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Survey

Innate immune activation of NFκB and its antagonism by poxviruses

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ABSTRACT

In recent years there has been an acceleration of discovery in the field of innate anti-viral immunity to the point that many of the key events in early virus sensing and the discrete anti-viral responses they trigger have been elucidated in detail. In particular, pattern recognition receptors (PRRs) that detect viruses at the plasma membrane, in endosomes, and within the cytosol have been characterized. Upon stimulation by viruses, most of these PRRs trigger signal transduction pathways culminating in NFκB activation. NFκB contributes both to type I interferon induction, and to production of pro-inflammatory cytokines from infected cells. Our understanding of host anti-viral innate immunity has been greatly aided by an appreciation of the ways in which poxviruses have evolved strategies to inhibit both innate sensing and effector responses. A recurring feature of poxviral immunomodulation is the apparent necessity for poxviruses to evolve multiple, non-redundant inhibitors of NFκB activation which often appear to act on the same innate signalling pathway. The reason for such apparent over-targeting of one transcription factor is not clear. Here we describe the current understanding of how host cells sense poxvirus infection to trigger signalling pathways leading to NFκB activation and pro-inflammatory cytokine induction, and the ways in which poxviruses have evolved to concisely antagonize these systems.

Contents

Abbreviations: NFκB, nuclear factor κB; MCV, *Molluscum contagiosum* virus; PPV, parapox virus; MYXV, myxoma virus; CMPV, camelpox virus; VARV, variola virus; VACV, vaccinia virus; MPV, monkeypox virus; ECTV, ectromelia virus; CPV, cowpox virus; PRR, pattern recognition receptor; PAMP, pathogen associated molecular pattern; DAMP, damage associated molecular pattern; TI-IFN, type I interferon; IL-1, interleukin-1; TNF, tumor necrosis factor; IRF, IFN regulatory factor; RHD, Rel Homology Domain; IKK, IκB kinase; NEMO, NFκB essential modulator; β-TrCP, β-transducin repeat-containing protein; MSK1, mitogen and stress-activated kinase-1; Vflip, FLICE-like protein; dsRNA, double stranded RNA; TIR, Toll-IL-1R; TRIF, TIR-domain-containing adapter-inducing interferon-β; RIP1, receptor-interacting protein 1; TRAF, TNF receptor-associated factor; TAK1, transforming growth factor beta-activated kinase 1; TLR, toll-like receptor; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus; cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; FLDC, Flt3 ligand-mobilized DC; MyD88, myeloid differentiation primary response gene 88; Mal, MyD88-adapter-like; TRAM, TRIF-related adapter molecule; VIPER, viral inhibitory peptide of TLR4; IRAK2, interleukin-1 receptor-associated kinase 2; MVA, modified vaccinia Ankara; RLRs, RIG-I-like receptors; MDA5, melanoma differentiation factor 5; RIG-I, retinoic acid-inducible gene; MAVS, mitochondrial antiviral signalling; STING, stimulator of IFN genes; TBK1, tank binding kinase 1; Cgamp, cyclic-GMP-AMP; cGAS, cGAMP synthase; DNA-PK, DNA-dependent protein kinase; CARD9, caspase-associated recruitment domain 9; AIM2, absent in melanoma 2; ASC, apoptotic speck protein..

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1. Poxviruses and innate immunity

Under the constant selective pressure of pathogens, immunity is the most rapidly evolving system in vertebrates. All viruses evolve strategies to inhibit or evade host immunity leading to minimally symptomatic and persistent infection once virus–host adaptation is optimally attuned. Poxviruses are particularly adept at targeting innate immunity in a precise and efficient manner. Poxviral immune evasion genes collectively form a toolbox of inhibitors and decoys targeting the rate-limiting steps in host anti-viral signalling pathways. Consequently, poxviruses have long served as unparalleled tutors of molecular immunity by pinpointing the genes and processes that hosts have evolved to combat them.

Poxviruses are large, enveloped, double-stranded DNA viruses, which exclusively replicate in the cytoplasm of infected cells. To date, the genomes of all sequenced poxviruses are between 134 and 365 kb and contain between 130 and 328 predicted open reading frames (ORFs) [1,2]. Poxviruses are grouped into two subfamilies: the *Chordopoxvirinae*, which infect vertebrates, and the *Entomopoxvirinae*, which infect invertebrates. *Chordopoxvirinae* are subdivided into ten distinct genera: orthopoxviruses, yatapoxviruses, leporipoxviruses, capripoxviruses, cervidpoxviruses, suipoxviruses, parapoxviruses, molluscipoxviruses, crocodylipoxviruses and avipoxviruses (Table 1). Chordopoxviruses can be also grouped into four phylogenetic categories in order of divergence [3]. Group I is the most divergent and includes the Avipoxvirus genera with Fowlpox (FPV) and Canarypox viruses. Group II, the second most divergent, includes Molluscipoxvirus with *Molluscum contagiosum* virus (MCV) and Parapoxvirus (PPV) genera. The remaining two groups III and IV are clustered together and are often referred as to as 'sister groups' based on the relative proximity of their grouping on the phylogenetic tree. Group III comprises members of Capripoxvirus, Leporipoxvirus such as myxoma virus (MYXV), Suipoxvirus and Yatapoxvirus genera and Group IV includes the seven members of the Orthopoxvirus genera such as camelpoxvirus (CMPV), variola virus (VARV), vaccinia virus (VACV), monkeypox virus (MPV), ectromelia virus (ECTV) and cowpox virus (CPV).

Chordopoxviruses exhibit striking variation in the range of host species that can be productively infected by a given virus. This 'host range' can be experimentally correlated with the presence of defined 'host range genes' which are commonly immunomodulators that block divergent host specific anti-viral responses [4]. These genes are under strong evolutionary selection and vary dramatically even between similar species within a genus [5]. For example, whilst the now extinct causative agent of smallpox, VARV, was strictly human-infectious, the closely related CPV and MPV naturally infect a wide variety of mammalian species. Despite this, most poxviruses can at least enter a wide variety of cells from many different animal species in a fashion that is mostly independent of species-specific receptors, but rather involves virion proteins that are conserved in all poxviruses [6]. However some poxviruses are incapable of replicating in hosts or even cells to which they are not strictly adapted, such as MCV which only appears to efficiently infect human keratinocytes [7].

Table 1
Representative viruses from the ten Chordopoxvirus genera.

Genus	Species
Avipoxvirus	Canarypox virus (CPV)[a]
	Fowlpox virus (FPV)
Capripoxvirus	Sheepox virus (SPV)[a]
Cervidpoxvirus	Deerpox virus (DPV)[a]
Crocodylipoxvirus	Crocodilepox virus
Leporipoxvirus	Myxoma virus (MYXV)
Molluscipoxvirus	*Molluscum contagiosum* virus (MCV)
Orthopoxvirus	Vaccinia virus (VACV)
	Variola virus (VARV)
	Camelpox virus (CMPV)
	Coxpox virus (CPV)
	Ectromelia virus (ECTV)
	Monkeypoxvirus (MPV)
Parapoxvirus	Orf virus
Suipoxvirus	Swinepox virus[a]
Yatapoxvirus	Yaba monkey tumour virus (YMTV)[a]

[a] Not discussed in this review

Approximately 50 relatively conserved genes are found in all sequenced poxviruses and another 40 genes are common to most chordopoxviruses [8]. These genes are chiefly located in the central region of the genomes and exhibit the least divergence between poxvirus family members. Unsurprisingly, such conserved genes control the essential activities of poxvirus biology such as transcription, RNA processing, replication and virion assembly. Conversely, genes that are involved in host–virus interactions generally map towards the terminal regions of the genome. These exhibit lower sequence identity to their orthologs in other poxviruses, with a more diverse distribution between lineages. Most are dispensable for virus replication in cell culture models yet their targeted deletion almost invariably results in viral attenuation in infection models where a complex in vivo environment and multi-cellular anti-viral immunity confront virus propagation and survival. As such, the majority of these 'non-essential' genes facilitate immune evasion and operate at a number of levels in inhibiting virus detection machinery, their signalling networks and downstream effector systems. It has long been known that poxviruses readily recombine with non-viral sequences in vivo [9] and viral immunomodulators thus likely originate from recombination events with host genetic information, as well as through mutation and evolution of pre-existing viral genes to new functionalities with high rates of diversifying evolution [10]. A recent study shed a fascinating new light on how poxvirus evolution functions by acquiring flexible genomic 'accordions' of tandem duplications after which copies undergo genetic drift and deletion once advantageous new variants are derived [11]. Identification of immune-regulatory sequences in some cases occurs when the viral genome has been sequenced and sequence homology to known host immune genes is observed. Functional characterization of putative immunomodulators thus proceeds along lines predicated on how well the host system is understood at the time of investigation. However, most sequences bear no obvious similarity to host sequences and require screening for immune-regulatory potential based on suspected viral activity during infection. Most poxviral immunoregulators are expressed immediately after entry to inhibit the early stages of anti-viral immunity before an effective response to the virus can be mounted.

There has been an acceleration in discoveries in the field of anti-viral innate immunity in the past decade which has led to an increasing awareness of the multitude of viral immunoregulators that exist to target innate immunity. Moreover, the wave of discovery of host innate immune mechanisms for pathogen detection has in no small part been aided by studying the ways in which poxviruses target these systems. The mechanism of early viral sensing in cells hinges on distinguishing host from viral products by germline-encoded pattern recognition receptors (PRRs) which survey extracellular and intracellular contents for pattern associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) produced as a consequence of viral infection [12]. These drive the innate anti-viral response which ultimately accelerates the acquisition of adaptive immunity leading to effective virus clearance and long term immunity. Sensing of viruses by PRRs leads to the induction of type I interferons (TI-IFNs) and of pro-inflammatory cytokines such as

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interleukin-1 (IL-1) and tumour necrosis factor (TNF) via the activation of transcription factors; the induction of TI-IFNs requires the combined activation of NFκB and IFN regulatory factors (IRFs) [13,14] whilst the regulation of pro-inflammatory genes is more directly under the control of the NFκB pathway [15]. Hence NFκB has a central role in controlling both the TI-IFN response and the inflammatory response, both of which are critical to anti-viral immunity.

The main poxviral PAMPs sensed by host PRRs, which cannot escape detection by viral evolution, are the virally-derived nucleic acids of genomes and transcripts present in the cytoplasm of infected cells, which are both chemically and/or compartmentally distinct from host RNA and DNA. In this review we describe the particular innate PRRs that have been linked to poxviral detection, and also the multiple strategies poxviruses have developed to suppress and avoid NFκB activation. Such anti-NFκB poxviral strategies target the NFκB complex directly, the proximal kinase activators of NFκB, and the upstream PRR and pro-inflammatory cytokine signalling pathways that converge on NFκB activation. The abundance of anti-NFκB strategies employed by poxviruses highlights the importance of NFκB activation in host anti-poxviral immunity.

2. Fundamental mechanisms of NFκB activation

The NFκB signalling system is one of the most ancient in animals and has retained a central role in immunity across the animal kingdom [16]. In mammals, the NFκB transcription factor complex consists of a homo- or heterodimeric association between constituent subunits RelA (p65), RelB, c-Rel, NFκB1 (p50) and NFκB2 (p52), via their N-terminal Rel Homology Domains (RHDs) [15]. The most commonly studied NFκB complex is p65-p50. In the absence of upstream activation, these dimeric complexes are pre-formed and sequestered in the cytoplasm in an inactive association with inhibitory family members of the IκB family. p50 and p52 are initially expressed as precursor proteins, p105 and p100 respectively, which themselves function as inhibitory IκB proteins until processed into mature forms.

NFκB activation broadly occurs via either a canonical or a non-canonical pathway, both of which control the proteolysis of the inhibitory IκB and IκB related proteins. The canonical pathway is activated by PRRs or pro-inflammatory cytokines such as IL-1 and TNFα leading to the activation of p65- or cRel-containing complexes [17], by proteasomal degradation of IκBα (Fig. 1). Thus PRR, IL-1 or TNF signalling activates the IκB kinase (IKK) complex composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit, NEMO (NFκB essential modulator or IKKγ) which phosphorylate both p65 and IκBα. Phosphorylation of IκBα induces its polyubiquitylation through the E3 ubiquitin ligase β-transducin repeat-containing protein (β-TrCP), leading to proteasomal degradation of IκB at the proteasome. This releases the active NFκB dimer from IκB inhibition, allowing NFκB to translocate into the nucleus and induce expression of target genes. The non-canonical pathway, involving either p105 or p100, is activated by other TNF family members such as lymphotoxin β1 and CD40 ligand [18]. This pathway activates IKKα to phosphoylate p100, causing it to be polyubuitylated by β-TrCP then partial proteolysed by the proteasome to produce mature p52. P52 then

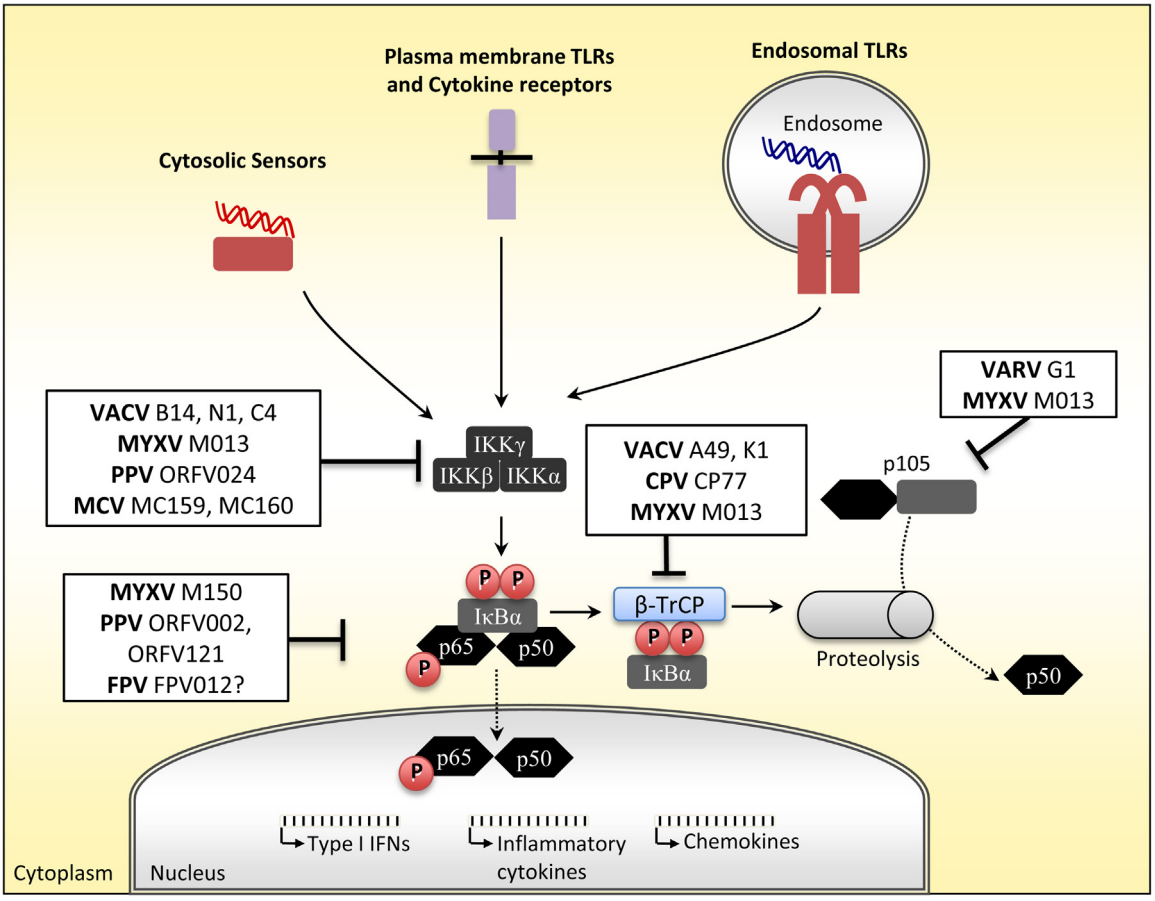

Fig. 1. Proximal inhibition of NFκB activation by poxviral proteins. Sensing of poxviruses by diverse upstream PRRs culminates in the activation of the IKK complex which phosphorylates both IκB, leading to its proteasomal degradation, and p65 which enhances transactivation. Poxviral proteins that target these proximal NFκB activation events are shown (see text for details).

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dimerizes predominantly with RelB to form an active NFκB complex which translocates into the nucleus [19]. Conversely, processing of p105 to produce the active, mature p50 subunit by the same mechanism is constitutive and does not require a stimulus. The activation of IKKβ by the canonical pathway causes IKKβ to phoshorylate p105 on the C-terminal destruction box which targets p105 for complete degradation and thus releases associated NFκB subunits to be phosphorylated by the canonical stimulus (Fig. 1).

3. Direct targeting of NFκB and its proximal kinases by poxviruses

Given that a diverse array of pathways that sense and respond to viral infection culminate in the activation of NFκB by IKKs, it might be presumed that the most efficient means of inhibiting NFκB from a virus point of view would be to directly target NFκB subunits or the proximal IKKs. It is therefore no surprise that this is the most common means by which poxviral inhibitors of this pathway exert their effect (Fig. 1).

Many Chordopoxviruses encode a proteins that are structurally similar to host Bcl-2 proteins. These were likely derived from a common ancestral gene encoding a protein with the bcl-2 fold, which may have been captured from the host or could have independently evolved, but was then duplicated and functionally diversified over time [20] whilst retaining the same bcl-2 structure. The viral Bcl-2 family amino acid sequences are highly divergent even though they share the same overall bcl-2-like structure, and they interact with an impressively diverse range of different host partners to bring about the same biological outcome (as described here and in Section 4). This suggests that the bcl-2 fold may represent a stable structural scaffold upon which diverse interaction interfaces have evolved. Interestingly, several VACV proteins either proven or predicted to adopt the Bcl-2 structure have been shown to inhibit activation of NFκB by directly targeting the IKK complex. Such bcl-2-like IKK antagonists include B14, which directly interacts with and inhibits the activity of IKKβ [21], N1, which associates with the IKK complex [21], and C4 which inhibits NFκB at the level of IKK activation [22].

Yet another VACV protein which proximally targets NFκB activation is A49. This protein displays molecular mimicry since it contains a motif similar to that in IκBα that is phosphorylated by IKKβ and subsequently binds to the E3 ligase β-TrCP. This allows A49 to preferentially bind β-TrCP and hence prevent it ubiquitylating IκBα [23]. Remarkably, although the VACV NFκB inhibitors described above would appear to be functioning redundantly, each inhibitor has been shown to contribute to virulence since viruses lacking just a single inhibitor are attenuated in vivo compared to wild type VACV [24].

All chordopoxviruses (with the notable exception of MCV and Crocodilepox virus) encode a family of proteins with multiple ankyrin repeats which, like the bcl-2 protein family described above, are involved in host-range and immunomodulation [25]. Some of these have also been shown to directly target NFκB, possibly by mimicking the ankyrin-repeats of the IκB family, which normally directly mediate the interaction of host IκB proteins with the nuclear localization sequence of p65 and the dimerization domains of both p50 and p65 [26,27]. For example, VACV K1 contains such repeats and prevents IκBα degradation [28]. CPV protein CP77 contains nine ankyrin repeats and an F-box like C-terminal domain that facilitate binding of CP77 to p65 and the E3 ubiquitin ligase complex that targets IκB, respectively. Both of these binding functions are essential for prevention of NFκB translocation into the nucleus suggesting that CP77 behaves like a surrogate IκB-like domain by binding p65 and β-TrCP to preventing proper translocation of NFκB [29]. CP77 might also target p65 for proteasomal degradation through these binding activities [30]. Other poxviral proteins demonstrated or suspected to directly interact with NFκB include: MYXV protein M150 which co-localises with NFκB and supresses inflammation [31]; VARV protein G1 which contains ankyrin repeats, is conserved in a number of orthopoxviruses (CPV, MYXV and ECTV) and has been shown to associate with p105 and block NFκB nuclear translocation [32]; MYXV protein M013 which also binds p105 to prevent its processing and subsequent NFκB activation [33].

Apart from the viral proteins described above which directly target IKKs or NFκB, other unique NFκB inhibitors exist that are more specific to certain vertebrate poxviruses, further emphasizing the need for diverse poxviruses to suppress NFκB activation in order to avoid elimination by host innate immunity. Thus ORFV024 protein from the parapoxvirus orf virus was shown to block IKK activation although the mechanism of its activity is not clear [34], while two other unique parapoxvirus proteins, ORFV002 and ORFV121, were recently shown to also inhibit NFκB activation. ORFV002 antagonized p65 phosphorylation by interfering with the association of p65 with the mitogen and stress-activated kinase-1 (MSK1) which prevented p65 recruiting the coactivator p300 to NFκB-dependent promoters, thus suppressing transactivation [35]. In contrast, ORFV121 interacts with p65 and prevents its nuclear translocation [36]. Very little is known about how the human-specific poxvirus MCV suppresses host innate immunity, yet the virus is known to encode two FLICE-like proteins (vFLIPs), MC159 and MC160, that antagonize NFκB. MC159 blocks IKKβ activation by interacting with IKKγ, while MC160 disrupts the IKK complex by stimulating IKKα degradation [37]. Given the density of NFκB inhibitors discovered in well-characterized poxviral genomes such as VACV, more poorly characterized poxviruses such as orf virus and MCV undoubtedly encode further NFκB inhibitors waiting to be discovered.

As noted above, activation of NFκB can occur through both PRRs and proinflammatory cytokine (IL-1 and TNFα) signalling. In the following sections, we describe the role of PRRs (see Table 2) and proinflammatory cytokines in anti-poxviral innate immunity, and discuss ways in which poxviruses target and inhibit these signalling pathways, which provides a further layer of anti-NFκB poxviral strategies that operate upstream of those already described that act proximal to NFκB.

4. TLRs link poxviral detection to NFκB activation

TLRs are prototypical PRRs for PAMPs and DAMPs and are conserved across the animal kingdom, having retained a key role in immunity in diverse species [38]. Contexts whereby TLRs could sense the presence of poxviruses include when cells come into contact with extracellular virus particles, within the endosomes of phagocytotic macrophages and dendritic cells at sites of infection or after endocytosis and uncoating of virus during infection of multiple cell types.

TLR3 recognizes viral double stranded RNA (dsRNA) in endosomes and signals via homotypic interactions between Toll-IL-1R (TIR) domains through the adapter TIR-domain-containing adapter-inducing interferon-β (TRIF), leading to recruitment and activation of receptor-interacting protein 1 (RIP1) and TNF receptor associated factor 6 (TRAF6), both of which are involved in transducing a signal to the IKK complex via activation the transforming growth factor beta-activated kinase 1 (TAK1) [38] (Fig. 2). TLR3 has been shown to respond to VACV but, rather than this response conferring protection on the host, TLR3 contributes to the immunopathology of the infection. Thus lower levels of VACV replication were observed in TLR3$^{-/-}$ mice compared to wild type animals, highlighting the critical balance between effective and excessive anti-viral responses [39]. Since the opposite result

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Table 2
PRRs implicated in poxviral detection leading to NFκB activation.

	PRR	PAMP	Cell types examined (virus used)
TLRs	TLR2	?	DC and T cells (VACV) [44]
	TLR3	dsRNA	Mouse, in vivo (VACV) [39]
	TLR4	?	Mouse, in vivo (VACV) [40]
	TLR7	ssRNA	pDC (MYXV and heat killed VACV) [47]
	TLR8	G-rich NTs	pDC (VACV) [48]
	TLR9	dsDNA	pDC and cDC (myxoma) [50]; mouse in vivo and FL-DC (ECTV and MVA) [49]
RLRs	RIG-I	PPP-RNA	Human fibroblasts and macrophages (MYXV) [69]; HeLa cells (VACV) [70]
	MDA5	dsRNA > 2000 nts	HeLa cells (VACV) [70]; Hela cells (VACV RNA) [71]; THP1 (MVA) [72]
	LGP2	dsRNA	Murine fibroblasts (MVA) [73]
Other cytosolic sensors	cGAS-STING	DNA	HEK293 cells (MVA) [85]; cDC and BMDC (MVA) [86]
	Rad50-CARD9	DNA	BMDCs (VACV) [90]

was seen in TRIF$^{-/-}$ mice, this suggested a TRIF-dependent TLR other than TLR3 was actually protective in VACV infections [40]. Consistent with this, TLR4 which also uses TRIF for signalling, but is best known as the signalling receptor for bacterial LPS, was shown to be protective during VACV pulmonary infection although the mechanism for this remains unclear [40]. Since TLR4 can also sense respiratory syncytial virus (RSV) [41] and vesicular stomatitis virus (VSV) glycoproteins [42], it is possible that it plays a direct or cooperative role in sensing VACV surface glycoproteins in the same way. Alternatively, TLR4 may respond to DAMPs produced by a viral infection, as is the case for influenza virus [43]. TLR2 has also been shown to be important for sensing VACV and Ectromelia infection [44] though other authors find no role for this PRR in combating VACV infection in vivo [45].

Apart from TLR3, other endosomal TLRs have been linked to poxviral responses (Table 2): TLR9, the first DNA-sensing PRR to be discovered [46], senses unmethylated CpG DNA, whilst TLR7 and TLR8 sense ssRNA [38]. These endosomal TLRs activate NFκB via myeloid differentiation primary response gene 88 (MyD88) and TRAF6 [38] (Fig. 2). Poxvirus infection is detected by TLR7 [47], TLR8 [48] and TLR9 [49,50] in endosomes of conventional and plasmacytoid dendritic cells (cDCs and pDCs). Survival of mice after a lethal ECTV infection was critically reliant on TLR9, while MVA infection protected mice from ECTV in a manner dependent

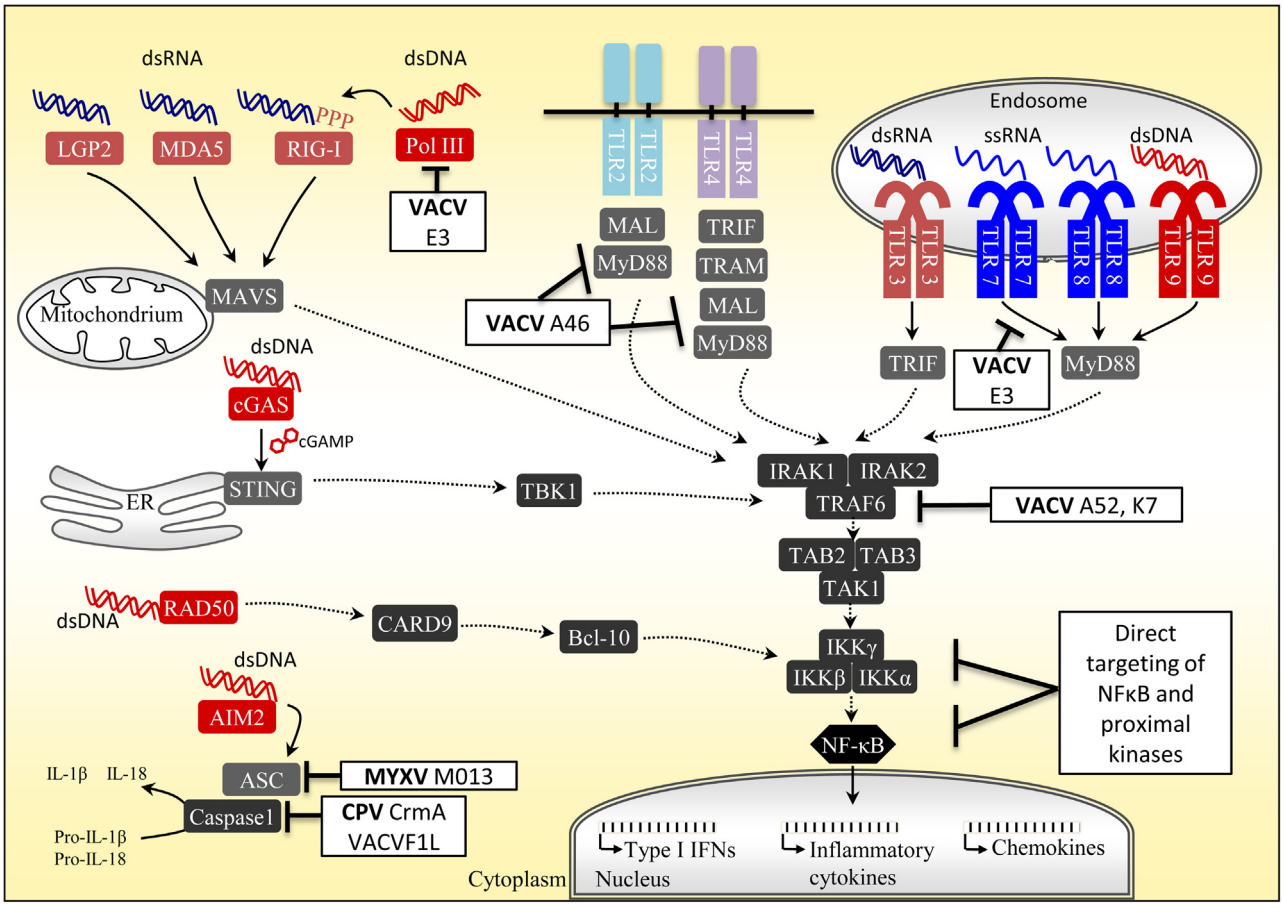

Fig. 2. Inhibition of PRR-stimulated NFκB activation by poxviruses. Poxviral nucleic acids are recognized by TLRs, at the plasma membrane and in endosome, and by RLRs (LGP2, RIG-I, MDA5) and DNA sensors (AIM2, cGAS, RAD50) in the cytosol. Poxviral proteins that target these PRR signalling pathways are shown and described in detail in the text.

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on TLR9 [49]. Recognition of poxvirus by TLR7 was proposed to involve detection of viral RNA transcripts [47], while surprisingly, TLR8-dependent responses were suggested to be mediated by recognition of poly(A)/T-rich poxviral DNA sequences [48]. Interestingly, although TLR3 and TLR9 are not normally expressed in skin [51,52], MCV-infected skin lesions exhibited increased expression of TLR3 and TLR9 in infected tissues, suggesting that these TLRs may be involved in the local host response to MCV in infected keratinocytes [52,53].

Consistent with the role of TLRs in sensing poxviruses, VACV has been shown to encode proteins that inhibit TLR signalling components, leading to suppression of TLR-dependent NFκB activation. The VACV TLR inhibitors A46 and A52 were initially identified as having sequence similarity to the TIR domain used by TLRs to signal to their adaptors [54], but were then proposed to be part of the poxviral bcl-2 family, which was confirmed by determination of their crystal structure [55,56]. Both were originally shown to inhibit TLR4-activated NFκB [54], while further work demonstrated that A52 inhibited all TLR pathways to NFκB activation by interacting with IL-1 receptor-associated kinase 2 (IRAK2), a finding that demonstrated a broad role for IRAK-2 in multiple TLR-stimulated NFκB activation pathways [57,58]. In contrast, A46 was shown to directly target TLR complexes by interacting with the TIR domain in both TLRs and the adaptor proteins MyD88, TRIF, MyD88-adapter-like (Mal) and TRIF-related adapter molecule (TRAM), and for TLR4 signalling to prevent adaptor recruitment to the TLR4 complex [59,60]. Indeed, inhibition of TLR4 could be reconstituted using a cell-permeable peptide (viral inhibitory peptide of TLR4, or VIPER) based on the surface of A46 that is required to disrupt TLR4 signalling to NFκB [61]. Yet another VACV bcl-2 protein, K7, which displays strong amino acid sequence similarity to A52, could also inhibit TLR-stimulated NFκB activation by binding to IRAK2 [62].

Most of the VACV NFκB inhibitors described in the previous section that act close to NFκB itself were also shown to inhibit TLR-stimulated NFκB. Thus, B14, C4, A49 and N1 were all shown to inhibit NFκB activation by a range of TLRs [21,22,63]. Further, the ankyrin repeat inhibitor of IκBα, K1, was shown to block TLR2, TLR4 and TLR9 stimulated NFκB in primary microglial cells [64]. Also, a recent screen of the avipoxvirus Fowlpox virus (FPV) identified an ankyrin repeat protein which blocked induction of chicken TI-IFN by the TLR3 ligand Poly (I:C), although the precise mechanism of its activity is still unclear [65]. However similar to other poxviral ankyrin repeat proteins, this FPV protein is likely to function by direct antagonism of NFκB.

Other poxviral proteins have unexpected roles in inhibiting TLRs. E3 is a key virulence factor for VACV, that has multiple activities in suppressing the host immune response, and contains an N-terminal Z-DNA/RNA binding motif as well as a C-terminal dsRNA binding domain [66]. The Z-DNA/RNA binding motif of E3 was shown to block TLR7 activation in pDCs [47]. This domain is not retained in the myxoma ortholog of E3, thus preventing it from evading TLR7, which suggests significant gain or loss of function between orthologous proteins in different poxviruses.

Altogether poxviruses are well equipped to suppress TLR-stimulated NFκB activation which attests to an important, thought diverse, role for TLRs in mobilizing host innate immune responses towards poxviruses.

5. NFκB activation by cytosolic detection of poxviral nucleic acids

Despite the ability of TLRs to sense poxvirus infection, the attenuated MVA can still induce TI-IFNs in a TLR-independent fashion [67] suggesting additional PRRs can sense poxviral infection. Consistent with this, further PRRs that act in the cytosol and detect both poxviral RNA and DNA have been defined (Table 2). The cytosolic RNA receptors melanoma differentiation factor 5 (MDA5) and retinoic acid-inducible gene (RIG-I) detect long dsRNA and dsRNA with a 5′ triphosphate group respectively in the cytoplasm of cells infected with RNA viruses [68]. Upon activation, these RIG-I-like receptors (RLRs) engage the adaptor protein mitochondrial antiviral signalling (MAVS) which activates the IKK complex and subsequently NFκB (Fig. 2). Several recent reports demonstrate how poxviruses are also sensed by RLRs. For example, MYXV stimulates NFκB-dependent TNFα and TI-IFNs in primary human macrophages via RIG-I [69], VACV induces TI-IFN in a RIG-I- and MDA5-dependent manner in different cell types, and MVA induced IFNβ and IFN-dependent chemokines via MDA-5 and MAVS but not RIG-I in macrophages, suggesting both virus and cell-type differences in these responses [70–72]. A third RLR, LGP-2, has also been shown to be important for the upregulation of NFκB-dependent genes in response to VACV DNA [73]. MVA infection also causes increased cellular expression of the RLRs thus increasing the sensitivity of DCs to aberrant RNA [74]. The importance of RLRs in managing poxviral infection is reflected in the fact that it has been suggested that historical species-specific poxviral infection has played a role in positive selection of RLR families in different mammalian species [75]. A rationale for how cytosolic dsRNA PRRs are involved in detecting poxviruses is provided by the fact that poxviruses produce large amounts of dsRNA during an infection due to simultaneous transcription of both strands of the dsDNA viral genome [76]. In fact VACV E3, which binds dsRNA, was shown to block RLR-driven NFκB-dependent gene induction in keratinocytes [77]. The requirement for RLRs in responding to poxviruses may also be partly explained by the RNA polymerase III intermediate system of cytosolic DNA detection whereby RNA polymerase III transcribes short RNA sequences from cytosolic AT-rich DNA that are direct ligands for RIG-I activation [78,79]. Interestingly, E3 can also antagonize AT-rich DNA sensing via the RNA polymerase III system [80].

Although the physiological relevance for poxviruses of AT-rich dsDNA sensing by RNA polymerase III is unclear, as DNA viruses that reside in the cytosol poxviruses would be likely to be sensed by other recently described cytosolic DNA sensors that account for the ability of DNA in the cytosol, whether from viral infection or from aberrant host DNA metabolism, to activate transcription factors and induce TNF and TI-IFNs [81]. Such DNA sensors in many cases strongly activate IRF3, via a well-defined stimulator of IFN genes (STING)-Tank binding kinase 1 (TBK1)-IRF3 signalling axis whereas the DNA sensing cytosolic pathways to NFκB activation are still less clear. Both genetic and biochemical studies have demonstrated the importance of STING in signalling a response to DNA viruses in the cytoplasm though how STING itself is activated by upstream DNA sensors was unclear until recently [82]. A series of elegant studies then showed that cyclic-GMP-AMP (cGAMP) synthase (cGAS) is a DNA sensor upstream of STING, whose enzyme activity is stimulated by direct binding of DNA, leading to production of the novel second messenger cGAMP (reviewed in [83]). cGAMP is a direct ligand for STING, which is initially localized in the endoplasmic reticulum, but on binding cGAMP translocates to TBK1-containing membrane-bound compartments leading to IRF3 activation. Interestingly, a recent study demonstrated that, after infection of cells with MVA, cGAMP can diffuse through cellular gap junctions to activate the TI-IFN response in adjacent, uninfected cells, implying that the cGAS/STING system may directly prepare bystander cells for resistance to incoming poxviral infection [84,85]. The cGAS/STING system was also shown to sense MVA DNA in the cytoplasm of conventional DCs during infection [86]. The STING pathway has recently been proven to also activate NFκB, at least in fibroblasts, via TRAF6 [87]. Some number of papers have demonstrated that TI-IFN induction by VACV in some

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cell types requires cGAS [85,86], although whether STING controls poxvirus-stimulated NFκB activation, as well as IRF3, remains to be determined. Whilst other viruses have been shown to target STING activation for immune evasion, no poxviral inhibitors of this system have yet been reported.

One cytosolic DNA sensing mechanism that exists in fibroblasts has been shown to be targeted by poxviruses for immune evasion: Ferguson et al. [88] showed that DNA-dependent protein kinase (DNA-PK) senses MVA, leading to STING-dependent IRF3 activation, which is subject to antagonism by the conserved VACV virulence factor C16, which directly bound the DNA-PK DNA sensing complex [89]. However, DNA-PK sensing of MVA did not lead to NFκB activation. A rare to date clear example of how cytosolic DNA sensing of poxviruses can cause NFκB activation was recently provided by Roth et al. [90]. This involved detection of cowpox and VACV DNA in murine DCs by direct binding to the DNA damage sensor Rad50, which somehow relocated from the nucleus to the cytoplasm in response to poxviral infection. Rad50 then recruited the innate immune signalling adaptor caspase-associated recruitment domain 9 (CARD9), which activated the IKK complex via Bcl-10, leading to induction of NFκB-dependent genes including TNFα and pro-IL-1β [90]. Importantly, Rad50/CARD9 sensing of poxviruses was also shown to operate in vivo.

In the future, based on what has been discovered for other PRR activators of NFκB, such as TLRs, one would expect that poxviral antagonists of DNA sensing pathways such as Rad50/CARD9 and cGAS will be discovered. As with other upstream activators of NFκB such as TLRs, some of the direct NFκB inhibitors (Section 3) would be predicted to block activation of NFκB through cGAS/STING and Rad50/Card9 sensing.

6. Poxviral targeting of pro-inflammatory cytokines

As described above, detection of poxviruses by PRRs leads to NFκB-dependent pro-inflammatory cytokine induction, which is a key host anti-viral protection mechanism. PRR stimulation causes intracellular expression of pro-IL-1β, which needs to be processed by a caspase 1-containing inflammasome into mature secreted IL-1β. Absent in melanoma 2 (AIM2) is a PYRIN and HIN200 domain-containing protein which forms an inflammasome that is activated by cytosolic DNA [91]. The HIN200 domain of AIM2 directly binds to dsDNA in the cytosol [92], leading to the recruitment of apoptotic speck protein (ASC) via the PYRIN domain, and subsequent recruitment and processing of pro-caspase 1 into caspase 1 [91] (Fig. 2). Importantly, the AIM2 inflammasome was shown to be required for VACV-stimulated IL-1β production in vivo. The NLRP3 inflammasome may also play a role in poxviral sensing since MVA-infected NALP3 knockout macrophages exhibited impaired processing of pro-IL1β [72]. Poxviral detection by inflammasome activation is targeted by a number of viral evasion strategies. The earliest reported inhibitor was CPV protein CrmA (and orthologs such as B13R in VACV) which inhibits IL1β processing by targeting Caspase 1 [93]. The MYXV protein M013, which was discussed earlier as a direct inhibitor of NFκB, is like many poxviral inhibitors multi-functional in that it also blocks caspase-1 activation and pro-IL1β processing by targeting ASC [33]. Another inflammasome, NLRP1, is also targeted by poxviruses: the VACV Bcl-2 family member, F1L was shown to bind NLRP1 thereby limiting pro-IL-1β processing though the precise mechanism by which poxvirus infection activates this type of inflammasome is not yet clear [94].

Thus through PRR stimulation and inflammasome activation by poxviruses, IL-1β and TNFα are produced by the host. This leads to autocrine and paracrine stimulation of distinct pathways through the IL1 and TNF receptors which activates a second wave of NFκB activation [95,96]. Pro-inflammatory cytokine stimulation of NFκB activation induces a wide range of chemokines, adhesion molecules and further pro-inflammatory cytokines that cause local inflammation and fever, facilitating virus clearance and long-term adaptive immunity.

Both IL-1 and TNFα signalling culminate in the activation of the IKK complex and of NFκB, but by distinct upstream receptor-proximal events (Fig. 3). For IL-1, binding to the IL1 receptor complex (containing the IL-1R and the IL-1R accessory protein) recruits the adapter MyD88, leading to the activation of a similar signalling axis as used by MyD88-dependent TLRs involving IRAK2, TRAK6 and TAK1. Thus the VACV inhibitors of TLR-stimulated NFκB activation, A46 and A52, also antagonize IL-1 signalling, which likely contributes to their role in virulence [57,59]. TNF binding to the TNF receptor causes receptor trimerization and recruits TRADD which in turn recruits TRAF2, cIAPs and receptor-interacting protein kinase (RIPK1). The IKKs are recruited to this complex and activate NFκB (Fig. 3). Both IL-1 and TNF-stimulated NFκB activation are targeted by most if not all of the proximal NFκB inhibitors described in Section 3.

Apart from inhibiting intracellular signalling to NFκB by IL-1 and TNFα, poxviruses also target these cytokines by expressing poxvirally-encoded secreted binding proteins and receptors from infected cells, which inhibit the cell surface binding of the IL-1 family (IL-1β and IL-18) and of the TNF family (TNFα, Lymphotoxin-α, CD153) [97]. Some poxvirally-encoded TNF-binding proteins resemble a soluble TNFR (vTNFRs) with varying degrees of ligand specificity and affinity [98] while some do not directly resemble the TNFR and are therefore referred to as 'TNF binding proteins' (vTNF-BPs) [99]. For IL-1, unlike the cellular receptor that binds both IL-1α and IL-1β, the poxviral soluble IL-1 receptor of VACV and CPXV binds only IL-1β, which is the main form produced and processed in poxvirus-infected cells.

TNF is particularly important in controlling MCV infections as evidenced by the exacerbation and spread of lesions in patients taking anti-TNF therapy [7], which is reflected in the immune

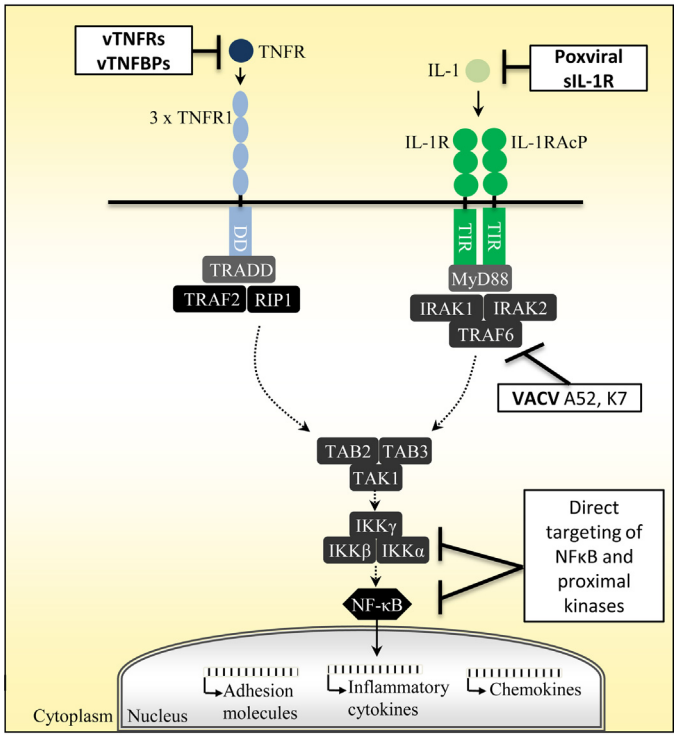

Fig. 3. Poxviral targeting of pro-inflammatory cytokine signalling to NFκB. Poxviruses target IL-1 and TNFα function by encoding both secreted extracellular cytokine binding proteins, and intracellular inhibitors of signal transduction (see text for details).

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evasion proteins known to be expressed by MCV: MC159 and MC160 block TNF receptor induced apoptosis and NFκB activation by distinct mechanisms [37]. As yet only 5 out of 182 MCV ORFs have been shown to possess immunomodulatory function and these all use strategies not employed by other poxviruses. Given the ability of this human poxvirus to block the local immune response to its presence and the density of such ORFs in better characterized poxviruses like VACV, further study of this virus would undoubtedly reveal many more.

Overall one can surmise the importance of IL1 and TNF in poxviral infections by the number of genes and strategies poxviruses devote to blocking their production, signalling and pro-inflammatory activity. Such strategies include inhibiting their induction and processing by blocking PRR signalling and inflammasome activation, soaking up IL1 and TNF through the production of secreted binding proteins, blocking their signal transduction to NFκB, and also preventing the activity of IL1- and TNF-induced genes, such as chemokines, with viral chemokine binding proteins.

7. Concluding remarks

We have described the current understanding of the ways in which poxviruses trigger anti-viral immunity to activate NFκB and the many different non-redundant ways in which they target and inhibit its activation. Poxviruses are particularly adept at blocking NFκB at many levels, although many of the known NFκB antagonists function proximal to the IKK complex or to NFκB itself. This allows effective inhibition of multiple PRR and cytokine signalling pathways that converge on NFκB by a small repertoire of viral proteins. However poxviruses invariably evolve multiple inhibitors of NFκB which do not seem to act redundantly based on data from in vivo challenges of animals with viruses lacking individual inhibitors. The importance of targeting NFκB activation in multiple ways, frequently on the same pathways and in the same cell, has not been clear. One possibility is that the complexity of upstream activating networks stimulated by poxvirus infection which lead to this key anti-viral and inflammatory transcription factor being activated makes distinct inhibitors necessary to ensure effective inhibition of every poxvirus sensing and activation pathway. In addition, the crucial importance of NFκB activation to an effective anti-viral response may have exerted evolutionary pressure on the host to diversify the processes of activation of this transcription factor in order to protect it against single, modular mechanisms of viral inhibition. It would be interesting to assess the combinatorial activity of these inhibitors to determine if their combined activity is required to fully cut through the 'checks and balances' of this pathway in vivo.

A recent study demonstrated that when all the known NFκB inhibitors were knocked out in VACV the virus was still able to inhibit NFκB-induced gene expression. This suggests other inhibitors remain to be found even in this relatively well characterized virus [100]. It is sensible to assume that other poxviruses may encode a similar amount of NFκB inhibitors that remain to be found. For example, only two have been discovered in the understudied human-specific poxvirus MCV which has an unsurpassed ability to inhibit local inflammation. Since MCV, more than any other poxvirus studied, is specifically adapted to target human innate immunity, it will be informative to elucidate further MCV strategies of NFκB antagonism, which may yield unique insights into the role of human NFκB in inflammation and immunity.

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