Poxviral TNFRs Encode a Novel FcγReceptor Interaction Domain

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Introduction

Poxviruses encode soluble TNF receptor (TNFR) homologs that block the activity of the pro-inflammatory cytokine TNF-α. Cowpox virus encodes four viral TNFRs, known as cytokine response modifiers (Crm) B, C, D and E. CrmB and CrmD exhibit binding properties virtually identical to myxoma T2, the first viral TNFR to be identified. T2 and all four Crm proteins contain a N-terminal TNF receptor homology domain that is necessary and sufficient to bind and sequester TNF-α. The C-terminus of T2, CrmB, and CrmD (T2-tail) contains no homology to cellular TNF receptors or other cellular proteins. One function of this domain is to bind chemokines and is named smallpox virus-encoded chemokine receptor (SECRET) domain. Other SECRET domain-containing proteins (SCP) were identified in certain strains of Cowpox, Vaccinia and Ectromelia viruses. The mousepox strain Ectromelia-Naval (EV-Naval) encodes a SECRET domain-containing gene (EVM008) with significant sequence and functional homology to the C-terminal region of CrmB and CrmD while lacking the cysteine-rich N-terminal portion. Surprisingly, EVM008 was found to bind to the surface of mouse monocyte cell lines, which could not be explained by it binding to chemokines. Identification of the cell surface binding partner of EVM008 by immunoprecipitation and mass spec identification revealed multiple Fcγ receptors (FcγRs). The FcγR binding domain is conserved in T2-tail-TNF-α binding proteins CrmB and CrmD.

Materials and Methods

The ectromelia EVM008 gene (Viral Protein ID, VPID 1241) was synthesized and fused to a streptavidin binding domain-containing affinity tag (HAC-tag) consisting of a hemagglutinin (HA) peptide, a C-Protein binding tag (CTAG) and two streptavidin binding peptides (SBP).
We performed Flow Activated Cell Scanning (FACS), Tandem Affinity Purification and Mass Spectrometry with the EVM008-HAC fusion protein to identify its binding partner on mouse monocyte cell lines. Subsequently, binding of EVM008 to its target was confirmed by FACS analysis on cells overexpressing FcγRs. Binding affinities of EVM008 were determined by Surface Plasmon Resonance Analysis (BIAcore). FACS based competition studies were performed to identify the functional properties of EVM008.

Results

Ectromelia EVM008-HAC (1241-HAC) contains a carboxy-terminal SECRET domain and binds to recombinant human FcγRs and to FcγRs expressed on the murine monocytic cell line Wehi265.1.

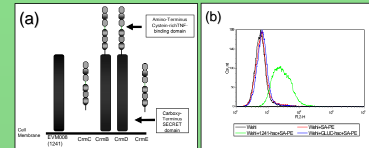

Figure 1.
a.Graphic illustration of ectromelia EVM008 (1241), cowpox CrmB CrmD, CrmC and CrmE. EVM008 contains a C-terminal (T2-tail) domain with high sequence similarity to the C-terminus of CrmB and CrmD. In contrast, CrmC and CrmE only contain the TNF-α-binding N-terminal domain.
b.FACS analysis for binding of EVM008-HAC fusion protein to mouse Wehi265.1 cells. The presence of FcγRs on the surface of Wehi265.1 cells was previously confirmed via FACS analysis using appropriate antibodies (data not shown). A moderate shift is observed with EVM008-HAC compared to the negative control (GLUC-HAC).

Immunoprecipitation/Mass spectrometry (IP/MS) technology identified FcγRs as targets for the vTNFR, EVM008 (1241)

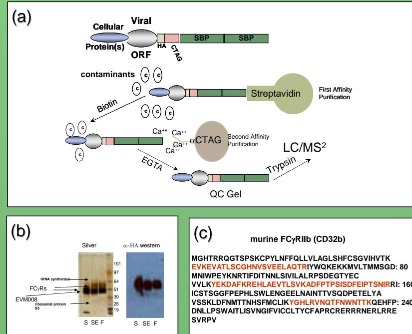

Figure 2.
a.Schematic representation of HAC-tag Tandem Affinity Purification.
b.EVM008-HAC Tandem Affinity Purification (Western Blot and SDS Page). EVM008-HAC was immunoprecipitated from 1x10⁹ cell equivalents of Jurkats, Wehi265.1, THP-1, BJAB, and P388 cells (lysed in 1% TX100/non reduced). S (Starting Material); SE (Streptavidin Elution); F (Final Elution).
c.Mass Spectrometry Identification of mouse FcγRIIb (CD32b) target peptides binding to EVM008 post tandem affinity purification. Unique tryptic peptides are denoted red. FcγRs I (CD64), III (CD16) and IV (CD16-2) were previously identified (data not shown).

Surface Plasmon Resonance (Biacore) Analysis shows high binding affinity of EVM008 (1241) to mouse FcγRs

KD (M)	Mouse CD64	Mouse CD32b	Mouse CD16	Mouse CD16-2
EVM008-HAC	4.0e-11	7.0e-12	9.0e-12	3.0e-11

Figure 3
Surface Plasmon Resonance (BIAcore) analysis of monomeric EVM008 binding to mouse FcγRs. Binding affinities of EVM008-HAC to mouse FcγRs were in the nanomolar to picomolar range.

Viral TNFR family members CrmB, CrmD and T2 bind to murine FcγRI (CD64) and IIb (CD32b)

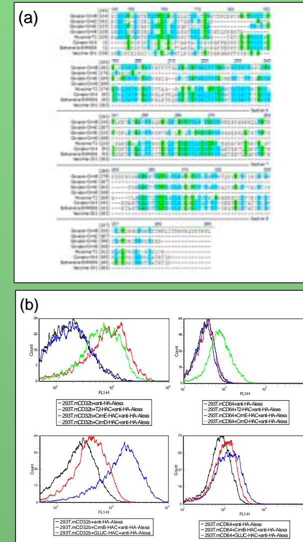

Figure 4.
Characterization of vTNFR family members.
a.T2-tail sequence alignment of CPX CrmB, CrmC, CrmD, CrmE, 014, Ectromelia EVM008 (1241) and myxoma T2. High sequence similarity in the C-terminal domain (T2-tail) could be observed across all vTNFRs.
b.FACS analysis to test for binding of T2-HAC, CrmE-HAC, CrmD-HAC and CrmB-HAC Conditioned Media to stable 293T. mouse CD64γ and 293T.mouse CD32b cell lines. Binding of CrmB-HAC and CrmD-HAC was observed on both, 293T.CD32b and CD64 cell lines. However, T2-HAC only showed binding to CD32b. CrmE-HAC failed to bind to either CD32b or CD64.

EVM008 (1241)-HAC blocks binding of murine IgG2a to FcγRs I (CD64) and IIb (CD32b)

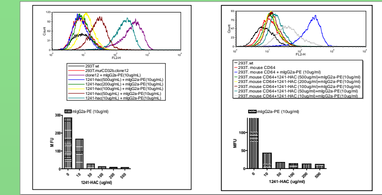

Figure 5.
FACS Competition Assay.
293T cells stably expressing mouse CD32b and mouse CD64 were pre-incubated with EVM008-HAC at concentrations ranging from 10μg/ml to 500μg/ml. Cells were subsequently treated with murine IgG2a-Phycoerythrin (PE) and subjected to FACS analysis.
Pre-treatment of 293T.CD32b and 293T.CD64 cells with EVM008-HAC dimer inhibited binding of murine IgG2a to CD32b (a) and CD64 (b).

Conclusion/Discussion

We identified a second function of the T2-tail/SECRET domain-containing protein, EVM008 (1241). In addition to binding chemokines, EVM008 binds murine FcγRs, CD64, CD32b, CD16 and CD16-2 with high affinity. This binding activity is shared with other proteins that contain a T2-tail/SECRET domain. Specifically, the cowpox viral TNFα-binding proteins CrmB and CrmD contain at T2-tail/SECRET domain and bind FcγRs. Consistent with the FcγR binding activity mapping to the T2-tail, CrmE lacks the T2-tail/SECRET domain and fails to bind to FcγR-expressing cells.

The biological consequences of this interaction remain to be elucidated. One possibility is that the FcγR-binding serves as a anchor to localizes the TNF and chemokine inhibitory activities of the T2-tail family of proteins to the surface of FcγR- expressing cells. This may allow poxviruses to increase the local concentration of chemokine or TNF inhibitor on the surface of invading immune cells. An additional possibility is that the FcγR binding activity serves to inhibit the effector functions of FcγR-bearing invading immune cells and inhibit antibody dependent cytotoxicity mediated lysis of infected cells. Consistent with this second possibility, EVM008 inhibits binding of IgG2a to FcγR-expressing cells.