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Short Communication

Poxvirus-encoded TNF decoy receptors inhibit the biological activity of transmembrane TNF

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Poxviruses encode up to four different soluble TNF receptors, named cytokine response modifier B (CrmB), CrmC, CrmD and CrmE. These proteins mimic the extracellular domain of the cellular TNF receptors to bind and inhibit the activity of TNF and, in some cases, other TNF superfamily ligands. Most of these ligands are released after the enzymic cleavage of a membrane precursor. However, transmembrane TNF (tmTNF) is not only a precursor of soluble TNF but also exerts specific pro-inflammatory and immunological activities. Here, we report that viral TNF receptors bound and inhibited tmTNF and describe some interesting differences in their activity against the soluble cytokine. Thus, CrmE, which does not inhibit mouse soluble TNF, could block murine tmTNF-induced cytotoxicity. We propose that this anti-tmTNF effect should be taken into consideration when assessing the role of viral TNF decoy receptors in the pathogenesis of poxvirus.

Poxviruses have evolved multiple mechanisms to evade the host immune response, including the mimicry of cytokines and their receptors (Alcami, 2003). A well-conserved strategy is the expression of viral TNF receptors (vTNFRs), secreted viral proteins that bind cytokines of the TNF superfamily (TNFSF), inhibiting their biological activities (Alejo *et al.*, 2011). Four different TNF inhibitors are included in the vTNFR family: cytokine response modifier B (CrmB), CrmC, CrmD and CrmE. These vTNFRs have been identified as TNF only, for CrmE and CrmC (Reading *et al.*, 2002; Saraiva & Alcami, 2001; Smith *et al.*, 1996), or as TNF and lymphotoxin α (LTα) inhibitors, for CrmB and CrmD (Hu *et al.*, 1994; Loparev *et al.*, 1998). In addition, we recently identified LTβ as a new ligand for CrmD and CrmB (Pontejo *et al.*, 2015). Importantly, CrmB and CrmD, but not CrmC and CrmE, contain an extended C-terminal chemokine-binding domain termed SECRET (Alejo *et al.*, 2006). Previous data support a role of vTNFRs in poxvirus pathogenesis. Using an intranasal mouse model of recombinant vaccinia virus (VACV), Reading *et al.* (2002) demonstrated that CrmB, CrmC and CrmE enhanced VACV virulence. Similarly, a cowpox virus (CPXV) deficient in CrmB but competent for other vTNFRs showed an increased LD_{50} in a mouse intracranial infection model (Palumbo *et al.*, 1994). Finally, M-T2, the TNF inhibitor encoded by the leporipoxvirus myxoma virus and homologue to the orthopoxvirus vTNFRs, was shown to be required for a fully symptomatic infection in rabbits (Upton *et al.*, 1991).

The contribution of vTNFRs to poxvirus pathogenesis suggests that their target ligands, TNF, LTα and LTβ, may play important roles in the antiviral response. TNF and LTα share the cellular TNF receptors 1 and 2 (TNFR1 and TNFR2), while LTβ signals through a distinct receptor, LTβR (Aggarwal, 2003; Crowe *et al.*, 1994). TNF and LTβ, but not LTα, like other TNFSF ligands, are first expressed as transmembrane cytokines (Aggarwal, 2003). These membrane ligands can be processed by a metalloproteinase to release the soluble cytokines. Thus, transmembrane TNF (tmTNF) is cleaved by the TNF-α converting enzyme releasing the soluble TNF (sTNF) to act at remote sites (Black *et al.*, 1997). However, tmTNF can act locally and mediate pro-inflammatory and immunological activities by itself (Perez *et al.*, 1990). While some of these tmTNF biological activities overlap with those of sTNF, it has been shown that tmTNF can mediate specific and distinct effects *in vitro* and *in vivo* (Mueller *et al.*, 1999), e.g. tmTNF has been shown to be sufficient to induce arthritis (Georgopoulos *et al.*, 1996). More importantly, tmTNF is a key mediator of the defence against different pathogens. For instance, transgenic mice expressing a non-cleavable form of TNF are able to mount efficient immune responses against bacterial infections such as *Listeria monocytogenes*, *Mycobacterium bovis* and *Mycobacterium tuberculosis* (Olleros *et al.*, 2002, 2005; Torres *et al.*, 2005). Similarly, tmTNF has been shown to be sufficient to eliminate parasitic infections of *Leishmania major* and *Trichinella spiralis*

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(Allenbach *et al.*, 2008; Ierna *et al.*, 2009). However, the role of tmTNF in the antiviral response remains poorly understood. Although sTNF, but not tmTNF, is required to limit the immunopathology upon influenza infection, no differences were found in control of the viral burden (DeBerge *et al.*, 2014). By contrast, tmTNF-expressing cells induce cell death of human immunodeficiency virus-infected CD4 cells *in vitro* (Lazdins *et al.*, 1997).

These differential activities between sTNF and tmTNF are thought to arise from differences in their molecular properties. While both TNF forms interact with TNFR1 and TNFR2, TNFR1 dominates the sTNF-induced intracellular signalling, whereas TNFR2 is thought to act as a mere binding partner to increase the sTNF concentration on the cell surface and pass the ligand to TNFR1 (Lu *et al.*, 2001). However, tmTNF can signal through both receptors, which makes tmTNF the main activating ligand of TNFR2 (Grell *et al.*, 1995). As TNFR1 and TNFR2 induce different signalling cascades (Cabal-Hierro & Lazo, 2012), this could explain the different biological activities found between sTNF and tmTNF.

Poxvirus-encoded vTNFRs mimic the ligand binding domain of cellular TNFRs, comprised of four repetitions of cysteine-rich domains (CRDs). The CrmE structure revealed that vTNFRs are more similar to TNFR2 than to TNFR1 (Graham *et al.*, 2007). This suggests that, despite being identified as inhibitors of sTNF, tmTNF could be one of the main targets of vTNFRs *in vivo*. Although Smith *et al.* (1996) showed that CrmC binds mouse tmTNF, its effect on biological activity was not addressed and no further efforts have been reported in this regard. Therefore, we decided to investigate whether other vTNFRs can interact with tmTNF and if this interaction was able to block the tmTNF-induced cytotoxicity.

vTNFRs are differentially expressed among poxvirus species. Variola virus (VARV), the causative agent of smallpox, and ectromelia virus (ECTV), which causes mousepox in mice, express only one vTNFR, CrmB or CrmD, respectively (Fenner, 1993; Mavian *et al.*, 2014; Ribas *et al.*, 2003). However, CPXV strains express three to four different vTNFRs (Alzhanova & Früh, 2010). We recently defined and compared the inhibitory and affinity properties of all these vTNFRs for their soluble TNFSF ligands (Pontejo *et al.*, 2015). Here, we tested the tmTNF binding and blocking ability of ECTV CrmD, VARV CrmB, CrmE from the CPXV strain Elephantpox and the three vTNFRs expressed by the CPXV strain Brighton Red, CrmB, CrmC and CrmD. All these genes were cloned in frame with a C-terminal V5-His tag for their expression using recombinant baculoviruses and purified as described previously (Pontejo *et al.*, 2015).

We first analysed whether vTNFRs were able to interact with tmTNF. To this end, the binding of recombinant vTNFRs to the surface of CHO-745 cells transfected or not with human TNF was assessed by FACS. To guarantee that the expressed TNF was retained on the cell surface, cells were transfected with pSP23, a pcDNA3.1 plasmid encoding a non-cleavable human TNF. The expression of tmTNF was confirmed using an anti-human TNF antibody (Fig. 1a). First, pSP23- and mock-transfected cells were incubated with ECTV CrmD. As shown in Fig. 1(b), CrmD was detected only on the surface of pSP23-transfected cells. The cell staining level achieved with CrmD was similar to the staining with the specific anti-human TNF antibody in Fig. 1(a), suggesting that CrmD bound to tmTNF-expressing cells. To confirm the specificity of this binding, we assayed the binding of the two CrmD domains, CRDs and SECRET, separately. These two domains were expressed and purified using the same cloning strategy and conditions applied to the full-length vTNFRs. The TNF-binding domain CRDs, but not the chemokine-binding domain SECRET, was detected on the surface of pSP23-transfected cells, suggesting that the observed binding of CrmD was mediated by tmTNF. For subsequent experiments, the SECRET protein was therefore included as a negative control. Next, we assayed the binding of all the other vTNFRs and, as shown in Fig. 1(c), all were detected on the cell surface when incubated with tmTNF-expressing cells but not with mock-transfected cells. These results demonstrated that vTNFRs interact with tmTNF. The summary of the mean fluorescent intensities presented in Fig. 1(d) shows that CPXV CrmC was the vTNFR with the lowest tmTNF binding capacity, which agrees with the lower affinity for human sTNF (VARV CrmB, K_D 0.28 nM; ECTV CrmD, K_D 0.41 nM; CPXV CrmC, K_D 2.42 nM) as reported previously by us (Pontejo *et al.*, 2015).

We investigated whether this binding of vTNFRs to tmTNF was able to block the biological activity of tmTNF. For this, we performed a cytotoxicity assay where the cell death of a TNF-susceptible cell line, L929, was induced by co-cultivation with tmTNF-bearing cells. Because human tmTNF-transfected cells did not display a consistent killing effect towards susceptible cells, we used the mouse cell line RAW 264.7 upon tmTNF induction with lipopolysaccharide (LPS) following the protocol outlined in Fig. 2(a) and adapted from published methods (Monastra *et al.*, 1996). The LPS-stimulated RAW 264.7 cells induced cytotoxicity of L929 cells when co-cultivated at a 5 : 1 cell number ratio (control, +LPS) (Fig. 2b). This L929 cell death was mediated exclusively by TNF, as cells were protected when LPS-induced RAW 264.7 cells were pre-incubated with a soluble TNF receptor (hTNFR2, +LPS) or a TNF neutralizing antibody (anti-TNF, +LPS) (Fig. 2b). The protein hTNFR2 was purified as described previously (Pontejo *et al.*, 2015). To check that this cytotoxicity was not induced by remaining traces of sTNF in the fixed RAW 264.7 cell preparation, an equivalent cell volume of supernatant from fixed LPS-induced RAW 264.7 cells after the PBS washes was added to L929 cells and, as expected, no cytotoxic effect was detected (medium, +LPS) (Fig. 2b). Under these conditions, increasing concentrations of recombinant vTNFRs were incubated with fixed LPS-activated RAW 264.7 cells before co-cultivation

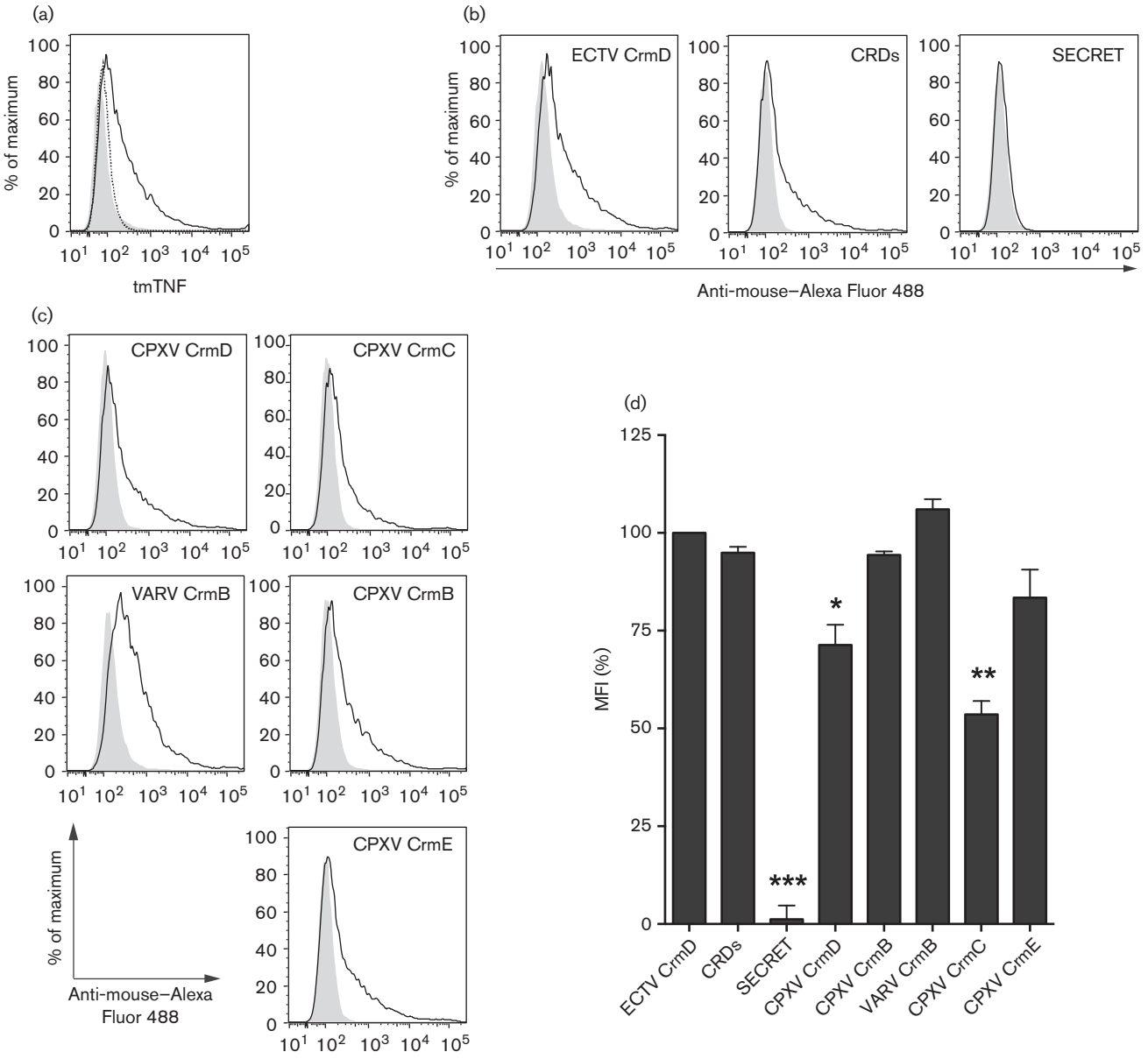

Fig. 1. vTNFRs bind human tmTNF. (a) FACS detection with an FITC-conjugated anti-TNF antibody (R&D) of human TNF expression after transfection of CHO-745 cells with pSP23 using Fugene HD (Promega). pSP23 was generated after deletion of the TNF-α converting enzyme cleavage site (^{77}VRSSSRTPSDKP^{88}) in the human TNF-pcDNA3.1 plasmid with the primers hTNF del77-88 (5′-CCTCTGGCCCAGGCAGTAGCCCATGTTGTA-3′) and hTNF del77-88 antisense (5′-TACAACATGGGCTACTGCCTGGGCCAGAGG-3′) using a QuikChange Lightning Site-Directed mutagenesis kit (Agilent Technologies). Grey shading, mock-transfected cells; dotted line, isotype control; solid line, pSP23-transfected cells. (b) FACS analysis of the interaction of ECTV CrmD full-length, CRDs and SECRET domain with mock-transfected (grey shading) and pSP23-transfected (solid line) cells. (c) FACS analysis of the binding of vTNFRs: VARV CrmB, and CPXV, CrmB, CrmC, CrmD and CrmE to mock-transfected (grey shading) and pSP23-transfected (solid line) CHO-745 cells. In (b) and (c), recombinant proteins were incubated on ice at 100 nM with 3×10^5 mock- and pSP23-transfected cells for 30 min before three PBS washes to remove unbound protein. Proteins were detected on the cell surface with a mouse anti-V5-His antibody (Qiagen) followed by an anti-mouse Alexa Fluor 488-conjugated secondary antibody (Life Technologies). The fluorescence histograms for 10 000 events analysed using FlowJo 7.5 software (Treestar) are depicted. Results are representative of three independent experiments. (d) Summary of the binding of recombinant proteins to pSP23-transfected cells. The mean fluorescence intensity (MFI) of the protein incubation with mock-transfected cells was subtracted from the corresponding pSP23-transfected MFI. Data are represented as mean percentage $\pm$ SD of ECTV CrmD MFI of three independent experiments. (Student's *t*-test: **P*<0.05, ***P*<0.01, ****P*<0.001).

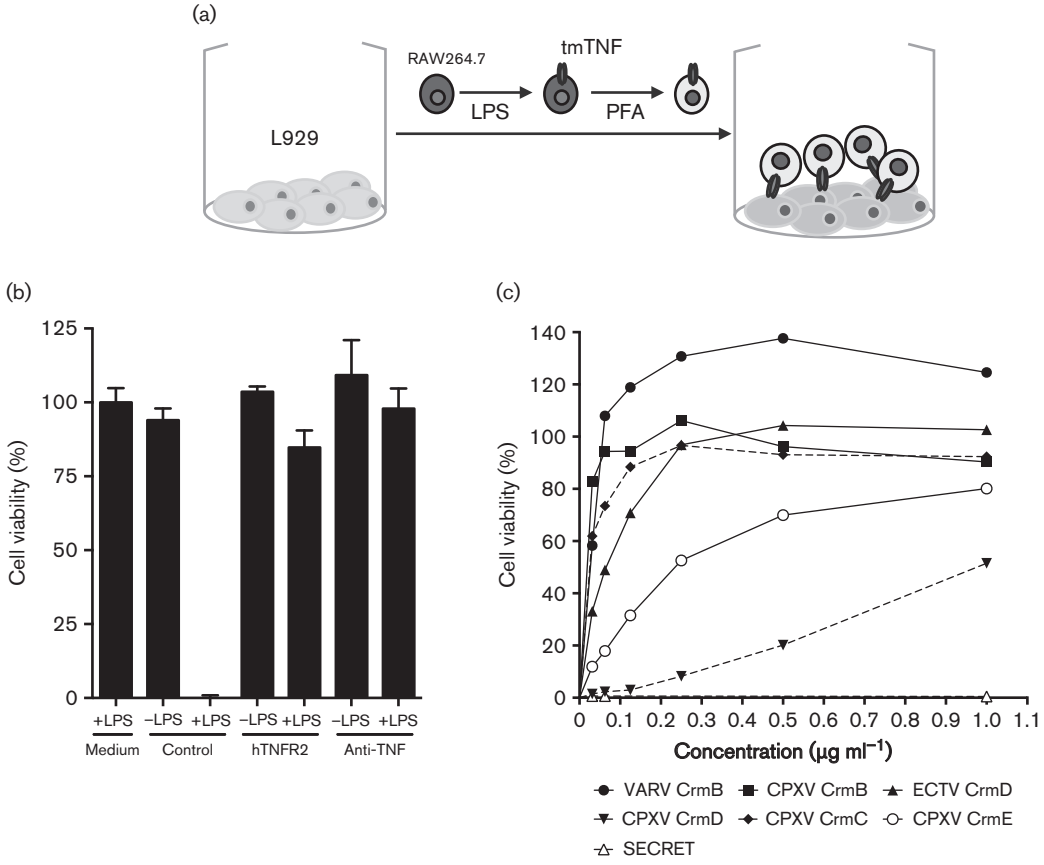

Fig. 2. vTNFRs block murine tmTNF-induced cytotoxicity. (a) Outline of the tmTNF-mediated cytotoxicity assay protocol. RAW 264.7 cells were stimulated for 5 h with 500 ng LPS ml^{-1} (*E. coli* strain O26 : B6; Sigma) to induce TNF. Subsequently, cells were fixed with 1 % paraformaldehyde (PFA) at room temperature for 15 min and washed profusely with PBS before being added to L929 cells in a 5 : 1 (RAW 264.7 : L929) cell number ratio. (b) L929 cell survival after addition of activated (+LPS) and non-activated (−LPS) RAW 264.7 cells, incubated or not (control) with a TNF neutralizing antibody (anti-TNF) or a soluble human TNF receptor (hTNFR2). In the Medium column, an equivalent cell volume of supernatant from fixed activated RAW264.7 cells was incubated with L929 cells. (c) Inhibition of the activated RAW 264.7-induced cytotoxicity of L929 cells by increasing concentrations of purified recombinant vTNFRs and SECRET protein. In (b) and (c), fixed RAW 264.7 cells were incubated in 100 μl Dulbecco's Modified Eagle Medium (DMEM) with 2 % FCS with a TNF neutralizing antibody or increasing amounts (0–1 μg ml^{-1}) of recombinant proteins for 30 min at room temperature. RAW 264.7 cells were then added to L929 cells on 96-well plates in the presence of 4 μg actinomycin D (Sigma) ml^{-1}. Plates were incubated at 37 °C during 18 h and cell viability was determined by the CellTiter Aqueous One Solution kit (Promega). Values were normalized with the absorbance value at 492 nm (A_{492}) recorded from samples containing only RAW 264.7 cells and these were set to zero. Data are represented as the percentage relative to the A_{492} value in the absence of RAW264.7 cells. Means ± SD of triplicate samples of three representative experiments are shown in (b).

with L929 cells. The SECRET domain from ECTV CrmD was included as a negative control (Fig. 2c). We found that CrmB orthologues ECTV CrmD and CrmC were efficient inhibitors of tmTNF-induced cytotoxicity, as only 0.125 μg ml^{-1} was sufficient to reach or exceed a 50 % survival level (Fig. 2c). The CrmD orthologue of CPXV was a weaker inhibitor as 1 μg ml^{-1} was required to protect 50 % of the cells (Fig. 2c). This weak inhibition of mouse tmTNF by CPXV CrmD agrees with its lower ability to inhibit murine sTNF than other vTNFRs (Pontejo *et al.*, 2015). Strikingly, CrmE prevented the killing effect of mouse tmTNF. CrmE protected 50 % of L929 cells at 0.25 μg ml^{-1}, being even more efficient than CPXV CrmD (Fig. 2c). We and others have shown that CrmE is a specific human TNF inhibitor (Pontejo *et al.*, 2015; Reading *et al.*, 2002; Saraiva & Alcami, 2001). Despite binding mouse sTNF with high affinity (K_D 0.26 nM), CrmE does not inhibit the mouse sTNF-mediated cytotoxicity. However, here we showed that CrmE was able to block the biological activity of murine tmTNF. Therefore, in the mouse host, CrmE would inhibit tmTNF in a specific way. Reading *et al.* (2002) showed that CrmE surprisingly enhanced the virulence of

the VACV vTNFR-deficient strain Western Reserve in an intranasal mouse model. One of the authors' hypotheses for this phenotype was that CrmE could be inhibiting a different TNFSF ligand. However, binding assays with many other TNFSF cytokines did not identify additional ligands for CrmE (Saraiva & Alcami, 2001). We propose that the observed CrmE contribution to VACV virulence in mice may be due to its ability to block mouse tmTNF.

Previous data suggest that the molecular mechanisms that drive the receptor binding and signalling of sTNF are not completely conserved in tmTNF. Thus, point mutations of Arg^{31}, Tyr^{87} and Tyr^{119}, which are known to be key residues for the sTNF biological activity (Zhang *et al.*, 1992), have little or no effect in tmTNF (Zheng *et al.*, 2009). Similarly, some mutations in the leader sequence, which anchors tmTNF to the cell membrane and is absent in sTNF, have deleterious effects on tmTNF bioactivity (Zheng *et al.*, 2009). These data suggest that the molecular structures of sTNF and tmTNF may be different. Therefore, it cannot be presumed that a sTNF inhibitor will block tmTNF and vice versa. One example is our finding that CrmE inhibited mouse tmTNF but not sTNF. There are two possible molecular explanations for this observation: (i) the ligand residues in contact with CrmE are different in tmTNF and sTNF; or (ii) the ligand residues in contact with CrmE are the same in sTNF and tmTNF, but these are key residues for the biological activity of tmTNF only. Further molecular studies will be needed to distinguish between these two possibilities.

Here, we reported that vTNFRs inhibits tmTNF, adding a new ligand to the TNFSF cytokines known to be blocked by vTNFRs (Fig. 3). It has been shown that the tmTNF–TNFR interaction may induce cell signalling not only within the receptor-bearing cells but also within the tmTNF-expressing cells, a phenomenon known as 'reverse signalling' (Eissner *et al.*, 2004). Although the biological effects of this signalling are not fully understood, some contributions of the reverse signalling in tmTNF-bearing T-cells, monocytes, macrophages and NK cells have been described (Horiuchi *et al.*, 2010). It will be interesting to evaluate whether the vTNFR–tmTNF interaction can trigger intracellular signals through reverse signalling and how this signalling modulates the immune response in poxvirus infections.

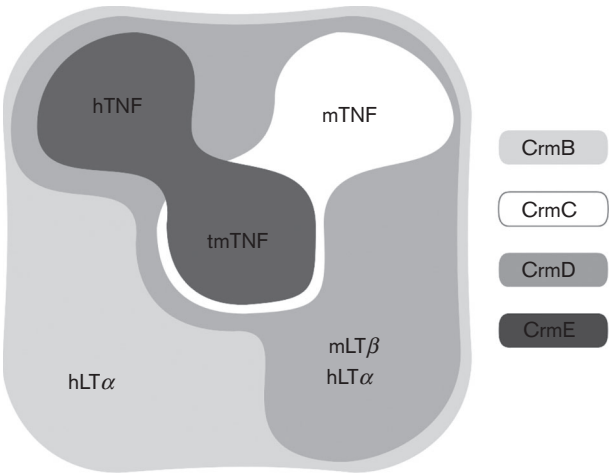

Fig. 3. Diagram of vTNFR inhibitory activities. The cytokines blocked by each vTNFR are indicated in the corresponding group. m, Mouse; h, human.

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