

***Mycobacterium tuberculosis* attenuates
Macrophage responses through
Pentose Phosphate Pathway activity**



A dissertation submitted to Trinity College Dublin in
candidature for the degree of Doctor of Philosophy

School of Biochemistry and Immunology,
Trinity Biomedical Sciences Institute,
Trinity College Dublin, the University of Dublin

2025

By John McGrath

Supervisor: Dr Frederick Sheedy, PhD

Declaration

I, John McGrath, declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so in my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Signed:

Date:

X_____

John McGrath

For my Mam and Dad

Summary

This thesis investigates the metabolic interplay between *Mycobacterium tuberculosis* (Mtb) and host macrophages, with a focus on the role of the pentose phosphate pathway (PPP) in modulating immune responses during infection. Mtb, the world's most successful pathogen, is known for its ability to evade host immune defences and establish a chronic infection within macrophages. Central to its survival strategy is the manipulation of host metabolism, which we show involves rerouting central carbon metabolism towards the induction of PPP activity, thereby creating a metabolic environment that promotes bacterial replication.

The findings of this work demonstrates that during infection, Mtb suppresses glycolytic activity in host macrophages through the upregulation of anti-inflammatory microRNA miR-21. This leads to reduced production of the chief antimycobacterial pro-inflammatory cytokine, IL-1 β . Building on this, we demonstrate that Mtb's attenuation of glycolysis results in the re-routing of central carbon metabolism towards the upregulation of PPP activity. This shift in metabolism results in a net loss of carbon through glycolysis and reinforces a permissive intracellular environment for Mtb replication.

To further understand the impact of PPP modulation on macrophage responses, we induced two alternative states of PPP activity: pentose insufficiency and pentose overflow. Our findings show that specifically during infection with viable Mtb, sufficient PPP activity is required to support macrophage pro-inflammatory responses, particularly the secretion of IL-1 β , TNF α , and IL-6 – a dependency not observed in cells stimulated with inactivated Mtb components. Pentose insufficiency, induced by the inhibitor 6-AN, dramatically reduced the pro-inflammatory cytokine profile of infected macrophages and linked glycolytic activity, while pentose overflow (achieved through miR-21 depletion) enhanced IL-1 β secretion, glycolytic responses and promoted bacterial clearance. These results indicate that in the face of repression of upper glycolysis by Mtb, the macrophage relies on PPP to support glycolysis and associated immunometabolic responses. Despite this, Mtb infection upregulates this pathway

further, suggesting PPP activity may also play a role in evasion of host metabolic responses.

Building on this, we examined the production of PPP-dependent signal, reactive nitrogen intermediates (RNI)/ NO. We found that prolonged exposure to elevated NO levels, reflective of chronic infection, actually enhances intracellular Mtb replication, by creating a positive feedback loop between PPP activity and NO generation. This heightened PPP activity is preferentially fuelled by glycogen breakdown rather than glucose uptake, leading to a decelerated bioenergetic state in the macrophages, that favors bacterial survival. Our study also reveals that NO accumulation during Mtb infection suppresses mitochondrial metabolism, including oxidative phosphorylation (OXPHOS), by inhibiting key enzymes in the tricarboxylic acid (TCA) cycle. This suppression of mitochondrial function is further exacerbated by the accumulation of itaconate, a metabolite that inhibits glycolysis and reduces IL-1 β production, promoting bacterial replication.

Importantly, we show that targeting PPP activity offers a potential therapeutic strategy for enhancing macrophage-mediated killing of Mtb. By inhibiting NADPH oxidase (Nox2), which regenerates the PPP cofactor NADP, we were able to disrupt Mtb-mediated metabolic reprogramming and restore mitochondrial polarization and OXPHOS activity. This shift in metabolism led to increased IL-1 β production and enhanced intracellular bacterial clearance, highlighting the critical role of mitochondrial metabolism in driving macrophage immune responses.

In summary, this work demonstrates that Mtb manipulates host macrophage metabolism by inducing PPP activity, and although this may initially play a host defence role, by upregulating flux through this pathway, Mtb creates an immunosuppressive environment that promotes its intracellular survival. Targeting PPP metabolism therefore represents a promising strategy for boosting host immunity and enhancing macrophage killing capacity against Mtb. These findings provide new insights into the metabolic strategies employed by Mtb to evade immune responses and suggest novel therapeutic approaches for combating tuberculosis.

Acknowledgements

The completion of my PhD journey would not have been possible without the support, guidance, and encouragement of many individuals to whom I am deeply grateful.

First and foremost, I would like to express my sincere gratitude to my supervisor Fred. Your dedication to science, keen insights, and unwavering support have not only shaped this project but also inspired me to grow as a scientist. Thank you for creating an environment that fosters curiosity, kindness and above all else, Taylor Swift puns. I am incredibly fortunate to have had you as a mentor and have valued every moment working in your lab. Your passion for science and the future of the Sheedy Lab truly knows “NO” bounds.

To all the past and present members of the Sheedy lab.

Anna – It simply cannot be put into words how fortunate I feel that we started and finished our PhD journeys together. It’s impossible to write in such a short paragraph how grateful I am for your friendship over the past four years. From grade A “yapper” coffee breaks to maratHon *in vivo* days, your constAnt encouraGement, and shared laughter is something I’ll cherish forever. As a wise individual once said.....name a more iconic duo....

CIANterferon – From the moment you joined the lab you’ve been nothing but a joy to be around....maybe a little less so when Liverpool or Sligo Rovers fall short. Your love of science (maybe it’s just IFNs?) and research is something I admire on a daily basis and I have no doubt that you’ve a bright future ahead of you. Thank you for being an amazing friend. Keep being Ween.

Stephen, Jamie M, Mimmi – You’ve all made the lab the most enjoyable place to work. Jamie, thank you for always making sure I wasn’t skiving when supposed to be thesis writing, trekking across the city to the blood bank and most importantly, being the sole pioneer in bringing Sambas back into fashion...it was all you babe. Stephen, thank you

for bringing the laughter to every *in vivo* day. I'm so grateful for your friendship and hope you continue to spread the important word on the regenerative capacity of the liver. Mimmi, you've been so great to work with. Thank you for all your words of wisdom over the years and helping me to troubleshoot when experiments didn't seem to going to be going to plan.

To past FJS members Hannah, Sarah, Emer, Tara and Hugo – You've all made this PhD one of the most enjoyable experiences. Hannah, you've been such a great friend. You're truly a fantastic scientist and I'm constantly blown away by your talent. Sarah, I don't think I've ever met someone more like me in my life. You can read my mind at the drop of a hat and I'll be forever grateful for all your advice over the years, thank you. Emer, my lab mom, thank you for teaching me everything I know in the lab. It definitely wasn't an easy job but I couldn't of asked for a better mentor and friend to teach me the ways of TB. Tara – thank you for being a fantastic friend and for always making my day with three simple words, "Oat flat white?". Hugo – Thank you for always being so kind and going above and beyond to help whenever you could. I miss the scarf.

To the past and present O'Farrelly lab members

Prof Cliona O'Farrelly – Thank you for always being so welcoming, for your keen insights into this project and for your advice throughout my PhD. I'm extremely grateful to have had the opportunity to learn from you and your amazing lab members, Mihai, Fede, Jamie S, Marie, Andrea and Valeria.

Mihai, I don't think I've ever met someone more enthusiastic about science. Your work ethic is something I truly admire. I really appreciate you always taking the time to talk through experiments and discuss results, especially when I hadn't a clue what was happening. Fede, it's been a pleasure to work alongside you. Thank you for always being a breath of fresh air and making me cry in laughter on an daily basis. Jamie, saying the walking pace on the fourth floor since you left has been abysmal would be an understatement.

Nik and Selina – the two best friends a gal could ask for. This thesis simply wouldn't exist if it wasn't for the both of you. Through thick and thin, I can always count on you two—not just to go above and beyond for me, but to drop some killer jokes while you're at it. And Selina, after 20 years of friendship it breaks my heart to tell you....Nik once told me she doesn't like your cooking. Love you both.

Clodagh and Hilary – From the moment we met you've been the two most welcoming people...to say I'll never forget our covid lockdown days is an understatement. Thank you for being a constant source of support throughout this PhD and making my move to Dublin the most enjoyable and fun filled time.

Peter – You're the most amazing partner and I'm so lucky to have you by my side. Your unwavering support, love, and sense of humour have kept me going. Thank you for everything you do for me. I'm so excited to start this next chapter with you, filled with new memories, trips and who knows – maybe even a sea swim or two now that I don't have the excuse that I'm too busy. I look forward to what the future holds and facing any challenges life may throw our way together. I love you.

To my amazing family. Mam, Dad, Pat, Amy....and of course Paddy. You've always been there for me no matter what. Mam and Dad, thank you for always teaching me to value my education. You've been the most amazing role models and have shown me the importance of hard work, perseverance, and kindness. Pat and Amy, your unique ability to annoy and support me simultaneously is truly an art form. Sarcasm aside, thank you all so much, I really appreciate everything you have done and continue to do for me. I love you all and I am very lucky to have you.

Publications

Recognition of yeast β -glucan particles triggers immunometabolic signalling required for trained immunity

Cian J H Horneck Johnston, Anna E Ledwith, Mimmi L Lundahl, Hugo Charles-Messance, Emer Hackett, Simon O'Shaughnessy, Jonah Clegg, Hannah Prendeville, **John P McGrath**, Aaron M Walsh, Sarah Case, Hollie Austen Byrne, Parth Gautam, Elaine Dempsey, Sinead C Corr, Frederick J Sheedy

iScience, 2024 (DOI: 10.1016/j.isci.2024.109030)

Table of Contents

Declaration	iii
Summary	v
Acknowledgements	viii
Publications	xi
List of Figures.....	xvi
List of Tables	xxii
Abbreviations.....	xxiv
Abstract	xxix
Chapter 1. Introduction	1
1.1 <i>Mycobacterium tuberculosis</i> and the Immune Response.....	2
1.1.1 Heterogeneity in human response to Mtb infection	3
1.1.2 Macrophages	5
1.1.3 Macrophage heterogeneity during Mtb infection	8
1.1.4 Macrophage recognition of Mtb.....	12
1.1.5 Innate cytokines during Mtb infection.....	14
1.1.6 Reactive oxygen and nitrogen species during Mtb infection	17
1.1.7 The phagosome during Mtb infection	21
1.1.8 Activation of the adaptive response during Mtb infection	22
1.2 Macrophage metabolism during Mtb infection	26
1.2.1 Glycolysis in Mtb infected macrophages	26
1.2.2 Glycogen metabolism	28
1.2.3 The Pentose Phosphate Pathway.....	29
1.2.4 Macrophage pentose phosphate pathway activity.....	31
1.2.5 Macrophage The TCA cycle.....	34
1.3 Nitric oxide and macrophage metabolism	36
1.3.1 Macrophage Nitric oxide generation	36
1.3.2 Nitric oxide and immunometabolism	40
1.3.3 The impact of NO on inflammatory cytokine production	44
1.3.4 NO and human macrophages	44
1.4 Thesis Aims	47
1.4.1 Specific Objectives:	47
Chapter 2. Materials and Methods	49

2.1	Materials.....	50
2.1.1	Chemicals and reagents	50
2.1.2	Molecular Assays and Kits.....	52
2.1.3	Equipment	53
2.1.4	Animals	54
2.1.5	Cell lines.....	54
2.1.6	Cell culture medium	54
2.1.6.1	Bone-marrow derived macrophages culture medium.....	54
2.1.6.2	Human monocyte-derived macrophages culture medium.....	55
2.1.6.3	L-929 cell culture medium	55
2.1.7	Cell culture stimulators/ inhibitors	55
2.1.8	Buffers.....	56
2.1.9	Flow cytometry antibodies / stains.....	56
2.1.9.1	Flow cytometry antibodies	56
2.1.9.2	Flow cytometry stains.....	56
2.1.10	PCR primers	57
2.2	Methods	58
2.2.1	Murine cell culture	58
2.2.1.1	Bone-marrow derived macrophages	58
2.2.1.2	BMDM purity assessment.....	59
2.2.1.4	Isolation and ex vivo culture of murine alveolar macrophages	61
2.2.1.5	Generation of L929-conditioned medium (LCM)	61
2.2.2	Infection: <i>Mycobacterium tuberculosis</i>	61
2.2.2.1	Culture of <i>Mycobacterium tuberculosis</i>	61
2.2.2.2	Preparation of Middlebrook 7H9 broth	62
2.2.2.3	Preparation of Middlebrook 7H11 agar	62
2.2.2.4	<i>Mycobacterial tuberculosis</i> bacterial growth assay	63
2.2.3	Benchtop sample analysis: RNA.....	64
2.2.3.1	Total RNA isolation from cells	64
2.2.3.2	RNA quantification/ quality assessment.....	65
2.2.3.3	Reverse transcription of mRNA to cDNA	65
2.2.3.4	Reverse transcription of microRNA to cDNA	66
2.2.3.5	Real-Time PCR.....	67
2.2.4	Benchtop sample analysis: Protein, Metabolite and Molecule assays ..	68

2.2.4.1 ELISA	68
2.2.4.2 Glucose-6-Phosphate Dehydrogenase activity quantification.....	69
2.2.4.3 Lactate quantification	70
2.2.4.4 Quantification of 6-phosphogluconic acid (6-PGA).....	71
2.2.4.5 NADP/NADPH quantification	72
2.2.4.6 Nitrite quantification	73
2.2.4.7 Glucose quantification	74
2.2.5 Metabolic phenotyping: Seahorse XF analyser	74
2.2.5.1 Cartridge hydration.....	74
2.2.5.2 Cellular metabolic phenotyping.....	74
2.2.6 Flow cytometry analysis	75
2.2.6.1 LysoTracker Red (LTR) staining.....	75
2.2.6.2 Mitotracker Green (MTG) / TMRM co-staining.....	76
2.2.6.3 LipidTOX green (LTG) staining	76
2.2.6.4 Live dead staining	76
2.2.6.5 Extracellular staining.....	76
Chapter 3. <i>Mycobacterium tuberculosis</i> infection attenuates macrophage central carbon metabolism, whilst promoting Pentose Phosphate Pathway activity.....	79
3.1 Introduction	80
3.2 Results	82
3.2.1 <i>Mycobacterium tuberculosis</i> attenuates macrophage pro-inflammatory cytokine secretion	82
3.2.2 <i>Mycobacterium tuberculosis</i> attenuates macrophage glycolytic reprogramming.....	87
3.2.3 <i>Mycobacterium tuberculosis</i> promotes pentose phosphate pathway activity during infection of macrophages	94
3.2.4 Pentose phosphate pathway activity supports macrophage immunometabolic responses	102
3.2.5 Pentose overflow promotes macrophage antimycobacterial function	119
3.3 Discussion	127
Chapter 4. Pentose Phosphate Pathway derived NO orchestrates macrophage immunometabolic responses	131
4.1 Introduction	132
4.2 Results	135
4.2.1 <i>Mycobacterium tuberculosis</i> promotes PPP derived NO generation...	135
4.2.2 Nitric oxide promotes macrophage PPP activity during Mtb infection	143

4.2.3	Nitric oxide generates an immuno-permissive environment for Mtb replication.....	156
4.2.4	Nitric oxide targets TCA cycle metabolism during Mtb infection of macrophages	161
4.2.5	Itaconate promotes metabolic phenotype associated with increased Mtb burden in macrophages	174
4.2.6	Inhibition of Succinate Dehydrogenase replicates metabolic phenotype associated with itaconate accumulation during Mtb infection	190
4.2.7	NOX2 inhibition promotes macrophage clearance of Mtb by disrupting PPP activity and in turn enhancing mitochondrial metabolism.....	196
4.3	Discussion	206
Chapter 5.	Final Discussion	211
Chapter 6.	Bibliography.....	223

List of Figures

Chapter 1. introduction

Figure 1. 1 Active and latent Mtb infection	7
Figure 1. 2 Major reactive oxygen species (ROS) generation pathways in macrophages	20
Figure 1. 3 The cellular immune response to Mtb	25
Figure 1. 4 Glycolysis and the Pentose Phosphate Pathway	33
Figure 1. 5 S-nitrosylation and peroxynitrite reaction pathways	39
Figure 1. 6 Nitric Oxide and classical activation of Macrophages	46

Chapter 2. Materials and Methods

Figure 2. 1 Sample gating strategy for assessing BMDM purity	60
---	----

Chapter 3. *Mycobacterium tuberculosis* attenuates macrophage central carbon metabolism, whilst promoting pentose phosphate pathway activity

Figure 3. 1 Mtb attenuates IL-1 β secretion during infection of murine and human macrophages	84
Figure 3. 2 Mtb attenuates early TNF α secretion during infection of murine and human macrophages	85
Figure 3. 3 Mtb attenuates early IL-6 secretion during infection of murine BMDMs	86
Figure 3. 4 Mtb infection attenuates lactate production in murine and human macrophages	89
Figure 3. 5 Mtb limits host glycolysis and IL1 β by restriction of PFK-M via miR-21	90
Figure 3. 6 Mtb promotes expression of miR-21 in human macrophages	91
Figure 3. 7 Mtb reduces lower glycolytic-associated gene expression in human macrophages	92
Figure 3. 8 Mtb infection reduces glycolytic rates in macrophages	93

Figure 3. 9 Mtb promotes expression of oxidative and non-oxidative PPP associated enzymes in murine BMDMs	96
Figure 3. 10 Mtb promotes expression of oxidative and non-oxidative PPP associated enzymes in human MDMs	97
Figure 3. 11 Mtb promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages.....	98
Figure 3. 12 Mtb infection results in heightened PPP metabolite accumulation in macrophages	99
Figure 3. 13 Mtb infection results in heightened NADP/H generation in macrophages	100
Figure 3. 14 Mtb infection results in heightened neutral lipid accumulation in macrophages	101
Figure 3. 15 6-AN inhibition decreases G6PD activity in macrophages	105
Figure 3. 16 PPP inhibition decreases IL-1 β expression & secretion during infection of murine BMDMs	106
Figure 3. 17 PPP inhibition decreases IL-1 β expression & secretion during infection of human MDMs.....	107
Figure 3. 18 PPP inhibition decreases TNF α expression & secretion during infection of murine BMDMs	108
Figure 3. 19 PPP inhibition decreases TNF α secretion during infection of human MDMs	109
Figure 3. 20 PPP inhibition decreases IL-1 β expression specifically during viable Mtb infection.....	110
Figure 3. 21 Alternative sources of glucose-6-phosphate promotes macrophage pro-inflammatory cytokine responses during viable Mtb infection	111
Figure 3. 22 PPP inhibition decreases lactate secretion during Mtb infection	112
Figure 3. 23 6-AN inhibition decreases lower glycolytic associated gene expression during Mtb infection of macrophages	113
Figure 3. 24 6-AN inhibition increases non-OxPPP associated gene expression during Mtb infection of macrophages	114
Figure 3. 25 PPP inhibition reduces glycolytic rates of murine and human macrophage	115

Figure 3. 26 PPP inhibition reduces maximum respiratory rates of murine and human macrophage OXPHOS	116
Figure 3. 27 PPP inhibition reduces NADPH in macrophages.....	117
Figure 3. 28 PPP inhibition promotes Mtb growth in macrophages.....	118
Figure 3. 29 Loss of miR-21 increases expression of PPP associated genes	121
Figure 3. 30 Loss of miR-21 increases G6PD enzyme activity.....	122
Figure 3. 31 Loss of miR-21 increases lactate secretion during Mtb infection	123
Figure 3. 32 Loss of miR-21 significantly increases IL-1 β and TNF α secretion during Mtb infection of macrophages	124
Figure 3. 33 PPP inhibition blocks heightened glycolytic activity in miR-21 ^{-/-} macrophages	125
Figure 3. 34 Loss of miR-21 significantly increases bacterial killing and is overcome by PPP inhibition	126
Figure 3. 35 PPP modes of action	130

Chapter 4. Pentose phosphate pathway derived NO orchestrates macrophage immunometabolic responses

Figure 4. 1 Mtb promotes NO accumulation in murine and human macrophages	137
Figure 4. 2 PPP inhibition decreases NO accumulation in macrophages.....	138
Figure 4. 3 Glycogenolysis supports NO production in macrophages	139
Figure 4. 4 Mtb infection promotes glycogenolysis.....	140
Figure 4. 5 Glycogen supports macrophage central carbon metabolism	141
Figure 4. 6 Loss of miR-21 increases NO during Mtb infection.....	142
Figure 4. 7 NO supplementation increases cellular nitrite in murine and human macrophages during infection	145
Figure 4. 8 NO promotes glycolytic activity in uninfected macrophages.....	146
Figure 4. 9 NO increases glycolytic capacity of uninfected murine and human macrophages	147

Figure 4. 10 NO increases lactate secretion in murine and human macrophages during Mtb infection	148
Figure 4. 11 NO does not alter glycolytic gene expression during Mtb infection.....	149
Figure 4. 12 NO promotes glycogenolysis as preferential carbon source during Mtb infection.....	150
Figure 4. 13 NO promotes PPP activity in uninfected macrophages	151
Figure 4. 14 NO increases PPP gene expression in murine macrophages during Mtb infection.....	152
Figure 4. 15 NO increases PPP gene expression in human macrophages during Mtb infection.....	153
Figure 4. 16 NO promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages during Mtb infection.....	154
Figure 4. 17 NO increases NADP:NADPH during Mtb infection.....	155
Figure 4. 18 NO attenuates IL-1 β secretion during Mtb infection of macrophages	158
Figure 4. 19 NO attenuates TNF α secretion during Mtb infection of macrophages ...	159
Figure 4. 20 NO promotes Mtb survival in murine and human macrophages	160
Figure 4. 21 Mtb promotes transcriptional signature associated with TCA cycle break	164
Figure 4. 22 Mtb reduces basal and maximum respiratory rates of macrophage oxphos	165
Figure 4. 23 NO alters maximum respiratory capacity of uninfected human macrophage oxphos	166
Figure 4. 24 NO promotes transcriptional signature in line with disruption to TCA cycling during Mtb infection.....	167
Figure 4. 25 NO sequestration alters TCA and PPP associated gene expression in murine macrophages during Mtb infection	168
Figure 4. 26 NO sequestration promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages	169
Figure 4. 27 NO sequestration decreases lactate secretion in murine and human macrophages during Mtb infection	170
Figure 4. 28 NO sequestration decreases IL-1 β in murine and human macrophages during Mtb infection.....	171

Figure 4. 29 NO sequestration decreases TNF α in murine and human macrophages during Mtb infection.....	172
Figure 4. 30 NO sequestration promotes Mtb survival in murine and human macrophages	173
Figure 4. 31 Itaconate decreases IL-1 β in murine and human macrophages during Mtb infection.....	177
Figure 4. 32 Itaconate decreases TNF α secretion during Mtb infection	178
Figure 4. 33 Itaconate promotes PPP-associated gene expression in murine BMDMs	179
Figure 4. 34 Itaconate promotes PPP-associated gene expression in human MDMs..	180
Figure 4. 35 Itaconate promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages.....	181
Figure 4. 36 Itaconate increases NADP:NADPH.....	182
Figure 4. 37 Itaconate promotes preferential use of glycogen over glucose during Mtb infection of macrophages	183
Figure 4. 38 Itaconate decreases nitrite accumulation	184
Figure 4. 39 Itaconate decreases glycolytic rate of macrophages during Mtb infection	185
Figure 4. 40 Itaconate promotes Mtb survival in murine and human macrophages ..	186
Figure 4. 41 Itaconate promotes Mtb survival in macrophages	187
Figure 4. 42 Itaconate alters macrophage central carbon metabolism and NO accumulation	188
Figure 4. 43 Itaconate accumulation promotes macrophage metabolic reprogramming	189
Figure 4. 44 SDH inhibition promotes macrophage PPP activity	191
Figure 4. 45 SDH inhibition decreases lactate accumulation in Mtb infected macrophages	192
Figure 4. 46 SDH inhibition decreases nitrite accumulation during Mtb infection of macrophages	193
Figure 4. 47 SDH inhibition decreases IL-1 β secretion during Mtb infection	194
Figure 4. 48 SDH inhibition promotes Mtb survival in murine and human macrophages	195

Figure 4. 49 NOX2 inhibition decreases lysosomal acidification of macrophages.....	198
Figure 4. 50 NOX2 inhibition decreases lactate secretion in Mtb infected macrophages	199
Figure 4. 51 NOX2 inhibition decreases glycolytic rates of murine and human macrophages	200
Figure 4. 52 NOX2 inhibition decreases activity of PPP rate-limiting enzyme G6PD during Mtb infection of macrophages	201
Figure 4. 53 NOX2 inhibition decreases nitrite accumulation during Mtb infection of macrophages	202
Figure 4. 54 NOX2 inhibition increases macrophage basal oxphos and maximum respiratory capacity	203
Figure 4. 55 NOX2 inhibition increases mitochondrial polarisation	204
Figure 4. 56 NOX2 inhibition increases macrophage Mtb clearance.....	205

Chapter 5. Final Discussion

Figure 5. 1 Mtb orchestrates central carbon metabolism by manipulating PPP-derived NO production	222
---	-----

List of Tables

Chapter 2. Materials and Methods

Table 2. 1	List of cell culture reagents and suppliers	50
Table 2. 2	List of infection reagents and suppliers.....	51
Table 2. 3	List of cell culture stimulants / inhibitors and suppliers.....	51
Table 2. 4	List of benchtop reagents and suppliers	52
Table 2. 5	List of assay/ kits and suppliers	52
Table 2. 6	List and concentrations of cell culture stimulants and inhibitors.....	55
Table 2. 7	List and concentrations of buffers	56
Table 2. 8	List of flow cytometry antibodies.....	56
Table 2. 9	List of flow cytometry stains	56
Table 2. 10	List of TaqMan real-time PCR gene expression assays	58
Table 2. 11	Controls and Samples for BMDM purity assessment.....	59
Table 2. 12	Components and volumes required for 100 ml of Middlebrook 7H9 broth	62
Table 2. 13	Components and volumes required for 100 ml of Middlebrook 7H11 agar	63
Table 2. 14	Components and volumes required for mRNA reverse transcription mix ..	66
Table 2. 15	Reverse transcription thermocycle program (mRNA to cDNA)	66
Table 2. 16	Components and volumes required for microRNA reverse transcription mix	67
Table 2. 17	Reverse transcription thermocycle program (microRNA to cDNA)	67
Table 2. 18	Components and volumes required for real-time PCR assay mix	68
Table 2. 19	Components and volumes required for G6PD assay master mix	70
Table 2. 20	Components and volumes required for Lactate assay master mix	71
Table 2. 21	Components and volumes required for 6-PGA assay master mix	72

Table 2. 22	Components and volumes required for NADP/NADPH assay master mix...	73
Table 2. 23	List and concentration of inhibitor used in Seahorse Analysis	75

Abbreviations

2-DG	2-deoxy-D-glucose
6-AN	6-aminonicotinamide
6-PGA	6-phosphogluconic acid
6PGD	6-phosphogluconate dehydrogenase
AA	Antimycin-A
ACO2	Aconitase decarboxylase 2
AM	Alveolar macrophage
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BCKDH	Branched-chain α -ketoacid dehydrogenase complex
BH4	Tetrahydrobiopterin
BMDM	Bone marrow-derived macrophage
CARD	C-terminal caspase recruitment domain
cDNA	Complementary DNA
CFU	Colony forming unit
CGD	Chronic granulomatous disease
cGMP	Cyclic guanosine monophosphate
CO ₂	Carbon dioxide
cPTIO	Carboxy-PTIO, potassium salt
Ct	Cycle threshold
DC	Dendritic cell
DetaNO	Deta NONOate
DMEM	Dulbecco's modified eagle's medium
DMM	Dimethyl malonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates

ECAR	Extracellular acidification rate
EEA1	Early endosomal autoantigen 1
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
F2,6BP	Fructose-2,6-biphosphate
F6P	Fructose-6-phosphate
FBP	Fructose 1,6-biphosphatase
FBS	Foetal bovine serum
FCCP	Fluoro-carbonyl cyanide phenylhydrazine
FH1	Fumarate hydratase-1
FPKM	Fragments Per Kilobase per Million
G3P	Glycerol-3-phosphate
G6PD	Glucose 6-phosphate dehydrogenase
GABA	Glutamine-derived gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT	Glucose transporter
GPD2	Glycerol-3-phosphate dehydrogenase 2
GPI	Glucose 6-phosphate isomerase
GSH	Glutathione
H ₂ SO ₄	Sulfuric acid
HDTs	Host-directed therapies
HIF-1	Hypoxia-inducible factor 1
HIV	Human Immunodeficiency Virus
HK2	Hexokinase 2
hMDM	Human monocyte-derived macrophage
HMOX1	Haem oxygenase 1
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRP	Horseradish peroxidase
HSP	Heat shock protein
IDH	Isocitrate dehydrogenase
IFN	Interferon
IFN γ	Interferon gamma

IL	Interleukin
IM	Interstitial macrophage
iMtb	Inactivated <i>Mycobacterium tuberculosis</i>
iNOS	Inducible nitric oxide synthase
IRG1	Aconitate decarboxylase 1
KEAP1	Kelch-like ECH-associated protein 1
KO	Knockout
L929	L929 conditioned medium
LAM	Lipoarabinomannan
LDH	Lactate dehydrogenase
LM	Lipomannan
LPS	Lipopolysaccharide
LTBI	latent tuberculosis infection
LTG	LipidTOX green
ManLAM	Lipoarabinomannan
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistant
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
miRNA	microRNA
MOI	Multiplicity of infection
MR	Mannose receptor
mRNA	Messenger RNA
Mtb	<i>Mycobacterium tuberculosis</i>
MTG	Mitotracker Green
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAMPT	Nicotinamide phosphoribosyl transferase
Ndufv2	NADH dehydrogenase ubiquinone flavoprotein 2
NFκB	Nuclear factor κ-light chain enhancer of activated B cells
NHP	Non-human primate
NLR	NOD-like receptor

nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
Non-oxPPP	Non-oxidative pentose phosphate pathway
NOS	Nitric oxide synthase
NOX	Nitric oxide oxidase
NRF2	Nuclear respiratory factor 2
OCR	Oxygen consumption rate
OGCD	α -ketoglutarate dehydrogenase
Oligo	Oligomycin
OXPHOS	Oxidative phosphorylation
oxPPP	Oxidative pentose phosphate pathway
PAMP	Pathogen-associated molecular pattern
PBMCs	Peripheral blood monocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PFA	Paraformaldehyde
PFK-1	Phosphofructokinase-1
PFK-I	Phosphofructokinase-1 liver isoform
PFK-m	Phosphofructokinase-1 muscle isoform
PFK-p	Phosphofructokinase-1 platelet isoform
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PGD	Phosphogluconate dehydrogenase
PGM1	Phosphoglucomutase-1
PI3K	Phosphatidylinositol 3-kinase
PIM	Mannoysylated phosphatidylinositol
PK	Pyruvate kinase
PKM2	Pyruvate kinase M2
PknG	Protein kinase G
PPP	Pentose phosphate pathway

PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
R5P	Ribulose 5-phosphate
RBC	Red blood cell
RD1	Region of difference
RET	Reverse electron transport
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rot	Rotenone
RPE	Ribulose 5-phosphate epimerase
RPI	Ribose 5-phosphatase isomerase
RPMI-1640	Roswell Park memorial institute-1640 medium
RT-qPCR	Quantitative reverse transcription PCR
SDH	Succinate dehydrogenase
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
SP-A	Surfactant protein-A
SR	Scavenger receptor
TALDO	Transaldolase
TCA	Tricarboxylic acid
TDM	Trehalose dimycolate
TGF- β	Transforming growth factor- β
TIR	Toll-IL-1R
TKT	Transketolase
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TRP	transient receptor potential
vMtb	Viable <i>Mycobacterium tuberculosis</i>
WT	Wild type
XDR	Extensively drug resistant

Abstract

Mycobacterium tuberculosis (Mtb), the causative agent of tuberculosis, is adept at evading host immune defences and establishing chronic infections, particularly within macrophages. Central to its survival is its ability to reprogram host metabolism. This work explores the metabolic interactions between Mtb and host macrophages, focusing on the role of the pentose phosphate pathway (PPP) in modulating immune responses during infection. Here, we demonstrate that central to Mtb's survival is its ability to reprogram host metabolism, notably rerouting central carbon metabolism towards PPP activity, which fosters a metabolic environment favourable for bacterial replication. Previous work within our group demonstrated that Mtb suppresses glycolytic activity in macrophages through upregulation of the anti-inflammatory microRNA miR-21, which targets the glycolytic enzyme PFK-m. This suppression reduces the production of IL-1 β , a critical antimycobacterial cytokine. Now, we further characterise this immuno-evasive phenomenon as a means to regulate PPP-derived NO and subsequently target mitochondrial-based metabolism. NO is a soluble endogenous gas produced in macrophages by the activity of iNOS and proposed to have direct bactericidal activity. However, this model is poorly understood and moreover disputed in the context of Mtb infection of macrophages. This work alongside other recent studies challenges this perspective, suggesting that NO serves a more immune-modulatory than anti-microbial role, including the orchestration of metabolic reprogramming. Moreover, we identify glycogen as a crucial substrate for Mtb-driven PPP activity. During infection, macrophages mobilize glycogen stores to sustain PPP-derived NO production, which inhibits mitochondrial respiration and suppresses immune function. Blocking glycogenolysis decreased NO accumulation and IL-1 β secretion, highlighting the importance of glycogen in maintaining Mtb's metabolic niche. Notably, chronic infection elevates NO levels, enhancing Mtb replication through a feedback loop between NO and PPP activity, further supported by glycogen breakdown. This suppresses mitochondrial metabolism, including oxidative phosphorylation (OXPHOS), through inhibition of key enzymes in the tricarboxylic acid (TCA) cycle and the accumulation of the metabolite itaconate, which further reduces IL-1 β production and facilitates bacterial survival. Finally, we show that targeting PPP activity can enhance macrophage-mediated killing

of Mtb. Inhibiting NADPH oxidase (Nox2), which regenerates the PPP cofactor NADP, restored mitochondrial function and OXPHOS activity, leading to increased IL-1 β production and bacterial clearance. These findings suggest that targeting metabolic pathways like the PPP offers a promising therapeutic strategy for boosting host immunity against tuberculosis.

Chapter 1. Introduction

1.1 *Mycobacterium tuberculosis* and the Immune Response

The World Health Organisation's ministerial conference, the Moscow Declaration of 2017 placed emphasis on dealing with one of the world's ongoing pandemics, *Mycobacterium tuberculosis* (Mtb) (1). The objective of this meeting was to formulate new strategies in dealing with this disease, resulting in approximately 10 million people falling ill in 2019. In the face of challenges since the emergence of COVID-19, Mtb remains as one of the top ten causes of death world-wide, killing 1.6 million people in 2021, remaining as the leading cause of death from a single infectious agent, outranking HIV / AIDS (1). It is estimated that 2 billion or one third of the global population carries latent Mtb infection with approximately 10 % of infections resulting in active (or re-activated) clinical disease, thus maintaining transmission (2). Equally of huge global concern is the escalating incident in multidrug resistant (MDR) strains of Mtb (i.e. resistance to rifampicin and isoniazid), with an increase of 20 % in MDR cases between 2006 and 2019 (3). In 2017, a total of 558,000 cases of TB were documented with rifampicin resistance, with only 1.1 % of these cases to be susceptible to isoniazid (4). Alarming, there is increasing emergence of extensively drug resistant (XDR) tuberculosis, displaying resistance to fluoroquinolone and second line intravenous treatment. MDR / XDR diagnosis requires bacteriological confirmation, however, only 61 % of bacteriologically detected cases of TB in 2019 were tested for the occurrence of a MDR strain (5). Empiric treatment with first line drugs without prior susceptibility testing has enhanced the rise in these MDR cases through drug specific resistance amplification (4). Efforts have intensified in closing the gap between incidence, diagnosis and effective treatment and requires a multifaceted approach including an increase in detection, bacteriological confirmation and MDR identification (5). Mtb prevalence is more pronounced in the developing world and is accompanied by poor treatment coverage e.g. > 50 % in Central Africa Republic and Nigeria. Globally, 69 % of diagnosed TB cases had a documented HIV test result. In the WHO Africa region, the rates of HIV-associated TB are the highest at 86%. It is estimated that one third of all HIV-related deaths are attributed to Mtb infection (2). Currently, successful treatment rates are estimated at 85

% for TB, 57 % for rifampicin resistant / MDR-TB and 76 % for TB patients diagnosed with HIV (5).

1.1.1 Heterogeneity in human response to Mtb infection

Heterogeneity within the global population plays a major role in how individuals respond to Mtb infection and moreover, influences the outcome of the disease. Key factors in human heterogeneity during Mtb infection include genetic variation, immune system diversity, socioeconomic and environmental factors and co-morbidities. When first exposed to Mtb, which is typically transmitted through aerosolized droplets from individuals with active infection, the pathogen is either immediately eliminated by the innate immune system or establishes infection. In most cases, this infection triggers both the innate and adaptive immune responses, leading to the formation of a granuloma. Within 90-95% of infections, individuals either successfully eliminate the pathogen or contain the bacteria in a latent state, termed latent tuberculosis infection (LTBI). Individuals with LTBI present as asymptomatic and do not transmit the disease (6). Studies carried out in both humans and non-human primates (NHP) have characterised a spectrum of LTBI, ranging from those who either successfully eliminate or contain the pathogen to those who present as high risk of reactivation (7-9). The mechanisms driving host susceptibility to disease progression or reactivation are still not fully understood and require further research, but are known to be multifactorial, significantly varied among individuals and populations and are influenced by distinct biological endotypes. Among these, the role of tumour necrosis factor (TNF) in Mtb immunity is highlighted as particularly critical, making individuals on TNF blockers—commonly prescribed for autoimmune diseases like rheumatoid arthritis and inflammatory bowel disease—highly susceptible to Mtb infection and reactivation. TNF is a key pro-inflammatory cytokine that facilitates granuloma formation and maintenance, enabling the host to contain latent Mtb infection. TNF blockers, such as infliximab, adalimumab, and etanercept, disrupt these mechanisms by inhibiting TNF signalling, leading to impaired granuloma integrity and reduced macrophage activation (10). Consequently, individuals on TNF blockers are at heightened risk for Mtb reactivation, even in regions with low Mtb

prevalence. This vulnerability is exacerbated by the inability to mount a robust Th1 immune response, characterized by inadequate production of interferon-gamma (IFN- γ), further compromising the host's ability to control Mtb. Intracellularly, TNF is essential for promoting phagolysosome fusion and autophagy, which are key for restricting Mtb replication. TNF inhibitors impair these processes, leading to decreased macrophage antimicrobial activity and enabling Mtb to persist and replicate within host cells (11). The intersection of TNF inhibition and latent Mtb underscores the importance of pre-screening and prophylactic treatment for latent Mtb infection (LTBI) in patients initiating TNF blocker therapy. Understanding the endotype defined by TNF dysregulation is essential for developing stratified host-directed therapies that mitigate Mtb risk while maintaining the efficacy of TNF blockade in managing autoimmune conditions.

Genetic polymorphisms are also linked to the development of active infection with reports of a distinct type I IFN transcriptional signature leading to disease activation in NHP models and has also been described as a marker in Mtb infection in humans (12, 13). Other factors that impact immune function, and subsequently influence disease outcomes, include co-morbidities such as diabetes/hyperglycaemia and HIV status. *In vivo* studies have demonstrated higher expression of TNF α and IL-1 β during hyperglycaemia and in turn lead to increased bacterial burden (14). In general, Th1-based cytokine secretion is crucial for activating macrophages and protecting against Mtb infection. However, in diabetic individuals, the infection is poorly controlled due to elevated levels of anti-inflammatory cytokines and IL-4, which inhibit the expression of IFN- γ . This inhibition impairs the immune response, leading to more rapid disease progression before the adaptive immune system can effectively respond (15, 16). With respect to HIV infection, the disease hallmark of irreversible CD4⁺ T lymphocyte depletion enables both establishment of Mtb infection and also the reactivation of LTBI. Here, during active infection and granuloma formation, a niche environment of permissive cells is provided for HIV to infect, replicate and use as 'Trojan horse' to invade surrounding environments. Such damage to surrounding tissue further promotes inflammatory immune cell activation and in turn creates a self-sustaining cycle for Mtb replication. From these observations, it is clear that understanding the factors that influence different disease outcomes of individuals exposed to Mtb is key to successfully

developing new treatment strategies including host-directed therapies and vaccines (17).

1.1.2 Macrophages

Arising from bone marrow hematopoietic cells, macrophages can differentiate from promonocytic cells to mature monocytes within the peripheral blood and finally terminally differentiated macrophages following tissue specific migration. Here, they undertake both homeostatic functions or are recruited during proinflammatory responses (18). Typical macrophage functions include phagocytosis of cellular debris and pathogens, antigen presentation and regulation of adaptive immune cells through cytokine secretion (19, 20). Macrophage activity and function is classically defined by two main parameters: (A) tissue of origin and (B) local environment. Naïve macrophages are plastic in nature with environmental factors resulting in differentiation towards a committed activation state. Opposing ends of these states cluster macrophages into a classical (M1) or alternative (M2) phenotypes. External microenvironmental factors governing where cells reside on this activation scale typically results from type and duration of cytokine exposure in addition to specificity in pattern recognition receptors (PRRs) activated (21, 22). Classically activated macrophages display effector functions that result in proinflammatory and Th-1 stimulatory properties, whilst alternatively activated macrophages demonstrate anti-inflammatory, angiogenic and Th-2 mediated responses (23) (24).

M1 commitment is classically induced *in-vitro* in response to TLR-4 activation via lipopolysaccharide (LPS) and Th-1 associated cytokines (IFN- γ TNF- α), with cell surface markers including TLR-2, TLR-4, CD80, CD86, iNOS and MHC-II. Secretion of pro-inflammatory cytokines (TNF α , IL-1 α , IL-1 β , IL-6, IL-12, CXCL9 and CXCL10) by classically activated macrophages results in positive feedback loops and skewing of noncommitted, naive neighbouring macrophages towards the M1 activation state through signalling of downstream transcription factors NF- κ B and STAT1 (25). Phenotypic cell surface markers associated with M2 macrophages include CD206, CD163, CD209, FIZZ1 and Ym1/2 with

polarisation occurring predominantly from exposure to cytokines IL-4 and IL-13. Whilst not directly inducing M2 commitment, cytokines such as IL-33 and IL-25 augment M2 characterisation through downstream induction of Th-2 cytokines (26, 27). Other cytokines and chemokines involved in the M2 skewing include IL-10, TGF- β , CCL1, CCL17, CCL18, CCL22 and CCL24, upregulating activity of downstream transcription factors STAT6, IRF4, JMJD3, PPAR δ and PPAR γ (28).

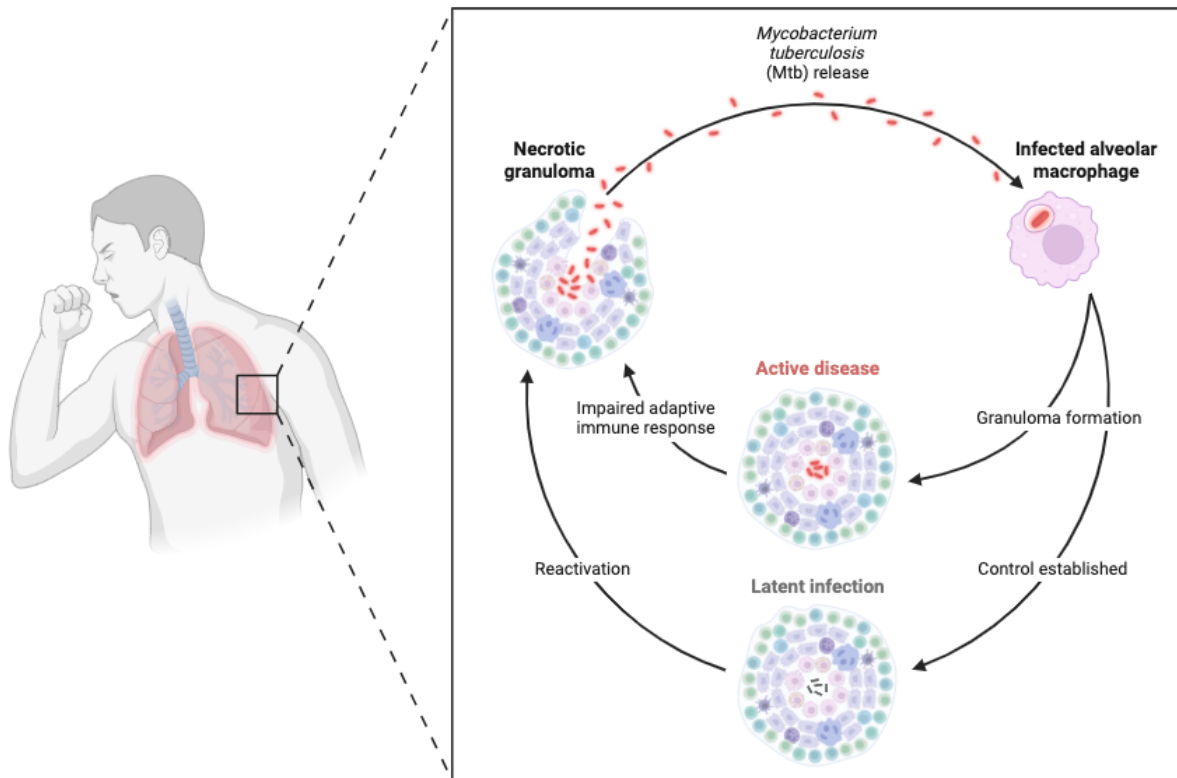


Figure 1. 1 Active and latent Mtb infection

Following inhalation of aerosolised bacilli, lung resident innate alveolar macrophages become infected with Mtb. Infection triggers both the innate and adaptive immune responses, leading to the formation of a granuloma. Within 90-95% of infections, individuals either successfully eliminate the pathogen or contain the bacteria in a latent state (LTBI). During active infection or reactivation of LTBI, an impaired adaptive immune response and excessive inflammation causes the granuloma to become necrotic and permits bacterial dissemination and infection of surrounding tissue.

1.1.3 Macrophage heterogeneity during Mtb infection

Within the lung compartment, the primary site of Mtb infection, distinct populations of macrophages and monocytes—alveolar macrophages (AMs), interstitial macrophages (IMs), and blood-recruited monocytes—play unique roles in shaping immune responses. Their ontogeny, localization, metabolic profiles, and functional characteristics dictate their contributions to the host-pathogen interaction in the lung, particularly in the context of granuloma formation, bacterial clearance, and tissue homeostasis.

Alveolar macrophages – AMs predominantly originate from embryologically distinct progenitors within the yolk sac where foetal monocytes proliferate locally during lung development to maintain resident populations (29). AMs exist in close association with alveolar epithelium and as a result are continually exposed to lipid rich and immunomodulatory surfactant. Consequently the lipoprotein content within surfactant that regulates lung function (30) also influences AM phenotype (31) and promotes disease tolerance, providing Mtb with a niche for survival. With continuous exposure to this surfactant, AMs commit to an alternative activation status, which is a contradictory phenotype when considering their high expression of pattern recognition receptor subsets including scavenger receptor A (SR) and toll-like receptors (TLRs) (32). Thus, AMs function as an immunological barrier, capable of effectively eliminating routinely encountered microbes. However, microenvironmental influence on AM phenotype often results in an inadequate response for uniquely adapted intracellular pathogens, such as Mtb.

AMs exhibit an M2-like metabolic profile under homeostatic conditions, relying primarily on oxidative phosphorylation (OXPHOS) for energy (33). This metabolic adaptation aligns with their role as phagocytic, long-lived, self-renewing cells, which require sustained energy without the anabolic demands of rapid proliferation. The lipid-rich lung environment, enriched by surfactant, provides AMs with diverse energy substrates, including fatty acids (FAs) and cholesterol. While glucose is a preferential fuel, its limited availability in lung microenvironments drives AMs to utilise fatty acid oxidation (FAO), a

hallmark of M2 macrophages (34-36). Mechanistically, IL-4 induces the expression of CD36, a receptor that facilitates fatty acid uptake and lipolysis, fuelling FAO and promoting anti-inflammatory cytokine production (37). Surfactant proteins such as SP-A may further drive CD36 expression and activity, reinforcing this M2-like phenotype and reliance on FAO (38). This metabolic reliance has implications during Mtb infection. Although Mtb can metabolise glucose, it preferentially utilises fatty acids as a carbon source (39). The lipid-rich environment of AMs, enriched with triacylglycerols, cholesterol, and fatty acids, provides an ideal niche for Mtb growth. CD36-mediated lipid uptake may serve as a double-edged sword: limiting inflammatory responses while supplying nutrients that facilitate bacterial replication. Indeed, *ex vivo* studies indicate that AMs provide a more permissive environment for Mtb replication compared to bone marrow-derived macrophages (BMDMs) or recruited lung interstitial macrophages (IMs), a phenomenon demonstrated as strongly dependent on FAO (39, 40).

While infection with attenuated or inactivated Mtb permits glycolytic activation in both human and murine AMs, live virulent Mtb inhibits glycolytic activity, forcing AMs to rely on OXPHOS for energy (39, 41). Transcriptomic studies of infected AMs reveal upregulation of FAO and lipid metabolism pathways, with minimal contributions from glycolysis (39, 42). Additionally, the lung microenvironment suppresses glycolysis in AMs, impairing their IL-4-mediated responses. This environmentally dictated metabolic rigidity of AMs, in contrast to the plasticity seen in monocyte-derived macrophages, may explain the higher threshold required for AM activation via glycolysis during Mtb infection. Targeting the regulatory mechanisms that restrict glycolysis in AMs could offer therapeutic strategies to shift their metabolism from OXPHOS toward glycolysis, thereby enhancing their inflammatory and bactericidal responses to Mtb.

Interstitial monocyte derived macrophages – The majority of research exploring the immunometabolic responses to Mtb infection has focused on macrophage models that simulate naïve, newly recruited macrophages at sites of inflammation. These models include human monocyte-derived macrophages (hMDMs), bone marrow-derived macrophages (BMDMs), and THP-1 cells. Across these systems, the findings consistently align with the metabolic reprogramming observed in M1-like macrophages,

characterized by a marked upregulation of glycolytic activity (43-47). Notably, this glycolytic shift is critical for macrophage-mediated control of *Mtb* infection. *In vitro* studies demonstrate that macrophages with enhanced glycolysis exhibit superior bacterial containment, with their antimicrobial efficacy being dependent on sufficient glycolytic activation (39, 45). Disruption of glycolysis in mice, either through pharmacological inhibition or genetic deletion, completely abolished IM ability to restrict *Mtb* growth (39). Similarly, poisoning glycolysis within *in vitro* systems—using BMDMs or hMDMs—yielded comparable results, with macrophages losing their capacity to control bacterial replication (39, 45, 48). This was also observed in BMDMs derived from mice lacking HIF-1 α , a key transcriptional regulator of the glycolytic response (46, 49). Collectively, these findings highlight glycolysis as a crucial metabolic pathway for effective macrophage-mediated immune defense against *Mtb*.

While the upregulation of glycolysis in *Mtb*-infected macrophages has been widely reported, recent evidence suggests that the pathogen may exploit this metabolic pathway to evade immune responses (43). An extensive metabolic screen conducted on hMDMs and THP-1 cells infected with various strains of *Mtb* revealed that virulent *Mtb* strains can suppress glycolytic flux, potentially dampening the pro-inflammatory immune response. This attenuation of glycolysis during successful infection aligns with *Mtb*'s known strategies of immune evasion, as it prevents the macrophage from mounting a robust inflammatory response. Further evidence of the importance of glycolysis in host defense comes from the role of Th1 derived IFN- γ in promoting glycolysis within *Mtb*-infected macrophages (41, 46). IFN- γ , a critical cytokine in the adaptive immune response to *Mtb*, drives glycolytic activation, thereby reinforcing the macrophage's pro-inflammatory and antimicrobial functions. Together, these findings underscore the dual role of glycolysis as both a critical host defense mechanism and a potential target for immune subversion by *Mtb*.

Initial studies proposed that glycolysis supports the production of pro-inflammatory IL-1 β , which regulates eicosanoid prostaglandin E2 (PGE2) synthesis to promote bacterial containment (45, 50). However, additional glycolysis-associated pathways contributing to host defense have since been identified. Among these, nitric oxide (NO) production

has emerged as a key mechanism and is associated increased glycolytic activity in macrophages (41, 46, 49). Elevated NO levels occur not only through IL-1 β signalling but also via the generation of NADPH through the glycolysis-associated pentose phosphate pathway (PPP) (51). This NO production serves dual functions: it enhances glycolysis by activating HIF-1 α and simultaneously modulates inflammation by inhibiting NF- κ B signalling (49).

While NO plays an important role in the host response to Mtb, its precise contribution to antimycobacterial activity remains complex and context-dependent. Some studies suggest that NO functions primarily as an immunomodulatory molecule, influencing the magnitude and resolution of inflammation rather than directly killing Mtb (52, 53). For example, NO has been shown to suppress NLRP3 inflammasome-mediated IL-1 β production, thereby mitigating neutrophil driven pulmonary tissue damage (54). Interestingly, NO also regulates neutrophil recruitment during Mtb infection, which paradoxically supports bacterial growth by providing a permissive niche (52). This dual role highlights NO's involvement in balancing inflammatory responses to protect lung tissue while limiting excessive damage. In the context of granuloma formation, NO appears to coordinate the recruitment of IFN- γ -producing T cells to infected lung tissue, facilitating the transition from host resistance to disease tolerance. Recent findings further suggest that NO-mediated regulation of inflammation may shape subsequent immunometabolic responses (53), underscoring a possible broader role in the immune response to Mtb.

It is important to note that much of our understanding of NO's role in Mtb infection stems from studies in mouse models, where NO production is significantly higher than in human macrophages, including AMs. Human macrophages produce relatively low levels of NO, which raises questions about whether findings from murine models fully translate to human systems. Despite these differences, the collective evidence underscores the importance of NO in regulating macrophage activity and coordinating pro-inflammatory mediators to balance bacterial control with disease tolerance. Future studies are needed to validate the immunometabolic roles of NO in human macrophages and clarify its relevance in the human host response to Mtb.

1.1.4 Macrophage recognition of Mtb

Toll-like receptors (TLRs) – present on macrophages, neutrophils, lymphocytes and DCs, TLRs comprise a family of highly conserved transmembrane receptors and are structurally defined by their PAMP recognising extracellular N-terminal leucine-rich repeat, interleukin-1 receptor homologous C-terminal and Toll-IL-1R (TIR) domains, whose composition enables the assembly of signalling complexes (55-59). Expressed on the cell surface, TLR2 and 4 play a significant role in macrophage recognition of Mtb, resulting in the induction of intracellular signalling cascades and differential activation of pro-inflammatory transcription factor NF- κ B (60). Major mycobacterial TLR2 agonists include lipoarabinomannan (LAM), its precursor lipomannan (LM), mannosylated phosphatidylinositol (PIM), lipoprotein LpqH and virulence factor ESAT-6 (61). Null mutations associated with TLR2 signalling has demonstrated increased susceptibility to Mtb with diminished TNF- α secretion (62). TLR2 also demonstrates the ability to heterodimerise with other members of the TLR family including TLR1 and 6 (63). TLR signalling efficacy is influenced by the lung microenvironment with surfactant protein-A (SP-A) resulting in increased TLR2 membrane localisation, however, dampening TLR2 and 4 pro-inflammatory responses (64).

Mannose receptor (MR) – The mannose receptor (CD206) is a key phagocytic receptor expressed through which Mtb gains intracellular access. MRs are expressed on AMs, monocyte-derived macrophages and DCs and contain eight lectin-like carbohydrate recognition domains and binds with high affinity to mycobacterial agonists - mannose-capped lipoarabinomannan (ManLAM) and phosphatidyl-myo-inositol mannosides (PIMs)(65-69). Positioning of mannosylated PAMPs on the cell surface is deemed a deliberate method of molecular mimicry in which Mtb attempts to bypass immune detection by presenting similar mannosylated glycoproteins native to the host cell molecules. MR signalling is associated with the secretion of anti-inflammatory cytokines such as IL-10 whilst also restricting pro-inflammatory IL-12 production (70, 71). MR associated phagocytosis of Mtb leads to reduced lysosomal fusion with moderated acidification, a consequence of diminished subunit recruitment for the formation of

vacuolar proton pump ATPase (66, 72-75). MR activation is also demonstrated to promote alternative macrophage activation through the induction of PPAR γ (76).

NOD-like receptors (NLRs) – NLRs are highly expressed intracellular receptors found predominantly within antigen-presenting cells (APCs), including macrophages and epithelial cells. With signalling promoting an inflammatory response, NLRs are structurally composed of three distinct domains: a N-terminal functional domain, a C-terminal ligand sensing domain and a central nucleotide-binding oligomerization domain (NOD). NOD2 is a highly expressed protein within human macrophages and functions in the recognition of muramyl peptides derived from bacterial cell wall peptidoglycan. NOD2 interaction with ligand has been implicated in myeloid production of proinflammatory cytokines during Mtb infection in vitro, with signalling resulting in the indirect downstream activation of NF- κ B and AP-1 through mitogen-activated protein kinases (MAPKs) (77-80). Through direct interaction with caspase-1 and the NALP1/NALP3 inflammasome, NOD2 promotes the cleavage of inflammatory pro-IL-1 β to its active form (81). During Mtb infection of human macrophages, NOD2 is demonstrated to specifically recognise an N-glycosylated muramyl dipeptide found in Mtb, with subsequent signalling influencing the nature of the pro-inflammatory response (82).

Complement and Fc γ receptors – Both monocytes and macrophages express various levels of complement C3 receptors CR1, CR3 and CR4, whose activity and expression pattern occurs in a tissue specific manner. AMs display preferential expression of CR4, a hallmark substitute from CR3 during monocyte differentiation (83). As well as conventional complement mediated opsonic phagocytosis, CRs play a role in the non-opsonic phagocytosis of Mtb during infection through interaction with bacterial cell wall components such as polysaccharides phosphatidylinositol mannosides (PIM) and glycopeptidolipids (84). Fc γ receptors require the engagement of an adaptive immune response in order to facilitate opsonising antibody mediated phagocytosis of Mtb. Phagocytosis through these targeted Mtb specific antibodies promotes heightened phago-lysosomal activity (85).

Other pathogen-associated molecular patterns (PAMPs) recognised during the innate response to Mtb included bacterial secretion of cyclic dinucleotides and subsequent host cell activation of STING (86, 87). STING signalling results in the production of type-I interferons and is a consequence of bacterial ESX-1 mediated perforation of vacuolar membranes following phagocytosis, an unfavourable outcome during host cell defense (88-90). In the context of Mtb infection, the interferon response is facilitated by bacterial secretion of cyclic-di-AMP and direct activation of STING without the need for upstream production of cyclic GMP-AMP (cGAMP) via cGAS activity (91). Engaging the antiviral response is one way Mtb manipulates host cell activity to promote pathogenesis where Type I IFNs have been shown to contribute to disease progression in animal models by reducing IL-1 responses, partly through disrupting macrophage glycolysis and mitochondrial respiration (92).

1.1.5 Innate cytokines during Mtb infection

TNF α – As a key modulator of the of the early immune response, TNF α is deemed a crucial cytokine in macrophage containment of Mtb. Through activation of membrane bound receptors TNFR1 and TNFR2, TNF α potentiates macrophages pro-inflammatory responses through transcription factor NF κ B and enzyme MAPK. TNF α is also abundantly produced by CD4 T-cells with secretion heightened in the presence of IFN- γ (93, 94). Murine models devoid of TNF α signalling have demonstrated increased disease susceptibility and bacterial burden, whilst humans treated with anti-TNF agents such as infliximab for inflammatory based diseases displaying increased vulnerability for reactivation of latent disease (95-99). Non-human primate models of infection have validated the importance TNF- α signalling in Mtb infection, with cytokine neutralisation resulting in bacterial dissemination as well as reactivation of previously established latent infection (100). Zebrafish models indicate an indirect role of TNF- α in promoting granuloma formation through restriction of bacterial expansion. However, excessive TNF- α has been shown to induce a state of hyperinflammation where initial microbicidal activities are increased through ROS but followed by immoderate necrotic cell death, thus potentiating dissemination (101-104).

IL-1 β – The IL-1 family of pro-inflammatory interleukins are primarily produced by monocytes, macrophages, DCs and neutrophils and consists of IL-1 α and IL-1 β , both of which bind the same receptor, IL-1R. IL-1 signalling is deemed crucial in protection against Mtb with murine models lacking IL-1R activity displaying increased susceptibility to disease (90, 105-107). Of note, elimination of IL-1R activity results in the independent loss of IL-1 signalling, with no effect on TNF- α , iNOS activity or IFN- γ responses (105). In the context of murine *in vivo* models of Mtb infection, greater disease burden has been associated with the loss of both IL-1 α and IL-1 β (108). Similar enlarged granulomas have also been identified with loss of IL-1R in mice (109). When examining the necessity for IL-1 signalling during Mtb infection, it was noted that the presence of either IL-1 α or IL-1 β was adequate in controlling acute infection, however, over time both family members were required to combat persistent infection (110). Of note, it has also been recorded that IL-1 β deficiency results in heightened bacterial burden despite compensatory secretion of IL-1 α (107).

The generation of IL-1 β during Mtb infection remains a disputed topic. Traditionally, production of mature IL-1 β requires cleavage of its precursor protein through inflammasome and caspase-1 activity. The NLRP3 inflammasome has been demonstrated as the primary inflammasome required for IL-1 β cleavage during Mtb infection, a host anti-microbial response triggered by detection of the ESX-1 secretion system (111-115). However, murine *Nlrp3*^{-/-} and *Csp1*^{-/-} models are still capable of mature IL-1 β production, indicating an inflammasome-independent mechanism of secretion (107, 114). NLRP3 inflammasome and caspase-1 deficient mice also demonstrate greater anti-Mtb responses when compared to mice with impaired IL-1 β or IL-1R activity (107, 114, 116-118). Transcription factors involved in IL-1 β expression include NF κ B and HIF-1 α . Nuclear translocation of NF κ B to the IL-1 β promoter is triggered through PRR signalling, such as TLR activation of phosphatidylinositol 3-kinase (PI3K) (119, 120). Whilst NF κ B activity is associated with the immediate induction of IL-1 β (0-3h), HIF-1 α has been identified as the transcription factor responsible for

maintained secretion (6-24h), with HIF-1 α binding loci identified in both human and murine IL-1 β promoter regions (121, 122).

Full activation of the NLRP3 inflammasome and subsequent production of mature IL-1 β requires the coordination of at least two intracellular signalling cascades: a priming signal and an activation signal. Priming signals involve those which generate the mobilisation of NF κ B such as TLR and TNFR and subsequent expression of pro-IL-1 β , as well as licensing/upregulation of NLRP3 expression itself. This pro-IL-1 β polypeptide is then subject to cleavage into its mature form through the activation signal, involving PAMP and DAMP mediated assembly of the adapter apoptosis-associated speck-like protein (ASC) to NLRP3 forming multi-protein complexes termed inflammasomes. ASC binds pro-caspase-1 through its C-terminal caspase recruitment domain (CARD) and results in catalytic activation and generation of mature IL-1 β (123). The AIM2 inflammasome has also been demonstrated to play a role in anti-mycobacterial responses, through recognition of bacterial DNA. AIM2 undergoes oligomerisation and subsequent conformational changes result in the exposure of a pyrin domain, functioning as a binding site for pro-caspase-1 (124). Murine models with deficient AIM2 activity demonstrate decreased bacterial containment with impaired IL-1 β signalling (125).

Many means of anti-mycobacterial activity have been attributed to IL-1 β signalling. These include promotion of IL-12p40 and IFN- γ production as well as enhanced expression and presentation of TNFR1 (109, 118, 126). Interestingly, IL-1 β has been shown to play an active role in prostaglandin PGE2 mediated suppression of the type-1-interferon response, often induced by Mtb during infection as means of immune evasion. PGE2 also promotes bacterial clearance through induction of apoptotic cell death of infected phagocytes, rather than necrosis which permits dissemination (127).

IL-10 – The role of anti-inflammatory IL-10 in the context of Mtb infection has been a point of controversy for some time. Secreted by T-cells, B-cells, neutrophils, DC subsets and macrophages, IL-10 results in the activation of STAT3 through binding its receptors

IL10R1 and IL10R2 and subsequent signalling through the Jak-STAT pathway (128, 129). IL-10 potentiates its immuno-modulatory effects through reduction in MHCII expression as well as diminishing production of Th1 cytokines, TNF- α , ROS and RNS (130-132).

Efforts to analyse the contribution of IL-10 contribution to host responses during Mtb infection has exhibited mixed outcomes, a proposed ramification of the murine genetic background used. Conflicting data includes knockout of IL-10 expression in a C57BL/6 murine infection model resulting in heightened bacterial burden and late-stage mortality, whilst loss of IL-10 on both C57BL/6 and BALB/C mice from an alternative study demonstrating reduced bacterial expansion within the lungs (133, 134). Interestingly, use of CBA/J mice, a background particularly know for Mtb susceptibility, revealed decreased bacterial burden within the spleen and lungs upon loss of IL-10 signalling during infection (135, 136).

1.1.6 Reactive oxygen and nitrogen species during Mtb infection

The generation of reactive oxygen (ROS) and nitrogen (RNS) species and subsequent activation of antioxidant defense systems is a key feature in macrophage antimicrobial responses to Mtb infection. The generation of these radical molecules is closely associated with multiple metabolic processes, highlighting the importance of metabolic-immune crosstalk. A key driver in the generation of these ROS and RNS is the coordinated activity of two key intracellular NADPH oxidases – NOS2 and NOX2. iNOS/NOS2 is a cytosolic enzyme responsible for catalysing the conversion of arginine to NO and L-citrulline. Induction of iNOS/NOS2 is prompted by a NADPH oxidase mediated respiratory burst, where the formation of radical oxygen molecules and subsequent association with iNOS/NOS2 generated NO, enables the formation of ROS and RNS. *Nos2* expression has been documented during murine models of Mtb infection (137). The induction of *Nos2* gene expression within macrophages has also been attributed to IFN- γ signalling during Mtb infection (138). The importance of NOS2 activity during Mtb infection is highlighted by the fact that NO can be detected in the breath of people

during active infection along with enhanced expression of *Nos2* within lung alveolar macrophages (139-141).

The NADPH oxidase complex reaction results in the production of superoxide which undergoes subsequent reactions to produce ROS molecules including H_2O_2 , HClO and hydroxyl radicals. Multiple lines of data suggest an important role for the activity of NADPH oxidase during Mtb infection, including patients with chronic granulomatous disease (CGD). These CGD individuals present with an inherited defect in NADPH oxidase and subsequently are susceptible to active Mtb infection as well as disseminated bacillus Calmette-Guérin (BCG) disease following routine vaccination (142). Additionally, when macrophages were isolated from these individuals, it was apparent that these cells were unable to restrain Mtb growth. Controversially, murine models lacking *Cybb*, subunit gp91 of the NADPH complex display no increase in bacterial burden upon Mtb infection (143, 144). However, these mice displayed increased weight-loss and mortality which was concluded a result of excessive neutrophil recruitment. Furthermore, inhibition of IL-1 signalling in these genetically altered mice lessened neutrophil infiltration, possibly demonstrating a role for ROS in limiting exorbitant inflammation (144). This ROS mediated monitor of inflammation may be a result of NOS2 induced NO production which limits IL-1 β production by both nitrosylation and inhibition of the NLRP3 inflammasome and also inhibition of NF- κ B signalling (49, 52, 54).

Another source of cellular ROS is mediated through reduced mitochondrial electron transport chain (ETC) activity, again, leading to the heightened generation of O_2^- and subsequent NOS2 induction. This reduction in mitochondrial activity is a hallmark feature of Warburg metabolism and a well-documented characteristic of classical macrophage polarisation in murine bone marrow-derived macrophages (BMDMs) (145). Other mitochondrial enzymes contributing to the cellular generation of O_2^- include glycerol-3-phosphate dehydrogenase 2 (GPD2) and succinate dehydrogenase (SDH). Through generation of dihydroxyacetone phosphate (DHAP) from glycolytically imported glycerol-3-phosphate (G3P), mitochondrial GPD2 contributes to an elevated reduction state of the ETCs coenzyme Q pool through coupled FAD-dependent reactions within the mitochondrial inner membrane. This is presumed to contribute to the mitochondrial

generation of O_2^- within macrophage with documented strong induction of *Gpd2* expression during Mtb infection. Similarly, the dual function enzyme SDH, playing a role in both the tricarboxylic acid cycle (TCA) and complex II of the ETC, also contributes to the coenzyme Q electron pool through FAD-dependent reactions. SDH activity and linked oxidation of succinate is demonstrated to result in the generation of ROS during classical activation of macrophages, suggested as a consequence of reverse electron transfer (146).

To compensate for the possible deleterious effects of the oxidative stress response, the employment of antioxidant defense systems is required. Investigation of superoxide dismutases (SOD) and catalases, responsible for driving antioxidant defense, are in agreement with the mitochondria as the primary site for ROS production during Mtb infection (147). Supporting evidence includes a moderate decrease in cytosolic *Sod1* expression, whilst mitochondrial associated *Sod2* is strongly upregulated. Increased NAD^+ formation by induction of nicotinamide phosphoribosyl transferase (NAMPT), serves as another means of redox homeostasis during Mtb infection (148, 149). The generation of intracellular glutathione (GSH) also functions to reduce levels of radical oxygen species. Mtb infection of macrophages had been demonstrated to increase cellular synthesis of GSH through induction of glutamate-cysteine ligase subunits *Gclc* and *Gclm*, catalytic and modifier subunits respectively (150). This ligase catalyses the first and rate limiting step of GSH synthesis, a process regulated by the availability of its substrate, cysteine (151). Cysteine is typically in low availability within cells, therefore, sufficient induction of its import system is deemed an important step in mounting an antioxidant response. Made up of SLC7A11 and chaperone SLC3A2, xC⁻ is the antiporter system that couples glutamine efflux with cellular uptake of cysteine (152). Infection models within murine macrophages have demonstrated upregulation of SLC7A11 in response to Mtb (153). Further evidence supporting the antioxidant contribution of cysteine during Mtb infection include *Slc7a11* deficient mice having the heightened ability to control Mtb infection when compared to their wild-type counterparts (154).

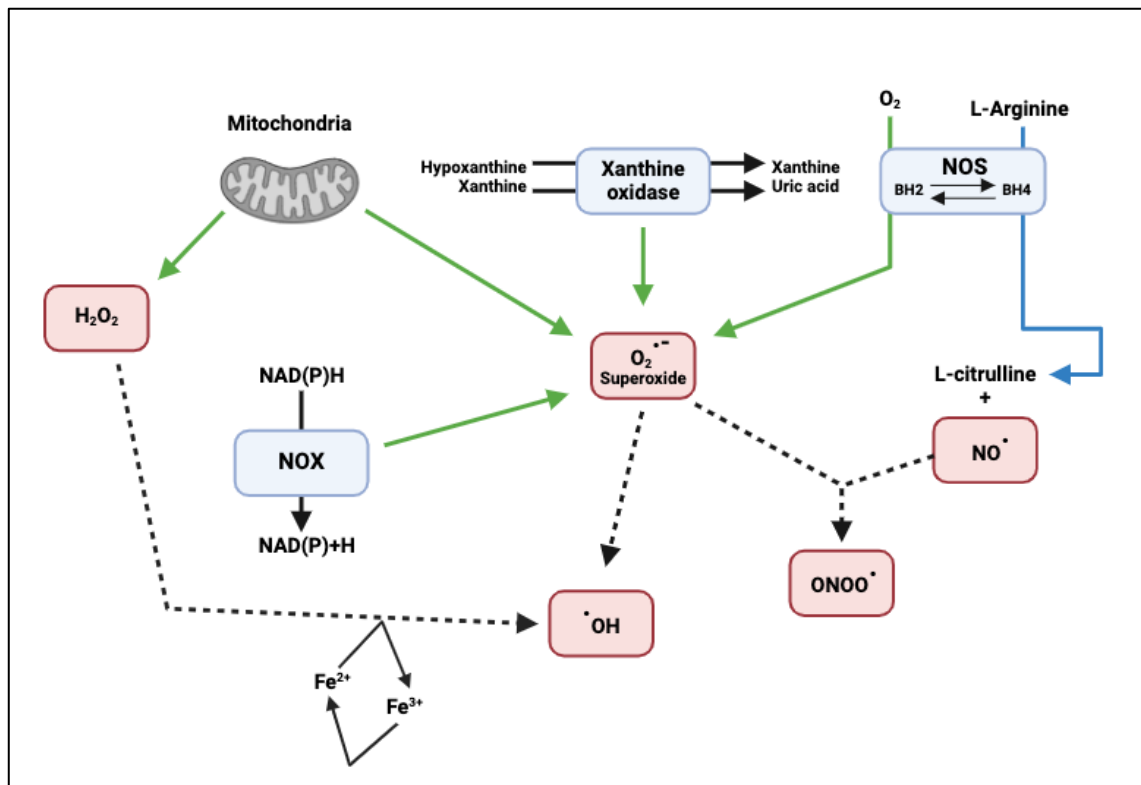


Figure 1. 2 Major reactive oxygen species (ROS) generation pathways in macrophages

Mitochondria produce superoxide through leak of electrons from respiratory chain complexes. Superoxide is also generated by the reduction of oxygen (O) by free electrons released by the activity of (1) NAD(P)H oxidase which oxidise NAD(P)H to NAD(P)+H, (2) xanthine oxidase, which catalyses the conversion of hypoxanthine to xanthine or xanthine to uric acid and (3) nitric oxide synthase, which switches from a coupled state, producing NO, to an uncoupled state, producing O^- . Under such conditions L-arginine is converted L-citrulline. Superoxide can also react with NOS derived NO and generate peroxynitrite (ONOO^-).

1.1.7 The phagosome during Mtb infection

Classical phagocytosis of bacteria by macrophage involves cytoskeletal rearrangement of actin to enable the cellular membrane to surround and engulf the target bacterium. Generation of the phagosome initiates the sequential recruitment of Rab GTPases and subsequently vacuolar ATPases to mediate acidification of the membrane bound compartment. Further maturation of the phagosome involves lysosomal fusion to merge the acidified phagosome with lysosomal acid hydrolases.

Evolutionary, Mtb has adopted multiple host-directed immune-evasive mechanisms in an effort to disrupt normal host-cell phago-lysosomal trafficking. After uptake of the bacteria, the early phagosome contains a Ca^{2+} concentration consistent with the extracellular milieu where further maturation is initiated by a rapid transient receptor potential (TRP) mediated Ca^{2+} efflux into the cytosol and subsequent activation of Ca^{2+} signalling pathways. Multiple studies have demonstrated that Mtb ManLAM, located on the bacterial cell surface, can inhibit the formation of these Ca^{2+} hotspots within the cytosol and subsequently disrupts calmodulin complex formation with PI3K and phosphatidylinositol 3-phosphate (PI3P) phagosomal recruitment (155-158). GTPase localisation to the phagosome is dependent on this PI3P lipid intermediate. Mtb mediated inhibition of sphingosine kinase also prevents phagosomal efflux of Ca^{2+} (159). Other defective components in phagosomal maturation documented during Mtb infection include the impaired recruitment of Rab5 effector proteins – early endosomal autoantigen 1 (EEA1) and hVps35 (160-162). EEA1 works in combination with Syntaxin-6 to deliver lysosomal hydrolases, vacuolar ATPases and cathepsins. Endosomal retention of EEA1 is dependent on a specific type III PI3K located on the phagosome, also demonstrated to be diminished during Mtb infection (75, 163). Ultimately, by targeting this fundamental bactericidal signalling pathway, Mtb prevents conversion of early lysosomal associated Rab5 to late marker Rab7 (164, 165).

Mtb components known to interfere with phagosomal maturation include SecA 1/2 proteins and eukaryotic-like serine/threonine protein kinase G (PknG) as well as

previously described ManLAM and ESAT-6, (166-169). Bacterial glycolipid trehalose dimycolate (TDM) has been demonstrated to disrupt membrane trafficking during phagosomal maturation (170). Further studies into the immune-evasive mechanisms employed by Mtb during infection have eluded to bacterial escape from the phagosome in a dependent on mycobacterial region of difference (RD1) and ESAT-6 dependent in both macrophages and neutrophils following several days *in vitro* (171, 172). However, a question remains over the driving force behind this phagosomal escape: does it truly represent a virulence strategy to avoid host antimicrobial responses and promote bacterial expansion or occur simply a consequence of membrane rupture during necrotic cell death and a means to infect neighbouring cells. Moreover, it has been postulated that the RD1 dependent ESX-1 secretion system functions to decrease the bactericidal capacity of the phagosome by perforating but not destroying the membrane, leading to a leaking of phagosomal components (173, 174).

1.1.8 Activation of the adaptive response during Mtb infection

As key APCs, dendritic cells play a central role in orchestrating the adaptive immune system in response to Mtb infection (175). Following initial uptake of Mtb, dendritic cells undergo IL-12(p40)2/IL-12p70 cytokine and CCL19/CCL21 chemokine mediated migration from the primary site of infection within the lung parenchymal space to the draining lymph node (175-178). Here, through MHC-mediated antigen presentation, dendritic cells enable naïve T-cell differentiation towards a Th1 phenotype (179, 180). Arrangement of adaptive responses mounted against Mtb require substantial time – documented at 12 days post infection in murine models and strikingly, 5-6 weeks in humans (181-183). This delay has been attributed to impaired trafficking of DC's carrying Mtb bacilli towards the lymph node (184). Delays in mounting T-cell responses provide opportunity for bacilli to replicate exponentially within the lung, resulting in a more established infection (185). The development of a sufficient adaptive response is critical in host antimycobacterial immunity, predominantly that mediated by CD4⁺ and CD8⁺ T-cells. In particular, the bactericidal capacity of macrophage has been attributed the CD4⁺ secretion of IFN- γ . Murine models of infection have demonstrated the importance of

this innate-adaptive crosstalk, with animals lacking either CD4⁺ cells or the ability produce IFN- γ displaying exponential susceptibility to Mtb infection (186, 187). The depletion of CD8⁺ T cells in a murine model during the chronic stage of Mtb infection led to an increased bacterial burden, suggesting that these cells play a role in the long-term control of the infection. Both CD4⁺ and CD8⁺ T cells have been demonstrated as producers of IFN- γ within the murine models, however, previous research has suggested that the presence of CD4⁺ cells is required for the sufficient production of IFN- γ by CD8⁺ cells (188). The activity of CD4⁺ T cells is essential for effective immune function during Mtb infection, demonstrated by CD4⁺ depletion and MHC II knockout models, which show that CD4⁺ antigen recognition is necessary for bacterial containment (189). Furthermore, CD4⁺ cells surpass CD8⁺ cells in recognition of Mtb infected macrophages, potentially explaining the relatively limited impact of CD8⁺ cells on infection outcomes (190).

The adaptive response of CD4⁺ T cells is more thoroughly characterised in murine models compared to human models. Clinical trials assessing responses to BCG vaccination reported the secretion of TNF α , IFN- γ , IL-17 and IL-12 from both CD4⁺ and CD8⁺ T cells did not differ between infants who were immunised and those who developed Mtb infection (191). Additionally, adults capable of eliciting antigen specific CD4⁺ and CD8⁺ cell responses are demonstrated to potential to still develop active Mtb infection (192). Previously mentioned observations of Mtb coinfection with HIV further highlight the importance of a robust CD4⁺ response during infection. Under these conditions, increased susceptibility to Mtb infection correlates with lower peripheral CD4⁺ T cell counts. Specifically, individuals co-infected with Mtb and HIV have reduced CD4⁺ T cell counts and lower IFN- γ levels in bronchoalveolar lavage (BAL) fluid compared to Mtb infected individuals who are HIV-negative (193-195).

Sufficient organisation of the adaptive immune response and associated cytokines/chemokines enables the generation of the granuloma (185, 196). To date, granulomas have been characterised as inflammatory lesions consisting of a central mass of infected immune cells, particularly macrophages, neutrophils, Langerhans's multinucleated giant cells and foam cells. This central core of infected cells becomes

encapsulated by lymphocytes and fibroblasts, both providing structure and preventing bacterial dissemination (185, 186, 196). Granulomas permit the containment and localisation of bacterial replication which in the majority of individuals results in latent, asymptomatic infection. Successful formation of the granuloma results in the contained bacilli entering a dormant, non-replicative state once the internalised nutrient availability diminishes (185, 197, 198). Here, immunocompetent individuals have high infiltration of IFN- γ producing CD4⁺ cells that facilitate bacterial killing and possibly even sterilisation of the granuloma. However, an estimated one-third of infected individuals will develop clinically active infection including immunocompromised individuals, such as those who contract HIV infection, where granulomas become larger in size and have fewer surrounding lymphocytes (199). Here, bacterial persistence is accompanied by cellular necrosis within the hypoxic core of the granuloma, leading to caseous cavitation of the necrotic core through encapsulating fibrotic boundary. In addition to human-to-human transmission, granulomatous dissemination facilitates infection of surrounding cells as well as haematogenous dissemination to other organs (185, 200). Development of active infection may result after initial infection with Mtb or may occur “spontaneously” at a later date (years to decades) following alterations to host immune function (98, 201). Thus, the granuloma represents a point of balance between the host and bacteria, where the progression of the infection is determined by the effectiveness of the individual's innate and adaptive immune responses. Due to significant differences in animal models, granuloma formation is challenging to accurately model both *in vivo* and *in vitro*. This has been a limiting factor in fully understanding the pathogenesis of Mtb and in the development of effective therapies and vaccines. As a result, many research groups focus on modelling the individual cellular interactions that occur early in Mtb infection, prior to granuloma formation. These models aim to elucidate the initial immune responses and cellular dynamics, which are critical for understanding the processes that ultimately lead to granuloma development.

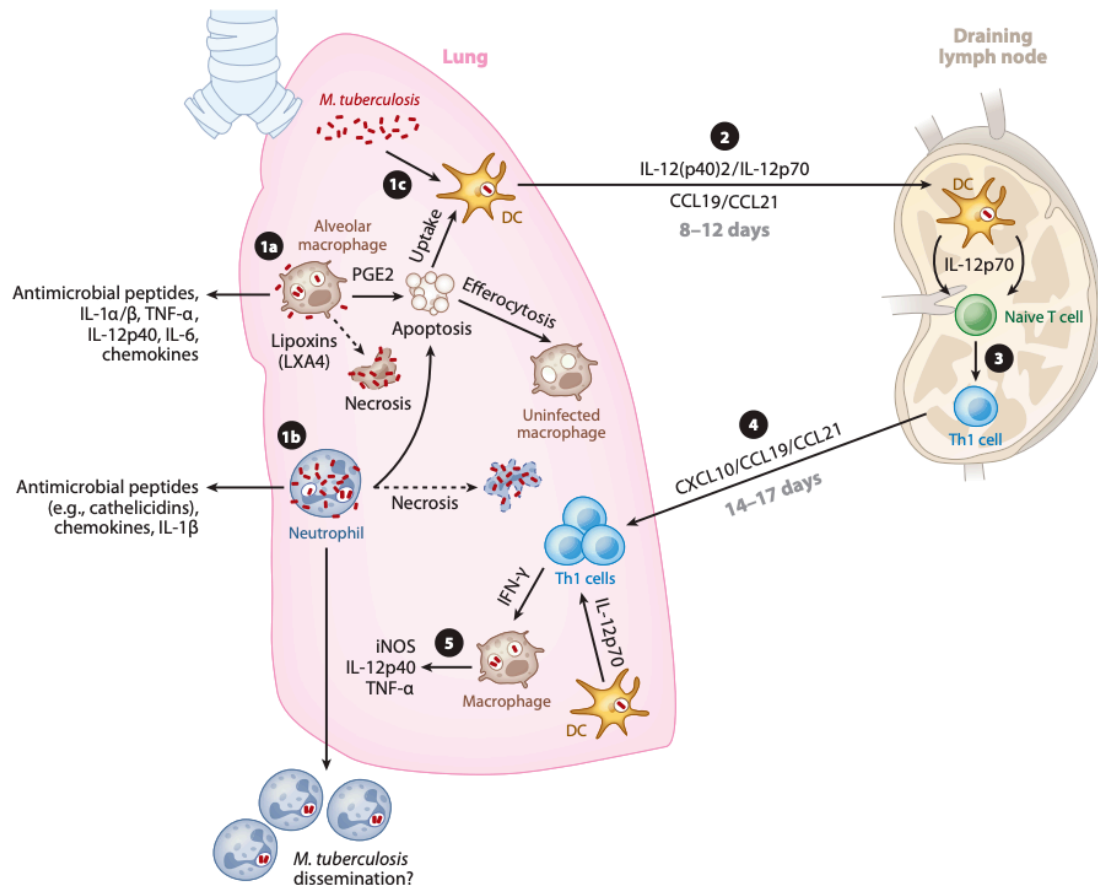


Figure 1. 3 The cellular immune response to Mtb

Following inhalation of aerosolised bacilli, lung resident innate immune cells become infected with Mtb: (1a) alveolar macrophages, (1b) neutrophils and (1c) DCs, resulting in the secretion of antimicrobial peptides, cytokines and chemokines. The balance of lipid mediators, such as prostaglandin E2 (proapoptotic) or lipoxin (LX) A4 (pronecrotic), within infected macrophages determine induction of downstream pathways leading to either apoptosis or necrosis. Apoptotic cells are taken up by DCs or efferocytosed by uninfected resident macrophages. (2) 8-12 post infection, Mtb containing DCs migrate to local draining lymph nodes, influenced through the production of IL-12(p40)2 and IL-12p70 and that of the chemokines CCL19 and CCL21 whilst also (3) promoting naïve T-cell differentiation towards the Th1 phenotype. (4) 14-17 days post infection, antigen specific Th1 cells migrate back to the lungs in a chemokine dependent manner, (5) enhancing macrophage activity through secretion of IFN- γ resulting in secretion of microbicidal factors such as cytokines and *Nos2* expression. Reproduced from O'Garra *et al.* (186).

1.2 Macrophage metabolism during Mtb infection

1.2.1 Glycolysis in Mtb infected macrophages

Classical activation of macrophages upon Mtb infection demonstrates the ability of these phagocytic cells to undergo glycolytic reprogramming in order to meet the increased energy demands required to mount an effective antimycobacterial response (122). Heightened availability of glucose is required to fuel Warburg metabolism, a process predominantly mediated in macrophages by the rate-limiting glucose transporter GLUT1, encoded by SLC2A1. Multiple studies have demonstrated upregulation of GLUT1 during Mtb infection (41, 46). Once inside the cell membrane, glucose is subject to the irreversible phosphorylation catalysed by a family of enzymes known as hexokinases, with hexokinase-2 (HK2) highlighted as the principal isoform upregulated in most cell immune types (202). Phosphorylation of glucose functions to sequester this carbon source within the cell as glucose-6-phosphate (G6P), which is now available to continue down the glycolytic pathway or be alternatively diverted towards the pentose phosphate pathway (PPP).

The glycolytic pathway consists of a series of enzyme mediated reactions, three of which (including HK2) are irreversible in nature. Phosphofructokinase-1 (PFK-1) catalyses the second irreversible and rate-limiting step of glycolysis, converting fructose-6-phosphate (F6P) and ATP to fructose-1,6-bisphosphate. As PFK-1 plays a major role in determining overall glycolytic flux, its activity is subject to multiple modes of allosteric regulation including activators AMP and fructose-2,6-bisphosphate (F2,6BP) and inhibitors ATP and TCA intermediate citrate (203-205). F2,6BP is generated from fructose-6-phosphate through the activity of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) which may also function to degrade F2,6BP through accompanied phosphatase activity. Mtb infection results in the upregulation of PFKFB3, which is further augmented in the presence of IFN- γ (36, 206, 207). Overall, PFKFB has greater kinase to phosphatase activity and thus supports glycolytic activity in an allosteric capacity through F2,6BP generation (208). Structurally, PFK-1 may be classified as a heterotetramer composed of different, tissue specific combinations of the three enzyme isoforms: muscle (M),

platelet (P) and liver (L). Transcriptionally, these isoforms are encoded by three different genes with L and P isoforms demonstrated to be upregulated following murine BMDM infection with Mtb (46). More recently, studies have begun to document the immune-evasive mechanisms employed by Mtb through bacterial mediated deceleration of cellular bioenergetics (43). Furthermore, pro-glycolytic isoform PFK-M has been demonstrated to play a critical role in this process where a repression in host cell glycolysis in Mtb infected macrophages was dependent on the sustained inhibition of PFK-M through bacterial mediated induction of anti-inflammatory microRNA-21 (41).

The final irreversible step in the glycolytic pathway is catalysed by pyruvate kinase (PK), which works to convert phosphoenolpyruvate (PEP) to pyruvate and ATP. Recent studies into the role of PK in classically activated macrophages has begun to unravel the complexities of immune-metabolic crosstalk, where PK splice variant, PKM2 has been identified as a key metabolic regulator in the activation of master pro-inflammatory transcription factor hypoxia-inducible factor 1 (HIF-1) and subsequent induction of IL-1 β (48). Specific inhibition of PKM2 during Mtb infection of macrophages results in decreased bacterial containment, highlighting the importance of this metabolic node during infection.

Lactate dehydrogenase (LDH) catalyses the interconversion of glycolytic end product pyruvate and lactate, with accompanied interconversion of NADH and NAD⁺. Structurally, LDH is composed of homo/heterotetramers with isoform LDH-A dominance preferentially converting pyruvate into lactate, whilst LDH-B preferentially works in reverse. Recent studies have begun to assess the consequences of lactate accumulation during Mtb infection with significant quantities recorded both intra- and extracellularly at the site of infection and within the granuloma (47, 209, 210). Mtb has shown to capacity to use lactate as its sole carbon source to promote bacterial expansion, however, at higher concentrations lactate may also be toxic towards the bacilli (211). Furthermore, generation of a mutant strain of Mtb that lacks the ability to process lactate through the lactate dehydrogenase gene (*Lld2*) results in increased bacterial sensitivity to the toxic effects of lactate. This limits bacterial exposure to lipid-derived carbon sources, resulting in upregulation of mycobacterial encoded *Lld2*, highlighting

the use of lactate as a substitute fuel source (211, 212). These observations support the notion of Mtb targeting lactate as a carbon source in an attempt to limit its accumulation to toxic concentrations.

Alternatively, studies have demonstrated the antimicrobial properties of lactate accumulation through upregulation of HIF-1 α during Mtb infection of macrophages (213). This metabolic axis is highlighted by the diminished induction of LDH-A in HIF-1 α deficient macrophages, with LDH-A deficient cells displaying heightened bacterial burden when compared to wild-type counterparts. Whether this is due to direct effects of lactate accumulation or an effect of increased glycolytic activity remains to be explored. NAD⁺ generation helps maintain cytosolic pH which impacts mitochondrial function and thus impacts the production of cytokines linked to TCA-responses. More recently, through the development of comparative infection models where live Mtb strains are examined with respect to their inactivated counterparts, evidence indicates that Mtb aims to decrease the accumulation of glycolytic metabolites as a means of immune evasion (41, 43).

1.2.2 Glycogen metabolism

Glycogen is a highly branched polysaccharide composed of glucose and stored intracellularly as a long-term energy reserve. Initially, it was primarily recognised as being produced by the liver and muscle tissues to supply energy to cells and regulate blood glucose levels. However, the role of glycogen is now emerging as more complex, especially in the context of immune cell function. Recent work has demonstrated a role for glycogen in the regulation of CD8⁺ memory T cell function, specifically in memory formation and regulation (214). Moreover and in the context of macrophage function, glycogenolysis is demonstrated as a key process that governs the acute inflammatory response, including the secretion of IL-6, TNF- α and IL-1 β (215). The study further demonstrated that glucose-6-phosphate derived from glycogen is directed toward supporting PPP activity rather than proceeding through traditional glycolysis, thereby enhancing inflammatory responses. Previous research had linked PPP driven

metabolism primarily to glucose uptake and its conversion to glucose-6-phosphate by hexokinase. However, it is now understood that glucose uptake is also directed towards glycogen storage, which later supports PPP based inflammatory responses.

1.2.3 The Pentose Phosphate Pathway

The PPP is a glucose metabolising pathway that runs analogous to glycolysis and consists of two distinct phases: the oxidative PPP (oxPPP) and non-oxidative PPP (non-oxPPP). The oxPPP diverts from glycolysis after HK generation of glucose 6-phosphate (G6P) and results in the generation of ribulose 5-phosphate (R5P). Additionally, the oxPPP generates two molecules of NADPH for every G6P consumed. In total, the oxPPP consists of three enzymatic reactions before carbon transfer into the non-oxPPP branch. The first committed and rate limiting step is catalysed by glucose 6-phosphate dehydrogenase (G6PD), converting G6P and co-factor NADP^+ into 6-phosphogluconolactone and NADPH. Notably, G6PD has been classified as the most commonly mutated enzyme in humans to date, resulting in the generation of frangible red blood cells (RBCs) and subsequently confers protection against malaria (216). The third irreversible step of the oxPPP generates R5P in combination with the second molecule of NADPH, following 6-phosphogluconate dehydrogenase (6PGD) activity.

The reversible nature of reactions undertaken in the non-oxPPP enable for interconversion of key metabolic metabolites including the oxPPP end-product, R5P. This flexibility also enables the efficient flow of carbon between glycolysis and the PPP, enabling the transfer of carbon through these key pathways to meet specific requirements of the cell. Glycolytic intermediates subject to consumption by non-oxPPP consumption include F6P and glyceraldehyde 6-phosphate (G6P). The interconversion into these pentoses are catalysed by a series of enzymes including ribulose 5-phosphate epimerase (RPE), ribose 5-phosphate isomerase (RPI), transaldolase (TALDO) and thiamine dependent transketolase (TKT).

The intertwined character of the PPP with other key metabolic pathways such as glycolysis enables this pathway to adopt a dynamic nature influenced by cellular requirements. Different modes of PPP carbon trafficking are classified as the following: pentose insufficiency, pentose overflow and pentose cycling. In pentose insufficiency, the cellular demand for ribose is greater than that of NADPH. Here, the non-oxPPP branch works to reroute glycolytic intermediates F6P and G6P towards the generation of R5P as it is insufficiently supplied by the oxidative branch. During pentose overflow, there is a greater demand for oxPPP generated NADPH with an associated overflow of R5P produced. In these circumstances, non-oxPPP enzymes work to consume and reroute this unnecessary carbon load back towards conventional glycolysis. Lastly, pentose cycling is employed when the cell requires access to a larger NADPH reservoir. During cycling the excessive carbon used to produce R5P is rerouted towards glycolytic intermediates, where it is redirected and recycled to generate G6P, now subject to re-enter the oxPPP to support further NADPH generation. Theoretically, with gluconeogenic enzymes fructose 1,6-bisphosphatase (FBP) and glucose 6-phosphate isomerase (GPI) active, pentose cycling has the potential to lead to complete oxidation of glucose into six molecules of CO₂, producing 12 NADPH molecules. This complete oxidation of glucose has yet to be observed in mammals, however, partial cycling has been recorded in immune cells such as neutrophils in instances that require a heightened oxidative burst to aid in pathogen killing (217).

Activity of the PPP is subject to multiple levels of regulation and correlates with both cellular bioavailability and requirements, such as the NADP/NADPH ratio and input of an adequate carbon source, primarily G6P. The oxPPP is greatly influenced by levels of NADP, acting as both a substrate and allosteric regulator of rate-limiting and entry point enzyme, G6PD (218, 219). Consequently, non-oxPPP activity adapts to consume or produce pentose phosphates to meet cellular demands and in turn determines direction of carbon flux. In addition to post-translational/allosteric regulation, the PPP may also be subject to transcriptional modulation during certain instances such as the demand for NADPH in response to oxidative stress. Under these conditions, transcription factor family nuclear respiratory factor 2 (NRF2) promotes upregulation of multiple PPP associated genes including G6PD, 6PGD, TKT and TALDO (220, 221). DNA damage may

also influence transcriptional upregulation through the combinatory activity of ataxia-telangiectasia mutated (ATM) serine/threonine kinase and heat shock protein 27 (HSP27) (222-224).

PPP contribution to cellular process may be readily required, such as the maintenance of redox homeostasis and phagocyte mediated pathogen killing. To overcome this rapid demand for pathway responsiveness, PPP enzymes display reserve capacity flux and are present in greater amounts than needed to facilitate basal pathway activity. Basally, activity is restricted by limited availability of NADP but may increase instantaneously when NADP levels increase during oxidative stress (217, 225, 226).

Due to the dynamic relationship between PPP and glycolysis, regulation of one pathway may be used to directly influence activity of the other. As such, under cellular oxidative stress, a reduction in glycolytic carbon flux supports G6P accumulation and consequently boosts oxPPP substrate availability. This primarily occurs through susceptibility of key glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase M2 (PKM2), to active site cysteine residue oxidation and subsequent reduction in their catalytic capacity (226-230). Alternatively, stimulation of glycolysis through development of small molecule activators of glycolytic gatekeeper PFK-L results in decreased PPP-derived oxidative burst in neutrophils, whilst loss of PFK promotes NADPH (231).

1.2.4 Macrophage pentose phosphate pathway activity

The contribution of PPP activity to cell fate and function makes it a key player in immune responses, namely impacting oxidative environments and cytokine production. Phagocytic cells, such as macrophages and neutrophils, employ PPP activity for the generation of an oxidative burst to aid in microbial killing. Here, PPP derived NADPH is used to support the activity of NOX and iNOS, leading to the production of superoxide and nitric oxide respectively. Classical activation of macrophages to a pro-inflammatory state through the use of lipopolysaccharide (LPS) and subsequent induction of Warburg

metabolism reveals and increase in PPP activity 1 hour post stimulation as per [1,2-¹³C] glucose isotope tracing (51, 232, 233). This in turn, increases NADPH contingent and NOX activity. Inhibition of NADPH accumulation from oxPPP activity leads to reduced generation of macrophage ROS and RNS (215, 234). However, the effects of oxPPP activity on expression of both iNOS and cytokines remains much more elusive and are yet to undergo proper investigation. Macrophage capacity to clear haem has been demonstrated as dependent on oxPPP activity. Although this requires further evaluation, is most likely a result of PPP derived NADPH acting as a required co-factor for haem oxygenase 1 (HMOX1), converting haem into biliverdin and carbon monoxide. Haem uptake has been demonstrated to promote macrophage uptake of extracellular glucose to promote PPP activity, a phenotype that can be also generated through treatment with carbon monoxide donor molecule tricarbonylchloro(glycinato)ruthenium. Altogether, these observations suggest HMOX1 derived carbon monoxide supports oxPPP activity in an effort to further promote haem clearance (235).

Overall, metabolism strongly influences effector functions of macrophages, however, further work is required to evaluate the role of PPP activity during Mtb infection. To date, studies have aimed to highlight glycolytic contribution during infection, but with more emerging data highlighting the importance of PPP activity in immunometabolic responses, such alternative carbon fates must now also be considered.

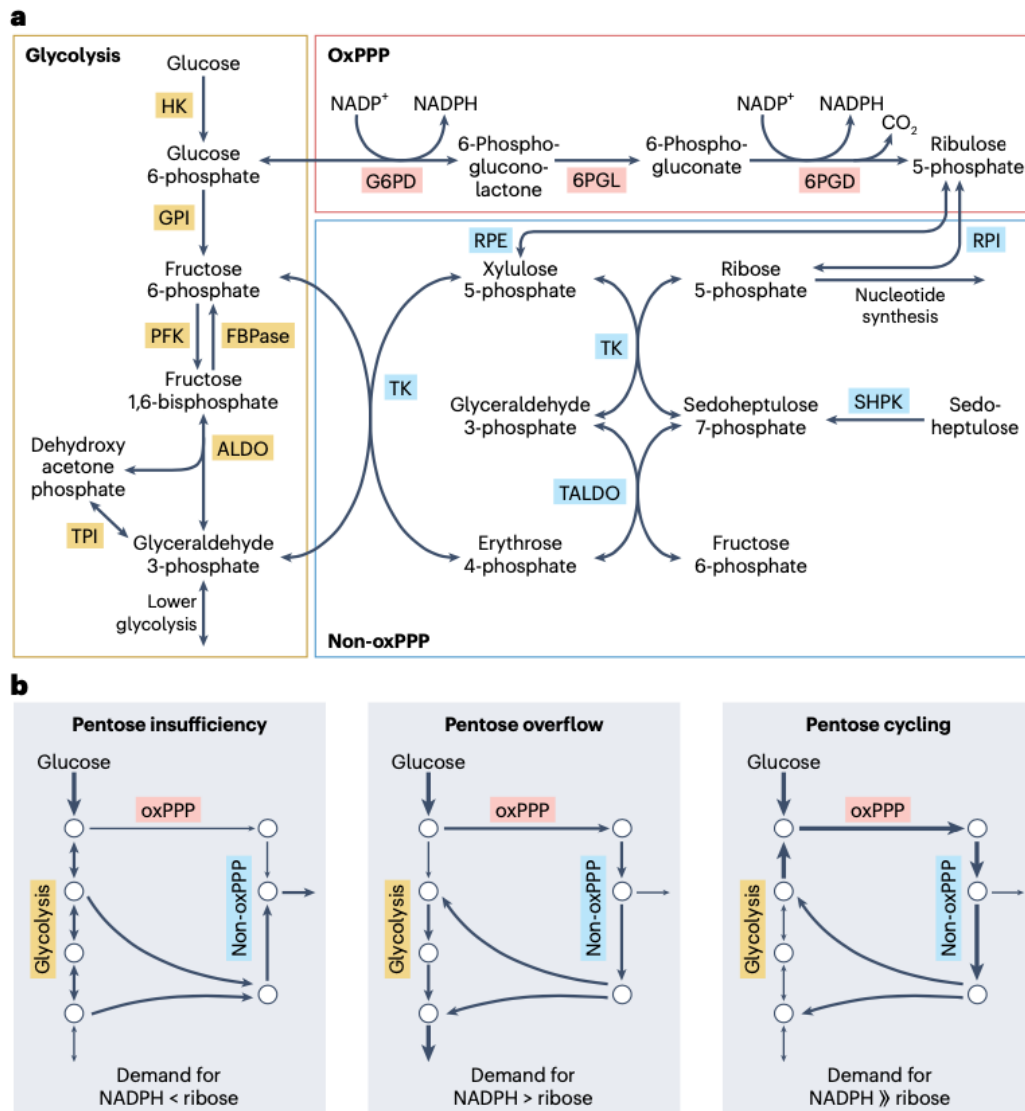


Figure 1. 4 Glycolysis and the Pentose Phosphate Pathway

(a) Once taken up, glucose is converted to glucose 6-phosphate and may continue through glycolysis or diverted towards the PPP. Phosphofructokinase (PFK) catalysis rate-limiting and committed step of glycolysis where carbon can continue through central carbon metabolism. G6PD is the rate limiting entry point into the OxPPP where reactions are irreversible and require NADP^+ as co-factor for generation of NADPH and Non-OxPPP entry point metabolite ribulose 5-phosphate. Non-OxPPP reactions are reversible and facilitate carbon re-entry into glycolysis via generation of fructose 6-phosphate and glyceraldehyde 3-phosphate. Non-OxPPP generates ribose 5-phosphate for nucleotide synthesis. (b) Modes of PPP operation. Pentose insufficiency: when unmet ribose demands leads to net Non-OxPPP flux towards ribose 5-phosphate generation. Pentose overflow: higher NADPH demand over ribose (after 2:1 stoichiometry) results in net OxPPP flux towards glycolysis. Pentose cycling: High NADPH requirements result in glycolytic glucose 6-phosphate isomerase (GPI) to work in reverse, regenerating glucose 6-phosphate, now available to re-enter the OxPPP. Reproduced from TeSlaa *et al.* (236).

1.2.5 The TCA cycle

The TCA cycle plays a central role in macrophage metabolic reprogramming. However, in addition to its traditional role in fulfilling cellular energy requirements, it is now appreciated that the TCA cycle and associated regulation of metabolite generation plays a key principal role in shaping immune cell responses, particularly that of macrophages. Classical activation of macrophages with agonists such as LPS results in the suppression of mitochondrial oxidative phosphorylation due to the introduction of two metabolic breaks within cycle, enabling the accumulation of two particularly immunomodulatory metabolites: itaconate and succinate. Itaconate generation occurs as a result of the first metabolic break, traditionally where isocitrate dehydrogenase (IDH) generates alpha-ketoglutarate from isocitrate. Here, activated macrophages downregulate IDH transcription in substitution for the induction of aconitate decarboxylase 1 (IRG1), mediating citrate and isocitrate derived cis-aconitate oxidation to itaconate. The second breakpoint occurs through downregulation of succinate dehydrogenase (SDH), leading to the subsequent accumulation of succinate rather than its conventional conversion to fumarate.

SDH mediated oxidation of succinate to fumarate contributes to macrophage inflammatory response through mitoROS generation in addition to stabilising HIF-1 α and thereby promoting transcription of IL-1 β . This observation is supported through murine injection of LPS in conjunction with a SDH inhibitor, dimethyl malonate, resulting in reduced inflammation, namely the secretion of pro-inflammatory cytokines TNF- α and IL-1 β (146). In the context of Mtb infection, studies have documented a decrease in transcription of multiple SDH subunits (SDHA, SDHC, SDHD), leading to the accumulation of cellular succinate (237). *In vivo* murine Mtb infection has also demonstrated the accumulation of succinate within the lungs (209). Furthermore, employment of isotope tracing in the context of Mtb infection of hMDMs also displays a reduction in oxidation of succinate to fumarate (43). Given the role of TCA SDH in macrophage production of bactericidal IL-1 β , further work is required to investigate the possibility of Mtb mediated accumulation of succinate within host cells as a means of immune evasion. Additionally,

succinate has been demonstrated to promote IL-1 β secretion through direct interaction with G-protein coupled receptor SUCNR1, with LPS stimulation leading to both enhanced expression of the receptor and metabolite accumulation (238, 239). However, SUCNR1 derived generation of IL-1 β has yet to be investigated in the context of Mtb infection and may provide further insight into possible bacterial immune evasive strategies through manipulation of host metabolic signalling.

SDH oxidation of succinate during classical activation of macrophages is further inhibited through *Irg1* mediated generation of itaconate from TCA intermediate cis-aconitate (240-242). The anti-inflammatory properties of itaconate have also been demonstrated to occur through its capacity to promote transcription factor Nrf2, leading to a reduced inflammatory transcriptional signature and the reduction of type 1 interferons (243). With respect to Mtb, itaconate accumulation has been observed within the murine lungs during infection with IRG1 also demonstrated to be massively upregulated in Mtb infected murine macrophages (153, 237). Alternative studies have demonstrated an upregulation of IRG1 as an important factor in the host response to infection, with knockout mice experiencing excessive inflammation and higher Mtb burden (244). Metabolic profiling of murine lungs over a time course of Mtb infection has demonstrated an initial (week 4) accumulation of succinate that reduces over time (week 9) with the concurrent increase in itaconate levels (245). However, further work is required to extrapolate whether these metabolic shifts are a result of host immunometabolic responses or alternatively, bacterial mediated manipulation of host cell central carbon metabolism in an attempt to evade bactericidal mechanisms.

1.3 Nitric oxide and macrophage metabolism

1.3.1 Macrophage Nitric oxide generation

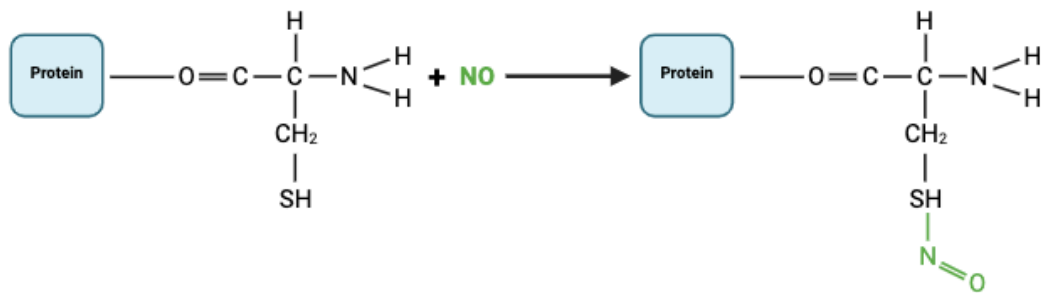
NO is a soluble, endogenous gas produced through catalytic activity of NO synthase (NOS) enzymes. During this reaction, L-arginine and O₂ are converted through intermediate N-hydroxy-L-arginine to L-citrulline and NO. This five carbon oxidation of L-arginine requires electron donation from reducing agent NADPH (246). Under homeostatic physiological conditions, NO is constitutively produced in nanomolar quantities through the activity of NOS1 and NOS3, located in endothelial and neuronal tissues respectively. In these tissues, any potentially harmful effects of NO signalling are mitigated by the favourable kinetics of its target reactions. For instance, high rate constants allow NO to rapidly interact with metal ions, such as the iron atoms in the heme groups of proteins like haemoglobin, myoglobin, soluble guanylate cyclase (sGC), cytochrome P450, and even NOS itself (247-252). NO-mediated physiological processes include muscle relaxation, inhibition of platelet aggregation, and neuronal transmission, all of which occur through the activation of sGC and the subsequent production of cyclic guanosine monophosphate (cGMP). Alternatively, NO has been demonstrated to play a key role in the generation of an effective immune response, functioning in anti-microbial defences or as a mediator of immune regulation (253-261). In these contexts, NO may need to be produced and maintained at micromolar levels for extended periods. To elicit such effects, employment of an inducible catalyst, iNOS/NOS2, is needed. The generation of NO during classical activation of macrophages (BMDMs) has been previously described as cytokine and stimuli specific and NO concentration may be classified as low, medium or high depending upon treatment with IFN- γ +IL-1 β , IFN- γ +TNF α and IFN- γ +LPS respectively (262, 263). Moreover, these varying NO concentrations were found to directly correlate with two key factors: the expression levels of *Nos2* and the density of cells expressing *Nos2*. Higher NO production is associated with increased *Nos2* expression and a greater number of cells expressing *Nos2*, indicating that both factors play a crucial role in regulating the extent of nitric oxide production during immune responses. With such an ability for macrophages to

produce a finely tuned NO response depending upon activation status or surrounding immune microenvironment, it is now important to consider how NO may play a pivotal role in modulating immune cell function and how this intersects with the metabolic state of the cell.

Nitrosylation is the term given to modifications involving the direct addition of NO. Here the NO group is added to a molecule, typically targeting proteins, lipids, or nucleic acids. One common form, S-nitrosylation, involves the attachment of an NO group to the thiol group of cysteine residues in proteins, which can alter their function, stability, or localization. Nitrosylation plays an important role in various physiological processes, including cell signalling, regulation of enzyme activity, and immune responses. It can also have pathological effects when dysregulated, contributing to conditions such as inflammation, neurodegeneration, and cardiovascular disease (264-266). Alternatively, the term 'nitrosation' is given to a chemical process in which a nitroso group ($-\text{NO}$) is added to a molecule, often targeting amines to form nitrosamines. This reaction typically occurs under acidic conditions, where nitrite (NO_2^-) reacts with amines to produce nitrosating agents. While potent oxidants, NO_2 and peroxynitrite (ONOO^-) cannot directly participate in nitrosation reactions but they serve as important precursors to nitrosating species. Peroxynitrite (ONOO^-) serves as a precursor to nitrosating species through its ability to decompose into reactive nitrogen intermediates. Formed by the rapid reaction of nitric oxide (NO) with superoxide (O_2^-), peroxynitrite can undergo protonation or react with carbon dioxide to generate reactive nitrogen species (RNS) like nitrogen dioxide (NO_2) and nitronium ion (NO_2^+), both of which are potent nitrosating agents. These species are capable of transferring nitroso groups ($-\text{NO}$) to proteins, lipids, and nucleic acids, resulting in nitrosation reactions. NO is known for its antimicrobial properties, exerting deleterious effects on bacterial and microbial growth by damaging microbial proteins, DNA, and cell membranes. However, at higher concentrations, NO can also have harmful effects on the host's own cells and proteins. The impact of NO on the host is closely tied to both its spatial and temporal concentration, as localised and transient bursts of NO are necessary for effective immune defense without damaging the host tissue. Prolonged or excessive NO production can result in oxidative stress, impairing host protein function and potentially contributing to tissue damage and

inflammation. Therefore, precise regulation of NO levels is crucial for balancing its protective role against pathogens with minimising harm to the host.

(A) S-nitrosylation



(B) Peroxynitrite reaction pathway

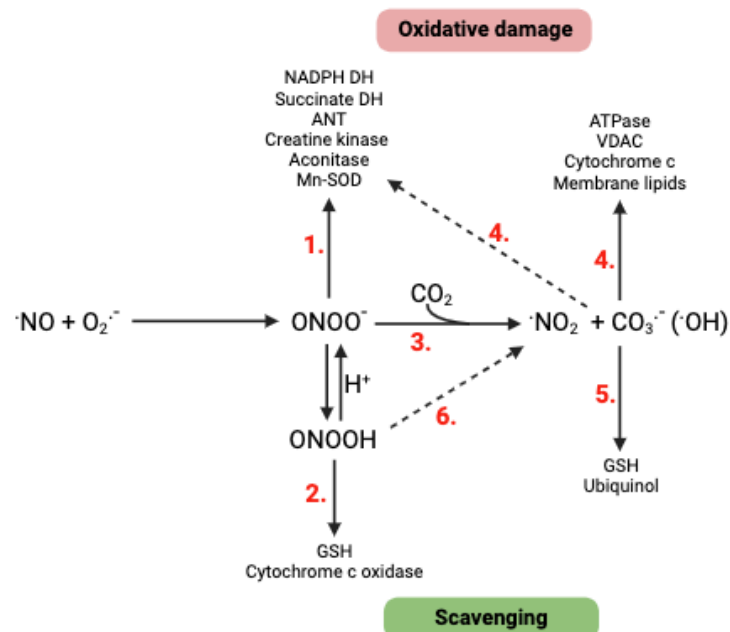


Figure 1. 5 S-nitrosylation and peroxynitrite reaction pathways

(a) S-nitrosylation at cysteine residues. After synthesis, nitric oxide (NO) reacts with cysteine residues of proteins forming a nitrosylated compound. (B) Peroxidation reaction pathway. The controlled reaction of $\cdot\text{NO}$ with $\text{O}_2^{\cdot-}$ result in the formation of peroxynitrite (ONOO^-). Here peroxynitrite can undergo biomolecular reactions with targets (1, 2) or alternatively react with CO_2 (3) resulting in the formation of $\text{CO}_3^{\cdot-}$ and $\cdot\text{NO}_2$ radicals and thus initiate free radical chemistry (4, 5). Minimal quantities of peroxynitrite undergoes proton-catalysed homolysis, resulting in the generation of $\cdot\text{OH}$ and $\cdot\text{NO}_2$ (6), which in turn can lead to lipid oxidation. Oxidative damage from peroxynitrite accumulation may be partially prevented by the activity of scavenging enzymes.

1.3.2 Nitric oxide and immunometabolism

As previously described, classical activation of macrophages is accompanied by alterations to traditional carbon metabolism in order to fulfil new cellular energy requirements and subsequently support immune effector functions, typically referred to as Warburg metabolism. This typically results in enhanced (aerobic) glycolysis with conversion of pyruvate to lactate and inhibition of TCA cycle and mitochondrial respiration. Following this, the effects of NO on central carbon metabolism during metabolic reprogramming to date are predominantly attributed to observations documenting NO mediated inhibition of electron transport chain complexes such as the competitive inhibition of Complex IV or s-nitrosylation of Complex I (267-269). Others postulate NO mediated s-nitrosation of other metabolic enzymes would alter enzyme kinetics and activity (270, 271).

Recent works have aimed to further investigate metabolic alterations accredited to NO signalling during classical activation of macrophages. Through utilisation of NOS2 inhibitors and NO donors in addition to knockout mouse models, such as *Nos2*^{-/-}, the interplay between NO signalling and central carbon metabolism is in its early stages of unravelment (53, 262, 272). It is not surprising that NO mediated effects on metabolic activity are variable, with early conflicting results attributed to differential activation states in individual cell types. For instance, during LPS induced activation of DCs, an increase in glycolytic activity was identified as a consequence of NO production by NOS2, leading to mitochondrial dysfunction, whilst DCs deficient in NOS2 did not exhibit the same glycolytic shift (273). Alternatively, another study found a transient increase in glycolytic activity in DCs even in the absence of *Nos2*, though this was a short-lived state, after which the cells returned to near-baseline metabolic activity (274). Together, these findings suggest an initial NO independent boost in glycolysis, but sustained upregulation relies on the induction of *Nos2* and subsequent NO production. This sustained commitment to glycolytic activity in DCs had been demonstrated as mTOR/HIF-1 α /NOS2 dependent (273, 275). With respect to activation of NO producing

DCs, it is also important to note that HIF-1 α activity is required during sustained glycolytic commitment rather than its initial induction (274).

Such results highlight a biphasic nature to the metabolic response which has been highlighted in both classical LPS-activated hMDMs and murine macrophages and details the following: initial upregulation glycolytic carbon consumption with associated TNF α and IL-1 β secretion. Following from this, second wave commitment to glycolysis requires downregulation of cellular oxygen consumption and is dependent on NOS2 derived NO (276).

The role of NO in regulating macrophage classical activation goes beyond its early recognized inhibition of OXPHOS. Recent studies have revealed that NO induces a metabolic break in the TCA cycle between citrate and α -ketoglutarate. This disruption was initially attributed to a temporary reduction in *Idh1* expression, which catalyses the conversion of isocitrate to α -ketoglutarate (36). However, further evidence suggests that this break is primarily due to NO's direct inhibition of aconitase (ACO2), the enzyme responsible for converting citrate to isocitrate through cis-aconitate, which occurs before IDH's involvement. Loss of NOS2 activity results in reduced carbon flux between citrate and α -ketoglutarate, leading to citrate accumulation (36). Interestingly, this NO-driven model is not essential in conditions where IDH activity is already diminished. Studies have shown that IDH activity is reduced in systems lacking the NOS cofactor tetrahydrobiopterin, with NO supplementation further enhancing this downregulation (272). It's important to note that many IDH activity assays fail to distinguish between IDH1 (cytoplasmic) and IDH2 (mitochondrial), with IDH1 unlikely to impact the TCA cycle. In contrast, NO has a well-documented inhibitory effect on the Fe-S cluster within ACO2, reducing its activity during macrophage polarization (53, 277-279). Additionally, activated macrophages exhibit impaired citrate respiration, while isocitrate-driven mitochondrial respiration remains unaffected (53).

Similar to its role in macrophages, NO can inhibit mitochondrial respiration in T-cells by targeting components of the ETC (280, 281). Here, it is proposed that NO-mediated repression of T-cell mitochondrial activity occurs as a result of NO being produced by

antigen-presenting cells or supplied 'in trans' by the microenvironment. This inhibition forces activated T-cells to rely on glycolysis, which supports their effector functions such as cytokine production and cytotoxic activity. However, the effects of NO on T-cells are highly context-dependent. In some cases, high concentrations of NO can suppress T-cell proliferation and function by inducing mitochondrial dysfunction and promoting apoptosis (282, 283). This immunosuppressive effect of NO is particularly relevant in chronic infections and cancer, where persistent inflammation and high NO levels can contribute to immune exhaustion and impaired T-cell responses (284). On the other hand, NO can enhance T-cell function by modulating key signalling pathways, such as NF- κ B and HIF-1 α . NO can activate NF- κ B in T-cells, leading to the expression of genes involved in T-cell activation, cytokine production, and survival (285, 286). Additionally, NO stabilizes HIF-1 α , which promotes glycolysis and enhances the effector functions of T-cells in hypoxic environments, such as inflamed tissues and tumours (287-289). Through these pathways, NO not only affects T-cell metabolism but also modulates their activation and differentiation into various functional subsets (283).

NO can inhibit mitochondrial respiration in DCs by targeting the ETC, similar to its effects in macrophages and T-cells. This inhibition drives DCs towards glycolysis, providing the energy required for antigen processing, migration, and the upregulation of co-stimulatory molecules necessary for T-cell activation (273). Additionally, NO influences the production of key inflammatory cytokines, such as IL-12, which is critical for promoting T-helper 1 (Th1) responses (290). By modulating both metabolism and cytokine production in DCs, NO plays a crucial role in linking innate and adaptive immune responses.

As NOS derived NO leads to introduction of the primary TCA breakpoint, anaplerotic pathways become upregulated in order to provide essential metabolites to support classical activation of macrophages, including glutaminolysis, pyruvate decarboxylation and malic enzyme activity. The extent at which such metabolic pathways are utilised is augmented during stimulation in an NO dependent manner (291). The engagement of alternative carbon sources has also been linked to NO's targeting of pyruvate dehydrogenase (PDH), particularly its E3 subunit. This subunit plays a critical role in re-

oxidising the reduced lipoyl groups of the E2 subunit through NAD⁺ reduction to NADH (53). Inhibition of PDH's E3 subunit has been associated with the activation of the glutamine-derived gamma-aminobutyric acid (GABA) shunt, involving key enzymes like α -ketoglutarate dehydrogenase (OGCD) and the branched-chain α -ketoacid dehydrogenase complex (BCKDH). This pathway allows the production of succinate from α -ketoglutarate or glutamate-derived GABA (122). However, these metabolic pathways are still in the early stages of understanding, and further research is needed to fully elucidate their role in macrophage activation

Classical activation of macrophages is characterised by a decline in mitochondrial ETC protein complexes, previously attributed to NO mediated post-translational modifications. However, further studies have shown that while NO is sufficient for this effect, the presence of iNOS is essential, pointing to a more specific role for NO in modulating Complex I activity (53). Here, It is proposed that NO's inhibition of the TCA enzyme aconitase (ACO2) leads to a reduced supply of substrates, such as low NADH levels that are necessary for Complex I function. This substrate deficiency causes Complex I to accumulate in its deactivated form, making it more susceptible to modification and eventual degradation (292-295). Accordingly, inhibition of NOS2 during macrophage stimulation can restore ETC function and maximal respiratory capacity, with subjection to low NO also resulting in recovery of respiratory capacity but not basal oxygen consumption (53, 262, 296). An alternative study suggests another potential mechanism for NO-mediated downregulation of Complex I activity. In this case, NO-producing macrophages showed decreased expression of NADH dehydrogenase ubiquinone flavoprotein 2 (Ndufv2), the catalytic subunit of Complex I responsible for binding NADH, compared to Nos2^{-/-} cells. Although this hypothesis remains unproven, the loss of this N-terminal subunit could impair Complex I's ability to transfer electrons from NADH to ubiquinone, potentially contributing to the overall reduction in Complex I activity. (272).

1.3.3 The impact of NO on inflammatory cytokine production

As NO plays a key functional role in the orchestration of TCA metabolism, it has also been demonstrated to influence pro-inflammatory cytokine secretion during macrophage activation. Introduction of metabolic breakpoints in TCA cycling are deemed vital in mounting an effective immune response within macrophages (297, 298). NO directly regulates cytokine expression by influencing signalling pathways like NF- κ B and hypoxia-HIF-1 α , which are crucial transcription factors in the production of pro-inflammatory cytokines. NO enhances the secretion of cytokines such as TNF- α , IL-6 and IL-12 (253, 299), all of which are vital for promoting inflammation and activating Th1 responses. However, NO also has immunosuppressive effects under certain conditions, where prolonged or excessive NO production can inhibit cytokine synthesis and dampen immune responses. Recent observations suggest a functional TCA promoted by loss of cellular NO results in the heightened stabilisation of the oxidative stress sensor Kelch-like ECH-associated protein 1 (KEAP1) and subsequent anti-inflammatory NRF2-mediated transcription (300). Such a result implicates NO as a mediator of the antioxidant response at the expense of full macrophage pro-inflammatory potential. Other works have also demonstrated classical macrophage polarisation in the absence of reprogramming of mitochondrial respiration (272, 301). In line with above observations, LPS stimulated *Nos2*^{-/-} macrophages displayed glycolytic reprogramming and heightened secretion of pro-inflammatory mediators IL-1 β , TNF α and IL-6. Furthermore, augmented levels of IL-1 β in *Nos2*^{-/-} BMDMs were found to be independent of HIF-1 α signalling (53). These observations underscore the immunomodulatory role of NO, suggesting that its effects may vary depending on its levels, potentially inhibiting inflammation while promoting antimicrobial activity or other processes during infection, possibly through mechanisms like NRF2 activation.

1.3.4 NO and human macrophages

The generation of NO and its subsequent contribution to metabolic responses within human monocytes and macrophages remains a heavily disputed topic. Many studies

have reported restricted expression of the macrophage *Nos2* locus due to its hypermethylated state resulting in a closed conformation (302, 303). However, within circumstances of acute and chronic lung inflammation such as *Mtb* infection, *Nos2* expression has been detected within human alveolar macrophages (139, 304-306). Additionally, both detection and measurement of exhaled NO is possible in *Mtb* infected patients (141), however, the source of this NO requires further investigation. Current evidence suggesting that alveolar epithelial cells may contribute to its production (307). Nonetheless, its mere presence raises the possibility that NO could play a role in modulating macrophage function. Moreover, alternative studies have demonstrated the potential of human derived macrophages to produce NO in the specific context of *Mtb* infection (308). Furthermore, supplementing key NOS2 cofactors, such as tetrahydrobiopterin (BH4) and L-arginine, not only boosted the cells' NO production but also promoted increased intracellular bacterial replication, raising questions about the bactericidal role of NO in the context of *Mtb* infection. An alternative hypothesis within this NO centric dilemma suggests that both human monocytes and macrophages are unable to synthesise BH4 and require its generation by surrounding cells, possibly lymphocytes (309, 310). Nevertheless, more evidence is required to support this model (311).

In contrast to classical activation of murine BMDMs, stimulation of hMDMs derived from human peripheral blood monocytes (PBMCs) results in heightened glycolytic commitment without displaying a disruption to TCA metabolism (276, 312). Such observations are in agreement with a lower NO environments. Isotope tracing has revealed continued incorporation of glucose into TCA metabolites downstream of conventional metabolic breakpoints such as α -ketoglutarate and malate during activation of hMDMs (313). Similarly, upon LPS stimulation, hMDMs display an unaltered oxygen consumption rate when compared to unstimulated controls (276). This data suggests the continued metabolism of glucose derived carbon through the TCA cycle in a low NO environment.

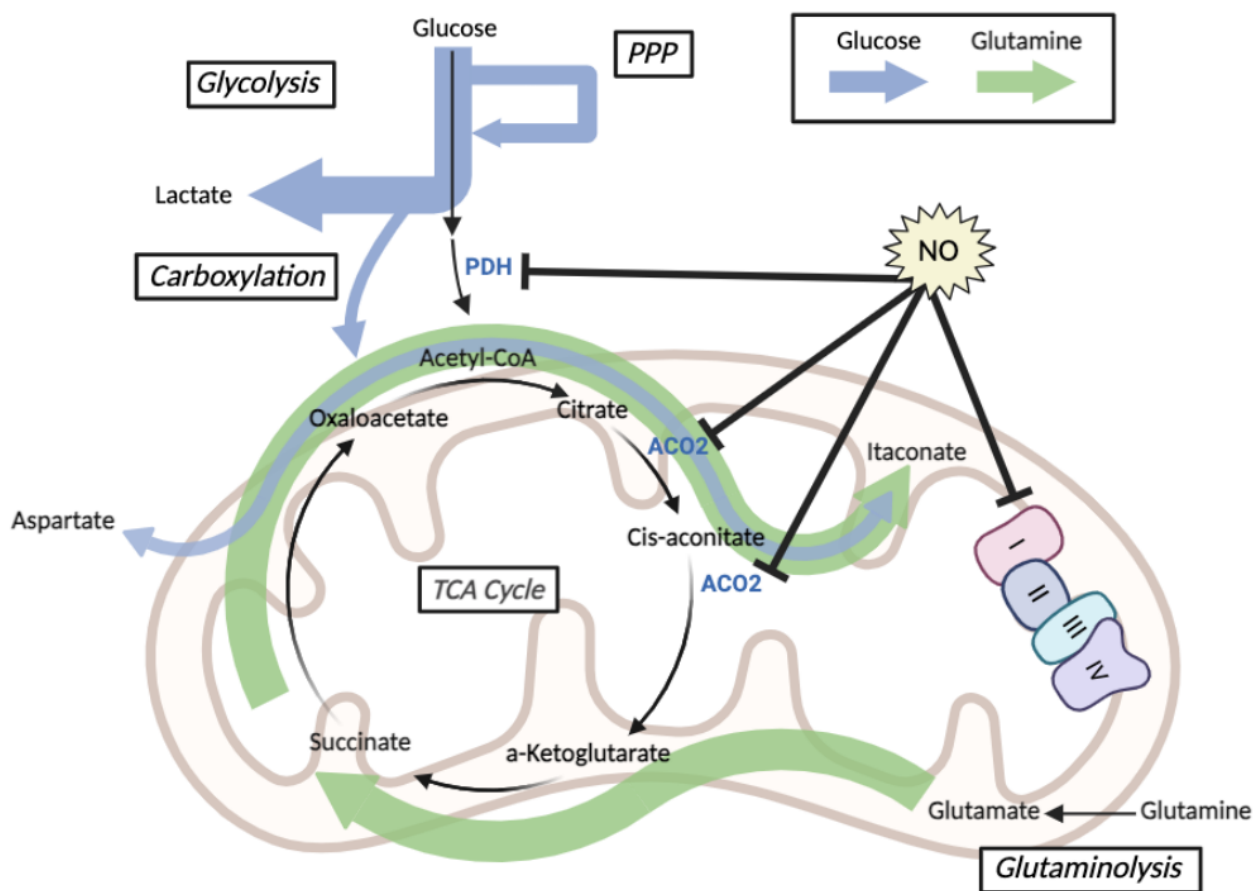


Figure 1. 6 Nitric Oxide and classical activation of Macrophages

During M1 polarisation of Macrophages, NO plays a major role in orchestration of metabolic rewiring. Firstly, NO inhibits activity of Aconitase 2 (ACO2), decreasing carbon entry into the TCA cycle through Pyruvate dehydrogenase (PDH) activity. Consequently, carboxylation and glutaminolysis are upregulated to compensate and result in accumulation of Citrate and Itaconate. The TCA cycle break and subsequent reduction in cellular NADH levels results in the inactivation of mitochondrial OXPHOS. Decreased mitochondrial oxidation results in heightened glycolytic commitment to meet cellular energy requirements. Adapted from *Palmieri et al, 2020 (53)*.

1.4 Thesis Aims

To date, Mtb remains as the world's most successful pathogen, demonstrating a unique capacity to evade innate immune responses, particularly that of its primary target cell of infection – the macrophage. Work within the Sheedy lab and others have shown that upon infection of macrophages, Mtb displays the ability to induce a state of host cell decelerated bioenergetic metabolism (41, 43). Furthermore, glycolytic metabolism has emerged as a key feature in macrophage antimicrobial responses against Mtb infection. However, little is known in how this process is regulated. Work within our group has demonstrated a Mtb mediated deceleration of macrophage glycolytic metabolism, a process dependent on the sustained upregulation of anti-inflammatory microRNA-21 and subsequent targeting of glycolytic gatekeeper PFK-m by proliferating bacteria (41). This suppression of central carbon metabolism facilitates bacterial expansion by limiting production of pro-inflammatory IL-1 β , a chief cytokine required for macrophage anti-mycobacterial responses. This thesis sets out to examine consequences of this Mtb induced metabolic breakpoint and **hypothesises bacterial employment of host macrophage Pentose Phosphate Pathway (PPP) activity in an effort to create an immuno-permissive environment, favouring bacterial replication.**

1.4.1 Specific Objectives:

1. To examine the role of the PPP with respect to activity and function of macrophages in the context of *Mycobacterium tuberculosis* infection.
2. To define the PPP as a target for Mtb mediated immune evasion and examine potential for targeting the PPP in a therapeutic manner.
3. To investigate possible rationale for Mtb induction of the PPP as a means of host cell macrophage immune evasion.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Reagent – Cell culture	Supplier
Phosphate buffered saline (PBS)	Merck
Dulbecco modified eagle medium (DMEM)	Bio-Sciences
Roswell Park Memorial Institute Medium (RPMI 1640)	Bio-Sciences
Foetal bovine serum	Bio-Sciences
Human serum	Merck
Human recombinant M-CSF	VWR International
L929 conditioned medium (L929)	Produced on site
Red blood cell lysis buffer	Merck
Trypan blue	Merck
Penicillin/streptomycin (pen/strep)	Bio-Sciences
Dimethyl sulfoxide (DMSO)	Merck
Seahorse XF DMEM media	Agilent
Seahorse XF calibrant solution	Agilent
L-Glutamine	Agilent
D-Glucose	Agilent

Table 2. 1 List of cell culture reagents and suppliers

Reagent – Infections (<i>Mycobacterium Tuberculosis</i>)	Supplier
Middlebrook 7H9 broth base	Merck
Middlebrook 7H10 agar base	Merck
ADC enrichment medium	Becton Dickinson
OADC enrichment medium	Becton Dickinson
Tween 80	Difco
L-asparagine	Merck
Glycerol	Difco

Cycloheximide	Merck
Paraformaldehyde (PFA)	Merck
Auramine-O	Becton Dickinson
Auramine-O decolouriser	Becton Dickinson
Hoechst 33358	Merck
Dako fluorescent mounting medium	Agilent
Triton-X	Merck

Table 2. 2 List of infection reagents and suppliers

Reagent – Cell Stimulants/ inhibitors	Supplier
6-aminonicotinamide (6-AN)	Merck
2-Deoxy-D-glucose	Merck
Oligomycin	Merck
Fluoro-carbonyl cyanide phenylhydrazone (FCCP)	Merck
Rotenone	Merck
Antimycin-A	Merck
Glycogen phosphorylase inhibitor (GPI)	Merk
Deta NONOate (DetaNO)	Cayman Chemical
Carboxy-PTIO, potassium salt (cPTIO)	Santa Cruz Biotech
Dimethyl malonate (DMM)	Merk
GSK2795039 (iNOX2)	GSK

Table 2. 3 List of cell culture stimulants / inhibitors and suppliers

Reagent – Laboratory benchtop work	Supplier
Molecular grade ethanol	VWR International
RNase-free water	Bio-Sciences
10X RT buffer	Bio-Sciences
100mM deoxyribonucleotide triphosphates (dNTPs)	Bio-Sciences
Random primers	Bio-Sciences

RNase inhibitor	Bio-Sciences
Multiscribe reverse transcriptase	Bio-Sciences
2X Taqman Fast Advanced Master Mix	Bio-Sciences
20X Taqman gene expression assay probes	Bio-Sciences
20X microRNA primers	Bio-Sciences
Tween-20	Santa-Cruz Biotech
3,3',5,5'-Tetramethylbenzidine (TMB)	Invitrogen
Sulfuric acid (H₂SO₄)	Merk
Fixation buffer	eBioscience

Table 2. 4 List of benchtop reagents and suppliers

2.1.2 Molecular Assays and Kits

Assay/Kit	Supplier
PureLink RNA Mini Kit	Invitrogen
Ready set go ELISA Kit	Invitrogen
MicroRNA reverse transcription kit	Applied Biosystems
Glucose-6-Phosphate Dehydrogenase activity assay (MAK015)	Merck
6-Phosphogluconic Acid (6-PGA) assay kit (BN00494)	Medical Supply Company
Lactate assay kit (MAK064)	Merck
NADP/NADPH assay kit (ab65349)	Abcam
Griess assay kit (ALX-400-004-L050)	Enzo Life Sciences
Glycogen assay kit (MAK016)	Merk
Glucose assay kit (EIAGLUC)	Invitrogen

Table 2. 5 List of assay/ kits and suppliers

2.1.3 Equipment

Cruinn Diagnostics Ltd.: Sterile tissue culture plates (6, 12, 24, 48, 96 well), Sterile tissue culture flasks (T25, T75, T175), Falcon tubes (15 ml, 50 ml), Eppendorf tubes (0.2 ml, 1.5 ml, 2.5ml), Cryovials (2 ml), serological pipettes (5 ml, 10 ml, 25 ml, 50 ml), pipette tips (p10, p200, p1000), sterile transfer pipettes (3 ml), syringes (1 ml, 2 ml, 5 ml, 10ml, 20 ml), ELISA plates (high binding 96 well)

Fisher Scientific: Sterile petri-dishes (10cm, 15cm), Non-sterile 96 well plates, cell strainers (70 μ M, 100 μ M), filter barrier pipette tips (10 μ l, 20 μ l, 200 μ l, 1000 μ l), dissection instruments (scissors, tweezers), needles (25 g), QuantStudio™ 5 real-time PCR system, StepOnePlus™ real time PCR system, Techne™ 5PrimeG Gradient Thermal Cycler

Thermo Fisher Scientific: Cell culture hood, steri-cycle CO₂ incubator, nanodrop 2000 UV spectrophotometer

Agilent Technologies: Seahorse Analyzer (XFp, XFe96), Seahorse cell culture plates, Seahorse cartridges

Medical Supplies Company: Water bath, vortex, mini-fuge, multi-channel pipettes

VWR: Mr. Frosty, Reagent reservoirs, Cell scrapers (20mm, 30mm), Scalpels, T-shaped spreaders (99.1mm x 39.9mm)

Eppendorf: Centrifuges (5424 R, 5425 R, 5804 R)

Gilson: Pipette sets (p2, p10, p20, p200, p1000)

Starstedt Ltd.: Screw-capped tubes (2 ml)

Applied Biosystems: MicroAmp 384-Well reaction plate, MicroAmp adhesive film

Hawksley: Haemocytometer

2.1.4 Animals

C57BL/6, miR-21 deficient and *Irg1* deficient mice used in the duration of this project were between 8 and 20 weeks of age. miR-21-deficient mice were generated by Taconic using Cre-loxP technology to delete the miR-21 coding region downstream of *Trem49*. After deletion, mice were backcrossed further to obtain full body miR-21-deficient mice as described in (314). Uncrossed mice were used as wild-type controls. *Irg1*^{-/-} mice (named C57BL/6N-Acod1em1(IMPC)J/J) were generated by CRISPR-targeted deletion of exon 4 of *Irg1* and were purchased from The Jackson Laboratory. Wild-type littermates were used as controls. Mice used for cell extractions were both age and sex-matched. Animals were bred in house by TCD comparative medicine unit (CMU) and maintained under specific pathogen-free conditions in accordance with both Irish and European Union regulations. Experiments requiring the use of mice were carried out in compliance under the Sheedy lab project licence and approved by both the Animal Research Ethics Committee from the Health Products Regulatory Authority (HPRA) and Trinity College Dublin university ethics committee. Mice were sacrificed by CO₂ asphyxiation, organs harvested within the CMU and carcasses disposed of in accordance with TCD CMU guidelines.

2.1.5 Cell lines

L-929, murine fibroblast cell line NCTC clone 929 of strain L, derived in 1984 from C3H/An male mouse was purchased from The American Type Culture Collection (ATCC).

2.1.6 Cell culture medium

2.1.6.1 Bone-marrow derived macrophages culture medium

Cell culture medium was prepared by supplementing DMEM with 10% FBS and 5% L929 culture medium.

2.1.6.2 Human monocyte-derived macrophages culture medium

Culture medium was prepared by supplementing RPMI with 10% human serum or 10 ng ml⁻¹ recombinant human M-CSF.

2.1.6.3 L-929 cell culture medium

Culture medium was prepared by supplementing DMEM with 10% FBS

2.1.7 Cell culture stimulators/ inhibitors

Reagent	Vehicle	Stock Concentration	Working Concentration
2DG	Seahorse Media	1M	30mM
Antimycin-A	Ethanol	5mM	4μM
FCCP	Ethanol	10.1μM	1μM
Oligomycin	Ethanol	2mM	2μM
Rotenone	DMSO	1mM	0.1μM
6-AN	DMSO	50mM	25-100μM
GPI	DMSO	100mM	25-50μM
DetaNO	H ₂ O	100mM	25-100μM
cPTIO	DMSO	100mM	50μM
DMM	Ethanol	5M	1-5mM
Itaconic acid	PBS	100mM	10mM
GSK2795039 (iNOX2)	DMSO	50mM	10-25μM

Table 2. 6 List and concentrations of cell culture stimulants and inhibitors

2.1.8 Buffers

Buffer	Composition
Freezing medium	10% DMSO in FBS
FACs buffer	50ml: 1.5 ml FBS + 48.5 ml PBS

Table 2. 7 List and concentrations of buffers

2.1.9 Flow cytometry antibodies / stains

2.1.9.1 Flow cytometry antibodies

Target	Colour	Clone	Supplier
CD11b	PE	M1/70	Biolegend
F4/80	PeCy7	BM8	Biolegend

Table 2. 8 List of flow cytometry antibodies

2.1.9.2 Flow cytometry stains

Stain	Stock concentration	Desired Concentration	Supplier
Zombie Aqua Fixable Viability Dye	1000X solution in DMSO	Dilute 1:1000	Biolegend
HSC LipidTOX™ green Neutral Lipid Stain	1000X solution in DMSO	Dilute 1:2000	Invitrogen
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	100µM	100nM	Invitrogen
MitoTracker™Green FM (MTG)	1mM	1µM	Invitrogen
LysoTracker™Red (LTR)	1mM	1µM	Invitrogen

Table 2. 9 List of flow cytometry stains

2.1.10 PCR primers

Gene	Species	Taqman Assay ID
18S	Mouse	Mm04277571_s1
Hk2	Mouse	Mm00443385_m1
Hprt	Mouse	Mm01545399_m1
IL-1β	Mouse	Mm00434228_m1
IL-10	Mouse	Mm00439614_m1
Ldha	Mouse	Mm01612132_g1
miR-21	Conserved	Hs04231424_s1
Pfk-m	Mouse	Mm01309576_m1
Sdhb	Mouse	Mm00458272_m1
RNU6B	Conserved	001093
Slc2a1	Mouse	Mm00441480_m1
Tnf-α	Mouse	Mm00443258_m1
G6pdx	Mouse	<u>Mm00656735_g1</u>
Pgd	Mouse	<u>Mm00503037_m1</u>
Taldo1	Mouse	<u>Mm00807080_g1</u>
Tkt	Mouse	<u>Mm00447559_m1</u>
Gpi1	Mouse	<u>Mm01962484_u1</u>
Pgm1	Mouse	Mm00804141_m1
Irg1	Mouse	Mm01224532_m1
Fh1	Mouse	Mm01321349_M1
Nos2	Mouse	Mm00440502_m1
G6PD	Human	Hs00166169_m1
Taldo1	Human	Hs00997203_m1
Ldha	Human	Hs01378790_g1
Pfk-m	Human	Hs01075411_m1
Hk2	Human	Hs00606086_m1
Slc2a1	Human	Hs00892681_m1

<i>Il-18</i>	Human	Hs01555410_m1
<i>Tnf-α</i>	Human	Hs00174128_m1
<i>IS6110</i>	Mycobacterium	Custom
<i>16S</i>	Bacteria	Ba04230899_s1

Table 2. 10 List of TaqMan real-time PCR gene expression assays

2.2 Methods

2.2.1 Murine cell culture

2.2.1.1 Bone-marrow derived macrophages

Following CO₂ asphyxiation and cervical dislocation, mice aged 8-12 weeks were pinned down and sprayed with ethanol. Using scissors and tweezers, the lower abdomen was opened without rupturing the peritoneum. Making a distal incision along the legs, skin was removed to expose the legs and hips. Without breaking the bones, the femur and tibia were isolated from the hip joint and placed in DMEM medium. In the cell culture hood, muscle was separated from the bones using a scissors and remaining tissue removed with an ethanol-soaked tissue. Bone-marrow was isolated using the scissors to clip the extremities of both the femur and tibia, followed by flushing the cavity of marrow with a 5 ml syringe of DMEM equipped with a 25G needle into a 50 ml falcon. The marrow was pelleted for 5 minutes at 400 g, supernatant removed and pellet resuspended in 1 ml red blood cell lysis buffer for 2 minutes. The lysis buffer was neutralised by adding 9 ml DMEM and the cell suspension was pelleted once again at 400 g for 5 minutes. Cells were resuspended in 10 ml BMDM differentiation media (DMEM, 20% L929-conditioned medium, 10% FBS and 1% penicillin-streptomycin) and passed through a 70 µm cell strainer. Cell number was calculated using Trypan Blue staining and a haemocytometer. 20 ml of differentiation media containing 1x10⁶ cells/ml was prepared and plated on a 15 cm sterile, non-culture treated dish and supplemented with an additional 20 ml media after 3 days. On day 7, mature BMDMs were washed with PBS and then detached from the petri-dish using ice cold PBS. Cells

were counted and seeded one day prior to experiments at a density of 1×10^6 cells/ml in BMDM culture media (DMEM, 5% L929-conditioned medium and 10% FBS).

2.2.1.2 BMDM purity assessment

Following differentiation, both purity and yield of mature BMDMs was assessed in wild-type and all knockout murine models used. Approximately 1×10^6 cells were washed with PBS and transferred to labelled FACs tubes. FACs tubes were coated on ice with 500 μ L FBS which was removed before the addition of cells in an effort to minimise adherence of cells to the tube. Cells were pelleted 1400 rpm for 5 minutes and supernatant discarded. Samples were resuspended in 50 μ L Fc receptor inhibitor antibody and left at room temperature for 5 minutes. Samples were washed in 1 mL FACs buffer, pelleted and resuspended in 50 μ L Zombie Aqua viability dye (diluted 1:1000 in PBS) and left to incubate for 15 minutes in the dark at room temperature. Live/dead stain was not added to unstained, or single stain compensation tubes, instead, 50 μ L PBS was added. Samples were washed, pelleted and resuspended in 50 μ L extracellular antibody master mix (diluted 1:200 in PBS). Unstained and live/dead samples were resuspended in PBS whilst single stain compensation tubes only received the desired antibody at the equivalent concentration. Samples were left to incubate in the dark at room temperature for 20 minutes. Samples were washed, pelleted resuspended in 150 μ L FACs buffer prior to sample acquisition using the LSR Fortessa or FACs Canto.

	Zombie Aqua	CD11b PE	F4/80 PeCy7
Unstained	-	-	-
Live/dead	+	-	-
CD11b Comp	-	+	-
F4/80 Comp	-	-	+
CD11b FMO	+	-	+
F4/80 FMO	+	+	-
Samples	+	+	+

Table 2. 11 Controls and Samples for BMDM purity assessment

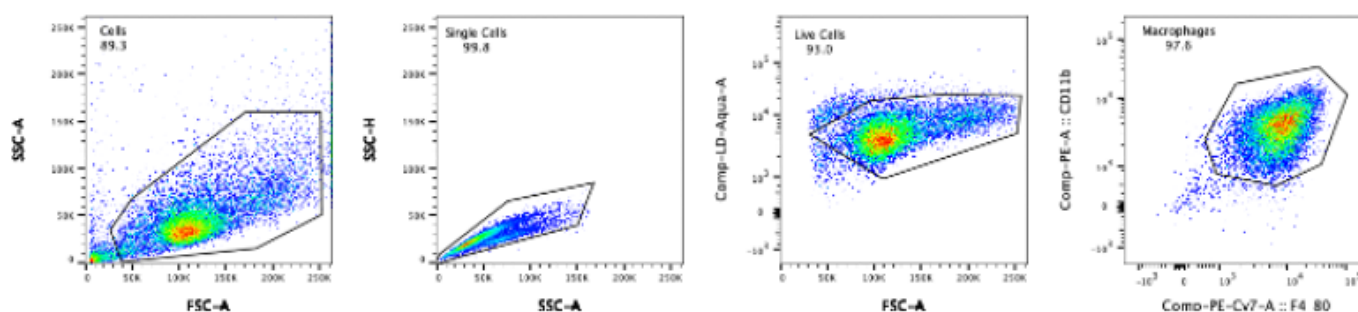


Figure 2. 1 Sample gating strategy for assessing BMDM purity

2.2.1.3 Isolation of human monocytes and macrophage differentiation

Primary human monocyte derived macrophages (hMDMs) were generated from isolated monocytes obtained from Buffy packs acquired from the Irish Blood Transfusion Service. Using a sterile scalpel, the Buffy packs were cut open and the blood was transferred to a sterile container. The blood was diluted 1:1 in PBS, mixed and slowly layered using a 25 mL serological pipette on top of 15 mL Lymphoprep in a 50 mL tube. The layered sample was then centrifuged at 400 rcf for 25 minutes with no breaks in order to isolate peripheral blood mononuclear cells (PBMCs). Using a Pasteur pipette, the buffy coat layer was gently removed and transferred to a new 50 mL tube. The sample volume was brought to 50 mL using PBS and pelleted at 300 rcf for 10 minutes with breaks on. The supernatant was removed the cell pellet was resuspended in 1 mL red blood cell lysis buffer for 2 minutes to lyse contaminating red blood cells. The sample volume was brought to 50 mL with PBS, pelleted at 300 rcf for 10 minutes and supernatant discarded. PBMCs were resuspended in 5 mL warm culture medium containing RPMI 1640 and 10% human serum or or 10 ng ml⁻¹ recombinant human M-CSF. Cells were counted using trypan blue and a haematocytometer and seeded at a density of 3x10⁶ cells/mL. Non-adherent cells were removed and adherent monocytes were selected for using PBS washes, with fresh medium added every two days. On day six, following PBS wash, the media was replaced, leaving differentiated macrophages now ready for experimentation.

2.2.1.4 Isolation and ex vivo culture of murine alveolar macrophages

Directly after CO₂ asphyxiation and in sterile conditions, the trachea was exposed using a scissors and forceps. Using a 20G IV catheter, the trachea was punctured between the rings of cartilage and the catheter was fed intratracheally, stopping above the trachea bifurcation. With a 1 ml syringe, 0.5 ml PBS was syringed through the catheter into the lungs and slowly withdrawn back into the syringe. This was repeated 10 times with fresh PBS to maximise cell yield. Lavage fluid was pelleted at 400 g for 5 minutes, resuspended in 1 ml red blood cell lysis buffer for 1 minute which was then neutralised by adding 2 ml of DMEM. The cell suspension was pelleted again at 400 g for 5 minutes and resuspended in culture medium (DMEM, 10% FBS and 1% P/S). After 24 hours non-adherent cells were washed and removed and cell culture medium was replaced without the addition of P/S prior to infection with *Mycobacterium tuberculosis*.

2.2.1.5 Generation of L929-conditioned medium (LCM)

M-CSF required for macrophage differentiation was generated through culturing the murine fibroblast cell line, L929. LCM was generated by seeding 20x10⁶ cells in 40 mL culture media (DMEM and 10% FBS) and left to become over-confluent. After 8 days the supernatant was collected and passed through a 0.22 µm, low protein binding filter. Filtered LCM was pooled and stored at -80°C.

2.2.2 Infection: *Mycobacterium tuberculosis*

2.2.2.1 Culture of *Mycobacterium tuberculosis*

Experiments requiring the use of Mtb were carried out in TBSI Biosafety level 2 facility. Live Mtb strain H37Ra was obtained from ATCC (25177) and stored at -80°C until use. As required, H37Ra was thawed, expanded at 37°C and maintained at log phase through sub-cultures in 10 ml Middlebrook 7H9 broth enriched with ADC (Table 2.12) in a T25 culture flask. Mtb was sub-cultured weekly for a minimum of 5 weeks before use in experiments.

2.2.2.2 Preparation of Middlebrook 7H9 broth

Table 2.12 lists the components required to make 100 mL of Middlebrook 7H9 broth. Once combined (except the ADC) in a glass bottle, the broth was autoclaved, cooled to 45°C and the ADC enrichment media was added. Middlebrook 7H9 broth was used within 4 weeks of preparation.

Component	Volume
Middlebrook 7H9 broth base	0.47 g
Tween 80	50 µl
Deionised H ₂ O	90 ml
ADC enrichment medium	10 ml

Table 2. 12 Components and volumes required for 100 ml of Middlebrook 7H9 broth

2.2.2.3 Preparation of Middlebrook 7H11 agar

Middlebrook 7H11 agar plates were prepared at least 1 day prior to use. Table 2.13 lists the components required to make 100 mL of Middlebrook 7H11 agar. Components were combined (except the OADC and cycloheximide) in a glass bottle, autoclaved, cooled to 55°C and placed in a water bath (55°C) for 45 minutes. Following this and under sterile conditions, the OADC and cycloheximide was added. Cycloheximide is a eukaryotic protein synthesis inhibitor that prevents the growth of moulds and yeasts on the agar plates. 15 ml of agar was immediately aliquoted onto petri dishes using a serological pipette, left to cool and solidify for 10 minutes. Agar plates were stored inverted at 4°C for a maximum of 4 weeks and placed in an incubator at 37°C with no CO₂ a day prior to use.

Component	Volume
Middlebrook 7H11 agar base	1.9 g
L-asparagine	0.1 g
Glycerol	0.5 ml
Deionised H ₂ O	90 ml

OADC enrichment medium	10 ml
Cycloheximide	50 µg/ml

Table 2. 13 Components and volumes required for 100 ml of Middlebrook 7H11 agar

2.2.2.4 *Mycobacterial tuberculosis* bacterial growth assay

Prior to infection of desired cells, the Mtb culture was prepared to a desired bacterial concentration to achieve the required MOI. 9 ml of the log phase culture was removed using a transfer pipette into a 50 ml falcon. The falcon was sealed within a centrifuge bucket within the BSC and then centrifuged at 3800 rpm for 10 minutes. Once complete, the centrifuge bucket was returned to the BSC before opening the lid. The supernatant was removed and the bacterial pellet was resuspended in 1 ml PBS and transferred to a sterile 2 ml screw-capped tube. To achieve a single cell suspension, the Mtb was syringed using a 25G needle 5- 10 times. To further remove any cellular clumps, the Mtb suspension was centrifuged at 800 rpm for 3 minutes and the top 900 µl was transferred to a fresh 2 ml tube. Using 2 ml cuvettes, a blank control (PBS) and a dilution of the single cell bacterial pellet was generated (typically 1:20). A portable cell density OD600 spectrophotometer was transferred into the BSC from which the bacterial density was assessed and volume required to reach the desired MOI was calculated (as per example below). The desired bacterial MOI was added to each well on the cell plate for 3 hours at 37 °C after which the remaining extracellular bacteria was removed by centrifuging the supernatant at 5000 rpm for 5 minutes, during which time, the wells were washed once with warm fresh media. Once the extracellular bacteria was removed, the supernatant was returned to its corresponding well on the cell plate.

OD600 = 1.0 (where 1.0 = 10^7 bacteria)	Desired MOI = 5
$1.0(10^7) \times 20$ (Dilution factor)(mL)	Macrophages/well = 5×10^5 (5)(MOI)
= 200×10^6 bacteria/mL	Bacteria/well = 2.5×10^6

$$\text{Volume/well} = 2.5 \times 10^6 / 200 \times 10^6 = 0.0125 \text{ mL} = 12.5 \mu\text{L}$$

At desired time points, cells were lysed in 0.1% Triton-X for 10 minutes. For 3 hour timepoints and representative of bacterial uptake, a serial dilution was performed by adding 50 µl lysate to 450 µl Middlebrook broth that was supplemented with ADC to create 10^{-1} , 10^{-2} and 10^{-3} dilutions. The dilutions were plated in triplicate on 7H10 Middlebrook agar and incubated at 37 °C without CO₂ for 14-21 days at which time the colony-forming units were counted. At later timepoints, the supernatant was removed and centrifuged at 10,000 g for 10 minutes and the bacterial pellet was added to the cellular lysate before being plated.

Bacterial replication was also assessed through quantification of Mtb IS6110 genomic DNA (gDNA), made relative to bacterial 16S ribosomal RNA (rRNA) in BMDMs and hMDMs. Mtb DNA was isolated as per protocol 2.2.3.1 and quantified by qPCR as per protocol 2.2.3.5 using custom designed TaqMan probe targeting Mt's IS6110 transposable element. This method of bacterial quantification was previously designed, optimised and published within the Sheedy lab (41).

2.2.3 Benchtop sample analysis: RNA

2.2.3.1 Total RNA isolation from cells

To minimise sample degradation, all RNA-based work was carried out on ice using filter barrier tips and RNase-free tubes. With a modified protocol, total RNA isolation was achieved using the PureLink RNA Mini Kit. Once the supernatant was removed, cells were lysed in 350 µl RNA lysis buffer, after which the cell culture plate was immediately placed in ice. Adherent cells were gently dethatched using a cell scraper, placed in an epi and stored at -80 °C until purification. When required, samples were thawed and 1.5 times the volume of 100% molecular grade ethanol was added. The samples were slowly pipetted up and down to disperse visible particulate and transferred to a spin cartridge containing the RNA purification filter with an attached collection tube below. Samples were centrifuged at 10,000 g for 30 seconds at 4 °C, flow through discarded and spin cartridge re-inserted into the same collection tube. 500 µl of wash buffer II was added to the spin cartridge, centrifuged at 10,000 g for 30 seconds and flow-through discarded

once again. This wash buffer II step was repeated once. The spin cartridge was placed into a fresh collection tube and centrifuged at 10,000 g for 5 minutes to remove and remaining contaminants within the spin cartridge filter, leaving behind the membrane bound RNA. The spin cartridge was placed into a RNase-free recovery tube and the RNA was eluted by adding 30 µl RNase-free water to the centre of the spin cartridge filter, incubated for 1 minute and centrifuging the sample at 12,000 g for 2.5 minutes. Flow-through was pipetted back into the spin cartridge and once again centrifuged at 12,000 g for 2.5 minutes to maximise the RNA yield. Extracted RNA samples were stored at – 80 °C.

2.2.3.2 RNA quantification/ quality assessment

The concentration and the quality of extracted RNA was assessed using a Nanodrop 2000 UV spectrophotometer. Before use, the machine was cleaned and blanked using the RNA solvent, RNase-free water. 2 µl of each sample was run to obtain concentration and the ratio of absorbance. The ratio of absorbance to assess the quality of both RNA and DNA is at 260 nm and 280 nm (260/280). A 260/280 value of approximately 2 is generally accepted as an indication of pure RNA. A lower absorption rate may be observed in the incidence of contaminants within the sample, such as the presence of protein. Higher than expected absorbance ratios may indicate a contaminated blank.

2.2.3.3 Reverse transcription of mRNA to cDNA

Prior to reverse transcription, all RNA sample concentrations were recorded and the measurements used to normalise the samples to a set total RNA value, typically 1 µg or lower for sample groups with a lower yield. RNase-free water was used to normalise the samples to a set concentration in a total final sample volume of 13.2 µl in 200 µl RNase-free microcentrifuge tubes. Reverse transcription master mix was prepared and added to each sample, bringing the total reaction volume to 20 µl. The components and quantities of reverse transcription master mix are outlined below. Samples were centrifuged at 500 g for 10 seconds to ensure master mix/ sample amalgamation, placed into a thermocycler and run at a set cycle as outlined below. Following cDNA generation, samples were stored at -20 °C until use in real-time PCR.

Component	Volume per reaction (μl)
10X reverse transcription buffer	2
25X 100mM dNTPs	0.8
10X random primers	2
RNase inhibitor	1
Reverse transcriptase	1
Total	6.8

Table 2. 14 Components and volumes required for mRNA reverse transcription mix

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (minutes)	10	120	5	∞

Table 2. 15 Reverse transcription thermocycle program (mRNA to cDNA)

2.2.3.4 Reverse transcription of microRNA to cDNA

Prior to reverse transcription, all RNA sample concentrations were recorded and the measurements used to normalise the samples to a set total RNA value of 2 ng/μl. RNase-free water was used to normalise the samples to a set concentration in a total final sample volume of 5 μl in 200 μl RNase-free microcentrifuge tubes. Reverse transcription master mix was prepared and added to each sample, bringing the total reaction volume to 15 μl. The components and quantities of reverse transcription master mix are outlined below. Samples were centrifuged at 500 g for 10 seconds to ensure master mix/sample amalgamation, placed into a thermocycler and run at a set cycle as outlined below. Following cDNA generation, samples were stored at -20 °C until use in real-time PCR.

Component	Volume per reaction (μl)
10X reverse transcription buffer	1.5
25X 100mM dNTPs	0.15

RNase-free water	6.41
RNase inhibitor	0.19
Reverse transcriptase	1
20X miR primer (specific to target)	0.75
Total	10

Table 2. 16 Components and volumes required for microRNA reverse transcription mix

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	16	42	85	4
Time (minutes)	30	30	5	∞

Table 2. 17 Reverse transcription thermocycle program (microRNA to cDNA)

2.2.3.5 Real-Time PCR

FAM-labelled, Taqman real-time PCR gene expression assay probes were used to quantify the frequency of sample mRNA and microRNA. cDNA at a concentration of 1 µg was diluted 1:10 using RNase-free water for mRNA samples whilst microRNA samples were used neat. 2 µl of each sample was pipetted in triplicate into a MicroAmp 384-Well reaction plate followed by 8 µl RT-PCR mix, composition outlined below. Housekeeping genes HPRT and 18S were used to normalise of mRNA samples, with RNU6B used for microRNA normalisation. The wells of the reaction plate were sealed with MicroAmp adhesive film after which the plate was centrifuged at 400 g for 1 minute. The plates were run on Applied Biosystems QuantStudio 5 Real-Time PCR System according to the protocol detailed below. Data obtained was analysed using the $\Delta\Delta$ Cycle Threshold ($\Delta\Delta Ct$) value method where ΔCt were generated by normalising the Ct value to the corresponding endogenous control. Using this, the $\Delta\Delta Ct$ was deduced through subtracting the control ΔCt from the sample ΔCt of the equivalent sample. Fold change in the target sample expression relative to the housekeeping sample control was generated using the formula $2^{(-\Delta\Delta Ct)}$.

Component	Volume per reaction (μl)
2X Taqman FAST advanced master mix	5
RNase free water	2.5
20X Taqman gene expression assay probe	0.5
Total	8

Table 2. 18 Components and volumes required for real-time PCR assay mix

2.2.4 Benchtop sample analysis: Protein, Metabolite and Molecule assays

2.2.4.1 ELISA

Supernatant cytokine concentration was quantified using uncoated Enzyme-linked immunosorbent assays (ELISAs). Using high-binding flat bottom 96-well plates, 50 μl capture antibody was added to each well and left to incubate at 4 °C overnight. The following day, the plate was washed three times by submerging the plate in ELISA wash buffer composed of 0.05% Tween20 and PBS. Following each wash step, the plates were dried by placing them upside down and banging them against tissue to remove remaining wash buffer. Any sites within the well that remained unbound by capture antibody were blocked by adding 150 μl ELISA diluent and leaving the plate to incubate on a rocker at room temperature for 1 hour. ELISA diluent was removed from the wells and the plate washed three times. To normalise protein concentration to the ELISA protocol, standards were generated through serial dilution of the assay standards made up in ELISA diluent. ELISA diluent alone serves as a blank reaction well. Both standards and samples were added to the plate in triplicate with the samples diluted to an appropriate concentration to fall within range of the standard curve. The plate was left to incubate at 4 °C overnight. The next day, the plate was washed three times, 50 μl detection antibody in ELISA diluent added to each well and left to incubate on a rocker at room temperature for 2 hours. The plate was washed three times and 50 μl streptavidin-horseradish peroxidase (HRP) conjugate diluted in ELISA diluent was added to each well and incubated on a rocker in the dark for 30 minutes at room temperature. Following this, the plate was washed seven times after which 50 μl TMB substrate was added to each well. HRP enzymatic activity results in the reduction of hydrogen peroxide

and oxidation of TMB, resulting in a colour change from clear to blue. This reaction is allowed to develop until the lowest standard concentration of the standard curve begins to turn blue, after which it is stopped by adding 25 µl of acidic H₂SO₄. This results in a blue to yellow colour change with an absorbance that can be read at 450 nm using a microplate reader, where colour intensity is relative to protein concentration. Cytokine quantity within the sample was determined through generation of a concentration vs A450 based slope.

2.2.4.2 Glucose-6-Phosphate Dehydrogenase activity quantification

Cellular glucose-6-phosphate dehydrogenase (G6PD) activity was analysed a Glucose-6-Phosphate Dehydrogenase Activity Colorimetric Assay (Table 2.5). In this kit, glucose-6-phosphahate is oxidised to generate gluconolactone that is specifically detected by colorimetric (450nm) assay. This is proportional to the activity of G6PD. A standard curve was generated ranging from 0 - 12.5 nmole NADH/well in 25 µl assay buffer in a 96-well plate. 5x10⁵ cells per condition were rapidly homogenised in 200 µl ice-cold PBS. Samples were centrifuged at 10,000 g for 10 minutes to remove any insoluble material and diluted adequately to fall within the range of standard curve. The volume of sample wells was brought to 50 µl with G6PD Assay Buffer. 50 µl reaction master mix was added to each sample and standard wells. The components and quantities of master mix are outlined below. After 2-3 minutes, initial absorbance (A450^{initial}) was measured at 450nm using a microplate reader pre-set to 37°C whilst also protecting the samples from light. Absorbance was continuously measured every 5 minutes until the value for the most active sample was greater than the value of the highest standard (12.5 nmole/well). The final measurement (A450^{Final}) for calculating the enzyme activity was the value before the most active sample was near the end of the linear range of the standard curve. G6PD activity was calculated by first subtracting the final measurement obtained for the blank reading from the final sample measurement of the standards, creating the NADH standard curve. Sample values were corrected to the slope of the standard curve and corrected sample values were then used in the following formula:

$$\text{G6PD Activity} = (\text{NADH}^{\text{calc}}) \times \text{Sample dilution factor} / (T^{\text{final}} - T^{\text{initial}}) \times V$$

$T^{\text{final}} - T^{\text{initial}}$ = time between initial and final measurement (minutes)

V = volume of sample added to the wells (ml)

$\text{NADH}^{\text{calc}} = \text{NADH generated between the initial and final readings and calculated from the standard curve (nmole)}$

G6PD activity was reported as nmole/min/ml (milliunit/ml). One unit correlates to the amount of enzyme that catalyses the conversion of 1.0 μmole of glucose-6-phosphate to 6-phosphoglucono- δ -lactone and generates 1.0 μmole of NADH per minute at 37°C.

Component	Volume per reaction (μl)
G6PD assay buffer	46
G6PD substrate mix (glucose-6-phosphate + NADP)	2
G6PD developer mix	2
Total	50

Table 2. 19 Components and volumes required for G6PD assay master mix

2.2.4.3 Lactate quantification

Extracellular L(+)-lactate released by cells into the supernatant was quantified by colorimetric assay (Table 2.5). Here, an enzymatically induced colour change is proportional to lactate concentration within the supernatant. To avoid lactate degradation induced during freeze thaw cycles, all samples were used fresh once harvested. A standard curve was generated ranging from 0 - 100 picomole/well in 25 μl assay buffer in a 96-well plate. Samples were diluted appropriately in order to find a sample concentration that falls within the detection range of the standard curve, generally 1:100 for BMDMs plated at a density of 5×10^5 cells/ml. Samples were added to the plate in duplicate and master was added to both sample and standard wells. The components and quantities of master mix are outlined below. The plate was placed on a rocker at room temperature and protected from light for 30 minutes. Following this, the plate was read using a microplate reader set to an absorbance of 570 nm where colour intensity reflects lactate concentration. The standard curve was then used to determine the lactate concentration within the samples.

Component	Volume per reaction (μl)
Lactate assay probe	23
Enzyme mix	1
Lactate probe	1
Total	25

Table 2. 20 Components and volumes required for Lactate assay master mix

2.2.4.4 Quantification of 6-phosphogluconic acid (6-PGA)

6-Phosphogluconic Acid (6-PGA) was measured using the colorimetric 6-Phosphogluconic Acid (6-PGA) Assay Kit (Table 2.5). 6-PGA is an intermediate of the PPP, catalysed by 6-phosphogluconolactonase and used to generate ribulose-5-phosphate and NADPH through the activity of 6-phosphogluconate dehydrogenase. In this assay, 6-PGA is converted to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase in the presence of NAD, resulting in the production of NADH which reduces the assays probe and generates absorbance at 450 nm.

A standard curve was generated ranging from 0 – 10 nmole/well in a 96-well plate. The volume of the well was adjusted to 25 μl using assay buffer. 5×10^5 cells per condition were homogenized in 200 μl ice cold assay buffer for 5 minutes in ice. Samples were centrifuged at 10,000 g, 4°C for 10 minutes to remove cellular debris. Samples were diluted to fall within the range of the standard curve and added to the 96-well plate. Sample wells were brought to 25 μl using 6-PGA assay buffer. 25 μl reaction mix per sample was generated. The components and quantities of master mix are outlined below. Samples were also plated for blank reactions to omit the sample NADH which generates background. Reaction mix for blank samples lacked 6-PGA enzyme. The plate was incubated for 60 minutes at 37°C and absorbance at 450 nm was recorded (A450). 6-PGA concentration was calculated firstly by subtracting the blank standard from all standard readings and plotting the standard curve. Sample values were corrected for by subtracting the value derived from the background control from all sample readings. Sample readings were corrected for by applying the values obtained to the standard

curve to get the amount of 6-PGA in the sample wells. The corrected values were then used in the following equation:

$$6\text{-PGA}(\text{mM}) = B(\text{nmol})/V(\mu\text{L}) \times \text{Dilution factor}$$

Where B is the amount of 6-PGA from the standard curve and V is the sample volume added to each reaction well. 6-PGA concentration was reported as nmole/ μL .

Component	Volume per reaction (μL)
6-PGA assay buffer	23
Enzyme	1
Substrate mix	1
Total	25

Table 2. 21 Components and volumes required for 6-PGA assay master mix

2.2.4.5 NADP/NADPH quantification

Levels of cellular NADP/NADPH were analysed using a NADP/NADPH Colorimetric Assay kit (Table 2.5). A standard curve was generated ranging from 0 - 100 pmole NADPH/well in 50 μL assay buffer in a 96-well plate. 1×10^6 cells per condition were washed in PBS and rapidly homogenised in 400 μL ice-cold extraction buffer and vortexed. Samples were put through two freeze-thaw cycles by placing them on dry ice for 20 minutes followed by 10 minutes at room temperature. Once thawed, samples were centrifuged at 14000 rpm to remove cellular debris and supernatants were split between two fresh sample tubes. In order to calculate NADP/NADPH, total NADP and NADPH (NADPt) levels were quantified in one half of the sample, whilst NADP was decomposed in the other sample half before quantifying NADPH alone (NADPd). For the NADPd sample, NADP was decomposed by heating the sample to 60 °C for 30 minutes, followed by rapid cooling on ice. Samples were diluted adequately in assay buffer to fall within the range of standard curve and 50 μL was added to the sample well. 100 μL reaction master mix was added to each sample and standard wells. The components and quantities of master mix are outlined below. Samples were also plated for blank reactions to omit the sample NADH which generates background. Reaction mix for blank samples lacked the NADP

cycling enzyme mix. The sample plate was left to incubate at room temperature for 5 minutes to convert NADP to NADPH. 10 µl of developer solution was added to each well and the sample plate was left to incubate at room temperature for 1-4 hours with multiple readings taken at absorbance 450 nm during this timeframe. Sample readings were corrected for by applying the values obtained to the standard curve to get the amount of NADPH in the sample wells. The corrected values were then used in the following equation:

$$\text{NADPt or NADPH} = (\text{corrected absorbance} - (y - \text{intercept}))/\text{slope}$$

$$\text{NADP/NADPH ratio} = \text{NADPt} - \text{NADPH/NADPH}$$

Component	Volume per reaction (µl)
NADP cycling buffer	98
NADP cycling enzyme mix	2
Total	100

Table 2. 22 Components and volumes required for NADP/NADPH assay master mix

2.2.4.6 Nitrite quantification

A Griess assay was used to quantify nitrite levels within the supernatants of stimulated macrophages. The Griess reagent system works through measurement of nitrite (NO_2^-), a stable, non-volatile breakdown product of nitric oxide (NO) and thus works as an indirect measurement of cellular NO levels. A standard curve was generated from a standard serial dilution of sodium nitrite generated through a 2-fold serial dilution ranging from 100 – 1.5 µM. 50 µl of the standard and each sample supernatant, diluted appropriately to fall within range of the standard curve was added a 96-well plate. 50 µl Griess reagent was added to each standard and sample well and the plate was left to incubate at room temperature for 15 minutes. The absorbance values were read at 540 nm using a microplate reader.

2.2.4.7 Glucose quantification

Levels of glucose within supernatants were analysed using a Glucose Colorimetric detection kit (Table 2.5). A standard curve was generated ranging from 0 – 32 mg/dL glucose/well in 20 µl assay buffer in a 96-well plate. Samples were diluted to fall within the range of the standard curve and 20 µl was added to the 96-well plate. 25 µl 1X HRP solution was added to each well. 25 µl substrate was added to each well. 25 µl 1X glucose oxidase was added to each well. The plate was incubated for 30 minutes at room temperature and absorbance at 560 nm was recorded. Glucose concentration was calculated firstly by subtracting the blank standard from all standard readings and plotting the standard curve. Sample values were corrected for by subtracting the value derived from the background control from all sample readings. Sample readings were corrected for by applying the values obtained to the standard curve to get the amount of glucose in the sample wells and multiplied by the appropriate dilution factor.

2.2.5 Metabolic phenotyping: Seahorse XF analyser

Using a Seahorse XFe-96 Analyser, extracellular acidification rate (ECAR) of media was used as a readout of glycolytic carbon flux and cellular oxygen consumption rate (OCR) was used as an indicator of oxphos

2.2.5.1 Cartridge hydration

A day prior to Seahorse analysis, a Seahorse XFe-96 sensor cartridge was hydrated overnight in a non-CO₂ incubator at 37°C with Agilent XF calibrant buffer.

2.2.5.2 Cellular metabolic phenotyping

On the day of analysis, culture media was replaced with Seahorse medium supplemented with 10 mM glucose and 2 mM glutamine, pH 7.4. This was completed in a stepwise manner to ensure total removal of any pH buffers that are present in culture medium. Seahorse media was added to all wells including blank wells and placed into a non-CO₂ incubator at 37°C while working stocks of metabolic inhibitors were prepared in Seahorse medium and added to the designated injection ports of the sensor cartridge.

The metabolic inhibitors and concentrations used are outlined in the table below. The loaded Seahorse cartridge and utility plate was placed into the Seahorse XFe-96 analyser and calibrated for 20 minutes. Upon completion, the utility plate was swapped out for the cell culture plate for analysis of real-time cellular metabolism.

Compound	Stock concentration	Final concentration	Vehicle (Seahorse media) (μl)	Inhibitor (μl)
Oligomycin	2mM	2μM	2970	30
FCCP	1mM	1μM	2980	30
Rot/Anti-A	1mM/5mM	0.1μM/4μM	2967	3.6/29
2DG	1M	30mM	1819	1181

Table 2. 23 List and concentration of inhibitor used in Seahorse Analysis

2.2.6 Flow cytometry analysis

Cells were harvested into pre-labelled FACs tubes. Cells were washed by adding 1 ml PBS and centrifugation for 5 min. Supernatants were removed, cell pellet disrupted and stained for analysis with the following controls: unstained for each treatment, live/dead, lysotracker, TMRM, Mitotracker Green (MTG), LipidTOX and singularly stained compensatory beads for each antibody used.

2.2.6.1 Lysotracker Red (LTR) staining

To quantify lysosomal acidification, cells were stained with LTR. LTR was diluted in warmed DMEM media (Table 2.10). Cells were removed from culture plates and transferred to labelled FACs tubes, washed with 1 ml 1X PBS, centrifuged for 5 min at 1400 RPM and resuspended in 200 μl of LTR containing media. Cells were left to incubate for 25 min at 37°C. Samples were washed with PBS and pelleted for further staining. Samples stained with LTR were acquired live (not fixed) using the FACs Canto cytometer.

2.2.6.2 Mitotracker Green (MTG) / TMRM co-staining

MTG and TMRM dyes were used in order to co-stain for quantification of mitochondrial membrane potential and mass. A master mix was prepared in warm DMEM containing both MTG / TMRM at the desired concentrations (Table 2.10). Cells were transferred into labelled FACs tubes, washed in 1X PBS, pelleted and resuspended in 200 µl of the MTG/TMRM master mix. Cells were left to stain at 37°C for 30 min. The stain was washed off by adding 1X PBS, after which the samples were pelleted for further staining. Samples were acquired live (not fixed) using the LSR Fortessa cytometer.

2.2.6.3 LipidTOX green (LTG) staining

LTG stain is used to determine total neutral lipid content of cells, including both membrane and intracellular lipids. LTG master mix was prepared in PBS (Table 2.10). Cells were resuspended within FACs tubes in 100 µl LTG master mix and stored at 4 °C until sample acquisition using the FACs Canto or LSR Fortessa cytometer. Unstained controls were resuspended in 100 µl PBS. LTG stain was not washed off prior to acquiring the samples.

2.2.6.4 Live dead staining

Live/dead staining was carried out to enable gating of live cells. Live dead stain was prepared at the desired concentration in PBS (Table 2.10). To stain, cells were resuspended in 50 µl of live/dead master mix and left in the dark for 20 min at room temperature. 50 µl of PBS was added to single-stain and unstained controls. Cells were then washed in 1X PBS and pelleted for further staining.

2.2.6.5 Extracellular staining

Extracellular antibodies with conjugated fluorochrome and Fc block were prepared in FACs buffer (Table 2.7). Samples were re-suspended in 50 µl master mix and incubated for 30 min at room temperature in the dark. Unstained controls were resuspended in 50 µl FACs buffer. Cells were washed with 1X PBS, pelleted and resuspended in 150 µl FACs buffer prior to sample acquisition using the LSR Fortessa or FACs Canto.

2.2.7 Assessing impact of treatment condition on macrophage viability

The impact of treatment conditions utilised (Table 2.24) on macrophage viability was assessed by flow cytometry. Cells were treated and left in culture for 24 hours. Cells were harvested into pre-labelled FACs tubes. Cells were washed by adding 1 ml PBS and centrifugation for 5 min. Supernatants were removed, cell pellet disrupted and stained for analysis using live dead staining as per 2.2.6.4. Percentage of live cells was quantified and graphed (Figure 2.2).

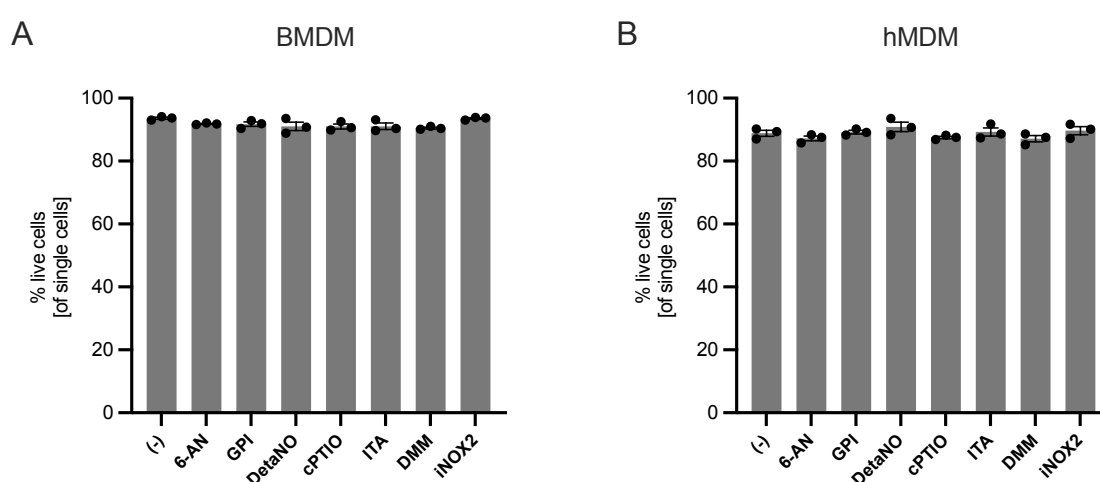


Figure 2. 2 Cell viability during experimental conditions

Compound	Concentration used: BMDM	Concentration used: hMDM
6-AN	100 μ M	25 μ M
GPI	25 μ M	25 μ M
DetaNO	50 μ M	25 μ M
cPTIO	50 μ M	25 μ M
Itaconic acid	10 mM	10 mM
DMM	1 mM	1 mM
GSK2795039 (iNOX2)	20 μ M	20 μ M

Table 2. 24 List and concentration of conditions used

Chapter 3. *Mycobacterium tuberculosis* infection
attenuates macrophage central carbon metabolism,
whilst promoting Pentose Phosphate Pathway
activity

3.1 Introduction

Pathogenesis of Mtb and bacterial mechanisms engaged to subvert host macrophage antimicrobial responses remain largely unexplored. However, increasing evidence suggests Mtb actively targets host immunometabolic programmes associated with macrophage classical activation in an effort to establish persistent infection (41).

Classical activation of macrophages and employment of antimicrobial processes is associated with Warburg metabolism, where host cells display increased reliance on glycolytic metabolism in order to support inflammatory activity such as cytokine secretion, antigen presentation and enhanced bacterial killing (36, 122, 206, 315, 316). In particular, these metabolic alterations have been strongly linked to the enhanced secretion of pro-inflammatory IL-1 β , a chief cytokine underpinning macrophage response to Mtb infection. Studies investigating the metabolic profile of host cells during infection, including human alveolar macrophages, monocyte derived macrophages and murine bone marrow derived macrophages, display a functional switch from cellular OXPHOS towards increased glycolytic metabolism (45). Moreover, inhibition of this rewired metabolic profile resulted in decreased secretion of IL-1 β whilst promoting secretion of anti-inflammatory IL-10, in addition to supporting increased intracellular bacterial replication.

Investigation of glycolytic contribution to macrophage antimycobacterial activity found that during infection, Mtb actively undermines host cells efforts to engage glycolysis through the sustained upregulation of anti-inflammatory miR-21 by the proliferating bacteria (41). This Mtb induced deceleration of bioenergetic metabolism is achieved through targeting glycolytic gatekeeper and rate limiting enzyme, PFK-m by miR-21, subsequently reducing the capacity for host macrophages to drive glycolysis and produce IL-1 β . These findings were further supported by studies demonstrating diminished energy metabolism through extracellular flux analysis on human monocyte derived macrophages during Mtb infection, enabling analysis of both glycolysis and mitochondrial respiration during real-time infection (43). Here, it was concluded that

Mtb induces a state of host macrophage quiescence whilst shifting cellular dependency to exogenous fatty acids as an energy source over that of both glucose and endogenous fatty acids.

Further work focusing on the relationship between glycolysis and the PPP in immune cell metabolism demonstrates that during neutrophil mediated phagocytosis, small molecule allosteric activation of the glycolytic enzyme phosphofructokinase-L (PFK-L) can enhance glycolytic flux, leading to a suppression of the oxidative burst during phagocytosis by diverting glucose away from the PPP (231). Since the PPP is essential for generating NADPH, a critical cofactor for producing ROS, increased glycolysis reduces PPP activity, thereby limiting NADPH production and diminishing the oxidative burst. The identification of PFK-L as a negative regulatory node in controlling phagocyte oxidative burst was further highlighted in *Pfkl*^{-/-} BMDMs, where enhanced PPP activity was observed at the expense of glycolytic metabolism (317).

Based on these previous findings, we have hypothesised that Mtb subverts host macrophage central carbon metabolism through targeting glycolytic gatekeeper PFK-m. This subsequently leads to accumulation of upper glycolytic metabolites and coerces employment of Pentose Phosphate Pathway activity as means to evade antimycobacterial metabolism and support bacterial mediated immune evasion. Thus this chapter examines PPP activity in the context of Mtb infection of macrophages – using macrophages stimulated with heat-killed/inactivated forms of the same strain as a control, to distinguish host from bacterial driven processes.

3.2 Results

3.2.1 *Mycobacterium tuberculosis* attenuates macrophage pro-inflammatory cytokine secretion

To study the effects of Mtb infection on macrophage metabolism and subsequent antimicrobial responses, we employed a model of comparative infection. Macrophages were infected with live Mtb, strain H37Ra (Live Mtb) and compared to that of cells infected with an equivalent amount of inactivated counterpart, heat-killed H37Ra (Dead Mtb). This allowed us to delineate the contribution of innate immune recognition of Mtb by PRRs with the immune-evasive processes employed during active Mtb infection. Similar models of comparative infection have been routinely used to examine host cell immunometabolic function during Mtb infection (41, 43).

Given the importance of pro-inflammatory cytokine secretion during macrophages antimicrobial responses, we first examined the generation of IL-1 β and TNF α within our system. Bone-marrow derived macrophages (BMDMs) and human-monocyte derived macrophages (hMDMs) were isolated and cultured as per respective protocols, 2.2.1.1 and 2.2.1.3. Once matured, macrophages were infected with live and dead Mtb strain H37Ra at a multiplicity of infection (MOI) of 5 mycobacteria per cell as described in protocol 2.2.2.4. After the indicated timepoints, RNA was harvested for qPCR analysis, whilst supernatant was collected for protein quantification through ELISA.

Firstly, we quantified gene expression and mature cytokine levels of IL-1 β within BMDMs following Mtb infection (Figure 3.1). As previously described within the literature and when compared to heat killed Mtb, live infection results in attenuated levels of IL-1 β at both the RNA and protein level over the time course of the infection (3-72 hours). The reduction of IL-1 β production by Mtb was subsequently verified in a hMDM infection model following 24 hours of infection (Figure 3.1). Next, we examined the impact of Mtb infection on TNF α secretion by macrophages.. BMDMs stimulated with inactivated mycobacteria generated significantly higher levels of TNF α both transcriptionally and

translationally during early 3 hour time points of infection (Figure 3.2). At later timepoints, 24 and 72 hpi, levels of TNF α produced in response to infection with viable Mtb surpassed that of its inactivated counterpart. Similar results were observed during infection of hMDMs 24 hours post infection (Figure 3.2). Finally, a comparable trend was observed in the quantification of IL-6 secretion (Figure 3.3). Treatment with inactivated Mtb led to significantly higher IL-6 production in BMDMs after 3 hours, whereas live Mtb infection resulted in greater IL-6 levels at 24 and 72 hours post infection. These results highlight the specific attenuation of IL-1 β during viable infection of macrophages.

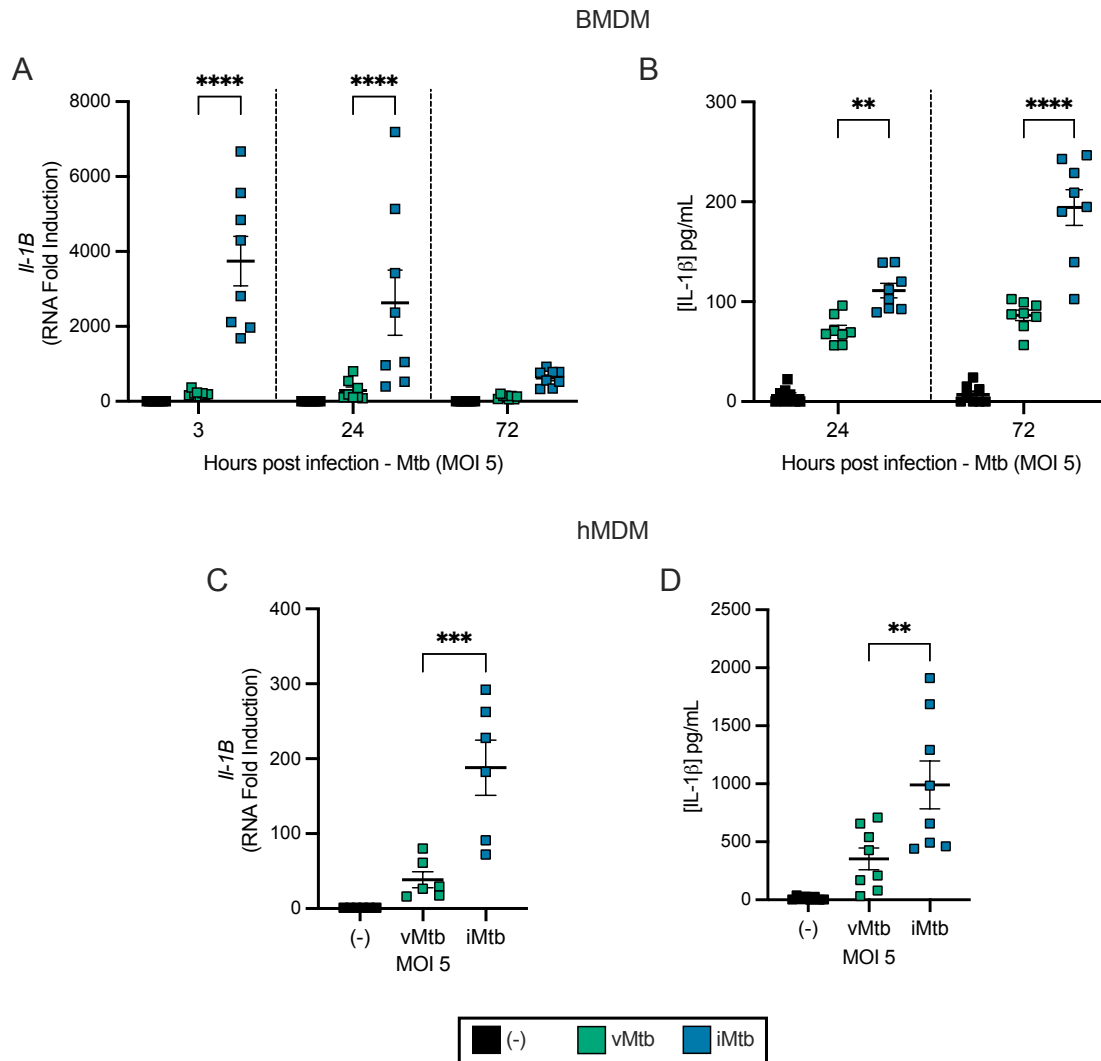
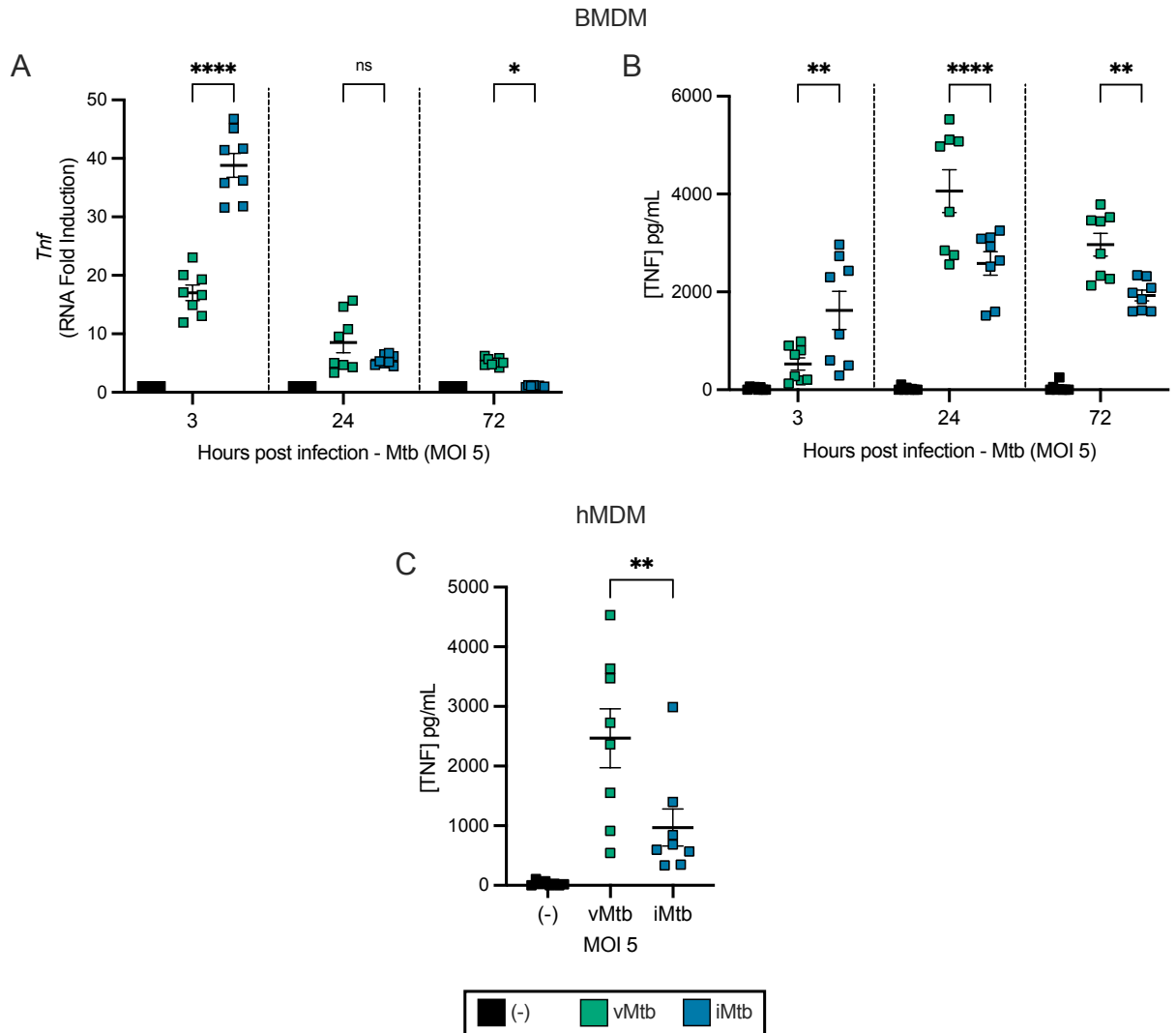


Figure 3. 1 IL-1 β secretion is attenuated during viable Mtb infection of murine and human macrophages compared to inactive Mtb

Murine BMDMs (A+B) human MDMs (C+D) were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3 (A+B), 24 (A-D) and 72 (A-B) hours. (A+C) qPCR was performed and expression of *Il-1β* relative to the untreated control was calculated. Data was normalised to *Hprt* (A) and *18S* (C). (B+D) Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an unpaired 2way ANOVA with Tukey's multiple comparisons test (A+B) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (C+D). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.



Murine BMDMs (A+B) human MDMs (C) were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3 (A+B), 24 (A-C) and 72 (A-B) hours. (A) qPCR was performed and expression of *Tnf* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B+C) Supernatants were collected and TNF α protein quantified using an ELISA. Statistical analysis was performed using an unpaired 2way ANOVA with Tukey's multiple comparisons test (A+B) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (C). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

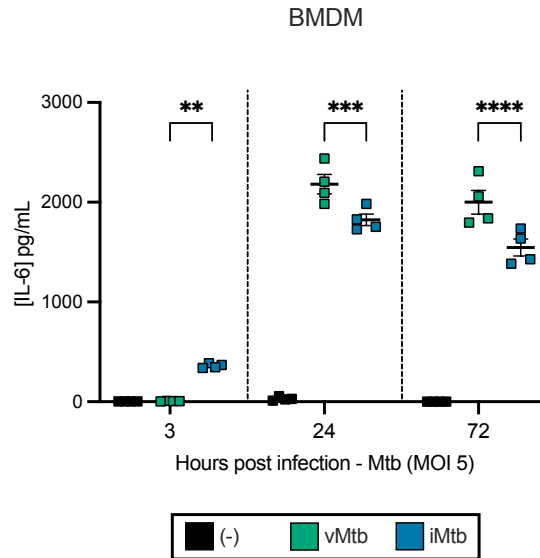


Figure 3. 3 IL-6 secretion is attenuated three hours post viable Mtb infection of murine macrophages and increased at later time points compared to inactive Mtb

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3, 24 and 72 hours. Supernatants were collected and IL-6 protein quantified using an ELISA. Statistical analysis was performed using an unpaired 2way ANOVA with Tukey's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

3.2.2 *Mycobacterium tuberculosis* attenuates macrophage glycolytic reprogramming

Our lab and others have highlighted IL-1 β as a vital cytokine necessary for macrophages to control Mtb intracellular infection (41, 105, 110). Its generation has been shown to increase the expression of eicosanoids, which subsequently limits type I interferon signalling and enhances bacterial containment through apoptotic cell death (105). Gleeson et al. demonstrated that effective induction of IL-1 β relies on increased glycolysis during metabolic reprogramming in order to promote bacterial containment (45). Building from this, our work in the Sheedy lab has identified a Mtb mediated negative induction of glycolytic metabolism by targeting expression of the pathways rate-limiting enzyme, PFK-1 (41). I confirmed this metabolic attenuation within our model and further characterise macrophage bioenergetics during infection.

As a result of metabolic reprogramming to support antimicrobial effector functions, macrophages produce increased amounts of the glycolytic by-product lactate. The quantification of lactate can be used to infer levels of glycolytic activity. Within the comparative infection model, we recorded a significant reduction in both BMDM and hMDM derived lactate secretion during live infection (strain H37Ra) when compared to treatment with an equivalent dose (MOI 5) of heat killed Mtb (Figure 3.4).

Next, we analysed glycolytic gene expression patterns within our infection model. Previous work highlights Mtb mediated metabolic attenuation through targeting of PFK-1 (41). Structurally, PFK-1 is a heterotetramer composed of different combinations of three isoforms: muscle (M), platelet (P) and (L) liver. Each isoform is encoded by a distinct gene and is differentially expressed across various tissues with RNA-seq analysis from BMDMs revealing upregulation of both PFK-L and PFK-P following Mtb infection, whilst PFK-M was downregulated (47). Following this, the Sheedy lab demonstrated that Mtb induced miR-21, which directly repressed *Pfk-m* expression. In turn, this reduced glycolytic induction and diminished antimicrobial responses, such as IL-1 β secretion (Figure 3.5) This mechanism of immune evasion was initially described within murine

models, therefore we sought to further evaluate this within human macrophages. Following comparative infection, we recorded a heightened expression of mature miR-21 in hMDMs infected with viable Mtb compared to cells stimulated with equivalent amounts of heat-killed Mtb or uninfected controls (Figure 3.6). Consistent with this, analysis of glycolytic-associated genes within hMDMs demonstrated an increased expression of upper pathway gene *Hk2* during live infection, with a reduction in the expression of miR-21 target *Pfk-m* and lower glycolysis-associated *Ldha* compared to heat killed Mtb (Figure 3.7).

To support both the lactate concentration and transcriptional profile recorded during infection, we used extracellular flux analysis to record the modulation of energy metabolism, specifically extracellular acidification rate (ECAR) to analyse glycolytic activity. Here, we observed that infection with viable Mtb leads to significantly less basal glycolysis and maximal glycolytic rates following inhibition of oxphos ATP synthesis with oligomycin than that seen in cells stimulates with heat-killed Mtb.

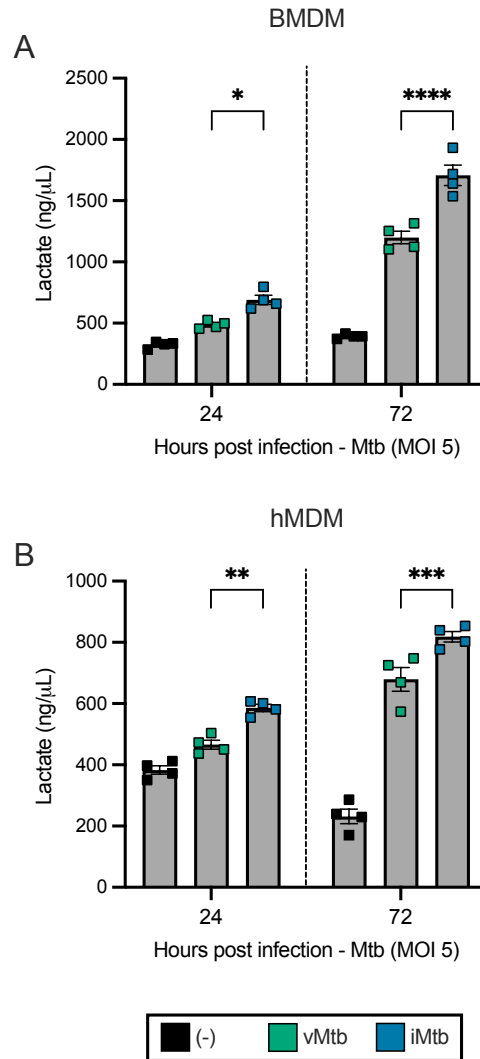


Figure 3. 4 Viable Mtb infection attenuates lactate production in murine and human macrophages compared to inactive Mtb

Murine BMDMs (A) human MDMs (B) were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and lactate metabolite levels quantified. Statistical analysis was performed using an unpaired 2way ANOVA with Tukey's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

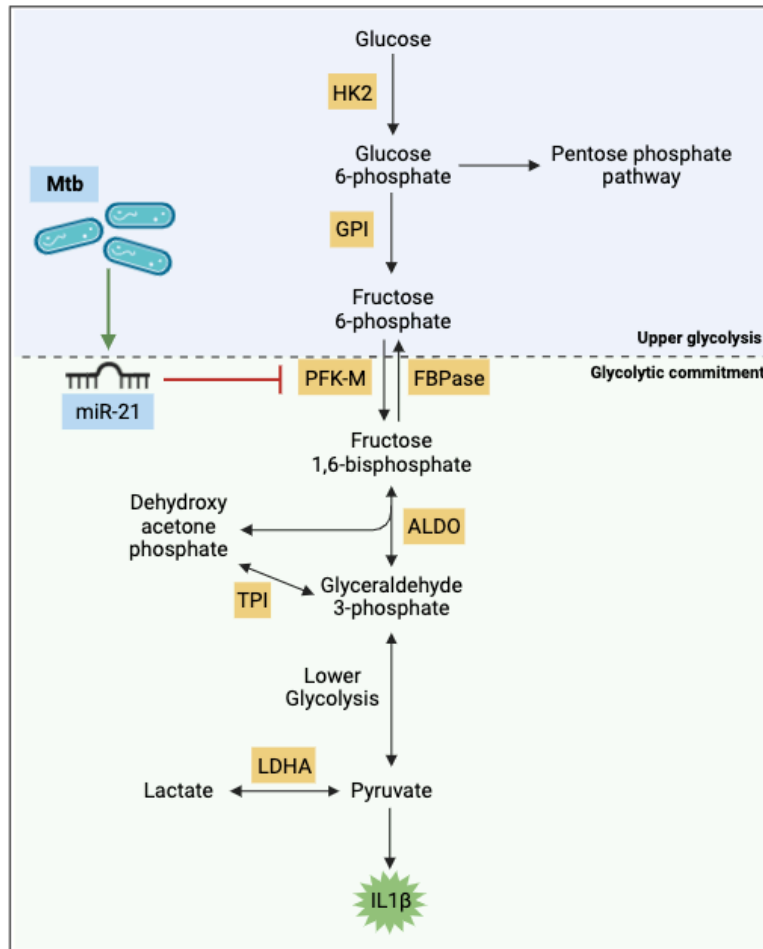


Figure 3. 5 Viable Mtb limits host glycolysis and IL1 β by restriction of PFK-M via miR-21

Repression of host glycolysis in Mtb-infected macrophages is dependent on the sustained upregulation of anti-inflammatory miR-21 by proliferating mycobacteria. MiR-21 mediates the dampening of glycolysis by targeting the phosphofructokinase muscle (PFK-M) isoform at the committed step of glycolysis, facilitating bacterial growth by limiting pro-inflammatory mediators, primarily IL-1 β .

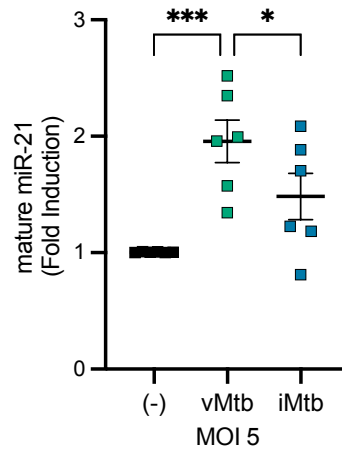


Figure 3. 6 Viable Mtb promotes heightened expression of miR-21 in human macrophages

Human MDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of miR-21 relative to untreated control was calculated. Data was normalised to RNURB. Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 6$ independent experiments.

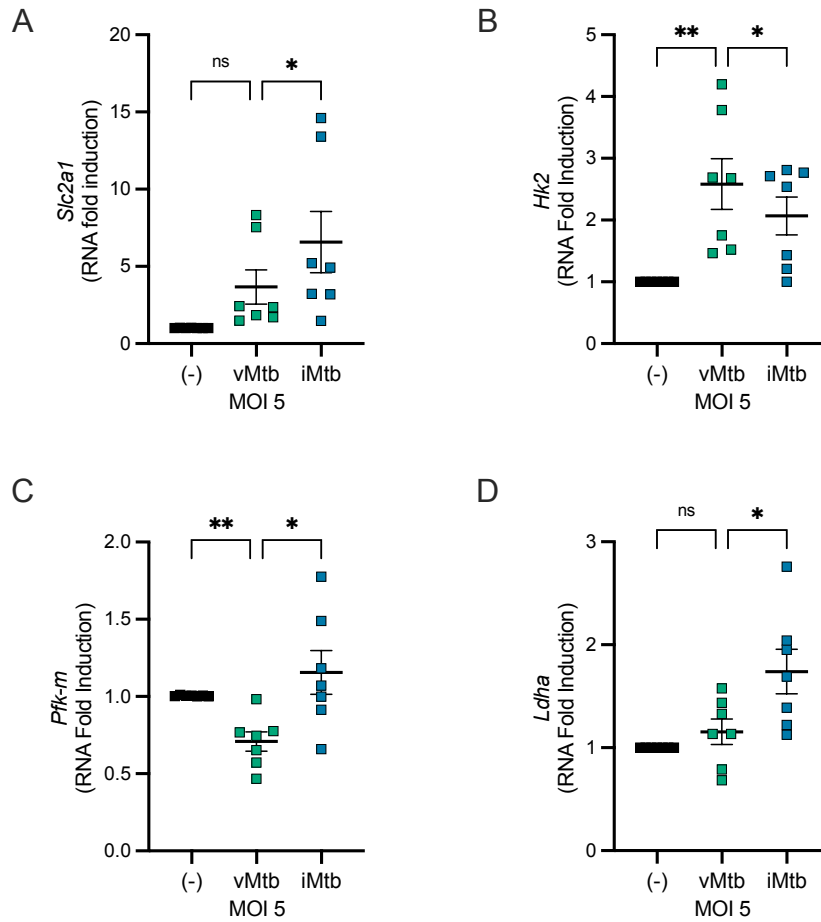


Figure 3. 7 Viable Mtb reduces lower glycolytic-associated gene expression in human macrophages compared to inactive Mtb

Human MDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of (A) *Slc2a1*, (B) *Hk2*, (C) *Pfk-m* and (D) *Ldha* relative to the untreated control was calculated. Data was normalised to *18S*. Statistical analysis was performed using an RM one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

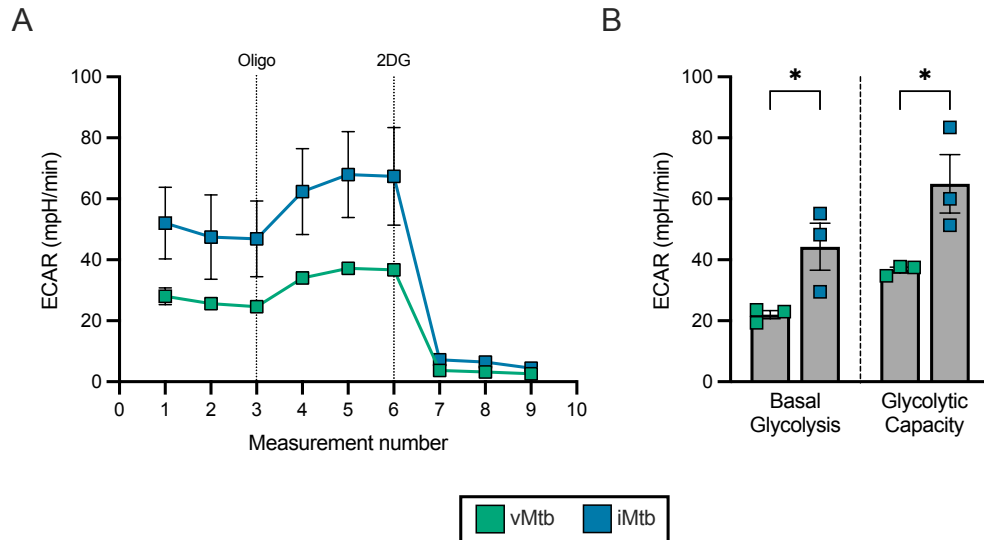


Figure 3. 8 Viable Mtb infection reduces glycolytic rates in macrophages compared to inactive Mtb

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Extracellular acidification rate (ECAR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo) and 2-deoxyglucose (2DG). Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, N=1, n = 3 independent experiments.

3.2.3 *Mycobacterium tuberculosis* promotes pentose phosphate pathway activity during infection of macrophages

Given that Mtb significantly attenuates central glycolytic metabolism during macrophage infection, we hypothesized that Mtb-derived miR-21, by targeting the rate-limiting enzyme PFK-M, could cause an accumulation and rerouting of upper glycolytic intermediates into alternative metabolic pathways. Therefore, we examined pentose phosphate pathway (PPP) activity during infection.

First, we analysed expression of PPP-associated enzymes within the comparative infection model, focusing on *G6pd* and *Pgd* in the irreversible oxidative phase and *Taldo1* and *Tkt* in the pathways non-oxidative reversible phase. Infection with viable Mtb resulted in a significant increase in expression of both *G6pdx* and *Pgd* in murine BMDMs, both of whose activity results in the generation of NADPH from enzymatic reduction of co-factor NADP. An increase in expression of the non-oxPPP enzymes *Taldo1* and *Tkt* was also recorded in BMDMs during Mtb infection when compared to the treatment with the heat-killed counterpart (Figure 3.9). These observations were replicated within human MDMs upon specific analysis of *G6pd* and *Taldo1* expression (Figure 3.10).

In order to validate the expression pattern observed was consistent at a protein level, we examined G6PD enzyme activity in both BMDMs and hMDMs following infection. G6PD catalyses the first entry point and serves as the rate-limiting step for PPP activity. Using a substrate-linked assay which specifically quantifies G6PD enzyme activity in cell lysates, we observed heightened enzyme activity during infection with viable Mtb within both murine and human macrophage models than that observed in macrophages treated with inactivated forms (Figure 3.11). These results were supported by increased levels of the PPP metabolite (6-phosphogluconate) during viable infection (Figure 3.12), measured by a specific metabolite detection kit. This was particularly pronounced at later time points post-infection, consistent with the decrease in glycolysis. These data are consistent with increased G6PD RNA and protein expression in these cells. Since the PPP is one of the primary producers of cellular NADPH in classically activated

macrophages, to more accurately measure PPP-linked activity, both NADPH and its oxidized form, NADP, were quantified during comparative Mtb infection (Figure 3.13). Consistent with our previous findings, we recorded a significantly higher concentration of both NADP and reduced NADPH with viable Mtb compared to inactive Mtb within murine BMDMs. As PPP-derived NADPH provides the reducing power necessary for the biosynthesis of fatty acids and other lipids, facilitating the conversion of acetyl-CoA into fatty acid chains, we assessed the neutral lipid content within Mtb infected macrophages. Here we found significantly increased lipid content in viable Mtb infected cells compared to those stimulated with comparable levels of inactivated Mtb (Figure 3.14). Such intracellular lipid accumulation in host cells may create a permissive yet nutrient-rich environment that promotes Mtb replication by providing essential lipids for bacterial growth and persistence.

Due to its dynamic nature and multiple links with glycolysis, the PPP can redirect central carbon metabolism by operating in three distinct modes: pentose insufficiency, pentose overflow, and pentose cycling, depending on the cell's requirements for ribose and NADPH (236). Here we observe an increase in central carbon metabolism with respect to PPP activity during viable Mtb infection, with a net loss of the glycolytic end product lactate. Such a discrepancy between carbon input and output suggests an alternative fate for PPP-derived carbon, possibly indicating the induction of a cyclic-PPP where intermediates re-entering upper glycolysis (as fructose-6-phosphate) from the non-OxPPP are recycled to regenerate glucose-6-phosphate via the reversible activity of glucose-6-phosphate isomerase (GPI) and recycled back into the pathway, supporting anabolic processes or redirecting carbon toward biosynthetic routes rather than energy production, ultimately modulating the metabolic environment to meet the pathogen's survival needs.

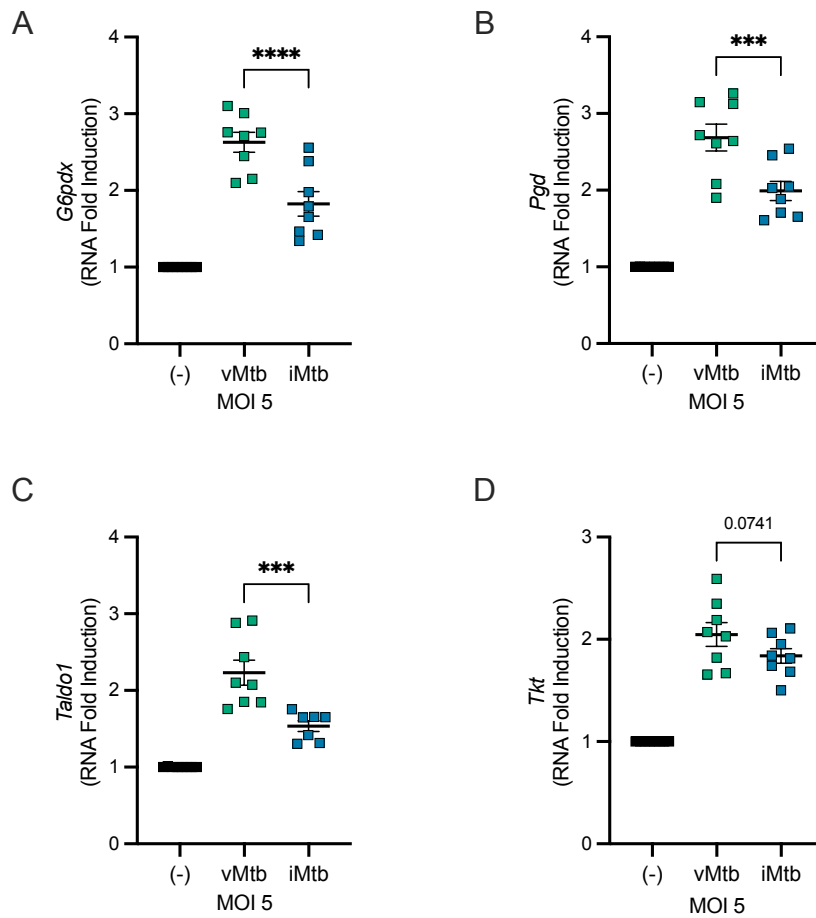


Figure 3. 9 Viable Mtb promotes expression of oxidative and non-oxidative PPP associated enzymes in murine BMDMs

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of *G6pdx* (A), *Pgd* (B), *Taldo1* (C) and *Tkt* (D) relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

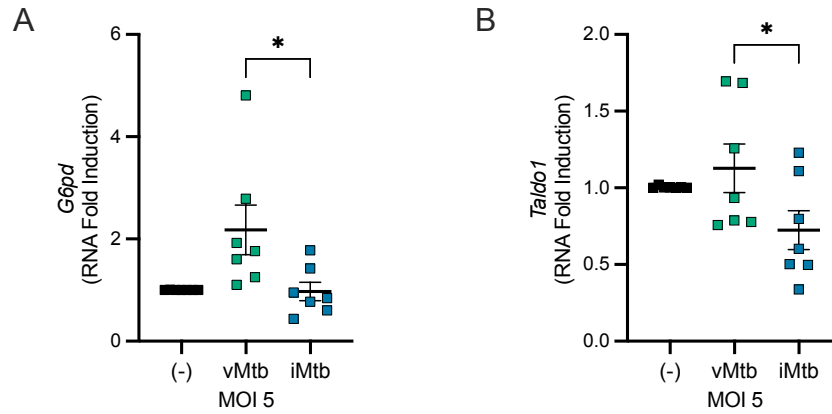


Figure 3. 10 Viable Mtb promotes expression of oxidative and non-oxidative PPP associated enzymes in human MDMs compared to inactive Mtb

Human MDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of *G6pd* (A) and *Taldo1* (B) relative to the untreated control was calculated. Data was normalised to *18S*. Statistical analysis was performed using a RM one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

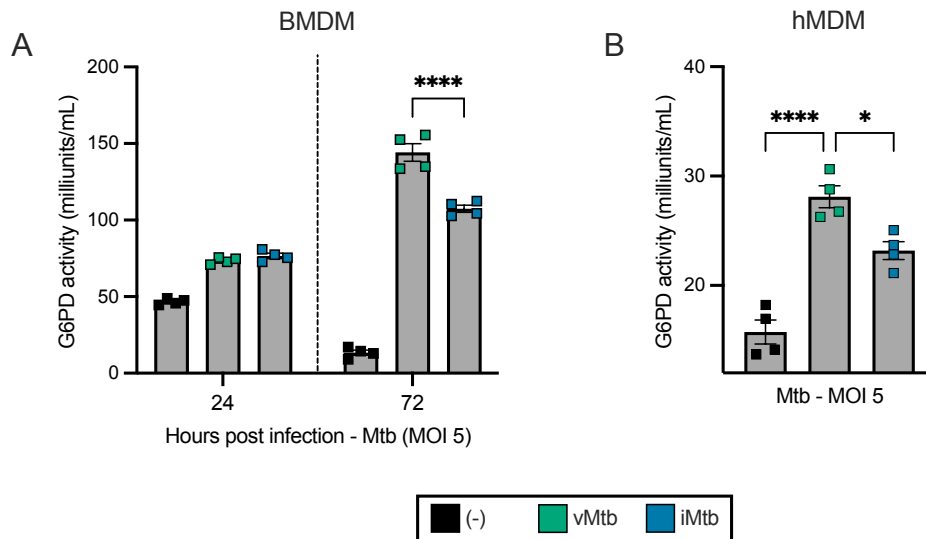


Figure 3. 11 Viable Mtb promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages

Murine BMDMs (A) human MDMs (B) were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A+B) and 72 (A) hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using a 2way ANOVA with Tukey test (A) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

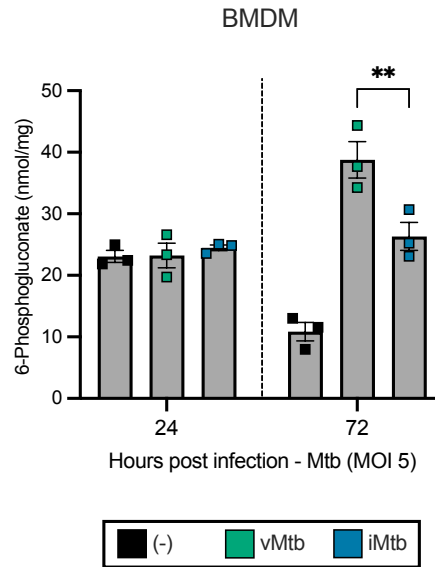


Figure 3. 12 Viable Mtb infection results in heightened PPP metabolite accumulation in macrophages

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify concentration of 6-phosphogluconate. Statistical analysis was performed using a 2way ANOVA with Tukey's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments.

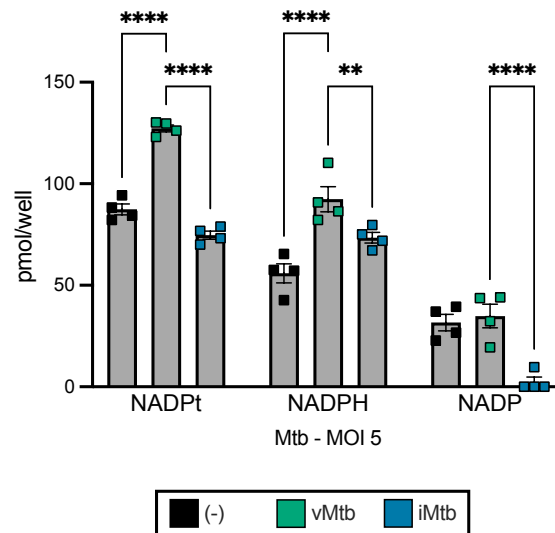


Figure 3. 13 Viable Mtb infection results in heightened NADP/H generation in macrophages

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify concentration of NADPH, NADP and total NADP/H (NADPt). Statistical analysis was performed using a 2way ANOVA with Tukey’s multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

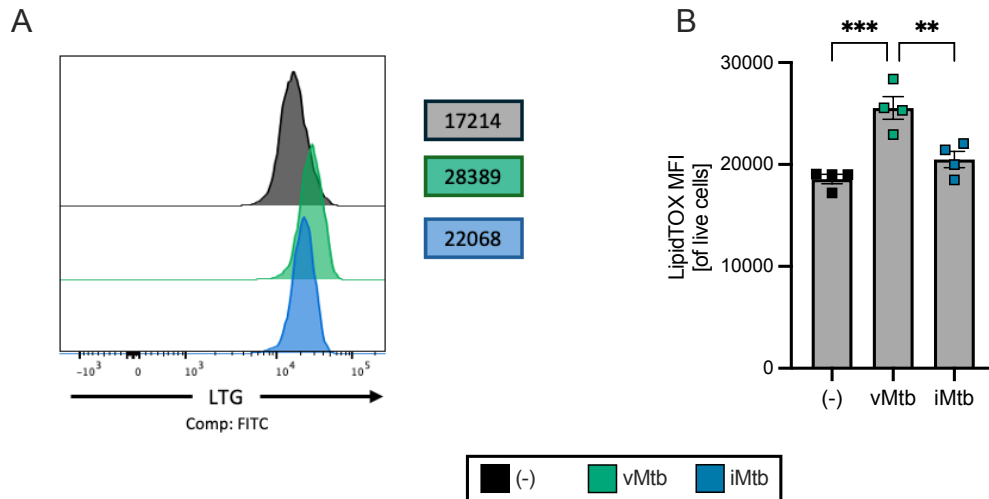


Figure 3. 14 Viable Mtb infection results in heightened neutral lipid accumulation in macrophages

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Graphs show LipidTOX green (LTG) representative histogram (A) and mean fluorescence intensity (MFI) of live macrophages (B). Statistical analysis was performed an ordinary one-way ANOVA with way Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments. .

3.2.4 Pentose phosphate pathway activity supports macrophage immunometabolic responses

The PPP is essential for regulating immune cell function, particularly in phagocytic cells like macrophages and neutrophils, where it supports processes such as the generation of an oxidative burst by producing the pathway's enzymatic co-product, NADPH. With our infection model revealing the preferential induction of a PPP activity in the specific context of viable Mtb infection, we next aimed to disrupt this metabolic activity and evaluate the effect of the alternative PPP modes, pentose insufficiency and pentose overflow, on both macrophage metabolism and bacterial survival.

First, we sought to induce a state of pentose insufficiency through the use of the small molecule inhibitor 6-aminonicotinamide (6-AN), blocking the activity of rate-limiting oxidative PPP enzyme, G6PD. Through irreversibly occupying the enzyme's NADP binding site, 6-AN prevents the contribution of oxidative-PPP activity towards the generation of ribose-5-phosphate, thus promoting a state of pentose insufficiency. Quantification of G6PD activity during Mtb infection of murine and human macrophages upon 6-AN inhibition verified the efficacy of various concentrations of this inhibitor within these cell types (Figure 3.15).

Evaluation of metabolically linked IL-1 β upon 6-AN inhibition demonstrated a surprising and significant decrease in cytokine at both the mRNA and mature protein level within Mtb infected BMDMs over the time course of infection (Figure 3.16). This inhibitory effect on IL-1 β was confirmed within human MDMs (Figure 3.17). A similar albeit less dramatic trend was recorded upon evaluation of TNF α secretion following 6-AN treatment, resulting in a significant decrease in cytokine levels within murine BMDMs (Figure 3.18) and in human MDMs (Figure 3.19), following 24 hours of infection. Notably, 6-AN treatment within the comparative infection model of viable versus inactivated Mtb revealed the requirement of 6-AN for IL-1 β secretion was specific to live/active infection (Figure 3.20), since no differences in cytokine production was measured during inactive Mtb treated BMDMs in the presence of 6-AN.

Following this stark contrast in 6-AN inhibition during comparative infection, we next aimed to evaluate the contribution of alternative sources of glucose-6-phosphate to macrophage pro-inflammatory cytokine profiles during viable Mtb infection. 2DG and GPI were used to inhibit incorporation of carbon from both glucose and glycogen respectively. During active infection, both 2DG and GPI inhibition significantly reduced the secretion of IL-1 β and TNF α (Figure 3.21). In contrast, in response to inactive Mtb, only 2DG reduced IL-1 β secretion, while glycogenolysis inhibition had no effect on either IL-1 β or TNF α levels. Notably, IL-6 were significantly decreased following GPI treatment during both live and inactive Mtb infection. These data suggest specificity to the immunometabolic pathways employed in response to Mtb infection of macrophages and suggests that during live infection with Mtb, when glycolytic and TCA activity is limited, there is a greater demand on the PPP to fuel host immunometabolic responses.

In line with this state of pentose insufficiency, 6-AN treatment significantly reduced lactate secretion normally associated with macrophage classical activation during Mtb infection, in both murine and human models (Figure 3.22). This metabolic phenotype was further supported by a transcriptional signature demonstrating a significant decrease in expression lower glycolytic genes *Pfk-m* and *Ldha* (Figure 3.23), with an increase in non-OxPPP associated *Taldo1* and *Tkt* after 6-AN treatment (Figure 3.24), implying an unmet ribose demand leads to a net non-OxPPP flux towards ribose-5-phosphate synthesis at the expense of glycolytic activity. This attenuated glycolytic activity was also recorded in basal murine and human macrophages using Seahorse extracellular flux analysis following PPP inhibition (Figure 3.25). 6-AN treatment had a no effect on basal oxphos but limits mitochondrial maximum respiratory rate (Figure 3.26). Thus, increased PPP activity during infection with viable Mtb may help fuel glycolytic/immunometabolic responses to support host defence.

Ox-PPP activity supports macrophage NADPH generation. G6PD inhibition by 6-AN results in a significant loss of enzymatic product NADPH and a trending accumulation in its oxidised equivalent and co-factor, NADP (Figure 3.27). Quantification of bacterial growth within Mtb infected macrophages resulted in a dose-dependent and significant

increase in CFUs following 6-AN inhibition (Figure 3.28). Together, these data suggest macrophage dependency on sufficient PPP activity to support traditional central carbon metabolism associated with metabolic reprogramming during Mtb infection. Furthermore, inhibition of ox-PPP activity with 6-AN remodels glycolytic metabolism mimicking a model of pentose insufficiency.

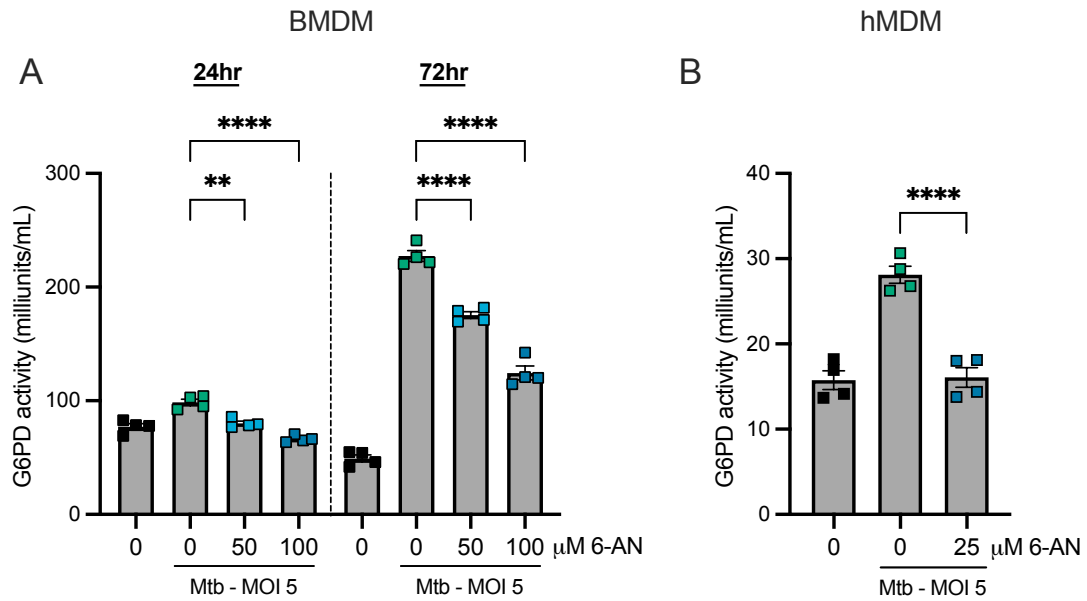


Figure 3. 15 6-AN inhibition decreases G6PD activity in macrophages

Murine BMDMs (A) and human MDMs (B) were pre-treated with 6-AN one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A+B) and 72 (A) hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

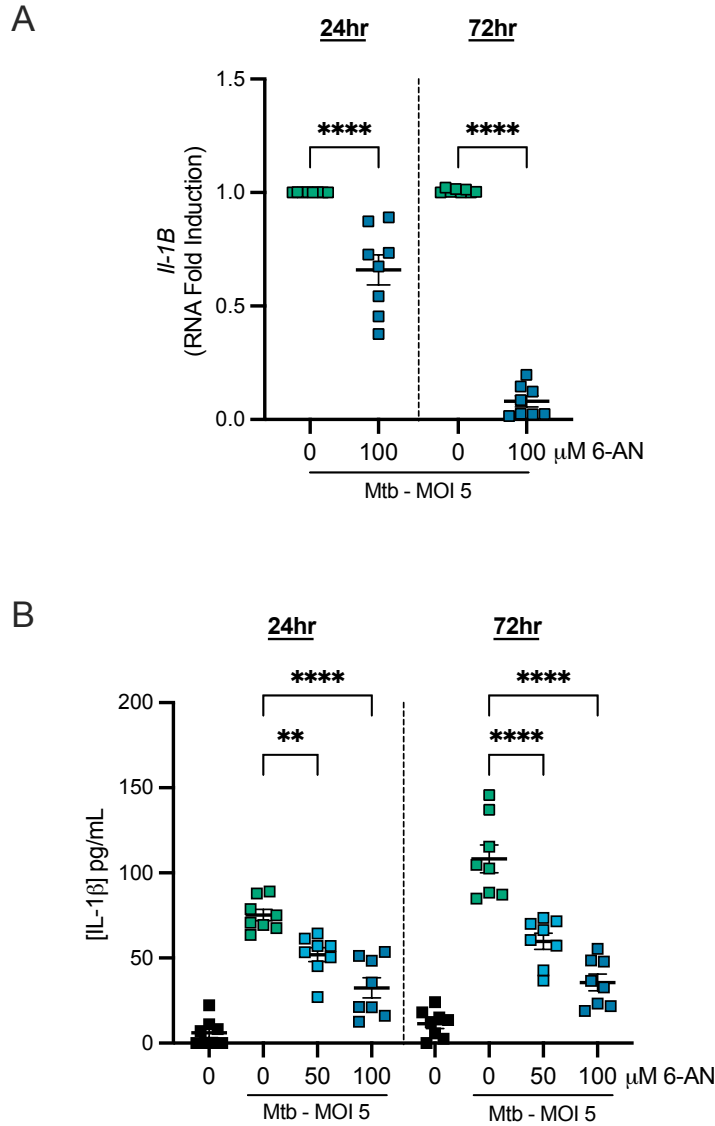


Figure 3. 16 PPP inhibition decreases IL-1 β expression & secretion during infection of murine BMDMs

Murine BMDMs were pre-treated with 6-AN (50 – 100 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. (A) qPCR was performed and expression of *Il-1 β* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B) Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

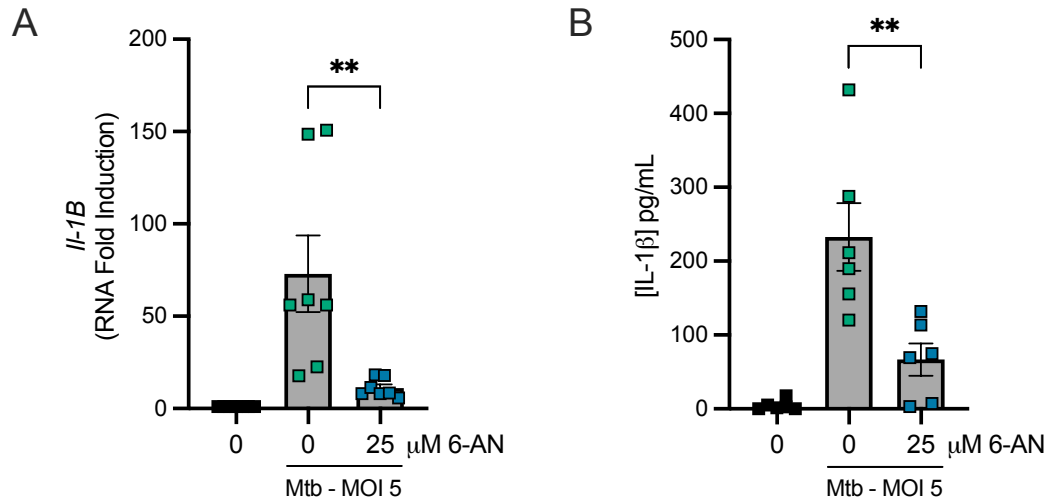


Figure 3. 17 PPP inhibition decreases IL-1 β expression & secretion during infection of human MDMs

Human MDMs were pre-treated with 6-AN (25 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. (A) qPCR was performed and expression of *IL-1 β* relative to the untreated control was calculated. Data was normalised to *18S*. (B) Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

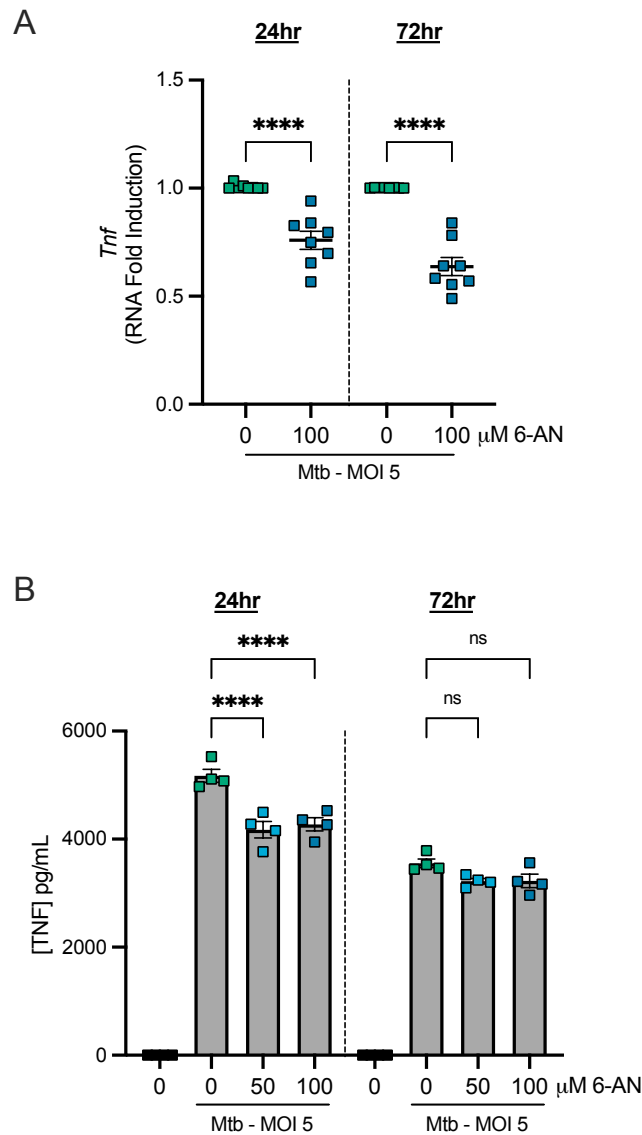


Figure 3. 18 PPP inhibition decreases TNF α expression & secretion during infection of murine BMDMs

Murine BMDMs were pre-treated with 6-AN (50 – 100 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. (A) qPCR was performed and expression of *Tnf* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B) Supernatants were collected and TNF α protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ (A), 4(B) independent experiments.

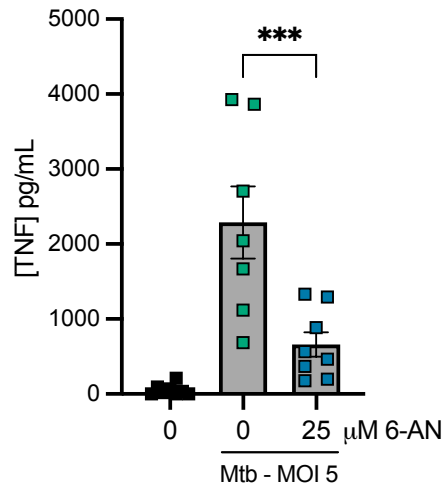


Figure 3. 19 PPP inhibition decreases TNF α secretion during infection of human MDMs

Human MDMs were pre-treated with 6-AN (25 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Supernatants were collected and TNF α protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

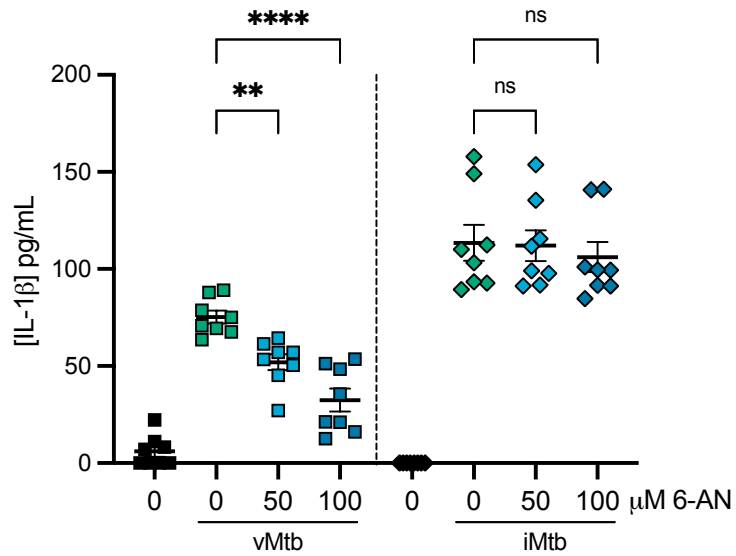


Figure 3. 20 PPP inhibition decreases IL-1 β expression specifically during viable Mtb infection

Murine BMDMs were pre-treated with 6-AN (50 – 100 μ M) one hour prior to infection with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

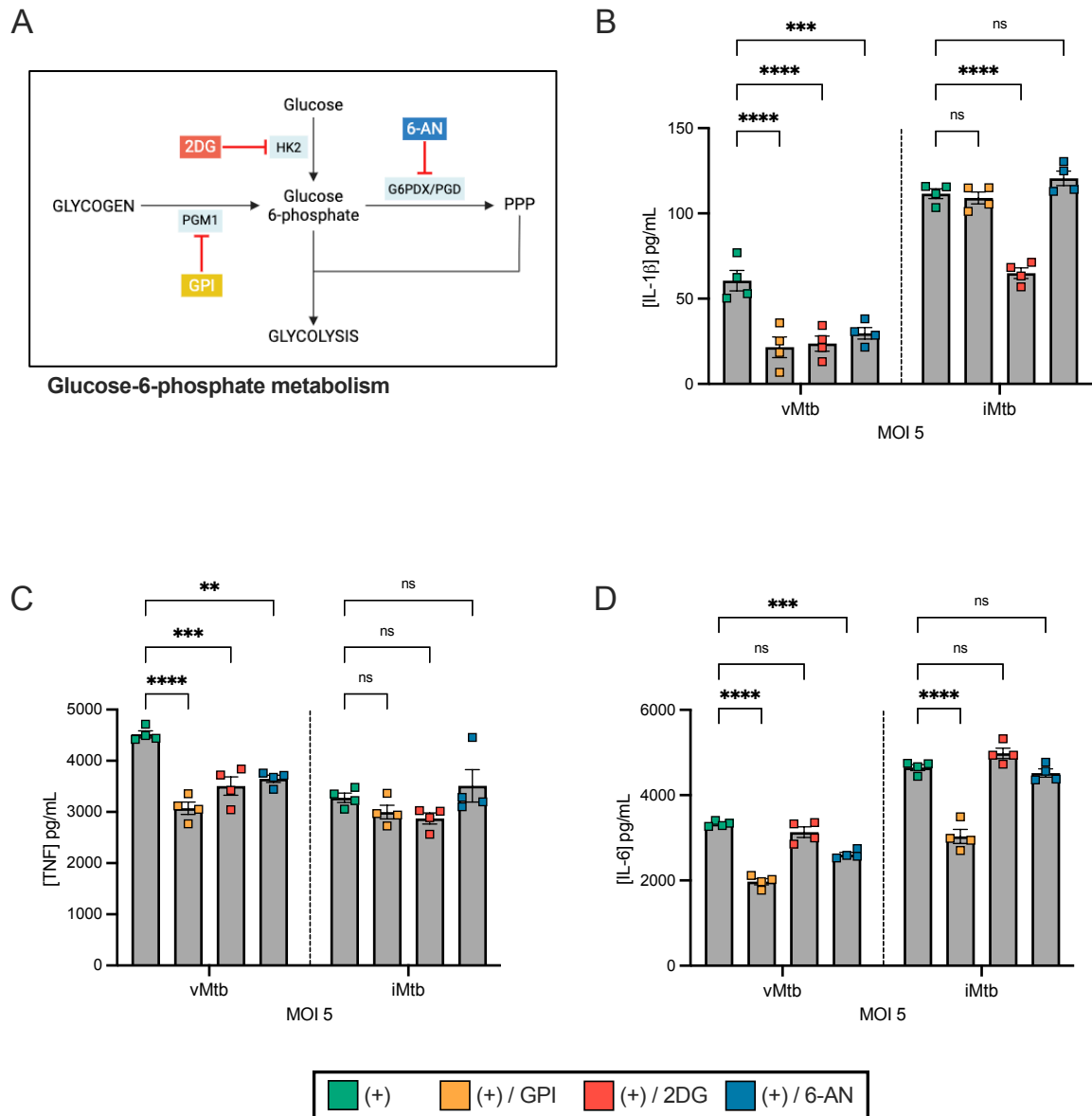


Figure 3. 21 Alternative sources of glucose-6-phosphate promotes macrophage pro-inflammatory cytokine responses during viable Mtb infection

Murine BMDMs were pre-treated with GPI (25 μ M), 2DG (1 mM) and 6-AN (50 μ M) one hour prior to infection with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Supernatants were collected and IL-1 β , TNF α and IL-6 protein was quantified using an ELISA. Statistical analysis was performed using a 2way ANOVA with Tukey's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

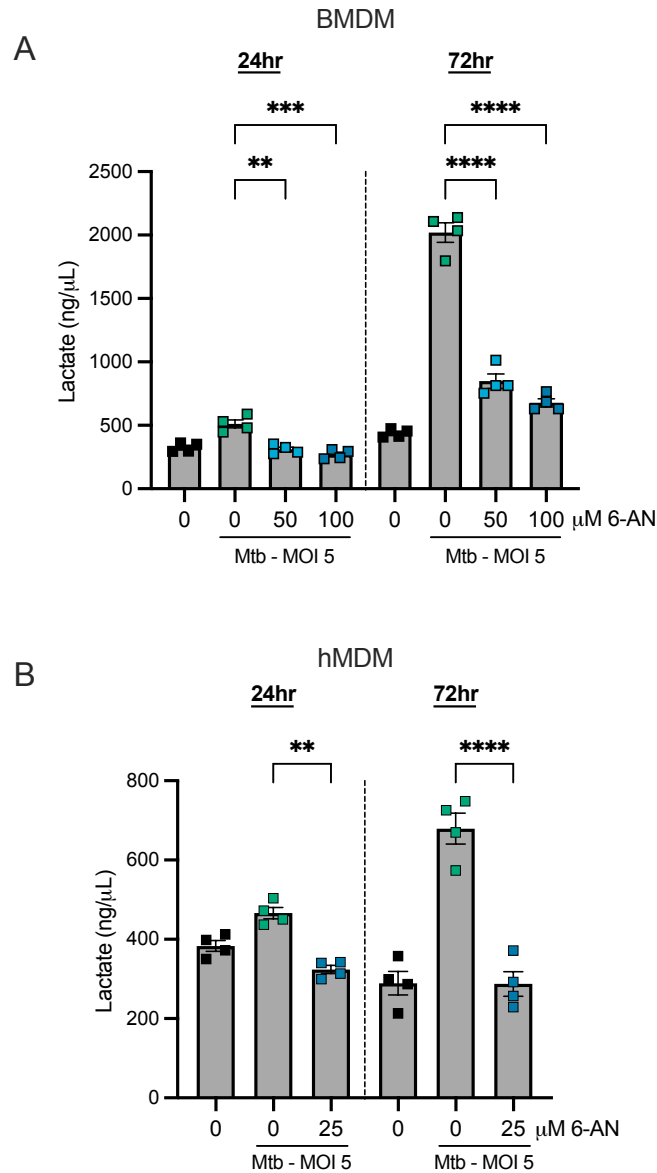


Figure 3. 22 PPP inhibition decreases lactate secretion during Mtb infection

Murine BMDMs (A) and human MDMs (B) were pre-treated with 6-AN one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and lactate metabolite levels quantified. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

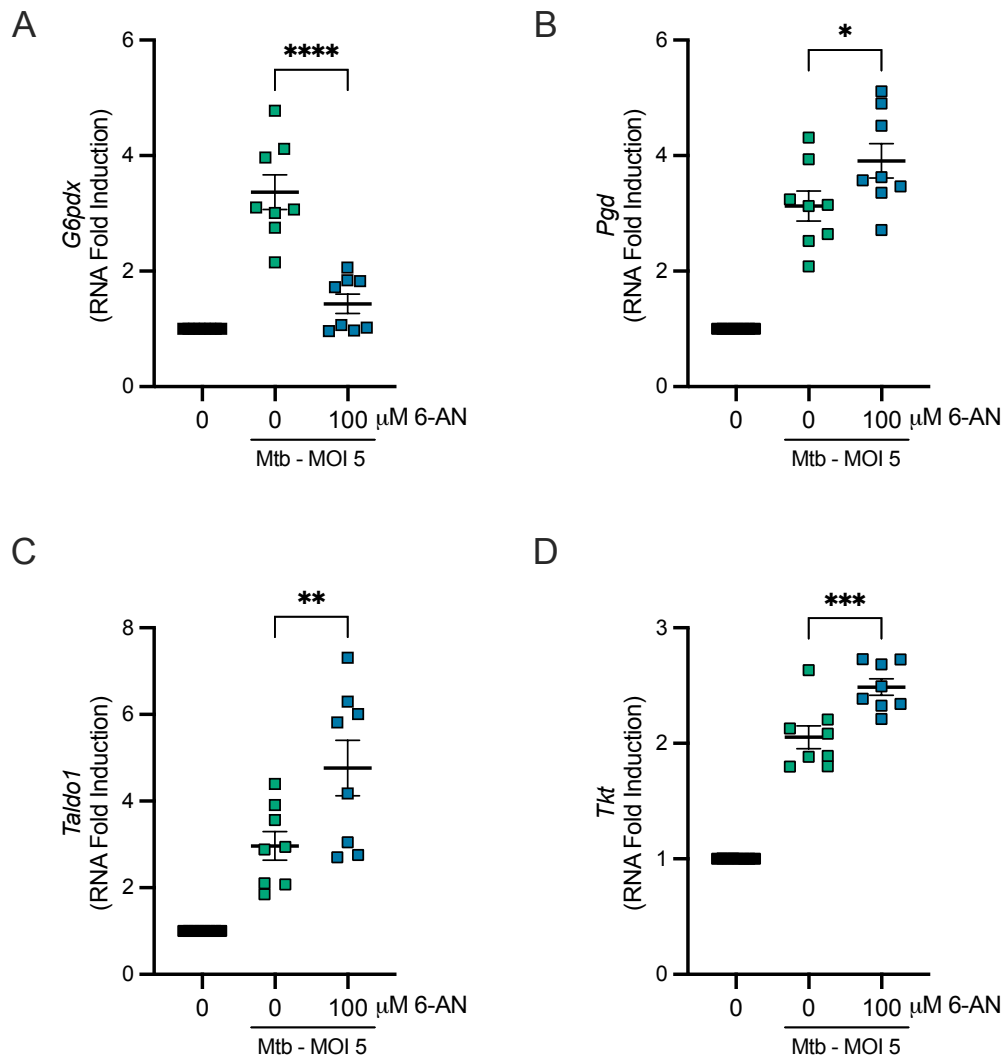


Figure 3. 23 6-AN inhibition increases non-OxPPP associated gene expression during *Mtb* infection of macrophages

Murine BMDMs were pre-treated with 6-AN one hour prior to infection with viable *Mtb* (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. qPCR was performed and expression of *Hk2* (A), *Gpi1* (B), *Pfk-m* (C) and *Ldha* (D) relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

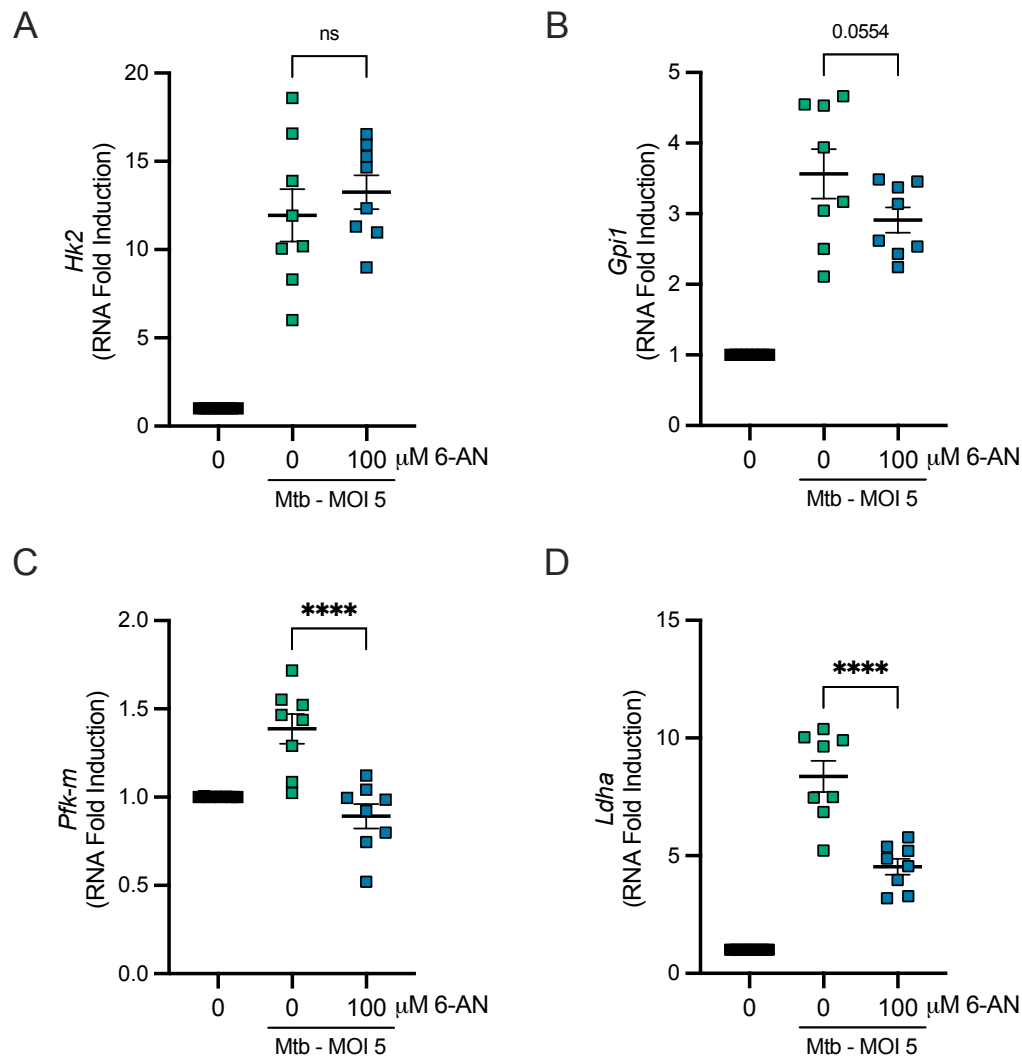


Figure 3. 24 6-AN inhibition decreases lower glycolytic associated gene expression during *Mtb* infection of macrophages

Murine BMDMs were pre-treated with 6-AN one hour prior to infection with viable *Mtb* (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. . qPCR was performed and expression of *G6pdx* (A), *Pgd* (B), *Taldo1* (C) and *Tkt* (D) relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

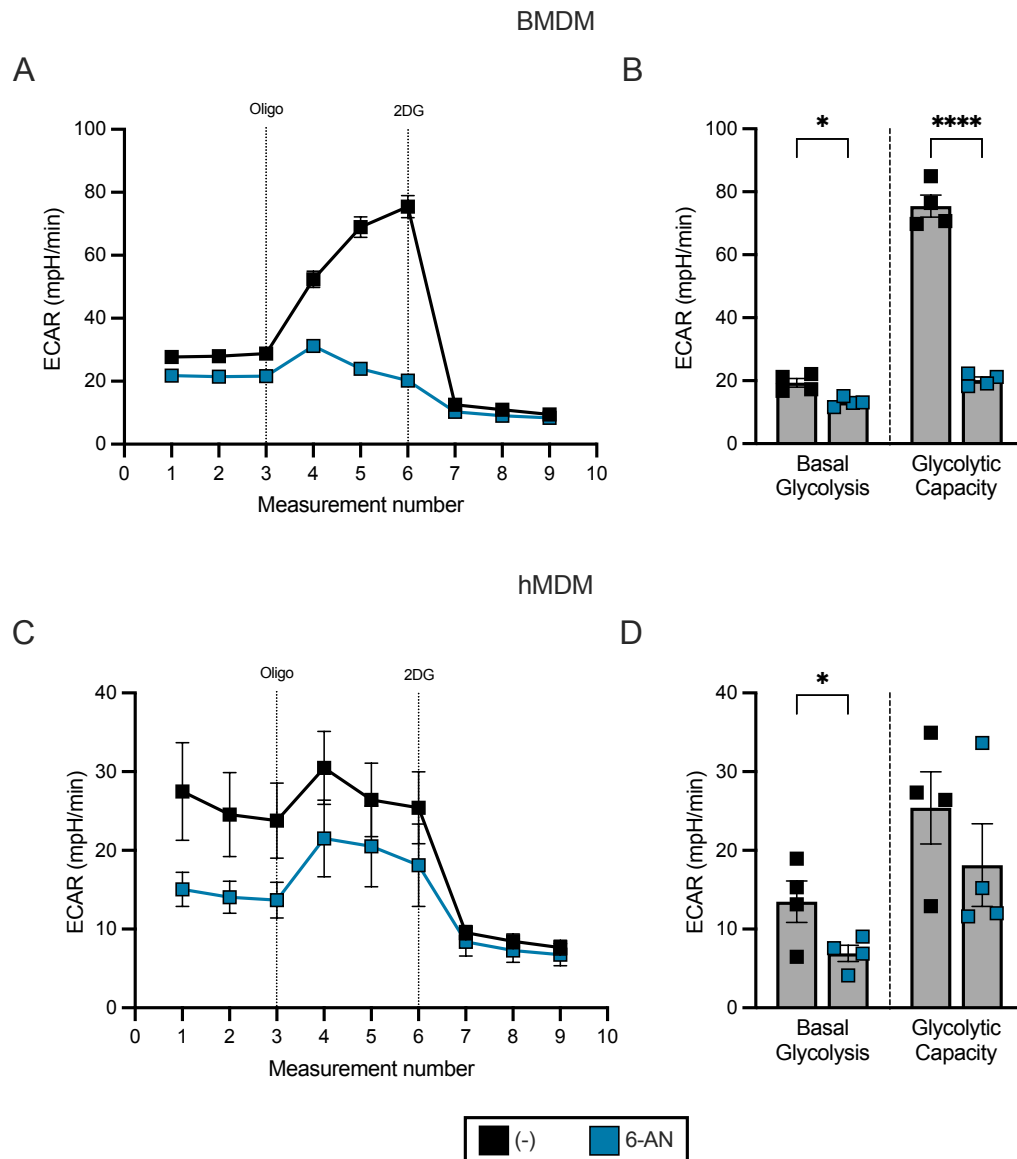


Figure 3. 25 PPP inhibition reduces glycolytic rates of murine and human macrophage

Murine BMDMs (A-B) and human MDMs (C-D) were treated with 6-AN (100 μ M A-B, 25 μ M C-D) for 24 hours Extracellular acidification rate (ECAR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo) and 2-deoxyglucose (2DG). Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test (B) and a RM one-way ANOVA with Fishers LSD test (D) . *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

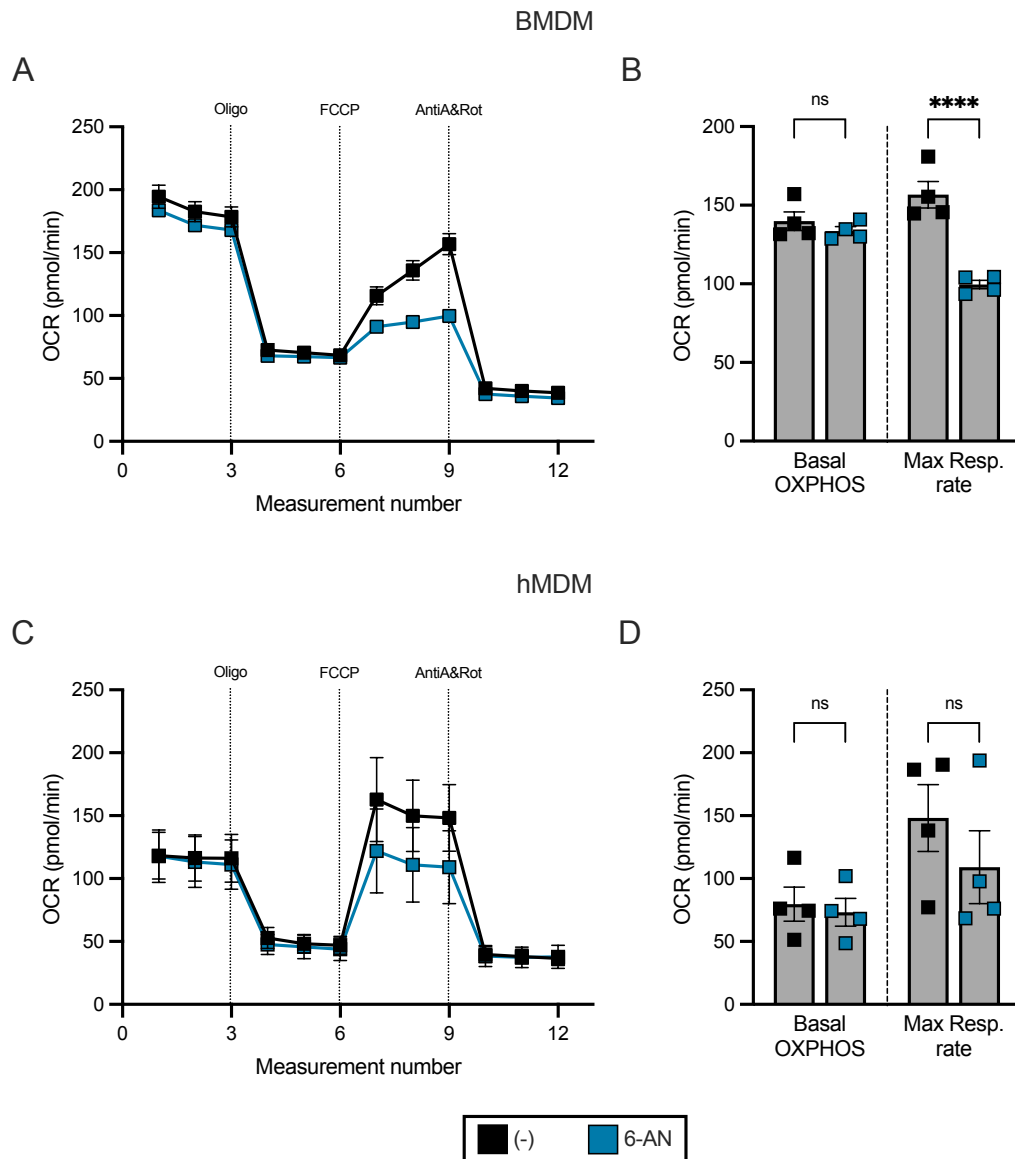


Figure 3. 26 PPP inhibition reduces maximum respiratory rates of murine and human macrophage OXPHOS

Murine BMDMs (A-B) and human MDMs (C-D) were treated with 6-AN (100 μ M A-B, 25 μ M C-D) for 24 hours. Oxygen consumption rate (OCR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo), FCCP, antimycin-A and rotenone (Anti-A&Rot). Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test (B) and a RM one-way ANOVA with Fishers LSD test (D). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

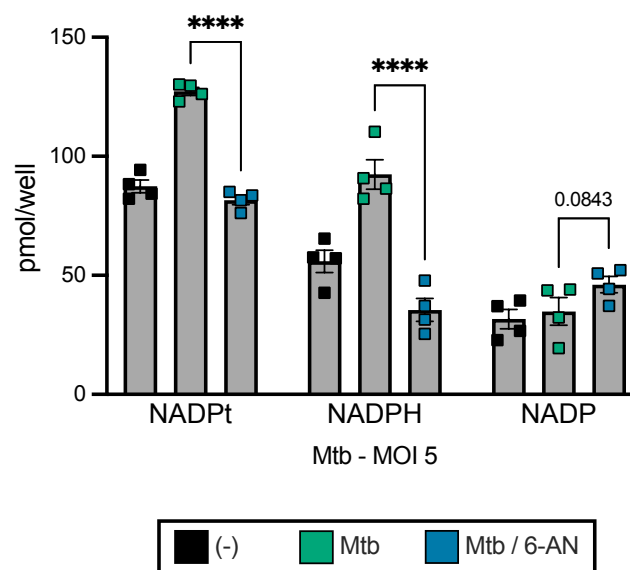


Figure 3. 27 PPP inhibition decreases NADPt and NADPH while increasing NADP during Mtb infection of macrophages

Murine BMDMs were pre-treated with 6-AN (100 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify concentration of NADPH, NADP and total NADP/H (NADPt). Statistical analysis was performed using a 2way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

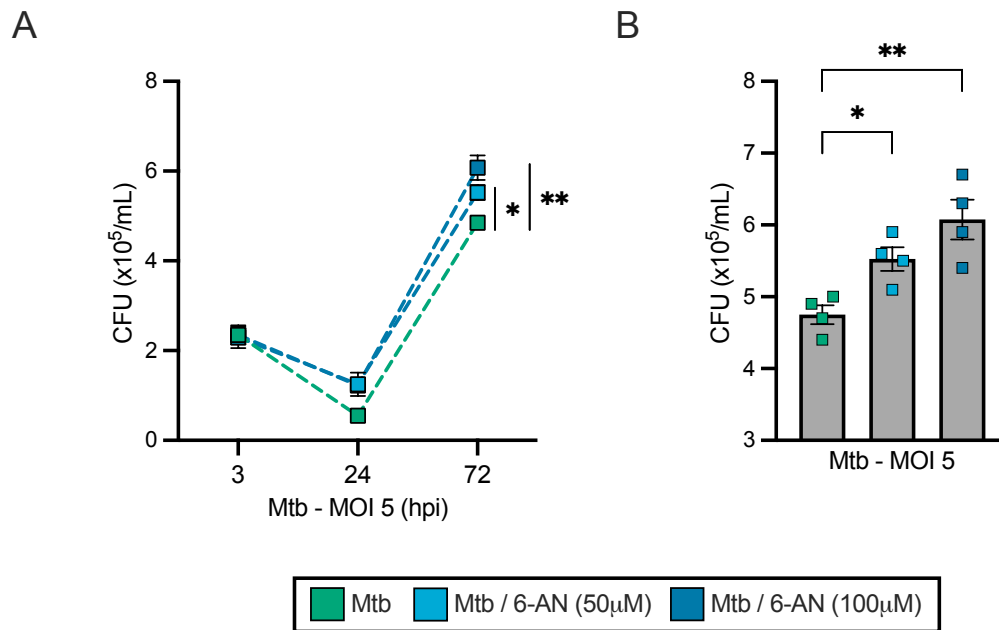


Figure 3. 28 PPP inhibition promotes Mtb growth in macrophages

Murine BMDMs were pre-treated with 6-AN (50 - 100 μM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3, 24 and 72 hours. Cells were lysed in 0.1% Triton X-100 at the indicated time-points (A) and 72 hours (B) and diluted lysates were streaked in triplicate on Middlebrook agar plates and incubated at 37°C for approximately 14 days. Colony forming units (CFUs) were counted and graphed. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

3.2.5 Pentose overflow promotes macrophage antimycobacterial function

To date, we have demonstrated different functional modes of the PPP during Mtb infection of macrophages. The first proposed mode is pentose cycling and is utilized by Mtb as a means of immunometabolic manipulation to evade host macrophage responses. The second mode, pentose insufficiency, involved the active inhibition of G6PD activity, demonstrating the PPP's contribution to macrophage immune function. Thus, we next looked to induce the third described state of PPP activity, pentose overflow, in a bid to overcome decelerated metabolic bioenergetics typically associated with Mtb infection of macrophages (41, 43). Here, we utilized a novel miR-21^{-/-} murine model, where Mtb is unable to impose a metabolic brake on glycolytic gatekeeper PFK-M and the PPP re-entry point to central carbon metabolism. Earlier research has pinpointed PFK-1 activity as a crucial regulatory node of PPP activity. PFK-1 agonists reduce cyclic signalling in activated neutrophils, resulting in lower NADPH levels (231). Conversely, PFK knockdown can increase PPP-derived NADPH production (317).

Following Mtb infection, we aimed to demonstrate increased PPP activity in the absence of miR-21. Analysis of both oxidative (*G6pdx* and *Pgd*) and non-oxidative (*Taldo1* and *Tkt*) PPP-associated genes revealed significantly elevated expression in miR-21^{-/-} macrophages during Mtb infection (Figure 3.29). Correspondingly, greater G6PD activity was observed following the loss of miR-21 (Figure 3.30). Removal of the metabolic brake enforced by Mtb during infection to promote PPP cycling resulted in restored glycolytic activity, as recorded by increased lactate accumulation in miR-21^{-/-} macrophages (Figure 3.31). This heightened metabolic profile supports pro-inflammatory activity, with significantly greater IL-1 β and TNF α secretion in miR-21^{-/-} macrophages during infection (Figure 3.32). Heightened lactate secretion associated with deletion of miR-21 was ablated following 6-AN treatment (Figure 3.33). Finally, evaluation of Mtb growth revealed heightened bacterial clearance in miR-21^{-/-} macrophages, a phenotype found to be dependent on sufficient PPP activity with 6-AN inhibition rescuing bacterial growth back to that of wild-type macrophages (Figure 3.34). Thus, this data using miR21^{-/-} macrophages and 6-AN inhibition support a model whereby PPP activity can support

host immunometabolic responses during Mtb infection, by rerouting Carbon back to glycolytic intermediates and promoting host responses. However, we suggest that Mtb promotes PPP activity over glycolysis in macrophages, leading to reduced glycolytic flux, which in turn attenuates the production of pro-inflammatory mediators such as lactate and cytokines, thereby dampening the host's inflammatory response and creating a more permissive environment for bacterial survival.

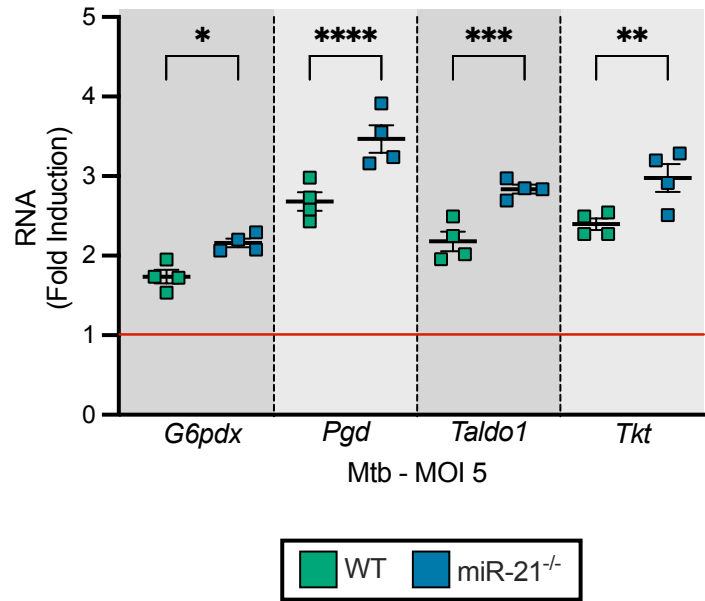


Figure 3. 29 Loss of miR-21 increases expression of PPP associated genes during Mtb infection of macrophages

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 72 hours. qPCR was performed and expression of *G6pdx*, *Pgd*, *Taldo1* and *Tkt* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

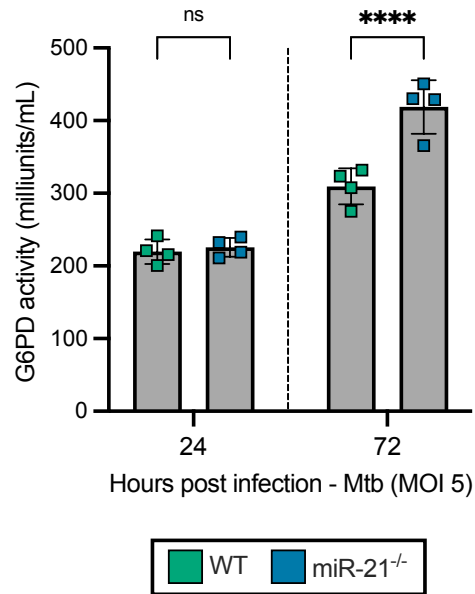


Figure 3. 30 Loss of miR-21 increases G6PD enzyme activity during Mtb infection of macrophages

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

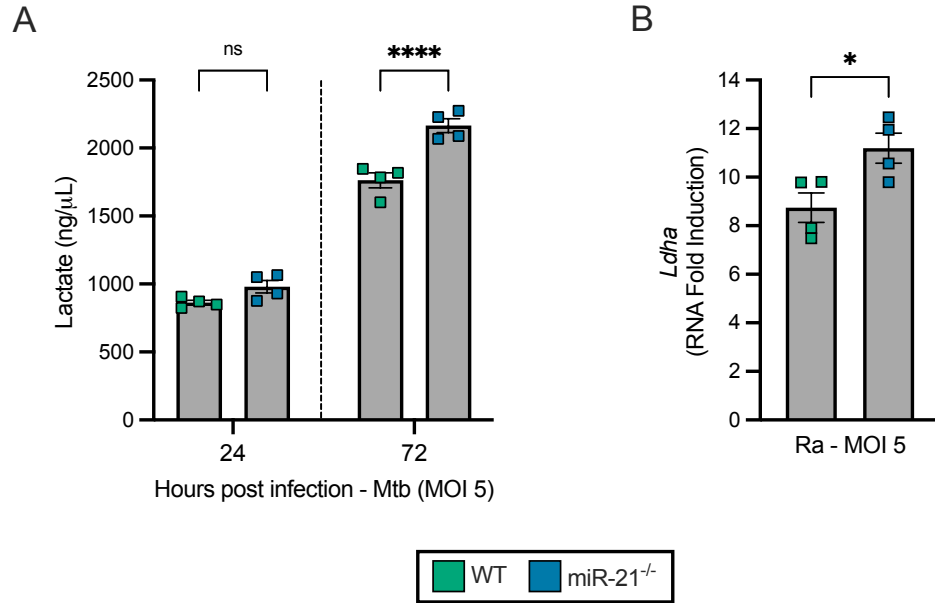


Figure 3.31 Loss of miR-21 increases lactate secretion during Mtb infection of macrophages

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A) and 72 (A-B) hours. (A) Supernatants were collected and lactate metabolite levels quantified. (B) qPCR was performed and expression of *Ldha* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test (A) and an unpaired t-test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

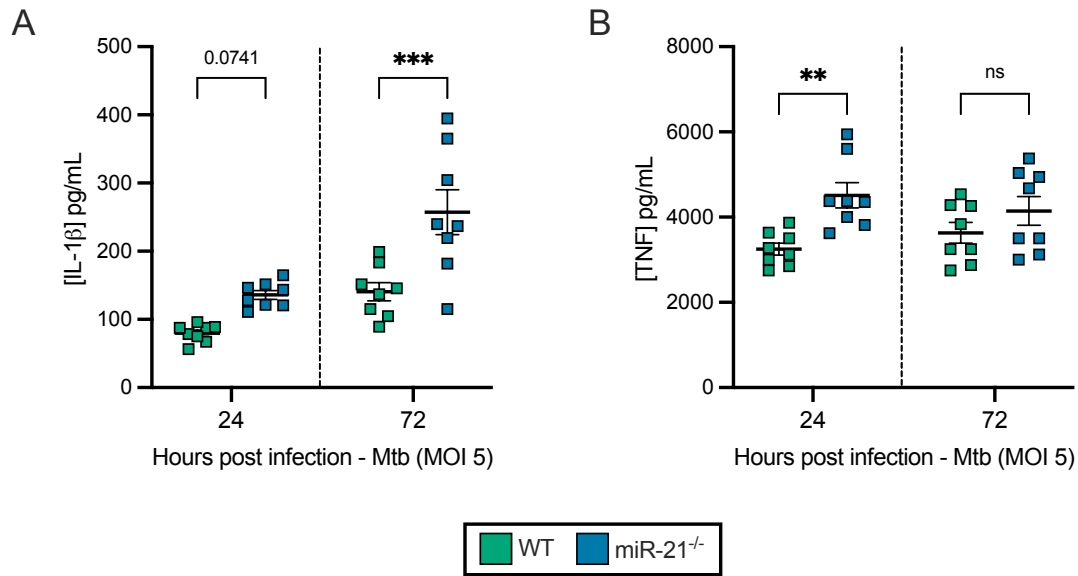


Figure 3. 32 Loss of miR-21 significantly increases IL-1 β and TNF α secretion during Mtb infection of macrophages

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and IL-1 β , and TNF α protein was quantified using an ELISA. Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments

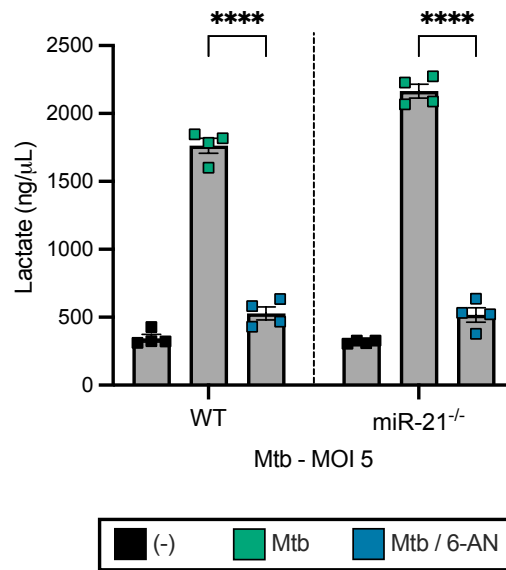


Figure 3. 33 PPP inhibition blocks heightened glycolytic activity in miR-21^{-/-} macrophages during Mtb infection

Murine BMDMs were pre-treated with 6-AN (100 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 72 hours. Supernatants were collected and lactate metabolite levels quantified. Statistical analysis was performed using a 2way ANOVA with Tukey's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments

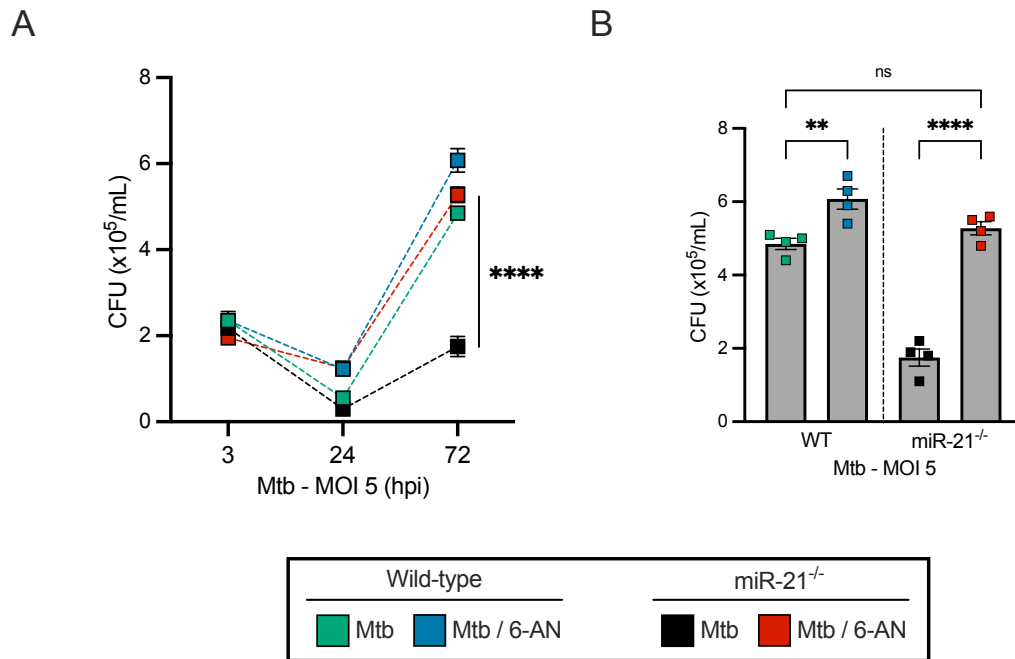


Figure 3. 34 Loss of miR-21 significantly increases bacterial killing and is overcome by PPP inhibition

Murine BMDMs were pre-treated with 6-AN (100 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3, 24 and 72 hours. Cells were lysed in 0.1% Triton X-100 at the indicated time-points (A) and 72 hours (B) and diluted lysates were streaked in triplicate on Middlebrook agar plates and incubated at 37°C for approximately 14 days. Colony forming units (CFUs) were counted and graphed. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

3.3 Discussion

Work from this chapter shows that Mtb can evade host macrophage immunometabolic responses during infection, by actively rerouting central carbon metabolism towards the induction of PPP activity. This favors generation of a permissive environment for bacterial replication. In addition, we found that by directly targeting PPP activity to induce alternative states of activity, namely pentose insufficiency and pentose overflow, we can modulate macrophage responses to infection and thus influence bacterial growth or clearance.

Mtb is termed the world's most successful pathogen, inducing a state of decelerated bioenergetics within its primary cell type of infection, the macrophage (43). Indeed, work from this project shows that during infection of both murine and human macrophages, Mtb lessens typical metabolic responses associated with infection. In particular, we show that Mtb decreases glycolytic activity through upregulation of anti-inflammatory miR-21 and subsequent targeting of glycolytic gatekeeper, PFK-M, which is consistent with previous work (41). Mtb's targeting of classical activation aligns with the observation of metabolically associated IL-1 β playing a central role in mediating bacterial clearance within macrophages. Inhibition of glycolytic reprogramming previously demonstrated to reduce levels of IL-1 β , increase anti-inflammatory IL-10 and promote intracellular bacillary survival (45). Here, though our model of comparative infection, we have demonstrated the extent to which Mtb is capable of blunting typical metabolically associated antimicrobial activity, including a significant reduction IL-1 β , ECAR and subsequent lactate secretion.

With respect to demand, cells direct nutrients through different metabolic routes to produce metabolites, co-factors, ATP or biosynthetic precursors. For example, glucose-6-phosphate utilization can yield two ATP and two pyruvate molecules if directed towards conventional glycolysis. Alternatively, its partial oxidation through the ox-PPP results in the generation of two NADPH and one ribose. In cells with a high demand for NADPH over that of ribose, a pentose cycle may be employed. Here, PPP-derived ribose can be

directed back towards glycolysis and recycled into glucose-6-phosphate for additional NADPH production. Such a cycle has only been described in bacteria and human skin cells, with a more recent demonstration within immune cells, specifically to support generation of the oxidative burst within neutrophils (217, 318, 319). Here, we describe the possible indirect induction of pentose cycling within host macrophages by proliferating Mtb as a means to evade metabolic responses. Contrastingly to the typical attenuated metabolic signature associated with central carbon metabolism, we found Mtb infection of human and murine macrophages to induce accelerated rates of ox-PPP metabolism, demonstrating heightened rates of enzyme expression and G6PD activity. This increased PPP activity, along with decreased lactate levels, indicates a net loss of carbon through glycolytic metabolism, reinforcing the concept of non-canonical cycling.

After confirming the heightened PPP activity during Mtb infection, we aimed to induce alternative modes of pathway activity, pentose insufficiency and pentose overflow, to investigate the effects on macrophage responses and bacterial survival. Previous work evaluating PPP activity in the context of hypercholesterolemia finds that sufficient pathway activity is required to support macrophage pro-inflammatory responses, particularly IL-6 secretion (320). It was found that hypercholesterolemia attenuated macrophages inflammatory phenotype independent of glycolytic or TCA reconfiguration, but rather through targeting *Pgd* expression. Here, we have expanded upon these observations to demonstrate that ox-PPP activity is required to support IL-1 β , TNF α AND IL-6 secretion with 6-AN induced pentose insufficiency resulting in a dramatic reduction in macrophage pro-inflammatory cytokine profile. Furthermore, we show PPP dependent inflammatory cytokine secretion as specific to viable infection with previous studies reporting no difference in cytokines such as TNF α following LPS simulation (320). This is more than likely due to macrophage requirement of PPP activity to support anabolic and antimicrobial processes employed during active infection that are not required to the same extent for TLR agonists such as LPS. This notion is further supported by our viable vs inactivated Mtb infection model demonstrating no effect on IL-1 β , TNF α or IL-6 secretion following inactivated Mtb stimulation during 6-AN inhibition. This specificity in the metabolic pathways that drive macrophage responses to Mtb infection indicates a potential specificity in the carbon sources that fuel these

processes. In line with this, we highlight the mobilization of macrophage glycogen stores during active infection with inhibition of glycogenolysis resulting in a reduced IL-1 β response. Building on previous findings that show a preferential rerouting of glycogen-derived carbon to support PPP activity, further research is needed to assess whether Mtb exploits this mechanism to enhance cellular anabolic processes, thereby promoting its own survival.

Through our induction of pentose insufficiency, we have demonstrated macrophage requirement on sufficient PPP activity not only to support cytokine responses, but also to maintain glycolytic flux with PPP inhibition having a dramatic effect on central carbon metabolism at the expense of ribose generation. This ultimately leads to immunometabolic paralysis and favors intracellular Mtb replication. Finally, through induction of a state of pentose overflow and removal of Mtb's potential to induce non-canonical activity, we have demonstrated how augmented PPP activity can support macrophage responses to Mtb infection leading to a heightened metabolic and inflammatory profile, particularly IL-1 β secretion, thus, promotes bacterial clearance. Disruption of pentose overflow through introduction of 6-AN completely abolishes this antimycobacterial effect and rescued Mtb replication.

Together, the data from this chapter demonstrates the role of PPP metabolism in macrophage immune responses. We have identified a novel mechanism by which bacteria induce PPP activity to disrupt host cell metabolism. Furthermore, targeting PPP activity emerges as a promising strategy to enhance macrophage-mediated killing of Mtb.

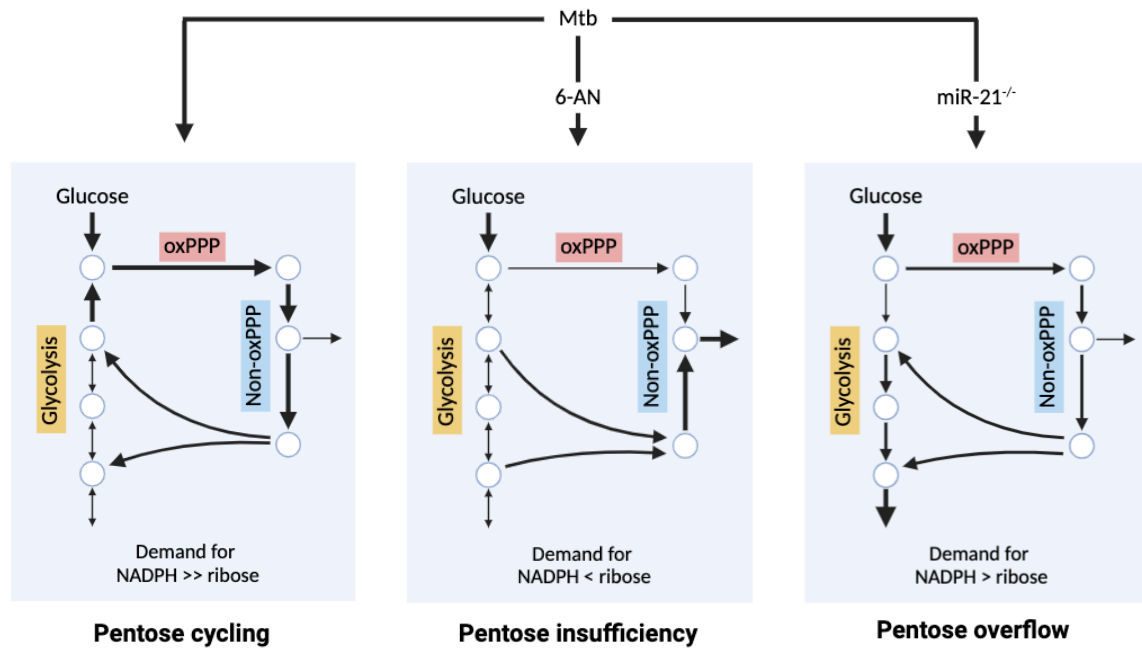


Figure 3. 35 PPP modes of action

Mtb infection leads to pentose cycling. Once pentoses re-enter glycolytic metabolism, glucose-6-phosphate isomerase functions in reverse to regenerate glucose-6-phosphate to feed oxPPP activity at the expense of glycolysis. Inhibition of oxPPP metabolism results in pentose insufficiency where unmet ribose demand lets to a net flux of glycolytic carbon towards non-oxPPP to support ribose-5-phosphate synthesis. In the absence of microRNA-21, glycolytic metabolism remains unchecked and supports induction of pentose overflow. Adapted from TeSlaa *et al*, 2022 (236).

Chapter 4. Pentose Phosphate Pathway
derived NO orchestrates macrophage
immunometabolic responses

4.1 Introduction

The PPP has emerged as crucial for the immune system, supporting a variety of functions across different cell types. For phagocytes, such as macrophages and neutrophils, the PPP works to produce an oxidative burst to support pathogen killing, whilst in T-cells, PPP activity is required to generate anabolic building blocks for rapid cell proliferation and cytokine production following activation (236). Such diversity in function highlights unique roles of the PPP in alternative cell types. The phagocytic response triggered during pathogen killing depends on the activity of NADPH-dependent enzymes, NADPH oxidase 2 (NOX2) and iNOS, which have K_M for NADPH of 40 μ M and 10 μ M respectively. Although much of the literature emphasises phagocyte NOX as a major consumer of NADPH, iNOS is less well appreciated but equally plays a critical role in immune responses. While NOX and ROS are key to neutrophil function, driving the respiratory burst for pathogen killing, NO production via iNOS and arginine metabolism are more strongly associated with macrophage activity, particularly in distinguishing M1 and M2 macrophage polarisation. M1 macrophages rely on NO for antimicrobial action, while M2 macrophages use arginase-driven pathways for tissue repair and anti-inflammatory roles. However, both enzymes rely on sufficient PPP activity as the primary source of NADPH with both NOX deficiency and severe PPP rate limiting G6PD mutations resulting in chronic granulomatous disease (321-323). Targeting PPP activity altering cellular ROS and RNS production has been demonstrated to modulate the expression of iNOS in addition to inflammatory IL-6 secretion (215, 324, 325). However, the contribution of PPP activity to redox based inflammatory responses and the extent to which these processes are regulated during infection, particularly that of Mtb, remain largely unexplored.

Building on the data from Chapter 3, which demonstrated bacterial-mediated induction of PPP activity, we next aimed to evaluate the functional consequences on macrophage metabolic responses due to increased cellular NADP/H levels, particularly that attributed to NO generation during infection. The induction of iNOS derived NO has been previously linked as a mediator of IFN- γ dependent control of Mtb infection, with iNOS^{-/-} murine models displaying heightened susceptibility to infection and accounting in part

to the increased Mtb susceptibility also recorded in IFN- γ deficient mice (137, 326, 327). While the role of NO as a direct messenger in regulating cell signalling is well established, its potential secondary roles, especially in the context of immunometabolic responses during Mtb infection, have been largely overlooked. As such, the bacterial burdens observed in iNOS^{-/-} models have been linked to the direct bactericidal properties of NO (328, 329), however, further studies highlight this model as incomplete. Firstly, microarray analysis found a portion of IFN- γ activation is dependent on both expression of iNOS and phagosome oxidase and likely impacted signalling during Mtb infection (330). Nonetheless, no direct or mechanistic transcriptional alterations were attributed to NO signalling. Secondly, an alternative study demonstrated the ability of NO to suppress inflammasome dependent generation of antimycobacterial IL-1. This resulted in decreased inflammatory responses and was found to contribute to immune pathology regardless of Mtb growth in mice (54). In agreement with this, a more recent study has identified NO as a necessary mediator of IFN- γ dependent stabilization of HIF-1 α , while simultaneously suppressing excessive inflammation by inhibiting the nuclear localization of NF- κ B (49). As a gas, NO itself is highly reactive, and while we often measure nitrite production as a proxy for NO activity, what we are truly aiming to quantify is the downstream effect of nitrosylation—post-translational modifications by NO groups—on proteins, DNA, and other molecules. This ability to modify various biomolecules grants NO diverse functions, as it can target both microbial and host proteins. During infections such as Mtb, macrophages use NO as part of their defense. However, Mtb has evolved to produce NO-scavenging molecules that allow it to evade these effects, underscoring the dynamic battle between pathogen and host. The concentration of NO is also critical to its function. In the context of this chapter, we're particularly interested in how NO operates when PPP activity is ramped up during the later stages of infection. While NO likely has a protective role early on, effectively targeting and inhibiting microbial growth, its sustained or increased production as the infection progresses might have mixed effects. As Mtb replicates and produces more evasion factors, the continued high levels of NO could potentially become detrimental to the host, contributing to tissue damage or impairing other immune responses. This balance between protective and harmful effects is a key aspect of NO's role in chronic infections like Mtb. Building on this and previous findings, in this chapter we look to further explore the role of NO in modulating

cellular inflammatory signalling, particularly in the context of macrophage immunometabolic activity in response to Mtb infection.

4.2 Results

4.2.1 *Mycobacterium tuberculosis* promotes PPP derived NO generation

To examine the generation of NO during Mtb infection, we employed Griess colorimetric assays to quantify nitrite levels within our macrophage infection model. The Griess assay is a well-established method for detecting nitrite, a stable end product of NO metabolism, thus serving as an indirect measure of NO production. In our comparative infection model, samples were collected from infected and control groups and treated with the Griess reagent, which reacts with nitrite to produce a purple azo dye. Here, we recorded a significant increase in nitrite accumulation in both murine and human macrophages during infection with viable Mtb compared to both uninfected controls which displayed similar levels to those infected with inactivated Mtb (Figure 4.1). In particular, this NO production associated with Mtb infection was most pronounced at later time points (72h post-infection) when Mtb is known to reprogramme macrophage responses and replicate intracellularly and the same points where we observed significant PPP activity in terms of gene induction, enzyme activity and 6-PGA levels. In agreement with NO accumulation, we recorded greater levels of *Nos2* mRNA expression in response to viable Mtb infection (Figure 4.1)

In order to verify that the increased NO production observed during infection was PPP dependent, we inhibited G6PD using 6-AN as before and demonstrate a dose dependant decrease in nitrite accumulation when PPP is inhibited, particularly prominent at 72 hours post-infection when pentose insufficiency is induced (Figure 4.2). Furthermore, PPP inhibition significantly decreased expression of *Nos2* during Mtb infection (Figure 4.2), suggesting a feed forward loop where PPP activity promotes iNOS transcription and enzyme activity. These results support previous observations of heightened PPP metabolism during live infection, including increased G6PD activity (Figure 3.13) and NADP/H levels (Figure 3.14) recorded in Chapter 3. Interestingly, blocking the contribution of glycogen to central carbon metabolism using the glycogenolysis inhibitor GPI led to reduced NO production (Figure 4.3), similar to G6PD inhibition seen with 6-

AN treatment. This suggests an alternative cellular source of glucose-6-phosphate that promotes PPP activity during Mtb infection. This is in line with recently published findings of preferential routing of glycogen derived carbon towards supporting PPP activity during macrophage activation (215). We thus examined intracellular glycogen levels during Mtb infection and observed a decrease in macrophage glycogen stores after 24 hours infection (Figure 4.4). Taken together, these data suggest Mtb mediated mobilisation of glycogen to support NO production. Seahorse extracellular flux analysis further demonstrated the significant contribution of glycogen derived carbon towards supporting central metabolism with GPI inhibition of glycogenolysis derived glucose-6-phosphate resulting in a reduction in both macrophage maximum glycolytic capacity and spare glycolytic reserve (Figure 4.5).

As we had measured nitrite accumulation after 6-AN treatment which mimics the PPP mode of pentose insufficiency, we next quantified nitrite within miR-21^{-/-} macrophages, previously demonstrated to employ a state of pentose overflow. Again, consistent with heightened PPP activity (Figure 3.30), we found a significant increase in both nitrite accumulation and *Nos2* expression in miR-21 deficient macrophages (Figure 4.6). Collectively, these findings establish the PPP as a key contributor to the increased NO accumulation observed during Mtb infection in macrophages. Additionally, our results demonstrate that glycogenolysis serves as a significant alternative source of carbon to support PPP effector functions, particularly NO production during Mtb infection.

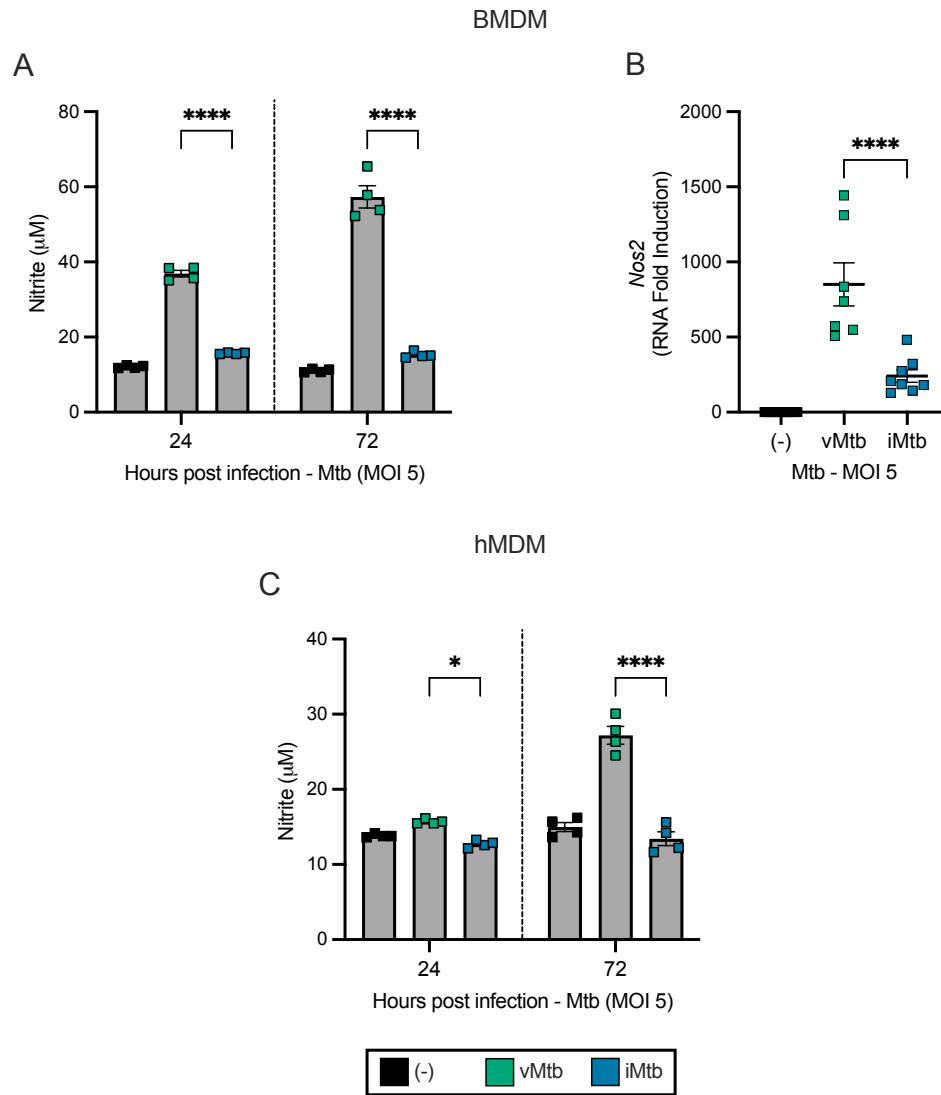


Figure 4. 1 Viable Mtb promotes NO accumulation in murine and human macrophages

Murine BMDMs (A+B) human MDMs (C) were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A-C) and 72 (A+C) hours. (A+C) Supernatants were collected and nitrite species quantified using colorimetric Griess assay. (B) qPCR was performed and expression of *Nos2* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using a 2way ANOVA with Tukey's multiple comparisons test (A+C) and two-way ANOVA with Šídák's multiple comparisons test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, n = 4 (A+C), 8 (B) independent experiments.

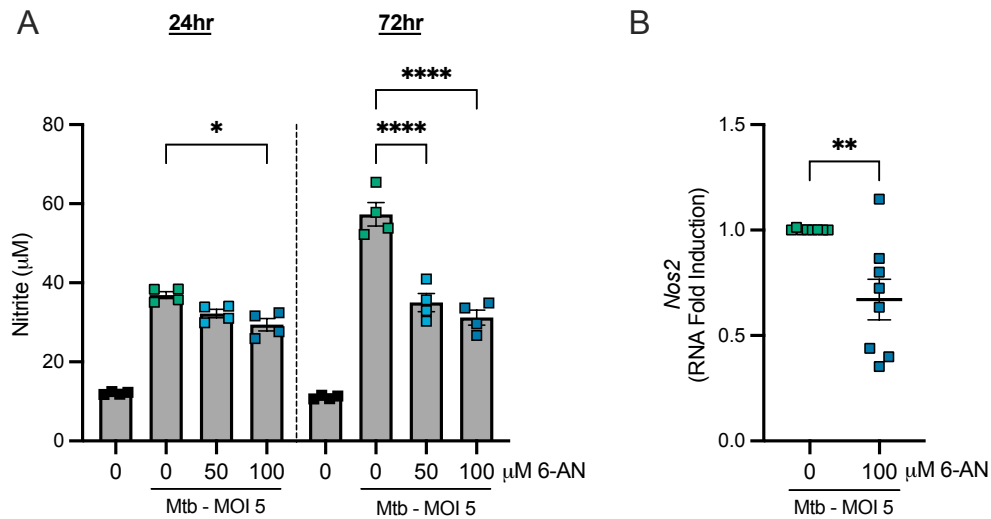


Figure 4. 2 PPP inhibition decreases NO accumulation during Mtb infection of macrophages

Murine BMDMs were pre-treated with 6-AN one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A+B) and 72 (A) hours. (A) Supernatants were collected and nitrite species quantified using colorimetric Griess assay. (B) qPCR was performed and expression of *Nos2* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test (A) and an unpaired t-test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ (A), 8 (B) independent experiments.

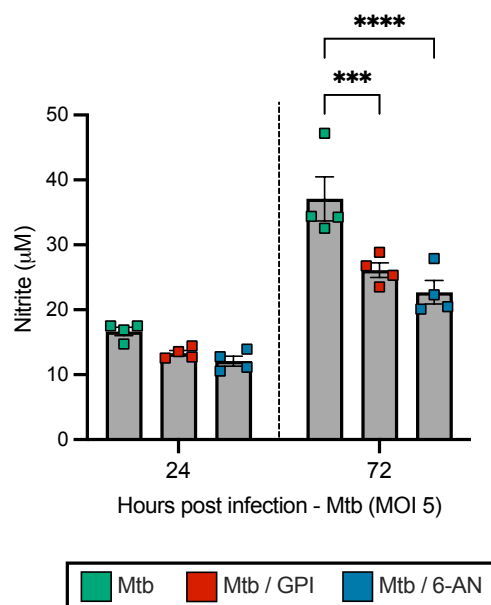


Figure 4. 3 Glycogenolysis supports NO production during Mtb infection of macrophages

Murine BMDMs were pre-treated with GPI (25 µM) 6-AN (50 µM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and nitrite species quantified using colorimetric Griess assay. Statistical analysis was performed 2way ANOVA with Tukey test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

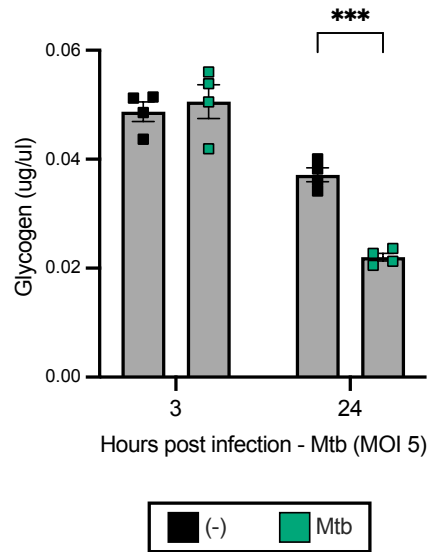


Figure 4. 4 Mtb infection promotes glycogenolysis

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3 and 24 hours. Cells were homogenised and colorimetric assay used to quantify glycogen levels. Statistical analysis was performed 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

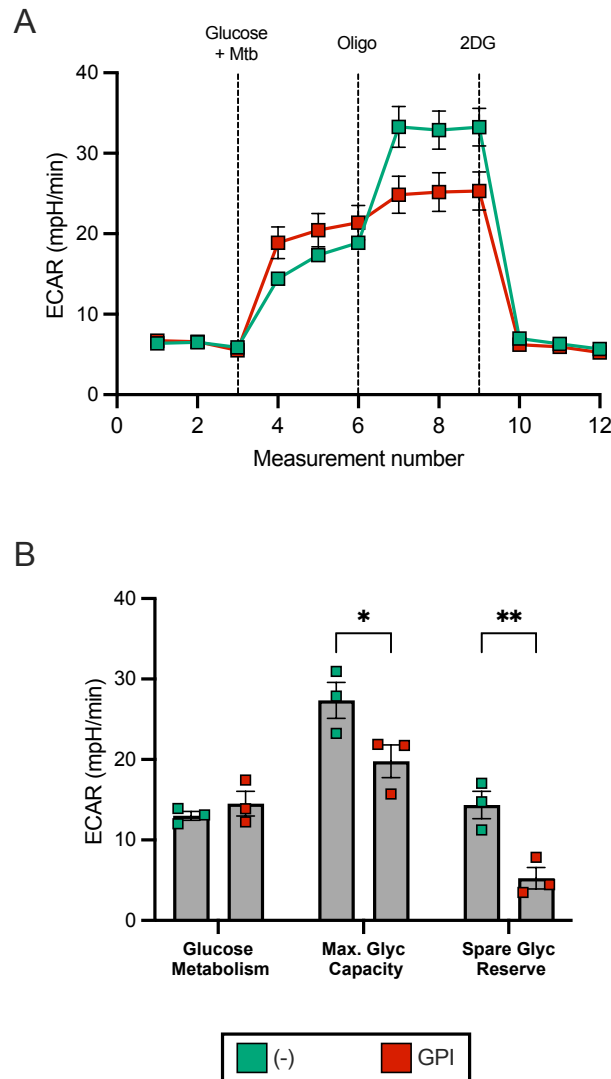


Figure 4. 5 Glycogen supports macrophage glycolytic metabolism

Murine BMDMs were pre-treated with GPI (25 μ M) for 1 hour. Extracellular acidification rate (ECAR) was recorded by Seahorse analysis following injection with glucose + Mtb (inactivated strain H37Ra), oligomycin (Oligo) and 2-deoxyglucose (2DG). Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments.

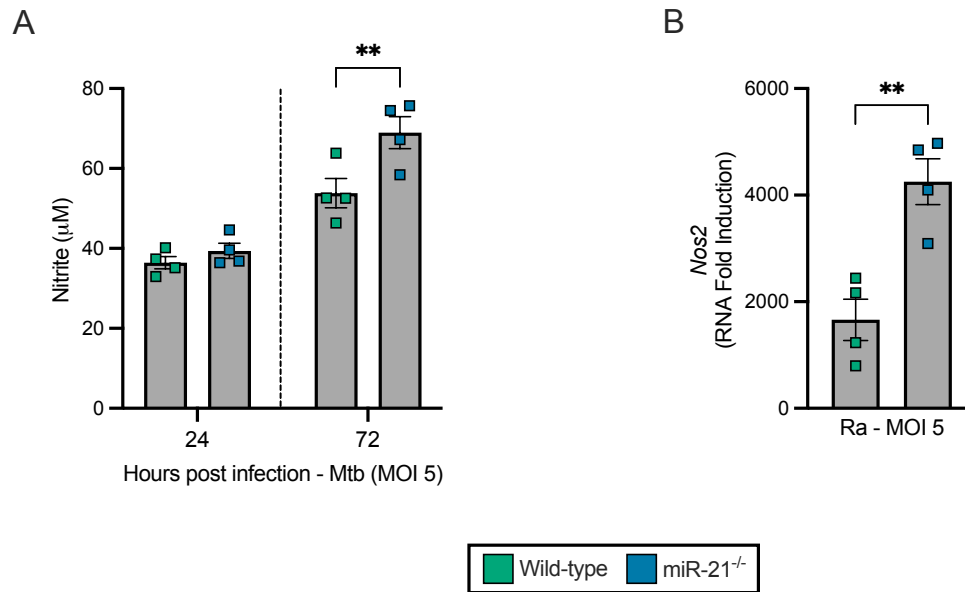


Figure 4. 6 Loss of miR-21 increases NO during Mtb infection of macrophages

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A) and 72 (A-B) hours. (A) Supernatants were collected and nitrite species quantified using colorimetric Griess assay. (B) qPCR was performed and expression of *Nos2* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test (A) and an unpaired t-test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

4.2.2 Nitric oxide promotes macrophage PPP activity during Mtb infection

After establishing that PPP activity is the primary contributor to NO production during Mtb infection in macrophages, we proceeded to assess the direct impact of increased NO on central carbon metabolism. To do so, we employed the use of a NO donor, DETA NONOate, commonly referred to as DetaNO. Here, we utilised a repeated supplementation model in which a moderate level of DetaNO, relative to comparable studies (53), was added to approximately double the amount that accumulates during viable Mtb infection of both murine BMDMs and hMDMs, with supplementation occurring at 24-hour intervals throughout the infection period. This model was used to address the short half-life of DetaNO (20 hours at 37°C) and to mimic the significant NO accumulation resulting from increased PPP activity observed during Mtb infection, in an effort to evaluate the role of NO on macrophage metabolism during infection.

Firstly, we confirmed increased NO levels within murine and human macrophage infection models using Griess assays following treatment with DetaNO (Figure 4.7). We measured nitrite levels at 24h post-supplementation (1x50µM BMDM, 1x25µM hMDM) or at 72h post-supplementation (3x50µM BMDM, 3x25µM hMDM) and found that this led to a significant increase in nitrite in Mtb infected cells at both 24h-72h. Supplementation with NO in uninfected macrophages resulted in a dose-dependent and significant increase in both lactate accumulation and *Ldha* expression (Figure 4.8), suggesting NO directly promotes heightened glycolytic activity. This increased activity was also found to rely on sufficient PPP signalling, as inhibition with 6-AN treatment abolished increased lactate levels in the presence of NO. These results were supported with extracellular flux analysis recording heightened glycolytic capacity in both murine BMDMs and human MDMs following NO supplementation, again, reduced by PPP inhibition (Figure 4.9). Finally, this heightened glycolytic output was confirmed following NO supplementation during Mtb infection by measuring lactate production as a surrogate of glycolysis (Figure 4.10).

Interestingly, we found repeated NO supplementation to have no effect on glycolytic gene expression and even significantly decreased *Hk2* expression (Figure 4.11), suggesting the use of an alternative fuel source, such as glycogen, which we have already demonstrated as a primary carbon source supporting NO production during Mtb infection of macrophages (Figure 4.3-4). We then confirmed that NO mediates the preferential use of glycogen stores over extracellular glucose during macrophage infection. Supplementation with DetaNO significantly reduced glucose uptake compared to cells infected with Mtb alone (Figure 4.12). Moreover, NO supplementation increased the expression of the glycogenolysis mediator *Pgm1* mRNA and reduced cellular glycogen stores (Figure 4.12). Such results support to notion of Mtb mediated induction of glycogenolysis in an attempt to support PPP activity. This observation, along with macrophage dependence on PPP-derived NADPH to support NO production, led us to explore the possibility that NOS2-derived NO and regeneration of ox-PPP enzyme cofactor, NADP, directly affects PPP activity and this in turn supports metabolic reprogramming. In agreement with this, NO addition resulted in a dose dependent increase in G6PD activity in basal macrophages (Figure 4.13). In the context of Mtb infection of murine and human macrophages, NO promoted the expression of all PPP-associated genes examined, including those supporting oxidative (*G6pdx*, *Pgd*) and non-oxidative (*Taldo1*, *Tkt*) branches (Figure 4.14 – 4.15). This increased expression was accompanied by heightened G6PD activity in both murine and human macrophages during infection (Figure 4.16), suggesting Mtb derived NO production in macrophages is used to support PPP activity. Finally, increased PPP activity during NO supplementation is accompanied by elevated levels of the Ox-PPP enzyme cofactor, NADP (Figure 4.17). Together, these data demonstrate the capacity of increased NO production to directly influence carbon metabolism by promoting glycogen fuelled PPP activity during Mtb infection of macrophages.

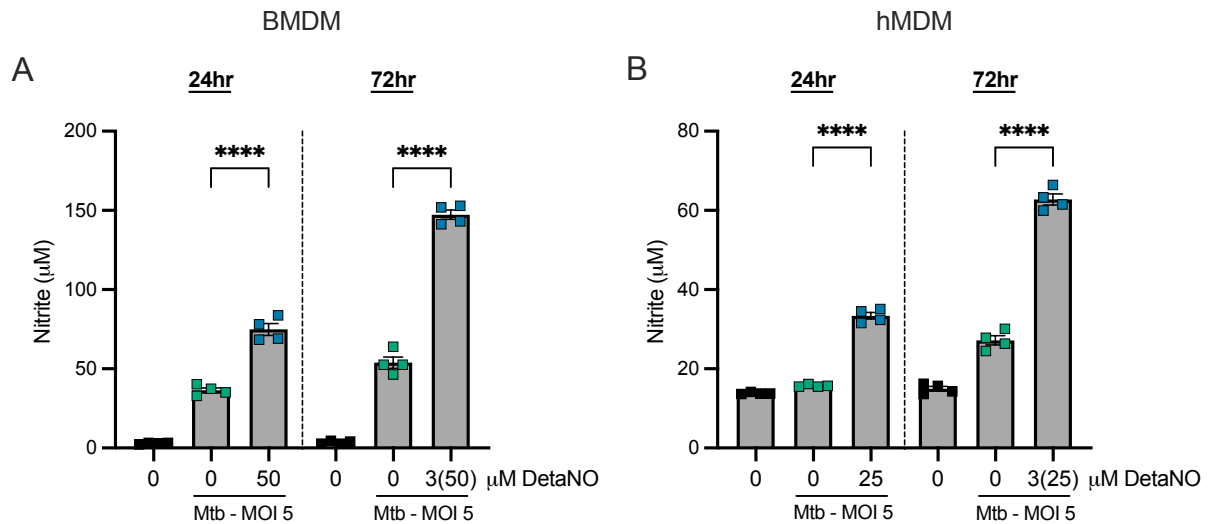


Figure 4. 7 NO supplementation increases cellular nitrite in murine and human macrophages during Mtb infection

Murine BMDMs (A) human MDMs (B) were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. NO donor DetaNO was added 3 hours post infection (50 μM (A), 25 μM (B)) and topped up every 24 hours for 72 hours. Supernatants were collected and nitrite species quantified using colorimetric Griess assay. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

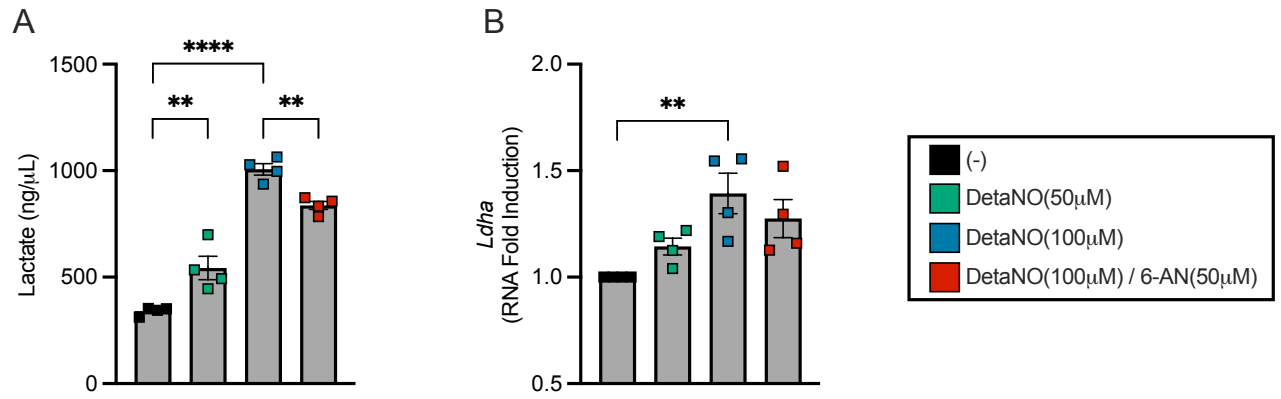


Figure 4. 8 NO promotes glycolytic activity in uninfected macrophages

Murine BMDMs were treated with a concentration range of DetaNO (50-100μM) and 6-AN (50μM) for 24 hours. (A) Supernatants were collected and lactate metabolite levels quantified. (B) qPCR was performed and expression of *Ldha* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

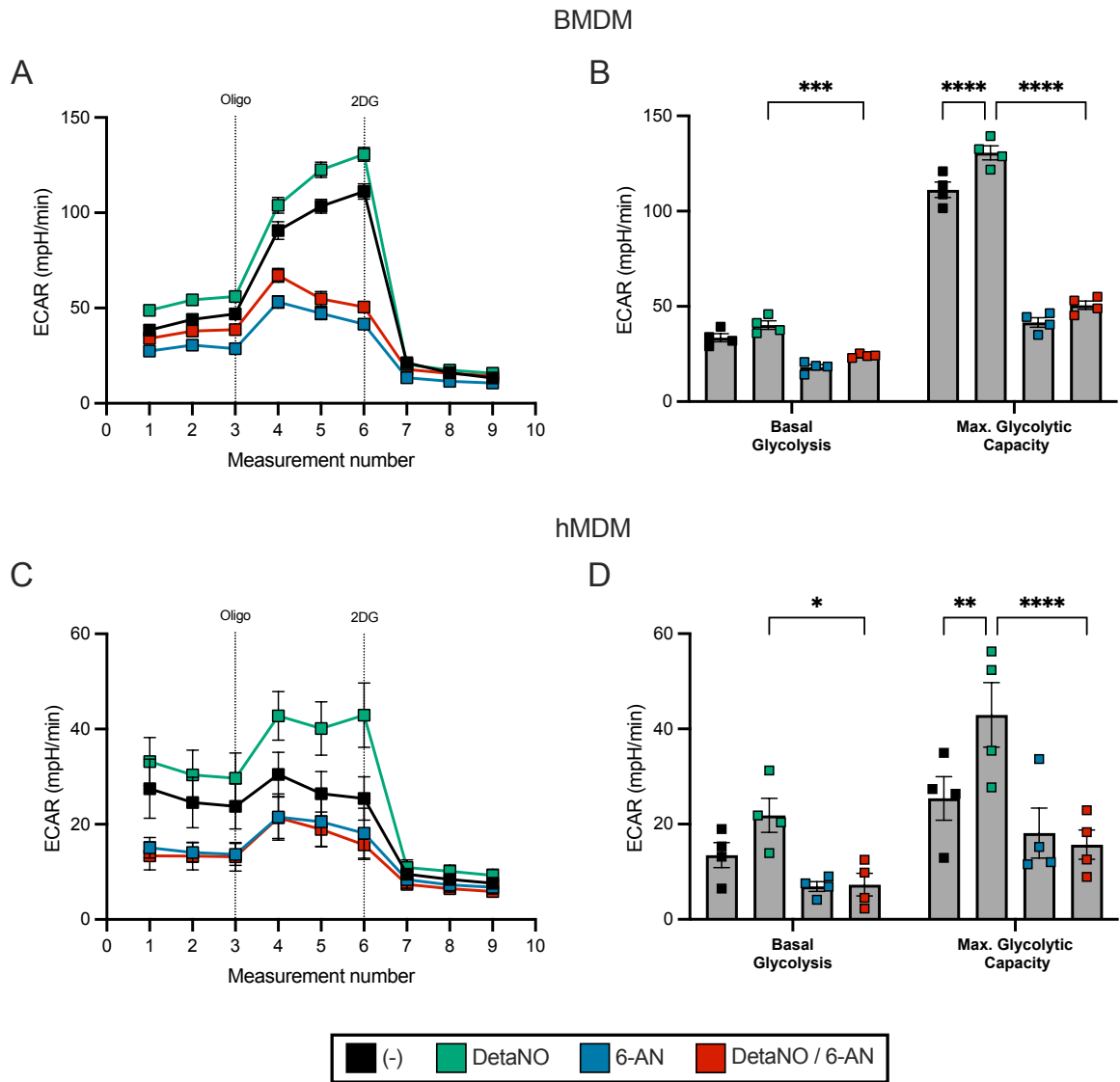


Figure 4.9 NO increases glycolytic capacity of uninfected murine and human macrophages

Murine BMDMs (A-B) and human MDMs (C-D) were treated with DetaNO (50 μ M A-B, 25 μ M C-D) and 6-AN (100 μ M A-B, 25 μ M C-D) for 24 hours. Extracellular acidification rate (ECAR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo) and 2-deoxyglucose (2DG). Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

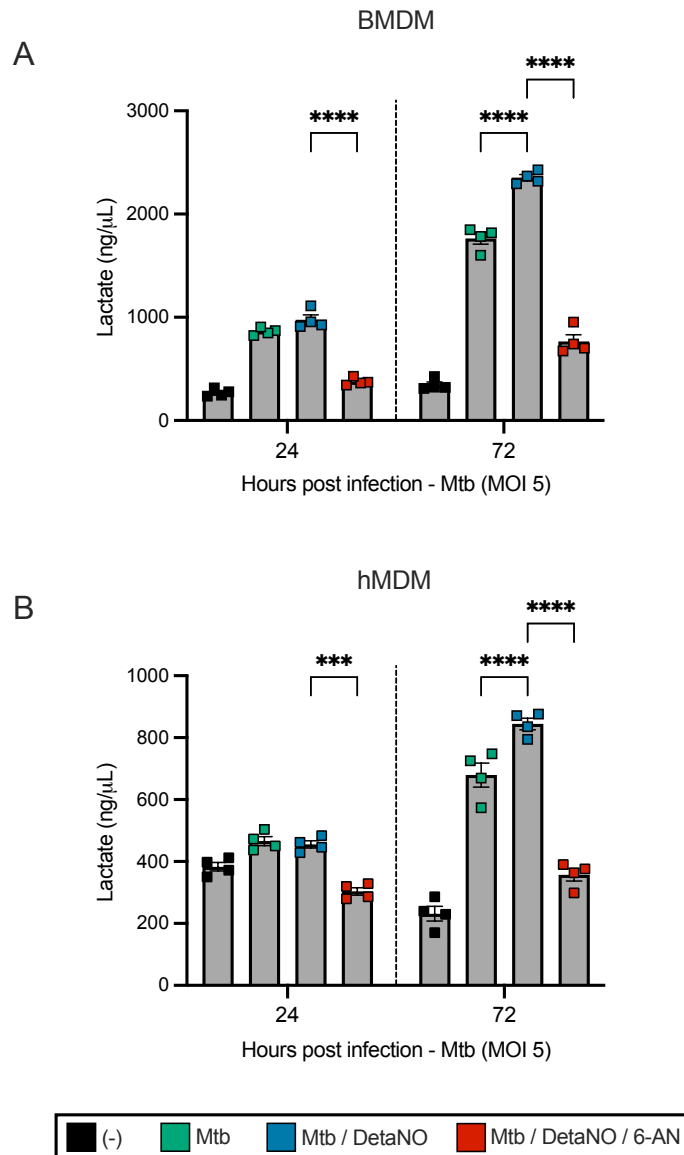


Figure 4. 10 NO increases lactate secretion in murine and human macrophages during Mtb infection

Murine BMDMs (A) and human MDMs (B) were pre-treated with 6-AN (100μM A, 25μM B) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. NO donor DetaNO was added 3 hours post infection (50μM A, 25μM B) and topped up every 24 hours for 72 hours. Supernatants were collected and lactate metabolite levels quantified. Statistical analysis was performed using a 2way ANOVA with Tukey test . *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, n = 4 independent experiments.

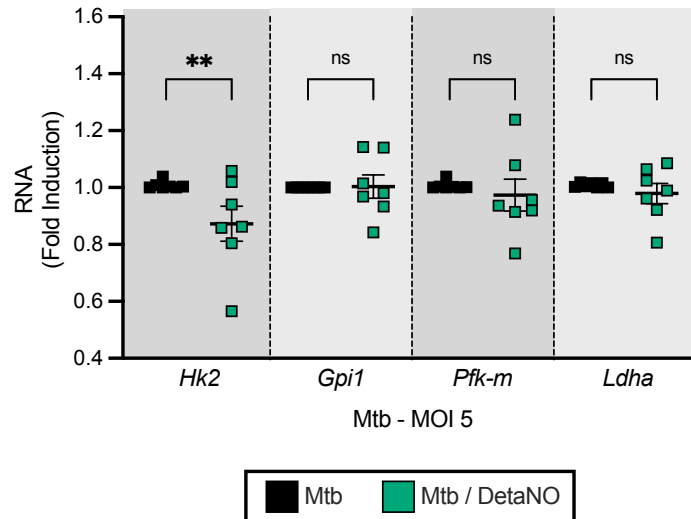


Figure 4. 11 NO does not alter glycolytic gene expression during Mtb infection

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DetaNO was added 3 hours post infection (50 μ M). qPCR was performed and expression of *Hk2*, *Gpi1*, *Pfk-m* and *Ldha* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

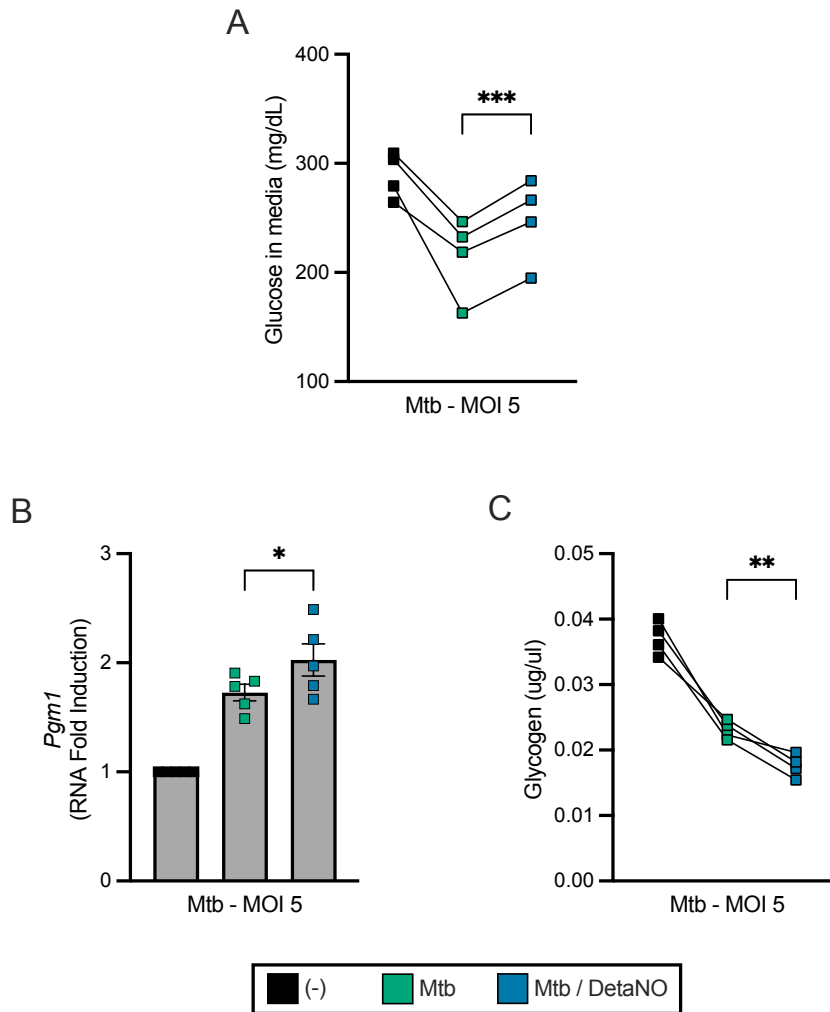


Figure 4. 12 NO decreases glucose uptake and promotes glycogenolysis during Mtb infection

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DetaNO was added 3 hours post infection (50 μ M). (A) Supernatants were collected and glucose levels quantified. (B) qPCR was performed and expression of *Pgm1* relative to the untreated control was calculated. Data was normalised to *Hprt*. (C) Cells were homogenised and colorimetric assay used to quantify glycogen levels. Statistical analysis was performed using an RM one-way ANOVA (A) and an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ (A+C), 5 (B) independent experiments.

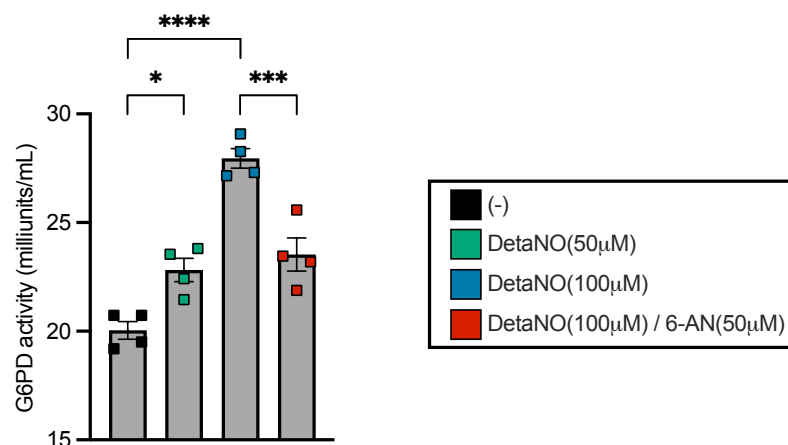


Figure 4. 13 NO promotes PPP activity in uninfected macrophages

Murine BMDMs were treated with a concentration range of DetaNO (50-100µM) and 6-AN (50µM) for 24 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

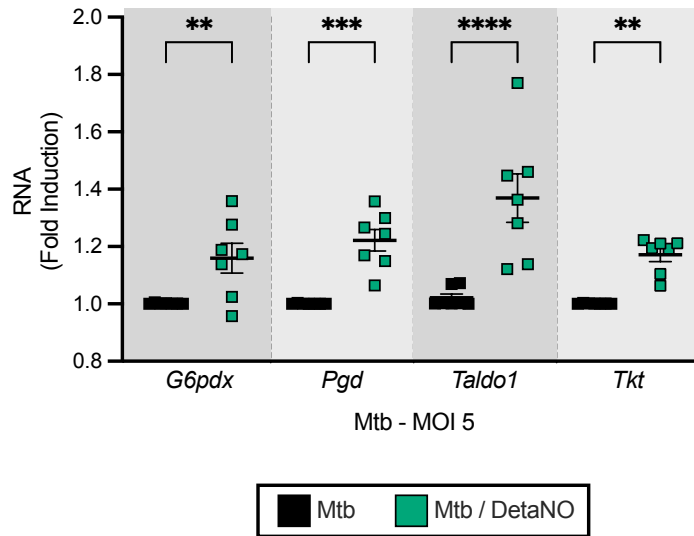


Figure 4. 14 NO increases PPP gene expression in murine macrophages during Mtb infection

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DetaNO was added 3 hours post infection (50 μ M). qPCR was performed and expression of *G6pdx*, *Pgd*, *Taldo1* and *Tkt* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

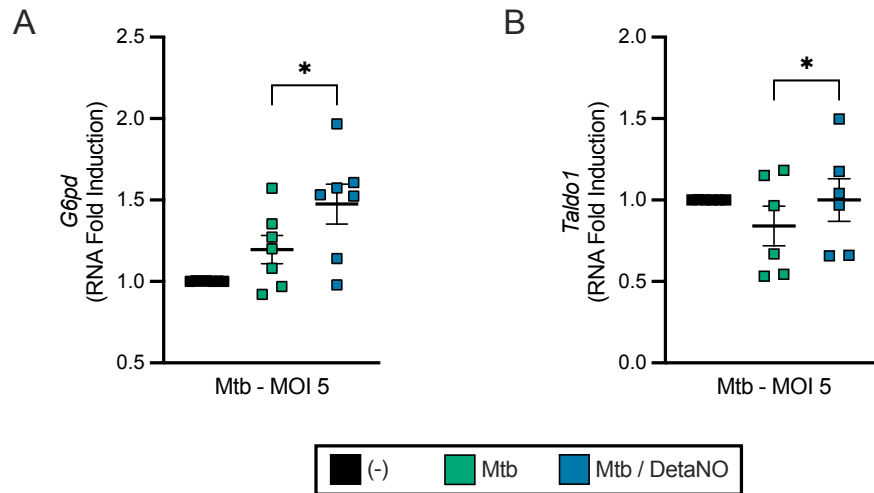


Figure 4. 15 NO supplementation increases PPP gene expression in human macrophages during Mtb infection compared to Mtb alone

Human MDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DetaNO was added 3 hours post infection (25 μ M). qPCR was performed and expression of *G6pdx* and *Taldo1* relative to the untreated control was calculated. Data was normalised to *18s*. Statistical analysis was performed using an RM one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

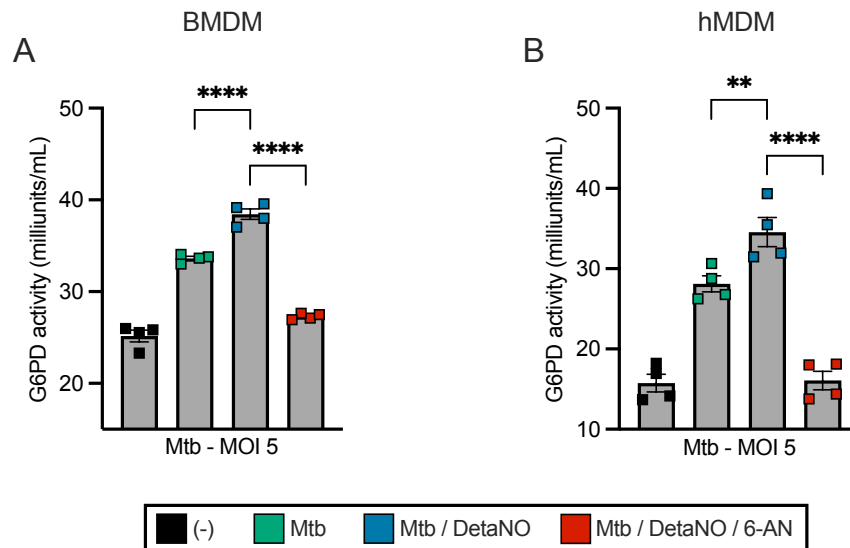


Figure 4. 16 NO promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages during Mtb infection

Murine BMDMs (A) and human MDMs (B) were pre-treated with 6-AN (100 μ M A, 25 μ M B) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DetaNO was added 3 hours post infection (50 μ M A, 25 μ M B). Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

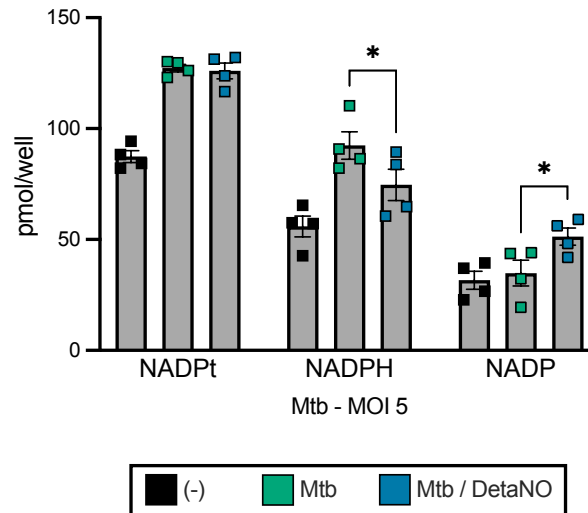


Figure 4. 17 NO increases NADP:NADPH during Mtb infection

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DETA-NO was added 3 hours post infection (50 μ M). Cells were homogenised and colorimetric assay used to quantify concentration of NADPH, NADP and total NADP/H (NADPt). Statistical analysis was performed using a 2way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

4.2.3 Nitric oxide generates an immuno-permissive environment for Mtb replication

Having analysed the effect of NO on central carbon metabolism, we next looked to evaluate pro-inflammatory cytokine secretion during NO accumulation. Recent studies in murine macrophages has identified a NOS2-dependent NRF2 transcriptional signature, indicating that NO-mediated alterations to metabolism and antioxidant action could potentially reduce the inflammatory capacity of macrophages (300). It was also demonstrated that *Nos2*-deficient murine macrophages produce elevated levels of IL-1 β and this was thought to occur in the absence of NO-mediated nitrosation of the inflammasome complex (54, 331). Consequently, we evaluated the effect of NO supplementation on pro-inflammatory cytokine secretion during Mtb infection of macrophages to replicate NO accumulation recorded during infection.

Following NO donation and in line with *Nos2*^{-/-} models (53), we recorded a significant decrease in the mRNA expression and secretion of mature IL-1 β protein in both murine and human macrophages following infection (Figure 4.18), again, particularly effective at later timepoints of infection (72 hrs). A similar attenuation of TNF α secretion was also observed following NO supplementation (Figure 4.19), likely a result of decreased IL-1 β production, as observed previously (48). Finally, using bacterial-chromosome-specific probes (*IS6110*) as a measure of mycobacterial replication, we found heightened expression in both murine and human macrophages in the presence of increased NO (Figure 4.20). This is in line with findings of increased bacterial growth within human macrophages where NO accumulation was supported through supplementation with L-Arginine and NO co-factor, tetrahydrobiopterin (BH4) (308). Importantly, this impact of high NO on bacterial growth was not direct and instead dependent on feedback to increased PPP activity, since treatment with 6-AN blocked this effect (Figure 4.20). Previous studies have demonstrated the role of early NO production in promoting bacterial clearance (49). Our work with collaborators (currently unpublished) has also demonstrated this effect. However, the NO supplementation in our current model is distinct and mimics the elevated NO levels associated with sustained PPP activity during

Mtb infection. This approach is potentially more relevant, as it reflects a more physiologically accurate response during Mtb infection of macrophages. In sum, these data demonstrate the immune-suppressive properties of NO accumulation, creating a permissive metabolic environment for Mtb growth.

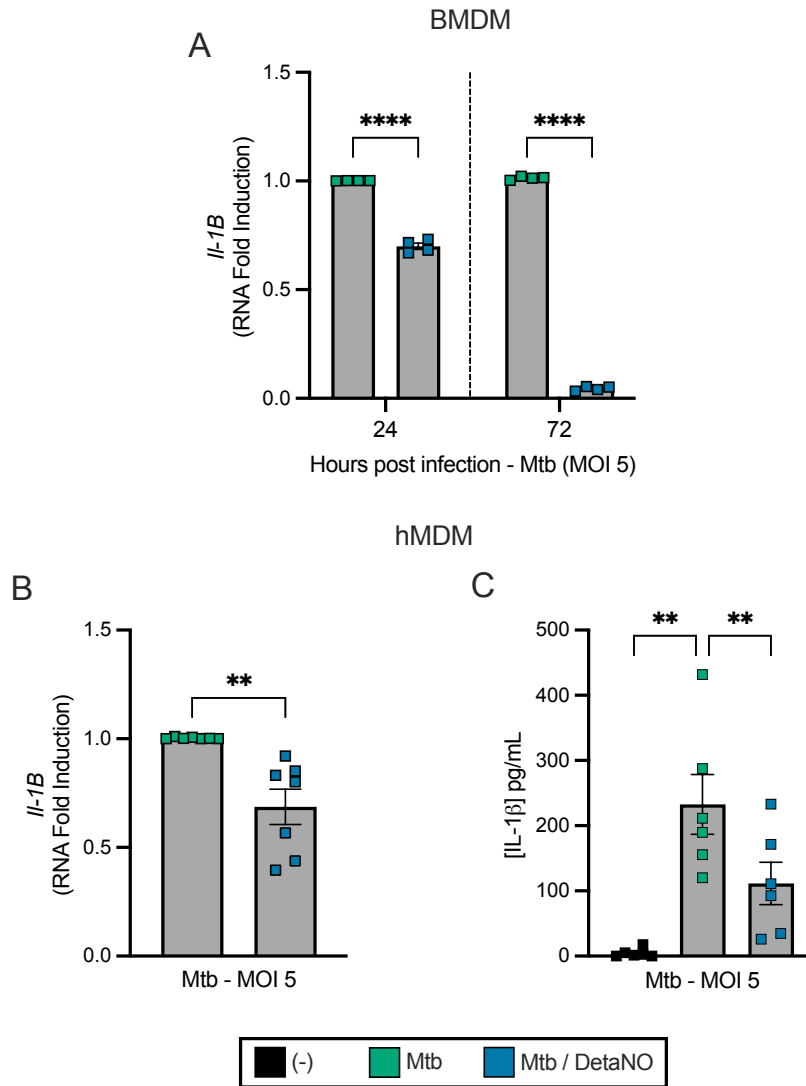


Figure 4. 18 NO attenuates IL-1 β secretion during Mtb infection of macrophages

Murine BMDMs (A) and human MDMs (B) were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. DetaNO was added 3 hours post infection (50 μ M A, 25 μ M B-C) and every 24 hours for infection duration. (A-B) qPCR was performed and expression of *Il-1 β* relative to the untreated control was calculated. Data was normalised to *Hprt* (A) *18S* (B). (C) Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using a 2-way ANOVA with Šídák's multiple comparisons test (A), an unpaired t-test (B) and a RM one-way ANOVA with way Šídák's multiple comparisons test (C). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ (A), 7 (B+C) independent experiments.

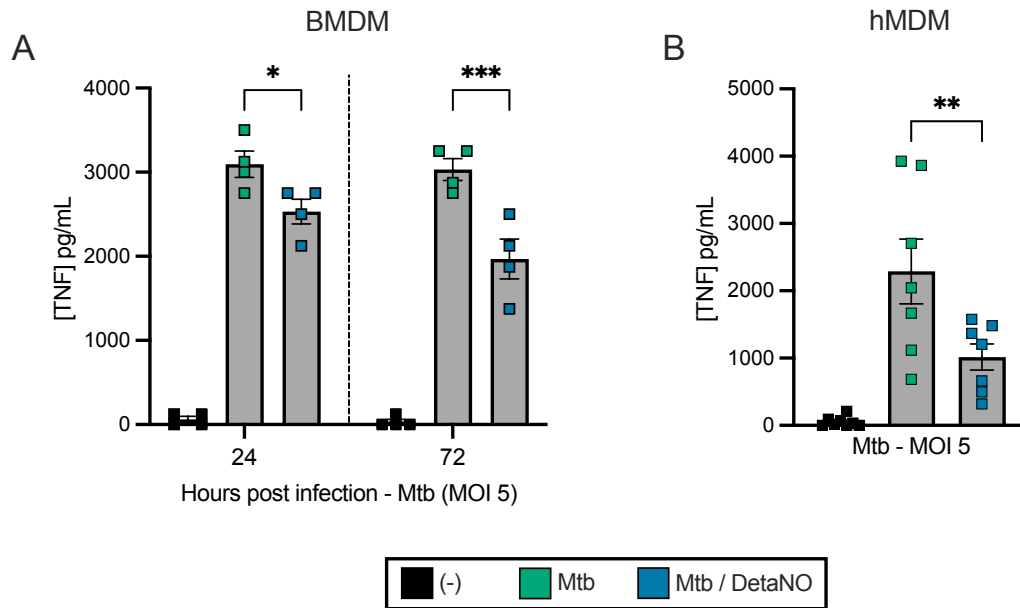


Figure 4. 19 NO attenuates TNF α secretion during Mtb infection of macrophages

Murine BMDMs (A) and human MDMs (B) were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. DetaNO was added 3 hours post infection (50 μ M A, 25 μ M B-C) and every 24 hours for infection duration. Supernatants were collected and TNF α protein quantified using an ELISA. Statistical analysis was performed using a 2-way ANOVA with Tukey test (A), and an ordinary one-way ANOVA with way Šídák's multiple comparisons test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ (A), 7 (B) independent experiments.

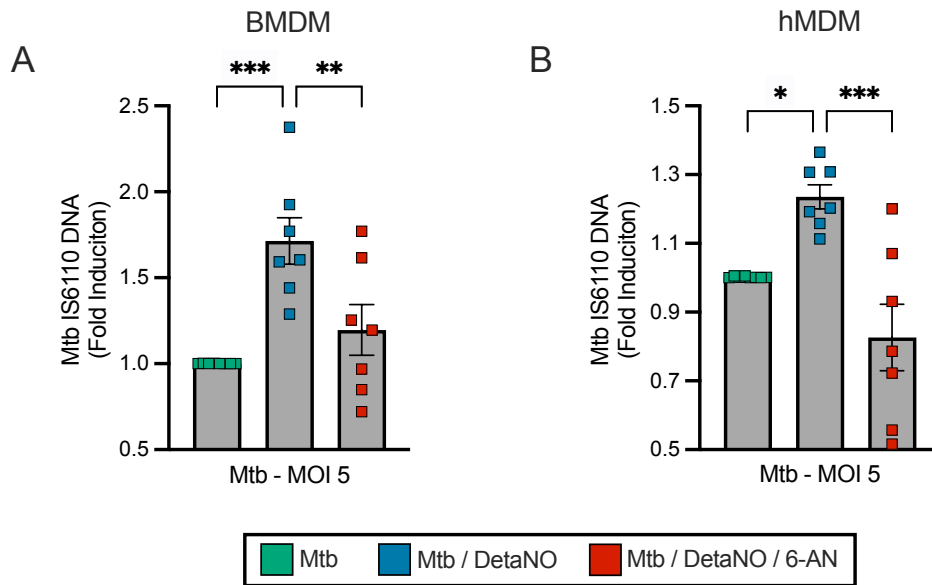


Figure 4. 20 DetaNO supplementation promotes Mtb survival in murine and human macrophages

Murine BMDMs (A) and human MDMs (B) were pre-treated with 6-AN (100 μ M A, 25 μ M B) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 72 hours. DetaNO was added 3 hours post infection (50 μ M A, 25 μ M B) and every 24 hours for infection duration. qPCR was performed and expression of bacterial *IS6110* relative to the untreated control was calculated. Data was normalised to bacterial *16S*. Statistical analysis was performed an ordinary one-way ANOVA with way Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

4.2.4 Nitric oxide targets TCA cycle metabolism during Mtb infection of macrophages

Having confirmed the negative effect of NO on inflammatory cytokine secretion, particularly IL-1 β , our next objective was to explore possible mechanisms through which NO facilitates the generation of an environment permissive to Mtb replication. As mentioned, previous work owes the immunosuppressive nature of NO to its regulation of inflammasome activity (54, 331). However, given the work from our lab and others emphasizing metabolic dependency of IL-1 β transcription, and now recognising the role of Mtb derived NO in influencing PPP metabolism, we hypothesized that the effect of NO on IL-1 β might also be rooted in metabolic processes.

Metabolomic analysis of *Nos2*^{-/-} deficient macrophages has previously highlighted the correlation between NO and the induction of a TCA cycle “break” (53). This was attributed to NO targeting the TCA enzyme aconitase-2 (ACO2), which mediates the generation of isocitrate from citrate, via the intermediate cis-aconitate. In line with this, we next looked to evaluate whether attenuated responses during Mtb infection of macrophages is a result of bacterial mediated induction of PPP derived NO and subsequent targeting of TCA cycle metabolism. Recent studies have shown that macrophage PPP activity supports itaconate production during macrophage activation. Here, the TCA cycle is rewired, leading to the accumulation of cis-aconitate, which is converted to itaconate by IRG1. The PPP sustains NADPH production, which maintains redox balance and supports both TCA cycle activity and *Irg1* expression, potentially through STAT1-mediated inflammatory signalling. Inhibition of the PPP reduces NADPH levels, impairs redox homeostasis, and limits *Irg1* expression, thereby decreasing itaconate synthesis. This highlights the critical role of PPP flux in regulating NADPH availability, *Irg1* expression, and itaconate production during macrophage immune responses (332). Here in the context of Mtb infection of macrophages, analysis of TCA associated genes displayed a signature consistent with the disruption to cycling obvious during infection with viable Mtb (Figure 4.21). This includes a significant increase in expression of *Irg1* in response to active infection, which catalyses the conversion of cis-

aconitate to itaconate. Similarly we observed a reduction in the later TCA-cycle enzyme *Fh1*, which is upregulated after treatment with inactivated Mtb. These observations were supported using Seahorse extracellular flux analysis, demonstrating a reduction in both basal oxphos and maximum respiratory capacity in response to viable infection relative to cells stimulated with inactive Mtb (Figure 4.22).

We next wanted to evaluate whether PPP derived NO may also play a role in promoting the TCA derived IRG1 – itaconate axis, in addition to the previously proposed STAT1-mediated inflammatory signalling (332). Here, we found DetaNO supplementation to further decrease the oxygen consumption rates of human MDMs (Figure 4.23), resulting in significant decrease in macrophage maximum respiratory capacity. Interestingly, this effect was not observed in murine BMDMs and may be a result of NO scavenging and peroxynitrite production at macrophage basal levels, an observation previously recorded in astrocytes (333). Differences between both systems in the context of manipulation of TCA/OXPHOS have been previously recorded (276) However, NO supplementation further amplified the transcriptional signature recorded during infection of BMDMs, specifically causing a significant increase in *Irg1* and corresponding decrease in *Fh1* expression within BMDMs. (Figure 4.24). To further implicate NO in disruption of TCA cycle metabolism, we used the NO scavenging molecule cPTIO, which converts all available NO into its metabolised form, nitrite. In agreement, cPTIO significantly reduced *Irg1* expression, implying a direct role for moderate NO levels in the generation of itaconate (Figure 4.25). Curiously, scavenging NO resulted in an increase in both PPP enzyme expression (Figure 4.25) and G6PD activity (Figure 4.26) which we hypothesise is an attempt to compensate for lost cellular NO due to cPTIO activity. In contrast to DetaNO supplementation, loss of NO decreased *Ldha* expression and attenuated lactate secretion of both murine BMDMs and human MDMs during infection (Figure 4.27). This suppressive effect of NO scavenging on macrophage classical reprogramming resulted in reduction of expression and mature protein levels of *Il-1 β* (Figure 4.28) and TNF α (Figure 4.29) and unexpectedly led to consequent increases in Mtb replication within murine and human macrophages (Figure 4.30). These results suggest that NO may be required to initiate Warburg metabolism; however, the sustained induction driven by Mtb's manipulation of PPP activity could ultimately

suppress inflammatory responses, creating an environment favourable for bacterial replication.

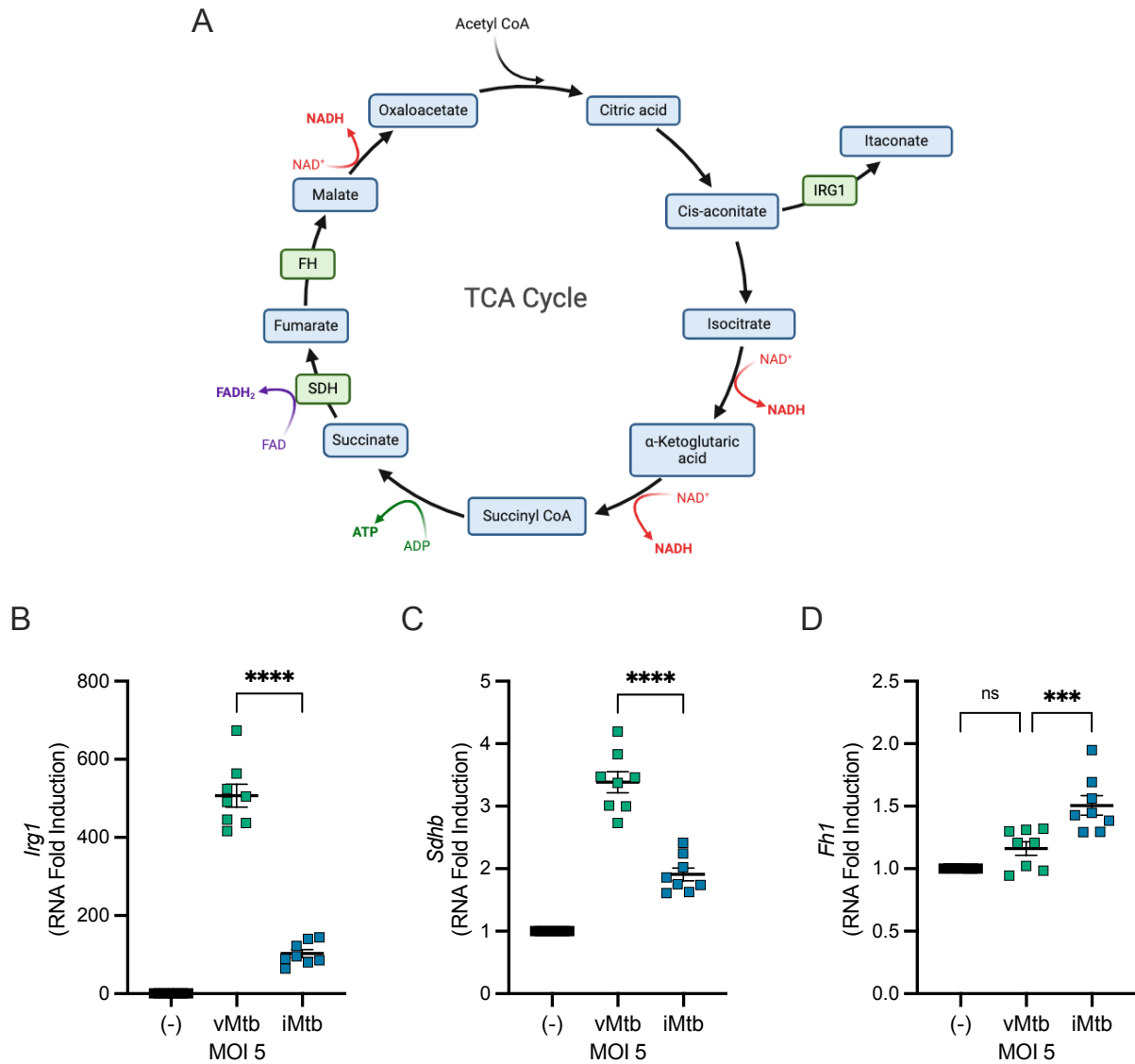


Figure 4. 21 Viable Mtb promotes transcriptional signature associated with TCA cycle break

(A) TCA cycle. (B-D) Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of *Irg1* (B), *Sdhb* (C) and *Fh1* (D) relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

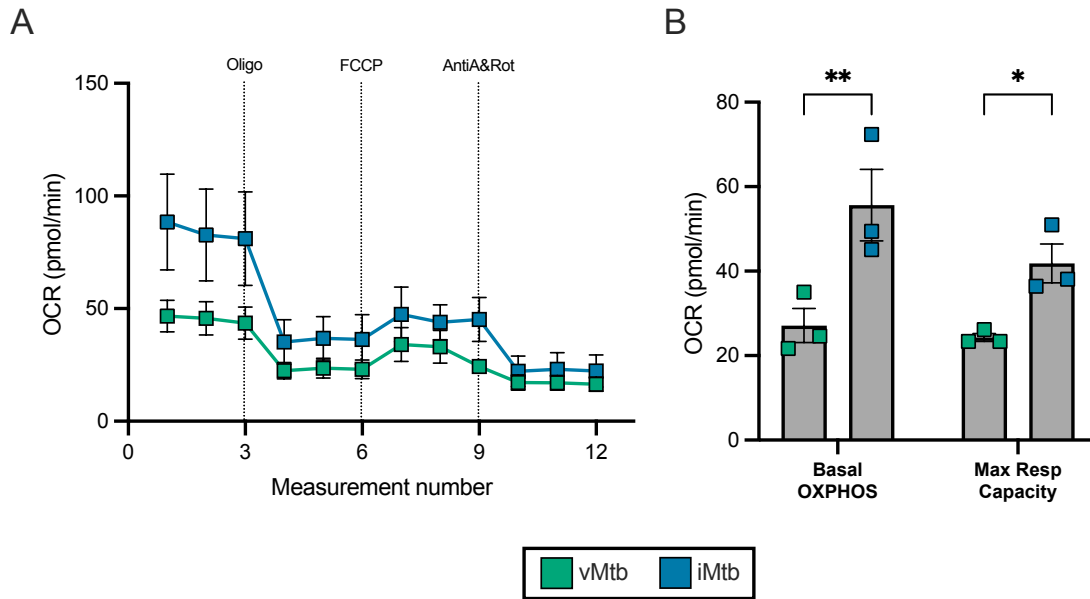


Figure 4. 22 Viable Mtb reduces basal and maximum respiratory rates of macrophage oxphos

Murine BMDMs were infected with viable (vMtb) and inactivated (iMtb) (heat-killed) Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Oxygen consumption rate (OCR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo), FCCP, antimycin-A and rotenone (Anti-A&Rot). Statistical analysis was performed using a two-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, N = 1, n = 3 independent experiments.

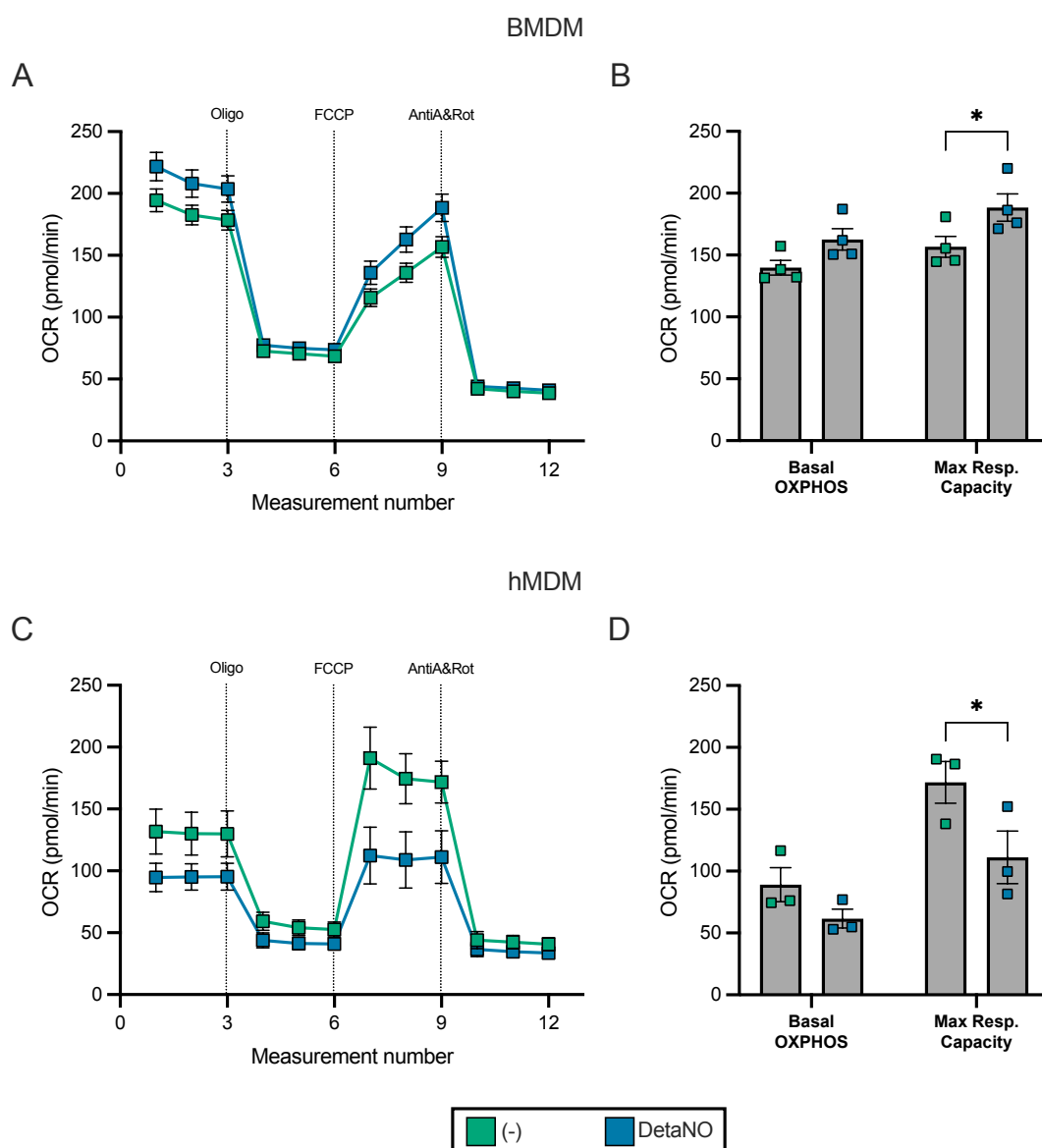


Figure 4. 23 NO alters maximum respiratory capacity of uninfected human macrophage oxphos

Murine BMDMs (A-B) and human MDMs (C-D) were treated with DetaNO (50 μ M A-B, 25 μ M C-D) for 24 hours. Oxygen consumption rate (OCR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo), FCCP, antimycin-A and rotenone (Anti-A&Rot). Statistical analysis was performed using a two-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ (A-B), 3(C-D) independent experiments.

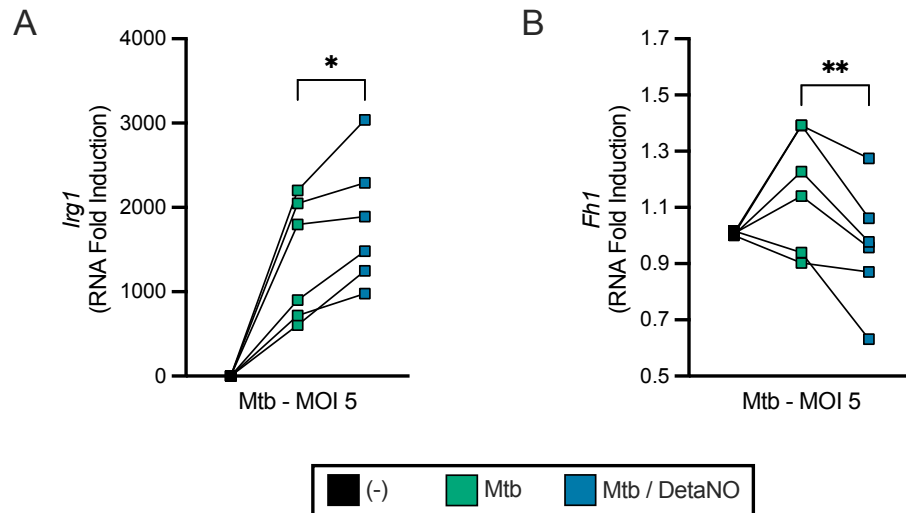


Figure 4.24 NO promotes transcriptional signature in line with disruption to TCA cycling during Mtb infection

Murine BMDMs were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. NO donor DETA-NO was added 3 hours post infection (50 μ M). qPCR was performed and expression of *Irg1* and *Fh1* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an RM one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 6$ independent experiments.

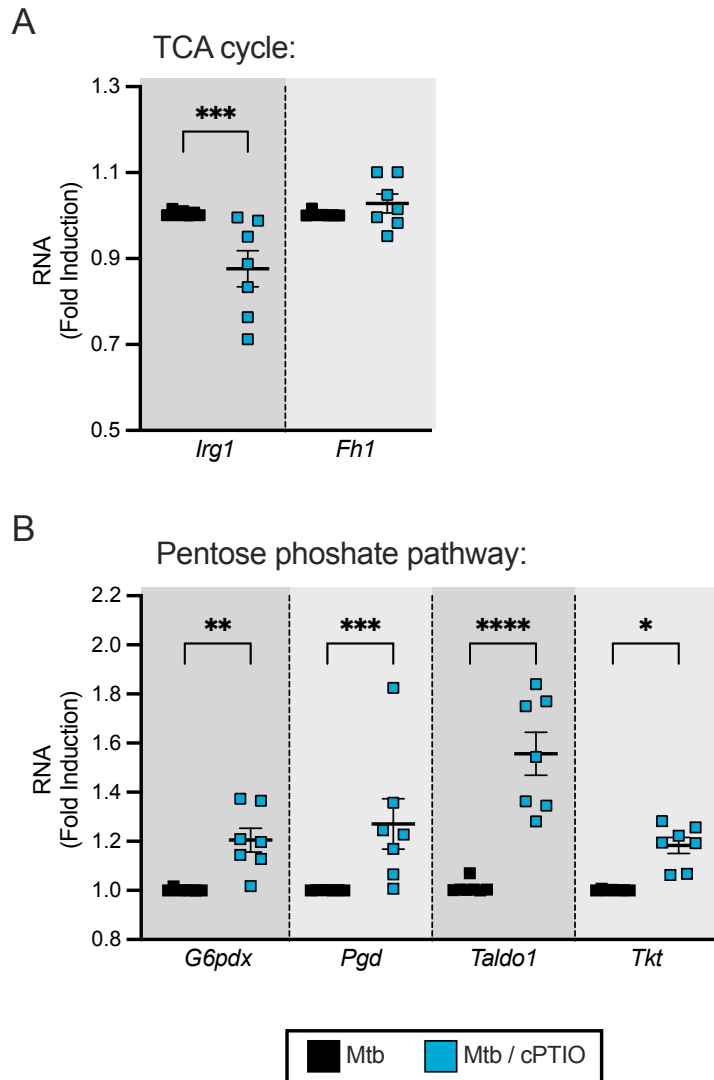


Figure 4. 25 NO sequestration alters TCA and PPP associated gene expression in murine macrophages during Mtb infection

Murine BMDMs were treated with NO scavenger cPTIO (50 μ M) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of *Irg1*, *Pgd* and *Fh1* (A) and *G6pdx*, *Pgd*, *Taldo1* and *Tkt* (B) relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

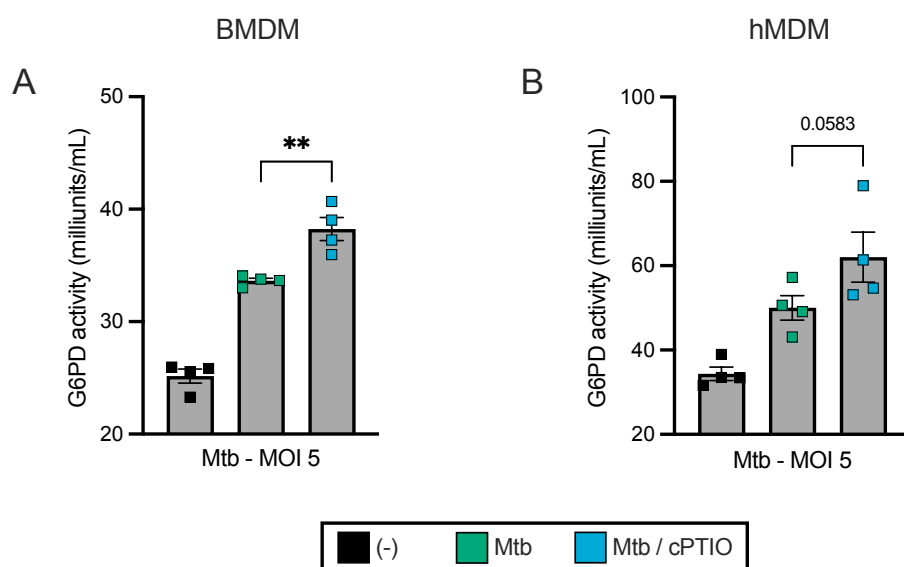


Figure 4. 26 NO sequestration promotes heightened activity of PPP rate-limiting enzyme G6PD in murine and human macrophages

Murine BMDMs (A) and human MDMs (B) were treated with NO scavenger cPTIO (50 μ M A, 25 μ M B) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

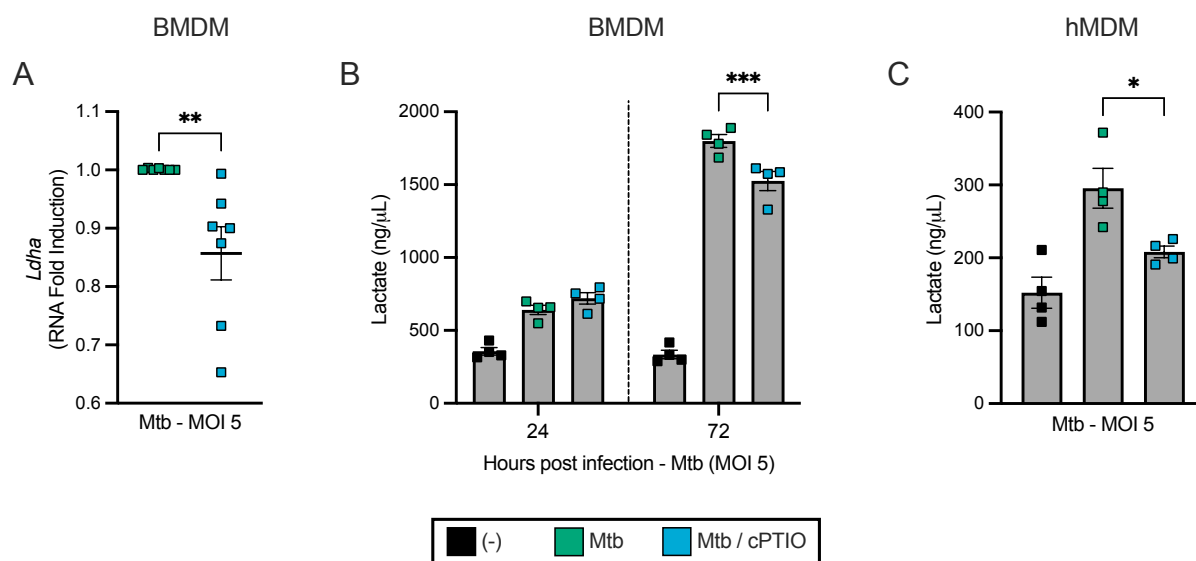


Figure 4. 27 NO sequestration decreases lactate secretion in murine and human macrophages during Mtb infection

Murine BMDMs (A-B) and human MDMs (C) were treated with NO scavenger cPTIO (50 μ M A-B, 25 μ M C) one hour prior to infection with viable (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. (A) qPCR was performed and expression of *Ldha* (A) relative to the untreated control was calculated. Data was normalised to *Hprt*. (B-C) Supernatants were collected and lactate metabolite levels quantified. Statistical analysis was performed using an unpaired t-test (A), a 2-way ANOVA with Tukey test (B) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (C). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ (A), 4 (B-C) independent experiments.

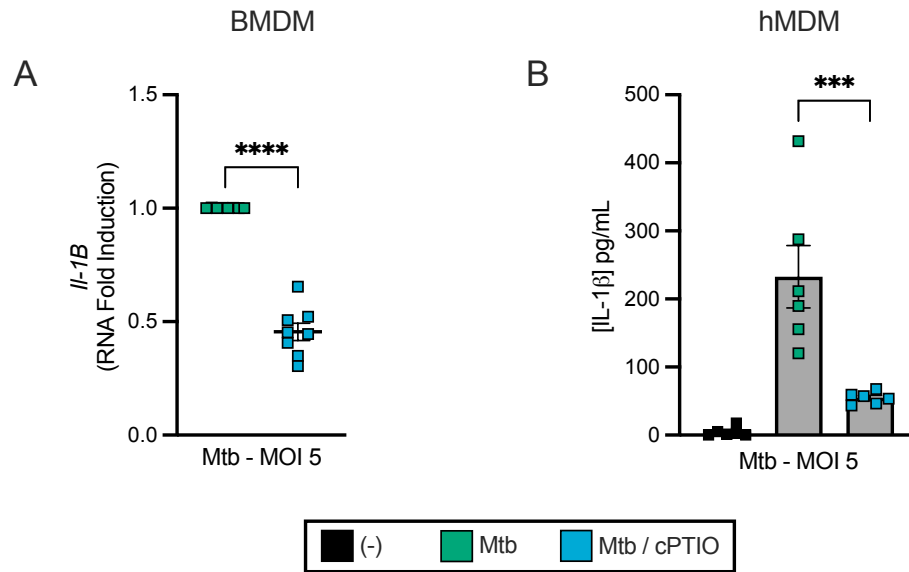


Figure 4. 28 NO sequestration decreases IL-1 β in murine and human macrophages during Mtb infection

Murine BMDMs (A-B) and human MDMs (C) were treated with NO scavenger cPTIO (50 μ M A-B, 25 μ M C) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. (A) qPCR was performed and expression of *Il-1 β* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B) Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an unpaired t-test (A) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ (A), 6 (B) independent experiments.

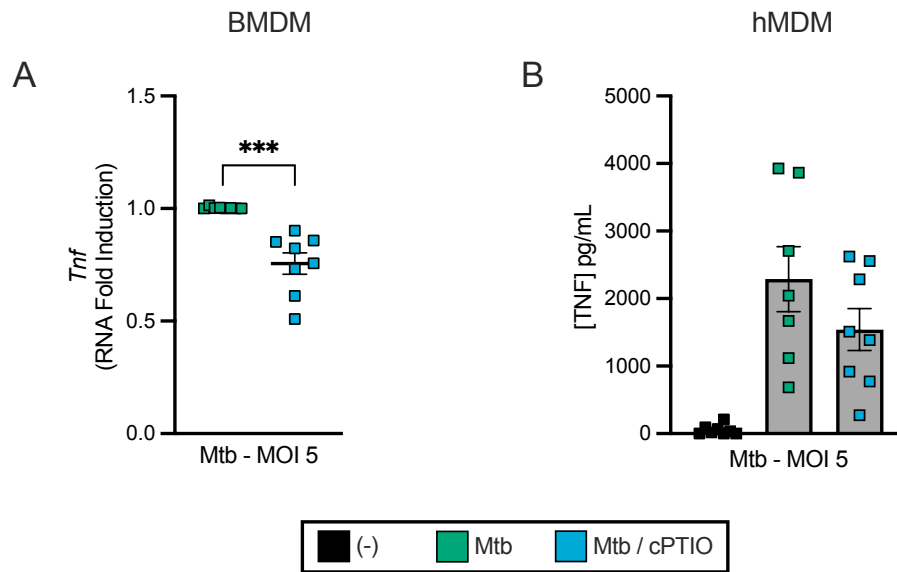


Figure 4. 29 NO sequestration decreases TNF α in murine and human macrophages during Mtb infection

Murine BMDMs (A-B) and human MDMs (C) were treated with NO scavenger cPTIO (50 μ M A-B, 25 μ M C) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. (A) qPCR was performed and expression of *Tnf* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B) Supernatants were collected and TNF α protein quantified using an ELISA. Statistical analysis was performed using an unpaired t-test (A) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

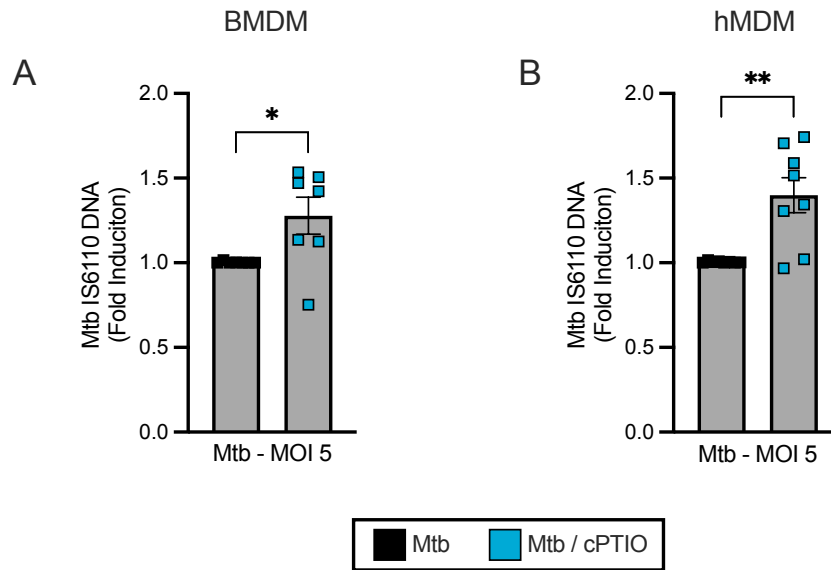


Figure 4. 30 NO sequestration promotes Mtb survival in murine and human macrophages

Murine BMDMs (A) and human MDMs (B) were pre-treated with cPTIO (50 μ M A, 25 μ M B) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 72 hours. qPCR was performed and expression of bacterial *IS6110* relative to the untreated control was calculated. Data was normalised to bacterial *16S*. Statistical analysis was performed an unpaired t-test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

4.2.5 Itaconate promotes metabolic phenotype associated with increased Mtb burden in macrophages

Having demonstrated the induction of a TCA cycle "break" during Mtb infection of macrophages—supported by bacterial-mediated PPP activity and subsequent NO generation—we next aimed to evaluate how this altered metabolic flux contributes to supporting bacterial growth. Previous data have shown a significant upregulation of *Irg1* during Mtb infection (Figure 4.21), which is further enhanced by NO supplementation (Figure 4.24) and attenuated by NO scavengers. Thus, we next looked to investigate whether NO accumulation during Mtb infection leads to a compensatory increase in itaconate production during infection. Such an observation would fall in line with previous reports of moderate NO levels targeting PDH activity, resulting in glycolytic lactate accumulation and accumulation of citrate and itaconate with compensatory glutaminolysis fuelling TCA cycle metabolism and accumulation of citrate and itaconate (53). The direct effects of itaconate on Mtb are complex and have yielded conflicting results. Some studies suggest that itaconate inhibits Mtb proliferation by targeting isocitrate lyase (ICL), a key enzyme in the glyoxylate cycle (334, 335). Notably, here itaconate has been shown to suppress Mtb growth when the bacteria are cultured in minimal medium supplemented with short-chain fatty acids (SCFAs), which serve as a preferred energy source for mycobacteria within host cells and rely on ICL enzymes for their metabolism (336-339). Conversely, other studies report that Mtb can metabolise significant quantities of itaconate into pyruvate and acetyl-CoA through the activity of the enzyme Rv2498c, highlighting a potential mechanism for itaconate resistance (340, 341).

To evaluate the potential of itaconate accumulation leading to the suppression of inflammatory responses during Mtb infection, we treated both human and murine-derived macrophages with itaconic acid prior to infection. Consistent with previous reports in LPS-treated macrophages (243), we found that itaconate significantly reduces both the mRNA expression and mature protein levels of IL-1 β during Mtb infection (Figure 4.31). Similarly, TNF α levels were reduced upon treatment with itaconate (Figure

4.32). Next , we examined the effect of elevated itaconate levels on PPP activity. Interestingly, itaconate supplementation resulted in enhanced expression of all PPP associated genes examined, namely *G6pdx*, *Taldo1* and *Tkt* within murine BMDMs (Figure 4.33) and *G6pd* and *Taldo1* in human MDMs (Figure 4.34). This expression pattern was validated at a translational level with itaconate resulting in greater G6PD activity in human and murine macrophages (Figure 4.35) and further supported by a higher NADP:NADPH when compared to untreated controls (Figure 4.36). In agreement with heightened PPP activity recorded during increased NO levels, itaconate supplementation resulted in a decrease in extracellular glucose uptake with a compensatory increase in glycogen breakdown (Figure 4.37).

Having established that increased PPP activity following itaconate supplementation is supported by greater levels of glycogenolysis and cellular NADP levels, we next looked to evaluate whether the heightened levels of this co-factor is a consequence of NOS2 activity. Interestingly, whilst itaconate supplementation led to an increase in *NOS2* expression during both LPS stimulation and Mtb infection, a significant reduction in nitrite accumulation was recorded in both murine and human derived macrophages (Figure 4.38). Also in contrast to NO supplementation, itaconate decreased glycolysis derived lactate accumulation over the time course of the infection (Figure 4.39). Previous studies have shown that by targeting PDH activity, moderate NO levels promote lactate and itaconate accumulation, while excessive NO depletes cis-aconitate, limiting *Irg1*-mediated itaconate generation. Here, we propose the formation of a bacterial-mediated negative feedback loop that autoregulates NO levels to sustain itaconate accumulation and subsequent repression of IL-1 β . This, in turn, maintains an attenuated environment favourable for bacterial replication

In agreement with maintenance of moderate intracellular levels of NO and attenuation of immunometabolic responses, itaconate supplementation resulted in increased bacterial burden in murine and human macrophages following Mtb infection (Figure 4.40). These results are further supported by increased bacterial clearance within *Irg1*^{-/-} murine BMDMs over a time course of infection – particularly pronounced at later times (Figure 4.41). An increase in bacterial burden was observed at earlier time points of

infection, such as 24 hours, potentially due to the reduced ability of these cells to induce a TCA cycle break and activate Warburg metabolism, a process shown to support inflammatory responses during this stage. Finally, consistent with itaconate production being the process driven by NO accumulation in Mtb infected macrophages which brings about the anti-inflammatory and immune evasive effects, we recorded increased G6PD activity, nitrite accumulation and decreased lactate production in *Irg1*^{-/-} macrophages after Mtb infection (Figure 4.42 – 4.43), consistent with accumulation of NO. However, the effects of itaconate accumulation on intracellular Mtb replication are negated by loss of IRG1 enzyme. Thus, these data highlight the potential ability of itaconate to potentiate the immunosuppressive effect exerted by regulated NO accumulation during Mtb infection of macrophages.

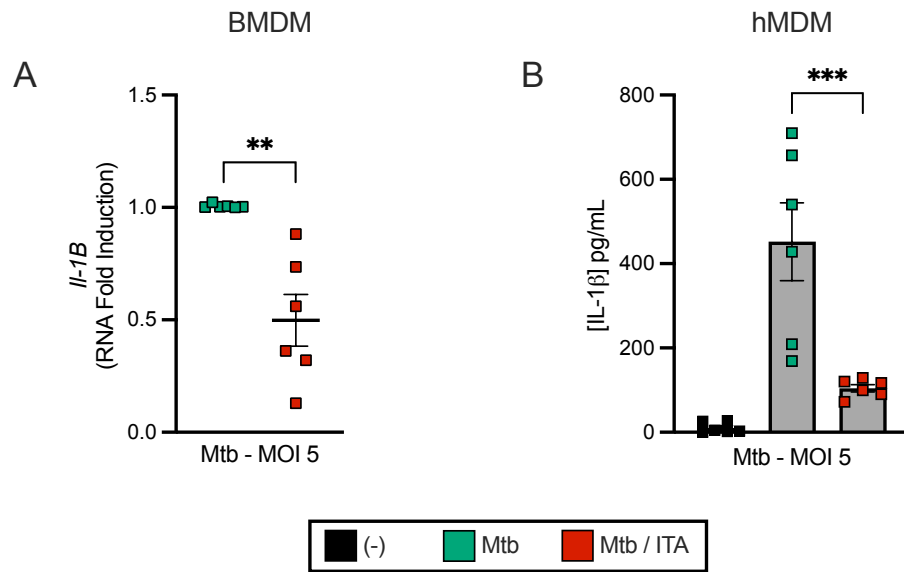


Figure 4. 31 Itaconate decreases IL-1 β in murine and human macrophages during Mtb infection

Murine BMDMs (A-B) and human MDMs (C) were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. (A) qPCR was performed and expression of *Il-1 β* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B) Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an unpaired t-test (A) and an ordinary one-way ANOVA with Šídák's multiple comparisons test (B). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 6$ independent experiments.

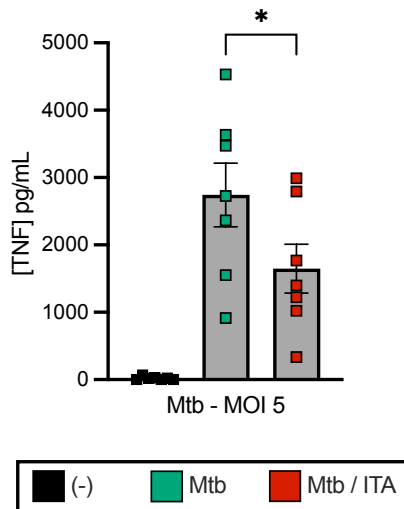


Figure 4. 32 Itaconate decreases TNF α secretion during Mtb infection

Human MDMs were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Supernatants were collected and TNF α protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

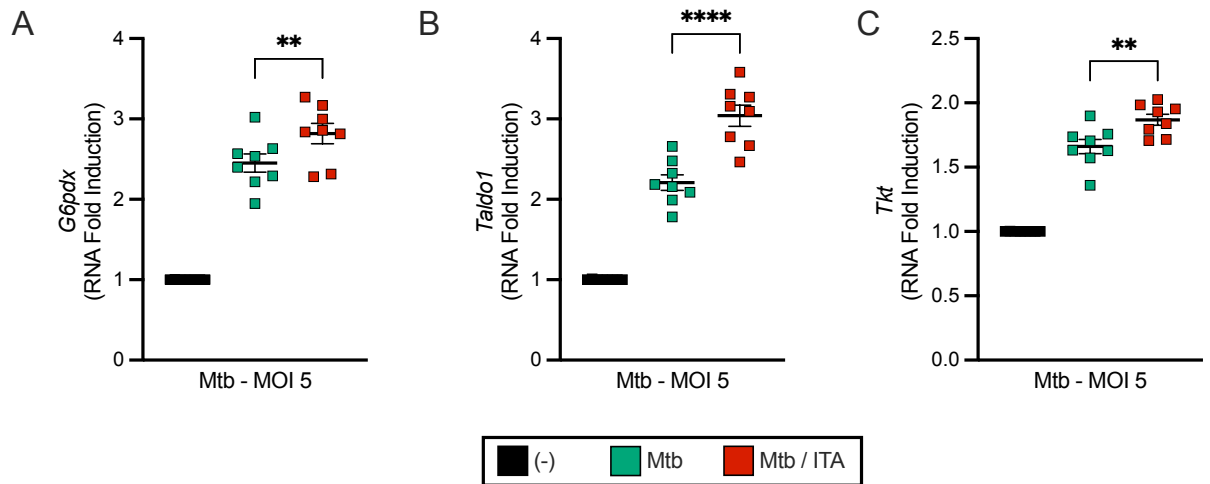


Figure 4. 33 Itaconate promotes PPP-associated gene expression during Mtb infection of murine BMDMs

Murine BMDMs were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of (A) *G6pdx*, (B) *Taldo1* and (C) *Tkt* relative to the untreated control was calculated. Data was normalised to *Hprt*. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ independent experiments.

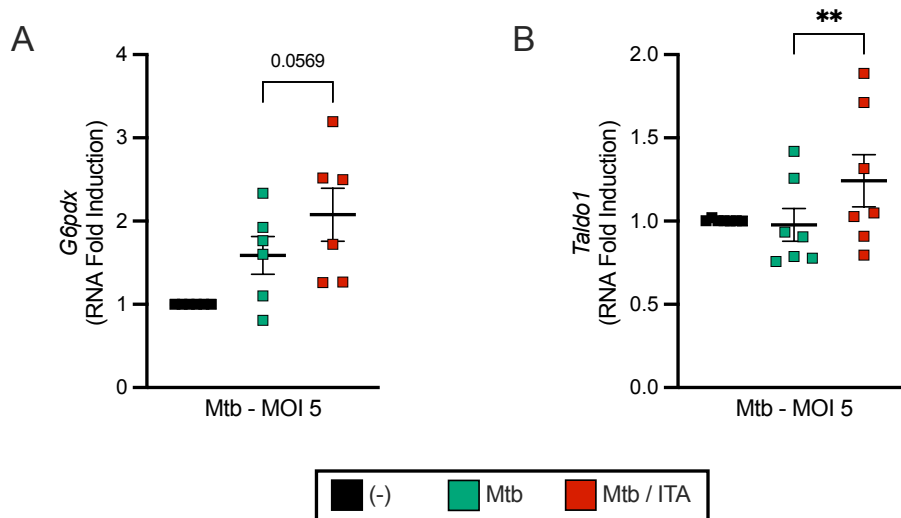


Figure 4. 34 Itaconate increases PPP gene expression in human macrophages during Mtb infection compared to Mtb alone

Human MDMs were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. qPCR was performed and expression of (A) *G6pdx* and (B) *Taldo1* relative to the untreated control was calculated. Data was normalised to *18S*. Statistical analysis was performed using a RM one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 7$ independent experiments.

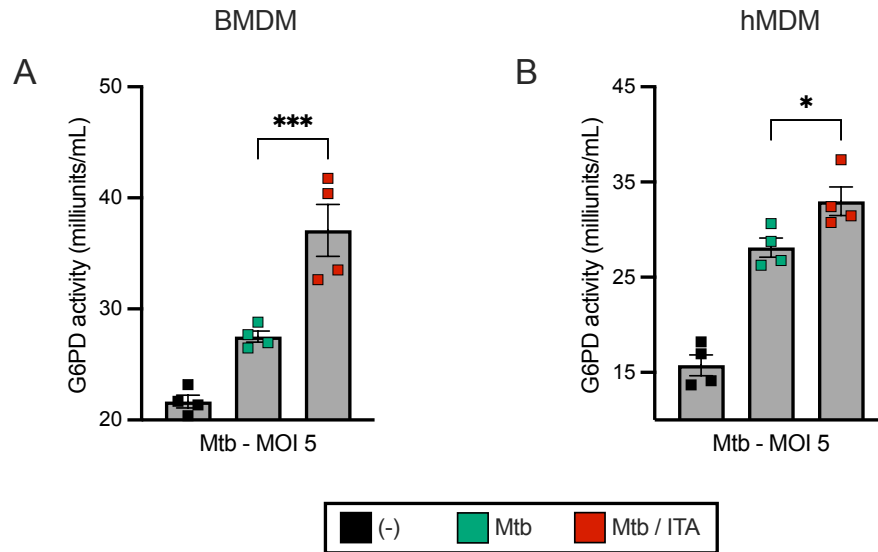


Figure 4.35 Itaconate promotes heightened activity of PPP rate-limiting enzyme G6PD during Mtb infection of murine and human macrophages

Murine BMDMs (A) and human MDMs (B) were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

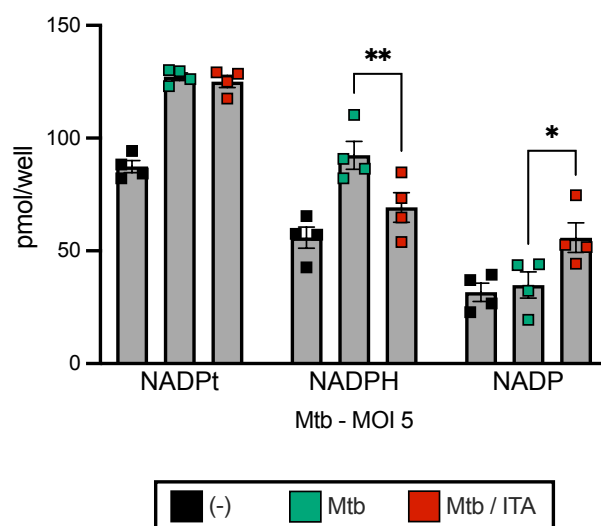


Figure 4. 36 Itaconate increases NADP:NADPH during Mtb infection of murine macrophages

Murine BMDMs were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify concentration of NADPH, NADP and total NADP/H (NADPt). Statistical analysis was performed using a 2way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

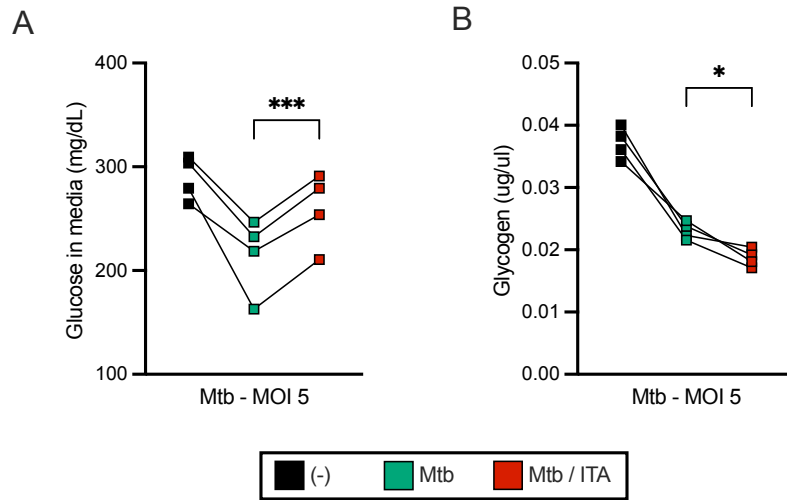


Figure 4. 37 Itaconate promotes preferential use of glycogen over glucose during Mtb infection of macrophages

Murine BMDMs were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb at an MOI of 5 bacteria per cell for 24 hours. (A) Supernatants were collected and glucose levels quantified. (B) Cells were homogenised and colorimetric assay used to quantify glycogen levels. Statistical analysis was performed using an RM one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

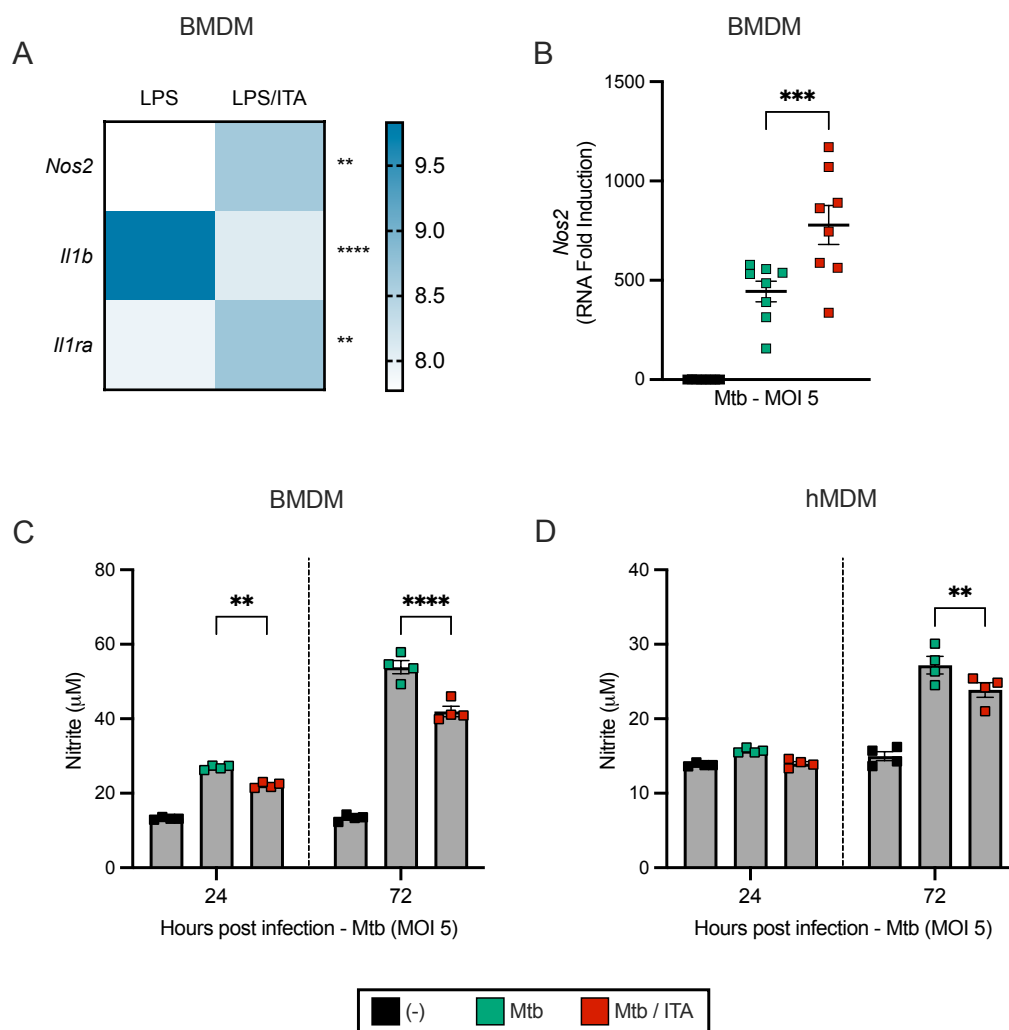


Figure 4. 38 Itaconate decreases nitrite accumulation during Mtb infection of human and murine macrophages

Murine BMDMs (A-C) and human MDMs (D) were pre-treated with Itaconic acid (10 mM) one hour prior to (A) stimulation with LPS (100 ng/mL) and (B-D) infection with live Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. (A) RNA was isolated and samples sent for sequencing. Results are displayed as Fragments Per Kilobase per Million mapped fragments (FPKM). (B) qPCR was performed and expression of *Nos2* relative to the untreated control was calculated. Data was normalised to *Hprt*. (C+D) Supernatants were collected and nitrite species quantified using colorimetric Griess assay. Statistical analysis was performed using an unpaired t-test (A), a RM one-way ANOVA with Šídák's multiple comparisons test (B) and a 2-way ANOVA with Tukey test (C+D). *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ (A), 8 (B), 4 (C+D) independent experiments.

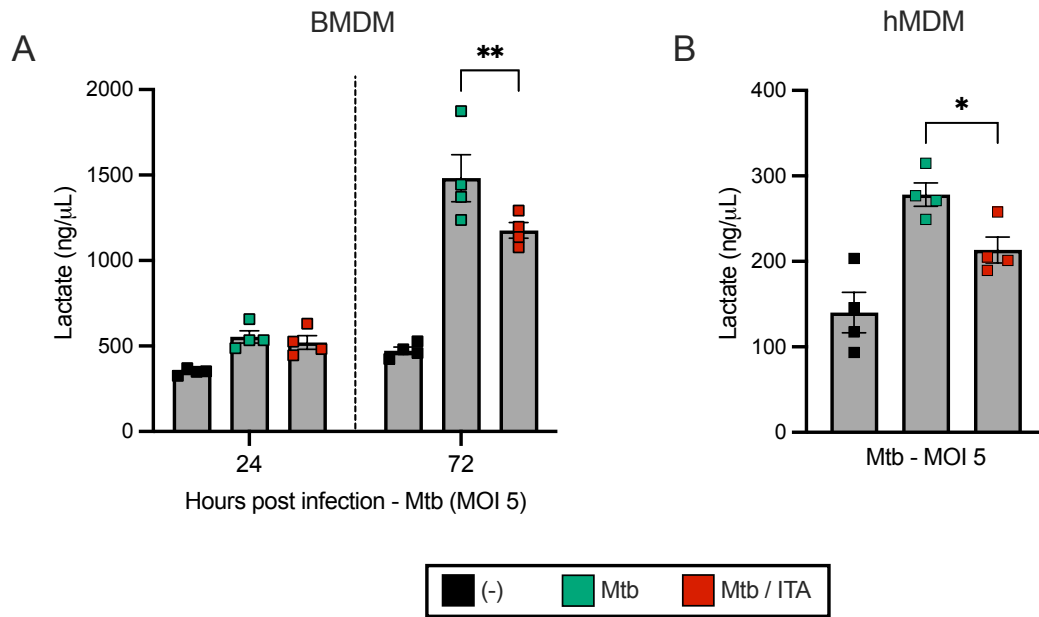


Figure 4. 39 Itaconate decreases lactate production during Mtb infection of murine and human macrophages

Murine BMDMs (A-B) and human MDMs (C-D) were treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb at an MOI of 5 bacteria per cell for 24 hours. Extracellular acidification rate (ECAR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo) and 2-deoxyglucose (2DG). Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

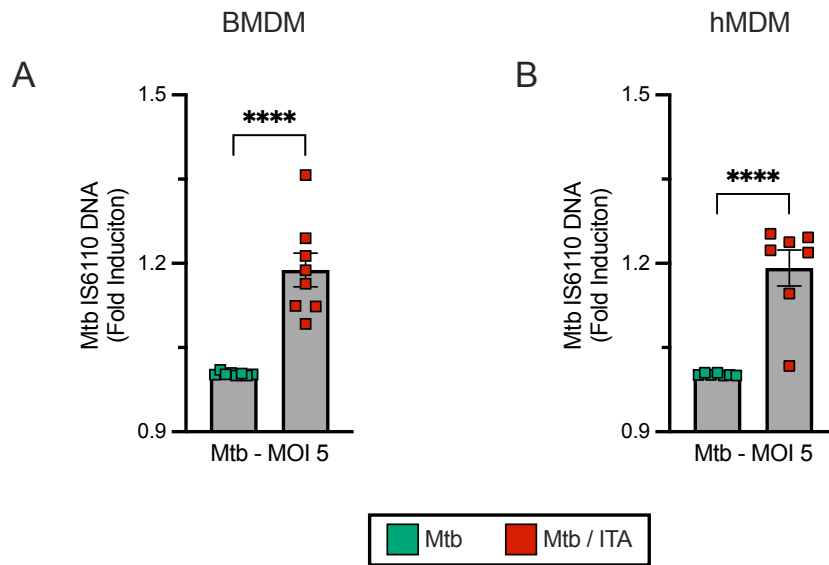


Figure 4. 40 Itaconate supplementation promotes Mtb survival in murine and human macrophages

Murine BMDMs (A) and human MDMs (B) were pre-treated with Itaconic acid (10 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 72 hours. qPCR was performed and expression of bacterial *IS6110* relative to the untreated control was calculated. Data was normalised to bacterial *16S*. Statistical analysis was performed using an unpaired t-test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ (A), 7 (B) independent experiments.

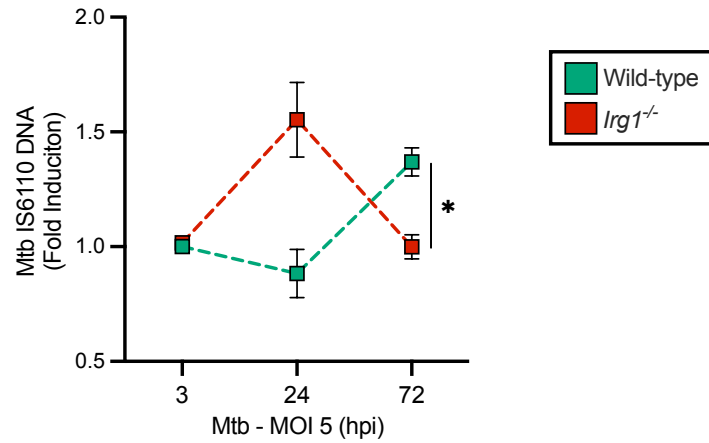


Figure 4. 41 Loss of itaconate promotes Mtb clearance at later time points of infection

Murine BMDMs (Wild-type and *Irg1*^{-/-}) were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 3, 24 and 72 hours. qPCR was performed and expression of bacterial *IS6110* relative to the untreated control was calculated. Data was normalised to bacterial *16S*. Statistical analysis was performed using an unpaired t-test. *, **, *** and **** represent p<0.05, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, n = 4 independent experiments.

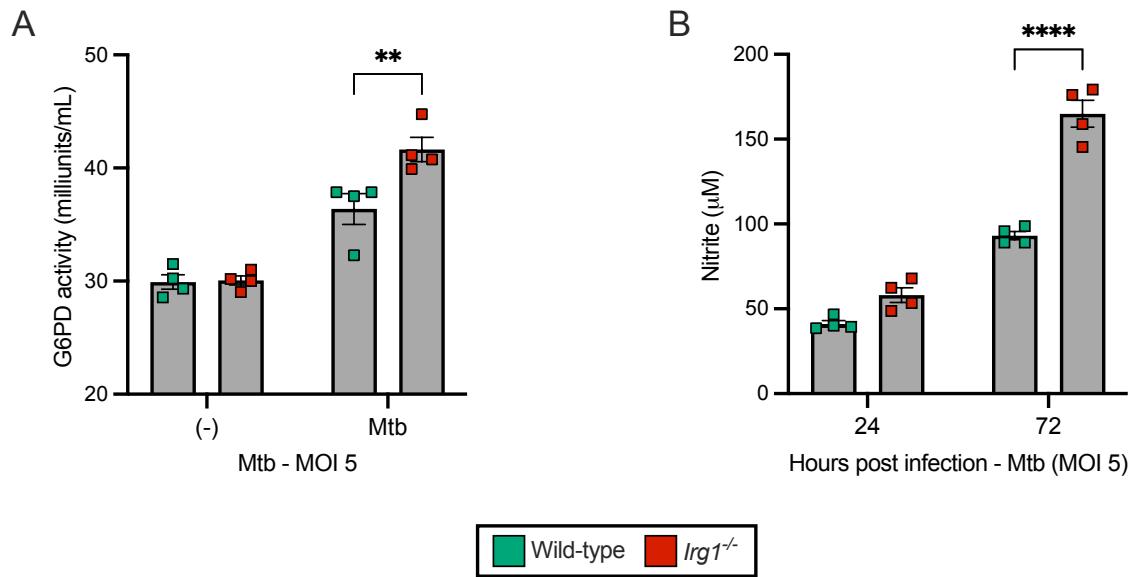


Figure 4. 42 Loss of itaconate promotes PPP metabolism and NO accumulation during Mtb infection of macrophages

Murine BMDMs (Wild-type and *Irg1*^{-/-}) were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A-C) and 72 (B) hours. (A) Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. (B) Supernatants were collected with (B) nitrite. Statistical analysis was performed using a 2-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

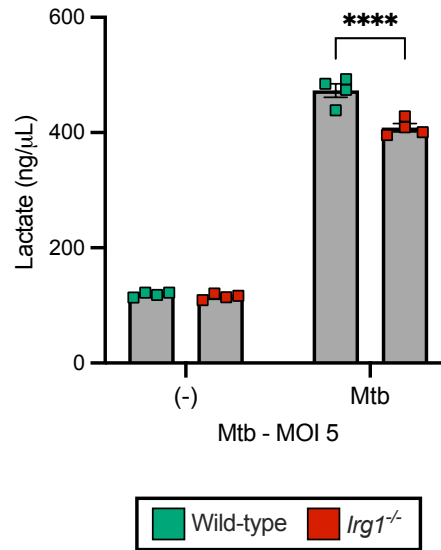


Figure 4. 43 Loss of itaconate accumulation decreases lactate production during Mtb infection of macrophages

Murine BMDMs (Wild-type and *Irg1*^{-/-}) were infected with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Supernatants were collected and lactate metabolite levels quantified. Statistical analysis was performed using a 2-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

4.2.6 Inhibition of Succinate Dehydrogenase replicates metabolic phenotype associated with itaconate accumulation during Mtb infection

Having demonstrated the capacity of itaconate to further potentiate the suppressive effect of PPP derived NO during Mtb infection of macrophages, we next proceeded to assess whether this effect results from succinate dehydrogenase (SDH) inhibition, a known target during itaconate accumulation (242). While it is known that metabolic reprogramming in Mtb infected macrophages promotes IL-1 β production, it remains unclear whether this is a direct effect of increased glycolysis, HIF-1 α activation, or other underlying processes. However, our data suggest that it may be driven by reverse electron transport (RET) from succinate oxidase activity, offering a potential link between mitochondrial function and IL-1 β regulation in Mtb infected cells. Here, we targeted SDH using dimethyl malonate (DMM) and observed that its inhibition resulted in a metabolic phenotype similar to that of itaconate accumulation, including increased G6DP activity (Figure 4.44). Additionally, DMM inhibition led to decreased lactate and nitrite accumulation over time (Figure 4.45-4.46). Finally, SDH inhibition resulted in decreased IL-1 β secretion (Figure 4.47) and heightened bacterial burden within Mtb infected murine and human derived macrophages which was overcome through PPP inhibition with 6-AN (Figure 4.48). In sum, these data suggest metabolic crosstalk between PPP and TCA cycle activity, with disruption to TCA cycle activity promoting Mtb survival during infection of macrophages.

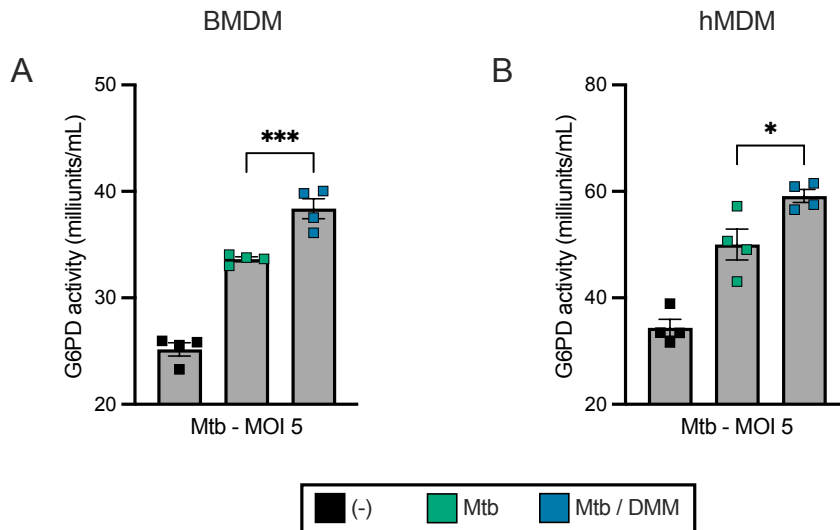


Figure 4. 44 SDH inhibition promotes macrophage PPP activity during Mtb infection of macrophages

Murine BMDMs (A) and human MDMs (B) were treated with DMM (1 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

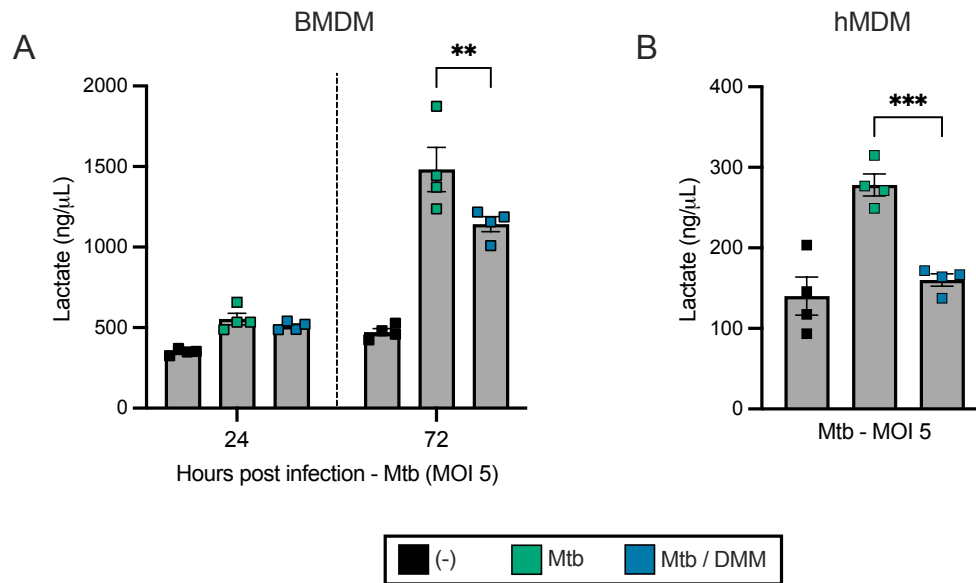


Figure 4. 45 SDH inhibition decreases lactate accumulation in Mtb infected macrophages

Murine BMDMs were treated with DMM (1 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and lactate levels quantified. Statistical analysis was performed using a 2-way ANOVA with Tukey test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

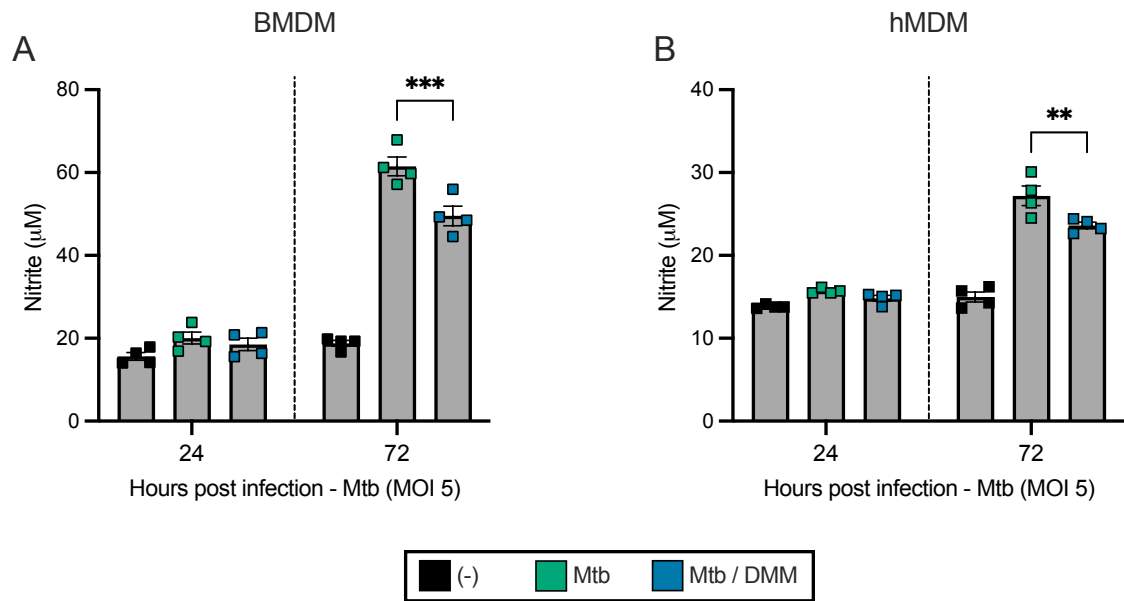


Figure 4. 46 SDH inhibition decreases nitrite accumulation during Mtb infection of macrophages

Murine BMDMs (A-B) and human MDMs (C-D) were treated with DMM (1 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and nitrite species quantified using colorimetric Griess assay. Statistical analysis was performed using a 2-way ANOVA with Tukey test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

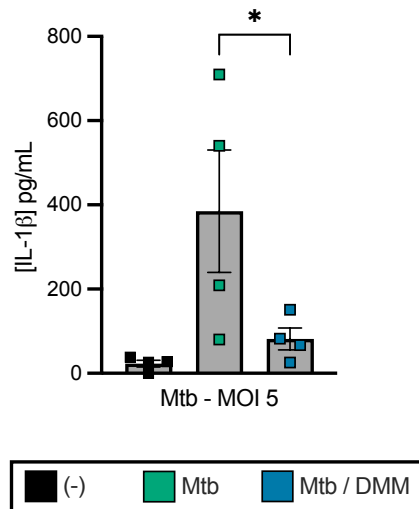


Figure 4. 47 SDH inhibition decreases IL-1 β secretion during Mtb infection of macrophages

Human MDMs were treated with DMM (1 mM) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Supernatants were collected and IL-1 β protein quantified using an ELISA. Statistical analysis was performed using an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

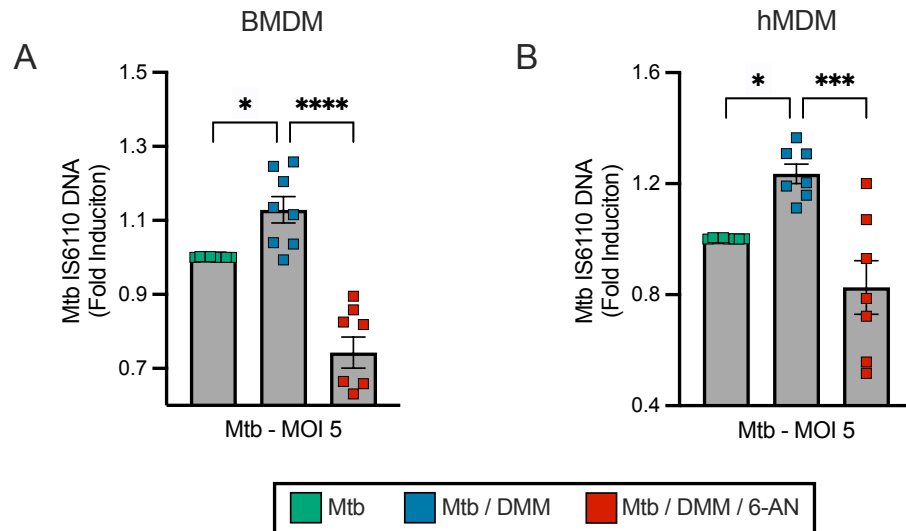


Figure 4. 48 SDH inhibition promotes Mtb survival in murine and human macrophages

Murine BMDMs (A) and human MDMs (B) were pre-treated with DMM (1 mM) and 6-AN (100 μ M A, 25 μ M B) one hour prior to infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 72 hours. qPCR was performed and expression of bacterial *IS6110* relative to the untreated control was calculated. Data was normalised to bacterial *16S*. Statistical analysis was performed an ordinary one-way ANOVA with way Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 8$ (A), 7 (B) independent experiments.

4.2.7 NOX2 inhibition promotes macrophage clearance of Mtb by disrupting PPP activity and in turn enhancing mitochondrial metabolism

With work to date demonstrating the capacity of PPP-derived NO in creating a permissive environment for Mtb replication by disrupting TCA cycle activity and thus impairing mitochondrial metabolism and linked induction of IL-1 β , we next aimed to support mitochondrial respiration by targeting PPP-derived NO production. To accomplish this, we aimed to target lysosomal NOX2 activity, thereby depleting cellular NADPH oxidation to enzymatic co-product NADP, which in turn would compromise the activity of NADP-dependent PPP enzymes, namely G6PD and PGD.

Inhibition of NOX2 using iNOX2, a small molecule inhibitor of the enzymes NADPH binding site resulted in decreased macrophage lysosomal acidification following stimulation with heat-killed Mtb (strain H37Ra) (Figure 4.49). Further evaluation of cellular metabolism revealed that NOX2 inhibition led to a decrease in lactate accumulation during Mtb infection (Figure 4.50). This observation was confirmed using Seahorse extracellular flux analysis to demonstrate decreased basal glycolytic activity following iNOX2 treatment (Figure 4.51). We subsequently linked this metabolic attenuation to reduced PPP activity, as NOX2 inhibition led to a significant and dose-dependent decrease in the activity of G6PD, the rate-limiting enzyme of the PPP metabolism (Figure 4.52). The interdependency between NO accumulation and PPP activity was further emphasized by the observation that iNOX2 treatment led to a reduction in macrophage NO levels during Mtb infection (Figure 4.53).

We next turned our attention to evaluate the impact of iNOX2 mediated inhibition of PPP activity on cellular respiration. Here, we recorded a dramatic increase in macrophage OCR following iNOX2 treatment, further demonstrated as a significant increase in both basal OXPHOS and maximum respiratory capacity (Figure 4.54). Following this, we examined macrophage mitochondrial dynamics following stimulation in the presence of iNOX2. While there was no difference in mitochondrial mass, iNOX2

resulted in increased levels of mitochondrial polarisation (Figure 5.55), suggesting greater levels of oxidative metabolism at the expense of PPP activity. Finally, this increased mitochondrial metabolism was demonstrated to promote the expression of pro-inflammatory *IL-1 β* and in turn decrease bacterial burden over the time course of infection (Figure 5.56). Together, our data demonstrate that NO orchestrates metabolic cross-talk, with increased PPP activity leading to the suppression of mitochondrial-based pro-inflammatory responses.

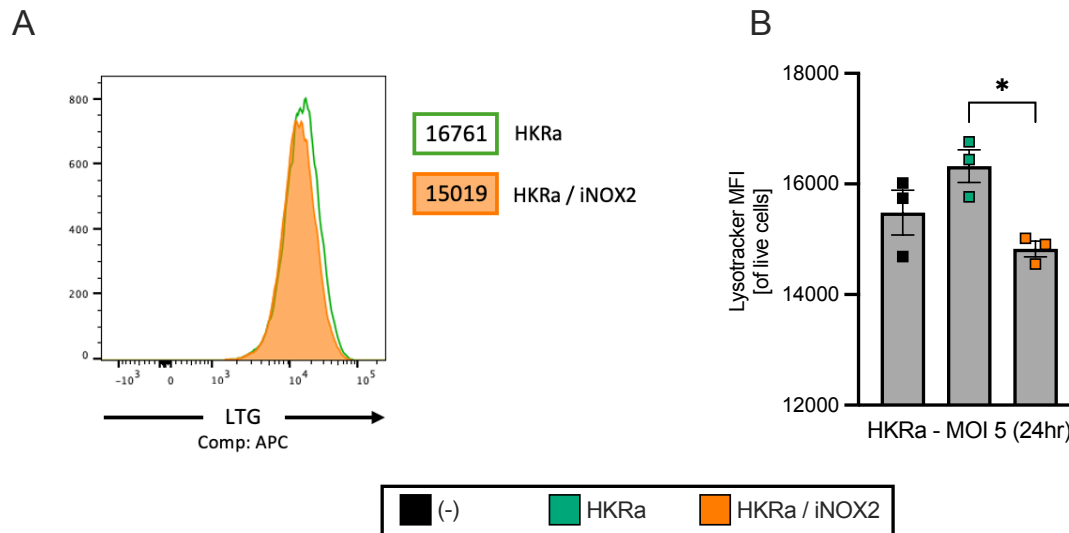


Figure 4. 49 NOX2 inhibition decreases lysosomal acidification of macrophages

Murine BMDMs were treated with iNOX2 (10 μ M) three hours after stimulation with heat-killed Mtb (HKRa - H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Graphs show Lysotracker Green (LTG) representative histogram (A) and mean fluorescence intensity (MFI) of live macrophages (B). Statistical analysis was performed an ordinary one-way ANOVA with way Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments.

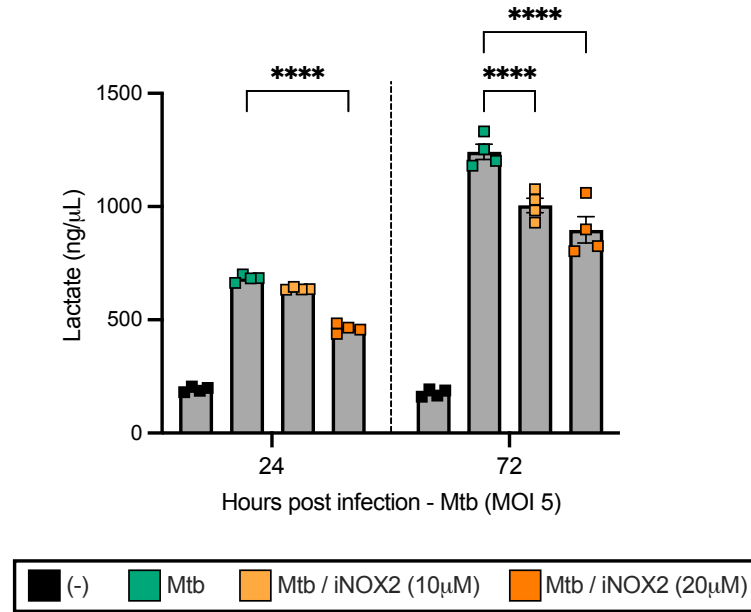


Figure 4. 50 NOX2 inhibition decreases lactate secretion in Mtb infected macrophages

Murine BMDMs were treated with iNOX2 three hours after infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Supernatants were collected and lactate levels quantified. Statistical analysis was performed using a 2-way ANOVA with Tukey test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

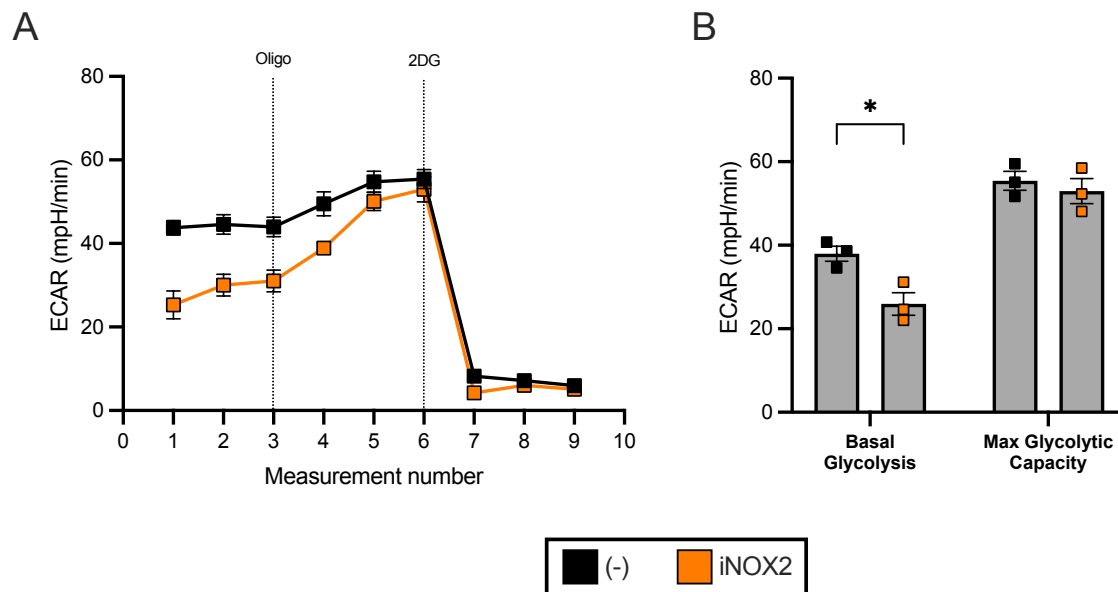


Figure 4. 51 NOX2 inhibition decreases glycolytic rates of murine and human macrophages

Murine BMDMs were treated with iNOX2 (10 μ M) three hours after stimulation with heat-killed Mtb (HKRa - H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Extracellular acidification rate (ECAR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo) and 2-deoxyglucose (2DG). Statistical analysis was performed using a 2way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments.

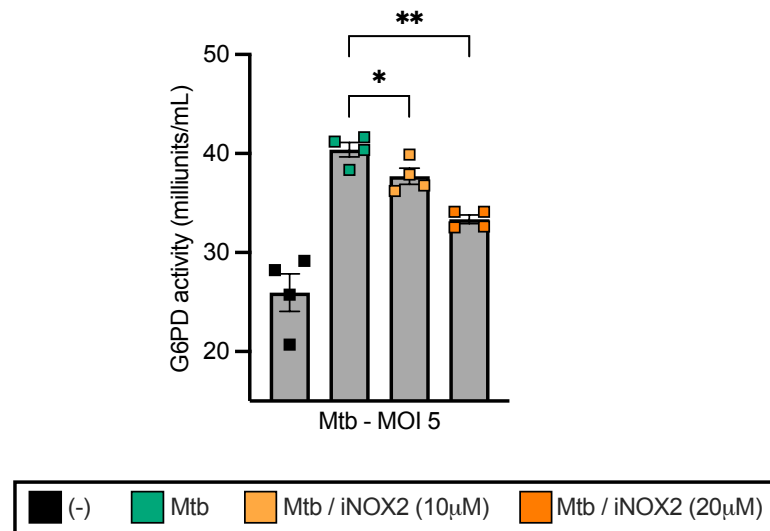


Figure 4. 52 NOX2 inhibition decreases activity of PPP rate-limiting enzyme G6PD during Mtb infection of macrophages

Murine BMDMs were treated with iNOX2 three hours after infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Statistical analysis was performed using an ordinary a RM one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

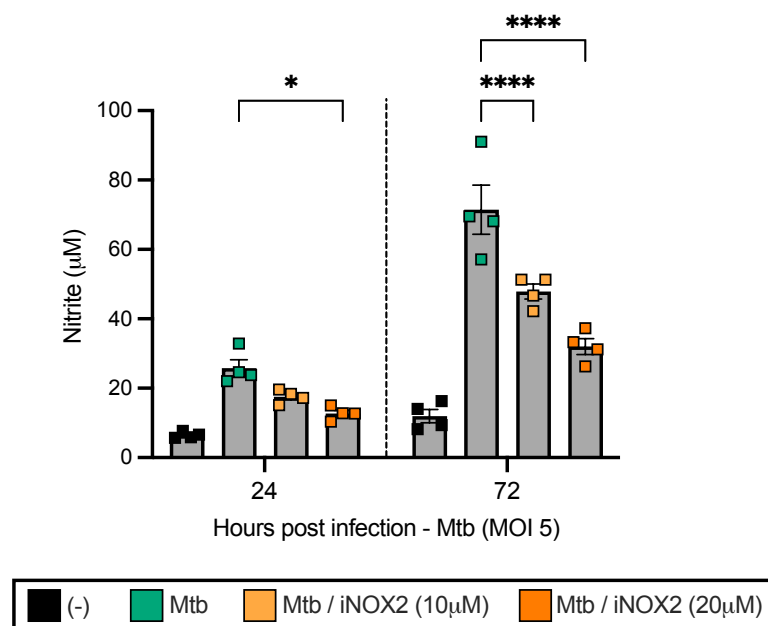


Figure 4. 53 NOX2 inhibition decreases nitrite accumulation during Mtb infection of macrophages

Murine BMDMs were treated with iNOX2 three hours after infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 and 72 hours. Cells were homogenised and colorimetric assay used to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Supernatants were collected and nitrite species quantified using colorimetric Griess assay. Statistical analysis was performed using a 2-way ANOVA with Tukey test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

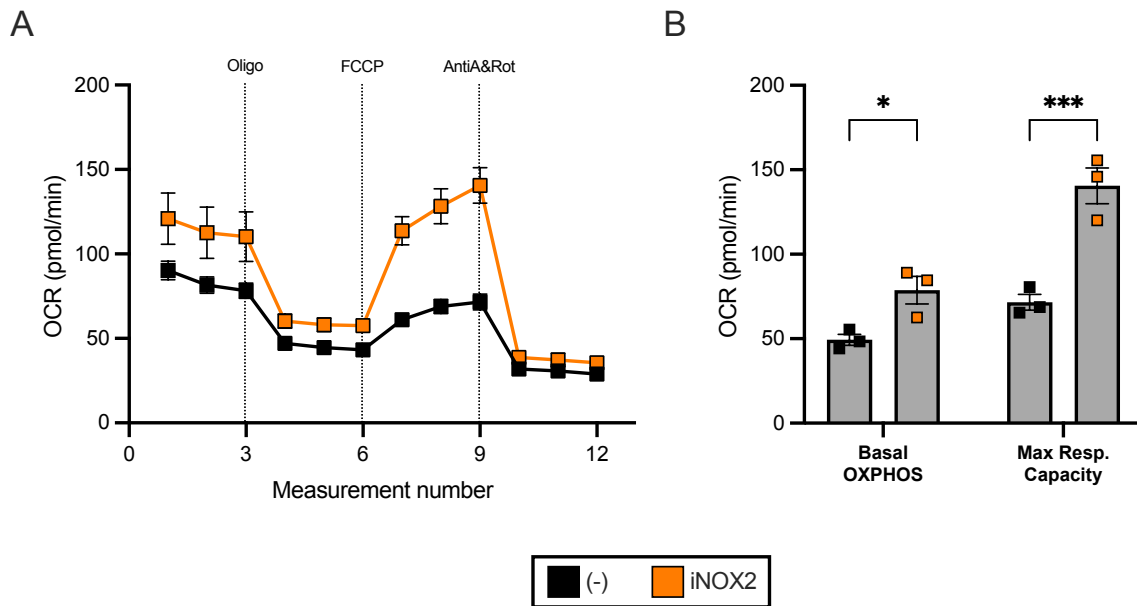
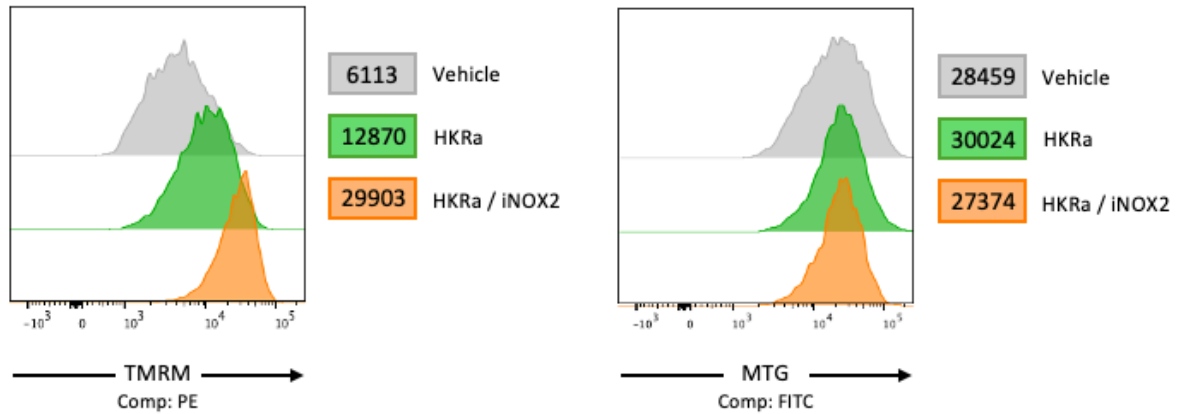


Figure 4. 54 NOX2 inhibition increases macrophage basal oxphos and maximum respiratory capacity

Murine BMDMs were treated with iNOX2 (10 μ M) three hours after stimulation with heat-killed Mtb (HKRa - H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Oxygen consumption rate (OCR) was recorded by Seahorse analysis following injection of metabolic inhibitors oligomycin (Oligo), FCCP, antimycin-A and rotenone (Anti-A&Rot). Statistical analysis was performed using a 2-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments.

A



B

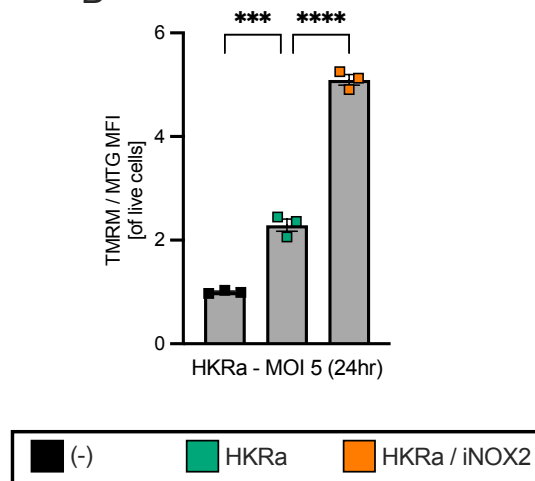


Figure 4. 55 NOX2 inhibition increases mitochondrial polarisation

Murine BMDMs were treated with iNOX2 (10 μ M) three hours after stimulation with heat-killed Mtb (HKRa - H37Ra strain) at an MOI of 5 bacteria per cell for 24 hours. Graphs show the mean fluorescence intensity (MFI) of TMRM / MTG of macrophages, relative to vehicle (B) and representative flow plot (A). Statistical analysis was performed using an ordinary an ordinary one-way ANOVA with Šídák's multiple comparisons test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 3$ independent experiments.

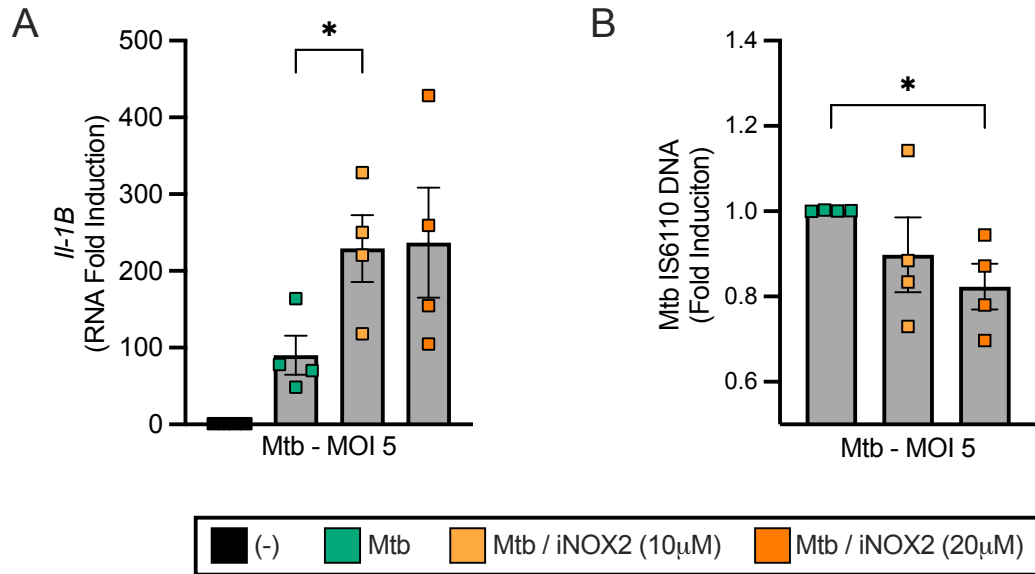


Figure 4.56 NOX2 inhibition increases macrophage Mtb clearance

Murine BMDMs were treated with iNOX2 three hours after infection with viable Mtb (H37Ra strain) at an MOI of 5 bacteria per cell for 24 (A) and 72 (B) hours. (A) qPCR was performed and expression of *Il-1β* relative to the untreated control was calculated. Data was normalised to *Hprt*. (B) qPCR was performed and expression of bacterial *IS6110* relative to the untreated control was calculated. Data was normalised to bacterial *16S*. Statistical analysis was performed using an ordinary a RM one-way ANOVA with Fishers LSD test. *, **, *** and **** represent $p < 0.05$, 0.01, 0.001 and 0.0001 respectively. Data shown as mean $\pm$ SEM, $n = 4$ independent experiments.

4.3 Discussion

This chapter demonstrates that NO derived from the PPP orchestrates macrophage immunometabolic responses. Specifically, it highlights how this creates an environment permissive to Mtb replication, essentially allowing the bacteria to evade host-immunometabolic responses, particularly the production of IL-1 β .

NO is a diffusible, free radical gas produced in mammals by three NOS isoforms, namely neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3) (138). The generation of NO by human macrophages has long been a subject of significant debate. However, over the years there has been growing evidence in support of NO production in human macrophages (139, 342-345). Moreover, in the specific context of Mtb, NOS2 was found to be more frequently expressed in the lungs of patients during tuberculosis infection compared to healthy controls and is in line with increased detection of NO in the exhaled breath of patients during Mtb infection (139, 346). These observations highlight the importance of evaluating macrophage activity within NO-enriched environments. Here, we show that NO has the capacity to promote both glycolytic and PPP based metabolism in basal macrophages. Furthermore, during active Mtb infection, there is an increased expression of *Nos2* and a corresponding moderate accumulation of cellular NO over the course of the infection in both murine and human-derived macrophages. This observation of NO accumulation aligns with previous studies that detected NO production during Mtb infection of human macrophages and demonstrated its role in promoting bacterial replication through promoting NOS activity (308). In our model, by repeatedly supplementing NO, we further support these findings, showing that NO accumulation enhances the rate of intracellular Mtb replication. With PPP metabolism serving as primary producer of cellular NADPH, we highlight elevated pentose phosphate pathway activity during Mtb infection as the metabolic driving force behind NOS2 derived NO. Interestingly, we also show that during Mtb infection, macrophages utilize glycogen stores as a primary source of carbon to generate and sustain NO production with inhibition of glycogenolysis resulting in decreased NO accumulation in addition to blunted glycolytic reprogramming. These observations of macrophage glycogenolysis highlights the importance of investigating alternative

pathways that influence macrophage inflammatory phenotype, challenging the common biased assumption that glucose is the sole driving force behind central carbon metabolism.

Having established the distinctive NO-replete environment associated with active Mtb infection of macrophages, and suggesting this as an evasive mechanism facilitated by bacterial re-routing of central carbon towards PPP metabolism, we sought to challenge the common assumption that NO supports Mtb clearance. The impact of NO on macrophage metabolism, particularly its role in repressing OXPHOS during classical activation, has been well recognised over the years. Specifically, NO has long been identified as an inhibitor of complexes I, II, III, and IV of the mitochondrial ETC (267, 278, 279, 347). After identifying PPP activity as a major source of macrophage NO during Mtb infection, we looked to investigate the potential effects of elevated NO levels on other aspects of central carbon metabolism. Our studies go beyond examining the early immune responses to Mtb infection by mimicking the later, high-level induction of NO production seen in chronic stages of infection. While much research has focused on the initial protective role of NO, our model simulates the prolonged exposure to elevated NO levels, revealing its potential to enhance intracellular Mtb replication. In collaborative work, we have demonstrated that early NO production is protective against Mtb, as targeting NO early on or supplementing with low amounts can effectively limit bacterial growth (unpublished to date, in collaboration with the Creagh Lab, TCD). However, our findings emphasize that as NO levels rise later in infection, its role shifts, potentially becoming detrimental and promoting bacterial survival and replication within macrophages. In a model to steadily increase cellular NO levels to mimic gradual accumulation, we identified the formation of a positive-feedback loop between PPP activity and NO generation with increased carbon consumption through the ox-PPP enhancing lower glycolytic activity and subsequent accumulation of lactate. Moreover, we observe that this heightened activity is preferentially supported by the consumption of glycogen stores over the uptake of extracellular glucose, consistent with our results of macrophage glycogen depletion during Mtb infection. These observations and the possible discrepancy between glucose uptake and glycogen breakdown may in part account for the decelerated bioenergetics typically associated with Mtb infection of

macrophages. Alternative studies have identified glycogen mobilization within immune cells primarily as a mechanism to support antioxidant defense by producing NADPH and reducing glutathione, rather than its traditionally assumed role in energy storage (214). Here, we propose that Mtb hijacks cellular carbon stores to support PPP-derived NO production, aiming to dampen downstream host macrophage immunometabolic activity, particularly the generation of IL-1 β .

Given the central role of IL-1 β during Mtb infection of macrophages, we are the first to demonstrate that, in response to Mtb infection, it is not solely the up-regulation of glycolysis that drives IL-1 β production, but rather the activity of succinate oxidase. Our findings suggest that succinate oxidase supports IL-1 β production through mitochondrial based mechanisms, possibly RET. This novel insight shifts the focus from glycolysis as the primary metabolic driver of IL-1 β in infected macrophages to mitochondrial processes, providing a deeper understanding of how immune responses are metabolically regulated during Mtb infection. Moreover, the up-regulation of aerobic glycolysis alters cytosolic pH, which in turn affects mitochondrial function. As a result, when the TCA cycle “brake” occurs, RET is facilitated (146). Together, these processes drive the production of pro-IL-1 β , contributing to the inflammatory response. Previous work has established the capacity of NO to disrupt TCA cycling through targeting ACO2 enzymatic conversion of citrate to cis-aconitate and subsequently to α -ketoglutarate (53). Moderate NO levels were demonstrated to suppress PDH activity, reducing glycolytic influx into the TCA cycle and promoting increased incorporation of glutamine into citrate and itaconate. As NO levels accumulate, ACO2 activity is targeted, leading to increased citrate export while itaconate production is limited by a decrease in cis-aconitate. Here, in the context of Mtb infection, we propose bacterial mediated moderation of NO levels to support itaconate production, leading to the suppression of metabolic activity and the reduced IL-1 β production typically associated with Mtb infection. We have supported these claims by showing that controlled NO supplementation during infection further decreases both mRNA and mature protein levels of IL-1 β , subsequently promoting bacterial survival. This effect was found to be dependent on sufficient PPP activity, as inhibition with 6-AN overcame NO's impact on bacterial survival.

Further evaluation of mitochondrial metabolism during infection revealed that Mtb significantly upregulates the expression of the itaconate-generating enzyme *Irg1* while simultaneously suppressing OXPHOS levels. In line with this, NO supplementation exacerbates this disruption, further increasing *Irg1* levels and dramatically reducing the OCR of hMDMs. Supplementation with itaconate resulted in a phenotype similar to that observed with moderate NO supplementation, characterized by heightened NADP ratios, increased PPP activity, a significant reduction in IL-1 β levels, thereby promoting bacterial survival. However, we observed a reduction in lactate accumulation, indicating a itaconate mediated suppression in NO influenced metabolism. In line with these observations, itaconate reduced levels of cellular NO despite promoting heightened *Nos2* expression, possibly suggesting the formation of a bacterial mediated negative feedback loop to autoregulate NO levels, and thus sustain itaconate accumulation. This hypothesis aligns with previous reports showing increased itaconate accumulation during moderate NO levels, however, a decrease in itaconate at higher NO concentrations (53). Such regulation suggests that maintenance of NO levels portraying itaconate mediated repression of IL-1 β during infection are maintained by the metabolite itself and thus sustain an attenuated environment for bacterial replication. Furthermore, this suppression of glycolytic activity suggests a role for itaconate in the previously reported attenuation of the glycolytic response during infection (41). To further implicate itaconate in the development of this Mtb-permissive macrophage state, we demonstrated that DMM-mediated inhibition of the known itaconate target, SDH (145), which leads to succinate accumulation, also promotes a PPP-centric environment. This shift also led to diminished NO-driven glycolytic reprogramming, characterized by a reduction in both NO accumulation and lactate level, also enhancing intracellular Mtb survival.

Having demonstrated the suppressive effect of PPP-derived NO on mitochondrial metabolism, we further underscored this metabolic crosstalk by indirectly targeting Mtb-derived NO through inhibition of NOX2, which facilitates the regeneration of the PPP enzymatic cofactor NADP. Here, by disrupting Mtb-mediated PPP activity, we overcame the bacterial repression of host macrophage mitochondrial metabolism and, in contrast, promoted mitochondrial polarization and OXPHOS. Restoring oxidative

metabolism supported the macrophage inflammatory response to infection, leading to increased IL-1 β production and enhanced intracellular Mtb killing capacity. Altogether, the data from this chapter highlight the role of NO, derived from glycogen-fuelled PPP signalling, in coordinating macrophage immunometabolic activity. We subsequently describe how Mtb actively targets PPP activity to exploit the suppressive effect of NO on mitochondrial metabolism, thereby promoting its own survival.

Chapter 5. Final Discussion

Mtb is the world's most successful pathogen and continues to persist as a global pandemic today, with over 10 million new cases and nearly 1.5 million deaths annually. Central to its pathogenesis is its interaction with its primary cell type of infection – the macrophage. Rather than being eradicated, Mtb subverts macrophage functions, residing in a latent state within phagosomes and evading immune detection. Understanding the complex interplay between Mtb and macrophage biology – particularly how Mtb manipulates host immune signalling pathways, lipid metabolism, and autophagy – is crucial for advancing therapeutic strategies. This knowledge is key for the development of host-directed therapies (HDTs), which aim to modulate the host's immune response to enhance macrophage antimycobacterial activity or prevent the pathological immune responses that contribute to tissue damage during infection. Through greater understanding of macrophage biology, the development of HDT's may be achieved by repurposing existing drugs – such as immunomodulators, metabolic regulators, or autophagy inducers – establishing novel interventions that may be used in combination with traditional antimicrobials to provide more effective, targeted treatment for Mtb.

Metabolism has emerged as a pivotal regulator of immune responses, particularly in macrophages, where metabolic reprogramming drives their functional response to infection. Understanding and targeting these metabolic pathways has profound therapeutic potential, particularly in the development of HDT's aimed at fine-tuning macrophage responses to enhance pathogen clearance, control inflammation, and overcome diseases like Mtb. Mtb has developed strategies to manipulate macrophage metabolism, enabling it to evade immune detection and persist within host cells. Upon infection, Mtb reprograms macrophage metabolic pathways to create a favourable environment for its survival. One key mechanism of inhibition is through targeting the normal shift toward glycolysis seen in pro-inflammatory macrophages, thereby dampening the production of key pro-inflammatory cytokines needed for bacterial clearance (43). Previous work from our lab has shown that this occurs due to the sustained upregulation of the anti-inflammatory microRNA, miR-21, by proliferating bacteria, which specifically targets the expression of the glycolytic gatekeeper PFK-m at

the rate-limiting step of glycolysis (41). Mtb also promotes a skewing of macrophage metabolism toward lipid accumulation and fatty acid oxidation, particularly by inducing the formation of lipid droplets, which it uses as a nutrient source (348). This metabolic shift not only supports the long-term survival of Mtb within the host but also suppresses key antimicrobial processes, such as autophagy and phagosome maturation. This work, in further examining how Mtb manipulates central carbon metabolism, has identified the bacterial mediated induction of the PPP and more specifically, we highlight how Mtb supports PPP derived NO production in an effort to suppress mitochondrial-based pro-inflammatory responses.

This work conclusively shows that by suppressing PFK-m, Mtb derived miR-21 reduces the flow of glucose through glycolysis, inducing a state of decelerated bioenergetics in macrophages. This alters metabolic flux towards induction of the PPP with our data providing compelling evidence of increased PPP activity during viable infection of both murine and human macrophages, including elevated expression of all PPP-associated enzymes and enhanced activity of the pathway's rate-limiting enzyme, G6PD. This Mtb-mediated redirection of macrophage metabolism towards the PPP occurs at the expense of the macrophage's pro-inflammatory response, specifically leading to a reduction in IL-1 β production, which is critical for effective immune defense against infection. Our observation of elevated pathway activity during Mtb infection is further reinforced by the increased accumulation of the PPP metabolite 6-phosphogluconate and the pathway's byproduct, NADPH. These findings underscore PPP activity as a key regulator of macrophage function, with its signalling demonstrating the capacity to influence metabolic flux.

Given our model of infection with viable Mtb infection showed increased PPP activity, this prompted us to explore how disrupting this pathway impacts macrophage metabolism and in turn bacterial survival. Through induction of pentose insufficiency using the small molecule inhibitor 6-AN and blocking activity of rate-limiting oxidative enzyme G6PD, we prevented oxidative PPP activity from contributing to ribose-5-phosphate production. By inducing a ribose-starved intracellular environment, we demonstrated that PPP activity sustains glycolysis, as 6-AN inhibition led to complete

glycolytic paralysis, redirecting normal carbon flux towards ribose generation via the non-oxidative branch of the PPP. This disruption to glycolysis was primarily demonstrated by reduced lactate secretion, a marker of classical macrophage activation, and downregulated key glycolytic genes such as *Pfk-m* and *Ldha*, while increasing non-oxidative PPP genes like *Taldo1* and *Tkt*, suggesting a shift toward ribose-5-phosphate synthesis at the expense of glycolysis. This was corroborated by extracellular flux analysis, showing reduced glycolytic activity and limited mitochondrial respiratory capacity following PPP inhibition. Additionally, G6PD inhibition led to a significant loss of NADPH and an increase in NADP, impairing macrophage bioenergetics and resulting in increased bacterial burden in Mtb-infected macrophages. Observations of heightened bacterial replication during PPP inhibition was supported by a significant reduction in IL-1 β in Mtb-infected macrophages. This effect was specific to viable infection, as cytokine levels were unchanged in inactivated Mtb-treated cells following 6-AN treatment. These findings highlight the critical balance between PPP activity and macrophage function for sustaining IL-1 β production during active Mtb infection where metabolic activity is the driving force behind the antimicrobial response, rather than metabolism acting as a secondary consequence that is typically associated with PRR activation.

Additionally, we demonstrate that Mtb specifically mobilises glycogen stores in macrophages to support increased PPP activity during infection. By using glycogen phosphorylase inhibitors and blocking glycogenolysis, we show a significant reduction in IL-1 β production during viable Mtb infection, while having no effect in the context of inactivated Mtb. This supports previous data which suggests that glycogen-derived glucose-6-phosphate is critical for sustaining PPP flux, which in turn supports pro-inflammatory cytokine production (215). Here we propose that during Mtb infection, increased PPP activity, driven by glycogenolysis and Mtb-derived miR-21 inhibition, results in a cyclic-PPP. In this cycle, carbon re-entering glycolysis from the non-oxidative branch of the PPP is impeded by the glycolytic bottleneck induced by Mtb-derived miR-21. Consequently, this carbon is diverted back into glucose-6-phosphate, where it may re-enter the PPP rather than proceeding through traditional glycolysis. While in this circumstance the PPP's role in supporting glycolysis is diminished, this increased PPP activity might benefit the pathogen by promoting the synthesis of essential metabolites

and other anabolic pathways. These findings reshape our understanding of immunometabolism by revealing how metabolic processes, particularly the PPP, are intricately linked to immune function and pathogen survival. Traditionally, immune responses have been viewed through the lens of pathogen recognition and cytokine signalling. However, this research highlights that metabolic shifts within immune cells, such as macrophages, are not merely consequences of infection but play an active role in shaping immune outcomes. The concept of a "cyclic-PPP," where Mtb-induced glycolytic bottlenecks force carbon back into the PPP, further deepens this understanding by showing how pathogens can manipulate host metabolism for their benefit. By redirecting metabolic flux to favour anabolic pathways, Mtb not only impairs the host's pro-inflammatory response but also supports its own survival and replication. This interplay between metabolic pathways and immune function underscores the importance of metabolism as a central regulator of immunity. It also shifts the focus of immunology towards the recognition that immune cell metabolism can be hijacked by pathogens to suppress effective immune responses. These insights open new avenues for therapeutic strategies that target metabolic pathways, such as the PPP, to enhance immune function or disrupt pathogen survival, positioning metabolism as a critical battleground in the immune response.

Given the heightened PPP activity and increased cellular NADPH levels observed during Mtb infection, we focused on how this metabolic shift might create a permissive environment for Mtb replication, particularly through NADPH-supported NO production. NADPH fuels NOS2 activity, driving NO generation, which, while typically associated as part of the host defense, we have now also demonstrated can modulate metabolic pathways to benefit Mtb survival. Moderate NO accumulation has previously been shown to inhibit key metabolic enzymes like PDH (53), leading to altered macrophage function, such as increased glutamine uptake and reduced pro-inflammatory cytokine production. Indeed, we support these findings by demonstrating NO accumulation can have a dramatic effect on the metabolic landscape of Mtb infected macrophages and in turn, significantly diminish IL-1 β production to promote bacterial replication. More specifically, our results revealed a significant increase in nitrite accumulation in both murine and human macrophages infected with viable Mtb,

particularly at 72 hours post-infection, compared to uninfected controls and macrophages infected with inactivated Mtb. This increase coincided with elevated *Nos2* expression, indicating enhanced NO production during active infection. The late-stage NO accumulation is notable because it occurs during a critical phase when Mtb reprograms macrophage metabolism to support its intracellular survival and replication, suggesting that Mtb may exploit the elevated NO levels to create a more permissive intracellular environment.

Through use of PPP inhibitor 6-AN, we demonstrate that PPP activity promotes both iNOS transcription and is a major contributor to macrophage NO production, establishing a metabolic feedback loop that sustains macrophage reprogramming during infection and suggesting NO shifts macrophage metabolism further toward a PPP-dependent state. In line with these claims, we demonstrate that NO supplementation promotes the expression of PPP-associated genes and increased G6PD activity, linking NO production to enhanced PPP flux during Mtb infection and supporting bacterial replication. Additionally, inhibiting glycogenolysis reduced NO production to levels similar to those seen with G6PD inhibition, suggesting that glycogen is a crucial alternative source of glucose-6-phosphate fuelling PPP activity during Mtb infection. Consistent with this, macrophage glycogen stores were depleted after 24 hours of infection and Seahorse analysis confirmed that glycogen-derived glucose-6-phosphate supports both glycolysis and the PPP. This data highlights a broader reconfiguration of carbon metabolism during Mtb infection, where glycogenolysis compensates for reduced glycolysis to sustain essential metabolic pathways. Moreover, further NO supplementation during Mtb infection reduced glucose uptake but increased glycogenolysis, as evidenced by decreased glycogen stores and increased *Pgm1* expression. This suggests that Mtb derived NO drives the preferential use of glycogen over glucose to fuel PPP activity. Overall, our results underscore the critical role of the PPP, supported by glycogenolysis, in driving NO production during Mtb infection, which, while intended to be protective, may paradoxically aid in bacterial persistence by creating a metabolic environment supporting Mtb survival.

While NO's immunosuppressive role is linked to its regulation of inflammasome activity, we have demonstrated that its impact on IL-1 β production also involves metabolic mechanisms. With previous work demonstrating NO mediated targeting of ETC complexes (267, 268), here we describe the effect of NO accumulation observed during Mtb infection on TCA cycle metabolism. Mtb infection results in a dramatic increase in *Irg1* expression, which drives itaconate production, and a reduction in the TCA enzyme *Fh1*. This disruption to mitochondrial based metabolism was confirmed through Seahorse analysis, showing reduced oxidative phosphorylation and respiratory capacity in Mtb-infected macrophages. Previous studies have shown that high NO supplementation disrupts the TCA cycle by targeting ACO2, leading to a "break" (53). Here, using a model of more moderate NO supplementation model, representative of that observed during Mtb infection, we further impaired ETC oxygen consumption rates and amplified the transcriptional disruption observed during infection, notably increasing *Irg1* and reducing *Fh1* expression. We confirm that NO influences *Irg1* expression, as scavenging NO with cPTIO significantly reduces *Irg1* levels, indicating that NO facilitates the metabolic reprogramming necessary for itaconate production during Mtb infection and aligning with reports of NO targeting PDH, causing lactate accumulation and compensatory glutaminolysis into the TCA cycle metabolism.

Our findings confirm previous reports of itaconate significantly suppressing pro-inflammatory cytokines, including IL-1 β and TNF α (146), in both human and murine macrophages during Mtb infection. It also enhances PPP activity by increasing the expression of key genes (*G6pd*, *Taldo1*, and *Tkt*) and elevating G6PD activity, which boosts NADPH production to support cellular redox balance. This metabolic shift results in decreased glycolysis-derived lactate accumulation and reduced glucose uptake, compensated by increased glycogen breakdown, highlighting the role of itaconate in reprogramming macrophage metabolism in favour of supporting Mtb replication. Interestingly, while itaconate upregulates *Nos2* expression, it paradoxically leads to a reduction in nitrite accumulation, suggesting a modulation of NO levels to prevents further metabolic manipulation associated with higher scale NO accumulation. This creates a favourable environment for Mtb survival by maintaining moderate NO levels that sustain itaconate accumulation, which in turn suppresses immune activation. The

immunosuppressive effect of itaconate is further evidenced by the increased bacterial burden observed in macrophages treated with itaconate, while *Irg1*-deficient macrophages show enhanced bacterial clearance. This indicates that *Mtb* leverages itaconate to dampen host immune responses and promote its persistence. The loss of itaconate in *Irg1*^{-/-} macrophages leads to increased NO and PPP activity but negates the immunosuppressive effects exerted by itaconate accumulation, enhancing bacterial killing. This work demonstrates the dual role of NO in the context of *Mtb* infection of macrophages, acting as both an antimicrobial agent and an immunomodulatory signal. As an antimicrobial, NO, produced by inducible nitric oxide synthase (iNOS), can directly kill *Mtb* and other pathogens through mechanisms such as the formation of reactive nitrogen species (RNS) and induction of oxidative stress. This potent antimicrobial activity is crucial for controlling infections, as elevated NO levels can hinder *Mtb* replication and promote pathogen clearance. However, our findings indicate that the role of NO extends beyond its direct antimicrobial effects; it also functions as an immunomodulatory signal that significantly influences macrophage behaviour. For instance, while moderate levels of NO are necessary for pro-inflammatory cytokine production and effective immune activation, excessive NO accumulation during *Mtb* infection has been shown to inhibit key metabolic enzymes and shift macrophage metabolism towards a pentose phosphate pathway (PPP)-dependent state, ultimately reducing IL-1 β production. This shift not only impairs macrophage activation but also promotes a permissive environment for *Mtb* replication. The interplay between NO and itaconate further complicates this dynamic, as itaconate, which is upregulated during *Mtb* infection, enhances PPP activity while simultaneously modulating NO levels to prevent further immune activation. Thus, understanding the balance between the antimicrobial and immunomodulatory roles of NO is crucial for elucidating its impact on *Mtb* infection

The potential role of the antioxidant response in mediating the biological effects of elevated NO levels, particularly in the context of itaconate during *Mtb* infection, presents an intriguing area of investigation. Our results demonstrated that *Mtb* infection leads to increased levels of itaconate, which modulates inflammation and immune responses while influencing redox homeostasis in macrophages. This accumulation of

itaconate correlates with elevated NO production, a process that, while typically associated with host defense, can also induce oxidative stress. In response to this stress, macrophages activate antioxidant pathways, upregulating various enzymes such as glutathione peroxidase and superoxide dismutase, which help mitigate the harmful effects of reactive nitrogen species. Notably, our findings indicated that elevated NO levels not only diminished IL-1 β production—crucial for effective immune responses—but also shifted metabolic flux towards the PPP, enhancing NADPH production. This increase in NADPH is vital for maintaining redox balance and supporting antioxidant defences. Therefore, itaconate may act as a metabolic regulator, ensuring that heightened NO levels contribute to beneficial immune responses while safeguarding cellular integrity through antioxidant mechanisms. In the context of Mtb infection, this interplay between itaconate, NO, and the antioxidant response could enable macrophages to sustain an environment conducive to their survival and function, while also shaping the metabolic landscape that favors Mtb persistence. Exploring these relationships further could unveil critical insights into how macrophages orchestrate their metabolic and inflammatory responses during Mtb infection, offering potential therapeutic strategies that target oxidative stress and inflammation to enhance host immunity against this pathogen.

We assessed whether these effects result from the inhibition of SDH, a known target of itaconate. Our findings suggest that metabolic reprogramming in Mtb-infected macrophages, which promotes IL-1 β production, may be linked to RET from succinate oxidase activity, rather than solely increased glycolysis or HIF-1 α activation, suggesting Mtb derived itaconate accumulation may be a bacterial means to suppress SDH contribution to IL-1 β generation during infection. Targeting SDH using dimethyl malonate (DMM) mimicked the metabolic effects of itaconate accumulation, including increased G6PD activity and reduced lactate and nitrite production in addition to increasing bacterial burden. Altogether, these results highlight the crucial role of SDH inhibition and PPP based metabolic crosstalk in promoting Mtb persistence during macrophage infection. Understanding this interaction offers potential therapeutic targets to enhance host immune responses against Mtb by modulating key metabolic pathways.

These findings regarding the manipulation of central carbon metabolism by Mtb and the subsequent restoration of mitochondrial respiration through inhibition of NOX2 opens new avenues for understanding macrophage immunometabolism and developing innovative therapeutic strategies. NOX2 inhibition and its effects on macrophage metabolism provide insights into potential therapeutic targets. By restoring mitochondrial respiration and enhancing OXPHOS, the inhibition of NOX2 presents a promising strategy for counteracting the metabolic adaptations that favour Mtb survival. This highlights the potential for pharmacological interventions that not only target the pathogen directly but also modulate the host's metabolic pathways to enhance immune responses. In this regard, developing small molecules that inhibit NOX2 may serve as a means to reprogram macrophage metabolism, thereby increasing their capacity to produce pro-inflammatory cytokines like IL-1 β and effectively combat Mtb replication. Additionally, these results contribute to the growing recognition of mitochondrial dysfunction as a hallmark of chronic infection. The restoration of mitochondrial function via NOX2 inhibition emphasizes the importance of mitochondrial health in the context of macrophage immunometabolism. As mitochondrial dysfunction can impair cellular bioenergetics and contribute to a skewed immune response, targeting metabolic pathways that promote mitochondrial respiration could be pivotal in designing effective therapies for chronic infections like tuberculosis. Moreover, the study enriches the dialogue surrounding immunometabolic plasticity in macrophages. It illustrates that macrophages possess the ability to dynamically adapt their metabolic pathways in response to environmental cues, particularly during pathogen infection. This adaptability is critical for maintaining a balance between host defense and pathogen persistence.

In conclusion, our findings show that NO drives a metabolic shift toward PPP activity, which suppresses mitochondrial respiration and dampens inflammation during Mtb infection. Additionally, we demonstrate that Mtb promotes glycogen-fuelled, PPP-centric metabolism by reducing glycolytic activity, creating an immunosuppressive environment that supports its intracellular survival. Finally, inhibiting NOX2 reverses this metabolic shift, presenting a potential therapeutic strategy to enhance macrophage

immunity by restoring mitochondrial-driven pro-inflammatory responses to Mtb infection.

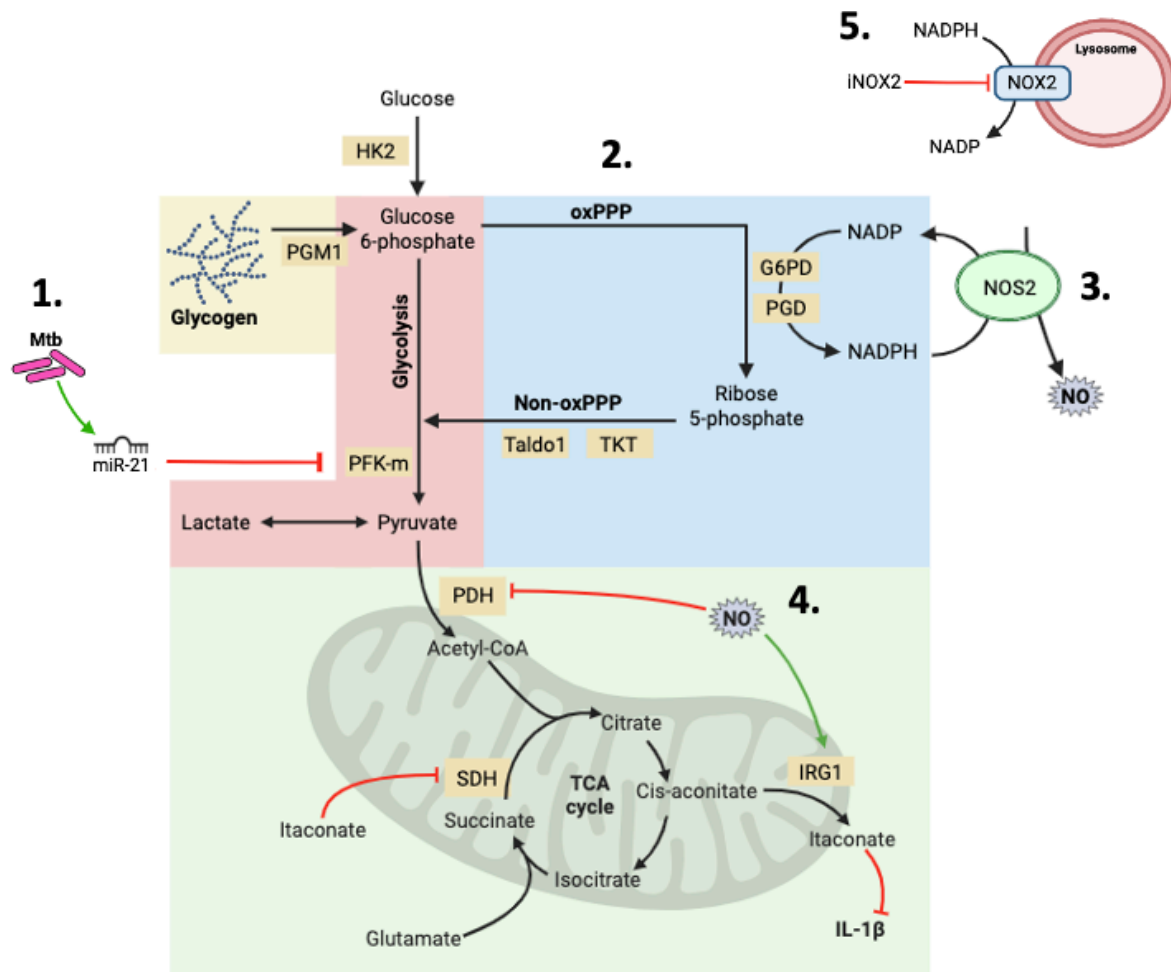


Figure 5. 1 Mtb orchestrates central carbon metabolism by manipulating PPP-derived NO production

(1) Mtb infection of macrophages leads to the upregulation of anti-inflammatory miR-21, which reduces glycolytic activity by targeting the key metabolic regulator, PFK-m. (2) The resulting decrease in glycolysis triggers a compensatory upregulation of PPP activity, enhancing NADPH production, with augmented PPP flux further supported by glycogenolysis. (3) Elevated PPP activity increases NO accumulation via NADPH-dependent NOS2 activity. (4) Moderate NO accumulation inhibits PDH activity, driving increased glutamine uptake and its incorporation into itaconate, thereby reducing IL-1 β production. (5) Therapeutically targeting NOX2 activity decreases NADPH oxidation, reducing PPP-derived NO production, restoring mitochondrial metabolism and promoting Mtb clearance.

Chapter 6. Bibliography

1. Petersen E, Blumberg L, Wilson ME, Zumla A. Ending the Global Tuberculosis Epidemic by 2030 - The Moscow Declaration and achieving a Major Translational Change in Delivery of TB Healthcare. *Int J Infect Dis.* 2017;65:156-8.
2. Ryndak MB, Laal S. Mycobacterium tuberculosis Primary Infection and Dissemination: A Critical Role for Alveolar Epithelial Cells. *Front Cell Infect Microbiol.* 2019;9:299.
3. Lange C, Chesov D, Heyckendorf J, Leung CC, Udwadia Z, Dheda K. Drug-resistant tuberculosis: An update on disease burden, diagnosis and treatment. *Respirology.* 2018;23(7):656-73.
4. Singh R, Dwivedi SP, Gaharwar US, Meena R, Rajamani P, Prasad T. Recent updates on drug resistance in Mycobacterium tuberculosis. *J Appl Microbiol.* 2020;128(6):1547-67.
5. Harding E. WHO global progress report on tuberculosis elimination. *Lancet Respir Med.* 2020;8(1):19.
6. Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, et al. Tuberculosis. *Nat Rev Dis Primers.* 2016;2:16076.
7. Esmail H, Lai RP, Lesosky M, Wilkinson KA, Graham CM, Coussens AK, et al. Characterization of progressive HIV-associated tuberculosis using 2-deoxy-2-[(18)F]fluoro-D-glucose positron emission and computed tomography. *Nat Med.* 2016;22(10):1090-3.
8. Capuano SV, 3rd, Croix DA, Pawar S, Zinovik A, Myers A, Lin PL, et al. Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection. *Infect Immun.* 2003;71(10):5831-44.
9. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect Immun.* 2009;77(10):4631-42.
10. DiNardo AR, Nishiguchi T, Grimm SL, Schlesinger LS, Graviss EA, Cirillo JD, et al. Tuberculosis endotypes to guide stratified host-directed therapy. *Med.* 2021;2(3):217-32.
11. Harris J, Hope JC, Keane J. Tumor necrosis factor blockers influence macrophage responses to Mycobacterium tuberculosis. *J Infect Dis.* 2008;198(12):1842-50.
12. Gideon HP, Skinner JA, Baldwin N, Flynn JL, Lin PL. Early Whole Blood Transcriptional Signatures Are Associated with Severity of Lung Inflammation in Cynomolgus Macaques with Mycobacterium tuberculosis Infection. *J Immunol.* 2016;197(12):4817-28.

13. Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*. 2010;466(7309):973-7.
14. Kumar NP, Moideen K, George PJ, Dolla C, Kumaran P, Babu S. Coincident diabetes mellitus modulates Th1-, Th2-, and Th17-cell responses in latent tuberculosis in an IL-10- and TGF-beta-dependent manner. *Eur J Immunol*. 2016;46(2):390-9.
15. Podell BK, Ackart DF, Obregon-Henao A, Eck SP, Henao-Tamayo M, Richardson M, et al. Increased severity of tuberculosis in Guinea pigs with type 2 diabetes: a model of diabetes-tuberculosis comorbidity. *Am J Pathol*. 2014;184(4):1104-18.
16. Kumar NP, Sridhar R, Banurekha VV, Jawahar MS, Nutman TB, Babu S. Expansion of pathogen-specific T-helper 1 and T-helper 17 cells in pulmonary tuberculosis with coincident type 2 diabetes mellitus. *J Infect Dis*. 2013;208(5):739-48.
17. Azevedo-Pereira JM, Pires D, Calado M, Mandal M, Santos-Costa Q, Anes E. HIV/Mtb Co-Infection: From the Amplification of Disease Pathogenesis to an "Emerging Syndemic". *Microorganisms*. 2023;11(4).
18. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J Exp Med*. 1968;128(3):415-35.
19. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. 2013;496(7446):445-55.
20. Sica A, Erreni M, Allavena P, Porta C. Macrophage polarization in pathology. *Cell Mol Life Sci*. 2015;72(21):4111-26.
21. Labonte AC, Tosello-Tramont AC, Hahn YS. The role of macrophage polarization in infectious and inflammatory diseases. *Mol Cells*. 2014;37(4):275-85.
22. Mege JL, Mehraj V, Capo C. Macrophage polarization and bacterial infections. *Curr Opin Infect Dis*. 2011;24(3):230-4.
23. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13:453-61.
24. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-69.
25. Murray PJ. Macrophage Polarization. *Annu Rev Physiol*. 2017;79:541-66.
26. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*. 2014;6:13.

27. Porta C, Riboldi E, Ippolito A, Sica A. Molecular and epigenetic basis of macrophage polarized activation. *Semin Immunol*. 2015;27(4):237-48.
28. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. *Immunity*. 2010;32(5):593-604.
29. Guillemins M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, et al. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med*. 2013;210(10):1977-92.
30. Goerke J. Pulmonary surfactant: functions and molecular composition. *Biochim Biophys Acta*. 1998;1408(2-3):79-89.
31. Nguyen HA, Rajaram MV, Meyer DA, Schlesinger LS. Pulmonary surfactant protein A and surfactant lipids upregulate IRAK-M, a negative regulator of TLR-mediated inflammation in human macrophages. *Am J Physiol Lung Cell Mol Physiol*. 2012;303(7):L608-16.
32. Williams MC. Alveolar type I cells: molecular phenotype and development. *Annu Rev Physiol*. 2003;65:669-95.
33. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;38(4):792-804.
34. Lawless SJ, Kedia-Mehta N, Walls JF, McGarrigle R, Convery O, Sinclair LV, et al. Glucose represses dendritic cell-induced T cell responses. *Nat Commun*. 2017;8:15620.
35. Baker EH, Clark N, Brennan AL, Fisher DA, Gyi KM, Hodson ME, et al. Hyperglycemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis. *J Appl Physiol (1985)*. 2007;102(5):1969-75.
36. Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*. 2015;42(3):419-30.
37. Huang SC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol*. 2014;15(9):846-55.
38. Dodd CE, Pyle CJ, Glowinski R, Rajaram MV, Schlesinger LS. CD36-Mediated Uptake of Surfactant Lipids by Human Macrophages Promotes Intracellular Growth of *Mycobacterium tuberculosis*. *J Immunol*. 2016;197(12):4727-35.
39. Huang L, Nazarova EV, Tan S, Liu Y, Russell DG. Growth of *Mycobacterium tuberculosis* in vivo segregates with host macrophage metabolism and ontogeny. *J Exp Med*. 2018;215(4):1135-52.

40. Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler JK, et al. Alveolar Macrophages Provide an Early Mycobacterium tuberculosis Niche and Initiate Dissemination. *Cell Host Microbe*. 2018;24(3):439-46 e4.
41. Hackett EE, Charles-Messance H, O'Leary SM, Gleeson LE, Munoz-Wolf N, Case S, et al. Mycobacterium tuberculosis Limits Host Glycolysis and IL-1beta by Restriction of PFK-M via MicroRNA-21. *Cell Rep*. 2020;30(1):124-36 e4.
42. Svedberg FR, Brown SL, Krauss MZ, Campbell L, Sharpe C, Clausen M, et al. The lung environment controls alveolar macrophage metabolism and responsiveness in type 2 inflammation. *Nat Immunol*. 2019;20(5):571-80.
43. Cumming BM, Addicott KW, Adamson JH, Steyn AJ. Mycobacterium tuberculosis induces decelerated bioenergetic metabolism in human macrophages. *Elife*. 2018;7.
44. Lachmandas E, Beigier-Bompadre M, Cheng SC, Kumar V, van Laarhoven A, Wang X, et al. Rewiring cellular metabolism via the AKT/mTOR pathway contributes to host defence against Mycobacterium tuberculosis in human and murine cells. *Eur J Immunol*. 2016;46(11):2574-86.
45. Gleeson LE, Sheedy FJ, Palsson-McDermott EM, Triglia D, O'Leary SM, O'Sullivan MP, et al. Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. *J Immunol*. 2016;196(6):2444-9.
46. Braverman J, Sogi KM, Benjamin D, Nomura DK, Stanley SA. HIF-1alpha Is an Essential Mediator of IFN-gamma-Dependent Immunity to Mycobacterium tuberculosis. *J Immunol*. 2016;197(4):1287-97.
47. Shi L, Salamon H, Eugenin EA, Pine R, Cooper A, Gennaro ML. Infection with Mycobacterium tuberculosis induces the Warburg effect in mouse lungs. *Sci Rep*. 2015;5:18176.
48. Palsson-McDermott EM, Curtis AM, Goel G, Lauterbach MAR, Sheedy FJ, Gleeson LE, et al. Pyruvate Kinase M2 Regulates Hif-1alpha Activity and IL-1beta Induction and Is a Critical Determinant of the Warburg Effect in LPS-Activated Macrophages. *Cell Metab*. 2015;21(2):347.
49. Braverman J, Stanley SA. Nitric Oxide Modulates Macrophage Responses to Mycobacterium tuberculosis Infection through Activation of HIF-1alpha and Repression of NF-kappaB. *J Immunol*. 2017;199(5):1805-16.
50. Mayer-Barber KD, Andrade BB, Oland SD, Amaral EP, Barber DL, Gonzales J, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature*. 2014;511(7507):99-103.

51. Haschemi A, Kosma P, Gille L, Evans CR, Burant CF, Starkl P, et al. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab.* 2012;15(6):813-26.
52. Mishra BB, Lovewell RR, Olive AJ, Zhang G, Wang W, Eugenin E, et al. Nitric oxide prevents a pathogen-permissive granulocytic inflammation during tuberculosis. *Nat Microbiol.* 2017;2:17072.
53. Palmieri EM, Gonzalez-Cotto M, Baseler WA, Davies LC, Ghesquiere B, Maio N, et al. Nitric oxide orchestrates metabolic rewiring in M1 macrophages by targeting aconitase 2 and pyruvate dehydrogenase. *Nat Commun.* 2020;11(1):698.
54. Mishra BB, Rathinam VA, Martens GW, Martinot AJ, Kornfeld H, Fitzgerald KA, et al. Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1 β . *Nat Immunol.* 2013;14(1):52-60.
55. Krutzik SR, Modlin RL. The role of Toll-like receptors in combating mycobacteria. *Semin Immunol.* 2004;16(1):35-41.
56. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood.* 2003;102(7):2660-9.
57. Dasari P, Nicholson IC, Hodge G, Dandie GW, Zola H. Expression of toll-like receptors on B lymphocytes. *Cell Immunol.* 2005;236(1-2):140-5.
58. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med.* 2001;194(6):863-9.
59. Basu S, Fenton MJ. Toll-like receptors: function and roles in lung disease. *Am J Physiol Lung Cell Mol Physiol.* 2004;286(5):L887-92.
60. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell.* 2006;124(4):783-801.
61. Stamm CE, Collins AC, Shiloh MU. Sensing of *Mycobacterium tuberculosis* and consequences to both host and bacillus. *Immunol Rev.* 2015;264(1):204-19.
62. Underhill DM, Ozinsky A, Smith KD, Aderem A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc Natl Acad Sci U S A.* 1999;96(25):14459-63.
63. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A.* 2000;97(25):13766-71.

64. Henning LN, Azad AK, Parsa KV, Crowther JE, Tridandapani S, Schlesinger LS. Pulmonary surfactant protein A regulates TLR expression and activity in human macrophages. *J Immunol.* 2008;180(12):7847-58.
65. Schlesinger LS, Hull SR, Kaufman TM. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. *J Immunol.* 1994;152(8):4070-9.
66. Torrelles JB, Azad AK, Schlesinger LS. Fine discrimination in the recognition of individual species of phosphatidyl-myo-inositol mannosides from *Mycobacterium tuberculosis* by C-type lectin pattern recognition receptors. *J Immunol.* 2006;177(3):1805-16.
67. Wileman TE, Lennartz MR, Stahl PD. Identification of the macrophage mannose receptor as a 175-kDa membrane protein. *Proc Natl Acad Sci U S A.* 1986;83(8):2501-5.
68. McGreal EP, Miller JL, Gordon S. Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr Opin Immunol.* 2005;17(1):18-24.
69. Stahl PD, Ezekowitz RA. The mannose receptor is a pattern recognition receptor involved in host defense. *Curr Opin Immunol.* 1998;10(1):50-5.
70. Chieppa M, Bianchi G, Doni A, Del Prete A, Sironi M, Laskarin G, et al. Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J Immunol.* 2003;171(9):4552-60.
71. Nigou J, Zelle-Rieser C, Gilleron M, Thurnher M, Puzo G. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol.* 2001;166(12):7477-85.
72. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol.* 1999;17:593-623.
73. Kang PB, Azad AK, Torrelles JB, Kaufman TM, Beharka A, Tibesar E, et al. The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. *J Exp Med.* 2005;202(7):987-99.
74. Singh CR, Moulton RA, Armitage LY, Bidani A, Snuggs M, Dhandayuthapani S, et al. Processing and presentation of a mycobacterial antigen 85B epitope by murine macrophages is dependent on the phagosomal acquisition of vacuolar proton ATPase and in situ activation of cathepsin D. *J Immunol.* 2006;177(5):3250-9.
75. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science.* 1994;263(5147):678-81.

76. Rajaram MV, Brooks MN, Morris JD, Torrelles JB, Azad AK, Schlesinger LS. Mycobacterium tuberculosis activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. *J Immunol*. 2010;185(2):929-42.
77. Yang Y, Yin C, Pandey A, Abbott D, Sassetti C, Kelliher MA. NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. *J Biol Chem*. 2007;282(50):36223-9.
78. Pandey AK, Yang Y, Jiang Z, Fortune SM, Coulombe F, Behr MA, et al. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis. *PLoS Pathog*. 2009;5(7):e1000500.
79. Juarez E, Carranza C, Hernandez-Sanchez F, Leon-Contreras JC, Hernandez-Pando R, Escobedo D, et al. NOD2 enhances the innate response of alveolar macrophages to Mycobacterium tuberculosis in humans. *Eur J Immunol*. 2012;42(4):880-9.
80. Chen CM, Gong Y, Zhang M, Chen JJ. Reciprocal cross-talk between Nod2 and TAK1 signaling pathways. *J Biol Chem*. 2004;279(24):25876-82.
81. Hsu LC, Ali SR, McGillivray S, Tseng PH, Mariathasan S, Humke EW, et al. A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide. *Proc Natl Acad Sci U S A*. 2008;105(22):7803-8.
82. Brooks MN, Rajaram MV, Azad AK, Amer AO, Valdivia-Arenas MA, Park JH, et al. NOD2 controls the nature of the inflammatory response and subsequent fate of Mycobacterium tuberculosis and M. bovis BCG in human macrophages. *Cell Microbiol*. 2011;13(3):402-18.
83. Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood*. 1990;75(5):1037-50.
84. Villeneuve C, Gilleron M, Maridonneau-Parini I, Daffe M, Astarie-Dequeker C, Etienne G. Mycobacteria use their surface-exposed glycolipids to infect human macrophages through a receptor-dependent process. *J Lipid Res*. 2005;46(3):475-83.
85. Armstrong JA, Hart PD. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual nonfusion pattern and observations on bacterial survival. *J Exp Med*. 1975;142(1):1-16.
86. Burdette DL, Monroe KM, Sotelo-Troha K, Iwig JS, Eckert B, Hyodo M, et al. STING is a direct innate immune sensor of cyclic di-GMP. *Nature*. 2011;478(7370):515-8.
87. Diner EJ, Burdette DL, Wilson SC, Monroe KM, Kellenberger CA, Hyodo M, et al. The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. *Cell Rep*. 2013;3(5):1355-61.

88. Stanley SA, Johndrow JE, Manzanillo P, Cox JS. The Type I IFN response to infection with *Mycobacterium tuberculosis* requires ESX-1-mediated secretion and contributes to pathogenesis. *J Immunol*. 2007;178(5):3143-52.
89. Manca C, Tsenova L, Freeman S, Barczak AK, Tovey M, Murray PJ, et al. Hypervirulent *M. tuberculosis* W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. *J Interferon Cytokine Res*. 2005;25(11):694-701.
90. Ji DX, Yamashiro LH, Chen KJ, Mukaida N, Kramnik I, Darwin KH, et al. Type I interferon-driven susceptibility to *Mycobacterium tuberculosis* is mediated by IL-1Ra. *Nat Microbiol*. 2019;4(12):2128-35.
91. Dey B, Dey RJ, Cheung LS, Pokkali S, Guo H, Lee JH, et al. A bacterial cyclic dinucleotide activates the cytosolic surveillance pathway and mediates innate resistance to tuberculosis. *Nat Med*. 2015;21(4):401-6.
92. Olson GS, Murray TA, Jahn AN, Mai D, Diercks AH, Gold ES, et al. Type I interferon decreases macrophage energy metabolism during mycobacterial infection. *Cell Rep*. 2021;35(9):109195.
93. Dorhoi A, Kaufmann SH. Tumor necrosis factor alpha in mycobacterial infection. *Semin Immunol*. 2014;26(3):203-9.
94. Valone SE, Rich EA, Wallis RS, Ellner JJ. Expression of tumor necrosis factor in vitro by human mononuclear phagocytes stimulated with whole *Mycobacterium bovis* BCG and mycobacterial antigens. *Infect Immun*. 1988;56(12):3313-5.
95. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity*. 1995;2(6):561-72.
96. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, et al. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J Immunol*. 1999;162(6):3504-11.
97. Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K, et al. Role of tumor necrosis factor-alpha in *Mycobacterium*-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. *Lab Invest*. 1999;79(4):379-86.
98. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwiertman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med*. 2001;345(15):1098-104.

99. Xie X, Li F, Chen JW, Wang J. Risk of tuberculosis infection in anti-TNF-alpha biological therapy: from bench to bedside. *J Microbiol Immunol Infect.* 2014;47(4):268-74.
100. Lin PL, Myers A, Smith L, Bigbee C, Bigbee M, Fuhrman C, et al. Tumor necrosis factor neutralization results in disseminated disease in acute and latent *Mycobacterium tuberculosis* infection with normal granuloma structure in a cynomolgus macaque model. *Arthritis Rheum.* 2010;62(2):340-50.
101. Tobin DM, Vary JC, Jr., Ray JP, Walsh GS, Dunstan SJ, Bang ND, et al. The *Ita4h* locus modulates susceptibility to mycobacterial infection in zebrafish and humans. *Cell.* 2010;140(5):717-30.
102. Clay H, Volkman HE, Ramakrishnan L. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity.* 2008;29(2):283-94.
103. Roca FJ, Whitworth LJ, Redmond S, Jones AA, Ramakrishnan L. TNF Induces Pathogenic Programmed Macrophage Necrosis in Tuberculosis through a Mitochondrial-Lysosomal-Endoplasmic Reticulum Circuit. *Cell.* 2019;178(6):1344-61 e11.
104. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. *Cell.* 2013;153(3):521-34.
105. Mayer-Barber KD, Andrade BB, Barber DL, Hieny S, Feng CG, Caspar P, et al. Innate and adaptive interferons suppress IL-1alpha and IL-1beta production by distinct pulmonary myeloid subsets during *Mycobacterium tuberculosis* infection. *Immunity.* 2011;35(6):1023-34.
106. Juffermans NP, Florquin S, Camoglio L, Verbon A, Kolk AH, Speelman P, et al. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. *J Infect Dis.* 2000;182(3):902-8.
107. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, et al. Caspase-1 independent IL-1beta production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. *J Immunol.* 2010;184(7):3326-30.
108. Yamada H, Mizumo S, Horai R, Iwakura Y, Sugawara I. Protective role of interleukin-1 in mycobacterial infection in IL-1 alpha/beta double-knockout mice. *Lab Invest.* 2000;80(5):759-67.
109. Sugawara I, Yamada H, Hua S, Mizuno S. Role of interleukin (IL)-1 type 1 receptor in mycobacterial infection. *Microbiol Immunol.* 2001;45(11):743-50.

110. Bourigault ML, Segueni N, Rose S, Court N, Vacher R, Vasseur V, et al. Relative contribution of IL-1alpha, IL-1beta and TNF to the host response to *Mycobacterium tuberculosis* and attenuated *M. bovis* BCG. *Immun Inflamm Dis*. 2013;1(1):47-62.
111. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, Moita LF, et al. *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell Microbiol*. 2010;12(8):1046-63.
112. Carlsson F, Kim J, Dumitru C, Barck KH, Carano RA, Sun M, et al. Host-detrimental role of Esx-1-mediated inflammasome activation in mycobacterial infection. *PLoS Pathog*. 2010;6(5):e1000895.
113. Wong KW, Jacobs WR, Jr. Critical role for NLRP3 in necrotic death triggered by *Mycobacterium tuberculosis*. *Cell Microbiol*. 2011;13(9):1371-84.
114. Dorhoi A, Nouailles G, Jorg S, Hagens K, Heinemann E, Pradl L, et al. Activation of the NLRP3 inflammasome by *Mycobacterium tuberculosis* is uncoupled from susceptibility to active tuberculosis. *Eur J Immunol*. 2012;42(2):374-84.
115. Abdalla H, Srinivasan L, Shah S, Mayer-Barber KD, Sher A, Sutterwala FS, et al. *Mycobacterium tuberculosis* infection of dendritic cells leads to partially caspase-1/11-independent IL-1beta and IL-18 secretion but not to pyroptosis. *PLoS One*. 2012;7(7):e40722.
116. McElvania Tekippe E, Allen IC, Hulseberg PD, Sullivan JT, McCann JR, Sandor M, et al. Granuloma formation and host defense in chronic *Mycobacterium tuberculosis* infection requires PYCARD/ASC but not NLRP3 or caspase-1. *PLoS One*. 2010;5(8):e12320.
117. Walter K, Holscher C, Tschopp J, Ehlers S. NALP3 is not necessary for early protection against experimental tuberculosis. *Immunobiology*. 2010;215(9-10):804-11.
118. Fremont CM, Togbe D, Doz E, Rose S, Vasseur V, Maillet I, et al. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobacterium tuberculosis* infection. *J Immunol*. 2007;179(2):1178-89.
119. Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, et al. NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J Immunol*. 1994;153(2):712-23.
120. Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur J Immunol*. 2003;33(3):597-605.
121. Fang HY, Hughes R, Murdoch C, Coffelt SB, Biswas SK, Harris AL, et al. Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood*. 2009;114(4):844-59.

122. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature*. 2013;496(7444):238-42.
123. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol*. 2016;13(2):148-59.
124. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol*. 2013;13(6):397-411.
125. Saiga H, Kitada S, Shimada Y, Kamiyama N, Okuyama M, Makino M, et al. Critical role of AIM2 in *Mycobacterium tuberculosis* infection. *Int Immunol*. 2012;24(10):637-44.
126. Jayaraman P, Sada-Ovalle I, Nishimura T, Anderson AC, Kuchroo VK, Remold HG, et al. IL-1 β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J Immunol*. 2013;190(8):4196-204.
127. Chen M, Divangahi M, Gan H, Shin DS, Hong S, Lee DM, et al. Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. *J Exp Med*. 2008;205(12):2791-801.
128. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010;10(3):170-81.
129. Williams L, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol*. 2004;172(1):567-76.
130. Koppelman B, Neefjes JJ, de Vries JE, de Waal Malefyt R. Interleukin-10 down-regulates MHC class II alphabeta peptide complexes at the plasma membrane of monocytes by affecting arrival and recycling. *Immunity*. 1997;7(6):861-71.
131. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med*. 1991;174(5):1209-20.
132. Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. *J Exp Med*. 1991;174(6):1549-55.
133. Higgins DM, Sanchez-Campillo J, Rosas-Taraco AG, Lee EJ, Orme IM, Gonzalez-Juarrero M. Lack of IL-10 alters inflammatory and immune responses during pulmonary *Mycobacterium tuberculosis* infection. *Tuberculosis (Edinb)*. 2009;89(2):149-57.
134. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, et al. Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10-deficient mice is

accompanied by early and enhanced Th1 responses in the lung. *Eur J Immunol*. 2010;40(8):2200-10.

135. Cyktor JC, Carruthers B, Kominsky RA, Beamer GL, Stromberg P, Turner J. IL-10 inhibits mature fibrotic granuloma formation during *Mycobacterium tuberculosis* infection. *J Immunol*. 2013;190(6):2778-90.

136. Beamer GL, Flaherty DK, Assogba BD, Stromberg P, Gonzalez-Juarrero M, de Waal Malefyt R, et al. Interleukin-10 promotes *Mycobacterium tuberculosis* disease progression in CBA/J mice. *J Immunol*. 2008;181(8):5545-50.

137. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A*. 1997;94(10):5243-8.

138. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol*. 1997;15:323-50.

139. Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR, Nathan C, Xie QW, Mumford R, et al. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med*. 1996;183(5):2293-302.

140. Nathan C. Inducible nitric oxide synthase in the tuberculous human lung. *Am J Respir Crit Care Med*. 2002;166(2):130-1.

141. Idh J, Westman A, Elias D, Moges F, Getachew A, Gelaw A, et al. Nitric oxide production in the exhaled air of patients with pulmonary tuberculosis in relation to HIV co-infection. *BMC Infect Dis*. 2008;8:146.

142. Bustamante J, Arias AA, Vogt G, Picard C, Galicia LB, Prando C, et al. Germline CYBB mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. *Nat Immunol*. 2011;12(3):213-21.

143. Cooper AM, Segal BH, Frank AA, Holland SM, Orme IM. Transient loss of resistance to pulmonary tuberculosis in p47(phox^{-/-}) mice. *Infect Immun*. 2000;68(3):1231-4.

144. Olive AJ, Smith CM, Kiritsy MC, Sasseti CM. The Phagocyte Oxidase Controls Tolerance to *Mycobacterium tuberculosis* Infection. *J Immunol*. 2018;201(6):1705-16.

145. Coleman MM, Ruane D, Moran B, Dunne PJ, Keane J, Mills KH. Alveolar macrophages contribute to respiratory tolerance by inducing FoxP3 expression in naive T cells. *Am J Respir Cell Mol Biol*. 2013;48(6):773-80.

146. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell*. 2016;167(2):457-70 e13.

147. Mills EL, Kelly B, O'Neill LAJ. Mitochondria are the powerhouses of immunity. *Nat Immunol.* 2017;18(5):488-98.
148. Rongvaux A, Andris F, Van Gool F, Leo O. Reconstructing eukaryotic NAD metabolism. *Bioessays.* 2003;25(7):683-90.
149. Skokowa J, Lan D, Thakur BK, Wang F, Gupta K, Cario G, et al. NAMPT is essential for the G-CSF-induced myeloid differentiation via a NAD(+)-sirtuin-1-dependent pathway. *Nat Med.* 2009;15(2):151-8.
150. Franklin CC, Backos DS, Mohar I, White CC, Forman HJ, Kavanagh TJ. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Mol Aspects Med.* 2009;30(1-2):86-98.
151. Lu SC. Regulation of glutathione synthesis. *Curr Top Cell Regul.* 2000;36:95-116.
152. Lewerenz J, Hewett SJ, Huang Y, Lambros M, Gout PW, Kalivas PW, et al. The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities. *Antioxid Redox Signal.* 2013;18(5):522-55.
153. Kang DD, Lin Y, Moreno JR, Randall TD, Khader SA. Profiling early lung immune responses in the mouse model of tuberculosis. *PLoS One.* 2011;6(1):e16161.
154. Cai Y, Yang Q, Liao M, Wang H, Zhang C, Nambi S, et al. xCT increases tuberculosis susceptibility by regulating antimicrobial function and inflammation. *Oncotarget.* 2016;7(21):31001-13.
155. Deretic V, Singh S, Master S, Harris J, Roberts E, Kyei G, et al. Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism. *Cell Microbiol.* 2006;8(5):719-27.
156. Fratti RA, Chua J, Vergne I, Deretic V. Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc Natl Acad Sci U S A.* 2003;100(9):5437-42.
157. Chua J, Vergne I, Master S, Deretic V. A tale of two lipids: Mycobacterium tuberculosis phagosome maturation arrest. *Curr Opin Microbiol.* 2004;7(1):71-7.
158. Vergne I, Chua J, Deretic V. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca²⁺/calmodulin-PI3K hVPS34 cascade. *J Exp Med.* 2003;198(4):653-9.
159. Malik ZA, Thompson CR, Hashimi S, Porter B, Iyer SS, Kusner DJ. Cutting edge: Mycobacterium tuberculosis blocks Ca²⁺ signaling and phagosome maturation in human macrophages via specific inhibition of sphingosine kinase. *J Immunol.* 2003;170(6):2811-5.

160. Fratti RA, Backer JM, Gruenberg J, Corvera S, Deretic V. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J Cell Biol.* 2001;154(3):631-44.
161. Kusner DJ. Mechanisms of mycobacterial persistence in tuberculosis. *Clin Immunol.* 2005;114(3):239-47.
162. Connolly SF, Kusner DJ. The regulation of dendritic cell function by calcium-signaling and its inhibition by microbial pathogens. *Immunol Res.* 2007;39(1-3):115-27.
163. Deretic V, Vergne I, Chua J, Master S, Singh SB, Fazio JA, et al. Endosomal membrane traffic: convergence point targeted by *Mycobacterium tuberculosis* and HIV. *Cell Microbiol.* 2004;6(11):999-1009.
164. Clemens DL, Lee BY, Horwitz MA. Deviant expression of Rab5 on phagosomes containing the intracellular pathogens *Mycobacterium tuberculosis* and *Legionella pneumophila* is associated with altered phagosomal fate. *Infect Immun.* 2000;68(5):2671-84.
165. Via LE, Deretic D, Ulmer RJ, Hibler NS, Huber LA, Deretic V. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J Biol Chem.* 1997;272(20):13326-31.
166. Hinchey J, Lee S, Jeon BY, Basaraba RJ, Venkataswamy MM, Chen B, et al. Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. *J Clin Invest.* 2007;117(8):2279-88.
167. Hou JM, D'Lima NG, Rigel NW, Gibbons HS, McCann JR, Braunstein M, et al. ATPase activity of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins and its importance for SecA2 function in macrophages. *J Bacteriol.* 2008;190(14):4880-7.
168. Tan T, Lee WL, Alexander DC, Grinstein S, Liu J. The ESAT-6/CFP-10 secretion system of *Mycobacterium marinum* modulates phagosome maturation. *Cell Microbiol.* 2006;8(9):1417-29.
169. Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, et al. Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science.* 2004;304(5678):1800-4.
170. Kan-Sutton C, Jagannath C, Hunter RL, Jr. Trehalose 6,6'-dimycolate on the surface of *Mycobacterium tuberculosis* modulates surface marker expression for antigen presentation and costimulation in murine macrophages. *Microbes Infect.* 2009;11(1):40-8.
171. van der Wel N, Hava D, Houben D, Fluitsma D, van Zon M, Pierson J, et al. *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. *Cell.* 2007;129(7):1287-98.

172. Houben D, Demangel C, van Ingen J, Perez J, Baldeon L, Abdallah AM, et al. ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cell Microbiol.* 2012;14(8):1287-98.
173. Manzanillo PS, Shiloh MU, Portnoy DA, Cox JS. Mycobacterium tuberculosis activates the DNA-dependent cytosolic surveillance pathway within macrophages. *Cell Host Microbe.* 2012;11(5):469-80.
174. Watson RO, Manzanillo PS, Cox JS. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell.* 2012;150(4):803-15.
175. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998;392(6673):245-52.
176. Khader SA, Partida-Sanchez S, Bell G, Jelley-Gibbs DM, Swain S, Pearl JE, et al. Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J Exp Med.* 2006;203(7):1805-15.
177. Marino S, Pawar S, Fuller CL, Reinhart TA, Flynn JL, Kirschner DE. Dendritic cell trafficking and antigen presentation in the human immune response to Mycobacterium tuberculosis. *J Immunol.* 2004;173(1):494-506.
178. Humphreys IR, Stewart GR, Turner DJ, Patel J, Karamanou D, Snelgrove RJ, et al. A role for dendritic cells in the dissemination of mycobacterial infection. *Microbes Infect.* 2006;8(5):1339-46.
179. Tascon RE, Soares CS, Ragno S, Stavropoulos E, Hirst EM, Colston MJ. Mycobacterium tuberculosis-activated dendritic cells induce protective immunity in mice. *Immunology.* 2000;99(3):473-80.
180. Tian T, Woodworth J, Skold M, Behar SM. In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. *J Immunol.* 2005;175(5):3268-72.
181. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. *Infect Immun.* 2002;70(8):4501-9.
182. Poulsen A. Some clinical features of tuberculosis. 1. Incubation period. *Acta Tuberc Scand.* 1950;24(3-4):311-46.
183. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol.* 2011;4(3):288-93.

184. Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, Takatsu K, et al. Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. *J Exp Med*. 2008;205(1):105-15.
185. Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat Immunol*. 2009;10(9):943-8.
186. O'Garra A, Redford PS, McNab FW, Bloom CI, Wilkinson RJ, Berry MP. The immune response in tuberculosis. *Annu Rev Immunol*. 2013;31:475-527.
187. North RJ, Jung YJ. Immunity to tuberculosis. *Annu Rev Immunol*. 2004;22:599-623.
188. Bold TD, Ernst JD. CD4⁺ T cell-dependent IFN-gamma production by CD8⁺ effector T cells in *Mycobacterium tuberculosis* infection. *J Immunol*. 2012;189(5):2530-6.
189. Srivastava S, Ernst JD. Cutting edge: Direct recognition of infected cells by CD4 T cells is required for control of intracellular *Mycobacterium tuberculosis* in vivo. *J Immunol*. 2013;191(3):1016-20.
190. Saunders BM, Briscoe H, Britton WJ. T cell-derived tumour necrosis factor is essential, but not sufficient, for protection against *Mycobacterium tuberculosis* infection. *Clin Exp Immunol*. 2004;137(2):279-87.
191. Kagina BM, Abel B, Scriba TJ, Hughes EJ, Keyser A, Soares A, et al. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns. *Am J Respir Crit Care Med*. 2010;182(8):1073-9.
192. Sutherland JS, Adetifa IM, Hill PC, Adegbola RA, Ota MO. Pattern and diversity of cytokine production differentiates between *Mycobacterium tuberculosis* infection and disease. *Eur J Immunol*. 2009;39(3):723-9.
193. Lawn SD, Myer L, Edwards D, Bekker LG, Wood R. Short-term and long-term risk of tuberculosis associated with CD4 cell recovery during antiretroviral therapy in South Africa. *AIDS*. 2009;23(13):1717-25.
194. Kalsdorf B, Scriba TJ, Wood K, Day CL, Dheda K, Dawson R, et al. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. *Am J Respir Crit Care Med*. 2009;180(12):1262-70.
195. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, et al. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *J Exp Med*. 2010;207(13):2869-81.

196. Peters W, Ernst JD. Mechanisms of cell recruitment in the immune response to *Mycobacterium tuberculosis*. *Microbes Infect.* 2003;5(2):151-8.
197. Peyron P, Vaubourgeix J, Poquet Y, Levillain F, Botanch C, Bardou F, et al. Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for *M. tuberculosis* persistence. *PLoS Pathog.* 2008;4(11):e1000204.
198. Robertson HE. The Persistence of Tuberculous Infections. *Am J Pathol.* 1933;9(Suppl):711-8 1.
199. Ahsan H, Halpern J, Kibriya MG, Pierce BL, Tong L, Gamazon E, et al. A genome-wide association study of early-onset breast cancer identifies PFKM as a novel breast cancer gene and supports a common genetic spectrum for breast cancer at any age. *Cancer Epidemiol Biomarkers Prev.* 2014;23(4):658-69.
200. Kaplan G, Post FA, Moreira AL, Wainwright H, Kreiswirth BN, Tanverdi M, et al. *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect Immun.* 2003;71(12):7099-108.
201. Selwyn PA, Hartel D, Lewis VA, Schoenbaum EE, Vermund SH, Klein RS, et al. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *N Engl J Med.* 1989;320(9):545-50.
202. Wilson JE. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol.* 2003;206(Pt 12):2049-57.
203. Hers HG, Van Schaftingen E. Fructose 2,6-bisphosphate 2 years after its discovery. *Biochem J.* 1982;206(1):1-12.
204. Jenkins CM, Yang J, Sims HF, Gross RW. Reversible high affinity inhibition of phosphofructokinase-1 by acyl-CoA: a mechanism integrating glycolytic flux with lipid metabolism. *J Biol Chem.* 2011;286(14):11937-50.
205. Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E. Energy metabolism in tumor cells. *FEBS J.* 2007;274(6):1393-418.
206. Rodriguez-Prados JC, Traves PG, Cuenca J, Rico D, Aragonés J, Martín-Sanz P, et al. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *J Immunol.* 2010;185(1):605-14.
207. Ruiz-Garcia A, Monsalve E, Novellásdemunt L, Navarro-Sabate A, Manzano A, Rivero S, et al. Cooperation of adenosine with macrophage Toll-4 receptor agonists leads to increased glycolytic flux through the enhanced expression of PFKFB3 gene. *J Biol Chem.* 2011;286(22):19247-58.

208. Sakakibara R, Uemura M, Hirata T, Okamura N, Kato M. Human placental fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase: its isozymic form, expression and characterization. *Biosci Biotechnol Biochem*. 1997;61(11):1949-52.
209. Shin JH, Yang JY, Jeon BY, Yoon YJ, Cho SN, Kang YH, et al. (1)H NMR-based metabolomic profiling in mice infected with *Mycobacterium tuberculosis*. *J Proteome Res*. 2011;10(5):2238-47.
210. Somashekar BS, Amin AG, Rithner CD, Troudt J, Basaraba R, Izzo A, et al. Metabolic profiling of lung granuloma in *Mycobacterium tuberculosis* infected guinea pigs: ex vivo 1H magic angle spinning NMR studies. *J Proteome Res*. 2011;10(9):4186-95.
211. Billig S, Schneefeld M, Huber C, Grassl GA, Eisenreich W, Bange FC. Lactate oxidation facilitates growth of *Mycobacterium tuberculosis* in human macrophages. *Sci Rep*. 2017;7(1):6484.
212. Baena A, Cabarcas F, Alvarez-Eraso KLF, Isaza JP, Alzate JF, Barrera LF. Differential determinants of virulence in two *Mycobacterium tuberculosis* Colombian clinical isolates of the LAM09 family. *Virulence*. 2019;10(1):695-710.
213. Osada-Oka M, Goda N, Saiga H, Yamamoto M, Takeda K, Ozeki Y, et al. Metabolic adaptation to glycolysis is a basic defense mechanism of macrophages for *Mycobacterium tuberculosis* infection. *Int Immunol*. 2019;31(12):781-93.
214. Ma R, Ji T, Zhang H, Dong W, Chen X, Xu P, et al. A Pck1-directed glycogen metabolic program regulates formation and maintenance of memory CD8(+) T cells. *Nat Cell Biol*. 2018;20(1):21-7.
215. Ma J, Wei K, Liu J, Tang K, Zhang H, Zhu L, et al. Glycogen metabolism regulates macrophage-mediated acute inflammatory responses. *Nat Commun*. 2020;11(1):1769.
216. Luzzatto L, Ally M, Notaro R. Glucose-6-phosphate dehydrogenase deficiency. *Blood*. 2020;136(11):1225-40.
217. Britt EC, Lika J, Giese MA, Schoen TJ, Seim GL, Huang Z, et al. Switching to the cyclic pentose phosphate pathway powers the oxidative burst in activated neutrophils. *Nat Metab*. 2022;4(3):389-403.
218. Wrigley NG, Heather JV, Bonsignore A, De Flora A. Human erythrocyte glucose 6-phosphate dehydrogenase: electron microscope studies on structure and interconversion of tetramers, dimers and monomers. *J Mol Biol*. 1972;68(3):483-99.
219. Garcia AA, Mathews, II, Horikoshi N, Matsui T, Kaur M, Wakatsuki S, et al. Stabilization of glucose-6-phosphate dehydrogenase oligomers enhances catalytic activity and stability of clinical variants. *J Biol Chem*. 2022;298(3):101610.

220. Kitamura H, Motohashi H. NRF2 addiction in cancer cells. *Cancer Sci.* 2018;109(4):900-11.
221. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. *Cancer Cell.* 2012;22(1):66-79.
222. Cosentino C, Grieco D, Costanzo V. ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. *EMBO J.* 2011;30(3):546-55.
223. Preville X, Salvemini F, Giraud S, Chaufour S, Paul C, Stepien G, et al. Mammalian small stress proteins protect against oxidative stress through their ability to increase glucose-6-phosphate dehydrogenase activity and by maintaining optimal cellular detoxifying machinery. *Exp Cell Res.* 1999;247(1):61-78.
224. Yan LJ, Christians ES, Liu L, Xiao X, Sohal RS, Benjamin IJ. Mouse heat shock transcription factor 1 deficiency alters cardiac redox homeostasis and increases mitochondrial oxidative damage. *EMBO J.* 2002;21(19):5164-72.
225. Christodoulou D, Kuehne A, Estermann A, Fuhrer T, Lang P, Sauer U. Reserve Flux Capacity in the Pentose Phosphate Pathway by NADPH Binding Is Conserved across Kingdoms. *iScience.* 2019;19:1133-44.
226. Ralser M, Wamelink MM, Latkolik S, Jansen EE, Lehrach H, Jakobs C. Metabolic reconfiguration precedes transcriptional regulation in the antioxidant response. *Nat Biotechnol.* 2009;27(7):604-5.
227. Eaton P, Wright N, Hearse DJ, Shattock MJ. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol.* 2002;34(11):1549-60.
228. Peralta D, Bronowska AK, Morgan B, Doka E, Van Laer K, Nagy P, et al. A proton relay enhances H₂O₂ sensitivity of GAPDH to facilitate metabolic adaptation. *Nat Chem Biol.* 2015;11(2):156-63.
229. Talwar D, Miller CG, Grossmann J, Szyrwił L, Schwecke T, Demichev V, et al. The GAPDH redox switch safeguards reductive capacity and enables survival of stressed tumour cells. *Nat Metab.* 2023;5(4):660-76.
230. Anastasiou D, Pouligiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science.* 2011;334(6060):1278-83.
231. Amara N, Cooper MP, Voronkova MA, Webb BA, Lynch EM, Kollman JM, et al. Selective activation of PFKL suppresses the phagocytic oxidative burst. *Cell.* 2021;184(17):4480-94 e15.

232. Galvan-Pena S, O'Neill LA. Metabolic reprogramming in macrophage polarization. *Front Immunol*. 2014;5:420.
233. Nagy C, Haschemi A. Time and Demand are Two Critical Dimensions of Immunometabolism: The Process of Macrophage Activation and the Pentose Phosphate Pathway. *Front Immunol*. 2015;6:164.
234. Koo SJ, Szczesny B, Wan X, Putluri N, Garg NJ. Pentose Phosphate Shunt Modulates Reactive Oxygen Species and Nitric Oxide Production Controlling *Trypanosoma cruzi* in Macrophages. *Front Immunol*. 2018;9:202.
235. Bories GFP, Yeudall S, Serbulea V, Fox TE, Isakson BE, Leitinger N. Macrophage metabolic adaptation to heme detoxification involves CO-dependent activation of the pentose phosphate pathway. *Blood*. 2020;136(13):1535-48.
236. TeSlaa T, Ralser M, Fan J, Rabinowitz JD. The pentose phosphate pathway in health and disease. *Nat Metab*. 2023;5(8):1275-89.
237. Shi L, Jiang Q, Bushkin Y, Subbian S, Tyagi S. Biphasic Dynamics of Macrophage Immunometabolism during *Mycobacterium tuberculosis* Infection. *mBio*. 2019;10(2).
238. He W, Miao FJ, Lin DC, Schwandner RT, Wang Z, Gao J, et al. Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. *Nature*. 2004;429(6988):188-93.
239. Littlewood-Evans A, Sarret S, Apfel V, Loesle P, Dawson J, Zhang J, et al. GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis. *J Exp Med*. 2016;213(9):1655-62.
240. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, et al. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. *Proc Natl Acad Sci U S A*. 2013;110(19):7820-5.
241. Strelko CL, Lu W, Dufort FJ, Seyfried TN, Chiles TC, Rabinowitz JD, et al. Itaconic acid is a mammalian metabolite induced during macrophage activation. *J Am Chem Soc*. 2011;133(41):16386-9.
242. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. *Cell Metab*. 2016;24(1):158-66.
243. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, et al. Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature*. 2018;556(7699):113-7.

244. Nair S, Huynh JP, Lampropoulou V, Loginicheva E, Esaulova E, Gounder AP, et al. Irg1 expression in myeloid cells prevents immunopathology during *M. tuberculosis* infection. *J Exp Med*. 2018;215(4):1035-45.
245. Fernandez-Garcia M, Rey-Stolle F, Boccard J, Reddy VP, Garcia A, Cumming BM, et al. Comprehensive Examination of the Mouse Lung Metabolome Following *Mycobacterium tuberculosis* Infection Using a Multiplatform Mass Spectrometry Approach. *J Proteome Res*. 2020;19(5):2053-70.
246. Coleman JW. Nitric oxide in immunity and inflammation. *Int Immunopharmacol*. 2001;1(8):1397-406.
247. Wink DA, Osawa Y, Darbyshire JF, Jones CR, Eshenaur SC, Nims RW. Inhibition of cytochromes P450 by nitric oxide and a nitric oxide-releasing agent. *Arch Biochem Biophys*. 1993;300(1):115-23.
248. Abu-Soud HM, Wang J, Rousseau DL, Fukuto JM, Ignarro LJ, Stuehr DJ. Neuronal nitric oxide synthase self-inactivates by forming a ferrous-nitrosyl complex during aerobic catalysis. *J Biol Chem*. 1995;270(39):22997-3006.
249. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43(2):109-42.
250. James SL. Nitric oxide in health and disease. *Parasitol Today*. 1998;14(12):504.
251. Ford PC, Lorkovic IM. Mechanistic aspects of the reactions of nitric oxide with transition-metal complexes. *Chem Rev*. 2002;102(4):993-1018.
252. Thomas DD, Heinecke JL, Ridnour LA, Cheng RY, Kesarwala AH, Switzer CH, et al. Signaling and stress: The redox landscape in NOS2 biology. *Free Radic Biol Med*. 2015;87:204-25.
253. Hibbs JB, Jr., Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science*. 1987;235(4787):473-6.
254. Liew FY, Li Y, Moss D, Parkinson C, Rogers MV, Moncada S. Resistance to *Leishmania major* infection correlates with the induction of nitric oxide synthase in murine macrophages. *Eur J Immunol*. 1991;21(12):3009-14.
255. Brunet LR. Nitric oxide in parasitic infections. *Int Immunopharmacol*. 2001;1(8):1457-67.
256. Langrehr JM, Hoffman RA, Lancaster JR, Jr., Simmons RL. Nitric oxide--a new endogenous immunomodulator. *Transplantation*. 1993;55(6):1205-12.
257. Bogdan C, Rollinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr Opin Immunol*. 2000;12(1):64-76.

258. van der Veen RC. Nitric oxide and T helper cell immunity. *Int Immunopharmacol*. 2001;1(8):1491-500.
259. Taylor-Robinson AW, Liew FY, Severn A, Xu D, McSorley SJ, Garside P, et al. Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *Eur J Immunol*. 1994;24(4):980-4.
260. Wei XQ, Charles IG, Smith A, Ure J, Feng GJ, Huang FP, et al. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*. 1995;375(6530):408-11.
261. Barnes PJ, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today*. 1995;16(3):128-30.
262. Somasundaram V, Gilmore AC, Basudhar D, Palmieri EM, Scheiblin DA, Heinz WF, et al. Inducible nitric oxide synthase-derived extracellular nitric oxide flux regulates proinflammatory responses at the single cell level. *Redox Biol*. 2020;28:101354.
263. Espey MG, Miranda KM, Pluta RM, Wink DA. Nitrosative capacity of macrophages is dependent on nitric-oxide synthase induction signals. *J Biol Chem*. 2000;275(15):11341-7.
264. Choi MS. Pathophysiological Role of S-Nitrosylation and Transnitrosylation Depending on S-Nitrosoglutathione Levels Regulated by S-Nitrosoglutathione Reductase. *Biomol Ther (Seoul)*. 2018;26(6):533-8.
265. Uehara T, Nakamura T, Yao D, Shi ZQ, Gu Z, Ma Y, et al. S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature*. 2006;441(7092):513-7.
266. Beigi F, Gonzalez DR, Minhas KM, Sun QA, Foster MW, Khan SA, et al. Dynamic denitrosylation via S-nitrosoglutathione reductase regulates cardiovascular function. *Proc Natl Acad Sci U S A*. 2012;109(11):4314-9.
267. Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett*. 1994;345(1):50-4.
268. Galkin A, Moncada S. S-nitrosation of mitochondrial complex I depends on its structural conformation. *J Biol Chem*. 2007;282(52):37448-53.
269. Chouchani ET, Methner C, Nadtochiy SM, Logan A, Pell VR, Ding S, et al. Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. *Nat Med*. 2013;19(6):753-9.

270. Doulias PT, Tenopoulou M, Greene JL, Raju K, Ischiropoulos H. Nitric oxide regulates mitochondrial fatty acid metabolism through reversible protein S-nitrosylation. *Sci Signal*. 2013;6(256):rs1.
271. Kelly B, O'Neill LA. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res*. 2015;25(7):771-84.
272. Bailey JD, Diotallevi M, Nicol T, McNeill E, Shaw A, Chuaiphichai S, et al. Nitric Oxide Modulates Metabolic Remodeling in Inflammatory Macrophages through TCA Cycle Regulation and Itaconate Accumulation. *Cell Rep*. 2019;28(1):218-30 e7.
273. Everts B, Amiel E, van der Windt GJ, Freitas TC, Chott R, Yarasheski KE, et al. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood*. 2012;120(7):1422-31.
274. Thwe PM, Amiel E. The role of nitric oxide in metabolic regulation of Dendritic cell immune function. *Cancer Lett*. 2018;412:236-42.
275. Amiel E, Everts B, Fritz D, Beauchamp S, Ge B, Pearce EL, et al. Mechanistic target of rapamycin inhibition extends cellular lifespan in dendritic cells by preserving mitochondrial function. *J Immunol*. 2014;193(6):2821-30.
276. Vijayan V, Pradhan P, Braud L, Fuchs HR, Gueler F, Motterlini R, et al. Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis. *Redox Biol*. 2019;22:101147.
277. Drapier JC, Hibbs JB, Jr. Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J Clin Invest*. 1986;78(3):790-7.
278. Drapier JC, Hibbs JB, Jr. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J Immunol*. 1988;140(8):2829-38.
279. Stadler J, Billiar TR, Curran RD, Stuehr DJ, Ochoa JB, Simmons RL. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. *Am J Physiol*. 1991;260(5 Pt 1):C910-6.
280. Beltran B, Quintero M, Garcia-Zaragoza E, O'Connor E, Esplugues JV, Moncada S. Inhibition of mitochondrial respiration by endogenous nitric oxide: a critical step in Fas signaling. *Proc Natl Acad Sci U S A*. 2002;99(13):8892-7.
281. Beltran B, Mathur A, Duchon MR, Erusalimsky JD, Moncada S. The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. *Proc Natl Acad Sci U S A*. 2000;97(26):14602-7.

282. Jianjun Y, Zhang R, Lu G, Shen Y, Peng L, Zhu C, et al. T cell-derived inducible nitric oxide synthase switches off Th17 cell differentiation. *J Exp Med*. 2013;210(7):1447-62.
283. Niedbala W, Cai B, Liew FY. Role of nitric oxide in the regulation of T cell functions. *Ann Rheum Dis*. 2006;65 Suppl 3(Suppl 3):iii37-40.
284. Navasardyan I, Bonavida B. Regulation of T Cells in Cancer by Nitric Oxide. *Cells*. 2021;10(10).
285. Hosoi A, Matsushita H, Shimizu K, Fujii S, Ueha S, Abe J, et al. Adoptive cytotoxic T lymphocyte therapy triggers a counter-regulatory immunosuppressive mechanism via recruitment of myeloid-derived suppressor cells. *Int J Cancer*. 2014;134(8):1810-22.
286. Marigo I, Zilio S, Desantis G, Mlecnik B, Agnellini AH, Ugel S, et al. T Cell Cancer Therapy Requires CD40-CD40L Activation of Tumor Necrosis Factor and Inducible Nitric-Oxide-Synthase-Producing Dendritic Cells. *Cancer Cell*. 2016;30(4):651.
287. Huang LE, Bunn HF. Hypoxia-inducible factor and its biomedical relevance. *J Biol Chem*. 2003;278(22):19575-8.
288. Colegio OR, Chu NQ, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature*. 2014;513(7519):559-63.
289. Palmer LA, Semenza GL, Stoler MH, Johns RA. Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. *Am J Physiol*. 1998;274(2):L212-9.
290. Jantsch J, Chakravorty D, Turza N, Prectel AT, Buchholz B, Gerlach RG, et al. Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. *J Immunol*. 2008;180(7):4697-705.
291. De Souza DP, Achuthan A, Lee MK, Binger KJ, Lee MC, Davidson S, et al. Autocrine IFN-I inhibits isocitrate dehydrogenase in the TCA cycle of LPS-stimulated macrophages. *J Clin Invest*. 2019;129(10):4239-44.
292. Nystrom T. Role of oxidative carbonylation in protein quality control and senescence. *EMBO J*. 2005;24(7):1311-7.
293. Timon-Gomez A, Sanfeliu-Redondo D, Pascual-Ahuir A, Proft M. Regulation of the Stress-Activated Degradation of Mitochondrial Respiratory Complexes in Yeast. *Front Microbiol*. 2018;9:106.
294. Tomin T, Schittmayer M, Honeder S, Heininger C, Birner-Gruenberger R. Irreversible oxidative post-translational modifications in heart disease. *Expert Rev Proteomics*. 2019;16(8):681-93.

295. Ugarte N, Petropoulos I, Friguet B. Oxidized mitochondrial protein degradation and repair in aging and oxidative stress. *Antioxid Redox Signal*. 2010;13(4):539-49.
296. Diers AR, Broniowska KA, Darley-Usmar VM, Hogg N. Differential regulation of metabolism by nitric oxide and S-nitrosothiols in endothelial cells. *Am J Physiol Heart Circ Physiol*. 2011;301(3):H803-12.
297. O'Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med*. 2016;213(1):15-23.
298. Noe JT, Mitchell RA. Tricarboxylic acid cycle metabolites in the control of macrophage activation and effector phenotypes. *J Leukoc Biol*. 2019;106(2):359-67.
299. Bogdan C. Nitric oxide and the immune response. *Nat Immunol*. 2001;2(10):907-16.
300. Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, et al. Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nat Commun*. 2016;7:11624.
301. Van den Bossche J, Baardman J, Otto NA, van der Velden S, Neele AE, van den Berg SM, et al. Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages. *Cell Rep*. 2016;17(3):684-96.
302. Gross TJ, Kremens K, Powers LS, Brink B, Knutson T, Domann FE, et al. Epigenetic silencing of the human NOS2 gene: rethinking the role of nitric oxide in human macrophage inflammatory responses. *J Immunol*. 2014;192(5):2326-38.
303. Albina JE. On the expression of nitric oxide synthase by human macrophages. Why no NO? *J Leukoc Biol*. 1995;58(6):643-9.
304. Choi HS, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *Am J Respir Crit Care Med*. 2002;166(2):178-86.
305. Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *J Clin Invest*. 1995;96(5):2357-63.
306. De Groot CJ, Ruuls SR, Theeuwes JW, Dijkstra CD, Van der Valk P. Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J Neuropathol Exp Neurol*. 1997;56(1):10-20.
307. Kwon S, Newcomb RL, George SC. Mechanisms of synergistic cytokine-induced nitric oxide production in human alveolar epithelial cells. *Nitric Oxide*. 2001;5(6):534-46.

308. Jung JY, Madan-Lala R, Georgieva M, Rengarajan J, Sohaskey CD, Bange FC, et al. The intracellular environment of human macrophages that produce nitric oxide promotes growth of mycobacteria. *Infect Immun*. 2013;81(9):3198-209.
309. Cameron ML, Granger DL, Weinberg JB, Kozumbo WJ, Koren HS. Human alveolar and peritoneal macrophages mediate fungistasis independently of L-arginine oxidation to nitrite or nitrate. *Am Rev Respir Dis*. 1990;142(6 Pt 1):1313-9.
310. Schoedon G, Troppmair J, Fontana A, Huber C, Curtius HC, Niederwieser A. Biosynthesis and metabolism of pterins in peripheral blood mononuclear cells and leukemia lines of man and mouse. *Eur J Biochem*. 1987;166(2):303-10.
311. Sakai N, Milstien S. Availability of tetrahydrobiopterin is not a factor in the inability to detect nitric oxide production by human macrophages. *Biochem Biophys Res Commun*. 1993;193(1):378-83.
312. Jones AE, Divakaruni AS. Macrophage activation as an archetype of mitochondrial repurposing. *Mol Aspects Med*. 2020;71:100838.
313. Palmieri EM, Menga A, Martin-Perez R, Quinto A, Riera-Domingo C, De Tullio G, et al. Pharmacologic or Genetic Targeting of Glutamine Synthetase Skews Macrophages toward an M1-like Phenotype and Inhibits Tumor Metastasis. *Cell Rep*. 2017;20(7):1654-66.
314. Johnston DGW, Kearney J, Zaslon Z, Williams MA, O'Neill LAJ, Corr SC. MicroRNA-21 Limits Uptake of *Listeria monocytogenes* by Macrophages to Reduce the Intracellular Niche and Control Infection. *Front Cell Infect Microbiol*. 2017;7:201.
315. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKK ϵ supports the anabolic demands of dendritic cell activation. *Nat Immunol*. 2014;15(4):323-32.
316. Garaude J, Acin-Perez R, Martinez-Cano S, Enamorado M, Ugolini M, Nistal-Villan E, et al. Mitochondrial respiratory-chain adaptations in macrophages contribute to antibacterial host defense. *Nat Immunol*. 2016;17(9):1037-45.
317. Graham DB, Becker CE, Doan A, Goel G, Villablanca EJ, Knights D, et al. Functional genomics identifies negative regulatory nodes controlling phagocyte oxidative burst. *Nat Commun*. 2015;6:7838.
318. Christodoulou D, Link H, Fuhrer T, Kochanowski K, Gerosa L, Sauer U. Reserve Flux Capacity in the Pentose Phosphate Pathway Enables *Escherichia coli*'s Rapid Response to Oxidative Stress. *Cell Syst*. 2018;6(5):569-78 e7.
319. Kuehne A, Emmert H, Soehle J, Winnefeld M, Fischer F, Wenck H, et al. Acute Activation of Oxidative Pentose Phosphate Pathway as First-Line Response to Oxidative Stress in Human Skin Cells. *Mol Cell*. 2015;59(3):359-71.

320. Baardman J, Verberk SGS, Prange KHM, van Weeghel M, van der Velden S, Ryan DG, et al. A Defective Pentose Phosphate Pathway Reduces Inflammatory Macrophage Responses during Hypercholesterolemia. *Cell Rep*. 2018;25(8):2044-52 e5.
321. Cooper MR, DeChatelet LR, McCall CE, LaVia MF, Spurr CL, Baehner RL. Complete deficiency of leukocyte glucose-6-phosphate dehydrogenase with defective bactericidal activity. *J Clin Invest*. 1972;51(4):769-78.
322. Roos D, van Zwieten R, Wijnen JT, Gomez-Gallego F, de Boer M, Stevens D, et al. Molecular basis and enzymatic properties of glucose 6-phosphate dehydrogenase volendam, leading to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. *Blood*. 1999;94(9):2955-62.
323. van Bruggen R, Bautista JM, Petropoulou T, de Boer M, van Zwieten R, Gomez-Gallego F, et al. Deletion of leucine 61 in glucose-6-phosphate dehydrogenase leads to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. *Blood*. 2002;100(3):1026-30.
324. Ham M, Lee JW, Choi AH, Jang H, Choi G, Park J, et al. Macrophage glucose-6-phosphate dehydrogenase stimulates proinflammatory responses with oxidative stress. *Mol Cell Biol*. 2013;33(12):2425-35.
325. Ghergurovich JM, Garcia-Canaveras JC, Wang J, Schmidt E, Zhang Z, TeSlaa T, et al. A small molecule G6PD inhibitor reveals immune dependence on pentose phosphate pathway. *Nat Chem Biol*. 2020;16(7):731-9.
326. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science*. 2003;302(5645):654-9.
327. Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science*. 2006;313(5792):1438-41.
328. Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med*. 1992;175(4):1111-22.
329. Darwin KH, Ehrt S, Gutierrez-Ramos JC, Weich N, Nathan CF. The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. *Science*. 2003;302(5652):1963-6.
330. Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras TR, et al. Reprogramming of the macrophage transcriptome in response to interferon-gamma and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J Exp Med*. 2001;194(8):1123-40.

331. Mao K, Chen S, Chen M, Ma Y, Wang Y, Huang B, et al. Nitric oxide suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock. *Cell Res.* 2013;23(2):201-12.
332. Beielstein AC, Izquierdo E, Blakemore S, Nickel N, Michalik M, Chawan S, et al. Macrophages are activated toward phagocytic lymphoma cell clearance by pentose phosphate pathway inhibition. *Cell Rep Med.* 2024:101830.
333. Bolanos JP, Peuchen S, Heales SJ, Land JM, Clark JB. Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J Neurochem.* 1994;63(3):910-6.
334. Luan HH, Medzhitov R. Food Fight: Role of Itaconate and Other Metabolites in Antimicrobial Defense. *Cell Metab.* 2016;24(3):379-87.
335. Cordes T, Michelucci A, Hiller K. Itaconic Acid: The Surprising Role of an Industrial Compound as a Mammalian Antimicrobial Metabolite. *Annu Rev Nutr.* 2015;35:451-73.
336. Ruetz M, Campanello GC, Purchal M, Shen H, McDevitt L, Gouda H, et al. Itaconyl-CoA forms a stable biradical in methylmalonyl-CoA mutase and derails its activity and repair. *Science.* 2019;366(6465):589-93.
337. Eoh H, Rhee KY. Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of *Mycobacterium tuberculosis* on fatty acids. *Proc Natl Acad Sci U S A.* 2014;111(13):4976-81.
338. Lee W, VanderVen BC, Fahey RJ, Russell DG. Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *J Biol Chem.* 2013;288(10):6788-800.
339. Munoz-Elias EJ, McKinney JD. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med.* 2005;11(6):638-44.
340. O'Neill LAJ, Artyomov MN. Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nat Rev Immunol.* 2019;19(5):273-81.
341. Wang H, Fedorov AA, Fedorov EV, Hunt DM, Rodgers A, Douglas HL, et al. An essential bifunctional enzyme in *Mycobacterium tuberculosis* for itaconate dissimilation and leucine catabolism. *Proc Natl Acad Sci U S A.* 2019;116(32):15907-13.
342. Azenabor AA, Kennedy P, York J. Free intracellular Ca²⁺ regulates bacterial lipopolysaccharide induction of iNOS in human macrophages. *Immunobiology.* 2009;214(2):143-52.
343. Blond D, Raoul H, Le Grand R, Dormont D. Nitric oxide synthesis enhances human immunodeficiency virus replication in primary human macrophages. *J Virol.* 2000;74(19):8904-12.

344. Panaro MA, Brandonisio O, Acquafredda A, Sisto M, Mitolo V. Evidences for iNOS expression and nitric oxide production in the human macrophages. *Curr Drug Targets Immune Endocr Metabol Disord*. 2003;3(3):210-21.
345. Weinberg JB, Misukonis MA, Shami PJ, Mason SN, Sauls DL, Dittman WA, et al. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. *Blood*. 1995;86(3):1184-95.
346. Wang CH, Liu CY, Lin HC, Yu CT, Chung KF, Kuo HP. Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. *Eur Respir J*. 1998;11(4):809-15.
347. Granger DL, Lehninger AL. Sites of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J Cell Biol*. 1982;95(2 Pt 1):527-35.
348. Daniel J, Maamar H, Deb C, Sirakova TD, Kolattukudy PE. Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS Pathog*. 2011;7(6):e1002093.