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T2 OPEN READING FRAME FROM THE SHOPE FIBROMA VIRUS ENCODES A SOLUBLE FORM OF THE TNF RECEPTOR

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A transcriptionally active open reading frame (T2) from Shope Fibroma Virus was recently shown to have striking sequence homology with members of a new superfamily of cell surface proteins, including a receptor for human tumor necrosis factor (1). Here we report that recombinant T2 protein expressed in COS cells is a soluble, secreted glycoprotein which specifically binds human TNFα and β, and inhibits binding of these cytokines to native TNF receptors on cells. T2 binding of TNF is not inhibited by nerve growth factor, although the nerve growth factor receptor is also a member of the same family, nor by nine other recombinant cytokines. Further, the repeating domain structure of T2 most closely resembles that of the type I TNF receptor (p75) and is significantly different from other family members, including the type II TNF receptor (p55). Since T2 possesses a leader sequence but lacks a transmembrane domain, these results confirm the original suggestion (1) that T2 represents a soluble form of the type I TNF receptor which is secreted from virally infected cells, and whose function is to immunosupress the host by abrogating the potentially destructive effects of TNF. This is the first such virally-encoded soluble cytokine receptor to be identified, and may represent a more general mechanism by which viruses subvert the host immune system.

The Shope Fibroma Virus (SFV), a 160 kb double stranded DNA virus of the Poxvirus family, was the first DNA tumor viruses described (2). SFV induces invasive malignancies in newborn or immunosuppressed rabbits, and benign tumors in adults (3) As with all large DNA viruses which productively infect eukaryotic hosts, SFV depends crucially on mechanisms to evade immune surveillance that would otherwise lead to its elimination (4). One approach to uncovering these mechanisms is to exploit the explosive growth of DNA sequences in data bases to search for homologies between open reading frames (ORFs) in viruses, perhaps 80% of which are of unknown function, and cellular proteins of known function. We recently detected one such unexpected homology in the course of cloning one form of the human receptor for tumor necrosis factor (TNFR I, p75), whose sequence revealed a new superfamily of integral

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membrane proteins (1). Sequence homology between family members is restricted to an extracellular, cysteine-rich region of about 170 amino acids, and the greatest similarity is between the type I TNF receptor and the T2 ORF from SFV. The T2 ORF has been shown to be transcriptionally active and encodes a putative protein of 325 amino acids (5) Unlike other family members, however, the predicted T2 protein lacks a transmembrane element, yet retains a 138 residue domain C-terminal to the canonical cysteine-rich moiety, and thus we suggested (1) that T2 may represent a virally-encoded, soluble TNF receptor. In this paper, we show that recombinant SFV T2 protein expressed in COS cells is a soluble, secreted glycoprotein that does, in fact, specifically bind TNF, and also inhibits TNF binding to cellular TNF receptors. The T2 protein should thus be a functional inhibitor of TNF-mediated activities, and may represent a more general mechanism by which eukaryotic viruses subvert the immune system.

MATERIALS AND METHODS

Recombinant Cytokines. Human IL1α, IL2-IL4, IL6-IL7, G-CSF, and GM-CSF were expressed in yeast and purified at Immunex, as described (6). Purified murine nerve growth factor (BioProducts, Inn., Ind.), human γ-IFN (Genzyme, Boston, Ma.) human TNFα and β (R&D Sciences, Minn., Minn.) were obtained commercially. For binding assays, TNFα was radioiodinated to a specific activity of $2x10^{15}$ cpm/mmole (1).

Recombinant SFV T2 Protein and Cytokine Receptors. The pDC302 vector (7) was utilized for transient expression of proteins in COS-7 cells. A construct in this vector expressing the SFV T2 ORF was synthesized from a Hae III fragment of the virus which had been subcloned into pUC19. A Spe I/Bam HI fragment from this subclone, which lacks the N-terminus of the T2 ORF, was isolated, and the 5' end reconstructed with synthetic oligonucleotides to generate a consensus translational initiation site (8) and an overhang appropriate for subcloning into Asp 718/Bgl II-cut pDC302. A cDNA clone encoding a full-length type I TNF receptor was isolated by direct expression (1). A secreted, soluble form the human type I TNF receptor (Δ^STNFRI) containing the entire 235 amino acids of the extracellular domain, but lacking transmembrane and cytoplasmic domains, was constructed utilizing the N-terminal Not I/Pvu II fragment of the cloned cDNA and synthetic oligonucleotides to introduce a termination codon following Asp 257. A full-length type II TNF receptor cDNA was isolated from a human placental cDNA library using synthetic DNA probes based on published amino acid sequence data of a TNF-binding protein (9). A full length human CD40 cDNA (10) in a CDM8 vector was a gift of Dr. Ed Clark (University of Washington, Seattle). Methods for expression of cDNAs in COS cells has been described (1). Supernatants from cells transfected with vector containing the T2 insert were used to purify the recombinant protein to homogeneity by affinity chromatography on TNF columns (9). Detergent solubilized lysates from adherent COS cells expressing surface-bound recombinant receptors were prepared as described (6).

Metabolic Radiolabeling and SDS PAGE. COS-7 cells (3 X 10^6) were electroporated with 5 ug of cDNA encoding SFV T2 protein, the soluble TNFRI receptor (Δ^STNFRI), or the vector lacking insert and cultured for 3 days as described (11). Cells were then washed twice with PBS and incubated at 37°C for 6 hours in 2.5 ml MEM without methionine or cysteine but containing 100 uCi of ^{35}S-methionine and ^{35}S-cysteine (Translabel, ICN Radiochemicals). The media containing labelled, secreted proteins was removed, centrifuged, SDS sample buffer added then electrophoresed through an 18% polyacrylamide gel. The gel was washed, dried, and autoradiographed. Purified recombinant T2 protein was electrophoresed through an identical SDS gel and silver stained.

TNF Binding Assays. For solid phase (nitrocellulose) binding assays (12), purified recombinant T2 protein, or detergent solubilized lysates of COS cells expressing surface TNFRI (1), TNFRII (13,14), or CD40 (1,10) were serially diluted 2-fold with PBS/1% Triton x-100, then dotted (2ul) onto nitrocellulose (Schleicher & Schuell, Keene, NH.). Blots were air dried, blocked overnight with 20mM Tris/3%BSA, probed with human ^{125}I-TNFα (1nM) and autoradiographed (12). Competition studies were performed identically, except for inclusion of

a 200-fold molar excess (each) of unlabeled ligands in the probe mixture. Detergent-solubilized lysates of COS cells expressing recombinant receptors contained 4 x 10^8 receptor equivalents/ul. Suspension inhibition assays used the human histiocytic line U937 which expresses 7500 TNFα binding sites/cell (Kd=0.5nM). U937 cells (5 x 10^7/ml) were incubated with ^{125}I-TNFα (0.5nM) and COS supernatants (source of T2 protein, Δ^STNFRI, or mock transfected control), unlabeled cytokines (200-fold molar excess each) or binding media for 4 hours (4 °C), and duplicate aliquots of the suspension centrifuged through a phtalate oil mixture as previously described (12) to separate free and bound ligand. Radioactivity was determined by counting gamma emissions. Percent inhibition was determined relative to that of binding media alone.

Computer-Assisted Sequence Analysis. Quantitative similarities between cysteine-rich regions of TNF receptor family members was assessed by scores derived using the NBRF ALIGN program (15). Residue boundaries for human TNFRI, human and rat NGF receptor, SFV ORF T2, human CD40 and murine clone 4-1BB are as described (1). Homologous regions of rat OX40 antigen (16) and human type II TNF receptor (14) are amino acids 25-164 and 14-167, respectively. Comparative residue alignments in domains are based on visual inspection aided by software from the University of Wisconsin Genetics Computer Group (17). Elements shown are: TNFRI (D1, 39-76; D2, 77-118; D3, 119-162; D4, 163-201); TNFRII (D1, 14-54; D2, 55-96; D3, 97-138; D4, 139-167); T2 (D1, 27-62; D2, 63-104; D3, 105-147;D4, 148-186).

RESULTS AND DISCUSSION

SDS-PAGE Analysis of Purified T2 Protein and ^{35}S-Labeled Proteins Secreted from Transfected COS Cells. Fig. 1A shows SDS-PAGE analysis followed by autoradiography of ^{35}S-labeled proteins secreted from COS-7 cells transfected with expression plasmids containing sequences encoding a soluble type I TNF receptor, SFV T2 ORF or plasmid lacking

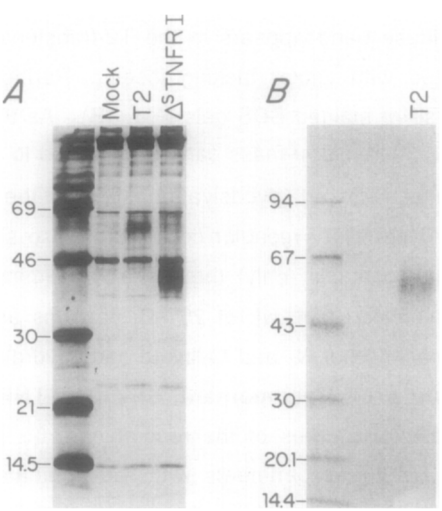


Fig. 1. (A). SDS-PAGE analysis and Autoradiography of ^{35}S-Labeled Proteins Secreted from COS-7 cells transfected with expression plasmids containing a soluble type I TNFR (Δ^STNFRI), SFV T2 (T2) or plasmid lacking inserts (MOCK). Vector and plasmid constructs used for transient expression of proteins, and methods for metabolically labelling cells are described in Materials and Methods. (B) SDS-PAGE (silver stained) of purified recombinant T2 protein. Purification was effected by TNF affinity chromatography (7) from supernatants of COS cells transfected with expression vector containing the SFV T2 insert. ^{35}S-labeled proteins and purified T2 were electrophoresed through an 18% polyacrylamide gel under reducing conditions.

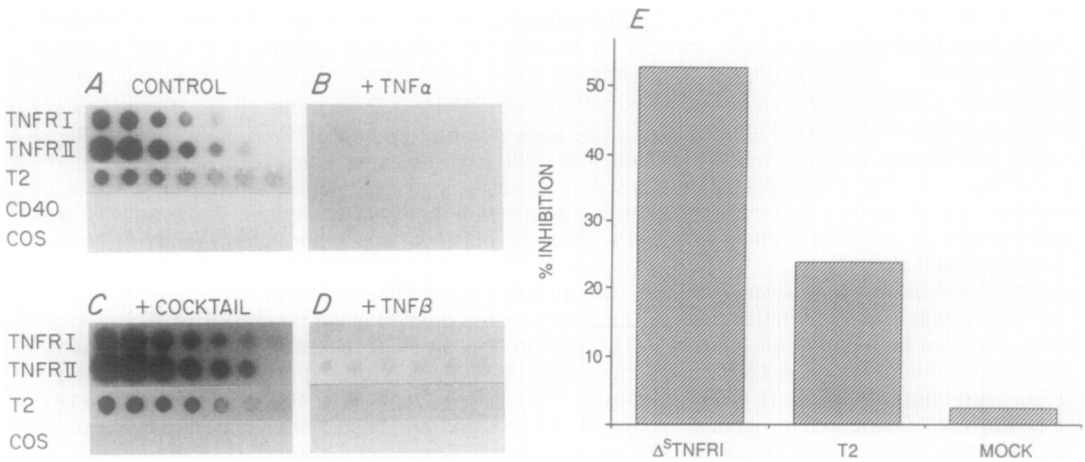


Fig. 2. TNF binding characteristics of purified recombinant T2 protein, and COS cell-expressed TNFRI, II, and CD40. **(A).** Solid phase (nitrocellulose) detection of ^{125}I-TNFα binding to purified T2, COS extracts expressing TNFRI, II, or CD40. Extracts of COS cells transfected with vector alone were also included. Blots were prepared with serially diluted (2X) samples and developed as described in Materials and Methods. **(B)** same conditions as (A) except a 200-fold molar excess of unlabeled TNFα was included with the radiolabeled TNF. (C), same conditions as (A) (CD40 omitted), except for inclusion of a 200-fold molar excess (each) of unlabeled human IL1α, IL2-4, IL6-7, G-CSF, GM-CSF, γ-IFN and NGF in the probe mixture. **(D)**, same as (A), except probe also contained a 200-fold excess of unlabeled human TNFβ. **(E)** Inhibition of ^{125}I-TNFα binding to U937 cells (in suspension) by COS cell supernatants containing soluble TNFRI (Δ^STNFRI), T2, or by supernatants from mock transfected COS cells (MOCK). U937 cells were incubated with ^{125}I-TNFα (0.5nM) and COS supernatants or binding media. Percent inhibition was determined relative to that of binding media alone. Details of assay described under Materials and Methods.

inserts. A prominent but diffuse band appears in the T2-transfected cells at 58 kD which is absent from cells transfected with vector lacking insert. Purified recombinant T2 protein displays a similar profile on silver stained SDS gels (Fig. 1B). As the calculated MW of the T2 protein is 34 kD, the ~24 kD difference in mass can be attributed to carbohydrate attached to at least some of the 4 potential N-linked glycoslyation sites in the protein (5). The protein product of a soluble, human type I TNF receptor construct is also shown for comparison. This truncated form of the receptor contains (only) the entire 235 amino acids of the extracellular domain and possesses a calculated mol. wt. of 25 kD, yet runs as a diffuse band at 41 kD, consistent with the known presence of N- and O-linked carbohydrate (1,18).

TNF Binding Characteristics of Recombinant SFV T2, TNFRI, TNFRII, AND CD40. To examine the ligand binding properties of the recombinant T2 protein, we first performed solid phase (nitrocellulose) binding experiments with radioiodinated TNFα (Fig. 2). Purified recombinant T2 protein and detergent-solubilized extracts of COS cells expressing the full-length TNFRI were serially diluted 2-fold, then dotted onto nitrocellulose and developed with human ^{125}I-TNFα. We also included extracts from COS cells transfected with human CD40 and a recently cloned human type II TNF receptor (13,14), both superfamily members. As with extracts containing TNFRI and II, T2 clearly bound ^{125}I-TNFα (Fig. 2A), and binding was completely inhibited by unlabeled TNFα (Fig. 2B). CD40-containing extracts did not bind ^{125}I-TNFα, nor did extracts of mock transfected COS cells. Specificity of TNFα binding to T2

	huTNFRI	huTNFRII	sfvT2	huNGFR	huCD40	mu4-1BB	raOX40
huTNFRI	- -	8.6	20.7	10.3	17.3	9.0	10.1
huTNFRII	30.6	- -	8.4	10.0	7.0	3.1	6.9
sfvT2	37.5	29.2	- -	9.9	10.8	8.8	7.7
huNGFR	27.1	32.2	32.0	- -	11.9	6.4	9.7
huCD40	38.5	31.2	25.6	30.3	--	11.6	12.5
mu4-1BB	36.2	26.1	31.2	30.0	31.7	- -	9.6
raOX40	36.6	30.2	30.0	30.9	34.3	30.0	- -

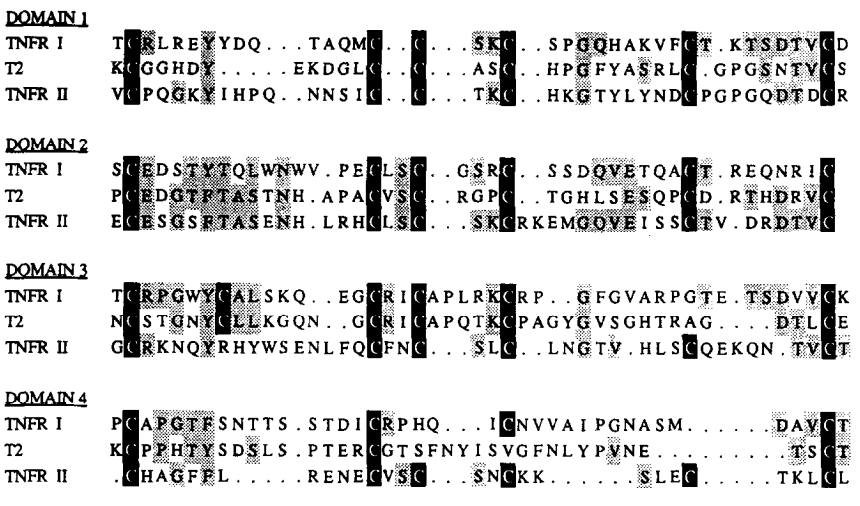
% IDENTITY

Fig. 3. ALIGN scores of (superfamily-defining) cysteine-rich regions of TNF receptor family members. Residue boundaries for human TNFRI, II, human NGF receptor, SFV ORF T2, human CD40, murine clone 4-1BB, rat OX40 antigen are as described in Materials and Methods. ALIGN scores >3 are considered significant.

was further underscored by the failure of a mixture of ten cytokines to inhibit this binding (Fig. 2C). The mixture contained interleukins 1-4, 6-7, γ-interferon, GM-CSF, G-CSF, and nerve growth factor (NGF). The NGF receptor is also a TNF receptor superfamily member (1). Further, ^{125}I-TNFα binding to T2, as well as to TNFRI and II, was also clearly inhibited by unlabeled human TNFβ (Fig. 2D). All these results, including purification of recombinant T2 by affinity chromatography, reinforce the specificity of TNF binding to T2, and underscore the functional similarities between the secreted viral protein and membrane-bound TNF receptors. Finally, T2 protein and the soluble TNFRI were tested for their ability to specifically inhibit human ^{125}I-TNFα binding to native TNF receptors on U937 cells (Fig. 2E). T2-containing COS supernatants produced 26% inhibition, mock-transfected supernatants 3%, and supernatants containing Δ^STNFRI, 51%. These results provide independent evidence that T2 does specifically bind TNF, and also demonstrate that the T2 protein should be a functional inhibitor of TNF-mediated activities.

Quantitative Sequence Similarities Between SFV T2 and TNF Receptor Superfamily Members. T2 is clearly most strongly related to type I, not type II TNF receptor, based on examination of the National Biomedical Research Foundation ALIGN scores between the (superfamily-defining) cysteine-rich regions of all seven presently known members, including the rat OX40 protein, a recently cloned induced T cell surface antigen (Fig. 3). ALIGN scores >3 are considered significant and suggest common ancestry. While the T2-TNFRI ALIGN score (20.7 S.D.; 38% identity) is the highest between any pair of family members, the T2-TNFRII score is one of the lowest (8.4 S.D.; 29% identity). Interestingly, the type I and II TNFRs appear *less* similar (8.6 S.D.; 31% identity) than most other family pairs, underscoring the difficulty of predicting ligand specificity on the basis of sequence similarity

A



B

		HTNFRI				HTNFRII				svfT2			
		1	2	3	4	1	2	3	4	1	2	3	4
HTNFRI	1	-	4.0	4.6	1.7	**4.0**	4.3	4.8	2.3	**7.7**	2.8	3.5	1.9
	2	29.7	-	2.4	2.4	2.4	**7.3**	5.1	3.9	4.2	**9.4**	4.6	2.3
	3	44.7	19.5	-	6.3	2.7	4.0	**2.5**	2.8	4.0	3.4	**9.7**	3.9
	4	25.0	15.8	33.3	-	2.9	3.0	3.5	**1.4**	2.8	4.2	5.1	**4.7**
HTNFRII	1	33.3	23.7	25.6	15.4	-	4.8	4.0	3.6	**6.2**	3.5	2.7	2.8
	2	37.8	39.0	34.1	26.3	34.2	-	4.3	4.6	3.7	**7.3**	3.4	3.1
	3	35.1	30.0	30.0	21.1	34.2	32.5	-	2.9	3.5	3.6	**2.6**	2.4
	4	28.6	39.3	24.1	20.7	31.0	50.0	37.9	-	2.1	3.8	2.5	**0.3**
svfT2	1	36.1	25.7	38.9	19.4	38.9	25.7	38.2	27.2	-	2.7	3.1	4.3
	2	29.7	40.5	26.8	31.6	23.1	41.5	17.5	39.3	28.6	-	2.8	1.5
	3	26.3	22.5	46.5	23.1	36.8	32.5	25.6	31.0	32.4	22.2	-	2.5
	4	22.2	21.1	36.8	25.6	25.0	21.1	26.3	24.1	41.2	18.4	15.8	-

PER CENT IDENTITY

Fig. 4. Comparison of pseudo-repeats in amino acid sequences of TNFRI, II and T2. **(A)**, consensus alignment of pseudo four-fold repeat elements (domains). Sequences corresponding to pseudo-repeat elements shown are listed under Materials and Methods. Cysteines are in bold and residues common to at least three domains are highlighted. **(B)**, quantitative alignment scores between domain-pairs. Analagous domain pairs of 3rd and 4th repeats are heavily boxed; those of 1st and 2nd, lightly boxed.

alone, and also suggesting the TNF receptor genes are quite ancient. Support for this proposal comes from the recently sequenced *murine* TNF receptor type I and II cDNAs. Both pairs of analogous murine and human TNF receptors show strong sequence conservation (65% identify) throughout the entire molecules (Goodwin et al., manuscript submitted), consistent with duplication and divergence of a proto-TNF receptor gene considerably predating speciation.

Comparative Pseudo-Repeats in TNFRI, TNFRII AND SFV T2. Members of the TNF receptor superfamily contain pseudo four-fold repeats of about 40 residues (and about 6 cysteines) in the cysteine-rich extracellular region (1,13,14). Alternatively, pseudo 2-fold repeats of about 80 residues and 12 cysteines can also be written, and generally give higher alignment scores. Either sets of repeats may represent discrete crystallographic domains, although the exon structure of the human NGF receptor gene does not correlate well with these

putative domains (19). The crystal structure of wheat germ agglutinin (20) solved at atomic resolution, however, may be relevant. This protein of 160 amino acids, although not homologous in sequence to TNF receptor family members, also contains four cysteine-rich pseuo-repeats of about 40 residues, and clearly consists of four discrete domains. In any case, the three-dimensional structures of these family members are no doubt quite similar. Analogous four-fold repeats between human TNFRI, II, and T2 are represented in Fig. 4A; ALIGN scores are shown in Fig. 4B. Although most domains are based on a motif of 6 cysteines and other conserved residues, the precise spacing and number of the cysteines, as well as the presence of other key residues, are variable, both within domain sets of a given protein and between domains of different proteins. The greatest divergence between corresponding repeats is with domains three and four, and both these sets of T2 elements show greater similarity with TNFRI than TNFRII. This is due not only to a better alignment of cysteines, but also to a more closely matched domain size (39 residues with TNFRI and T2 vs. 29 for TNFRII). This further supports the assignment of T2 as an acquired Type I TNF receptor. Although the origin of poxvirus genes is unclear, it seems likely that recombination event(s) between host TNFRI and the viral genome which created T2, or subsequent selection pressure, deleted the receptor transmembrane element, and perhaps part of the cytoplasmic domain. The vestigial 'cytoplasmic' domain of T2 contains only 129 residues (174 for TNFRI) and has diverged to an unrecognizable form: this region of T2 shows no sequence homology with any known proteins, including the cytoplasmic domains of TNFRI and II. Interestingly, the C-terminal domains of all 3 proteins contain the same number of cysteines (five), although the spacings appear quite different. T2 also possesses one potential C kinase phosphorylation site (Ser 221), similar to TNFRI (21); TNFRII, in contrast, possesses three. The construction and functional evaluation of viral deletion mutants of T2 will greatly aid in understanding its role in viral pathogenesis.

The most obvious function of T2 is to bind host-derived TNF, preventing the ligand from reaching cellular targets, and thereby dampening the potential antiviral effects of the cytokine. Such a 'soluble' receptor would no doubt confer a selective advantage to the pathogen. In this regard, it is notable that other members of the *Leporipoxvirus* family also encode T2 homologues (G. McFadden, unpublished data), including myxoma virus and malignant rabbit fibroma virus, both of which aggressively immunosuppress adult immunocompetent hosts, and are uniformly fatal (3) That TNF may be an especially important cytokine in the immune response to viral infection is also consistent with Adenovirus expression of a 14.7 kd protein (22) in the E3 region, unrelated to T2, which confers resistance to TNF-mediated lysis of infected cells and whose presence strongly augments viral propagation. Naturally occuring soluble receptors have been discovered for a number of cytokines (7, 9, 23-26), particularly in biological fluids of patients. Two of these, murine IL4 and human IL7 receptors, are encoded by discrete transcripts, consistent with an immunological function for these forms.

SFV T2 represents the first soluble cytokine receptor to be identified in a virus. Only three other viral ORFs have been identified which show homology to host-related immune molecules: the epidermal growth factor-like protein encoded by Shope and Vaccinia (3) viruses, a Vaccinia complement-like protein (27) and an IL10-like cytokine (28) encoded by the EBV genome. These may well represent general mechanisms of viral subversion of the host immune system.

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