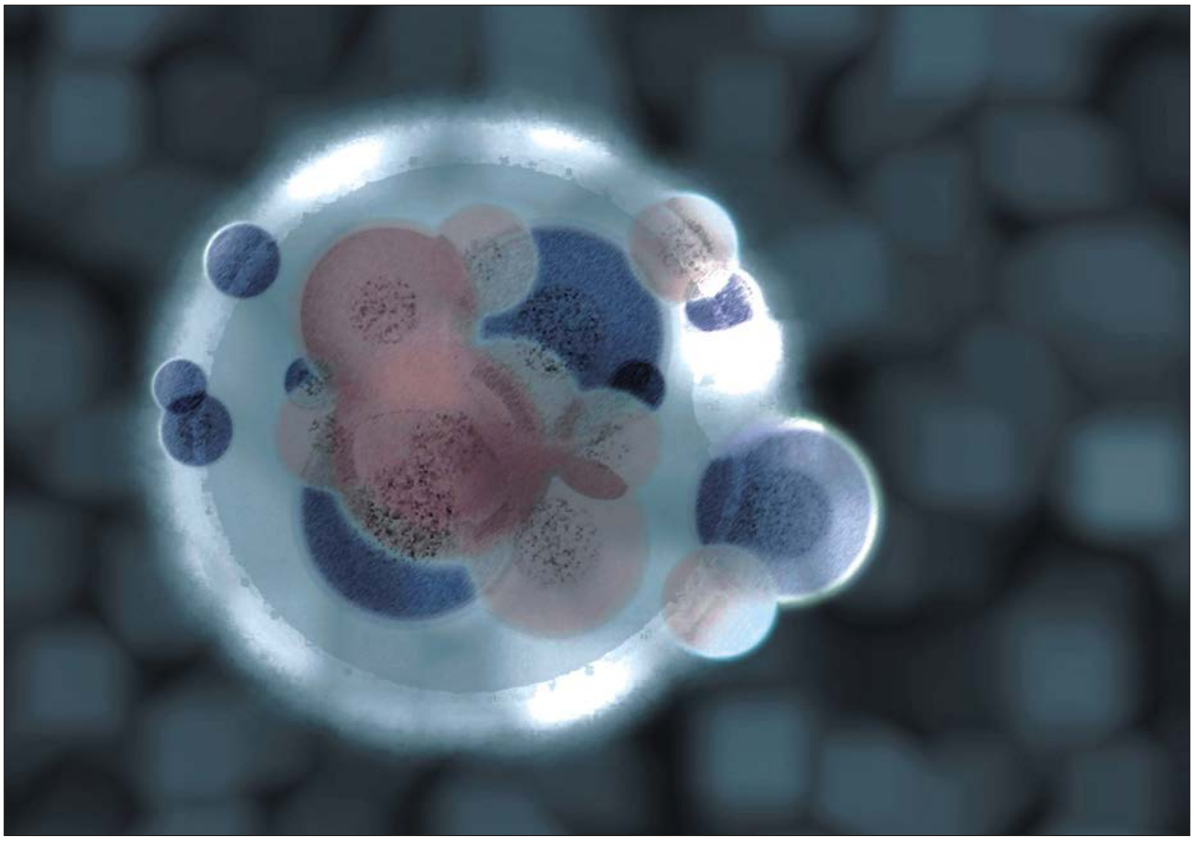

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A Growing Family of Poxvirus Innate Immune Inhibitors

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Abstract: The variola and vaccinia orthopoxviruses express key proteins that can inhibit the host innate immune response. For example, orthopoxviruses encode a family of evolutionarily conserved poxvirus proteins that inhibit signaling by the Toll-Like Receptor (TLR) family. Two of these poxvirus proteins have been identified as robust determinants of vaccinia virus virulence-N1L and A52R-which are both vaccinia virus proteins that inhibit TLR signaling. The TLRs mediate the innate immune recognition of pathogens. Once engaged by a bacterial or viral pathogen, the TLRs ultimately activate the NF-κB and MAPK signaling pathways and drive cytokine expression. Other TLRs eventually activate the interferon regulatory factor-3 (IRF3) signaling pathway and NF-κB to drive interferon production. These innate immune responses stimulate a vigorous adaptive immune response. This review explores the known and hypothesized mechanisms of action of the identified inhibitors of TLR signaling and contrasts their function with other poxviral inhibitors of innate immunity. Finally, potential functions for other incompletely characterized members of this growing gene family are discussed.

Key words: Toll-Like Receptor (TLR), interferon regulatory factor-3 (IRF3), NF-κB, poxvirus vaccinia virus, signal transduction

INTRODUCTION

Orthopoxviruses are re-emerging threats to human health because of the renewed concerns about deliberate dissemination of the lethal variola (smallpox) virus[1]. One strategy to prevent smallpox involves the widespread inoculation of humans with another orthopoxvirus-the vaccinia virus-as a human vaccine[2]. Smallpox vaccination using vaccinia virus is sometimes associated with serious side effects[3]. Nevertheless, vaccinia virus is a component of gene therapy for cancer[4] and use of vaccinia virus vectors was proposed as part of worldwide childhood vaccine initiatives[5]. This highlights the need for continued study of orthopoxviruses, both pathogens and vaccine species.

The orthopoxvirus genome contains over 200 genes and encodes viral homologs of several components of the innate and adaptive immune system. The related swinepox virus has a genome numbered 5' to 3'[6]. In contrast, the vaccinia virus genome is nominally divided into Open Reading Frames (ORFs) using Hind III restriction endonuclease fragments. These fragments are assigned a letter, followed by open reading frames that are assigned a numeral and finally a designation of R or L for the direction of transcription. Thus, the Hind III A fragment, 52nd ORF, Rightward reading frame is designated A52R. This open reading frame encodes a protein that inhibits signaling by the innate immune system, including the Toll-like receptors (TLRs)[7]. TLRs are the key pattern-recognition element of the innate immune response against infection.

TLRs mediate an innate immune response against pathogens: The TLRs are a superfamily of pattern recognition molecules that respond to infection, as exemplified by the prototypic Toll receptor that is central to the *Drosophila* antifungal response. TLR signaling is triggered by binding of the components of pathogens to the extracellular leucine-rich repeat regions of the TLRs[8]. Following ligand-dependent engagement of the TLRs, signal transduction is thought to occur via homotypic interactions of TLRs and Toll/interleukin-1 receptor (TIR) - adaptor signaling proteins, which physically associate with the cytoplasmic domain of the receptors, the Toll/IL-1 receptor (TIR) domain[9,10]. This evolutionarily conserved signaling pathway is also implicated in signal transduction by the IL-1R. Signaling involves the ligation of TLRs followed by clustering of

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TIR adapters, such as Mal/TIRAP, TRIF/TICAM-1, MyD88[10-14] and TRAM/TICAM-2[15,16]. TIR adapter clustering results in recruitment of the IL-1 receptor associated kinases (IRAK), tumor necrosis factor-associated factor 6 (TRAF6) and the I-κB kinase complex (IKK)[10]. Utilization of this pathway at a minimum leads to NF-κB activation and the activation of various MAPK pathways, including p38, JNK and ERK pathways[14].

Another way in which the host innate immune system responds to pathogens is via the interferon response pathway that is induced by viruses, double stranded RNA (dsRNA) and lipopolysaccharide (LPS)[17]. Ligation of TLR3 or TLR4 ultimately leads to signaling via the IKK complex to NF-κB and IRF3 (Fig. 2). Signaling via the TIR and Tumor Necrosis Factor-α (TNF-α) signal transduction pathways ultimately activates the IKK complex leading to the phosphorylation of I-κBα. Phosphorylated I-κBα remains associated with NF-κB while it is ubiquitinated. Ubiquitinated I-κBα is degraded by the proteasome, permitting NF-κB to translocate to the nucleus. Phosphorylation of I-κBα is carried out by IKK-β as part of a complex with the kinases IKK-α/-β/-γ. Two related kinases, IKKε (also designated IKK-I) and TANK-binding Kinase 1 (TBK1) (alternatively designated NAK and T2K), are part of a multiprotein complex that also contains IKK-α, IKK-β, IKK-γ and the TRAF family member associated NF-κB activator, TANK[18]. TBK1 and IKKε phosphorylate IRF3, which drives promoters that produce proteins that are part of the Type I interferon response[14,17,19]. TBK1 activates NF-κB[20], likely as part of a complex that phosphorylates p65 to transactivate NF-κB[21]. IRF3 and NF-κB drive transcription of the antiviral genes in the interferon response pathway[22], forming a critical part of a potent innate antiviral response.

The innate immune response activates the adaptive immune response to viral infections: Viral nucleic acids and glycoproteins trigger TLRs, inducing the transcription factors NF-κB and IRF3, followed by the prompt production of cytokines, chemokines and type I interferons[22]. In addition, TLR signaling also primes dendritic cells (DCs) to present antigens, eventually resulting in a potent adaptive immune response[9]. This TLR signaling pathway is MyD88-independent and is thought to involve the TIR adapters TRAM and TRIF[16]. The presentation of antigen to T cells expressing the T-cell receptor and CD28 is enhanced by the expression of MHC and co-stimulatory molecules CD80/CD86 on the surface of DCs-expression that is stimulated by Toll receptor signaling[23,24]. Immature peripheral DCs are incapable of presenting antigen to T cells. DCs stimulated by viral components migrate to the T cell zones of lymphatic tissue, but fail to mature without the interferon signal that depends upon co-ordinate gene activation induced by TLR signaling to the promoters for IFN-α/-β[19]. Furthermore, an age-related decline in TLR expression correlates with declines in adaptive immune function[25], suggesting that innate and adaptive immune function are functionally linked at a population level. Thus, the innate immune response appears to be required for the maturation of an adaptive immune response[23,24]. For all these reasons, studies of poxviral inhibitors of the innate immune response promise to provide much insight into the details of the adaptive immune response.

Poxviral innate immune inhibitors: Poxviruses perturb the host innate immune response in many ways[2]. For example, the soluble IFN-α-β-receptor of vaccinia virus interdicts the type I interferon response at the receptor level (Fig. 2)[26]. Although this appears to duplicate the E3L-and N1L-induced interdiction of IRF3 signaling to the ISRE, the soluble interferon receptors interdict a distinct ISRE-binding transcription factor (Fig. 2). Other poxviral inhibitors of interferon signaling include the proteins encoded by the E3L and K3L genes of vv that bind double stranded RNA (dsRNA) and can inhibit IRF3 phosphorylation and IFN production[27]. Expression of the E3L gene can inhibit the dsRNA-binding PKR directly[28], whereas K3L cannot[27]. The precise mechanism whereby E3L inhibits IFN production is unknown, but it has been shown that E3L is important to *in vivo* pathogenesis[29]. Finally, the TLR response is central to the inhibition of vaccinia virus infections because TRIF-a TIR-adapter which can mediate the dsRNA TLR response-also mediates a 20-fold reduction in poxvirus titer *in vitro*[30]. Since TLRs inhibit the replication of poxviruses, it is not surprising that evolutionary pressure has selected for several poxvirus proteins that interdict TLR signaling[7,31].

The transcription of many IRF3 dependent genes often depends upon the co-ordinate action of another transcription factor, NF-κB. Vaccinia virus encodes several inhibitors of TLR/IL-1R signaling which result in NF-κB driven transcription. The first vaccinia TLR inhibitors to be described were the proteins A46R and A52R that inhibit TLR/IL-1R signaling[7] and N1L[31]. A52R inhibits IL-1R and IL-18R signaling[7]. Furthermore, A52R also inhibits TLR -1, -2, -4 and -6 signaling via NF-κB[32]. A46R inhibits IL-1 and IL-18 activation of NF-κB signaling[7]. The mechanistic implications of these functional differences in TLR/IL-1R inhibitors will be discussed below, as will the identity of novel members of the A52R family of TLR/IL-1R inhibitors.

Table 1: Identified vaccinia virus A52R gene family members with proposed functions

Vaccinia virus gene	Swinepox virus homolog	Amino acids	Mechanisim	Function
A52R	SPV135	188	Targets IRAK2 and TRAF6	Inhibits IL-1β, 1L-18 and TLR activation of NF-κB
A46R	SPV133	214	Unknown	Inhibits IL-1β activation of NF-κB
N1L	None	117	Targets TBK1 in the IKK complex	Inhibits IL-1β, TNF-α, lymphotoxin and TLR signaling
lymphotoxin				
C16L/B22R	SPV001/SPV150	181	Unknown	May inhibit TLR signaling

Table 2: Alignment of candidate vaccinia innate immune suppressor proteins via clustal 1.8[4-5]

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vvN1L      1 ----------------------MRTLLIRYILWRNDNDQTYYNDDFRKLMLLDELVDD----
vvA46R     1 ---MAFDISVNASKTINALVYFSTQQNKLVIRNEVNDTHYTVEFDRDKVVDTFISYNRHN
vvA52R     1 -MDIKIDISISGDK-FTVTTRRENEERKKYLPLQKEKTTDVIKFDYLEYDDLLDRD----
C16L       1 MISLSFLIHNPLKKWKLKPSISINGYRSTFTMAFPCAQFRPCHCHATFDSLNTVADVR--

vvN1L     37 ------------------------------------------------------------
vvA46R    58 DTIEIRGVLPEETNIGCAVNTPVSMTYLYNKYSFKLILAEYIRHRNTISGNIYSALMTLD
vvA52R    55 ----------EMSTILEEYFMYRGLLGLRIKYG-----------------RLENEIKKFD
C16L      59 -------------HCLTEYILWVSHRMTFRESAG----------------------SLYR

vvN1L     37 -------GDVCTLIKNMRMTL----SDGPLLLDRLNQPVNNIEDAKRMIAISAKWARDIGE
vvA46R   118 DLAIKQYGDIDLLFNEKLKVD----SDSGLFDFVNFVKDMICCDSRIVVAISSLVSKHWE
vvA52R    88 NDAEEQFGTIEELKQKLRLNSEEG-ADNFIDYIKVQKQDIVKLTVYDCISMIGLCACVVD
C16L      84 LLISFRTDATELFGGELKDSLPWDNIDNCVEIIKCFIRNDSMKTAEELRAIIGLCTQSAI

vvN1L     86 RS-----EIRWEESFTILFRMIETY-FDDLMIDLYGEK------
vvA46R   174 LTNKKYRCMALANIYLIVFQYLSYLDYDTIYVSTYAGTIRA---
vvA52R   147 VWRNEKLFSRWKYCLRAIKLFINDHMLDKIKSIIQNRLVYVEMS
C16L     144 VSG-RVFNDKYIDILLMLRKILNENDYLTLLDHIRTAKY-----
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Vaccinia virus also encodes several novel A52R family members many of which are incompletely characterized. N1L is one of these novel TLR inhibitors. The N1L protein inhibits NF-κB signaling via TLRs and the receptors for interleukin-1 β, lymphotoxin and TNF-α. N1L inhibits signaling by: TIR adapter molecules, several downstream components of the NF-κB signaling pathway and IRF3-dependent signaling. The C16L ORF belongs to the A52R family and possesses an unknown function. Duplicate copies of C16L are present in variola virus inverted terminal repeats. The B22R gene represents the duplicate copy in certain vaccinia virus strains, but only one copy of C16L/B22R is present in highly the attenuated vaccinia virus strain known as modified vaccinia virus Ankara. C16L also inhibits innate immune signaling (our unpublished observations). The precise target of C16L is unknown, as is the significance of its absence from the modified vaccinia virus Ankara.The swinepox genome encodes at least five A52R homologs in the swinepox virus genome[6], yet only four homologs are identified in the vaccinia virus genome (Table 1). Since there is not an identified homolog in vaccinia virus corresponding to the SPV007 A52R family member (Table 1), it is logical to hypothesize that the genome of vaccinia virus may encode more uncharacterized A52R family members. Thus, much remains to be learned about the number and function of poxviral inhibitors of innate immune signaling.

As is evident from inspection of Fig. 1 and 2, many innate signaling pathways are targeted by more than one immunomodulatory protein encoded by vaccinia virus. Examples include poxviral targeting of TNF-α and IL-1β signaling at multiple steps in the signal transduction pathway. IL-1β mediates numerous antiviral inflammatory responses[33]. Four separate vaccinia virus proteins-soluble IL-1 receptor, A46R, A52R and N1L-are known to interdict the host IL-1 signaling pathway, many via targeting individual components of the NF-κB signaling pathway (Fig. 1). TNF-α represents another potent antiviral cytokine[34,35], which is inhibited by several poxviral genes. Several distinct soluble and/or membrane-bound TNFRs are encoded by Orthopoxviruses[36], possibly reflecting differences in selection pressure upon the poxviral genome[37]. TNF-α is also inhibited by the TLR inhibitor N1L that is present in several poxviruses[31], but apparently is not encoded by the swinepox virus genome (Table 1). Thus, distinct selection pressures from the innate immune system may have differentially shaped the evolution of several poxviral immunomodulatory genes.

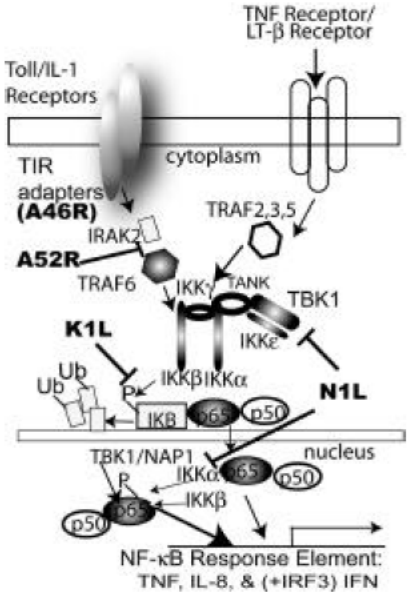

Fig. 1: Toll-like receptor ligands, TNFα and light all induced signal transduction that is targeted by vaccinia virus proteins. Vaccinia virus proteins K1L, N1L, A52R and A46R inhibit the NF-κB signaling pathway. While specific signal transduction components of A46R are still unknown, A52 associates with TRAF6 and IRAK2, ultimately inhibiting the phosphorylation of IκBα. K1L target prevents the ubiquitination and degradation of IκB, thus inhibiting the translocation and the transactivation of NF-κB. N1L targets TBK1 of the IKK complex and potentially inhibits either inhibits the translocation of NF-κB and/or TBK/NAP1 pathways which phosphorylates p65 to transactive the NF-κB transcription factor

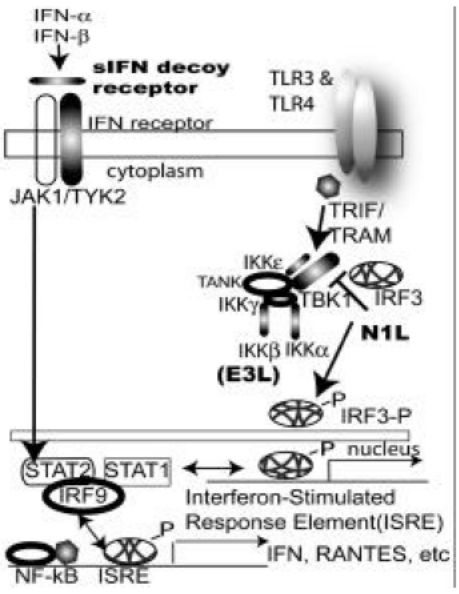

Fig. 2: N1L and E3L viral proteins inhibit IRF3 signal transduction pathways. Ligands dsRNA and LPS, respective of TLR3 and TLR4, induces signaling via the IKK complex to IRF3. N1L vaccina virus protein targets TBK1 inhibiting the phosphorylation of IRF3 and translocation of IRF3. Soluble interferon receptors (sINRF), which are independent to TLRs, simulate the JAK/STAT complex that ultimately phosphorylates STAT2/STAT1/IRF9, permitting the STAT2/STAT1/IRF9 complex to bind to the ISRE

Two different TLR inhibitors, A52R and N1L are known to independently target most TLR signaling. However, signaling via the TLR3 pathway is only inhibited by N1L[31]. Signaling via certain lymphotoxins is only inhibited by N1L and no other poxviral antagonist of similar lymphotoxins has been identified to date[36]. One hypothesis to explain the salient existence of only one poxvirus inhibitor of lymphotoxin and TLR 3 signaling pathways is that the these signaling pathways may be inhibited by uncharacterized vaccinia virus proteins[37]. Alternatively, the evolution of innate immunomodulatory proteins may represent gene duplication followed by mutation of the duplicated gene(s), with partial retention of the duplicated genes' original function. Thus, certain innate immune pathways may only be targeted by a single poxviral inhibitor, because the interdiction of these signaling pathways represents a distant, divergent evolutionary event (alignment Table 2). Other mechanistic constraints upon immunomodulation via vaccinia virus-encoded soluble Toll receptors might potentially include the large number of Toll receptors-currently numbering eleven-relative to the number of viral virulence genes potentially devoted to interdicting the TLR response (Table 1). We previously hypothesized that other poxvirus innate immune inhibitors possessing amino acid sequence similarity to A52R would be identified[38]. The analysis in this review suggests the hypothesis that at least two more uncharacterized A52R family members are encoded by vaccinia virus.

Mechanisms of innate immune modulation by poxviruses: Poxviral inhibitors of TLR/IL-1R signaling function by several different mechanisms. A52R inhibits NF-κB signaling triggered by stimulation of TLRs signaling via targeting both TRAF6 and IRAK 2 (Fig. 1)[32]. A46R inhibits IL-1R signaling, but A46R does not inhibit TLR

signaling via NF-κB[7]. Thus, A46R appears to inhibit signaling upstream of A52R. N1L co-immunoprecipitated along with members of the IKK complex, specifically targeting TANK-binding kinase 1 (TBK1)[31]. These findings are consistent with the hypothesis that N1L disrupts the IRF3/NF-κB signaling pathway by targeting the IKK kinase complex (Fig. 1 and 2). Orthopoxviruses, except for the modified vaccinia virus Ankara strain, have been hypothesized to encode a novel inhibitor of TNF-mediated signaling to NF-κB-[39]. This inhibitor is encoded by the K1L ORF and the K1L protein halted degradation of IkBα[40]. As noted earlier the degradation of IkBα and its dissociation from p50:p65 NF-κB is a required step for NF-κB translocation and activation of NF-κB-responsive promoters. Thus, there are several mechanisms whereby poxvirus proteins inhibit the translocation of p50:p65 NF-κB.

Inhibition of transactivation of the p65 subunit of NF-κB is another hypothetical mechanism to partly explain the inhibition of NF-κB signaling by poxviruses. Phosphorylation of the p65 subunit of NF-κB enhances the potential of NF-κB to bind to many NF-κB sensitive promoters, thereby driving the transcription of many immune regulatory and inflammatory genes of the immune system[41]. This process, known as transactivation, is common to all the signaling pathways inhibited by N1L (Fig. 1), including signaling by IL-1β, Toll ligands, TNF-α and LIGHT. Furthermore, TBK-1 and IKKϵ may not promote NF-κB translocation by phosphorylating IκBα and permitting the translocation of NF-κB. Instead, TBK1 is thought to phosphorylate and transactivate p65[21]. Because N1L associates with TBK1 and inhibits TBK1 activation of NF-κB (Fig. 1), we hypothesize that inhibition of transactivation of p65 is responsible for N1L-mediated inhibition of NF-κB.

Future directions in the investigation of poxvirus innate immune inhibitors: Poxviral inhibition of the innate immune response may suppress the adaptive immune response, consistent with a growing body of literature linking innate to adaptive immune responses[23,24,42]. A study of priming of dendritic cells infected with wild type or N1L-deficient vaccinia virus, would be useful to test the hypothesis that N1L and other poxviral innate immune inhibitory molecules may inhibit priming of DCs or APCs *in vitro*. Suppression of innate immune signaling by N1L (Fig. 1 and 2), has been suggested to explain the increased viral load and an inhibited adaptive immune response seen with infection using wild type, N1L-encoding, vaccinia virus[30].

Virus deficient in innate immune inhibitors, such as A52R and N1L, is attenuated[43,44]. The immunogenicity of N1L-deficient vaccinia virus is comparable to wild type vaccinia virus[43]. A46R and A52R are upstream of and more potent inhibitors of NF-κB signaling than N1L; thus, experiments to test the phenotype of vaccinia virus deficient in both N1L and A52R may yield insight into the role of innate immune inhibition in vaccine immunogenicity and attenuation. Such experiments should quantitate the combined effects of A52R and N1L upon adaptive and innate immunity. Such studies would advance understanding of poxvirus mechanisms that perturb innate immune signaling and increase knowledge about a potent poxvirus virulence factor.

Several distinct mechanisms have been identified whereby vaccinia virus evades the immune response via modulation of innate immune signaling including: decoy receptors for cytokines and interferons, IKK kinase complex inhibitors, inhibitors of PKR and inhibitors of TRAF6/IRAK2. Some studies suggest that vaccinia viruses that do not encode such immunoevasion genes may represent superior[43], or at least safer[29,44], vaccines. Ultimately, investigation of the interdiction of innate immune signaling pathways by poxviruses promises to yield a better understanding of the immune response.

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