JBC EDITORS' PICK HIGHLIGHT

# Harnessing poxviral know-how for anti-cytokine therapies

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**Andrew G. Bowie**[1]

*From the School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland*

Edited by Charles E. Samuel

**Poxviruses have evolved efficient proteins that bind mammalian cytokines and chemokines to suppress host immunity. Here Pontejo *et al.* examine in detail how one such poxviral protein, CrmD, that has activity against both mammalian tumor necrosis factor and chemokines, interacts with its host targets. They apply their findings to refine a human anti-cytokine therapeutic and increase its specificity, providing an elegant example of the benefits of mining viral proteins for therapeutically useful information.**

Cytokines are critical regulators of innate and adaptive immunity during an anti-pathogen response. However, cytokine dysregulation leads to autoimmune and inflammatory diseases. For example, uncontrolled activity of tumor necrosis factor (TNF)[2] is linked to numerous immune disorders including rheumatoid arthritis. For such immune disorders, anti-cytokine therapies are at the forefront of treatment options, and anti-TNF therapies based on monoclonal antibodies (mAbs) or soluble TNF receptors (TNFRs) have been extensively employed. One such commonly used therapy is etanercept, a fusion protein containing the ligand-binding domain of TNFR2 fused to the Fc domain of a human IgG1 (1). There is always a need to improve such therapies, to increase their specificity and reduce unwanted side effects. Pontejo *et al.* (2) now show that one innovative way to improve anti-cytokine therapy is to turn to viruses, and specifically poxviruses, for inspiration. Poxviruses encode proteins that antagonize host-immune responses, including virally encoded cytokine-binding proteins that are secreted from infected cells (3). Elucidating the mechanisms whereby viral proteins antagonize their host targets sheds light on viral pathogenesis but can also reveal important details about the host proteins themselves. Since viruses have been engaging with our immune system for millennia, highly evolved host-targeting viral proteins can contain useful information that could potentially be used therapeutically to target inflammation (4). The study reported herein (2), which focuses on a soluble poxvirus TNF decoy receptor called cytokine response modifier D (CrmD) from the mouse-specific ectromelia virus (ECTV), is a case in point.

CrmD has been known for some time (3), yet information on how exactly its protein domains are involved in ligand binding of TNF superfamily (TNFSF) cytokines has been very limited. It has been presumed that, like other TNFRs, CrmD binds to TNFSF cytokines via its cysteine-rich domains (CRDs), which are characteristic of both viral and host TNFRs. The authors previously showed that CrmD binds to and neutralizes hTNF, but—unusually for a soluble TNFR—is not capable of neutralizing the closely related TNFSF member lymphotoxin-$\alpha$ (hLT$\alpha$) (5). This was of interest since understanding the molecular determinants explaining hTNF and hLT$\alpha$ targeting by TNFRs could be useful in refining etanercept, which binds to both hTNF and hLT$\alpha$. A new version of etanercept with reduced affinity for hLT$\alpha$ could reduce side effects due to LT$\alpha$ binding and also maintain the host-defense activity of LT$\alpha$ against pathogens such as *Mycobacterium tuberculosis* for those patients taking anti-TNF therapy (6).

Pontejo *et al.* (2) now follow this discovery by first defining the motifs and residues required for ligand binding, and then very elegantly using molecular information from CrmD, in terms of why it targets hTNF but not hLT$\alpha$, to redesign etanercept to make it more specific for hTNF compared with hLT$\alpha$. To do this, they expressed and purified CrmD and associated mutant proteins from recombinant baculoviruses and used surface plasmon resonance (SPR) to assay the binding of CrmD to mouse and human TNFSF cytokines and to mouse chemokines. The authors also used functional assays to check whether changes in binding affinity correlated with changes in inhibition of TNFSF-mediated cytotoxicity or chemokine-mediated chemotaxis. These approaches yielded a number of new insights into how CrmD antagonizes both TNFSF cytokines (through its N-terminal CRD domains) and chemokines (through its C-terminal domain) (2). In terms of the TNFSF targets, it was known that CRD2 and CRD3 contain the main ligand-binding determining residues of TNFRs (7), and the mutagenesis analysis of CrmD focused on two regions within these domains—the 50s loop in CRD2 and the 90s loop in CRD3. Thus, mutations of 50s and 90s loop residues were assayed by SPR for binding affinities for four different CrmD ligands—mTNF, hTNF, mLT$\alpha$, and hLT$\alpha$. Key discoveries from these experiments included showing the importance of a groove in CRD2 formed under the 50s loop, and of specific residues in CRD3, for binding to all four CrmD ligands tested. Importantly, the authors also found residues in CRD3 that when mutated decreased the binding affinity for all ligands except hLT$\alpha$. Interestingly, other ligand-specific differences

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[1] To whom correspondence should be addressed. Tel.: 353-1-8962435; E-mail: agbowie@tcd.ie.

[2] The abbreviations used are: TNF, tumor necrosis factor; TNFSF, TNF superfamily; CRD, cysteine-rich domain; Crm, cytokine-response modifier; ECTV, ectromelia virus; hLT$\alpha$, human lymphotoxin $\alpha$; hTNF, human tumor necrosis factor; LT, lymphotoxin; mAb, monoclonal antibody; mLT$\alpha$, mouse lymphotoxin $\alpha$; mTNF, mouse tumor necrosis factor; SPR, surface plasmon resonance.

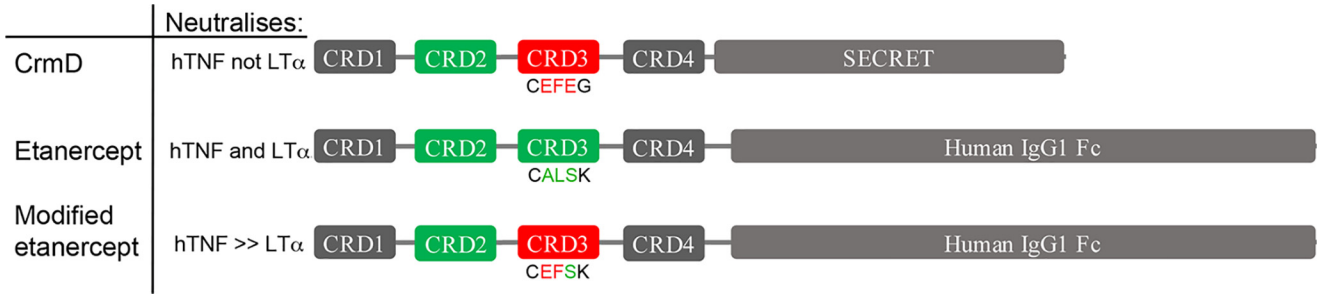


**Figure 1. Virus-directed modification of etanercept.** Domain structure of the viral decoy TNF receptor CrmD and the anti-TNF therapeutic etanercept showing the CRDs involved in binding of TNF superfamily cytokines such as hTNF and hLTα. The SECRET domain of CrmD that binds mouse chemokines and the Fc region from human IgG1 contained in etanercept are also shown. CRD2 and CRD3 contain amino acids that define ligand specificity in terms of the ability of proteins to neutralize hLTα and/or hTNF. Residues shown in *red* from the 90s loop in CRD3 prevent CrmD from targeting hLTα. Substituting two of these residues into the analogous region of etanercept reduces its ability to neutralize hLTα and thus increases its specificity for hTNF.

between the CrmD mutants included the observation that altering residues in the 50s and 90s loops, and the connecting region between them, affected mTNF binding without significantly impinging on the binding to the other ligands, suggesting that CrmD was optimized for mTNF binding. They also noticed that many of the mTNF-specific binding determinants appeared to obstruct hLTα binding, suggesting a selective pressure on CrmD to maintain mTNF, but not hLTα binding, consistent with its role in the mouse-specific pathogen ectromelia virus (ECTV). Reduced binding activity against TNFSF cytokines often, but not always, was found to correlate with reduced functional activity in blocking TNFSF-mediated cytotoxicity.

Of relevance to etanercept was the observation that one 90s loop CrmD mutant, E116A/F117A/E118A, displayed a strong gain of both anti-hLTα activity and hLTα binding affinity. It was then hypothesized that transfer of this EFE motif into etanercept might reduce its affinity for hLTα. Sequence alignment showed that the EFE motif mapped to the residues $Ala^{127}$-$Leu^{128}$-$Ser^{129}$ in the etanercept 90s loop (Fig. 1). However, examination of a TNFR2:TNF co-crystal structure showed that the $Ser^{129}$ residue did not face the ligand interface, which was also the case for $Glu^{118}$ in the CrmD model. Thus, in order to potentially transfer a minimal hLTα-blocking motif into etanercept, the authors made an etanercept A127E/L128F mutant. Remarkably, this mutant did indeed display strongly reduced neutralizing activity toward hLTα (60-fold reduced compared with WT protein), and only very mildly reduced neutralizing activity toward hTNF (3-fold reduced compared with WT protein), even though the binding affinity for hLTα was not significantly affected in the mutant. Thus, they successfully transferred viral molecular information into etanercept to favorably modify its activity profile.

This paper demonstrates the value of understanding highly evolved viral cytokine-binding proteins in order to refine anti-cytokine therapeutics. Poxviruses and clinicians "want" the same thing, namely to be able to dampen down the host's inflammatory response, and in both cases it makes sense to have a diversity of tools at one's disposal for this. Clinical anti-TNFs are mainly mAbs, which are effective, but do have limitations. Etanercept represents an alternative TNF-targeting strategy, and "viral refinement" may make it more mainstream, and a similar approach could be used for other anti-cytokine therapies. It is likely that there is a lot that viruses can still teach us about targeting cytokines, and this paper should encourage a renewed interest in re-examining other known viral cytokine-binding proteins to mine them for useful therapeutic information.

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