UNIVERSITY OF TECHNOLOGY, SYDNEY

Interactions of viral and cellular Tumour Necrosis Factor Receptor molecules

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy: Science

Alexander Douglas Gale

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Certificate of original authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student:

Date:

Presentations

Hunter Cell Biology Meeting LoveDale NSW, March 16-20th, 2015
Alexander Gale*, Michael Johnson, Michael F McDermott & Lisa M Sedger.
Abstract: Viral and cellular Tumour Necrosis Factor Receptor (TNFR) interactions and implications for inhibition of TNF and TNFR-related biology. (Poster)

8th Australasian Virology Society (AVS) Meeting. October 30th 2015
Lisa M Sedger*, Alexander D Gale, Ralph Reboblado, Khonder Rufaka Hossain, Michael S Johnson, Michael F McDermott
Abstract: Are viral Tumour Necrosis Factor-Receptors (vTNFRs) a driver for the existence of TNFR-Associated Periodic fever Syndrome (TRAPS) in humans? (Oral presentation)

EMBL – Australia Masterclass on protein analysis, October 21 -25th 2013

Garvan Institute of Medical Research, (Oral)

The 11th Hunter Meeting. March 22-25th, 2011.
Gale A*, Johnson M, Sherwood S, McDermott M, & Sedger LM.
Abstract: Visualisation of viral and cellular TNFRs with mutations in the pre-ligand assembly domain (PLAD), required for viral:cellular TNFR co-association and inhibition of TNFR signalling. (Poster)

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Abbreviations

CFP – Cyan Fluorescent protein

CMV- Cytomegalovirus

CRD – Cysteine Rich Domain

CRM- – Cytokine Response Modifying Protein

DD – Death Domain

DED – Death Effector Domain

FADD – Fas-associated Death Domain Protein

FHF - Familial Hibernian Fever

IKK – Inhibitor of Nuclear Factor Kappa B Kinase

IκB – Inhibitor of Nuclear Factor Kappa B

JNK – c-Jun N Terminal Kinase

LTα - Lymphotoxin alpha

MPV – Monkeypox virus

MPVJ2R – Monkeypox virus J2R

MYXV – Myxoma virus

MYXT2 – Myxoma virus T2

NFκB – Nuclear Factor Kappa B

PLAD – Pre-Ligand Assembly Domain

RIP – Receptor Interacting Protein

SECRET – Smallpox virus-Encoded Chemokine REcepTor domain

SODD – Silencer of Death Domain

TACE - Tumour necrosis Factor Alpha Converting Enzyme

TNF - Tumour Necrosis Factor Alpha

TNFR – Tumour Necrosis Factor Receptor

TNFRSF – Tumour Necrosis Factor Receptor Super Family

TNFSF - Tumour Necrosis Factor Super Family

TRADD - Tumour Necrosis Factor Receptor Associated Death Domain Protein

TRAF - Tumour Necrosis Factor Associated Factor

TRAPS - Tumour Necrosis Factor Receptor Associated Periodic Syndrome

VARV – Variola virus

VARG4R – Variola virus G4R

vTNFR – viral TNFR

YFP – Yellow fluorescent protein

Abstract

Tumour necrosis factor (TNF) is potent pro-inflammatory and anti-viral cytokine, acting via two cellular receptors, TNFR1 and TNFR2 that induces apoptosis and inflammation. Poxviruses encode homologues of TNF-receptors (viral TNFRs) that independently interact with both TNF, and simultaneously with cellular TNFRs, to subvert TNF-induced anti-viral apoptosis. The vTNFRs are expressed during poxvirus infection and are considered as bona fide virulence factors. The recently discovery of a "Pre-ligand Assembly Domain (PLAD)" within the N-terminus of the cellular TNFRs is shown to be required for receptor trimerisation and efficient cell death signalling. Whilst it has previously shown that the rabbit-trophic Myxoma (MYX) viral TNFR also contains a PLAD required for viral TNFR:cellular TNFR interactions, little is known about the human-trophic poxvirus TNFRs, nor physical characteristics of the interactions of vTNFRs and cellular TNFRs.

To assess the importance of the PLAD domain in TNFR structure, function and viral subversion of TNFRs, this study focused on naturally occurring mutations in the TNFR PLAD domain, that occur in transient periodic fevers (TRAPS) – a clinical syndrome of febrile attacks of inflammation. TRAPS PLAD domain mutations were generated in a TNFR1-YFP in plasmids by site-directed mutagenesis and cloning. WT and TRAPS mutant TNFR1 constructs were transfected into U20S cells and TNFR1 location was determined by confocal microscopy. Neither WT TNFR1 nor TRAPS TNFRs were unable to be detected at the cell surface by both widefield and confocal microscopy despite published data on surface expression of WT TNFR1. WT TNFR1-YFP fusion proteins were found to be expressed within endocytic vesicles known as receptosomes and also as aggregates

in a membranous structure resembling Golgi/ER. In addition it was found that TRAPS mutations in particular those affecting critical amino acids such as cysteines in disulphide bonds, display reduced TNFR-induced cell death as determined by flow cytometry.

To better understand the biology of the vTNFR association with cellular TNFRs, and with WHO Smallpox committee approval, the human tropic poxviral TNFRs from Variola (Smallpox) (VAR) and Monkeypox (MPV) were synthesised and cloned as CFP/YFP and MycHis expression plasmids. Using multi-colour flow cytometry we have shown that, like the MYXT2 vTNFR, VARG4R and MPVJ2R TNFRs are potent intracellular inhibitors of TNFR1-induced cell death. As each vTNFR was able to inhibit TNFR-induced cell death, an assay was developed by flow cytometry to measure the intracellular abundance of the vTNFRs in the presence of cellular TNFR overexpression. MYXT2 was found to increase in intracellular abundance however for unknown reasons VARG4R and MPVJ2R did not convincingly increase in abundance. A structure for each of the vTNFRs was then attempted to be determined by X-ray crystallography, however bacterial expression of the both the cellular TNFRs and viral TNFRs proteins were unable to be obtained.

Lastly to determine the structural orientations and conformations of cellular vTNFR interactions, a method of fluorescence resonance energy transfer (FRET) was established by flow cytometry. Using the generated C-terminal fusion -CFP and -YFP TNFRs, interactions were assessed between each of the cellular and vTNFRs. It was found that in addition to the reduced cell death TRAPS TNFRs when expressed with WT TNFR1, TRAPS mutations also cause reduced FRET possibly due to altered conformations

in the receptor. Again mutations affecting more critical structural amino acids were found to have a more dramatic effect. Moreover differences were observed between mutations in distribution of FRET histograms further indicating altered network formations of higher order complexes. Next the FRET method was used to assess interactions between each of the vTNFRs with WT human TNFRs as well as with themselves and other vTNFRs. However no FRET was detectable between each of the molecules despite evidence of MYXT2 associating with human TNFR1 and TNFR2. Thus Comparative homology modelling and automated docking simulations were performed to explain possible orientations of the interactions tested in FRET. These data suggest that the interactions of vTNFRs with cellular TNFRs may possibly occur in a C-N anti-parallel orientation and not the previously predicted PLAD-PLAD interactions.

Taken together, these data further our understanding of basic TNFR biology as well as for the first time characterise an entire panel of PLAD TRAPS mutations. It also furthers the characterisation of the very limited evidence of vTNFR subversion of TNFRs for the human trophic viral proteins VARG4R and MPVJ2R. Overall these results show the importance of PLAD interactions to TNFR biology and a possible new avenue in which TNFR signalling may be exploited in the development of new therapeutics.