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The vaccinia virus C12L protein inhibits mouse IL-18 and promotes virus virulence in the murine intranasal model

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A bioassay that measured the interleukin (IL)-12-induced production of interferon (IFN)-γ from mouse splenocytes was used to identify a soluble factor in the supernatants of vaccinia virus (VV)-infected cells that inhibited the production of IFN-γ. This soluble factor was expressed by 14 out of 16 VV strains including the Western Reserve (WR) strain, but strains Copenhagen and Tashkent and a mutant of strain WR called 6/2 lacked this activity. The gene encoding this activity was identified as C12L by transferring DNA present in VV WR but missing in VV WR 6/2 into VV Copenhagen and testing for expression of the soluble factor. The C12L protein shows amino acid similarity to IL-18 binding proteins that are encoded by poxviruses, mice and humans, and C12L protein produced from VV or baculovirus inhibited the biological activity of mouse IL-18 *in vitro*. Thus the inhibition of IL-12-induced IFN-γ production was due to indirect effects of C12L on IL-18, illustrating the synergistic action of these pro-inflammatory cytokines. To study the role of the C12L protein in the virus life-cycle, we constructed a deletion mutant lacking the C12L gene and a revertant virus in which the gene was reinserted into the deletion mutant. *In vitro* the replication and plaque size of these viruses were indistinguishable. However, infection of BALB/c mice by the intranasal route showed that the deletion mutant was attenuated and induced lower weight loss and signs of illness compared to controls.

Introduction

Large DNA viruses such as herpesviruses and poxviruses have evolved many strategies to modulate the host response to infection (for review see Alcamí & Koszinowski, 2000; Tortorella *et al.*, 2000). One strategy is to secrete soluble factors from infected cells that bind host proteins and inhibit components of the host response to infection (Smith, 2000). Vaccinia virus (VV), the vaccine used to eradicate smallpox, secretes proteins that bind complement factors (Kotwal & Moss, 1988a; Kotwal *et al.*, 1990), interferon (IFN)-γ (Alcamí & Smith, 1995, 2002; Mossman *et al.*, 1995; Symons *et al.*, 2002), IFN-α/β (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Alcamí *et al.*, 2000), interleukin (IL)-1β (Alcamí & Smith, 1992, 1996; Spriggs *et al.*, 1992), CC chemokines (Graham *et al.*, 1997; Smith *et al.*, 1997; Alcamí *et al.*, 1998), tumour necrosis factor (TNF) (Alcamí *et al.*, 1999; Reading *et al.*, 2002), plexins (Comeau *et al.*, 1998; Gardner *et al.*, 2001) and IL-18 (Smith *et al.*, 2000). Other proteins are secreted and contribute to virulence but the ligands are unknown (Ng *et al.*, 2001).

Poxvirus immunomodulators have been identified by several methods. Most commonly, computational comparisons of the sequences of proteins deduced from poxvirus genome sequences revealed amino acid similarity with host protein(s) of known function. Soluble inhibitors of TNF (Smith *et al.*, 1990), IL-1β (Smith & Chan, 1991) and complement factors

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The nucleotide sequence of the vaccinia virus strain Western Reserve C12L gene has been deposited at GenBank and assigned accession no. AF510447.

(Kotwal & Moss, 1988a) were identified in this manner. Alternatively, proteins that are predicted or demonstrated to be secreted from infected cells have been studied and their ligands sought (Upton *et al.*, 1992; Smith *et al.*, 1997; Comeau *et al.*, 1998; Ng *et al.*, 2001). Lastly, functional assays using the supernatants of virus-infected cells have identified virus proteins that can bind to host factors (Graham *et al.*, 1997) or inhibit their biological activity (Symons *et al.*, 1995). In this way, virus proteins that bind chemokines or type I IFNs were identified and subsequently the encoding gene was mapped.

In this report, we screened the supernatants of orthopoxvirus-infected cells for a soluble inhibitor of IL-12 by testing the ability of these supernatants to inhibit the IL-12-induced production of IFN-γ from mouse splenocytes. IL-12 was selected because this is an important pro-inflammatory cytokine that promotes the Th1 immune response via induction of IFN-γ (Gately *et al.*, 1998). IL-12 acts synergistically with another pro-inflammatory cytokine, IL-18 (Robinson *et al.*, 1997; Yoshimoto *et al.*, 1998), which was originally designated IFN-γ-inducing factor (reviewed by Nakanishi *et al.*, 2001; Sims, 2002). Both IL-12 and IL-18 bind to specific receptors called IL-12R and IL-18R, respectively. No poxvirus protein with amino acid sequence similarity to the cytokine binding subunits of IL-12R or IL-18R has been identified, but when the human and mouse soluble inhibitors of IL-18, called IL-18 binding protein (IL-18 bp), were identified (Novick *et al.*, 1999) related proteins were reported to be encoded by several poxviruses including molluscum contagiosum virus (MCV) (gene MC54L) (Xiang & Moss, 1999a, b), ectromelia virus, cowpox virus and VV (Born *et al.*, 2000; Smith *et al.*, 2000), Yaba-like disease virus (Lee *et al.*, 2001), monkeypox virus (Shchelkunov *et al.*, 2001) and swinepox virus (Afonso *et al.*, 2002). Mutagenesis indicated that the human and MCV IL-18 bps have similar functional epitopes (Xiang & Moss, 2001a, b).

Here we report the identification and mapping of a VV inhibitor of IL-12-induced IFN-γ from mouse splenocytes and the mapping of the activity to VV gene C12L that encodes a protein with amino acid similarity to IL-18 bps. Recombinant C12L protein was shown to inhibit mouse IL-18 *in vitro*. Finally, the VV WR C12L gene is shown to contribute to virus virulence in a murine intranasal model.

Methods

■ **Cells and viruses.** Human TK$^-$143B and D98OR cells, and monkey BS-C-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated foetal bovine serum (FBS). CBA/ca mice were bred and maintained in specific pathogen-free conditions and single cell suspensions of mouse splenocytes were obtained by mechanical disruption in RPMI 1640 containing 10% FBS. *Spodoptera frugiperda* (Sf)21 cells were cultured as described previously (Symons *et al.*, 1995). VV strain Western Reserve (WR) was grown, titrated and purified as described previously (Mackett *et al.*, 1985) and the source of other VV strains was as described (Symons *et al.*, 1995).

■ **Construction of recombinant vaccinia viruses.** A virus deletion mutant lacking 40% of the C12L ORF was constructed using transient dominant selection (Falkner & Moss, 1990). A plasmid was assembled that contained the DNA flanking the 5′ and 3′ regions of the C12L ORF. These fragments were amplified by PCR using *Pyrococcus furiosus* DNA polymerase and VV WR DNA as template. The left flanking region was amplified with oligonucleotides 5′ CAAGGATCCG TTTCTAATATAATCTGCC 3′ (C12L1F) and 5′ GTTGAGCTCGGA TATGAGATCGGACGAG 3′ (C12L1R), which contain *Bam*HI and *Sac*I sites, respectively (underlined). The right flanking region was amplified with oligonucleotides 5′ GATGAGCTCCTTGCCAAAATATCACTA GA 3′ (C12L2F) and 5′ AAAGAATTCTAGGTGGTAATACTAT GTTC 3′ (C12L2R), which contain *Sac*I and *Eco*RI sites (underlined), respectively. These fragments were digested with the appropriate restriction enzymes and cloned sequentially into plasmid pSJH7 (Hughes *et al.*, 1991) that had been cut with the same enzymes. The resultant plasmid, termed pΔC12L, contained 331 and 315 nucleotides of the 5′ and 3′ flanking sequence, respectively, including 9 and 216 nucleotides of coding sequence at the 5′ and 3′ ends of the gene, respectively. All cloned PCR fragments were sequenced to check fidelity.

Plasmid pΔC12L was transfected into VV-infected cells and mycophenolic acid (MPA)-resistant recombinant viruses were isolated as described (Falkner & Moss, 1988). These were grown on hypoxanthine guanine phosphoribosyltransferase-negative D980R cells in the presence of 6-thioguanine (TG) (Kerr & Smith, 1991) and isolates corresponding to WT (vC12L) or deletion mutant (vΔC12L) were identified by PCR.

A revertant virus (vC12L-R) in which the C12L locus was restored to WT was constructed by transfecting a plasmid (pC12L-R) containing the entire WT C12L gene into cells infected with vΔC12L. Plasmid pC12L-R was constructed by amplifying the entire C12L ORF and flanking regions using primers C12L1F and C12L2R (above). The resulting 677 bp fragment was digested with *Bam*HI and *Eco*RI and cloned into pSJH7. MPA-resistant intermediate viruses were isolated as above and resolved into deletion mutant and revertant viruses (vC12L-R) on D980R cells in the presence of 6-TG as described. The genome structures of vC12L, vΔC12L and vC12L-R were analysed by PCR and Southern blotting and were found to be as predicted.

To generate a recombinant VV expressing high levels of the C12L protein the C12L ORF was cloned downstream of a strong VV promoter and inserted into the thymidine kinase (TK) locus of VV strain Copenhagen, a virus that lacks the IL-18 bp. The C12L ORF was excised from pAcC12L (see below) using *Bam*HI and *Sty*I and the resulting 400 bp fragment was cloned into pMJ601 (Davison & Moss, 1990) that had been cut with *Bam*HI and *Nhe*I, generating pMJ601/C12L. Plasmid pMJ601/C12L was transfected into VV strain Copenhagen-infected cells and TK-negative, β-galactosidase-positive recombinant viruses were isolated as described (Chakrabarti *et al.*, 1985).

■ **Construction of recombinant baculovirus expressing C12L.** The C12L protein was expressed in recombinant baculovirus (*Autographa californica* nuclear polyhedrosis virus; AcNPV) using methods described previously (Ng *et al.*, 2001). Briefly, the C12L ORF with or without six histidine residues at the C terminus was amplified by PCR using as primers oligonucleotides C12LF (5′ AGTAAGCTTGGCAAGATGAG AATCC 3′) and C12LR (5′ AAACTCGAGCACGCACTACTTCAGCC 3′), which contain *Hin*dIII and *Xho*I sites (underlined) respectively, or C12LF and C12LRhis (5′ GCACCTCGAGCTTCAGCCAAATATTC 3′), which contains a *Xho*I site (underlined), and VV WR DNA as template. The resultant DNA fragments were digested with *Hin*dIII and *Xho*I and cloned into transfer vector pBAC1 that had been digested with the same enzymes. The resultant plasmids, pAcC12L and pAcC12L-his, were used to construct baculovirus recombinants AcC12L and AcC12L-his, respectively.

■ **Preparation of supernatants from virus-infected cells.**

Cultures of TK⁻143B cells were infected with orthopoxviruses at 5 p.f.u. per cell. Alternatively, Sf21 cells were infected with baculoviruses at 10 p.f.u. per cell. Supernatants from poxvirus or baculovirus-infected cells were harvested at 1 or 3 days post-infection (p.i.), respectively, centrifuged at 3000 r.p.m. for 10 min at 4 °C and the pellet was discarded. Virus particles were removed by centrifugation at 16500 r.p.m. in an SW41 Ti rotor for 60 min at 4 °C. Supernatants were stored at −20 °C until use.

■ **IL-12-induced production of IFN-γ.** Microtitre plates (Falcon) were coated overnight at 4 °C with 50 µl of non-neutralizing monoclonal antibodies (mAb) against mouse IL-12 (C15.1.2 and C15.6.7) (gifts from G. Trinchieri, Wister Institute, Philadelphia, USA) each at 15 µg/ml in bicarbonate buffer. Plates were washed three times with PBS and blocked with 100 µl of 10% FBS in PBS for 2 h at 37 °C. After further washing, recombinant mouse IL-12 (R&D Systems) was added and incubated overnight at 4 °C. Plates were washed in PBS and 100 µl of a single cell suspension of mouse (CBA/ca) spleen cells (5×10^6 cells/ml) were added in RPMI 1640 medium containing 10% FBS in the presence or absence of 100 µl of medium alone or medium from mock- or virus-infected cells or neutralizing mAb to IL-12. Plates were incubated for 48 h at 37 °C and the medium was then harvested and assayed for mouse IFN-γ by ELISA.

■ **Anti-CD3 induced production of IFN-γ.** Microtitre plates (Falcon) were coated for 2 h at 37 °C with 50 µl of anti-CD3 mAb KT3 (Tomonari, 1988) at 1 µg/ml. The plates were washed three times with PBS. Single cell suspensions of mouse (CBA/Ca) spleen cells, which had been incubated in plastic vessels for 2 h to deplete macrophages, were added to the wells as above with either medium alone or medium from mock- or virus-infected cells at various concentrations. Plates were incubated for 48 h at 37 °C and the medium was then harvested and assayed for mouse IFN-γ by ELISA.

■ **IL-18-induced production of IFN-γ.** Splenocytes from CBA mice were cultured in RPMI 1640 medium supplemented with 10% FBS and were stimulated with 200 ng/ml concanavalin A and 12·5 ng/ml murine IL-18 for 24 h at 37 °C. The IFN-γ level in the culture medium was determined by ELISA. To test for inhibition of IL-18 by the VV C12L protein, murine IL-18 was incubated for 1 h at room temperature with clarified supernatants from TK⁻143 cells that had been infected with recombinant VVs or Sf21 cells that had been infected with recombinant baculoviruses.

■ **Measurement of IFN-γ by ELISA.** Microtitre plates were coated with anti-mouse IFN-γ mAb R4-6A2 (Pharmingen) at 10 µg/ml in bicarbonate buffer for 1 h. Plates were washed with PBS containing 0·5% Tween 20 and blocked with PBS containing 10% FBS for 1 h. Supernatants from IL-12, IL-18 or anti-CD3 stimulated splenocytes were then added in duplicate and incubated for 2 h at room temperature. A standard curve of mouse IFN-γ (R&D Systems) was included in each experiment. Plates were washed and bound IFN-γ was detected by incubation with the biotinylated anti-IFN-γ mAb XMG-1.2 (Pharmingen) for 1 h. The plates were washed extensively and then extravidin–peroxidase conjugate (Sigma; diluted 1:1000) was added for 1 h. Finally, the ELISA was developed by addition of OPD substrate for 0·5 h, the reaction was stopped by addition of 3 M H_2SO_4 and the absorbance was read at 492 nm.

■ **Assays for virus virulence.** Groups of female BALB/c mice, between 6 and 8 weeks of age, were anaesthetized and infected intranasally with 10^4 p.f.u. of VV in 20 µl PBS. Each day, mice were weighed individually and monitored for signs of illness as described previously (Alcamí & Smith, 1992), and those suffering a severe infection or having lost >30% of their original body weight were sacrificed. Alternatively, mice were infected by injection of virus into the ear pinna and the lesion size was measured daily as described previously (Tscharke & Smith, 1999; Tscharke *et al.*, 2002).

Results

Poxviruses express an inhibitor of IL-12-induced IFN-γ

Poxviruses encode several proteins that are secreted from infected cells and bind to host proteins involved in the innate response to infection (Introduction). Given that several of these proteins target Th1 cytokines such as IFN-γ, TNF and IL-1β, we were interested to determine if these viruses also expressed an inhibitor of IL-12. To search for an IL-12 inhibitor, supernatants from TK⁻143 cells infected with different VV strains or other orthopoxviruses were tested in a bioassay that measured the IL-12-induced production of IFN-γ from mouse splenocytes (Fig. 1a). These splenocytes produced IFN-γ if stimulated with IL-12 or if incubated with IL-12 in the presence of supernatants from mock-infected cells, but the production of IFN-γ was inhibited by supernatants taken from cells infected with 13 out of 15 strains of VV, two strains of cowpox virus (Brighton Red and elephantpox virus) and to a lesser extent camelpox virus (CMPV). Notably, VV strains Tashkent and Copenhagen lacked this activity. In addition, supernatants of chick embryo fibroblasts (CEF) infected with VV strain modified vaccinia Ankara (MVA) express an inhibitor of IL-12-induced IFN-γ (Fig. 1b).

The inhibition of IFN-γ production by supernatants from WR-infected cells in a dose-dependent manner (Fig. 2a) was not due to a general inhibition of the ability of these cells to produce IFN-γ because splenocytes stimulated with anti-CD3 mAb produced IFN-γ in the presence or absence of supernatants from infected cells (Fig. 2b). Supernatants from cells infected with VV Copenhagen or VV WR strain v6/2 did not inhibit IFN-γ production induced by either IL-12 or anti-CD3.

Mapping the gene encoding the inhibitory activity

The sequences of the genomes of VV strains Copenhagen, MVA, WR and Tian Tan did not predict a protein related to the IL-12R alpha chain, which binds IL-12, and so the gene encoding the inhibitor of IL-12-induced IFN-γ production was mapped by molecular genetics. Previously, the gene encoding the VV soluble type I IFN inhibitor was mapped using VV mutants that contained genome deletions adjacent to either terminus (Symons *et al.*, 1995). A similar approach was used here. Fortunately, one such deletion mutant of VV strain WR, called v6/2 and containing a large deletion near the left end of the genome (Moss *et al.*, 1981), lacked the activity encoded by the parent virus (Fig. 2a), whereas other mutants, vGS100 and vSSK2, with deletions towards the right terminus (Symons *et al.*, 1995) expressed the activity (Fig. 3b). This indicated that the gene encoding the inhibitory factor was likely to be present in the left end of the VV WR genome in the region deleted from VV 6/2 (Fig. 3a, second line).

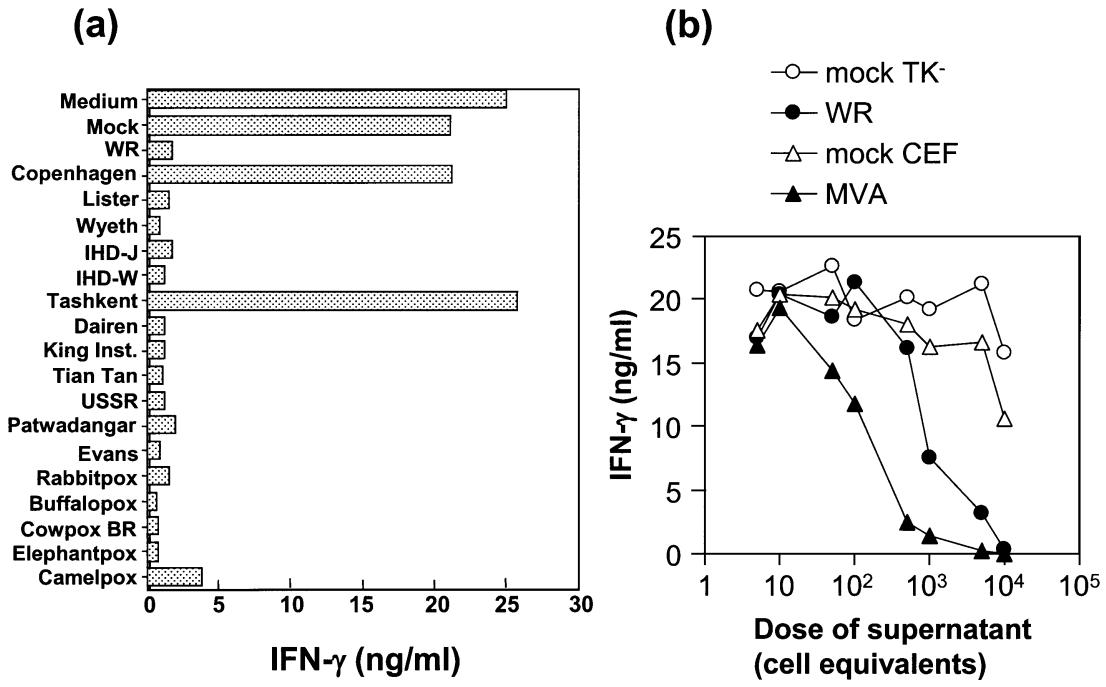

Fig. 1. Many orthopoxviruses express a soluble factor that inhibits the IL-12-induced production of IFN-γ by mouse splenocytes. (a) Human TK$^-$143 cells were mock-infected or infected with the indicated orthopoxviruses and supernatants were prepared as described in Methods. Supernatants derived from 10^4 cells were added to splenocytes together with IL-12 and the production of IFN-γ was measured 24 h later by ELISA. (b) Human TK$^-$143 cells (TK$^-$) were mock-infected or infected with VV WR and chick embryo fibroblasts were mock-infected or infected with VV MVA. Supernatants were prepared and the indicated doses of supernatants were then used in bioassay as in (a).

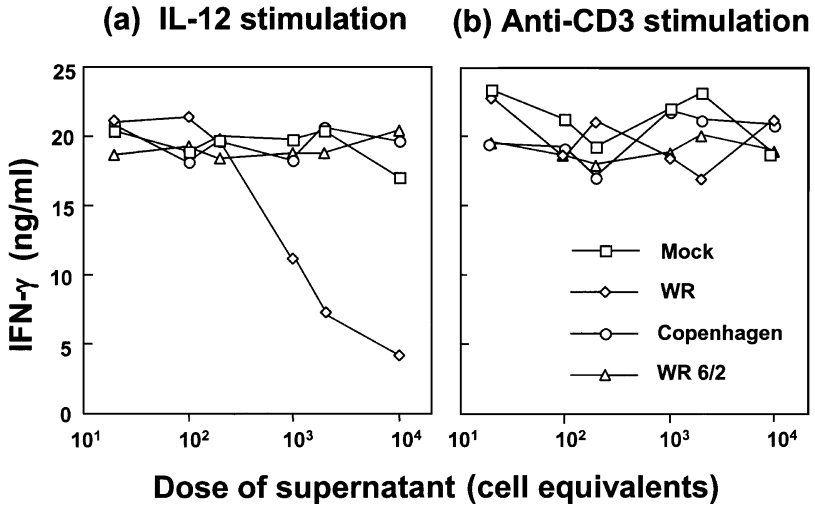

Fig. 2. Production of IFN-γ by mouse splenocytes. Splenocytes were stimulated with either IL-12 (a) or mAb to CD3 (b) in the presence of the indicated doses of supernatants from TK$^-$143 cells that had been mock-infected or infected with the indicated viruses. After 24 h the level of IFN-γ was measured by ELISA.

To identify the encoding gene, *Eco*RI fragments of DNA from this region were cloned into plasmid pGS50 (Chakrabarti *et al.*, 1985) within the TK gene and, via this vector, were inserted into the TK locus of VV strain Copenhagen (which lacks the activity). The supernatants taken from cells infected with these recombinant viruses were then tested for the biological activity as above. A Copenhagen virus containing an *Eco*RI fragment of 6·7 kb from the left end of the region deleted in v6/2 (Cop/E6.7) was found to contain the activity, whereas Copenhagen recombinants containing other *Eco*RI

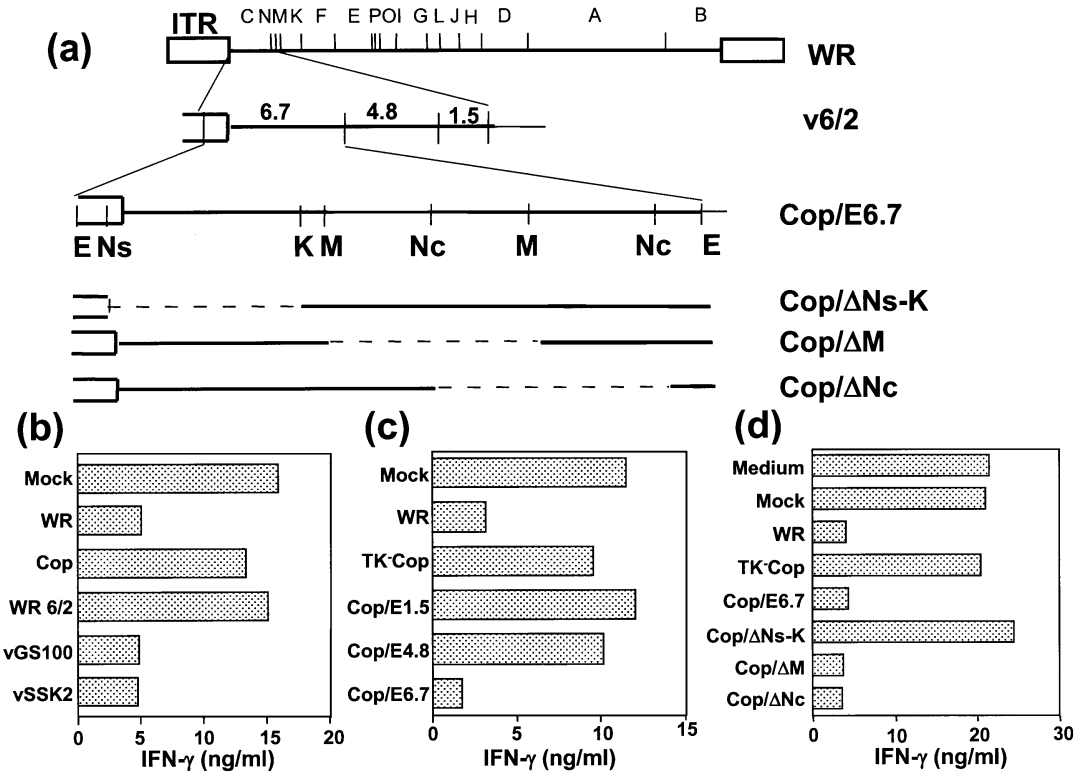

Fig. 3. Mapping of the VV WR gene encoding the inhibitor of the IL-12-induced IFN-γ production. (a) The top row shows the *Hin*dIII restriction map of the VV WR genome with the inverted terminal repeats (ITR) shown as open boxes. The second row shows the region of the WR genome that is deleted from VV WR strain 6/2 and the position of the *Eco*RI restriction fragments of 6·7, 4·8 and 1·5 kb. The third row shows a more detailed restriction map of the 6·7 kb *Eco*RI fragment (E, *Eco*RI; Ns, *Nsi*I; K, *Kpn*I; Nc, *Nco*I; M, *Mlu*I) and the name of the recombinant Copenhagen virus containing this fragment (Cop/E6.7). The next three rows show the regions of the 6·7 kb *Eco*RI restriction fragment that were deleted (dashed line) and the names of the recombinant Copenhagen viruses containing these truncated regions (Cop/ΔNs-K, Cop/ΔM and Cop/ΔNc). (b–d) Bioassays measuring the production of IL-12-induced IFN-γ from mouse splenocytes in the presence of supernatants from TK$^-$143 cells that had been mock-infected or infected with the indicated viruses, or incubated with medium alone.

fragments of 4·8 kb and 1·5 kb (Cop/E1.5 and Cop/E4.8) did not (Fig. 3c). The 6·7 *Eco*RI fragment was analysed further by deletion of sequences from the left, centre or right end. Recombinant Copenhagen viruses that lacked an internal *Nco*I fragment from the right end of this fragment (Cop/ΔNc), or an internal *Mlu*I fragment from the central region (Cop/ΔM), both retained the activity, whereas a virus lacking a *Nsi*I–*Kpn*I fragment from the left end (Cop/ΔNs-K) did not (Fig. 3d).

The published sequence of this region from VV WR (Kotwal & Moss, 1988b) indicated that there was no open reading frame (ORF) likely to encode a secreted protein of greater than 5 kDa, but gel filtration analysis of the supernatants of VV WR-infected cells had revealed that the inhibitor was a polypeptide of approximately 15 kDa (data not shown). Therefore, the *Nsi*I–*Kpn*I fragment that encoded the inhibitor was sequenced. This analysis detected differences from the published sequence. In particular, additional nucleotides were detected at two positions that caused frameshift changes and created a larger ORF that we called C12L (accession no. AF510447). This gene was predicted to encode a 126 amino acid protein of 14·5 kDa that contained an N-terminal signal peptide followed by a more hydrophilic domain that included a single putative *N*-linked glycosylation site (NFS). Without the signal peptide the predicted polypeptide size was 12·6 kDa. Subsequently, the sequences of human and mouse IL-18 bps were published and these showed amino acid similarity to C12L (Novick *et al.*, 1999) and related proteins encoded by other poxviruses. A comparison of the WR C12L protein sequence with that of Lister, MVA and cowpox virus GRI-90 showed that whereas the Lister and MVA sequences were virtually identical, the WR sequence differed at 19 amino acids scattered throughout the protein. At 17 of these 19 positions the cowpox virus GRI-90 sequence was identical to WR.

To test if gene C12L encoded the inhibitor detected by bioassays, the C12L ORF was amplified by PCR, cloned into VV transfer plasmid pMJ601 (Davison & Moss, 1990) and expressed from VV strain Copenhagen (Cop/C12L). The C12L ORF was also expressed from recombinant baculovirus with or without a C-terminal tag of six histidine residues (AcC12Lhis and AcC12L, respectively). A protein of 13 kDa was detected in the supernatant of Cop/C12L-infected cells but was absent from the supernatants of cells infected with Cop/β-gal or from

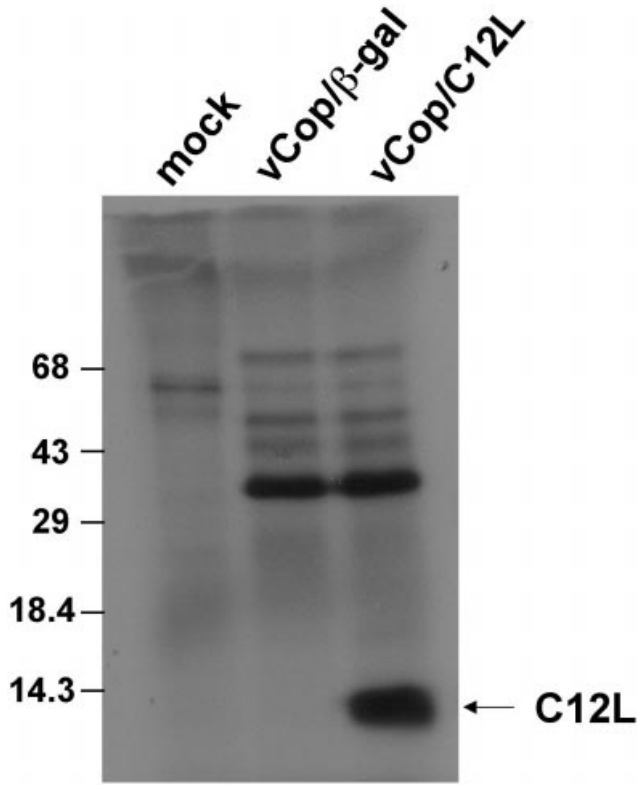

Fig. 4. Expression of C12L by Cop/C12L. TK⁻143 cells were either mock-infected or infected with either Copenhagen (Cop) or Cop/C12L at 10 p.f.u. per cell. At 4 h p.i. the cells were pulse-labelled with [^{35}S]methionine and [^{35}S]cysteine as described previously (Bartlett *et al*., 2002) and at 8 h p.i. the supernatants and cells were harvested and samples were analysed by SDS–PAGE (15% gel). The gel was dried and an autoradiograph was prepared. The sizes of molecular mass markers are shown in kDa.

mock-infected cells (Fig. 4). Additionally, a protein of 13 kDa was detected in the supernatant of Sf21 cells infected with AcC12L but was absent from cells infected with AcNPV, Ac35K or mock-infected cells (data not shown). Moreover, VV strains WR and Cop/C12L (Fig. 5a) and baculovirus strains AcC12L and AcC12L-his (Fig. 5b) each expressed a factor that inhibited the IL-12-induced IFN-γ production from mouse splenocytes in a dose-dependent manner. In contrast, a control Copenhagen virus made with the empty transfer vector (Cop/β-gal) (Fig. 5a), AcNPV or recombinant baculovirus AcB15R expressing the VV WR B15R gene (Fig. 5b) did not express this activity. The level of inhibitor expressed by Cop/C12L was greater than VV WR, consonant with the use of a strong synthetic promoter to drive C12L in the Cop/C12L virus.

IL-12 and IL-18 act synergistically

To assess the relative contribution of IL-12 or IL-18 to the induction of IFN-γ following the stimulation of mouse splenocytes with IL-12, the splenocytes were incubated with increasing concentrations of IL-12 in the presence of 10 μg/ml of mAb specific for IL-12, IL-18, both mAbs or an isotype-matched control. After 24 h incubation the level of IFN-γ was determined by ELISA (Fig. 5c). These results showed that mAb to IL-12 was more effective than mAb to IL-18 in inhibiting the formation of IFN-γ, but both mAbs together reduced the level of IFN-γ close to background. This shows that IL-12 can induce IFN-γ directly and via induction and release of IL-18 as reported previously (Fantuzzi *et al*., 1999).

Gene C12L is expressed early during infection

The phase during infection at which the C12L gene was expressed by VV WR was determined by preparing supernatants from cultures that had been infected with VV WR in the presence or absence of 40 μg/ml AraC, an inhibitor of virus DNA replication and therefore late gene expression, and measuring whether these samples could inhibit IL-12-induced IFN-γ production in bioassay. The levels of IFN-γ produced from mouse splenocytes incubated with IL-12 and supernatants from cells mock-infected in the presence or absence of AraC were 5·3 and 5·2 ng/ml, respectively. In comparison, the levels of IFN-γ produced by splenocytes incubated with IL-12 and supernatants from cells infected with VV WR in the presence or absence of AraC were 0·61 and 0·93 ng/ml, respectively. In a second experiment, the supernatants from cells infected with VV WR in the presence or absence of AraC reduced the levels of IFN-γ produced from 12·1 ng/ml (supernatant from mock-infected cells) to 0·36 or 0·47 ng/ml, respectively. These data indicate the C12L gene is expressed early during infection.

The C12L gene is non-essential for virus replication

To explore the role of the C12L protein in virus replication we used VV strain WR to make a deletion mutant lacking the C12L gene. A control virus, in which the C12L gene was reinserted into its original locus, was also constructed. Analysis of the genomes of vC12L, vΔC12L and vC12L-R viruses by PCR confirmed that most of the C12L gene had been deleted from vΔC12L only, and that the genes flanking C12L were unchanged in each of the viruses (data not shown). Furthermore, no differences in the plaque morphology (data not shown) or yield of intracellular virus were noted following infection of BS-C-1 cells with vC12L, vΔC12L or vC12L-R (Fig. 6), indicating that C12L is not essential for replication of VV strain WR.

Inhibition of IL-18 by the C12L protein

The ability of the C12L protein to inhibit the biological activity of mouse IL-18 was investigated. IL-18 was added to mouse splenocytes in the presence or absence of various doses of supernatants from VV- or baculovirus-infected cells and the levels of IFN-γ in the supernatants were measured 24 h later by ELISA (Fig. 7). VV strains vC12L, vC12L-R and Cop/C12L inhibited IFN-γ-production whereas the deletion mutant

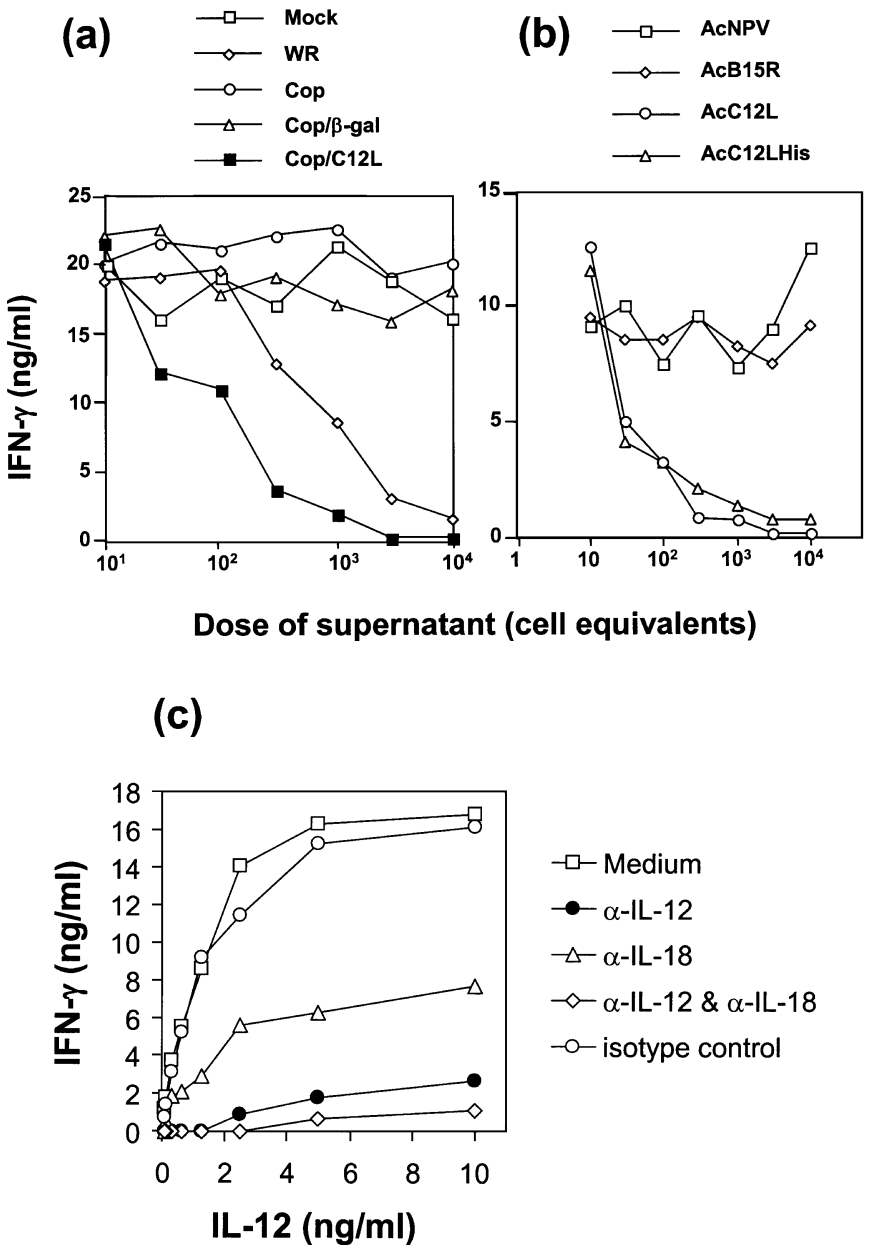

Fig. 5. (a, b) Inhibition of IL-12-induced IFN-γ production from mouse splenocytes by the VV WR C12L gene product. Splenocytes were incubated with IL-12 in the presence of the indicated doses of supernatants from TK$^-$143 cells (a) or Sf21 cells (b) that had been mock-infected or infected with the indicated vaccinia viruses (a) or baculoviruses (b). After 24 h the level of IFN-γ present in the culture supernatant was determined by ELISA. (c). Effect of mAbs to IL-12 or IL-18 on the production of IFN-γ by mouse splenocytes. Splenocytes were incubated with the indicated doses of mouse IL-12 in the presence of 10 μg/ml of mAb against mIL-12 (Clone C17.8; a gift from G. Trinchieri; Wister Institute, Philadelphia, PA, USA), mIL-18 (Clone 51817.111; R&D Systems), both cytokines, an isotype-matched control or medium. The level of IFN-γ was determined 24 h later by ELISA.

vΔC12L and vCop/β-gal did not (Fig. 7a). Similarly, AcC12L and AcC12L-his inhibited IFN-γ production in this assay, whereas AcNPV, AcB15R and Ac35K-his did not (Fig. 7b).

Deletion of C12L attenuates VV virulence in mice

The virulence of recombinant VVs with or without the C12L protein was assessed in two murine models of infection. In the intradermal model, the deletion mutant produced the same lesion size as control viruses (data not shown), but in the intranasal model a phenotype was apparent. Groups of five mice were infected with 10^4 p.f.u. of vC12L, vΔC12L or vC12L-R and each animal was monitored daily for weight loss (Fig. 8a) and signs of illness such as ruffled fur, arched backs, reduced mobility or evidence of pneumonia (Fig. 8b). Mice

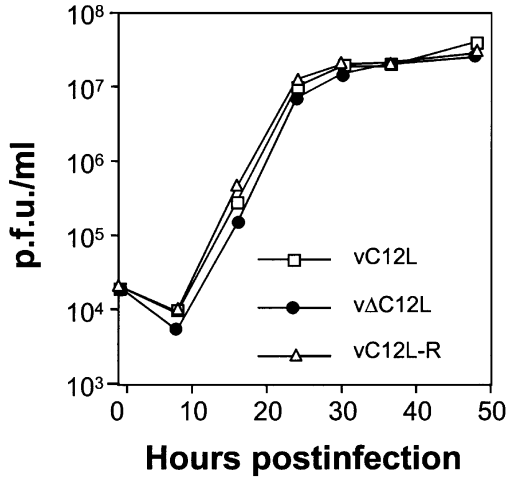

Fig. 6. Growth curves for strains of VV WR with or without the C12L gene. BS-C-1 cells were infected with the indicated viruses at 0·01 p.f.u. per cell and the infectious titres of the virus in the cells were determined at the indicated times p.i. by plaque assay in duplicate monolayers of BS-C-1 cells.

infected with vΔC12L showed significantly milder signs of illness ($P = < 0{\cdot}05$, Student's *t*-test) and lost a maximum of 15% of their body weight. In contrast, animals infected with vC12L or vC12L-R showed more severe signs of illness and lost up to 20–25% of their initial body weight. The attenuated phenotype of the deletion mutant was also indicated by reduced mortality data at 14 days p.i. After infection with

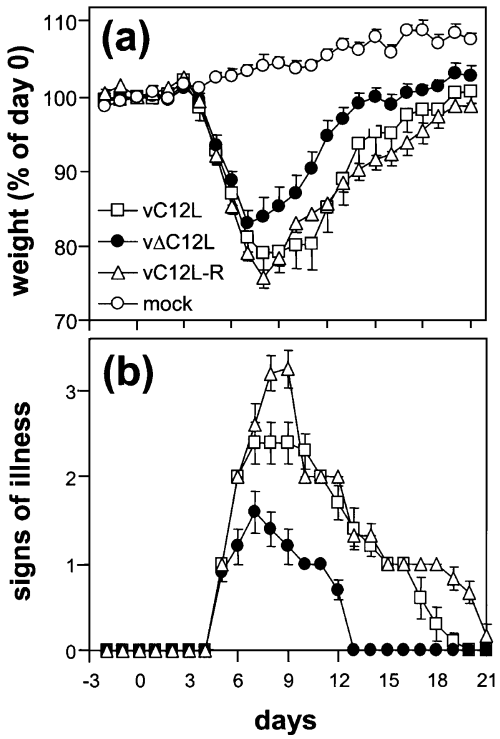

Fig. 8. Virulence of recombinant viruses in the murine intranasal model. Groups of five BALB/c mice were mock-infected or infected with 10^4 p.f.u. of the indicated viruses. (a) Mice were weighed daily and the results are expressed as the mean percentage weight of each group ± SEM, compared with the weight immediately prior to infection. (b) Animals were monitored daily for signs of illness, scored from 1 to 4 (Methods). Data from each day are expressed as the mean ± SEM from five mice. Where error bars are not evident they are within the dimensions of the symbol.

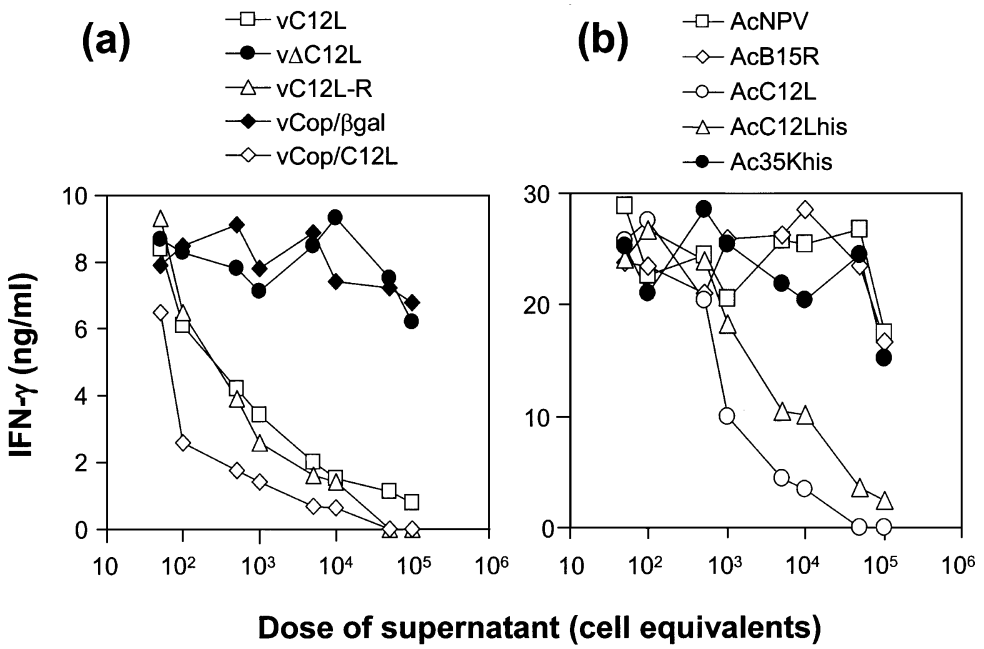

Fig. 7. Inhibition of mouse IL-18-induced IFN-γ production by mouse splenocytes. Splenocytes were treated with IL-18 (Methods) in the presence of the indicated doses of supernatants from TK$^-$143 cells (a) or SF21 cells (b) that had been infected with the indicated VV strains (a) or baculovirus strains (b). The level of IFN-γ in the culture supernatant was determined by ELISA 24 h later.

10^5 p.f.u. all animals (5/5) infected with vC12L and vC12L-R were sacrificed at humane endpoints, whereas 2/5 animals infected with vΔC12L survived. Together, these findings indicate that the IL-18 bp contributes to VV virulence in this model.

Discussion

The screening of supernatants of VV-infected cells revealed an inhibitor of IL-12-mediated IFN-γ production from mouse splenocytes. The gene encoding this activity was mapped by molecular genetics to C12L, a gene encoding a protein related to IL-18 bps. Recombinant C12L protein inhibited IFN-γ production from splenocytes stimulated with IL-12 or IL-18, and a deletion mutant lacking C12L was attenuated in a mouse model compared to wild-type and revertant controls.

Several poxvirus immunomodulators were identified by computational comparison of proteins predicted from DNA sequence with protein databases. However, biological assays to screen the supernatants of infected cells for inhibitory activity have also been employed (Symons *et al.*, 1995). Here we have used the latter method to identify an inhibitor of IL-12-induced IFN-γ production in the supernatant of VV-infected cells. Identification of the gene encoding this inhibitor was aided by a deletion mutant of VV WR called v6/2 that lacked a 12 kb region near the left terminus of the genome (Kotwal & Moss, 1988b) and which, unlike its parent virus (WR), did not express the activity. By transferring smaller and smaller fragments of DNA from the region deleted in v6/2 to VV Copenhagen and testing for expression of the biological activity, the gene encoding the protein was identified as C12L. The sequence of VV WR DNA from this region indicated that the region corresponding to the C12L ORF was broken into three pieces (Kotwal & Moss, 1988b). However, we found the sequence of VV WR to contain an additional nucleotide at two positions and to have a complete ORF that encoded a biologically active protein of 13 kDa. Surprisingly, the VV WR C12L protein showed several differences from the corresponding sequences of VV strains MVA (Antoine *et al.*, 1998) and Lister (Smith *et al.*, 2000) but was very similar to the sequence of cowpox virus strain GRI-90 (Shchelkunov *et al.*, 1998).

The C12L protein is closely related to IL-18 bps from other poxviruses, man and mouse, and data presented here show that the C12L protein inhibits the biological activity of IL-18 in bioassays. However, the C12L protein did not prevent the binding of ^{125}I-IL-12 to cells bearing IL-12Rs and could not be chemically cross-linked to ^{125}I-IL-12 (data not shown). Previously, VV WR was shown to encode a protein that binds to human and murine IL-18, but the VV protein, unlike the related protein from ectromelia virus, was not used in bioassays (Smith *et al.*, 2000). The identification of an IL-18 bp by mapping an inhibitor of IL-12-induced IFN-γ illustrates the synergy between IL-18 and IL-12 and this was shown further by using mAbs to IL-12, IL-18 or both cytokines in assays measuring IFN-γ production. Either mAb reduced the level of IFN-γ produced but maximum inhibition required both mAbs (Fig. 5c). Supernatants from VV WR- or Copenhagen-infected cells were only able to inhibit the IL-12-induced IFN-γ production if the C12L protein was expressed, indicating that these viruses do not encode another IL-12 soluble inhibitor.

The IL-18 bp is widely distributed in VV strains (14/16 tested), other orthopoxviruses and other poxvirus genera. This is in contrast to some other immunomodulators such as TNF-binding proteins (Alcamí *et al.*, 1999) and the intracellular inhibitor of caspase 1 (Kettle *et al.*, 1995, 1997) that are present in only 3/16 and 5/14 strains, respectively. The distribution of the IL-18 bp is more like that of soluble inhibitors of IFN-γ (Alcamí & Smith, 1995) and IFN-α/β (Symons *et al.*, 1995), which are expressed by the great majority of VV strains. This implies that IL-18 bp is important for virus replication (see below).

The importance of defensive strategies against IFN is illustrated further by the number of proteins encoded by VV that interfere with IFN production or function. Within VV-infected cells, the B13R protein inhibits the activity of caspase 1 (Kettle *et al.*, 1997), an enzyme required for the cleavage of pro-IL-1β and pro-IL-18 into the mature cytokines IL-1β and IL-18 (IFN-γ inducing factor). Outside the cell, IL-18 is bound and inhibited by the C12L protein so that IFN-γ production is restricted. Moreover, the binding of IFN-γ to its receptor is inhibited by the B8R protein (Alcamí & Smith, 1995), and the action of type I IFNs is inhibited by the VV B18R protein in solution and on the cell surface (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Alcamí *et al.*, 2000). Within infected cells the E3L (Chang *et al.*, 1992) and K3L (Beattie *et al.*, 1991) proteins inhibit the activity of IFN-induced antiviral proteins. The IL-18 bp represents another VV-encoded protein that is likely to combat IFN by restricting IFN-γ production and thereby diminish the Th1 response to infection. Notably, the IL-18 bp and all the above proteins that combat IFNs are expressed early during infection, consistent with the need for VV to prevent the potent antiviral activity of IFNs as soon as possible. Some other immunomodulators made by VV such as the CC chemokine binding protein (Alcamí *et al.*, 1998) and the related secreted protein encoded by A41L (Ng *et al.*, 2001) are expressed early but also later during infection, and the IL-1β receptor (Alcamí & Smith, 1992) and TNF binding proteins are expressed late in infection (Alcamí *et al.*, 1999; Reading *et al.*, 2002).

The role of the C12L protein in virus replication was assessed *in vitro* and *in vivo*. *In vitro* the plaque size and growth kinetics (Fig. 6) were unaltered, but *in vivo* the virulence of the deletion mutant was reduced in the murine intranasal model compared to wild-type and revertant controls (Fig. 8). The importance of IL-18 in combating poxvirus infection is illustrated by the reduced pock formation on the tails of mice inoculated intravenously with VV, as well as augmenting NK and CTL activity (Tanaka-Kataoka *et al.*, 1999). Previously, an

ectromelia virus mutant engineered to lack the IL-18 binding protein (p13) was injected intraperitoneally into mice (Born *et al.*, 2000). An enhanced local NK cell response was reported compared to parental virus but a revertant control was not used and the virulence of the virus was not reported.

The attenuation observed here in the intranasal model may be contrasted with that resulting from deletion of the B13R (Kettle *et al.*, 1995) and B8R (Symons *et al.*, 2002) genes, which gave no phenotype in this model. In the case of B13R, although no phenotype was observed in the intranasal model, the deletion mutant caused an enhanced lesion size in the intradermal model (Tscharke *et al.*, 2002). For B8R, the lack of attenuation in the mouse intranasal model is consistent with the low affinity of this protein for mouse IFN-γ (Symons *et al.*, 2002) and the failure to inhibit the biological activity of mouse IFN-γ (Alcamí & Smith, 1995).

In summary, we have used a bioassay to identify an inhibitor of IL-12-induced IFN-γ production and molecular genetics to map the inhibitor to gene C12L, which encodes the VV IL-18 bp. This demonstrates the synergy in action of IL-12 and IL-18. A virus deletion mutant grew normally *in vitro* but was attenuated in a mouse intranasal model illustrating the importance of controlling IL-18 in a systemic poxvirus infection.

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