

Investigation of miR-652 in Host Immunity Against Intracellular Bacterial Pathogens

by Maxwell T. Stevens

Thesis submitted in fulfilment of the requirements for
the degree of

Doctor of Philosophy

under the supervision of Associate Professor Bernadette
Saunders and Dr Matthew Padula

University of Technology Sydney
Faculty of Science

July 2022

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Maxwell Stevens, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution. This research is supported by the Australian Government Research Training Program.

Signature:

Production Note:
Signature removed prior to publication.

Date:

30 July 2022

Acknowledgments

The last 4 years have been an endurance event, and there are many people who I need to thank for helping me through.

Firstly, I would like to acknowledge and thank my supervisor Associate Professor Bernadette Saunders and my co-supervisor Dr Matthew Padula. Your support and guidance throughout my PhD have instilled me with the ethical and scientific base I needed to really delve into my study, continually learning and developing as a research scientist. You provided sound advice when I needed it most, but also gave me the reigns to drive my project and make the most of this opportunity. Also, thank you to Dr Niles Bokil for the technical introduction to kick off the whole project.

A big thank you to everyone who has been a part of the Saunders lab with me, with special thanks to Dr Jessica Pedersen and Giang Le. Your physical assistance and effervescent characters were the essential ingredients to get through arduous days in the PC3, toiling for hours through flow cytometry data, or carefully deciphering my most perplexing results. So much of this work would not have been accomplished without you all.

Thank you to Professor Warwick Britton and all the members of the mycobacteria group at the Centenary Institute. The technical expertise you've all shared has been amazing, and the opportunity to regularly present my work to you all has been invaluable. Presentation opportunities have been few and far between these 2 years, and I am very grateful. Your fresh perspectives and weekly discussion fostered my scientific thinking, which really made this thesis what it has finally become.

My parents, family, and friends have been a constant, not just through my PhD, but through all my work and education. Thank you so much for all your encouragement and support. To Matthew Hamlyn, thank you for your constant positivity, it is a very welcome light when things feel rough. To Dr Marley Pulbrook, thanks for your wit, humour, and encouragement as we've both been slogging through what feels like endless study.

And most importantly, thank you to my wife Emily. These years have been hard and I could not have done it without you. There were many months where you were literally the only person I saw, which is lucky because you are the best support I could wish for. Your love, friendship, and encouragement have kept me sane through long nights in the lab and prolonged weeks writing. Thank you so, so much; I love you!

Thesis format

This is a thesis by compilation, consisting of two published peer-reviewed papers, two results chapters, and a general discussion chapter.

Publications associated with this thesis

Stevens, M. T., B. D. Nagaria, W. J. Britton, and B. M. Saunders. 2021. Macrophages of different tissue origin exhibit distinct inflammatory responses to mycobacterial infection. *Immunol. Cell Biol.* 99: 1085-1092.

Stevens, M. T., and B. M. Saunders. 2021. Targets and regulation of microRNA-652-3p in homoeostasis and disease. *J. Mol. Med.* 99: 755-769.

Table of Contents

Acknowledgments.....	iii
Thesis format.....	v
Publications associated with this thesis.....	v
Table of Contents	vi
List of figures	xiii
List of tables	xvii
Abbreviations	xviii
Abstract	xx
Chapter 1. Targets and regulation of microRNA-652-3p in homeostasis and disease.	2
1.1. Preamble to Chapter 1	2
1.2. Chapter 1 – Declaration.....	5
1.3. Abstract	6
1.4. Introduction.....	6
1.5. Characteristics of miR-652	7
1.6. miR-652-3p in cardiovascular disease.....	8
1.7. miR-652-3p in cancer	12
1.7.1 Lung cancer	12
1.7.2 Breast cancer.....	14
1.7.3 Gastrointestinal cancers.....	15
1.7.4 Other cancers	17
1.8. miR-652-3p in mental illnesses and the central nervous system	19
1.9. miR-652-3p in other indications.....	21
1.10. miRNAs regularly associated with dysregulated miR-652-3p	23
1.11. Interspecies conservation of miR-652-3p and its validated target genes	25
1.12. Conclusion	27
1.13. Declarations.....	28

1.14. Acknowledgements	28
1.15. Online resources.....	29
Chapter 2. Macrophages of different tissue origin exhibit distinct inflammatory responses to mycobacterial infection	31
2.1. Chapter 2 – Declaration.....	31
2.2. Abstract	32
2.3. Introduction.....	33
2.4. Results	34
2.4.1 Macrophages from distinct origins retain control of mycobacterial growth 34	
2.4.2 iNOS activity is influenced by macrophage tissue origin	36
2.4.3 Increased proinflammatory cytokines expression in alveolar macrophages 36	
2.4.4 Surface phenotype of alveolar macrophages indicates stronger proinflammatory response to mycobacterial infection	37
2.5. Discussion	39
2.6. Methods	42
2.6.1 Cell culture	42
2.6.2 Bacterial cultures	42
2.6.3 Macrophage infections with mycobacteria	43
2.6.4 Cytometric bead array	43
2.6.5 Nitrite assay.....	43
2.6.6 Flow cytometry	43
2.6.7 Cell viability	44
2.6.8 Statistical analysis	44
2.7. Acknowledgements	44

Chapter 3. General materials and methods.....	46
3.1. Materials.....	46
3.1.1 General solutions	46
3.1.2 Bacterial culture media	46
3.1.3 Bacteria	47
3.1.4 Animals.....	47
3.1.5 Cell lines	48
3.1.6 Plasmids	48
3.1.7 miRNA mimics	50
3.1.8 Tissue culture media	50
3.1.9 Western blotting solutions.....	50
3.1.10 Western blot staining antibodies.....	51
3.1.11 Primer oligonucleotides for RT-qPCR.....	51
3.1.12 Flow cytometry staining antibodies.....	53
3.1.13 Flow cytometer	54
3.1.14 Reagents for protein isolation and processing	54
3.2. Methods	55
3.2.1 Mammalian cell line tissue culture	55
3.2.2 Bacterial culture	56
3.2.3 Enumeration of bacterial colony forming units	56
3.2.4 Tissue homogenisation to determine bacterial load	57
3.2.5 Histology.....	57
3.2.6 Tissue dissociation to single cells for flow cytometry.....	57
3.2.7 Flow Cytometry	58
3.2.7.1 Surface antigen staining	58
3.2.7.2 Intracellular staining	58

3.2.8	Resazurin metabolic activity assay.....	59
3.2.9	Cytometric bead array cytokine assay	59
3.2.10	Western blot for protein phosphorylation	60
3.2.10.1	Assessing protein concentration	60
3.2.10.2	Separation by gel electrophoresis	61
3.2.10.3	Western blot membrane transfer	61
3.2.10.4	Antibody staining and stripping.....	62
3.2.10.5	Western blot band quantitation	63
3.2.11	RNA purification	63
3.2.12	Determining mRNA expression by quantitative PCR	64
3.2.12.1	RNA purification and cDNA synthesis from mRNA template	64
3.2.12.2	Quantitative real-time quantitative polymerase chain reaction (RT-qPCR)	65
3.2.12.3	Relative expression calculation	65
3.2.13	Determining miRNA expression by quantitative PCR	66
3.2.13.1	cDNA synthesis from miRNA template	66
3.2.13.2	Quantitative RT-qPCR for miRNA expression	67
3.2.13.3	Relative miRNA expression calculation	67
3.2.14	Statistical analyses	68
Chapter 4.	The impact of miR-652 during mycobacterial infection	70
4.1.	Introduction.....	70
4.2.	Methods	72
4.2.1	Isolation and culture of primary mouse bone marrow cells.....	72
4.2.2	<i>In vitro</i> mycobacterial infection	72
4.2.3	<i>In vivo</i> mycobacterial infection	73
4.2.4	Flow cytometry gating	74

4.3. Results	77
4.3.1 <i>In vitro</i> cytokine expression by infected macrophages is impaired by miR-652 knockout.....	77
4.3.2 Inflammatory pathways are downregulated in infected miR-652 ^{-/-} macrophages.....	78
4.3.3 miR-652 knockout in macrophages does not impair control of mycobacterial growth	79
4.3.4 miR-652 ^{-/-} mice capably control <i>M. tuberculosis</i> bacterial load	83
4.3.5 Lung immune cell populations are not affected by miR-652 knockout....	87
4.4. Discussion	94
Chapter 5. The impact of miR-652 during <i>Listeria monocytogenes</i> infection	103
5.1. Introduction.....	103
5.2. Methods	105
5.2.1 Isolation and culture of primary mouse peritoneal macrophages	105
5.2.2 <i>In vitro</i> <i>L. monocytogenes</i> infection	105
5.2.3 <i>In vivo</i> <i>L. monocytogenes</i> infection.....	106
5.2.4 Flow cytometry gating	107
5.2.1 Proteomics analysis of protein expression	111
5.2.1.1 Protein isolation and alkylation	111
5.2.1.2 Protein digestion and clean-up.....	111
5.2.1.3 Protein normalisation by STAGE-tip desalting.....	111
5.2.1.4 LC/MS/MS	112
5.2.1.5 Data analysis	112
5.2.2 Mammalian cell transient transfection.....	113
5.2.3 Firefly luciferase luminescence assay	114
5.3. Results	115

5.3.1	miR-652 ^{-/-} mice are highly susceptible to <i>L. monocytogenes</i> infection..	115
5.3.2	Liver inflammation is increased in susceptible miR-652 ^{-/-} mice	120
5.3.3	miR-652 ^{-/-} macrophages capably control <i>L. monocytogenes</i> bacterial load <i>in vitro</i>	132
5.3.4	Proinflammatory pathways are downregulated in miR-652 ^{-/-} macrophages	134
5.3.5	miR-652 targeting of <i>Capzb</i> in mouse macrophages	140
5.4.	Discussion	143
Chapter 6.	General Discussion	152
6.1.	Tuberculosis and miR-652 as a prospective therapeutic	152
6.1.1	TB as a continuing problem	152
6.1.2	Targeting miR-652 as a host-directed therapy	153
6.1.3	Known miR-652 targets in TB therapy	154
6.1.3.1	ARRB1.....	154
6.1.3.2	KLF9.....	154
6.1.3.3	RORα	155
6.1.3.4	ZEB1	156
6.1.3.5	HOXA9.....	157
6.1.3.6	ENPP1.....	158
6.2.	Metabolism and antimicrobial defence	159
6.2.1	Deconvolution of metabolism and leukocyte action	159
6.2.2	Cell metabolism and tuberculosis.....	160
6.2.3	Metabolic targets as tuberculosis therapeutics.....	163
6.2.4	Host-microbe metabolic interactions – targets in the microbiome?	165
6.3.	Developing miRNA molecules for infectious disease treatment	166
6.3.1	Feasibility of miRNA as therapeutic targets.....	166

6.3.1.1	Population variability	167
6.3.1.2	Off-target effects	169
6.4.	Limitations	171
6.5.	Future Studies.....	172
6.6.	Conclusion	172
Chapter 7.	Bibliography	174
Appendix 1	232
Appendix 2	248
Appendix 3	259
Appendix 4	268
Appendix 5	274
Appendix 6	278

List of figures

Chapter 1

Figure 1.1. Regulation of cell polarity and Notch signalling by miR-652-3p.....	11
Figure 1.2. Human miRNAs reported dysregulated with hsa-miR-652-3p in lung cancer, breast cancer, and gastrointestinal cancers.	14
Figure 1.3. Human miRNAs reported dysregulated with hsa-miR-652-3p in cardiovascular disease, cancer, and mental health and central nervous system diseases.	21
Figure 1.4. mir-652-3p target sequences are conserved between humans and mice. .	27

Chapter 2

Figure 2.1.....	35
Figure 2.2.....	37
Figure 2.3.....	39

Chapter 3

Figure 3.1. The western blot transfer stack.	62
---	----

Chapter 4

Figure 4.1. Flow cytometry gating strategy for the analysis of myeloid lineage cells in tissue of <i>M. tuberculosis</i> -infected mice.	75
Figure 4.2. Flow cytometry gating strategy for the analysis of T cells in tissue from <i>M. tuberculosis</i> -infected mice.	76
Figure 4.3. Proinflammatory cytokine expression is reduced in miR-652 ^{-/-} macrophages after mycobacterial infection.....	78

Figure 4.4. Activation of the AKT-mTOR pathway is decreased in miR-652 ^{-/-} macrophages following mycobacterial infection.....	80
Figure 4.5. Notch receptor expression is not impaired in miR-652 ^{-/-} cells after mycobacterial infection.	81
Figure 4.6. Wild type and miR-652 ^{-/-} BMDMs control <i>M. tuberculosis</i> growth.	82
Figure 4.7. Wild type and miR-652 ^{-/-} macrophages are metabolically active 24 hours after mycobacterial infection.....	82
Figure 4.8. <i>In vivo Mycobacterium tuberculosis</i> infection in mice.....	85
Figure 4.9. Wild type and miR-652 ^{-/-} mice control <i>M. tuberculosis</i> bacterial load.....	85
Figure 4.10. Lung inflammation progresses in both wild type and miR-652 ^{-/-} mice following <i>M. tuberculosis</i> infection.....	86
Figure 4.11. Myeloid leukocyte populations are not affected by miR-652 expression in <i>M. tuberculosis</i> -infected mice.....	89
Figure 4.12. Lymphocyte populations are altered during chronic <i>M. tuberculosis</i> infection of miR-652 ^{-/-} mice.	90
Figure 4.13. miR-652 deficiency does not affect activation of effector T cell populations in <i>M. tuberculosis</i> -infected mice.....	91
Figure 4.14. Helper T cell populations are similar in wild type and miR-652 ^{-/-} mice during <i>M. tuberculosis</i> infection.	92
Figure 4.15. Memory cytotoxic T cell populations are decreased during acute <i>M. tuberculosis</i> infection of miR-652 ^{-/-} mice.....	93

Chapter 5

Figure 5.1. Flow cytometry gating strategy for analysis of T cell populations in the spleen of <i>L. monocytogenes</i> -infected mice.	108
Figure 5.2. Flow cytometry gating strategy for analysis of myeloid lineage cell populations in the spleen of <i>L. monocytogenes</i> -infected mice.....	109
Figure 5.3. Flow cytometry gating strategy for analysis of T cell cytokine expression, following intracellular staining of spleen cells from <i>L. monocytogenes</i> -infected mice.	110
Figure 5.4. <i>In vivo Listeria monocytogenes</i> infection in mice.	115
Figure 5.5. miR-652 ^{-/-} are mortally susceptible to <i>Listeria</i> infection.	117

Figure 5.6. Infection-induced weight loss is increased and prolonged in miR-652 ^{-/-} mice.	118
Figure 5.7. Bacterial growth was uncontrolled in moribund ET mice.	119
Figure 5.8. Cytokine expression is increased in miR-652 ^{-/-} mice and moribund ET mice from both groups.	122
Figure 5.9. Wild type mice contain <i>L. monocytogenes</i> in compact liver lesions.	123
Figure 5.10. <i>L. monocytogenes</i> induces large necrotic liver lesions in miR-652 ^{-/-} mice.	124
Figure 5.11. Spleen CD8 ⁺ T cell expansion was diminished in miR-652 ^{-/-} mice after <i>L. monocytogenes</i> infection.....	127
Figure 5.12. CD4 ⁺ effector T cell populations are elevated in miR-652 ^{-/-} mice early during <i>L. monocytogenes</i> infection.....	128
Figure 5.13. Activation marker expression is similar on wild type and miR-652 ^{-/-} effector T cells during <i>L. monocytogenes</i> infection.....	129
Figure 5.14. Cytokine-expressing T cell populations are similar in <i>L. monocytogenes</i> -infected wild type and miR-652 ^{-/-} mice.....	130
Figure 5.15. Single, double- and triple-positive cytokine expressing T cells are comparable between wild type and miR-652 ^{-/-} mice.	131
Figure 5.16. miR-652 expression does not affect mouse macrophage metabolic activity or control of <i>Listeria</i> infection.	133
Figure 5.17. TNF expression is not suppressed in <i>Listeria</i> -infected miR-652 ^{-/-} macrophages.....	133
Figure 5.18. Protein expression patterns are altered by miR-652 knock-out.	135
Figure 5.19. Essential cellular pathways downregulated in miR-652 ^{-/-} macrophages..	135
Figure 5.20. STRING network shows interacting pathways with known anti-bacterial activity were dysregulated in miR-652 ^{-/-} macrophages.	137
Figure 5.21. Gene expression trends infected macrophages correlated with differential protein expression.	139
Figure 5.22. miR-652 overexpression did not impact <i>Capzb</i> transcription.	141
Figure 5.23. miR-652 overexpression did not cause decreased translation of CAPZB protein.....	141
Figure 5.24. miR-652 does not target to predicted site in the <i>Capzb</i> 3'UTR.	142

Appendices

Figure A 1. pIS0 luciferase expression plasmid map.....	275
Figure A 2. pIS0-Capzb-WT luciferase reporter plasmid map.....	276
Figure A 3. pIS0-Capzb-mut luciferase reporter plasmid map.....	277
Figure A 4. A small minority of mycobacteria are removed in culture supernatant, leaving the majority internalised by macrophages, or adherent to the assay plate....	279
Figure A 5. Internalised mycobacteria have small effect on resazurin metabolic activity assay fluorescence readout 24 hours post-infection.....	280

List of tables

Chapter 3

Table 3.1. Oligonucleotide gene fragments for insertion into pISO.....	49
Table 3.2. Antibodies used in western blot staining procedure.	51
Table 3.3. Primers sequences used in mRNA qPCR reactions.	52
Table 3.4. Forward primer sequences used in miRNA qPCR reactions.	52
Table 3.5. Fluorescent antibodies for flow cytometry staining.	53
Table 3.6. Fortessa X20 lasers and detectors.....	54
Table 3.7. cDNA synthesis reaction for messenger RNA template.....	64
Table 3.8. mRNA qPCR reaction reagent volumes.	65
Table 3.9. cDNA synthesis reaction for microRNA template.	66
Table 3.10. miRNA qPCR reaction reagent volumes.	67

Chapter 5

Table 5.1. Euthanasia times for <i>L. monocytogenes</i> -infected mice.	118
Table 5.2. KEGG pathways enriched in differentially expressed proteins from <i>Listeria</i> -infected peritoneal macrophages.	138

Chapter 6

Table 6.1. Single nucleotide polymorphisms identified in the miR-652 binding sequence of target genes.	168
---	-----

Abbreviations

3'UTR	3' untranslated region
ADC	Albumin dextrose catalase
ANOVA	Analysis of variance
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CBA	Cytometric bead array
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
cGAMP	Cyclic GMP-AMP
CNS	Central nervous system
CVD	Cardiovascular disease
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ET	Ethical threshold
FBS	Foetal bovine serum
LB	Lysogeny broth
LPS	Lipopolysaccharide
MDR TB	Multidrug-resistant
miRNA	microRNA
MLN	Mediastinal lymph node
MOI	Multiplicity of infection
NK cell	Natural killer cell
NP	Nanoparticle
OADC	Oleic acid albumin dextrose catalase
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC3	Physical containment level 3
PEG	Polyethylene glycol
PEI	Polyethylenimine
Pre-miR	Pre-microRNA
Pri-miR	Primary microRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROC curve	Receiver operating characteristic curve
RT-qPCR	Real time quantitative polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
TBST	Tris-buffered saline Tween 20
TCA cycle	Tricarboxylic acid cycle
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TLR	Toll-like receptor
TP	Time point

Abstract

Tuberculosis (TB) is an infectious respiratory disease caused by the bacterial pathogen *Mycobacterium tuberculosis*. Each year, 1.5 million deaths are attributable to TB and survivors are prone to increased all-cause mortality, due to excessive TB-associated pulmonary inflammation. Recent literature indicated the microRNA hsa-miR-652-3p (miR-652) was downregulated in plasma of Chinese TB patients, and further decreased in patients who failed to clear the bacteria after antibiotic therapy. This thesis investigated the activities of miR-652 during *in vitro* and *in vivo* infections with intracellular bacterial pathogens, with a special focus on the macrophage response to infection.

My initial study aimed to characterise the phenotypic differences between murine alveolar (AMJ2-C11) and peritoneal (IC-21) macrophage cell lines during *in vitro* mycobacterial infections, in order to illustrate the influence of tissue origin on macrophage function. Both cell lines were able to control *M. bovis* BCG and *M. tuberculosis* H37Rv bacterial loads. However, AMJ2-C11 cells exhibited a more inflammatory phenotype, with significantly increased cytokine release and nitric oxide generation. Additionally, expression of inflammatory cell surface markers was increased on AMJ2-C11 cells relative to IC-21 cells. These data suggest that whilst tissue origin can influence macrophage phenotype, cell plasticity ensures diverse macrophages can respond to invading pathogens.

Chapter 4 investigated the impact of miR-652 on the murine immune response to *M. tuberculosis*. Bone marrow macrophages from miR-652^{-/-} C57BL/6 mice were able to control bacterial growth over 6 days *in vitro*, though IL-6, TNF, MIP-1 α , and KC expression was significantly lower than in their wild type counterparts. Western blot results indicated AKT and mTOR activation was attenuated in miR-652^{-/-} macrophages. miR-652^{-/-} mice infected aerogenically with *M. tuberculosis* were able to control the bacterial load in the lungs and spleen equal to wild type mice over 13 weeks. Leukocyte populations were comparable between mouse strains, however, early CD8⁺ effector T cell numbers were elevated in the lung and lymph node miR-652^{-/-} mice, suggesting miR-652 may have some impact on T cell differentiation during bacterial infection.

Chapter 5 investigated this question in a CD8⁺ T cell-focused infection model; intraperitoneal *Listeria monocytogenes* infection. miR-652^{-/-} mice were highly susceptible to a low-dose infection of 2000 CFU/mouse, exhibiting significantly increased weight loss and high morbidity. The early onset of morbidity indicated a deficiency in the innate immune response. Highly necrotic liver lesions in miR-652^{-/-} mice displayed intense recruitment of neutrophils and macrophages, but bacterial load was uncontrolled in these mice. To investigate the antimicrobial phenotype of miR-652^{-/-} macrophages, primary peritoneal macrophages were infected with *L. monocytogenes in vitro*. A proteomic analysis highlighted dysregulation of key immune pathways, including the lysosome pathway and the pentose phosphate pathway. Also downregulated was the *in silico*-predicted miR-652 target CAPZB. Transfection experiments using luciferase reporter constructs indicated miR-652 does not target a predicted sequence in the CAPZB 3'UTR. Further, CAPZB mRNA and protein were unaffected by transfection with a miR-652 mimic in IC-21 mouse peritoneal macrophage cells, indicating CAPZB expression is unaffected by miR-652.

This thesis demonstrates miR-652 plays clear roles in the proper innate immune response to acute infection with an intracellular bacterial pathogen. The pathways impacted in miR-652^{-/-} macrophages position miR-652 as an important regulator of immune function, potentially regulating inflammation and cell metabolism. Host-directed therapies possess amazing potential as a complement to existing antimicrobial drugs. microRNA-based therapeutics for infectious diseases are progressing well through clinical trials. Analysis of the genes validated as targets for miR-652 underscores the promise for a miR-652 mimic as a therapeutic in chronic TB, particular when administered with a cell-targeted delivery mechanism. Additional holistic research is needed to evaluate the impacts of miR-652 in macrophages to realise the potential of miR-652 as a therapeutic miRNA.