Ectromelia, vaccinia and cowpox viruses encode secreted interleukin-18-binding proteins

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Interleukin–18 (IL-18) is a proinflammatory cytokine that plays a key role in the activation of natural killer and T helper 1 cell responses principally by inducing interferon-γ (IFN-γ). Human and mouse secreted IL-18-binding proteins (IL-18BPs) have recently been described which block IL-18 activity but have no sequence similarity to membrane IL-18 receptors. Several poxvirus genes encode proteins with sequence similarity to IL-18BPs. Here we show that vaccinia, ectromelia and cowpox viruses secrete from infected cells a soluble IL-18BP (vIL-18BP) that may modulate the host antiviral response. The ectromelia virus protein was found to block NF-κB activation and induction of IFN-γ in response to IL-18. The highly attenuated vaccinia virus modified virus Ankara encodes IL-18-binding activity, and thus deletion of the vIL-18BP may improve further the safety and immunogenicity of this promising human vaccine candidate. We confirm that molluscum contagiosum virus, a molluscipoxvirus that produces small skin tumours in immunocompetent individuals and opportunistic infections in immunodeficient AIDS patients, also encodes a related, larger vIL-18BP (gene MC54L). This protein may contribute to the lack of inflammatory response characteristic of molluscum contagiosum virus lesions. The expression of vIL-18BPs by distinct poxvirus genera that cause local or general viral dissemination, or persistent or acute infections in the host, emphasizes the importance of IL-18 in response to viral infections.

Introduction

Poxviruses are a family of complex DNA viruses which infect a wide variety of mammalian species, but only two species exclusively infect humans (Fenner et al., 1989; Moss, 1996). These are the orthopoxvirus variola virus which, before its eradication, caused smallpox, and the distantly related molluscipoxvirus molluscum contagiosum virus (MCV), which causes papular tumours in the skin of immunocompetent children and young adults and more serious opportunistic infections in immunodeficient individuals such as AIDS patients. Other species of orthopoxvirus are: vaccinia virus (VV), the vaccine used for smallpox eradication which is of unknown origin and natural host; cowpox virus, probably a rodent virus that causes sporadic skin infections in cows, cats and humans; and ectromelia virus (EV), a highly virulent natural pathogen of mice that causes mousepox and has been isolated from outbreaks in laboratory mouse colonies.

Molecular mimicry of cytokines and cytokine receptors is an immune evasion strategy adopted by large DNA viruses (herpesviruses and poxviruses) (Dairaghi et al., 1998; Nash et al., 1999; Smith et al., 1997; Spriggs, 1996). Poxviruses express a unique set of secreted, soluble receptors for tumour necrosis factor, interleukin (IL)-1β, interferon (IFN)-γ, IFN-α/β and chemokines, which modulate the immune response and viral pathogenesis. The genomic sequence of MCV has not revealed homologues of known orthopoxvirus cytokine receptors (Senkevich et al., 1996). This is surprising because immune responses to MCV are weak, with little or no inflammatory reaction in the virus-filled epidermal lesions. The MCV anti-inflammatory mechanisms are not fully understood, but MCV probably encodes several other cytokine inhibitors. For example, rather than producing a chemokine-binding protein like the orthopoxviruses, MCV encodes a chemokine antagonist that may block leukocyte recruitment to sites of virus replication (Damon et al., 1998; Krathwohl et al., 1997).

IL-18 synergizes with co-stimulants such as IL-12 to induce IFN-γ production from T, B and natural killer cells (Dinarello, 1999). Binding of this cytokine to specific cell-surface receptors induces NF-κB activation (Kojima et al., 1999). In vivo, IL-18 is critically important for production of IFN-γ and inflammatory
responses, and may contribute to chronic inflammatory diseases. The production of IL-18 is important in the antiviral response since administration of this cytokine protects mice against herpes simplex virus 1 and VV infection (Fujikawa et al., 1999; Tanaka-Kataoka et al., 1999). IL-18 is related to IL-1: the ligands share some sequence similarity, their mature forms are processed by caspase-1, their receptors are Ig superfamily members related at the sequence level, and both induce NF-κB activity in cells in culture and in mice injected with lipopolysaccharide (LPS) (Aizawa et al., 1999; Novick et al., 1999). The IL-18-binding proteins (BPs) have amino acid sequences unrelated to the IL-18 receptor and are unlikely to derive from post-translational or post-transcriptional modification of a membrane form. Intriguingly, the IL-18BP were found to be related, at the amino acid sequence level, to a family of putative poxvirus proteins encoded by single open reading frames (ORFs) in the orthopoxviruses EV, VV, cowpox virus and variola virus (ORF D7L in variola virus Bangladesh-1975), and the suipoxvirus swinepox virus, and by three ORFs in MCV (MC51L, MC53L and MC54L) (Novick et al., 1999; Xiang & Moss, 1999a). A recent paper by Xiang & Moss (1999b) reported that MCV MC53L and MC54L encode IL-18-binding activity.

Here we report that the orthopoxviruses VV, EV and cowpox virus express a soluble IL-18BP (vIL-18BP), encoded by homologues of the variola virus D7L ORF, that is secreted from infected cells and may modulate the host antiviral response. We also confirm that MCV encodes a related, larger vIL-18BP (gene MC54L) which may contribute to the lack of inflammatory response characteristic of MCV lesions.

Methods

Cells and viruses. Human KG-1 cells were obtained from the European Collection of Cell Cultures. VV strains Lister and Western Reserve (WR), cowpox virus and EV were grown in BSC-1 cells, and VV modified virus Ankara (MVA) was grown in BHK-21 cells. VV strains WR, Lister and MVA, and cowpox virus Bright Red, were obtained from Geoffrey L. Smith (St. William Dunn School of Pathology, University of Oxford, UK). EV isolates were obtained from the following sources: Hampstead and Moscow (original stocks from Keith Dunbell) from John Williamson (St. Mary’s Hospital, Imperial College School of Medicine, London, UK); Ishibashi-I-111 from Y. Ishibashi (Faculty of Medicine, Niigata University, Japan); Naval Medical Research Institute (Naval) and plaque-purified Moscow (mos-3-P2) from R. M. L. Butler (School of Medicine, Saint Louis University, MO, USA); MP-1, -2, -3, -4 and -5 from Hermann Meyer (Institute of Microbiology, Federal Armed Forces Medical Academy, Munich, Germany); and the attenuated strain Hampstead egg from A. Mullbacher (John Curtin School of Medical Research, Australia). The viral species of all orthopoxviruses was confirmed by a diagnostic test based on the amplification by PCR of the gene encoding the A-type inclusion body protein followed by restriction enzyme analysis (not shown) (Meyer et al., 1997). Skin biopsies containing MCV were obtained from Javier Ortiz (Hospital 12 Octubre, Madrid, Spain) and the virus isolate was designated MCV Madrid 99. This sample was confirmed to contain MCV subtype 1 by PCR and restriction nuclease analysis of specific viral DNA sequences (data not shown) (Nuñez et al., 1996). Spodoptera frugiperda (SF) 21 insect cells and Autographa californica nuclear polyhedrosis virus (AcNPV) were cultured in TC100 medium containing 10% foetal calf serum (FCS).

Reagents. Recombinant human and mouse IL-18 were obtained from R&D Systems. Human IL-15 and IFN-γ were from Peprotech. Human IL-18 was radiiodinated using 125I-Bolton–Hunter reagent (Amersham Pharmacia) to a specific activity of 2 x 10^6 c.p.m./μg.

Extraction of viral DNA and sequence of viral genes. MCV DNA was prepared from skin lesions (Nuñez et al., 1996) and viral DNA from BSC-1 cells infected with EV or VV was extracted from viral cores (Esposito et al., 1981). The vIL-18BP genes from EV strains Hampstead and Naval and VV Lister were PCR amplified with Taq DNA polymerase and oligonucleotides flanking the ORF, based on the known sequence of the gene in EV mos-3-P2 and VV WR (accession nos PXX01161 and M22812, respectively), and the PCR fragment sequenced. The MCV gene MC54L was PCR amplified with Taq DNA polymerase with oligonucleotides based on the MCV genomic sequence (Senkevich et al., 1996). MC54L-1 (5’ CCCGATATGTAAGGATTTCTGGA 3’) and MC54L-2 (5’ CCGAACTTCTCGATCATGTGATCTTCTGGG 3’), corresponding to both ends of the ORF. The PCR fragment was cloned into pBAC-1 (Novagen) and the sequence determined in two independent clones, which were identical. Note that the 21 nucleotides at each end of the ORF correspond to the published MCV4L sequence (Senkevich et al., 1996). DNA sequencing was carried out by the DNA Sequencing Service of the Department of Biochemistry (University of Cambridge) and the sequence data were analysed using the GCG computer programs.

Expression in the baculovirus system. Recombinant proteins, fused to a C-terminal 6-His tag, were produced in the baculovirus system. vIL-18BP genes from orthopoxviruses were amplified by PCR with Pfu DNA polymerase using viral DNA as template. MCV genes were amplified with Taq DNA polymerase from skin samples. The oligonucleotides corresponding to the 5’ and 3’ ends of the ORFs provided BamHI and HindIII/PstI sites, respectively. The oligonucleotides used for EV Naval and VV Lister were P16-3 (5’ CCCGATATGTAAGGATTTCTGGA 3’) and P16-4 (5’ CCGAACTTCTCGATCATGTGATCTTCTGGG 3’), and for EV mos-3-P2 were P16-3 and P16-1 (5’ CCGGTCGACCTAGGCAAAATATCCTTTTGG 3’), and were based on the DNA sequence of each virus. The MC54L gene was amplified with oligonucleotides MC54L-1 and MC54L-2. DNA fragments were cloned into pBAC-1 creating plasmids pNB1 (EV mos-3-P2), pNB2 (EV Naval), pNB6 (VV Lister) and pNB2 (MC54L). The DNA sequence of the inserted was confirmed not to contain mutations. The recombinant baculoviruses were produced as described (Alcamí et al., 1998a), and named Acp11Moscow (AcNb1), Acp9Naval (AcNb5), Acp9Lister (AcNb6) and AcCMC54L (AcNb2). The recombinant baculoviruses AcB15R and Ac35K have been described (Alcamí & Smith, 1992; Alcamí et al., 1998a). A recombinant baculovirus expressing the EV IL-1β receptor, fused to a C-terminal 6-His tag, will be described elsewhere (V. P. Smith & A. Alcamí, unpublished). Recombinant EV IL-18BP and IL-1β receptor were purified by metal affinity chromatography by using TALON Metal Affinity Resin (Clontech).

Metabolic labelling of proteins and electrophoretic analysis. SF21 cells were infected with baculoviruses at high m.o.i. At the
indicated time post-infection, cultures were pulse-labelled with 150 μCi/ml [35S]methionine (Amersham; 1200 Ci/mmol) and 150 μCi/ml [35S]cysteine (DuPont–New England Nuclear, 600 Ci/mmol) in methionine- and cysteine-free TC100 medium in the absence of serum. Cells or media were dissociated in sample buffer and analysed by SDS-PAGE in 12% gels and fluorography with Amplify (Amersham).

■ Binding assays. Supernatants from orthopoxvirus-infected BSC-1 cells or baculovirus-infected SF cells were harvested at 2 or 3 days post-infection, respectively, and were concentrated and dialysed against PBS as described (Alcamí & Smith, 1992; Alcamí et al., 1998a). Infectious VV, EV and cowpox virus present in supernatants was inactivated with 4.5, 8-trimethylpsoralen and exposure to UV light (Alcamí et al., 1998a). Binding medium was RPMI 1640 containing 20 mM HEPES (pH 7.4) and 0.1% BSA. Cross-linking to 0.4 mM 125I-IL-18 was performed in a volume of 25 μl with 5 mM bis(sulfosuccinimidyl)suberate (BS3, Pierce) as the cross-linker (Alcamí et al., 1998a; Novick et al., 1999). Samples were analysed by 12% acrylamide SDS–PAGE.

■ Electrophoretic mobility shift assay. KG-1 cells (5 × 10⁶ cells in 1 ml) were stimulated for 20 min at 37 °C with 10 ng/ml of human IL-18, preincubated for 1 h at room temperature with purified vIL-18BP or control medium. Nuclear extracts were then prepared as described (Bacherie et al., 1991) and incubated for 20 min at room temperature with a [γ-32P]ATP-labelled probe corresponding to the NF-κB-binding element (Promega), together with poly(dI–dC). The mixtures were analysed by non-denaturing PAGE in 0.5 × TBE (40 mM Tris–HCl, 45 mM boric acid and 5–5 mM EDTA) and autoradiography. Binding specificity was assessed in the presence of 100-fold excess unlabelled oligonucleotide and antibodies (goat polyclonal IgG) specific for the p50 subunit of NF-κB (Santa Cruz Biotechnology).

■ Assay of IL-18 in murine splenocytes. Freshly isolated splenocytes of BALB/c mice in RPMI 1640–10% foetal calf serum (2.5 × 10⁶ cells/ml) were stimulated with 1 μg/ml LPS and 15 ng/ml murine IL-18 (Novick et al., 1999). IL-18 was preincubated for 2 h at room temperature with EV IL-18BP or IL-1α receptor expressed in the baculovirus system and purified by metal affinity chromatography. The production of murine IFN-γ in the culture supernatant after 24 h was determined by ELISA (Diaclone Research). Purified recombinant proteins were confirmed not to interfere with the detection of IFN-γ by ELISA (not shown).

Results

IL-18-binding activity in the supernatant of cultures infected with orthopoxviruses

We investigated the presence of soluble IL-18BPs in supernatants from orthopoxvirus-infected cells in cross-linking experiments with recombinant human 125I-IL-18 and BS3 (Alcamí et al., 1998a; Novick et al., 1999). We detected a 27–28 kDa complex between 125I-IL-18 and a secreted viral protein expressed by VV strains WR, Lister and MVA, cowpox virus strain Brighton Red and EV strain Naval (Fig. 1a). No such complexes were detected with EV strains Moscow (not shown), Hampstead or plaque-purified Moscow (mos-3-P2), VV Copenhagen, or mock-infected cells (Fig. 1a). Screening of further EV strains revealed that strains MP-1, -2, -3, -4 and -5 expressed a vIL-18BP whereas strains Ishibashi and Hampstead Egg, which was derived from strain Hampstead by passage in chicken embryos, did not (Fig. 1a). The binding specificity of vIL-18BP encoded by orthopoxviruses was further investigated by cross-linking to human 125I-IL-18 in the presence of unlabelled cytokines. vIL-18BPs specifically bound both human and mouse IL-18 but not human and mouse IL-1β and IFN-γ (Fig. 1b). The protein encoded by cowpox virus and EV bound mouse IL-18 better than human IL-18, consistent with rodents being the natural host of these viruses.

Sequence of vIL-18BP genes in orthopoxviruses

The vIL-18BP genes in VV Lister and EV strains Hampstead and Naval were amplified by PCR, with oligonucleotides based on the known sequence of EV mos-3-P2, and sequenced. The vIL-18BPs encoded by different orthopoxviruses are very similar (Fig. 2a). Sequence analysis predicted a shorter polypeptide in EV Naval relative to the EV mos-3-P2 and Hampstead ORFs, which have a 3' end mutation that extends the ORF 12 amino acids and may be responsible for the lack of activity in this cross-linking assay (Fig. 2a). The vIL-18BP ORF is intact in VV Lister, deleted in VV Copenhagen (accession no. M35027) and truncated in the EV WR sequence available (accession no. M22812), but we observed IL-18-binding activity in our EV WR strain. The MVA gene sequence (Antoine et al., 1998) predicts a deletion of six amino acids that does not affect protein activity. These amino acids are also absent in the mammalian and MCV MC54L IL-18BPs (Fig. 2b).

Expression of vIL-18BPs from orthopoxviruses and MCV in the baculovirus system

To formally demonstrate that the viral IL-18-binding activity is encoded by the ORFs related to human IL-18B, recombinant baculoviruses expressing the predicted vIL-18BP from EV Naval, EV Moscow and VV Lister, fused to a C-terminal 6 × His tag, were constructed. A recombinant baculovirus expressing the MCV ORF MC54L, the closest homologue to human IL-18BP in MCV (Novick et al., 1999; Xiang & Moss, 1999a), was also constructed. The MC54L ORF was amplified by PCR with oligonucleotides based on the DNA sequence of the MCV type I prototype isolate (Senkevich et al., 1996). Since MCV does not replicate in tissue culture, the MCV DNA used as a template was extracted from skin biopsies from a patient clinically diagnosed with MCV, an isolate we designated MCV Madrid 99. PCR and restriction endonuclease analysis of specific viral DNA sequences showed that this isolate is MCV subtype 1 (data not shown) (Nunez et al., 1996). DNA sequence analysis showed that this sequence has 92.9% amino acid identity with the published MC54L sequence. One of the few differences was a deletion of 24 amino acids in a region with direct repeats, which may represent variability among MCV isolates (Fig. 2b) (Moratilla et al., 1997; Senkevich et al., 1996).
Pulse-labelling experiments with $[^{35}S]$methionine and $[^{35}S]$cysteine of insect cells infected with recombinant baculoviruses showed secretion of all recombinant proteins (Fig. 3a). The apparent sizes of the vIL-18BP from EV Naval and VV Lister were smaller (9 kDa) than predicted from the sequence (14 kDa). The larger molecular mass of the EV mos-3-P2 protein (11 kDa) was consistent with the predicted C-terminal extension of the polypeptide. The 28 kDa size of the MCV protein MC54L, ranging from 26 to 29 kDa, was smaller than that reported after expression from a VV recombinant (Xiang & Moss, 1999a), probably due to deficient glycosylation in insect cells. Binding experiments to human $[^{125}I]$-IL-18 followed by cross-linking showed that the recombinant proteins encoded by EV Naval, VV Lister and MCV MC54L encoded IL-18-binding activity (Fig. 3b). Cross-linking of human $[^{125}I]$-IL-18 in the presence of excess unlabelled human and mouse IL-18, or a 200-fold molar excess of unlabelled human or mouse IL-18, or a 200-fold molar excess of unlabelled human (h) or mouse (m) IL-1$\beta$ and IFN-γ showed binding specificity for IL-18 (not shown).

Inhibition of IL-18 biological activity by recombinant EV vIL-18BP

The recombinant vIL-18BP from EV strain Naval purified by metal affinity chromatography was tested for its ability to inhibit the biological activity of IL-18 in vitro. Two different assays were performed: the IL-18-mediated activation of NF-$\kappa$B in human KG-1 cells (Kojima et al., 1999) and the induction of IFN-γ in murine splenocytes in response to IL-18 and LPS (Novick et al., 1999).

Human IL-18 activated NF-$\kappa$B in KG-1 cells, as determined by electrophoretic mobility shift assay with nuclear extracts and a radiolabelled oligonucleotide of the NF-$\kappa$B consensus sequence (Fig. 4a). Binding specificity was confirmed in the presence of a 100-fold excess of unlabelled oligonucleotide, by supershift in the presence of antibodies specific for the p50 subunit of NF-$\kappa$B, and by the induction of a similar band in response to IL-1$\beta$, known to activate NF-$\kappa$B. Purified EV Naval
vIL-18BP inhibited, in a dose dependent manner, the activation of NF-κB in response to IL-18 (Fig. 4a).

Recombinant purified vIL-18BP from EV Naval blocked the production of IFN-γ by murine splenocytes in response to murine IL-18 and LPS (Fig. 4b). IFN-γ levels were reduced to the limit of detection, indicating that the lower levels of IFN-γ produced in response to LPS alone, probably co-induced by endogenous IL-18 produced by splenocytes, were also inhibited by the vIL-18BP. Addition of the EV IL-18 receptor, produced in the baculovirus system and also purified by metal affinity chromatography, had minor effects on IFN-γ production, indicating that possible contaminants present in the preparations of purified proteins had no effect on the IL-18 activity. The EV vIL-18BP inhibited IL-18 activity in a second
experiment in which splenocytes were incubated with a higher dose of IL-18 (75 ng/ml) and produced 1-5 ng/ml of IFN-γ (not shown).

**Discussion**

We report that soluble vIL-18BPs are encoded by a single ORF in the orthopoxviruses EV, VV and cowpox virus. We also confirm that the related, but distinct, ORF MC54L from the molluscipoxvirus MCV encodes IL-18-binding activity (Xiang & Moss, 1999b). The vIL-18BP encoded by EV strain Naval blocks the biological activity of IL-18 in vitro, suggesting that the vIL-18BPs may modulate the immune response in the infected host.

IL-18 and IL-1, and their respective receptors, are related proteins. The binding and biological activity experiments with recombinant proteins included the orthopoxvirus IL-1β receptor, encoded by gene B15R in the VV WR strain (Alcamí & Smith, 1992; Spriggs et al., 1992), and showed that it does not bind IL-18. In addition, competition studies showed that natural and recombinant vIL-18BPs do not bind IL-1β. The cowpox protein CrmA and its VV homologue inhibit caspase-1 and block maturation of IL-1β (Smith et al., 1997). Poxviruses may also inhibit IL-18 cleavage by caspase-1 within infected cells as a second mechanism to block IL-18 activity during viral infection.

VV represents a potential recombinant human vaccine. One of the best candidates is VV MVA due to its restricted host range, immunogenicity and avirulence in animal models, and excellent safety record as a smallpox vaccine. VV MVA does not express the soluble cytokine receptors found in other VV strains, with the exception of the IL-1β receptor (Antoine et al., 1998; Blanchard et al., 1998). Here we show that VV MVA produces IL-18-binding activity, and thus deletion of vIL-18BP from VV MVA may improve further the safety and immunogenicity of this virus vector.

The variola virus vIL-18BP has 95-2 and 93-6% amino acid identity to the active genes encoded by EV and VV, respectively. Thus, it is likely that the human pathogen which caused smallpox also utilized this immune evasion strategy. The discovery of vIL-18BP in the highly virulent EV, which causes a generalized infection known as mousepox and represents a model for human smallpox (Fenner et al., 1989), will allow determination of the role of vIL-18BP in vivo. These studies will also be relevant for the vIL-18BP encoded by MC54L since there is no animal model available for MCV.
The MCV MC54L polypeptide has an extended C terminus which is approximately 70 and 100 amino acids longer than the host and the orthopoxvirus IL-18BPs, respectively, and may have additional binding properties. MCV encodes two other secreted proteins, MC53L and MC51L, related to a lesser extent to IL-18BPs (Novick et al., 1999; Xiang & Moss, 1999a). The MC53L ORF has recently been shown to encode IL-18-binding activity (Xiang & Moss, 1999b) and the MC51L protein may bind other host immune molecules related to IL-18.

The expression of vIL-18BPs by MCV is particularly relevant. The complete DNA sequence of MCV revealed immune evasion strategies different from those identified in other poxviruses (Senkevich et al., 1996). MCV persists for months in the skin of the immunocompetent host (Moss, 1996), and may require immune evasion molecules different to those evolved by other poxviruses that produce acute infections. The vIL-18BPs are the first soluble cytokine-binding proteins identified in MCV and may account in part for the lack of inflammatory reaction at the site of viral replication in the skin. Resolution of MCV lesions depends upon the host immune system. Thus, neutralization of MCV-encoded IL-18-binding activity constitutes a potential target for therapeutic intervention.

Poxvirus-encoded soluble cytokine inhibitors normally represent secreted versions of the binding domain of membrane-bound receptors, with the exception of two VV soluble proteins that bind type I IFNs and chemokines and have no cellular counterpart (Alcamí et al., 1998b; Smith et al., 1998). Interestingly, the vIL-18BPs described here have sequence similarity to a host IL-18BP distinct from IL-18 receptors, suggesting that poxviruses may have also incorporated into their genomes genes that mimic host cytokine inhibitors.

The vIL-18BP is conserved among members of the poxvirus family. We detected by cross-linking that an active protein is expressed by six out of nine strains of EV isolated from outbreaks in laboratory mouse colonies, suggesting evolutionary pressure to keep this activity in poxviruses that replicate in their natural host. The conservation of the activity in all orthopoxvirus species studied and in MCV, which does not utilize the poxvirus cytokine receptors previously identified, emphasizes the important role of IL-18 in antiviral host defence and immune regulation.

IL-18 is required in vivo for induction of IFN-γ, as indicated by the low levels of IFN-γ produced in mice deficient in IL-18 (Takeda et al., 1998). Therefore, the inhibition of IL-18 activity by vIL-18BP represents a novel poxvirus mechanism for inhibiting IFN-γ induction, further emphasizing the importance of counteracting IFNs as an immune evasion strategy. These viruses are already known to inhibit the binding of IFNs to cellular receptors via their expression of soluble, high affinity receptors for IFN-γ and IFN-α/β, and also to encode intracellular proteins that block IFN-induced antiviral pathways in the infected cell (Smith et al., 1998).

vIL-18BP represents a novel viral immune evasion strategy and another poxvirus secreted protein that binds host immune regulatory molecules. These studies provide insights into
mechanisms of viral pathogenesis and new strategies of immune modulation.

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References


