions as a co-factor with factor I in promoting cleavage of C3b and C4b and thereby blocks activation of the complement cascade by either the classical or alternative pathway (McKenzie *et al.*, 1992). VCP contributes to virus virulence because a VACV strain engineered to lack the *VCP* gene is attenuated *in vivo* (Isaacs *et al.*, 1992a). More recently, it was reported that VCP binds to the A56 protein (also called the haemagglutinin), which is present on the surface of infected cells and EEV particles (DeHaven *et al.*, 2010). This interaction helps defend cells and EEV particles against complement. A second VACV protein, B5, is also a member of the complement protein family and has four short consensus repeats (Takahashi-Nishimaki *et al.*, 1991). B5 is a type I integral membrane glycoprotein and is present in the EEV outer envelope (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992b), but no role in protection against complement has been described. Instead, B5 is needed for the formation of EEV and thereby promotes virus dissemination (Engelstad & Smith, 1993; Wolffe *et al.*, 1993; reviewed by Smith *et al.*, 2002; Roberts & Smith, 2008). VACV has another defence against complement that is mediated by acquisition of host complement control proteins CD46, CD55 and CD59 in the EEV envelope (Vanderplasschen *et al.*, 1998). The presence of CD55, in particular, helps the EEV particle escape destruction by complement and explains the greater resistance of EEV to complement compared with IMV particles, which lack these host complement proteins and are very sensitive to destruction by complement.

These virus defences against complement are beneficial but not absolute. For instance, it is known that, whilst the EEV particle is more resistant to complement than IMV in the absence of specific antibody (Vanderplasschen *et al.*, 1998), the EEV particle can be neutralized by antibody directed against the A33 protein if complement is present (Lustig *et al.*, 2004), and neutralization via antibodies to B5 is enhanced by, or dependent on, complement (Benhnia *et al.*, 2009). Furthermore, the protective efficacy of antibody to EEV is mediated by complement and Fc receptors (Cohen *et al.*, 2011).

Interferons

Interferons (IFNs) are species-specific, secreted glycoproteins with potent antiviral effects. They were discovered in 1957 (Isaacs & Lindenmann, 1957; Isaacs *et al.*, 1957) and are grouped into three classes. Type I IFNs include IFN-α and IFN-β, and others such as IFN-κ, IFN-δ and IFN-ω, and all exert their effects via the type I IFN receptor, which is expressed ubiquitously. Type II IFN (IFN-γ) is secreted by activated immune cells [principally natural killer (NK) and T-cells], and activates macrophages and promotes a cell-mediated [T-helper 1 (Th1)] adaptive immune response. Type III IFNs (IFN-λs) are also secreted in response to virus infection, but the type III IFN receptor

has a more limited tissue distribution. Type I, II and III IFNs each play important roles in anti-VACV defence, as illustrated by the different *in vivo* responses to infection when these IFNs, or their receptors (IFNRs), are either absent from the host or expressed by the virus (Kohonen-Corish *et al.*, 1990; van den Broek *et al.*, 1995; Bartlett *et al.*, 2005).

The IFN response is initiated upon sensing of viral pathogen-associated molecular patterns (PAMPs) by host cell pattern recognition receptors (PRRs) (Fig. 1). These include membrane-associated Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), such as RIG-I and melanoma differentiation antigen 5 (MDA5) that detect foreign cytoplasmic RNA, and putative cytosolic dsDNA sensors, such as absent in melanoma 2 (AIM2), RNA polymerase III, DNA-dependent activator of IFN-regulatory factors (DAI), IFN-inducible factor 16 (IFI16), DNA protein kinase (DNA-PK) and cyclic GMP-AMP synthase (cGAS) (reviewed by Paludan & Bowie, 2013). Engagement of PRRs induces signalling cascades that culminate in the activation of transcription factors, such as IFN-regulatory factors (IRF) 3 and IRF7, NF-κB and activator protein 1 (AP-1), their translocation into the nucleus and transcription of genes encoding type I IFNs (notably IFN-β), cytokines and chemokines. IRF3, NF-κB and AP-1 together form a complex called the enhanceosome that binds to the IFN-β promoter and stimulates transcription (Wathelet *et al.*, 1998). IRF7 induces the transcription of IFN-α genes as well as the IFN-β gene (Marie *et al.*, 1998), and is responsible for the high levels of IFN-αs secreted by specialized plasmacytoid dendritic cells (Kato *et al.*, 2005). IFNs are secreted from the cell and then engage their cognate receptor on the same cell (autocrine signalling) or neighbouring cells (paracrine signalling), and thereby initiate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling cascades. Type I and III IFNs activate signalling pathways that lead to the formation of a complex called IFN-stimulated gene factor 3 (ISGF3) (comprising STAT1, STAT2 and IRF9) (Fig. 1). ISGF3 activates transcription of IFN-stimulated genes (ISGs) by binding to IFN-stimulated response elements (ISREs) present in the promoters of these genes. Type II IFN (IFN-γ) signals via STAT1 homodimers that activate transcription by binding to γ-activated sequences (GAS) in a subset of ISG promoters. For a review of JAK/STAT signalling, see Stark & Darnell (20120. Thus, IFN induces the co-ordinated expression of several hundred ISGs [including protein kinase R (PKR), 2′-5′-oligoadenylate synthase (OAS), ISG15 and Mx proteins] that together confer an antiviral state. PKR is activated by dsRNA and then phosphorylates the eukaryotic translation initiation factor (eIF)2α, thereby causing inhibition of protein synthesis. OAS synthesizes 2′-5′-oligoadenylates upon its activation by dsRNA, and these activate RNase L, which degrades viral and cellular mRNAs (Silverman, 2007). The ubiquitin-like protein ISG15 becomes conjugated to a range of proteins, including IRF3, RIG-I, human MxA and PKR, and stabilizes these targets against degradation (Zhao *et al.*, 2005; Lu *et al.*, 2006; Kim *et al.*, 2008), thus modulating the IFN-stimulated antiviral response. ISG15 may also be secreted from cells and contribute to defence against microbial infection (Bogunovic *et al.*, 2012). For a review of IFNs, their antiviral activity and virus evasion strategies see Randall & Goodbourn (2008).

Early studies indicated that VACV was able to interfere with IFNs or their mechanism(s) of action. Forty years ago, it was noted that vesicular stomatitis virus (VSV) replication was inhibited by prior treatment of susceptible cells with IFN. However, if the cells were treated with IFN and then co-infected with VSV and VACV, VSV replication was restored (Thacore & Youngner, 1973). This implied that VACV was providing some factor(s) *in trans* that overcame the IFN-mediated blockade of VSV replication. Later, it was demonstrated that this factor prevented PKR and OAS activation (Whitaker-Dowling & Youngner, 1983; Paez & Esteban, 1984), bound dsRNA and was encoded by the *E3L* gene (Chang *et al.*, 1992). Other early studies revealed that VACV mutants with large deletions in the terminal regions of the virus genome had increased sensitivity to IFN, and that the presence of IFN would prevent formation of such deletion mutant viruses (Paez & Esteban, 1985), suggesting that these regions encode proteins that antagonize the antiviral action of IFNs.

It is now known that viruses inhibit the IFN response at multiple levels, and many of these are illustrated by VACV (Fig. 1). First, VACV restricts IFN production by minimizing the production or recognition of PAMPs, expressing proteins that block the PRR-induced signalling pathways that activate transcription factors leading to IFN induction, and by inhibiting host protein synthesis. Secondly, VACV secretes proteins from the infected cell to capture IFNs in solution or on the cell surface, and prevent engagement of IFN receptors by IFNs. Thirdly, VACV blocks signal transduction induced by IFNs binding to their receptors. Finally, additional VACV proteins act within the infected cell to inhibit the antiviral action of IFN-induced proteins. Individual VACV proteins can possess multiple anti-IFN activities, as exemplified by the E3 protein, which inhibits B-DNA-stimulated IFN-β transcription in mouse embryonic fibroblasts (Wang *et al.*, 2008), sensing of RNAs produced by DNA-dependent RNA polymerase transcription of AT-rich DNA (Marq *et al.*, 2009; Valentine & Smith, 2010) and the antiviral action of ISG15 in mouse embryonic fibroblasts (Guerra *et al.*, 2008).

Blocking induction of IFNs

The simplest way of avoiding the antiviral activity of IFNs is to prevent their production, and VACV utilizes several mechanisms to accomplish this. The first is to minimize the production of dsRNA, an important PAMP leading to IFN production. To do this, the VACV genome is arranged with

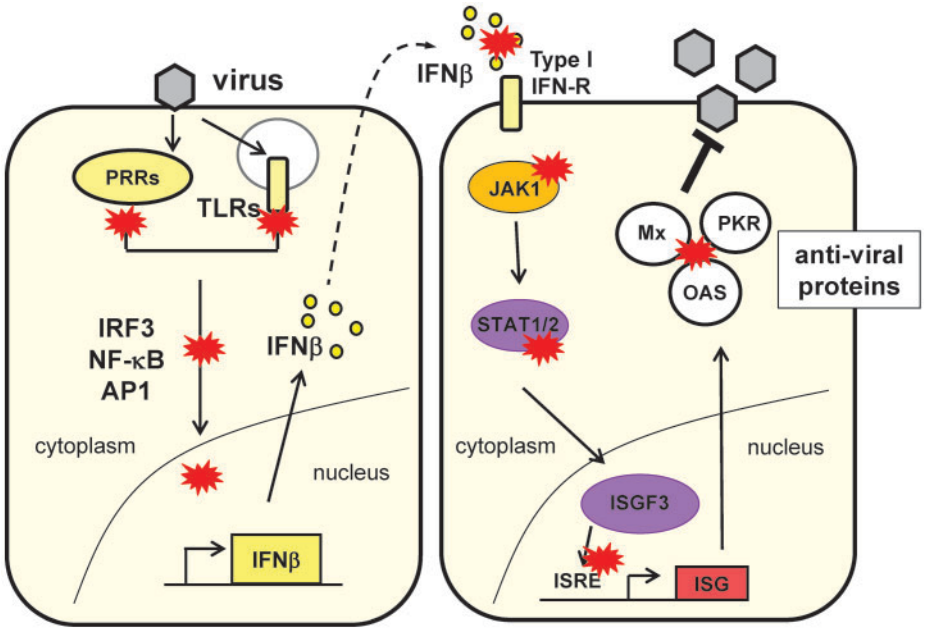

Fig. 1. IFN signalling and its antagonism by VACV. A virus infection is sensed by PRRs within the cytosol or in endosomes, and these trigger signalling cascades leading to activation of transcription factors NF-κB, IRF3 and AP-1. These enter the nucleus and stimulate transcription of the *IFN*-β gene. IFN-β is then secreted from the cell and binds to the type I IFNR on the same or adjacent cells. This triggers activation of the JAK/STAT pathway leading to assembly of the ISGF3 complex in the nucleus and the transcription of hundreds of ISGs. The expression of these proteins, such as Mx, PKR and OAS, in the cytoplasm renders the cell more resistant to subsequent virus infection. The positions at which viral proteins can inhibit the production or action of IFN are shown by red stars, and many of these are illustrated by VACV proteins. This figure is adapted from an excellent review on IFNs and their antiviral activities by Haller *et al.* (2006). See main text for abbreviations.

genes located at the left or right terminal region of the genome being transcribed predominantly outward towards the genomic terminus. With transcription from only one DNA strand in these regions, dsRNA formation is prevented. In the central region of the genome, although genes are transcribed equally from both strands, genes are generally arranged in blocks with leftward and rightward transcribed blocks of genes alternating. Where there is convergent transcription, especially early during infection, there are multiple intergenic transcriptional termination sequences to prevent the formation of overlapping transcripts and so diminish dsRNA formation (Smith *et al.*, 1998).

A second strategy is to inhibit host protein synthesis, so that production of host IFNs and other pro-inflammatory molecules is diminished shortly after infection. VACV inhibits host protein synthesis within a few hours of infection (Moss & Salzman, 1968). A contributory factor is the increased degradation of host mRNAs (Rice & Roberts, 1983) mediated by the D9 and D10 proteins, which recognize mRNAs and cleave off their 5′ methylated caps (Parrish & Moss, 2006, 2007; Parrish *et al.*, 2007). These proteins also cause turnover of VACV mRNAs, thereby promoting a switch from early to intermediate and late protein synthesis. Although most host mRNAs are down-regulated during infection (Brum *et al.*, 2003; Guerra *et al.*, 2004), VACV can preserve or increase some cellular functions by activating host gene expression, for instance, genes controlled by the transcription factor hypoxia-inducing factor (HIF)-1α. Early after infection, VACV strain Western Reserve (WR) protein C16 binds and inhibits prolyl hydroxylase domain-containing protein 2 (PHD2) (Mazzon *et al.*, 2013). PHD2 is an oxygen sensor and under normoxic conditions can hydroxylate and induce proteosomal degradation of HIF-1α. However, once PHD2 is inhibited by C16, HIF-1α remains stable and moves into the nucleus to activate genes containing a HIF-responsive element, so mimicking a hypoxic response under normoxic conditions (Mazzon *et al.*, 2013).

A third strategy is to prevent binding of PAMPs by PRRs. The VACV E3 protein sequesters dsRNA via a C-terminal dsRNA-binding domain and thereby prevents activation of dsRNA-binding PRRs (Chang *et al.*, 1992). E3 also blocks the production of IFN following sensing of AT-rich dsDNA by RNA polymerase III, because this leads to RNA formation, which is bound by E3 (Marq *et al.*, 2009; Valentine & Smith, 2010). E3 has an N-terminal Z-DNA-binding domain of unknown function, and both this and the C-terminal RNA-binding domain contribute to virulence (Brandt & Jacobs, 2001; Kim *et al.*, 2003). VACV also encodes a protein to block the recognition of

cytosolic DNA by DNA-PK, which was shown recently to be a PRR for IRF3-dependent innate immunity (Ferguson *et al.*, 2012). The C-terminal region of VACV strain WR protein C16 binds the Ku complex (part of DNA-PK) and thereby blocks the recruitment of DNA-PK to DNA and inhibits the induction of IRF3-dependent chemokines, cytokines and IFN (Peters *et al.*, 2013).

A fourth strategy is to express proteins that act downstream of PRR binding to PAMPs to block the intracellular signalling pathways leading to IRF3 or NF-κB activation that are required for IFN induction (see Figs 2 and 3, discussed below). There are many such proteins and they are grouped according to the pathway they target.

Inhibitors of NF-κB activation

VACV intracellular proteins that inhibit NF-κB activation are numerous and include A46, A49, A52, B14, C4, E3, K1, K7, M2 and N1. These proteins are all expressed early during infection but inhibit NF-κB activation at differing stages in the signalling pathway (Fig. 2). Protein A46 binds to several Toll/IL-1R (TIR) domain-containing adaptor molecules [myeloid differentiation primary response gene 88 (MyD88), Myd88 adaptor-like (MAL), TIR-domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM)] that associate with the cytoplasmic tails of TLRs (Bowie *et al.*, 2000; Stack *et al.*, 2005). Binding to these multiple ligands enables A46

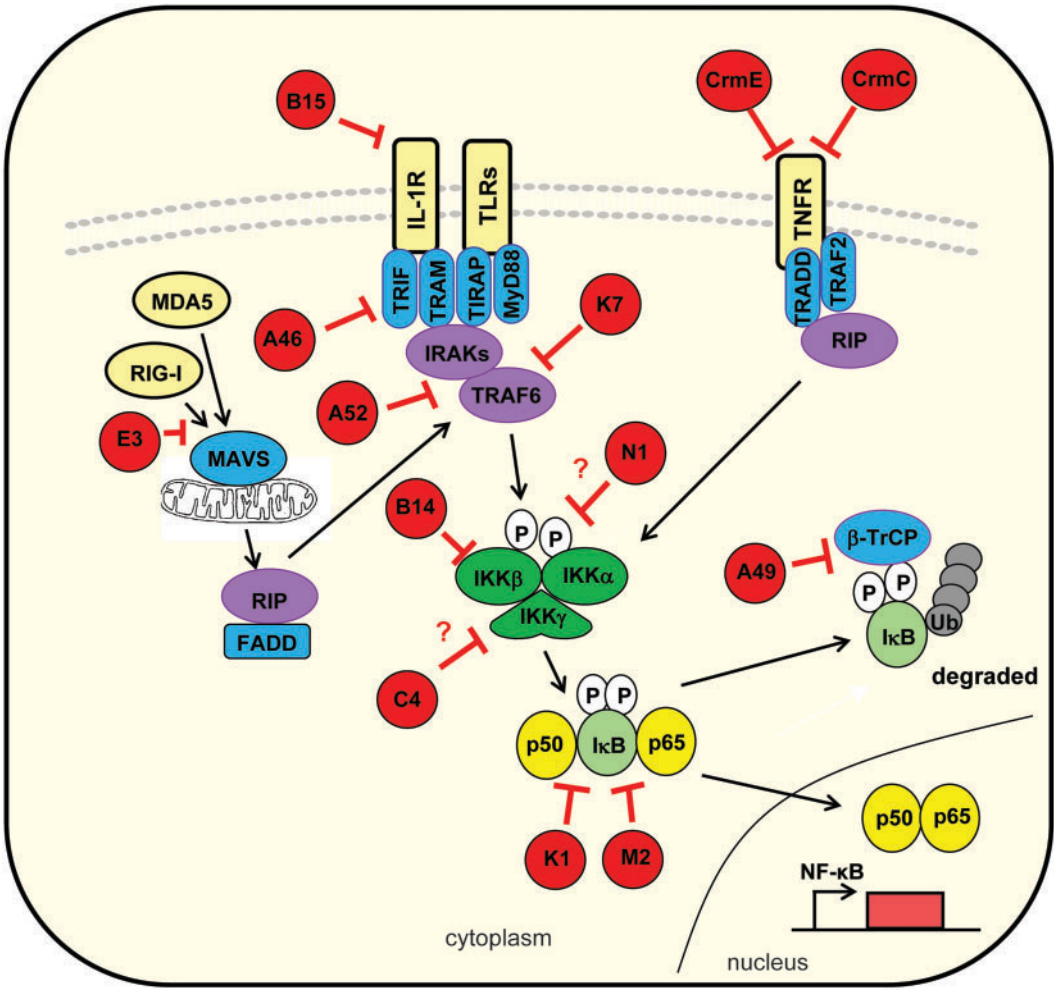

Fig. 2. NF-κB signalling pathway and its antagonism by VACV. The pathway leading to activation of NF-κB can be activated by external or internal signals. The pathway illustrated here is activated by TNF binding to the TNFR, IL-1 binding to the IL-1R or TLR ligands binding to TLRs. Engagement of these receptors by their ligands leads to signalling cascades that converge on the IKK complex and results in phosphorylation of IKKα or IKKβ on serine residues in their activation loops. Once IKKβ (in particular) is activated, it phosphorylates the inhibitor of κB (IκBα) leading to its recognition by the E3 ubiquitin ligase β-TrCP, and its ubiquitination and degradation by the proteasome. This releases the NF-κB subunits, which then translocate into the nucleus and induce transcription from NF-κB-responsive genes, such as those encoding cytokines, chemokines and IFNs. The sites of action of VACV proteins B15, CrmC, CrmE, E3, A46, A52, K7, B14, N1, C4, A49, K1 and M2 that inhibit the NF-κB pathway are shown and are described further in the text.

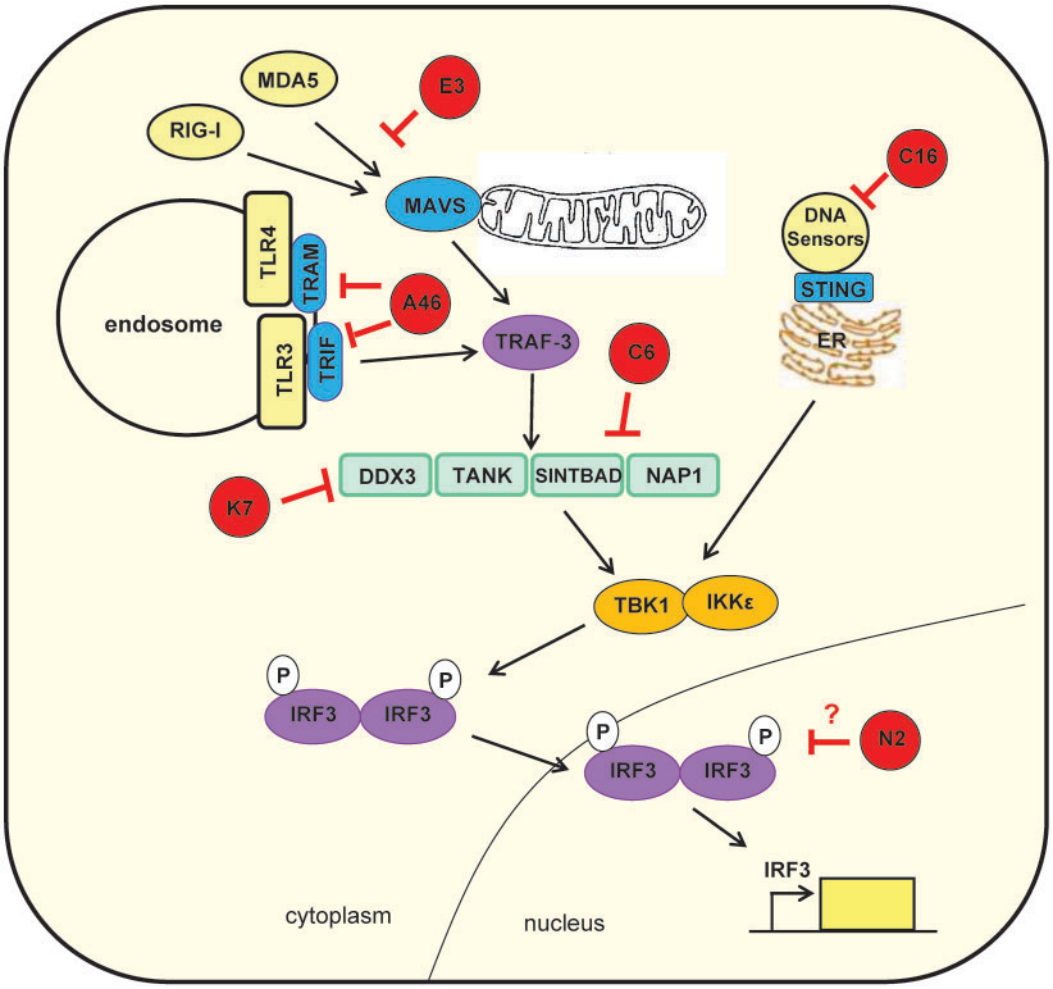

Fig. 3. IRF3 signalling pathway and its antagonism by VACV. RNA sensing by RIG-I or MDA5, and DNA sensing by RNA polymerase III induces signalling via the mitochondrial-associated adaptor MAVS (also known as IPS-1, CARDIF and VISA). Signalling from other cytoplasmic DNA sensors such as DNA-PK and IFI16 involves the adaptor protein STING. Signalling from TLR3 (that is endosomal) and TLR4 (found at the plasma membrane and in endosomes) requires the adaptor TRIF, whilst TRAM acts as a bridging adaptor between TRIF and TLR4. The IRF3 kinases, TBK1 and IKKε, are activated subsequent to activation of TRAF3, and form complexes with the proteins NAP1, SINTBAD, TANK and DDX3. Upon phosphorylation, IRF3 undergoes dimerization and nuclear translocation. Within the nucleus, activated IRF3 binds to IRF3-binding sites within the promoters of IFN-β and other IRF3-dependent genes. The sites of action of VACV proteins E3, C16, A46, K7, C6 and N2, which inhibit the IRF3 pathway, are shown and are described further in the text.

to inhibit activation of mitogen-activated protein (MAP) kinases, NF-κB and IRF3 and, therefore, the induction of IFN-β. Interactions with TRIF and TRAM also enable A46 to block TLR3/4-mediated activation of IRF3 (Fig. 3). The A52 protein also acts early in the activation pathway and inhibits IL-1 and TLR-induced activation of NF-κB by binding to IL-1R-associated kinase 2 (IRAK2) and tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Bowie *et al.*, 2000; Harte *et al.*, 2003). The structure of protein A52 has been solved and revealed a B-cell lymphoma-2 (Bcl-2) fold (Graham *et al.*, 2008) that is conserved in several other VACV proteins that function as inhibitors of innate immune signalling pathways (see below). Protein K7 is also a member of the Bcl-2 family (Kalverda *et al.*, 2009) and binds to IRAK2 and TRAF6 and suppresses TLR-dependent NF-κB activation (Schröder *et al.*, 2008; Oda *et al.*, 2009). K7 also binds the DEAD-box RNA helicase 3 (DDX3) and inhibits PRR-induced IFN-β induction by preventing IRF3 activation (Schröder *et al.*, 2008). These three proteins (A46, A52 and K7) inhibit TLR- and IL-1β-mediated NF-κB activation, but not NF-κB activation mediated by TNF (Fig. 2). In contrast, all the other VACV proteins targeting NF-κB activation act at, or downstream of, the convergence of IL-1- and TNF-mediated signalling and so inhibit both pathways.

Protein N1 inhibits NF-κB activation (DiPerna *et al.*, 2004), but the report that it binds to the inhibitor of κB

kinase (IKK) complex has been disputed (Chen *et al.*, 2008) and its mechanism of action remains uncertain. N1 is dimeric (Bartlett *et al.*, 2002) and has a Bcl-2 fold (Aoyagi *et al.*, 2007; Cooray *et al.*, 2007), and mutagenesis of the N1 dimer surface inhibits both dimerization and the ability to block NF-κB activation, suggesting that either N1 acts as a dimer or it uses the dimer interface to block NF-κB (Maluquer de Motes *et al.*, 2011).

Protein B14 binds to IKKβ and prevents its phosphorylation and activation by upstream kinases, thereby blocking IKKβ-mediated phosphorylation of the inhibitor of κB (IκBα) (Chen *et al.*, 2008). The structure of B14 also revealed a Bcl-2 fold (Graham *et al.*, 2008), and targeted mutation of an exposed hydrophobic surface eliminated binding of IKKβ and inhibition of NF-κB activation (Benfield *et al.*, 2011). Protein C4 inhibits activation of NF-κB at, or downstream of, the IKK complex by an unknown mechanism (Ember *et al.*, 2012). Protein E3 inhibits NF-κB activation in both PKR-dependent and -independent manners (Myskiw *et al.*, 2009) and also by antagonizing the RNA polymerase III–dsDNA sensing pathway (Marq *et al.*, 2009; Valentine & Smith, 2010).

Recently, protein A49 was shown to stabilize phosphorylated IκBα by molecular mimicry (Mansur *et al.*, 2013). Near the N terminus of A49, there is a short sequence containing conserved serine residues that are present in IκBα and that, in IκBα, are phosphorylated by IKKβ. Normally, once IκBα is phosphorylated by IKKβ, p-IκBα is recognized and ubiquitinated by the E3 ubiquitin ligase β-transducing repeat-containing protein (β-TrCP) leading to p-IκBα degradation by the proteasome. A49 functions by binding to β-TrCP so that, even if IκBα is phosphorylated, it is not recognized and ubiquitinated by β-TrCP, and p-IκBα remains bound to NF-κB in the cytoplasm (Mansur *et al.*, 2013). Protein K1 was also reported to inhibit NF-κB signalling in rabbit kidney epithelial cells by preventing the degradation of IκBα, but the mechanism remains unclear (Shisler & Jin, 2004). Finally, protein M2 reduces extracellular signal-regulated kinase 2 (ERK2) phosphorylation induced by phorbol myristate acetate and prevents p65 nuclear translocation (Gedey *et al.*, 2006).

Despite inhibiting the NF-κB activation pathway at multiple places, these VACV proteins are non-redundant, because loss of any individual protein gives an *in vivo* phenotype (see below).

Inhibitors of IRF3 activation

The kinases TANK-binding kinase 1 (TBK1) and IKKε lie at the convergence of several pathways leading to IRF3 activation (Fig. 3), and thus provide an attractive target for viral antagonism of IFN production. For example, the VACV C6 protein interacts with the scaffold adaptor proteins for these kinases, NAK-associated protein 1 (NAP1), TRAF family member-associated NF-κB activator (TANK) and similar to NAP1 TBK1 adaptor (SINTBAD), and prevents TBK1/IKKε-dependent activation of IRF3 and IRF7 (Unterholzner *et al.*, 2011). A related VACV protein, K7, binds to DDX3, and led to the discovery that DDX3 is an adaptor of TBK1/IKKε and can also directly bind the IFN-β promoter (Schröder *et al.*, 2008; Soulat *et al.*, 2008; Oda *et al.*, 2009). Thus, K7 antagonizes both TLR-dependent and -independent IFN-β activation. As noted earlier, protein C16 inhibits IRF3 activation by disrupting recognition of dsDNA by DNA-PK (Peters *et al.*, 2013). In addition, as mentioned above, protein A46 binds to TRIF, TRAM, MAL and Myd88 and so can also block IRF3 activation (Stack *et al.*, 2005). Lastly, protein N2 inhibits IRF3 activation downstream of IRF3 phosphorylation and nuclear translocation via an unknown mechanism (Ferguson *et al.*, 2013).

Collectively, this wide array of proteins that block IRF3 and NF-κB activation provides a profound block on IFN-β production from VACV-infected cells.

Blocking IFN reaching its receptors

The strategies outlined above are effective means by which VACV can restrict the production of IFN from infected cells. To combat IFNs produced from uninfected cells, VACV has another strategy, namely the release of decoy IFN receptors from the infected cells (Fig. 4).

VACV strain WR protein B18 is a type I IFN-binding protein and acts both in solution and when associated with the cell surface via glycosaminoglycans (GAGs) to sequester type I IFNs (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Alcamí *et al.*, 2000; Montanuy *et al.*, 2011). B18 binds type I IFNs from a broad range of species (Symons *et al.*, 1995); however, it only inhibits IFN-α efficiently and defence against IFN-β relies more on intracellular proteins (Waibler *et al.*, 2009). This strategy is logical because IFN-α (leukocyte IFN) is produced largely, and in high levels, by activated dendritic cells that are recruited to sites of inflammation and so require neutralization outside the cell by a decoy receptor. In contrast, IFN-β (fibroblast IFN) is also produced by fibroblasts and epithelial cells before leukocytes are recruited to the site of infection and so can be targeted by inhibition of signalling pathways leading to IFN-β promoter activation. The ability of B18 to bind to the surface of cells enables it to prevent the IFN-mediated antiviral state in uninfected cells, which therefore remain susceptible to virus replication as it spreads.

Type II IFN is also neutralized via a VACV soluble decoy IFN receptor, protein B8, which binds IFN-γ outside cells and, unlike the cellular IFN-γR, can also dimerize in the absence of IFN-γ (Alcamí & Smith, 2002). The B8 protein shares amino acid similarity to the cellular IFN-γR but lacks the transmembrane and cytoplasmic domains found in the cellular IFN-γR (Upton *et al.*, 1992; Alcamí & Smith, 1995; Mossman *et al.*, 1995), and this enabled its function to be predicted. A structural study of the closely related IFN-γ-binding protein from ectromelia virus (ECTV, the causative agent of mousepox) showed that the complex of

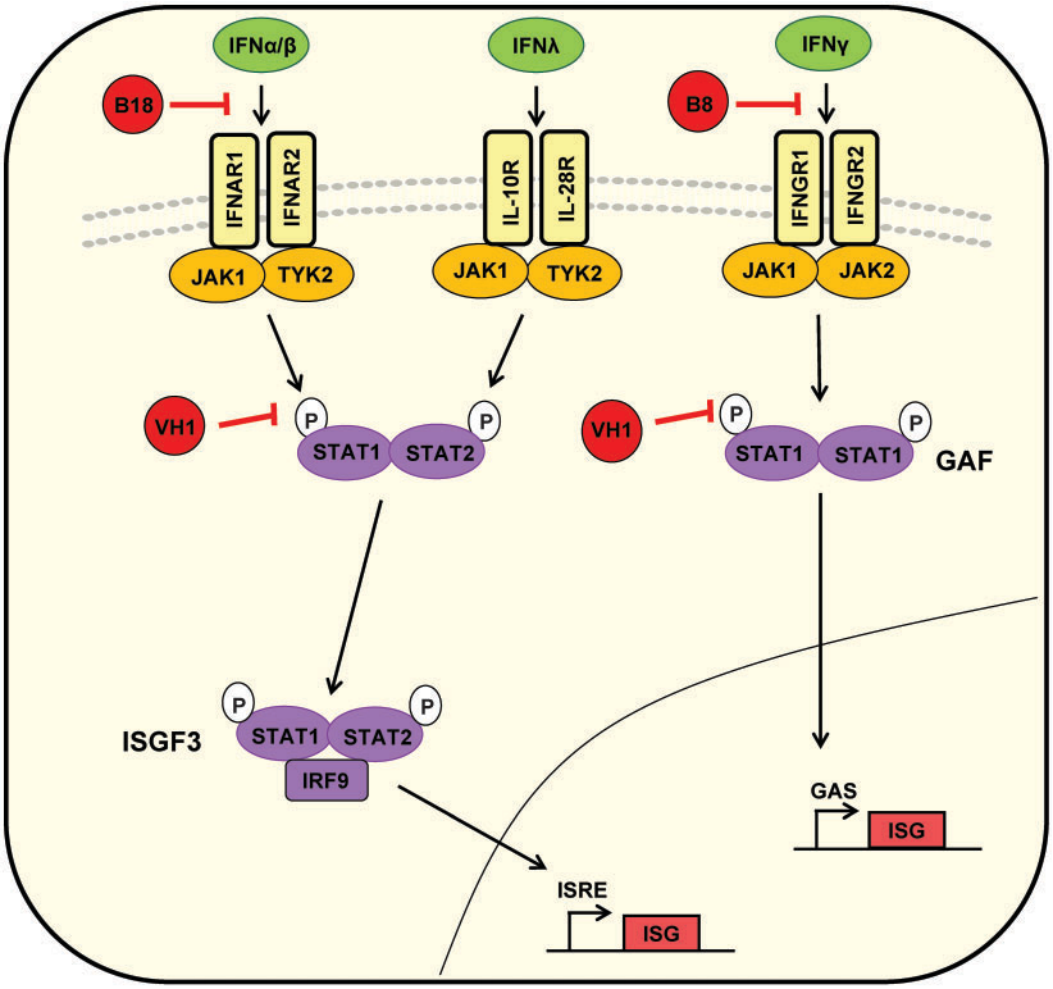

Fig. 4. IFN-induced signalling pathways and their inhibition by VACV. Type I, II and III IFNs bind to their receptors on cells and induce signalling via the JAK/STAT pathways. Binding of type I or type III IFN causes activation of the JAK1/TYK2 kinases, and these then recruit and phosphorylate STAT1 and STAT2. Once phosphorylated, STAT1 and STAT2 form a heterodimer that associates with IRF9 forming ISGF3, which activates transcription of hundreds of ISGs with an ISRE. Type II IFN-stimulated phosphorylation of JAK1/JAK2 causes phosphorylation of STAT1. STAT1 homodimers (GAF complex) translocate into the nucleus and bind to GAS elements. VACV inhibits the actions of IFNs both intracellularly and extracellularly. The mechanisms of action of VACV extracellular proteins B8 and B18 and intracellular protein VH1 are illustrated and described in the text.

the ECTV IFN-γ-binding protein and IFN-γ consisted of four molecules of the ECTV protein in complex with two IFN-γ dimers, and that tetramerization of the ECTV IFN-γ-binding protein is mediated by a C-terminal helix–turn–helix domain (Nuara *et al.*, 2008).

The study of these decoy IFN receptors provided an opportunity to investigate the unknown origin of VACV because of the species-specific nature of IFNs and their receptors. It seemed logical that VACV would have evolved proteins to bind and neutralize IFNs from its natural host species, and therefore studying the binding of B18 and B8 to type I or type II IFNs from different species might indicate a natural host for VACV. However, both B18 and B8 bound type I or type II IFNs from many different species, although they bound less well to murine IFNs (Alcamí & Smith, 1995; Mossman *et al.*, 1995; Symons *et al.*, 1995). This broad IFN specificity did not reveal a likely host for VACV but did indicate that these proteins would be useful to help VACV combat IFNs in many hosts and thereby help facilitate a broad host range (Alcamí & Smith, 1996b). It has been established that both B18 (Symons *et al.*, 1995) and B8 (Symons *et al.*, 2002b) contribute to VACV virulence where the host IFN is bound by the virus protein. Interestingly, the yatapoxvirus, Yaba-like disease virus, encodes protein Y136, which is related to VACV WR B18 but can inhibit both type I and type III IFNs (Huang *et al.*, 2007).

Inhibiting IFN-induced signal transduction

Another mechanism to block the action of IFNs is to inhibit signal transduction induced by IFN binding to IFN

receptors. Signalling events within the JAK/STAT pathway that are elicited in response to the binding of type I, II or III IFNs are shown in Fig. 4.

The VACV protein VH1 is a virion-associated phosphatase that is brought into infected cells with the infecting virion and rapidly dephosphorylates STAT1 and STAT2, thereby inhibiting signalling from all IFN receptors (Najarro *et al.*, 2001; Mann *et al.*, 2008). Given the redundancy in inhibitors of the IRF3 and NF-κB activation pathways, it is quite possible that other VACV inhibitors of the JAK/STAT signalling pathway exist.

Inhibiting IFN-induced antiviral proteins

Finally, VACV also encodes proteins that counter the effects of proteins encoded by ISGs. The E3 dsRNA-binding protein sequesters dsRNA to prevent activation of PKR, OAS and other dsRNA sensors. E3 also binds ISG15 and prevents its antiviral action (Guerra *et al.*, 2008). Another example is VACV protein K3, which has amino acid similarity to eIF2α, and acts as a non-phosphorylatable pseudo-substrate for PKR and thus competitively inhibits the phosphorylation of eIF2α by PKR (Beattie *et al.*, 1991; Carroll *et al.*, 1993). Lastly, the ability of the host-range genes C7 and K1 to support VACV replication in permissive cell lines is thought to be due to antagonism of currently undefined IFN-induced antiviral factor(s) (Meng *et al.*, 2009).

Cytokines

Pro-inflammatory cytokines such as TNF, IL-1 and IL-18 amplify the innate immune response to virus infection and shape the adaptive immune response to follow. TNF also has direct antiviral activity by the induction of apoptosis of infected cells (see below). VACV has evolved strategies to block these cytokines at multiple levels in a manner analogous to the inhibition of IFN. These include the inhibition of proteolytic maturation of specific cytokines, expression of soluble decoy receptors and inhibition of cytokine-induced signal transduction. These principles are illustrated with proteins targeting IL-1, IL-18 and TNF.

Targeting IL-1

IL-1 is a pro-inflammatory cytokine that activates NF-κB and leads to induction of inflammatory mediators such as cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). It exists in three forms, IL-1α and IL-1β, which can both activate IL-1R-mediated signalling, and the IL-1R antagonist protein, which binds to the IL-1R without signalling (reviewed by Dinarello, 2006). The production and secretion of IL-1β usually requires two signals; a first signal, often NF-κB activity, induces the transcription and synthesis of an inactive intracellular form, pro-IL-1β. VACV can therefore inhibit the synthesis of pro-IL-1β by inhibiting NF-κB (see above). The second signal is required to activate caspase-1; this can cleave pro-IL-1β to form IL-1β, which is then secreted from the cell. In response to virus infection, this second signal is provided by the activation of inflammasomes, large protein complexes containing PRRs, which activate caspase-1. Various inflammasomes, which are activated by different molecular stimuli, can detect VACV infection and contribute to IL-1β production (Hornung *et al.*, 2009; Gerlic *et al.*, 2013), and VACV has evolved mechanisms to inhibit this process. The F1 protein can bind directly to the nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein 1 (NLRP1) inflammasome and reduce the amount of active caspase-1 and IL-1β secretion (Gerlic *et al.*, 2013). In addition, to prevent pro-IL-1β cleavage within infected cells, the VACV strain WR protein B13 [also called serine proteinase inhibitor 2 (SPI-2) and cytokine response modifier A (CrmA) in CPXV] binds caspase-1 and blocks pro-IL-1β cleavage (Ray *et al.*, 1992; Dobbelstein & Shenk, 1996; Kettle *et al.*, 1997). This has the additional function of preventing caspase-1-induced cell death. Not all VACV strains have a functional *B13R* gene, and in several strains, including Copenhagen and Lister, the B13 protein is not produced (Kettle *et al.*, 1995).

The strategy of preventing IL-1β formation is restricted to infected cells, and so uninfected cells recruited to infected tissue can produce IL-1β freely. To combat IL-1β from this source and any IL-1β that escapes blocking strategies from infected cells, VACV strain WR expresses a soluble IL-1R that is released from infected cells to capture IL-1β in solution (Alcamí & Smith, 1992; Spriggs *et al.*, 1992). Protein B15 shows amino acid similarity to the extracellular ligand-binding domain of the cellular IL-1R (Smith & Chan, 1991) and binds IL-1β with high affinity, preventing IL-1β from binding its natural receptor (Alcamí & Smith, 1992; Spriggs *et al.*, 1992). A study of the contribution of the B13 and B15 proteins to virus virulence showed that loss of the B13 protein did not alter virulence in the murine intranasal model (Kettle *et al.*, 1995) but caused an increase in lesion sizes after intradermal infection (Tscharke *et al.*, 2002). In contrast, a virus lacking the *B15R* gene had reduced virulence after intracranial infection (Spriggs *et al.*, 1992) and caused an accelerated weight loss and the induction of fever after intranasal infection (Alcamí & Smith, 1996a). B15 only bound IL-1β, and not IL-1α, TNF or IFNs, and therefore the ability to block fever was due to inhibition of IL-1β only. This finding contributed to the conclusion that IL-1β is the principal endogenous pyrogen (Alcamí & Smith, 1996a).

In addition to preventing the production of IL-1β from infected cells and the capturing of IL-1β in solution, VACV expresses multiple proteins that can block IL-1-induced activation of NF-κB. These have been described already under IFNs. A remarkable feature of the multiple inhibitors of NF-κB activation is that the removal of A46, A49, A52, B14, C4 and N1 separately causes an *in vivo* phenotype (Bartlett *et al.*, 2002; Harte *et al.*, 2003; Stack *et al.*, 2005; Chen *et al.*, 2006; Ember *et al.*, 2012; Mansur *et al.*, 2013),

indicating that these proteins have non-redundant functions. This may be due to a protein having multiple functions (for instance, N1 can block apoptosis and NF-κB activation; Maluquer de Motes *et al.*, 2011), multiple binding partners (e.g. A46; Stack *et al.*, 2005) or being able to inhibit the pathway at different stages. In the latter case, cross-talk between signalling pathways can lead to other signalling pathways being inhibited, or remaining unaffected, depending upon where inhibition of NF-κB activation is mediated.

Targeting IL-18

IL-18 (originally termed IFN-γ-inducing factor) is also a member of the IL-1 family and is a pleiotropic, pro-inflammatory cytokine (Gracie *et al.*, 2003). Like IL-1β, IL-18 is synthesized as an inactive precursor that requires cleavage by caspase-1, and the intracellular signalling pathway induced by binding of IL-18 to the IL-18R has similarity with IL-1β-induced signalling. IL-18 facilitates clearance of virus-infected cells by inducing IFN-γ synthesis and activating NK and $CD8^+$ T-cells (Gracie *et al.*, 2003), and its importance for defence against viruses is illustrated by IL-18 administration enabling recovery from infection with VACV or herpes simplex virus type 1 (Fujioka *et al.*, 1999; Tanaka-Kataoka *et al.*, 1999). VACV has been predicted, or shown, to inhibit IL-18 action in three ways. First, the formation of mature IL-18 is predicted to be inhibited by B13, which inhibits caspase-1. Secondly, VACV and several other orthopoxviruses secrete a soluble IL-18-binding protein (IL-18BP), called protein C12, that binds IL-18 in solution and thereby stops IL-18 reaching the IL-18R on cells (Born *et al.*, 2000; Smith *et al.*, 2000; Calderara *et al.*, 2001; Symons *et al.*, 2002a). Unlike protein B15, which is related to the IL-1R, the VACV IL-18BP is unrelated to the cellular IL-18R but shows amino acid similarity to the host IL-18BP, a negative regulator of IL-18 action. Protein C12 enhances VACV virulence by diminishing the production of IFN-γ, leading to reduced NK cell and VACV-specific $CD8^+$ T-cell responses (Reading & Smith, 2003). Thirdly, VACV inhibits IL-18-induced signal transduction by expressing intracellular proteins that block NF-κB or IRF activation (see above).

Targeting TNF

TNF-α and TNF-β, also called lymphotoxin α (LT-α), are related cytokines that have pro-inflammatory activity and also can cause cell death. TNF-α is the most studied member, and has been implicated in tumour regression, rheumatoid arthritis, septic shock and cachexia (Locksley *et al.*, 2001). The predominant source of TNF-α is thought to be macrophages and activated T-cells, and its pleiotropic activity is attributed to the promotion of an antiviral state in uninfected neighbouring cells, selective cytolysis of virus-infected cells, apoptosis and recruitment of lymphocytes to foci of infection (Smith *et al.*, 1994). Therefore, it is unsurprising that viruses have evolved anti-TNF strategies. These include the secretion of VACV proteins that serve as decoy TNF receptors (vTNFRs) to sequester extracellular TNF and preclude cellular receptor binding (reviewed by Alcamí, 2003), and also proteins that interfere with intracellular signalling downstream of host-cell TNFRs (described above). In addition, TNF is itself an NF-κB target gene and thus intracellular VACV inhibitors of NF-κB also affect TNF production.

The majority of VACV strains, including Copenhagen and WR, encode gene fragments related to *vTNFR* genes but do not encode functional TNF-binding proteins (Goebel *et al.*, 1990; Howard *et al.*, 1991; Smith *et al.*, 1991). However, VACV strains USSR, Lister and Evans express soluble and cell-surface vTNFRs encoded by the *CrmC* (A53R) and *CrmE* genes (Alcamí *et al.*, 1999). These decoy vTNFRs exhibit amino acid similarity to the extracellular domains of cellular TNFRs but lack a membrane anchor and the C-terminal cytoplasmic domain necessary for signal transduction (Alcamí *et al.*, 1999). The crystal structure of CrmE confirmed that it has the canonical TNFR fold but showed that it is closer to the type II TNFR (p75) rather than type I TNFR (p55) and contains only one of the two ligand-binding loops of TNFRSF1A, suggesting an explanation for the higher affinity of poxvirus TNFRs for TNF-α than LT-α (Graham *et al.*, 2007). Furthermore, VACV CrmE is a virulence factor, because VACV strain USSR mutants lacking CrmE were attenuated in a mouse model of infection, and a recombinant strain of VACV WR expressing CrmE had increased virulence in the same model (Reading *et al.*, 2002). Although the expression of vTNFRs is restricted to a few strains of VACV, CPXV strains express multiple TNFRs, some of which have additional properties (see below) (Hu *et al.*, 1994; Smith *et al.*, 1996).

Chemokines

Chemokines are small chemoattractant cytokines that recruit leukocytes to the site of infection or inflammation (Rot & von Andrian, 2004; Charo & Ransohoff, 2006). Chemokines are produced in response to infection or inflammation and bind to GAGs on the surface of adjacent endothelial cell walls, where a concentration gradient is established. Circulating leukocytes express chemokine receptors on their cell surface that bind to chemokines via their chemokine receptor-binding site, and thereby recruit leukocytes to sites of infection (Fig. 5). There are many different chemokines, which are grouped according to the position of conserved cysteine residues (C, CC or CXC, where X is another amino acid), and also many chemokine receptors. A single chemokine may bind to several different chemokine receptors and, equally, a single chemokine receptor may bind several different chemokines. Chemokine production from the first cells infected by an invading virus is important for induction of the host response to infection, and without chemokine-mediated recruitment of leukocytes, the ability of a host to fight infection is diminished.

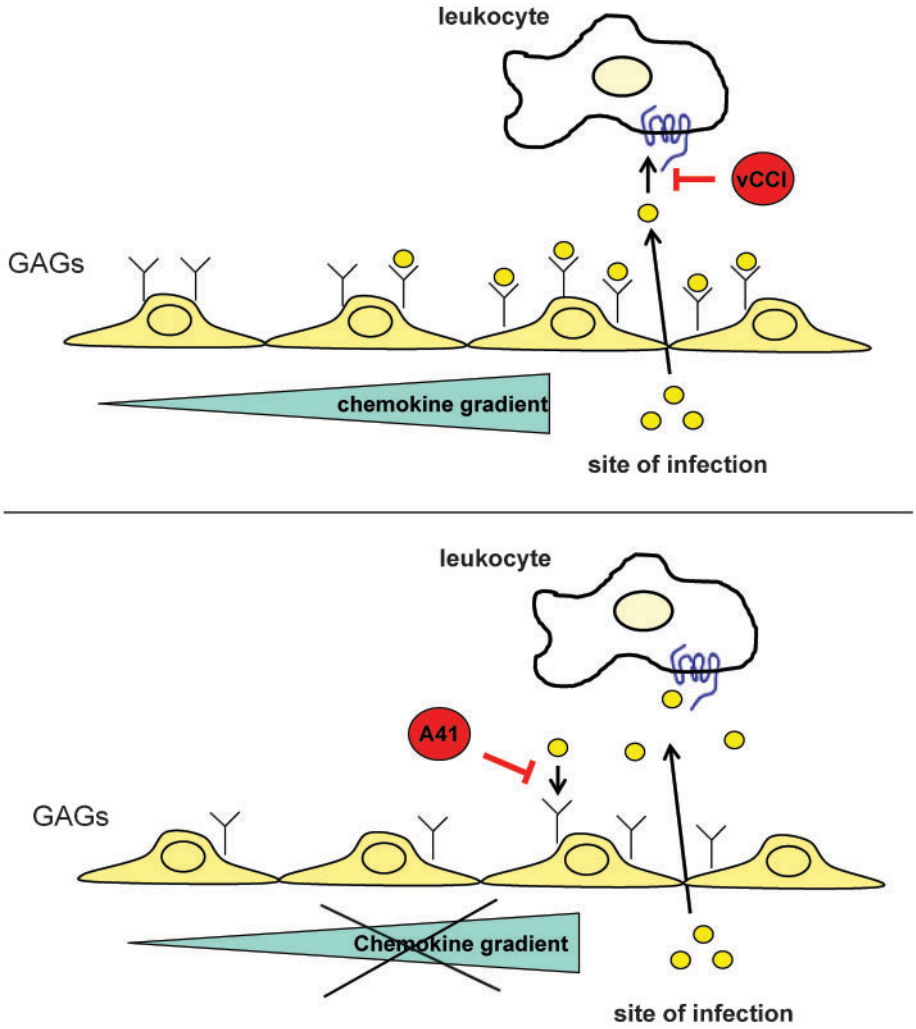

Fig. 5. Mechanisms of chemokine inhibition by VACV. Chemokines are released from sites of infection and establish concentration gradients on the endothelial cell wall by binding to GAGs. The chemokines then bind to and activate circulating leukocytes, which are recruited to the site of infection. VACV protein vCCI prevents binding of CC chemokines to chemokine receptors on leukocytes (top), whereas protein A41 prevents the establishment of the chemokine concentration gradient (bottom). See text for details.

VACV has several ways to inhibit the production or function of chemokines. After infection, PRR engagement leads to IRF3 and NF-κB activation and the transcription of many chemokine genes driven by these transcription factors. For instance, whilst infection with VACV strain MVA induces the expression of several chemokines (Lehmann *et al.*, 2009), infection with VACV strains WR, Wyeth and Lister (Elstree) does not (Lehmann *et al.*, 2009), owing to their expression of immunomodulatory proteins that block intracellular signalling (see above) leading to chemokine production, such as of CXCL10 (Ferguson *et al.*, 2012).

Secondly, VACV expresses chemokine-binding proteins. The first to be identified, called VACV chemokine-binding protein (vCKBP) or VACV CC chemokine inhibitor (vCCI), binds CC chemokines and is secreted from infected cells early during infection (Graham *et al.*, 1997; Smith *et al.*, 1997a; Alcamí *et al.*, 1998). vCCI is made by some but not all VACV strains and binds a range of CC chemokines with pM affinity, such that it is able to prevent these chemokines binding to their receptors (Alcamí *et al.*, 1998). The structures of the orthologue of vCCI from CPXV (Carfi *et al.*, 1999) and from VACV strain rabbitpox in complex with CCL4 (macrophage inflammatory protein 1β) have been solved (Zhang *et al.*, 2006). The latter study provided insight into how vCCI binds CC chemokines with high affinity and broad specificity. A second protein, A41, shows amino acid similarity to vCCI and is also secreted from infected cells early during infection (Ng *et al.*, 2001). A41 binds chemokines (CCL21, CCL25, CCL26 and CCL28) with lower affinity (between 8 and 118 nM) and is unable to prevent chemokines binding to chemokine

receptors due to this low affinity (Bahar *et al.*, 2008). A41 has a similar structure to vCCI, but there are surface charge differences and an extended acidic loop that may contribute to chemokine-binding specificity (Bahar *et al.*, 2008). A41 binding to chemokines was inhibited by heparin, indicating that the heparin and chemokine-binding sites overlap and suggesting that the mechanism of action of A41 is via the disruption of chemokine concentration gradients on endothelial cells (Bahar *et al.*, 2008) (Fig. 5). A parallel study with the closely related ECTV protein E163 came to broadly similar conclusions (Ruiz-Arguello *et al.*, 2008).

The role of A41 and vCCI *in vivo* is to diminish inflammation by preventing recruitment of leukocytes to the site of infection. Thus, when vCCI is expressed from VACV strain WR, which lacks this protein normally (Alcamí *et al.*, 1998), the recombinant virus is less virulent in a murine intranasal model in which pathology is associated with excessive inflammation (Reading *et al.*, 2003b). For A41, a VACV WR strain lacking this gene produced larger lesion sizes than controls in a murine intradermal model, but the infectious virus was cleared more rapidly due to greater recruitment of leukocytes (Ng *et al.*, 2001).

VACV strain WR proteins B7 and B23 were predicted to have chemokine-binding activity because they share sequence similarity with protein E184 from ECTV, protein V216 from CPXV and the C-terminal domain from TNFRs CrmB and CrmD from CPXV and variola virus, which have been demonstrated to bind chemokines (Alejo *et al.*, 2006). Interestingly, the structure of the chemokine-binding domain of CrmD from ECTV in complex with CX3CL1 showed that this domain shares structural similarity with the vCCI and A41 proteins (Xue *et al.*, 2011).

Apoptosis

Apoptosis is an irreversible cascade of biochemical events orchestrated by caspase proteases that culminates in cell death and represents a powerful mechanism to eliminate virus-infected cells (Tait & Green, 2010). Activation of apoptosis by the intrinsic pathway is controlled by a complex network of protein–protein interactions between pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins. In response to apoptotic signals, pro-apoptotic proteins such as Bad or Bid bind the effector proteins Bax and Bak, which oligomerize in the outer mitochondrial membrane inducing release of cytochrome *c* into the cytoplasm and formation of the caspase activation platform or apoptosome (Fig. 6). VACV directly subverts the intrinsic pathway by expressing proteins F1 and N1. Protein F1 adopts a Bcl-2-like fold (Kvansakul *et al.*, 2008) and binds Bak at the mitochondrion (Wasilenko *et al.*, 2003, 2005; Postigo *et al.*, 2006; Kvansakul *et al.*, 2008). F1 also reduces the inflammatory response by binding NLRP-1, an upstream activator of caspase-1 (Gerlic *et al.*, 2013). Protein N1 also has a Bcl-2 fold (Aoyagi *et al.*, 2007; Cooray *et al.*, 2007) and contains a surface groove that enables it to bind pro-apoptotic Bcl-2 proteins Bid and Bad and to inhibit apoptosis (Cooray *et al.*, 2007; Maluquer de Motes *et al.*, 2011). N1 was also reported to bind peptides containing Bcl-2 homology domain 3 (BH3) domains of pro-apoptotic Bcl-2 proteins Bid, Bim and Bak *in vitro* (Aoyagi *et al.*, 2007). Other studies found no interaction between N1 and Bax or N1-mediated inhibition of apoptosis (Banadyga *et al.*, 2009; Postigo & Way, 2012). Nonetheless, mutagenesis of N1 showed that loss of binding to Bid and Bad correlated with loss of anti-apoptotic activity whilst retaining anti-NF-κB activity (Maluquer de Motes *et al.*, 2011). Interestingly, for both F1 and N1, inhibition of innate immune signalling was more important for virus virulence than inhibition of apoptosis (Maluquer de Motes *et al.*, 2011; Gerlic *et al.*, 2013).

In addition to these viral Bcl-2-like proteins, VACV strain WR B13 (SPI-2) (Smith *et al.*, 1989; Kettle *et al.*, 1995) inhibits caspase-1 activity as well as extrinsic apoptosis triggered by TNF-α or Fas ligand (Dobbelstein & Shenk, 1996; Kettle *et al.*, 1997). The CPXV orthologue of B13 (CrmA) inhibits caspases from group I (caspase-1, -4 and -5) and group III (caspase-8, -9 and -10) (Ray *et al.*, 1992; Zhou *et al.*, 1997; García-Calvo *et al.*, 1998), and similar specificity is expected for VACV protein B13 due to conservation of the reactive site. This may explain how B13 inhibits both extrinsic apoptosis and the host inflammatory response. Furthermore, VACV inhibitors such as the dsRNA-binding protein E3 can show protective effects on cells when neutralizing pro-apoptotic effects of external stimuli like dsRNA (Kibler *et al.*, 1997; García *et al.*, 2002). Lastly, a few VACV strains, such as Evans, and also camelpox virus (Gubser & Smith, 2002) express a protein called viral Golgi anti-apoptotic protein (vGAAP) that is closely related (73 % amino acid identity) to a human orthologue, hGAAP (Gubser *et al.*, 2007). GAAPs are highly conserved throughout evolution, being present in all eukaryote genomes examined, and are part of the transmembrane Bax inhibitor-containing motif (TMBIM) family, which includes other anti-apoptotic and Ca^{2+}-modulating membrane proteins such as Bax inhibitor I. vGAAP and hGAAP have a six-membrane topology (Carrara *et al.*, 2012), form oligomers (Saraiva *et al.*, 2013a) and modulate calcium in intracellular stores (de Mattia *et al.*, 2009). hGAAP also promotes cell adhesion and migration via the stimulation of store-operated Ca^{2+} entry and calpain 2 (Saraiva *et al.*, 2013b). hGAAP and vGAAP can protect cells from apoptosis deriving from both intrinsic and extrinsic pathways (Gubser *et al.*, 2007). vGAAP and B13 are only expressed by the minority of VACV strains, whereas proteins F1 and N1 are more widely conserved. Nonetheless, deletion of any of these proteins gives an *in vivo* phenotype (Bartlett *et al.*, 2002; Tscharke *et al.*, 2002; Gubser *et al.*, 2007; Gerlic *et al.*, 2013).

NK cells

NK cells are a specialized population of granular lymphocytes displaying high cytolytic activity against virus-infected and tumour cells. They serve as a first line of defence against

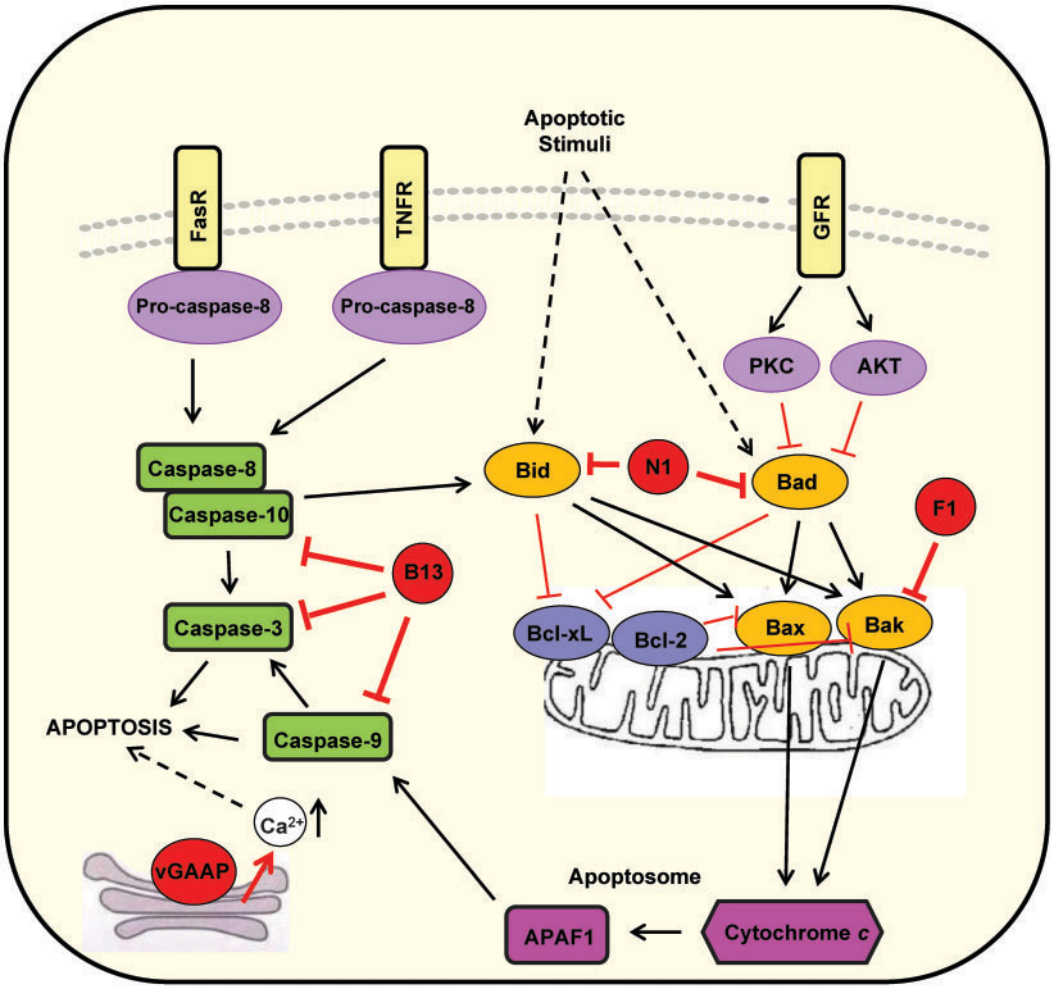

Fig. 6. Pathways of apoptosis and their inhibition by VACV. Apoptosis can be induced by signals from either outside (extrinsic) or inside (intrinsic) the cell. The release of cytochrome *c* and activation of the apoptotic protease activator factor 1 (APAF1) from the mitochondrion to form the apoptosome is central in the induction of apoptosis. The apoptosome then activates a series of initiator and executioner caspases that lead to apoptosis. External stimuli like the Fas ligand or TNF can activate caspases directly. In non-apoptotic cells, the engagement of Fas receptor (FasR) or TNF receptor (TNFR), or intrinsic signals resulting from the withdrawal of growth factors, lead to activation of the pro-apoptotic proteins Bid and Bad. These then sequester anti-apoptotic proteins Bcl-2 and Bcl-$_{xL}$ enabling formation of Bax/Bak-mediated mitochondrial pores. This causes cytochrome *c* release from the mitochondrion. VACV protein N1 targets Bid and Bad upstream in the signalling cascade, whereas VACV protein F1 neutralizes Bak in the mitochondrial membrane. VACV protein B13 inhibits caspase-1 and the apoptotic processes where it is involved. vGAAP is an integral membrane protein present in the Golgi and causes a reduction in Ca^{2+} loading of intracellular stores, so that less Ca^{2+} can be released by pro-apoptotic pathways.

viral infections before the development of a virus-specific antibody and CTL response. NK cell activation depends on cytokines such as IFN-α, IFN-β, IL-12 and IL-18 but is mostly regulated by a repertoire of activating and inhibitory receptors that integrate signals emanating from the surface of target cells (Lanier, 2005). Most NK inhibitory receptors recognize MHC class I molecules, and this ensures tolerance against self-antigens. In contrast, CD16, NKG2D and the natural cytotoxicity receptors NKp46, NKp44 and NKp30 are mostly involved in the activation of NK cells (Lanier, 2005).

It is well known that NK cells play an important role against poxvirus infections: for instance, the resistance of B6 mice to ECTV maps to genes involved in activation and regulation of NK cell responses (Delano & Brownstein, 1995), and depletion of NK cells with anti-asialo GM1 or anti-NK1.1 antibodies increases susceptibility to VACV (Bukowski *et al.*, 1983; Martinez *et al.*, 2010). In addition, VACV infection induces proliferation and accumulation of NK cells at the site of infection (Natuk & Welsh, 1987; Dokun *et al.*, 2001; Jacobs *et al.*, 2006), and VACV can modulate NK cell activation by modulating production of

cytokines and IFN (see above). VACV infection increases cell susceptibility to NK lysis (Brutkiewicz *et al.*, 1992; Baraz *et al.*, 1999; Chisholm & Reyburn, 2006), and in specific cases this has been attributed to selective downregulation of HLA-E or C-type lectin related protein b (Clr-b) (Brooks *et al.*, 2006; Williams *et al.*, 2012). Protection against VACV challenge can also be mediated by passive transfer of NK cells from VACV-infected mice lacking classical adaptive T- and B-lymphocytes (Gillard *et al.*, 2011).

Susceptibility to NK cells occurs after virus-mediated downregulation of MHC class I, a process employed by viruses to escape recognition by CTLs. Whereas VACV is known to modestly decrease MHC class I expression (Brutkiewicz *et al.*, 1992; Kirwan *et al.*, 2006), it seems that only downregulation of HLA-E is sufficient to induce lysis by NK cells (Brooks *et al.*, 2006). Studies *in vitro* have shown that NK cell recognition of VACV-infected cells depends on the natural cytotoxicity receptor (Chisholm & Reyburn, 2006). Recently, the VACV haemagglutinin (protein A56) was identified as a novel viral ligand for the activating receptors NKp30 and NKp46 (Jarahian *et al.*, 2011). Upon binding to A56, NKp46 triggered NK cytotoxicity, but NKp30 activity was neutralized, which exemplifies the delicate interplay between VACV and its host. The same study revealed minimal alteration of NKG2D ligands after VACV infection of human cells. This is in contrast to other studies demonstrating a key role for NKG2D in the control of VACV (Martinez *et al.*, 2010) or ECTV (Fang *et al.*, 2008, 2011) infection *in vivo*. This indicates that different NK receptors are responsible for the control of genetically similar poxvirus species and strains in different hosts. To date, A56 is the only described VACV direct modulator of NK cell activity, although removal of several VACV proteins, including N1, F3, C12 and K7, altered the NK response to infection and this is probably due to their ability to block intracellular signalling cascades leading to production of cytokines or chemokines (Reading & Smith, 2003; Froggatt *et al.*, 2007; Jacobs *et al.*, 2008; Benfield *et al.*, 2013). Other VACV mechanisms to evade NK recognition may exist.

The influence of VACV immunomodulators on virulence and immunogenicity

Virulence

Many of the VACV immunomodulatory genes described above are non-essential for virus replication in cell culture but affect virulence *in vivo*, where the innate immune response plays an important role in determining the outcome of infection. Two murine models of infection are used routinely to study VACV virulence: the intradermal model where inoculation of the ear pinnae causes a localized infection resulting in the formation of a lesion (Tscharke & Smith, 1999), and the intranasal infection route, which results in a systemic infection causing mice to lose weight (Williamson *et al.*, 1990). In addition, virulence has also been investigated using intracranial and intraperitoneal injection, dermal scarification to the base of the tail, or by using rabbits and guinea pigs. The removal or inactivation of most immunomodulatory genes leads to reduced virus virulence in either the intranasal or intradermal infection model (Table 1). In many instances, attenuation is accompanied by increased immune cell infiltrates to the site of infection and/or decreased viral titres after the initial virus replication (see Table 1 for references). In some cases deletion of immunomodulatory genes leads to attenuation in more than one model of infection (Tscharke *et al.*, 2002). Further work is required to understand why these differences exist. In certain instances, the removal of an immunomodulatory gene can lead to an increase in virulence; for example, VACV strain WR viruses engineered to lack the A41 (Ng *et al.*, 2001; Clark *et al.*, 2006) or B15 (Alcamí & Smith, 1992; 1996a) proteins, or to express vCCI/vCKBP (Reading *et al.*, 2003b), produce immune pathology.

Immunogenicity

The adaptive immune system includes antibody ('humoral') and cell-mediated responses that are mediated by antibody-secreting B-lymphocytes and cytotoxic ($CD8^+$) and Th1 ($CD4^+$) T-lymphocytes, respectively. Adaptive immunity is characterized by its delayed onset, targeting of pathogen-specific antigens and immunological memory, and is the basis for vaccination. Both humoral and $CD4^+$ and $CD8^+$ T-cell responses are induced by VACV infection in mice (Xu *et al.*, 2004) and humans (Ennis *et al.*, 2002; Crotty *et al.*, 2003; Hammarlund *et al.*, 2003; Kennedy *et al.*, 2004; Pütz *et al.*, 2005, 2006; Moutaftsi *et al.*, 2010). Efforts to dissect the relative roles of these responses in the mouse model using antibody depletion and gene knockout showed that the $CD4^+$ T-cell-dependent antibody response is more important for viral clearance, whilst $CD8^+$ T-cells limit disease severity and mediate protective memory upon reinfection (Xu *et al.*, 2004). T-cell responses are evidently important for the response to infection with VACV because individuals with defects in cellular immunity are at risk of serious complications from vaccination (Lane *et al.*, 1969). For a review of the response to smallpox vaccination, see Moss (2011).

VACV is being studied intensely as a vaccine vector and, in particular, the highly attenuated VACV strains MVA and NYVAC (Tartaglia *et al.*, 1992) are being tested as vaccines for several diseases including AIDS, malaria, tuberculosis and some cancers (Gomez *et al.*, 2008; Walsh & Dolin, 2011). MVA is a promising vaccine vector due to its safety record, efficient expression of foreign antigens and ability to induce a robust immune response (Sutter & Moss, 1992). MVA does not replicate fully in most mammalian cell lines (Sutter & Moss, 1992; Blanchard *et al.*, 1998; Okeke *et al.*, 2006) and is a safe vaccine even in immune-compromised mammals (Wyatt *et al.*, 2004).

MVA was generated by the passage of chorioallantois VACV Ankara more than 500 times in chicken embryo fibroblasts, resulting in the loss of large portions of the

genome and substantial attenuation of the virus (Meyer *et al.*, 1991; Antoine *et al.*, 1998). Despite these deletions, a significant number of immunomodulatory genes remain in the MVA genome (Antoine *et al.*, 1998; Blanchard *et al.*, 1998). However, retention of an ORF does not ensure that the function of the encoded protein is retained, as illustrated with protein 183 (equivalent to VACV WR protein B14), which is rendered unstable by an internal deletion of 5 aa (McCoy *et al.*, 2010). Removal of the chemokine-binding protein encoded by *A41L* (Clark *et al.*, 2006) or the IL-1β-binding protein encoded by *B15R* (WR) (Staib *et al.*, 2005) led to enhanced VACV-specific total $CD8^+$ T-cell responses and enhanced protection against a challenge with virulent VACV WR. The deletion of *A35R*, encoding an inhibitor of MHC class II antigen presentation, from MVA also resulted in enhanced immunogenicity, with increased numbers of VACV-specific IFN-γ-secreting splenocytes, enhanced immunoglobulin production and class switching to IgG isotypes (Rehm & Roper, 2011). Deletion of the IL-18-binding protein, *C12L* (also from MVA), led to increased $CD8^+$ and $CD4^+$ T-cell responses to various VACV epitopes and provided enhanced protection against WR challenge (Falivene *et al.*, 2012). Furthermore, a deletion mutant of *C6L* from the WR strain of VACV led to enhanced VACV-specific cytotoxicity of T-cells and provided better protection against challenge with a lethal dose of WR (Sumner *et al.*, 2013).

Recently, several VACV vectors have been generated for immunization trials against HIV infection and their immunogenicity has been investigated. These include MVA-B, a vector expressing four HIV antigens and engineered to lack either both *A41L* and *B15R* (García-Arriaza *et al.*, 2010) or the IRF3 inhibitor *C6L* (García-Arriaza *et al.*, 2011). These viruses induced HIV-1-specific $CD4^+$ and $CD8^+$ T-cell immune responses of enhanced magnitude and polyfunctionality. For the *C6L* deletion mutant, enhanced levels of antibodies against the HIV Env protein were also observed. Furthermore, individual and double deletion mutants of the type I (B18) and type II (B8) IFN-binding proteins from NYVAC-C (which expresses the HIV-1 Env, Gag, Pol, and Nef antigens) improved HIV-1-specific $CD8^+$ T-cell responses (Gomez *et al.*, 2012). Finally, an MVA vector expressing codon-optimized HIV subtype C Env and Gag antigens and lacking four or five viral immunomodulators, including C12, B15, a dominant negative Toll/IL-1 signalling adaptor, a CC chemokine-binding protein and a gene encoding uracil-DNA glycosylase, elicited higher frequencies of HIV-specific $CD8^+$ and $CD4^+$ T-cells and higher titres of Env-specific antibodies in immunized rhesus macaques (Garber *et al.*, 2012). Whether these VACV-based HIV vaccine vectors provide enhanced protection against infection remains to be determined.

Despite promising data with the recombinant VACVs described above, deletion of immunomodulatory genes does not always enhance immunogenicity. For instance, a VACV WR lacking *C16L* (Fahy *et al.*, 2008) or *N2L* (Ferguson *et al.*, 2013) and a VACV Wyeth lacking regions encoding the *B5R*, *B8R*, *B12R*, *B13R*, *B14R*, *B16R*, *B18R* and *B19R* genes, a number of which are immunomodulators (Jackson *et al.*, 2005), showed unaltered immunogenicity under the conditions tested. It is likely that immunogenicity will reflect a balance between efficient recognition of virus antigens and the level of antigens expressed during infection (Wyatt *et al.*, 2008). Understanding why the removal of certain immunomodulators enhances immunogenicity, whilst that of others does not, remains a goal of future research and will aid in the rational design of more efficacious vaccine vectors.

Future prospects

Therapeutics

As well as having applications for the development of vaccines, progress towards understanding how VACV evades and disrupts the immune system has important implications for the design and production of novel therapeutics. These can be split into three categories: (i) using modified forms of the virus; (ii) using recombinant viral proteins; and (iii) designing compounds that mimic the specific activity of viral proteins. The first two categories have examples that are now in clinical trials, whilst the third category is at a less advanced stage.

Other than as a vaccine, the therapeutic use of VACV as an oncolytic agent shows promise. Oncolytic viruses are those that can selectively, or preferentially, replicate in and kill tumour cells (Thorne, 2011). VACV has several advantages for this type of therapy, such as its broad tissue tropism, its ability to spread rapidly, the considerable experience with its use clinically during the eradication of smallpox, and the fact that it does not integrate its DNA into the host genome (Kirn & Thorne, 2009). Use of unmodified strains of VACV, such as WR and Lister, has proved less effective than other species of oncolytic viruses due to their lack of tumour specificity (Kirn & Thorne, 2009). However, a greater understanding of VACV immunogenicity and immune evasion mechanisms is enabling the design of modified viruses that have greater efficacy against cancer (Kirn & Thorne, 2009; Schmidt, 2011; Thorne, 2011). For example, the JX-594 virus, which is a thymidine kinase-negative VACV engineered to express granulocyte–macrophage colony stimulating fctor (GM-CSF), specifically lyses neoplastic cells and has undergone phase II clinical trials for the treatment of solid tumours such as advanced hepatocellular carcinoma (Breitbach *et al.*, 2011; Heo *et al.*, 2013). Furthermore, the removal of certain VACV immune evasion genes, such as B18, which inhibits type I IFN, improved its oncolytic potential, suggesting that immune modulation plays an important part in the activity of these viruses when attacking tumours (Kirn *et al.*, 2007; Thorne, 2011). It is probable, therefore, that this promising approach will be improved by further modifications of VACV. For example, deletion of additional immune

Table 1. VACV immune modulators affecting virulence

Strain	Protein	Function	Intranasal	Intradermal	Intracranial	References
WR	A35	Inhibitor of MHC class II antigen presentation	Attenuated			Roper (2006)
Cop (WR)	A39	Secreted glycoprotein, aa similar to semaphorins	Attenuated	Smaller lesion		Gardner *et al.* (2001)
WR	A40	Surface glycoprotein. Immune modulator	No phenotype	Smaller lesion		Wilcock *et al.* (1999); Tscharke *et al.* (2002)
WR	A41	Secreted chemokine-binding protein	Increased virulence	Larger lesion, more rapid virus clearance		Ng *et al.* (2001); Clark *et al.* (2006)
WR	A44	3β-Hydroxysteroid dehydrogenase	Attenuated	Smaller lesion		Moore & Smith (1992); Tscharke *et al.* (2002); Reading *et al.* (2003a)
WR	A46	NF-κB/IRF3 inhibitor	Attenuated			Stack *et al.* (2005)
WR	A49	NF-κB inhibitor	Attenuated	No phenotype		Mansur *et al.* (2013)
WR	A52	NF-κB inhibitor	Attenuated			Harte *et al.* (2003)
WR	A53	Soluble TNF receptor (CrmC)	Attenuated	No phenotype (rabbit)	Attenuated	Dai *et al.* (2008)
WR	A55	Intracellular kelch protein immunomodulator	No phenotype	Larger lesion		Beard *et al.* (2006)
WR	A56	NK cell agonist/antagonist	No phenotype		Attenuated	Flexner *et al.* (1987); Smith *et al.* (2002)
WR	B7	ER protein, putative chemokine-binding protein	No phenotype	Smaller lesion		Price *et al.* (2000)
WR	B8	Secreted IFNγ binding protein	No phenotype/attenuated	Histological changes in rabbit skin		Verardi *et al.* (2001); Symons *et al.* (2002b)
WR	B13	Inhibitor of caspases	No phenotype/attenuated	Larger lesion		Kettle *et al.* (1995); Tscharke *et al.* (2002); Legrand *et al.* (2004)
WR	B14	NF-κB inhibitor	No phenotype	Smaller lesion, more rapid virus clearance		Chen *et al.* (2006)
WR	B15	Secreted IL-1β binding protein	Increased virulence (high virus dose)/ attenuated (low virus dose)	No phenotype	Attenuated	Alcamí & Smith (1992, 1996a); Spriggs *et al.* (1992); Tscharke *et al.* (2002)
WR	B18	Type I IFN binding protein	Attenuated			Symons *et al.* (1995)
WR	B22	Serine protease inhibitor	No phenotype/attenuated			Kettle *et al.* (1995); Legrand *et al.* (2004)
WR	C2	Intracellular kelch protein immunomodulator	No phenotype	Larger lesion		Pires de Miranda *et al.* (2003)
WR	C4	NF-κB inhibitor	Attenuated	No phenotype		Ember *et al.* (2012)
WR	C6	IRF3 inhibitor	Attenuated	Smaller lesion		Unterholzner *et al.* (2011); Sumner *et al.* (2013)
WR & Tian Tan	C12	IL-18-binding protein	Attenuated	Smaller lesion (rabbits)	Attenuated	Symons *et al.* (2002a); Reading & Smith (2003); Dai *et al.* (2008)
WR	C16	Binds DNA-PK	Attenuated	No phenotype		Fahy *et al.* (2008)

Table 1. cont.

Strain	Protein	Function	Intranasal	Intradermal	Intracranial	References
WR	C21 VCP	Secreted complement regulatory protein		Smaller lesion (guinea pigs)		Isaacs *et al.* (1992a)
COP	E3	dsRNA-binding protein	Attenuated		Attenuated	Brandt & Jacobs (2001)
WR	F1	Anti-apoptotic, inflammasome inhibitor	Attenuated			Gerlic *et al.* (2013)
WR	F3	Intracellular kelch protein immunomodulator	No phenotype	Attenuated		Froggatt *et al.* (2007)
WR	K3	eIF2α mimic	Intratracheal infection; reduced virus titres and dissemination			Rice *et al.* (2011)
WR	K7	NF-κB/IRF3 inhibitor	Attenuated	Smaller lesion		Benfield *et al.* (2013)
WR	N1	NF-κB inhibitor/anti-apoptotic	Attenuated	Smaller lesion	Attenuated	Kotwal *et al.* (1989); Bartlett *et al.* (2002)
WR	N2	IRF3 inhibitor	Attenuated	Smaller lesion, more rapid virus clearance		Ferguson *et al.* (2013)
USSR WR	CrmE	TNF receptor	Attenuated			Reading *et al.* (2002)
Evans	vGAAP	Anti-apoptotic (Ca^{2+} regulator)	Attenuated			Gubser *et al.* (2007)

evasion genes may help the destruction of specific tumours and optimize the adaptive immune response to provide long-term remission (Wang *et al.*, 2012).

As described above, VACV secretes proteins from infected cells that bind to cytokines, chemokines and IFNs, such as B18, which binds type I IFNs (Colamonici *et al.*, 1995; Symons *et al.*, 1995). The secreted nature of such proteins makes them attractive therapeutics because they are stable outside the cell and are easy to express and purify. Such proteins may be used to treat both acute and chronic inflammatory conditions such as rheumatoid arthritis, where hyperactive or dysregulated inflammatory processes results in tissue destruction (Fallon & Alcamí, 2006), although as foreign proteins their repeated administration will eventually induce an antibody response that will restrict their activity. As an example, vCCI binds a wide range of CC chemokines (Alcamí *et al.*, 1998; Burns *et al.*, 2002) and, in an *in vivo* inflammatory model, can mediate effective reductions of eotaxin-induced eosinophilia (Alcamí *et al.*, 1998). The potential of these proteins as therapeutics is best illustrated by SERP-1, a poxvirus serine protease inhibitor (Upton *et al.*, 1990), which reduces vascular inflammation in patients with acute coronary syndromes and has undergone phase II clinical trials in patients receiving cardiac stents (Tardif *et al.*, 2010).

VACV intracellular immunomodulators are harder to deliver as therapeutic agents. The functions of several of these proteins, however, represent attractive therapeutic targets. For example, inhibitors of IKKβ are sought as therapy for treatment of inflammatory diseases such as rheumatoid arthritis and chronic obstructive pulmonary disease, as well as cancer, thanks to their potential to block NF-κB activation (Luo *et al.*, 2005; Lee & Hung, 2008). Indeed, several IKKβ kinase inhibitors are in clinical trials (Lee & Hung, 2008). As VACV WR protein B14 binds to and inhibits the activity of IKKβ (Chen *et al.*, 2008), its function might be mimicked by a small molecule to provide a novel therapeutic. This approach relies on a more detailed structural understanding of the interaction between IKKβ and B14, but as B14 is not a kinase inhibitor and instead blocks the phosphorylation of IKKβ itself (Chen *et al.*, 2008), this information may reveal a novel allosteric target site for IKKβ inhibition. In a similar vein, the function of C6 could be mimicked to provide a novel TBK1 inhibitor (Unterholzner *et al.*, 2011). TBK1 is considered a therapeutic target for rheumatoid arthritis (Hammaker *et al.*, 2012) and certain specific cancers (Migita & Nakamura, 2012). Proof of principle for this approach has been provided by the development of peptide mimetics of the VACV A52 (McCoy *et al.*, 2005) and A46 (Lysakova-Devine *et al.*, 2010) proteins. These peptides have a cell-penetrating peptide at the N terminus to allow cell entry and inhibit TLR signalling, thereby blocking inflammatory responses both *in vitro* and *in vivo* (McCoy *et al.*, 2005; Lysakova-Devine *et al.*, 2010). TLR inhibitors are sought as targets for many inflammatory disorders (Kanzler *et al.*, 2007) and compounds such as these

peptides can provide the specificity needed to inhibit specific aspects of TLR signalling necessary for such treatments.

Vaccines

Following the development of VACV as an expression vector in 1982, its potential as a vaccine vector has been investigated extensively. Although attenuated VACV strains have a good safety profile, improving immunogenicity remains a priority (Jacobs *et al.*, 2009), and this depends on a deeper understanding of the effects of VACV immunomodulators on the immune response. Deletion of immunomodulators is one strategy to increase VACV immunogenicity. As outlined above, VACV encodes many immunomodulator genes, and in several cases their deletion has increased the immune response to VACV and provided better protection against challenge.

An alternative attenuation strategy is to express host genes encoding immunomodulatory proteins in VACV. For example, expression of IL-2 (Flexner *et al.*, 1987), IFN-γ (Kohonen-Corish *et al.*, 1990), IFN-λs (Bartlett *et al.*, 2005) and IL-15 (Perera *et al.*, 2007) in VACV WR caused a reduction in virus virulence, and in the latter case there was also a greater $CD8^+$ T-cell memory response and an enhanced cellular and humoral response (Perera *et al.*, 2007). However, this strategy requires care because of the potential of some cytokines to increase virus virulence, as illustrated by the expression of IL-4 in VACV (Sharma *et al.*, 1996) and ECTV (Jackson *et al.*, 2001). A further possibility is the expression of immune-stimulating proteins that can selectively enhance recruitment and activation of specifically targeted immune cells, such as antigen-presenting cells, and thereby induce a more selective and specific immune response. For instance, increased anti-MVA antibody titre and an increased cellular immune response were obtained with an MVA strain expressing GM-CSF or CCL20 in mice (Chavan *et al.*, 2006).

In spite of the large number of studies and applications of VACV, a clear understanding of the immunological basis of its protective properties is lacking (Moss, 2011). The immunogenicity of VACV is striking considering the large number of immunosuppressive proteins expressed by the virus. Recent studies have started unravelling the fundamental mechanisms by which the innate immune system orchestrates the adaptive response and protective response to vaccination (Pulendran & Ahmed, 2006). The study of VACV immune evasion may help identify specific molecular signatures in the early response to vaccination that correlate with the development of a protective immune response, or indicate how different subsets of T-cells or antibodies influence protection. Such studies may also illustrate how their induction can be fine tuned, and how different components of the innate immune system interact to integrate signals that impact on adaptive immunity. Recent advances in virus immunology and the application of vaccine engineering, proteomics and systems biology to the study of vaccines can help address these issues and provide information on how the immune system responds to vaccination (Querec *et al.*, 2009; Pulendran *et al.*, 2010). A deeper understanding of VACV and its multitude of immunomodulators has the potential to reveal the underlying signalling networks that underpin development of immunological protection, and to enable more rational vaccine design.

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