



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

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2016

## Certificate of original authorship

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## 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 30<sup>th</sup> 2015**

Lisa M Sedger\*, Alexander D Gale, Ralph Rebolado, 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 11<sup>th</sup> 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)

## Acknowledgements

As I begin to look back at the journey this project has lead me down, both within this project and also the events that have occurred outside of this project over the last few years have been the hardest challenges I've had to face, and without all the help and support of my friends, family and mentors along the way, this all would not have been possible. This has been one of my greatest achievements and can't thank all who have enough, so I dedicate this to you all.

To my supervisor Lisa Sedger, thank you for being accepting me on this project. From my naive beginnings, I have taken with me not only the skills to be more analytical of myself as well as others, but to also further myself in many ways to become a much stronger person. Despite all the challenges we have both faced I thank you for all your support as well as help in the development of the FRET methodology.

To Michael Johnson, I thank you for support and guidance both practical and morally. Your perception and inclusion of all thing technical on the project has inspired my interest in the work you do, and is reflected by the many paths taken in this in this project. I'd also like to acknowledge your input and training with all the microscopy experiments

To Michael Wallach, you undertook me as your student when times proved difficult and have continued to support me until the end. I am in debt for all your generosity and many avenues made possible. To Sonja Frolich, I also want to thank you for welcoming me into your lab and making my stay at UTS much more enjoyable.

Sarah Sherwood, you have taught me and mentored me in the lab to enable me with many of the skills to take on this project. As well as that you have always taken the time to ensure my time in the lab was always enjoyable, for this I am extremely lucky and grateful. Joyce Tran you have been my saviour in the lab and I cannot express how much you have helped me along the way. Your continuing support and guidance has pushed me through some of the hardest times that have challenged me. To all my friends and fellow PhD students I have made along the way; Rita (Monahan), Sam Burns, Raquel Alvarado, Amelia Hynan, Maria Lund, Mike Strauss, Charmain Castel, Erin Gloag, Pdraig Winter and Akane Tanaka, your patience, reassurance, guidance, and mental distractions have kept me sane to continue on a path to complete my journey. Your friendships are all unique and is something I am extremely lucky to have gained during my time as a student. For the house of Jumbunna I only wish I could provide the care and welcome that you have provided me. You make me proud to represent the indigenous community in the field of science and I hope that I can inspire more to follow in my path.

My parents, although you may not understand what I have embarked on, you have unconditionally supported my decisions. Lastly to my endearing Wife Kylie. I know that you wish only the best of me and have unconditionally loved and supported me through the most difficult times in my lifetime. However through this we have become so much stronger together and I am now proud to call you my wife. I am excited to begin the next chapter in our lives together.

I would also like to acknowledge the financial assistance of the Faculty of Science as well as the house of Jumbunna, for who this project would not be possible.

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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 U2OS cells and TNFR1 location was determined by confocal microscopy. Neither WT TNFR1 nor TRAPS TNFRs were able 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.