Supporting Methods

Cloning of Viral Genes for Expression in the Baculovirus System. The genes of interest were amplified by PCR with specific oligonucleotides, *Pfu* or *Vent* DNA polymerase and viral DNA as template. The VaV *CrmB* gene was generated by site-directed mutagenesis of the camelpox virus orthologue (1). Genes were cloned into pBAC-1 (Novagen) or pFastBac1 (Invitrogen), and the proteins were expresed fused to a C-terminal 6×His tag. The correct sequence of all DNA fragments cloned or mutagenized was confirmed by DNA sequencing.

Cloning of the EV CrmD gene. The oligonucleotides CrmD7 (5'-CGCGTTTAAACGGATCCATGATGAAGATGACACCATCATA-3') and CrmD9 (5'-CGCCTCGAGATCTCTTTCACAATCATTTGGTGG-3') were used, and the PCR products were cloned into BamHI/XhoI-digested pBAC-1 to generate the plasmid pMS1 (pBac-CrmDHis). The NotI/SphI restriction fragment of pIgPlus (Novagen) bearing the human Fc coding fragment was subcloned into pMS1 to generate pMS40 (pBac-CrmDFc).

Cloning of the CPV CrmB, CrmC, and CrmE genes. The genes were cloned into BamHI/NotI-digested pBAC-1 by using the following oligonucleotides: (*i*) CPVBamHICrmB (5'-CGCGGATCCATGAAGTCATATATATTGCTA-3') and CPVCrmBNotI (5'-GCGGCGGCCGCTAAAAAGTGGGTGGGATACTG-3') for CPV strain Brighton Red CrmB to generate the plasmid pCPVCrmB; (*ii*) CPVBamHICrmC (5'-CGCGGATCCATGGATATAAAGAATT-3') and CPVCrmCNotI (5'-GCGGCGGCCGCATTACATTTAGATAGTTTGCATGG-3') for

CPV strain Brighton Red CrmC to generate pCPVCrmC; and (*iii*) K3R8 (5'-CGCGGATCCGCTAGCATGACGAAAGTTATCATCATCTTAG-3') and K3R9 (5'-CGCGCGGCCGCTCTTGTCATTGGTTTACATTGATC-3') for CPV strain Elephantpox CrmE to generate pMS3.

Cloning of camelpox virus CrmB gene. The ORF264 of the camelpox virus strain CMS (1), corresponding to the *CrmB* gene, was amplified by PCR with oligonucleotides CMLV264Eco (5'-GCGGAATTCATGAAGTCCGTATTATACTCG-3') and CMLV264Xho (5'-GCGCTCGAGTAAAAAGTGGGTGGGTTTGG-3'). The PCR product was cloned into EcoRI/XhoI-digested pBAC-1 to generate plasmid pRA1.

Cloning of VaV CrmB gene. The DNA corresponding to the VaV (strain Bangladesh 1975) ORF188 was obtained by multiple-site directed mutagenesis of plasmid pRA1 with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) and following the manufacturer's instructions. Oligonucleotides used for the generation of VaV *CrmB* gene by site-directed mutagenesis of the camelpox virus orthologue are the following:

Mutation(s) introduced	**Oligonucleotide sequence**
S7L	5'-CCGTATTATACTTGTATATATTGTTTC-3'
ΔI16	5'-CTCTCATGTATAATAAACGGAAGAGATG-3'
V22A, T23A	5'-CGGAAGAGATGCAGCACCGTATGCACC-3'

T23A, A26T, S28P	5'-GCACCGTATACACCACCCAATGGAAAG-3'
N35T 2	5'-GGAAAGTGTAAAGACACCGAATACAAACGCCATAATCTG-3'
D91N 2	5'-GTTGTAACGGAAGATGCAATAGTAATCAGGTAGAGACG-3'
I117L 2	5'-CTCCCGGATATTATTGTCTTCTCAAAGGATCATCCG-3'
A145V	5'-GGACACACGTCTGTCGGAGACGTCATC-3'
L155F	5'-CTCCGTGTGGTTTCGGAACATATTCTC-3'
R160H, L155F 2	5'-GTTTCGGAACATATTCTCACACCGTCTCTTCCGC-3'
S173N 2	5'-ATGCGAACCCGTACCCAACAATACATTTAACTATATCG-3'
N184T 2	5'-CGATGTGGAAATTACCCTGTATCCAGTCAACGAC-3'
I201L	5'-GACCACTACCGGTCTCAGCGAATCCATC-3'
S206L	5'-GCGAATCCATCTTGACGTCGGAACTAAC-3'
K217T, D220N 3	5'-CGAAATACGGGATTGCAATCTGTATGATTCATAGTAATAGTTA-GTTCCG-3'
S268F 2	5'-AACAAAGGTTCTTCCTTCAAACAGCTAACGAAAGCAAAG-3'
I280M, P282S 2	5'-GAATGATGATGGTATGATGTCGCATTCGGAGACGG-3'
V290A	5'-CGGTAACTCTAGCGGGCGACTGTCTATC-3'
T308A 2	5'-CTATATAGTAATACCAATGCTCAAGACTACGAAACTGATAC-3'
H319R, A320V 2	5'-CTGATACAATCTCTTATCGTGTGGGTAATGTTCTCGATG-3'
V326D 2	5'-GGGTAATGTTCTCGATGACGATAGCCATATGCC-3'
D335N, L339P 2	5'-GCCCGGTAGTTGCAATATACATAAACCGATCACTAATTCC-3'
H347R 3	5'-GGTGCTCGAGTAAAAAGCGGGTGGGTTTGG-3'

After several consecutive rounds of site-directed mutagenesis, plasmid pRA105 was obtained, containing the sequence coding for VaV CrmB.

Cloning of VaV CrmB, CrmB-CRD, *and* CrmB-CTD *genes.* The full length VaV *CrmB* gene fused to a C-terminal 6×His tag was subcloned into EcoRI/SphI-digested pFastBac1 to generate pRA107 (pFastBac-CrmBHis). The N-terminal CRDs of VaV CrmB, corresponding to residues M1 to C192, was amplified by PCR with oligonucleotides VaV188Eco (5'-GCGGAATTCATGAAGTCCGTATTATACTTG-3') and VaV188CRDs1-4Xho (5'-GCGCTCGAGACACGATGTGTCGTTAACTGG-3') using pRA107 as a template. The amplified fragment was cloned into EcoRI/XhoI-digested pBAC-1 to generate pRA99. pRA99 was digested with EcoRI/SphI and the fragment carrying the VaV CrmB-CRD fused to a C-terminal 6×His tag cloned into pFastBac1 to generate pRA106 (pFastBac-CrmB-CRDHis). The C-terminal fragment of VaV CrmB (residues T194 to L348) was PCR-amplified with oligonucleotides VaV188Cter-PflMI (5'-CGCCCACCCAATGGAACTAGGACGACCACTACCGG-3') and H347R3 by using pRA107 as a template. This fragment was digested with PflMI and XhoI and cloned into pRA107 digested with the same enzymes. This generated pRA108, a plasmid that encodes a fusion protein composed of the 29 N-terminal residues of VaV CrmB (which includes the predicted signal peptide) followed by the CTD of CrmB and a C-terminal 6×His tag.

Cloning of CrmC-CrmB CTD and CrmE-CrmB CTD fusion proteins. CPV strain Brighton Red CrmC ORF was PCR-amplified by using primers CrmC1 (5'-CGCGGATCCATGGATATAAAGAATTTGCTGAC-3') and CrmC6 (5'-CGCCCATTGGGTGGATTACATTTAGATAGTTTGCATGG-3') and viral DNA as a template. CPV strain Elephantpox CrmE was PCR-amplified with primers K3R8 and K3R15 (5'-CGCCCATTGGGTGGTCTTGTCATTGGTTTACATTGATC-3') by using plasmid pMS3 as a template. VaV CrmB CTD was amplified with primers

VaV188CterPflMI and H347R3 by using pRA108 as template. The PCR products were annealed to each other (overlap at *PflMI* site), and the fusion product was amplified with oligos CrmC1 and H347R3 (for CrmC fusion) or K3R8 and H347R3 (for the CrmE fusion). These PCR products were cloned into BamHI/XhoI-digested pRA106 to generate pRA112 (pFastBac-CrmC-CTDHis) or pRA113 (pFastBac-CrmE-CTDHis), respectively.

Cloning of SCP-1, *SCP-2*, *and SCP-3.* CPV V218 (corresponding to SCP-1) was amplified by using oligonucleotides 5'V218EcoRI (5'-CGCGAATTCATGATGATATACGGATTAATAGC-3') and 3'V218SalI (5'-GCGGTCGAGACCATCGACACCACTCATC-3') and cloned into EcoRI/XhoI-digested pRA106 to generate pAH17 (pFastBac-V218His). EV ORF E12 (corresponding to SCP-2) was PCR-amplified by using oligonucleotides E1 (5'-GCGGGATCCATGATAAACATAAACATAAACACAATAC-3') and E2 (5'-GCGGCGGCCGCATTAATAGTTCTAGTAGCGCAAG-3') and cloned into BamHI/NotI-digested pBAC-1. The resulting plasmid, pMS51, was digested with BamHI/XhoI, and the fragment containing the E12 gene subcloned into BamHI/XhoI-digested pRA106 to generate pAH18 (pFastBac-E12His). EV ORF E184 (corresponding to SCP-3) was amplified by using oligonucleotides 5'E184EcoRI (5'-GCGGAATTCATGTATAAAAAACTAATAACGTTT-3') and 3'E184XhoI (5'-CGCCTCGAGAAAATCATATTTTGAATAATATGTA-3') and cloned into EcoRI/XhoI-digested pRA106 to generate pAH11 (pFastBac-E184His).

Generation of Recombinant Baculoviruses. The pBAC-1-based recombinant plasmids were cotransfected with linearized baculovirus DNA (BacPAK6, Clontech)

into Hi5 cells to generate recombinant baculoviruses, which were plaque-purified in three consecutive steps and amplified as described (2). For those genes cloned into pFastBac1, recombinant baculoviruses were constructed by using the Bac-to-Bac expression system (Invitrogen) as described by the manufacturer. Briefly, plasmids were transformed into competent DH10Bac bacteria, where a transposition event generated the corresponding recombinant bacmids. These bacmids were purified and transfected into Hi5 insect cells, and the recombinant baculoviruses were harvested from the cell culture supernatants 3–5 days after transfection. These viruses were further amplified in one single step to generate a high-titer recombinant virus stock for protein production.

Construction of Recombinant VVs. The EV strain Naval CrmD protein and a truncated version lacking the CTD (CrmD-CRD) were expressed in VV Western Reserve, a strain that does not encode TNF or chemokine binding activities (2, 3). EV CrmD was amplified by PCR with virus DNA as template, *Pfu* DNA polymerase, and oligonuceotides CrmD7 and CrmD15 (5'-CGCGGTACCTCAATCTCTTTCACAATCATTTGG-3'). EV CrmD-CRD was PCR-amplified with the oligonucleotides CrmD7 and CrmD24 (5'-GCGAAGCTTTTACCATGGACAAGAGGTCTTGTTAACAGGATAC-3'). The DNA fragments were cloned into BamHI/KpnI-digested pMJ601, creating plasmids pMS11 (EV CrmD) and pMS22 (EV CrmD-CRD). The recombinant VV was produced as described (2) and termed VV-EVCrmD and VV-EVCrmD-CRD.

Protein Purification. Hi5 cells were infected (multiplicity of infection of 2–5 plaque-forming units per cell) with recombinant baculovirus. Cell supernatant was harvested

3–4 days after infection, clarified at 1,000 × *g* for 5 min and then concentrated to 2.5 ml by using Stirred Ultrafiltration Cell 8200 (Amicon). Supernatant was then desalted and buffer exchanged against phosphate buffer containing 10 mM imidazole (PD-10 desalting columns, Amersham Biosciences). Proteins were purified by metal chelate affinity chromatography (Ni-NTA resin, Qiagen). Purified recombinant proteins were concentrated and dialyzed against PBS by using a Vivaspin500 device (VivaScience), and analyzed by SDS/PAGE and Coomassie blue staining.

TNF Binding Assays. Binding assays in solution to human ^{125}I-TNF (100–250 pM) were performed by precipitation of the ligand–receptor complexes with polyethylene glycol and filtration as described in ref. 2.

1. Gubser, C. & Smith, G. L. (2002) *J. Gen. Virol.* **83,** 855–872.

2. Alcami, A., Khanna, A., Paul, N. L. & Smith, G. L. (1999) *J. Gen. Virol.* **80,** 949–959.

3. Alcami, A., Symons, J. A., Collins, P. D., Williams, T. J. & Smith, G. L. (1998) *J. Immunol.* **160,** 624–633.