Variation in Ligand Binding Specificities of a Novel Class of Poxvirus-encoded Tumor Necrosis Factor-binding Protein*§

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The Yatapoxviruses encode a distinct class of secreted TNF-binding protein (TNF-BP) that resembles an MHC class I heavy chain but distinct from any other known TNF inhibitor. Characterization of these viral TNF inhibitors from Tanapox virus, Yaba monkey tumor virus (YMTV) and a closely related version from Swinepox virus revealed dramatically differential TNF binding specificities for different mammalian species. The Tanapox virus 2L protein (TPV-2L) formed inhibitory complexes with human TNF, and interacted with monkey and canine TNF with high affinity but rabbit TNF with low affinity. On the other hand, YMTV-2L bound human and monkey TNF with high affinity but rabbit TNF with only low affinity. The TNF-BP from swinepox virus (SPV003/148) only interacted with porcine TNF with high affinity. The observed TNF binding analysis mirrored the biological activity of these TNF-binding proteins to block TNF-induced cellular cytolysis. TPV-2L and YMTV-2L also inhibited the human TNF-mediated signaling in cells but TPV-2L exhibited higher affinity for human TNF (K_D 43 pm) compared with monkey (K_D 120 pm) whereas for YMTV-2L, the affinities were reversed (human TNF K_D 440 pm; monkey TNF K_D 230 pm). The interaction domain of human TNF with TNF-binding proteins is significantly different from that of TNFRs, as determined using human TNF mutants. We conclude that these poxvirus TNF-binding proteins represent a new class of TNF inhibitors and are distinct from the viral TNF receptor homologues characterized to date.

The complex and ever evolving interactions between host and pathogens has led to an evolutionary interplay between the host immune system and countermeasures by the pathogens. Large DNA viruses such as poxviruses, which replicate exclusively within the cytoplasm of infected cells, encode diverse immunomodulatory proteins to modulate, avoid or diminish multiple antiviral responses mediated by hosts (1–3).

Tumor necrosis factor (TNF), secreted primarily by macrophages and monocytes, is a potent mediator cytokine of inflammation and the immune response to various pathogens (4, 5). TNFα, the prototype member of the TNF superfamily, forms trimers and is expressed as both a membrane-bound 26-kDa and a soluble 17-kDa form, both of which are biologically active. The biological effects of TNFα (here called TNF) are mediated by binding to two receptors, TNFR1 (p55/p60) or TNFR2 (p75/80), members of the TNFR superfamily of proteins (6, 7). TNF is also directly involved in the pathogenesis of inflammatory and autoimmune diseases such as rheumatoid arthritis, septic shock, Crohn disease and other syndromes (6).

The broad inhibitory effects of TNF have forced many viruses to acquire specific strategies to neutralize TNF or TNF-mediated responses. Of particular interest are poxvirus-encoded molecules that bind and sequester extracellular TNF prior to TNFR engagement (8–10). Two classes of viral-encoded anti-TNF proteins have been described: secreted homologues of TNFRs and secreted proteins that bind TNF, termed vTNFRs and vTNF-BP, respectively. Among the poxvirus-encoded vTNFRs, two major categories have been described: the T2-like inhibitors encoded by leporipoxviruses and the cytokine response modifier (Crm)-like orthologs encoded by orthopoxviruses (8). The first identified such immunomodifier targeted to TNF was the T2 TNFR homolog in Shope Fibroma virus (SFV), a leporipoxvirus, which causes tumors in rabbits (11, 12). The function of such a soluble viral receptor mimic is to bind and sequester host-elicited TNF and LTA, thereby preventing activation of cellular TNF receptors and TNF-mediated responses that lead to downstream antiviral effects. The closely related myxoma virus encoded M-T2 is an early glycosylated, dimeric, secreted protein that specifically binds to, and inhibits rabbit TNF (11, 12). Additionally, the M-T2 protein is also able to inhibit apoptosis in virus-infected lymphocytes (13). M-T2 knock-out myxoma virus exhibited significantly reduced infectivity in domestic rabbits susceptible to wild-type virus infection and M-T2 was the first described “viroceptor” (14).

Orthopoxvirus-encoded vTNFRs, denoted CrmB, CrmC, CrmD, and CrmE, vary in ligand binding specificities and pat-

* This work was supported by the National Cancer Institute of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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2 The abbreviations used are: TNF, tumor necrosis factor; TNF-BP, TNF-binding protein; vTNFR-BP, viral TNF-BP; TPV, Tanapox virus; YMTV, Yaba monkey tumor virus; SVF, swinepox virus; YLDV, Yaba-like disease virus; TNFR, TNF receptor; LTA, lymphotoxin-α; Crm, cytokine response modifier; CRD, cysteine-rich domain; DPV, deerpox virus; SPF, surface plasmon resonance; NTA, nitrilotriacetic acid; hTNF, human TNF; ORF, open reading frame.
Poxvirus-encoded TNF-binding Proteins

terms of expression. Cowpox virus encodes all four Crm paralogues (15–18), Ectromelia virus encodes only CrmD (19) and variola (VaV) and monkeypox virus, which causes smallpox-like disease in humans, encode CrmB only. Although all of the above vTNFrs possess the conserved TNF binding cysteine-rich domains (CRDs), CrmC and CrmE lack the 150-residue C-terminal domain present in CrmB and CrmD. Recently, it has been demonstrated that the VaV-encoded CrmB also binds to chemokines in addition to TNF (20). The TNF binding CRD-containing domain remains solely in the N-terminal region of CrmB, whereas the C-terminal domain that binds chemokines represents a newly identified family of poxvirus chemokine inhibitors (20). Among the vaccinia virus (VV) strains, Lister, USSR, and Evans encode CrmC and CrmE; however other strains such as Western Reserve, Copenhagen, DryVax (Wyeth), and Tian–Tan encode truncated vTNFrs that are not functional (21). Cowpox and mousepox virus also encode an ortholog of another TNFR family member, vCD30 that binds human cytokines or members of TNF superfamily of proteins (25). Similarly, TPV-2L protein exhibits some sequence similarity (25% identity) to the

Cloning of Viral Genes for Expression in the Baculovirus System

The genes of interest were amplified by PCR with specific oligonucleotides, Pfu and viral DNA as template. Genes were cloned into pcDNA3.1/Myc/His (Invitrogen) or pFastBac1 (Invitrogen), and the proteins were expressed fused to the C-terminal Myc and His, tag. The correct sequence of all DNA fragments cloned was confirmed by DNA sequencing. Cloning of TPV-2L and construction of recombinant baculoviruses expressing TPV-2L has been described previously (25).

Generation of Recombinant Baculoviruses

The genes YMTV-2L and SPV 003/148 from pcDNA-YMTV-2LMyc/His and pcDNA-SPV 003Myc/His, respectively, were cloned into pFastBac1 (Invitrogen), and recombinant baculoviruses referred to as AcYMTV-2LMyc/His and AcSVP003Myc/His were produced by using the Bac-to-Bac expression system following the manufacturer’s protocols (Invitrogen). Briefly, plasmids were transformed into competent DH10Bac bacteria, where a transposition event generated the corresponding recombinant bacmids. These were purified and transfected into SF21 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 step to generate a higher titer recombinant virus stock for protein production.

Protein Purification

SF21 cells were infected (multiplicity of infection of 5–10 plaque-forming units/cell) with the recombinant baculoviruses. Cell supernatants were harvested at 3–4 days postinfection (pi), clarified by centrifugation at 2,000 × g for 10 min and then at high speed at 30,000 × g for 30 min, and then concentrated 10-fold using a 10,000-dalton ultrafiltration disc (Pall). Supernatants were then desalted and buffer-exchanged against phosphate buffer, pH 7.0, containing 10 mM imidazole. Protein was purified by metal chelate affinity chromatography (Ni²⁺/Co²⁺) following the manufacturer’s protocol (Invitrogen). The eluted protein was concentrated using Nanosep 10K omega microconcentrator (Pall) and further purified by applying to a Superdex 200 (Amersham Biosciences) fast protein liquid chromatography 60-ml column, and 2-ml fractions were collected.

EXPERIMENTAL PROCEDURES

Reagents

Recombinant human, rhesus monkey, canine, porcine, and murine TNF and human LTα were obtained from R&D biosystems. Soluble human TNFR1 and TNFR2 were obtained from BioSource International. Rabbit TNF was produced, concentrated and quantified using methods described previously (12). Swinepox virus genomic DNA was provided from the laboratory of Dr. Richard Moyer (UF, Gainesville). Human TNF mutants hTNFR32W-S86T and hTNFD143N-A145R were produced as described before (29, 30).
The peak fraction was further checked by Western blot analysis, concentrated, and quantified. Purified protein was analyzed by 12% acrylamide SDS-PAGE stained with Coomassie Blue R250. The protein concentration was measured by the Bradford assay or absorbance measurements at 280 nm.

Cytolytic Assays

Human, monkey, canine, rabbit, and murine TNF-mediated cytotoxicity for mouse L929 cells and porcine TNF-mediated cytotoxicity for porcine PK15 cells was examined by using a crystal violet staining method (16). Briefly, 10^5 cells per well were plated in 12-well plates in a total volume of 500 μl of cell growth medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum) and incubated overnight at 37 °C. Spent medium was removed, and fresh medium containing TNF (1 ng/ml for human, monkey, rabbit, and mouse TNF, 10 ng/ml for canine TNF, and 0.075 ng/ml for porcine TNF) with 5 μg/ml actinomycin D in the presence or absence of serial dilutions of inhibitors was added. The constant amount of all these TNFs produced 100% cytolysis in this assay. After 18 h of incubation, cells were washed three times with phosphate-buffered saline and stained for 10 min with 200 μl of 0.5% crystal violet in water. Plates were washed with distilled water and the indicator was solubilized with 2% sodium deoxycholate and the absorbance (A) at 570 nm was determined. All assays were performed in triplicate. Percentage of cytolysis was quantified by Equation 1.

\[
%C = \frac{A_{\text{control well}} - A_{\text{test}}}{A_{\text{control well}}} \times 100 \quad \text{(Eq. 1)}
\]

Analysis of TNF Binding Specificity and Affinity Constants

Cytokine binding specificity and affinity constants were estimated by Surface Plasmon Resonance (SPR) using a BIACore X biosensor. Screening of cytokines was done using the NTA (nitrilotriacetic acid) sensor chip. The sensor chip surface was activated with 500 μM NiCl₂ solution in eluent buffer (10 mM HEPES, 0.15 mM NaCl, 50 μM EDTA, 0.005% surfactant P20, pH 7.4) followed by immobilization of the protein by injection of 50 nM His-tagged protein at a flow rate of 2–5 μl/min until the RU reached >3000. To monitor the binding, various cytokines were injected at a rate of 30 μl/min for 3 min. The sensor chip surface was regenerated by stripping nickel from the surface by injection of regeneration solution containing EDTA (10 mM HEPES, 0.15 mM NaCl, 0.35 mM EDTA, 0.005% surfactant P20, pH 8.3).

For kinetic analysis the recombinant proteins were immobilized at low densities of ~500 RU on CM5 chips using standard amine-coupling chemistry. Different concentrations of the corresponding cytokine were then injected at a flow rate of 50 μl/min over a period of 2 min and allowed to dissociate for an additional 5 min by allowing the running buffer HBS-EP to flow. The sensor was regenerated after each injection using 10 mM acetate, pH 4.0 or 10 mM glycine-HCl, pH 1.5. Bulk refractive index changes were removed by subtracting the reference flow cell responses and the average response of a blank injection was subtracted from all analyte sensograms to remove systematic artifacts. The data were analyzed globally with the BIAevaluation 3.0 software by using a 1:1 Langmuir model.

| TABLE 1 |
|---------|
| Identity/similarity matrix (percent identity (above diagonal) and percent similarity (below diagonal)) of poxvirus MHC class I-like vTNF-BPs |
| TPV-2L | YLDV-2L | YMTV-2L | SPV003 | DPV008 |
| TPV-2L | 100 | 97 | 73 | 37 | 37 |
| YLDV-2L | 97 | 100 | 81 | 34 | 37 |
| YMTV-2L | 73 | 81 | 100 | 50 | 57 |
| SPV003 | 37 | 34 | 50 | 100 | 51 |
| DPV008 | 37 | 37 | 48 | 51 | 100 |

Cell Assays and Immunoblotting

TNF-mediated IκBα depletion was tested in HeLa cells. 2 ng/ml TNF (the lowest amount that depleted maximum IκBα) was incubated with different concentration of recombinant inhibitors for 2 h at room temperature in cell growth media (Dulbecco’s modified Eagle's medium with 10% fetal bovine serum). The mixture was then added to 5 × 10^5 cells in 6-well plates with 1 μg/ml actinomycin D for 30 min. Cells were collected and processed for Western blot analyses as described before (25). Loading of equal amounts of protein from each sample was confirmed by detection of the housekeeping gene actin.

Cleavage of the fluorescently labeled caspase substrate DEVD-R110 (Molecular Probes) was used to monitor TNF-induced caspase activation in HeLa cells. To measure TNF-mediated caspase activation, cells were incubated for 6 h with human TNF or LTA (as control) or TNF mixed with increasing concentrations of TPV-2L or YMTV-2L. The cells were harvested and processed...
according to manufacturer's protocol. Fluorescence was determined using Fluorimeter (Thermo Labsystems) and further calibrated against a standard curve generated with free R110.

RESULTS

Expression of TPV-2L Homologues—TPV-2L encodes a high affinity human TNF-binding protein (25). The closest relatives of this protein are present in other members of the Yatapox viruses, the single member of the Suipoxvirus (swinepox) and the recently sequenced, deepervirus (27). Amino acid sequence alignment showed considerable divergence (~31% identity) among these newly identified class of poxvirus-encoded MHC class I-like TNF-binding proteins. TPV-2L is most similar to YLDV-2L (97%) and YMTV-2L (73%) and 37% identical to SPV003 and DPV008. SPV003 and DPV008 share 57% identity between them (Table 1 and supplementary Fig. S2). To characterize the 2L family of vTNF-BPs, we have cloned and expressed TPV-2L, YMTV-2L, and SPV003 as Myc-His-tagged recombinant proteins using the baculovirus expression system (BES). All the recombinant proteins were secreted from baculovirus-infected cells and have similar molecular mass of about 47 kDa (Fig. 1).

Analysis of vTNF-BPs Using SPR—Although the evolutionary host of the Yatapox viruses is unknown, based on the pathogenicity to primates and the presence of 2L-related genes in multiple poxviruses that infect other mammalian species, we speculated that the 2L family of proteins might bind to TNF from other species. Previous studies using SPR have demonstrated that recombinant TPV-2L expressed using BES, bound to human TNF with high affinity (KD,4 3 pm); however, it did not bind to rat or mouse TNF and other members of the TNF superfamily of proteins (25). To screen for potential interacting TNF from other species and other cytokines, we immobilized recombinant TPV-2L, YMTV-2L, and SPV003 at high density (~2000–3000 RU) on NTA sensor chips. Various TNF ligands and cytokines were injected over the control (flow cell 1) and recombinant protein surface (flow cell 2). The interacting ligands that were positive on NTA sensor chips were further confirmed using CM5 chips. TPV-2L, in addition to human TNF, also bound to rhesus monkey, canine, and rabbit TNF (Fig. 2A). The recombinant YMTV-2L bound to human, rhesus monkey, and rabbit TNF but not to canine, porcine, murine, or rat TNF (Fig. 2B). Neither one could interact with cell 1) and recombinant protein surface (flow cell 2). The interacting ligands that were positive on NTA sensor chips were further confirmed using CM5 chips. TPV-2L, in addition to human TNF, also bound to rhesus monkey, canine, and rabbit TNF (Fig. 2A). The recombinant YMTV-2L bound to human, rhesus monkey, and rabbit TNF but not to canine, porcine, murine, or rat TNF (Fig. 2B). Neither one could interact with

FIGURE 2. Screening of interacting TNFs by SPR. Purified recombinant TPV-2L, YMTV-2L, and SPV003 were immobilized individually on one flow cell of a CM5 sensor chip. The other flow cell acted as a blank control surface. Over the immobilized sensor chip was passed 100 μl of each different cytokine including TNF. Sensograms showing the binding of canine (6.2 nM), human (6.2 nM), monkey (6.2 nM), rabbit (25 nM), porcine (100 nM) TNF to TPV-2L (A), the binding of monkey (50 nM), human (50 nM), rabbit (50 nM), canine (100 nM), porcine (100 nM), and murine (100 nM) TNF to YMTV-2L (B), and the binding of porcine (25 nM), canine, human, monkey, rabbit, and murine TNF of 100 nM each to SPV003 (C) analyzed by SPR (BIAcore X). Arrows indicate end of injection.
TABLE 2
Kinetic binding parameters and affinity constants of vTNF-BPs to various TNF

| TNF-BP  | TNF          | $k_a$           | $k_d$           | $K_D$       | $\chi^2$ |
|---------|--------------|-----------------|-----------------|-------------|----------|
| TPV-2L  | Human TNF    | $5.26 \times 10^{-6}$ (0.25e-6) | $2.25 \times 10^{-7}$ (0.31e-4) | 0.043 | 1.6 |
|         | Monkey TNF   | $1.70 \times 10^{-3}$ (0.13e-6) | $2.14 \times 10^{-7}$ (0.012e-4) | 0.120 | 1.9 |
|         | Canine TNF   | $2.26 \times 10^{-1}$ (0.21e-6) | $5.76 \times 10^{-7}$ (0.062e-4) | 0.250 | 2.0 |
|         | Rabbit TNF   | $2.38 \times 10^{-3}$ (0.10e-6) | $9.47 \times 10^{-7}$ (0.021e-4) | 4.00  | 0.8 |
|         | Monkey TNF   | $2.71 \times 10^{-3}$ (0.032e-6) | $1.20 \times 10^{-7}$ (0.010e-3) | 0.440 | 2.0 |
|         | Rabbit TNF   | $8.00 \times 10^{-4}$ (0.041e-6) | $9.36 \times 10^{-7}$ (0.064e-4) | 0.230 | 1.9 |
|         | Porcine TNF  | $2.59 \times 10^{-3}$ (0.12e-6) | $1.03 \times 10^{-7}$ (0.020e-3) | 4.00  | 1.2 |
| YMTV-2L | Human TNF    | $1.01 \times 10^{-6}$ (0.48e-6) | $2.38 \times 10^{-7}$ (0.29e-4) | 0.240 | 2.0 |

other cytokines tested including LTα, IL-2, IL-5, or IFNγ. The recombinant SPV003, on the other hand bound to only porcine TNF with high affinity (Fig. 2C).

**Kinetic and Affinity Analysis of the TPV-2L Family Members with TNF**—To more accurately assess the affinity of the recombinant TPV-2L family of proteins with TNF, we performed individual kinetic binding analysis of various species of TNF. The recombinant proteins were immobilized at low densities of ~500 RU on CM5 chips and varying concentrations of TNF applied on them. Following an association period of 120 s, running buffer HBS-EP was injected to monitor the dissociation phase of binding. Table 2 summarizes the kinetic binding parameters of TPV-2L, YMTV-2L, and SPV003 to various species of TNF. TPV-2L bound with high affinity to TNF from rhesus monkey ($K_{a}$, 120 pM) and dogs (canine; $K_{a}$, 250 pM) and with moderate affinity to rabbit TNF ($K_{a}$, 4 nM) (Fig. 3A). Although we observed some TPV-2L bound to porcine TNF at high concentration on NTA sensor chip surface, the interaction was kinetically insignificant (data not shown). Kinetic analysis demonstrated that human TNF exhibits a more rapid association rate (on-rate) than TNF from other species, while the dissociation rate (off-rate) of human, monkey, and canine TNF are all very slow. The $K_D$ of human TNF was also 3- and 6-fold lower than monkey and canine TNF, respectively. In contrast, rabbit TNF demonstrated slower association and relatively fast dissociation rate resulting lower affinity with a $K_D$ of 4 nM.

SPR analysis using a CM5 chip demonstrated that YMTV-2L bound with high affinity to both human ($K_{a}$, 440 pM) and monkey TNF ($K_{a}$, 230 pM) but the affinity for monkey TNF was about 2-fold higher than for human TNF (Fig. 3B). This difference is explained because monkey TNF has a relatively rapid association and slower dissociation rate than human TNF. However, compared with TPV-2L, YMTV-2L has a 10-fold faster dissociation rate with human TNF that resulted in about a 10-fold higher $K_D$. Rabbit TNF also bound to YMTV-2L with moderate affinity ($K_D$ of 4 nM). Canine TNF bound to YMTV-2L at high density on NTA sensor chip surface, but the interaction was kinetically insignificant (data not shown).

To determine the affinity of porcine TNF with SPV003, the recombinant protein was immobilized on a CM5 sensor chip and varying concentrations of porcine TNF were applied. Porcine TNF bound with high affinity to SPV003 with a $K_D$ of 240 pM (Fig. 3C). Porcine TNF bound with a relatively fast association rate and a very slow dissociation rate, which is similar to binding of human and monkey TNF with TPV-2L.

**Inhibition of TNF-mediated Cytotoxicity by TPV-2L Family Members**—The inhibition of TNF-mediated biological activity by TPV-2L, YMTV-2L, and SPV003 was tested by cytotoxicity assay using murine L929 (for human, monkey, canine, and rabbit TNF) cells and porcine PK15 cells (for porcine TNF). In addition to human TNF, TPV-2L was also able to inhibit the cytlosis caused by rhesus monkey and canine TNF but not by rabbit or porcine TNF (Fig. 4A). The IC$_{50}$ of TPV-2L to neutralize human and monkey TNF was less than 1 nM, whereas for canine TNF it was somewhat higher (24 nM). To quantitatively neutralize human and monkey TNF (60 pM), about 18-fold molar excess of TPV-2L was required, whereas in the case of canine TNF (600 pM) about 40-fold molar excess of the recombinant protein was required. The inhibition of TNF-mediated cytotoxicity by TPV-2L family members closely matched their interaction kinetics.

YMTV-2L was able to inhibit the cytlosis caused by human and monkey TNF but not by rabbit or canine TNF (Fig. 4B). The IC$_{50}$ of YMTV-2L to neutralize human TNF (19 nM) was slightly higher than the monkey TNF (11 nM), reflecting the better affinity for monkey TNF, as observed with kinetic analysis. To neutralize human TNF (60 pM), about 300-fold molar excess of YMTV-2L was required while for monkey TNF (60 pM) it was about 200-fold molar excess of the protein. However, YMTV-2L has about 10-fold lower inhibitory activity than TPV-2L with human and monkey TNF. To determine whether SPV003 could inhibit the biological activity of porcine TNF, the cytotoxicity assay was carried out using porcine PK15 cells (Fig. 4C). SPV003 was able to inhibit the cytlosis caused by porcine TNF but not by TNF from other species. The IC$_{50}$ of SPV003 to neutralize porcine TNF was 6 nM, which is about 1300-fold molar excess to porcine TNF (4.5 pM) used.

**Inhibition of TNF-mediated Responses**—TNF can activate two major signaling pathways downstream of TNF receptors, induction of NF-κB pathway, or caspase activation (28). We therefore tested the ability of these TNF-binding proteins to act as inhibitors by measuring their capacity to block TNF activity in cell-based assays. We incubated TNF (2 ng/ml) with increasing concentrations of vTNF-BPs. We then monitored depletion of the inhibitor of NF-κB (IkBα) or caspase activation induced by these mixtures following addition to HeLa cells. Both TPV-2L and YMTV-2L exhibited comparable potency for inhibiting TNF-mediated stimulation of IkBα degradation in HeLa cells (Fig. 5A). The IC$_{50}$ value of TPV-2L and YMTV-2L was about 4 nM and 16 nM, respectively. They were also able to...
inhibit TNF but not LTα-mediated caspase activation at similar concentrations (Fig. 5B and data not shown).

Interaction of Human TNF Mutants with TPV-2L and YMTV-2L—Human TNF interacts with TNFR1 and TNFR2 with different affinity to mediate its biological response. Previous studies using cell based binding analysis demonstrated that human TNF has significantly higher affinity for TNFR1 than TNFR2 under physiological conditions (36). Mutational studies in human TNF have identified several critical residues required for binding to either or both TNF receptors. Mutations located in or near the three loops (positions 30–36, 84–88, and 138–150), which surround the cleft between two subunits, at the broader half of the pyramid-shaped, trimeric molecule are important (29, 30). Mutant R32W-S86T (hTNF32–86) selectively bound only to TNFR1, whereas D143N-A145R (hTNF143–145) bound only to TNFR2 (29, 30).

We have tested these two key human TNF (hTNF) mutants, for their ability to bind vTNF-BPs and compared their affinity with soluble human TNFR1 and TNFR2 using SPR. Our kinetic binding analysis of human TNF with soluble TNFR1 and TNFR2 using SPR established that human TNF has about 10-fold higher affinity to TNFR1 ($K_D$, 49 pM) than TNFR2 ($K_D$, 500 pM).

FIGURE 3. Binding affinity of vTNF-BPs to TNF. A, binding affinity of TPV-2L to TNF. Top, middle, and bottom panels show binding curves of monkey TNF (0.8, 1.6, 3.1, 6.2, and 12.5 nM), canine TNF (0.8, 1.6, 3.1, 6.2, and 12.5 nM) and rabbit TNF (6.2, 12.5, 25, and 50 nM) to TPV-2L. B, binding affinity of YMTV-2L to TNF. Top, middle, and bottom panels show binding curves of human TNF (1.6, 3.1, 6.2, 12.5, and 25 nM), monkey TNF (1.6, 3.1, 6.2, 12.5, and 25 nM) and rabbit TNF (6.2, 12.5, 25, and 50 nM) to YMTV-2L. C, binding affinity of SPV003 to porcine TNF at various concentrations (3.1, 6.2, 12.5, 25, and 50 nM). Solid lines correspond to the global fitting using a 1:1 Langmuir model. Arrows indicate end of injection.
Human TNF mutant R32W-S86T bound to soluble TNFR1 with slightly reduced affinity ($K_D$, 209 pM) but did not bind to TNFR2 even at higher concentrations. On the other hand, mutant D143N-A145R bound only to soluble TNFR2 with reduced affinity of about 30-fold ($K_D$, 13 nM) than native TNF but did not bind to TNFR1 at all (Table 3 and supplementary Fig. S3).

Both the TNF mutants bound to TPV-2L with different binding affinities, as determined by SPR using CM5 chip (Fig. 6, A and B). The hTNF32–86 exhibited reduced binding by 14-fold, because of faster dissociation rate when compared with native hTNF. In contrast, hTNF143–145 demonstrated only a modest 4-fold change compared with native hTNF. This suggests that neither of these mutations are very critical in terms of binding to TPV-2L, compared with the dramatic effects on TNFR1 or TNFR2. Surprisingly, hTNF32–86 did not exhibit any binding to YMTV-2L, whereas hTNF143–145 exhibited reduced binding by 3-fold compared with native hTNF (Fig. 6, C and D). This suggests that residues at positions 32 and 86 are as important for interaction with YMTV-2L as they are for TNFR2, but not critical in terms of binding with TPV-2L. Like the native human TNF, both the mutant human TNFs were unable to bind to SPV003 (data not shown).

**DISCUSSION**

TNF is a key mediator in host innate and adaptive immune response against various pathogens, including viruses (10). So, it is not surprising that viruses would acquire strategies to counter the TNF response (8, 9). The identification of a new class of TNF-binding protein (25) further exemplifies the complexity and sophistication of viral countermeasures to TNF through different mechanisms. Among the poxvirus genomes sequenced so far, homologues of the 2L family of TNF-BP are present only in members of the yatapox viruses, swinepox virus, and deerpox virus. Phylogenetic analysis of poxviruses suggested that these viruses form a distinct pox-virus clade with the 2L orthologs likely a gene acquired by a common ancestor (31). At the protein level, this group of vTNF-BPs is, nevertheless, quite diverged. Although TPV-2L and YMTV-2L share 78% identity for 2L, SPV003 and DPV008 are only 38% identical to TPV-2L (Table 1). We have shown here that TPV-2L, YMTV-2L, and SPV003 all exhibit TNF binding properties, which are biologically significant but quite diverged from each other. Among the vTNF-BPs tested, TPV-2L exhibits the broadest TNF binding species specificity, as well as the greatest inhibition of TNF-mediated bioactivity. On the other hand, SPV003 has a much more restricted species selectivity, in terms of both binding affinity and inhibition of TNF-mediated bioactivity. This suggests that all of the vTNF-BPs possess similar structural motifs, thereby allowing them to recognize TNF, but their specific ligand-interacting amino acid residues will dictate their precise TNF species specificity. The differences in binding to TNF from different species by these vTNF-BPs can also be attributed to amino acid changes among the TNF species. TPV-2L and YMTV-2L bind both human and monkey TNF (96% identical) with very high affinity. However, only rabbit TNF bound to both TPV-2L and YMTV-2L although mouse and rabbit TNF are 78 and 79% identical with their human counterpart (supplementary Table S1). This observation suggests that residues critical for binding or recognition of vTNF-BP might be variable among the different TNFs and explain the species specificity. Collectively these results can be interpreted as reflecting how naturally evolved TNFs adapt to their particular host immune system.

The species variability of the mammalian TNFs may explain why poxviruses have acquired diverse inhibitors to inhibit the TNF pathway. Poxvirus vTNFRs, the T2-like proteins and Crm family members, significantly vary in binding to TNF or TNF-related molecules from different species and the inhibition of TNF-mediated cytotoxicity (1, 20). These viral TNF inhibitors also exhibit different levels of similarity to host proteins and unique species specificity. The myxoma virus M-T2 protein for example, binds and inhibits rabbit TNF only (12). Although the orthopoxvirus-encoded Crm
family of proteins bind human and mouse TNF with different affinities, they vary primarily in their inhibition of TNF-mediated cytotoxicity. CrmB (CPV and VaV) and CrmD (CPV and EV), both bind and inhibit TNF and LTα-mediated cytotoxicity (15, 17, 20). Although both CrmD and CrmE bind TNF from rat, murine and human, they only inhibit human TNF-mediated cytolysis (17, 18). In contrast, we show that the 2L-family of vTNF-BPs exhibit a close correlation between binding TNFs and inhibiting its cytotoxic bioactivity.

The trimeric TNF can bind to either TNFR1 or TNFR2 to mediate biological responses. Mutational studies with human and murine TNF have identified several structural regions, and residues, important for binding with either of these receptors (32, 33, 34). The three TNF loops (positions 30–36, 84–88, and 138–150) that cluster around the interface between each of any two subunits of the trimeric hTNF structure are important for binding. Mutational studies have identified additional residues that are important for binding selectively to either of the receptors (29, 30). Mutations introduced between residues 29 and 34 and at residue 86 tend to perturb binding of hTNF to TNFR2 to a greater extent than binding to TNFR1. By combining the R32W and S86T mutations, the double mutant (R32W-S86T) TNF completely lost its binding activity for TNFR2 but retained all the binding activity for TNFR1 (29). The lack of YMTV-2L binding to this double mutant TNF suggests that either or both of these two residues are in intimate contact with YMTV-2L, possibly in a manner similar to their interaction with YMTV-2L, and contribute to the free energy of binding. In contrast, mutations of residues 143–145 of hTNF generally interfere more with binding to TNFR1 than TNFR2. This may indicate a closer interaction of this TNF surface loop with TNFR2 and contribute to the free energy of binding. Based on the binding with human and other TNF species and their inhibition of human TNF-mediated biological responses, TPV-2L is a more potent inhibitor of human TNF than YMTV-2L and is more effective at blocking hTNF-mediated cytotoxicity at lower (nM) concentrations. On the other hand, YMTV-2L exhibits higher affinity for rhesus monkey TNF.

As mentioned, all the members of this new class of TNF-binding proteins show some identity with the α1, α2, and α3 domains of cellular MHC class I molecule. Molluscum contagiosum virus (MCV), a human

![FIGURE 5. Inhibition of human TNF-mediated signaling. A, inhibition of lκBα depletion. HeLa cells were treated with increasing concentration of human TNF (top panel) or TNF (2 ng/ml) incubated with increasing concentrations of TPV-2L (second panel) or YMTV-2L (third panel) for 30 min and lκBα level was detected by Western blot analysis. Actin was used as a loading control (bottom panel). B, inhibition of caspase activation. HeLa cells were treated with increasing concentrations of human TNF or LTα (left panel) or TNF (2 ng/ml) incubated with increasing concentrations of TPV-2L or YMTV-2L (right panel) for 6 h to induce caspase activation. Caspase activity, arbitrary units normalized to V_max.](image-url)

### TABLE 3
Kinetic binding parameters and affinity constants of TNFR1 and TNFR2 to human TNF and mutants

| TNFR   | TNF/mutants | $K_\text{a}$ ($\text{ms}^{-1}$) | $K_\text{d}$ ($\text{s}^{-1}$) | $K_D$ ($\text{nM}$) | $\chi^2$ |
|--------|--------------|-------------------------------|-----------------------------|---------------------|---------|
| TNFR1  | Human TNF    | 4.60e+5 ± 0.23e5             | 2.25e+5 ± 0.12e-5           | 0.049               | 1.8     |
|        | hTNF32/86   | 4.50e+5 ± 0.88e5             | 9.44e+4 ± 1.82e-3           | 0.209               | 2.0     |
|        | hTNF143/145 | NDB                          | NDB                        | NDB                 | NDB     |
| TNFR2  | Human TNF    | 8.84e+5 ± 0.21e5             | 4.21e+4 ± 0.15e-3           | 0.470               | 1.6     |
|        | hTNF32/86   | NDB                          | NDB                        | NDB                 | NDB     |
|        | hTNF143/145 | 9.17e+5 ± 0.20e5             | 1.20e+3 ± 0.03e-2           | 13.10               | 1.7     |

NDB, no detectable binding.
Poxvirus, also encodes a MHC class I homolog (MC80R) that forms a complex with β2-microglobulin, however, its role in protection of infected cells against cytolysis by natural killer (NK) cells has not been reported (35). The 2L family of MHC class I-like TNF-binding proteins described here, however, exhibit no sequence similarity to MCV encoded MHC class I homologs, suggesting that these distinct immunomodulators have been acquired independently.

The results presented here represent a direct comparison of a novel class of viral TNF inhibitor and its diversity in interaction with TNF, one of the most potent proinflammatory cytokines. Future studies involving the site directed mutagenesis to identify the critical residues and crystal structure resolution of the interaction between vTNF-BP and TNF complex will further elucidate the molecular details of interaction of TNF and this class of molecules.
Acknowledgments—We thank S. Nazarian and R. Singh for invaluable discussions and suggestions and D. Hall for assistance with the manuscript preparation.

REFERENCES

1. Seet, B. T., Johnston, J. B., Brunetti, C. R., Barrett, J. W., Everett, H., Cameron, C., Syypula, J., Nazarian, S. H., Lucas, A., and McFadden, G. (2003) Annu. Rev. Immunol. 21, 377–423
2. Alcami, A. (2003) Nat. Rev. Immunol. 3, 36–50
3. Nazarian, S., and McFadden, G. (2006) Future Virol. 1, 123–132
4. Aggarwal, B. B. (2003) Nat. Rev. Immunol. 3, 745–756
5. Ware, C. F. (2005) Annu. Rev. Immunol. 23, 787–819
6. Mocellin, S., Rossi, C. R., Pilati, P., and Nitti, D. (2005) Cytokine Growth Factor Rev. 16, 35–53
7. Hehlgans, T., and Pfeffer, K. (2005) Immunology 115, 1–20
8. Cunnion, K. M. (1999) Mol. Genet. Metab. 67, 278–282
9. Benedict, C. A. (2003) Cytokine Growth Factor Rev. 14, 349–357
10. Rahman, M., and McFadden, G. (2006) PLoS Pathogens 2, 66–77
11. Schreiber, M., Rajarathnam, K., and McFadden, G. (1996) J. Biol. Chem. 271, 13333–13341
12. Schreiber, M., and McFadden, G. (1994) Virology 204, 692–705
13. Schreiber, M., Sedger, L., and McFadden, G. (1997) J. Virol. 71, 2171–2181
14. Upton, C., Macen, J. L., Schreiber, M., and McFadden, G. (1991) Virology 184, 370–382
15. Hu, F.-Q., Smith, C. A., and Pickup, D. I. (1994) Virology 204, 343–356
16. Smith, C. A., Hu, F.-Q., Smith, T. D., Richards, C. L., Smolak, P., Goodwin, R. G., and Pickup, D. I. (1996) Virology 223, 132–147
17. Loparev, V. N., Parsons, J. M., Knight, J. C., Panus, J. F., Ray, C. A., Buller, R. M. L., Pickup, D. I., and Esposito, J. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3786–3791
18. Saraiva, M., and Alcami, A. (2001) J. Virol. 75, 226–233
19. Smith, V. P., Bryant, N. A., and Alcami, A. (2000) J. Gen. Virol. 81, 1223–1230
20. Alejo, A., Ruiz-Arguello, M. B., Ho, Y., Smith, V. P., Saraiva, M., and Alcami, A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 5995–6000
21. Alcami, A., Khanna, A., Paul, N. L., and Smith, G. L. (1999) J. Gen. Virol. 80, 949–959
22. Panus, J. F., Smith, C. A., Ray, C. A., Smith, T. D., Patel, D. D., and Pickup, D. I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8348–8353
23. Saraiva, M., Smith, P., Fallon, P. G., and Alcami, A. (2002) J. Exp. Med. 196, 829–839
24. Lee, H.-J., Essani, K., and Smith, G. L. (2001) Virology 281, 170–192
25. Brunetti, C. R., Paulose-Murphy, M., Singh, R., Qin, J., Barrett, J. W., Tardivel, A., Schneider, P., Essani, K., and McFadden, G. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 4831–4836
26. Afonso, C. L., Tulman, E. R., Lu, Z., Zsak, L., Osorio, F. A., Balinsky, C., Kutish, G. F., and Rock, D. L. (2002) J. Virol. 76, 783–790
27. Afonso, C. L., Delhon, G., Tulman, E. R., Lu, Z., Zsak, A., Becerra, V. M., Zsak, L., Kutish, G. F., and Rock, D. L. (2005) J. Virol. 79, 966–977
28. Chen, G., and Goeddel, D. V. (2002) Science 296, 1634–1635
29. Loetscher, H., Stueber, D., Banner, D., Mackay, F., and Lesslauer, W. (1993) J. Biol. Chem. 268, 26350–26357
30. Van Ostade, X., Vandenabeele, P., Tavernier, J., and Fiers, W. (1994) Eur. J. Biochem. 220, 771–779
31. Guibser, C., Hue, S., Kellam, P., and Smith, G. L. (2004) J. Gen. Virol. 85, 105–117
32. Van Ostade, X., Tavernier, J., Prange, T., and Fiers, W. (1991) EMBO J. 10, 827–836
33. Barbara, J. A., Smith, W. B., Gamble, J. R., Van Ostade, X., Vandenabeele, P., Tavernier, J., Fiers, W., Vadas, M. A., and Lopez, A. F. (1994) EMBO J. 13, 843–850
34. Ameloot, P., Declercq, W., Fiers, W., Vandenabeele, P., and Brouckaert, P. (2001) J. Biol. Chem. 276, 27098–27103
35. Senkevich, T. G., and Moss, B. (1998) Virology 250, 397–407
36. Grell, M., Wajant, H., Zimmermann, G., and Scheurich, P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 570–575