



Trinity College Dublin

Coláiste na Tríonóide, Baile Átha Cliath

The University of Dublin

Early Immune Responses to Emerging and Enduring Respiratory Pathogens

A Thesis Submitted to Trinity College Dublin, the University of Dublin,
for the Degree of Doctor of Philosophy in the Faculty of Medicine

Dr. Conor Grant, MB BCH BAO BA DTMH

Conferred 2024

Supervisors:

Prof. Colm Bergin, Prof. Joe Keane, Dr. Mary O'Sullivan
Department of Clinical Medicine,
Trinity Translational Medicine Institute,
St. James's Hospital,
Trinity College Dublin,
University of Dublin.

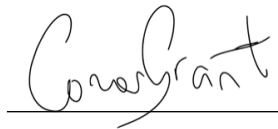
Thesis Declaration

I, Conor Grant, 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 on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish (EU GDPR May 2018)

Conor Grant

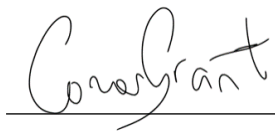
A handwritten signature in cursive script that reads "Conor Grant". The signature is written in black ink and is positioned above a horizontal line.

Date: July 5th, 2023

Plagiarism Statement

I, Conor Grant, declare that the work in this thesis is my own and is original, unless otherwise stated. Sections of this thesis have been copied directly or adapted from a published review paper, which was written during the course of this research, on which I am joint first author (1). Our collaborator Dr. Hossam Gewaid performed the cellular infections with *live* SARS-CoV-2, viral plaque and viral titre assays which were necessary for figures 5.11 and 5.12. My supervisor Dr. Mary O’Sullivan ran the MSD multiplex analyser assays. Full and informed consent was obtained from donors, and samples were obtained under pre-existing ethics approved by the St. James’s Hospital/Tallaght University Hospital Joint Research Ethics Committee.

Conor Grant

A handwritten signature in black ink, reading 'Conor Grant', is written over a horizontal line.

Date: July 5th, 2023

Acknowledgements

This research was made possible by the participation of blood donors at the Irish Blood Transfusion Service and patients undergoing bronchoscopy at St. James's Hospital. Their selfless contributions to science are greatly appreciated and have helped advance the understanding of the immunology of infectious diseases.

I am so fortunate to have three exceptional supervisors. Dr. Mary O'Sullivan exhibited extraordinary patience with me, never let me get away with sloppy thinking and had the vision that crystalised into a coherent project. Prof. Joe Keane's passion and energy are more infectious than tuberculosis – qualities that drive all who are lucky enough to work with him, myself included. Prof. Colm Bergin has been much more than a principal investigator or a supervisor, but also a mentor, a role model, a life coach, a friend, a therapist at times and someone who saw my potential and always pointed me towards opportunities to grow. I could not ask for better supervisors than Mary, Joe and Colm.

It takes a village to raise a PhD student, and my laboratory colleagues in the TB immunology group were that village. In particular, Emily worked like a Trojan to keep the lab and the supply of alveolar macrophages going. My fellow clinician students, Lorraine and Kevin, made the lab feel like home. I owe my new lab skills in large part to Donal, James, Gina and Karl. Sharee, Derv, Sarah, Laura, Gráinne, Anjali and Aenea all shaped this research and were fantastic company on my journey.

In St. James's Hospital, my other family has been the staff of the GUIDe department, who have always been encouraging and supportive of my plans. Dr. Ciaran Bannan, Prof. Susan Clarke, Prof. Ceppie Merry, Prof. Fiona Lyons, Dr. Emma Devitt and Prof. Fiona Mulcahy all helped guide and inspire me in my career. The staff of the post-COVID clinic, including Dr. Ciaran Bannan, Dr. Brian Kent and Dr. Parthiban Nadarajan were a strong support during these last three years. Dr. Parthiban Nadarajan and Dr. Finbarr O'Connell made an invaluable contribution to this research, and continue to support the TB immunology group, by performing the bronchoalveolar lavages that make this research possible. Further afield in Galway University Hospital, Prof. Catherine Fleming was an influential and valuable source of wisdom at a critical moment when I decided to pursue

the goal of becoming a clinician scientist. Our conversations solidified my confidence in the path I was taking.

Funding for this research was granted by The Wellcome Trust, The Health Research Board and the Health Service Executive through the vehicle of the Irish Clinical Academic Training (ICAT) Programme. I will be forever grateful of having been given this remarkable opportunity by the ICAT programme and its funders, and hope to live up to their expectations and the challenge I've accepted. The Science Foundation Ireland and The Royal City of Dublin Hospital Trust also contributed funding to my research, and those of my laboratory colleagues. This research would be impossible without the patronage of these funders.

To my amazing siblings, Kevin, Julie (and Sarah too) and our parents, Dermot and Áine, who continue to demonstrate the dignity of hard work, how to blissfully ignore life's perceived limits and chart one's own course, and how to love and seize each day like the last.

My greatest discovery during my PhD was, without any doubt or comparison, my wonderful partner Ján. Ján's boundless positivity and humour, even in the face of life's challenges, were the perfect antidotes to any mundane anxieties, doubts or moments of gloomy seriousness of mine. Ján was my sounding board, and is now more fluent in immunology than I am in Slovak. Ďakujem láska.

I was shaped and inspired by three scientist-heroes in my youth: To Carl Sagan for giving me perspective (*"The cosmos is within us. We are made of star-stuff. We are a way for the universe to know itself"*), to Richard Dawkins for *'unweaving the rainbow'* and to Richard Feynman for teaching me *'the pleasure of finding things out'*.

Table of Contents

THESIS DECLARATION	3
PLAGIARISM STATEMENT	4
ACKNOWLEDGEMENTS	5
TABLE OF CONTENTS	7
OUTPUTS.....	12
PUBLICATIONS.....	12
MANUSCRIPTS AWAITING SUBMISSION	12
ORAL PRESENTATIONS	12
POSTER PRESENTATIONS	14
LIST OF ACRONYMS	15
LIST OF FIGURES	21
LIST OF TABLES	23
ABSTRACT	24
LAY ABSTRACT.....	26
AIMS AND HYPOTHESES	28
VALUE OF RESEARCH	30
CHAPTER 1.....	32
1 INTRODUCTION	32
1.1 ANCIENT AND MODERN THREATS	32
1.2 HOST DEFENCE AGAINST INFECTION IN THE LUNG	34
1.3 VITAMIN A AS A HOST-DIRECTED THERAPY FOR MYCOBACTERIAL INFECTIONS	36
1.3.1 <i>Mycobacterium tuberculosis: Epidemiology, Microbiology and Pathophysiology</i>	36
1.3.2 <i>Mycobacterium tuberculosis Infections: Clinical Challenges</i>	37
1.3.3 <i>Nontuberculous Mycobacterial Infections: Microbiology and Epidemiology</i>	39
1.3.4 <i>Transmission of Nontuberculous Mycobacteria</i>	40
1.3.5 <i>Risk factors for Nontuberculous Mycobacterial Infections</i>	41
1.3.6 <i>Clinical Aspects of Nontuberculous Mycobacterial Infections</i>	42
1.3.7 <i>Treatment of Nontuberculous Mycobacterial Infections</i>	44
1.3.8 <i>Host-Directed Therapies for Nontuberculous Mycobacterial Infectious</i>	45
1.3.9 <i>Host-Directed Therapies for TB</i>	46
1.3.10 <i>ATRA as a Promising Host-Directed Therapy for TB</i>	47

1.3.11	<i>ATRA Mechanisms of Action</i>	49
1.3.12	<i>Clinical Trials of Vitamin A for TB</i>	51
1.3.13	<i>Why Clinical Trials Have Failed</i>	52
1.5	VITAMIN A DEFICIENCY AND DEFECTS IN HOST RESPONSES TO INFECTION.....	56
1.5.1	<i>Epidemiology of Vitamin A Deficiency</i>	56
1.5.2	<i>Vitamin A Deficiency in Infectious Diseases</i>	57
1.5.3	<i>Vitamin A Deficiency in Active Tuberculosis</i>	58
1.5.4	<i>Vitamin A Absorption and Storage</i>	59
1.5.5	<i>Vitamin A Transport</i>	60
1.5.6	<i>Vitamin A Cell Entry</i>	61
1.5.7	<i>Enzymes Involved in Vitamin A Metabolism</i>	62
1.5.8	<i>Carrier Proteins</i>	63
1.5.9	<i>Degradation by Cytochrome P450</i>	64
1.6	ALVEOLAR MACROPHAGE RESPONSES TO SARS-CoV-2.....	66
1.6.1	<i>SARS-CoV-2 and the COVID-19 Pandemic</i>	66
1.6.2	<i>Transmission of SARS-CoV-2</i>	66
1.6.3	<i>Clinical Aspects of SARS-CoV-2 Infections</i>	67
1.6.4	<i>Age and Other Risk Factors in SARS-CoV-2 Infections</i>	68
1.6.5	<i>Pathogenesis of SARS-CoV-2 Infections: Virology</i>	69
1.6.6	<i>Pathogenesis of SARS-CoV-2 Infections: Immunology</i>	70
1.6.7	<i>Alveolar Macrophages and SARS-CoV-2</i>	72
1.6.8	<i>SARS-CoV-2 Proteins are PAMPs</i>	74
1.6.9	<i>Early Events in SARS-CoV-2 Infections</i>	76
1.6.10	<i>Ageing and Alveolar Macrophages</i>	76
1.6.11	<i>Alveolar Macrophage Responses to SARS-CoV-2: Clinical and Research Needs</i>	78
1.7	OUTLINE OF RESULTS CHAPTERS	80
CHAPTER 2		81
2	METHODS AND MATERIALS.....	81
2.1	CELL CULTURE.....	81
2.1.1	<i>Human Monocyte-Derived Macrophages</i>	81
2.1.2	<i>Human Alveolar Macrophages</i>	82
2.1.3	<i>THP-1 Macrophages</i>	83
2.2	MACROPHAGE TREATMENTS AND PRE-TREATMENTS.....	83
2.2.1	<i>Retinoic Acid Receptor Inhibitors & Ligands</i>	83
2.2.2	<i>Cytokines</i>	84
2.2.3	<i>Antibodies</i>	85
2.2.4	<i>Other Treatments</i>	85

2.3	INFECTIONS AND STIMULATIONS	88
2.3.1	<i>Determination of the Multiplicity of Infection</i>	88
2.3.2	<i>Mycobacterium tuberculosis Infections and Stimulations</i>	89
2.3.3	<i>Mycobacterium chimaera Infections</i>	91
2.3.4	<i>Mycobacterium abscessus Infections</i>	91
2.3.5	<i>Stimulation of Macrophages with TLR Ligands</i>	92
2.3.6	<i>Stimulation of Macrophages with SARS-CoV-2 Antigens</i>	93
2.3.7	<i>Infection of Macrophages with SARS-CoV-2</i>	93
2.4	ASSESSMENT OF MACROPHAGE ATRA PRODUCTION AND TRANSPORT GENES.....	98
2.4.1	<i>Gene Expression Analysis</i>	98
2.4.2	<i>Western Blotting</i>	100
2.5	MACROPHAGE FUNCTIONAL ANALYSES	101
2.5.1	<i>Cytokine Analyses</i>	101
2.5.2	<i>Cholesterol Assay</i>	102
2.5.3	<i>Intracellular Bacillary Killing Assay</i>	102
2.5.4	<i>Axenic Culture Assay</i>	103
2.5.5	<i>Cell Viability</i>	104
2.6	METABOLIC ANALYSES	104
2.6.1	<i>Assessment of Mitochondrial Function using the Mito Stress Test</i>	104
2.6.2	<i>Assessment of Metabolic Flux During Acute Infection or Stimulation</i>	107
2.6.3	<i>Assessment of Metabolic Flux During Treatment with RARI and/or ATRA</i>	108
2.6.4	<i>Normalisation using Crystal Violet Assay</i>	108
2.6.5	<i>Glycolytic Gene Analyses</i>	109
2.7	STATISTICAL ANALYSIS	109
CHAPTER 3.....		110
3	ENDOGENOUS MACROPHAGE ATRA PRODUCTION AND MYCOBACTERIUM TUBERCULOSIS	110
3.1	INTRODUCTION	110
3.3	AIMS AND HYPOTHESES	112
3.4	RESULTS	113
3.4.1	<i>Human MDMs and AMs express vitamin A oxidation enzymes and carrier proteins</i>	113
3.4.2	<i>Mtb infection reduces the expression of vitamin A oxidation enzymes and carrier proteins</i>	114
3.4.4	<i>GM-CSF supports the expression of vitamin A oxidation enzymes during Mtb infection</i>	118
3.4.5	<i>IL-4 supports the expression of vitamin A oxidation enzymes during Mtb infection</i>	119
3.4.6	<i>Blocking the nuclear signalling of endogenously produced ATRA alters human MDM metabolic responses to Mtb</i>	121
3.4.7	<i>Blocking the nuclear signalling of endogenously produced ATRA does not alter human MDM cytokine production in response to Mtb</i>	140

3.4.9	<i>Blocking the nuclear signalling of endogenously produced ATRA impairs human MDMs' ability to kill Mtb</i>	142
3.5	DISCUSSION	144
3.6	CONCLUSION	151
3.7	APPENDIX FOR CHAPTER 3	152
CHAPTER 4		154
4	ALL TRANS RETINOIC ACID AS A HOST-DIRECTED THERAPY FOR NONTUBERCULOUS MYCOBACTERIA	154
4.1	INTRODUCTION	154
4.3	AIMS AND HYPOTHESES	156
4.4	RESULTS	157
4.4.1	<i>ATRA does not affect the growth of NTMs in axenic culture</i>	157
4.4.2	<i>ATRA does not affect the viability of uninfected macrophages</i>	158
4.4.4	<i>ATRA does not affect the viability of M. abscessus infected or uninfected THP-1 macrophages</i>	160
4.4.5	<i>ATRA is an effective HDT in THP-1 macrophages infected with M. chimaera but not M. abscessus</i>	162
4.4.7	<i>ATRA does not affect THP-1 macrophage intracellular cholesterol concentrations during infection with M. chimaera and M. abscessus</i>	164
4.4.8	<i>ATRA alters THP-1 macrophage cytokine responses to M. chimaera</i>	165
4.4.9	<i>ATRA induces the Warburg effect in THP1 macrophages, an effect which is abrogated by inhibition of RARα</i>	167
4.4.10	<i>ATRA prevents the early glycolytic response of AMs to M. chimaera</i>	169
4.4.11	<i>ATRA prevents the early glycolytic response of AMs to M. abscessus</i>	172
4.5	DISCUSSION	174
4.7	CONCLUSION	179
4.8	APPENDIX FOR CHAPTER 4	180
CHAPTER 5		181
5	HUMAN ALVEOLAR MACROPHAGE RESPONSES TO SARS-COV-2 ANTIGENS: THE INFLUENCE OF AGE	181
5.1	INTRODUCTION	181
5.2	AIMS AND HYPOTHESES	184
5.3	RESULTS	186
5.3.1	<i>SARS-CoV-2 envelope protein, but not spike protein, induces the secretion of cytokines from human AMs</i>	186
5.3.2	<i>TLR4, and not TLR2, recognises SARS-CoV-2 envelope protein on human AMs</i>	189

5.3.3	<i>AMs from older donors have higher cytokine responses to SARS-CoV-2 envelope protein than AMs from younger donors.....</i>	<i>192</i>
5.3.4	<i>AMs from older donors do not have significantly different cytokine responses to LPS than AMs from younger donors.....</i>	<i>194</i>
5.3.5	<i>AMs from smokers had lower cytokine responses to SARS-CoV-2 envelope protein than AMs from never- or ex-smokers</i>	<i>196</i>
5.3.6	<i>AMs from smokers did not have significantly different cytokine responses to LPS than AMs from never- or ex-smokers</i>	<i>198</i>
5.3.7	<i>SARS-CoV-2 does not induce the secretion of cytokines from human AMs.....</i>	<i>200</i>
5.3.8	<i>SARS-CoV-2 does not productively infect human AMs.....</i>	<i>202</i>
5.5	DISCUSSION.....	204
5.6	CONCLUSION	211
CHAPTER 6.....		212
6	DISCUSSION.....	212
6.1	RELEVANCE OF CHAPTER 3 TO THE RESEARCH AND CLINICAL DOMAINS	212
6.2	RELEVANCE OF CHAPTER 4 TO THE RESEARCH AND CLINICAL DOMAINS	215
6.3	RELEVANCE OF CHAPTER 5 TO THE RESEARCH AND CLINICAL DOMAINS	220
6.4	CONCLUDING REMARKS.....	222
7	REFERENCES	224

Outputs

Publications

Bahloul AZ, **Grant C**, Cryan SA, Keane J, O'Sullivan MP. **All trans retinoic acid as a host-directed immunotherapy for tuberculosis**. Current Research in Immunology. 2022 Jan 1;3:54-72. <https://doi.org/10.1016/j.crimmu.2022.03.003>

Manuscripts awaiting submission

Grant C, Nadarajan P, Bergin C, Keane J, O'Sullivan MP. **Endogenous Macrophage ATRA Production and *Mycobacterium tuberculosis***

Grant C, Duffin E, Bergin C, Keane J, O'Sullivan MP. **All *trans* Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria**

Grant C, Gewaid H, Bergin C, Keane J, Bowie AG, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age and Smoking**

Oral Presentations

Grant C, Bergin C, Keane J, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age**. DMHG Research and Poster Competition, St. James's Hospital, Dublin, May 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age**. COVID-19 Research Hub Meeting, St. James's Hospital, Dublin, May 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age**. Infectious Diseases Society of Ireland conference, Croke Park, Dublin, May 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Effect of Age on Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens**. European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, April 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age**. Trinity Translational Medicine Institute Immunology Meeting, St. James's Hospital, Dublin, December 2022.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age**. ICAT Retreat, The Grand Hotel Malahide, Dublin, December 2022.

Grant C, Bergin C, Keane J, O'Sullivan MP. ***M. tuberculosis* Infection Reduces Macrophage Expression of Vitamin A Metabolism and Transport Genes**. Keystone Symposium, Breckenridge, Colorado, August 2022.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Vitamin A Signalling Enables Macrophage Metabolic Responses to *Mycobacterium tuberculosis***. Infectious Diseases Society of Ireland conference, Croke Park, Dublin, May 2022.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Vitamin A Signalling Enables Macrophage Metabolic Responses to *Mycobacterium tuberculosis***. Trinity Translational Medicine Institute Immunology Meeting, St. James's Hospital, Dublin, May 2022.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Exploiting Immunometabolism in Tuberculosis**. ICAT Retreat, The Grand Hotel Malahide, Dublin, November 2020.

Poster Presentations

Grant C, Bergin C, Keane J, O'Sullivan MP. **All *Trans* Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacterial Disease.** Dublin Academy of Pathogenomics and Infection Biology Meeting, University College Dublin, Dublin, June 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age.** DMHG Research and Poster Competition, St. James's Hospital, Dublin, May 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Effect of Age on Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens.** European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, April 2023.

Grant C, Bergin C, Keane J, O'Sullivan MP. ***M. tuberculosis* Infection Reduces Macrophage Expression of Vitamin A Metabolism and Transport Genes.** Keystone Symposium, Breckenridge, Colorado, August 2022.

Grant C, Bergin C, Keane J, O'Sullivan MP. **Endogenous Vitamin A Signaling is Important for Macrophage Metabolic Responses to *Mycobacterium tuberculosis*.** ICAT Retreat, The Grand Hotel Malahide, Dublin, December 2021.

List of Acronyms

AA	Antimycin A
ABC	ATP-binding cassette transporter
ACE2	Angiotensin-converting enzyme 2
ADC	Albumin, dextrose, catalase
ADH	Alcohol dehydrogenases
ALDH	Aldehyde dehydrogenase
AM	Alveolar macrophage
AMP	5' adenosine monophosphate-activated protein
ANOVA	Analysis of variance
AP1	Activator protein 1
APL	Acute promyelocytic leukaemia
ARDS	Acute respiratory distress syndrome
ART	Antiretroviral therapy
AT1	Alveolar type 1 cell
AT2	Alveolar type 2 cell
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATRA	All <i>trans</i> retinoic acid
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette–Guérin
BMDM	Bone marrow derived macrophage
BMI	Bone marrow index
BSA	Bovine serum albumin

CAR T	Chimeric antigen receptor T lymphocyte
CCL	Chemokine ligand
CCR2	C-C motif chemokine receptor
CD	Cluster of differentiation
CF	Cystic fibrosis
CFU	Colony-forming unit
CHX	Cycloheximide
COVID-19	Coronavirus disease 2019
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
Ct	Cycle threshold
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
CYP	Cytochrome P450
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin
DEAB	N,N-diethylaminobenzaldehyde
DHRS	Dehydrogenase/Reductase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rate
ELISA	Enzyme-linked immunosorbent assay

ERK	Extracellular signal-regulated kinases
ESR	Erythrocyte sedimentation rate
ETC	Electron transport chain
FABP	Fatty acid binding protein
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
FDA	United States Food and Drug Administration
FXR	Farnesoid X Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT1	Glucose transporter 1
GPL	Glycopeptidolipid
HDT	Host-directed therapy
HEK	Human Embryonic Kidney cell
HHC	Household contacts
HIV	Human immunodeficiency virus
HK2	Hexokinase 2
HLA-DR	Human leukocyte antigen – DR isotype
HSV	Herpes simplex virus
IFN	Interferon
IGRA	Interferon gamma release assay
IL	Interleukin
IRF	Interferon regulatory transcription factor
IU	International units
JAK	Janus kinase
LAL	limulus amebocyte lysate
LPS	Lipopolysaccharide

LRAT	Lecithin retinol acyltransferase
LRP1	Low density lipoprotein receptor-related protein 1
LTBI	Latent tuberculosis infection
MAC	<i>Mycobacterium avium</i> complex
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation-associated protein 5
MDM	Monocyte derived macrophage
MDR	Multidrug resistant
MDSC	Myeloid derived suppressor cells
MEK	Mitogen activated protein kinase kinase
MERS	Middle East respiratory syndrome–related coronavirus
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSD	Meso Scale Discovery
Mtb	<i>Mycobacterium tuberculosis</i>
NDV	Newcastle disease virus
NF-κB	Nuclear transcription factor-κB
NLRP3	NOD-like receptor family pyrin domain containing 3
NOS	Nitric oxide synthase
NPC2	Niemann–Pick intracellular cholesterol transporter 2
NTM	Nontuberculous mycobacteria
OADC	Oleic acid, albumin, dextrose, catalase
OCR	Oxygen consumption rate
OR	Odds ratio
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid responsive element
RARI	Retinoic acid receptor inhibitor
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
RIG-I	retinoic acid-inducible gene 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSV	Respiratory syncytial virus
RXR	Retinoid X receptor
SAA	Serum amyloid A
SARS	Severe acute respiratory syndrome
SDR	Short-chain dehydrogenase/reductases
SEAP	Secreted embryonic alkaline phosphatase
SEM	Standard error of the mean
SHM	Seahorse media

SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer and activator of transcription
TACO	Tryptophan–aspartate-containing coat protein
TB	Tuberculosis
TBST	Tris-buffered saline with tween
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease, serine 2
TNF α	Tumour necrosis factor alpha
TRAF6	TNF receptor associated factor 6
TTR	Transthyretin
VAD	Vitamin A deficiency
VDR	Vitamin D receptor
VLDL	Very low density lipoprotein
WFI	Water for injection
WHO	World Health Organization
XDR	Extensively drug resistant

List of Figures

FIGURE 1.1: THE PRESERVED, UNDERWATER, SKELETAL REMAINS OF A MOTHER AND CHILD AT ATLIT-YAM	33
FIGURE 1.2: ATRA'S MECHANISMS OF ACTION AS A HDT AGAINST MTB	51
FIGURE 1.3: ATRA HAS PLEIOTROPIC EFFECTS THAT PROTECT THE HOST AGAINST MTB.....	55
FIGURE 1.4: VITAMIN A IS DELIVERED TO TARGET CELLS IN SEVERAL WAYS	65
FIGURE 2.1: BRIGHT FIELD MICROSCOPY IMAGES OF PLATED ALVEOLAR MACROPHAGES	83
FIGURE 2.2: COMPOSITE FLUORESCENT MICROSCOPY IMAGE OF ALVEOLAR MACROPHAGES	89
FIGURE 2.3: METHODS FOR EXPERIMENTS IN WHICH HUMAN MDMs AND AMs WERE INFECTED	90
FIGURE 2.4: METHODS FOR SEAHORSE EXPERIMENTS IN WHICH HUMAN MDMs WERE STIMULATED WITH IH37Rv.	91
FIGURE 2.5: METHODS FOR EXPERIMENTS IN WHICH HUMAN AMs WERE STIMULATED WITH	93
FIGURE 2.6: MITO STRESS TEST REAGENTS MECHANISMS OF ACTION.	106
FIGURE 2.7: MITO STRESS TEST – REPRESENTATIVE GRAPH OF OCR AND DERIVATION OF THE MEASURES	107
FIGURE 3.1: MTB INFECTION REDUCES THE EXPRESSION OF ENZYMES NEEDED TO OXIDISE RETINOL TO ATRA.....	116
FIGURE 3.2: MTB INFECTION REDUCES THE mRNA EXPRESSION OF THE CARRIER PROTEINS NEEDED	117
FIGURE 3.3: GM-CSF PREVENTS THE MTB-INDUCED REDUCTION IN MACROPHAGE EXPRESSION OF ENZYMES.....	120
FIGURE 3.4: THE VIABILITY OF MDMs WAS NOT AFFECTED BY 24- OR 96-HOUR TREATMENT WITH Ro 41-5253	122
FIGURE 3.5: THE RAR α INHIBITOR Ro 41-5253 DOES NOT ALTER THE MITOCHONDRIAL FUNCTION	124
FIGURE 3.6: ILLUSTRATIVE GRAPHIC DEMONSTRATING HOW AUC WAS CALCULATED FOR EACH CONDITION	126
FIGURE 3.7: THE RAR α INHIBITOR Ro 41-5253 PREVENTED THE GLYCOLYTIC RESPONSE OF MDMs TO MTB.....	128
FIGURE 3.8: THE RAR α INHIBITOR Ro 41-5253 DOES NOT ALTER THE MITOCHONDRIAL FUNCTION	130
FIGURE 3.9: BLOCKING PPAR γ OR MEK1 PRIOR TO TREATING MDMs WITH Ro 41-5253.....	132
FIGURE 3.10: AN ALTERNATIVE RAR α INHIBITOR, ER 50891, REPLICATED THE EFFECTS OF Ro 41-5253	134
FIGURE 3.11: A PAN-RAR α INHIBITOR, AGN 194310, AND AN ALDH INHIBITOR, DEAB, DID NOT REPLICATE	136
FIGURE 3.12: RAR α INHIBITION DID NOT SIGNIFICANTLY REDUCE THE mRNA EXPRESSION OF GLYCOLYTIC GENES.....	139
FIGURE 3.13: RAR α INHIBITION DOES NOT AFFECT THE CYTOKINE RESPONSE OF MDM'S INFECTED WITH MTB	141
FIGURE 3.14: RAR α INHIBITION IMPAIRS MDMs' ABILITY TO KILL MTB.	143
FIGURE 3.15: PROPOSED NEW MECHANISM CONTRIBUTING TO THE ASSOCIATION BETWEEN VAD AND TB.....	146
FIGURE 3.16: NORMALISED, NON-BASELINED TRACINGS FOR FIGURES 3.7, 3.9, 3.10.	152
FIGURE 3.17: NORMALISED, NON-BASELINED TRACINGS FOR FIGURE 3.11.	153
FIGURE 4.1: ATRA DOES NOT KILL RAPID OR SLOW GROWING NTMs IN AXENIC CULTURE.....	158
FIGURE 4.2: THE VIABILITY OF MDMs WAS NOT AFFECTED BY 24- OR 96-HOUR TREATMENT WITH ATRA	159
FIGURE 4.3: THE VIABILITY OF THP-1s WAS NOT AFFECTED BY M. ABSCESSUS INFECTION	161
FIGURE 4.4: ATRA REDUCES CFUs IN M. CHIMAERA INFECTED THP-1 MACROPHAGES	163
FIGURE 4.5: ATRA DOES NOT AFFECT THE INTRACELLULAR CHOLESTEROL CONCENTRATION.....	164
FIGURE 4.6: ATRA REDUCES MACROPHAGE IL-10 SECRETION IN RESPONSE TO NTM INFECTION	166
FIGURE 4.7: ATRA INDUCED THE WARBURG EFFECT IN THP-1 MACROPHAGES	168
FIGURE 4.8: M. CHIMAERA AND M. ABSCESSUS INFECTIONS INDUCE GLYCOLYSIS IN HUMAN AMs.....	170

FIGURE 4.9: ATRA PREVENTED THE GLYCOLYTIC RESPONSE OF AMs TO M. CHIMAERA	171
FIGURE 4.10: ATRA PREVENTED THE GLYCOLYTIC RESPONSE OF AMs TO M. ABSCESSUS.....	173
FIGURE 4.11: NORMALISED, NON-BASELINED TRACINGS FOR FIGURES 4.7, 4.8, 4.9, 4.10.....	180
FIGURE 5.1: FOLLOWING DROPLET TRANSMISSION, SARS-CoV-2 ENTERS THE AIRWAYS	183
FIGURE 5.2: SARS-CoV-2 ENVELOPE PROTEIN, BUT NOT SPIKE PROTEIN, INDUCES THE SECRETION	188
FIGURE 5.3: ANTIBODIES AGAINST TLR4 DIMINISH AM CYTOKINE RESPONSES TO LPS.	190
FIGURE 5.4: BLOCKING ANTIBODIES AGAINST TLR2, BUT NOT TLR4, ALTER THE CYTOKINE.....	191
FIGURE 5.5: ALVEOLAR MACROPHAGES FROM DONORS AGED OVER 75 YEARS OLD HAD HIGHER	193
FIGURE 5.6: ALVEOLAR MACROPHAGES FROM DONORS AGED OVER 75 YEARS OLD	195
FIGURE 5.7: ALVEOLAR MACROPHAGES FROM SMOKERS HAD LOWER CYTOKINE RESPONSES	197
FIGURE 5.8: ALVEOLAR MACROPHAGES FROM SMOKERS DID NOT HAVE LOWER CYTOKINE RESPONSES	199
FIGURE 5.9: SARS-CoV-2 OF AMs DID NOT INDUCE CYTOKINE SECRETION FROM AMs.....	201
FIGURE 5.10: SARS-CoV-2 INFECTION OF AMs DID NOT RESULT IN A TYPE-1 OR TYPE-3 INTERFERON RESPONSE	203

List of Tables

TABLE 1.1: IN VITRO AND IN VIVO STUDIES OF ATRA AS A HDT AGAINST MTB.	50
TABLE 2.1: MACROPHAGE TREATMENTS AND PRE-TREATMENTS.	87
TABLE 2.2: MACROPHAGE INFECTIONS AND STIMULATIONS.	97
TABLE 2.3: TAQMAN PRIMERS USED FOR PCR.	99
TABLE 2.4: MITOCHONDRIAL FUNCTION PARAMETERS AND EQUATIONS FOR THEIR DERIVATION	106
TABLE 5.1: AM DONOR CHARACTERISTICS.	187

Abstract

For most of recent history, Tuberculosis (TB) has been the leading cause of death by an infective agent worldwide. Tuberculosis is caused by *Mycobacterium tuberculosis* (Mtb), which infects human lungs following respiratory transmission. Epidemiological studies have estimated up to a tenfold higher risk of active TB infection among those with vitamin A deficiency (VAD), but the mechanism of this increased risk is currently undefined. All *trans* retinoic acid (ATRA) is the active metabolite of vitamin A and has previously been found to improve the ability of human macrophages to kill Mtb when given exogenously, by affecting macrophage cytokine production, autophagy and cholesterol pathways. However clinical trials of vitamin A, in the form of retinol, as an adjunct to TB therapy have failed to show any measurable clinical benefit. We show that Mtb infected human macrophages likely have a reduced capacity to oxidise retinol to ATRA, and a reduced capacity to deliver ATRA to its nuclear receptors due to the reduced expression of key enzymes and carrier proteins. This may partly explain why clinical trials of retinol have failed and suggests that ATRA itself is worthy of investigation in clinical trials. We also show that blocking the signalling of *endogenously* produced ATRA within human macrophages impairs early metabolic responses to Mtb and impairs the ability of macrophages to kill Mtb. This may partly explain why those with VAD are at increased risk of TB.

Nontuberculous mycobacteria (NTM) also cause respiratory infections, typically in patients with underlying structural lung disease or immunodeficiencies. NTM infections have increasing incidence worldwide and have worse treatment outcomes than TB due in large part to frequent drug resistance and relapse. It is unknown if ATRA, which is an effective host-directed therapy (HDT) for Mtb *in vitro* and *in vivo* in animal models of TB, might also improve host responses to NTMs, such as the slow grower *Mycobacterium chimaera* and the fast grower *Mycobacterium abscessus*. We found that ATRA is an effective HDT for *M. chimaera* *in vitro*, but not for *M. abscessus*. ATRA altered macrophage metabolic responses to NTMs and altered macrophage cytokine responses to *M. chimaera*. ATRA treated macrophages secreted less anti-inflammatory IL-10 when

infected with *M. chimaera* which may support phagolysosomal maturation. ATRA may hold promise as an adjunctive HDT for slow growing NTM infections.

In more recent years, TB has been overtaken as the leading infectious killer by COVID-19. SARS-CoV-2 also infects human lungs via respiratory transmission. Older adults, in particular those over the age of 75, are at far higher risk of severe infections and mortality due to COVID-19. Little is known about human alveolar macrophage (AM) responses to SARS-CoV-2 antigens, such as the spike protein and envelope protein. We found that human AMs do not sense SARS-CoV-2 spike protein but do sense envelope protein via the pattern recognition receptor TLR4, secreting IL-1 β , IFN γ , IL-12p70, IL-6 and TNF α in response. AMs from donors over the age of 75 years produced significantly more cytokines than those from younger patients following stimulation with SARS-CoV-2 envelope protein. This may partly explain why older adults are at such high risk of severe lung inflammation in COVID-19.

In this thesis we demonstrate how studying the early immune responses of human macrophages to a diverse set of emerging and enduring respiratory pathogens – Mtb, NTMs and SARS-CoV-2 – can reveal findings that are relevant to future laboratory and clinical research.

Lay Abstract

Tuberculosis (TB) is a lung infection that kills more people worldwide than any other infection. TB is caused by bacteria that spread from person to person through the air by coughing. People who have lower vitamin A levels are more likely to get TB infections, but we don't know exactly why. In laboratory experiments, an 'activated' type of vitamin A was previously shown to help human immune cells, called macrophages, to kill TB bacteria. But when vitamin A was given to patients with TB, it did not improve their treatment. We did laboratory experiments and found that macrophages that are infected with TB bacteria are less able to activate and deliver vitamin A. This might explain why vitamin A didn't improve treatment for patients with TB. Giving activated vitamin A to patients with TB might work better. In another experiment, we found that macrophages activate vitamin A and use it to boost their energy use when infected by TB bacteria, and use activated vitamin A to help them kill TB bacteria. This might explain why people with lower vitamin A levels are more likely to get TB infections.

We also studied infections caused by bacteria that are closely related to TB bacteria, called nontuberculous mycobacteria (NTM). NTM infections are becoming more common, can be more difficult to treat than TB infections and usually affect people who have problems with their lungs or immune systems. It is not known if activated vitamin A helps macrophages to kill NTM bacteria, like it does for TB bacteria. We found that activated vitamin A does help macrophages kill a slow growing type of NTM bacteria, but not for a rapid growing type of NTM bacteria. We found that activated vitamin A changes how macrophages use energy and what immune messengers, called cytokines, they produce when infected with NTM bacteria. Activated vitamin A might improve the treatment of patients with infections due to slow growing NTM bacteria.

More recently, COVID-19 has killed more people than TB. Old people are more likely to get severe COVID-19 lung infections and are more likely to die from COVID-19. It is unknown if human lung macrophages can sense and respond to parts of the COVID-19 virus, such as the spike protein or the envelope protein. In our experiments, we found that human lung macrophages sense the envelope protein of the COVID-19 virus and that

human lung macrophages from older people produce more cytokines than those from younger people. This might help explain why older people get worse COVID-19 infections.

Aims and Hypotheses

Aim 1: Defining the role of endogenous macrophage production of ATRA in Mtb infection.

Hypothesis 1: Endogenous macrophage ATRA production supports macrophage defences during Mtb infection.

In chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) we aimed to determine the effect of Mtb infection on human macrophage vitamin A pathways and to determine the effects of *endogenously* produced ATRA on macrophage responses to Mtb infection. We investigated whether human macrophages express the enzymes needed to produce ATRA from retinol and the carrier proteins needed to deliver ATRA to its nuclear receptors, and if their expression is altered by Mtb infection. We also investigated whether macrophage responses to Mtb were dependent on the signalling of *endogenously* produced ATRA.

Aim 2: Investigating exogenous ATRA as a potential HDT for NTM infections.

Hypothesis 2: ATRA is an effective HDT for NTM infections, *in vitro*.

In chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) we aimed to determine if *exogenous* ATRA is an effective HDT against NTMs *in vitro*, and if so by what mechanisms. Specifically, we determined whether ATRA inhibited the growth of *M. chimaera* or *M. abscessus* in a macrophage dependent manner. Additionally, we tested the hypotheses that ATRA would improve the microbiocidal activity of macrophages by reducing macrophage intracellular cholesterol concentrations and altering their cytokine and metabolic responses to infection with these NTMs.

Aim 3: Determining the cytokine responses of human AMs to SARS-CoV-2 antigens.

Hypothesis 3: Human AMs mount a cytokine response to SARS-CoV-2 antigens via engagement with TLRs but are not productively infected by the virus.

In chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) we aimed to determine if and how human AMs sense SARS-CoV-2 antigens, and to understand what their cytokine responses are and if these responses vary by age and smoking status. We hypothesised that SARS-CoV-2 spike protein or envelope protein induce the secretion of cytokines from human AMs, and those cytokine responses are dependent on TLR2 or TLR4. We also aimed to investigate if human AMs can be infected by or produce a cytokine response to *live* SARS-CoV-2.

Value of Research

In chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) many of our research findings are novel. We are the first to demonstrate the expression of the vitamin A oxidation enzymes DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1 and the ATRA carrier protein FABP5 in human monocyte-derived macrophages (MDMs) and AMs. We are the first to report that the macrophage expression of these vitamin A enzymes and ATRA carrier proteins is reduced by Mtb infection and LPS stimulation. We are the first to show that the signalling of endogenously produced ATRA is important in human macrophage metabolic responses to Mtb and their ability to kill Mtb. Our chapter 3 findings are relevant to the clinical domain, and potentially impactful, on several fronts. Our finding that macrophage production of ATRA is impaired by Mtb suggests that, despite negative clinical trials using retinol, vitamin A should not yet be ruled out and support the clinical investigation of exogenous ATRA (not retinol), as a HDT in TB infection. Our finding that impaired signalling of endogenously produced ATRA results in impaired energetic responses to Mtb and impaired macrophage killing of Mtb may partially explain why VAD confers such a high risk of active TB. Our research supports the pursuit of clinical trials examining the efficacy of ATRA as a prophylactic agent against active TB in certain risk groups. Using ATRA for TB prophylaxis or as an adjunct to TB therapy might go some way to address several clinical challenges for TB – the leading infectious cause of death – such as disease prevention in high-risk patients, drug resistance, treatment durations and TB immunopathology.

In chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) our results were novel and potentially impactful in several ways. Ours is the first direct evidence of an effective HDT for an NTM infection. Our experiments are also the first examining the *real time* cellular metabolic responses during an infection with *live* mycobacteria (including Mtb) and validate the findings of other researchers who used irradiated mycobacteria. We are the first to document the metabolic effects of ATRA on AMs, and the first describing ATRA's effects on cellular metabolism during an infection with a live pathogen. NTM infections have rising incidence, affect the

immunosuppressed or those with underlying lung disease, and have high treatment failure and relapse rates. Our chapter 4 results should be built upon using murine models of NTM infection and then, perhaps, clinical trials. If effective, adjunctive ATRA might reduce the long durations of treatment currently required for NTM infections. An effective HDT might reduce the high rates of relapse if given after antimycobacterial therapy or prevent the onset of disease if given to culture positive patients without a diagnosis of NTM *disease*.

In chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) we provide novel evidence that human AMs produce cytokines in response to SARS-CoV-2 antigens and we correlate those cytokine responses with clinical risk factors. Ours is the first evidence of age affecting cytokine responses of *human* AMs. In fact, while others have reported age differences with human monocytes, this is the first evidence of age affecting the cytokine responses of human *macrophages* of any kind, to any stimulus. We therefore uncover a potential mechanism by which the innate pulmonary response to COVID-19 in the elderly differs from younger adults, in a manner that could plausibly be pathological. Our findings would strongly support future research initiatives investigating envelope protein-containing vaccines, envelope protein-targeting therapeutic antibodies, TLR4 pathway antagonists or inhaled therapies targeting the AM inflammasome. Such novel preventative or therapeutic applications of our research, used in conjunction with existing vaccines and therapies, might prevent clinical infections, reduce immunopathology, improve clinical outcomes and reduce the suffering of those with COVID-19 infections.

Chapter 1

1 Introduction

1.1 Ancient and Modern Threats

In the late 20th century, beneath the eastern Mediterranean, the remains of a young mother and her infant child were excavated from a now-submerged, 9,000-year-old neolithic settlement named Atlit-Yam. Palaeopathological features of tuberculosis (TB) were evident on inspection of both skeletons, and bone samples were analysed using polymerase chain reaction (PCR). The DNA of *Mycobacterium tuberculosis* (Mtb) was identified in both individuals (2) (*Figure 1.1*). Genetic modelling studies have projected the common ancestry of Mtb subspecies back millions of years to east Africa, at the right time and place to encounter early hominids – the first gunshots before a long stalemate (3). Mtb is a truly ancient foe whose story has been interlaced with *Homo sapiens*' story since the first paragraph of chapter one.

TB is an ancient threat but not a historical one. In fact, TB remains unparalleled to the present day, consistently killing more than any other pathogen outside the exceptional pandemic years. In 2021, Mtb infected 10.6 million and killed 1.6 million people – both rising over time (4). Mtb's first cousins, the nontuberculous mycobacteria (NTM), also cause significant morbidity and mortality and are on the rise too. NTMs predominantly afflict the most vulnerable as opportunistic infections and are more difficult to treat than TB (5).

In contrast to Mtb, SARS-CoV-2 is not ancient but rather the most modern of human pathogens (6). Despite its short existence, having caused the first confirmed COVID-19 case in late December 2019, SARS-CoV-2 has dethroned Mtb as the leading killer for the time being. In three years, SARS-CoV-2 has been responsible for *billions* of infections and millions of excess deaths (7,8). Those costs are in spite of unprecedented mitigation measures which have exacted their own unmeasurable economic, social and political costs – not to mention untold individual human isolation and quiet misery (8–10). The pandemic shockwave blasted through healthcare infrastructure. TB notifications and

treatment enrolment crashed into freefall and are only now starting to recover – a conspiracy of new and old foes (11).

At first glance, the slow intractability of the ancient TB pandemic seems quite distinct to the blistering force of the modern SARS-CoV-2 pandemic. But both point to a unique human vulnerability – a chink in the armour of the immune system and a zero-day bug in the network firewall – in the form of the human lungs.



Figure 1.1: The preserved, underwater, skeletal remains of a mother and child at Atlit-Yam, that tested positive for Mtb DNA (yellow arrow points to child's remains). Dating back over 9,000 years, this is the earliest indisputable evidence of TB in humans, although TB has likely co-evolved with its human hosts for much longer (2). Indirect palaeopathological evidence that is suggestive of, but not specific for TB meningitis in a Homo erectus skeleton that is half a million years old, and genetic modelling studies push the co-evolution of TB and hominids back much further still (12). Photo credit: HersHKovitz et al. (2).

1.2 Host Defence against Infection in the Lung

Each day, over ten thousand litres of air flow in and out of our lungs. This air, together with its suspended particles and pathogens, is brought into direct contact with the inner lung surface, which has an area of over 100 m² (13–15). Understandably, this has been described as a 'critical immune interface'. Evolution has bestowed the host with multiple layers of defence to prevent and fight infections.

The physical barrier of airway mucous is continuously cleared by mucociliary transport, sweeping back trapped invaders. Structural lung diseases such as bronchiectasis interfere with this important clearance mechanism (16). Pathogens that survive and penetrate the airway mucous encounter the mucosal barrier made of tightly packed epithelial cells. Epithelial cells secrete IgAs and other effector molecules which neutralise viruses and block bacterial invasion mechanisms (13,14).

Innate immune cells patrol the airways, in particular the native, tissue resident alveolar macrophages (AMs). AMs are the most prevalent immune cell in the resting airways and function as phagocytes, as antigen presenters and as sentinel immune cells that signal to other immune cells. This signalling function is mediated through the production of cytokines and chemokines that summon other innate and adaptive immune cells to the lung and affect their activation (13–15). Given that AMs are the first *professional* immune cells to interact with pathogens in the earliest moments of natural infection, and given their role in summoning and activating other innate and adaptive cells, it is perhaps unsurprising that AMs make a critical contribution to host defence against viruses (17,18), bacteria (19) and fungi (20).

Immune cells that traffic to the lung in response to AM signals include innate cells (neutrophils, monocytes, dendritic cells) as well as adaptive cells (CD4 and CD8 T cells). These recruited cells contribute to the cytokine milieu and to host defence through NETosis (neutrophils), phagocytosis (monocytes), activation of AMs (IFN γ secreted by CD4 T cells), presentation of antigens in lymph nodes (dendritic cells) and inducing apoptosis of infected cells (CD8 T cells), among many other mechanisms (13–15,21).

The cumulative result of these many processes is pulmonary inflammation. While these immune responses to infection have evolved to protect the host, they too contribute to pathology. Counterintuitively, some models of viral infection (22) (but not others (23–26)) suggest that AMs are on balance *net contributors* to disease, as their depletion reduces pathology. Similarly, depletion of AMs reduces Mtb burden in mouse models of infection (27,28). Excessive pulmonary inflammation on occasion results in the acute respiratory distress syndrome (ARDS). ARDS is characterised by alveolar epithelial cell death, oedema and dysregulated coagulation. In this setting, AMs play another crucial role – in tissue repair and the resolution of ARDS (29).

Whether during the first encounters or during the resolution of late-stage pulmonary inflammation and pathology, AMs are influential in the host defence against infection. This is certainly the case for SARS-CoV-2, and for mycobacterial infections – the subjects of this thesis.

1.3 Vitamin A as a Host-Directed Therapy for Mycobacterial Infections

1.3.1 *Mycobacterium tuberculosis*: Epidemiology, Microbiology and Pathophysiology

As already mentioned, Mtb infected 10.6 million and killed 1.6 million people in 2021 (4). These cases and deaths are geographically concentrated – the Central African Republic, South Africa and the Philippines have incidences of active TB greater than 500 per 100,000 of population per year while Ireland, the United States and Australia all have incidences lower than 10 per 100,000 (4). For most years over the last two decades, TB has been the leading killer by an infectious agent and is still the leading cause of death among people with HIV, globally (30). HIV increases the risk of active TB, like other risk factors such as malnutrition, smoking status, alcohol dependence, diabetes mellitus and the use of immunosuppressive medications such as TNF α inhibitors (4,31,32). Certain social cohorts are more likely to acquire TB or develop active TB infections, including contacts of known TB cases, healthcare workers, migrants from high incidence countries, people who are homeless, prisoners and those who inject drugs (31,33). Animal contact confers a risk of TB infection due to the zoonotic transmission of *Mycobacterium bovis* (34).

Mtb is a slow-growing, acid-fast, non-motile, aerobic bacillus with a waxy outer cell wall, rich in mycolic acid. The Mtb complex includes several species causing human infection. Those include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti* – the progenitor species from which other Mtb species evolved (35). Mtb is transmitted via respiratory droplets. Upon entry in the airways, it is phagocytosed by AMs. ‘Early clearance’ of Mtb by innate immune cells occurs in a significant fraction of those infected, before the adaptive immune system has had a chance to record an Mtb-specific immune memory (19). Killing of Mtb by AMs occurs through the fusion of phagosomes with acidic lysosomes via the endolysosomal system or autophagy, through oxidative stress, through the production of antibacterial proteins, and through the apoptotic death of Mtb-containing AMs (19). However, Mtb produces virulence factors that can interfere with these processes and allow it to persist within the phagosome and even escape to the cytoplasm and extracellular space (35). There is even some evidence that Mtb-containing AMs migrate into the interstitium and so assist in its dissemination (36). In more recent

years, the immunometabolic responses of macrophages to infection have been linked with enhanced effector functions such as phagocytosis and cytokine production (37). More specifically, macrophages infected with Mtb rapidly upregulate glycolysis, and this increased glycolysis is necessary for them to secrete IL-1 β and contain Mtb growth (28,38,39). Like for other host defence processes, virulent Mtb strains may interfere with macrophage immunometabolic responses (40).

Where the innate immune system fails in early clearance, a primary active TB infection can ensue in about 10%, with an associated risk of onward transmission (35). Alternatively, the Mtb can persist within granulomas – a cooperation between innate and adaptive cells to contain the bacillus. This quiescent, *latent* TB infection (LTBI) is identifiable in asymptomatic individuals by the release of interferon gamma by CD4 T cells stimulated with Mtb antigens. This latent infection confers a 5-10% lifetime risk of reactivation to an active TB infection, a risk which is much higher in the immunosuppressed (4,35). Approximately one quarter of the global population is estimated to have LTBI (41).

1.3.2 *Mycobacterium tuberculosis* Infections: Clinical Challenges

A confluence of factors in TB combines to present extraordinary clinical and public health challenges:

- TB infection frequently occurs in the immunosuppressed, more often presenting with disseminated and extrapulmonary disease in these patients (32,42).
- TB infection frequently occurs in hard-to-reach members of the population, complicating access to care and the management of cases (31).
- TB infection frequently occurs in patients with multiple co-morbidities and co-medications, complicating management and prescribing (31,43).
- TB is transmissible. Exactly how transmissible is difficult to estimate given its long incubation period, among other factors (44). Transmissibility complicates patient management (need for isolation), leads to outbreaks and the spread of resistance, and demands the follow-up of close contacts in many settings. This

factor also complicates efforts to eliminate TB, as a large reservoir of asymptomatic individuals with LTBI continually spawns new active TB disease and transmission.

- Increased global travel puts travellers from lower incidence countries at risk of exposure to TB and migrants arriving from higher incidence countries at risk of importing TB and drug resistance (45).
- Delays in TB treatment are typical as infections can involve any body site, mimic other common diseases, and therefore present a diagnostic challenge. Stigma also is a factor in delayed presentations. Even when clinically suspected, the microbiological diagnosis of TB is often difficult (43).
- TB therapies are relatively toxic, interact with other many other medications, and treatment durations are long compared with other infections (46). As a result, adherence and treatment success can suffer, with one in seven patients starting TB treatment not attaining a successful outcome (4).
- Mtb readily acquires drug resistance if not treated with multiple effective drugs under careful, specialised supervision. This places high demands on healthcare infrastructures, most often in low-resource countries. Resistance to even the most recently developed antituberculous agents has emerged – the supply of new agents through the drug development pipeline can barely quench the thirst for new therapies (47,48).
- In 2021 there were an estimated 450,000 cases of MDR TB, with only one third prescribed treatment. Traditionally, treatment of drug resistant TB has required even longer treatment durations, although recent innovations have reduced durations for these patients (4,49).

To overcome several of these challenges, many researchers have been developing therapies that target the host's ability to kill Mtb, rather than the bacillus itself (50). If the promise of such host-directed therapies (HDTs) is vindicated by future research, they could complement antimycobacterials to reduce treatment durations, reduce pathology, and would be effective even in highly drug resistant Mtb infections. Because they target the host, they may also be effective in other infections such as

those caused by nontuberculous mycobacteria, which present many challenges of their own.

1.3.3 Nontuberculous Mycobacterial Infections: Microbiology and Epidemiology

‘Nontuberculous mycobacteria’ (NTM) is a term used to classify different species of mycobacteria that do not cause tuberculosis or leprosy. NTMs are subdivided into ‘rapid growers’ and ‘slow growers’, depending on whether they produce mature colonies on agar by 7 days or less, or more than 7 days. Both rapid and slow-growing NTM species can cause human disease (51). Rapid growers were the evolutionary ancestors to all NTMs, and mycobacteria causing tuberculosis and leprosy. A single evolutionary split resulted in the development of all slow-growing mycobacteria, including slow-growing NTMs and *M. tuberculosis* (52). In the environment, NTMs can infect and survive in amoeba trophozoites. This equips them to be able to survive within macrophages and is postulated to be an important evolutionary milestone in the transition from purely environmental to pathogenic mycobacteria (51). There is variation in the species causing NTM infections across different geographies (53). Some notable NTM species that cause human disease include: species of the *M. avium* complex (MAC), which account for approximately half of global NTM lung disease (53); *M. ulcerans*, the cause of Buruli ulcer; and *M. marinum*, which causes a sporotrichoid skin and soft tissue infection, associated with exposure to aquaria (51).

The global burden of NTM infections is difficult to estimate given the difficulty in diagnosing NTM infections, the confounding factor of NTM colonisation and the fact that NTM infections are not notifiable in most countries. However, in the United States (US), the incidence of NTM infections has been estimated at 4.73 per 100,000 person-years and was found to be increasing (54). This compares with a falling incidence of active TB of 2.4 per 100,000 person-years in the United States in 2021 (55). This increasing incidence is being observed globally (5).

M. abscessus is an example of a rapid-growing NTM species complex, consisting of three subspecies: *M. abscessus* subsp. *abscessus*; *M. abscessus* subsp. *bolletii* and *M. abscessus*

subsp. *massiliense*. This complex can manifest 'smooth' and 'rough' colony morphologies, engendered by glycopeptidolipid (GPL) expression (smooth), or non-expression (rough), on the nontuberculous mycobacterial cell wall (GPL is not expressed by Mtb). These two morphologies are associated with differing experimental phenotypes: rough variants are phagocytosed in clumps, whereas smooth variants are taken up singly; smooth variants inhibit phagosomal maturation, whereas the rough variant-containing phagosomes fuse readily with lysosomes; smooth variants escape the phagolysosome and induce granuloma formation whereas rough variants induce apoptosis and undergo extracellular replication; and rough variants are more virulent than smooth variants in zebrafish infection models (51,56). *M. abscessus* bacilli can irreversibly transition from smooth to rough *in vivo* via genetic mutations, which leads to a step change in virulence (51).

M. chimaera is an example of a slow-growing NTM species. It is the newest identified member of the *M. avium* complex and sits alongside *M. avium* and *M. intracellulare*. MAC species can produce rough or smooth colonies, and sequencing techniques are required to distinguish *M. chimaera* from other members of the complex (57).

Both *M. abscessus* and *M. chimaera*, like other mycobacteria, are acid-fast, non-motile aerobic bacilli with a waxy outer cell wall, rich in mycolic acid (51). *M. abscessus* and *M. chimaera* are the subject NTM species of our research in chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) of this thesis. *M. abscessus* was chosen because it is the most common cause of pulmonary disease due to rapidly growing mycobacteria, and because it is associated with particularly poor clinical outcomes and therefore represents a major clinical challenge. *M. chimaera* was chosen because it is a member of the *M. avium* complex, which together are the most common cause of pulmonary disease due to slow-growing mycobacteria, and because it has been associated with iatrogenic outbreaks (57–59).

1.3.4 Transmission of Nontuberculous Mycobacteria

NTMs are sometimes called environmental mycobacteria as they are, unlike Mtb, ubiquitous in soil and water. As such, regular exposure to NTMs is the norm: one third of

participants in a random-household survey in Florida had a positive *M. avium* sensitin skin test (60). The most ubiquitous NTM species in garden soil are also those that most commonly cause human disease (61). Humans acquire NTM infections predominantly through environmental exposure, via inhalation of aerosols from contaminated soil or water sources in the cases of *M. abscessus* and members of the *M. avium* complex (such as *M. chimaera*), or via percutaneous inoculation in the cases of *M. marinum* or *M. ulcerans*. Although evidence of human-to-human transmission of NTM infections is scant (in contrast to *M. tuberculosis* and *M. leprae*), comparative phylogenetic analysis of *M. abscessus* strains between patients attending cystic fibrosis clinics is suggestive of some human-to-human transmission in this specific situation (62,63).

Iatrogenic *M. abscessus* outbreaks have also been reported among ‘lipotourists’, presumably resulting from contaminated surgical equipment (64). *M. chimaera* has been similarly linked with iatrogenic outbreaks due to contaminated equipment – specifically airborne transmission from heater-cooler devices used to regulate body temperature as part of extracorporeal membrane oxygenation in patients undergoing cardiothoracic surgery. This global outbreak has resulted in over 120 identified cases of *M. chimaera* infection, with mortality rates reaching 50-60% (57,59). The propensity of both *M. chimaera* and *M. abscessus* to be transmitted from contaminated surfaces is attributable to their ability to form robust biofilms (51,57).

1.3.5 Risk factors for Nontuberculous Mycobacterial Infections

Risk factors for NTM infections, such as those caused by *M. chimaera* and *M. abscessus* include immunocompromise, structural lung disease (e.g. cystic fibrosis (CF) and non-CF bronchiectasis) or a particular exposure history (e.g. cardiothoracic surgery, ‘lipotourism’).

While pulmonary NTM infections can occur in those without apparent underlying risk factors, disseminated infection (in the absence of an iatrogenic infection history) is a definite sign of an underlying immune defect (65). In those with HIV, the risk of NTM infections has sharply declined as ART has become available, is seen predominantly in

those with a CD4 count <50 cells/mm³ and is reduced by prophylactic azithromycin (66,67). Azithromycin prophylaxis is now recommended in clinical guidelines for those with CD4 counts <50 cells/mm³ (68). Apart from HIV, inherited defects of IFN γ or IL-12 and treatment with immunosuppressive therapies such as corticosteroids or TNF α inhibitors have also been associated with NTM infections and Mtb (51,65,69).

Structural lung diseases such as CF and non-CF bronchiectasis are characterised by defective physical immunity – impaired ciliary clearance of inhaled pathogens (69). Routine exposure to environmental NTMs that are capable of biofilm formation can result in airway colonisation in these patients, much in the same way that biofilm-forming *Pseudomonas aeruginosa* can (51,70). One meta-analysis calculated an NTM prevalence of 9.3% in patients with bronchiectasis (71). Not all bronchiectatic patients with sputa culturing positive for NTM species will meet the diagnostic criteria for pulmonary NTM infections, and some will merely be colonised without evidence of disease. One retrospective study of 182 patients with non-CF bronchiectasis found that 68 (37%) had NTM isolates and 55 (30%) met the diagnostic criteria for infection (58,72).

Patients with pulmonary nontuberculous mycobacterial disease are more likely to be hyporetinolaemic than healthy controls, although there is no *strong* evidence to link micronutrient deficiencies with NTM infection risk (73).

1.3.6 Clinical Aspects of Nontuberculous Mycobacterial Infections

Several factors collude to make the management of NTM infections challenging – frequently more challenging than the treatment of tuberculosis: unlike with TB, rapid diagnostics are not available for NTM infections; NTM species are typified by inherent and acquired drug resistance; high bacillary burdens are cloistered within biofilms in structurally damaged airways – at the outer reaches of the antimycobacterial concentration gradient; NTM infections almost always occur in those with underlying immune defects or an irreversible structural lung abnormality; and environmental re-exposure to NTMs is inevitable. Unsurprisingly, patients with confirmed NTM infections have a higher mortality rate than those with NTM colonisation, without NTM colonisation

but with comparable lung disease, and compared to the general population. One study in Korea, which captured the vast majority of the population, derived a 5-year mortality rate of 17.8%, 2.13 times the background rate of the general population (74–76).

In addition to infecting those with non-CF bronchiectasis, 10-20% of patients with cystic fibrosis develop NTM infections. *M. abscessus* is the most common cause in these patients and is usually ineradicable despite treatment (51,77). *M. abscessus* pulmonary infection is characterised radiologically by reticulonodular opacities, unilateral or bilateral cavitation and consolidation (78). *M. abscessus* colonisation and infection are also considered strong relative contraindications for lung transplant in patients with cystic fibrosis, as it can result in dissemination with post-transplant immunosuppression, though transplants have been successful in some cases (79–81). In addition to the aforementioned, iatrogenic soft tissue infections in ‘lipotourists’, *M. abscessus* can also cause skin and soft tissues infections and even osteomyelitis in immunocompetent hosts following penetrating trauma (82). Disseminated *M. abscessus* infections are unusual but have resulted from prosthetic valve endocarditis and immunosuppression secondary to IFN γ auto-antibodies, corticosteroid use and the use of immunosuppressants following solid organ transplant. HIV is notably absent as a predisposing factor for disseminated *M. abscessus* infections, in contrast to the risk HIV confers for disseminated *slow-growing* mycobacterial infections (82,83).

M. chimaera pulmonary infection is also characterised by cavitations and necrotising pneumonia and treatment is as for other MAC species (57,59,84). Disseminated *M. chimaera* infection has occurred following iatrogenic transmission associated with cardiothoracic surgery. The latent period between exposure and symptom onset is 21 months, with a range of up to 5 years (85). Manifestations have included *M. chimaera* prosthetic valve endocarditis, sternotomy wound infection, mediastinitis, vascular graft infection, mycobacteraemia, septic arthritis, osteomyelitis, bone marrow infection, pulmonary septic emboli, chorioretinitis and encephalitis (57,59,84,86–88). Mortality in these outbreak cases is estimated at 50-60% (85). In patients with HIV, disseminated MAC infections usually follow intestinal colonisation and infection, but the lungs can also be the port of entry. Unlike *M. avium* and *M. intracellulare*, *M. chimaera* has yet to be reported as a cause of disseminated infection in patients with HIV, although it has been

described in a haematology patient (89,90). Sequencing techniques are needed to distinguish *M. chimaera* from other MAC species, so the true proportion of disseminated MAC in HIV patients that are caused by *M. chimaera* is unknown.

1.3.7 Treatment of Nontuberculous Mycobacterial Infections

In light of the fact that both contamination and colonisation can occur, the joint Infectious Diseases Society of America and American Thoracic Society guidelines recommend treatment only when diagnostic criteria for pulmonary NTM disease are met. Those diagnostic criteria include symptoms consistent with pulmonary NTM disease, radiologically characteristic findings of pulmonary NTM disease and two microbiologically positive sputum samples (or one bronchoscopic sample) (58). In disseminated infections, when NTM species are grown in normally sterile sites such as blood or bone marrow, there is no such ambiguity and treatment is universally recommended.

Pulmonary *M. abscessus* infections are treated with an intensive phase which includes three or four active agents selected by *in vitro* susceptibility testing, followed by a continuation phase containing two or three agents. The optimal duration of *M. abscessus* treatment is not well evidenced, but most patients are treated initially for over a year. Agents used include parenteral amikacin, imipenem and tigecycline, in addition to oral macrolides, fluoroquinolones, linezolid and clofazimine. *M. abscessus* is inherently resistant to both rifampicin and ethambutol. The presence of a functional *erm* gene confers inducible macrolide resistance in *M. abscessus*, or alternatively, mutational macrolide resistance can occur. Macrolide resistance is a strong predictor of poor treatment outcomes, with only 8% of macrolide-resistant cases achieving culture conversion in one study. Given the low probability of sustained culture conversion with current therapies, some experts recommend repeated, shorter, intense courses of therapy for select patients. In clinical trials, good treatment outcomes were observed in only 23% of patients with pulmonary infections due to *M. abscessus* subsp. *abscessus*, highlighting the enormous clinical need for new treatment strategies (58). Disseminated *M. abscessus* infections are exceptionally rare, are usually associated with

immunosuppression (although they are not reported with HIV) and often have poor outcomes (82,91). One case report describes a post-transplant cystic fibrosis patient who developed a treatment-refractory, disseminated *M. abscessus* infection that resolved following an experimental bacteriophage treatment (81).

Pulmonary *M. chimaera* infections are diagnosed with the same criteria as pulmonary *M. abscessus* infections and are treated the same as other members of the *M. avium* complex. Choice of therapy is guided by susceptibility testing and consists of at least three drugs. Macrolides (azithromycin, in particular) are recommended for inclusion if susceptibility testing supports their use, as they improve treatment outcomes. As for *M. abscessus*, macrolide resistance can result from mutations of the 23S rRNA (*rrl*) gene. Other first-line therapies for pulmonary MAC infections are ethambutol, rifampicin and intravenous amikacin (in particular as a fourth agent if cavities or severe bronchiectasis are present). The standard treatment duration is 12 months after culture conversion (58). Disseminated MAC infections in HIV patients are treated similarly, with the additional recommendation of secondary prophylaxis following treatment completion until the CD4 count is greater than 100 cells/mm³ for at least 6 months. Drug interactions with antiretroviral therapy complicate management in such patients (92). Disseminated *M. chimaera* infections following cardiothoracic surgery are distinguished from disseminated MAC infections in the immunosuppressed by the importance of surgical debridement and removal of contaminated prosthetic material – inability to achieve source control necessitates treatment beyond 12 months. These patients also are distinguished by their poor outcomes, with mortality approaching 60% (93,94). Surgery is also advised as an adjunct to medical therapy for select patients with *pulmonary* NTM infections, such as those failing medical management due to drug resistance (58).

1.3.8 Host-Directed Therapies for Nontuberculous Mycobacterial Infections

In short, NTM infections caused by rapid-growing species such as *M. abscessus* and slow-growing species such as *M. chimaera* have rising incidence, necessitate long and complicated treatment courses with relatively intolerable drugs, frequently have inherent

or acquired drug resistance, are often uneradicable despite treatment, relapse frequently and have poor treatment outcomes. Innovation in NTM infection management is desperately needed.

The experimental phage therapy mentioned above is an example of a pathogen-directed therapeutic innovation (95). Other innovators have targeted the host and aim to improve bacillary killing by augmenting host immune functions. One example of a host-directed therapy (HDT) tried in NTM infections is inhaled nitric oxide (96). Clinical guidelines also lend support to this concept in recommending macrolides for pulmonary NTM infections even in the presence of macrolide resistance – macrolides have an immunomodulatory effect, reducing neutrophil numbers and function and the resulting tissue damage (58,97). While many pathways have been proposed as targets for HDTs in NTM infections, the concept is more fully developed, and more data exist for TB (98).

1.3.9 Host-Directed Therapies for TB

Despite an estimated one quarter of the world's population having LTBI, only 10.6 million people had active TB infections in 2021 (4). Thus, the host immune system is capable of successfully controlling the infection in the majority of those infected and it should not be underestimated. The concept of HDTs describes therapeutic strategies that target host immune responses to augment beneficial features of fighting the bacteria and reduce harmful effects of tissue damage (50). The most attractive features of this treatment strategy are the lower potential of developing drug resistance by Mtb, the possibility to use a HDT as a vaccine adjuvant or as a prophylactic for close contacts, and HDT's potential to improve the overall pathology of TB disease by limiting excessive inflammation.

Additionally, the repurposing of licensed medications as HDTs is considered as a faster approach for market access and lowering industrial development-related costs (99,100). In parallel with the interest in drug repurposing and HDT, there has been great interest in new drug delivery approaches to facilitate drug targeting to the site of primary Mtb infection in the lungs and alveolar macrophages (101–103). A new chemical entity (NCE)

can take up to 20 years to gain market authorisation under the current regulations. There has been a renewed focus on the benefits of drug repurposing as part of the global efforts to tackle COVID-19. The repurposing of approved drugs could be an answer to reduce the long drug development timelines that the TB community cannot afford.

Potential HDTs for Mtb infection include a broad range of compounds targeting a number of pathways including but not limited to; autophagy inducers such as rapamycin and the antihyperglycemic agent metformin (104–107), metabolic regulators such as the lipid-lowering statins (108), cytokine modulators (109), corticosteroids (110) and protein kinase inhibitors such as Imatinib (111). Clinical practice guidelines recommend multi-drug combination regimens to effectively treat TB, reduce the risk of relapse and reduce the development of resistance (49). Therefore, any new HDT formulation could be considered as an adjunctive treatment to the current regimens.

The host immune response to Mtb infection is very complex and many host functions that are important in the early stages are considered detrimental at later stages (112). Genetic variation is also an important factor to be considered in the era of personalised medicine. These variations in host immunity may significantly impact the response to HDT to a greater extent than classical antimicrobials (113,114). Thus, it is important to have a comprehensive understanding of the mechanisms of action of HDTs and their interaction with the host immune system in order to properly implement them in the current treatment regimens and prevent any underestimation of their clinical effects.

1.3.10 ATRA as a Promising Host-Directed Therapy for TB

All *trans* retinoic acid (ATRA), known as tretinoin, is the active metabolite of vitamin A. Tretinoin is currently used as treatment for acne and acute promyelocytic leukaemia (APL) (115). Our laboratory has previously reported that human alveolar macrophages display ALDH1a activity and produce ATRA and this induces FoxP3⁺ regulatory T-cells that suppress inflammation and thus reduce unwanted tissue damage caused by the immune system (116). Three vitamin A metabolites; ATRA, 13-*cis* retinoic acid and retinyl acetate resulted in dose-dependent inhibition of the growth of several mycobacterium species *in*

vitro. ATRA and 13-*cis* retinoic acid were the most effective against Mtb (117). One of the earliest reports of the role of retinoic acid in TB was in 1989 and showed that RA was bacteriostatic against Mtb in human MDMs if added before infection at physiologic concentration or at pharmacological concentrations after infection (118).

Retinoids regulate gene transcription, which is mediated by binding to the retinoic acid receptor (RAR) and retinoid X receptor (RXR) (119). Each family of these receptors is associated with three subtypes (alpha, beta, gamma), and each one of them can present in several isoforms (120). Human RARs can be activated by the ligands all-trans RA, 9-*cis* RA, 13-*cis* RA, etretinate and acitretin, while the ligands for human RXRs are only 9-*cis* RA and bexarotene (119). RXRs can form homodimers or heterodimers with RAR, and other receptors such as vitamin D receptor (VDR), bile acids Farnesoid X Receptor (FXR) and fatty acids peroxisomal proliferator-activated receptors (PPAR) (121). Therefore, several signalling pathways can be activated. The RAR/RXR heterodimer is mainly responsible for the biological activity of RA, and it is believed that most of the RA effects are mediated by this receptor dimer (122). The RXR-RAR heterodimers bind to certain retinoic acid-responsive elements (RAREs) located in target gene promoters and regulate gene expression (123–125). In addition to the genomic mechanisms, non-genomic mechanisms of RA have been discussed. Studies have reported that RA can activate ERK1/2 kinases (126–128) which may have a role in cytoskeletal rearrangement and neurite outgrowth (129). Also, retinoylation (RA acylation) – a post-translational alteration of proteins by eukaryotic cells (120) which may have a role in cell differentiation (130).

Laboratories, including our own, have demonstrated that exogenous ATRA improves the ability of human monocyte-derived macrophages (MDMs) and alveolar macrophages (AMs) to kill Mtb *in vitro* (131,132) and *in vivo* (133). We and others have also shown that exogenous ATRA reduces bacillary burdens and pathology in animal models of TB infection (134–137) (*Table 1.1*).

1.3.11 ATRA Mechanisms of Action

A recent paper used Mtb CRISPR interference screening in a human macrophage infection model to identify the Mtb genes required to survive in ATRA-treated macrophages (138). It showed that ATRA treatment starves Mtb of cholesterol and its downstream metabolite propionyl coenzyme A. ATRA did this in part by upregulating macrophage expression of ABCA1 and ABCG1, two cholesterol efflux pumps that have previously been shown to be upregulated in human MDMs by ATRA (138,139). Another cholesterol transporter, NPC2, has also previously been shown to be relevant to ATRA's ability to kill Mtb (132). ATRA's other mechanisms of action include improved phagosome-lysosome fusion (131,140), a reduction of intracellular iron (141), improved cytokine responses (134,137), depletion of myeloid-derived suppressor cells (136), increased CD4+ and CD8+ T cell counts, (137), IgA switching by B cells (142) and improved antigen-presentation by macrophages and DCs (143) (*Figure 1.2*).

Initiation of glycolytic metabolism by human macrophages is crucial for them to effectively respond to infection with Mtb (38). The switch to glycolytic metabolism during infection allows for more rapid ATP generation and for the production of biosynthetic intermediates which allow the macrophage to better carry out its effector functions (37). Interfering with the glycolytic response of macrophages being infected with Mtb results in impaired cytokine responses and higher Mtb growth (28,38,39). Data exist linking *exogenous* ATRA with glycolytic responses to lipopolysaccharide (LPS) stimulation (144), but none exist to indicate whether *exogenous* ATRA or indeed *endogenously* produced ATRA signalling is a relevant factor in macrophage metabolic responses to Mtb infection (144). The effect of *exogenous* ATRA on macrophage metabolic responses to NTM infection is explored in chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*). The impact of *endogenously* produced ATRA on macrophage metabolic responses to Mtb infection is investigated in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*).

	Author	ATRA Dose (Route)	Mycobacterial Strain Used	Cell Type or Animal	Findings	Ref
In Vitro	Coleman et al.	ATRA 5 x10 ⁻⁶ M	H37Ra BCG	Murine BMDM Human MDM	Promotion of autophagy Reduced Mtb burden	(131)
	Crowle et al.	ATRA 1 x10 ⁻⁵ M	Mtb Erdman (ATCC 35801)	Human MDM	Reduced Mtb burden	(118)
	Wheelwright et al.	ATRA 1 x10 ⁻⁸ M	H37Ra H37Rv	Human monocytes Human MDM	Reduced intracellular cholesterol Reduced Mtb burden	(132)
	Babunovic et al.	ATRA 1, 10 x10 ⁻⁶ M	H37Rv	Murine BMDM, THP-1, Human MDM	Reduced intracellular cholesterol Reduced Mtb burden	(138)
	Estrella et al.	ATRA 1 x10 ⁻⁶ M with vitamin D ₃	H37Ra H37Rv	THP-1	Increased antigen presentation Promotion of autophagy Increased ROS production Reduced Mtb burden	(145)
	Anand et al.	ATRA 0.5 x10 ⁻⁶ M with chenodeoxycholic acid	H37Rv	THP-1	Promotion of autophagy Reduced Mtb burden	(140)
	Abd-Nikfargam et al.	13-cis-RA 500-2000 x10 ⁻⁶ M	H37Ra	U937	Increased antigen presentation Increased NO production Reduced Mtb burden	(143)
	O'Connor et al.	ATRA 17 x10 ⁻⁶ M	H37Ra	THP-1	Reduced IL-10 Reduced Mtb burden	(134)
In Vivo	O'Connor et al.	ATRA 2.5mg/kg (intratracheal)	H37Rv	BALB/c mice	Reduced pathology Reduced Mtb burden	(134)
	Yamada et al.	ATRA 1g/kg (oral)	H37Rv	LEW/CrIcrIj rats	Reduced pathology Reduced Mtb burden Increased CD4, CD8 T cells Increased TNF α , IL-1 β	(137)
	Mourik et al.	ATRA 2mg/kg (subcutaneous) with vitamin D ₃ and α -Galactosylceramide	H37Rv	BALB/c mice	Reduced Mtb burden Reduced relapse Increased CD8 T cells, Reduced MDSCs Increased TNF α	(135)
	Knäul et al.	ATRA 5mg pellet (subcutaneous)	H37Rv BCG	C57BL/6 mice 129S2 mice	Reduced MDSCs Reduced Mtb burden Increased CD4, CD8 T cells	(136)
	Riccomi et al.	ATRA 300 μ g (oral) with subunit vaccine	H37Rv BCG	CB6F1 mice	Increased CD4 T cells Increased mucosal IgA Increased IFN γ , IL-17 Limited later inflammation	(146)

Table 1.1: In vitro and in vivo studies of ATRA as a HDT against Mtb.

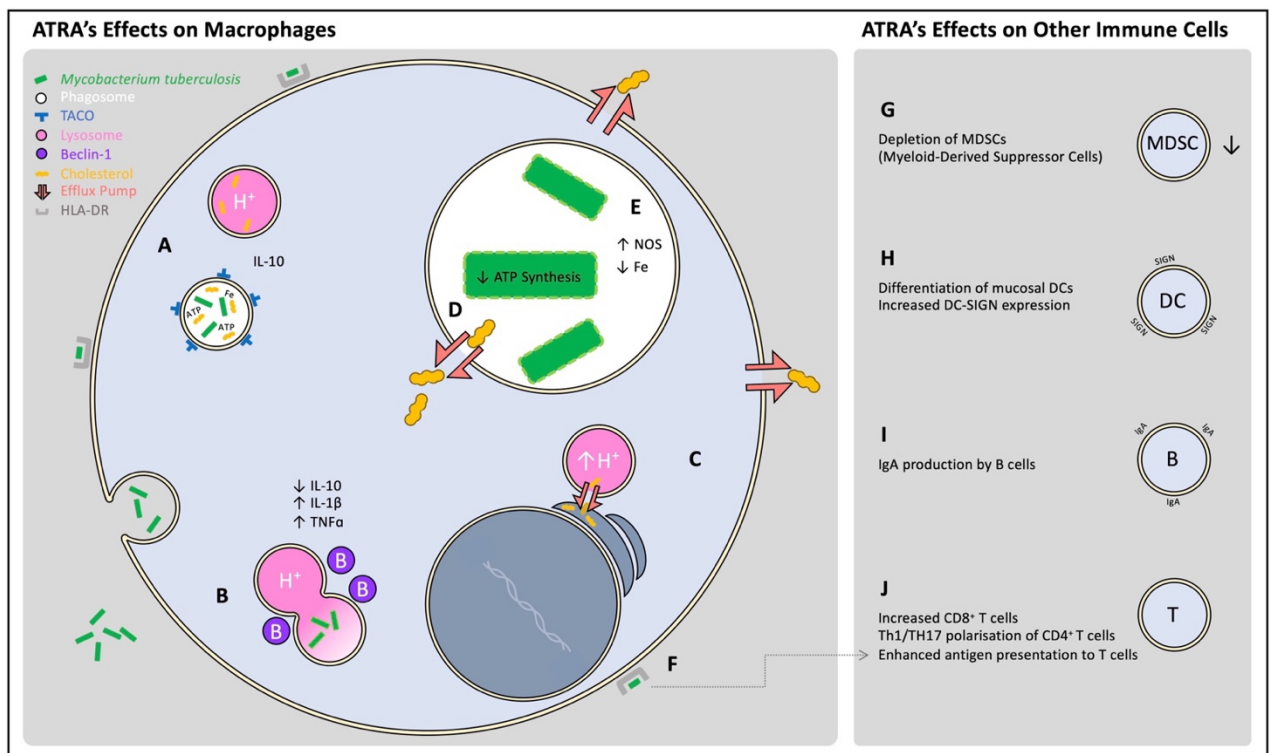


Figure 1.2: ATRA's mechanisms of action as a HDT against Mtb. (A) Mtb is phagocytosed by macrophages and contained in phagosomes but survives by utilising host-derived cholesterol and iron for its metabolism and inhibiting fusion with lysosomes. (B) ATRA increases macrophage expression of a key pro-autophagy protein, Beclin-1. ATRA also reduces the expression of tryptophan–aspartate-containing coat protein (TACO) and IL-10 and increases the expression of cytokines IL-1β and TNFα. These factors enable phagolysosomal maturation. (C) ATRA upregulates macrophage expression of multiple cholesterol efflux pumps, such as NPC2, ABCA1 and ABCG1. NPC2 shuttles cholesterol from lysosomes to the endoplasmic reticulum, increasing lysosomal acidity. (D) ABCA1 and ABCG1 reduce intracytoplasmic and intraphagosomal cholesterol concentrations. This starves Mtb of its fuel source, reducing its ability to synthesise ATP. (E) ATRA reduces macrophage intracellular iron concentrations, reducing the availability of a key Mtb nutrient, and increases cellular NOS production. (F) ATRA increases macrophage HLA-DR expression, increasing their ability to present Mtb antigens to T helper cells. (G) ATRA induces maturation and functional depletion of regulatory MDSCs. (H) ATRA increases dendritic cell expression of DC-SIGN, augmenting their antigen-presentation ability. (I) ATRA leads to isotype switching of IgA antibodies in B cells. (J) ATRA increases the counts of CD4⁺ and CD8⁺ T cells in vivo and regulates CD4⁺ polarisation.

1.3.12 Clinical Trials of Vitamin A for TB

No human clinical trials evaluating ATRA as a TB HDT have been undertaken. Only one relevant case report has been published, which describes a patient prescribed ATRA for acute promyelocytic leukaemia in addition to Mtb therapy, and who developed hypercalcemia as a possible adverse effect of ATRA (147). However, many trials

evaluating a range of oral doses of retinol supplementation (typically 5,000 IU daily or 200,000 IU as a single dose) as esters as an adjunct to TB therapy have taken place. These trials in Mexico (148), Nigeria (149), Indonesia (150,151), South Africa (152,153), Malawi (154), China (155) and India (156) have included both adult and paediatric (HIV-seropositive and HIV-seronegative) patients. While some trials have indicated a trend towards earlier sputum conversion in the retinol-supplemented group (148–152), a 2016 meta-analysis found that overall, oral retinol supplementation did not significantly affect sputum positivity at 2 weeks, 1 month or 2 months. No significant difference in mortality was found (157). No trials of retinol or ATRA prescribed for the prevention of active TB in contacts, LTBI, or other high-risk groups have been undertaken.

1.3.13 Why Clinical Trials Have Failed

Both retinol and ATRA enhance the Mtb-killing ability of human macrophages *in vitro* (116,118,158–161) and are suggested to hold promise as a HDT for Mtb (50). There are several possible explanations for why retinol has failed to improve outcomes in clinical trials of Mtb infection (*Figure 1.3*):

- Absorption of oral retinol supplementation is negatively correlated with fever (162) and requires a diet containing lipids.
- Baseline liver vitamin A reserves of TB patients may be severely depleted by the subacute Mtb infection itself, the associated anorexia, other infections and malnutrition (163). This is supported by the low baseline retinol levels, low body mass index and the lack of difference in retinol trajectory between intervention and placebo arms observed in many of the trials (148–155).
- Infection and fever are strongly associated with urinary retinol excretion (164,165). This may counteract supplementation efforts.
- It is possible that, in practice, active TB patients present at too late a stage and that retinol would be most effective at enhancing the early clearance of the bacillus.

- The serum carrier proteins that transport retinol to its sites of action, RBP4 and TTR, are both significantly reduced in TB patients (166,167). RBP4 is a negative acute phase reactant that declines with inflammation (168,169). Baseline C-Reactive Protein (CRP) and Erythrocyte Sedimentation Rate (ESR) were elevated among trial patients (149–152,155).
- Rifampicin is a potent CYP450 enzyme-inducer. As has been shown for other CYP450-inducers, rifampicin may significantly increase the clearance of ATRA and thereby reduce retinol's effectiveness (170,171).
- Inflammation downregulates murine intestinal macrophage expression of ALDH enzymes which are required for the production of ATRA from retinol (172). In addition, ALDH1a2 and DHRS9 expression are reduced in human TB granulomata (173). The macrophages of TB patients may not be capable of effectively producing ATRA from retinol.
- ATRA is promising as a TB HDT as it both enhances autophagy and contributes to immune regulation, reducing unwanted tissue damage. Nevertheless, specific HDTs may be effective only in patients with specific TB phenotypes or endotypes (174,175).

Two compatible strategies might sidestep several of these obstacles. The first is to use ATRA rather than retinol as an adjunct to TB-therapy. This would remove the need for retinol absorption, storage, mobilisation and the intracellular oxidation of retinol to ATRA. The second is to target the lung with ATRA directly rather than systemically by using an inhaled formulation. This could increase the concentrations of ATRA at the site of interest while reducing systemic effects. Randomised controlled trials of ATRA as an adjunct to TB therapy could first be undertaken in patients with extensively drug-resistant TB failing therapy (*Figure 1.3*).

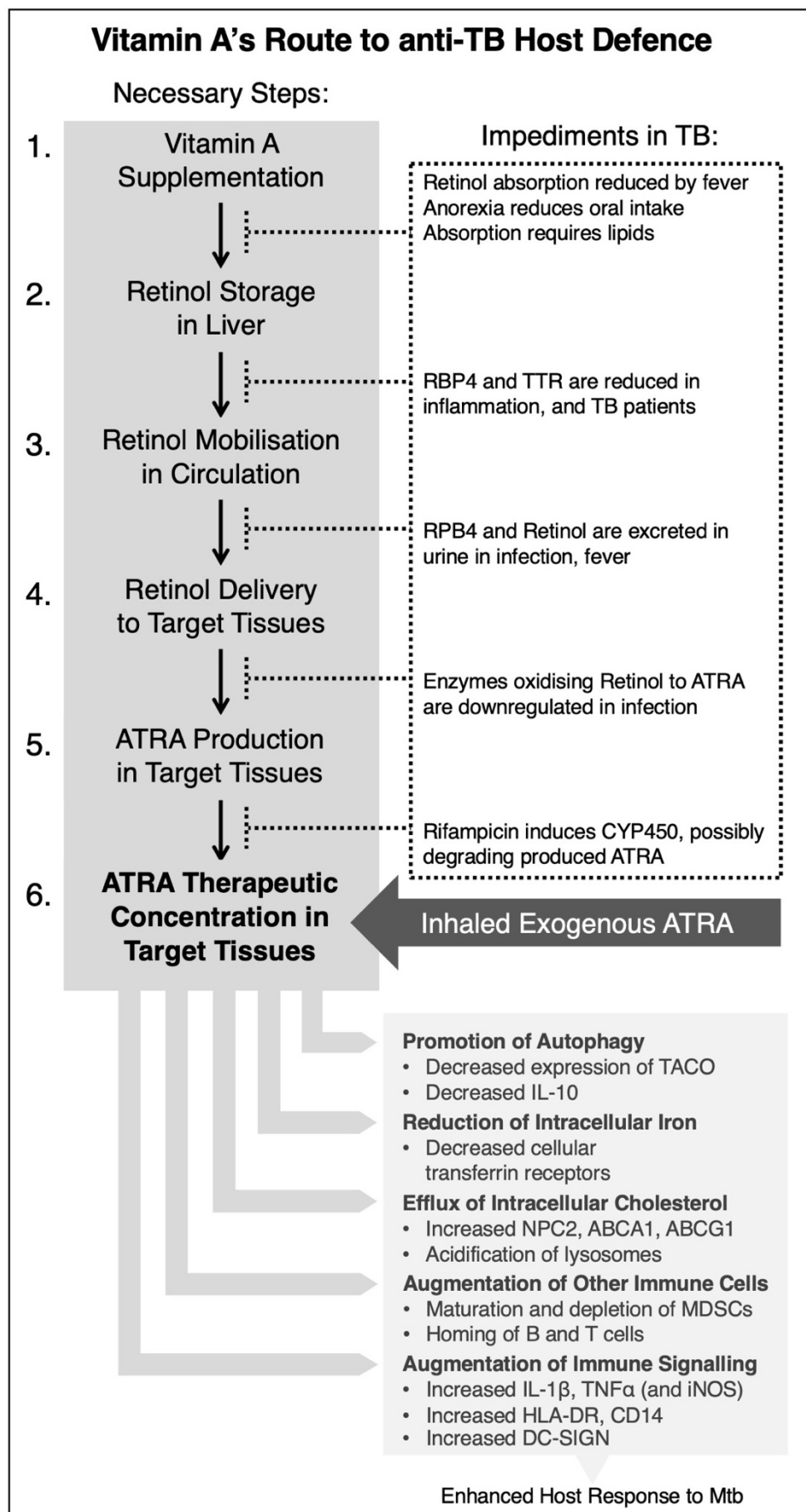


Figure 1.3 (legend below)

Figure 1.3: ATRA has pleiotropic effects that protect the host against Mtb (lower-right box). These include: the co-localisation of Mtb with autophagic vesicles and the promotion of phagolysosomal maturation through a reduction in the expression of TACO and a reduction in IL-10; a reduction in the cellular expression of transferrin receptors, reducing the supply of a vital nutrient of Mtb – iron; the efflux of another Mtb nutrient, cholesterol, and the acidification of lysosomes via increased NPC2 expression; a functional depletion of MDSCs and improvement of the homing capacity of B and T cells; and the augmentation of innate immune signalling, characterised by an increase in HLA-DR, CD14, DC-SIGN and pro-inflammatory cytokine secretion. There are many necessary steps that dietary vitamin A must undertake in order to exert these host-protective effects (upper-left box). First, supplemented retinoids must be absorbed by enterocytes, secreted in chylomicrons via the lymphatics into circulation and endocytosed by the liver for processing and storage. Retinol must then be secreted with its carrier proteins into circulation and delivered to its target tissues. Once intracellular, retinol must be converted to its active metabolites in a two-step oxidation reaction before it can be transported to its nuclear receptors. However, TB patients present many obstacles to these steps (upper-right box). For example, retinoid absorption can be reduced by fever, anorexia, or a lipid-poor diet. Retinol's circulatory carrier proteins (RBP4 and TTR) are negative acute-phase proteins that are suppressed in TB patients. Retinol is excreted from the circulation in urine during fever and infection. The macrophages of TB patients may not be capable of producing ATRA from retinol, and anti-tuberculous medications may increase the degradation of ATRA that is locally produced. Many of these obstacles might be bypassed by using inhaled exogenous ATRA as a HDT.

In summary, there is already strong support for the use of ATRA as a HDT in TB. While there has been no research to date examining the use of ATRA in NTM infections, there is good reason to think that it could be effective, especially for slow-growing NTM species. There is an enormous clinical need for innovations in NTM management. There is a research need to understand if ATRA is an effective HDT for NTMs *in vitro* and if so, for which species and by what mechanisms. This question is addressed in chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) of this thesis. Chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) examines the *endogenous* production of vitamin A in Mtb infection.

1.5 Vitamin A Deficiency and Defects in Host Responses to Infection

1.5.1 Epidemiology of Vitamin A Deficiency

Vitamin A cannot be synthesised *de novo* in the body and must be obtained from diet (176). In developed, high-resource settings such as Europe, dietary surveys indicate that few children or adults have a vitamin A intake lower than what is recommended (900 mcg for adult men and 700 mcg for adult women) (177). Unsurprisingly, the sequelae of vitamin A deficiency (VAD), which are rarely seen in these settings, are usually the result of restrictive diets or malabsorption and are the subject of case reports (178,179). It is estimated that 1.1% of all global mortality, and 1 in 5 deaths from diarrheal illness are attributable to VAD (180). Likewise, 1.5% of global disability adjusted life years have been attributed to VAD. VAD causes xerophthalmia and is the leading cause of acquired blindness in children. Night-blindness affects 2% of African and 0.5% of South East Asian children (180–182).

Vitamin A has a central role in development, growth, cell proliferation, epithelial integrity and immunity (183). High-risk populations are those in low-resource settings where crop yields and infrastructure are poor, where diets are low in fat and where populations depend on rice, which lacks carotenoids. Within these populations, young children following weaning and pregnant women are at particularly high risk, given their increased requirements (180,182). Consequently, high-dose vitamin A supplementation is recommended by the World Health Organization for all children aged 6-59 months and pregnant women in settings where vitamin A deficiency is prevalent (184,185). In fact, meta-analyses evaluating the efficacy of vitamin A supplementation in these children have estimated a relative risk reduction in all-cause mortality of about one quarter (186,187). Vitamin A supplementation is also recommended in the treatment of severe acute malnutrition, as it reduces mortality (188).

1.5.2 Vitamin A Deficiency in Infectious Diseases

Plasma retinol levels are lower in patients with infectious disease than in other disease types (189). Whether this is a cause or an effect of infection (or more likely both) is difficult to unpick. As discussed, supplementation has successfully reduced infection-related mortality in certain populations, which supports the hypothesis that VAD results in immune dysfunction and infection (186,187). However, inflammation also reduces plasma retinol and retinol binding protein-4 (RBP4) levels (168,169). Retinol is excreted in the urine of patients with infections (164,165) and certain infections, giardiasis or ascariasis for example, might reduce intestinal absorption of vitamin A (190,191). Also, in the absence of inflammation, low circulating retinol levels only reflect liver vitamin A stores when they are severely depleted, making their levels' interpretation more difficult (168).

Notwithstanding, hyporetinolaemia and VAD have been associated with many types of infections: a large, prospective, longitudinal cohort study of Colombian children found significant relationships between plasma retinol levels and reductions in the risks of gastroenteritis, respiratory tract infections, otitis and need for medical attention (192). VAD is associated with an increased severity of measles infection (193). A Cochrane systematic review found that two doses of vitamin A significantly reduced the mortality of young children with measles (193,194). Hyporetinolaemia was not associated with progression of HIV infection in the 1990s, but is an independent predictor of non-response to interferon therapy for Hepatitis C virus infection, unlike vitamin D (195,196). Giardiasis, ascariasis, or infection with any soil-transmitted helminth have repeatedly been found to be associated with VAD (190,191,197). While retinol supplementation supports host anti-helminthic cytokine responses in children with *Ascaris* infections, routine supplementation following deworming does not appear to reduce reinfection rates or intensity in endemic settings (198,199). Oh et al., report that VAD was significantly more prevalent in patients with pulmonary nontuberculous mycobacterial disease than healthy controls (73) .

1.5.3 Vitamin A Deficiency in Active Tuberculosis

Many case-control studies have found an association between TB and low retinol levels or VAD. These studies have a wide geographic spread, and were undertaken in South Korea (200), Ethiopia (201), India (202), Tanzania (203) and South Africa (204). While our understanding of vitamin A's immunological role and promising *in vitro* results suggest a direction of causality, the systemic inflammatory responses of TB patients complicate the interpretation of these case-control studies as providing strong evidence for VAD causing TB-risk. However, two important prospective cohort studies following high-risk groups over time lend strong support to the theory that hyporetinolaemia is a risk factor for active TB. Aibana et al., followed 6751 HIV-negative household contacts (HHCs) of TB cases in Peru for one year. Even after adjusting for many confounders, the authors found that HHCs with baseline VAD had more than a 10-fold increased risk of developing TB, or 20-fold for those aged between 10 and 19 years. Notably, there was a stepwise increase in TB risk with each vitamin A quartile, even for contacts who were not deficient (205). In addition, Tenforde and colleagues followed HIV positive patients starting antiretroviral therapy (ART) in nine countries. When comparing those who developed active TB within 96 weeks, and after adjusting for confounders, pre-ART VAD was associated with a 5.3 hazard ratio of developing active TB. This risk remained significant even after adjusting for Vitamin D status (206). Two studies have investigated the association of vitamin A deficiency and severity of TB diseases. In Indonesia, a cross sectional study of 300 smear positive TB patients, found VAD in 64% of patients with severe disease but only 37% of patients with mild disease (207). However, in Morocco, Qrafli et al., examined 44 smear positive TB cases and did not find an association between plasma retinol and TB disease severity (208). As noted before, vitamin A has a central role in development, growth, cell proliferation, epithelial integrity and immunity (183). Among the many physiological effects of VAD, those on epithelial integrity and cellular immune function are most relevant to *Mtb* pathogenesis and perhaps best explain the increased risk of TB observed in those with VAD (183).

In summary, multiple lines of evidence suggest that infection is associated with hyporetinolaemia and that, reciprocally, VAD is associated with an increased risk of several infections affecting the respiratory or gastrointestinal mucosa in children, and an increased risk of developing active tuberculosis in adults. Randomised controlled trials that intervene to correct VAD and measure the incidence of active TB are needed to determine if VAD is causally associated with TB and if correction of VAD is a clinically effective prophylactic strategy for TB. These trials could focus on high-risk groups with VAD such as patients starting antiretroviral therapy for HIV, as studied by Tenforde et al. (206), or household contacts of TB cases, as studied by Aibana et al. (205). Indeed, supplementation of vitamin A should be trialled irrespective of vitamin A levels or deficiency status, given the inverse correlation between baseline serum retinol level and risk of active TB even among contacts who are vitamin A 'sufficient'.

1.5.4 Vitamin A Absorption and Storage

Dietary vitamin A takes the forms of preformed retinoids, retinol or retinyl esters, found in animal food sources and pro-retinoid carotenoids, such as β -carotene found in plant food sources. Vitamin A absorption is negatively correlated with fever (162). Retinyl esters are converted to retinol in the intestinal lumen and carotenoids are converted to retinol in enterocytes. Retinol is then esterified in the enterocyte by lecithin retinol acyltransferase (LRAT) and secreted in chylomicrons into circulation via the lymphatic system – a process important to host defence in a murine model of TB infection (209–212). Hepatocytes endocytose circulating chylomicron remnants. Retinol-binding protein 4 (RBP4) is synthesised in hepatocytes and binds to retinol in the endoplasmic reticulum, at which point retinol-RBP4 is either shipped to stellate cells for storage or secreted into circulation for transport to target tissues (211). Almost all of the body's total retinoid is stored in hepatic stellate cells in retinyl ester form (209,210).

1.5.5 Vitamin A Transport

There are several pathways through which retinol is delivered to target tissues, including as dietary retinoids in chylomicrons (before liver processing) or as liver-secreted retinyl esters bound to VLDL (209,213). However, nearly all of circulating retinoids are in the form of retinol bound to Retinol Binding Protein 4 (RBP4) and a second protein, transthyretin (TTR) in a 1:1 ratio (209,210). RBP4 is a 21kDa lipocalin protein that chaperones hydrophobic retinol in the circulation (214,215) (*Figure 1.4*). RBP4 is required for deployment of liver retinoid stores, and retinol is required for the secretion of RBP4 from hepatocytes (209). Low circulating RBP4 levels can indicate VAD, however only after liver stores are depleted (210). RBP4 mutations are associated with visual defects, obesity, cardiovascular disease and hypertriglyceridemia (213). RBP4 is a negative acute phase response protein that is downregulated in inflammation, such as in the postoperative period (169). Retinol and RBP4 were inversely correlated with CRP in Cameroonian women and children, but positively correlated with haemoglobin (216). This complicates the interpretation of retinol or RBP4 levels as indicators of vitamin A sufficiency or deficiency in inflammatory states. Unsurprisingly, RBP4 levels are lower in TB patients (166). A recent proteomic study by Jarsberg et al., found that in African patients, TTR levels improved faster with TB treatment than RBP4, which depends on liver retinoid stores for its secretion. This suggests that vitamin A deficiency, and not just the inflammatory response, causes low RBP4 in this group of TB patients (217). In the inflammatory state, another retinol-binding protein may take over the function of RBP4: Serum amyloid A (SAA) was shown to be responsible for transport of retinol to myeloid cells during bacterial infection (218,219). SAA delivers retinol to RA-producing intestinal myeloid cells, where it binds to the transmembrane receptor LRP1 (220).

Another, unexplored factor that may reduce retinol-RBP4 levels in TB patients is urinary excretion. While only trace amounts are excreted in healthy adults and children, substantial quantities are excreted in patients with fever, pneumonia, sepsis and rotaviral diarrhea (164,165). RBP4 levels are also correlated with obesity, hepatic steatosis, triglyceride levels and VLDL-cholesterol levels. In obese patients undergoing bariatric

surgery, weight loss was independently associated with a reduction in RBP4 (221–223). While weight loss is emblematic of TB disease, and it is tempting to hypothesise that TB-induced weight loss is another factor reducing circulating retinol-RBP4, these cohorts are quite distinct.

Transthyretin (TTR) is a 55kDa protein that prevents renal excretion of retinol-RBP4 by binding the complex in circulation (213,214). Interestingly, TTR also serves as a transporter of thyroxine and tri-iodothyronine. Like RBP4, TTR is a negative acute phase protein that is suppressed in inflammatory states. Agranoff et al., analysed the serum of 179 culture-confirmed TB cases and 170 controls with other inflammatory or infectious conditions. They found that TTR was one of the most discriminatory proteins between cases and controls (167).

1.5.6 Vitamin A Cell Entry

TTR needs to dissociate with the retinol-RBP4 complex before the retinol-RBP4 complex can associate with its cell surface receptor (STRA6). STRA6 binds RBP4, and facilitates the transmembrane channelling of retinol to an intracellular acceptor, the cellular retinol binding protein (CRBP) (211,213). Retinol-RBP4 also phosphorylates STRA6, triggering a signalling cascade that ends in STAT3 and STAT5 activation and SOCS3 signalling (*Figure 1.4*). This retinol-RBP ‘sensing’ is thought to underlie the known link between vitamin A homeostasis and insulin resistance, and represents an effector pathway of vitamin A in addition to those mediated by retinal and retinoic acid (224,225). However, most cellular uptake of retinol from the retinol-RBP4-TTR complex is not through STRA6, and retinol is thought to diffuse freely across the cell membrane (225). In fact, while retinol has beneficial anti-TB effects in human monocyte-derived macrophages (MDM), they do not express STRA6 (104,226). As STRA6 is not necessary for cellular entry of retinol, its primary function is thought to be that of ‘sensing’ and signalling (225).

1.5.7 Enzymes Involved in Vitamin A Metabolism

While retinol itself is inert, its metabolite retinal is essential for vision and its metabolite retinoic acid exerts many effects by activating nuclear receptors in its target cells. These active metabolites are manufactured in situ in target cells from retinol, or alternatively retinoic acid diffuses from neighbouring cells. Retinoic acid has three isoforms: all-trans retinoic acid (ATRA), 9-cis RA and 13-cis RA, with ATRA being the most abundant and active isoform (227). These metabolites are produced in a two-step oxidation reaction in the target tissues. Firstly, retinol is reversibly oxidised to retinal by the widely expressed alcohol dehydrogenases (ADH), and short-chain dehydrogenase/reductases (SDR) when retinol is protein-bound (228). Secondly, members of the aldehyde dehydrogenase (ALDH) family irreversibly oxidise retinal to ATRA or its isomers. These include ALDH1a1, ALDH1a2 and ALDH1a3 (*Figure 1.4*). While oxidation of retinol to retinal is not tissue restricted, the tissue-specific expression of the ALDH1a enzymes determines which tissues are capable of producing vitamin A's active metabolites (229–231).

There is some evidence suggesting that human macrophages express these enzymes and are capable of producing ATRA. DHRS9, an SDR, is highly expressed in regulatory macrophages that suppress T cell proliferation, and reduced in interferon gamma-stimulated macrophages (232). In keeping with this, the expression of DHRS9 and ALDH1a2 were significantly lower in caseous TB lung tissue than in unaffected tissue, although the cells expressing these enzymes were not identified (173). In a rabbit model of TB infection, ALDH1a2 was significantly reduced in lung tissue following infection (173). Similarly, mice infected with *Trichuris muris* had reduced ALDH activity in their intestinal macrophages (233). ALDH1a2 expression was detected in alternatively activated macrophages in murine liver during *Schistosoma mansoni* infection and IL-4 also induced its expression in peritoneal macrophages *in vivo* and in bone marrow derived macrophages (BMDM) *in vitro* (234). Niu et al. reported that resting murine BMDM do not express ALDH1a1, ALDH1a2 or ALDH1a3 but rather synthesise ATRA using the enzyme ALDH3b1 (235). Human intestinal macrophages express ALDH1a1 and ALDH1a2, but not ALDH1a3 (236,237). ALDH1a activity was detected in human alveolar macrophages using

a low-specificity Aldefluor assay (116). Overall, there is a paucity of data identifying which vitamin A-metabolising enzymes are expressed in the human macrophage populations.

Blocking the oxidation of retinol to ATRA in human macrophages with the ALDH inhibitor N,N-diethylaminobenzaldehyde (DEAB) undermines their ability to kill Mtb (104).

Enhancing the cellular production of ATRA in human macrophages by increasing the expression of these enzymes may benefit the host response to Mtb, acting as a host-directed therapy (HDT). ALDH1a-family enzyme induction by IL-4, GM-CSF and PPAR- γ agonists has been observed in human monocyte-derived dendritic cells (DCs) (173,238,239). This approach may be constrained by a negative feedback inhibition of ATRA on ALDH1a1 expression (240).

1.5.8 Carrier Proteins

Intracellular retinoid-binding proteins chaperone the hydrophobic retinoids in the cytosol. Cellular Retinol-Binding Proteins (CRBP) bind retinol and retinal. Cellular Retinoic Acid-Binding Proteins, ubiquitous CRABP1 and more selectively-expressed CRABP2, bind ATRA (121). CRABP2 is expressed in human MDMs (241). It has been suggested that the expression of these carrier proteins, and not the oxidation enzymes, is the rate-limiting step in the production of ATRA (235). In addition, there is evidence to suggest that the relative expression of CRABP1, CRABP2 and fatty acid binding protein 5 (FABP5) may determine the fate of ATRA – whether it is degraded and which nuclear receptors are activated. In summary, CRABP1 may target ATRA for degradation by cytochrome P450 enzymes, CRABP2 delivers ATRA to the retinoic acid receptor (RAR) nuclear receptor and FABP5 delivers ATRA to the PPAR δ/β nuclear receptor (242–244). PPAR- γ agonists increase CRABP2 expression, and thus may increase the protective effects resulting from ATRA delivery to the RAR receptor (239) (*Figure 1.4*).

1.5.9 Degradation by Cytochrome P450

ATRA is degraded to polar metabolites by members of the Cytochrome P450 26 family (CYP26A1, CYP26B1 and CYP26C1) (230,245) (*Figure 1.4*). The expression of these enzymes is strongly increased by ATRA, which may cause treatment-resistance if ATRA is used as a HDT (246). CYP26 inhibitors used alone, or in combination with ATRA, are worth considering when exploring ATRA as a HDT for Mtb. In summary, there is a need to understand better how these oxidising enzymes and carrier proteins are affected by Mtb infection and to understand better how impaired signalling of endogenously produced ATRA contributes to macrophage susceptibility to Mtb. Both of these topics are explored in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*). Chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) of this thesis is focused on host responses to SARS-CoV-2.

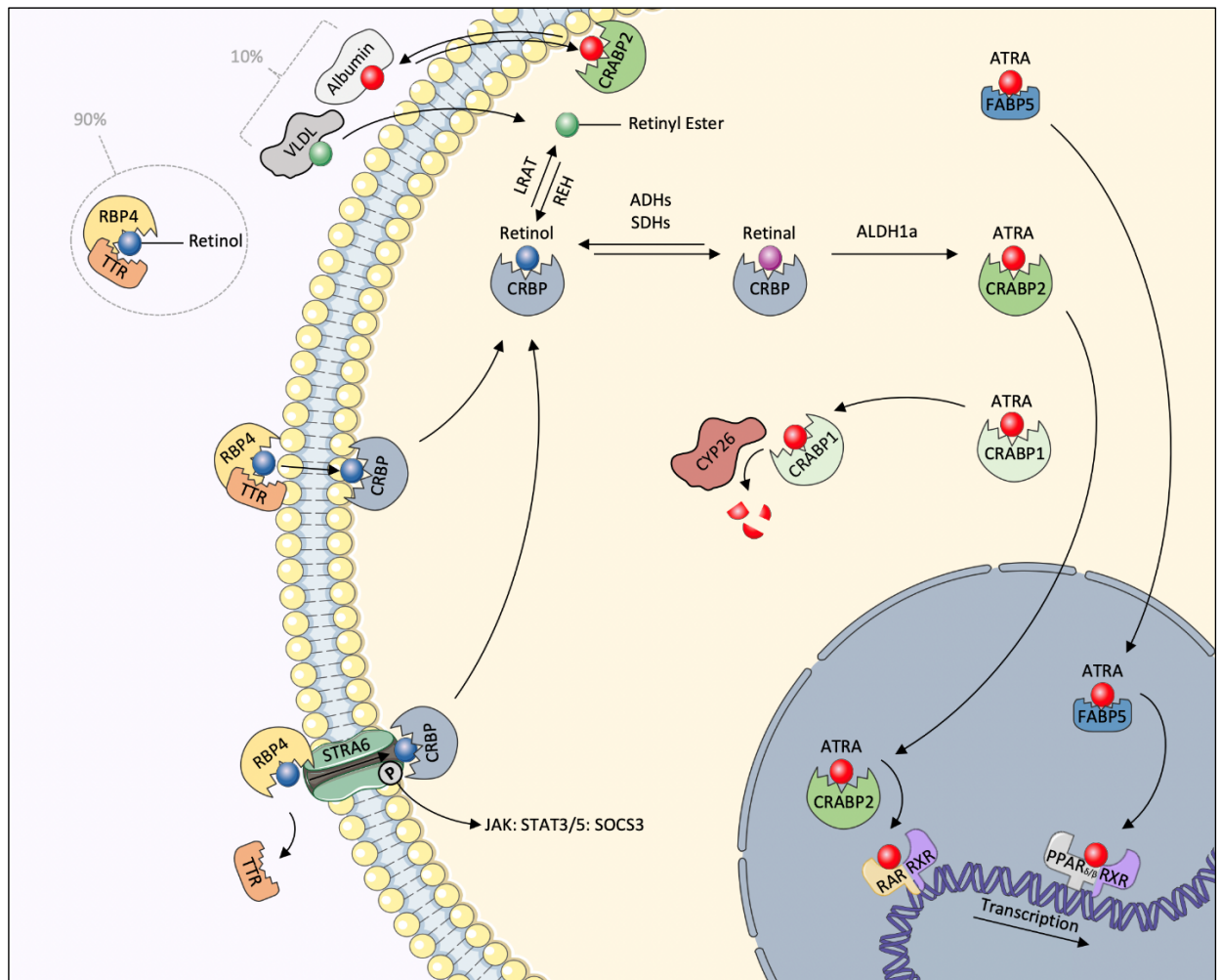


Figure 1.4: Vitamin A is delivered to target cells in several ways. Retinyl esters (in green) can be delivered within chylomicrons post-prandially or within liver-secreted VLDL. Retinoic acid (in red), can be produced by neighbouring cells and delivered, bound to albumin. However, 90% of all circulating retinoids are in the form of retinol (in blue), bound to RBP4 and TTR. Retinol can diffuse freely across cell membranes or in certain cells, following dissociation of TTR, can also be channelled through the surface receptor STRA6, which triggers a JAK-STAT3/5 signalling cascade. Intracellularly, retinol is bound to Cellular Retinol Binding Protein (CRBP), which also binds retinal (in purple). If not esterified by LRAT, retinol can be reversibly oxidised to retinal by short-chain dehydrogenases or alcohol dehydrogenases. Retinal can then be irreversibly oxidised to retinoic acid by members of the aldehyde dehydrogenase 1a family. ATRA (in red), the predominant isoform of retinoic acid, is bound to Cellular Retinoic Acid Binding Protein 1 or 2 (CRABP1, CRABP2), or Fatty Acid Binding Protein 5 (FABP5). CRABP1 preferentially delivers ATRA to Cytochrome P450 26 (CYP26) for degradation to polar metabolites, whereas CRABP2 delivers ATRA to its primary nuclear receptor, the RAR:RXR heterodimer, to activate transcription of its target genes. If the ratio of FABP5 to CRABP2 is high, ATRA is preferentially delivered to an alternative nuclear receptor, the PPAR δ /6:RXR heterodimer.

1.6 Alveolar Macrophage Responses to SARS-CoV-2

1.6.1 SARS-CoV-2 and the COVID-19 Pandemic

COVID-19 is an infection caused by a novel, single-stranded, positive-sense RNA beta-coronavirus named SARS-CoV-2. The virus is closely related to SARS-CoV, which caused the SARS (or Severe Acute Respiratory Syndrome) pandemic in the early years of the 21st century, and more distantly related to MERS-CoV and the four other coronaviruses known to infect humans – HCoV-229E, HCoV-HKU1, HCoV-NL63 and HCoV-OC43. SARS-CoV, SARS-CoV-2 and MERS-CoV can cause severe pneumonitis, while the others cause only mild upper respiratory tract infections in humans (247). The first cases of COVID-19 were identified in Wuhan, Hubei province, China, in December 2019. Following its emergence in China, a global pandemic has ensued, affecting every country (7). At the time of writing (9th February 2023), 755 million confirmed COVID-19 infections and 6.8 million deaths associated with a confirmed SARS-CoV-2 infection had been reported to the WHO (248). However, excess global mortality during the first two years of the COVID-19 pandemic is estimated at 14.8 million deaths, indicating both an undercount of direct COVID-19 deaths and a significant indirect toll of the pandemic (7).

1.6.2 Transmission of SARS-CoV-2

SARS-CoV-2 is primarily transmitted between humans via respiratory droplets, expelled from the lungs during coughing, speech, singing etc. These droplets fall under the influence of gravity within approximately 2 metres of their source, and as such, proximity is a strong determinant of the epidemiologically-observed transmission risk (249). It is perhaps unsurprising, therefore, that outbreaks within congregated settings such as nursing homes have been a major feature of the pandemic and have resulted in disproportionate morbidity and mortality in these settings (250). Airborne transmission via droplets smaller than 5µm, which can linger, suspended in the air for longer periods and travel greater distances, has also been documented. However, airborne transmission is thought to contribute less to overall transmission of SARS-CoV-2 and may be limited to

specific circumstances, such as following aerosolising procedures on COVID-19 patients in healthcare settings.

In addition to respiratory droplets and aerosols, SARS-CoV-2 is excreted in saliva and faeces and can persist in bodily fluids and on surfaces of various materials. Despite this, there is no strong evidence that direct inoculation from physical contact or fomite transmission contributes significantly to overall COVID-19 transmission (251,252). In broad terms, SARS-CoV-2 has a natural reproduction number (R_0) which is higher than influenza virus but lower than the highly transmissible measles virus (253).

Following infection with SARS-CoV-2, and a median incubation period of 4 to 5 days, most people (approximately 60%) develop symptomatic COVID-19 infections (247).

Epidemiological evidence indicates that the median infectious period in human COVID-19 infection spans from approximately 2 days pre-symptom onset to 7 days post-symptom onset, with peak infectiousness coinciding with the apex of viral loads the day before symptom onset. While SARS-CoV-2 RNA can be detected in respiratory samples for some time beyond this point, this does not translate to the isolation of viable, infectious virus *in vitro* or to an ongoing transmission risk in observational studies. Importantly, this duration of infectiousness can be much longer in immunocompromised hosts (249,254).

1.6.3 Clinical Aspects of SARS-CoV-2 Infections

While COVID-19 is a multisystem disease, approximately 40% of people infected with SARS-CoV-2 are asymptomatic for the duration of infection (255,256). Asymptomatic patients have similar SARS-CoV-2 viral loads to symptomatic patients and contribute, along with pre-symptomatic patients, to about 40% of overall transmission (256,257).

Most patients with symptomatic COVID-19 infections report mild symptoms such as fever, cough, headache, myalgia, sore throat, coryza, anosmia and diarrhoea. In the pre-vaccination period of the pandemic, severe disease, critical disease and the crude case fatality rate were estimated at 14%, 5% and 2.3%, respectively (258). Vaccination is associated with more than a 10-fold decrease in risk of hospitalisation and severe

outcomes, and different SARS-CoV-2 variants pose varying severity risks (259,260). Severe disease tends to manifest approximately one week following symptom onset, most significantly as respiratory failure associated with acute respiratory distress syndrome (ARDS) in approximately one quarter of hospitalised patients, which frequently necessitates mechanical ventilation (261). Apart from pneumonitis, severe COVID-19 disease commonly includes thromboembolic complications of endothelial dysfunction as well as cardiac injury and dysfunction.

Some therapies for COVID-19 target the host. Corticosteroids are the mainstay of COVID-19 treatment, as they improve mortality in hospitalised patients requiring oxygen. IL-6 monoclonal antibodies and Janus kinase inhibitors are also recommended in certain subgroups (262). Other therapies for COVID-19 target SARS-CoV-2 itself. Remdesivir blocks viral RNA transcription. Nirmatrelvir is a SARS-CoV-2 protease inhibitor. Both are recommended in the early phase of infection in patients at a high risk of progression to severe disease. Neutralising antibody therapies targeting SARS-CoV-2 spike protein were previously recommended for COVID-19 pre-exposure prophylaxis, post-exposure prophylaxis and treatment. However, their efficacy has markedly declined due to the evolutionary drift of SARS-CoV-2, and therefore their use is no longer advised in clinical guidelines (263). Despite the availability of these various therapies in a high-resource country such as Ireland, with almost universal vaccination uptake among those at highest risk, there were 2,321 Irish COVID-19 deaths recorded in 2022 (264).

1.6.4 Age and Other Risk Factors in SARS-CoV-2 Infections

Booth et al. found in a meta-analysis including 17 million patients' data that co-morbidities such as cardiovascular disease, chronic lung disease, active cancer, immunosuppression, chronic kidney disease and obesity increased the risk of severity and mortality due to COVID-19. Surprisingly, active smoking did not significantly increase the risk of severe COVID-19 in their multivariate analysis (265).

Age, however, was a much more profound determinant of risk than any of these clinical diagnoses or criteria. Booth et al. report an odds ratio of severe COVID-19 of 1.69 in those

over the age of 60, 1.76 in those over the age of 65 and 1.93 in those over the age of 75. This compares with 1.69 for obesity, 1.09 for cardiovascular disease and 1.48 for active cancer. Regarding the risk of mortality, they report an odds ratio of 3.15 for those over the age of 60, 3.79 for those over the age of 65 and 5.82 for those over the age of 75. This compares with 1.45 for obesity, 1.53 for cardiovascular disease and 2.15 for active cancer (265). One of the most prevalent risk factors, advanced age, is therefore also the most potent. In Ireland in 2020, the central statistics office estimated that 306,936 of the 5,203,265 population (5.9%) were 75 years of age or older (266). Almost three quarters of recorded Irish COVID-19 deaths are among this small minority (267).

In addition to these common risk factors, which account for the bulk of severe COVID-19 cases, rarer risk factors have been identified that may shed light on the pathogenesis of severe COVID-19 – genetic defects affecting TLR7 (268,269), TLR3 (270), IRF7 (270), CCR9 (271), CXCR6 (271) or the ABO group locus (271). Similarly, autoantibodies targeting type 1 interferons have been associated with severe COVID-19, are detectable in 4% of the elderly population but in 18% of those who died from COVID-19 – suggesting such antibodies are responsible for a sizable fraction of overall mortality, and highlighting the importance of functional innate immune responses in determining disease outcomes (272,273).

1.6.5 Pathogenesis of SARS-CoV-2 Infections: Virology

The SARS-CoV-2 genome encodes structural proteins, non-structural proteins and accessory proteins. The structural proteins consist of the membrane, nucleocapsid, envelope and spike proteins. The non-structural and accessory proteins consist of those required for RNA replication, transcription and immune evasion (247,274). A host-derived lipid bilayer and the four structural proteins form the virion capsule that contains the SARS-CoV-2 genome.

The SARS-CoV-2 spike protein is a 1273 amino acid-long glycosylated protein that forms trimers on the virion surface and has close analogues in other *Coronaviridae*. Importantly, the spike protein contains the receptor-binding domain (why vaccines have targeted the

spike protein), which facilitates the binding of the SARS-CoV-2 virion with its target receptor: the angiotensin-converting enzyme 2 (ACE2) receptor. ACE2 is widely expressed in many human tissues including by type 2 alveolar epithelial (AT2) cells – the primary target of SARS-CoV-2 in the lower respiratory tract (247,275,276) – and by vascular endothelial cells, explaining the association of COVID-19 with thromboembolic disease (277).

Both AT2 cells and vascular endothelial cells also express another critical surface protein: TMPRSS2. Following the binding of the spike protein with ACE2, it is cleaved by TMPRSS2 – a transmembrane serine protease. This leads to a conformational change of the spike protein, allowing the SARS-CoV-2 virion membrane to fuse with the host cell's membrane and the SARS-CoV-2 genome to enter the host cytoplasm. The positive-sense RNA of SARS-CoV-2 acts initially as an mRNA for the transcription of a replication-transcription complex, which then facilitates the production of negative-sense RNA copies and then positive-sense RNA fragments which associate with host ribosomes to produce SARS-CoV-2 proteins. The structural proteins self-assemble in the endoplasmic reticulum to form daughter virions which are transported to the cell membrane and ejected by exocytosis. Daughter virions can then infect neighbouring cells, setting off an exponential chain reaction of viral replication, or even be expectorated in respiratory droplets to complete the lifecycle (274). SARS-CoV-2 is directly toxic to host cells and induces cell death in AT2 cells, perhaps mediated by accessory proteins such as ORF3a (278,279).

At a length of only 76 amino acids, the SARS-CoV-2 envelope protein is the smallest structural protein. It is, however, a SARS-CoV-2 virulence factor and serves a function by forming cation channels in the host endoplasmic reticulum membrane, improving viral replicative fitness *in vitro* and leading to pathology *in vivo* (274,280,281).

1.6.6 Pathogenesis of SARS-CoV-2 Infections: Immunology

In addition to the pathology caused directly by SARS-CoV-2, much of the pathogenesis of COVID-19 is immunopathological. SARS-CoV-2 is initially sensed by host MDA5, a cytoplasmic pattern recognition receptor (PRR), which detects SARS-CoV-2 double-

stranded RNA, provoking the transcription and secretion of type 1 and type 3 interferons. In keeping with what was suggested by the epidemiological work on type 1 interferon autoantibodies, type 1 interferon responses have been found to be protective but suppressed in early SARS-CoV-2 infection (273,282). These released interferons cause paracrine, bystander effects on neighbouring epithelial cells and resident macrophages which (perhaps in addition to the direct sensing of SARS-CoV-2 by macrophages) triggers the production of chemokines (e.g. CCR2, CCL7, CCL8) and pro-inflammatory cytokines (e.g. IL-6, TNF α , pro-IL-1 β). These chemokines and cytokines trigger the homing and activation of monocytes, neutrophils and T cells, which respond by further producing cytokines and chemokines. Of particular clinical importance, IL-6 and TNF α levels are independently associated with COVID-19 severity and mortality (283). Although raised alongside acute phase reactants like CRP and ferritin, the circulating levels of these and other pro-inflammatory cytokines are much lower than in patients with sepsis, ARDS from traditional causes, or the 'cytokine release syndrome' seen in patients undergoing CAR T cell therapy (284). Nevertheless, the pro-inflammatory cascade set off in the alveoli results in alveolar epithelial cell death, oedema and dysregulated coagulation and fibrinolysis (leading to microvascular thrombosis) – a common pathway on the developmental route to ARDS (247).

Both monocyte-derived macrophages and native, resident alveolar macrophages display markers of aberrant activation and dysfunction in severe COVID-19. AMs were observed to have markedly suppressed expression of the AXL receptor, which is critical in their functions of efferocytosis and tissue repair (285). Monocytes and macrophages are a source of IL-1 β and IL-6 in the lungs of patients with COVID-19, and higher levels of IL-1 β are expressed by lung monocytes and macrophages in COVID-19 versus other respiratory infectious like influenza or pneumonia. Locally-produced pro-inflammatory cytokines like IL-1 β contribute to the pathogenesis of ARDS in part by interrupting the transition of AT2 cells to AT1 cells – an appropriate feature of lung regeneration following damage (285).

While IL-6 and TNF α were the cytokines associated with COVID-19 severity and mortality in clinical studies, their secretion from SARS-CoV-2 -infected monocytes was completely dependent on IL-1 β *in vitro*. Importantly, IL-1 β requires inflammasome activation for its own activation and secretion and, blocking components of the inflammasome, such as

NLRP3, also reduces IL-6 and TNF α secretion (286). IL-1 β is extremely short-lived and is not consistently detected in the serum of patients with COVID-19. Evidence for the relevance of inflammasome activation as a central feature of COVID-19 pathogenesis exists: circulating IL-18 (which also requires inflammasome activation) is associated with severity (287); IL-1 β has been detected in bronchoalveolar lavage fluid and pathological specimens (288,289); and mouse models of SARS-CoV-2 infection confirm inflammasome activation in infected lung macrophages, and inhibition of the macrophage inflammasome resulted in higher viral titres (290). Interestingly, SARS-CoV envelope protein activates the NLRP3 inflammasome in Vero E6 cells, mediated by its function as a cation channel. Alternatively, indirect inflammasome activation via damage-associated molecular patterns (DAMPs) may be dominant (291). Yalcinkaya and colleagues found that envelope protein suppresses NLRP3 inflammasome activation in naïve macrophages but increases it in LPS-primed macrophages (292). Once the NLRP3 inflammasome is activated, and IL-1 β is released, a positive feedback loop can develop with amplification of IL-6 secretion, cell death and further inflammasome activation (291).

1.6.7 Alveolar Macrophages and SARS-CoV-2

Alveolar macrophages (AMs) are the native, tissue-resident immune cell of the lung. In addition to their defining role in host-defence against bacteria such as *Mycobacterium tuberculosis*, AMs are also central players in the host's battle against viral infections such as influenza A (17,18). Being the most numerous immune cells of the lung, and given their location, AMs are likely the first *professional* immune cell to encounter SARS-CoV-2 following transmission and can exert an influence before any homing monocytes, neutrophils, or T cells arrive on the scene.

There is equipoise on the question of whether human AMs can be productively infected by SARS-CoV-2. Evidence from murine models of SARS-CoV-2 infection indicates that M1-polarised (IFN γ -pre-treated) murine AMs uptake SARS-CoV-2 by phagocytosis (in a non-ACE2-dependent manner) and permit its replication and active release (22). Humanised mouse lung macrophages permit SARS-CoV-2 replication (290).

A single-cell RNA sequencing study of BAL fluid from COVID-19 patients found both positive- and negative-sense SARS-CoV-2 RNA in AMs, indicating possible viral replication or perhaps merely a signal of epithelial phagocytosis. AMs from these patients, who were intubated with ARDS (i.e. late-stage disease), display an increased expression of cytokine and chemokine genes, as could be expected (293). However, much less is known about the early interactions of human AMs with SARS-CoV-2.

Dalskov et al. infected human AMs from BAL fluid with SARS-CoV-2 *in vitro* and could not detect any induction of type 1 or type 3 interferons, in contrast to influenza A. Other cytokines were not assessed. They could detect intracellular SARS-CoV-2 RNA at similar levels to the control, influenza A-infected cells, suggesting that SARS-CoV-2 enters the cell but somehow evades detection by AMs. Dalskov and colleagues also could not detect ACE2 mRNA expression by human AMs, whereas expression was strong in alveolar epithelial cells (294). This finding was replicated in a recent paper by Labzin et al. (295). This is in contrast to other reports which suggest that human AMs do, in fact, express ACE2: lung macrophages from humanised mice express ACE2, and blocking ACE2 reduces viral uptake in these macrophages (290); single-cell RNA sequencing data indicates ACE2 expression in a minority of alveolar macrophages of healthy individuals (296). Despite the minority of AMs expressing ACE2, many AMs contain SARS-CoV-2 RNA *in vivo*, suggesting ACE2-independent uptake in the majority of AMs (297). Uptake via phagocytosis rather than via ACE2 by MDMs and AMs led to an unproductive, abortive infection, according to Labzin's recent paper, whereas ACE2-expressing THP-1 macrophages could be productively infected (295). Importantly, there was inter-individual variability in the expression of ACE2 among healthy donors' AMs, and lung fibrosis was associated with higher expression – suggesting that AM ACE2 expression is not fixed but dependent on other factors (296). Perhaps that factor is macrophage polarisation, as suggested by the previously-mentioned murine studies (22).

Nevertheless, even if human AMs can only be abortively infected or are not infectible at all, they may still play an important role by recognising and responding to SARS-CoV-2 antigens. By analogy, human AMs cannot be productively infected with seasonal influenza A but yet are known to be critical and central in host defence against influenza A – by serving as a 'dead end' for influenza replication, through Fc-mediated cytokine

responses and through the suppression of autocrine production of cysteinyl leukotriene D4 by type 1 alveolar epithelial cells (17,18,298).

1.6.8 SARS-CoV-2 Proteins are PAMPs

SARS-CoV-2 double-stranded RNA is sensed by epithelial cell cytosolic pattern recognition receptors (PRRs) MDA5 and RIG-I, resulting in type 1 and type 3 interferon signalling – which, as discussed, is defective in human AMs (247,294). However, SARS-CoV-2 surface proteins may also be pathogen-associated molecular patterns (PAMPs) that are recognised by PRRs present on the cell membrane of human AMs and other myeloid cells. In particular, there is evidence that SARS-CoV-2 spike and envelope proteins are PAMPs, recognised by the innate immune system's toll-like receptors (TLRs).

TLR2 is a cell surface PRR that forms heterodimers with TLR1 or TLR6 and is widely expressed, including by macrophages and epithelial cells. Ligands for TLR2 include bacterial or protozoal proteins such as lipoarabinomannan expressed by *Mycobacterium tuberculosis*, porin expressed by *Neisseria meningitidis* and glycosphosphatidylinositol expressed by *Plasmodium falciparum*. Certain viral proteins are also recognised by TLR2, such as glycoprotein B expressed by herpes simplex virus (HSV). Following the binding of a ligand with TLR2, an intracellular cascade is triggered involving MyD88, TRAF6 and the transcription factors NF κ B and AP1. This results in increased gene expression of TNF α , pro-IL-1 β and IL-8, among others (299). There are conflicting reports as to whether SARS-CoV-2 spike protein is a TLR2 ligand. Certain studies report that THP-1 macrophages or human monocyte-derived macrophages respond to spike protein via TLR2 (300,301). Others report that spike protein does not provoke a cytokine reaction in murine BMDMs or human PBMCs, in contrast to the TLR2 ligand Pam3CSK4 (302). The same study found that SARS-CoV-2 envelope protein could induce TNF α , IL-6 and IL-1 β secretion in these cells, which was attenuated in BMDMs from TLR2 knockout mice. This finding that envelope protein binds to TLR2 was replicated in human embryonic kidney (HEK) cell lines (303). It is interesting that SARS-CoV-2 envelope protein might act as both signal 1 (as a ligand for a TLR) and signal 2 (as discussed above, by its function as a cation channel) in

inflammasome activation under certain circumstances, and enable the release of activated IL-1 β on its own.

TLR4 is another widely expressed cell surface PRR that detects bacterial proteins such as lipopolysaccharide (LPS) expressed by *Escherichia coli* as well as viral proteins such as the fusion protein of respiratory syncytial virus (RSV). Binding of a ligand with TLR4 results in proinflammatory cytokine transcription (e.g. IL-6, TNF α , pro-IL-1 β and IL-12) via NF- κ B and type 1 interferon transcription via IRF3 (304). Multiple studies have found SARS-CoV-2 spike protein to be a TLR4 ligand, although others have not, as is well summarised in a review by Halajian et al. (304). This variability may be the result of reagent contamination in some instances, differences in experimental design in others, or perhaps may reflect a true variability of response according to cell type. Whether envelope protein is a ligand for TLR4 is not yet clear. This was not specifically assessed by Zheng et al., who found that envelope activates TLR2 (302). Planès et al., who replicated the finding, observed that envelope protein-stimulated HEK cells responded less if pre-treated with an antagonist of TLR4, but blocking antibodies against TLR2 had a stronger effect (303). The strongest evidence that envelope protein is a TLR4 ligand comes from an impressive study by Anand et al. Envelope protein induced lung inflammation when inhaled by mice, induced reactive oxygen species (ROS) production in murine alveolar macrophages and induced interferon and NF- κ B signalling in both THP-1 and murine RAW macrophage cell lines. Importantly, this effect was not abrogated by polymyxin B (a control for LPS contamination) but was abrogated by knocking out TLR4 (305). This pivotal study places envelope protein-stimulated lung macrophages at the centre of pathological inflammation in COVID-19.

No studies have assessed whether human AMs, specifically, recognise SARS-CoV-2 spike protein or envelope protein, what the cytokine responses might be and which TLR is responsible for their detection (if any). Given the findings of Anand et al., this is an important knowledge gap that should be addressed.

1.6.9 Early Events in SARS-CoV-2 Infections

The presence of AMs during the critical, earliest encounters in natural SARS-CoV-2 infection – early in the exponential viral growth curve – opens the possibility that they play a role in summoning and co-ordinating other immune cells, in conducting their antiviral responses, in restraining the viral load trajectory and ultimately in determining the disease course.

The early events of tuberculosis infection are well understood to be critical in determining the disease course, and AMs are heavily implicated in these early events (36,306). AMs have also been shown to be critical in the early events of viral infections like those caused by respiratory syncytial virus (RSV) (23,307), influenza virus (24,26,308) and Newcastle disease virus (NDV) (25). Depletion or impairment of AMs results in worse disease outcomes in mice infected with RSV, influenza virus and NDV infection (23–26). AMs are the primary producers of type 1 interferons in these three viral infections (23,25,308) and are also early producers of other pro-inflammatory cytokines and chemokines such as TNF α , IFN γ , IL-6, CXCL8 and CXCL10 in these infections (25,307,308). While human AMs fail to produce type 1 interferons in reaction to SARS-CoV-2 (the reason SARS-CoV-2 is so successful, perhaps) (294), they might produce other relevant pro-inflammatory cytokines and chemokines in the critical early phase. This has not been assessed in humans but is true in mice (22). These early AM responses would likely be dependent on the recognition of SARS-CoV-2 by PRRs such as TLRs. Strong epidemiological evidence for the importance of early innate responses in determining COVID-19 outcomes is presented in studies of TLR genetic defects (268–270).

1.6.10 Ageing and Alveolar Macrophages

Early innate immune responses to infection may be deficient in older people. Ageing adversely affects core macrophage functions like autophagy via the hypermethylation of autophagy genes (309,310). AMs from aged mice also display impaired phagocytosis and

a baseline enrichment of proinflammatory gene transcription. These older mice are more susceptible to influenza A (26). The same study found that AMs were less numerous in aged mice, although other studies dispute that (26,311). The ontogeny of AMs may be relevant here: AMs are derived primarily from the foetal yolk sac and liver, self-repopulate at steady state but can be replaced by macrophages of monocyte origin following severe pulmonary insult such as irradiation or infection with influenza (312,313). Over time, repeated environmental insults and infections may gradually lead to a replacement of 'true' AMs by monocyte-derived macrophages – which may have a distinct phenotype (314,315). McQuattie-Pimentel et al. recently reported, however, that the alveolar microenvironment, and not ontogeny, results in the dysfunction observed in aged mice AMs (316). In any case, AMs from older mice display an M1 polarised, proinflammatory phenotype (317–319). No studies examining the effect of ageing on *human* AM functions have been published.

Differences in TLR expression or pathway function with age might explain this proinflammatory phenotype. Circulating monocytes from older adults display baseline activation and increased responsiveness to LPS (320). TLR4 surface expression is increased on AMs from aged mice, whereas TLR2 expression is not affected by age (311,321). However, downstream signalling is impaired following TLR2 activation of AMs in aged mice (321). Circulating monocytes from older humans also have preserved TLR2 surface expression but, similarly, demonstrate impaired TLR2 pathway functioning – secreting less TNF α and IL-6 in response to Pam3CSK4 (322–325). Thus, if SARS-CoV-2 antigens are TLR4 ligands, a hyperresponsive reaction by AMs from older adults might be expected. On the other hand, if SARS-CoV-2 antigens are TLR2 ligands, a hyporesponsive reaction by AMs from older adults might be expected. Given that severe COVID-19 is more associated with an inappropriate, hyperinflammatory phenotype, we might predict the former to be more likely.

In addition to differences in TLR expression or pathway function, variance in baseline inflammasome activation with age could be relevant. Indeed, alveolar macrophages from aged mice are known to have increased NLRP3 activation (326), something that has been identified as driving pathological inflammation in COVID-19 disease, especially in the elderly (290,327).

1.6.11 Alveolar Macrophage Responses to SARS-CoV-2: Clinical and Research Needs

An understanding of how AMs from older and younger adults respond to SARS-CoV-2, which antigens are recognised, and by what pathway should be of great interest to both researchers and clinicians. Clarity is needed on whether human AMs can be productively infected with SARS-CoV-2, to answer a fundamental question of SARS-CoV-2 immunobiology on which some equipoise remains (22,290,294,295). The antigens, receptors or downstream pathways that drive inappropriate host immune responses in the elderly might be good therapeutic targets for drug development or the redeployment of already-existing therapies. Furthermore, while every licensed vaccine to-date is designed to induce antibodies against the SARS-CoV-2 spike protein, inhibiting ACE2-dependent cell entry, breakthrough infections do occur: despite almost universal vaccination coverage, 2,321 COVID-19 deaths were reported in Ireland in 2022, almost all in the elderly (264). If the SARS-CoV-2 envelope protein is a trigger of immunopathology in human AMs, anti-envelope antibodies induced by novel vaccines which included this antigen (in addition to spike protein) might intercept the envelope protein, reduce the resulting immunopathology, improve vaccine efficacy and help to prevent these severe breakthrough infections. Indeed, the inclusion of envelope protein in COVID-19 vaccines has been proposed as a suitable vaccine target before and even explored in preliminary studies (328–330). As the SARS-CoV-2 spike protein has mutated over the course of the pandemic, the efficacy of vaccines and monoclonal antibody therapies directed at spike protein have declined with more recent variants (263,331). On the other hand, envelope protein is highly genetically conserved between SARS-CoV and SARS-CoV-2, with 95% sequence similarity, while less pathogenic *Coronaviridae* show much less similarity, pointing to its role in virulence (332–334). Envelope protein was found to be more genetically stable than spike protein during the first two years of the pandemic, further supporting its exploration as a viable vaccine target (335).

In summary, there is both a research and clinical need to better understand the interactions between specific SARS-CoV-2 proteins and host receptors to characterise human AM responses in the earliest phase of SARS-CoV-2 infection and to define how these responses vary by age.

1.7 Outline of Results Chapters

In chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) we seek to examine the relationships between endogenous vitamin A pathways and macrophage responses to Mtb infection. We aim to determine the effect of Mtb infection on human macrophage vitamin A pathways and to determine the effects of *endogenously* produced ATRA on macrophage responses to Mtb infection. In pursuing these aims, we hope to better understand why clinical trials of retinol in TB have failed and understand how VAD confers a risk of TB.

In chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) we investigate ATRA as a potential HDT for NTMs. We aim to determine if ATRA is an effective HDT against NTMs *in vitro* and to determine ATRA's mechanisms of action as a HDT against NTMs. This research is a proof of concept for a novel, host-directed therapeutic strategy for NTM disease.

In chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) we assess if and how human AMs respond to SARS-CoV-2 antigens and the SARS-CoV-2 virus itself. We aim to determine if human AMs produce cytokines in response to SARS-CoV-2 antigens, by which TLR and to understand if the clinical risk factors of age and smoking are associated with different cytokine responses of human AMs to SARS-CoV-2 antigens. We also aim to determine how human AMs respond to SARS-CoV-2 itself. A better understanding of how advanced age leads to severe COVID-19 may arise from this research.

Chapter 2

2 Methods and Materials

We employed *in vitro* cellular models of infection to investigate our hypotheses. We used both primary human macrophages and human macrophage cell lines, and a variety of stimuli and live mycobacterial species to infect these human macrophages. This approach allowed us to probe, with high cell specificity and temporal resolution, the early host-pathogen interactions relevant to our research aims.

2.1 Cell Culture

2.1.1 Human Monocyte-Derived Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of healthy donors by density gradient centrifugation using Lymphoprep (STEMCELL Technologies), washed and resuspended at 3×10^6 cells/ml in RPMI (ThermoFisher) supplemented with 10% AB human serum (Sigma-Aldrich).

MDMs were selected using adherence separation by plating the PBMCs onto non-treated plates (Corning Costar). For donors that were planned to be infected, the PBMCs were simultaneously plated onto 8-well glass Labtek slides at the same density to allow for the determination of the multiplicity of infection (MOI). Cells were incubated at 37 °C and 5% CO₂ for 7-10 days. Non-adherent cells were removed by washing every 2-3 days.

For Seahorse metabolic experiments, MDMs were selected as before. Cells were washed on day 6, and then gently scraped and reseeded in fresh media onto XF24 Seahorse Cell Culture Plates (Agilent) at 1×10^5 cells per well and simultaneously onto 8-well glass Labtek slides at the same density to allow for the determination of the MOI. Cells were incubated 37 °C and 5% CO₂ for 2 days, washed and supplemented with fresh media immediately prior to experimentation.

2.1.2 Human Alveolar Macrophages

Human AMs were retrieved from bronchoalveolar lavage (BAL) fluid of consenting patients undergoing routine bronchoscopy at St. James's Hospital, Dublin. Ethics was approved by St. James's Hospital/Tallaght University Hospital Joint Research Ethics Committee. Exclusion criteria for collection included a diagnosis of lung cancer, sarcoidosis, HIV or any active infection. BAL fluid was filtered through a 100 µm nylon strainer (BD Falcon; BD Bioscience, Erembodegem, Belgium).

The AMs were pelleted by centrifugation at 150 g for 15 minutes and resuspended at 2.5×10^5 cells/ml in RPMI (ThermoFisher) supplemented with 10% foetal calf serum (Sigma-Aldrich), 2.5 µg/ml Fungizone and 5 µg/ml cefotaxime (Invitrogen). For chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) experiments, AMs were plated onto untreated 12-well plates (Corning Costar) at 2.5×10^5 cells/ml and simultaneously onto 8-well glass Labtek slides at the same density to allow for the determination of the MOI. For chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) experiments, AMs were plated onto untreated 8-well Seahorse miniplates (Agilent) at 8×10^4 cells per well and simultaneously onto 8-well glass Labtek slides. For chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) antigen stimulation experiments, AMs were plated onto untreated 48-well plates (ThermoFisher) at 2.5×10^5 cells/ml. For chapter 5 SARS-CoV-2 infection experiments, AMs were plated onto untreated 12-well plates (Corning Costar) at 2.5×10^5 cells/ml.

AMs were incubated at 37 °C, 5% CO₂ for 24 hours and then washed to remove non-adherent cells, at which point chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) Seahorse experiments started. Otherwise, cells were incubated for a further 2-4 days prior to experimentation, replacing with fresh media on the morning of experimentation (*Figure 2.1*).

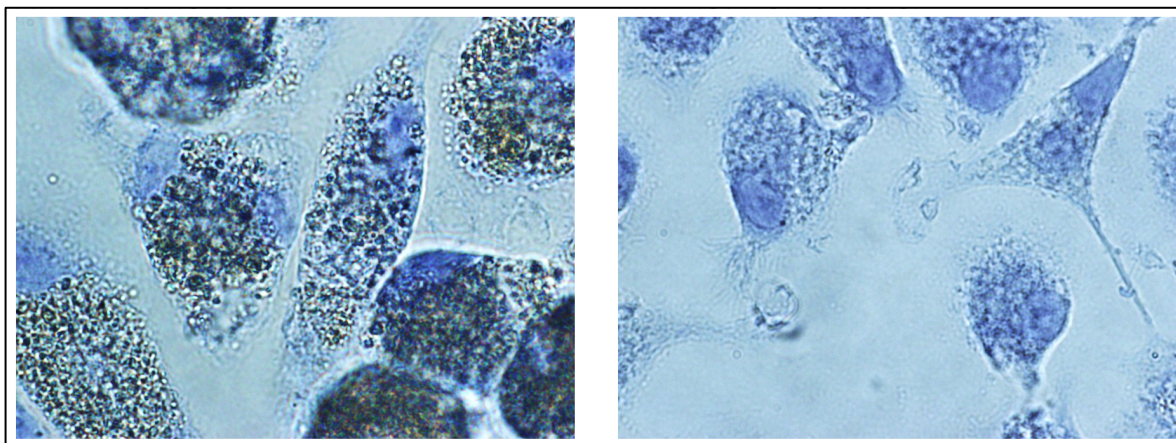


Figure 2.1: *Bright field microscopy images of plated alveolar macrophages from a smoker (left) and a non-smoker (right). The smoker's macrophages contain phagocytosed products of smoke inhalation.*

2.1.3 THP-1 Macrophages

THP-1 cells were cultured in RPMI with 10% foetal calf serum and incubated at 37 °C, 5% CO₂. 3-4 days prior to infection, the cells were centrifuged and counted. The THP-1s were diluted to a density of 1×10^5 cells per ml with 100nM phorbol 12-myristate 13-acetate (PMA) and plated onto untreated 12-well plates (Corning Costar) or XF24 Seahorse Cell Culture Plates (Agilent) as well as an 8-well glass Labtek slide. The THP-1s were allowed to differentiate for 3-4 days, at which point the PMA-containing medium was removed and replaced with fresh medium on the morning of experimentation.

2.2 Macrophage Treatments and Pre-treatments

2.2.1 Retinoic Acid Receptor Inhibitors & Ligands

Three retinoic acid receptor inhibitors (RARIs) were used. Ro 41-5253 (Sigma-Aldrich; SML0573) is a RAR α antagonist which was reconstituted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D2650) and stored at -30 °C. ER 50891 (R&D; 3823) is also a RAR α antagonist which was reconstituted in DMSO and stored at -30 °C. AGN 193109 (R&D; 5758) is a pan-RAR antagonist that was reconstituted in DMSO and stored at -30 °C. The

RAR ligand all *trans* retinoic acid (ATRA) (Sigma; R2625) was reconstituted in DMSO and stored at -30 °C. ATRA is photosensitive, and so was shielded from the light, and was sonicated before use. For all experiments, cells were first washed and fresh media was applied. The cells were then treated with 2 µM of Ro 41-5253, 10 µM of ER 50891, 1 µM of AGN 193109 or 10 µM of ATRA (*Table 2.1*).

For most experiments, the cells were treated for 3 hours before infection. The fresh media applied at the end of the infection (sections 2.3.2-4) also contained the RAR α or ATRA at the same concentration. For 'acute infection' Seahorse experiments, the cells were treated for 2 hours before the non-CO₂ incubation in Seahorse medium step (section 2.6.2). This Seahorse medium also contained the RAR α or ATRA at the same concentration. The RAR α or ATRA remained in situ for the duration of experimentation and were present at the moment of infection. In total, counting the 2 hour pre-treatment, the incubation in Seahorse medium step and the baseline ECAR and OCR measurements, the cells were treated for approximately 3 hours prior to infection. For 'Mito Stress Test' Seahorse experiments, the cells were treated for 3 hours before the non-CO₂ incubation in Seahorse medium step, but this Seahorse medium did not contain the treatments. In all cases, the same concentration of DMSO that was present in the treatment condition was used as a negative control (*Table 2.1*).

2.2.2 Cytokines

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immunotools; 11343125) was reconstituted in water for injection (WFI) and stored at -30 °C. Recombinant human interleukin 4 (IL-4) (Immunotools; 11340045) was reconstituted in WFI and stored at -30 °C. Cells were treated with 50 ng/ml of GM-CSF or 20 ng/ml of IL-4 for 24 hours prior to infection. As before, the fresh media applied at the end of infection also contained GM-CSF or IL-4 at the same concentration, and these cytokines remained in place for the duration of experimentation (*Table 2.1*).

2.2.3 Antibodies

Monoclonal anti-human TLR2 IgG2a kappa (anti-TLR2) (ThermoFisher; 16-9922), monoclonal anti-human TLR4 IgG2a kappa (anti-TLR4) (ThermoFisher; 16-9917) and isotype control anti-mouse IgG2a kappa (isotype control) (ThermoFisher; 16-4724) were reconstituted in sterile PBS and stored at 4 °C prior to experimentation. For TLR inhibition experiments, cells were first washed and then fresh media was applied. The cells were then treated with 10 µg/ml of anti-TLR2, anti-TLR4 or the isotype control antibody for 1 hour prior to stimulation. Following stimulation, the cells were not washed before the supernatant was collected i.e., the antibodies remained in place for the duration of the experiment (*Table 2.1*).

2.2.4 Other Treatments

The mitogen activated protein kinase kinase (MEK) inhibitor, PD98059 (ThermoFisher; PHZ1164), and the peroxisome proliferator-activated receptor gamma (PPAR γ) inhibitor, GW 9662 (Tocris; 1508), were each reconstituted in DMSO, and used at final concentrations of 10µM. For the Seahorse experiments in which these reagents were used, the cells were pre-treated with the MEK inhibitor or PPAR γ inhibitor for one hour before being treated with the RARI for two hours as before. When the media was replaced with Seahorse medium, this media also contained the MEK inhibitor or PPAR γ inhibitor in addition to the RARI, at the same concentrations and these treatments remained in place for the duration of experimentation (*Table 2.1*).

DEAB (Sigma-Aldrich; D86256), an inhibitor of aldehyde dehydrogenases was reconstituted in DMSO and used at a concentration of 100 µM, sonicating before use. Metformin (Sigma-Aldrich; 317240) was reconstituted in WFI and used at a concentration of 5 mM. For 'acute infection' Seahorse experiments utilising DEAB or metformin, the cells were treated for 2 hours before the non-CO₂ incubation in Seahorse medium step (see section 2.6.2). This Seahorse medium also contained the DEAB or metformin at the same concentration. The DEAB or metformin remained in situ for the duration of experimentation and were present at the moment of infection. For 'Mito Stress Test'

Seahorse experiments, the cells were treated for 3 hours before the non-CO₂ incubation in Seahorse medium step, but this Seahorse medium did not contain the treatments. DMSO was used as a vehicle control for DEAB (*Table 2.1*).

Amikacin (Sigma-Aldrich; A1774) was reconstituted in WFI and used at 250 µg/ml. Its use is described in section 2.3.4 (*Table 2.1*).

Treatment/Pre-Treatment	Cat. #	Conc.	Duration
<i>(Unless otherwise stated)</i>			
<u>RAR Inhibitors & Ligands</u>			
Ro 41-5253 (RAR α antagonist)	SML0573	2 μ M	3 hours pre-inf.
ER 50891 (RAR α antagonist)	3823	10 μ M	3 hours pre-inf.
AGN 193109 (pan-RAR antagonist)	5758	1 μ M	3 hours pre-inf.
All <i>Trans</i> Retinoic Acid	R2625	10 μ M	3 hours pre-inf.
<u>Cytokines</u>			
GM-CSF	11343125	50 ng/ml	3 hours pre-inf.
IL-4	11340045	20 ng/ml	3 hours pre-inf.
<u>Antibodies</u>			
TLR2 IgG	16-9922	10 μ g/ml	1 hour pre-stim.
TLR4 IgG	16-9917	10 μ g/ml	1 hour pre-stim.
Isotype Control IgG	16-4724	10 μ g/ml	1 hour pre-stim.
<u>Other</u>			
DMSO	D2650	(Control)	3 hours pre-inf.
Metformin	317240	5 mM	3 hours pre-inf.
DEAB	D86256	100 μ M	3 hours pre-inf.
PD98059 (MEK antagonist)	PHZ1164	10 μ M	1 hour pre-RAR
GW 9662 (PPAR γ antagonist)	CAYM70785	10 μ M	1 hour pre-RAR
Amikacin	A1774	250 μ g/ml	1 hour post-inf.

Table 2.1: Macrophage treatments and pre-treatments.

2.3 Infections and Stimulations

2.3.1 Determination of the Multiplicity of Infection

To control for inter-donor variability in phagocytosis, every infection or stimulation of macrophages with mycobacteria was preceded by a multiplicity of infection (MOI) assay. When cells were plated onto cell culture plates, they were also simultaneously plated in parallel onto 8-well glass Labtek slides at the same seeding density. Whenever the cells were washed and fresh media applied, both the cell culture plate and the Labtek slides were treated identically. On the morning of infection or stimulation, the cells in the Labtek slide wells were infected first with a range of volumes (a different volume in each well) of the log-phase mycobacterial preparation. Three hours later, the media was removed, the cells were washed with PBS and then fixed with 4% paraformaldehyde. The slides were stained for acid fast bacilli using an auramine stain, counterstained with Hoechst 33342, and the slides were then examined using fluorescent microscopy. The number of bacilli phagocytosed per cell and the percentage of infected cells were counted in at least 3 fields for each well. The volume needed to achieve the desired MOI in the cell culture plates was then calculated, using the ratio of the well surface areas as a multiplication factor (*Figure 2.2*).

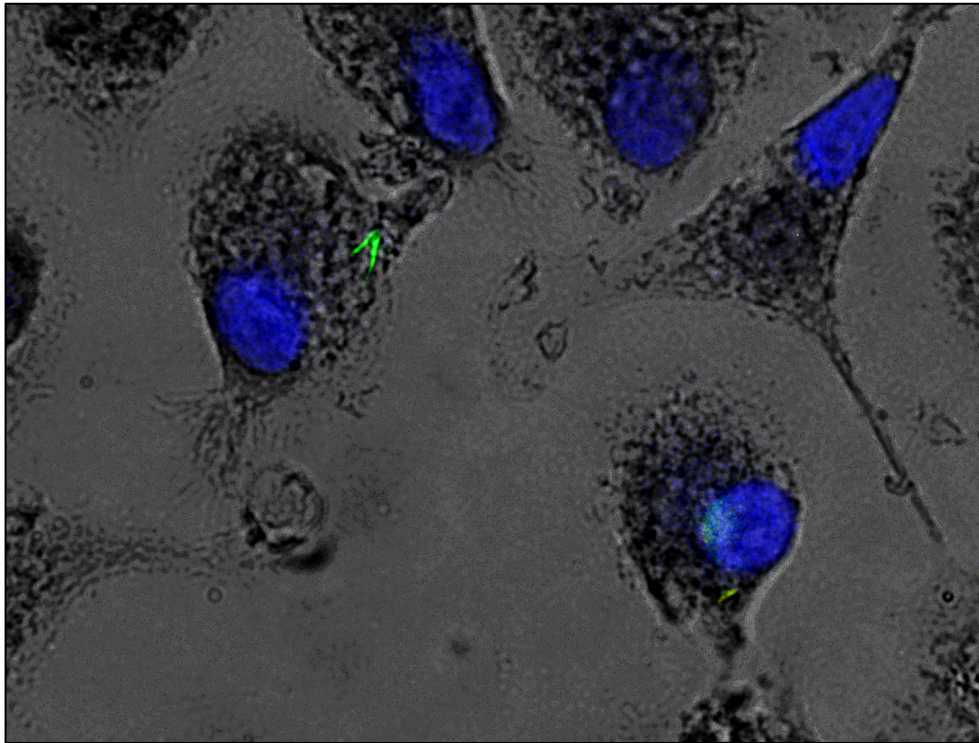


Figure 2.2: Composite fluorescent microscopy image of alveolar macrophages infected with Mycobacterium tuberculosis (H37Ra) in vitro. Nuclei stained with Hoechst 33342 are in blue and bacilli stained with auramine are in green. Fluorescent microscopy was used to determine the multiplicity of infection for each donor. In this image, the macrophage in the upper left quadrant is infected with at least two bacilli. The macrophage in the lower right quadrant is also infected with at least one bacillus (more exact counting requires the dynamic use of focusing and higher resolutions that can be achieved using a fluorescent microscope in person).

2.3.2 *Mycobacterium tuberculosis* Infections and Stimulations

Mycobacterium tuberculosis H37Ra was obtained from the American Type Culture Collection ATCC 2517, (Manassas, VA) and propagated from frozen stocks to log phase in Middlebrook 7h9 medium (Difco; 271310) containing WFI (Merck; 4.86505.1000), 0.05% Tween 80 (Difco; 231181) and 10% Middlebrook albumin, dextrose, catalase (ADC) enrichment (Difco; 211887) for approximately 7 days. On the morning of infection, log phase bacilli (growing in Middlebrook 7h9 medium) were centrifuged at 3,000 g for 10 minutes and then resuspended in the same media as that used for the macrophages being infected. To remove clumps, the bacilli were first passed through a 25 gauge needle six times and then the sample was centrifuged at 100 g for 3 minutes. The volume of the sample needed to achieve the desired MOI was then calculated as per section 2.3.1 (336).

The MOI was determined for each individual donor. Unless otherwise stated, MDM and AM were infected at an MOI of 1-10 bacilli/cell for 3 hours. Extracellular bacteria were then removed by washing with PBS, fresh media applied, and cells incubated at 37 °C and 5% for 24 hours (Figure 2.3, Table 2.2).

For Seahorse experiments, gamma-irradiated *M. tuberculosis* H37Rv (whole-cells) (BEI Resources; NR-49098) was used. On the morning of stimulation, this irradiated H37Rv (iH37Rv) was defrosted from stocks frozen at -30 °C and sonicated before use. In these experiments, macrophages were stimulated with an amount of iH37Rv sufficient to achieve an MOI of 5-10 bacilli/cell during the course of the metabolic measurements – the volume needed to achieve the desired MOI was determined as per section 2.3.1. The stimulating volume of iH37Rv preparation was introduced via the Seahorse cartridge injection port. These iH37Rv stimulations were timed to occur after three baseline metabolic measurement had been taken. An equal volume of Seahorse medium was injected as a negative control (Figure 2.4, Table 2.2).

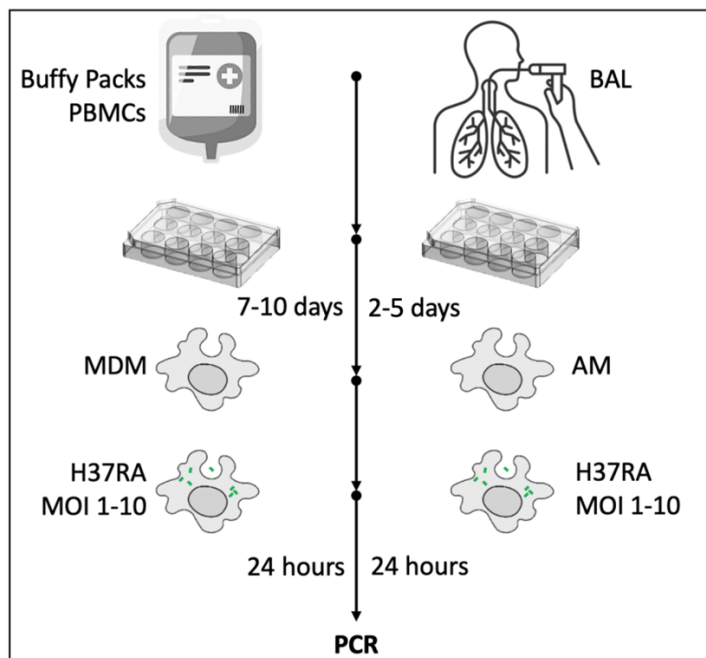


Figure 2.3: Methods for experiments in which human MDMs and AMs were infected with H37Ra.

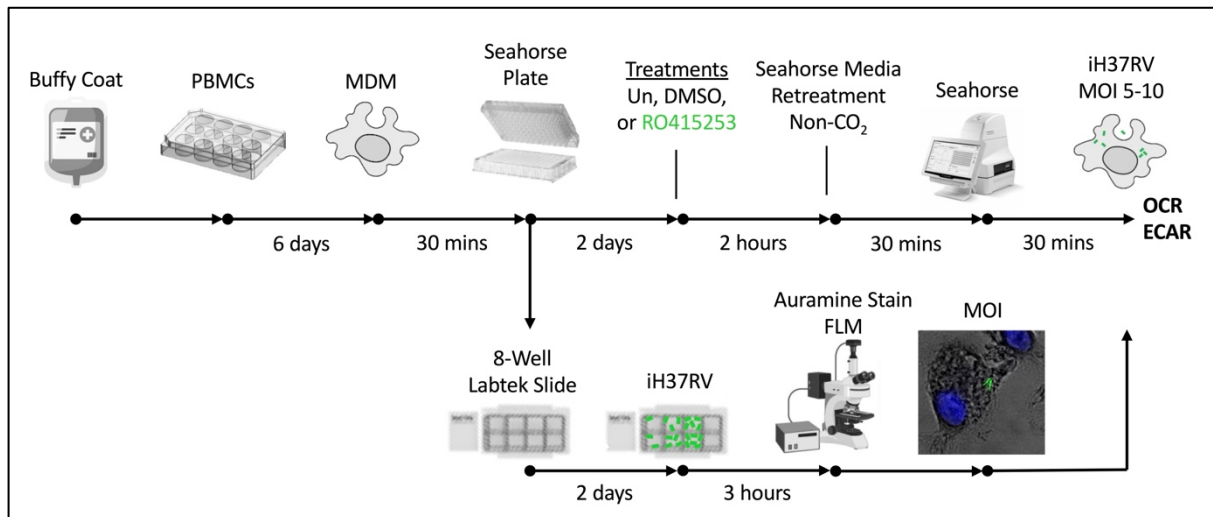


Figure 2.4: Methods for Seahorse experiments in which human MDMs were stimulated with iH37Rv.

2.3.3 *Mycobacterium chimaera* Infections

Mycobacterium chimaera (DSMZ, German Collection of Microorganisms and Cell Cultures; 44623) was propagated to log phase in Middlebrook 7h9 medium (same as for *M. tuberculosis*, described above). THP-1s were infected with *M. chimaera* at an average MOI of 1-2 bacilli per cell. 3 hours following infection, the cells were washed and fresh media was applied.

For Seahorse experiments, AMs were infected with *M. chimaera* at an MOI of 5-10 bacilli/cell during the course of the metabolic measurements – the infecting volume of the *M. chimaera* preparation was introduced via the Seahorse cartridge injection port after three baseline metabolic measurements. An equal volume of Seahorse medium was injected as a negative control (Table 2.2).

2.3.4 *Mycobacterium abscessus* Infections

Mycobacterium abscessus was obtained from the American Type Culture Collection (ATCC 19977, Manassas, VA) and propagated to log phase in Middlebrook 7h9 medium (same as for *M. tuberculosis*, described above).

THP-1s were infected with *M. abscessus* at an average multiplicity of infection (MOI) of 1-2 bacilli per cell. 3 hours following infection, the cells were washed, and fresh media was applied. At this point for experiments using *M. abscessus*, an additional 1h treatment step with amikacin (250 mg/ml) (A1774, Sigma-Aldrich) was undertaken to kill any remaining extracellular bacilli. The same step was also undertaken for uninfected conditions. The cells were again washed and fresh media was applied.

For Seahorse experiments, AMs were infected with *M. abscessus* at an MOI of 5-10 bacilli/cell during the course of the metabolic measurements – the infecting volume of the *M. abscessus* preparation was introduced via the Seahorse cartridge injection port after three baseline metabolic measurements. An equal volume of Seahorse medium was injected as a negative control (*Table 2.2*).

2.3.5 Stimulation of Macrophages with TLR Ligands

The TLR4 ligand lipopolysaccharide (LPS) (Sigma-Aldrich; L5418) was used at a concentration of 100 ng/ml. LPS stimulation was used in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) as a model of gram-negative bacterial infection, and in chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) as a positive control and for the validation of the anti-TLR4 blocking antibodies.

The TLR2/1 ligand Pam3CSK4 (Invivogen; tlr1-pms) was used at concentrations of 1 µg/ml and 2.5 µg/ml and used to validate the anti-TLR2 blocking antibodies in chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*).

Macrophages were stimulated with these TLR ligands for 24 hours before supernatants or cell lysates were retrieved (*Table 2.2*).

2.3.6 Stimulation of Macrophages with SARS-CoV-2 Antigens

Alveolar macrophages were retrieved and selected as described above. On the morning of the experiment, fresh media was applied to the AMs. The AMs were then treated for 1 hour with blocking antibodies against TLR2 or TLR4 or an isotype control antibody as described above. The AMs were then stimulated with SARS-CoV-2 spike protein (R&D; 10549-CV) at 1 µg/ml, envelope protein (ABclonal; RP01263) at 1 µg/ml or left unstimulated. An untreated, LPS-stimulated (100 ng/ml) condition was used as a positive control. Supernatant was collected at 6- and 24-hours post-stimulation, and cell lysate was taken at 24 hours post-stimulation for RNA extraction (*Figure 2.5, Table 2.2*).

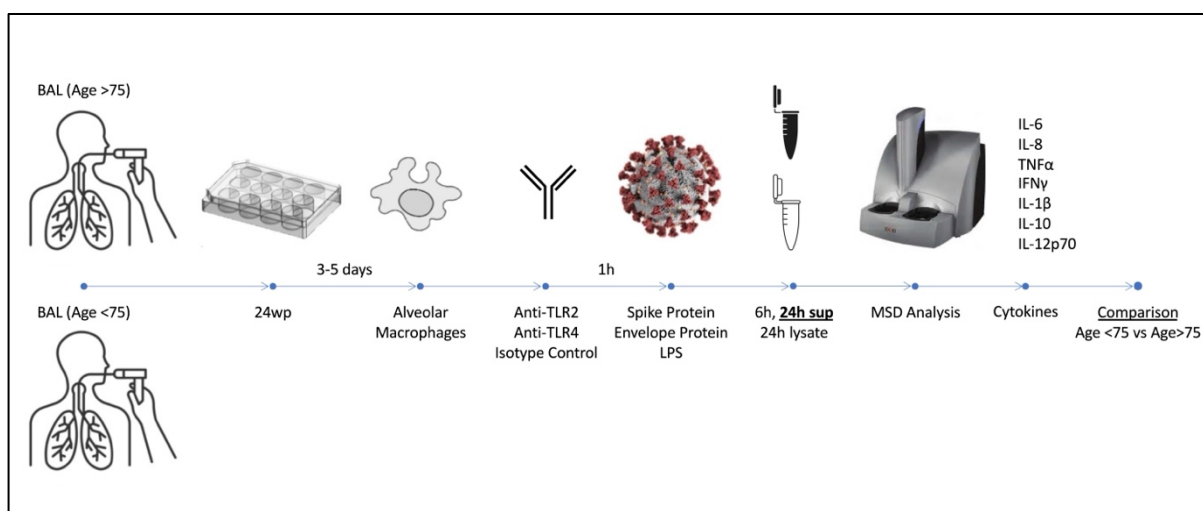


Figure 2.5: Methods for experiments in which human AMs were stimulated with SARS-CoV-2 antigens.

2.3.7 Infection of Macrophages with SARS-CoV-2

We worked in collaboration with Professor Andrew Bowie and Dr. Hossam Gewaid from the Trinity Biomedical Science Institute (TBSI) to infect AMs with live SARS-CoV-2 in a containment level 3 laboratory at their institute. Human AMs were retrieved and selected as described in section 2.1.2. Experiments using live SARS-CoV-2 (sections 2.3.7.1-2.3.7.8) were done by Dr. Gewaid as follows:

2.3.7.1 Cell Cultures:

HEK-Blue IFN- α/β reporter cells were purchased from InvivoGen and grown in DMEM, supplemented with 10% FBS (v/v), 10 $\mu\text{g/ml}$ penicillin-streptomycin, Blasticidin (30 $\mu\text{g/ml}$), Zeocin (100 $\mu\text{g/ml}$) and Normocin (100 $\mu\text{g/ml}$). Vero E6 Cells were a gift from Dr. Kim Robert (Trinity College Dublin).

2.3.7.2 SARS-CoV-2 Propagation:

Vero E6 cells were seeded (1:3 ratio) and incubated in the growth media (DMEM, 10% FBS, 1% Penicillin-streptomycin (Pen/Strep)), until 80% confluency. Cells were then washed with PBS on the day of infection. Ten μl of SARS-CoV-2 virus (delta variant; B.1.617.2) was diluted into 10ml DMEM, containing 4% BSA and added into the cells, and the flask was incubated for 90 minutes and shaken every 10 minutes. The inoculum was removed and replaced with DMEM, 2% FBS, Pen/Strep. The culture medium was collected 48- and 72-hours post-infection, passed through a 0.45 μm filter, aliquoted, and kept at -80°C .

2.3.7.3 Infection with SARS-CoV-2:

AMs isolated were infected with SARS-CoV-2 virus (at 0.01, 0.1, 1, or 5 MOI, diluted in 2% FBS RPMI). The inoculum was removed after 1 hour, and cells were washed twice with PBS and fresh media were added to the cells. Supernatants were collected at 48 hours post-infection and divided into two parts. One part was kept at -80°C for plaque assay while the other part was decontaminated by ultraviolet cross-linking and then kept at -20°C for ELISA analysis. The cells were washed with PBS and TRIZOL was added to each well and kept at -80°C for RNA extraction (*Table 2.2*).

2.3.7.4 Generation of RNA Transcript Standard for RT-qPCR:

Standard RNA used in the quantification of SARS-CoV-2 RNA genome copy numbers by RT-qPCR was synthesised and quantified as previously described (337). In brief, extracted RNA (using TRIZOL) from SARS-CoV-2-infected Vero E6 cells was used as a template for cDNA synthesis using SuperScript III and reverse primer (N-Std-Rev, TGTCTCTGCGGTAAGGCTTG). cDNA was used as a template for N gene amplification using

N-St-Rev primer and the forward primer (N-Std-T7-Fwd, TAATACGACTCACTATAGGGGAATTGTGCGTGGATGAGGC). After gel electrophoresis, the PCR product was purified using QIAquick PCR Purification Kit (Qiagen). The purified template was in vitro transcribed using Megascript T7 kit (ThermoFisher Scientific). In vitro transcribed RNA product was purified using Qiaamp Viral RNA extraction kit (Qiagen). Extracted RNA was quantified using nanodrop and the number of viral RNA copies/ μL was calculated using Avogadro's number as previously mentioned (337).

2.3.7.5 SARS-CoV-2 RNA Quantification by RT-qPCR Assay:

SARS-CoV-2 RNA quantification by RT-qPCR assay was performed using QuantiNova Probe RT-PCR kit (Qiagen) in a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland). The total reaction mixture was 20 μL reaction mixture containing 10 μL of 2 $\times$ QuantiNova Probe RT-PCR Master Mix (Qiagen), 0.2 μL of QuantiNova Probe RT-Mix (Qiagen), 1.6 μL each of forward and reverse primer, 0.4 μL of 10 μM probe, and 6.2 μL of extracted RNA or 10 times serially diluted standard as template. The reactions were incubated at 45 °C for 10 minutes, 95 °C for 5 minutes for denaturation, followed by 45 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds.

2.3.7.6 IFN-I Bioassay:

Collected supernatants, diluted (1:5) in the test medium (DMEM, 10% FBS, 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 100 $\mu\text{g}/\text{ml}$ normocin), and IFN standard (10 times diluted) were added in 20 $\mu\text{L}/\text{well}$ in 96 well plate. HEK-Blue IFN α/β reporter cells were seeded (180 $\mu\text{L}/\text{well}$) at 2.8×10^5 cells/ml in 96-well plates containing diluted supernatant, standard and blank, and incubated at 37 °C for 20-24 hours. Secreted SEAP activity that reflects INF-I activity was analysed by QUANTI-Blue (Invivogen), and absorbance was measured at 620 nm.

2.3.7.7 Plaque Assay for Titration of SARS-CoV-2:

To titrate the SARS-CoV-2 virus stock or the virus in the supernatants of SARS-CoV-2-infected AM cells, Vero E6 cells were seeded into a 12-well plate at a density of 3×10^5 cells per well. The next day, the cells were incubated with 8 serial 10-fold dilutions (in

DMEM, 2% FBS) of the virus stock or the virus in the collected supernatants from AM-infected cells (200 µl volume per well) for 90 min. at 37°C, overlaid with 2ml carboxymethyl cellulose 1%/well and incubated at 37 °C for 72 hours. The cells were fixed with 8% paraformaldehyde (4% final concentration) and incubated for at least 1 hour at room temperature and then stained with 0.1% crystal violet. The number of plaques was counted, and the virus titre was calculated.

2.3.7.8 RNA Analysis by RT-qPCR:

Genes expression was analysed as described previously (338). Total RNA was extracted from cells using Direct-zol™ RNA Miniprep (Zymo Research) and reverse transcribed with random hexamers (IDT) using SuperScript III (Invitrogen) according to the manufacturer's instructions. Relative expression of mRNA was quantified with SYBR Green using primer pairs targeting IFNL1, IFNL2/3, IL6, IL-1β, and TNFα. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, normalising the gene of interest to the housekeeping gene β-actin, and analysing the data as fold induction compared to that of the control sample.

2.3.7.9 Analysis of Secreted Cytokines Following SARS-CoV-2 Infection:

Supernatant was transported back to our laboratory, where we carried out the secreted cytokine analyses using an MSD multisport assay, as described in section 2.5.1.

Infection/Stimulation	Cat. #	MOI/Conc.	Duration
(Unless otherwise stated)			
Mycobacteria			
<i>M. tuberculosis</i> (H37Ra)	ATCC 2517	1-2 (CFUs)	0, 2, 5 days
		1-10 (Others)	24 hours
<i>M. tuberculosis</i> (irradiated H37Rv)	NR-49098	5-10 (Seahorse)	3 hours
<i>M. chimaera</i>	44623	1-2 (CFUs)	0, 2, 5 days
		5-10 (Seahorse)	3 hours
<i>M. abscessus</i>	ATCC 19977	1-2 (CFUs)	0, 18 hours
		5-10 (Seahorse)	3 hours
SARS-CoV-2			
Spike Protein	10549-CV	1 µg/ml	24 hours
Envelope Protein	RP01263	1 µg/ml	24 hours
SARS-CoV-2		0.01-5	48 hours
TLR ligands			
LPS	L5418	100 ng/ml	24 hours
Pam3CSK4	tlrl-pms	1, 2.5 µg/ml	24 hours

Table 2.2: Macrophage infections and stimulations.

2.4 Assessment of Macrophage ATRA Production and Transport Genes

2.4.1 Gene Expression Analysis

The gene expression of enzymes needed to oxidise retinol to retinal, enzymes needed to oxidise retinal to ATRA, ATRA carrier proteins and degradation enzymes was assessed at 24 hours post-infection or stimulation. The media was removed and the cells were washed once with PBS. Cells were lysed in Buffer RLT Plus containing β -mercaptoethanol (RNeasy Plus Mini kit, QIAGEN), according to the manufacturer's instructions, and then scraped from the plate. RNA extraction was performed using the RNeasy Plus Mini Kit (QIAGEN), and cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

Quantitative PCR (qPCR) was performed using TaqMan primers for oxidation enzymes (DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1), carrier proteins (CRABP1, CRABP2 and FABP5) and degradation enzymes (CYP26A1, CYP26B1) (ThermoFisher). Data were normalised to β -actin (ACTB) (ThermoFisher) expression. The messenger RNA (mRNA) expression of the genes of interest in infected and/or treated macrophages relative to uninfected, untreated macrophages was calculated using the $2^{-\Delta\Delta C_t}$ method. All genes were probed in MDMs. In AMs, a candidate approach was adopted, and only DHRS9, ALDH1a1, ALDH1a2, ALDH1a3, CRABP2 and FABP5 were probed (*Table 2.3*).

Gene	Supplier	Cat #	Dye
<u>Oxidation of Retinol to Retinal</u>			
DHRS3	ThermoFisher	Hs00191073_m1	FAM-MBG
DHRS9	ThermoFisher	Hs00608375_m1	FAM-MBG
RDH10	ThermoFisher	Hs00416907_m1	FAM-MBG
<u>Oxidation of Retinal to ATRA</u>			
ALDH1a1	ThermoFisher	Hs00946916_m1	FAM-MBG
ALDH1a2	ThermoFisher	Hs00180254_m1	FAM-MBG
ALDH1a3	ThermoFisher	Hs00167476_m1	FAM-MBG
ALDH3b1	ThermoFisher	Hs00997594_m1	FAM-MBG
<u>ATRA Carrier Proteins</u>			
CRABP1	ThermoFisher	Hs01011034_m1	FAM-MBG
CRABP2	ThermoFisher	Hs00275636_m1	FAM-MBG
FABP5	ThermoFisher	Hs02339439_g1	FAM-MBG
<u>ATRA Degradation Enzymes</u>			
CYP26A1	ThermoFisher	Hs00175627_m1	FAM-MBG
CYP26B1	ThermoFisher	Hs01011223_m1	FAM-MBG
<u>Glycolysis</u>			
HK2	ThermoFisher	Hs00606086_m1	FAM-MBG
PFKFB3	ThermoFisher	Hs00998698_m1	FAM-MBG
PKM	ThermoFisher	Hs00761782_s1	FAM-MBG
GAPDH	ThermoFisher	Hs99999905_m1	FAM-MBG
<u>Housekeeping</u>			
ACTB	ThermoFisher	Hs99999903_m1	FAM-MBG

Table 2.3: TaqMan primers used for PCR.

2.4.2 Western Blotting

In separate experiments, MDM lysates were used for Western Blot to characterise the protein expression of DHRS9, ALDH1a1, ALDH1a2, ALDH1a3. After experimental treatment the medium was removed from the plates and replaced with sodium dodecyl sulphate (SDS) lysis buffer (50mM Tris/HCl, pH 6.8 (Sigma-Aldrich), 2% SDS (Sigma-Aldrich), 10% glycerol (Scharlau) containing Pierce protease inhibitors (+ EDTA, 100 µl/ml). The protein lysate was scraped from the plates and heated to 95 °C for 10 minutes to inactivate proteases. Equal amounts (5ug) of the lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris glycine gels and a Criterion Electrophoresis Cell (Bio-Rad). A Criterion blotter (Bio-Rad) was used to transfer the separated proteins to a 0.2µm (pore size) polyvinylidene fluoride membrane (Immun-Blot PDVF membrane; Bio-Rad, Hercules, CA).

The membranes were blocked with 5% milk powder (Marvel) in Tris-buffered saline with Tween-20 (0.1% (v/v)) (TBST) at room temperature for one hour. Subsequently, the immunoblot was incubated with purified mouse anti-human DHRS9 (1:1000) (Sigma-Aldrich), mouse anti-human ALDH1a1 (1:1000) (Invitrogen), rabbit anti-human ALDH1a2 (1:1000) (Invitrogen) and ALDH1a3 (1:1000) (Invitrogen) at 4 °C overnight in 5% milk and TBST. β -actin was used as a loading control and detected using mouse anti-human β -actin (1:1000) (Sigma-Aldrich). This was followed by washing (x4 in TBST) followed by incubation with secondary goat anti-mouse (1:5000) (Millipore) or anti-rabbit IgG horseradish peroxidase conjugated antibody (1:2000) (Cell Signalling Technology) in 5% milk and TBST for one hour. The immunoblot signals were developed using enhanced chemiluminescence (Advansta, Sirius (for DHRS9, ALDH1a1, ALDH1a2, ALDH1a3) or ThermoFisher, West Pico (for β -actin)) and further visualised using a Chemiluminescence imaging system (Fusion FX). Blots were stripped between probes with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific (Life Technologies)).

2.5 Macrophage functional analyses

2.5.1 Cytokine Analyses

The concentrations of cytokines secreted by macrophages were measured using a Meso Scale Discovery multiplex analyser (MD, USA). Supernatant was collected at 48 hours post-infection with *M. tuberculosis* (H37Ra) and *M. chimaera*, 18 hours post-infection with *M. abscessus* and 24 hours post-stimulation with SARS-CoV-2 antigens and TLR ligands. The following cytokines were assessed using a 7-plex multi-spot assay (Human ProInflammatory 7, K15008B-02): IFN γ , IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α .

The assay was performed according to the manufacturer's instructions. Briefly, a standard curve (range 2.4 - 10,000 pg/ml) was prepared by four-fold dilution of a 10 μ g/ml solution of Calibrator 2 mix and the IFN γ calibrator in Diluent 1. Then 25 μ l of calibrator or sample was pipetted into separate wells of a 96 well T-plex MSD plate. The sealed plate was shaken on a microplate shaker (Cellestis, Victoria, Australia) for 1 hour at room temp and then incubated overnight at 4 °C. Detection Antibody Solution was diluted in Diluent 100, 25 μ l added to each well and the plate was incubated for 2h at room temperature on the shaker. The plate was washed 3 times with PBS containing 0.05% Tween and, using reverse pipetting to avoid bubbles, 150 μ l of 2X Read Buffer T was added to each well. The plate was read on a MESO Quick Plex SQ 120MM instrument, and the results were analysed using MSD Discovery 4.0 Workbench Analysis software.

For chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) experiments, in which MDMs were infected with *M. tuberculosis*, the cytokine secretions of RARI-treated macrophages were compared with DMSO-treated control cells.

For chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) experiments, in which THP-1 macrophages were infected with *M. chimaera* or *M. abscessus*, the cytokine secretions of ATRA-treated macrophages were compared with DMSO-treated control cells.

For chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) experiments, in which alveolar macrophages were stimulated with SARS-CoV-2 spike protein or envelope protein, the cytokine secretions of TLR2 or TLR4 antibody-treated macrophages were compared with isotype control antibody-treated cells. In addition, the cytokine secretions from macrophages originating from donors over the age of 75 were compared with those from patients under the age of 75.

2.5.2 Cholesterol Assay

The intracellular concentration of cholesterol within macrophages was measured using the Cholesterol/Cholesterol Ester-Glo assay (Promega). THP-1 macrophages, differentiated in a 96-well plate (Corning Costar), were treated with ATRA or DMSO as a control and infected with *M. chimaera* or *M. abscessus* or uninfected as before. 24 hours following infection, the media was removed, and the cells were washed twice with 100µl of PBS. 50µl of Cholesterol Lysis Solution (Promega) was added per well. The plate was briefly shaken on a plate shaker and then incubated for 30 minutes at 37 °C. 25 µl of each sample was then added to a well of an opaque, white-walled 96-well plate. 25µl of Cholesterol Detection Reagent with Esterase (Promega) was also added to each well. Again, the plate was briefly shaken and then incubated for 1 hour at room temperature. The luminescence was recorded on a plate-reading luminometer, and the total cholesterol concentration was calculated by comparison of the luminescence of samples and standards prepared under the same conditions. The intracellular cholesterol concentration was compared between ATRA-treated and DMSO-treated infected macrophages.

2.5.3 Intracellular Bacillary Killing Assay

An intracellular bacillary killing assay (CFU assay) was used to measure mycobacterial growth. A CFU assay was undertaken to understand if human MDMs depend on the signalling of endogenously produced ATRA to kill *M. tuberculosis*. MDMs were treated

with a RARI (Ro 41-5253) or DMSO and infected with H37Ra as before. Colony-forming units (CFUs) were assessed at 0 hours, 48 hours and 120 hours. A CFU assay was also undertaken to understand if ATRA is an effective host-directed therapy for macrophages infected with NTMs *in vitro*. THP-1 macrophages were treated with ATRA or DMSO and infected with *M. chimaera* or *M. abscessus* as before. CFUs were assessed at 0 hours, 48 hours and 120 hours for *M. chimaera* (same timepoints as slow-growing Mtb); and at 0 hours and 18 hours for *M. abscessus* (339). In each case, the CFU was determined as follows: Macrophages were lysed with 0.1% Triton-X (Sigma-Aldrich; T9284) and pooled with bacterial pellets (at all except the first timepoint, when the medium was discarded to exclude extracellular bacteria) from the centrifugation of supernatants. Bacteria were diluted in Middlebrook 7H9 broth (same as for propagation of *M. tuberculosis*, described above) and plated onto Middlebrook 7H10 agar (Difco; 262710) containing 10% Middlebrook OADC enrichment (Difco; 211886), 1g/l L-asparagine (Sigma-Aldrich; A4159), 0.5% glycerol (Difco; 228220) and 0.05% cycloheximide (Sigma-Aldrich; C09341).

CFU counts were performed 21 days post-incubation at 37 °C for *M. tuberculosis* and *M. chimaera*, and at 14 days post-incubation for *M. abscessus*.

The number and fold changes of CFUs was compared between treated and control macrophages.

2.5.4 Axenic Culture Assay

In addition to assessing intracellular growth, an axenic culture assay was performed to understand if ATRA directly kills *M. chimaera* or *M. abscessus*, in the absence of macrophages. For these axenic culture experiments, 500 µL of BACT/ALERT Mycobacterial Antimicrobial Supplement (Biomérieux; 414997) was injected into BACT/ALERT MP Culture Bottles (Biomérieux; 419744). This was followed by 500 µL of either ATRA (final concentration 10 µM), DMSO or clarithromycin (final concentration 4 µg/ml). Finally, 500 µL of *M. abscessus* or *M. chimaera* preparation were injected into each BACT/ALERT MP culture bottle. The bottles were incubated in a BACT/ALERT 3D

Automated Microbial Detection System (Biomérieux). The time to reach positivity was recorded and compared between conditions.

2.5.5 Cell Viability

The viability of human MDMs treated with Ro 41-5253 (concentrations: 10 μ M, 2 μ M, 0.4 μ M and 0.08 μ M) was assessed at 24 hours and 96 hours post-treatment. The viability of THP-1 macrophages infected with *M. abscessus* (at a 'Low MOI' of 1-2 bacilli per cell or a 'High MOI' of 4-6 bacilli per cell) was assessed at 24 hours post-infection.

To measure cell viability, the plasma membrane integrity of cells was assessed using the propidium iodide (PI) exclusion method (PI concentration 5 μ g/ml), and the nuclei were counterstained with Hoechst (Hoechst 33258 at 50 μ g/ml and Hoechst 33342 at 20 μ g/ml) (340). Cycloheximide (100 μ g/ml) (Sigma-Aldrich) was used as a positive control. The number of PI-positive cells relative to the total number of nuclei per field was counted by automated fluorescence microscopy using the Lionheart FX Automated Microscope (Agilent) and Gen5 software (Biotek). Each condition was assayed in triplicate, and four fields were counted in each well.

2.6 Metabolic Analyses

2.6.1 Assessment of Mitochondrial Function using the Mito Stress Test

The mitochondrial function of human MDMs treated with RARIs, Metformin, ATRA or DEAB was assessed using a Seahorse XF Mito Stress Test assay (Agilent), as per the manufacturer's instructions. DMSO was used as a vehicle control for RARIs, ATRA and DEAB. MDMs were plated onto XF24 Seahorse Cell Culture Plates (Agilent) and treated as described above. The XFe/XF24 sensor cartridges were pre-hydrated overnight at 37 °C in a non-CO₂ incubator with Seahorse calibrant (Agilent). The media in the XF24 Seahorse Cell Culture Plates was replaced with Seahorse medium and incubated for 45-60 minutes at 37 °C in a non-CO₂ incubator as per the manufacturer's instructions (341). The

extracellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured by the Seahorse XFe24 Analyzer (Seahorse Biosciences, Agilent Technologies United Kingdom Ltd.) at regular intervals as per the default Mito Stress Test programme. OCR is correlated with mitochondrial respiration and ECAR is correlated with the production of lactate – the downstream product of glycolysis. Measuring the OCR and ECAR in real-time can therefore indicate how cells use energy over time and in response to stimulation or pharmacological intervention. The Mito Stress Test allows for the interrogation of mitochondrial function by sequentially injecting inhibitors of specific parts of the mitochondrial electron transport chain (ETC) (*Figure 2.6, 2.7*).

Oligomycin blocks complex V (ATP synthase) of the electron transport chain (ETC) and reduces the OCR that is related to ATP production. Oligomycin was loaded into 'port A' of the XFe/XF24 sensor cartridge and injected after the 3rd OCR measurement into the plate wells by the Seahorse analyser at a final concentration of 1 μ M. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) disrupts the mitochondrial membrane potential, which causes the flow of electrons through the ETC to be uninhibited – OCR is increased to its maximum. FCCP was loaded into 'port B' and injected after the 6th OCR measurement, at a final concentration of 1 μ M. Rotenone blocks complex I and antimycin A (AA) blocks complex III of the ETC. The combination (R/AA) completely stops the ETC, and any ongoing oxygen consumption is the result of extra-mitochondrial processes. R/AA was loaded into 'port C' and injected after the 9th OCR measurement, at a final concentration of 1 μ M. Results were normalised to cell seeding density using a crystal violet assay as is described below (*Figure 2.6, 2.7*).

The measures of mitochondrial function and the equations for their derivations from Mito Stress Test OCR readouts are listed in *Table 2.4*.

Parameter	Equation
Non-mitochondrial OCR	Minimum OCR after R/AA injection
Basal respiration	[OCR just before Oligomycin] - [Non-mitochondrial OCR]
Maximal respiration	[Maximum OCR after FCCP] - [Non-mitochondrial OCR]
Proton leak	[Minimum OCR after Oligomycin] - [Non-mitochondrial OCR]
ATP production	[OCR just before Oligomycin] - [Minimum OCR after Oligomycin]
Spare respiratory capacity	[Maximal respiration] - [Basal respiration]
Spare respiratory capacity (%)	[Maximal respiration] / [Basal respiration] x 100

Table 2.4: Mitochondrial function parameters and equations for their derivation from Mito Stress Test OCR readouts (341).

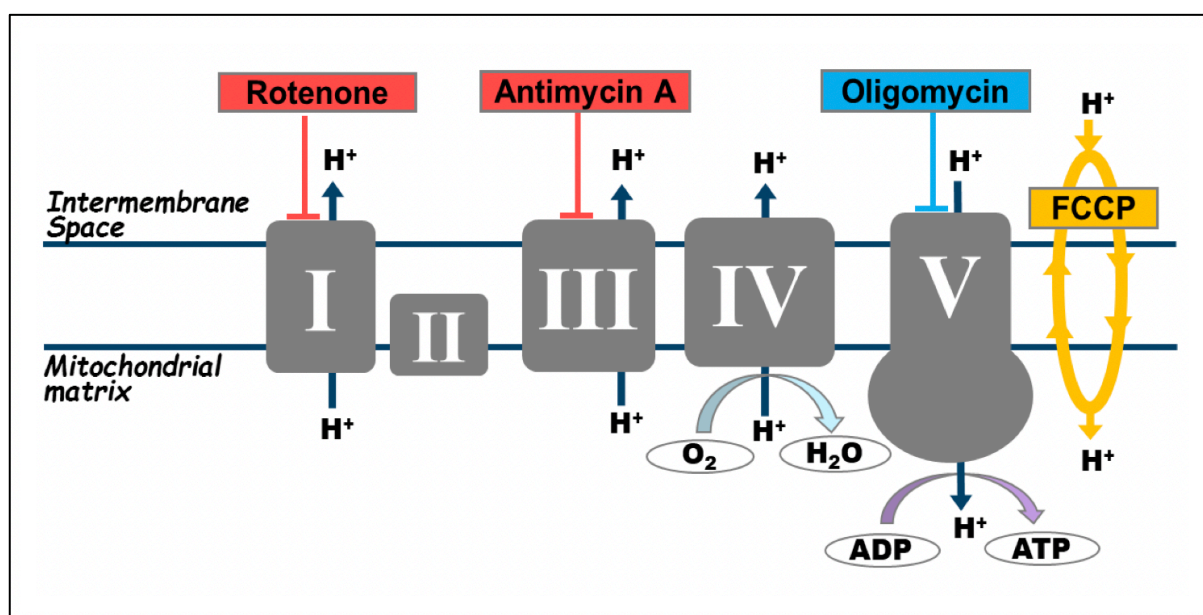


Figure 2.6: Mito Stress Test reagents mechanisms of action. Figure credit: Agilent (341).

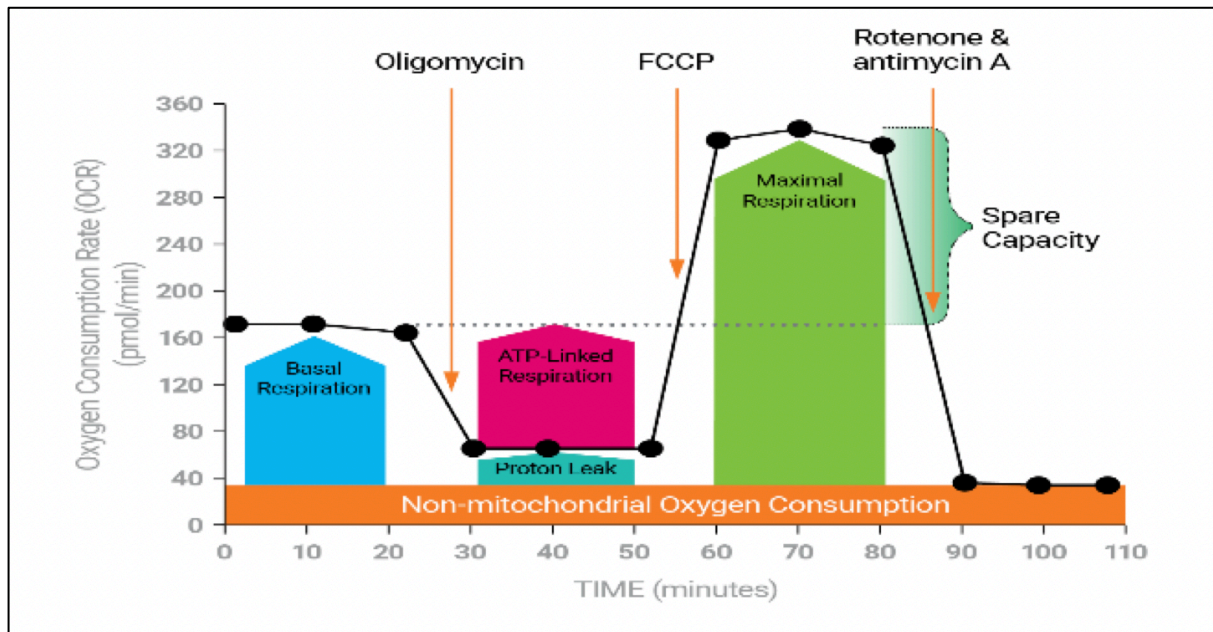


Figure 2.7: Mito Stress Test – representative graph of OCR and derivation of the measures of mitochondrial function (basal respiration, maximal respiration, spare respiratory capacity etc.). Figure credit: Agilent (341).

2.6.2 Assessment of Metabolic Flux During Acute Infection or Stimulation

In addition to assessing mitochondrial function in uninfected macrophages, the real-time metabolic responses of macrophages during an acute infection or stimulation were assessed (hereafter referred to as ‘acute infection experiments’). The metabolism of RARI-, DEAB- and metformin-treated MDMs was assessed in response to an acute stimulation with *M. tuberculosis* (iH37Rv). In other experiments the metabolism of ATRA-treated AMs was assessed in response to an acute infection with *M. chimaera* or *M. abscessus*. The MOI used in these ‘acute infection experiments’ was 5-10 bacilli per cell.

In ‘acute infection experiments’, macrophages were treated for 2 hours before the non-CO₂ incubation in Seahorse medium step. This Seahorse medium also contained the treatments, which remained in situ for the duration of experimentation and were present at the moment of infection or stimulation. As before, DMSO was used as a vehicle control for RARI-, DEAB- and ATRA-treated macrophages. At least three baseline OCR and ECAR measurements were taken prior to infection or stimulation, and OCR and ECAR measurements were repeatedly taken for at least two hours following infection or

stimulation (measurement interval for H37Ra experiments was 10 minutes, for NTM experiments was 7 minutes). Results were normalised to cell seeding density using a crystal violet assay as is described below.

2.6.3 Assessment of Metabolic Flux During Treatment with RARI and/or ATRA

To demonstrate that any metabolic effects of ATRA are being mediated through its nuclear receptor, and not through off-target effects, Seahorse experiments were undertaken in which THP-1 macrophages were sequentially injected with the RARI, Ro-41-5253 and then with ATRA. A tailored programme was used in these experiments as follows: three baseline ECAR and OCR readings were taken before the first injection (Ro-41-5253 or DMSO). ECAR and OCR readings continued for two hours before the second injection (ATRA or DMSO), and then readings continued for a further two hours (measurement interval was 8 minutes). Results were normalised to cell seeding density using a crystal violet assay as is described below.

2.6.4 Normalisation using Crystal Violet Assay

Upon completion of a Seahorse experiment, the Seahorse medium was removed, and the cells were fixed with 4% PFA. The PFA was then removed and the cells washed twice with PBS. 0.1% crystal violet was added to the wells for 30 minutes at room temperature, then discarded and the plate washed gently with water. The plate was allowed to air dry. 50 µl of 1% Triton-X (Sigma-Aldrich; T9284) solution was added to each well and the plate was put on a plate shaker for 15 minutes. The solution from each well was transferred to the wells of a new 96-well plate (Corning Costar). Absorbance was read at 590 nm and the absorbance values were used to normalise metabolic readings measured by the Seahorse analyser.

2.6.5 Glycolytic Gene Analyses

The expression of glycolytic genes in RARI-treated or untreated MDMs was assessed at 3- and 48-hours post-infection. As before, the media was removed and the cells were washed once with PBS. RNA extraction was performed using the RNeasy Plus Mini Kit (QIAGEN), and cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

Reverse transcriptase quantitative PCR (RT-qPCR) was performed using TaqMan primers for glycolytic enzymes (HK2, PFKFB3, PKM, GAPDH) (ThermoFisher). Data were normalised to β -actin (ACTB) (ThermoFisher) expression. The mRNA expression of the genes of interest in infected and/or RARI-treated macrophages relative to uninfected, untreated macrophages was calculated using the $2^{-\Delta\Delta C_t}$ method for each timepoint.

2.7 Statistical Analysis

All data were analysed, and graphs generated using GraphPad Prism software (version 9.5.0) (GraphPad Software, LLC.). Statistically significant differences between two groups were determined using two-tailed, unpaired student t tests, or paired student t tests. Where three or more groups were compared a one-way ANOVA was used, or a two-way ANOVA if more than one variable within each group was being compared, using repeated measures where appropriate. Corrections for multiple comparisons were performed as appropriate. Results are expressed as mean $\pm$ standard error of the mean (SEM). A p value < 0.05 was considered statistically significant.

Chapter 3

3 Endogenous Macrophage ATRA Production and

Mycobacterium tuberculosis

3.1 Introduction

Despite the ambitious goal to ‘End-TB’, in 2021 the global incidence of TB and the global mortality from TB increased. The number of multidrug-resistant TB (MDR TB) cases is increasing. A minority of such MDR TB patients are diagnosed and enrolled in treatment, though treatment is not successful in forty percent of cases (4). All *trans* retinoic acid (ATRA), the active metabolite of vitamin A, has been identified as a promising candidate for a novel host-directed therapy (HDT) for TB (134).

Prospective epidemiological studies indicate a 10-fold higher relative risk of TB in those with vitamin A deficiency (VAD), and an association between serum retinol and TB-risk exists even in those who are vitamin A sufficient (342). Laboratories, including our own, have demonstrated that exogenous ATRA improves the ability of human monocyte-derived macrophages (MDMs) and alveolar macrophages (AMs) to kill *Mycobacterium tuberculosis* (Mtb) *in vitro* (118,131,132). ATRA’s mechanisms of action include, among others, an increase in cholesterol efflux (132), improved phagosome-lysosome fusion (131,140), a reduction of intracellular iron (141) and improved cytokine responses (134,137). We and others have shown that exogenous ATRA reduces bacillary burdens and pathology in animal models of TB infection (134–137).

Despite the promising epidemiological, *in vitro* and *in vivo* evidence supporting vitamin A’s use as a HDT for TB, human trials of adjunctive retinol have failed (343). Retinol is the circulating form of vitamin A but is itself biologically inert; and its active metabolite – ATRA – must be produced intracellularly. Firstly, retinol is reversibly oxidised to retinal by short-chain dehydrogenases (e.g. DHRS3, DHRS9) or alcohol dehydrogenases (e.g. RDH10). Secondly, retinal is irreversibly oxidised to ATRA by members of the aldehyde dehydrogenase 1a enzyme family (ALDH1a1, ALDH1a2, ALDH1a3) (344). Niu et al. reported that ALDH3b1 is also capable of synthesising ATRA. The variable, cell-specific

expression of the ALDH enzymes determines which tissues are capable of producing ATRA (345). ATRA must then be delivered to its nuclear receptors by carrier proteins CRABP2 or FABP5 before it can alter the transcription of its target genes (346) (*Figure 1.4*).

DHRS9 is known to be expressed in human 'regulatory macrophages' (monocytes differentiated to macrophages with M-CSF and stimulated with IFN γ) (347). ALDH1a1 and ALDH1a2 are known to be expressed in human intestinal macrophages (348,349).

Otherwise, it is unknown which specific enzymes needed to produce ATRA are expressed in human MDMs or human AMs. Also unknown is what effect Mtb infection has on the expression of these enzymes and the carrier proteins needed to transport ATRA to its nuclear receptors.

While the evidence for the beneficial effects of *exogenous* ATRA at pharmacological concentrations on macrophage responses to Mtb is strong, data indicating the relevance or irrelevance of *endogenously* produced ATRA in macrophage responses to Mtb are lacking. Our laboratory published the first evidence that the initiation of glycolytic metabolism by human MDMs was crucial for them to effectively respond to infection with Mtb (38). Data exist linking *exogenous* ATRA with macrophage glycolytic responses to LPS, and with macrophage cytokine responses that are dependent on ATRA-induced glycolysis (144). However no data exist to indicate whether the signalling of *endogenously* produced ATRA influences macrophage metabolic responses to Mtb infection.

We aimed to examine the relationships between *endogenous* vitamin A pathways and macrophage responses to Mtb. We hypothesised that human MDMs and AMs express the enzymes needed to produce ATRA, and the carrier proteins needed to deliver ATRA to its nuclear receptors. We now show that these enzymes and carrier proteins are present and that they have reduced expression as a result of Mtb infection – possibly impairing the production and delivery of ATRA during infection. Finally, we show that blocking the signalling of *endogenously* produced ATRA is detrimental to human macrophage responses to Mtb.

3.3 Aims and Hypotheses

Overall Aim

To examine the relationships between endogenous vitamin A pathways and macrophage responses to Mtb infection.

Specific Aims

Aim 1: To determine the effect of Mtb infection on human macrophage vitamin A pathways.

Research Question 1: What vitamin A oxidising enzymes and ATRA carrier proteins are expressed in human macrophages, and what is the effect of Mtb infection on their expression?

Hypothesis 1: Human macrophages express the enzymes needed to produce ATRA from retinol and the carrier proteins needed to deliver ATRA to its nuclear receptors. Their expression is reduced by Mtb infection.

Aim 2: To determine the effects of *endogenously* produced ATRA on macrophage responses to Mtb infection.

Research Question 2: What is the effect of blocking the signalling of *endogenously* produced ATRA on human macrophage metabolic and cytokine responses to Mtb, and their ability to kill Mtb?

Hypothesis 2: Blocking the signalling of *endogenously* produced ATRA prevents the switch to glycolytic metabolism by human MDMs following infection with Mtb, alters their cytokine responses and impairs their ability to kill Mtb.

3.4 Results

3.4.1 Human MDMs and AMs express vitamin A oxidation enzymes and carrier proteins

Apart from evidence of DHRS9 expression in human 'regulatory macrophages' (347) and of ALDH1a1 and ALDH1a2 expression in human intestinal macrophages (348,349), it is unknown whether and which vitamin A-oxidising enzymes are expressed in human MDMs and AMs. While our group have published indirect evidence that human MDM can oxidise *exogenous* retinol to ATRA (131), others have suggested that human macrophages do not express vitamin A-oxidising enzymes (350). We aimed to identify which vitamin A-oxidising enzymes are expressed by human MDM and AMs, if any.

MDMs and AMs were obtained and cultured as described in sections 2.1.1 and 2.1.2. The mRNA expression of enzymes that oxidise retinol to retinal (DHRS3, DHRS9 and RDH10) and of enzymes that oxidise retinal to ATRA (ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1) were analysed using RT-qPCR, and their relative expression was calculated as a fold change versus the housekeeping gene ACTB (β -actin) using the $\Delta\Delta C_t$ method (as described in section 2.4.1). Uninfected human MDMs had evidence of mRNA expression for each of the genes probed (DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1) (*Figure 3.1 A*). Using a candidate approach in AMs, we probed for the mRNA expression of DHRS9, ALDH1a1, ALDH1a2 and ALDH1a3 and found that they were expressed in uninfected AMs (*Figure 3.1 B*). We then examined the protein expression of the enzymes DHRS9, ALDH1a1, ALDH1a2 and ALDH1a3 in MDMs by Western Blot (as described in section 2.4.2). We observed the protein expression of these enzymes in MDMs (*Figure 3.3 A*). From this we conclude that uninfected human MDMs and AMs are capable of oxidising retinol to ATRA.

It has been suggested that the expression of ATRA carrier proteins, and not the production of ATRA itself, is the rate-limiting step in ATRA's activation of its nuclear receptors (351). CRABP2 is expressed in human MDMs and AMs (352,353). FABP5 is expressed in human monocytes and in macrophages from preterm neonatal lung autopsy specimens (354,355). However, there are no data relating to the expression of FABP5 in

adult human MDMs and AMs. We performed RT-qPCR for CRABP2 and FABP5 mRNA expression in human MDMs and AMs and found that both CRABP2 and FABP5 were expressed in uninfected MDM and AM (*Figure 3.2*). Human macrophages are therefore capable of transporting *endogenously* produced ATRA to its nuclear receptors. The expression of CRABP1, which preferentially targets ATRA for degradation by cytochrome P450, was not detected in MDMs or AMs, nor were the ATRA degradation enzymes CYP26A1 and CYP26B1 (*data not shown*).

3.4.2 Mtb infection reduces the expression of vitamin A oxidation enzymes and carrier proteins

DHRS9 and ALDH1a2 have reduced expression in caseous granulomatous tissue from TB patients and ALDH1a2 is reduced in lung homogenates in a rabbit model of TB infection (350). Murine intestinal macrophages show reduced ALDH expression following infection with the nematode *Trichuris muris* (356). We wondered whether the enzymes responsible for ATRA production, that we found to be expressed in human MDMs and AMs, have reduced expression as a result of infection with Mtb. The mRNA and protein expression of the vitamin A oxidising enzymes examined in section 3.4.1 were also analysed in, and compared with, MDMs and AMs infected for 24 hours with Mtb (H37Ra at an MOI of 1-10 bacilli/cell, unless otherwise stated).

In MDMs, DHRS3, DHRS9, RDH10, ALDH1a1 and ALDH1a2 had reduced mRNA expression as a result of Mtb infection (*Figure 3.1 A*). ALDH1a3 and ALDH3b1 were unchanged by Mtb infection. In AMs infected at a low MOI (1-5 bacilli/cell), DHRS9 had reduced mRNA expression (*Figure 3.1 B*). In AMs infected at a high MOI (5-10 bacilli/cell), DHRS9 and ALDH1a3 had reduced mRNA expression (*Figure 3.1 B*). ALDH1a1 and ALDH1a2 were unchanged in AMs infected with Mtb versus their uninfected controls. A decrease in DHRS9, ALDH1a1, ALDH1a2 and ALDH1a3 protein expression was also evident in Mtb infected MDMs analysed by Western Blot (*Figure 3.3 A*). These data indicate that the enzymes required to produce ATRA from retinol are expressed in human MDMs and AMs, however their expression is reduced by Mtb infection.

We next sought to understand if this effect was specific to Mtb infection, or a more general response to infection. LPS stimulation was used as a model of gram-negative bacterial infection. In MDMs, we probed for DHRS9 and ALDH1a2 mRNA expression 24 hours following LPS stimulation and found that LPS stimulation resulted in a reduction in the expression of DHRS9 but not ALDH1a2. In AMs we probed for DHRS9, ALDH1a1 and ALDH1a2 mRNA expression 24 hours following LPS stimulation and found that DHRS9 expression, but not that of ALDH1a1 or ALDH1a2, was reduced by LPS stimulation. These data suggest that the reduction in expression of vitamin A-metabolising enzymes is not specific to Mtb infection but is a more general effect of infection (*Figure 3.1 C, D*).

We then wondered whether the delivery of ATRA to its nuclear receptors by its carrier proteins, CRABP2 and FABP5, might also be impaired by Mtb. Indeed, we found that both CRABP2 and FABP5 had reduced mRNA expression in human MDM infected with Mtb versus their uninfected controls (*Figure 3.2 A*). While in AMs, the mRNA expression of these carrier proteins was not reduced by Mtb infection, their expression was reduced following stimulation with LPS. These data indicate that in addition to impaired macrophage production of ATRA, the delivery of ATRA to its nuclear receptors may also be impaired in infection (*Figure 3.2 B,C*).

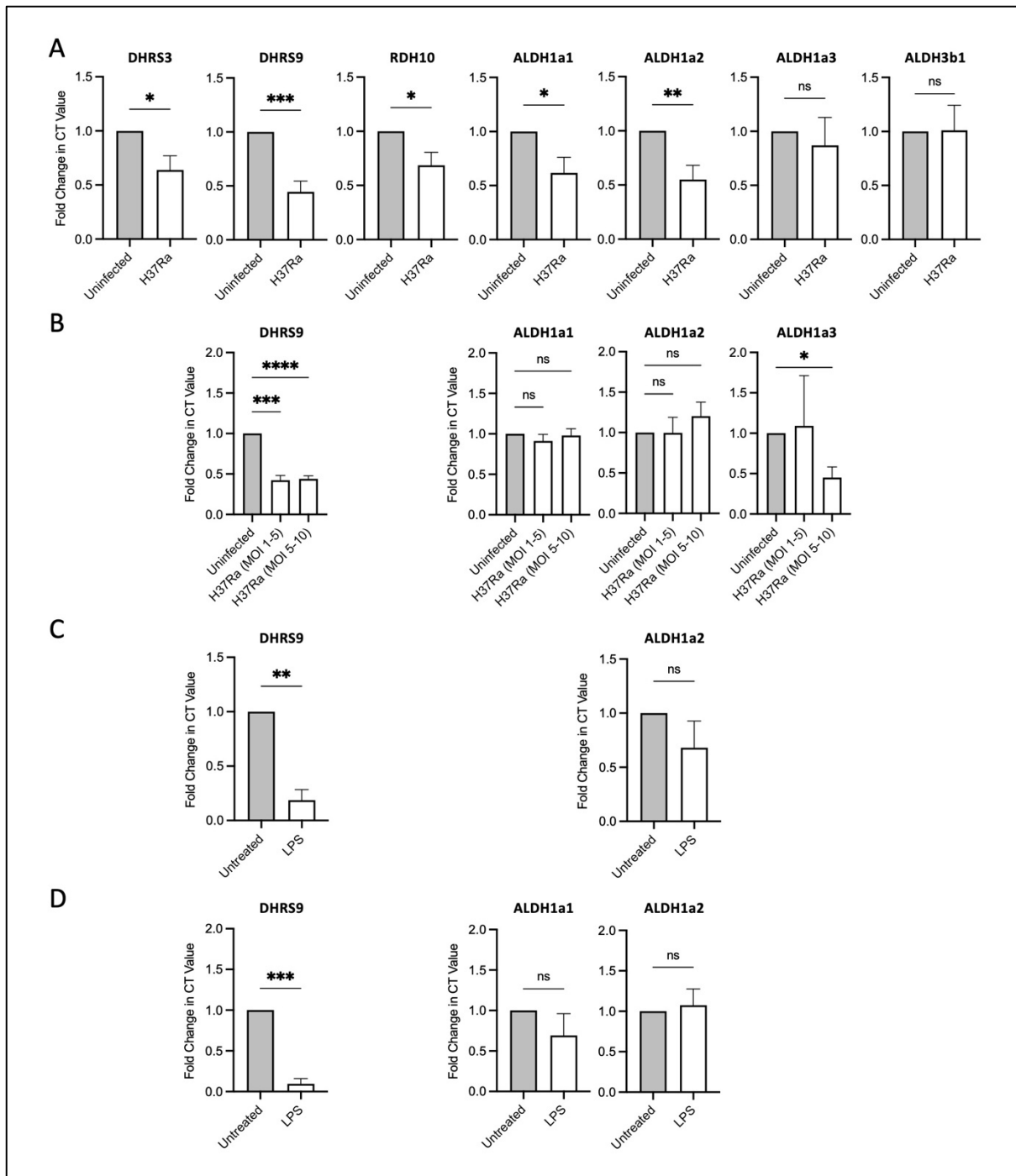


Figure 3.1: *Mtb* infection reduces the expression of enzymes needed to oxidise retinol to ATRA in human macrophages. (A) Human MDMs were selected from PBMCs of healthy donors and then infected for 24 hours with *Mtb* (H37Ra) at an MOI of 1-10 bacilli/cell or left uninfected. DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1 mRNA expression was measured by PCR (N=9-11). (B) Human AMs (N=7) isolated from BAL of donors undergoing routine bronchoscopy were infected with *Mtb* (H37Ra) at a low or high MOI (1-5 or 5-10 bacilli/cell) or left uninfected for 24 hours. The mRNA expression of DHRS9, ALDH1a1, ALDH1a2 and ALDH1a3 was measured by PCR. (C) MDMs were stimulated with LPS (100ng/ml) for 24 hours. DHRS9 and ALDH1a2 mRNA expression was measured by PCR (N=4). (D) AMs (N=3) were stimulated with LPS (100ng/ml) for 24 hours. DHRS9, ALDH1a1 and ALDH1a2 mRNA expression was measured by PCR. Statistical comparisons made by paired t test (A, C, D) or one-way ANOVA (B).

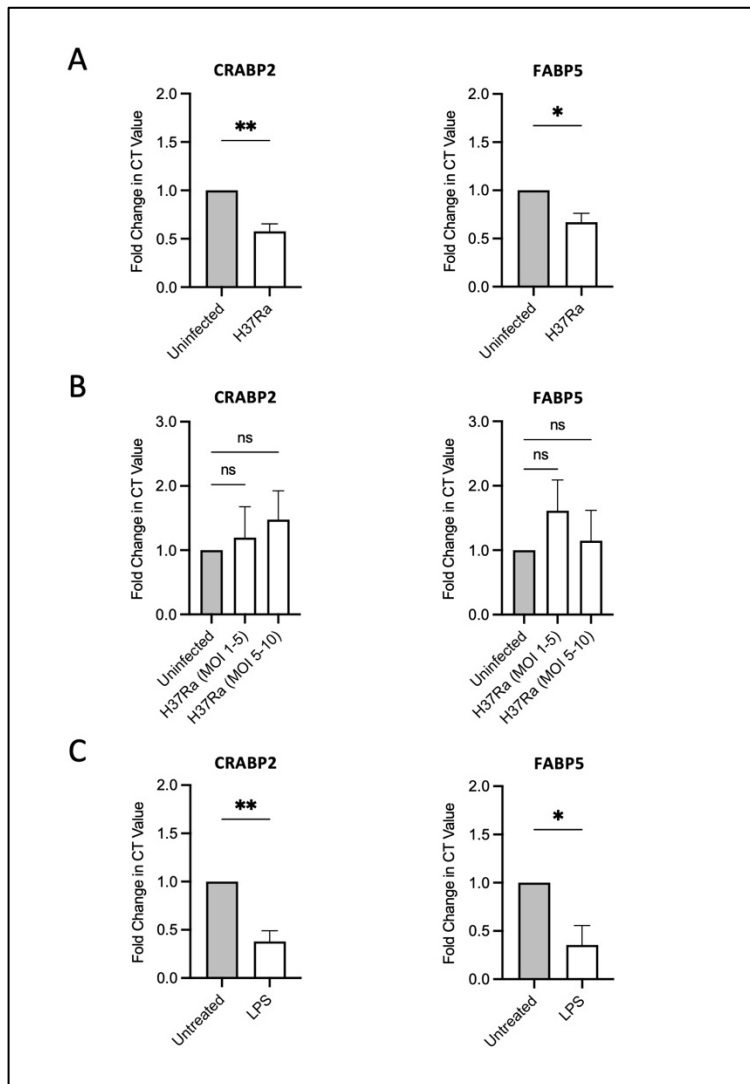


Figure 3.2: *Mtb* infection reduces the mRNA expression of the carrier proteins needed to deliver ATRA to its nuclear receptors in human MDMs. (A) Human MDMs were infected with *Mtb* (H37Ra) at an MOI of 1-10 bacilli/cell or left uninfected for 24 hours. The mRNA expression of both ATRA carrier proteins, CRABP2 and FABP5, was measured by PCR (N=4). (B) Human AMs were infected with *Mtb* (H37Ra) at a low or high MOI (1-5 or 5-10 bacilli/cell) or left uninfected for 24 hours. The mRNA expression of CRABP2 and FABP5 was measured by PCR (N=4,3,4). (C) Human AMs were stimulated with LPS (100ng/ml) for 24 hours. The mRNA expression of CRABP2 and FABP5 was measured by PCR (N=4). Statistical comparisons made by paired t test (A, C) or one-way ANOVA (B).

3.4.4 GM-CSF supports the expression of vitamin A oxidation enzymes during Mtb infection

Recombinant GM-CSF, named sargramostim, is an FDA-licensed drug used in patients following bone marrow transplantation, and is being investigated as a host-directed therapy candidate for mycobacterial disease (357). Alveolar macrophages from GM-CSF knockout mice have defective phagocytosis and bacterial killing (358). Published literature also suggests that GM-CSF might support macrophage production of ATRA. Yokota et al. found that GM-CSF induced the expression of ALDH1a2 in murine dendritic cells (DCs) (359). Kim et al. found that GM-CSF induced the expression of DHRS9 and ALDH1a2 in human DCs (350). We wondered if GM-CSF might also increase the expression of DHRS9 and ALDH1a2 in Mtb-infected human MDMs.

MDMs were pre-treated with GM-CSF for 24 hours before being infected for 24 hours with Mtb (H37Ra at an MOI of 1-10 bacilli/cell) as described in sections 2.2.2 and 2.3.2. The mRNA and protein expression of vitamin A oxidising enzymes was measured as described in sections 2.4.1 and 2.4.2, respectively, comparing GM-CSF-treated with untreated macrophages.

GM-CSF treatment prior to and during Mtb infection prevented the decrease in DHRS9 and ALDH1a3 mRNA expression caused by Mtb infection (*Figure 3.3 B*). Assessing the protein expression of DHRS9, ALDH1a1, ALDH1a2 and ALDH1a3 in cell lysates confirmed that GM-CSF pre-treatment increases the protein expression of these enzymes in GM-CSF treated versus untreated human MDMs infected with Mtb (*Figure 3.3 A*). These data support the candidacy of GM-CSF as a HDT for TB (perhaps by the inhaled route as was trialled for pulmonary alveolar proteinosis (27)) as it may support endogenous macrophage ATRA production during infection (*Figure 3.3*).

3.4.5 IL-4 supports the expression of vitamin A oxidation enzymes during Mtb infection

GM-CSF polarises macrophages to a classical M1 phenotype (360). Interestingly, there is also evidence that IL-4, which polarises macrophages to an alternative M2 phenotype, may also support ATRA production: ALDH1a2 expression was detected in alternatively activated macrophages in murine liver during *Schistosoma mansoni* infection and IL-4 also induced its expression in peritoneal macrophages *in vivo* and in bone marrow derived macrophages (BMDM) *in vitro* (234); ALDH1a-family enzyme induction by IL-4 has been observed in human monocyte-derived dendritic cells (DCs) (173). We wondered whether IL-4 also increased the mRNA and protein expression of vitamin A oxidising enzymes in human MDMs, like GM-CSF. If so, GM-CSF's support of vitamin A pathways is likely independent of its effects on macrophage polarisation (complementing our finding that LPS, an M1 polariser, *impaired* macrophage vitamin A pathways).

MDMs were pre-treated with IL-4 for 24 hours before being infected for 24 hours with Mtb (H37Ra at an MOI of 1-10 bacilli/cell) as described in sections 2.2.2 and 2.3.2. The mRNA and protein expression of vitamin A oxidising enzymes was measured as described in sections 2.4.1 and 2.4.2, respectively, comparing IL-4-treated with untreated macrophages.

IL-4 treatment prior to and during Mtb infection prevented the decrease in DHRS3 mRNA expression caused by Mtb infection and increased the mRNA expression of ALDH1a2 in Mtb infected macrophages (*Figure 3.3 C*). IL-4 also appeared to increase the protein expression of DHRS9, ALDH1a1, ALDH1a2 and ALDH1a3 versus untreated human MDMs infected with Mtb (*Figure 3.3 A*). From these data we conclude that GM-CSF's support of ATRA production is independent of any effects on macrophage polarisation.

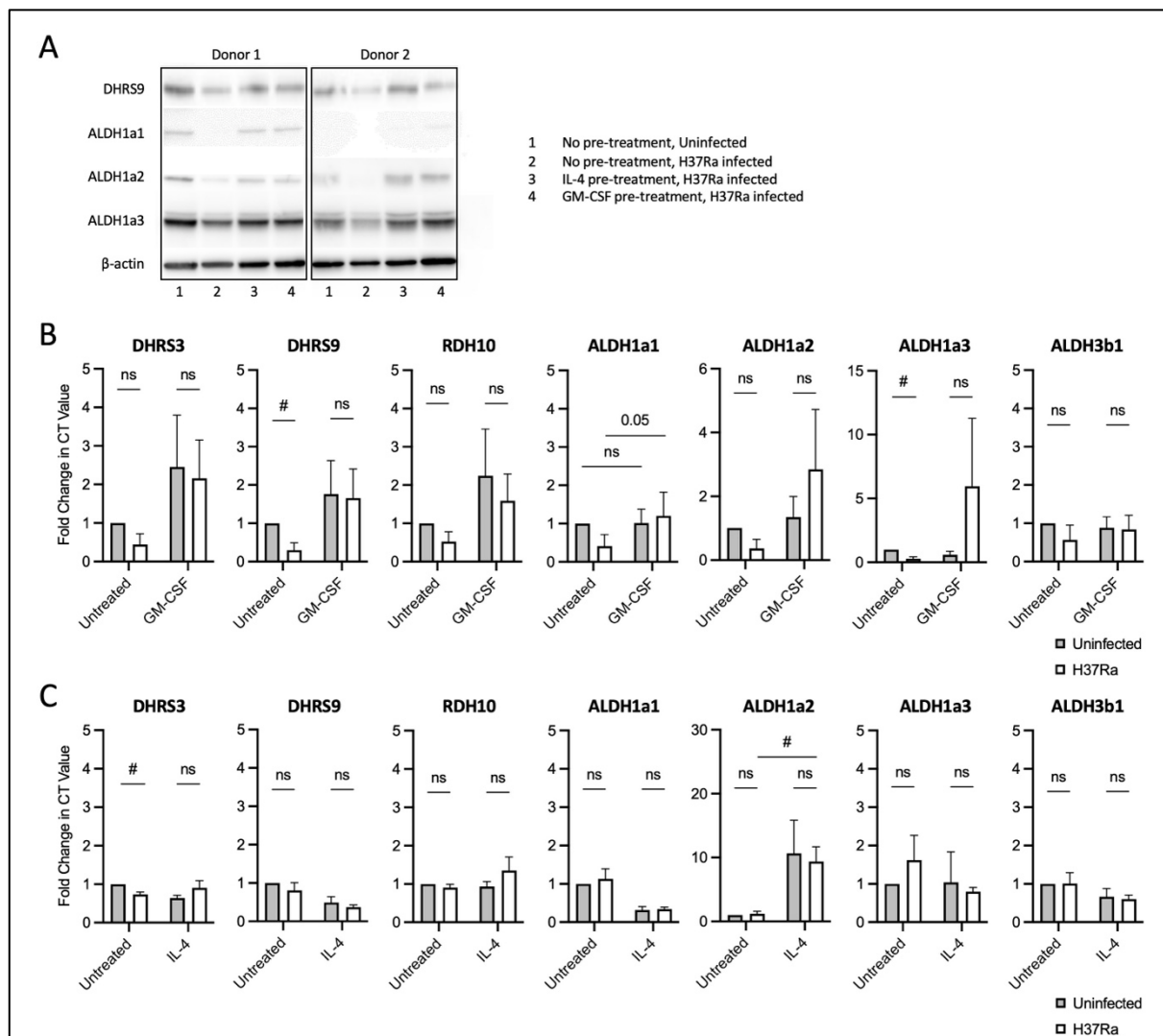


Figure 3.3: GM-CSF prevents the *Mtb*-induced reduction in macrophage expression of enzymes needed to oxidise retinol to ATRA. (A) Western Blot: Human MDMs were infected with *Mtb* (H37Ra) at an MOI of 1-10 bacilli/cell (condition 2) or left uninfected (condition 1). In two other conditions, MDMs were pre-treated with IL-4 (20ng/ml) (condition 3) or GM-CSF (50ng/ml) (condition 4) for 24 hours prior to infection with *Mtb*, and the treatment was continued for the duration of the infection (24 hours). MDMs were lysed and a western blot was performed, assessing the protein expression of DHRS9, ALDH1a1, ALDH1a2, ALDH1a3 and β-actin (ACTB) (N=2). (B) MDMs were pre-treated with GM-CSF (50ng/ml) for 24 hours prior to infection with H37Ra at an MOI of 1-10 bacilli/cell, and the GM-CSF treatment was continued for the duration of the infection (24 hours). The mRNA expression of DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1 was measured using PCR (N=7-9). (C) MDMs were pre-treated with IL-4 (20ng/ml) for 24 hours prior to infection with H37Ra at an MOI of 1-10 bacilli/cell, and the IL-4 treatment was continued for the duration of the infection (24 hours). The mRNA expression of DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1 was measured using PCR (N=7-9). Statistical comparisons made by two-way ANOVA (B,C) or with paired t test (significance denoted by #).

3.4.6 Blocking the nuclear signalling of endogenously produced ATRA alters human MDM metabolic responses to Mtb

Once ATRA has been produced within macrophages by vitamin A oxidising enzymes, and then chaperoned to the nucleus by its carrier proteins, it can activate its nuclear receptor and affect the transcription of its target genes. We have shown in section 3.4.2 that Mtb infected human macrophages have a reduced capacity to produce ATRA *endogenously* and transport ATRA to its nuclear receptors. While it is known that pharmacological concentrations of *exogenous* ATRA improve macrophage responses to Mtb (104,134), it is unknown if the nuclear signalling of *endogenously* produced ATRA is an important factor in macrophage responses to Mtb. If the signalling of *endogenously* produced ATRA is a determinant of macrophage responses to Mtb, it would suggest that the rehabilitation of defective ATRA production and delivery pathways may be a HDT strategy worthy of exploration. Perhaps more importantly, it could partly explain exactly why people with VAD are at higher risk of TB – separate to any effects of VAD on mucosal integrity, humoral or adaptive immunity. To understand whether the signalling of ATRA, produced *endogenously* by macrophages, is important to macrophage responses to Mtb we used retinoic acid receptor inhibitors (RARIs) in the absence of any *exogenous* retinol or ATRA. Retinoic acid receptor alpha (RAR α) is the predominant nuclear receptor for ATRA and is blocked by the specific inhibitor Ro 41-5253 (361,362).

We first examined metabolic responses. Our group has published evidence supporting the importance of alterations of macrophage energy metabolism as a critical response to Mtb infection (38,363–369). There is no evidence as to whether the signalling of *endogenously* produced ATRA is necessary for these glycolytic responses to occur. We wondered whether inhibiting the nuclear signalling of *endogenously* produced ATRA would prevent the ramping up of macrophage glycolysis that follows Mtb infection.

First, to check that RAR α inhibition did not adversely affect cell viability, MDMs were treated with Ro 41-5253 for 24 and 96 hours and the viability assessed as per section 2.5.5. Inhibition of RAR α did not cause a significant degree of cell death after 24 or 96 hours of treatment at the 2 μ M concentration used in subsequent experiments (*Figure 3.4 A*).

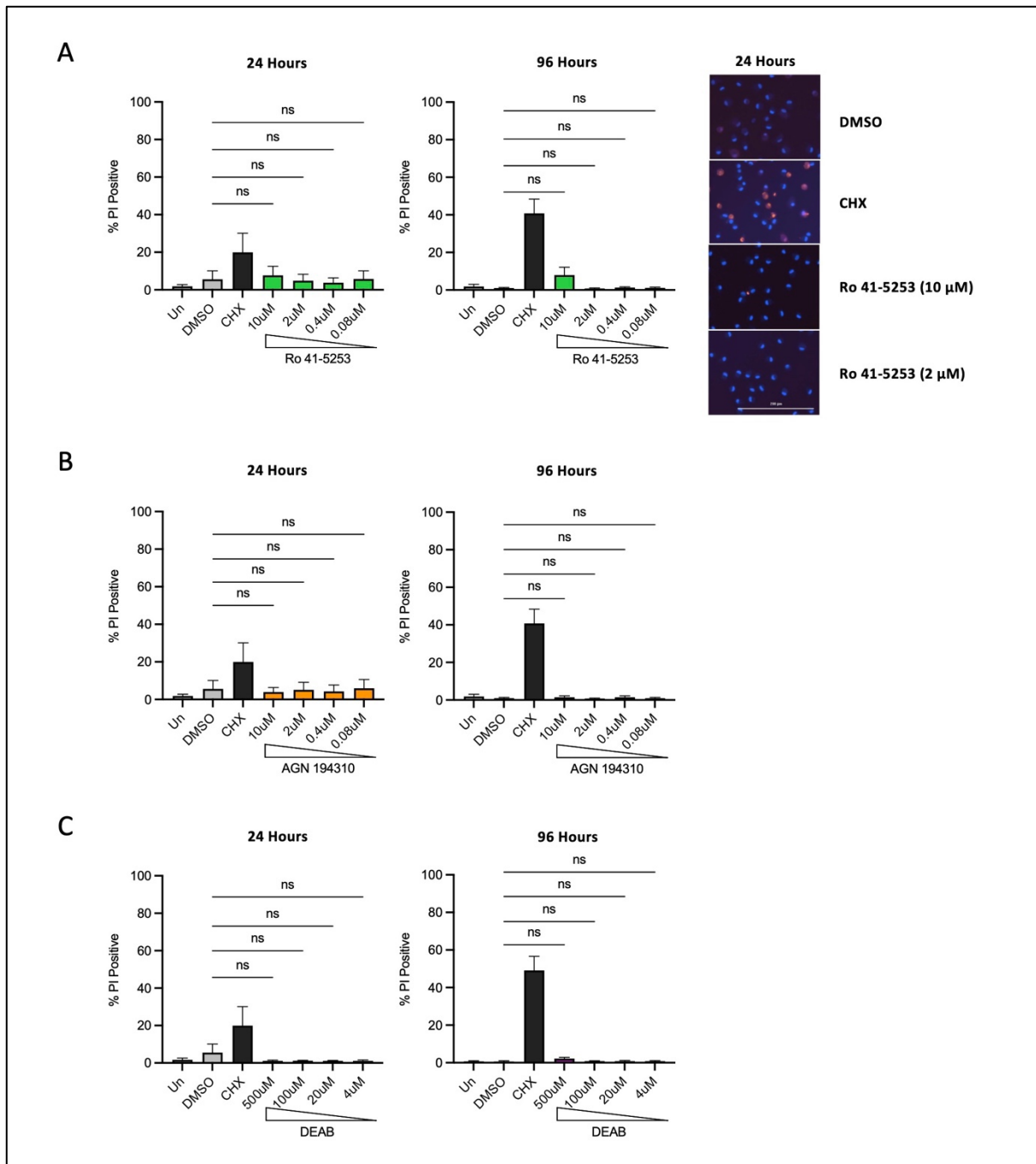


Figure 3.4: The viability of MDMs was not affected by 24- or 96-hour treatment with Ro 41-5253, AGN 194310 or DEAB. (A) MDMs were treated with the RAR α inhibitor Ro 41-5253 at doses 10 μ M, 2 μ M, 0.4 μ M and 0.08 μ M for 24 or 96 hours or left untreated or treated with a vehicle control (same DMSO concentration as the 10 μ M Ro 41-5253 condition). Cycloheximide (CHX) (100 μ g/ml) was used as a positive control. The cells were stained with propidium iodide (PI) at 5 μ g/ml, Hoechst 33258 at 50 μ g/ml and Hoechst 33258 at 20 μ g/ml. Using automated fluorescence microscopy, the proportion of PI-positive cells was calculated (percentage of non-viable cells) (N=4). (B) MDMs were treated with the pan-RAR inhibitor AGN 194310 at doses 10 μ M, 2 μ M, 0.4 μ M and 0.08 μ M for 24 or 96 hours or left untreated or treated with a vehicle control (DMSO). (C) MDMs were treated with the ALDH inhibitor DEAB at doses 500 μ M, 100 μ M, 20 μ M and 4 μ M for 24 or 96 hours or left untreated or treated with a vehicle control (DMSO). (N=4). Statistical

comparisons were made using one-way ANOVA, comparing the treatment conditions with the DMSO vehicle control condition.

To understand if RAR α inhibition affected the metabolic parameters of unstimulated MDMs, we analysed the effect of Ro 41-5253 on mitochondrial functions using a Mito Stress Test, as per section 2.6.1. Briefly, the OCR and ECAR were measured by a Seahorse analyser over time, with sequential injections of inhibitors of the mitochondrial electron transport chain after every third measurement. Those inhibitors are oligomycin (complex V inhibitor – first injection), FCCP (disrupts the mitochondrial membrane potential – second injection) and rotenone with antimycin A (complex I and III inhibitors – third injection). Measuring the effect of these inhibitors on the OCR permits the calculation of measures of mitochondrial function such as the spare respiratory capacity, as detailed in *Table 2.4*. After the Seahorse assay, a crystal violet assay was performed, as per section 2.6.4, to allow for the normalisation of OCR and ECAR readouts according to cell density. In these experiments, metformin was used as a positive control. Metformin is a known inhibitor of complex 1 of the ETC, activates AMP kinase and has been proposed as a potential HDT for TB (370,371). As per section 2.6.1, the mitochondrial functions of unstimulated MDMs were examined following a 3-hour treatment with metformin. When compared with untreated controls, metformin reduced the basal and maximal respiration, but did not affect the spare respiratory capacity. Metformin also increased basal ECAR. Treating MDMs with the RAR α inhibitor Ro 41-5253 for 3 hours did not affect their basal respiration, maximal respiration or spare respiratory capacity and also did not affect their baseline ECAR (*Figure 3.5*).

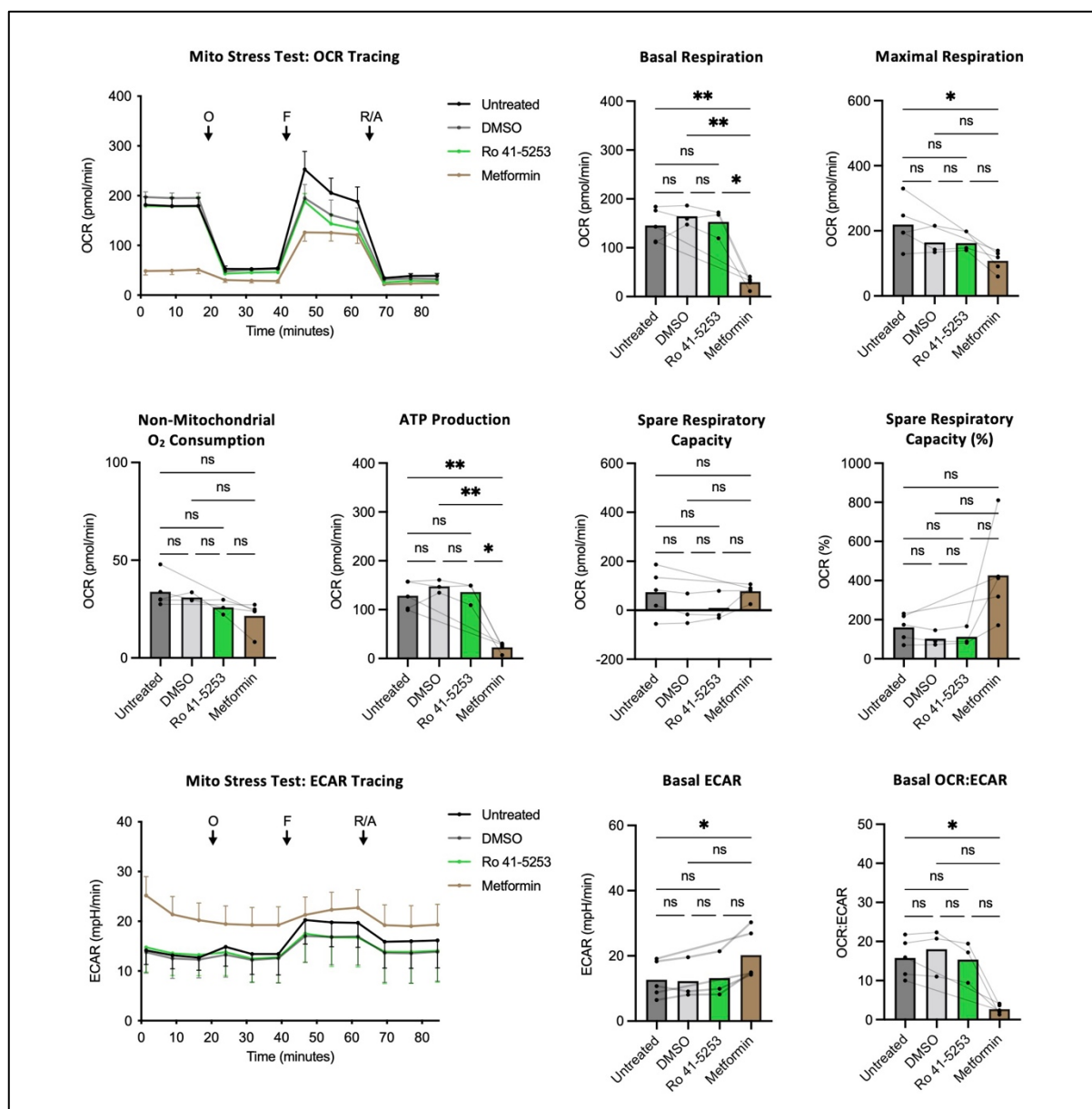


Figure 3.5: The *RAR α* inhibitor Ro 41-5253 does not alter the mitochondrial function of uninfected human MDMs. Human MDMs were differentiated for 5 days in 6-well plates, detached and reseeded onto Seahorse Cell Culture Plates as described in section 2.1.1. MDMs were treated with Ro 41-5253 (2 μ M), DMSO, Metformin (5mM) or untreated for three hours (described in section 2.2.4). OCR and ECAR were measured by the Seahorse analyser, with sequential injections of Mito Stress Test mitochondrial inhibitors as described in section 2.6.1 (O=oligomycin, F=FCCP (Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazine), R/A=rotenone/antimycin A). The measures of mitochondrial function (basal respiration, maximal respiration, non-mitochondrial O₂ consumption, ATP production and spare respiratory capacity) were derived from the OCR values, according to the manufacturer's instructions. The basal OCR and ECAR measurements were taken as the third measured – the measurement just preceding the injection of oligomycin (N=5,3,3,5). Statistical comparisons were made using one-way ANOVA.

We then sought to examine the metabolic effects of RAR α inhibition on human MDMs as they are responding to Mtb stimulation in real time. We chose this model for three reasons. Firstly, because it would allow for an understanding of the contribution of *endogenously* produced ATRA to important early metabolic responses of stimulated MDMs – responses that have been linked with their ability to kill Mtb (38). Secondly, because the reduction in enzymes and carrier proteins we observed at 24 hours post-infection might confound our results if a later timepoint was chosen. And thirdly, as a model of early responses to Mtb infection *in VAD*, where signalling of *endogenously* produced ATRA is likely to be limited or absent. As before, metformin was used as a positive control. We stimulated metformin treated and untreated (from 3 hours pre-infection) MDMs with Mtb, as described in section 2.6.2. Briefly, the volume of iH37Rv needed to achieve the desired MOI of 5-10 bacilli/cell was determined for each donor, by first performing a mock infection with a range of volumes, on cells plated at the same density on 8-well Labtek slides. This calculated volume of iH37Rv was later injected into the Seahorse plate wells through the injection ports, as OCR and ECAR were being measured over time. Alternatively, Seahorse medium (SHM) was used as a negative control for the stimulation. OCR and ECAR measurements were normalised according to cell density, determined using a crystal violet assay after the Seahorse assay was complete, as described in section 2.6.4. To calculate the change in OCR and ECAR from baseline, the fourth metabolic measurement – the measurement just preceding the injection of iH37Rv or SHM – was taken as the baseline and set at 100%. This also allowed for the derivation of the area under the curve (AUC) for each condition and each donor, calculated (using GraphPad Prism software) as the net area above (positive area) and below (negative area) this baseline (*Figure 3.6*).

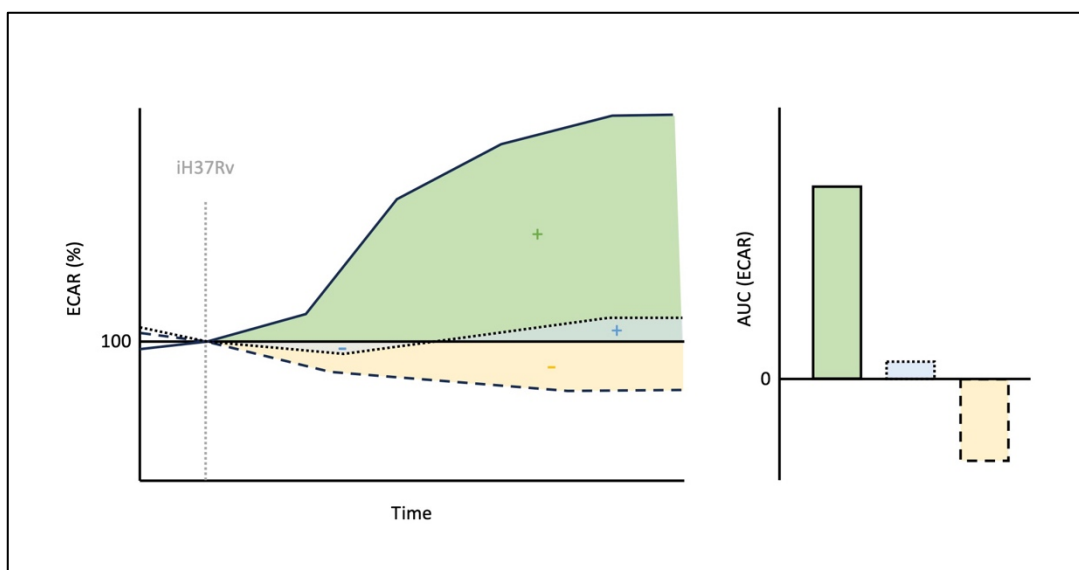


Figure 3.6: Illustrative graphic demonstrating how AUC was calculated for each condition, for each donor. The area is calculated from the time of the injection to the time of the last measurement.

In absolute terms, the positive control, metformin, resulted in an increased ECAR and reduced OCR in Mtb stimulated MDMs. In relative terms, the changes in ECAR and OCR following infection were not as great as in untreated MDMs (from different baselines). Inhibition of RAR α with Ro 41-5253 (from 3 hours pre-infection) prevented the increase in ECAR at 150 minutes following acute Mtb infection. This increase in ECAR was observed in DMSO treated (vehicle) control MDMs responding to Mtb infection (*Figure 3.7*).

In addition to an increase in ECAR, a reduction of OCR is observed in MDMs responding to Mtb – the second component of the Warburg effect. In our experiments, this was observed in DMSO treated control MDMs responding to Mtb and was also seen with Ro 41-5253 treated MDMs responding to Mtb. Taken together with its effects on ECAR, this indicates that Ro 41-5253 causes a (possibly inappropriate) shift to a ‘quiescent state’ of energy metabolism in stimulated MDMs, rather than the ‘glycolytic state’ seen in controls. These data suggest that the signalling of *endogenously* produced ATRA was needed to appropriately alter macrophage energy metabolism in response to Mtb infection (*Figure 3.7*).

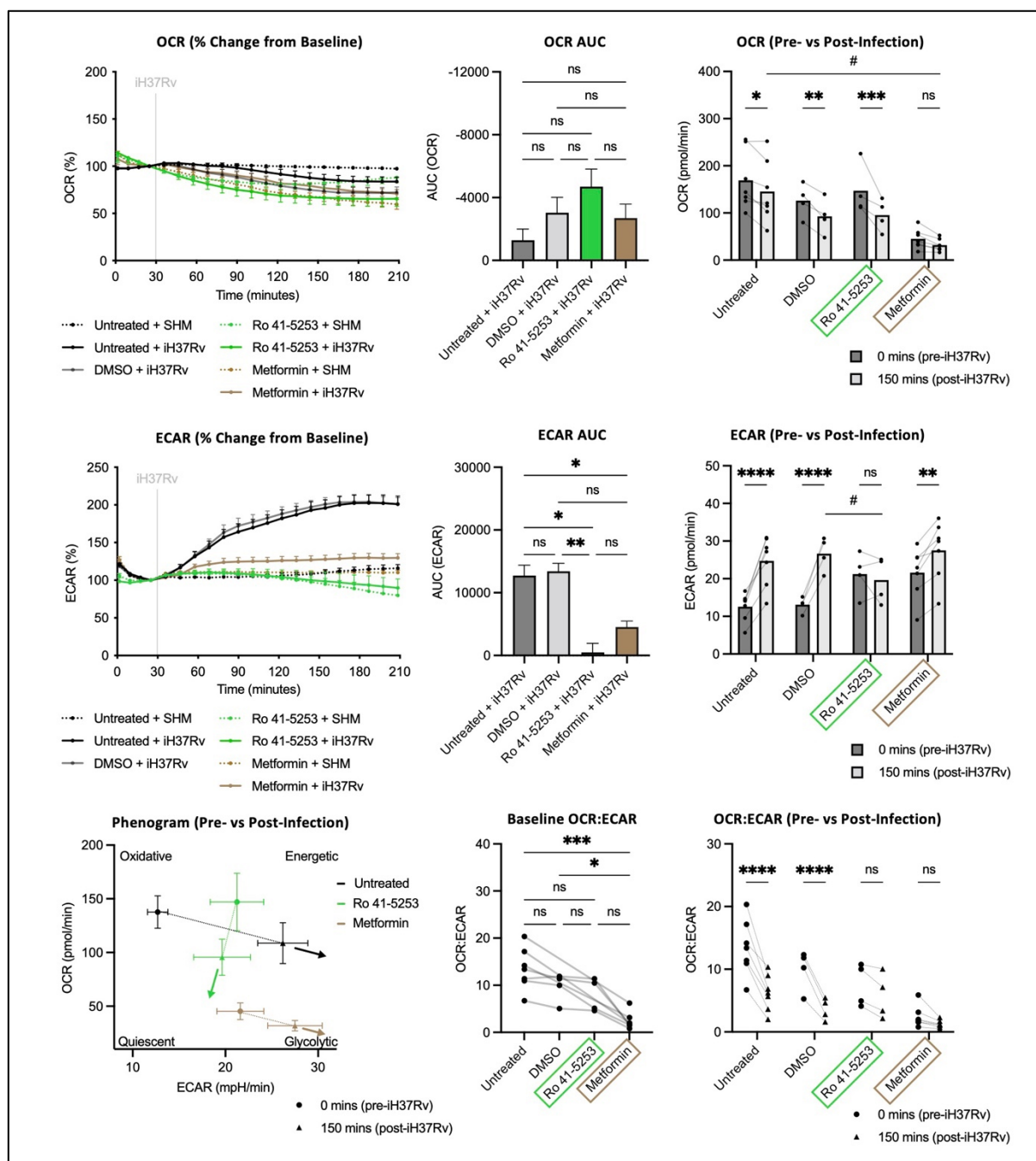


Figure 3.7: The *RARα* inhibitor Ro 41-5253 prevented the glycolytic response of MDMs to *Mtb*. Human MDMs were differentiated for 5 days in 6-well plates, detached and reseeded onto Seahorse Cell Culture Plates as described in section 2.1.1. MDMs were treated with Ro 41-5253 (2μM), DMSO, Metformin (5mM) or untreated as described in section 2.2.1 – MDMs were treated for 3 hours prior to stimulation with *Mtb* (ih37Rv, MOI 5-10 bacilli/cell) or Seahorse medium (SHM) in the Seahorse analyser, and the inhibitor remained present for the duration of the experiment (N=7,4,4,7). Statistical comparisons were made using one-way ANOVA (OCR AUC, ECAR AUC and Baseline OCR:ECAR) or two-way ANOVA ('Pre- vs. Post-Infection' OCR, ECAR and OCR:ECAR) or paired t test (significance denoted by #). Error bars represent the SEM. Normalised, non-baselined tracings available in the appendix for chapter 3 (Figure 3.16)

We examined the measures of mitochondrial function again using a Mito Stress Test, but this time 150 minutes after MDMs were stimulated with iH37Rv – the timepoint used in the previous analyses. We did this to understand if RAR α inhibition altered mitochondrial functions of Mtb stimulated macrophages, as we already had established (in *Figure 3.5*) that Ro 41-5253 did not affect mitochondrial functions of *unstimulated* macrophages. MDMs were treated with Ro 41-5253, DMSO or untreated for three hours (as described in section 2.2.4) and then stimulated with iH37Rv (MOI 5-10 bacilli/cell) for 150 minutes. The media was exchanged for SHM containing the same treatments, and then OCR and ECAR were measured by the Seahorse analyser, with sequential injections of Mito Stress Test mitochondrial inhibitors as described in section 2.6.1. Ro 41-5253 treated, iH37Rv stimulated MDMs were compared with DMSO treated, iH37Rv stimulated controls. Ro 41-5253 did not alter the parameters of mitochondrial function in Mtb stimulated MDMs including basal respiration, maximal respiration and spare respiratory capacity (*Figure 3.8*). The metabolic effects of RAR α inhibition on Mtb stimulated macrophages do not include mitochondrial toxicity.

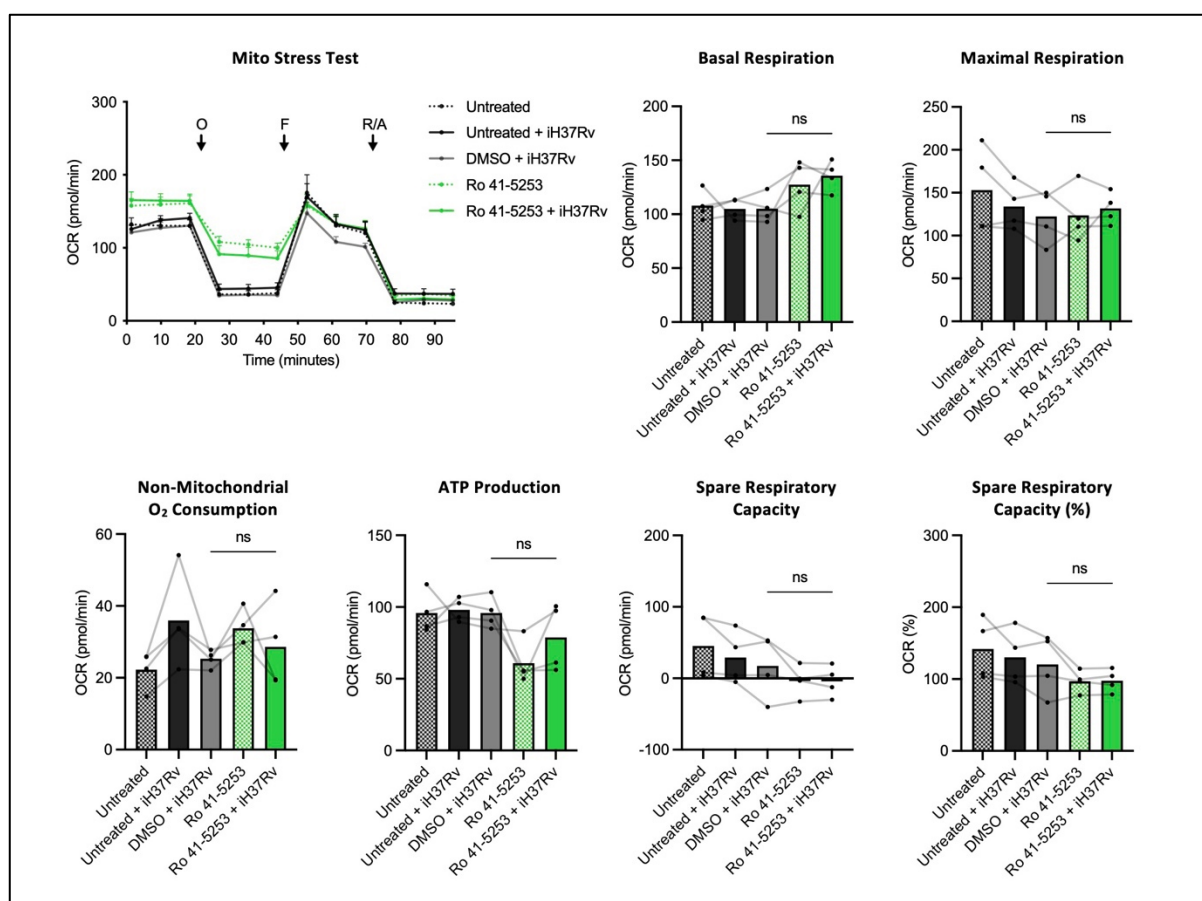


Figure 3.8: The *RAR α* inhibitor Ro 41-5253 does not alter the mitochondrial function of *Mtb* stimulated human MDMs. Human MDMs were differentiated for 5 days in 6-well plates, detached and reseeded onto Seahorse Cell Culture Plates as described in section 2.1.1. MDMs were treated with Ro 41-5253 (2 μ M), DMSO or untreated for three hours (described in section 2.2.4) and then stimulated with iH37Rv (MOI 5-10 bacilli/cell) for 150 minutes. The media was exchanged for SHM containing the same treatments and then OCR and ECAR were measured by the Seahorse analyser, with sequential injections of Mito Stress Test mitochondrial inhibitors as described in section 2.6.1 (O=oligomycin, F=FCCP (Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazine), R/A=rotenone/antimycin A). The measures of mitochondrial function (basal respiration, maximal respiration, non-mitochondrial O₂ consumption, ATP production and spare respiratory capacity) were derived from the OCR values, according to the manufacturer's instructions. The basal OCR and ECAR measurements were taken as the third measured – the measurement just preceding the injection of oligomycin (N=4). Ro 41-5253 treated, iH37Rv stimulated MDMs were compared with DMSO treated, iH37Rv stimulated controls by one-way ANOVA.

We wondered whether the metabolic effects of Ro 41-5253 could be caused by off-target effects, unrelated to its inhibition of RAR α . Ro 41-5253 has been found to be an agonist of PPAR γ in murine adipocytes (372). If the metabolic effects of Ro 41-5253 in preventing MDM glycolytic responses to Mtb are being mediated through activation of PPAR γ instead of RAR α inhibition, then blocking PPAR γ before Ro 41-5253 treatment should abrogate its effects – PPAR γ inhibited MDMs treated with Ro 41-5253 should more closely resemble DMSO treated MDMs in their metabolic responses to Mtb. GW 9662 is an inhibitor of PPAR γ . MDMs were treated with GW 9662 for 1 hour prior to being treated with Ro 41-5253 as before (as per section 2.2.4). GW 9662 pre-treatment did not restore the glycolytic response of MDMs stimulated with Mtb. These data suggest that the metabolic effects of Ro 41-5253 on MDMs are not being mediated through the activation of PPAR γ (*Figure 3.9*).

Other literature suggests that Ro 41-5253 may activate the extracellular signal-regulated kinases (ERK) signalling pathway, through a mechanism that involves conformational change of RAR α and phosphorylation of ERK (373). ERK is phosphorylated by the enzyme MEK1. If the metabolic effects of Ro 41-5253 in preventing the glycolytic response of MDMs to Mtb are being mediated through the phosphorylation of ERK by MEK1, then blocking MEK1 before Ro 41-5253 treatment should abrogate its effects – MEK1 inhibited MDMs treated with Ro 41-5253 should more closely resemble DMSO treated MDMs in their metabolic responses to Mtb. PD98059 is a MEK1 inhibitor. MDMs were treated with PD98059 for 1 hour prior to being treated with Ro 41-5253 as before (as per section 2.2.4). PD98059 pre-treatment did not restore the glycolytic response of MDMs stimulated with Mtb. These data suggest that the metabolic effects of Ro 41-5253 on MDMs are not being mediated through the phosphorylation of ERK (*Figure 3.9*).

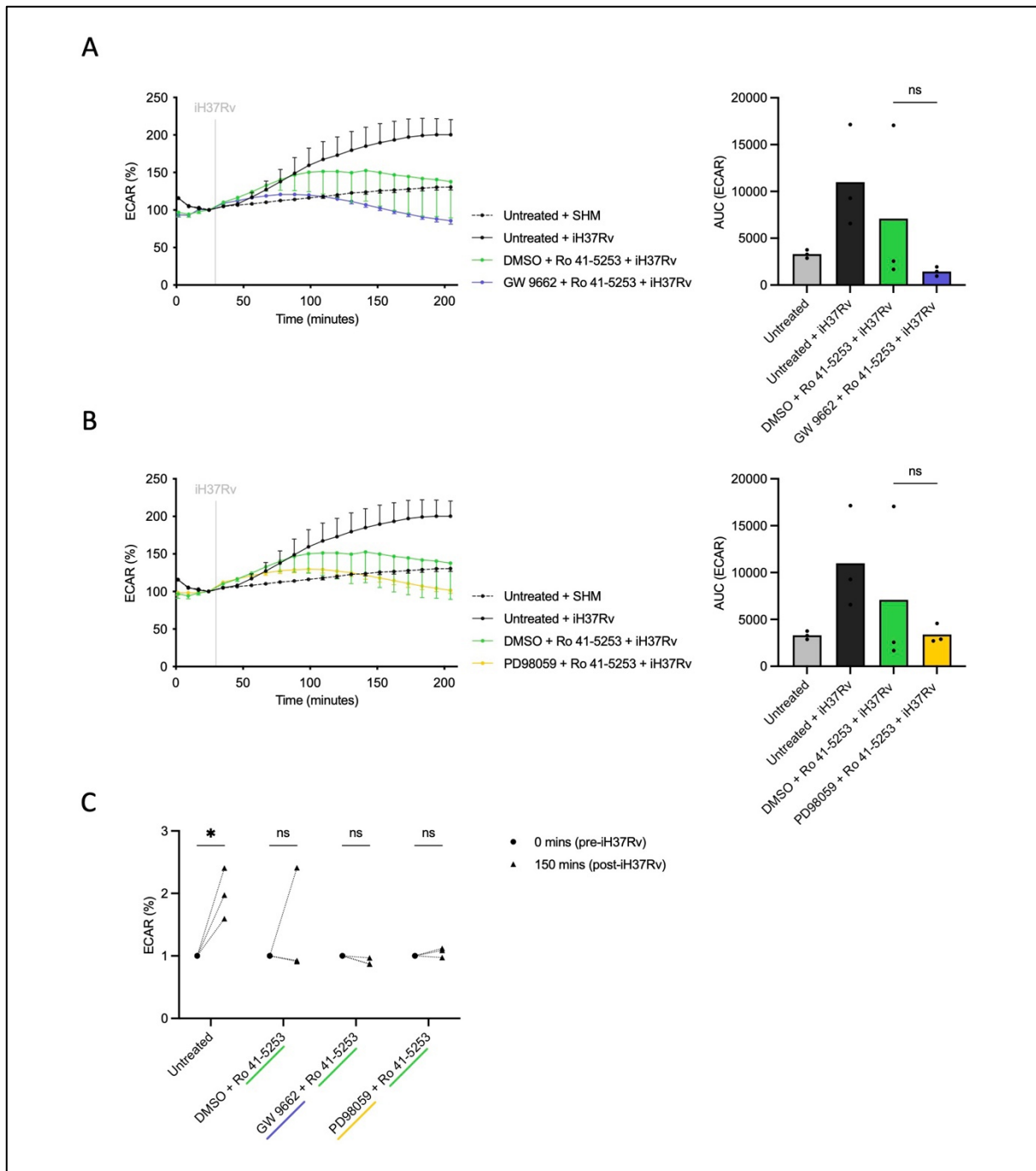


Figure 3.9: Blocking PPAR γ or MEK1 prior to treating MDMs with Ro 41-5253 does not restore their glycolytic responses to Mtb. (A) MDMs were treated with the PPAR γ inhibitor GW 9662 (10 μ M) or DMSO for one hour before being treated with the RAR α inhibitor Ro 41-5253 (2 μ M) and stimulated with iH37Rv (MOI 5-10 bacilli/cell) or Seahorse medium (SHM) in the Seahorse analyser as before (N=3). (B) MDMs were treated with the MEK1 inhibitor PD98059 (10 μ M) or DMSO for one hour before being treated with the RAR α inhibitor Ro 41-5253 (2 μ M) and stimulated with iH37Rv (MOI 5-10 bacilli/cell) or Seahorse medium (SHM) as before (N=3). (C) Change in ECAR from pre-infection to 150 minutes post-infection (N=3). GW 9662 or PD98059 pre-treated MDMs were compared with DMSO pre-treated controls by one-way ANOVA (A,B). MDMs were compared pre-infection versus post-infection by two-way ANOVA (C). Normalised, non-baselined tracings available in the appendix for chapter 3 (Figure 3.16).

Crucially, we also investigated an alternative inhibitor of RAR α , ER 50891, to see if our findings using Ro 41-5253 could be replicated with an entirely different antagonist. As described in section 2.6.2, MDMs were treated with ER 50891 for 3 hours prior to stimulation with iH37Rv, as for Ro 41-5253. Inhibition of RAR α with the alternative antagonist ER 50891 replicated the effects of Ro 41-5253 exactly and prevented the increase in ECAR that follows Mtb stimulation (*Figure 3.10*). Interestingly, a pan-RAR inhibitor AGN 193109 (which also did not affect MDM viability (*Figure 3.4 B*)) did not reproduce the findings of Ro 41-5253 and ER 50891 (*Figure 3.11 A*). From these data we conclude that the signalling of *endogenously* produced ATRA through its nuclear receptor RAR α is necessary for MDMs to alter their metabolism in response to Mtb.

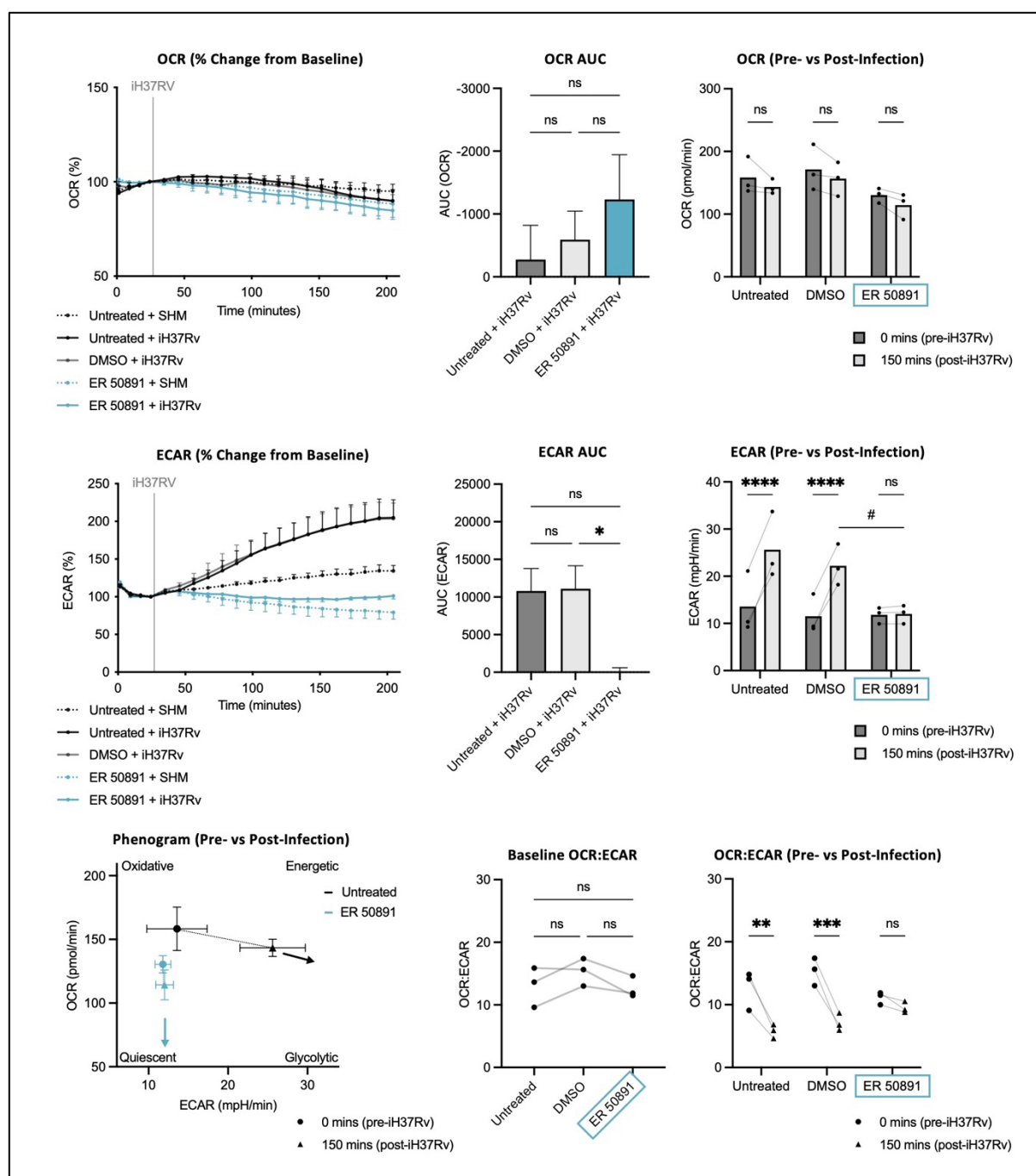


Figure 3.10: An alternative *RARα* inhibitor, ER 50891, replicated the effects of Ro 41-5253 (Figure 3.7) and prevented the glycolytic response of MDMs to *Mtb*. Human MDMs were differentiated for 5 days in 6-well plates, detached, and reseeded onto Seahorse Cell Culture Plates and treated as before, as described in section 2.1.1. MDMs were treated with ER 50891 (10 μ M) or DMSO using the same protocol as for experiments with Ro 41-5253 (described in section 2.2.1) – MDMs were treated for 3 hours prior to stimulation with *Mtb* (iH37Rv, MOI 5-10 bacilli/cell) or Seahorse medium (SHM) in the Seahorse analyser, and the inhibitor remained present for the duration of the experiment (N=3). Statistical comparisons were made using one-way ANOVA (OCR AUC, ECAR AUC and Baseline OCR:ECAR) or two-way ANOVA ('Pre- vs. Post-Infection' OCR, ECAR and OCR:ECAR) or paired t test (significance denoted by #). Error bars represent the SEM. Normalised, non-baselined tracings available in the appendix for chapter 3 (Figure 3.16).

We wondered whether these early, acute metabolic responses of macrophages to Mtb were dependent on signalling by ATRA that was preformed, or ATRA that was being actively and acutely manufactured during these earliest moments of infection. DEAB, an inhibitor of the ALDH enzymes (which did not affect MDM viability (*Figure 3.4 C*)) did not replicate the metabolic effects of Ro 41-5253 and ER 50891. This suggests that in the earliest moments of infection, pre-formed ATRA is sufficient for MDMs to respond energetically to Mtb (*Figure 3.11 B*).

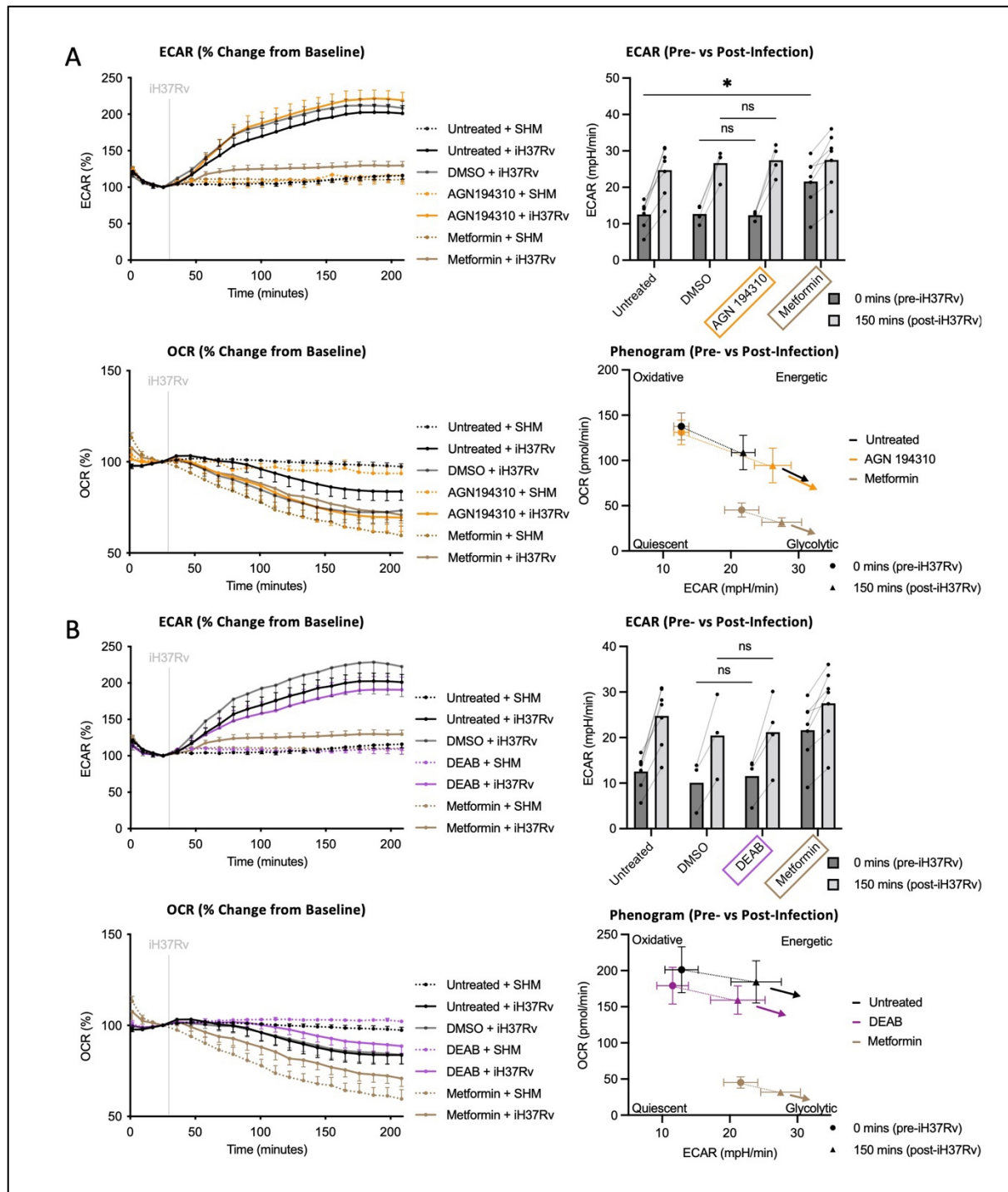


Figure 3.11: A pan-RAR α inhibitor, AGN 194310, and an ALDH inhibitor, DEAB, did not replicate the effects of Ro 41-5253 and did not alter the energetics of MDMs responding to Mtb stimulation. Human MDMs were differentiated for 5 days in 6-well plates, detached, and reseeded onto Seahorse Cell Culture Plates and treated as before, as described in section 2.1.1. MDMs were treated with AGN 194310 (1 μ M) (presented in A), DEAB (presented in B), DMSO, Metformin (5mM) or untreated using the same protocol as for experiments with Ro 41-5253 (described in section 2.2.1) – MDMs were treated for 3 hours prior to stimulation with Mtb (ih37Rv, MOI 5-10 bacilli/cell) or Seahorse medium (SHM) in the Seahorse analyser and the inhibitor remained present for the duration of the experiment (N=7,4,4,7). Statistical comparisons

were made using two-way ANOVA. Error bars represent the SEM. Normalised, non-baselined tracings available in the appendix for chapter 3 (Figure 3.17).

Finally, we also measured the mRNA expression of glycolytic genes (HK2, PFKFB3, PKM and GAPDH) to see if their expression correlated with the findings from our functional metabolic experiments. Ro 41-5253 treatment did not significantly reduce the expression of these glycolytic genes in uninfected or Mtb infected MDMs, although a trend towards decreased expression is evident for PFKFB3, PKM and GAPDH at 3- and 48-hours post-infection (*Figure 3.12*).

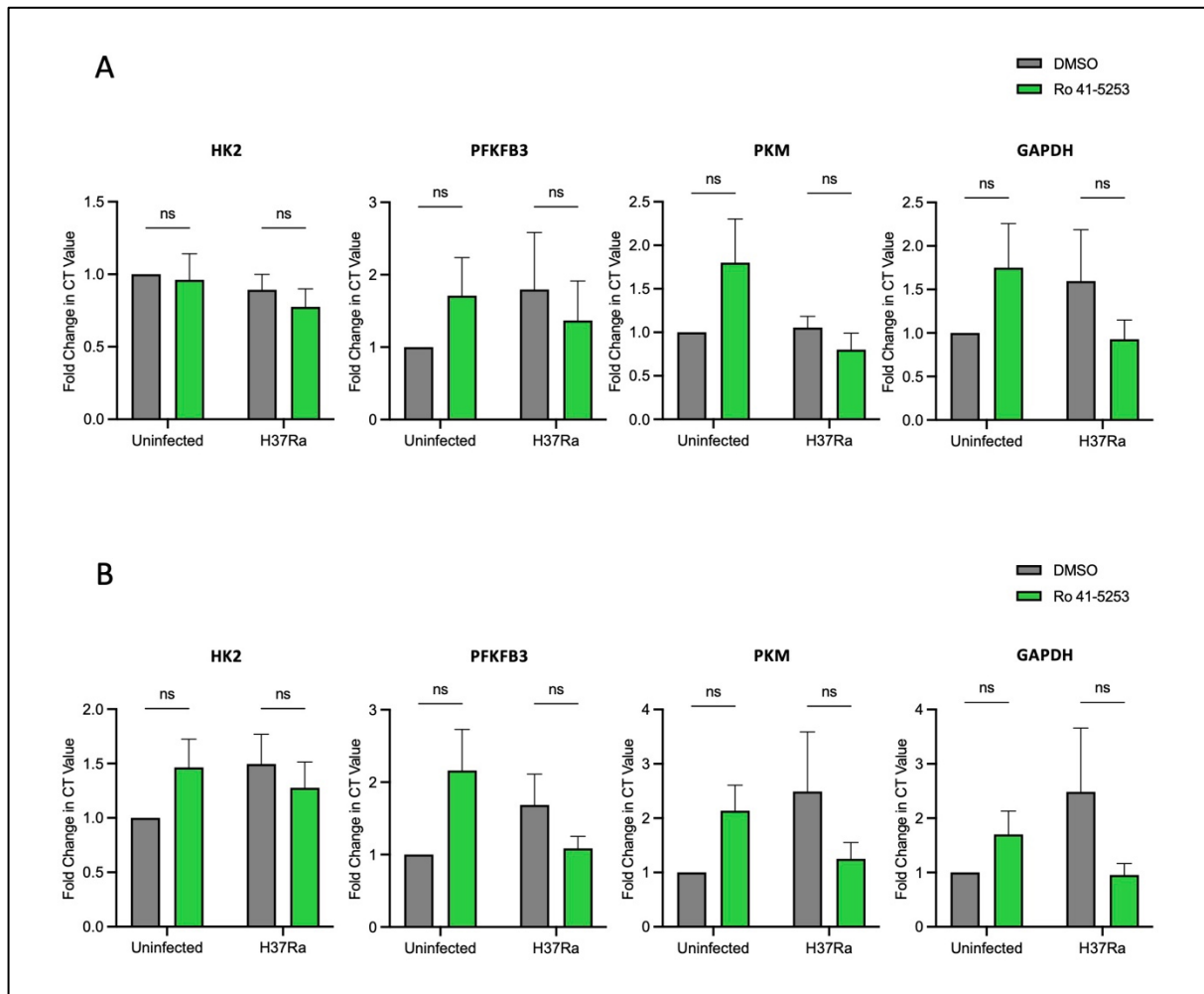


Figure 3.12: *RAR*α inhibition did not significantly reduce the mRNA expression of glycolytic genes in *Mtb* infected MDMs. Human MDMs were treated with Ro 41-5253 (2μM) or DMSO and infected with H37Ra at an MOI of 1-2 bacilli/cell (as described in sections 2.1.1 and 2.3.2). The expression of glycolytic genes at 3 hours (A) and 48 hours (B) post-infection was measured as described in section 2.6.5 and expressed as a fold change in Ct value versus the uninfected, DMSO treated control condition. Statistical comparison between the treatment groups was performed by two-way ANOVA (N=8).

3.4.7 Blocking the nuclear signalling of endogenously produced ATRA does not alter human MDM cytokine production in response to Mtb

In addition to examining the effect of RAR α inhibition on macrophage metabolic responses to Mtb, we also examined its effect on macrophage cytokine production. Our laboratory has previously shown that the secretion of mature IL-1 β by MDMs infected with Mtb is dependent on glycolysis (38). Our lab has also previously found that *exogenous* ATRA decreases IL-10 production by Mtb infected THP-1 macrophages (134). We wondered whether the impaired glycolytic response of MDMs caused by impaired signalling of *endogenously* produced ATRA was also associated with impaired IL-1 β secretion, or higher IL-10 secretion.

MDMs were treated with Ro 41-5253 for three hours prior to infection with Mtb as described in sections 2.2.1 and 2.3.2. Supernatant was taken at 3 hours and 48 hours post-infection. The concentrations of IFN γ , IL-6, IL-8, IL-1 β , TNF α , IL-10 and IL-12p70 were measured as per section 2.5.1.

RAR α inhibition did not significantly alter the secretion of any of the cytokines that were measured by uninfected or Mtb-infected MDMs at either 3- or 48-hours post-infection. These data indicate that the cytokine responses of human MDMs to infection are not dependent on the signalling of *endogenously* produced ATRA (*Figure 3.13*).

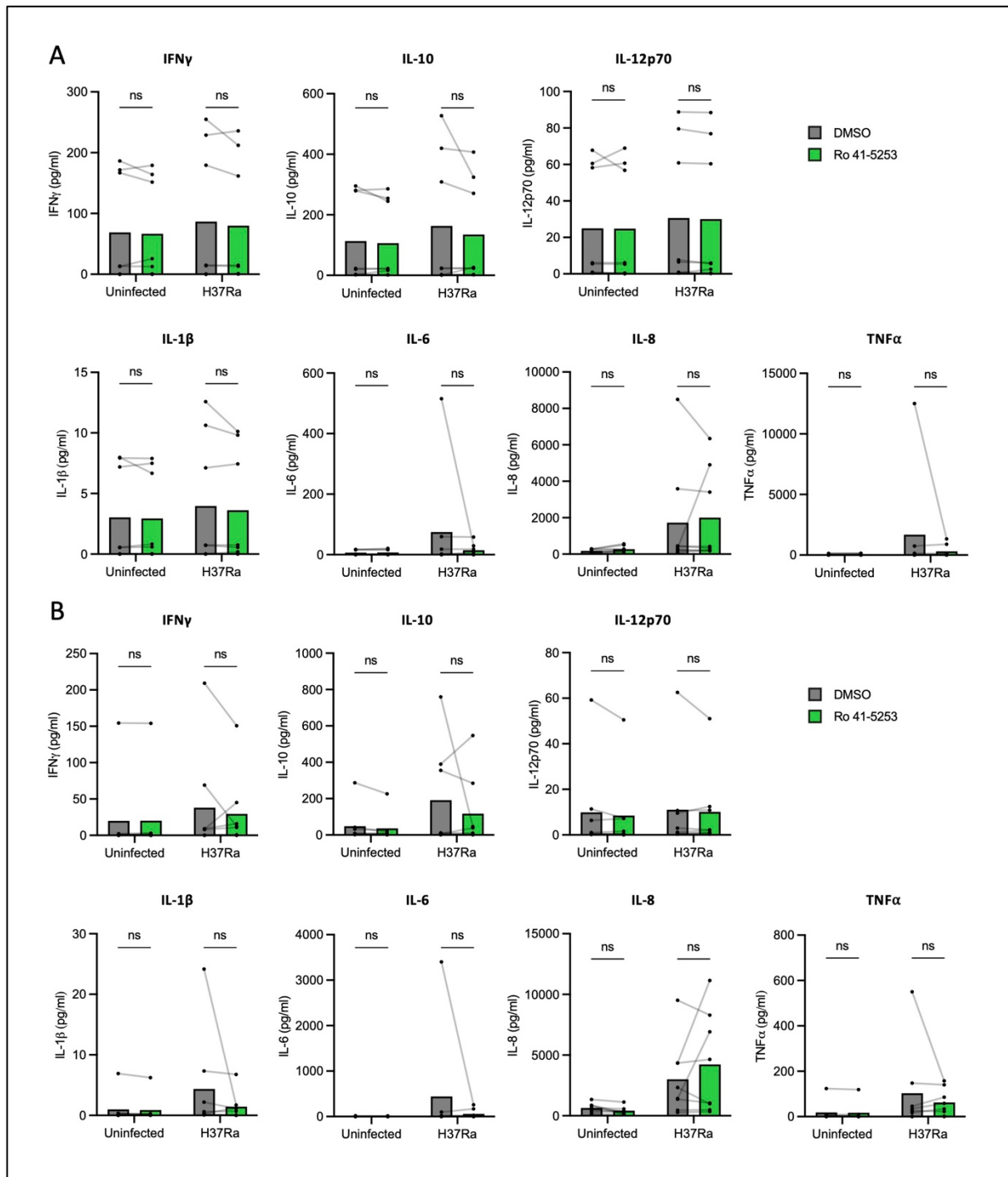


Figure 3.13: *RAR α* inhibition does not affect the cytokine response of MDM's infected with *Mtb*. Human MDMs were treated with Ro 41-5253 (2 μ M) or a vehicle control (DMSO) and then infected with H37Ra at an MOI of 1-2 bacilli/cell (as described in sections 2.1.1 and 2.3.2) or uninfected. Supernatant was collected at 3h (A) and 48h (B) post-infection. The concentration of IFN γ , IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α were measured using an MSD multiplex assay as per section 2.5.1 (N=8). Statistical comparisons were made using two-way ANOVA.

3.4.9 Blocking the nuclear signalling of endogenously produced ATRA impairs human MDMs' ability to kill Mtb

Our laboratory has previously shown that MDM's ability to kill Mtb is at least partly dependent on their ability to increase glycolysis (38). We asked if the impaired energy metabolic responses of MDMs responding to Mtb that we observed following RAR α inhibition were also associated with impaired *killing* of Mtb by human MDMs.

As described in sections 2.2.1 and 2.3.2, human MDMs were treated with Ro 41-5253 for 3 hours and then infected with H37Ra at an MOI of 1-2 bacilli/cell. A colony-forming units (CFU) assay was performed as per section 2.5.3. Briefly, Mtb growth was assessed at 0h, 48h and 120h post-infection. The inhibitor was not washed off and was present in the media for the duration of infection. MDMs were lysed and pooled with bacterial pellets (at all except the first timepoint) from the centrifugation of supernatants. Bacteria were diluted and plated on Middlebrook agar. CFUs were counted at 21 days post-incubation at 37 °C. The CFU counts of RAR α inhibited MDMs were compared with DMSO treated controls.

We found that the inhibition of RAR α led to higher CFUs than DMSO treated controls. These data indicate that MDM's ability to kill Mtb is, to a certain degree, dependent on the signalling of *endogenously* produced ATRA (*Figure 3.14*).

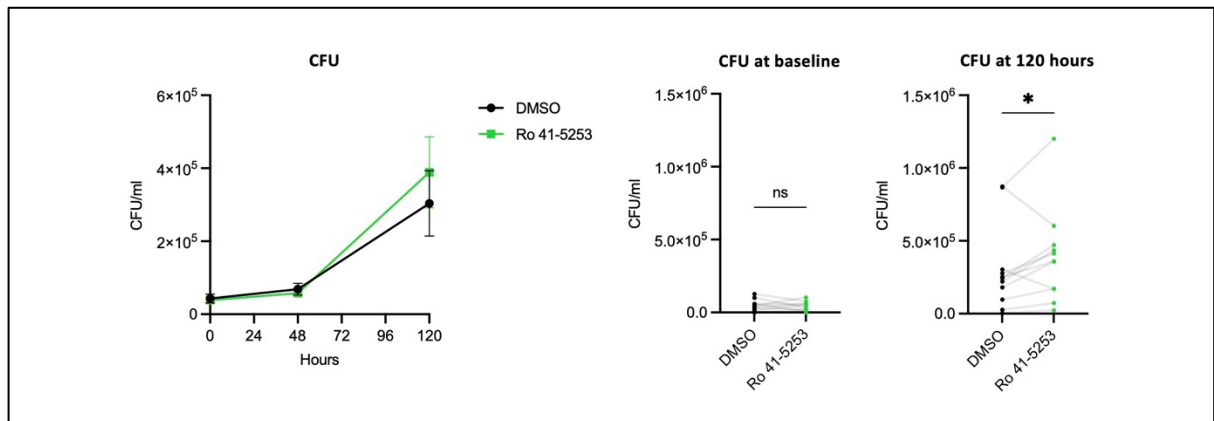


Figure 3.14: *RARα* inhibition impairs MDMs' ability to kill *Mtb*. Human MDMs were treated with Ro 41-5253 (2μM) and infected with H37Ra at an MOI of 1-2 bacilli/cell (as described in sections 2.2.1 and 2.3.2). CFUs were counted at 0h, 48h and 120h post-infection (as described in section 2.5.3). There was no difference in baseline CFUs between the two groups. At 120 hours post-infection, there was a significant difference between DMSO and Ro 41-5253 treated groups (ratio paired t test) (N=11).

3.5 Discussion

Here we report that in Mtb infected human macrophages, the endogenous production of ATRA from retinol and delivery of ATRA to its nuclear receptors may be significantly impaired due to the reduced expression of key enzymes and carrier proteins. Inhibiting the signalling of *endogenously* produced ATRA resulted in an impairment of macrophages' ability to kill Mtb. This impaired Mtb killing may be a consequence of the significantly impaired early glycolytic responses of macrophages to Mtb that we observed.

In Mtb infected human macrophages, we found that the enzymes needed to oxidise retinol to ATRA, and the carrier proteins needed to deliver ATRA to its nuclear receptor had significantly reduced expression. There have been many randomised controlled trials that have compared retinol with placebo as an adjunct to TB therapy. However, despite the encouraging laboratory evidence, and the plausibility lent by the epidemiological associations between VAD and TB, the results of these trials have been disappointing. Grobler et al. summarise these trials in their meta-analysis, and demonstrate that adjunctive retinol did not reduce mortality or sputum conversion rate in patients taking standard TB therapy (343). While there are likely several reasons why oral retinol supplementation is not an effective HDT in patients with active TB, including impaired absorption (374), mobilisation by retinol-binding proteins (375,376) and renal retention (377) of retinol in TB patients, here we identify two additional points of mechanistic interference: In Mtb infected macrophages themselves, the necessary oxidation of retinol to ATRA and the delivery of ATRA to its nuclear receptors are likely impaired. Potentially, this could be overcome by combining retinol with GM-CSF – which we have shown supports the expression of these enzymes in MDM. Interestingly, GM-CSF is known to also support glycolytic responses of macrophages. Whether this is in part through its support of endogenous ATRA production was not a question we explored (378,379). Perhaps more practically, exogenous ATRA itself (rather than retinol with GM-CSF) could bypass these obstacles. ATRA has established efficacy in the treatment of promyelocytic leukaemia, and its isomer 13-cis retinoic acid is used routinely for the treatment of acne vulgaris. Several pilot trials of ATRA for non-infectious diseases have taken place, and

established ATRA's clinical safety profile (380–385). To date, no trials comparing ATRA with placebo as an adjunctive treatment for TB have been conducted.

We show that the early glycolytic responses of human macrophages are dependent on the signalling of *endogenously* produced ATRA. In addition to the association between VAD and active TB (342,386), hyporetinolaemia is associated with pulmonary nontuberculous mycobacterial infections (387) and VAD is associated with other infections in paediatric populations – in particular those involving the gastrointestinal and respiratory tracts (388–391). The suggested mechanisms explaining the relationship between VAD and these infections include effects on secretory IgA antibodies (392), effects on the differentiation of DCs and CD4⁺ T-cells (393,394) and effects on epithelial integrity (395). In cellular models of Mtb infection, *exogenous* ATRA results in an increase in cholesterol efflux (132), improved phagosome-lysosome fusion (131,140), a reduction of intracellular iron (141) and improved cytokine responses (134,137) – although it is uncertain to what extent these mechanisms of *exogenous* ATRA are relevant in VAD. Here we suggest an additional mechanism that might partly explain the association between VAD and TB: We hypothesise that when people with pre-existing VAD are exposed to TB, their macrophages cannot effectively trigger a switch to glycolytic metabolism in response to Mtb and fail to contain Mtb as a result. This might lead to the higher risk of active TB observed in those with VAD (*Figure 3.15*). Regarding mechanism, ATRA pre-treated MDMs stimulated with LPS demonstrate a significantly upregulated hexokinase 2 (HK2) – the rate limiting glycolytic enzyme (144). While we observed that the inhibition of RAR α resulted in a trend towards decreased mRNA expression of PFKFB3, PKM and GAPDH in Mtb infected MDMs, this reduction was not statistically significant. We did not examine the protein expression of these glycolytic genes. Other genes involved in glycolysis that we did not investigate may still be affected by RAR α inhibition. The genes for the GLUT1 glucose transporter, and for the glycolytic enzymes phosphofructokinase (L and M subunits) and phosphopyruvate hydratase were found to be within 10kbp of retinoic acid responsive elements (RAREs) in one human genetic study, whereas the glycolytic genes we measured were not (396). This may be a fruitful avenue for further research.

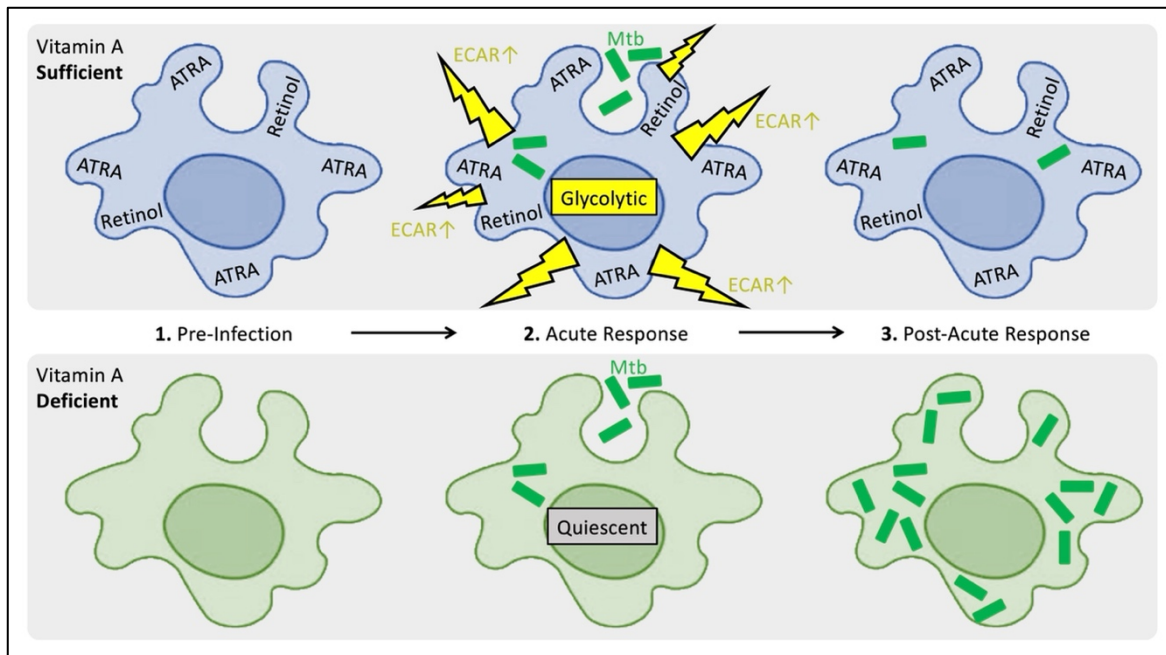


Figure 3.15: Proposed new mechanism contributing to the association between VAD and TB: (1: Pre-Infection) Macrophages from people who are vitamin A sufficient contain retinol and pre-formed ATRA, whereas macrophages from people who are vitamin A deficient do not. (2: Acute Response) In the early moments of Mtb infection, macrophages from people who are vitamin A sufficient rapidly increase their rate of glycolysis, an effect that has previously been shown to contribute to Mtb killing (38). Macrophages from people with vitamin A deficiency do not contain pre-formed ATRA and cannot ramp up glycolysis, in a similar way to what we demonstrated using RAR α inhibitors. They remain in an inappropriately quiescent energetic state during infection. (3: Post-Acute Response) Macrophages from people who are vitamin A sufficient, in part because of their early energetic response, can better control bacillary growth compared with quiescent macrophages from people who are vitamin A deficient. We observed higher CFUs at day 5 in human macrophages treated with a RAR α inhibitor.

When we blocked PPAR γ in advance of treating MDMs with Ro 41-5253, we did not restore their glycolytic responses to Mtb. Schupp et al. treated murine adipocyte cell lines with Ro 41-5253 for 24 hours and observed an increased *activation* of the PPAR γ receptor and suggested that Ro 41-5253 is a PPAR γ agonist (372). However, the known PPAR γ agonist pioglitazone has been shown to *decrease* ECAR in *unstimulated* lung cancer cell lines after a 2 hour treatment, whereas we saw no difference in baseline ECAR following our use of Ro 41-5253 in unstimulated MDMs after a similar treatment duration (*Figure 3.5, 3.7*) (397). In our experiments, pre-blocking PPAR γ did not abrogate the metabolic effects of Ro 41-5253. Taken together, these indicate that the effect of Ro 41-5253 on glycolytic responses is not due to activation of PPAR γ . Other literature suggests that both ATRA and Ro 41-5253 may activate the extracellular signal-regulated kinases (ERK) signalling pathway, through a mechanism that involves conformational change of extranuclear RAR α and the subsequent phosphorylation of ERK (242,373). In our experiments, pre-blocking MEK1 did not abrogate the metabolic effects of Ro 41-5253. This suggests that the effect of Ro 41-5253 on glycolytic responses is not due to extranuclear effects that cause ERK phosphorylation. Most significantly, an alternative RAR α antagonist, which is not known to activate PPAR γ or to phosphorylate ERK, had precisely the same metabolic effects as Ro 41-5253. This strongly suggests that it is the inhibition of RAR α itself that prevents the glycolytic response of human macrophages to Mtb. While a pan-RAR inhibitor did not replicate the effects of either RAR α inhibitor, this difference might be explained by technical factors such as differing inhibitor potencies or may indicate that different RAR receptor subtypes mediate different effects.

RAR α inhibition did not affect the secretion of IFN γ , IL-6, IL-8, IL-1 β , TNF α , IL-10 or IL-12p70 by human MDMs at 3- or 48-hours post-infection with Mtb. Our lab has previously found that *exogenous* ATRA decreases IL-10 production by Mtb infected THP-1 macrophages (134). Other researchers found that *exogenous* ATRA increased the secretion of IL-1 β and IL-6 from LPS stimulated MDMs (144). Our laboratory has previously shown that the secretion of mature IL-1 β by MDMs infected with Mtb is dependent on glycolysis (38). Despite preventing any increase in ECAR by macrophages in response to Mtb, there was no significant disruption in IL-1 β secretion by Ro 41-5253 treated cells. This suggests that the relationship between glycolysis and IL-1 β secretion is

not straightforward, or that multiple effects are at play. However, it should also be noted that we observed very low cytokine secretion even in DMSO treated MDMs following infection with Mtb. This is probably a consequence of the low MOI that we used. Repeating this experiment using a higher infecting MOI may result in higher cytokine secretion and may reveal an effect of RAR α inhibition on cytokines.

A systematic review by Yang et al. found that two doses of vitamin A significantly reduced the mortality from measles in a paediatric population (398). While trials of retinol in patients with active TB failed, this is relatively late in the disease course. It is possible that retinol (or perhaps even more so, ATRA) might be more effective if given earlier in the disease course – to support macrophage early clearance of the pathogen. ATRA could be applied to prevent the development of active TB in household contacts who are VAD. Indeed, given that contacts exhibit a stepwise decrease in risk of active TB with increasing baseline serum retinol levels – even in those who are deemed vitamin A ‘sufficient’ – this could be extended to all household contacts (342). Another high-risk group that might benefit from prophylactic retinol or ATRA (‘host-directed *prophylaxis*’) is patients newly diagnosed with HIV who have VAD (386).

Both Mtb infection and LPS stimulation reduced macrophage expression of vitamin A oxidation enzymes and carrier proteins. This suggests that a common pathway links the recognition of pathogen associated molecular patterns (PAMPs) and the downregulation of these vitamin A pathways. Counter to this hypothesis, there is evidence that stimulation of TLR2 results in an increased expression of ALDH1a2 in murine DCs (399). LPS is a TLR4 ligand, and Mtb stimulates both TLR2 and TLR4 (400). The pathway by which LPS and Mtb infection reduces the expression of these enzymes and carrier proteins remains unknown, and an indirect effect of the post-infection cytokine milieu could be implicated.

There are several limitations of this research. We did not measure ATRA or the activation of RAR α directly. ATRA is difficult to measure due to its instability, low intracellular concentrations and similar structure to other isomers (401). Likewise, we did not validate the inhibition of RAR α by Ro 41-5253 (although figure 4.7 indicates it is effective at abrogating ATRA’s effects). Importantly, another RAR α inhibitor, ER 50891, had the

same effect as Ro 41-5253 on glycolytic flux (Figure 3.10). While we show a reduction in the enzymes that produce ATRA and a reduction in the carrier proteins that deliver ATRA to its nuclear receptors, the consequence of this is only inferred. Yet blocking ALDH with diethylaminobenzaldehyde reduces the effectiveness of *exogenous* retinal in improving macrophage killing of Mtb (131), which supports our conclusion that ATRA produced by human macrophages can affect their ability to kill Mtb. We also report that inhibiting RAR α resulted in impaired macrophage metabolic responses to Mtb and reduced the ability of MDM to kill Mtb. However, while people with VAD could be assumed to have a low intracellular concentration of ATRA within their macrophages encountering Mtb, the complicated clinical state of VAD cannot be fully represented in a cell-based model, and ATRA binds to receptors other than RAR α . While we report that inhibition of RAR α stunts macrophage metabolic responses to Mtb, we did not see a statistically significant corresponding decrease in the mRNA expression of glycolytic genes. We did not measure the protein expression of these glycolytic or assess metabolism at later timepoints and these limitations could be addressed with future research. However, our laboratory has previously published this acute injection model of Mtb infection (368) which tallied with a longer term commitment to glycolysis by macrophages observed at 24 hours after challenge with Mtb (38). We interpreted ECAR as a correlate of glycolysis, however more specific metabolic assays could be done to ensure that the changes in ECAR we observed are reflective of glycolysis. Non glycolytic acidification can also contribute to ECAR and occurs due to production of CO₂ via the TCA cycle. The CO₂ produced is hydrated to carbonic acid which can reduce the extracellular pH. Lactate secretion closely mirrors glycolytic ECAR (402) and future studies could include an end point lactate measurement to confirm an increase in glycolytic flux. In addition, Agilent's Glycolytic Rate Assay, which uses sequential introduction of rotenone/antimycin A to inhibit OXPHOS and 2-DG to inhibit glycolysis, could be used to evaluate the relative contributions of both pathways to ECAR. Another limitation is that we did not directly investigate the link between ATRA's glycolytic effects and its effects on Mtb growth inhibition. This may be a subject of our future research.

This research adds to the few mechanistic publications linking vitamin A with TB. We believe that clinical trials should be considered, examining the efficacy of ATRA as a

prophylactic agent against active TB in household contacts of TB cases, and in patients starting antiretroviral therapy for HIV (in whom diagnostics for latent TB infection are known to be less effective (403,404)) who are vitamin A deficient. We also believe that clinical trials of ATRA as an adjunct to TB therapy should be considered. Finally, it is our opinion that further laboratory research should be conducted examining the mechanisms linking ATRA with energy metabolism in Mtb infection, ATRA's efficacy as a HDT or chemoprophylaxis in other infections, and further exploring the potential of GM-CSF as a HDT for TB.

3.6 Conclusion

In Mtb infected human macrophages, the production of ATRA from retinol and the delivery of ATRA to its nuclear receptors may be significantly impaired due to reduced gene expression of key enzymes and carrier proteins. This may partly explain the failure of trials of retinol as an adjunct to TB therapy. GM-CSF may act as a HDT for TB by supporting macrophage vitamin A metabolism. Inhibiting the signalling of endogenously produced ATRA prevented necessary macrophage metabolic responses to Mtb and impaired their ability to kill Mtb. This may partly explain why VAD confers susceptibility to TB.

3.7 Appendix for Chapter 3

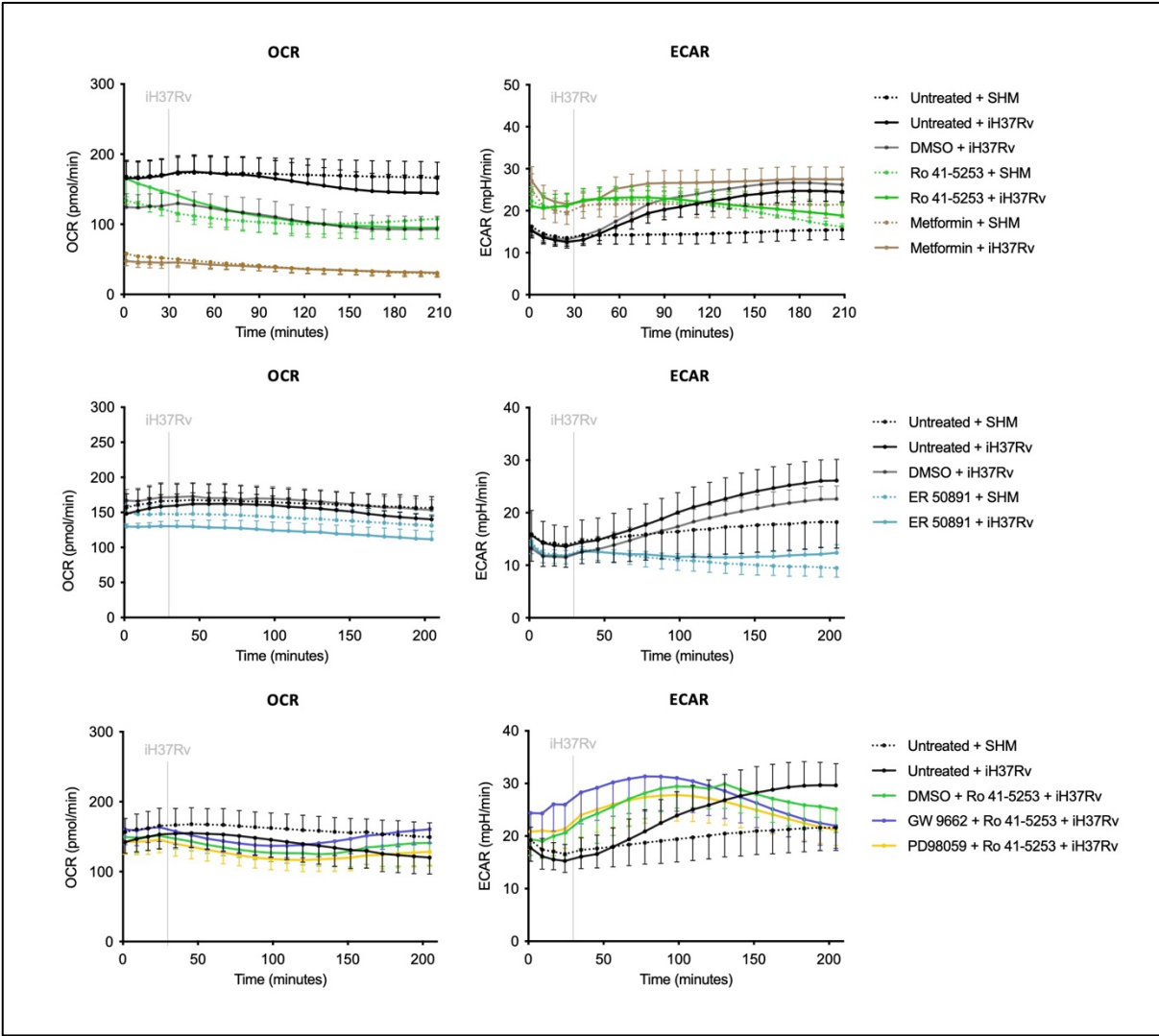


Figure 3.16: Normalised, non-baselined tracings for figures 3.7, 3.9, 3.10.

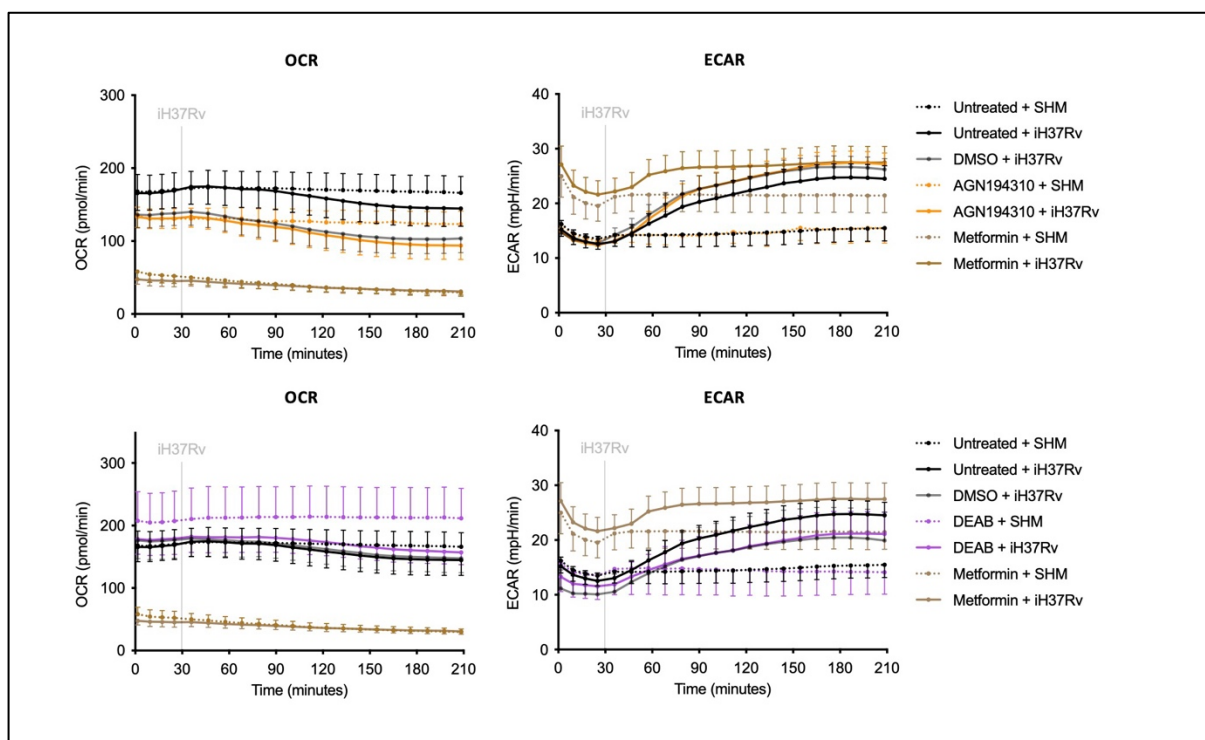


Figure 3.17: Normalised, non-baselined tracings for figure 3.11.

Chapter 4

4 All *trans* Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria

4.1 Introduction

NTM infections are increasing in incidence, globally (5). NTM species are numerous and diverse, but can be broadly categorised into rapid growers, like *M. abscessus*, and slow growers, like *M. avium*. *M. chimaera* is a subspecies of *M. avium* (51,56,57). While *M. abscessus* and *M. avium* differ in their speed of replication, the infections they cause are similar in many respects. Both most commonly cause pulmonary disease, in keeping with their usual mode of transmission, inhalation (62,63). Both almost always cause pulmonary disease in people with pre-existing structural lung disease, such as cystic fibrosis (CF) and non-CF bronchiectasis (69). Where a pulmonary NTM infection occurs in someone without an underlying structural lung disease, this instead indicates an underlying defect of the immune system (65).

The management of NTM infections is difficult. Treatment demands the combination use of multiple antibiotics with considerable associated toxicities, for durations of several months. Despite treatment, persistent colonisation and relapse are common (51,57,59,77,84). Drug resistance is another challenge of NTM infection treatment. Macrolide resistance is associated with an 8% culture conversion rate in pulmonary *M. abscessus* infections (58). Five-year mortality of patients with NTM infections was reported as 17.8% in one study (74). As such, innovation in the management of pulmonary NTM infections is needed.

Treatments that target the host's response – host-directed therapies (HDTs) – may present such an opportunity for innovation. ATRA, the active metabolite of vitamin A, is known to improve host responses during infections with *Mtb* *in vitro* and *in vivo* (104,134,138) (Table 1.1). However, it cannot be assumed that the same will hold true for infections caused by NTMs. There is no strong, relevant clinical evidence to support vitamin A as a treatment in these patients. Patients with NTM infections do have lower

circulating vitamin A – retinol – than healthy controls (73). However, this correlation may describe an effect rather than a cause – circulating retinol declines with the acute phase response (168,169). While the evidence from *in vitro* and *in vivo* experiments lends strong support for the use of ATRA as a HDT in Mtb infections, there are no published experiments investigating ATRA, or any other member of the vitamin A family, as a HDT in NTM infections. Like Mtb, NTMs are phagocytosed by host macrophages, express ESX secretion systems, suppress phagolysosome fusion, escape the phagosome, can persist intracellularly and induce granuloma formation by immunocompetent hosts (51). However, NTMs differ from Mtb in several fundamental ways that might reduce ATRA's efficacy.

We aimed to investigate the potential of ATRA as a HDT for NTM infections by understanding if ATRA improves the ability of human macrophages to kill *M. chimaera* and *M. abscessus in vitro*. *M. abscessus* was chosen because it is the most common cause of pulmonary disease due to rapidly growing mycobacteria, and because it is associated with particularly poor clinical outcomes and therefore represents a major clinical challenge. *M. chimaera* was chosen because it is a member of the *M. avium* complex (MAC), which together are the most common cause of pulmonary disease due to slow-growing mycobacteria, and because it has been associated with iatrogenic outbreaks (57–59).

We also aimed to determine the effects of ATRA on macrophage viability, macrophage intracellular cholesterol concentrations, macrophage cytokine production and macrophage metabolic responses during infection with *M. chimaera* and *M. abscessus* – pathways that might be relevant to ATRA's mechanism of action as a HDT in these infections.

4.3 Aims and Hypotheses

Overall Aim

To investigate ATRA as a potential HDT for NTMs.

Specific Aims

Aim 1: To determine if ATRA is an effective HDT against NTMs *in vitro*.

Research Question 1: Does ATRA improve NTM killing in a macrophage dependent manner?

Hypothesis 1: ATRA does not kill *M. chimaera* and *M. abscessus* in axenic culture, but will improve the ability of THP-1 macrophages to kill *M. chimaera* or *M. abscessus*.

Aim 2: To determine ATRA's mechanisms of action as a HDT against NTMs.

Research Question 2: Does ATRA affect the intracellular cholesterol concentration, cytokine secretion or metabolic responses of macrophages infected with NTMs?

Hypothesis 2: ATRA treatment will reduce the intracellular cholesterol concentration, alter cytokine secretion or alter the metabolic responses of human macrophages infected with *M. chimaera* or *M. abscessus*.

4.4 Results

4.4.1 ATRA does not affect the growth of NTMs in axenic culture

We first sought to understand if ATRA was directly toxic to NTMs. ATRA was previously found not to be directly toxic to *Mtb* at a concentration of 5 μ M (405). The growth of *M. chimaera* and *M. abscessus* in the presence of the antibiotic clarithromycin (4 μ g/ml), DMSO (vehicle control) or ATRA (10 μ M), were measured by the BACT/ALERT 3D Automated Microbial Detection System as per section 2.5.4. The times to positivity of each condition were recorded. For *M. chimaera* treated with the positive control clarithromycin, no growth was detected by the 42-day cut-off, and so growth was assumed to be detected at 42 days for the statistical analyses. Clarithromycin inhibited the growth of *M. chimaera*, whereas there was no difference in the times to positivity between ATRA and DMSO conditions. For *M. abscessus*, clarithromycin also slowed growth, and there was no difference in the times to positivity between ATRA and DMSO conditions. From these results we conclude that ATRA does not affect the growth of *M. chimaera* or *M. abscessus* in axenic culture, and therefore is not itself directly toxic to these NTMs (Figure 4.1).

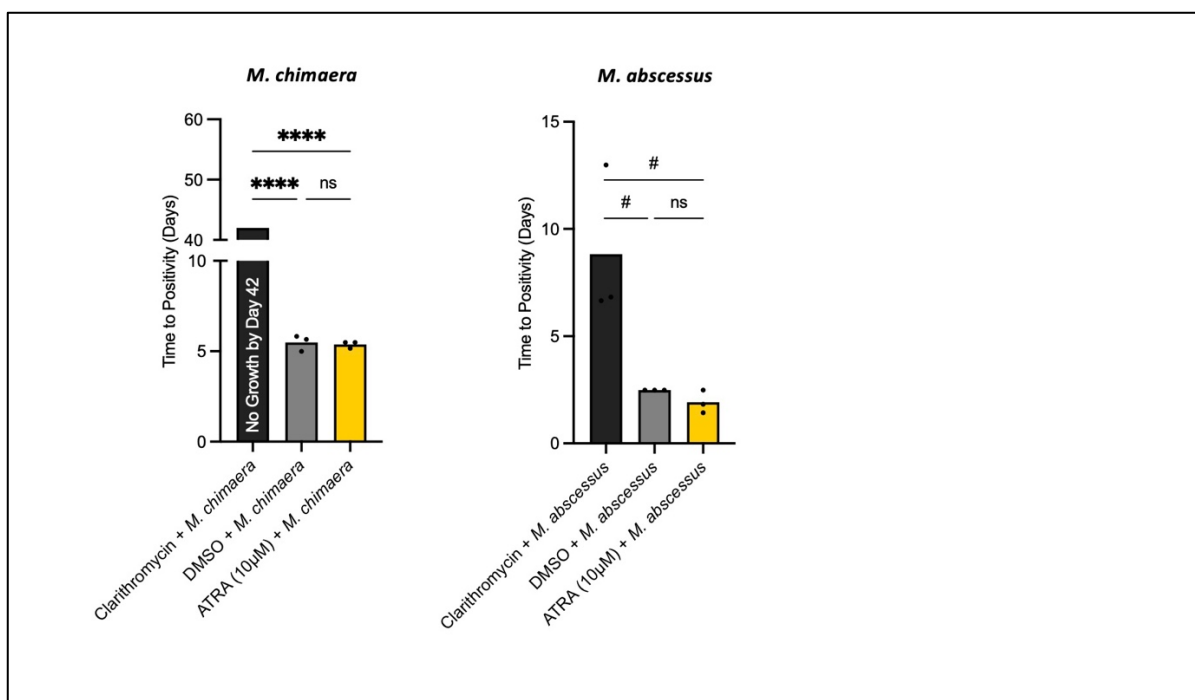


Figure 4.1: ATRA does not kill rapid or slow growing NTMs in axenic culture. The times to positivity of *M. chimaera* (left) and *M. abscessus* (right), in the presence of the antibiotic clarithromycin (4 µg/ml), DMSO (vehicle control) or ATRA (10µM), were measured by the BACT/ALERT 3D Automated Microbial Detection System (N=3). Statistical comparisons were made using one-way ANOVA or unpaired t test (significance denoted by #).

4.4.2 ATRA does not affect the viability of uninfected macrophages

We next sought to understand if ATRA was toxic to human macrophages. ATRA was previously found not to induce cell death in MDMs at a concentration of 5µM (405). MDMs were treated with ATRA at doses 10µM, 5µM and 1µM for 24 or 96 hours or left untreated or treated with a vehicle control (same DMSO concentration as the 10µM ATRA condition). Cycloheximide (CHX) (100 µg/ml) was used as a positive control for cell death. The cells were stained with propidium iodide (PI) at 5µg/ml, Hoechst 33258 at 50µg/ml and Hoechst 33342 at 20µg/ml. Using automated fluorescence microscopy, the proportion of PI-positive cells was calculated (a measure of the proportion of non-viable cells). ATRA did not adversely affect the viability of MDMs after 24 or 96 hours at of the concentrations. For subsequent experiments, ATRA was used at a concentration of 10µM (Figure 4.2).

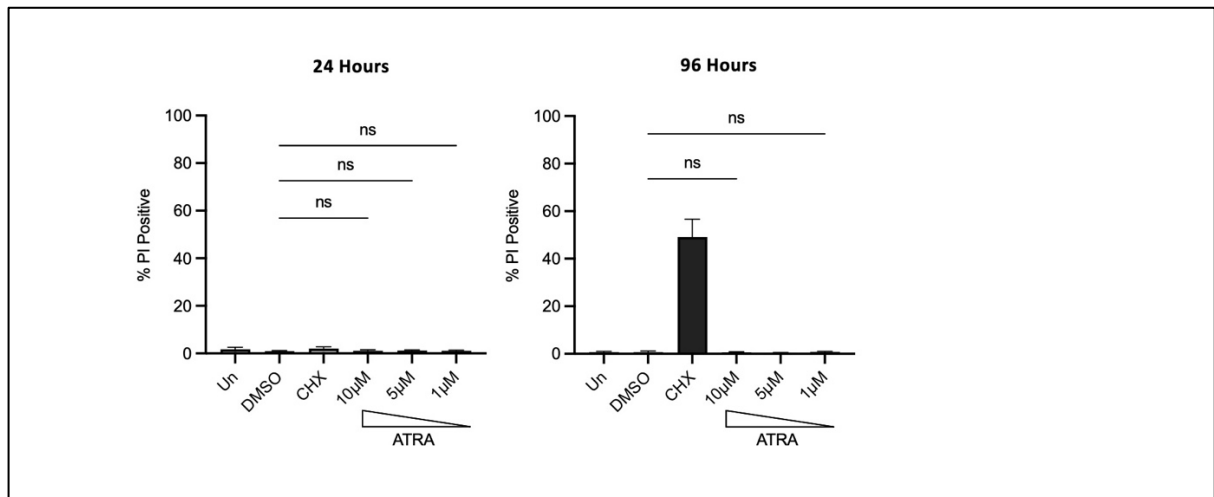


Figure 4.2: The viability of MDMs was not affected by 24- or 96-hour treatment with ATRA. MDMs were treated with ATRA at doses 10µM, 5µM and 1µM for 24 or 96 hours or left untreated or treated with a vehicle control (same DMSO concentration as the 10µM ATRA condition). Cycloheximide (CHX) (100 µg/ml) was used as a positive control. The cells were stained with propidium iodide (PI), Hoechst 33258 and Hoechst 33342. Using automated fluorescence microscopy, the proportion of PI-positive cells was calculated (percentage of non-viable cells) (24 hours: N=4,3,4,4,4,4. 96 hours: N=4,3,4,4,2,4). Statistical comparisons between ATRA treated conditions and DMSO control were made using one-way ANOVA.

4.4.4 ATRA does not affect the viability of *M. abscessus* infected or uninfected THP-1 macrophages

We also examined the effect of ATRA (at 10 μ M) on the viability of THP-1 macrophages, the cell type used in most experiments in this chapter. We used this as an opportunity to also examine the effect of *M. abscessus* infection on the viability of THP-1 macrophages, in advance of subsequent experiments assessing intracellular bacillary killing (CFU assays), as *M. abscessus* is a rapidly growing NTM species. A cell viability assay was performed as before. While the positive control CHX increased the proportion of non-viable cells, ATRA did not affect the viability of uninfected THP-1 macrophages after 24 or 48 hours. *M. abscessus* infection (at MOIs of 1-2 bacilli/cell or 4-6 bacilli/cell) did not itself increase cell death over the uninfected control at either timepoint. Importantly, ATRA did not alter the viability of *M. abscessus* infected THP-1 macrophages versus the vehicle control at either timepoint. From these experiments we conclude that neither ATRA, *M. abscessus* nor their combination affect the viability of THP-1 macrophages within the timeframe studied in subsequent experiments (*Figure 4.3*).

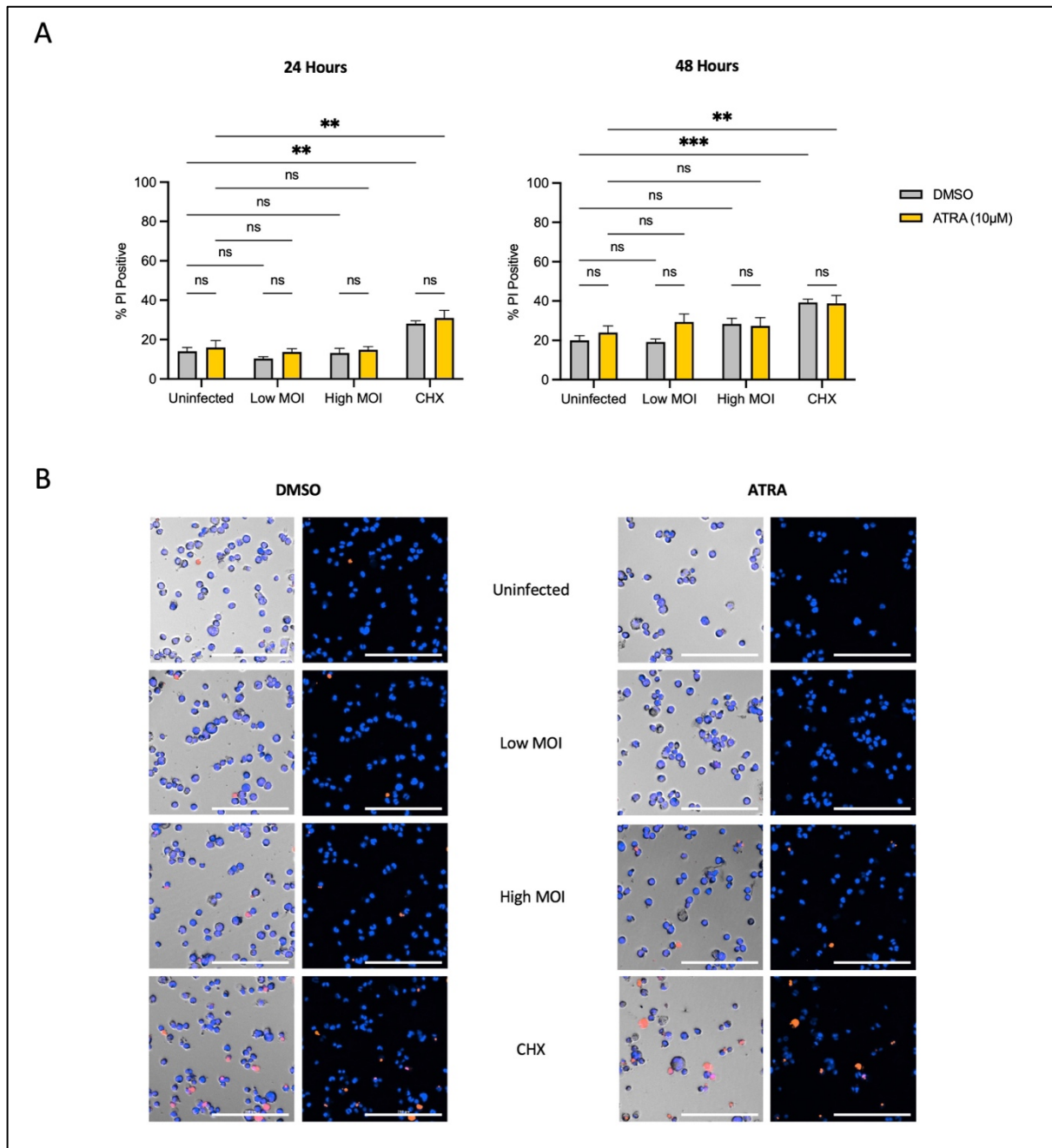


Figure 4.3: The viability of THP-1s was not affected by *M. abscessus* infection at 24 or 48 hours post-infection, and was not affected by ATRA. THP-1 macrophages were treated with ATRA at 10µM for 3 hours or treated with a vehicle control (DMSO). The cells were then infected with *M. abscessus* at a low MOI (1-2 bacilli/cell) or high MOI (4-6 bacilli per cell) as per section 2.3.4. Cycloheximide (CHX) (100 µg/ml) was used as a positive control. 24 or 48 hours after infection, the cells were stained with propidium iodide (PI), Hoechst 33258 and Hoechst 33342. (A) Using automated fluorescence microscopy, the proportion of PI-positive cells was calculated (percentage of non-viable cells) (N=4). Statistical comparisons were made using two-way ANOVA. (B) Representative fluorescent microscopy images taken at 24 hours post-infection. Blue channel = Hoechst nuclear stains, orange channel = PI. White line represents 200 µm. Left = bright field image with blue and orange channels overlaid, right = blue and orange channels only.

4.4.5 ATRA is an effective HDT in THP-1 macrophages infected with *M. chimaera* but not *M. abscessus*

We then examined whether ATRA improved the ability of THP-1 macrophages to kill *M. chimaera* or *M. abscessus*. ATRA is already known to improve the ability of MDMs, AMs and THP-1 macrophages to kill Mtb (104,134). THP-1 macrophages were treated with ATRA at 10 μ M for 3 hours or a vehicle control (DMSO). The cells were then infected with *M. chimaera* or *M. abscessus* at an MOI of 1-2 bacilli/cell as per sections 2.3.3 and 2.3.4. CFUs were counted at 0h, 48h and 120h post-infection for *M. chimaera* or 0h and 18h post-infection for *M. abscessus* (as described in section 2.5.3). It was found that ATRA reduced the growth of *M. chimaera* but did not affect the growth of *M. abscessus* at these timepoints. From this we conclude that ATRA is an effective HDT *in vitro* for the slow growing NTM species, *M. chimaera*, but not the rapid growing NTM species, *M. abscessus* (Figure 4.4).

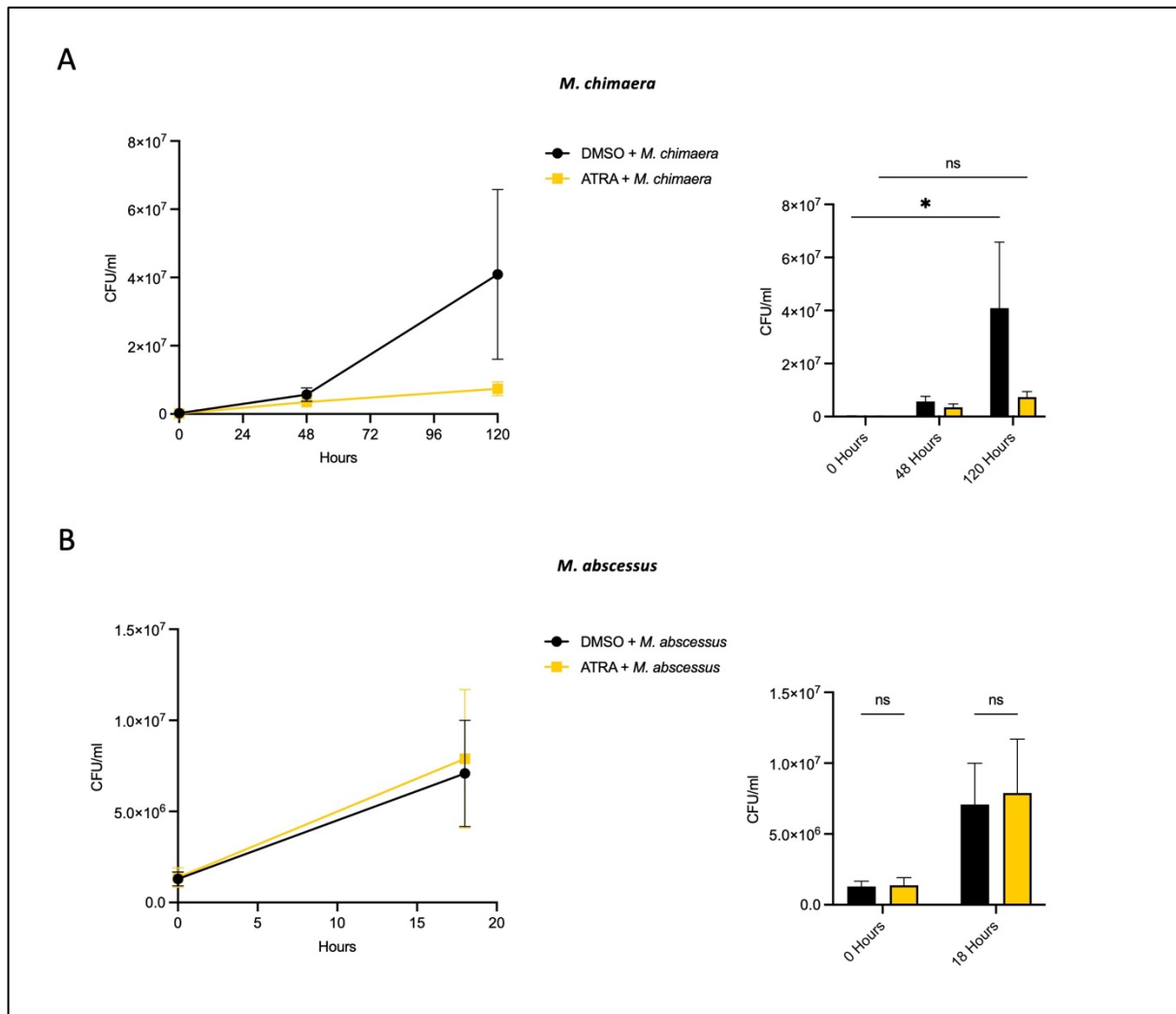


Figure 4.4: ATRA reduces CFUs in *M. chimaera* infected THP-1 macrophages, but not those infected with *M. abscessus*. THP-1 macrophages were treated with ATRA at 10 μ M for 3 hours or a vehicle control (DMSO). The cells were then infected with *M. chimaera* or *M. abscessus* at an MOI of 1-2 bacilli/cell as per sections 2.3.3 and 2.3.4. CFUs were counted at 0h, 48h and 120h post-infection for *M. chimaera* or 0h and 18h post-infection for *M. abscessus* (as described in section 2.5.3) (*M. chimaera*: N=5, *M. abscessus*: N=4). Statistical comparisons were made using two-way ANOVA.

4.4.7 ATRA does not affect THP-1 macrophage intracellular cholesterol concentrations during infection with *M. chimaera* and *M. abscessus*

We sought to understand how ATRA affects macrophages infected with *M. chimaera* and *M. abscessus*, in part to understand how it might be effective as a HDT in NTM infections. We began by measuring the intracellular cholesterol concentrations of THP-1 macrophages infected with *M. chimaera* and *M. abscessus* in the presence or absence of ATRA. Previous research has demonstrated that ATRA upregulates cholesterol transporters ABCA1, ABCG1 and NPC2 (132,138,139). Furthermore, ATRA reduces the intracellular cholesterol concentration of MDMs differentiated from monocytes in the presence of GM-CSF, restricting the access of Mtb to its cholesterol fuel source (138). We found in THP-1 macrophages (GM-CSF not used) that ATRA did not significantly reduce the total intracellular cholesterol concentration in either *M. chimaera* or *M. abscessus* infection. The availability of cholesterol to mycobacteria within the lysosomal compartment was not measured but might still be altered by ATRA. However, from this we conclude that the total intracellular concentration of cholesterol is not altered by ATRA in uninfected or NTM infected THP-1 macrophages (Figure 4.5).

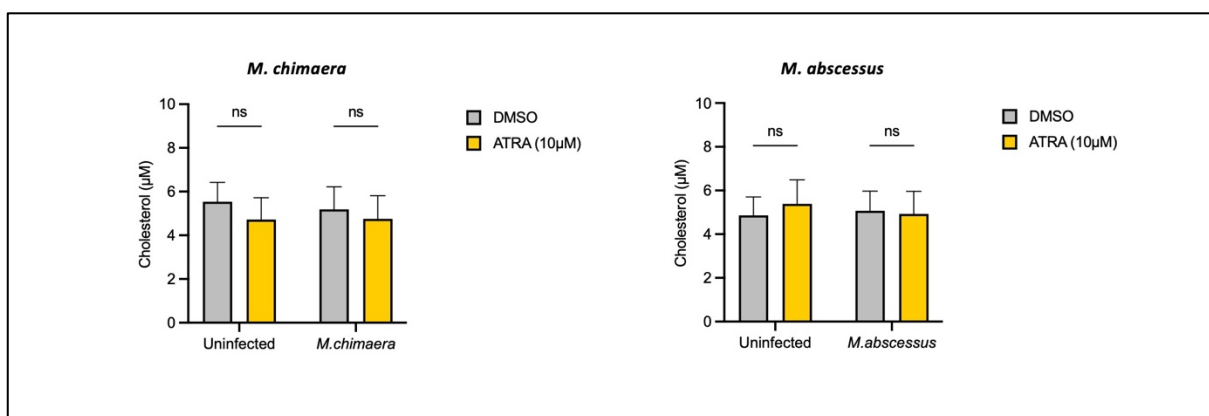


Figure 4.5: ATRA does not affect the intracellular cholesterol concentration of uninfected THP-1 macrophages, or those infected with *M. chimaera* or *M. abscessus*. THP-1 macrophages were treated with ATRA at 10µM for 3 hours or a vehicle control (DMSO). The cells were then infected with *M. chimaera* or *M. abscessus* at an MOI of 1-2 bacilli/cell as per sections 2.3.3 and 2.3.4 for 24 hours. The total intracellular cholesterol concentration was then measured using a luminescence assay described in section 2.5.2 (*M. chimaera*: N=5, *M. abscessus*: N=4). Statistical comparisons were made using two-way ANOVA.

4.4.8 ATRA alters THP-1 macrophage cytokine responses to *M. chimaera*

While we found that ATRA improved the ability of THP-1 macrophages to kill *M. chimaera*, it did not affect the total intracellular cholesterol concentrations. We wondered whether ATRA may improve the cytokine responses of THP-1 macrophages infected with NTMs. ATRA was previously found to reduce IL-10 secretion in THP-1 macrophages infected with *Mtb* (134). IL-10 is known to inhibit phagosome maturation, allowing *Mtb* growth (406). Other researchers found that ATRA increased the secretion of IL-1 β and IL-6 from LPS stimulated MDMs (144).

In our experiments, *M. chimaera* infection significantly induced the secretion of IFN γ , IL-10, IL-12p70, IL-1 β , IL-6, IL-8 and TNF α from DMSO treated THP-1 macrophages at 48 hours post-infection, whereas *M. abscessus* did not *significantly* induce any cytokine at 18 hours post-infection. *M. chimaera* significantly increased the secretion of IFN γ , IL-1 β , IL-6 and TNF α in ATRA treated macrophages, but not IL-10, IL-12p70 and IL-8. We found that ATRA reduced the concentration of IL-10 secreted by *M. chimaera* infected THP-1 macrophages. ATRA did not alter the secretion of cytokines from *M. abscessus* infected macrophages (*Figure 4.6*).

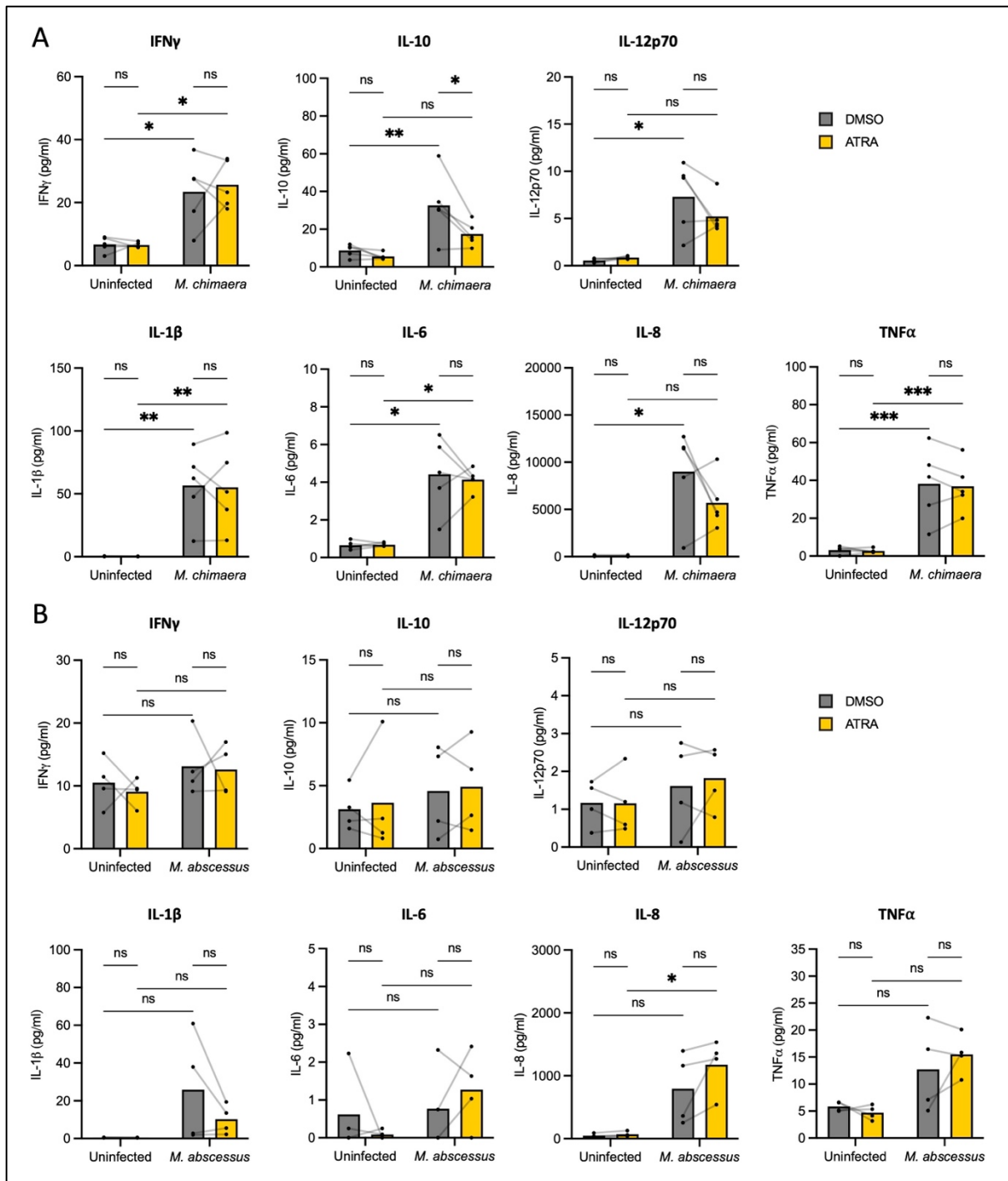


Figure 4.6: ATRA reduces macrophage IL-10 secretion in response to NTM infection. THP-1 macrophages were treated with ATRA at 10 μ M for 3 hours or a vehicle control (DMSO). The cells were then infected with *M. chimaera* (A) or *M. abscessus* (B) at an MOI of 1-2 bacilli/cell as per sections 2.3.3 and 2.3.4. Supernatant was collected at 48 hours or 18 hours post-infection for *M. chimaera* and *M. abscessus*, respectively. The concentration of IFN γ , IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α were measured using an MSD multiplex assay as per section 2.5.1 (N=5). Statistical comparisons were made using two-way ANOVA.

4.4.9 ATRA induces the Warburg effect in THP1 macrophages, an effect which is abrogated by inhibition of RAR α

We then wondered if ATRA was affecting the metabolism of human macrophages responding to NTMs. Macrophages undergo the Warburg effect when stimulated with Mtb, and blocking glycolytic responses to Mtb infection reduces the ability of macrophages to kill Mtb (38). A previous study found that MDMs treated with ATRA for 4 hours, then stimulated with LPS for 6 hours had a higher rate of glycolysis than untreated, LPS stimulated cells (144). We wanted to understand the effect of ATRA on the earliest metabolic responses of macrophages during infection with NTMs. Before doing so, we sought to first determine what the acute metabolic effects of ATRA on human macrophages are, and to determine if these metabolic effects are mediated through the RAR α nuclear receptor. The OCR and ECAR of THP-1 macrophages were measured over time by the Seahorse analyser using a bespoke protocol (described in section 2.6.3): OCR and ECAR were measured 39 times over approximately five hours (~every 8 minutes for 288 minutes). After the 3rd measurement, either DMSO (vehicle control) or the RAR α inhibitor, Ro 41-5253 were injected – Injection (1). After the 21st measurement, either DMSO (vehicle control) or ATRA were injected – Injection (2). In total, four conditions were studied: DMSO followed by DMSO; DMSO followed by ATRA; Ro 41-5253 followed by DMSO; and Ro 41-5253 followed by ATRA. The RAR α inhibitor Ro 41-5253 (introduced at ‘Injection (1)’) caused a modest increase in the ECAR from baseline after 140 minutes (8.0% +/- SEM 1.3%) but did not significantly affect the relative OCR (2.8% +/- SEM 2.1%), the raw ECAR values or the OCR:ECAR ratio. ATRA (introduced at ‘Injection (2)’) caused a large increase in the ECAR from baseline after 140 minutes (37.8% +/- SEM 8.7%) and decreased the OCR from baseline versus the vehicle control (-14.3% +/- SEM 6.5%). Importantly, pre-treatment with the RAR α inhibitor Ro 41-5253 (at ‘Injection (1)’) abrogated the increase in ECAR and the reduction in OCR caused by ATRA. This suggests that ATRA’s metabolic effects are being mediated at least in part through the RAR α receptor (*Figure 4.7*).

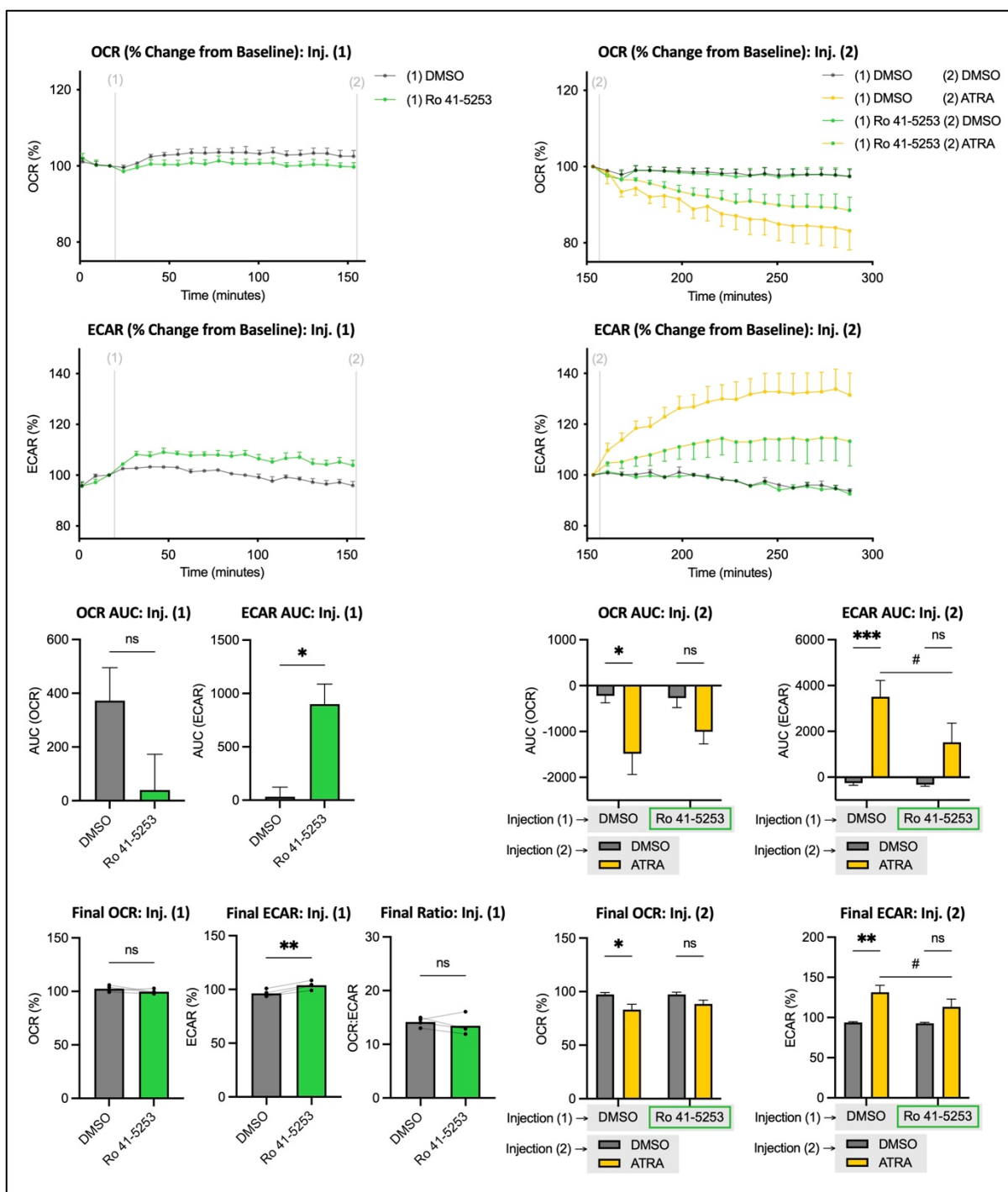


Figure 4.7: ATRA induced the Warburg effect in THP-1 macrophages, an effect which was abrogated by inhibition of RAR α . THP-1 macrophages were plated onto Seahorse plates as described in section 2.1.3. The OCR and ECAR were measured by the Seahorse analyser 39 times over approximately five hours (~every 8 minutes for 288 minutes). After the 3rd measurement, either DMSO (vehicle control) or the RAR α inhibitor, Ro 41-5253 were injected – Injection (1) ('Inj. (1)'). After the 21st measurement, either DMSO (vehicle control) or ATRA were injected – Injection (2) ('Inj. (2)'). In total, four conditions were studied: DMSO followed by DMSO; DMSO followed by ATRA; Ro 41-5253 followed by DMSO; and Ro 41-5253 followed by ATRA (N=4). For statistical analysis of Injection (1) results, paired t tests were used. Normalised, non-baselined tracings available in the appendix for chapter 4 (Figure 4.11). For statistical analysis of Injection (2) results, two-way ANOVA or paired t test (significance denoted by #) were used.

4.4.10 ATRA prevents the early glycolytic response of AMs to *M. chimaera*

Having demonstrated that ATRA induces the Warburg effect in uninfected THP-1 macrophages– most likely through its nuclear receptor RAR α – we next sought to understand the effects of ATRA on the metabolic responses of human alveolar macrophages being infected with NTMs, in real time (as per section 2.6.2). On their own, both *M. chimaera* and *M. abscessus* infections increased ECAR (Figure 4.8), which concords with prior experiments examining the metabolic responses of human macrophages to Mtb (iH37Rv), such as those presented in figure 3.7 and published studies from our laboratory (368).

We then measured the effect of ATRA on these glycolytic responses. Starting with *M. chimaera*, AMs were treated with ATRA or DMSO for three hours prior to infection in the Seahorse analyser as described in section 2.3.3. Surprisingly, ATRA reduced the ECAR at 140 minutes post-infection with *M. chimaera* versus DMSO treated, infected AMs, but did not affect the OCR. From this we conclude that ATRA prevents the increase in glycolysis of AMs responding to infection with *M. chimaera* (Figure 4.9).

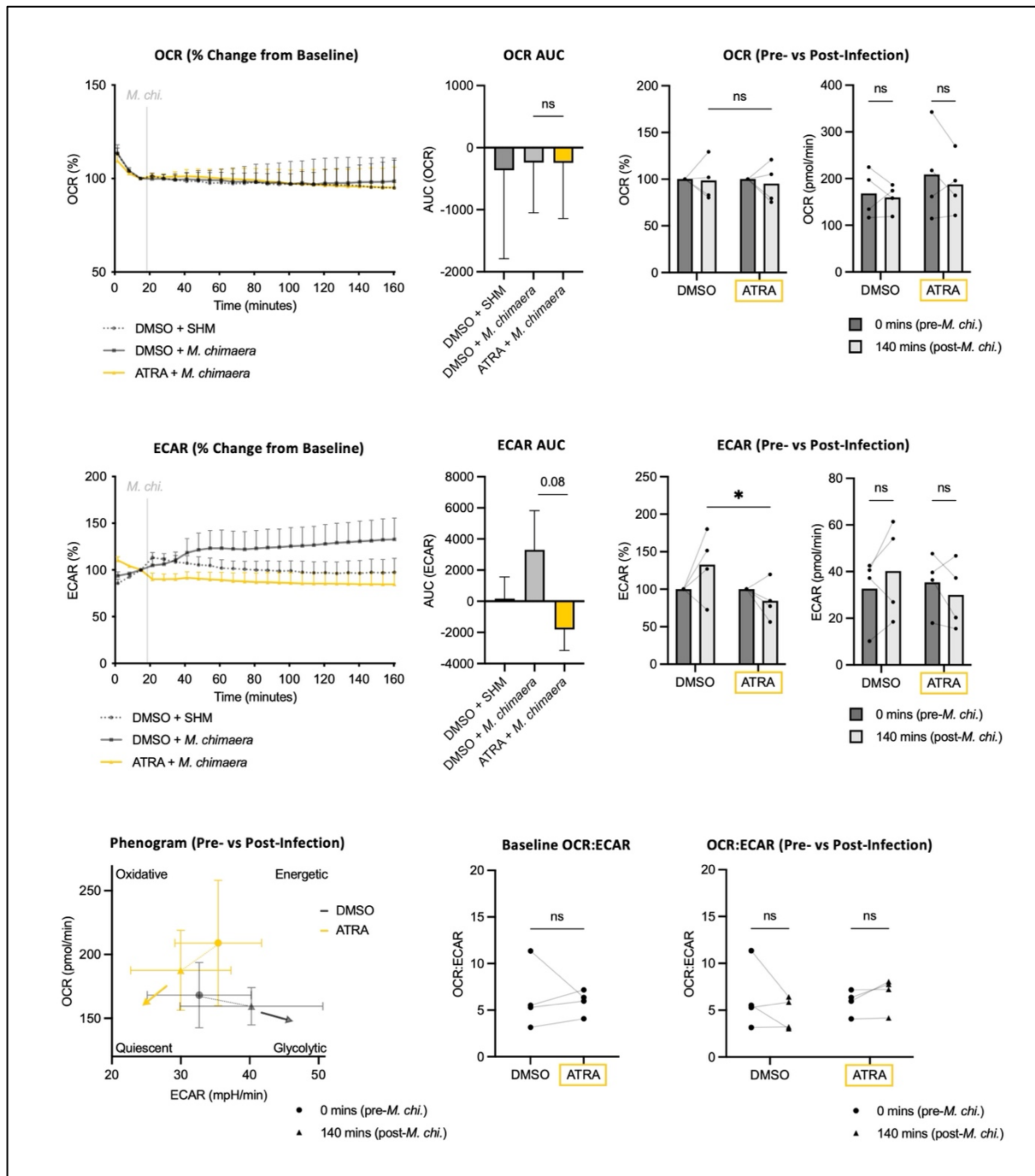


Figure 4.9: ATRA prevented the glycolytic response of AMs to *M. chimaera*. Human AMs were seeded onto Seahorse plates as described in section 2.1.2. AMs were treated with ATRA (10 μ M) or DMSO (vehicle control) as described in section 2.2.1 – AMs were treated for 3 hours prior to stimulation with *M. chimaera* (MOI 5-10 bacilli/cell) or Seahorse medium (SHM) in the Seahorse analyser (N=4). Normalised, non-baselined tracings available in the appendix for chapter 4 (Figure 4.11). Statistical comparisons were made using one-way ANOVA (OCR AUC, ECAR AUC, Baseline OCR:ECAR) or two-way ANOVA ('Pre- vs. Post-Infection' OCR, ECAR and OCR:ECAR). Error bars represent the SEM.

4.4.11 ATRA prevents the early glycolytic response of AMs to *M. abscessus*

We then examined AMs during infection with *M. abscessus*. Again, AMs were treated with ATRA or DMSO for three hours prior to infection in the Seahorse analyser as described in section 2.3.3. As for infection with *M. chimaera*, ATRA did not affect the OCR in *M. abscessus* infected AMs. However, ATRA did reduce the ECAR at 140 minutes post-infection with *M. abscessus* versus DMSO treated AMs and versus the pre-infection baseline ECAR. ATRA also resulted in an increase in the OCR:ECAR ratio with infection. From this we conclude that ATRA prevents the increase in glycolysis of AMs responding to infection with *M. abscessus* (Figure 4.10).

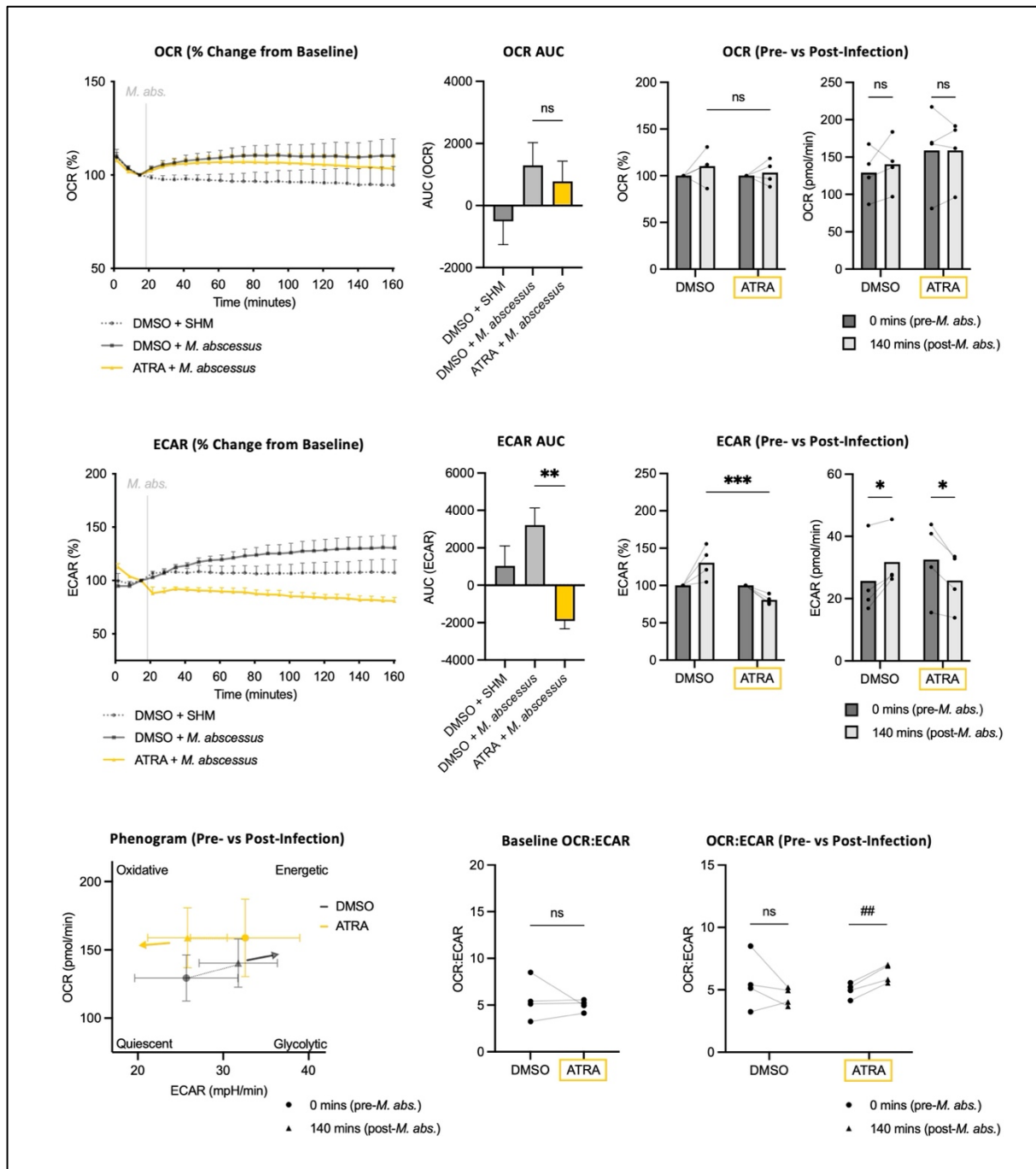


Figure 4.10: ATRA prevented the glycolytic response of AMs to *M. abscessus*. Human AMs were seeded onto Seahorse plates as described in section 2.1.2. AMs were treated with ATRA (10 μ M) or DMSO (vehicle control) for 3 hours prior to stimulation with *M. abscessus* (MOI 5-10 bacilli/cell) or Seahorse medium (SHM) in the Seahorse analyser (N=4). Normalised, non-baselined tracings available in the appendix for chapter 4 (Figure 4.11). Statistical comparisons were made using one-way ANOVA (OCR AUC, ECAR AUC, Baseline OCR:ECAR) or two-way ANOVA ('Pre- vs. Post-Infection' OCR, ECAR and OCR:ECAR) or paired t test (significance denoted using #). Error bars represent the SEM.

4.5 Discussion

In this chapter we show that ATRA is not directly toxic to either *M. chimaera* or *M. abscessus*, and that ATRA improves the ability of macrophages to kill *M. chimaera*, but not *M. abscessus*. Stated another way, ATRA was an effective HDT for *M. chimaera* *in vitro*. While indirect evidence suggests that macrolides may benefit host responses to *Pseudomonas aeruginosa*, and by extension macrolides are considered for their immunomodulatory effects in pulmonary NTM infections, this is the first direct evidence of efficacy of a *purely host-directed* therapy in an NTM infection (58,97,407). There are several possible explanations as to why ATRA was not an effective HDT for *M. abscessus* but is for *M. chimaera* and Mtb. Unlike Mtb, *M. abscessus* has been predicted to lack the ability to catabolise cholesterol. While most MAC species can catabolise cholesterol, *M. chimaera* was not specifically tested (408). If cholesterol fuel source restriction is the primary mechanism through which ATRA acts as a HDT against Mtb and *M. chimaera*, it could be expected that ATRA will be less effective against *M. abscessus* which does not depend on cholesterol catabolism for its energy. While we did not show a reduction in the total intracellular cholesterol concentration by ATRA of *M. chimaera* or *M. abscessus* infected THP-1 macrophages, we did not investigate intracellular redistribution of cholesterol. Other researchers have demonstrated a reduction in total intracellular cholesterol concentration by ATRA, although this was in MDMs differentiated from monocytes in the presence of GM-CSF (138) or in monocytes themselves (132). ATRA upregulates macrophage cholesterol transporters ABCA1 and ABCG1, which pump intracellular cholesterol to the extracellular space, and NPC2 which is a lysosome to endoplasmic reticulum lipid transporter (132,138,139).

Pathogenic rapid-growing mycobacteria like *M. abscessus* have a much shorter doubling time than slow growers like *M. chimaera* or Mtb (51). In Middlebrook liquid media, the generation time of *M. abscessus* is 4-5 hours, compared with 10-12 hours for certain *M. avium* species (409,410). ATRA acts through the transcription of genes and may therefore take some time to exert its effects. It's possible that ATRA may not operate on a relevant timescale before there has been overwhelming growth of rapid growers. This might be overcome with ATRA pre-treatment durations longer than those we investigated.

NTMs such as *M. abscessus* can transition from a smooth to a rough phenotype. The rough phenotype promotes apoptosis of its host cell, resulting in the extracellular replication of released bacteria in large 'serpentine cords' (51). Because ATRA works by improving macrophage killing of intracellular bacilli, it is likely less effective against rough NTM variants, which migrate to the extracellular space. That being said, we investigated the cell viability of THP-1 macrophages infected with *M. abscessus*, with or without ATRA, and did not observe a significant increase in cell death even at 48 hours post-infection. These data would argue against apoptosis-driven cell escape and extracellular replication as the reason why ATRA was ineffective in *M. abscessus* infection. This could be further explored by examining the efficacy of ATRA in improving macrophage killing of *M. abscessus*, with extracellular antibiotics (e.g. amikacin) present in the media for the duration of the CFU assay.

ATRA reduced IL-10 secretion from THP-1 macrophages infected with *M. chimaera*. This is in concordance with what has been seen with Mtb (134). IL-10 is known to inhibit phagosome maturation, allowing Mtb growth. The reduction in IL-10 may allow this process to proceed, and may be partly responsible for ATRA's efficacy in *M. chimaera* infection (406). The lack of IL-10 reduction in *M. abscessus* infected THP-1 macrophages could partially explain the differing CFU results that we observed in figure 4.4. Other researchers found that ATRA increased the secretion of IL-1 β and IL-6 from LPS stimulated MDMs (144). ATRA also increased TNF α in animal models of TB infection (135,137). We did not observe any change in IL-1 β , IL-6 or TNF α secretion following infection of THP-1 macrophages with NTMs.

ATRA induced Warburg metabolism in THP-1 macrophages. ATRA is known to increase cellular glucose uptake (411). Otherwise, the only published evidence of ATRA's effects on cellular metabolism comes from Alatshan et al, who investigated the metabolism of ATRA treated MDMs (differentiated from monocytes in the presence of M-CSF) that were unstimulated or stimulated with LPS for 6 hours. They also observed an increase in glycolysis but did not observe a reduction in oxidative phosphorylation in the ATRA treated, unstimulated condition at this later timepoint (144).

Importantly, we found that inhibiting RAR α in advance of ATRA treatment abrogated the Warburg effect. This suggests that ATRA's effects on cellular metabolism are mediated through the binding and activation of its nuclear receptor. This is useful because ATRA can also have non-genomic effects: ATRA can activate ERK1/2 kinases (126–128); ATRA can activate p38 MAPK (412); ATRA can activate PI3K/Akt (413,414); and 'retinoylation' is a post-translational alteration of proteins by eukaryotic cells by retinoic acid (120) which may have a role in cell differentiation (130). This result also supports chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) experiments using Ro 41-5253, as they confirm the efficacy of this inhibitor of ATRA's receptor. Furthermore, it could be proposed that the metabolic effects of RAR α inhibition that we observed in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) might not be due to poor signalling through RAR α , but rather due to enhanced signalling of ATRA through other receptors, due to a greater relative availability of ATRA for those receptors. This result argues against that hypothesis, as one would expect an *accentuation* of the Warburg effect if this were the case.

Despite inducing the Warburg effect immediately following injection in uninfected THP-1 macrophages, ATRA pre-treatment prevented the increase in glycolysis in human alveolar macrophages that were infected with *M. chimaera* or *M. abscessus*. This differs from the findings of Alatshan et al, who reported increased glycolysis and an associated increase in HK2 expression in MDMs treated with ATRA, 6 hours after stimulation with LPS (144). This divergence of findings might be explained by the different timepoints, cell types or stimuli used. We chose to examine the acute metabolic changes of alveolar macrophages responding to NTMs in real time, as we believe this best represents the important early events of infection. Additionally, the divergence might be explained by the different stimuli: Alatshan et al. used LPS whereas we infected AMs with live *M. chimaera* or *M. abscessus*. In fact, while our group has previously investigated the real time metabolic responses of human macrophages to irradiated mycobacteria, there are no published reports of the *real time* metabolic responses of macrophages infected with *live* mycobacteria (369). Our metabolic findings using live mycobacteria recapitulate those of other researchers (and ours in chapter 3) using irradiated mycobacteria (369) – DMSO

treated alveolar macrophages that were infected with NTMs raised their ECAR by approximately 2 hours. The increase in glycolysis of macrophages responding to Mtb has been shown to be important in bacillary killing (38). However, we observed that while ATRA prevented the glycolytic response of AMs infected with *M. chimaera*, it also improved their ability to kill *M. chimaera*. This suggests that other effects of ATRA, such as effects on cholesterol or cytokines, are more determinative here. Future research could compare the role of glycolysis in NTM versus Mtb-infected macrophages.

Both NTMs and Mtb can form biofilms. However, NTMs can survive in water sources in the environment, infect prosthetic material more readily than Mtb and can permanently colonise airways despite treatment, unlike Mtb. These observations hint at divergent biofilm behaviours and capabilities between Mtb and NTM species, which could affect ATRA's efficacy *in vivo* – HDTs which augment macrophage functions are likely less effective for bacilli that are not within macrophages (51,415). We present the results of experiments using a cellular infection model, which cannot address these questions. Another confounding factor that may affect the clinical efficacy of ATRA as a HDT for NTMs, and which is better studied *in vivo*, is ATRA's effects on other immune cells (116).

There are several limitations of this research. This is a cellular infection model, and so cannot account for all the interactions and complications that can be studied *in vivo*. Using a longer pre-treatment duration before infection, or measuring CFUs for longer after infection, may show that ATRA is also effective in *M. abscessus* infection.

Surprisingly, unlike Babunovic et al, we did not show an effect of ATRA on total intracellular cholesterol in infected macrophages (138). The redistribution of cholesterol between different cellular compartments is probably more relevant to cholesterol fuel source restriction and should be further examined. We interpreted ECAR as a correlate of glycolysis, however more specific metabolic assays could be done to ensure that the changes in ECAR we observed are reflective of glycolysis. Non glycolytic acidification can also contribute to ECAR and occurs due to production of CO₂ via the TCA cycle. The CO₂ produced is hydrated to carbonic acid which can reduce the extracellular pH. Lactate secretion closely mirrors glycolytic ECAR (402) and future studies could include an end point lactate measurement to confirm an increase in glycolytic flux. In addition, Agilent's Glycolytic Rate Assay, which uses sequential introduction of rotenone/antimycin A to

inhibit OXPHOS and 2-DG to inhibit glycolysis, could be used to evaluate the relative contributions of both pathways to ECAR. Finally, we did not examine the alterations of macrophage gene expression that may underlie ATRA's metabolic effects – mechanistic insight into *how* ATRA alters macrophage metabolic responses would be desirable. Although more work will be needed to determine how ATRA boosts macrophage microbicidal activity against *M. chimaera*, the contrast between its effects on *M. abscessus* and *M. chimaera* provides some clues. ATRA inhibited the metabolic shift induced by both NTM species suggesting that glycolysis may not be a factor. In contrast, IL-10 levels were decreased by ATRA only in *M. chimaera*-infected macrophages suggesting that reduced IL-10 may play a role in this process. CFU experiments examining the effect of ATRA plus glycolysis inhibitors and ATRA plus IL-10 supplementation or ablation (e.g. using BMDMs from IL-10 deficient mice) would provide some mechanistic insight.

4.7 Conclusion

We present the first direct evidence of an effective HDT for an NTM infection. ATRA improved the ability of human macrophages to kill the slow growing NTM species *M. chimaera*, an effect that was macrophage dependent. ATRA altered macrophage cytokine and metabolic responses to *M. chimaera*. While similar metabolic effects were observed for the rapid grower *M. abscessus*, ATRA did not reduce *M. abscessus* CFUs. There is strong rationale to investigate ATRA as a HDT for infections due to other MAC strains, and to investigate the efficacy and effects of ATRA as a HDT *in vivo* in animal models of infection due to slow growing NTM species.

4.8 Appendix for Chapter 4

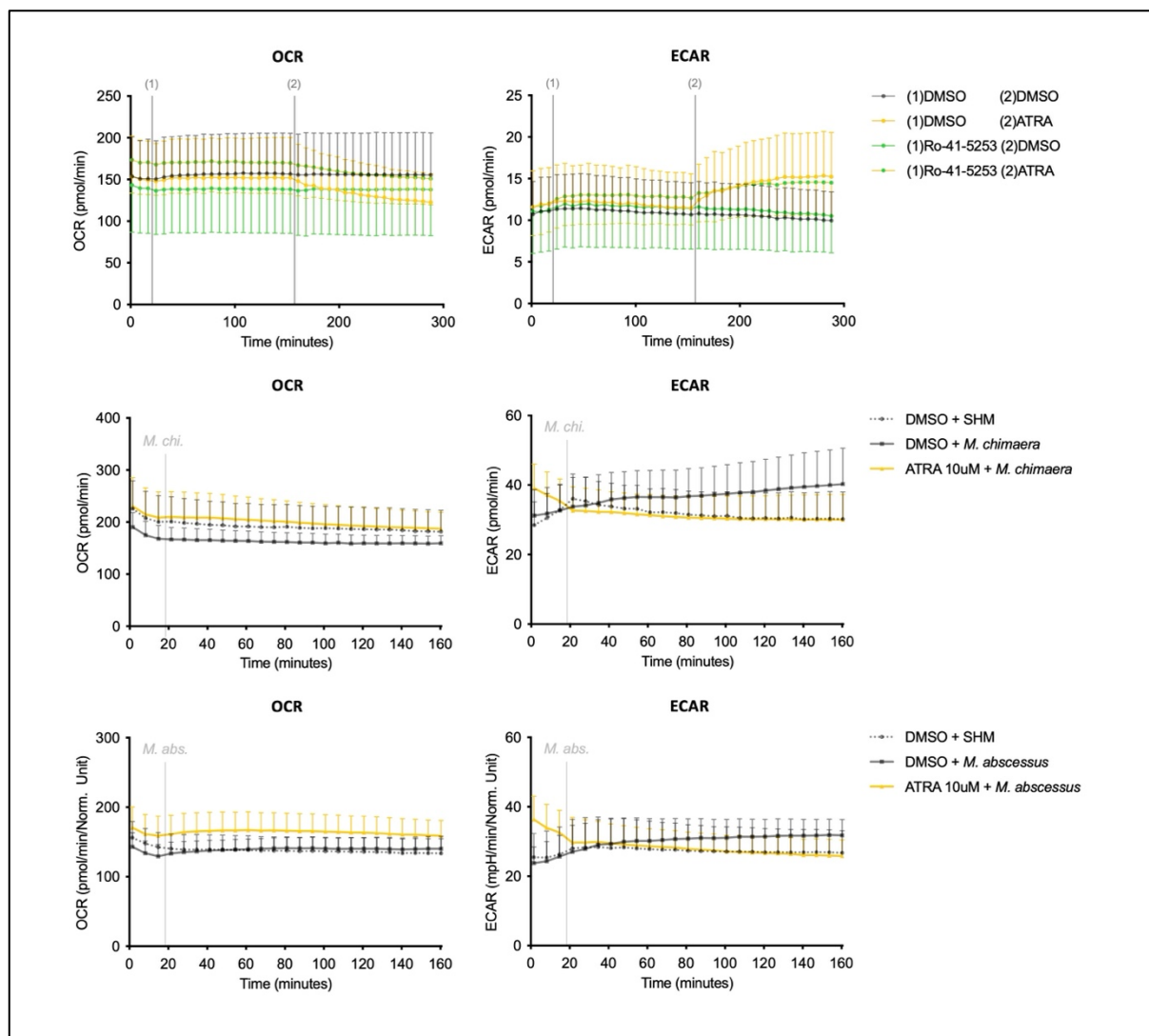


Figure 4.11: Normalised, non-baselined tracings for figures 4.7, 4.8, 4.9, 4.10.

Chapter 5

5 Human Alveolar Macrophage Responses to SARS-CoV-2

Antigens: The Influence of Age

5.1 Introduction

Advanced age is the strongest clinical risk factor for poor outcomes in COVID-19. Booth et al. collated the clinical data from over 17 million patients with COVID-19 and found that being over 75 years of age conferred an adjusted odds ratio (OR) of 1.93 for severity, and 5.82 for mortality from COVID-19. These risks were considerably higher than for other well documented risk factors such as cardiovascular disease and obesity (265). These worse outcomes in the elderly are associated with an increased risk of pulmonary hyperinflammation and ARDS (416). Another clinical variable – smoking – not only impairs pulmonary function over time, but has been associated with an increased risk of hospitalisation and death from influenza (417,418). It is perhaps surprising, therefore, that Booth et al. found active smoking to confer quite modest risks in COVID-19 (265).

AMs are the most numerous immune cells of the lung and are the resident, sentinel immunocytes of this compartment that summon other innate and adaptive cells and instruct their activation. AMs are known to be important in the host's defence against viral infections such as influenza A (17,18). It is therefore likely that AMs are the first *professional* immune cells to encounter SARS-CoV-2 following transmission. Because AMs can exert their influence so early in natural infection, before any homing monocytes, neutrophils, or T cells arrive on the scene, it is possible that they play an outsized role in determining the subsequent disease course. Indeed, RNA sequencing of the alveolar compartment in COVID-19 patients supports the centrality of AMs in the immune circuits that drive pulmonary inflammation in this condition – SARS-CoV-2 infected AMs have increased expression of cytokines and chemokines including IL-1 β , CCL2 and CXCL10 and recruit memory T cells to the lung. These recruited T cells secrete IFN γ , activating these infected AMs, with subsequent death of these SARS-CoV-2 infected AMs (293).

Despite the intriguing possibility of AMs' influence on the clinical trajectory in COVID-19, they are relatively understudied. Only three papers have been published in which *alveolar* macrophages have been infected *in vitro* with SARS-CoV-2. Dalskov et al. infected human AMs with SARS-CoV-2 and found no upregulation of type 1 or 3 interferons. Other cytokines such as IL-6 and TNF α , which are independently associated with poor outcomes in COVID-19, were not assessed by Dalskov and colleagues (283). They also observed that human AMs did not express ACE2, whereas other researchers have found ACE2 expression in a minority of AMs in healthy individuals and at higher frequencies in pulmonary fibrosis (294,296). Lv et al. infected humanised mouse AMs with SARS-CoV-2 *in vitro* and observed the secretion of IFN γ , IL-6 and TNF α . They found that M1 polarised AMs were more permissive to the replication of SARS-CoV-2 than M2 polarised AMs. Interestingly, depletion of AMs was associated with reduced lung pathology in these mice following infection *in vivo* (22). Labzin et al. infected human AMs with SARS-CoV-2 and found they did not support the replication of SARS-CoV-2. They found that ACE2 was needed to support SARS-CoV-2 replication and that ACE2 negative THP-1 macrophages and MDMs did not secrete cytokines in response to SARS-CoV-2. The secretion of cytokines from AMs, specifically, was not tested by Labzin et al. (295). Most recently, Pahari et al. found that SARS-CoV-2 persistence in human AMs was higher than in human MDMs, although AMs were not permissive to viral growth, like a Vero E6 cell line was (419). Overall, the weight of the evidence suggests that human AMs are, on the whole, not supportive of SARS-CoV-2 replication.

However, even if human AMs cannot be *productively* infected by SARS-CoV-2, they may still produce cytokines in response, which influence the disease course. There are no experimental data to indicate whether AMs secrete cytokines (other than the negative data for type 1 and 3 interferons mentioned above) following contact with the SARS-CoV-2 virus. AMs express surface pattern recognition receptors (PRRs) such as TLR2 and TLR4 which may detect SARS-CoV-2 antigens such as spike protein or envelope protein. Multiple studies have investigated these SARS-CoV-2 antigens and PRRs in THP-1 macrophages, human MDMs, HEK cells, murine BMDMs and murine RAW macrophages and have produced variable and conflicting results (300–305). Importantly, none of these studies examined the sentinel, resident immune cell of the lung – the human AM.

We aimed to understand if human AMs recognise SARS-CoV-2 antigens, and if so by what surface PRR and what cytokines are produced as a result. We also aimed to discover if the cytokine responses of human AMs to these antigens vary according to the clinical risk factors of age and smoking status – a possible mechanism underlying the different disease trajectories followed by these patient cohorts.

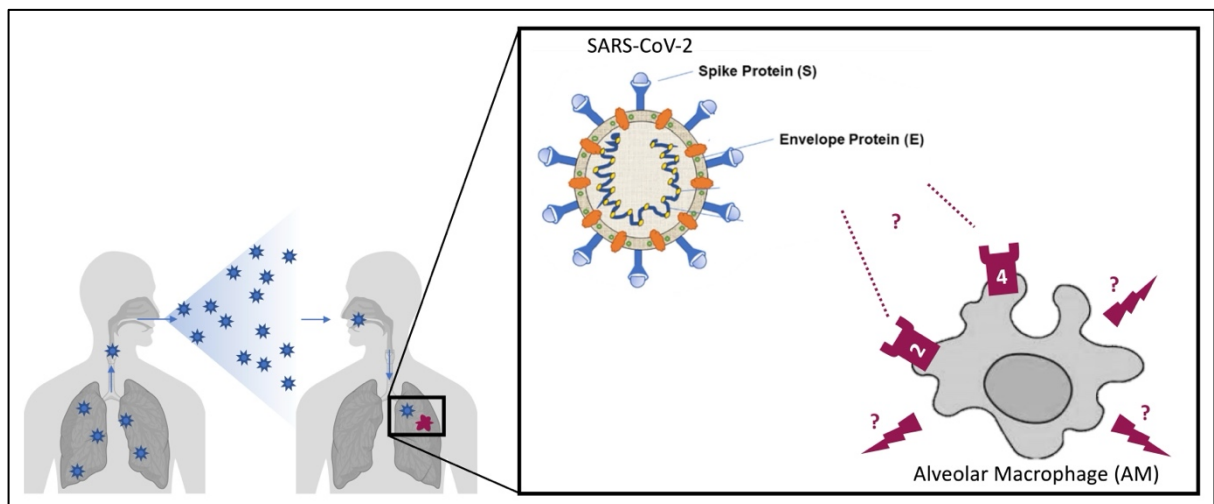


Figure 5.1: Following droplet transmission, SARS-CoV-2 enters the airways where it encounters its first professional immune cell – the human alveolar macrophage (AM). AMs may sense SARS-CoV-2 surface proteins such as spike protein or envelope protein by toll-like receptors such as TLR2 or TLR4 and respond by secreting cytokines and chemokines. These AM responses may be determinants of subsequent immune cell migration and responses that result in severe COVID-19 disease. These AM responses may also vary with age, which could partly explain the divergent clinical courses observed in young and old patients.

5.2 Aims and Hypotheses

Overall Aim

To assess if and how human AMs respond to SARS-CoV-2 antigens and the SARS-CoV-2 virus itself.

Specific Aims

Aim 1: To determine if human AMs produce cytokines in response to SARS-CoV-2 antigens, and by which TLR.

Research Question 1: Do human AMs produce cytokines in response to SARS-CoV-2 spike protein or envelope protein, and are those cytokine responses dependent on TLR2 and/or TLR4?

Hypothesis 1: SARS-CoV-2 spike protein or envelope protein will induce cytokine secretion from human AMs, and those cytokine responses will be diminished by blocking antibodies against TLR2 or TLR4.

Aim 2: To understand if the clinical risk factors of age and smoking are associated with different cytokine responses of human AMs to SARS-CoV-2 antigens.

Research Question 2: Do AMs from donors over the age of 75 years old, or from current smokers, have different cytokine responses when stimulated with SARS-CoV-2 antigens than AMs from donors under the age of 75 years old, or from never- and ex-smokers?

Hypothesis 2: AMs from donors over the age of 75 years old will have a more pro-inflammatory cytokine response when stimulated with SARS-CoV-2 antigens than those from donors under the age of 75 years old. AMs from current smokers will have a less pro-inflammatory cytokine response when stimulated with SARS-CoV-2 antigens than those from never- and ex-smokers.

Aim 3: To determine how human AMs respond to SARS-CoV-2.

Research Question 3: Can human AMs be productively infected with SARS-CoV-2, and do they mount a cytokine response to the SARS-CoV-2 virus?

Hypothesis 3: Human AMs are not productively infected with SARS-CoV-2, but do mount a cytokine response to the virus.

5.3 Results

5.3.1 SARS-CoV-2 envelope protein, but not spike protein, induces the secretion of cytokines from human AMs

We first aimed to establish whether the SARS-CoV-2 spike protein or envelope protein induced a cytokine response from human AMs. AMs were isolated from BAL fluid of patients undergoing routine bronchoscopy, as described in section 2.1.2, selected by adherence purification overnight and used within 2-4 days (*Table 5.1*). The AMs were then treated with blocking antibodies against TLR2, TLR4 or an isotype control antibody (control IgG) (all 10 µg/ml) and then stimulated with SARS-CoV-2 spike protein or envelope protein (both 1 µg/ml) or unstimulated (as per sections 2.2.3 and 2.3.6). Untreated AMs that were stimulated with LPS (100 ng/ml), or unstimulated, were used as positive and negative controls, respectively. Cytokine concentrations were measured in supernatant that was collected 24 hours following stimulation, using an MSD multiplex assay.

The two unstimulated negative control conditions (untreated and control IgG treated) had statistically equivalent concentrations of all cytokines. For all cytokines except IL-8, the background cytokine concentrations were negligible. SARS-CoV-2 spike protein did not induce the secretion of any of the tested cytokines above the levels of the negative controls. However, stimulation of human AMs with SARS-CoV-2 envelope protein resulted in a significant secretion of IFN γ , IL-12p70, IL-1 β , IL-6 and TNF α . From this we conclude that human AMs detect and mount a cytokine response to the SARS-CoV-2 envelope protein, but not the spike protein (*Figure 5.2*).

Donor #	Sex	Age (years)	Smoking status	Fig. 5.2, 5.4-5.8	Fig. 5.3	Fig. 5.10	Fig. 5.9
1	M	70.2	smoker	X			
2	M	43.7	smoker	X			
3	M	79.9	ex	X			
4	F	76.0	smoker	X			
5	F	42.1	never	X			
6	F	49.3	smoker	X			
7	F	31.9	never	X			
8	F	85.0	ex	X		X	
9	M	55.0	smoker		X		
10	F	63.6	smoker		X		
11	F	42.7	ex		X		
12	F	68.4	smoker			X	
13	F	68.5	ex			X	
14	F	64.8	smoker			X	X
15	M	76.9	ex			X	X

Table 5.1: AM Donor characteristics.

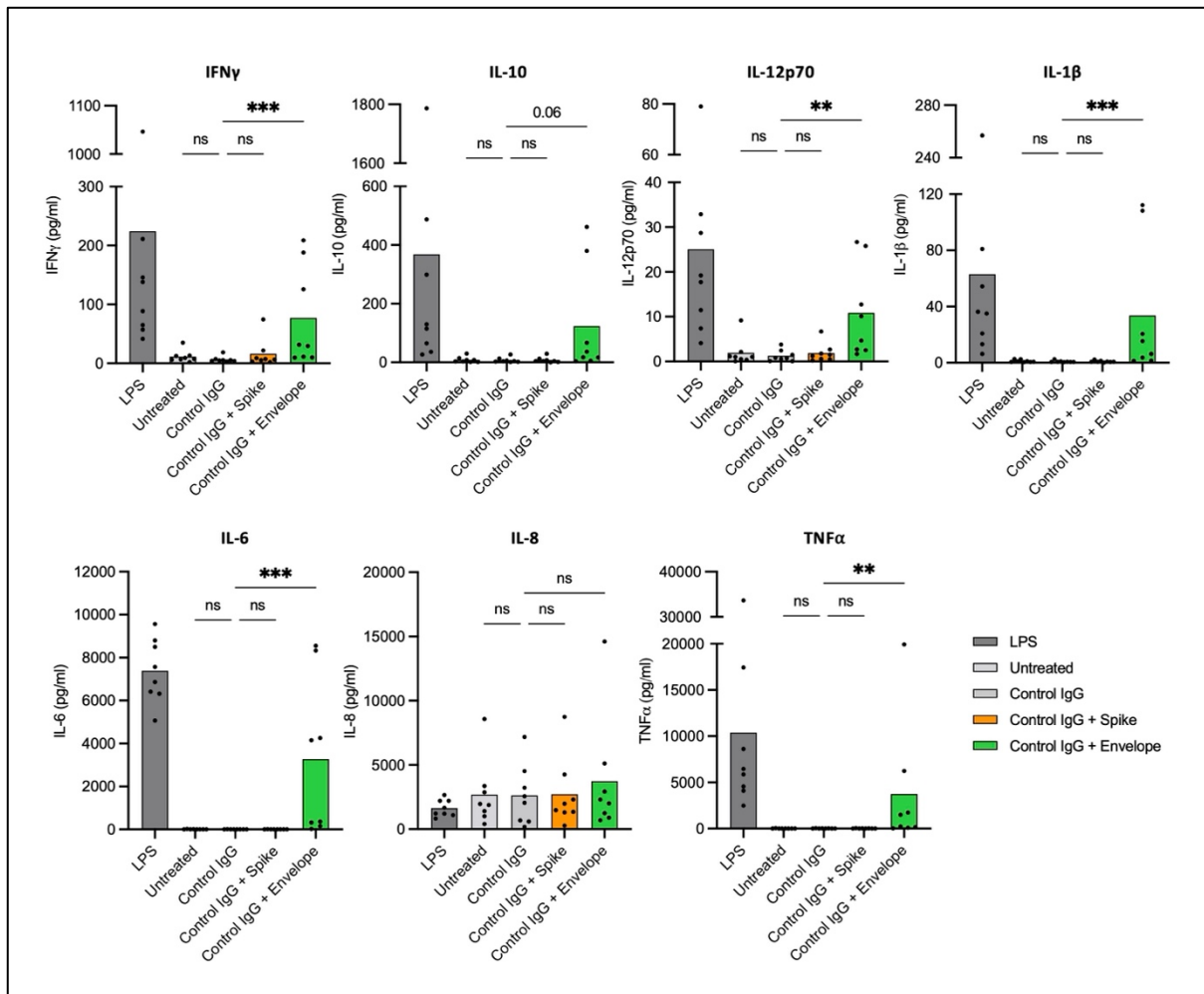


Figure 5.2: SARS-CoV-2 envelope protein, but not spike protein, induces the secretion of cytokines from human alveolar macrophages. AMs were isolated from BAL fluid of patients undergoing routine bronchoscopy, selected by adherence purification over 2-4 days (washing twice prior to experimentation). AMs were treated with blocking antibodies against TLR2, TLR4 or an isotype control antibody (control IgG) (all 10 μ g/ml) or untreated for one hour and then stimulated with SARS-CoV-2 spike protein or envelope protein (both 1 μ g/ml). Untreated AMs that were stimulated with LPS (100 ng/ml) or unstimulated and were used as positive and negative controls, respectively. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. The statistical comparison presented here is between the untreated and different control IgG treated groups (n=8). Statistical comparisons were made using Friedman test with Dunn's multiple comparisons test.

5.3.2 TLR4, and not TLR2, recognises SARS-CoV-2 envelope protein on human AMs

We next sought to understand if the SARS-CoV-2 envelope protein was detected by either the TLR2 or TLR4 receptors. We first validated the effectiveness of blocking antibodies against TLR2 and TLR4 by treating human AMs (*Table 5.1*) with these antibodies or an isotype control antibody (control IgG) for one hour, and then stimulated the cells with known ligands for TLR2 (PAM3CSK4 at 1 or 2.5µg/ml) or TLR4 (LPS at 100 ng/ml). 24 hours later, the cytokine concentrations were measured as before. LPS stimulation significantly induced IFN γ , IL-12p70, IL-6 and TNF α secretion from AMs and the blocking antibody against TLR4 resulted in a reduction of IFN γ , IL-10 and TNF α secretion. PAM3CSK4 did not *significantly* induce the secretion of any cytokine from AMs. This is consistent with prior reports that human AMs have significantly lower TLR2 expression than monocytes, and do not produce a cytokine response following stimulation with PAM3CSK4 (420). It would be desirable to validate the TLR2 blocking antibody using another cell type that expresses higher levels of TLR2 and responds to TLR2 ligands, for example MDMs (*Figure 5.3*).

We used these same blocking antibodies to investigate whether the SARS-CoV-2 envelope protein signalled through TLR2 or TLR4. Again, human AMs were treated with the blocking antibodies or an isotype control antibody for one hour before stimulation with SARS-CoV-2 envelope protein. Blocking TLR2 did not significantly reduce the secretion of any cytokine. Blocking TLR4 resulted in a significant reduction in both IL-6 and TNF α secretion from human AMs, with a non-significant trend towards decreased secretion of IFN γ , IL-10, IL12p70 and IL-1 β also evident. Interestingly, blocking TLR4 resulted in increased IL-8 secretion. We concluded from this data that SARS-CoV-2 envelope protein is recognised by TLR4 on human AMs (*Figure 5.4*).

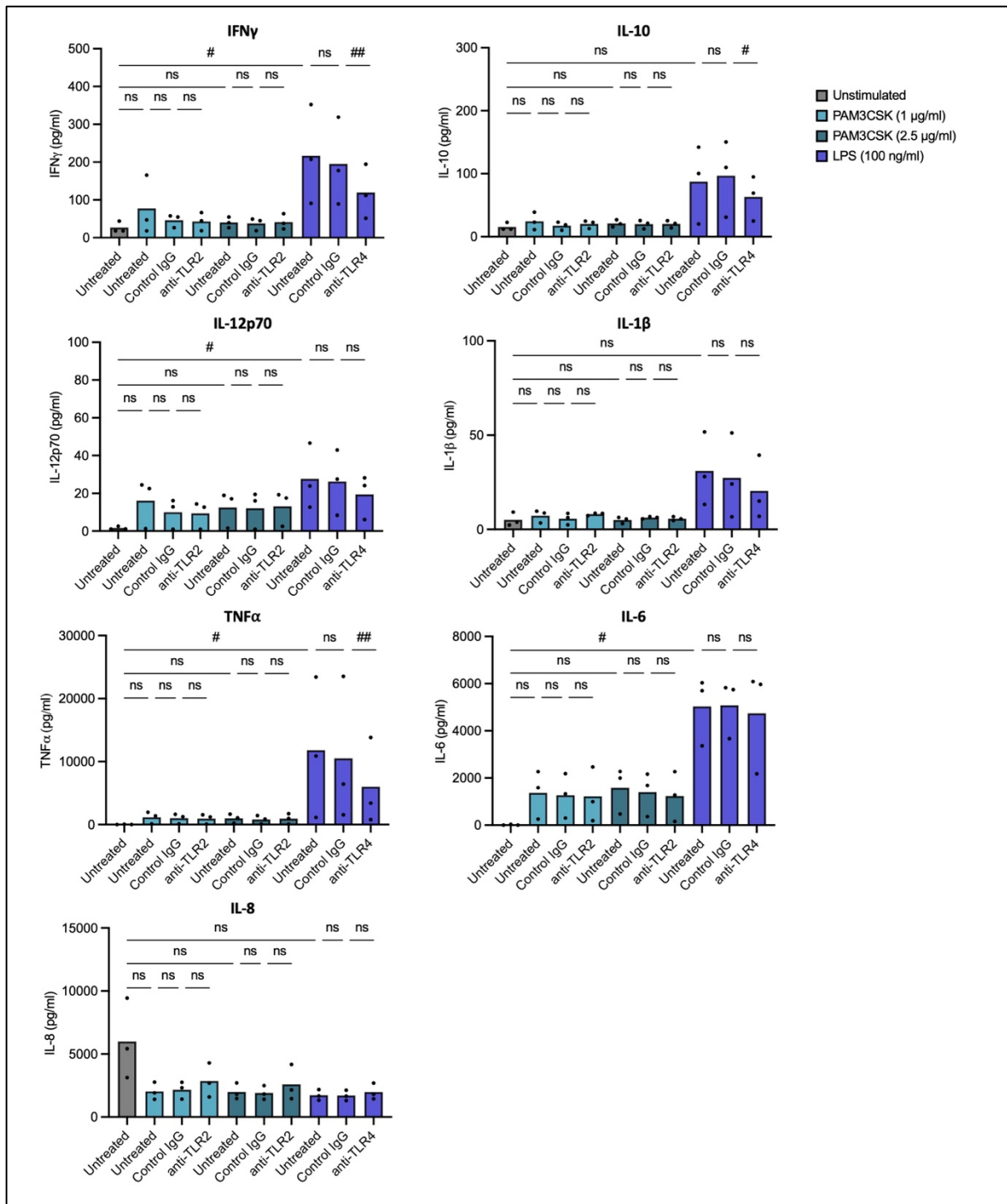


Figure 5.3: Antibodies against TLR4 diminish AM cytokine responses to LPS. AMs were isolated from BAL fluid as previously described. AMs were treated with blocking antibodies against TLR2, TLR4 or an isotype control antibody (control IgG) (all 10 μ g/ml) or untreated for one hour and then stimulated with PAM3CSK4 1 μ g/ml, PAM3CSK4 2.5 μ g/ml, LPS 100 ng/ml or unstimulated. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. (n=3). Statistical comparisons were made using one-way ANOVA or ratio paired t tests (significance denoted by #).

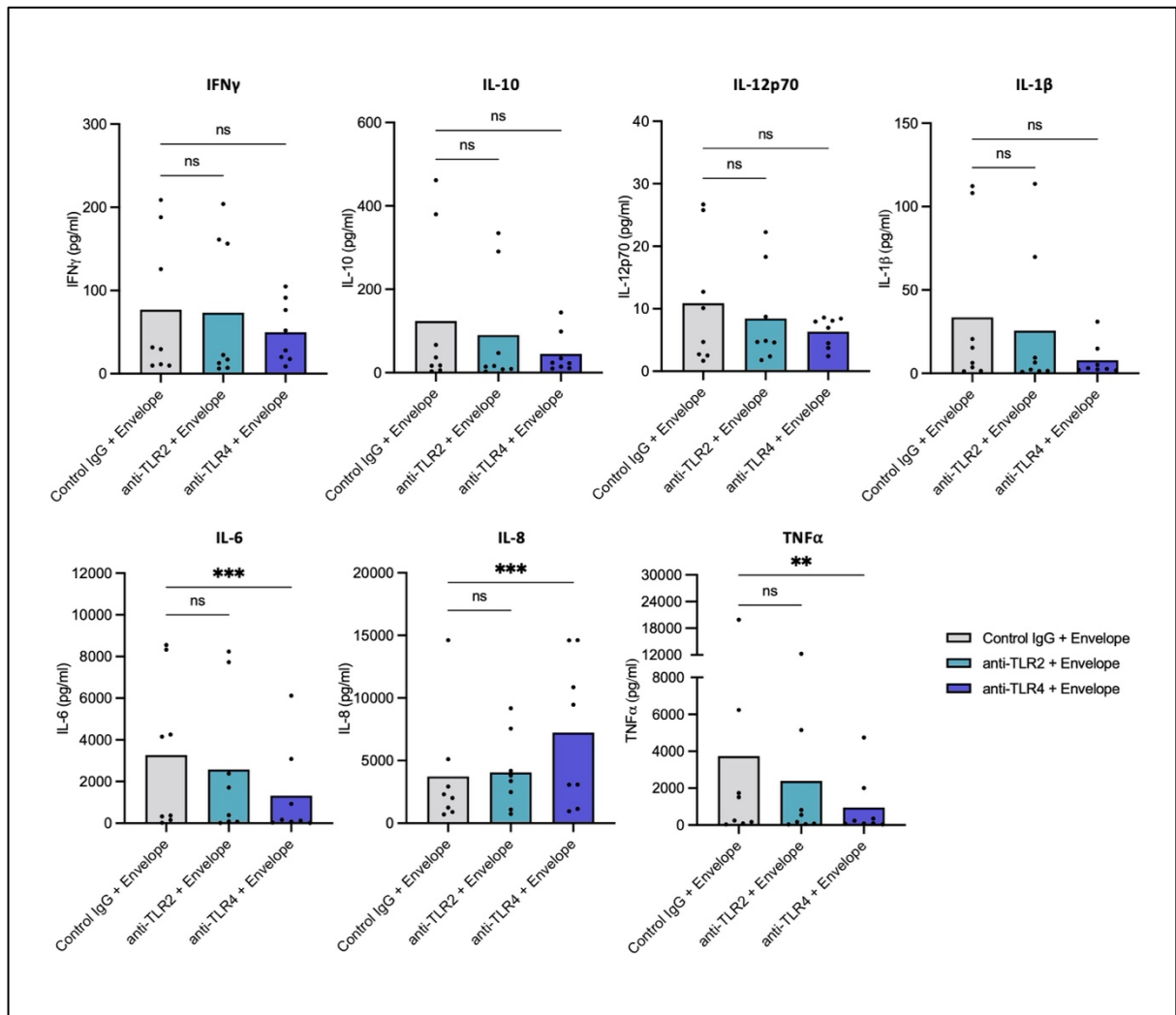


Figure 5.4: Blocking antibodies against TLR2, but not TLR4, alter the cytokine responses of human alveolar macrophages to SARS-CoV-2 envelope protein. AMs were isolated, treated and stimulated as previously described. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. The statistical comparison presented here is between the different envelope protein stimulated groups (n=8). Statistical comparisons were made using Friedman test with Dunn's multiple comparisons test.

5.3.3 AMs from older donors have higher cytokine responses to SARS-CoV-2 envelope protein than AMs from younger donors

Those older than 75 years of age are at far higher risk of severity and mortality due to COVID-19 (265). We compared the cytokine secretion of AMs responding to envelope protein from donors older or younger than 75 years. For unstimulated AMs, there was no difference between younger and older donors for any cytokine – older and younger donor's AMs were indistinguishable at rest. Stimulation of the AMs with SARS-CoV-2 envelope protein resulted in a significantly higher concentration of IFN γ , IL-10, IL-12p70, IL-1 β , IL-6 and TNF α in donors over the age of 75 compared with those under 75 years old. From this we conclude that older donors' AMs are more responsive to SARS-Cov-2 envelope protein than younger donors AMs, producing more pro-inflammatory cytokines (in addition to more IL-10) (*Figure 5.5*).

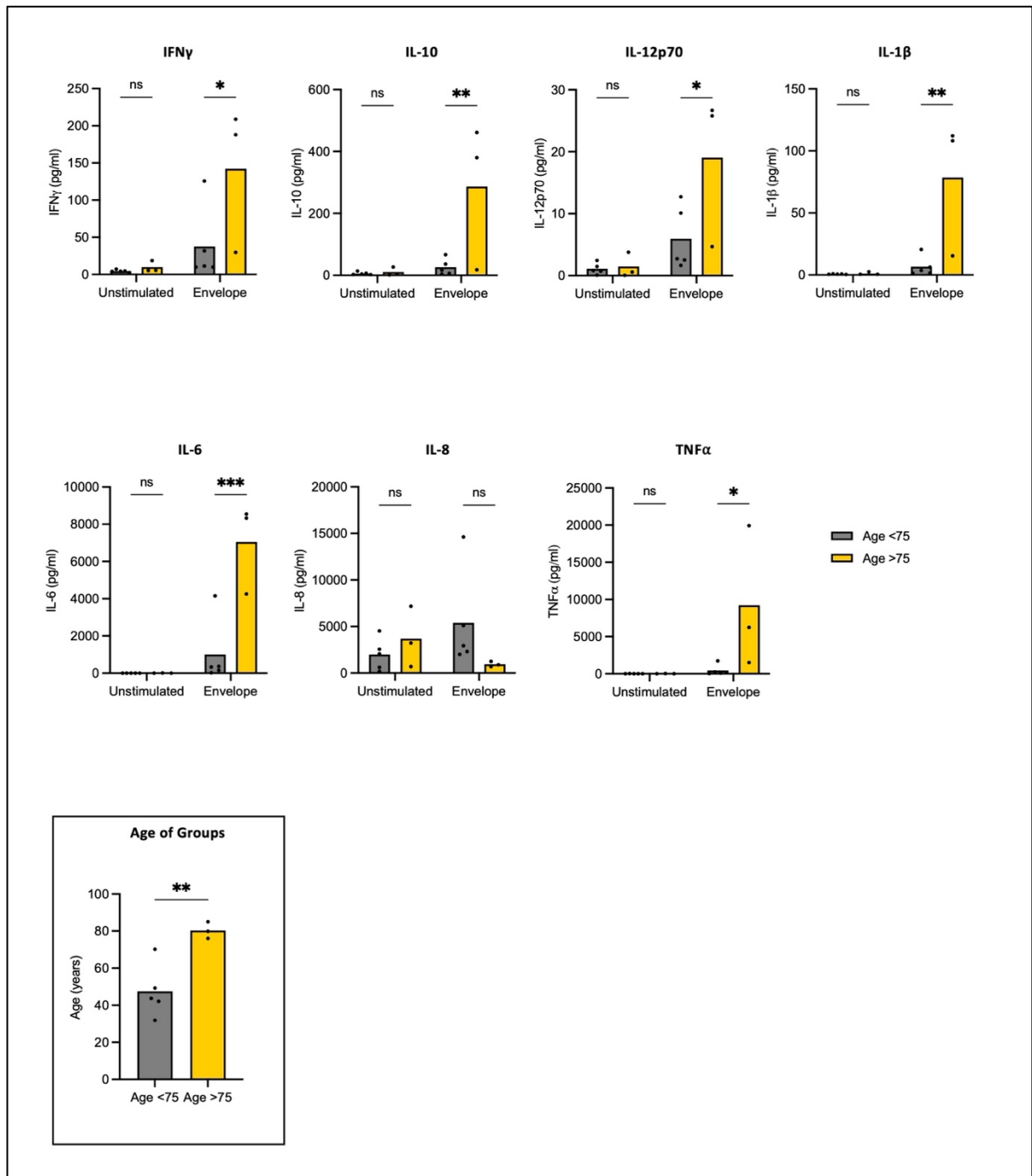


Figure 5.5: Alveolar macrophages from donors aged over 75 years old had higher cytokine responses to SARS-CoV-2 envelope protein than younger donors. AMs were isolated, treated and stimulated as previously described. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. The statistical comparison presented here is younger (n=5) and older (n=3) donor AMs treated with control IgG then stimulated with envelope protein, or unstimulated. Statistical comparisons were made using two-way ANOVA.

5.3.4 AMs from older donors do not have significantly different cytokine responses to LPS than AMs from younger donors

We also compared the cytokine secretion of AMs responding to our positive control, LPS, from donors older or younger than 75 years old. LPS stimulation did not result in a significantly different secretion of any cytokines between younger and older donors. IL-6 in particular, which has been independently associated with COVID-19 severity and mortality, had nearly identical secretion by younger and older donors' AMs in response to LPS, whereas we observed a stark age difference following stimulation of human AMs with the envelope protein (283). This suggests that the age differences observed with envelope protein might be a specific effect rather than a general effect of AM stimulation (*Figure 5.6*).

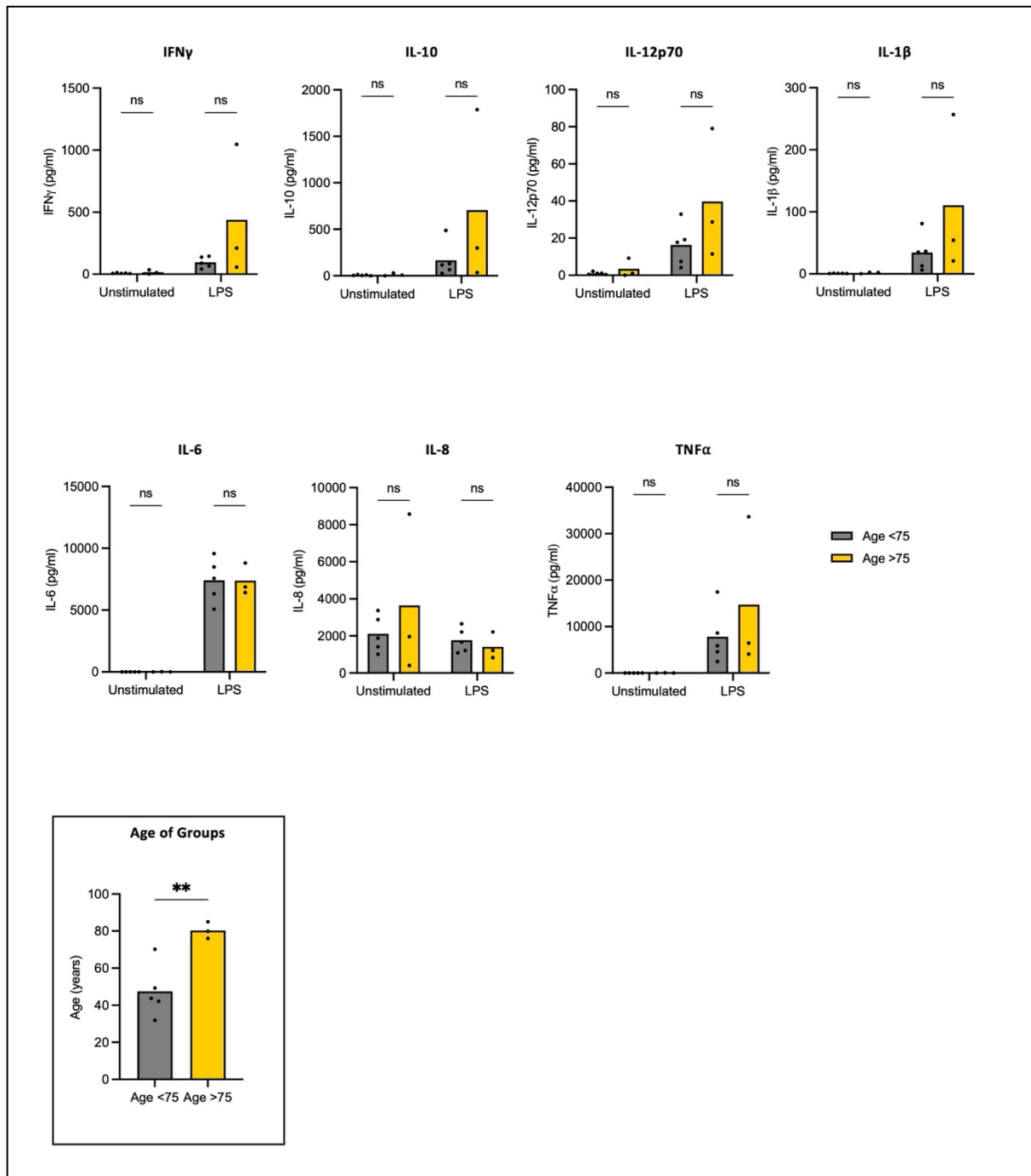


Figure 5.6: Alveolar macrophages from donors aged over 75 years old did not have higher cytokine responses to LPS in than younger donors. AMs were isolated, treated and stimulated as previously described. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. The statistical comparison presented here is younger (n=5) and older (n=3) donor untreated AMs stimulated with LPS, or unstimulated. Statistical comparisons were made using two-way ANOVA.

5.3.5 AMs from smokers had lower cytokine responses to SARS-CoV-2 envelope protein than AMs from never- or ex-smokers

Our laboratory has previously reported impaired TNF α , IFN γ and IL-1 β secretion in smokers' AMs responding to Mtb (421). We wondered whether the same impairment could be observed in smokers' AMs responding to the SARS-CoV-2 envelope protein. We found that following stimulation with envelope protein, AMs from current smokers produced significantly less IFN γ , IL-10, IL-12p70, IL-1 β , and IL-6 than AMs from never- or ex-smokers. Importantly, current smokers had the same age as never- or ex-smokers, so this result cannot be due to a younger mean age of smokers (mean age 59.7 versus 59.8 years – no statistically significant difference). This finding aligns well with previous research indicating that smoking impairs human AMs' cytokine responses in infection (*Figure 5.7*).

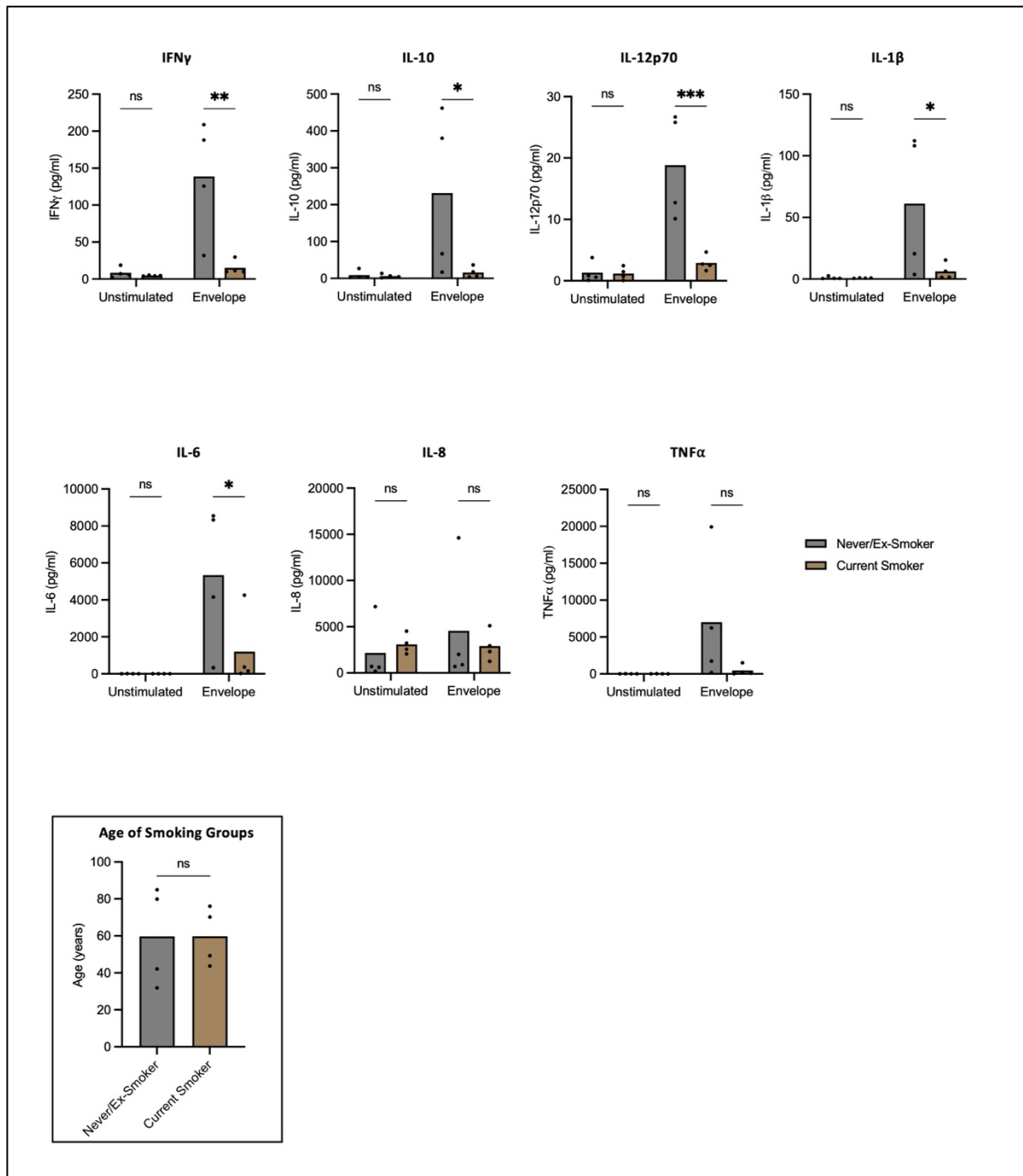


Figure 5.7: Alveolar macrophages from smokers had lower cytokine responses to SARS-CoV-2 envelope protein than non-smokers. AMs were isolated, treated and stimulated as previously described. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. The statistical comparison presented here is smokers' (n=4) and non-smokers' (n=4) AMs treated with control IgG then stimulated with envelope protein, or unstimulated. Statistical comparisons were made using two-way ANOVA.

5.3.6 AMs from smokers did not have significantly different cytokine responses to LPS than AMs from never- or ex-smokers

We investigated if smoking also impaired human AM cytokine responses to LPS. Interestingly, as was also true for age, current smoking did not significantly affect human AM cytokine responses to LPS. Other researchers have found that LPS stimulation results in diminished cytokine secretion from murine and human AMs exposed to cigarette smoke (422–424). Our findings could support the hypothesis that the effect of envelope protein we observed is somewhat specific (*Figure 5.8*).

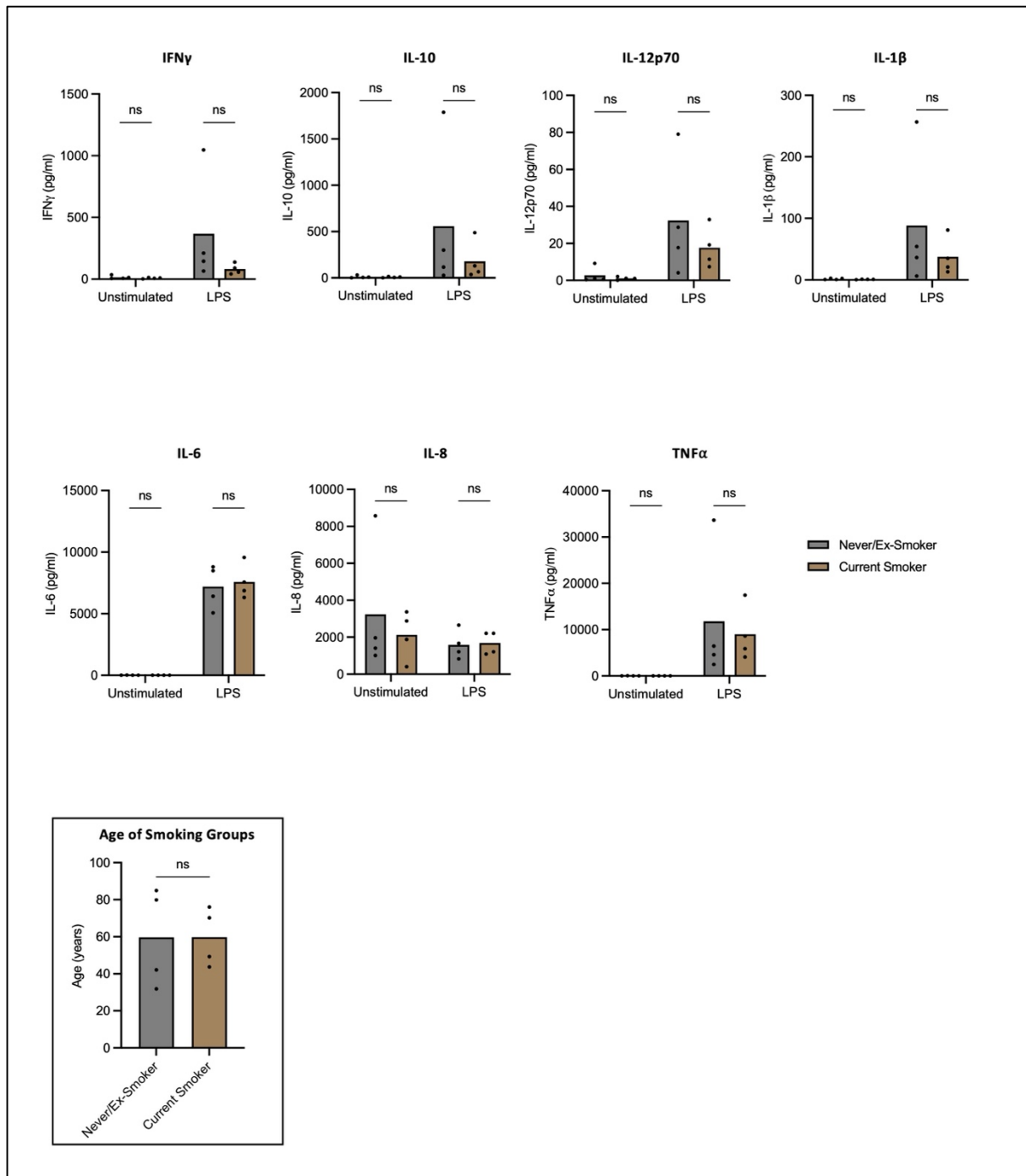


Figure 5.8: Alveolar macrophages from smokers did not have lower cytokine responses to LPS than non-smokers. AMs were isolated, treated and stimulated as previously described. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay. The statistical comparison presented here is smokers' ($n=4$) and non-smokers' ($n=4$) untreated AMs stimulated with LPS, or unstimulated. Statistical comparisons were made using two-way ANOVA.

5.3.7 SARS-CoV-2 does not induce the secretion of cytokines from human AMs

Following our observations that SARS-CoV-2 envelope protein induces a cytokine response from human AMs, we then investigated if the SARS-CoV-2 virus itself also induced a cytokine response from human AMs. Human AMs were obtained as before. Human AMs were infected with SARS-CoV-2 at an MOI of 1 or 5 virions/cell, or uninfected, by our colleagues Dr. Hossam Gewaid and Prof. Andrew Bowie. The supernatant was collected at 48 hours post-infection. We measured the cytokine concentrations as before using an MSD multiplex assay. Two donors' AMs were infected, and the supernatants assessed. *In vitro* infection of AMs with SARS-CoV-2 did not result in the increased secretion of cytokines (*Figure 5.9*).

In separate experiments with additional donors, secreted IFN-I was measured using a bioassay, as well as the mRNA expression of IFN- λ 1, IFN- λ 2/3, TNF α and IL-1 β using PCR. *In vitro* infection of AMs did not induce IFN-I secretion, or IFN- λ 1, IFN- λ 2/3 or TNF α mRNA expression. A trend towards increased IL-1 β mRNA expression with increasing SARS-CoV-2 MOI was observed, although only two donors were assessed, so this requires further investigation (*Figure 5.10*).

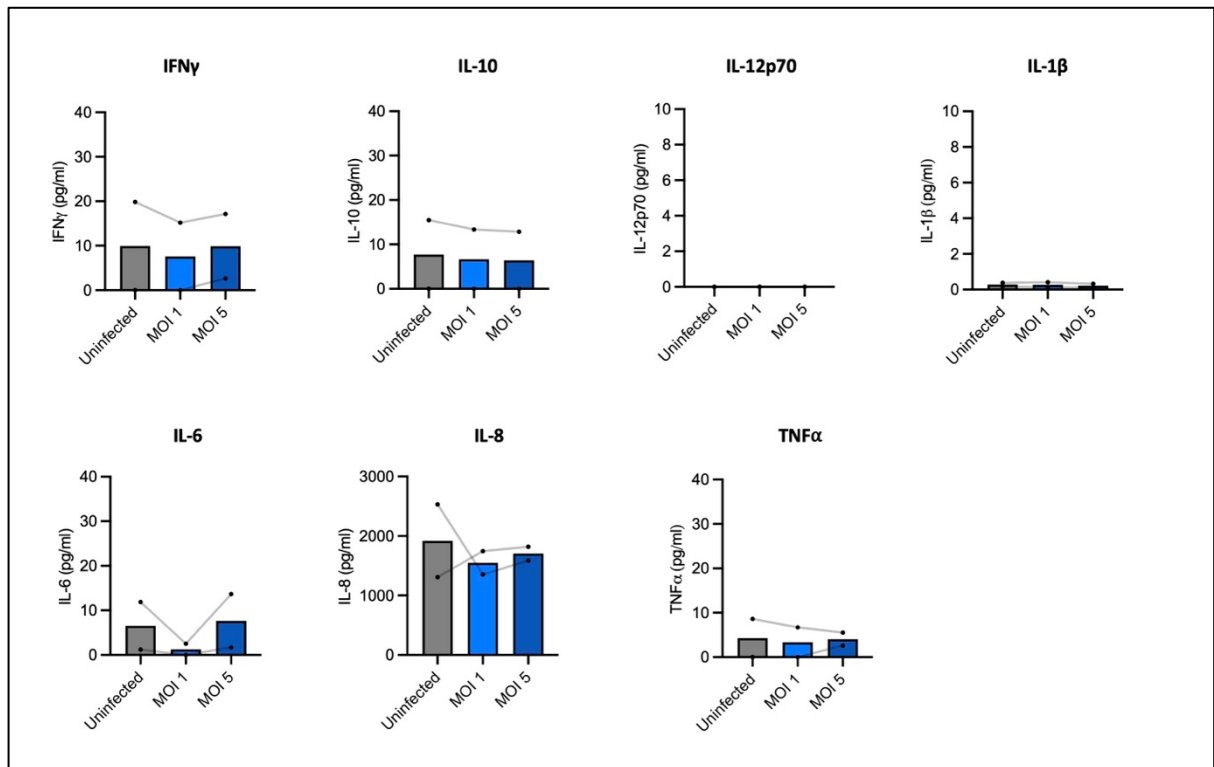


Figure 5.9: SARS-CoV-2 of AMs did not induce cytokine secretion from AMs. AMs were isolated from BAL fluid as before and infected with SARS-CoV-2 at an MOI of 1 or 5 virions/cell, or uninfected. Supernatant was collected at 24 hours post-stimulation. The concentration of cytokines was measured using an MSD multiplex assay (N=2).

5.3.8 SARS-CoV-2 does not productively infect human AMs

Finally, we sought to understand if human AMs could be productively infected with SARS-CoV-2. Human AMs were obtained as before and then infected by our colleagues Dr. Hossam Gewaid and Prof. Andrew with SARS-CoV-2 at a range of MOIs (0.01, 0.1, 1 or 5 virions/cell) or uninfected for 48 hours. A viral plaque assay was performed, and viral RNA was quantified using PCR. When compared with the positive control (Calu-3 human epithelial cell line), AMs infected with SARS-CoV-2 resulted in lower plaque-forming units, even at high MOIs. Infecting AMs also resulted in low RNA copies of SARS-coV-2 per microgram of total RNA, consistent with viral persistence rather than a productive infection of human AMs by SARS-CoV-2 (*Figure 5.10*).

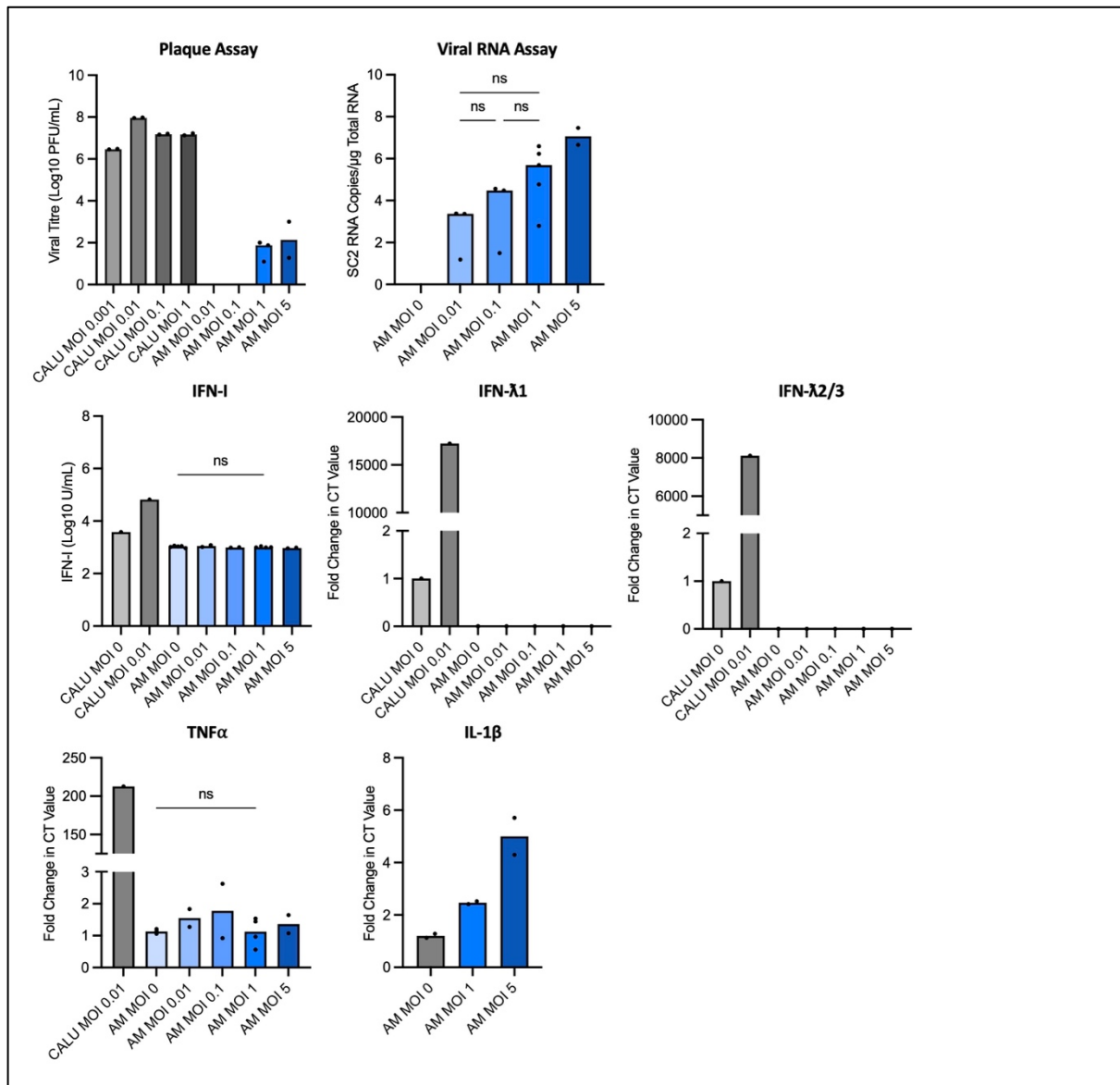


Figure 5.10: SARS-CoV-2 infection of AMs did not result in a type-1 or type-3 interferon response. AMs were isolated from BAL fluid as before and infected with SARS-CoV-2 at a range of MOIs, or uninfected. As a positive control, Calu-3 cells were infected with SARS-CoV-2 at an MOI of 0.001 to 1 virions/cell. At 48 hours post-infection a plaque assay, viral RNA quantification using PCR, measurement of mRNA expression of IFN-λ1, IFN-λ2/3, TNFα, IL-1β using PCR and an IFN-I bioassay were performed (N=1, 5, 2, 2, 4, 2). Statistical comparisons were made using one-way ANOVA.

5.5 Discussion

We found that SARS-CoV-2 envelope protein is recognised by TLR4 on human AMs, leading to the production of proinflammatory cytokines. AMs from older donors had higher cytokine secretion, and AMs from current smokers had lower cytokine secretion. This is the first report of human AMs producing cytokines in response to SARS-CoV-2 antigens, and the first to correlate those responses with clinical risk factors.

Blocking antibodies against TLR4 significantly reduced the secretion of IL-6 and TNF α from envelope protein stimulated AMs, compared with isotype control antibody treated AMs. This suggests that SARS-CoV-2 envelope protein is sensed by TLR4 on human AMs. Other viral glycoproteins like the RSV fusion protein are known to be TLR4 ligands (304). Other researchers have also found that SARS-CoV-2 envelope protein signals through TLR4 (303,305). Anand et al. observed NF- κ B signalling in THP-1 and murine RAW macrophage cell lines stimulated with envelope protein, which was abrogated by knocking out TLR4. Zheng et al. reported that envelope protein induced cytokine secretion from BMDMs that was diminished by knocking out TLR2, but did not examine TLR4 knockouts as a comparison (302). Previous research has suggested human AMs have significantly lower TLR2 expression than monocytes, and do not produce a cytokine response following stimulation with PAM3CSK4 (420).

Envelope protein induced the secretion of the cytokines IL-6, TNF α , IL-1 β , IFN γ and IL-12p70. These cytokines have wide-ranging effects on other innate and adaptive immune cells, including but not limited to neutrophil production and recruitment to the tissues (IL-6), cell death (TNF α), activation of monocytes and neutrophils (IL-1 β), activation of macrophages and other phagocytes (IFN γ) and the differentiation of T helper cells to a Th1 phenotype (IL-12p70) (425). IL-6 and TNF α are independently associated with severity and mortality in COVID-19 (283). However, the secretion of IL-6 and TNF α from monocytes infected with SARS-CoV-2 *in vitro* was entirely dependent on IL-1 β (286). IL-1 β has been detected in bronchoalveolar lavage fluid and pathological specimens from COVID-19 patients (288,289). Unlike the other cytokines we measured, IL-1 β requires inflammasome activation for its own activation and secretion. Unusually, in addition to its triggering of 'signal 1' of the NLRP3 inflammasome - TLR4 (as we have observed) - SARS-

CoV-2 envelope protein may also act as 'signal 2' through its function as a cation channel (274,280,281,291,292). That being said, LPS also induced IL-1 β secretion in our AMs, so both LPS and envelope protein appear to be acting both as signal 1 and 2 in human AMs.

We found that SARS-CoV-2 spike protein did not induce the secretion of any cytokines from human AMs. Zheng et al. also found that spike protein does not stimulate cytokine secretion from murine BMDMs or human PBMCs, in contrast to envelope protein (302). Van der Donk et al. found that spike protein did not induce cytokine production by monocyte derived dendritic cells (426). On the other hand, Khan et al. report that S1 and S2 subunits of the spike protein induced cytokine secretion by THP-1 macrophages and Theobald et al. found that spike protein induced cytokine secretion from human monocyte-derived macrophages (300,301). This variability may be the result of reagent contamination in some instances, differences in experimental design or reagents used in others, or perhaps may reflect a true variability of response according to cell type. Indeed, tissue resident AMs like those we studied, are well described to be less pro-inflammatory than monocytes or macrophages of monocyte origin (315,427).

We did not observe cytokine secretion from the two donors' AMs infected with SARS-CoV-2 that we assessed. We also did not observe IFN-I secretion or IFN- λ 1, IFN- λ 2/3 mRNA upregulation in response to SARS-CoV-2 infection. This concords well with what others have found and probably reflects a lack of viral replication in these cells (294). Interestingly, in the two donors that were assessed, SARS-CoV-2 infection was associated with an MOI-dependent increase in IL-1 β mRNA expression. AMs could be primed by non-productive infection (signal 1) inducing mRNA expression of IL-1 β , thereby increasing their propensity to secrete active IL-1 β if exposed to signal 2 in an inflammatory milieu, for example in the form of DAMPs or PAMPs. It is possible that *in vivo*, antigens such as the envelope protein accumulate during infection, activating AMs as was observed in murine studies (305).

We observed that AMs from donors over the age of 75 years old had higher cytokine secretion than younger donors. This is the first evidence of age affecting human AM cytokine responses. There are multiple possible mechanisms that could explain this difference. The first is a higher baseline NLRP3 inflammasome activation in AMs from

older donors. Stout-Delgado et al. observed higher inflammasome activation in AMs from aged mice (326). Other authors have proposed increased baseline inflammasome activation resulting from higher mitochondrial ROS or DNA as an explanation for hyperinflammation in older patients with COVID-19 (290,327). IL-1 β potentiates the release of IL-6 and TNF α from SARS-CoV-2 infected monocytes, so its release could amplify the secretion of other cytokines (286). As mentioned above, the SARS-CoV-2 envelope protein may activate the NLRP3 inflammasome through its function as a cation channel and TLR4 ligand. Therefore, in the early moments of SARS-CoV2 infection, or perhaps later in the disease course, AMs from the elderly – that have increased baseline NLRP3 activation – might respond more vigorously to SARS-CoV-2 envelope protein and secrete more activated IL-1 β , which can further amplify the production and potentiate the effects of other pro-inflammatory cytokines like IL-6 and TNF α . Perhaps this underlies some of the variance in COVID-19 severity that divides the old from the young. This variance suggests that inhibitors of the AM inflammasome might alleviate hyperinflammatory responses to SARS-CoV-2 envelope protein in the elderly.

There are other possibilities beyond the inflammasome. The second possible mechanism is differing baseline AM polarisation. Canan et al. reported that lung macrophages from aged mice had an activated phenotype (319). Boe et al. found that AMs from aged mice had higher TLR4 expression – a third possible mechanism (311). A fourth possible mechanism relates to the ontogeny of AMs. AMs are derived from the foetal yolk sac and are phenotypically distinct from MDMs. Over time, after repeated pulmonary immunological insults and infections, these native AMs can be replaced by AMs that originate from the bone marrow. These AMs of bone marrow origin make up a higher proportion of total AMs in the elderly and may have a distinct, pro-inflammatory phenotype (315). Although, other research suggests that the lung microenvironment rather than the ontogeny of AMs dictate their behaviour (316). Finally, ageing affects monocyte metabolism, which may have downstream effects on cellular responses to infection such as cytokine production, in particular IL-1 β (38,428). Unpublished data from our own laboratory, courtesy of Dr. Donal Cox, suggests that AMs from older donors may be less energetic (lower ECAR and OCR) at baseline, although this might be expected to be associated with *lower* IL-1 β secretion (38). These five mechanisms could possibly

explain the differences between older and younger donors' AMs' cytokine responses to envelope protein and could be pursued with future research. Whatever the mechanism, the augmented cytokine responses in older donors may be relevant in explaining the worse disease trajectories older patients follow.

We also observed lower cytokine responses in AMs from active smokers following stimulation with envelope protein, when compared with never or ex-smokers. Both groups had the same mean age. Our own laboratory has previously reported impaired $\text{TNF}\alpha$, $\text{IFN}\gamma$ and $\text{IL-1}\beta$ secretion in smokers' AMs, and observed diminished energetics in smokers' AMs responding to Mtb (363,421). This new finding concurs with that prior research and paints a picture of impaired pulmonary cytokine responses in smokers during SARS-CoV-2 infection. Cigarette smoking results in chronic obstructive pulmonary disease, emphysema, impaired pulmonary function and a risk of clinical decompensation with respiratory failure. As such, it is perhaps surprising that smoking has *not* been found to be a strong risk factor for severity in COVID-19, unlike in influenza (265,417,418). One speculative explanation could be that the increased risk of severe disease attributable to chronic lung disease in smokers is counterbalanced by a protective effect – the blunted cytokine response of smokers AMs responding to SARS-CoV-2 envelope protein that we observed.

It could be suggested that the effects we observed might be due to a contamination of our envelope protein reagent with LPS, rather than the envelope protein itself. Several factors cast doubt on this proposition. The first is that in our experiments, neither smoking nor age significantly affected the responses of AMs to LPS, unlike with envelope protein, suggesting a distinct effect. The second is that other researchers have used the exact same envelope protein reagent (same source and catalogue number) in zebrafish and murine experimental models and found results that are concordant with ours. Zheng et al. stimulated human PBMCs with envelope protein and observed $\text{TNF}\alpha$, $\text{IFN}\gamma$, IL-6, and $\text{IL-1}\beta$ mRNA expression upregulation, and found that envelope protein induced lung inflammation and pathology when inhaled by mice (302). Tyrkalska et al. stimulated human PBMCs and neutrophils with envelope protein and observed $\text{TNF}\alpha$ and $\text{IL-1}\beta$ mRNA expression upregulation. The authors also injected envelope protein into the hindbrain of zebrafish, and observed immune cell recruitment and cytokine upregulation,

especially IL-1 β (429). The third is that Anand et al. used a different, but similarly manufactured, envelope protein reagent that also aligned with our results. Anand et al. stimulated THP-1 and RAW macrophages with envelope protein and observed increased NF- κ B signalling, which was not affected by polymyxin B – a test for LPS contamination. Knocking out TLR4 entirely abrogated the envelope protein induced NF- κ B signalling, bolstering our findings that TLR4 blocking antibodies diminished AM responses to envelope protein. They also administered envelope protein intratracheally to mice, inducing lung inflammation and reactive oxygen species (ROS) production in murine alveolar macrophages (305). Finally, this envelope protein reagent has been tested for LPS contamination – both by the manufacturer using a LAL assay (<0.1 endotoxin units/ μ g of envelope protein) and by colleagues in our department using a V8 protease assay (data not shown) – neither of which suggested LPS contamination. If these results were due to LPS and not envelope protein, this would be the first evidence of LPS causing different responses in AMs from older donors. How Envelope protein might activate TLR4 is unclear. The hydrophobic Lipid A moiety of LPS binds to MD2 leading to dimerisation of an LPS/MD2/TLR4 complex to stimulate downstream signalling pathways. Viral proteins known to activate TLR4 include VSV G protein, RSV F protein, Dengue virus NS1, and Ebola virus glycoprotein. It is not known how these proteins interact with MD2/TLR4 but they have several things in common including being membrane-bound, glycosylated and containing hydrophilic domains (430). The coronavirus envelope proteins contain a hydrophobic transmembrane domain and a C-terminal domain containing motifs for glycosylation and palmitoylation (431). Detailed crystallographic and binding studies will be required to establish whether SARS-CoV-2 envelope protein directly interacts with the TLR4/MD2 complex.

These results may increase our understanding of COVID-19 immunopathology and why older people are at such increased risk of severe disease and poor outcomes. In addition to these insights, there are several possible practical applications of this research. One is the development of treatments: If AMs are a primary source of proinflammatory cytokines and chemokines in the lungs of COVID-19 patients, then targeting AMs directly – perhaps through inhaled therapies – may reduce COVID-19 immunopathology.

Another potential application of this research relates to prevention and vaccine research: We have shown that the envelope protein, and not the spike protein, provokes a cytokine response from human AMs, and is perhaps therefore partly responsible for downstream immune cell recruitment and immunopathology. It follows that antibodies (therapeutic or vaccine induced) targeting the envelope protein might diminish this cytokine response. An advantage of targeting the envelope protein in COVID-19 vaccine development is that it is highly genetically conserved, more stable than the spike protein (335). Antibodies against the SARS-CoV-2 envelope protein can develop in response to natural infection (432). Despite this, research exploring the potential of envelope protein as a candidate antigen for COVID-19 vaccines has been limited to computational analyses of envelope protein epitopes, and no clinical trials have been run (328,330,433–435). There is only one published, small laboratory study of an envelope protein-containing vaccine. The researchers vaccinated mice with an experimental DNA based vaccine containing the envelope protein antigen. This envelope protein vaccine did not affect SARS-CoV-2 viral loads, which could be expected given that spike protein is responsible for cell entry. However, the envelope protein vaccine induced T and B cell immunity and prevented lung pathology following an inhalational challenge with SARS-CoV-2. This reduction in pathology by an envelope protein vaccine strengthens the case that our (and other researchers) use of recombinant envelope proteins is relevant to natural infection (436). The inclusion of the envelope protein as an antigen in COVID-19 vaccines, alongside the spike protein, may improve their efficacy in preventing lung pathology in the elderly.

Many vaccine adjuvants such as aluminium salts act through NLRP3 inflammasome activation (437). As previously discussed, the SARS-CoV-2 envelope protein forms cation channels in the host endoplasmic reticulum membrane, that result in NLRP3 inflammasome activation (274,280,281,291,292). Therefore, pairing the envelope protein with spike protein in COVID-19 vaccines may not only induce adaptive T cell responses (or perhaps anti-envelope protein antibodies that could block its binding with TLR4), but also act as an adjuvant.

There are several limitations with this research. There are inherent difficulties in collecting BAL samples. This scarcity of AM donors combined with inter-donor variability and low cell counts manifest in several ways in this research. It is possible that our

experiments are underpowered, and that the addition of donors would reveal that the effects of LPS are also dependent on age or smoking status. We could not stratify the results of the live SARS-CoV-2 infection experiments by age or smoking status. The degree to which experiments using SARS-CoV-2 antigens are relevant to natural infection is difficult to gauge. However, support for the relevance of envelope protein in SARS-CoV-2 pathogenesis comes from the findings that envelope protein alone causes lung inflammation in mice when inhaled and that an experimental envelope protein vaccine reduced lung pathology in mice (305,436). *In vivo* models of SARS-CoV-2 infection would also be better placed to examine the downstream consequences of AM responses to envelope protein on immune cell recruitment as well as examining these responses in aged mice. A feasible and informative extension of our research, that we plan on pursuing next, is to assess if anti-envelope antibodies are effective at diminishing the cytokine responses of human AMs *in vitro*.

5.6 Conclusion

In the earliest phase of SARS-CoV-2 infection, AMs of older adults might produce significantly more proinflammatory cytokines following the recognition of SARS-CoV-2 envelope protein by the receptor TLR4. This effect may partly explain the worse clinical trajectories followed by these older patients. The inclusion of envelope protein as an antigen in COVID-19 vaccines alongside spike protein may improve their efficacy in reducing pathology in older patients.

Chapter 6

6 Discussion

In this thesis, we present our research investigating early immune responses to emerging and enduring respiratory pathogens. Those pathogens include *Mycobacterium tuberculosis*, nontuberculous mycobacteria such as *M. chimaera* and *M. abscessus*, and SARS-CoV-2. Here we summarise those findings, discuss their relevance to the research and clinical domains, and make suggestions for how this research could be built upon in the future.

6.1 Relevance of Chapter 3 to the Research and Clinical Domains

In chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*), we aimed to examine the relationships between endogenous vitamin A pathways and macrophage responses to Mtb infection. First, we aimed to understand if human macrophages expressed vitamin A oxidation enzymes and ATRA carrier proteins and to determine the effect of Mtb infection their expression. Secondly, we aimed to determine the effects of *endogenously* produced ATRA on macrophage responses to Mtb infection. These were important questions to investigate because they are relevant to understanding why people with VAD are at higher risk of active TB, why previous trials of retinol as an adjunct to TB therapy have failed and how ATRA may be an effective HDT for TB.

Our results in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) are novel and push the boundaries of scientific knowledge in several respects. We are the first to demonstrate the expression of the vitamin A oxidation enzymes DHRS3, DHRS9, RDH10, ALDH1a1, ALDH1a2, ALDH1a3 and ALDH3b1 and the ATRA carrier protein FABP5 in human MDMs and AMs. We are the first to report that the macrophage expression of these vitamin A enzymes and ATRA carrier proteins is reduced by Mtb infection and LPS stimulation. We are the first to find that GM-CSF and IL-4 increase vitamin A oxidation enzymes in human MDMs. We are the first to show that the

signalling of endogenously produced ATRA is important in human macrophage metabolic responses to Mtb and their ability to kill Mtb.

Our findings in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) also support those of other scientists. While we found that vitamin A oxidation enzymes have reduced expression in human MDMs and AMs as a result of Mtb infection or LPS stimulation, others have found reduced expression of ALDH1a2 in caseous granulomatous tissue from TB patients and in lung homogenates in a rabbit model of TB infection (350). Our findings pinpoint the defect to the macrophage and suggest a more general phenomenon that can be caused by other PAMPs. Other researchers reported that ALDH1a2 expression in murine or human dendritic cells could be increased by IL-4 or GM-CSF, and we suggest the same occurs in the macrophage (350,359).

In chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*), none of our results directly contradict those of other researchers. However, a systematic review of *retinol* as an adjunct to TB therapy concludes that ‘there is no evidence that (*vitamin A*) supplementation in doses up to three times the daily recommended intake has a beneficial effect on tuberculosis treatment outcomes, or nutritional recovery’ (157). We found that the production of ATRA from retinol, and the delivery of ATRA to its nuclear receptors may be significantly impaired in macrophages infected with Mtb. Our findings therefore suggest that vitamin A should not yet be ruled out and support the investigation of exogenous ATRA (not retinol), as a HDT in TB infection.

Our findings in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) could be developed and support future research in several ways. *In vitro* studies could attempt to measure the concentration of ATRA being produced by uninfected and Mtb infected macrophages, although this is technically difficult. Another approach that would also present challenges would be the measurement of the activation of ATRA’s receptor. While we did not see a significant change in the RNA expression of glycolytic enzymes, the protein expression of these enzymes could also be measured. We found that blocking ATRA’s receptor resulted in diminished macrophage

glycolytic responses to Mtb and was also associated with decreased killing of Mtb. Boosting glycolysis might restore Mtb killing and could be explored. *In vivo* models could compare the cellular energetic responses of AMs and BMDMs from vitamin A deficient and sufficient animals during infection with Mtb. The expression of vitamin A oxidation enzymes and ATRA carrier proteins could also be assessed in AMs *in vivo* using an animal infection model of Mtb infection, or potentially even in TB patients.

Chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) has two broad clinical implications. The first concerns vitamin A deficiency. Many researchers have found epidemiological associations between vitamin A deficiency and active TB. Here we have identified multiple specific defects at the level of the Mtb infected macrophage that result from a state of low signalling of endogenously produced ATRA. Those defects include impaired glycolytic responses and impaired mycobacterial killing. If present *in vivo*, these macrophage defects may explain why those with vitamin A deficiency are at such high risk of active TB, and why even those who are classified as vitamin A sufficient have a risk of active TB that is inversely proportional to their serum vitamin A levels (205,206). We believe that clinical trials should be considered, examining the efficacy of ATRA as a prophylactic agent against active TB in household contacts of TB cases, and in patients in high prevalence settings starting antiretroviral therapy for HIV with VAD (especially considering that diagnostics for LTBI are known to be less sensitive in HIV patients (403,404)). Indeed, given that lower retinol levels are associated with active TB even in those who are 'sufficient', trials should not limit participation based on standard 'deficiency' cut-offs. Outcome measures in such trials could include both the incidence of active TB as well as the rate of IGRA conversion, as ATRA may increase the likelihood of early clearance by innate immune cells like macrophages. The second clinical implication relates to the use of vitamin A as a TB treatment adjunct. Our research supports the consideration of ATRA, not retinol, as an adjunct to TB therapy. We showed that in Mtb infected macrophages, at the very site where it is most needed, the activation and transport of vitamin A to its nuclear receptors is impaired. It is therefore unsurprising that retinol was ineffective in clinical trials to date – ATRA should not have these shortcomings. Multiple animal studies of ATRA in TB infection report promising results (*Table 1.1*). Initial pilot studies might be conducted in patients with XDR TB in whom

antimycobacterial options have run out. In addition to assessing sputum clearance, treatment success and mortality as outcomes, such trials could include radiological or histological measures of pulmonary inflammation and pathology as these were ameliorated by ATRA in animal studies. In summary, ATRA is a promising candidate for a host-directed prophylactic agent in high-risk groups and as a HDT adjunct to TB therapy.

6.2 Relevance of Chapter 4 to the Research and Clinical Domains

In chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) we aimed to investigate ATRA as a potential HDT for NTMs. First, we aimed to determine if ATRA is an effective HDT against NTMs *in vitro*. Secondly, we aimed to determine ATRA's mechanisms of action as a HDT against NTMs. These topics were worth researching because ATRA has shown great promise *in vitro* and *in vivo* as an effective HDT for Mtb. If ATRA is also effective in NTM infections, it could represent an important development in the treatment of NTM infections – perhaps reducing treatment durations or relapse.

Chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) contributes to the understanding of NTM infections on a few fronts. At the time of writing (May 2023), our work is the first direct evidence of an effective HDT for an NTM infection. Other researchers have investigated mesenchymal stromal stem cells as an innovative therapy for MAC. However, this therapy was directly toxic to MAC and is therefore a pathogen-directed, host-derived therapy (438). We found that ATRA killed *M. chimaera* in a host (macrophage) dependent manner, confirming it is a *true* HDT. We measured macrophage metabolism during a real-time infection with live *M. chimaera* and *M. abscessus* and show that both NTMs induce glycolysis in AMs but did not affect oxidative phosphorylation. There are no published reports to date of *real time* cellular metabolic responses during an infection with *live* mycobacteria (including Mtb). Our results with real-time metabolic analysis using live mycobacteria generally concord with those of previous researchers using irradiated mycobacteria, and are a useful addition to our understanding of these early metabolic events in infection (368,369). In addition to

being the first analysis of these real-time metabolic responses with *live* mycobacteria, ours is also the first investigation of metabolic responses to *M. chimaera* or *M. abscessus* at any timepoint. Our laboratory has previously shown that macrophage glycolytic responses to Mtb were needed for them to kill Mtb and it is plausible that the same could hold true for NTMs (38). Furthermore, in chapter 3 (*Endogenous Macrophage ATRA Production and Mycobacterium tuberculosis*) we observed that the inhibitor Ro 41-5253 reduced glycolytic responses to Mtb and increased CFUs. While this further strengthens the link between glycolytic responses and bacillary killing, the association we found needs to be further investigated before conclusions about causality can be drawn. While one previously published paper reports that ATRA affected the metabolic responses of MDMs to LPS stimulation, our data is the first to document the metabolic effects of ATRA on AMs, and the first describing ATRA's effects on cellular metabolism during an infection with a live pathogen (144). In finding that ATRA was not an effective HDT for *M. abscessus*, our research highlights that not all NTMs are created equal and that newly discovered HDTs that are effective for one species are likely ineffective for others – perhaps especially so for rapid growers.

Our chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) results bolster prior findings of other scientists. We saw that human macrophages rapidly switch to glycolytic metabolism following infection with live mycobacteria, which validates the findings of those who have used irradiated mycobacteria. Predecessors who investigated ATRA as a HDT for Mtb found that ATRA reduced IL-10 in Mtb infected THP-1 macrophages. We found the same result with *M. chimaera*. On a broader level, this research adds to the growing body of evidence supporting the use of ATRA as a HDT for mycobacterial infections.

Our chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) results failed to reproduce findings of other researchers in some areas. Despite observing a HDT effect in *M. chimaera* infected macrophages, we did not see a change in total intracellular cholesterol concentration with ATRA treatment. ATRA has been found by multiple researchers to increase the transcription of multiple cholesterol transporters and this is thought to be an important mechanism of action for ATRA (132,138,139). Wheelwright et al. found that ATRA reduces the intracellular cholesterol

concentration of MDMs at 3 days (132). The timepoint we studied was 24 hours post-ATRA treatment, so longer timepoints would be worth investigating. Babunovic et al. found that ATRA reduces the intracellular cholesterol concentration of MDMs at 24 hours, although they used MDMs differentiated from monocytes in the presence of GM-CSF (138). GM-CSF polarises macrophages to an M1 phenotype, so it is possible that cholesterol restriction plays a greater role in M1 polarised macrophages and that other mechanisms of action, like IL-10 reduction, effects on cell energetics or phagolysosome maturation are more dominant in our model. We could not measure the intracellular redistribution of cholesterol caused by ATRA, so cholesterol restriction may still be part of its mechanism of action in our model. Other research led us to expect ATRA to increase the glycolytic response of macrophages to NTMs (144). However, we instead saw a blunting of the glycolytic response in AMs. This divergence may be due to the different stimuli (LPS versus live NTMs), cell types (MDMs versus human AMs), ATRA concentrations (1 versus 10 μ M) or time points used (6 versus 2.4 hours). The same researchers found that ATRA increased IL-1 β and IL-6 secretion in LPS stimulated MDMs, whereas we did not see a significant increase in the secretion of these cytokines from THP-1 macrophages infected with NTMs.

Future research could build upon our chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) findings in several directions. *In vitro* experiments could investigate the intracellular compartmentalisation and redistribution of cholesterol in NTM infected macrophages treated with ATRA and could examine the expression of the cholesterol transporters ABCA1, ABCG1 and NPC2. ATRA's effect on autophagy and phagolysosome maturation during NTM infections, and on the expression of glycolytic genes during NTM infection could also be investigated. Various clinical strains of *M. chimaera* could be compared. A longer pre-treatment of macrophages with ATRA prior to infection with *M. abscessus*, or a longer duration of infection before CFU measurement, may reveal that ATRA is also effective for rapid growers. *In vivo*, ATRA could be examined as a HDT in a murine model of NTM infection, as it was for Mtb (134). If the same holds true for NTM infections as for Mtb, we should expect ATRA to reduce NTM burden, reduce pathology, increase CD4 and CD8 T cells, reduce MDSCs and increase TNF α in these mouse studies (Table 1.1). Short of undertaking clinical trials with

ATRA in NTM patients, one could get relevant and informative human data using an *ex vivo* approach: Isotretinoin is an isomer of ATRA and is, like ATRA, an effective HDT in Mtb infected macrophages. Isotretinoin is commonly prescribed for acne vulgaris (143). Profiling the responses of PBMCs and magnetically sorted immune cells to infection with Mtb and NTMs, from patients prior to and after starting isotretinoin treatment, may validate the findings from mouse studies of ATRA. The immune responses of IGRA-negative patients prescribed isotretinoin could be profiled in skin biopsies before and after a tuberculin skin test (439).

From a clinical perspective, an effective HDT could address multiple challenges at once. An effective HDT might allow for the reduction in the long durations of treatment, with relatively toxic antimycobacterials, currently recommended for NTM infections. An effective HDT might reduce the high rates of relapse if given after antimycobacterial therapy or prevent the onset of disease if given to culture positive patients without a diagnosis of NTM *disease*. An effective HDT like ATRA should be effective regardless of the pattern of antimycobacterial resistance and should itself have a high genetic barrier to resistance as ATRA targets the host, and simultaneously affects multiple independent, fundamental conditions for mycobacterial replication – for example cholesterol fuel source availability and phagolysosome maturation.

Regarding implementation, both ATRA and 13-*cis*-RA (isotretinoin) are FDA approved drugs in common clinical use and are administered orally (ATRA and 13-*cis* retinoic acid) or intravenously (ATRA). The feasibility of pulmonary delivery of ATRA has been proven by our laboratory (1,134). Pulmonary drug delivery directly to the site of infection leads to higher concentrations and less systematic toxicity than oral or parenteral delivery (440,441). While no inhaled TB treatment has made it to the market yet, the FDA recently approved nebulised amikacin liposome inhalation suspension for *Mycobacterium avium* complex (MAC) (442). A phase I clinical trial of capreomycin dry powder formulation for drug resistant TB demonstrated safety in healthy adult volunteers (443). A study examining the delivery of rifampicin via inhalation in pigs found a 7-9 fold increase in the concentration of rifampicin in the lungs compared to delivery via other routes (444). Delivering ATRA by inhalation may increase its potential as a HDT for pulmonary infections.

ATRA may also hold promise for infections other than those caused by mycobacteria. ATRA was investigated as a HDT for the opportunistic fungal pathogen *Pneumocystis jiroveci* in immunosuppressed mice and rats. The rodents were commenced on ATRA orally 3 weeks post-inoculation with *Pneumocystis*. It was observed that ATRA treatment could cure pneumocystis pneumonia and it greatly reduced lung inflammation but that a prolonged course of therapy was required. Combining ATRA with primaquine was as effective as the more toxic first-line therapy, co-trimoxazole (445). Another animal study found that intraperitoneal ATRA pre-treatment greatly improved the survival of BALB/c mice when challenged with an intravenous infection of the intracellular gram-positive rod, *Listeria monocytogenes*. By day 5 post-infection, none of the control mice but 66.6% of the ATRA-treated mice survived. It was found that ATRA-treated mice had decreased circulating pro-inflammatory cytokines and reduced visceral burdens of bacilli (446). However, two clinical trials undertaken in the 1990s of oral *retinol* as an adjunctive therapy for pneumonia in paediatric patients did not observe a clinical benefit to retinol treatment (447,448).

In addition to ATRA's established efficacy in the treatment of acute promyelocytic leukaemia and 13-*cis*-RA's established efficacy in the treatment of acne vulgaris, ATRA has been trialled as a treatment in several non-infectious diseases. These include emphysema (449,450), solid tumours (451), pancreatic cancer (452), breast cancer (453), renal cell cancer (454) and prostate cancer (455). These studies have established ATRA's pharmacokinetics and pharmacodynamics properties, as well as its toxicities in a range of patients. While ATRA is generally well tolerated, the adverse effects most frequently observed are headache, xeroderma, hypertriglyceridemia and cough. RA is also teratogenic and contraindicated in pregnancy, limiting its potential use as a HDT in this population (456). Overall, ATRA and 13-*cis*-RA have been extremely well characterised in humans through decades of clinical experience and clinical trials – its implementation is feasible. *In vitro* and *in vivo* experiments support their further investigation with human clinical trials for TB, and for NTM infection if our chapter 4 (*All trans Retinoic Acid as a Host-Directed Therapy for Nontuberculous Mycobacteria*) results are supported in murine models of MAC infection.

6.3 Relevance of Chapter 5 to the Research and Clinical Domains

In chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) we aimed to assess if and how human AMs respond to SARS-CoV-2 antigens and the SARS-CoV-2 virus itself. First, we aimed to determine if human AMs produce cytokines in response to SARS-CoV-2 antigens, and by which TLR. Secondly, we aimed to understand if the clinical risk factors of age and smoking are associated with different cytokine responses of human AMs to SARS-CoV-2 antigens. We believe this research was necessary to address several fundamental knowledge gaps about human AM responses to this pathogen, and to identify AM responses that distinguished older from younger donors and that might be targetable with future therapies.

Our findings in chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) advance the research field in several ways. We provide the first evidence of human AMs producing cytokines in response to SARS-CoV-2 antigens and correlate those cytokine responses with clinical risk factors. Ours is the first evidence of age affecting cytokine responses of *human* AMs. In fact, while others have reported age differences with human monocytes, this is the first evidence of age affecting the cytokine responses of human *macrophages* of any kind, to any stimulus (322–325).

Our chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) findings also support discoveries by other researchers. We found that SARS-CoV-2 envelope protein is a TLR4 ligand that provokes an inflammatory response from macrophages, which aligns well with what Anand et al. observed (305). Smoking diminished the cytokine responses of human AMs to envelope protein, reconfirming an effect described by others, including results from our own laboratory (363,421).

There are certain areas where our results do not directly concord with prior research. Other researchers have found that SARS-CoV-2 spike protein induces a cytokine response from human macrophages, although many other studies did not (300–302,304). None of these studies examined alveolar macrophages. Other researchers found that SARS-CoV-2 envelope protein is a ligand for *TLR2*. Zheng et al. found that murine BMDMs' cytokine

response to envelope protein is diminished by knocking out TLR2, but TLR4 was not tested (302). Planès et al. studied HEK cell lines rather than macrophages and observed a reduction in cytokine production with TLR4 antibodies and a TLR4 antagonist, although blocking TLR2 had a stronger effect in these TLR2 over-expressing cells (303). We also saw a minor reduction in cytokine secretion by blocking TLR2, although this was not significant. Notably, Juarez et al. found that human AMs have significantly lower TLR2 expression than monocytes, and do not produce a cytokine response following stimulation with PAM3CSK4 (420). It could be possible that, like BCG lipomannan, SARS-CoV-2 envelope protein is a ligand for both TLR2 and TLR4, or that the cell type is determinative of response (457). Labzin et al. found that human AMs do not express ACE2 and that ACE2 expression was required for THP-1 macrophages to mount a cytokine response to SARS-CoV-2. They did not examine cytokine secretion by AMs specifically. (295). Our research fills in this knowledge gap. While we did not observe the secretion of cytokines following SARS-CoV-2 infection of human AMs, we did observe IL-1 β mRNA upregulation in the two donors tested, and therefore may respond to subsequent stimulation with higher IL-1 β release.

Future research could build upon our chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) findings in several directions. *In vitro* research could explore the molecular mechanisms underlying SARS-CoV-2 envelope protein's effects on human AMs, in particular regarding inflammasome activation. The baseline phenotypic differences between AMs from younger and older donors could be explored, to understand why they respond differently to envelope protein. Other stimuli or infections (by Mtb or NTMs, for example) could be studied in AMs from older adults to understand if the effects we observed with envelope protein are generalisable. The effect of ageing on other cell types could be explored. As a proof of concept for an envelope protein-containing vaccine or envelope protein-targeting therapeutic antibodies, antibodies directed at the envelope protein could be administered *in vitro* to see if its effects on alveolar macrophages are diminished. *In vivo* animal studies could explore envelope protein-containing vaccines, envelope protein-targeting therapeutic antibodies, TLR4 pathway antagonists or inhaled therapies targeting the AM inflammasome to investigate if lung pathology is reduced by these interventions.

Despite extremely high COVID-19 vaccine coverage in countries such as Ireland, breakthrough infections causing severe disease and death continue to occur, almost always in the elderly or immunocompromised (264). In chapter 5 (*Human Alveolar Macrophage Responses to SARS-CoV-2 Antigens: The Influence of Age*) we uncovered a mechanism by which the innate pulmonary response to COVID-19 in the elderly differs from younger adults, in a manner that could plausibly be pathological. Future clinical research could examine the effects and clinical efficacy of envelope protein-containing vaccines, envelope protein-targeting therapeutic antibodies, or inhaled therapies targeting the AM inflammasome. These novel preventative and therapeutic applications of our research, used in conjunction with existing vaccines and therapies, might prevent clinical infections, improve clinical outcomes and reduce the suffering of those with COVID-19 infections.

6.4 Concluding remarks

This thesis began with a contrast between an ancient, creeping pandemic and a modern pandemic that moves at breakneck speed. The TB and COVID-19 pandemics may differ in timescale and pace, but both have claimed millions of human lives since I began this research three years ago. Mtb, its cousins the NTMs, and SARS-CoV-2 are also linked by their exploitation of the human lungs as a mode of transmission, as a portal of entry and as a site of disease. It is not a coincidence that these most prolific killers among the list of human pathogens take advantage of this common vulnerability. The study of pulmonary immunology in infection is therefore vital. These diverse invaders confront the same defenders in the trenches at the alveolar frontline. Employing similar research tools, studying the same cell types can therefore yield useful insights for mycobacterial, bacterial and viral infections. In this thesis, these common tools have enabled us to learn that Mtb infection inhibits macrophage vitamin A activation, that vitamin A is closely linked with macrophage metabolic responses to Mtb and NTMs, that *exogenous* ATRA is an effective host-directed therapy for a species of the *M. avium* complex, and that including SARS-CoV-2 envelope protein in COVID-19 vaccines may improve their efficacy in reducing pathology in older adults. The conclusions of this research have been arrived

at by investigating hypotheses informed by an understanding of the clinical challenges posed to patients and clinicians by TB, NTM and COVID-19 infections. It is my hope that the results of this research may one day, in turn, provide a clinical benefit to such patients.

7 References

1. Bahloul AZ, Fattah S, O'Sullivan A, Cavanagh B, MacLoughlin R, Keane J, et al. Development of Inhalable ATRA-Loaded PLGA Nanoparticles as Host-Directed Immunotherapy against Tuberculosis. *Pharmaceutics*. 2022 Aug 21;14(8):1745.
2. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OYC, Gernaey AM, et al. Detection and Molecular Characterization of 9000-Year-Old *Mycobacterium tuberculosis* from a Neolithic Settlement in the Eastern Mediterranean. *PLoS ONE*. 2008 Oct 15;3(10):e3426.
3. Gutierrez MC, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, et al. Ancient Origin and Gene Mosaicism of the Progenitor of *Mycobacterium tuberculosis*. *PLOS Pathog*. 2005 Aug 19;1(1):e5.
4. Global tuberculosis report 2022 [Internet]. [cited 2023 Feb 21]. Available from: <https://www.who.int/publications-detail-redirect/9789240061729>
5. Dahl VN, Mølhave M, Fløe A, van Ingen J, Schön T, Lillebaek T, et al. Global trends of pulmonary infections with nontuberculous mycobacteria: a systematic review. *Int J Infect Dis*. 2022 Dec 1;125:120–31.
6. Roberts DL, Rossman JS, Jarić I. Dating first cases of COVID-19. *PLOS Pathog*. 2021 Jun 24;17(6):e1009620.
7. Msemburi W, Karlinsky A, Knutson V, Aleshin-Guendel S, Chatterji S, Wakefield J. The WHO estimates of excess mortality associated with the COVID-19 pandemic. *Nature*. 2023 Jan;613(7942):130–7.
8. Barber RM, Sorensen RJD, Pigott DM, Bisignano C, Carter A, Amlag JO, et al. Estimating global, regional, and national daily and cumulative infections with SARS-CoV-2 through Nov 14, 2021: a statistical analysis. *The Lancet*. 2022 Jun 25;399(10344):2351–80.
9. Cutler DM, Summers LH. The COVID-19 Pandemic and the \$16 Trillion Virus. *JAMA*. 2020 Oct 20;324(15):1495–6.

10. da Silva Neto RM, Benjamim CJR, de Medeiros Carvalho PM, Neto MLR. Psychological effects caused by the COVID-19 pandemic in health professionals: A systematic review with meta-analysis. *Prog Neuropsychopharmacol Biol Psychiatry*. 2021 Jan 10;104:110062.
11. Global tuberculosis report 2021. [cited 2021 Dec 7]; Available from: <https://www.who.int/publications/i/item/9789240037021>
12. Kappelman J, Alçiçek MC, Kazanci N, Schultz M, Ozkul M, Sen S. First Homo erectus from Turkey and implications for migrations into temperate Eurasia. *Am J Phys Anthropol*. 2008 Jan;135(1):110–6.
13. Nicod LP. Lung defences: an overview. *Eur Respir Rev*. 2005 Dec 1;14(95):45–50.
14. Gopallawa I, Dehinwal R, Bhatia V, Gujar V, Chirmule N. A four-part guide to lung immunology: Invasion, inflammation, immunity, and intervention. *Front Immunol* [Internet]. 2023 [cited 2023 Jun 14];14. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1119564>
15. Hartl D, Tirouvanziam R, Laval J, Greene CM, Habel D, Sharma L, et al. Innate Immunity of the Lung: From Basic Mechanisms to Translational Medicine. *J Innate Immun*. 2018 Feb 13;10(5–6):487–501.
16. King PT. The pathophysiology of bronchiectasis. *Int J Chron Obstruct Pulmon Dis*. 2009 Jul 20;4:411–9.
17. Somerville L, Cardani A, Braciale TJ. Alveolar Macrophages in Influenza A Infection Guarding the Castle with Sleeping Dragons. *Infect Dis Ther*. 2020 May;1(1):10.31038/idt.2020114.
18. He W, Chen CJ, Mullarkey CE, Hamilton JR, Wong CK, Leon PE, et al. Alveolar macrophages are critical for broadly-reactive antibody-mediated protection against influenza A virus in mice. *Nat Commun*. 2017 Oct 10;8(1):846.

19. Verrall AJ, G. Netea M, Alisjahbana B, Hill PC, van Crevel R. Early clearance of *Mycobacterium tuberculosis*: a new frontier in prevention. *Immunology*. 2014;141(4):506–13.
20. Xu S, Shinohara ML. Tissue-Resident Macrophages in Fungal Infections. *Front Immunol*. 2017 Dec 12;8:1798.
21. Schmidt ME, Varga SM. The CD8 T Cell Response to Respiratory Virus Infections. *Front Immunol* [Internet]. 2018 [cited 2023 Jun 15];9. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.00678>
22. Lv J, Wang Z, Qu Y, Zhu H, Zhu Q, Tong W, et al. Distinct uptake, amplification, and release of SARS-CoV-2 by M1 and M2 alveolar macrophages. *Cell Discov*. 2021 Apr 13;7(1):24.
23. Goritzka M, Makris S, Kausar F, Durant LR, Pereira C, Kumagai Y, et al. Alveolar macrophage–derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *J Exp Med*. 2015 Apr 20;212(5):699–714.
24. Cardani A, Boulton A, Kim TS, Braciale TJ. Alveolar Macrophages Prevent Lethal Influenza Pneumonia By Inhibiting Infection Of Type-1 Alveolar Epithelial Cells. *PLOS Pathog*. 2017 Jan 13;13(1):e1006140.
25. Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, Morii E, et al. Alveolar Macrophages Are the Primary Interferon- α Producer in Pulmonary Infection with RNA Viruses. *Immunity*. 2007 Aug 24;27(2):240–52.
26. Wong CK, Smith CA, Sakamoto K, Kaminski N, Koff JL, Goldstein DR. Aging Impairs Alveolar Macrophage Phagocytosis and Increases Influenza-Induced Mortality in Mice. *J Immunol Baltim Md 1950*. 2017 Aug 1;199(3):1060–8.
27. Leemans JC, Juffermans NP, Florquin S, van Rooijen N, Vervoordeldonk MJ, Verbon A, et al. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. *J Immunol Baltim Md 1950*. 2001 Apr 1;166(7):4604–11.

28. 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 Apr 2;215(4):1135–52.
29. Huppert LA, Matthay MA, Ware LB. Pathogenesis of Acute Respiratory Distress Syndrome. *Semin Respir Crit Care Med*. 2019 Feb;40(1):31–9.
30. Global HIV & AIDS statistics — Fact sheet [Internet]. [cited 2023 Jun 19]. Available from: <https://www.unaids.org/en/resources/fact-sheet>
31. Narasimhan P, Wood J, MacIntyre CR, Mathai D. Risk Factors for Tuberculosis. *Pulm Med*. 2013 Feb 12;2013:e828939.
32. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med*. 2001 Oct 11;345(15):1098–104.
33. Roycroft E, Fitzgibbon MM, Kelly DM, Scully M, McLaughlin AM, Flanagan PR, et al. The largest prison outbreak of TB in Western Europe investigated using whole-genome sequencing. *Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis*. 2021 Jun 1;25(6):491–7.
34. Vayr F, Martin-Blondel G, Savall F, Soulat JM, Deffontaines G, Herin F. Occupational exposure to human *Mycobacterium bovis* infection: A systematic review. *PLoS Negl Trop Dis*. 2018 Jan 16;12(1):e0006208.
35. Sakamoto K. The pathology of *Mycobacterium tuberculosis* infection. *Vet Pathol*. 2012 May;49(3):423–39.
36. Cohen SB, Gern BH, Delahaye JL, Adams KN, Plumlee CR, Winkler J, et al. Alveolar macrophages provide an early *Mycobacterium tuberculosis* niche and initiate dissemination. *Cell Host Microbe*. 2018 Sep 12;24(3):439–446.e4.
37. O'Neill LAJ, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol*. 2016 Sep;16(9):553–65.

38. Le G, Fj S, Em PM, D T, Sm O, Mp O, et al. Cutting Edge: Mycobacterium tuberculosis Induces Aerobic Glycolysis in Human Alveolar Macrophages That Is Required for Control of Intracellular Bacillary Replication. *J Immunol Baltim Md 1950* [Internet]. 2016 Mar 15 [cited 2022 Jan 4];196(6). Available from: <https://pubmed.ncbi.nlm.nih.gov/26873991/>
39. Sheedy FJ, Divangahi M. Targeting immunometabolism in host defence against Mycobacterium tuberculosis. *Immunology*. 2021;162(2):145–59.
40. Hackett EE, Charles-Messance H, O’Leary SM, Gleeson LE, Muñoz-Wolf N, Case S, et al. Mycobacterium tuberculosis Limits Host Glycolysis and IL-1 β by Restriction of PFK-M via MicroRNA-21. *Cell Rep*. 2020 Jan 7;30(1):124-136.e4.
41. Cohen A, Mathiasen VD, Schön T, Wejse C. The global prevalence of latent tuberculosis: a systematic review and meta-analysis. *Eur Respir J*. 2019 Sep;54(3):1900655.
42. Jones BE, Young SMM, Antoniskis D, Davidson PT, Kramer F, Barnes PF. Relationship of the Manifestations of Tuberculosis to CD4 Cell Counts in Patients with Human Immunodeficiency Virus Infection. *Am Rev Respir Dis* [Internet]. 2012 Dec 17 [cited 2023 Jun 19]; Available from: <https://www.atsjournals.org/doi/epdf/10.1164/ajrccm/148.5.1292?role=tab>
43. Grant C, McHugh J, Ryan C, Scarry M, O’Regan A, Tuite H, et al. Symptoms to script: delays in tuberculosis treatment in the west of Ireland. *Ir J Med Sci* 1971 -. 2022 Feb 1;191(1):295–300.
44. Ma Y, Horsburgh CR, White LF, Jenkins HE. Quantifying TB transmission: a systematic review of reproduction number and serial interval estimates for tuberculosis. *Epidemiol Infect*. 2018 Sep;146(12):1478–94.
45. Jassal MS, Bishai WR. Epidemiology and Challenges to the Elimination of Global Tuberculosis. *Clin Infect Dis*. 2010 May 15;50(Supplement_3):S156–64.

46. Nahid P, Dorman SE, Alipanah N, Barry PM, Brozek JL, Cattamanchi A, et al. Executive Summary: Official American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America Clinical Practice Guidelines: Treatment of Drug-Susceptible Tuberculosis. *Clin Infect Dis*. 2016 Oct;63(7):853–67.
47. Mlambo CK, Warren RM, Poswa X, Victor TC, Duse AG, Marais E. Genotypic diversity of extensively drug-resistant tuberculosis (XDR-TB) in South Africa. *Int J Tuberc Lung Dis*. 2008 Jan 1;12(1):99–104.
48. Mallick JS, Nair P, Abbew ET, Van Deun A, Decroo T. Acquired bedaquiline resistance during the treatment of drug-resistant tuberculosis: a systematic review. *JAC-Antimicrob Resist*. 2022 Apr 1;4(2):dlac029.
49. Nahid P, Mase SR, Migliori GB, Sotgiu G, Bothamley GH, Brozek JL, et al. Treatment of drug-resistant tuberculosis an official ATS/CDC/ERS/IDSA clinical practice guideline. *Am J Respir Crit Care Med*. 2019 Nov 15;200(10):E93–142.
50. O'Connor G, Gleeson LE, Fagan-Murphy A, Cryan SA, O'Sullivan MP, Keane J. Sharpening nature's tools for efficient tuberculosis control: A review of the potential role and development of host-directed therapies and strategies for targeted respiratory delivery. Vol. 102, *Advanced Drug Delivery Reviews*. Elsevier B.V.; 2016. p. 33–54.
51. Johansen MD, Herrmann JL, Kremer L. Non-tuberculous mycobacteria and the rise of *Mycobacterium abscessus*. *Nat Rev Microbiol*. 2020 Jul;18(7):392–407.
52. Bachmann NL, Salamzade R, Manson AL, Whittington R, Sintchenko V, Earl AM, et al. Key Transitions in the Evolution of Rapid and Slow Growing Mycobacteria Identified by Comparative Genomics. *Front Microbiol*. 2020 Jan 21;10:3019.
53. Hoefsloot W, Ingen J van, Andrejak C, Ängeby K, Bauriaud R, Bemer P, et al. The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. *Eur Respir J*. 2013 Dec 1;42(6):1604–13.

54. Winthrop KL, Marras TK, Adjemian J, Zhang H, Wang P, Zhang Q. Incidence and Prevalence of Nontuberculous Mycobacterial Lung Disease in a Large U.S. Managed Care Health Plan, 2008–2015. *Ann Am Thorac Soc*. 2020 Feb;17(2):178–85.
55. CDC. TB Incidence in the U.S. | Data & Statistics | TB | CDC [Internet]. 2022 [cited 2023 Feb 19]. Available from: <https://www.cdc.gov/tb/statistics/tbcases.htm>
56. Roux AL, Viljoen A, Bah A, Simeone R, Bernut A, Laencina L, et al. The distinct fate of smooth and rough *Mycobacterium abscessus* variants inside macrophages. *Open Biol*. 2016 Nov 30;6(11):160185.
57. Riccardi N, Monticelli J, Antonello RM, Luzzati R, Gabrielli M, Ferrarese M, et al. *Mycobacterium chimaera* infections: An update. *J Infect Chemother*. 2020 Mar 1;26(3):199–205.
58. Daley CL, Iaccarino JM, Lange C, Cambau E, Wallace RJ Jr, Andrejak C, et al. Treatment of Nontuberculous Mycobacterial Pulmonary Disease: An Official ATS/ERS/ESCMID/IDSA Clinical Practice Guideline. *Clin Infect Dis*. 2020 Aug 14;71(4):e1–36.
59. Sommerstein R, Hasse B, Marschall J, Sax H, Genoni M, Schlegel M, et al. Global Health Estimate of Invasive *Mycobacterium chimaera* Infections Associated with Heater–Cooler Devices in Cardiac Surgery. *Emerg Infect Dis*. 2018 Mar;24(3):576–8.
60. Reed C, von Reyn CF, Chamblee S, Ellerbrock TV, Johnson JW, Marsh BJ, et al. Environmental Risk Factors for Infection with *Mycobacterium avium* Complex. *Am J Epidemiol*. 2006 Jul 1;164(1):32–40.
61. De Groote MA, Pace NR, Fulton K, Falkinham JO. Relationships between *Mycobacterium* Isolates from Patients with Pulmonary Mycobacterial Infection and Potting Soils. *Appl Environ Microbiol*. 2006 Dec;72(12):7602–6.
62. Bryant JM, Grogono DM, Rodriguez-Rincon D, Everall I, Brown KP, Moreno P, et al. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science*. 2016 Nov 11;354(6313):751–7.

63. Hashish E, Merwad A, Elgaml S, Amer A, Kamal H, Elsadek A, et al. *Mycobacterium marinum* infection in fish and man: epidemiology, pathophysiology and management; a review. *Vet Q*. 2018 Jan 1;38(1):35–46.
64. Furuya EY, Paez A, Srinivasan A, Cooksey R, Augenbraun M, Baron M, et al. Outbreak of *Mycobacterium abscessus* wound infections among “lipotourists” from the United States who underwent abdominoplasty in the Dominican Republic. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2008 Apr 15;46(8):1181–8.
65. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An Official ATS/IDSA Statement: Diagnosis, Treatment, and Prevention of Nontuberculous Mycobacterial Diseases. *Am J Respir Crit Care Med*. 2007 Feb 15;175(4):367–416.
66. Henkle E, Winthrop KL. Nontuberculous mycobacteria infections in immunosuppressed hosts. *Clin Chest Med*. 2015 Mar;36(1):91–9.
67. Havlik JA Jr, Horsburgh CR Jr, Metchock B, Williams PP, Fann SA, Thompson SE III. Disseminated *Mycobacterium avium* Complex Infection: Clinical Identification and Epidemiologic Trends. *J Infect Dis*. 1992 Mar 1;165(3):577–80.
68. EACS Guidelines [Internet]. [cited 2023 Feb 20]. Primary Prophylaxis of OIs. Available from: <https://eacs.sanfordguide.com/ois/primary-prophylaxis-of-ois-according-to-stage-of-immunodeficiency>
69. Lake MA, Ambrose LR, Lipman MCI, Lowe DM. “Why me, why now?” Using clinical immunology and epidemiology to explain who gets nontuberculous mycobacterial infection. *BMC Med*. 2016 Mar 23;14(1):54.
70. Finch S, McDonnell MJ, Abo-Leyah H, Aliberti S, Chalmers JD. A Comprehensive Analysis of the Impact of *Pseudomonas aeruginosa* Colonization on Prognosis in Adult Bronchiectasis. *Ann Am Thorac Soc*. 2015 Nov;12(11):1602–11.
71. Chu H, Zhao L, Xiao H, Zhang Z, Zhang J, Gui T, et al. Prevalence of nontuberculous mycobacteria in patients with bronchiectasis: a meta-analysis. *Arch Med Sci AMS*. 2014 Aug 29;10(4):661–8.

72. Mirsaeidi M, Hadid W, Ericsson B, Rodgers D, Sadikot RT. Non-tuberculous mycobacterial disease is common in patients with non-cystic fibrosis bronchiectasis. *Int J Infect Dis.* 2013 Nov 1;17(11):e1000–4.
73. Oh J, Park HD, Kim SY, Koh WJ, Lee SY. Assessment of Vitamin Status in Patients with Nontuberculous Mycobacterial Pulmonary Disease: Potential Role of Vitamin A as a Risk Factor. *Nutrients.* 2019;11(2).
74. Park SC, Kang MJ, Han CH, Lee SM, Kim CJ, Lee JM, et al. Prevalence, incidence, and mortality of nontuberculous mycobacterial infection in Korea: a nationwide population-based study. *BMC Pulm Med.* 2019 Aug 1;19(1):140.
75. Marras TK, Campitelli MA, Lu H, Chung H, Brode SK, Marchand-Austin A, et al. Pulmonary Nontuberculous Mycobacteria–Associated Deaths, Ontario, Canada, 2001–2013. *Emerg Infect Dis.* 2017 Mar;23(3):468–76.
76. Andréjak C, Thomsen VØ, Johansen IS, Riis A, Benfield TL, Duhaut P, et al. Nontuberculous Pulmonary Mycobacteriosis in Denmark. *Am J Respir Crit Care Med.* 2010 Mar;181(5):514–21.
77. Qvist T, Gilljam M, Jönsson B, Taylor-Robinson D, Jensen-Fangel S, Wang M, et al. Epidemiology of nontuberculous mycobacteria among patients with cystic fibrosis in Scandinavia. *J Cyst Fibros.* 2015 Jan 1;14(1):46–52.
78. Han D, Lee KS, Koh WJ, Yi CA, Kim TS, Kwon OJ. Radiographic and CT Findings of Nontuberculous Mycobacterial Pulmonary Infection Caused by Mycobacterium abscessus. *Am J Roentgenol.* 2003 Aug;181(2):513–7.
79. Weill D. Lung transplantation: indications and contraindications. *J Thorac Dis.* 2018 Jul;10(7):4574–87.
80. Gilljam M, Scherstén H, Silverborn M, Jönsson B, Hollsing AE. Lung transplantation in patients with cystic fibrosis and Mycobacterium abscessus infection. *J Cyst Fibros.* 2010 Jul 1;9(4):272–6.

81. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat Med*. 2019 May;25(5):730–3.
82. Petrini B. *Mycobacterium abscessus*: an emerging rapid-growing potential pathogen. *APMIS*. 2006;114(5):319–28.
83. Lee MR, Sheng WH, Hung CC, Yu CJ, Lee LN, Hsueh PR. *Mycobacterium abscessus* Complex Infections in Humans. *Emerg Infect Dis*. 2015 Sep;21(9):1638–46.
84. Miskoff JA, Chaudhri M. *Mycobacterium Chimaera*: A Rare Presentation. *Cureus*. 10(6):e2750.
85. Ninh A, Weiner M, Goldberg A. Healthcare-Associated *Mycobacterium chimaera* Infection Subsequent to Heater-Cooler Device Exposure During Cardiac Surgery. *J Cardiothorac Vasc Anesth*. 2017 Oct 1;31(5):1831–5.
86. Lau D, Cooper R, Chen J, Sim VL, McCombe JA, Tyrrell GJ, et al. *Mycobacterium chimaera* Encephalitis Following Cardiac Surgery: A New Syndrome. *Clin Infect Dis*. 2020 Feb 3;70(4):692–5.
87. Kohler P, Kuster SP, Bloemberg G, Schulthess B, Frank M, Tanner FC, et al. Healthcare-associated prosthetic heart valve, aortic vascular graft, and disseminated *Mycobacterium chimaera* infections subsequent to open heart surgery. *Eur Heart J*. 2015 Oct 21;36(40):2745–53.
88. Sax H, Bloemberg G, Hasse B, Sommerstein R, Kohler P, Achermann Y, et al. Prolonged Outbreak of *Mycobacterium chimaera* Infection After Open-Chest Heart Surgery. *Clin Infect Dis*. 2015 Jul 1;61(1):67–75.
89. Corti M, Palmero D. *Mycobacterium avium* complex infection in HIV/AIDS patients. *Expert Rev Anti Infect Ther*. 2008 Jun 1;6(3):351–63.
90. de Melo Carvalho R, Nunes AL, Sa R, Ramos I, Valente C, Saraiva da Cunha J. *Mycobacterium chimaera* Disseminated Infection. *J Med Cases*. 2020 Feb;11(2):35–6.

91. Fukui S, Sekiya N, Takizawa Y, Morioka H, Kato H, Aono A, et al. Disseminated Mycobacterium abscessus Infection Following Septic Arthritis. *Medicine (Baltimore)*. 2015 May 29;94(21):e861.
92. EACS Guidelines [Internet]. [cited 2023 Feb 21]. Non-Tuberculous Mycobacteria (NTM). Available from: <https://eacs.sanfordguide.com/ois/non-tuberculous-mycobacteria>
93. Kasperbauer SH, Daley CL. Mycobacterium chimaera Infections Related to the Heater–Cooler Unit Outbreak: A Guide to Diagnosis and Management. *Clin Infect Dis*. 2019 Mar 19;68(7):1244–50.
94. Mason M, Gregory E, Foster K, Klatt M, Zoubek S, Eid AJ. Pharmacologic Management of Mycobacterium chimaera Infections: A Primer for Clinicians. *Open Forum Infect Dis*. 2022 Jun 15;9(7):ofac287.
95. Nick JA, Dedrick RM, Gray AL, Vladar EK, Smith BE, Freeman KG, et al. Host and pathogen response to bacteriophage engineered against Mycobacterium abscessus lung infection. *Cell*. 2022 May 26;185(11):1860-1874.e12.
96. Wu ML, Aziz DB, Dartois V, Dick T. NTM drug discovery: status, gaps and the way forward. *Drug Discov Today*. 2018 Aug 1;23(8):1502–19.
97. Zimmermann P, Ziesenitz VC, Curtis N, Ritz N. The Immunomodulatory Effects of Macrolides—A Systematic Review of the Underlying Mechanisms. *Front Immunol* [Internet]. 2018 [cited 2023 Feb 21];9. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.00302>
98. Crilly NP, Ayeh SK, Karakousis PC. The New Frontier of Host-Directed Therapies for Mycobacterium avium Complex. *Front Immunol* [Internet]. 2021 Jan 22 [cited 2023 Feb 21];11. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.623119/full>
99. Fatima S, Bhaskar A, Dwivedi VP. Repurposing Immunomodulatory Drugs to Combat Tuberculosis. *Front Immunol*. 2021 Apr 13;12.

100. Oprea TI, Bauman JE, Bologa CG, Buranda T, Chigaev A, Edwards BS, et al. Drug repurposing from an academic perspective. Vol. 8, Drug Discovery Today: Therapeutic Strategies. Elsevier Ltd; 2011. p. 61–9.
101. Batalha IL, Bernut A, Schiebler M, Ouberaï MM, Passemar C, Klapholz C, et al. Polymeric nanobiotics as a novel treatment for mycobacterial infections. *J Controlled Release*. 2019 Nov 28;314:116–24.
102. Kalombo L, Lemmer Y, Semete-Makokotlela B, Ramalapa B, Nkuna P, Booysen LLLIJ, et al. Spray-dried, nanoencapsulated, multi-drug anti-tuberculosis therapy aimed at once weekly administration for the duration of treatment. *Nanomaterials*. 2019 Aug 1;9(8).
103. Dua K, Rapalli VK, Shukla SD, Singhvi G, Shastri MD, Chellappan DK, et al. Multi-drug resistant *Mycobacterium tuberculosis* & oxidative stress complexity: Emerging need for novel drug delivery approaches. Vol. 107, *Biomedicine and Pharmacotherapy*. Elsevier Masson SAS; 2018. p. 1218–29.
104. Coleman MM, Basdeo SA, Coleman AM, Cheallaigh CN, Peral de Castro C, McLaughlin AM, et al. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial Infection. *Am J Respir Cell Mol Biol*. 2018 May 4;59(5):548–56.
105. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*. 2004 Dec 17;119(6):753–66.
106. Singhal A, Jie L, Kumar P, Hong GS, Leow MKS, Paleja B, et al. Metformin as adjunct antituberculosis therapy. *Sci Transl Med*. 2014 Nov 19;6(263):263ra159-263ra159.
107. Vashisht R, Brahmachari SK. Metformin as a potential combination therapy with existing front-line antibiotics for Tuberculosis. *J Transl Med*. 2015 Mar 7;13(1):83.
108. Parihar SP, Guler R, Khutlang R, Lang DM, Hurdal R, Mhlana MM, et al. Statin Therapy Reduces the *Mycobacterium tuberculosis* Burden in Human Macrophages and

- in Mice by Enhancing Autophagy and Phagosome Maturation. *J Infect Dis*. 2014 Mar 1;209(5):754–63.
109. Murray HW. Interferon-gamma and host antimicrobial defense: Current and future clinical applications. Vol. 97, *The American Journal of Medicine*. 1994. p. 459–67.
 110. Critchley JA, Young F, Orton L, Garner P. Corticosteroids for prevention of mortality in people with tuberculosis: A systematic review and meta-analysis. *Lancet Infect Dis*. 2013 Mar 1;13(3):223–37.
 111. Napier RJ, Rafi W, Cheruvu M, Powell KR, Zaunbrecher MA, Bornmann W, et al. Imatinib-Sensitive tyrosine kinases regulate mycobacterial pathogenesis and represent therapeutic targets against tuberculosis. *Cell Host Microbe*. 2011 Nov 17;10(5):475–85.
 112. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. *Cell*. 2013 Apr 25;153(3):521–34.
 113. Tobin DM, Roca FJ, Oh SF, McFarland R, Vickery TW, Ray JP, et al. Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. *Cell*. 2012 Feb 3;148(3):434–46.
 114. Olaru ID, Lange C, Heyckendorf J. Personalized medicine for patients with MDR-TB: Table 1. *J Antimicrob Chemother*. 2016 Apr 1;71(4):852–5.
 115. Alizadeh F, Bolhassani A, Khavari A, Bathaie SZ, Naji T, Bidgoli SA. Retinoids and their biological effects against cancer. Vol. 18, *International Immunopharmacology*. *Int Immunopharmacol*; 2014. p. 43–9.
 116. Coleman MM, Ruane D, Moran B, Dunne PJ, Keane J, Mills KHG. Alveolar macrophages contribute to respiratory tolerance by inducing FoxP3 expression in naive T cells. *Am J Respir Cell Mol Biol*. 2013 Jun 3;48(6):773–80.
 117. Greenstein RJ, Su L, Brown ST. Vitamins A & D Inhibit the Growth of Mycobacteria in Radiometric Culture. *PLOS ONE*. 2012 Apr 23;7(1):e29631.

118. Crowle AJ, Ross EJ. Inhibition by retinoic acid of multiplication of virulent tubercle bacilli in cultured human macrophages. *Infect Immun*. 1989;57(3):840–4.
119. Huen AO, Kim EJ. The Role of Systemic Retinoids in the Treatment of Cutaneous T-Cell Lymphoma. *Dermatol Clin*. 2015 Oct 1;33(4):715–29.
120. Das BC, Thapa P, Karki R, Das S, Mahapatra S, Liu TC, et al. Retinoic acid signaling pathways in development and diseases. Vol. 22, *Bioorganic and Medicinal Chemistry*. Bioorg Med Chem; 2014. p. 673–83.
121. Bushue N, Wan YJY. Retinoid pathway and cancer therapeutics. Vol. 62, *Advanced Drug Delivery Reviews*. *Adv Drug Deliv Rev*; 2010. p. 1285–98.
122. Lei M, De Thé H. Retinoids and retinoic acid receptor in cancer. *Eur J Cancer Suppl*. 2003 Sep 1;1(2):13–8.
123. Rochette-Egly C, Chambon P. F9 embryocarcinoma cells: a cell autonomous model to study the functional selectivity of RARs and RXRs in retinoid signaling. *Histol Histopathol*. 2001;16(3):909–22.
124. Chatagnon A, Veber P, Morin V, Bedo J, Triqueneaux G, Sémon M, et al. RAR/RXR binding dynamics distinguish pluripotency from differentiation associated cis-regulatory elements. *Nucleic Acids Res*. 2015 Apr 8;43(10):4833–54.
125. Rochette-Egly C, Germain P. Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). *Nucl Recept Signal*. 2009;7.
126. Bruck N, Vitoux D, Ferry C, Duong V, Bauer A, De Thé H, et al. A coordinated phosphorylation cascade initiated by p38MAPK/MSK1 directs RAR α to target promoters. *EMBO J*. 2009 Jan 7;28(1):34–47.
127. Waetzig V, Haeusgen W, Andres C, Frehse S, Reinecke K, Bruckmueller H, et al. Retinoic acid-induced survival effects in SH-SY5Y neuroblastoma cells. *J Cell Biochem*. 2019 Apr 1;120(4):5974–86.

128. Cañón E, Cosgaya JM, Scsucova S, Aranda A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. *Mol Biol Cell*. 2004 Dec 15;15(12):5583–92.
129. Pan J, Kao YL, Joshi S, Jeetendran S, DiPette D, Singh US. Activation of Rac1 by phosphatidylinositol 3-kinase in vivo: role in activation of mitogen-activated protein kinase (MAPK) pathways and retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *J Neurochem*. 2005 May;93(3):571–83.
130. Takahashi N, Breitman TR. Retinoic Acid Acylation (Retinoylation) of a Nuclear Protein in the Human Acute Myeloid Leukemia Cell Line HL60*. *J Biol Chem*. 1989;264(9):5159–63.
131. Coleman MM, Basdeo SA, Coleman AM, Cheallaigh CN, Peral de Castro C, McLaughlin AM, et al. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial Infection. *Am J Respir Cell Mol Biol*. 2018 May 4;59(5):548–56.
132. Wheelwright M, Kim EW, Inkeles MS, De Leon A, Pellegrini M, Krutzik SR, et al. All-trans retinoic acid triggered antimicrobial activity against *Mycobacterium tuberculosis* is dependent on NPC2. *J Immunol Baltim Md 1950*. 2014 Apr 19;192(5):2280–90.
133. Tallieux L, Schwartz O, Herrmann JL, Pivert E, Jackson M, Amara A, et al. DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J Exp Med*. 2003 Jan;197(1):121–7.
134. O'Connor G, Krishnan N, Fagan-Murphy A, Cassidy J, O'Leary S, Robertson BD, et al. Inhalable poly(lactic-co-glycolic acid) (PLGA) microparticles encapsulating all-trans-Retinoic acid (ATRA) as a host-directed, adjunctive treatment for *Mycobacterium tuberculosis* infection. *Eur J Pharm Biopharm*. 2019 Jan 1;134:153–65.
135. Mourik BC, Leenen PJM, De Knecht GJ, Huizinga R, Van Der Eerden BCJ, Wang J, et al. Immunotherapy added to antibiotic treatment reduces relapse of disease in a mouse model of tuberculosis. *Am J Respir Cell Mol Biol*. 2017 Feb 1;56(2):233–41.

136. Knaul JK, Jörg S, Oberbeck-Mueller D, Heinemann E, Scheuermann L, Brinkmann V, et al. Lung-residing myeloid-derived suppressors display dual functionality in murine pulmonary tuberculosis. *Am J Respir Crit Care Med*. 2014 Nov 1;190(9):1053–66.
137. Yamada H, Mizuno S, Ross AC, Sugawara I. Retinoic Acid Therapy Attenuates the Severity of Tuberculosis While Altering Lymphocyte and Macrophage Numbers and Cytokine Expression in Rats Infected with *Mycobacterium tuberculosis*. *J Nutr*. 2007 Dec 1;137(12):2696–700.
138. Babunovic GH, DeJesus MA, Bosch B, Chase MR, Barbier T, Dickey AK, et al. CRISPR Interference Reveals That All- Trans-Retinoic Acid Promotes Macrophage Control of *Mycobacterium tuberculosis* by Limiting Bacterial Access to Cholesterol and Propionyl Coenzyme A. *mBio*. 2022 Jan 18;13(1).
139. Costet P, Lalanne F, Gerbod-Giannone MC, Molina JR, Fu X, Lund EG, et al. Retinoic Acid Receptor-Mediated Induction of ABCA1 in Macrophages. *Mol Cell Biol*. 2003 Nov 1;23(21):7756–66.
140. Anand PK, Kaul D. Downregulation of TACO gene transcription restricts mycobacterial entry/survival within human macrophages. *FEMS Microbiol Lett*. 2005 Sep 1;250(1):137–44.
141. Iturralde M, Vass JK, Oria R, Brock JH. Effect of iron and retinoic acid on the control of transferrin receptor and ferritin in the human promonocytic cell line U937. *BBA - Mol Cell Res*. 1992 Feb 3;1133(3):241–6.
142. Lee JM, Jang YS, Jin BR, Kim SJ, Kim HJ, Kwon BE, et al. Retinoic acid enhances lactoferrin-induced IgA responses by increasing betaglycan expression. *Cell Mol Immunol*. 2016 Nov 1;13(6):862–70.
143. Abd-Nikfarjam B, Nassiri-Asl M, Hajiaghayi M, Naserpour Farivar T. Role of Chicoric Acid and 13-Cis Retinoic Acid in *Mycobacterium tuberculosis* Infection Control by Human U937 Macrophage. *Arch Immunol Ther Exp (Warsz)*. 2018 Oct 1;66(5):399–406.

144. Alatshan A, Kovács GE, Aladdin A, Czimmerer Z, Tar K, Benkő S. All-Trans Retinoic Acid Enhances both the Signaling for Priming and the Glycolysis for Activation of NLRP3 Inflammasome in Human Macrophage. *Cells* 2020 Vol 9 Page 1591. 2020 Jul 1;9(7):1591.
145. Estrella JL, Kan-Sutton C, Gong X, Rajagopalan M, Lewis DE, Hunter RL, et al. A Novel in vitro Human Macrophage Model to Study the Persistence of Mycobacterium tuberculosis Using Vitamin D3 and Retinoic Acid Activated THP-1 Macrophages. *Front Microbiol.* 2011 Apr 18;2(APR):67.
146. Riccomi A, Piccaro G, Christensen D, Palma C, Andersen P, Vendetti S. Parenteral Vaccination With a Tuberculosis Subunit Vaccine in Presence of Retinoic Acid Provides Early but Transient Protection to M. Tuberculosis Infection. *Front Immunol* [Internet]. 2019 [cited 2021 Apr 23];10. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.00934/full>
147. Abdullah AS, Adel AM, Hussein RM, Abdullah MAJ, Yousaf A, Mudawi D, et al. Hypercalcemia and acute pancreatitis in a male patient with acute promyelocytic leukemia and pulmonary tuberculosis. *Acta Bio Medica Atenei Parm.* 2018 Apr 23;89(Suppl 3):23–7.
148. Armijos RX, Weigel MM, Chacon R, Flores L, Campos A. Adjunctive micronutrient supplementation for pulmonary tuberculosis. *Salud Publica Mex.* 2010;52(3):185–9.
149. Lawson L, Thacher TD, Yassin MA, Onuoha NA, Usman A, Emenyonu NE, et al. Randomized controlled trial of zinc and vitamin A as co-adjuvants for the treatment of pulmonary tuberculosis. *Trop Med Int Health.* 2010 Dec 1;15(12):1481–90.
150. Karyadi E, West CE, Schultink W, Nelwan RHH, Gross R, Amin Z, et al. A double-blind, placebo-controlled study of vitamin A and zinc supplementation in persons with tuberculosis in Indonesia: effects on clinical response and nutritional status. *Am J Clin Nutr.* 2002;75(4):720–7.

151. Pakasi TA, Karyadi E, Suratih NMD, Salean M, Darmawidjaja N, Bor H, et al. Zinc and vitamin A supplementation fails to reduce sputum conversion time in severely malnourished pulmonary tuberculosis patients in Indonesia. *Nutr J*. 2010 Apr 21;9:41.
152. Visser ME, Grewal HMSS, Swart EC, Dhansay MA, Walzl G, Swanevelder S, et al. The effect of vitamin A and zinc supplementation on treatment outcomes in pulmonary tuberculosis: a randomized controlled trial. *Am J Clin Nutr*. 2011 Apr 21;93(1):93–100.
153. Hanekom WA, Potgieter S, Hughes EJ, Malan H, Kessow G, Hussey GD. Vitamin A status and therapy in childhood pulmonary tuberculosis. *J Pediatr*. 1997;131(6):925–7.
154. Semba RD. Vitamin A and immunity to viral, bacterial and protozoan infections. *Proc Nutr Soc*. 1999;58(3):719–27.
155. Wang J, Xiong K, Wang Q, Zhao S, Liu Y, Ma A. Adjunctive vitamin A and D during pulmonary tuberculosis treatment: a randomized controlled trial with a 2 × 2 factorial design. *Food Funct*. 2020;11(5):4672–81.
156. Ginawi I. Effect of Zinc and EFFECT OF ZINC AND VITAMIN A SUPPLEMENTATION ALONG WITH INTER-TUBERCULAR TREATMENT IN PULMONARY TUBERCULOSIS IN NORTH INDIAN PATIENTS | INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH. 2021.
157. Grobler L, Nagpal S, Sudarsanam TD, Sinclair D. Nutritional supplements for people being treated for active tuberculosis. *Cochrane Database Syst Rev*. 2016 Apr 23;(6).
158. Anand PK, Kaul D. Downregulation of TACO gene transcription restricts mycobacterial entry/survival within human macrophages. *FEMS Microbiol Lett*. 2005 Sep 1;250(1):137–44.
159. Wheelwright M, Kim EW, Inkeles MS, De Leon A, Pellegrini M, Krutzik SR, et al. All-trans retinoic acid triggered antimicrobial activity against *Mycobacterium tuberculosis* is dependent on NPC2. *J Immunol Baltim Md 1950*. 2014 Apr 19;192(5):2280–90.

160. Long J, Basu Roy R, Zhang YJ, Antrobus R, Du Y, Smith DL, et al. Plasma Membrane Profiling Reveals Upregulation of ABCA1 by Infected Macrophages Leading to Restriction of Mycobacterial Growth. *Front Microbiol.* 2016 Apr 23;7.
161. da Cunha MSB, Siqueira EMA, Trindade LS, Arruda SF. Vitamin A deficiency modulates iron metabolism via ineffective erythropoiesis. *J Nutr Biochem.* 2014 Apr 23;25(10):1035–44.
162. Aklamati EK, Mulenga M, Dueker SR, Buchholz BA, Pearson JM, Kafwembe E, et al. Accelerator mass spectrometry can be used to assess vitamin A metabolism quantitatively in boys in a community setting. *J Nutr.* 2010;140(9):1588–94.
163. Stephensen CB. Vitamin A, infection, and immune function. *Annu Rev Nutr.* 2001;21:167–92.
164. Alvarez JO, Salazar-Lindo E, Kohatsu J, Miranda P, Stephensen CB. Urinary excretion of retinol in children with acute diarrhea. *Am J Clin Nutr.* 1995;61(6):1273–6.
165. Stephensen CB, Alvarez JO, Kohatsu J, Hardmeier R, Kennedy Jr JI, Gammon Jr RB. Vitamin A is excreted in the urine during acute infection. *Am J Clin Nutr.* 1994 Apr 9;60(3):388–92.
166. Keicho N, Matsushita I, Tanaka T, Shimbo T, Hang NT Le, Sakurada S, et al. Circulating levels of adiponectin, leptin, fetuin-A and retinol-binding protein in patients with tuberculosis: markers of metabolism and inflammation. *PLOS ONE.* 2012 Apr 9;7(6):e38703.
167. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *Lancet Lond Engl.* 2006;368(9540):1012–21.
168. Larson LM, Namaste SM, Williams AM, Engle-Stone R, Addo OY, Suchdev PS, et al. Adjusting retinol-binding protein concentrations for inflammation: Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. *Am J Clin Nutr.* 2017;106(Suppl 1):390S-401S.

169. Louw JA, Werbeck A, Louw ME, Kotze TJ, Cooper R, Labadarios D. Blood vitamin concentrations during the acute-phase response. *Crit Care Med*. 1992;20(7):934–41.
170. Fex G, Larsson K, Andersson A, Berggren-Söderlund M. Low serum concentration of all-trans and 13-cis retinoic acids in patients treated with phenytoin, carbamazepine and valproate. *Arch Toxicol*. 1995 Apr 26;69(8):572–4.
171. Nau H, Tzimas G, Mondry M, Plum C, Spohr HL. Antiepileptic drugs alter endogenous retinoid concentrations: A possible mechanism of teratogenesis of anticonvulsant therapy. *Life Sci*. 1995 Apr 26;57(1):53–60.
172. Hurst RJMM, Else KJ. The retinoic acid-producing capacity of gut dendritic cells and macrophages is reduced during persistent *T. muris* infection. *Parasite Immunol*. 2013 Apr 19;35(7–8):229–33.
173. Kim EW, De Leon A, Jiang Z, Radu RA, Martineau AR, Chan ED, et al. Vitamin A Metabolism by Dendritic Cells Triggers an Antimicrobial Response against *Mycobacterium tuberculosis*. *mSphere*. 2019 Apr 26;4(3).
174. DiNardo AR, Nishiguchi T, Grimm SL, Schlesinger LS, Graviss EA, Cirillo JD, et al. Tuberculosis endotypes to guide stratified host-directed therapy. *Med*. 2021 Apr 26;2(3):217–32.
175. DiNardo AR, Rajapakshe K, Gandhi T, Grimm SL, Nishiguchi T, Heyckendorf J, et al. Discerning divergent tuberculosis endotypes: A meta-analysis and systematic review of individual patient data. *medRxiv*. 2020 May 11;2020.05.13.20100776.
176. Palace VP, Khaper N, Qin Q, Singal PK. Antioxidant potentials of vitamin A and carotenoids and their relevance to heart disease. Vol. 26, *Free Radical Biology and Medicine*. Free Radic Biol Med; 1999. p. 746–61.
177. Mensink GBM, Fletcher R, Gurinovic M, Huybrechts I, Lafay L, Serra-Majem L, et al. Mapping low intake of micronutrients across Europe. *Br J Nutr*. 2013;110(4):755–73.

178. Lin P, Fintelman RE, Khalifa YM, Bailony MR, Jeng BH. Ocular surface disease secondary to vitamin A deficiency in the developed world: it still exists. *Arch Ophthalmol Chic Ill 1960*. 2011;129(6):798–9.
179. Simkin SK, Tuck K, Garrett J, Dai S. Vitamin A deficiency--an unexpected cause of visual loss. *Lancet Lond Engl*. 2016;387(10013):93–4.
180. Organization WH. Global health risks : mortality and burden of disease attributable to selected major risks. World Health Organization; 2009. vi, 62 p. p.
181. Sommer A, Vyas KS. A global clinical view on vitamin A and carotenoids. *Am J Clin Nutr*. 2012;96(5):1204S-6S.
182. Sherwin JC, Reacher MH, Dean WH, Ngondi J. Epidemiology of vitamin A deficiency and xerophthalmia in at-risk populations. *Trans R Soc Trop Med Hyg*. 2012;106(4):205–14.
183. Wiseman EM, Bar-El Dadon S, Reifen R. The vicious cycle of vitamin a deficiency: A review. *Crit Rev Food Sci Nutr*. 2017;57(17):3703–14.
184. Guideline: vitamin A supplementation in infants and children 6-59 months of age. 2021.
185. WHO recommendations on antenatal care for a positive pregnancy experience. 2021.
186. Imdad A, Yakoob MY, Sudfeld C, Haider BA, Black RE, Bhutta ZA. Impact of vitamin A supplementation on infant and childhood mortality. *BMC Public Health*. 2011;11 Suppl 3:S20.
187. Mayo-Wilson E, Imdad A, Herzer K, Yakoob MY, Bhutta ZA. Vitamin A supplements for preventing mortality, illness, and blindness in children aged under 5: systematic review and meta-analysis. *The BMJ*. 2011 Mar 15;343.
188. Organization WH. Guideline: updates on the management of severe acute malnutrition in infants and children. 2021.

189. Chau N, Tébi A, Créton C, Belbraouet S, Debry G. Relationship between plasma retinol and infectious diseases in the elderly. A case-control study. *Ann Nutr Metab.* 2000;44(5–6):256–62.
190. Al-Mekhlafi HM, Surin J, Sallam AA, Abdullah AW, Mahdy MAK. Giardiasis and Poor Vitamin A Status among Aboriginal School Children in Rural Malaysia. *Am J Trop Med Hyg.* 2010 Apr 7;83(3):523–7.
191. de Gier B, Campos Ponce M, Van De Bor M, Doak CM, Polman K. Helminth infections and micronutrients in school-age children: a systematic review and meta-analysis. *Am J Clin Nutr.* 2014;99(6):1499–509.
192. Thornton KA, Mora-Plazas M, Marín C, Villamor E. Vitamin A Deficiency Is Associated with Gastrointestinal and Respiratory Morbidity in School-Age Children¹²³. *J Nutr.* 2014 Apr 7;144(4):496–503.
193. Frieden TR, Sowell AL, Henning KJ, Huff DL, Gunn RA. Vitamin A levels and severity of measles. New York City. *Am J Dis Child* 1960. 1992;146(2):182–6.
194. Yang HM, Mao M, Wan C. Vitamin A for treating measles in children. *Cochrane Database Syst Rev.* 2005 Apr 7;2005(4).
195. Bitetto D, Bortolotti N, Falletti E, Vescovo S, Fabris C, Fattovich G, et al. Vitamin A deficiency is associated with hepatitis C virus chronic infection and with unresponsiveness to interferon-based antiviral therapy. *Hepatol Baltim Md.* 2013;57(3):925–33.
196. Tang AM, Graham NM, Semba RD, Saah AJ. Association between serum vitamin A and E levels and HIV-1 disease progression. *AIDS Lond Engl.* 1997;11(5):613–20.
197. Suchdev PS, Davis SM, Bartoces M, Ruth LJ, Worrell CM, Kanyi H, et al. Soil-Transmitted Helminth Infection and Nutritional Status Among Urban Slum Children in Kenya. *Am J Trop Med Hyg.* 2014 Apr 7;90(2):299–305.
198. Long KZ, Estrada-Garcia T, Rosado JL, Santos JI, Haas M, Firestone M, et al. The Effect of Vitamin A Supplementation on the Intestinal Immune Response in Mexican

Children Is Modified by Pathogen Infections and Diarrhea. *J Nutr.* 2006 Apr 7;136(5):1365–70.

199. Al-Mekhlafi HM, Anuar TS, Al-Zabedi EM, Al-Maktari MT, Mahdy MAK, Ahmed A, et al. Does vitamin A supplementation protect schoolchildren from acquiring soil-transmitted helminthiasis? A randomized controlled trial. *Parasit Vectors.* 2014;7:367.
200. Oh J, Choi R, Park HD, Lee H, Jeong BH, Park HY, et al. Evaluation of vitamin status in patients with pulmonary tuberculosis. *J Infect.* 2017;74(3):272–80.
201. Keflie TS, Samuel A, Woldegiorgis AZ, Mihret A, Abebe M, Biesalski HK. Vitamin A and zinc deficiencies among tuberculosis patients in Ethiopia. *J Clin Tuberc Mycobact Dis.* 2018;12:27–33.
202. Ramachandran G, Santha T, Garg R, Baskaran D, Iliayas SA, Venkatesan P, et al. Vitamin A levels in sputum-positive pulmonary tuberculosis patients in comparison with household contacts and healthy ‘normals’
203. Mugusi FM, Rusizoka O, Habib N, Fawzi W. Vitamin A status of patients presenting with pulmonary tuberculosis and asymptomatic HIV-infected individuals, Dar es Salaam, Tanzania. *Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis.* 2003;7(8):804–7.
204. Plit ML, Theron AJ, Fickl H, van Rensburg CE, Pendel S, Anderson R. Influence of antimicrobial chemotherapy and smoking status on the plasma concentrations of vitamin C, vitamin E, beta-carotene, acute phase reactants, iron and lipid peroxides in patients with pulmonary tuberculosis. *Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis.* 1998;2(7):590–6.
205. Aibana O, Franke MF, Huang CC, Galea JT, Calderon R, Zhang Z, et al. Impact of Vitamin A and Carotenoids on the Risk of Tuberculosis Progression. *Clin Infect Dis Off Publ Infect Dis Soc Am.* 2017;65(6):900–9.
206. Tenforde MW, Yadav A, Dowdy DW, Gupte N, Shivakoti R, Yang WT, et al. Vitamin A and D deficiencies associated with incident tuberculosis in HIV-infected patients

- initiating antiretroviral therapy in multinational case-cohort study. *J Acquir Immune Defic Syndr* 1999. 2017 Apr 6;75(3):e71–9.
207. Pakasi TA, Karyadi E, Wibowo Y, Simanjuntak Y, Suratih NMD, Salean M, et al. Vitamin A deficiency and other factors associated with severe tuberculosis in Timor and Rote Islands, East Nusa Tenggara Province, Indonesia. *Eur J Clin Nutr*. 2009 Apr 6;63(9):1130–5.
 208. Qrafli M, El Kari K, Aguenau H, Bourkadi JE, Sadki K, El Mzibri M. Low plasma vitamin A concentration is associated with tuberculosis in Moroccan population: a preliminary case control study. *BMC Res Notes*. 2017 Apr 6;10(1):421.
 209. Blaner WS, Li Y, Brun PJ, Yuen JJ, Lee SA, Clugston RD. Vitamin A Absorption, Storage and Mobilization. *Subcell Biochem*. 2016;81:95–125.
 210. Li Y, Wongsiriroj N, Blaner WS. The multifaceted nature of retinoid transport and metabolism. *Hepatobiliary Surg Nutr*. 2014 Apr 8;3(3):126–39.
 211. Kono N, Arai H. Intracellular Transport of Fat-Soluble Vitamins A and E. *Traffic*. 2015 Apr 9;16(1):19–34.
 212. Trasino SE, Tang XH, Trujillo C, Andres J, Ehrt S, Gudas LJ. Mycobacterium tuberculosis infection is exacerbated in mice lacking lecithin:retinol acyltransferase. *Biochim Biophys Acta BBA - Mol Basis Dis*. 2020 Nov 1;1866(11):165909.
 213. Steinhoff JS, Lass A, Schupp M. Biological Functions of RBP4 and Its Relevance for Human Diseases. *Front Physiol*. 2021 Apr 8;12.
 214. Berry DC, Croniger CM, Ghyselinck NB, Noy N. Transthyretin blocks retinol uptake and cell signaling by the holo-retinol-binding protein receptor STRA6. *Mol Cell Biol*. 2012;32(19):3851–9.
 215. Berry DC, O’Byrne SM, Vreeland AC, Blaner WS, Noy N. Cross Talk between Signaling and Vitamin A Transport by the Retinol-Binding Protein Receptor STRA6. *Mol Cell Biol*. 2012 Apr 8;32(15):3164–75.

216. Engle-Stone R, Haskell MJ, Ndjebayi AO, Nankap M, Erhardt JG, Gimou MM, et al. Plasma retinol-binding protein predicts plasma retinol concentration in both infected and uninfected Cameroonian women and children. *J Nutr*. 2011;141(12):2233–41.
217. Jarsberg LG, Kedia K, Wendler J, Wright AT, Piehowski PD, Gritsenko MA, et al. Nutritional markers and proteome in patients undergoing treatment for pulmonary tuberculosis differ by geographic region. *PloS One*. 2021;16(5):e0250586.
218. Hu Z, Bang YJ, Ruhn KA, Hooper L V. Molecular basis for retinol binding by serum amyloid A during infection. *Proc Natl Acad Sci U S A*. 2019 Sep 17;116(38):19077–82.
219. Derebe MG, Zlatkov CM, Gattu S, Ruhn KA, Vaishnava S, Diehl GE, et al. Serum amyloid A is a retinol binding protein that transports retinol during bacterial infection. *eLife*. 2014 Jul 29;3(July2014):1–18.
220. Bang YJ, Hu Z, Li Y, Gattu S, Ruhn KA, Raj P, et al. Serum amyloid A delivers retinol to intestinal myeloid cells to promote adaptive immunity. *Science*. 2021 Sep 17;373(6561).
221. Broch M, Gómez JM, Auguet MT, Vilarrasa N, Pastor R, Elio I, et al. Association of retinol-binding protein-4 (RBP4) with lipid parameters in obese women. *Obes Surg*. 2010;20(9):1258–64.
222. Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Schleicher E, et al. High circulating retinol-binding protein 4 is associated with elevated liver fat but not with total, subcutaneous, visceral, or intramyocellular fat in humans. *Diabetes Care*. 2007;30(5):1173–8.
223. von Eynatten M, Lepper PM, Liu D, Lang K, Baumann M, Nawroth PP, et al. Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease. *Diabetologia*. 2007;50(9):1930–7.
224. Muenzner M, Tuvia N, Deutschmann C, Witte N, Tolkachov A, Valai A, et al. Retinol-binding protein 4 and its membrane receptor STRA6 control adipogenesis by

- regulating cellular retinoid homeostasis and retinoic acid receptor α activity. *Mol Cell Biol.* 2013;33(20):4068–82.
225. Berry DC, Jin H, Majumdar A, Noy N. Signaling by vitamin A and retinol-binding protein regulates gene expression to inhibit insulin responses. *Proc Natl Acad Sci U S A.* 2011 May 5;108(11):4340–5.
 226. Norseen J, Hosooka T, Hammarstedt A, Yore MM, Kant S, Aryal P, et al. Retinol-Binding Protein 4 Inhibits Insulin Signaling in Adipocytes by Inducing Proinflammatory Cytokines in Macrophages through a c-Jun N-Terminal Kinase- and Toll-Like Receptor 4-Dependent and Retinol-Independent Mechanism. *Mol Cell Biol.* 2012 Apr 9;32(10):2010–9.
 227. Gonçalves A, Estevinho BN, Rocha F. Formulation approaches for improved retinoids delivery in the treatment of several pathologies. Vol. 143, *European Journal of Pharmaceutics and Biopharmaceutics*. Elsevier B.V.; 2019. p. 80–90.
 228. Blomhoff R, Blomhoff HK. Overview of retinoid metabolism and function. *J Neurobiol.* 2006 Mar 15;66(7):606–30.
 229. Duester G, Mic FA, Molotkov A. Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. *Chem Biol Interact.* 2003;143–144:201–10.
 230. Isoherranen N, Zhong G. Biochemical and physiological importance of the CYP26 retinoic acid hydroxylases. *Pharmacol Ther.* 2019;204:107400.
 231. Stevison F, Jing J, Tripathy S, Isoherranen N. Role of retinoic acid metabolizing cytochrome P450s, CYP26, in inflammation and cancer. *Adv Pharmacol San Diego Calif.* 2015 Apr 21;74:373–412.
 232. Riquelme P, Amodio G, Macedo C, Moreau A, Obermajer N, Brochhausen C, et al. DHRS9 Is a Stable Marker of Human Regulatory Macrophages. *Transplantation.* 2017;101(11):2731–8.

233. Erkelens MN, Mebius RE. Retinoic Acid and Immune Homeostasis: A Balancing Act. Vol. 38, Trends in Immunology. Elsevier Ltd; 2017. p. 168–80.
234. Broadhurst MJ, Leung JM, Lim KC, Girgis NM, Gundra UM, Fallon PG, et al. Upregulation of retinal dehydrogenase 2 in alternatively activated macrophages during retinoid-dependent type-2 immunity to helminth infection in mice. PLoS Pathog. 2012;8(8):e1002883.
235. Niu H, Hadwiger G, Fujiwara H, Welch JS. Pathways of retinoid synthesis in mouse macrophages and bone marrow cells. J Leukoc Biol. 2016 Jun;99(6):797–810.
236. Sanders TJ, McCarthy NE, Giles EM, Davidson KLM, Haltalli MLR, Hazell S, et al. Increased production of retinoic acid by intestinal macrophages contributes to their inflammatory phenotype in patients with Crohn's disease. Gastroenterology. 2014;146(5):1272–8.
237. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, et al. Functional specializations of intestinal dendritic cell and macrophage subsets that control TH-17 and T regulatory responses is dependent on the T:APC ratio, source of mouse strain and regional localization. J Immunol Baltim Md 1950. 2011 Apr 19;187(2):733–47.
238. Agace WW, Persson EK. How vitamin A metabolizing dendritic cells are generated in the gut mucosa. Trends Immunol. 2012;33(1):42–8.
239. Gyöngyösi A, Szatmari I, Pap A, Dezso B, Pos Z, Széles L, et al. RDH10, RALDH2, and CRABP2 are required components of PPAR γ -directed ATRA synthesis and signaling in human dendritic cells. J Lipid Res. 2013 Apr 19;54(9):2458–74.
240. Ito K, Zolfaghari R, Hao L, Ross AC. Inflammation rapidly modulates the expression of ALDH1A1 (RALDH1) and vimentin in the liver and hepatic macrophages of rats in vivo. Nutr Metab. 2014 Apr 19;11.

241. Kreutz M, Fritsche J, Andreesen R, Krause SW. Regulation of Cellular Retinoic Acid Binding Protein (CRABP II) during Human Monocyte Differentiation in Vitro. *Biochem Biophys Res Commun*. 1998 Apr 20;248(3):830–4.
242. Al Tanoury Z, Piskunov A, Rochette-Egly C. Vitamin A and retinoid signaling: genomic and nongenomic effects. *J Lipid Res*. 2013;54(7):1761–75.
243. Larange A, Cheroutre H. Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System. *Annu Rev Immunol*. 2016;34:369–94.
244. Napoli JL. Cellular retinoid binding-proteins, CRBP, CRABP, FABP5: Effects on retinoid metabolism, function and related diseases. *Pharmacol Ther*. 2017;173:19–33.
245. Guo Y, Brown C, Ortiz C, Noelle RJ. Leukocyte Homing, Fate, and Function Are Controlled by Retinoic Acid. *Physiol Rev*. 2015 Apr 19;95(1):125–48.
246. Chen C, Perry TL, Chitko-McKown CG, Smith AD, Cheung L, Beshah E, et al. The regulatory actions of retinoic acid on M2 polarization of porcine macrophages. *Dev Comp Immunol*. 2019;98:20–33.
247. Lamers MM, Haagmans BL. SARS-CoV-2 pathogenesis. *Nat Rev Microbiol*. 2022 May;20(5):270–84.
248. WHO Coronavirus (COVID-19) Dashboard [Internet]. 2023 [cited 2023 Feb 9]. Available from: <https://covid19.who.int>
249. Meyerowitz EA, Richterman A, Gandhi RT, Sax PE. Transmission of SARS-CoV-2: A Review of Viral, Host, and Environmental Factors. *Ann Intern Med*. 2021 Jan 19;174(1):69–79.
250. Schultze A, Nightingale E, Evans D, Hulme W, Rosello A, Bates C, et al. Mortality among Care Home Residents in England during the first and second waves of the COVID-19 pandemic: an observational study of 4.3 million adults over the age of 65. *Lancet Reg Health – Eur* [Internet]. 2022 Mar 1 [cited 2023 Feb 10];14. Available from: <https://www.thelancet.com/journals/lanep/article/PIIS2666-7762%2821%2900281-7/fulltext>

251. Aboubakr HA, Sharafeldin TA, Goyal SM. Stability of SARS-CoV-2 and other coronaviruses in the environment and on common touch surfaces and the influence of climatic conditions: A review. *Transbound Emerg Dis*. 2021;68(2):296–312.
252. Gonçalves J, da Silva PG, Reis L, Nascimento MSJ, Koritnik T, Paragi M, et al. Surface contamination with SARS-CoV-2: A systematic review. *Sci Total Environ*. 2021 Dec 1;798:149231.
253. Rahman S, Montero MTV, Rowe K, Kirton R, Kunik F. Epidemiology, pathogenesis, clinical presentations, diagnosis and treatment of COVID-19: a review of current evidence. *Expert Rev Clin Pharmacol*. 2021 May 4;14(5):601–21.
254. Beran A, Zink E, Mhanna M, Abugharbyeh A, Hanrahan J, Duggan J, et al. Transmissibility and viral replication of SARS-COV-2 in immunocompromised patients. *J Med Virol*. 2021 Jul;93(7):4156–60.
255. Ma Q, Liu J, Liu Q, Kang L, Liu R, Jing W, et al. Global Percentage of Asymptomatic SARS-CoV-2 Infections Among the Tested Population and Individuals With Confirmed COVID-19 Diagnosis: A Systematic Review and Meta-analysis. *JAMA Netw Open*. 2021 Dec 1;4(12):e2137257.
256. Oran DP, Topol EJ. The Proportion of SARS-CoV-2 Infections That Are Asymptomatic : A Systematic Review. *Ann Intern Med*. 2021 May;174(5):655–62.
257. Ra SH, Lim JS, Kim G un, Kim MJ, Jung J, Kim SH. Upper respiratory viral load in asymptomatic individuals and mildly symptomatic patients with SARS-CoV-2 infection. *Thorax*. 2021 Jan 1;76(1):61–3.
258. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA*. 2020 Apr 7;323(13):1239–42.
259. Scobie HM, Johnson AG, Suthar AB, Severson R, Alden NB, Balter S, et al. Monitoring Incidence of COVID-19 Cases, Hospitalizations, and Deaths, by Vaccination

Status - 13 U.S. Jurisdictions, April 4-July 17, 2021. MMWR Morb Mortal Wkly Rep. 2021 Sep 17;70(37):1284–90.

260. Hyams C, Challen R, Marlow R, Nguyen J, Begier E, Southern J, et al. Severity of Omicron (B.1.1.529) and Delta (B.1.617.2) SARS-CoV-2 infection among hospitalised adults: a prospective cohort study in Bristol, United Kingdom. *Lancet Reg Health – Eur* [Internet]. 2023 Feb 1 [cited 2023 Feb 12];25. Available from: [https://www.thelancet.com/journals/lanepa/article/PIIS2666-7762\(22\)00252-6/fulltext](https://www.thelancet.com/journals/lanepa/article/PIIS2666-7762(22)00252-6/fulltext)
261. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, et al. Factors associated with hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City: prospective cohort study. *BMJ*. 2020 May 22;369:m1966.
262. Dexamethasone in Hospitalized Patients with Covid-19. *N Engl J Med*. 2021 Feb 25;384(8):693–704.
263. Bhimraj A, Morgan RL, Shumaker AH, Baden L, Cheng VC, Edwards KM, Gallagher JC, Gandhi RT, Muller WJ, Nakamura MM, O'Horo JC, Shafer RW, Shoham S, Murad MH, Mustafa RA, Sultan S, Falck-Ytter Y. Infectious Diseases Society of America Guidelines on the Treatment and Management of Patients with COVID-19. *Infectious Diseases Society of America* 2022; Version 10.2.0. [Internet]. 2023 [cited 2023 Feb 12]. Available from: <https://www.idsociety.org/practice-guideline/covid-19-guideline-treatment-and-management/>
264. Health Protection Surveillance Centre. COVID-19 deaths reported in Ireland - Health Protection Surveillance Centre [Internet]. [cited 2023 Feb 15]. Available from: <https://www.hpsc.ie/a-z/respiratory/coronavirus/novelcoronavirus/surveillance/covid-19deathsreportedinireland/>

265. Booth A, Reed AB, Ponzo S, Yassaee A, Aral M, Plans D, et al. Population risk factors for severe disease and mortality in COVID-19: A global systematic review and meta-analysis. *PLOS ONE*. 2021 Mar 4;16(3):e0247461.
266. Central Statistics Office. Population Estimates - CSO - Central Statistics Office [Internet]. CSO; 2021 [cited 2023 Feb 12]. Available from: <https://www.cso.ie/en/releasesandpublications/fp/fp-ipeads/irishpopulationestimatesfromadministrativedatasources2020/populationestimates/>
267. COVID-19 deaths reported in Ireland - Health Protection Surveillance Centre [Internet]. [cited 2023 Feb 12]. Available from: <https://www.hpsc.ie/a-z/respiratory/coronavirus/novelcoronavirus/surveillance/covid-19deathsreportedinireland/>
268. van der Made CI, Simons A, Schuurs-Hoeijmakers J, van den Heuvel G, Mantere T, Kersten S, et al. Presence of Genetic Variants Among Young Men With Severe COVID-19. *JAMA*. 2020 Aug 18;324(7):663–73.
269. Asano T, Boisson B, Onodi F, Matuozzo D, Moncada-Velez M, Maglorius Renkilaraj MRL, et al. X-linked recessive TLR7 deficiency in ~1% of men under 60 years old with life-threatening COVID-19. *Sci Immunol*. 2021 Aug 19;6(62):eabl4348.
270. Zhang Q, Bastard P, Liu Z, Le Pen J, Moncada-Velez M, Chen J, et al. Inborn errors of type I IFN immunity in patients with life-threatening COVID-19. *Science*. 2020 Oct 23;370(6515):eabd4570.
271. Genomewide Association Study of Severe Covid-19 with Respiratory Failure. *N Engl J Med*. 2020 Oct 15;383(16):1522–34.
272. Bastard P, Rosen LB, Zhang Q, Michailidis E, Hoffmann HH, Zhang Y, et al. Autoantibodies against type I IFNs in patients with life-threatening COVID-19. *Science*. 2020 Oct 23;370(6515):eabd4585.

273. Bastard P, Gervais A, Le Voyer T, Rosain J, Philippot Q, Manry J, et al. Autoantibodies neutralizing type I IFNs are present in ~4% of uninfected individuals over 70 years old and account for ~20% of COVID-19 deaths. *Sci Immunol*. 2021 Aug 20;6(62):eabl4340.
274. Yadav R, Chaudhary JK, Jain N, Chaudhary PK, Khanra S, Dhamija P, et al. Role of Structural and Non-Structural Proteins and Therapeutic Targets of SARS-CoV-2 for COVID-19. *Cells*. 2021 Apr 6;10(4):821.
275. Aguiar JA, Tremblay BJM, Mansfield MJ, Woody O, Lobb B, Banerjee A, et al. Gene expression and in situ protein profiling of candidate SARS-CoV-2 receptors in human airway epithelial cells and lung tissue. *Eur Respir J* [Internet]. 2020 Sep 1 [cited 2023 Feb 12];56(3). Available from: <https://erj.ersjournals.com/content/56/3/2001123>
276. Hikmet F, Méar L, Edvinsson Å, Micke P, Uhlén M, Lindskog C. The protein expression profile of ACE2 in human tissues. *Mol Syst Biol*. 2020 Jul;16(7):e9610.
277. Kumar A, Narayan RK, Kumari C, Faiq MA, Kulandhasamy M, Kant K, et al. SARS-CoV-2 cell entry receptor ACE2 mediated endothelial dysfunction leads to vascular thrombosis in COVID-19 patients. *Med Hypotheses*. 2020 Dec;145:110320.
278. Zhu Z, Shi J, Li L, Wang J, Zhao Y, Ma H. Therapy Targets SARS-CoV-2 Infection-Induced Cell Death. *Front Immunol* [Internet]. 2022 [cited 2023 Feb 12];13. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.870216>
279. Katsura H, Sontake V, Tata A, Kobayashi Y, Edwards CE, Heaton BE, et al. Human Lung Stem Cell-Based Alveolospheres Provide Insights into SARS-CoV-2-Mediated Interferon Responses and Pneumocyte Dysfunction. *Cell Stem Cell*. 2020 Dec 3;27(6):890-904.e8.
280. Nieto-Torres JL, DeDiego ML, Verdiá-Báguena C, Jimenez-Guardeño JM, Regla-Nava JA, Fernandez-Delgado R, et al. Severe acute respiratory syndrome coronavirus envelope protein ion channel activity promotes virus fitness and pathogenesis. *PLoS Pathog*. 2014 May;10(5):e1004077.

281. Xia B, Shen X, He Y, Pan X, Liu FL, Wang Y, et al. SARS-CoV-2 envelope protein causes acute respiratory distress syndrome (ARDS)-like pathological damages and constitutes an antiviral target. *Cell Res.* 2021 Aug;31(8):847–60.
282. Hadjadj J, Yatim N, Barnabei L, Corneau A, Boussier J, Smith N, et al. Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. *Science.* 2020 Aug 7;369(6504):718–24.
283. Del Valle DM, Kim-Schulze S, Huang HH, Beckmann ND, Nirenberg S, Wang B, et al. An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat Med.* 2020 Oct;26(10):1636–43.
284. Leisman DE, Ronner L, Pinotti R, Taylor MD, Sinha P, Calfee CS, et al. Cytokine elevation in severe and critical COVID-19: a rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. *Lancet Respir Med.* 2020 Dec 1;8(12):1233–44.
285. Melms JC, Biermann J, Huang H, Wang Y, Nair A, Tagore S, et al. A molecular single-cell lung atlas of lethal COVID-19. *Nature.* 2021 Jul;595(7865):114–9.
286. Ferreira AC, Soares VC, de Azevedo-Quintanilha IG, Dias S da SG, Fintelman-Rodrigues N, Sacramento CQ, et al. SARS-CoV-2 engages inflammasome and pyroptosis in human primary monocytes. *Cell Death Discov.* 2021 Mar 1;7(1):1–12.
287. Satış H, Özger HS, Aysert Yıldız P, Hızıl K, Gulbahar Ö, Erbaş G, et al. Prognostic value of interleukin-18 and its association with other inflammatory markers and disease severity in COVID-19. *Cytokine.* 2021 Jan 1;137:155302.
288. Liao M, Liu Y, Yuan J, Wen Y, Xu G, Zhao J, et al. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat Med.* 2020 Jun;26(6):842–4.
289. Li S, Zhang Y, Guan Z, Li H, Ye M, Chen X, et al. SARS-CoV-2 triggers inflammatory responses and cell death through caspase-8 activation. *Signal Transduct Target Ther.* 2020 Oct 9;5(1):1–10.

290. Sefik E, Qu R, Junqueira C, Kaffe E, Mirza H, Zhao J, et al. Inflammasome activation in infected macrophages drives COVID-19 pathology. *Nature*. 2022 Jun;606(7914):585–93.
291. Vora SM, Lieberman J, Wu H. Inflammasome activation at the crux of severe COVID-19. *Nat Rev Immunol*. 2021 Nov;21(11):694–703.
292. Yalcinkaya M, Liu W, Islam MN, Kotini AG, Gusarova GA, Fidler TP, et al. Modulation of the NLRP3 inflammasome by Sars-CoV-2 Envelope protein. *Sci Rep*. 2021 Dec 24;11(1):24432.
293. Grant RA, Morales-Nebreda L, Markov NS, Swaminathan S, Querrey M, Guzman ER, et al. Circuits between infected macrophages and T cells in SARS-CoV-2 pneumonia. *Nature*. 2021 Feb;590(7847):635–41.
294. Dalskov L, Møhlenberg M, Thyrssted J, Blay-Cadanet J, Poulsen ET, Folkersen BH, et al. SARS-CoV-2 evades immune detection in alveolar macrophages. *EMBO Rep*. 2020 Dec 3;21(12):e51252.
295. Labzin LI, Chew KY, Eschke K, Wang X, Esposito T, Stocks CJ, et al. Macrophage ACE2 is necessary for SARS-CoV-2 replication and subsequent cytokine responses that restrict continued virion release. *Sci Signal*. 2023 Apr 25;16(782):eabq1366.
296. Muus C, Luecken MD, Eraslan G, Sikkema L, Waghray A, Heimberg G, et al. Single-cell meta-analysis of SARS-CoV-2 entry genes across tissues and demographics. *Nat Med*. 2021 Mar;27(3):546–59.
297. Delorey TM, Ziegler CGK, Heimberg G, Normand R, Yang Y, Segerstolpe Å, et al. COVID-19 tissue atlases reveal SARS-CoV-2 pathology and cellular targets. *Nature*. 2021 Jul;595(7865):107–13.
298. Short KR, Brooks AG, Reading PC, Londrigan SL. The fate of influenza A virus after infection of human macrophages and dendritic cells. *J Gen Virol*. 2012 Nov;93(Pt 11):2315–25.

299. Oliveira-Nascimento L, Massari P, Wetzler LM. The Role of TLR2 in Infection and Immunity. *Front Immunol*. 2012 Apr 18;3:79.
300. Khan S, Shafiei MS, Longoria C, Schoggins J, Savani RC, Zaki H. SARS-CoV-2 spike protein induces inflammation via TLR2-dependent activation of the NF- κ B pathway. *BioRxiv Prepr Serv Biol*. 2021 Mar 17;2021.03.16.435700.
301. Theobald SJ, Simonis A, Georgomanolis T, Kreer C, Zehner M, Einfeld HS, et al. Long-lived macrophage reprogramming drives spike protein-mediated inflammasome activation in COVID-19. *EMBO Mol Med*. 2021 Aug 9;13(8):e14150.
302. M Z, R K, Ep W, D Y, E F, P V, et al. TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. *Nat Immunol* [Internet]. 2021 Jul [cited 2022 Mar 23];22(7). Available from: <https://pubmed.ncbi.nlm.nih.gov/33963333/>
303. Planès R, Bert JB, Tairi S, BenMohamed L, Bahraoui E. SARS-CoV-2 Envelope (E) Protein Binds and Activates TLR2 Pathway: A Novel Molecular Target for COVID-19 Interventions. *Viruses*. 2022 May 8;14(5):999.
304. Halajian EA, LeBlanc EV, Gee K, Colpitts CC. Activation of TLR4 by viral glycoproteins: A double-edged sword? *Front Microbiol* [Internet]. 2022 [cited 2023 Feb 14];13. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.1007081>
305. Anand G, Perry AM, Cummings CL, St. Raymond E, Clemens RA, Steed AL. Surface Proteins of SARS-CoV-2 Drive Airway Epithelial Cells to Induce IFN-Dependent Inflammation. *J Immunol Author Choice*. 2021 Jun 15;206(12):3000–9.
306. Cadena AM, Flynn JL, Fortune SM. The Importance of First Impressions: Early Events in *Mycobacterium tuberculosis* Infection Influence Outcome. *mBio*. 2016 Apr 5;7(2):e00342-16.
307. Pribul PK, Harker J, Wang B, Wang H, Tregoning JS, Schwarze J, et al. Alveolar Macrophages Are a Major Determinant of Early Responses to Viral Lung Infection but Do Not Influence Subsequent Disease Development. *J Virol*. 2008 May;82(9):4441–8.

308. Wang J, Nikrad MP, Travanty EA, Zhou B, Phang T, Gao B, et al. Innate Immune Response of Human Alveolar Macrophages during Influenza A Infection. *PLOS ONE*. 2012 Mar 2;7(3):e29879.
309. Khalil H, Tazi M, Caution K, Ahmed A, Kanneganti A, Assani K, et al. Aging is associated with hypermethylation of autophagy genes in macrophages. *Epigenetics*. 2016 May 3;11(5):381–8.
310. Stranks AJ, Hansen AL, Panse I, Mortensen M, Ferguson DJP, Puleston DJ, et al. Autophagy Controls Acquisition of Aging Features in Macrophages. *J Innate Immun*. 2015;7(4):375–91.
311. Boe DM, Hulsebus HJ, Najarro KM, Mullen JE, Kim H, Tan AC, et al. Advanced age is associated with changes in alveolar macrophages and their responses to the stress of traumatic injury. *J Leukoc Biol*. 2022;112(6):1371–86.
312. 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 Apr 18;38(4):792–804.
313. van de Laar L, Saelens W, De Pijck S, Martens L, Scott CL, Van Isterdael G, et al. Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop into Functional Tissue-Resident Macrophages. *Immunity*. 2016 Apr 19;44(4):755–68.
314. Joshi N, Walter JM, Misharin AV. Alveolar Macrophages. *Cell Immunol*. 2018;330:86–90.
315. Mould KJ, Barthel L, Mohning MP, Thomas SM, McCubbrey AL, Danhorn T, et al. Cell Origin Dictates Programming of Resident versus Recruited Macrophages during Acute Lung Injury. *Am J Respir Cell Mol Biol*. 2017 Sep;57(3):294–306.
316. McQuattie-Pimentel AC, Ren Z, Joshi N, Watanabe S, Stoeger T, Chi M, et al. The lung microenvironment shapes a dysfunctional response of alveolar macrophages in aging. *J Clin Invest*. 131(4):e140299.

317. Veldhuizen RAW, McCaig LA, Pape C, Gill SE. The effects of aging and exercise on lung mechanics, surfactant and alveolar macrophages. *Exp Lung Res.* 2019 Jul 3;45(5–6):113–22.
318. Brandenberger C, Kling KM, Vital M, Mühlfeld C. The Role of Pulmonary and Systemic Immunosenescence in Acute Lung Injury. *Aging Dis.* 2018 Aug 1;9(4):553–65.
319. Canan CH, Gokhale NS, Carruthers B, Lafuse WP, Schlesinger LS, Torrelles JB, et al. Characterization of lung inflammation and its impact on macrophage function in aging. *J Leukoc Biol.* 2014;96(3):473–80.
320. Hearps AC, Martin GE, Angelovich TA, Cheng WJ, Maisa A, Landay AL, et al. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. *Aging Cell.* 2012;11(5):867–75.
321. Boyd AR, Shivshankar P, Jiang S, Berton MT, Orihuela CJ. Age-related Defects in TLR2 Signaling Diminish the Cytokine Response by Alveolar Macrophages during Murine Pneumococcal Pneumonia. *Exp Gerontol.* 2012 Jul;47(7):507–18.
322. van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, et al. Age-associated defect in human TLR-1/2 function. *J Immunol Baltim Md 1950.* 2007 Jan 15;178(2):970–5.
323. Bailey KL, Smith LM, Heires AJ, Katafiasz DM, Romberger DJ, LeVan TD. Aging leads to dysfunctional innate immune responses to TLR2 and TLR4 agonists. *Aging Clin Exp Res.* 2019 Sep;31(9):1185–93.
324. Seidler S, Zimmermann HW, Bartneck M, Trautwein C, Tacke F. Age-dependent alterations of monocyte subsets and monocyte-related chemokine pathways in healthy adults. *BMC Immunol.* 2010 Jun 21;11(1):30.
325. Nyugen J, Agrawal S, Gollapudi S, Gupta S. Impaired Functions of Peripheral Blood Monocyte Subpopulations in Aged Humans. *J Clin Immunol.* 2010 Nov 1;30(6):806–13.

326. Stout-Delgado HW, Cho SJ, Chu SG, Mitzel DN, Villalba J, El-Chemaly S, et al. Age-Dependent Susceptibility to Pulmonary Fibrosis Is Associated with NLRP3 Inflammasome Activation. *Am J Respir Cell Mol Biol*. 2016 Aug;55(2):252–63.
327. Lara PC, Macías-Verde D, Burgos-Burgos J. Age-induced NLRP3 Inflammasome Over-activation Increases Lethality of SARS-CoV-2 Pneumonia in Elderly Patients. *Aging Dis*. 2020 Jul 23;11(4):756–62.
328. Bhattacharya S, Banerjee A, Ray S. Development of new vaccine target against SARS-CoV2 using envelope (E) protein: An evolutionary, molecular modeling and docking based study. *Int J Biol Macromol*. 2021 Mar 1;172:74–81.
329. Dai L, Gao GF. Viral targets for vaccines against COVID-19. *Nat Rev Immunol*. 2021 Feb;21(2):73–82.
330. Kibria KMK, Faruque MdO, Islam MdS bin, Ullah H, Mahmud S, Miah M, et al. A conserved subunit vaccine designed against SARS-CoV-2 variants showed evidence in neutralizing the virus. *Appl Microbiol Biotechnol*. 2022 Jun 1;106(11):4091–114.
331. Castelli JM, Rearte A, Olszevicki S, Voto C, Juarez MDV, Pesce M, et al. Effectiveness of mRNA-1273, BNT162b2, and BBIBP-CorV vaccines against infection and mortality in children in Argentina, during predominance of delta and omicron covid-19 variants: test negative, case-control study. *BMJ*. 2022 Nov 30;379:e073070.
332. Duart G, García-Murria MJ, Grau B, Acosta-Cáceres JM, Martínez-Gil L, Mingarro I. SARS-CoV-2 envelope protein topology in eukaryotic membranes. *Open Biol*. 2020 Sep 9;10(9):200209.
333. Castaño-Rodríguez C, Honrubia JM, Gutiérrez-Álvarez J, DeDiego ML, Nieto-Torres JL, Jimenez-Guardeño JM, et al. Role of Severe Acute Respiratory Syndrome Coronavirus Viroporins E, 3a, and 8a in Replication and Pathogenesis. *mBio*. 2018 May 22;9(3):e02325-17.
334. Jimenez-Guardeño JM, Nieto-Torres JL, DeDiego ML, Regla-Nava JA, Fernandez-Delgado R, Castaño-Rodríguez C, et al. The PDZ-Binding Motif of Severe Acute

Respiratory Syndrome Coronavirus Envelope Protein Is a Determinant of Viral Pathogenesis. *PLOS Pathog*. 2014 Aug 14;10(8):e1004320.

335. Troyano-Hernández P, Reinos R, Holguín Á. Evolution of SARS-CoV-2 in Spain during the First Two Years of the Pandemic: Circulating Variants, Amino Acid Conservation, and Genetic Variability in Structural, Non-Structural, and Accessory Proteins. *Int J Mol Sci*. 2022 Jan;23(12):6394.
336. O'Sullivan MP, O'Leary S, Kelly DM, Keane J. A caspase-independent pathway mediates macrophage cell death in response to *Mycobacterium tuberculosis* infection. *Infect Immun*. 2007 Apr;75(4):1984–93.
337. Vogels CBF, Brito AF, Wyllie AL, Fauver JR, Ott IM, Kalinich CC, et al. Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT–qPCR primer–probe sets. *Nat Microbiol*. 2020 Oct;5(10):1299–305.
338. Gu L, Casserly D, Brady G, Carpenter S, Bracken AP, Fitzgerald KA, et al. Myeloid cell nuclear differentiation antigen controls the pathogen-stimulated type I interferon cascade in human monocytes by transcriptional regulation of IRF7. *Nat Commun*. 2022 Jan 10;13(1):14.
339. Poerio N, Riva C, Olimpieri T, Rossi M, Lorè NI, De Santis F, et al. Combined Host- and Pathogen-Directed Therapy for the Control of *Mycobacterium abscessus* Infection. *Microbiol Spectr*. 2022 Jan 26;10(1):e02546-21.
340. Ryan RCM, O'Sullivan MP, Keane J. *Mycobacterium tuberculosis* infection induces non-apoptotic cell death of human dendritic cells. *BMC Microbiol*. 2011 Oct 24;11:237.
341. Seahorse XF Cell Mito Stress Test Kit User Guide.
342. Aibana O, Franke MF, Huang CC, Galea JT, Calderon R, Zhang Z, et al. Impact of Vitamin A and Carotenoids on the Risk of Tuberculosis Progression. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2017;65(6):900–9.
343. Grobler L, Nagpal S, Sudarsanam TD, Sinclair D. Nutritional supplements for people being treated for active tuberculosis. *Cochrane Database Syst Rev* [Internet].

2016 [cited 2021 Apr 23];(6). Available from:

<https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD006086.pub4/full>

344. Blomhoff R, Blomhoff HK. Overview of retinoid metabolism and function. *J Neurobiol.* 2006 Mar 15;66(7):606–30.
345. Duester G, Mic FA, Molotkov A. Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. *Chem Biol Interact.* 2003 Feb 1;143–144:201–10.
346. Napoli JL. Cellular retinoid binding-proteins, CRBP, CRABP, FABP5: Effects on retinoid metabolism, function and related diseases. *Pharmacol Ther.* 2017;173:19–33.
347. Riquelme P, Amodio G, Macedo C, Moreau A, Obermajer N, Brochhausen C, et al. DHRS9 Is a Stable Marker of Human Regulatory Macrophages. *Transplantation.* 2017;101(11):2731–8.
348. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, et al. Functional specializations of intestinal dendritic cell and macrophage subsets that control TH-17 and T regulatory responses is dependent on the T:APC ratio, source of mouse strain and regional localization. *J Immunol Baltim Md 1950.* 2011 Apr 19;187(2):733–47.
349. Sanders TJ, McCarthy NE, Giles EM, Davidson KLM, Haltalli MLR, Hazell S, et al. Increased production of retinoic acid by intestinal macrophages contributes to their inflammatory phenotype in patients with Crohn’s disease. *Gastroenterology.* 2014;146(5):1272–8.
350. Kim EW, De Leon A, Jiang Z, Radu RA, Martineau AR, Chan ED, et al. Vitamin A Metabolism by Dendritic Cells Triggers an Antimicrobial Response against *Mycobacterium tuberculosis*. *mSphere.* 2019 05;4(3).
351. Niu H, Hadwiger G, Fujiwara H, Welch JS. Pathways of retinoid synthesis in mouse macrophages and bone marrow cells. *J Leukoc Biol.* 2016 Jun;99(6):797–810.

352. Kreutz M, Fritsche J, Andreessen R, Krause SW. Regulation of Cellular Retinoic Acid Binding Protein (CRABP II) during Human Monocyte Differentiation in Vitro. *Biochem Biophys Res Commun*. 1998 Apr 20;248(3):830–4.
353. Lavalett L, Rodriguez H, Ortega H, Sadee W, Schlesinger LS, Barrera LF. Alveolar macrophages from tuberculosis patients display an altered inflammatory gene expression profile. *Tuberc Edinb Scotl*. 2017 Dec;107:156–67.
354. Padmos RC, Schloot NC, Beyan H, Ruwhof C, Staal FJT, de Ridder D, et al. Distinct Monocyte Gene-Expression Profiles in Autoimmune Diabetes. *Diabetes*. 2008 Oct;57(10):2768–73.
355. Ghelfi E, Karaaslan C, Berkelhamer S, Akar S, Kozakewich H, Cataltepe S. Fatty acid-binding proteins and peribronchial angiogenesis in bronchopulmonary dysplasia. *Am J Respir Cell Mol Biol*. 2011 Sep;45(3):550–6.
356. Hurst RJM, Else KJ. The retinoic acid-producing capacity of gut dendritic cells and macrophages is reduced during persistent *T. muris* infection. *Parasite Immunol*. 2013 Jul;35(7–8):229–33.
357. Scott JP, Ji Y, Kannan M, Wylam ME. Inhaled granulocyte-macrophage colony-stimulating factor for *Mycobacterium abscessus* in cystic fibrosis. *Eur Respir J*. 2018 Apr;51(4):1702127.
358. Ballinger MN, Paine R, Serezani CHC, Aronoff DM, Choi ES, Standiford TJ, et al. Role of granulocyte macrophage colony-stimulating factor during gram-negative lung infection with *Pseudomonas aeruginosa*. *Am J Respir Cell Mol Biol*. 2006 Jun;34(6):766–74.
359. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, et al. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int Immunol*. 2009 Apr;21(4):361–77.
360. Hamilton TA, Zhao C, Pavicic PG, Datta S. Myeloid Colony-Stimulating Factors as Regulators of Macrophage Polarization. *Front Immunol* [Internet]. 2014 [cited 2023

Mar 27];5. Available from:

<https://www.frontiersin.org/articles/10.3389/fimmu.2014.00554>

361. Das BC, Thapa P, Karki R, Das S, Mahapatra S, Liu TC, et al. Retinoic acid signaling pathways in development and diseases. *Bioorg Med Chem*. 2014 Jan 15;22(2):673–83.
362. Ke Q, Li R, Cai L, Wu S dong, Li C mei. Ro41-5253, a selective antagonist of retinoic acid receptor α , ameliorates chronic unpredictable mild stress-induced depressive-like behaviors in rats: Involvement of regulating HPA axis and improving hippocampal neuronal deficits. *Brain Res Bull*. 2019 Mar 1;146:302–9.
363. Gleeson LE, O’Leary SM, Ryan D, McLaughlin AM, Sheedy FJ, Keane J. Cigarette Smoking Impairs the Bioenergetic Immune Response to *Mycobacterium tuberculosis* Infection. *Am J Respir Cell Mol Biol*. 2018;59(5):572–9.
364. Phelan JJ, McQuaid K, Kenny C, Gogan KM, Cox DJ, Basdeo SA, et al. Desferrioxamine Supports Metabolic Function in Primary Human Macrophages Infected With *Mycobacterium tuberculosis*. *Front Immunol*. 2020;11:836.
365. Cox DJ, Coleman AM, Gogan KM, Phelan JJ, Ó Maoldomhnaigh C, Dunne PJ, et al. Inhibiting Histone Deacetylases in Human Macrophages Promotes Glycolysis, IL-1 β , and T Helper Cell Responses to *Mycobacterium tuberculosis*. *Front Immunol*. 2020;11:1609.
366. Cahill C, Phelan JJ, Keane J. Understanding and Exploiting the Effect of Tuberculosis Antimicrobials on Host Mitochondrial Function and Bioenergetics. *Front Cell Infect Microbiol*. 2020;10:493.
367. Leisching G, Keane J. Bystander Macrophage Metabolic Shift after *Mycobacterium tuberculosis* Infection. *Am J Respir Cell Mol Biol*. 2020 Dec;63(6):863–5.
368. Ó Maoldomhnaigh C, Cox DJ, Phelan JJ, Malone FD, Keane J, Basdeo SA. The Warburg Effect Occurs Rapidly in Stimulated Human Adult but Not Umbilical Cord Blood Derived Macrophages. *Front Immunol*. 2021;12:657261.

369. Ó Maoldomhnaigh C, Cox DJ, Phelan JJ, Mitermite M, Murphy DM, Leisching G, et al. Lactate Alters Metabolism in Human Macrophages and Improves Their Ability to Kill *Mycobacterium tuberculosis*. *Front Immunol*. 2021;12:663695.
370. Fontaine E. Metformin-Induced Mitochondrial Complex I Inhibition: Facts, Uncertainties, and Consequences. *Front Endocrinol [Internet]*. 2018 [cited 2023 Mar 28];9. Available from: <https://www.frontiersin.org/articles/10.3389/fendo.2018.00753>
371. Naicker N, Sigal A, Naidoo K. Metformin as Host-Directed Therapy for TB Treatment: Scoping Review. *Front Microbiol [Internet]*. 2020 [cited 2023 Mar 28];11. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.00435>
372. Schupp M, Curtin JC, Kim RJ, Billin AN, Lazar MA. A widely used retinoic acid receptor antagonist induces peroxisome proliferator-activated receptor-gamma activity. *Mol Pharmacol*. 2007 May;71(5):1251–7.
373. Quintero Barceinas RS, García-Regalado A, Aréchaga-Ocampo E, Villegas-Sepúlveda N, González-De la Rosa CH. All-Trans Retinoic Acid Induces Proliferation, Survival, and Migration in A549 Lung Cancer Cells by Activating the ERK Signaling Pathway through a Transcription-Independent Mechanism. *BioMed Res Int*. 2015;2015:404368.
374. Aklamati EK, Mulenga M, Dueker SR, Buchholz BA, Peerson JM, Kafwembe E, et al. Accelerator mass spectrometry can be used to assess vitamin A metabolism quantitatively in boys in a community setting. *J Nutr*. 2010 Sep;140(9):1588–94.
375. Keicho N, Matsushita I, Tanaka T, Shimbo T, Hang LNT, Sakurada S, et al. Circulating levels of adiponectin, leptin, fetuin-A and retinol-binding protein in patients with tuberculosis: markers of metabolism and inflammation. *PLOS ONE*. 2012 Apr 9;7(6):e38703.
376. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *Lancet Lond Engl*. 2006 Sep 16;368(9540):1012–21.

377. Stephensen CB, Alvarez JO, Kohatsu J, Hardmeier R, Kennedy Jr JI, Gammon Jr RB. Vitamin A is excreted in the urine during acute infection. *Am J Clin Nutr*. 1994 Apr 9;60(3):388–92.
378. Na YR, Gu GJ, Jung D, Kim YW, Na J, Woo JS, et al. GM-CSF Induces Inflammatory Macrophages by Regulating Glycolysis and Lipid Metabolism. *J Immunol*. 2016 Nov 15;197(10):4101–9.
379. Singh P, González-Ramos S, Mojena M, Rosales-Mendoza CE, Emami H, Swanson J, et al. GM-CSF Enhances Macrophage Glycolytic Activity In Vitro and Improves Detection of Inflammation In Vivo. *J Nucl Med Off Publ Soc Nucl Med*. 2016 Sep;57(9):1428–35.
380. Mao JT, Goldin JG, Dermand J, Ibrahim G, Brown MS, Emerick A, et al. A pilot study of all-trans-retinoic acid for the treatment of human emphysema. *Am J Respir Crit Care Med*. 2002 Mar 1;165(5):718–23.
381. Roth MD, Connett JE, D’Armiento JM, Foronjy RF, Friedman PJ, Goldin JG, et al. Feasibility of retinoids for the treatment of emphysema study. *Chest*. 2006 Nov;130(5):1334–45.
382. Conley BA, Egorin MJ, Sridhara R, Finley R, Hemady R, Wu S, et al. Phase I clinical trial of all-trans-retinoic acid with correlation of its pharmacokinetics and pharmacodynamics. *Cancer Chemother Pharmacol*. 1997 Apr 22;39(4):291–9.
383. Kocher HM, Basu B, Froeling FEM, Sarker D, Slater S, Carlin D, et al. Phase I clinical trial repurposing all-trans retinoic acid as a stromal targeting agent for pancreatic cancer. *Nat Commun*. 2020;11(1):4841.
384. Agadir A, Cornic M, Lefebvre P, Gourmel B, Jérôme M, Degos L, et al. All-trans retinoic acid pharmacokinetics and bioavailability in acute promyelocytic leukemia: intracellular concentrations and biologic response relationship. *J Clin Oncol Off J Am Soc Clin Oncol*. 1995 Oct;13(10):2517–23.

385. Trump DL, Smith DC, Stiff D, Adedoyin A, Day R, Bahnson RR, et al. A phase II trial of all-trans-retinoic acid in hormone-refractory prostate cancer: a clinical trial with detailed pharmacokinetic analysis. *Cancer Chemother Pharmacol*. 1997 Apr 22;39(4):349–56.
386. Tenforde MW, Yadav A, Dowdy DW, Gupte N, Shivakoti R, Yang WT, et al. Vitamin A and D deficiencies associated with incident tuberculosis in HIV-infected patients initiating antiretroviral therapy in multinational case-cohort study. *J Acquir Immune Defic Syndr* 1999. 2017 Jul 1;75(3):e71–9.
387. Oh J, Park HD, Kim SY, Koh WJ, Lee SY. Assessment of Vitamin Status in Patients with Nontuberculous Mycobacterial Pulmonary Disease: Potential Role of Vitamin A as a Risk Factor. *Nutrients*. 2019 Feb 5;11(2):343.
388. Thornton KA, Mora-Plazas M, Marín C, Villamor E. Vitamin A Deficiency Is Associated with Gastrointestinal and Respiratory Morbidity in School-Age Children123. *J Nutr*. 2014 Apr 7;144(4):496–503.
389. de Gier B, Campos Ponce M, van de Bor M, Doak CM, Polman K. Helminth infections and micronutrients in school-age children: a systematic review and meta-analysis. *Am J Clin Nutr*. 2014 Jun;99(6):1499–509.
390. Al-Mekhlafi HM, Surin J, Sallam AA, Abdullah AW, Mahdy MAK. Giardiasis and Poor Vitamin A Status among Aboriginal School Children in Rural Malaysia. *Am J Trop Med Hyg*. 2010 Sep;83(3):523–7.
391. Suchdev PS, Davis SM, Bartoces M, Ruth LJ, Worrell CM, Kanyi H, et al. Soil-Transmitted Helminth Infection and Nutritional Status Among Urban Slum Children in Kenya. *Am J Trop Med Hyg*. 2014 Apr 7;90(2):299–305.
392. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. 2006 Nov 17;314(5802):1157–60.

393. Cassani B, Villablanca EJ, De Calisto J, Wang S, Mora JR. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. *Mol Aspects Med.* 2012 Feb;33(1):63–76.
394. Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, et al. Essential role for retinoic acid in the promotion of CD4+ T cell effector responses via retinoic acid receptor alpha. *Immunity.* 2011 Mar;34(3):435–47.
395. Wiseman EM, Bar-El Dadon S, Reifen R. The vicious cycle of vitamin a deficiency: A review. *Crit Rev Food Sci Nutr.* 2017 Nov 22;57(17):3703–14.
396. Lalevée S, Anno YN, Chatagnon A, Samarut E, Poch O, Laudet V, et al. Genome-wide in silico identification of new conserved and functional retinoic acid receptor response elements (direct repeats separated by 5 bp). *J Biol Chem.* 2011 Sep 23;286(38):33322–34.
397. Ciaramella V, Sasso FC, Di Liello R, Corte CMD, Barra G, Viscardi G, et al. Activity and molecular targets of pioglitazone via blockade of proliferation, invasiveness and bioenergetics in human NSCLC. *J Exp Clin Cancer Res CR.* 2019 Apr 26;38(1):178.
398. Yang HM, Mao M, Wan C. Vitamin A for treating measles in children. *Cochrane Database Syst Rev.* 2005 Apr 7;2005(4).
399. Manicassamy S, Ravindran R, Deng J, Oluoch H, Denning TL, Kasturi SP, et al. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat Med.* 2009 Apr;15(4):401–9.
400. Biyikli OO, Baysak A, Ece G, Oz AT, Ozhan MH, Berdeli A. Role of Toll-Like Receptors in Tuberculosis Infection. *Jundishapur J Microbiol.* 2016 Sep 14;9(10):e20224.
401. Arnold SLM, Amory JK, Walsh TJ, Isoherranen N. A sensitive and specific method for measurement of multiple retinoids in human serum with UHPLC-MS/MS. *J Lipid Res.* 2012 Mar;53(3):587–98.

402. Mookerjee SA, Goncalves RLS, Gerencser AA, Nicholls DG, Brand MD. The contributions of respiration and glycolysis to extracellular acid production. *Biochim Biophys Acta*. 2015 Feb;1847(2):171–81.
403. Klautau GB, da Mota NVF, Salles MJC, Burattini MN, Rodrigues DS. Interferon- γ release assay as a sensitive diagnostic tool of latent tuberculosis infection in patients with HIV: a cross-sectional study. *BMC Infect Dis*. 2018 Nov 19;18:585.
404. Stout JE, Wu Y, Ho CS, Pettit AC, Feng PJ, Katz DJ, et al. Evaluating latent tuberculosis infection diagnostics using latent class analysis. *Thorax*. 2018 Nov 1;73(11):1062–70.
405. Coleman MM, Basdeo SA, Coleman AM, Cheallaigh CN, Peral de Castro C, McLaughlin AM, et al. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial Infection. *Am J Respir Cell Mol Biol*. 2018 May 31;59(5):548–56.
406. O’Leary S, O’Sullivan MP, Keane J. IL-10 blocks phagosome maturation in *Mycobacterium tuberculosis*-infected human macrophages. *Am J Respir Cell Mol Biol*. 2011 Jul;45(1):172–80.
407. Pollock J, Chalmers JD. The immunomodulatory effects of macrolide antibiotics in respiratory disease. *Pulm Pharmacol Ther*. 2021 Dec 1;71:102095.
408. van Wyk R, van Wyk M, Mashele SS, Nelson DR, Syed K. Comprehensive Comparative Analysis of Cholesterol Catabolic Genes/Proteins in *Mycobacterial* Species. *Int J Mol Sci*. 2019 Feb 27;20(5):1032.
409. Cortes MAM, Nessar R, Singh AK. Laboratory maintenance of *Mycobacterium abscessus*. *Curr Protoc Microbiol*. 2010 Aug;Chapter 10:Unit 10D.1.
410. Bannantine JP, Zhang Q, Li LL, Kapur V. Genomic homogeneity between *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* belies their divergent growth rates. *BMC Microbiol*. 2003 May 9;3:10.
411. Lee YM, Lee JO, Jung JH, Kim JH, Park SH, Park JM, et al. Retinoic Acid Leads to Cytoskeletal Rearrangement through AMPK-Rac1 and Stimulates Glucose Uptake

- through AMPK-p38 MAPK in Skeletal Muscle Cells *. *J Biol Chem.* 2008 Dec 5;283(49):33969–74.
412. Piskunov A, Rochette-Egly C. A retinoic acid receptor RAR α pool present in membrane lipid rafts forms complexes with G protein α Q to activate p38MAPK. *Oncogene.* 2012 Jul 12;31(28):3333–45.
 413. Masiá S, Alvarez S, de Lera AR, Barettino D. Rapid, nongenomic actions of retinoic acid on phosphatidylinositol-3-kinase signaling pathway mediated by the retinoic acid receptor. *Mol Endocrinol Baltim Md.* 2007 Oct;21(10):2391–402.
 414. García-Regalado A, Vargas M, García-Carrancá A, Aréchaga-Ocampo E, González-De la Rosa CH. Activation of Akt pathway by transcription-independent mechanisms of retinoic acid promotes survival and invasion in lung cancer cells. *Mol Cancer.* 2013 May 21;12:44.
 415. Esteban J, García-Coca M. Mycobacterium Biofilms. *Front Microbiol.* 2018 Jan 18;8:2651.
 416. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern Med.* 2020 Jul 1;180(7):934–43.
 417. Han L, Ran J, Mak YW, Suen LKP, Lee PH, Peiris JSM, et al. Smoking and Influenza-associated Morbidity and Mortality: A Systematic Review and Meta-analysis. *Epidemiology.* 2019 May;30(3):405.
 418. Wong CM, Yang L, Chan KP, Chan WM, Song L, Lai HK, et al. Cigarette smoking as a risk factor for influenza-associated mortality: evidence from an elderly cohort. *Influenza Other Respir Viruses.* 2013;7(4):531–9.
 419. Pahari S, Arnett E, Simper J, Azad A, Guerrero-Arguero I, Ye C, et al. A new tractable method for generating human alveolar macrophage-like cells in vitro to study lung inflammatory processes and diseases. *mBio.* 2023 Jun 8;0(0):e00834-23.

420. Juárez E, Nuñez C, Sada E, Ellner JJ, Schwander SK, Torres M. Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respir Res.* 2010;11(1):2.
421. O’Leary SM, Coleman MM, Chew WM, Morrow C, McLaughlin AM, Gleeson LE, et al. Cigarette Smoking Impairs Human Pulmonary Immunity to *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med.* 2014 Dec 15;190(12):1430–6.
422. Gaschler GJ, Zavitz CCJ, Bauer CMT, Skrtic M, Lindahl M, Robbins CS, et al. Cigarette Smoke Exposure Attenuates Cytokine Production by Mouse Alveolar Macrophages. *Am J Respir Cell Mol Biol.* 2008 Feb;38(2):218–26.
423. Ohta T, Yamashita N, Maruyama M, Sugiyama E, Kobayashi M. Cigarette smoking decreases interleukin-8 secretion by human alveolar macrophages. *Respir Med.* 1998 Jul 1;92(7):922–7.
424. Soliman DM, Twigg HL. Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. *Am J Physiol-Lung Cell Mol Physiol.* 1992 Oct;263(4):L471–8.
425. Mangalmurti N, Hunter CA. Cytokine Storms: Understanding COVID-19. *Immunity.* 2020 Jul 14;53(1):19–25.
426. van der Donk LEH, Eder J, van Hamme JL, Brouwer PJM, Brinkkemper M, van Nuenen AC, et al. SARS-CoV-2 infection activates dendritic cells via cytosolic receptors rather than extracellular TLRs. *Eur J Immunol.* 2022 Apr;52(4):646–55.
427. Hou F, Xiao K, Tang L, Xie L. Diversity of Macrophages in Lung Homeostasis and Diseases. *Front Immunol [Internet].* 2021 [cited 2023 Jun 28];12. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.753940>
428. Pence BD, Yarbro JR. Aging impairs mitochondrial respiratory capacity in classical monocytes. *Exp Gerontol.* 2018 Jul 15;108:112–7.

429. Tyrkalska SD, Martínez-López A, Arroyo AB, Martínez-Morcillo FJ, Candel S, García-Moreno D, et al. Differential proinflammatory activities of Spike proteins of SARS-CoV-2 variants of concern. *Sci Adv*. 2022 Sep 16;8(37):eabo0732.
430. Olejnik J, Hume AJ, Mühlberger E. Toll-like receptor 4 in acute viral infection: Too much of a good thing. *PLoS Pathog*. 2018 Dec;14(12):e1007390.
431. Bai Z, Cao Y, Liu W, Li J. The SARS-CoV-2 Nucleocapsid Protein and Its Role in Viral Structure, Biological Functions, and a Potential Target for Drug or Vaccine Mitigation. *Viruses*. 2021 Jun 10;13(6):1115.
432. Hu WT, Howell JC, Ozturk T, Benameur K, Bassit LC, Ramonell R, et al. Antibody Profiles According to Mild or Severe SARS-CoV-2 Infection, Atlanta, Georgia, USA, 2020. *Emerg Infect Dis*. 2020 Dec;26(12):2974–8.
433. Abdelmageed MI, Abdelmoneim AH, Mustafa MI, Elfadol NM, Murshed NS, Shantier SW, et al. Design of a Multiepitope-Based Peptide Vaccine against the E Protein of Human COVID-19: An Immunoinformatics Approach. *BioMed Res Int*. 2020 May 11;2020:2683286.
434. Jakhar R, Gakhar SK. An Immunoinformatics Study to Predict Epitopes in the Envelope Protein of SARS-CoV-2. *Can J Infect Dis Med Microbiol J Can Mal Infect Microbiol Medicale*. 2020;2020:7079356.
435. Susithra Priyadarshni M, Isaac Kirubakaran S, Harish MC. In silico approach to design a multi-epitopic vaccine candidate targeting the non-mutational immunogenic regions in envelope protein and surface glycoprotein of SARS-CoV-2. *J Biomol Struct Dyn*. 2022 Dec 19;40(23):12948–63.
436. Chen J, Deng Y, Huang B, Han D, Wang W, Huang M, et al. DNA Vaccines Expressing the Envelope and Membrane Proteins Provide Partial Protection Against SARS-CoV-2 in Mice. *Front Immunol*. 2022;13:827605.
437. Facciola A, Visalli G, Laganà A, Di Pietro A. An Overview of Vaccine Adjuvants: Current Evidence and Future Perspectives. *Vaccines*. 2022 May 22;10(5):819.

438. Bonfield TL, Sutton MT, Fletcher DR, Folz MA, Ragavapuram V, Somoza RA, et al. Donor-Defined Mesenchymal Stem Cell Antimicrobial Potency Against Nontuberculous Mycobacterium. *Stem Cells Transl Med*. 2021 Aug 1;10(8):1202–16.
439. Tomlinson GS, Cashmore TJ, Elkington PTG, Yates J, Lehloenya RJ, Tsang J, et al. Transcriptional profiling of innate and adaptive human immune responses to mycobacteria in the tuberculin skin test. *Eur J Immunol*. 2011;41(11):3253–60.
440. Wauthoz N, Deleuze P, Saumet A, Duret C, Kiss R, Amighi K. Temozolomide-based dry powder formulations for lung tumor-related inhalation treatment. *Pharm Res*. 2011 Apr 30;28(4):762–75.
441. O'Connor G, Krishnan N, Fagan-Murphy A, Cassidy J, O'Leary S, Robertson BD, et al. Inhalable poly(lactic-co-glycolic acid) (PLGA) microparticles encapsulating all-trans-Retinoic acid (ATRA) as a host-directed, adjunctive treatment for Mycobacterium tuberculosis infection. *Eur J Pharm Biopharm*. 2019 Jan 1;134:153–65.
442. Hoy SM. Amikacin Liposome Inhalation Suspension in Refractory Mycobacterium avium Complex Lung Disease: A Profile of Its Use. *Clin Drug Investig*. 2021 Apr 1;41(4):405–12.
443. Dharmadhikari AS, Kabadi M, Gerety B, Hickey AJ, Fourie PB, Nardell E. Phase I, single-dose, dose-escalating study of inhaled dry powder capreomycin: A new approach to therapy of drug-resistant tuberculosis. *Antimicrob Agents Chemother*. 2013 Jun;57(6):2613–9.
444. Garcia Contreras L, Sung J, Ibrahim M, Elbert K, Edwards D, Hickey A. Pharmacokinetics of Inhaled Rifampicin Porous Particles for Tuberculosis Treatment: Insight into Rifampicin Absorption from the Lungs of Guinea Pigs. *Mol Pharm*. 2015 Aug 3;12(8):2642–50.
445. Lei GS, Zhang C, Shao S, Jung HW, Durant PJ, Lee CH. All-Trans Retinoic Acid in Combination with Primaquine Clears Pneumocystis Infection. *PLOS ONE*. 2013 Apr 22;8(1):e53479.

446. Castillo Y, Tachibana M, Nakatsu Y, Watanabe K, Shimizu T, Watarai M. Combination of Zinc and All-Trans Retinoic Acid Promotes Protection against *Listeria monocytogenes* Infection. *PLOS ONE*. 2015 Apr 22;10(9):e0137463.
447. Kjolhede CL, Chew FJ, Gadomski AM, Marroquin DP. Clinical trial of vitamin A as adjuvant treatment for lower respiratory tract infections. *J Pediatr*. 1995 Apr 22;126(5):807–12.
448. Stephensen CB, Franchi LM, Hernandez H, Campos M, Gilman RH, Alvarez JO. Adverse Effects of High-dose Vitamin A Supplements in Children Hospitalized With Pneumonia. *Pediatrics*. 1998 Apr 22;101(5):e3–e3.
449. Mao JT, Goldin JG, Dermand J, Ibrahim G, Brown MS, Emerick A, et al. A Pilot Study of All-trans-Retinoic Acid for the Treatment of Human Emphysema. *Am J Respir Crit Care Med*. 2002 Apr 22;165(5):718–23.
450. Roth MD, Connett JE, D’Armiento JM, Foronjy RF, Friedman PJ, Goldin JG, et al. Feasibility of retinoids for the treatment of emphysema study. *Chest*. 2006;130(5):1334–45.
451. Conley BA, Egorin MJ, Sridhara R, Finley R, Hemady R, Wu S, et al. Phase I clinical trial of all-trans-retinoic acid with correlation of its pharmacokinetics and pharmacodynamics. *Cancer Chemother Pharmacol*. 1997 Apr 22;39(4):291–9.
452. Kocher HM, Basu B, Froeling FEM, Sarker D, Slater S, Carlin D, et al. Phase I clinical trial repurposing all-trans retinoic acid as a stromal targeting agent for pancreatic cancer. *Nat Commun*. 2020;11(1):4841.
453. Sutton LM, Warmuth MA, Petros WP, Winer EP. Pharmacokinetics and clinical impact of all-trans retinoic acid in metastatic breast cancer: a phase II trial. *Cancer Chemother Pharmacol*. 1997;40(4):335–41.
454. Mirza N, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ, et al. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res*. 2006 Sep 15;66(18):9299.

455. Trump DL, Smith DC, Stiff D, Adedoyin A, Day R, Bahnson RR, et al. A phase II trial of all-trans-retinoic acid in hormone-refractory prostate cancer: a clinical trial with detailed pharmacokinetic analysis. *Cancer Chemother Pharmacol*. 1997 Apr 22;39(4):349–56.
456. Rothman KJ, Moore LL, Singer MR, Nguyen USDT, Mannino S, Milunsky A. Teratogenicity of High Vitamin A Intake. <http://dx.doi.org/101056/NEJM1995112333332101>. 2009 Aug 20;333(21):1369–73.
457. Mishra AK, Driessen NN, Appelmek BJ, Besra GS. Lipoarabinomannan and related glycoconjugates: structure, biogenesis and role in *Mycobacterium tuberculosis* physiology and host–pathogen interaction. *FEMS Microbiol Rev*. 2011 Nov 1;35(6):1126–57.