

Cellular and molecular defence mechanisms against *Legionella* infection

Lara Marie Oberkircher

ORCID ID:

0000-0003-4370-6428

from Homburg (Saar), Germany

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1. Supervisor: Prof. Dr. Natalio Garcia Garbi
2. Supervisor: Prof. Dr. Ian van Driel

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Abbreviations

2-DG	2-Deoxy-D-glucose
ADP	Adenosine diphosphate
AEC	Alveolar epithelial cells
ALI	Air-liquid interface
AM	Alveolar macrophages
AMP	Anti-microbial peptides
APC	Antigen-presenting cell
ARF1	ADP ribosylation factor 1
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BCYE	Buffered charcoal yeast extract
BFP	Blue fluorescent protein
BiP	Binding immunoglobulin protein
Cas-9	CRISPR-associated protein 9
CCL	CC-motif ligand
CCR	CC-motif ligand receptor
CD	Cluster of differentiation
CFU	Colony forming units
cGAS	Cyclic GMP-AMP synthase
CHOP	CCAAT-enhancer-binding protein homologous protein
ciEpC	ciliated epithelial cell
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF	Colony-stimulating factor
CTL	Cytotoxic T cell
CXCL	CXC-motif ligand
CXCR	CXC-motif ligand receptor

VII

DHR123	Dihydrorhodamine 123
DMSO	Dimethyl sulfoxide
DNA	desoxyribonucleic acid
Dot/ Icm	Defect in organelle trafficking/ intracellular multiplication
EDTA	Ethylenediamine tetra acetic acid
eIF2 α	Eukaryotic translation initiation factor 2 α
EpC	Epithelial cells
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FCS	Foetal calf serum
Fig.	Figure
fMLP	N-Formyl methionine-leucyl-phenylalanine
G-CSF	Granulocyte CSF
GDP	Guanosine diphosphate
GEF	GDP-GTP exchange factor
GM-CSF	Granulocyte-macrophage CSF
GTP	Guanosine triphosphate
HBEC	Human bronchiolar epithelial cells
I-CAM	Intracellular adhesion molecule
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
IFN	Interferon
IL	Interleukin

VIII

IL-18R	IL-18 receptor
IRE1	Inositol-requiring enzyme type 1
IRF	Interferon-regulating factor
ISG	Interferon-induced gene
KO	Knockout
<i>L. lo</i>	<i>L. longbeachae</i>
<i>L. pn</i>	<i>L. pneumophila</i>
LCP	<i>Legionella</i> -containing phagosome
LCV	<i>Legionella</i> -containing vacuole
LD	Legionnaire's disease
LPS	Lipopolysaccharide
Ly-6C	Lymphocyte antigen 6 complex locus C
Ly-6G	Lymphocyte antigen 6 complex locus G
M-CSF	Macrophage CSF
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
mBD	Murine- β -defensin
MC	monocyte-derived cells
mCRAMP	Murine catechilidin-related AMP
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NAIP5	NLR family apoptosis inhibitor protein 5

NET	Neutrophil extracellular traps
nEV	Neutrophil-derived extracellular vesicles
NF- κ B	Nuclear factor- κ B
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP	NLR protein
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
O ₂	oxygen
O ₂ ⁻	superoxide anion
OD	Optical density
ON	Overnight
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PERK	Protein kinase-like ER kinase
PI(3)P	Phosphatidylinositol-3-phosphate
PMA	Phorbol myristate acetate
PMN	Polymorph-nuclear cells
PRR	Pattern recognition receptor
qPCR	quantitative polymerase chain reaction
Rab1	Ras-associated binding 1
RIDD	regulated IRE1-dependent decay
RIG-I	Retinoic acid-inducible gene-I
rIL-18	Recombinant IL-18
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAseq	RNAsequencing

ROS	Reactive oxygen species
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
S1P	Site 1 protease
SCENITH	Single-cell energetic metabolism by profiling translation inhibition
SERCA	Sarcoplasmatic/ endoplasmic reticulum calcium ATPase
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
spp.	Species
STAT	Signal transducer and activator of transcription
T4SS	Type IV secretion system
Tab.	Table
TCR	T cell receptor
Th cell	T helper cell
TLR	Toll-like receptor
TNF α	Tumour necrosis factor α
TRAF	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor protein
UMAP	Uniform manifold approximation and projection for dimension reduction
UPR	Unfolded protein response
V-CAM	Vascular cell adhesion molecule
WT	Wildtype
XBP1	X-box binding protein 1
XBP1s	Spliced XBP1

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Abstract

The two most prevalent causes of Legionnaires' disease are *L. pneumophila* and *L. longbeachae*. The rising incidence of the Legionnaires' disease over the past two decades together with the increasing prevalence of *L. longbeachae* in the Northern hemisphere highlights the necessity to gain a more detailed insight into the *L. longbeachae*-induced immune response. The aim of this thesis was to investigate the cellular and molecular mechanisms required for protection, focussing on immune cell responses and intracellular host-bacterial interactions.

By combining a murine mouse model, a novel fluorescent reporter to track *L. longbeachae*, and cell depletion experiments, I uncovered the differential contribution of tissue-resident alveolar macrophages (AM) and infiltrating neutrophils to the defence against *L. longbeachae*. Early during infection, AM contained most of the bacteria. AM numbers sharply decreased during infection, which was accompanied by a large influx of neutrophils that also internalized bacteria. Comparative analysis of bacterial viability revealed that neutrophils were more efficient at killing and clearing of *L. longbeachae* than AM. In contrast, the results presented here indicated that lung-resident AM promoted infection, most likely by serving as a replicative niche.

Defence against *L. pneumophila* is known to require IFN- γ but not IL-18, a strong IFN- γ inducer. However, *Il18r1*^{-/-} mice were less able to clear *L. longbeachae* and had significantly impaired IFN- γ levels in the lung. Furthermore, IL-18R signalling was critical for the efficient bacterial killing by neutrophils via production of reactive oxygen species. Previous bone marrow chimera experiments in our laboratory suggested that IL-18R expression in epithelial cells was necessary and sufficient for protection against *L. longbeachae*. However, genetic *in vivo* experiments using the Cre-lox-system revealed that IL-18R⁺ NK cells and T cells were central players in the IL-18-dependent anti-*L. longbeachae* defence via IFN- γ secretion, whereas IL-18R expression by ciliated bronchiolar epithelial cells did not confer protection.

Finally, our results highlighted key mechanisms by which Legionella subverts host macrophages to form an intracellular endoplasmic reticulum (ER)-like

vacuole as its intracellular replicative niche. Establishment of the *Legionella*-containing vacuole (LCV) by recruitment of ER-derived vesicles induces ER stress. Although the relevance of ER stress in this process is unclear, effector proteins secreted by *L. pneumophila* are known to inhibit onset of the ER stress response. Using the pharmacological ER stress inducer thapsigargin, we showed that ER stress induces a protective host response promoting the secretion of pro-inflammatory cytokines, limiting intracellular *L. pneumophila* replication, and improving host survival. Mechanistically, ER stress induced a novel non-canonical activation of the transcription factor STAT1 via the IRE1 kinase driving transcription of the IFN- γ -induced chemokine CXCL10. These results highlighted a potential role of the host ER stress response in the initiation of a protective cellular immune response towards *L. pneumophila*.

Declaration

The work presented in this thesis was conducted at The University of Monash, The University of Melbourne, and The Rheinische Friedrich-Wilhelms-Universität Bonn in the laboratories of Prof. Elizabeth Hartland (Hudson Institute of Medical Research; Department of Microbiology and Immunology) and Prof. Natalio Garbi (Institute of Molecular Medicine and Experimental Immunology).

This is to certify that

- (i) this thesis comprises only my original work towards the Ph.D except where indicated in the preface,
- (ii) due acknowledgment has been made in the text to all other material used,
- (iii) the thesis is less than 100,000 words in length, exclusive of tables, graphs, bibliographies, and appendices.

Lara Marie Oberkircher

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Preface

I would like to acknowledge the funding by The University of Melbourne in form of a Melbourne Research Scholarship (Fee Offset) and funding for the graduate school IRTG2168/1 and IRTG2168/2 by the DFG (German Research Society).

My contribution to the experiments within each chapter was as follows:

Chapter 3: 95 %

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Chapter 5: 60 %

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willing to wake up insanely early to catch up with me – it was a great meeting you and made me look forward a lot for my time in Melbourne! From that moment on you also joined all our meetings, giving input from another point of view – as a Microbiologist joining an Immunology project! And it was great that during my time here I could also obtain part of this expertise from another field of research. Additionally, you did not hesitate to invite all your lab to your beautiful country house giving me a first taste of the stunning Australian nature. Summing up: Big thanks to you, Liz, for jumping onto the boat of my Ph.D. journey!

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#AGGarbii #youneverworkalone #somuchlove

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#totaleclipseofmylung #partyshakersshakinglungs

#supportedbyBirthdayEscalation

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#it'sAmsterdambitches #dontwealllovesurprises #tikibarimwinter

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#raveinthemiddleofnoweher #pinkcaféforlunch #secondhome

But a Ph.D. is much more than science and work and especially as part of a graduate school you meet an uncountable number of amazing people:

- ♥ The first and biggest thank you gooooes to: The one and only **Marcii**! I remember the first time we talked: You were on the way to a date and needed a chewing gum – this nicely describes our friendship :D I remember the first time we went to Starbucks together – doing this amazing (yes because this is an official document here) mandatory online course about good scientific practice, four hours we will never get back but you made the time fly. I remember the moment when we realized we both love the BVB ♥♥ – sitting in the sun in front of, of course, Starbucks. I remember all the times you told me that talking always helps – and how right you are with this. I remember all the nice moments we had together – involving Tatort, plant shopping, soccer, Scotch&Soda, WG parties, electro dancing sessions, TastySumo, breakfast escalations, birthday escalations, a new tennis addict, and I could go on forever. I remember how scared and lonely I felt after our old friend Harry kicked me out of my room – and how you just stayed calm and said: ‘Let’s find a flat together’. I remember how we moved into 47 Caroline Street – and how it became our new home. I remember watching the sunset in Perth – and then running to get the best Pad Thai ever. I will remember writing this in tears – and I will always keep you as my best friend!

#supportedbyHarry #BlackandYellow #Scotchiiaddicts #creatorofκκΔ

#getthistattoonow #isittimeforthebowleagain? #Pluschiiefamily

#Jimmy+Donnnie+Hugo #Jacky’sgodparents #6tageammeer #it’scharliebaby

#Tiesto #therapycouch #pinkehölle #abenteuerland

#ginmarketakasuvivaltraining #zeitfüreinelixir #guessishouldstopnow

- ♥ **Anjii**, I was never good at making the first step when meeting new people – but I am glad I did it when I met you. And I am glad you really were new too, glad you really did not know anyone in Bonn yet, glad you really wanted to

become friends – glad we became best friends! God, we had sooo many cooking sessions, sooo many good drinks, sooo many great party nights, sooo many deep talks, sooo many relaxed TV nights, sooo many huge sushi boxes – and most important: We always had each other's back! During the COVID lockdown, during every crisis (afraid I had more of them than you :D), during the rainy penguin walk at Phillip Island, during the initial phase and the final phase of the Ph.D. – and everything in between. I can't wait to see what's still to come!

#bestfriendsatfirstsight #alterzollsummernights #makimangroßbestellung
 #ginmarketfirstedition #wievielevondenennkommendennoch?
 #yourfirstcarnival #kölschejung #rutunwiess #ginpalacetheplacetobe
 #crazydavy #couldnotwatch/understanddarkwithoutyou
 #venusbergwalks=therapywalks #fressorgieaufderautumnschool

- ♥ κκΔ – the best student union ever! We went to Cyprus together to celebrate **Marcii** and nothing was the same as before :D Thanks for this unforgettable trip to: **Lisa, Nina, Helena, Kostas, Patricia, Maria, and Rafa!** This trip was all about celebrating our birthday boy with good food (provided by a number of amazing Cypriot restaurants but also Rafa's family, this was so delicious!), and good drinks (learnt to love Cypriot gin), with trying not to freeze to death in the pool, trying not to laugh to death during Cards against Humanity, and trying not to drink to death when watching Pokemon. I will never forget this time (at least the parts I can remember) and I am looking forward to a reunion!

#κκΔ #icantfeelmybodyanymore #doesanyonecare? #aquaknarre
 #staryouakamindblown #seafoodfeast
 #coastalsunshinewalks #dumusstmehrnachrechts!
 #oopsiedavornehättenwirabbiegenmüssen #iwannabetheverbest
 #pinkfluffyunicorns #lookatmyhorse #donthavethe30inthephoto

- ♥ And now I can hardly think of a creative group name to describe my Australian crew :D Thanks to **Leonie, Lena and Alex, Andrea and Caro**, for being such amazing friends so far away from home. I will never forget how we celebrated my 27.5th birthday together (and how you only made a little bit of fun about me for that), how we met to celebrate our first AFL final day together (and how

hopefully nobody realized that I fell asleep), how we enjoyed the Australian Open together (and how you suffered under my non-existing tennis-knowledge), how we celebrated Christmas at the beach (and had an amazing photo session guided by Marcel and Caro), how we discovered together the backdoor-jungle of the best Thai restaurant in Melbourne (still remember when Marcel told us about it for the first time), how you joined the Gin Market experience (and how even those who hate Gin couldn't resist to buy three bottles), how we competed very well at HoleyMoley (and how you learned to love my favourite rooftop bar). I guess there is still so much more to come!

#notcreativeenough #weareGeelong #howaretheseplantsevengrowinghere?
 #kokkunderwater #summerchristmas=BBQchristmas #anyonewantsthehat?
 #likedyourpresibutitwastoopink #PadThaivs.PubFood #Idon'tlikeGin
 #fireshow

- ♥ And as I cannot go on forever: biiiig thanks to everyone at the IRTG, at the IMMMEI, and at the Hudson – guys, you are all incredible and it was an honour meeting you!

And of course it is also not only the people that you meet during the Ph.D. that help you on the journey – therefore I also want to thank all my amazing family and friends back home in Saarland:

- ♥ Schinkenschrippcher, of course this word could never be missing in my acknowledgments – **Julia and Sandra**, you are the longest and best friends I have. I cannot even remember a time when I did not know you! In all this time together we went on the best trips: to Barcelona (involving Marina and her melons), to London (where a cheap mask was so much better than some wax figures), to Stockholm (where a PJ saved one of us from freezing), to Amsterdam (where our angel was not yellow and only had one wing), to Lisbon (where we played detectives investigating a drug deal), to Paris (where they say merci a bit different than back home) and finally (but hopefully not the last one) to Australia – where we met Gerd and fell in love, where we went through heaven (aka the Blue Berry Farm) and hell (aka the Cottage or however you might call it), where we watched tennis together (cheering for

Yannick, the fucking man), where we enjoyed the wild life (kangaroos, koalas, dolphins, turtles, water dragons, even a, luckily dead, huge spider) – where we bonded even more! We have been forever and we will be forever the absolutely best friends – XOXO

- ♥ Jetzt kurz auf Deutsch – den größten Dank an meine Familie, die Oberkirchers und co.! Danke an **Mama, Papa und Lena**, danke an **Oma und Opa**, danke an **Markus und Birgit, Dagmar und Uli, Elisabeth und Alwin**. Danke dass ihr von Anfang an (bis auf Lenii :D) an meiner Seite wart, dass ihr immer an mich geglaubt und mich immer unterstützt habt (auch wenn mein Weg nicht Mathe war), danke dass ihr mich so oft in Bonn besucht habt (manchmal sogar spontan abgeholt), danke dass ihr immer fleißig meine Berichte aus Australien verfolgt und mich nicht vergessen habt, danke dass ihr während Corona mit mir Karneval gefeiert habt (obwohl ihr das gar nicht mögt), danke dass ihr mich so liebt wie ich bin – ihr seid die beste Familie, die man sich wünschen kann!

Chapter 1: Literature review

1.1. Lung function

In humans gas exchange is conducted via the lung as the primary respiratory organ (Ince, Pariollaud & Gibbs 2018; Guillot et al. 2013; Murray 2010). The main function of the lung is to allow oxygen uptake by and carbon dioxide egress from erythrocytes in the alveolar capillaries across a thin layer of squamous type I alveolar epithelial cells (AEC) (Guillot et al. 2013; Murray 2010). Most microorganisms and inert material are filtered while the air passes through the conducting airways (Ince, Pariollaud & Gibbs 2018; Murray 2010), including the nose, pharynx, larynx, trachea, bronchi and conducting bronchioles. Additionally, the air is warmed and moistened thereby preparing it for efficient oxygen uptake in the respiratory area, formed by terminal bronchioles and alveoli (Murray 2010). The alveoli are very small in diameter (< 2 mm) which, combined with their large number of around 100 million alveoli in a human lung, maximizes the surface area of the epithelium ($\approx 70\text{m}^2$) and, thereby, the oxygen uptake per breath (Murray 2010). In addition, cuboidal AEC type II cells (AECII) produce a complex mixture of lipoproteins collectively termed surfactant which, primarily, prevent the lung from collapsing by reducing the alveolar inner surface tension (Guillot et al. 2013; Murray 2010; Ince, Pariollaud & Gibbs 2018). They further contribute to surfactant homeostasis by producing GM-CSF (granulocyte-macrophage colony-stimulating factor) that promotes alveolar macrophages to remove excess surfactant (Sallese et al. 2017).

Gas exchange is enabled in the respiratory unit comprised of AECl and the surrounding network of pulmonary capillaries (Murray 2010; Guillot et al. 2013). The thin layer of AECl and the capillary-lining endothelium allow for gas diffusion and thereby oxygen uptake by the haemoglobin in the erythrocytes (Murray 2010).

The constant airflow through the lungs introduces not only fresh air but also potentially harmful environmental particles and pathogens. To defend the body against those challenges, the lungs need to fulfill an important barrier function including the presence of tissue resident immune cells as well as autonomous

defence mechanisms of the lung epithelium (Murray 2010; Ince, Pariollaud & Gibbs 2018; Rohmann et al. 2011). The immune response will be explained with a focus on the role during bacterial pneumonia.

1.1.1. Immune response in the lung

1.1.1.1. Bronchiolar epithelial cells

Stromal cells such as the lung epithelium can serve as a critical barrier fulfilling a range of protective roles. As the main conducting airways in the lung, bronchioles form a barrier between air and lung interstitium before the former reaches the alveoli. The bronchiolar epithelium is comprised of three differentiated epithelial cell types, with all of them fulfilling different protective functions: mucus-producing goblet cells, ciliated epithelial cells (ciEpC) and secretory club cells that release anti-microbial peptides (AMP) (Leiva-Juárez, Kolls & Evans 2018).

Goblet cells and ciliated cells act together to confer protection by a process termed **mucociliary clearance** (Whitsett & Alenghat 2014). The mucus produced by goblet cells traps pathogens, thereby limiting their spread. The two major pulmonary mucus proteins are Muc5AC and Muc5B. The former is upregulated in allergic reactions (Thai et al. 2008; Bonser & Erle 2017) whereas Muc5B is mainly induced during bacterial infection (Thai et al. 2008; Roy et al. 2014). Once pathogens are trapped by mucus, they can be expelled from the airways by upward beating of the cilia (Whitsett & Alenghat 2014).

In addition to the mucociliary clearance, the airway epithelium contributes to the host defence against infections by club cells producing a wide array of **anti-microbial peptides** (Leiva-Juárez, Kolls & Evans 2018). There are different peptides that are involved in the defence against bacterial pneumonia including defensins, like murine β -defensin-1 and -3 (mBD-1 and -3) (Bals et al. 1999; Moser et al. 2002), the catechilidin mCRAMP (murine catechilidin-related AMP) (Kovach et al. 2012), and lysozyme M (Shimada et al. 2008). Recently, it was shown that enteric neuronal-derived Interleukin-18 (IL-18) can confer anti-microbial protection via an epithelial cell-autonomous mechanism in the intestine

(Jarret et al. 2020). This protective process involves stimulation of the IL-18R on intestinal club cells inducing a subsequent upregulation of AMP-production.

In addition to epithelial cell-autonomous mechanisms, lung epithelial cells can **communicate with the immune system** (Whitsett & Alenghat 2014). It was recently shown by the Shin lab that AECII can act as intermediary signal transducers between alveolar macrophages and downstream myeloid cells (Liu et al. 2020). The group revealed defence against *L. pneumophila* to require such an intermediary signalling: a novel IL-1R⁺ AECII-dependent pathway in which AM-derived IL-1 α induces AECII to secrete GM-CSF resulting in an elevated production of pro-inflammatory cytokines by infiltrating monocytes. The intermediary signalling of epithelial cells, thereby, served to enhance protective immune stimulation of bystander myeloid cells by a low number of infected AM. In this context, bronchiolar epithelial cells can secrete a broad range of inflammatory cytokines including GM-CSF (Willart et al. 2012; Liu et al. 2020), CCL5 (CC-motif ligand 5) (Thomas et al. 1998; Gern et al. 2003), CXCL10 (CXC-motif ligand 10) (Sauty et al. 1999; Frangogiannis et al. 2000), and IL-8 (DiMango et al. 1995; Alcorn et al. 2001; Casola et al. 2000; Standiford et al. 1990).

1.1.1.2. Recognition of bacterial pathogens

If the epithelial cell-autonomous defence mechanisms of the lung fail to prevent a bacterial infection, the immune system will become activated. Recognition of invading pathogens via pattern recognition receptors (PRR) is the first step to induce a successful immune response. PRRs detect conserved motifs, so-called pathogen-associated molecular patterns (PAMP), and are located in specific locations of the cell both on immune cells like AM and dendritic cells as well as on stromal cells including epithelial cells (Moldoveanu et al. 2009; Kaisho & Akira 2006; Leiva-Juárez, Kolls & Evans 2018). PRR can be subdivided further dependent on their respective activating ligand: Toll-like receptors (TLR), NOD (nucleotide-binding oligomerization domain)-like receptors (NLR), and cytosolic nucleic acid receptors such as RIG-I (retinoic acid-inducible gene-I)-like receptors (RLR) and cGAS (cyclic GMP-AMP synthase) (Rohmann et al. 2011).

The human organism expresses ten different TLRs with each of them recognizing a distinct PAMP including microbial lipoproteins (TLR1/2), double stranded RNA (ribonucleic acid) (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), and CpG-rich DNA (deoxyribonucleic acid) (TLR9) (Moldoveanu et al. 2009; Menendez & Torres 2007). Signal transduction downstream of TLRs involves MyD88 (myeloid differentiation primary response 88)-NF- κ B (nuclear factor- κ B) or TRIF (TIR domain-containing adaptor protein)-IRF (Interferon-regulating factor) pathways (Kaisho & Akira 2006) enhancing expression of pro-inflammatory genes, like cytokines, chemokines, and AMP, as well as upregulating major histocompatibility complex (MHC) receptors and co-stimulatory molecules involved in antigen presentation (Moldoveanu et al. 2009; Leiva-Juárez, Kolls & Evans 2018).

NLRs include NLR proteins (NLRP) and the non-NLRPs AIM2 and Pyrin, that are components of the inflammasome, a multi-protein complex (Vladimer et al. 2013). The inflammasome is required to translate an infectious stimulus into production of the two pro-inflammatory cytokines IL-1 β and IL-18 as well as the induction of pyroptosis, the inflammatory form of regulated cell death (Moldoveanu et al. 2009; Vladimer et al. 2013). Mechanistically, a NLRP senses its specific PAMP which leads to oligomerization, recruitment of the adaptor protein Asc, and finally activation of caspase-1 (Barksby et al. 2007; Rohmann et al. 2011; Vladimer et al. 2013). Caspase-1 cleaves gasdermin D to form pores in the cell membrane leading to regulated death of infected cells as well as causing release of biologically active IL-1 β and IL-18 (Barksby et al. 2007; Rohmann et al. 2011; Weber, Wasiliew & Kracht 2010; Vladimer et al. 2013). Therefore, the secretion of IL-1 β is regulated via a two signal-process (van de Veerdonk et al. 2011). The first signal resulting in transcription and translation of pro-IL-1 β and is initiated by TLR stimulation (Netea et al. 2009) or via a feedback loop by IL-1 β (Warner, Auger & Libby 1987; Granowitz et al. 1992). The second signal is the assembly of the inflammasome and subsequent caspase-1 activation leading to the cleavage of proIL-1 β into its biologically active form and its subsequent

secretion (Sansonetti et al. 2000; Mariathasan et al. 2005; Moayeri et al. 2010; Netea et al. 2009; Greten et al. 2008).

Cytosolic nucleic acid receptors are important for type-I IFN (interferon) production, most importantly but not only during antiviral immunity. Amongst these receptors RIG-I-like receptors and cGAS are the most prominent, detecting non-self RNA and cytosolic DNA, respectively, thereby, providing a fast IFN-I response during infection (Lee, Chiang & Gack 2019; Ni, Ma & Damania 2018).

Pathogen recognition leads to production of a variety of inflammatory cues ultimately resulting in the activation of a cellular immune response (Moldoveanu et al. 2009). In general, a successful immune response requires the onset of both the innate and the adaptive immune system. The innate immunity confers protection against intruding pathogens by providing an immediate unspecific response while the adaptive immunity requires time to be activated but then offers highly pathogen-specific protection that can, upon secondary re-infection, be rapidly activated via generation of memory immune cells (Andersen et al. 2006). Both branches of the immune system do not exist in isolation but interact with each other: While antigen-presenting innate immune cells (APC) are required to stimulate adaptive immune cells, those can, in return and subsequent to activation, secrete inflammatory cytokines enhancing the anti-microbial capacity of innate immune cells (Andersen et al. 2006). One cytokine promoting the switch from innate to adaptive immunity is the pleiotropic cytokine IL-6 (Moldoveanu et al. 2009; Rose-John 2020).

1.1.1.3. Innate immune response

Alveolar macrophages are the most important tissue resident immune cell population in the lung. AM are phagocytes that patrol the luminal side of alveoli constantly probing the air for potentially harmful particles and pathogens (Garbi & Lambrecht 2017; Kopf, Schneider & Nobs 2015). Their development is strongly regulated via the pro-inflammatory growth factor GM-CSF (Chen, Mueller & Chou 1988; Guilliams et al. 2013; Schneider et al. 2014) derived from AECII (Blau et al. 1994; Sheih, Parks & Ziegler 2017; Cakarova et al. 2009). During homeostasis, AM mainly show an anti-inflammatory phenotype with a high

capacity for self-renewal. On the other hand, during infection they can acquire a pro-inflammatory phenotype resulting in the expression of inflammation-related genes (Zhu et al. 2021; Garbi & Lambrecht 2017). Phagocytosis and recognition of pathogens by AM activates inflammatory pathways resulting in the expression of cytokines and chemokines required to recruit and subsequently activate immune cells (Hussell & Bell 2014; Steinmüller et al. 2000; De Filippo et al. 2013). For example, AM secrete GM-CSF which during infection promotes the recruitment of bystander leukocytes such as neutrophils (De Alessandris et al. 2019; Weisbart, Golde & Gasson 1986) and monocytes (Hamilton 2019; Zhan et al. 1998). In addition, AM express direct effector mechanisms to kill internalized pathogens, including production of reactive oxygen species (ROS) and nitric oxide (NO) (Steinmüller et al. 2000) which can be stimulated again by GM-CSF (Coleman et al. 1988) as well as $\text{TNF}\alpha$ (tumour necrosis factor α) (Flynn et al. 1995), and $\text{IL-1}\beta$ (Yamada et al. 2000). Nevertheless, it is known that certain pathogens, including *L. pneumophila*, have developed strategies to evade degradation after internalization by AM (Mondino et al. 2020). In case the effector mechanisms of AM fail to control intracellular bacterial infections, $\text{TNF}\alpha$ can further limit replication of intracellular bacteria by inducing apoptosis in AM and thereby eliminating the bacterial host (Keane et al. 1997; Wong & Goeddel 1986).

Among the innate immune cells infiltrating the lung from the circulation during infection, two major types are neutrophils and monocytes. **Neutrophils** are highly abundant in the blood and bone marrow, and thereby rapidly react upon microbial invasion (Döhrmann, Cole & Nizet 2016; Moldoveanu et al. 2009). Neutrophils infiltrate infected tissues via stimulation of the highly expressed chemokine receptors CXCR1 (CXC-motif ligand receptor 1) and CXCR2 through release of receptor-specific chemokines, including CXCL1 and CXCL2, after pathogen recognition by e.g. epithelial cells (De Filippo et al. 2013). In humans, neutrophils are mainly recruited by CXCL8-CXCR1/2-interaction (Zeilhofer & Schorr 2000; Remick 2005) while mice lack a homologue of this chemokine (Remick 2005; Mukaida 2003). CXCL8 or alternatively termed IL-8 can be produced upon infection by a range of cell types including AM (Strieter et al. 1990), bronchiolar epithelial cells (Alcorn et al. 2001; Standiford et al. 1990), and also neutrophils

themselves (Braun et al. 1993). During pneumonia, the pleiotropic cytokine IL-6 first promotes recruitment and transmigration of neutrophils (Hoge et al. 2013; Romani et al. 1996; Modur et al. 1997; Rabe et al. 2008) via induction of e.g. IL-8 (Hurst et al. 2001) while later, in order to promote the switch from innate to adaptive immunity, IL-6 induces apoptosis in neutrophils (Chalaris et al. 2007).

Important anti-microbial mechanisms of neutrophils are to phagocytose and subsequently kill internalized bacteria via production of NADPH (nicotinamide adenine dinucleotide phosphate)-dependent ROS (Nguyen, Green & Mecsas 2017; Moldoveanu et al. 2009; Manda-Handzlik & Demkow 2015) or nitric oxide synthase-dependent NO (Manda-Handzlik & Demkow 2015). In addition, neutrophils can release DNA into the extracellular space to trap invading pathogens in NET (neutrophil extracellular traps) structures. Extracellular pathogens can, furthermore, be killed by neutrophil degranulation and release of AMP and enzymes (Döhrmann, Cole & Nizet 2016). In addition to the chemotactic activity, IL-8 can enhance the effector function of mainly neutrophils. It can boost their microbial killing capacity (Nibbering et al. 1993) by priming (Nibbering et al. 1993; Thelen et al. 1988) and directly stimulating (Jones et al. 1996) ROS production as well as inducing degranulation (Schröder et al. 1987) and the release of neutrophilic elastases (Webb et al. 1993).

Two other classical pro-inflammatory cytokines stimulating effector functions of neutrophils are $\text{TNF}\alpha$ and $\text{IL-1}\beta$ secreted by AM (Fiorentino et al. 1991; McNab et al. 2014; Greten et al. 2008; Perkins et al. 2015), monocytes (Takashima et al. 1990; de Waal Malefyt et al. 1991; Novikov et al. 2011), and neutrophils (Fäldt, Dahlgren & Ridell 2002; Cassatella et al. 1993). $\text{TNF}\alpha$ has strong anti-bacterial effects (Rothe et al. 1993; O'Brien et al. 1999; Skerrett et al. 1997; Crum, Lederman & Wallace 2005) by promoting bacterial phagocytosis (Collins & Bancroft 1992; Roilides et al. 1998; Rainard et al. 2000) and subsequent bacterial killing via ROS production in neutrophils (Nathan 1989; Wiemer et al. 2010; Roilides et al. 1998; Rainard et al. 2000). $\text{IL-1}\beta$ exerts strong anti-bacterial functions (Mayer-Barber et al. 2010, 2014, 2011; Yamada et al. 2000; Juffermans et al. 2000) by e.g. promoting production of ROS in neutrophils (Di Paolo et al. 2015).

It is becoming clear that production of ROS is a key effector function of neutrophils during bacterial pneumonia. NADPH oxidase-dependent production of anti-microbial ROS in neutrophils occurs in two steps: priming and activation of the NADPH oxidase complex (Döhrmann, Cole & Nizet 2016). During homeostasis there is a local separation of the five NADPH oxidase components: The heterodimer consisting of the gp91^{phox} electron transferase and the p22^{phox} regulatory subunit are located within intracellular membranes while the regulatory trimer p40^{phox}/ p47^{phox}/ p67^{phox} resides in the cytosol (Nunes, Demareux & Dinauer 2013). The local separation prevents spontaneous activation to limit tissue damage in homeostasis (Döhrmann, Cole & Nizet 2016). During the priming step, expression of NADPH oxidase components is enhanced (Marchi et al. 2014) and the regulatory complex is activated via phosphorylation (Brown et al. 2004; Guichard et al. 2005; Dang et al. 2006). Subsequently, the recruitment of the regulatory trimer to the plasma membrane brings it into closer contact with the NADPH oxidase heterodimer (Guichard et al. 2005). Priming of the NADPH oxidase can be driven by pro-inflammatory cytokines including IFN- γ (Marchi et al. 2014), TNF α (Brown et al. 2004; Dang et al. 2006), GM-CSF (Dang et al. 2006; Yuo et al. 1990; Nathan 1989) and, IL-8 (Guichard et al. 2005; Thelen et al. 1988). After priming, the actual consumption of oxygen (O₂) to generate the highly reactive superoxide anion (O₂⁻), termed oxidative burst, is easily triggered by a variety of ligands including the bacterial-peptide analogue fMLP (N-Formyl methionine-leucyl-phenylalanine) as well as ligands of integrin and Fc receptors (Döhrmann, Cole & Nizet 2016) or via further stimulation by pro-inflammatory cytokines such as GM-CSF (Yuo et al. 1990; Nathan 1989; Kowanko et al. 1991), TNF α (Nathan 1989; Wiemer et al. 2010; Roilides et al. 1998; Rainard et al. 2000), IL-8 (Jones et al. 1996), and IL-1 β (Di Paolo et al. 2015).

Monocytes, next to neutrophils, make up a vast proportion of tissue infiltrating innate immune cells during pneumonia. In mice, monocytes can be classified as inflammatory (Ly-6C^{high} CCR2^{high}) and resident (Ly-6C^{low} CX3CR1^{high}) (Lauvau, Loke & Hohl 2015). During bacterial infections, Ly-6C^{high} (Lymphocytes antigen 6 complex locus C) bone marrow monocytes are promptly recruited to the infected tissue by chemokines, mainly CCR2-ligands, such as CCL2, CCL7, and

CCL12 (Serbina & Pamer 2006). CCL2 is a well-known chemotactic agent for monocytes (Matsushima et al. 1989; Jia et al. 2008) being produced by a range of cell types including macrophages (Kitamura 1997; Fantuzzi et al. 2003) and monocytes themselves (Mengozi et al. 1999; Ansari, Meyer-Olson & Schmidt 2013). Next to these classical monocyte-specific chemokines, the recruitment of their can be further promoted by CCL5 and CXCL10, two chemokines traditionally known to recruit T cells (Schall et al. 1990; Schlecker et al. 2012; Taub et al. 1993; Lloyd et al. 1996).

After infiltration, monocytes contribute to innate immunity by cytokine secretion, including $\text{TNF}\alpha$ (Serbina et al. 2003; Kang et al. 2008), that leads to activation of bystander immune cells. In addition, monocytes participate in the anti-microbial defence by antigen-presentation (Cheong et al. 2010) and direct microbiocidal activities (Lauvau, Loke & Hohl 2015) including production of NO (Serbina et al. 2003; Kang et al. 2008) and ROS (Narni-Mancinelli et al. 2007) which are promoted by $\text{IFN-}\gamma$ (Kang et al. 2008; Soudja et al. 2014) or MyD88-dependent via IL-1R or TLR stimulation (Serbina et al. 2003). During the switch from innate to adaptive immunity IL-6 initially promotes neutrophil-recruitment, but, subsequently, drives monocyte-recruiting chemokines like CCL2 (Dube et al. 2004; Romano et al. 1997) as well as the growth factor M-CSF in order to induce their differentiation into macrophages (Romani et al. 1996; Chomarat et al. 2000). In this way, IL-6 supports antigen presentation and the subsequent induction of the adaptive immunity (Moldoveanu et al. 2009; Rose-John 2020).

Besides phagocytes, also lymphocytes, such as **NK cells** (natural killer cells), contribute to the innate immunity. NK cells in mice can be classified into four subgroups according to their maturation state depending on expression of CD27 (cluster of differentiation 27) and CD11b (Cong & Wei 2019). These cells can become activated after recognizing targets with decreased MHC class I (MHC-I) expression, enhanced expression of activating ligands such as CD16 (Lanier 2005; Cong & Wei 2019), or in a bystander fashion by a variety of pro-inflammatory cytokines including IL-2, IL-12, and IL-18 (Walzer et al. 2005; Ni et al. 2012). After activation, NK cells become protective either by directed cytotoxicity or by secretion of immune mediators such as $\text{IFN-}\gamma$ (Cong & Wei

2019). Taking into account these functions, NK cells were originally thought to mainly play a role in anti-viral defence but there is increasing evidence that they confer protection against bacterial infection (Cong & Wei 2019). Studies on *Mycobacterium tuberculosis* (Junqueira-Kipnis et al. 2003; Feng et al. 2006) and *Klebsiella pneumoniae* showed NK cells to be important producers of IFN- γ during bacterial pneumonia (Ivin et al. 2017). Using *M. tuberculosis* indicated that NK cells fulfill a redundant protective role together with T cells as only in T cell-deficient mice NK cell-depletion led to an impaired anti-bacterial defence (Junqueira-Kipnis et al. 2003; Feng et al. 2006).

1.1.1.4. Adaptive immune response

Adaptive immunity is driven by rearranged receptors that bear exquisite specificity for a given antigen. Accordingly, it can be subdivided into humoral adaptive response by antibody-producing B cells and cellular adaptive response by T helper (Th) cells and cytotoxic T lymphocytes (CTL) (Andersen et al. 2006). Following generation in the thymus, naïve T cells express the T cell receptor (TCR), to recognize MHC-bound peptide antigens (Moss, Rosenberg & Bell 1992). MHC molecules can be subdivided in two distinct classes. MHC-I is constitutively present on all nucleated cells and interacts with CD8 as a co-receptor. MHC-II, on the other side, is constitutively expressed only on so-called professional APC, including dendritic cells, macrophages, and B cells, but can be upregulated on a range of cell types, and interacts with CD4 as co-receptor (Andersen et al. 2006). This explains the distinct functions of CD8⁺ CTL and CD4⁺ Th cells. CTL kill infected or transformed cells that present the respective cytosolic peptides onto MHC-I, whereas Th cells recognize peptides generated in the endolysosomal compartment bound to MHC-II. In response, Th cells produce cytokines to stimulate antibody-production by B cells as well as effector functions of CTL and innate immune cells upon recognition of cells infected with extracellular or vesicular pathogens (Andersen et al. 2006; Berger 2000; Moldoveanu et al. 2009).

Th cells can be further subdivided into different subpopulations according to their transcription factor and cytokine secretion profile (Zhu & Paul 2010).

Cytokines produced by Th1 cells include IFN- γ (Berger 2000; Moldoveanu et al. 2009) that, as mentioned before, is a potent stimulator of microbiocidal functions of innate leukocytes such as neutrophils and monocytes. Again, IL-6 fulfills important functions in stimulating the onset of the adaptive immune response by promoting the expression of T cell-specific chemokines (CCL4, -5, -11, -17, CXCL10) (McLoughlin et al. 2005), the expression of their according receptors (CCR3, -4, -5, CXCR3) on T cells (McLoughlin et al. 2005), as well as subsequent T cell differentiation (Sofi et al. 2009; Rincón et al. 1997; Bettelli et al. 2006).

In addition to the classical adaptive function, T cells can further contribute to immunity in an innate-like fashion. For instance, phagocyte-derived IL-12 or IL-18 can instruct NK cells and T cells to produce IFN- γ independent of antigen-specific TCR activation (Bohn et al. 1998; Lauw et al. 1999; Neighbors et al. 2001; Kinjo et al. 2002; Mastroeni et al. 1999).

1.1.1.5. Interleukin-18

IL-18 was originally recognized as a factor inducing IFN- γ secretion mainly from activated macrophages (Bohn et al. 1998; Okamura et al. 1995; Nakamura et al. 1989). Later studies demonstrated that, in addition to different macrophage types, including alveolar macrophages, also non-immune cells, such as epithelial cells, can produce IL-18 (Nakanishi et al. 2001a; Ferrero et al. 2020; Jarret et al. 2020). Production of IL-18 is required for defence against a variety of bacterial pathogens (Sansonetti et al. 2000) by inducing not only Th1 but also Th2 responses as well as direct effects on a range of effector functions in innate immune cells (Vecchié et al. 2021; Boraschi et al. 2018).

IL-18 is expressed as an inactive pro-protein transferred into its biologically active form by proteolytical cleavage catalysed by caspase-1 (Ghayur et al. 1997). Caspase-1 is mainly activated via inflammasome pathways (Arend, Palmer & Gabay 2008) which also induce cleavage of gasdermin D resulting in plasma membrane pores required for release of IL-18 in a process similar to IL-1 β (He et al. 2015). In addition, a caspase-1- and inflammasome-independent cleavage of IL-18 has been reported that involves Fas-signalling (Tsutsui et al. 1999). In this study, treatment of macrophages with *Propionibacterium acnes* and

IFN- γ induced the expression of functional Fas and subsequent treatment with Fas ligand (FasL) drove secretion of mature IL-18, in both WT (wildtype) and *caspase1*^{-/-} macrophages (Tsutsui et al. 1999).

The functional heterodimeric IL-18 receptor (IL-18R) combines a ligand-binding α -chain (IL-18R1) and a signal-inducing β -chain (IL-18R β) (Torigoe et al. 1997; Born et al. 1998; Vecchié et al. 2021). IL-18R1 is constitutively expressed on certain cell types in contrast to the strictly regulated IL-18R β (Boraschi et al. 2018). Pro-inflammatory cytokines like IL-12 or IL-2 can upregulate IL-18R β expression whereas IL-4 downregulates it (Boraschi et al. 2018). During inflammation, the IL-18R complex is expressed on a variety of immune and non-immune cells with NK cells and Th1 cells being main expressors as well as epithelial cells to a lesser extent (Boraschi et al. 2018).

Even though, IL-18 was originally discovered to induce IFN- γ production by Th1 cells, it can also stimulate Th2 responses. Both effects, on Th1 and Th2 cells, are dependent on co-stimulation with other cytokines (Vecchié et al. 2021; Nakanishi et al. 2001b). IL-18 together with IL-12 triggers the production of large amounts of IFN- γ from Th1 cells independent of an additional antigen-specific TCR stimulation (Bohn et al. 1998; Lauw et al. 1999; Neighbors et al. 2001; Kinjo et al. 2002; Mastroeni et al. 1999; Nakanishi et al. 2001b). In contrast, combination of IL-18 with IL-2 can induce secretion of classical Th2 cytokines such as IL-4 and IL-13 from Th2 cells (Hoshino, Wiltout & Young 1999; Kinoshita, Kuranaga, et al. 2006; Leite-de-Moraes et al. 2001; Yoshimoto et al. 2000). The effect of IL-18 on Th2 cells can be further enhanced by TCR stimulation (Nakanishi et al. 2001b). Both responses can also be elicited in NK cells: treatment of NK cells with IL-12 and IL-18 promotes IFN- γ (Lertmemongkolchai et al. 2001; Kinoshita, Kuranaga, et al. 2006) whereas NK cells treated with IL-2 and IL-18 produce IL-4 and IL-13 (Hoshino, Wiltout & Young 1999; Kinoshita, Kuranaga, et al. 2006).

In addition to its influence on type-1 and -2 T cell responses, IL-18 upregulates I-CAM1 (intracellular adhesion molecule 1) and V-CAM1 (vascular cell adhesion molecule 1) on endothelial cells and thereby promotes adhesion of monocyte-like cells to the endothelium *in vitro* (Morel, Park, Woods, et al. 2001). As further pro-

inflammatory effects, it stimulates expression of TNF α (Neighbors et al. 2001; Leung et al. 2001) and a range of chemokines, including IL-8 (Morel, Park, Kumar, et al. 2001; Leung et al. 2001) and CCL2 (Amin et al. 2007). In addition, IL-18 can directly stimulate leukocyte effector functions including ROS production (Leung et al. 2001; Liao et al. 2021) and NET formation (Liao et al. 2021) by neutrophils, NO production by macrophages (Neighbors et al. 2001), as well as FasL- (Tsutsui et al. 1996) and perforin-dependent (Hyodo et al. 1999) NK cell cytotoxicity.

Taken together, IL-18 evokes a strong anti-bacterial activity especially towards intracellular bacteria (Vecchié et al. 2021) including *Burkholderia pseudomallei* (Lertmemongkolchai et al. 2001), *Mycobacterium tuberculosis* (Sugawara et al. 1999; Kinjo et al. 2002), *Listeria monocytogenes* (Lertmemongkolchai et al. 2001; Neighbors et al. 2001), and *Salmonella typhimurium* (Mastroeni et al. 1999; Dybing, Walters & Pascual 1999).

Despite the described multi-faceted mechanisms, it is generally accepted that IL-18 fulfils its immune functions mainly by induction of IFN- γ and subsequent enhancement of microbiocidal functions of myeloid cells (Nakanishi et al. 2001a; Ellis & Beaman 2004; Schroder et al. 2004) illustrated in Figure 1. IFN- γ stimulates both the receptor-mediated phagocytosis and the microbial killing capacity by induction of NO and ROS production as well as up-regulation of lysosomal enzymes (Ellis & Beaman 2004; Schroder et al. 2004; Marchi et al. 2014) and has been shown to be a key anti-bacterial cytokine stimulating for example the defence against *L. pneumophila* (Brown et al. 2016) and *L. longbeachae* (Massis et al. 2017).

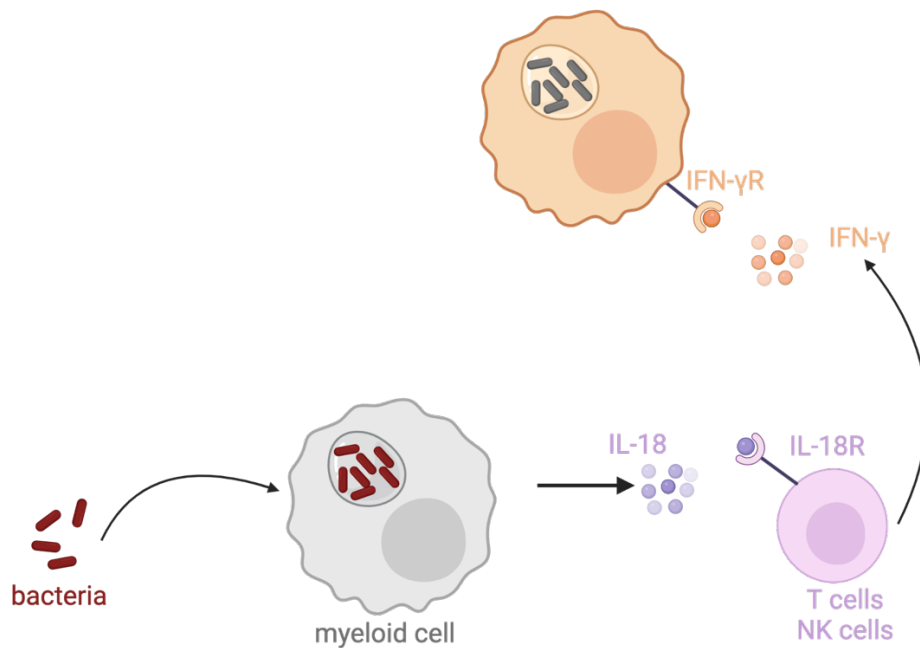


Figure 1: Schematic illustration of the generally expected canonical pathway of IL-18-dependent anti-bacterial defence.

Bacteria (red) are taken up by myeloid cells which induces secretion of IL-18. Subsequently, IL-18 stimulates its receptor on NK cells and/ or T cells and thereby instructs downstream secretion of IFN- γ . IFN- γ can bind to its receptor on myeloid cells and enhance their anti-microbial capacity. IL-18 = Interleukin-18, IFN- γ = Interferon- γ , IFN- γ R = IFN- γ receptor.

1.2. *Legionella*

Legionella spp. (species) include over 50 species of environmental gram-negative bacilli first identified in 1976 after an American Legion convention. Following this convention a number of attendees suffered from severe pneumonia and *Legionella* was discovered as the causing pathogen (Newton et al. 2010; Mondino et al. 2020). *Legionella* spp. are mainly known for inducing this so-called Legionnaire's disease (LD), a rare but severe form of bacterial pneumonia. The symptoms of infection with bacteria of these species range from mild non-pneumonic Pontiac fever to pneumonic LD which can ultimately lead to multi-organ failure (Fields, Benson & Besser 2002). The most common causative of LD is *L. pneumophila*, causing approximately 95 % of all cases in Europe and the US. In addition, *L. longbeachae* causes about 50 % of all cases of LD in Australia, New Zealand and East Asia (Whiley & Bentham 2011; Mondino et al. 2020).

Even though symptomatic infection with *Legionella* spp. are rare they still have a strong impact especially in susceptible individuals including immunocompromised patients (Cunha, Burillo & Bouza 2016; Chambers et al. 2021). And taking into account the rising trend of *L. longbeachae* in the northern hemisphere (Chambers et al. 2021), an until now highly under-diagnosed and under-investigated pathogen (Cunha, Burillo & Bouza 2016; Chambers et al. 2021), further strengthens the necessity of more extensive research towards *L. longbeachae*.

1.2.1. Epidemiology of *Legionella*

1.2.1.1. Natural hosts and human transmission

Legionella spp. are environmental bacteria that naturally colonize amoeba and infections of human hosts occur accidentally via inhalation of infectious aerosols. Human infections are apparently a dead-end infection as human-to-human transmission is believed to not have a relevant impact on bacterial spread (Cunha, Burillo & Bouza 2016; Mondino et al. 2020). The vast majority of *Legionella* spp., including *L. pneumophila*, colonize aquatic protozoa and human infections occur related to contaminated water resources, like air conditioning cooling towers or water pipes (Cunha, Burillo & Bouza 2016; Mondino et al. 2020). In contrast, the natural habitat of *L. longbeachae* is not aquatic but associated to soil, and infections are mainly related to contaminated potting mixes and compost (Steele, Lanser & Sangster 1990; Newton et al. 2010; Mondino et al. 2020). The variations in the natural habitat result from differences in the genome as *L. longbeachae* encodes for a range of products enabling the bacteria to degrade plant material and to parasitize plant protozoa, instead of aquatic protozoa (Cazalet et al. 2010). Furthermore, the different compositions of potting mixes in Europe and Australia might explain the different incidences between the two hemispheres (Whiley & Bentham 2011). In Australia potting mixes mainly consist of pine needles which serve as an important ecological niche for *L. longbeachae* (Chambers et al. 2020; Mondino et al. 2020).

As *L. pneumophila* is the main causative agent of LD in the global context, it has been the subject of most research on *Legionella* and will be the focus during the following description of general aspects.

1.2.1.2. *Legionella* life cycle

Bacteria of the *Legionella* spp. naturally replicate within phagocytic amoeba and use this capacity to turn mammalian macrophages into host cells. It is well understood that *L. pneumophila* are inhaled in aerosols and then phagocytosed by AM to cause disease (Isberg, O'Connor & Heidtman 2009; Newton et al. 2010; Mondino et al. 2020). After phagocytosis, bacteria use their natural capability for intracellular replication to turn the host cell into a replicative niche (Mondino et al. 2020). Using a Type-IV secretion system (T4SS), namely Dot/ Icm (defect-in-organelle-trafficking/ intracellular-multiplication), *L. pneumophila* translocate a variety of virulence factors, termed effector proteins, into the macrophage cytoplasm with the event of phagocytosis being essential for the onset of translocation (Charpentier et al. 2009). The Dot/ Icm system is particularly conserved between distinct *Legionella* spp. underpinning its importance for intracellular replication. Nevertheless, the translocated effector proteins differ largely between species (Gomez-Valero et al. 2019; Cazalet et al. 2010).

These proteins are required for different processes crucial to the establishment of an intracellular infection, including the formation of a *Legionella*-containing vacuole (LCV). In this vacuole the bacteria are able to replicate while being protected from phagocytic degradation (Swart & Hilbi 2020) and it was demonstrated that establishment of the LCV is crucial for bacterial replication (Swanson & Isberg 1995; Tilney et al. 2001; Kagan & Roy 2002; Mondino et al. 2020). *L. pneumophila* temporarily prevents acidification of the LCV early after infection by binding of the secreted effector SidK to and thereby blockage of the vacuolar ATPase proton pump (Xu et al. 2010). The blockage of the vacuolar ATPase creates a pH neutral environment required for efficient replication (Chauhan & Shames 2021). Nevertheless, at later stages bacterial replication requires acidification of the LCV and bacteria are able to develop an acid-resistant phenotype (Sturgill-Koszycki & Swanson 2000).

In addition to the regulation of the pH within the LCV, *L. pneumophila* effectors inhibit fusion of the vacuole with lysosomes by blocking endocytic maturation (Chauhan & Shames 2021). One example is VipD, a secreted phospholipase A1, that catalyses depletion of the phospholipid PI(3)P (Phosphatidylinositol 3-phosphate) (Gaspar & Machner 2014), in turn, inhibits recruitment of fusion factors (Kutateladze 2007) and, thereby, prevents lysosomal fusion (Chauhan & Shames 2021).

To further protect the developing LCV, *L. pneumophila* effectors promote membrane fusion between the LCV and host cellular membranes. One effector protein well-known for its involvement in the establishment of the LCV is SidM, a GDP-GTP exchange factor (GEF) (Murata et al. 2006; Ingmundson et al. 2007). SidM attracts and activates Rab1 (Ras-associated binding 1), a small GTPase, to the early plasma membrane-derived LCV in order to recruit secretory vesicles (Murata et al. 2006; Ingmundson et al. 2007; Machner & Isberg 2006). Then, SidM in concert with Rab1 mediates the interaction between plasma membrane-derived syntaxin proteins and secretory vesicle-derived SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins initiating membrane fusion (Arasaki, Toomre & Roy 2012; Dorer et al. 2006). This membrane fusion helps the LCV to establish features of the secretory compartments in order to avoid lysosomal fusion and degradation (Celli & Tsolis 2015; Arasaki, Toomre & Roy 2012) as well as to enable subsequent fusion with the ER membrane (Tilney et al. 2001; Robinson & Roy 2006; Kagan & Roy 2002).

This second fusion event is mediated by another secreted effector GEF namely RalF that navigates recruitment and activation of ARF1 (ADP ribosylation factor 1) (Nagai et al. 2002) required for membrane fusion between the developing LCV and the ER membrane, an essential process to establish the LCV as a replicative niche (Kagan & Roy 2002). This fusion explains the location of ER-derived ribosomal structures found on the surface of the LCV (Horwitz 1983; Tilney et al. 2001; Robinson & Roy 2006). The membrane of the replication vacuole formed by *L. longbeachae* also shows ER-like features even though these bacteria induce different LCV maturation processes involving late endosomal vesicles instead of secretory vesicles (Asare & Abu Kwaik 2007).

L. pneumophila is not only able to form an ER membrane-like vacuole but also features many ER-localised host factors including many protein-folding chaperons and degradation factors. The recruitment of components of the ER-associated degradation (ERAD) to the LCV mediates translocation of proteins into the cytoplasm for turnover by the host proteasomal degradation apparatus (Celli & Tsolis 2015). For example, the effector protein AnkB activates proteasomal protein degradation leading to the generation of amino acids as an essential nutrient source for *L. pneumophila* (Price et al. 2011).

Taken together, it becomes clear that *L. pneumophila* is able to manipulate ER pathways to establish an ER-like replicative niche (Alshareef, Hartland & McCaffrey 2021). In addition to hijacking the secretory pathway to establish its intracellular replication vacuole, *L. pneumophila* is likely to drive a plethora of cellular stress responses and immune signalling cascades. For example, *L. pneumophila* actively inhibits the onset of the unfolded protein response (UPR) within the macrophage host (Treacy-Abarca & Mukherjee 2015; Hempstead & Isberg 2015). The UPR or ER stress response is a cellular signalling pathway stimulated by the conglomeration of un- or misfolded proteins within the ER.

1.2.1.3. ER stress during *Legionella* infection

The ER fulfills a range of essential cellular functions ranging from protein folding and assembly, lipid and sterol synthesis to the storage of calcium, a key second messenger within cellular pathways (Zhang 2010). Therefore, its processes are tightly controlled and misfunction, resulting from misfolded proteins or Ca^{2+} depletion, leads to the induction of ER stress mechanisms including the unfolded protein response (Di Conza & Ho 2020; Celli & Tsolis 2015). The ER stress response is initiated by one or several of the three key ER stress sensors: **(1)** IRE1 (inositol-requiring enzyme type 1) (Tirasophon, Welihinda & Kaufman 1998), **(2)** PERK (protein kinase-like ER kinase) (Harding, Zhang & Ron 1999), and **(3)** ATF6 (activating transcription factor 6) (Haze et al. 1999). All three sensors are kept inactive during homeostasis by tight association of their luminal domain with a protein-folding chaperon called BiP (binding immunoglobulin protein) (Bertolotti et al. 2000; Shen et al. 2005). Aggregation of

mis- or unfolded proteins within the ER lumen drives dissociation of BiP for it to fulfill its protein-folding functions and, thereby, subsequent activation of the UPR sensors (Li et al. 2008; Bertolotti et al. 2000; Shen et al. 2005). Furthermore, the transmembrane domains of IRE1 and PERK are able to perceive changes in the ER membrane lipid composition initiating stimulation independent of the UPR (Di Conza & Ho 2020; Volmer, Van Der Ploeg & Ron 2013). These processes involve altered lipid composition-induced blockage of the SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) and thereby passive Ca^{2+} depletion from the ER lumen (Fu et al. 2011).

Subsequent of BiP dissociation, IRE1 and PERK form homo-oligomers leading to auto-phosphorylation and thereby activation of their cytoplasmic domains (Bertolotti et al. 2000). ATF6, on the other hand, gets proteolytically cleaved forming an active transcription factor able to drive expression of ER stress-response genes (Di Conza & Ho 2020). The downstream signalling induced by the three ER stress sensors is illustrated in Figure 2.

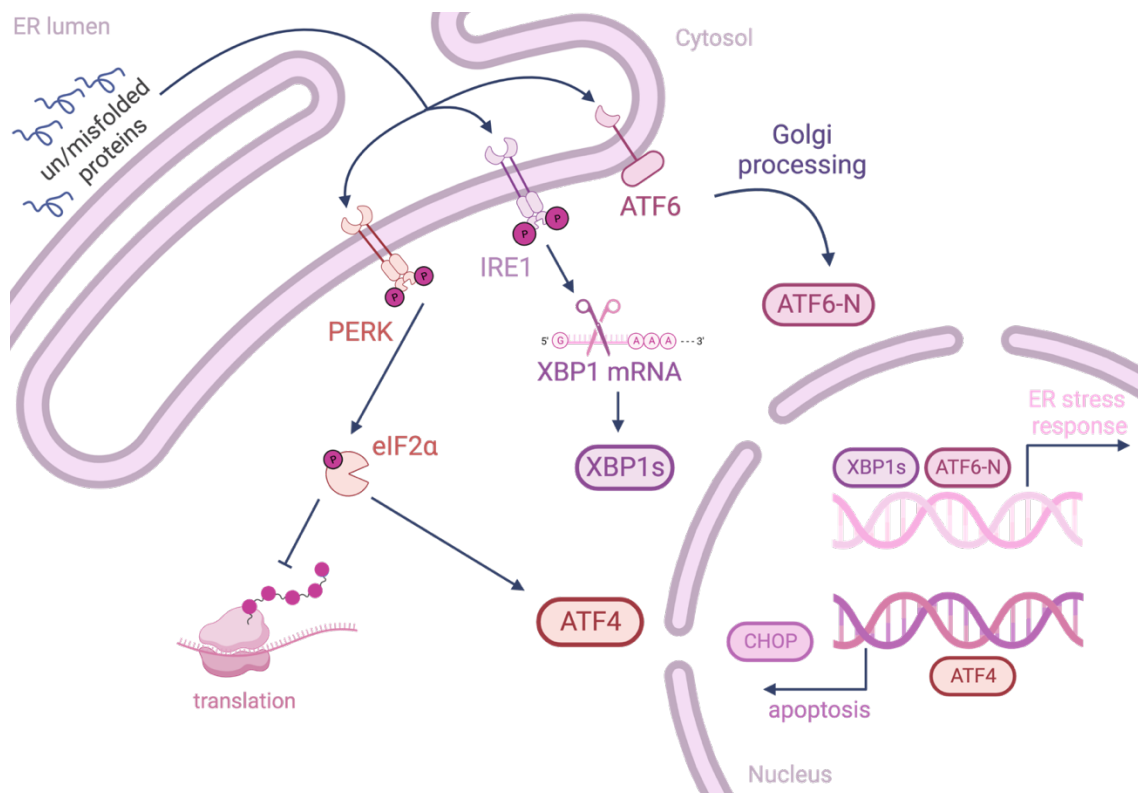


Figure 2: Schematic illustration of the downstream signalling of the three UPR sensors.

The cytoplasmic tail of the IRE1 combines a protein kinase and a RNase domain (Morl et al. 1993; Korennykh et al. 2009; Tirasophon, Welihinda & Kaufman 1998). While IRE1 activation requires auto-phosphorylation by its kinase domain (Bertolotti et al. 2000) the RNase domain promotes IRE1-dependent decay (RIDD) of mRNA in order to reduce the number of new proteins entering the ER and thereby allowing the ER time to restore homeostasis (Di Conza & Ho 2020; Alshareef, Hartland & McCaffrey 2021; Hollien et al. 2009; Hollien & Weissman 2006). In addition, the IRE1 RNase leads to unconventional splicing of XBP1 (X-box binding protein 1) mRNA and the subsequent expression of the transcription factor XBP1s (XBP1 spliced) which drives transcription of ER stress response genes (Lee et al. 2002; Lee, Iwakoshi & Glimcher 2003). XBP1s is further required for induction of the folding machinery and ER lipid synthesis during the UPR (Lee et al. 2002; Lee, Iwakoshi & Glimcher 2003). In cooperation with ATF6, IRE1 induces the expression of enzymes involved in the ERAD-mediated elimination of accumulating mis-/ unfolded proteins (Yamamoto et al. 2007; Celli & Tsolis 2015).

The ER luminal domain of PERK resembles that of IRE1 while the cytosolic kinase domain resembles its main interaction partner eIF2 α (Eukaryotic translation initiation factor 2 α) (Di Conza & Ho 2020; Bertolotti et al. 2000). PERK mainly phosphorylates eIF2 α (Harding, Zhang & Ron 1999; Harding, Zhang, et

Figure 2: Schematic illustration of the downstream signalling of the three UPR sensors.

Un- and misfolded protein accumulation in the ER lumen activates the luminal sensor domain of IRE1, PERK, and ATF6. Activation of the sensor domain initiates autophosphorylation and thereby activation of IRE1 and PERK. The RNase domain of IRE1, beyond inducing general mRNA degradation, alternatively splices the XBP1 mRNA resulting in XBP1s, an active transcription factor. XBP1s drives transcription of ER response genes involved in maximizing the ER protein folding capacity. PERK mainly phosphorylates eIF2 α leading to a general translational arrest but the specific upregulation of translation of proteins involved in UPR, such as ATF4. ATF4 drives expression of CHOP which is responsible to induce apoptosis in case ER stress is not resolvable. Activation of the sensor domain of ATF6 induces its proteolytical cleavage. The N-terminal domain of ATF6 is an active transcription factor that, after being processed in the Golgi, is translocated into the nucleus driving the transcription of ER stress response genes involved in maximizing the ER protein folding capacity. IRE1 = inositol-requiring enzyme type 1, PERK = protein kinase-like ER kinase, ATF = active transcription factor, XBP1 = X-box binding protein 1, XBP1s = spliced XBP1.

al. 2000; Koumenis et al. 2002) with eIF2 α subsequently being able to block protein synthesis (Harding, Zhang, et al. 2000; Koumenis et al. 2002; Harding, Novoa, et al. 2000; Scheuner et al. 2001). eIF2 α inhibits translation initiation to limit the number of newly synthesized proteins which, as mentioned above, allows the ER to restore homeostasis (Di Conza & Ho 2020). While eIF2 α blocks general translation, the expression of a selected range of proteins is not inhibited but selectively increased, including ATF4 (Harding, Novoa, et al. 2000). ATF4 subsequently upregulates the transcription of UPR mediators such as CHOP (CCAAT-enhancer binding protein homologous protein) (Harding, Novoa, et al. 2000) with CHOP being another protein not translationally inhibited by eIF2 α (Palam, Baird & Wek 2011). CHOP, in turn, induces apoptosis-related genes leading to cell death in case ER stress might not be resolvable (Endo et al. 2005; Marciniak et al. 2004). The short half-life of ATF4 and CHOP mRNAs ensures that only prolonged or severe ER stress induces apoptosis (Rutkowski et al. 2006).

ATF6, as the third ER stress sensor, consists of a transmembrane and a cytosolic domain (Di Conza & Ho 2020). Stimulation of the ER luminal sensor results in proteolytical cleavage of ATF6 by the two proteases S1P (site 1 protease) and S2P (Alshareef, Hartland & McCaffrey 2021; Yoshida et al. 1998) leading to translocation of the cytosolic domain into the nucleus where it stimulates the expression of UPR-related genes (Haze et al. 1999; Adachi et al. 2008; Wu et al. 2007). ATF6 is able to induce all major chaperones and protein folding enzymes to maximize the ER protein folding capacity (Okada et al. 2002; Yamamoto et al. 2007; Yoshida et al. 2000, 1998) eventually resulting in restorage of the ER homeostasis (Yamamoto et al. 2007; Yoshida et al. 2000; Wu et al. 2007). In addition, ATF6 induces transcription of XBP1 (Yoshida et al. 2001, 2000; Lee et al. 2002) and CHOP (Yoshida et al. 2001, 2000) to support the other arms of the UPR.

L. pneumophila effector proteins known to block host translation (Lgt1–3, SidL, SidL, LegK4, RavX) (Belyi et al. 2006; Shen et al. 2009; Ngwaga, Chauhan & Shames 2021) also interfere with UPR onset (Treacy-Abarca & Mukherjee 2015; Hempstead & Isberg 2015). Although the UPR primarily functions to restore ER

protein folding capacity, it also plays an important role in host immunity to infections with intracellular bacteria (Alshareef, Hartland & McCaffrey 2021). For example, *Brucella abortus* replicates within an ER-derived vacuole, like *Legionella*. Hijacking the ER compartment is essential for *Brucella* replication but also causes host ER stress (de Jong et al. 2013) and downstream activation of UPR-dependent pro-inflammatory responses required for bacterial restriction within the host (Keestra-Gounder et al. 2016). UPR-dependent inflammatory responses are, furthermore, important for restricting *Chlamydia* and *Francisella* infections (Martinon et al. 2010; Pham et al. 2020). Although *L. pneumophila* inhibits UPR signalling during infection, the significance of ER stress induction for intracellular bacterial growth and cytokine secretion remains to be elucidated.

1.2.2. Immune response to *Legionella* spp.

In general, human infections with *Legionella* are a rare event making the chance of re-infection very unlikely. Therefore, the immunity against *Legionella* that shapes the progress of infection and determines the development of severe LD is believed to be mainly dependent on innate immunity (Chauhan & Shames 2021). Adaptive immune responses to *Legionella* are, therefore, beyond the scope of this thesis and will not be described in detail here but are subject of numerous excellent reviews elsewhere (Chauhan & Shames 2021; Schuelein et al. 2011; Friedman, Yamamoto & Klein 2002; Brown, van Driel & Hartland 2013). The innate cellular and molecular immune response towards *L. pneumophila* has been extensively investigated (key points illustrated in **Fig. 3**) while responses to *L. longbeachae* still remain an under-investigated area of research.

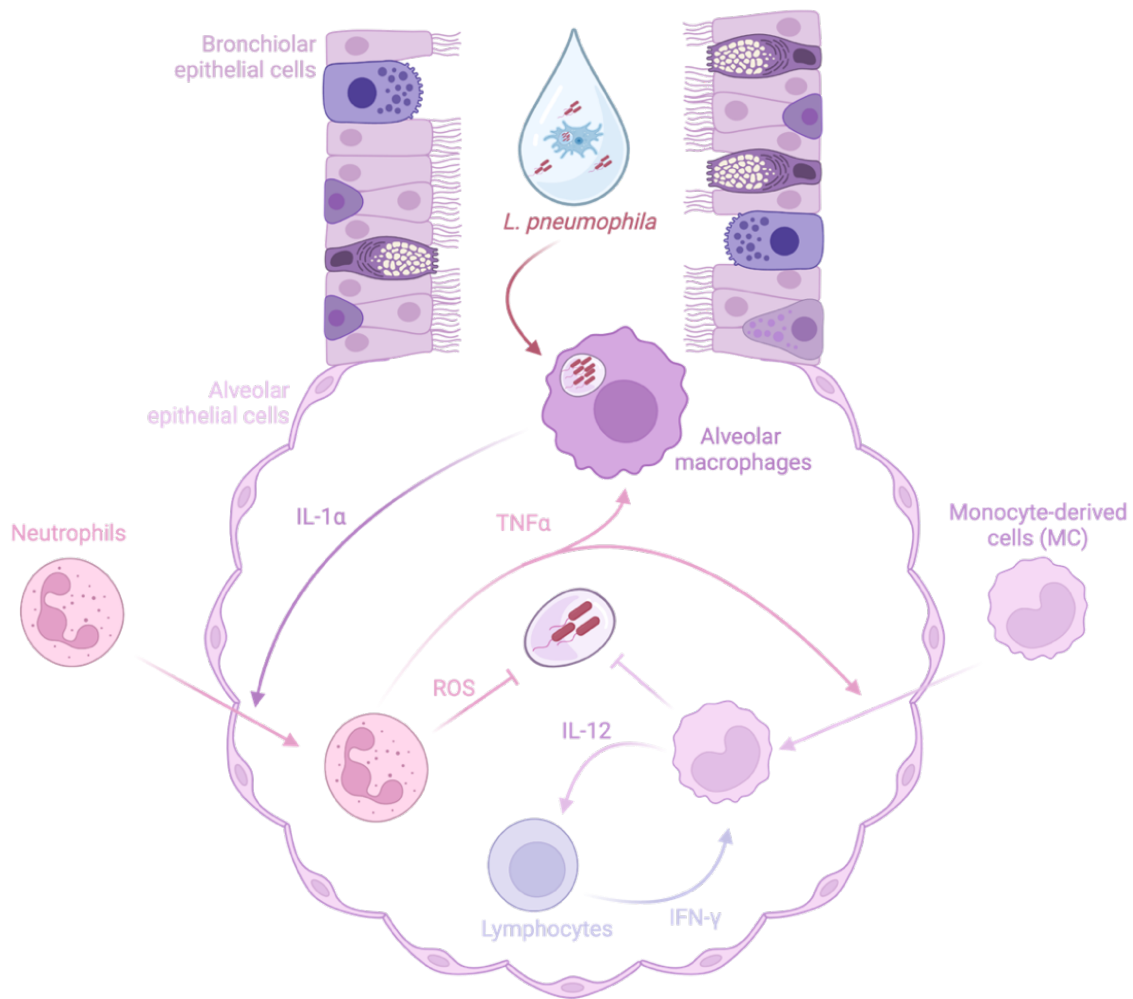


Figure 3: Schematic illustration of the innate immune response towards *L. pneumophila*.

L. pneumophila enter the human lung in infectious aerosols and are first encountered by AM in the alveoli. AM phagocytose the bacteria but *L. pneumophila* turns the phagocytes into a host cell and establishes its intracellular replicative niche, the LCV. In response to infection AM secrete IL-1 α shown to recruit neutrophils to the infection site. Neutrophils protect against *L. pneumophila* by direct bactericidal activity via production of ROS and indirectly by secretion of TNF α . TNF α in turn promotes bactericidal activity of AM and recruits monocyte-derived cells (MC) to the lung. MC protect against *L. pneumophila* via direct bactericidal mechanisms and secretion of IL-12. IL-12 drives IFN- γ secretion by lymphocytes, including NK cells and T cells, which via a positive feedback loop enhances the bactericidal capacity of MC. IL = interleukin, TNF α = tumour necrosis factor α , IFN- γ = interferon- γ , LCV = *Legionella*-containing vacuole, ROS = reactive oxygen species.

1.2.2.1. Macrophage host-intrinsic immunity

As explained above *L. pneumophila* enter the lung via contaminated aerosols and, subsequent to phagocytosis by AM, secrete a range of effector proteins to turn AM into its replicative niche. Accordingly, AM contain the highest number of

bacteria per cell beyond the different phagocytes present in the lung (Ziltener, Reinheckel & Oxenius 2016; Brown et al. 2016; Copenhaver et al. 2014) and are considered to be only poorly microbiocidal towards *L. pneumophila* (Brown et al. 2017). Nevertheless, AM are still able to recognize the bacteria via a range of different receptors leading to the recruitment of bystander immune cells (Brown et al. 2017).

Amongst the TLR *L. pneumophila* stimulates TLR2 (bacterial lipoproteins), TLR5 (flagellin), and TLR9 (unmethylated CpG DNA motifs) (Brown et al. 2017; Chauhan & Shames 2021). However, only TLR2 is clearly required for efficient bacterial clearance in mice (Hawn et al. 2006; Archer & Roy 2006) while TLR5 signalling contributes to the production of $\text{TNF}\alpha$ and the subsequent recruitment of neutrophils (Hawn et al. 2007). *L. pneumophila*-induced TLR signalling activates the adapter protein MyD88 which was demonstrated to be crucial for cytokine secretion as well as leukocyte recruitment (Hawn et al. 2006; Mascarenhas et al. 2015; Archer & Roy 2006). Lack of MyD88 strongly impaired *L. pneumophila* clearance from the mouse lung and their recovery from infection (Hawn et al. 2006; Mascarenhas et al. 2015; Archer & Roy 2006; Archer et al. 2009).

Next to TLR, intracellular recognition of *L. pneumophila* also induces inflammasome assembly. While murine macrophages can efficiently restrict infection with *L. pneumophila* (Amer et al. 2006; Zamboni et al. 2006), human macrophages are extremely permissive for intracellular growth of *L. pneumophila* (Kagan & Roy 2002; Tilney et al. 2001). This discrepancy can most likely be explained by the fact that only in murine macrophages but not in their human counterparts *L. pneumophila* infection drives caspase-1 activation (Amer et al. 2006; Zamboni et al. 2006). Caspase-1 in murine macrophages gets activated via stimulation of the NLRC4 which inhibits establishment of the LCV (Amer et al. 2006). The NAIP5 (NLR family apoptosis inhibitor protein 5)/ NLRC4 (NLR family CARD domain-containing protein 4) heterodimer is known to recognize bacterial flagellin leading to the assembly of the NLRC4 inflammasome (Pereira, Morgantetti, et al. 2011). The fact that non-flagellated mutant *L. pneumophila* and *L. longbeachae*, which naturally lack a flagellum (Cazalet et al. 2010), are able to

more efficiently replicate during *in vivo* infection even when compared to TLR5-deficient mice demonstrates the importance of the NAIP5/ NLRC4 pathway for anti-*Legionella* defence (Hawn et al. 2007; Pereira, Marques, et al. 2011). Activation of the NLRC4 inflammasome in infected AM induces pyroptosis (Molofsky et al. 2006; Ren et al. 2006; Case, Shin & Roy 2009) which is essential to restrict intracellular replication (Ren et al. 2006; Case, Shin & Roy 2009; Pereira, Morgantetti, et al. 2011; Wright et al. 2003; Miao et al. 2010). NLRC4 does not only induce host cell death but also recruitment of bystander immune cells via secretion of IL-1 β (Molofsky et al. 2006; Case, Shin & Roy 2009; Pereira, Morgantetti, et al. 2011). Therefore, the NAIP5/ NLRC4 heterodimer is crucial for anti-*L. pneumophila* defence (Molofsky et al. 2006; Case, Shin & Roy 2009; Zamboni et al. 2006; Berrington et al. 2013) especially early during infection (Ataide et al. 2023).

The presence of *L. pneumophila* alone was demonstrated not to be sufficient to trigger a downstream immune response in the host but requires effector-mediated recognition (Ngwaga, Chauhan & Shames 2021). A group of secreted effectors, namely Lgt1 – 3, SidI, SidL, LegK4, and RavX, do not only efficiently inhibit host translation (Belyi et al. 2006; Shen et al. 2009; Ngwaga, Chauhan & Shames 2021) but also initiate pro-inflammatory signalling (Fontana et al. 2011; Barry et al. 2013; Shin et al. 2008). Thereby, the efficient translocation of *L. pneumophila* effector proteins into AM (Copenhaver et al. 2014, 2015) minimizes the cytokine production in general but specifically induces the production of IL-1 α and IL-1 β (Barry et al. 2013; Fontana et al. 2011; Asrat, Dugan & Isberg 2014; Ivanov & Roy 2013). The bypassing of the translational inhibition by the macrophage host requires the presence of the MyD88 adapter protein (Asrat, Dugan & Isberg 2014) and the activation of the MAPK (mitogen-activated protein kinase) pathway by the bacterial effector proteins (Fontana et al. 2011; Matsunaga et al. 2003; Fontana, Shin & Vance 2012). The secreted bacterial effectors do, furthermore, inhibit mTOR (mammalian target of rapamycin) signalling to induce pro-inflammatory cytokines (Ivanov & Roy 2013) and just recently the effector protein LegC4 has been shown to stimulate cytokine expression (Ngwaga et al. 2019).

1.2.2.2. Immune response of infiltrating phagocytes

Even though there is a dampened cytokine production by *Legionella*-infected AM there is still a strong recruitment of bystander immune cells, mainly neutrophils and monocytes, during *L. pneumophila* infection (Ziltener, Reinheckel & Oxenius 2016; Brown et al. 2016; Copenhaver et al. 2014; Mascarenhas et al. 2015; Barry et al. 2013; Cai et al. 2016). Neutrophils and monocytes both ingest *L. pneumophila* (Brown et al. 2016) but the bacteria are only able to translocate effector proteins into the cytoplasm of neutrophils (Copenhaver et al. 2014, 2015).

The secreted bacterial effectors are required for the efficient bactericidal activity of neutrophils rather than pro-inflammatory cytokines like $\text{TNF}\alpha$ or $\text{IFN-}\gamma$ (Ziltener, Reinheckel & Oxenius 2016). Secretion of effector proteins by *L. pneumophila* induces metabolic reprogramming towards aerobic glycolysis (Price et al. 2021) required for the production of NADPH oxidase-dependent ROS (Price et al. 2021; Ziltener, Reinheckel & Oxenius 2016). Their high bacterial killing capacity makes neutrophils an essential leukocyte population for clearance of bacteria and recovery of infected mice (Mascarenhas et al. 2015; Barry et al. 2013; Copenhaver et al. 2015; LeibundGut-Landmann et al. 2011). Neutrophils are recruited to the lung via AM-derived $\text{IL-1}\alpha$ and, next to their direct anti-bacterial effector functions, promote the protective immune response by secretion of immune modulators such as $\text{TNF}\alpha$ and IL-12 (Ziltener, Reinheckel & Oxenius 2016; Copenhaver et al. 2015).

Neutrophil-derived $\text{TNF}\alpha$ is essential to instruct monocyte recruitment to the infection site (Skerrett et al. 1997). Monocyte-derived cells (MC) are protective against *L. pneumophila* infection (Ataide et al. 2023; Brown et al. 2016; Copenhaver et al. 2014) especially during later timepoints (Ataide et al. 2023). MC fulfill their protective function not only via $\text{IFN-}\gamma$ -dependent direct bacterial killing (Brown et al. 2016) but also via secretion of pro-inflammatory cytokines including $\text{TNF}\alpha$ (Ziltener, Reinheckel & Oxenius 2016; Copenhaver et al. 2015) and IL-12 (Brown et al. 2016).

1.2.2.3. Cytokines during *L. pneumophila* infection

Especially when bacteria are able to hijack the bactericidal functions of AM as the first line of pulmonary defence, the recruitment and activation of bystander leukocytes is crucial for the bacterial clearance (Brown et al. 2017). AM overcome the translational blockade by *L. pneumophila* and secrete IL-1 α and IL-1 β and both cytokines were demonstrated to be essential for the host defence against *L. pneumophila* (Ziltener, Reinheckel & Oxenius 2016; Mascarenhas et al. 2015; Copenhaver et al. 2015; LeibundGut-Landmann et al. 2011). IL-1 β instructs uninfected bystander immune cells to secrete cytokines such as TNF α (Fontana et al. 2011; Asrat, Dugan & Isberg 2014; Ivanov & Roy 2013; Copenhaver et al. 2015) and IL-12 (Copenhaver et al. 2015). IL-1 α , on the other hand, recruits neutrophils (Mascarenhas et al. 2015; Copenhaver et al. 2015) but also stimulates the secretion of protective GM-CSF from AECII (Liu et al. 2020). AECII-derived GM-CSF subsequently promotes the cytokine production in monocyte-derived cells by inducing a metabolic switch towards aerobic glycolysis (Liu et al. 2020).

Next to IL-1 α and IL-1 β another key cytokine for defence against *L. pneumophila* is TNF α which is essential for bacterial clearance and recovery of infected mice (Ziltener, Reinheckel & Oxenius 2016; Copenhaver et al. 2015). TNF α can be produced by neutrophils, MC, and uninfected AM during *L. pneumophila* infection (Ziltener, Reinheckel & Oxenius 2016; Copenhaver et al. 2015) and recruits both neutrophils and monocytes to the infected lung (Fujita et al. 2008; Skerrett et al. 1997). Furthermore, TNF α can promote anti-bacterial effector functions of infected AM *in vivo* (Ziltener, Reinheckel & Oxenius 2016) even though TNF α -stimulated AM are still less bactericidal than neutrophils (Brown et al. 2016). *In vitro* infection experiments revealed that TNF α is able to drive fusion of the lysosome with and subsequent acidification of the LCV in macrophages (Ziltener, Reinheckel & Oxenius 2016).

The most studied pro-inflammatory cytokine during anti-*L. pneumophila* defence, however, is IFN- γ . Intracellular IFN- γ signalling has been implemented into anti-bacterial functions of macrophages as it induces expression of multiple

IFN-stimulated genes (ISG) connected to the LCV (Brown et al. 2017). One example is IRG1 which is expressed in close proximity of the LCV and important to produce itaconate, a metabolite with broad anti-bacterial activity that can restrict *L. pneumophila* replication within its vacuole (Naujoks et al. 2016). The upregulation of ISG restricts intracellular *L. pneumophila in vitro* (Price et al. 2019; Naujoks et al. 2016) while IFN- γ was unable to improve the bactericidal capacity of AM *in vivo* (Brown et al. 2016). Nevertheless, IFN- γ is still crucial for bacterial clearance and recovery of infected mice (Cai et al. 2016; Archer et al. 2009; Brown et al. 2016). During infection with *L. pneumophila*, various cell types are able to produce and secrete IFN- γ including the best-known producers NK cells and T cells (Brown et al. 2016; Archer et al. 2009). IFN- γ significantly enhances anti-bacterial effector functions of MC via a positive feedback loop (Brown et al. 2016): MC produce IL-12 capable of driving IFN- γ expression from NK cells and T cells and the secreted IFN- γ in turn stimulates the bactericidal capacity of MC (Brown et al. 2016; Archer et al. 2009; Casson et al. 2017). Therefore, IL-12, next to IFN- γ , is required for bacterial clearance while the second IFN- γ -inducer IL-18 does not play an essential role during defence against *L. pneumophila* (Brown et al. 2016).

Next to pro-inflammatory cytokines, chemokines are crucial for defence against bacterial infections in general. During *L. pneumophila* infection chemokines have not been extensively investigated yet but the use of *Ccr2*^{-/-} mice implicated a crucial role for CCR2 ligands, e.g. CCL2, in recruitment of monocytes and overall bacterial clearance during *L. pneumophila* infection (Ataide et al. 2023).

1.2.2.4. Immune response towards *L. longbeachae*

Due to the lower prevalence, the immune determinants of *L. longbeachae* infection are not well understood but there were some first studies investigating that field. These studies revealed a strong neutrophil influx in response to *L. longbeachae* infection (Massis et al. 2017; Gobin et al. 2009). Therefore, neutrophils were suggested to play a protective role (Massis et al. 2017) which

has been recently demonstrated for MC (Ataide et al. 2023). Similar to *L. pneumophila* infection, MC protect against *L. longbeachae* during later timepoints after infection (Ataide et al. 2023). The study by Zamboni and colleagues further demonstrated the importance of $\text{TNF}\alpha$ and $\text{IFN-}\gamma$ for anti-*L. longbeachae* defence (Massis et al. 2017) underpinning the similarities to the immune response against *L. pneumophila*.

1.3. Hypotheses and aims

LD is a disease of growing significance especially for susceptible patients and globalization may lead to an increased prevalence of *L. longbeachae* in the northern hemisphere. My project aimed at unveiling cellular and molecular mechanisms underlying the host immune responses against *L. longbeachae*. Furthermore, I sought to gain a more detailed insight into the pathogen-host interaction during *Legionella* infection. Taken together, the key aims of this study were to investigate:

- (1) The role of tissue-resident and infiltrating immune cells during defence against *L. longbeachae*.
- (2) The molecular mechanisms underlying the anti-*L. longbeachae* defence.
- (3) The relevance of ER stress induction in host innate immune response to *Legionella* infection.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Equipment

Table 1: Equipment

Device	Manufacturer
1x 2 teeth curved Graefe forceps	Fine Science Tools, Foster City, CA, USA
Anaesthetic machine	Harvard Apparatus, Holliston, MA, USA
Centrifuges	Eppendorf, Hamburg, Germany
Dissection Equipment	Fine Science Tools, Foster City, CA, USA
EasySep™ magnet	STEMCELL technologies, Vancouver, Canada
FACSAria™ Fusion	Becton-Dickinson, Franklin Lakes, NJ, USA
FlexMap 3D System	Luminex, Seattle, WA, USA
HERAcell 240 incubator	Heraeus, Braunschweig, Germany
HERAsafe workbench	Heraeus, Braunschweig, Germany
IVC mouse cages	Tecniplast, Hohenpeißenberg, Germany
IX71 inverted microscope	Olympus, Hamburg, Germany
LSM 710 confocal microscope	Carl Zeiss Microscopy, Jena, Germany
LSRFortessa™ flow cytometer	Becton-Dickinson, Franklin Lakes, NJ, USA
Magnetic Plate Washer	Thermo Fisher Scientific, Waltham, MA, USA
Mastercycler nexus gradient	Eppendorf, Hamburg, Germany
NanoDrop One	Thermo Fisher Scientific, Waltham, MA, USA
Neubauer cell chamber	BRAND, Wertheim, Germany

ORCA-Flash4.0 V3, C13220-20 CU	Hamamatsu, Hamamatsu City, Japan
PCO Dimax camera	Excelitas PCO GmbH, Kelheim, Germany
Precellys (R) 24	Bertin Technologies, Montigny-le-Bretonneux, France
QuantStudio 6 RT- PCR system	Thermo Fisher Scientific, Waltham, MA, USA
Rodent laryngoscope Model LS-2-M	Penn Century, Wyndmoor, PA, USA
Scale Adventurer Pro	OHAUS
Small animal laryngoscope	Penn century, Wyndmoor, PA, USA
Spectrophotometer Ultraspec 10	Biochrom
Stainless Steel Beads, 3 mm	Qiagen, Hilden, Germany
Tecan Safire2	Tecan, Männedorf, Switzerland
Tissue Lyser LT	Qiagen, Hilden, Germany
VT1000S vibrating- blade microtome (vibratome)	Leica Biosystems, Nußloch, Germany

2.1.2. Consumables

Table 2: Consumables

Consumable	Manufacturer
2-Desoxyglucose	Merck & Co., Kenilworth, NJ, USA
BD Discardit™ Syringes, 1 ml	Becton-Dickinson, Franklin Lakes, NJ, USA
BD Microlance injection needles (23G, 25G, 27G)	Becton-Dickinson, Franklin Lakes, NJ, USA
Buffered charcoal yeast extract (BCYE) Agar plates	Thermo Fisher Scientific, Waltham, MA, USA

Cryo-tubes, 2 ml	TPP Techno Plastic Products AG, Transadingen, Switzerland
Cuvettes for photometer	SARSTEDT AG + Co., Nümbrecht, Germany
FACS tubes polystyrene, 5 ml	SARSTEDT AG + Co., Nümbrecht, Germany
Falcon® 40 µl Cell Strainer	Corning, Corning, USA
Falcon® Round-Bottom Tubes with Cell Strainer Cap, 5 ml	Corning, Corning, USA
Inoculation loop, 10 µl	Greiner bio-one, Solingen, Germany
Micro haematocrit tubes, L = 75+/- 0.5 mm	Brand, Wertheim, Germany
Microtiter plates (6 well, 96 well)	Greiner bio-one, Solingen, Germany
Microtome blades, ultra-thin, 0.076 mm	Ted Pella Inc., Redding, CA, USA
Polypropylene tubes (15 ml, 50 ml)	Greiner bio-one, Solingen, Germany
Reaction tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Precellys lysing kit CK14	Bertin Technologies, Montigny-le-Bretonneux, France
MicroAmp® Optical 384-Well Reaction Plate with Barcode	Applied Biosystems, Waltham, MA, USA
Microscope slides	Paul Marienfeld GmbH & Co. KG, Lauda- Königshofen, Germany
Microscope cover glasses	Paul Marienfeld GmbH & Co. KG, Lauda- Königshofen, Germany
S-Monovette® K3 EDTA, 7.5 ml	SARSTEDT AG + Co., Nümbrecht, Germany

2.1.3. Chemicals and reagents

Table 3: Chemicals and reagents

Reagent	Manufacturer
1-Propanol/ Isopropanol	Roth, Karlsruhe, Germany
4',6-Diamindino-2phenylindole (DAPI)	BioLegend, San Diego, CA, USA
4 μ 8C	Tocris Bioscience, Bristol, UK
ACK buffer	Lonza, Cologne, Germany
Agarose, low-temperature melting	Promega GmbH, Madison, WI, USA
Animal Component-Free Cell Dissociation Kit	STEMCELL technologies, Vancouver, Canada
Antigenfix	Diapath S.p.A., Martinengo, Bergamo, Italy
Bovine serum albumin fraction V	Roth, Karlsruhe, Germany
Brain Heart Broth (BHI)	Sigma-Aldrich, St. Louis, MO, USA
CaliBRITE™ Beads	Becton-Dickinson, Franklin Lakes, NJ, USA
Ceapin-A7	Tocris Bioscience, Bristol, UK
Chloroform	Roth, Karlsruhe, Germany
Clodronate liposomes	Liposoma, Amsterdam, Netherlands
Collagenase type IV	Roche, Berlin, Germany
Control liposomes (PBS)	Liposoma, Amsterdam, Netherlands
Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix	Corning Inc., Corning, NY, USA
Cultrex 3D Culture Matrix	R&D Systems, Minneapolis, MN, USA
Collagen, Type I	
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe, Germany
DNase I	Roche, Mannheim, Germany
Donkey gamma globulin	Jackson ImmunoResearch Laboratories, West Grove, PA, USA

Dulbecco's modified eagle medium (DMEM)	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Dulbecco's modified phosphate buffered saline	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
EasySep™ Direct Human Neutrophil Isolation Kit	STEMCELL technologies, Vancouver, Canada
Ethanol	Roth, Karlsruhe, Germany
Ethanol 99,5 %	Roth, Karlsruhe, Germany
Ethylene diamine tetra acetic acid (EDTA)	Merck, Darmstadt, Germany
Foetal calf serum (FCS)	Life Technologies, Carlsbad, CA, USA
Fixable Viability Dye eFluor® 780	eBioscience, San Diego, CA, USA
Foxp3/ Transcription Factor Staining Buffer Set	eBioscience, San Diego, CA, USA
Gelatine from cold water fish skin (CWFG)	Sigma-Aldrich, St. Louis, MO, USA
Gentamicin	Thermo Fisher Scientific, Waltham, MA, USA
Glycerol	Sigma-Aldrich, St. Louis, MO, USA
GSK2606414	Tocris Bioscience, Bristol, UK
Harringtonin	Abcam, Cambridge, UK
Heparin Solution, 0.2%	STEMCELL technologies, Vancouver, Canada
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific, Waltham, MA, USA
HOECHST33528	Sigma-Aldrich, St. Louis, MO, USA
Human CBA Flex Set	Becton-Dickinson, Franklin Lakes, NJ, USA
Hydrocortisone Stock Solution, 200 x	STEMCELL technologies, Vancouver, Canada
Iron (III) pyrophosphate hydrate	Sigma-Aldrich, St. Louis, MO, USA

Isoflurane	Piramal Critical Care Limited, West Drayton, UK
ISOLATE II RNA Mini Kit	Meridian Bioscience, Cincinnati, OH, USA
ISRIB	Tocris Bioscience, Bristol, UK
Ketamine 10 %	Wirtschaftsgenossenschaft deutscher Tierärzte eG, Garbsen, Germany
KIRA6	Tocris Bioscience, Bristol, UK
L-Cysteine hydrochloride monohydrate, ≥98%	Sigma-Aldrich, St. Louis, MO, USA
Lipopolysaccharide (LPS, Serotype O26:B6)	Sigma-Aldrich, St. Louis, MO, USA
Mouse CBA Flex Set	Becton-Dickinson, Franklin Lakes, NJ, USA
Mouse gamma globulin	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Mouse MUC5AC ELISA Kit (Colorimetric)	Novus Biologicals, Centennial, CO, USA
Mouse MUC5B ELISA Kit (Colorimetric)	Novus Biologicals, Centennial, CO, USA
N-(2-acetamido)-2-(aminoethane sulfonic) acid (ACES buffer)	MPU (media preparation unit), Department of microbiology & immunology, University of Melbourne
NucleoSpin RNA, Mini kit for RNA purification	Macherey-Nagel, Düren, Germany
Octagam® 10 % normal human IgG	Octapharma, Langenfeld, Germany
Oligomycin A	Merck & Co., Kenilworth, NJ, USA
Paraformaldehyde 16 % (PFA), methanol-free	Thermo Fisher Scientific, Waltham, MA, USA
Penicillin/ Streptomycin	Merck, Darmstadt, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, St. Louis, MO, USA

PneumaCult™ Airway Organoid Basal Medium	STEMCELL technologies, Vancouver, Canada
PneumaCult™ Airway Organoid Differentiation Supplement	STEMCELL technologies, Vancouver, Canada
PneumaCult™ Airway Organoid Seeding Supplement	STEMCELL technologies, Vancouver, Canada
PneumaCult™ ALI-Medium + 48 Costar® 12 mm Transwell® (0.4 µm Pore Polyester Membrane Inserts)	STEMCELL technologies, Vancouver, Canada
PneumaCult™ -Ex Plus Basal Medium	STEMCELL technologies, Vancouver, Canada
Polyacryl Carrier™	Molecular Research Centre, Cincinnati, OH, USA
<i>Power</i> SYBR(R) Green PCR Master Mix	Thermo Fisher Scientific, Waltham, MA, USA
PowerTrack SYBR Green Master Mix	Life Technologies, Carlsbad, CA, USA
ProcartaPlex Mouse 12-Plex Mix&Match Kit	Thermo Fisher Scientific, Waltham, MA, USA
Propidium iodide (PI)	Sigma-Aldrich, St. Louis, MO, USA
Puromycin	Merck & Co., Kenilworth, NJ, USA
Rat gamma globulin	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Recombinant human IFN- α	Sigma-Aldrich, St. Louis, MO, USA
Recombinant human IFN-g	BioLegend, San Diego, CA, USA
Recombinant human IL-12	BioLegend, San Diego, CA, USA
Recombinant human IL-18	BioLegend, San Diego, CA, USA
Recombinant human TNF α	Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA
Recombinant mouse CCL2	R&D Systems, Minneapolis, MN, USA
Recombinant mouse IL-18	BioLegend, San Diego, CA, USA

Rompun® 2 % (Xylazine)	Bayer Vital GmbH, Leverkusen, Germany
Roswell Park memorial institute medium 1640 (RPMI)	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Saponin	Sigma-Aldrich, St. Louis, MO, USA
Sodium azide (NaN ₃)	Roth, Karlsruhe, Germany
SuperScript™ III First-Strand Synthesis System for RT-PCR	Life Technologies, Carlsbad, CA, USA
Thapsigargin	Sigma-Aldrich, St. Louis, MO, USA
Triton-X 100	Sigma-Aldrich, St. Louis, MO, USA
Trizol	Life Technologies, Carlsbad, CA, USA
Trypan blue (0.4 %)	Lonza, Cologne, Germany
Tween® 20	Roth, Karlsruhe, Germany
Yeast extract	Oxoid Limited, Hampshire, UK
α-Ketoglutarate	Sigma-Aldrich, St. Louis, MO, USA

2.1.4. Buffers and media

Table 4: Buffers and media

Buffer	Reagents
ALI Maintenance medium	in PneumaCult™ -ALI Basal medium: 1x PneumaCult™ -ALI 10X supplement, 1x PneumaCult™ -ALI 100X Maintenance Supplement 50x, 0.00032 % Heparin, 0.8x Hydrocortison, 100 U/ ml Penicillin-Streptomycin
AYE broth	in H2Obidest: 1 % ACES buffer, 1 % Yeast extract, 0.1 % α-Ketoglutarate → autoclave, before use add 0.025 % iron pyrophosphate, 0.04 % L-cysteine
Basal medium (HBEC)	in PneumaCult™ -ExPlus Basal medium: 1x PneumaCult™ -Ex Plus 50X Supplement, 0.2x Hydrocortison, 100 U/ ml Penicillin-Streptomycin

Confocal Blocking Buffer	In PBS: 1 % CWFG (w/v), 1 % FCS (v/v), 0.3 % Triton-X 100 (v/v), relevant Ig
Confocal Washing Buffer	In PBS: 0.3 % Triton-X 100 (v/v)
Digestion Buffer	In PBS: 1 mg/ ml Collagenase IV, 1 mg/ ml DNase
FACS Blocking Buffer	FACS Buffer + 0.05 % Octagam [®] normal IgG
FACS Buffer	In PBS: 3 % FCS (v/v), 0.1 % NaN ₃ (v/v)
FACS Fix	in PBS: 100 mM Glucose, 0.1 % NaN ₃ , 1 % PFA
Organoid Differentiation medium	in PneumaCult [™] Organoid Basal medium: 1x PneumaCult [™] Organoid Differentiation Supplement, 0.00032 % Heparin, 0.8x Hydrocortisone, 100 U/ ml Penicillin-Streptomycin
Organoid Seeding medium	in PneumaCult [™] Organoid Basal medium: 1x PneumaCult [™] Organoid Seeding Supplement, 100 U/ ml Penicillin-Streptomycin

2.1.5. Antibodies

Table 5: Antibodies for flow cytometry

Antigen	Clone	Conjugate	Manufacturer
Mouse antigen			
CD11b	M1/70	BUV395	BD Biosciences, Franklin Lakes, NJ, USA
	M1/70	FITC	BD Biosciences, Franklin Lakes, NJ, USA
CD11c	N418	PB	BioLegend, San Diego, CA, USA
	N418	FITC	BioLegend, San Diego, CA, USA

CD19	6D5	FITC	BioLegend, San Diego, CA, USA
	6D5	BV786	BioLegend, San Diego, CA, USA
CD192/ CCR2	SA203G11	PE	BioLegend, San Diego, CA, USA
CD218a/ IL-18R1	P3TUNYA	PE	Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA
CD31	MEC 13.3	FITC	BioLegend, San Diego, CA, USA
CD326/ EpCAM	G8.8	AF647	BioLegend, San Diego, CA, USA
CD3ε	145-2C11	FITC	BioLegend, San Diego, CA, USA
	145-2C11	PE	BioLegend, San Diego, CA, USA
	17A2	BV650	BioLegend, San Diego, CA, USA
CD4	GK1.5	BUV737	BD Biosciences, Franklin Lakes, NJ, USA
CD44	IM7	PB	BioLegend, San Diego, CA, USA
CD45	30-F11	AF700	BioLegend, San Diego, CA, USA
	30-F11	PO	Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA
CD45.2	104	AF488	BioLegend, San Diego, CA, USA
	104	PB	BioLegend, San Diego, CA, USA
CD64	X54-5/7.1	PE	BioLegend, San Diego, CA, USA
	X54-5/7.1	BV605	BioLegend, San Diego, CA, USA
CD8a	53-6.7	BUV805	BD Biosciences, Franklin Lakes, NJ, USA
Ly-6G	1A8	BV711	BioLegend, San Diego, CA, USA
	1A8	PE	BioLegend, San Diego, CA, USA
	1A8	FITC	BD Biosciences, Franklin Lakes, NJ, USA
MHC-II	M5/114.15.2	PE/Cy7	BioLegend, San Diego, CA, USA
NK1.1	PK136	FITC	BioLegend, San Diego, CA, USA
	PK136	AF647	BioLegend, San Diego, CA, USA
	PK136	PE/ Cy7	BioLegend, San Diego, CA, USA

Puromycin	12D10	AF488	Merck & Co., Kenilworth, NJ, USA
SiglecF	E-50-2440	AF647	BD Biosciences, Franklin Lakes, NJ, USA
	E-50-2440	PE	BD Biosciences, Franklin Lakes, NJ, USA
TCR β	H57-597	AF700	BioLegend, San Diego, CA, USA
TCR γ/δ	GL3	PE/Cy7	BioLegend, San Diego, CA, USA
Ter119	TER-119	FITC	BioLegend, San Diego, CA, USA
Thy1.2	30-H12	BV785	BioLegend, San Diego, CA, USA
	53-2.1	PB	BioLegend, San Diego, CA, USA

Human antigen

CD11b	ICRF44	PE	BioLegend, San Diego, CA, USA
CD45	HI30	AF647	BioLegend, San Diego, CA, USA
CD62L	DREG-56	AF647	BioLegend, San Diego, CA, USA
CD66b	G10F5	FITC	BioLegend, San Diego, CA, USA

Table 6: Antibodies for confocal microscopy

Antigen	Clone	Conjugate	Manufacturer
AqP5	Rabbit polyclonal	Purified	Abcam, Cambridge, UK
CD45	30-F11	AF594	BioLegend, San Diego, CA, USA
CD45.2	104	AF488	BioLegend, San Diego, CA, USA
EpCAM/CD326	G8.8	AF647	BioLegend, San Diego, CA, USA
Rabbit	Donkey polyclonal	DL405	Jackson ImmunoResearch Laboratories, West Grove, PA, USA

Table 7: Antibodies for *in vivo* administration

Antigen	Clone	Species, isotype	Manufacturer
Depletion antibody			
CCL2	2H5	Armenian Hamster, IgG	BioXCell, Lebanon, NH, USA
Ly-6G	1A8	Rat, IgG2a	BioXCell, Lebanon, NH, USA
NK1.1	PK136	Mouse, IgG2a	BioXCell, Lebanon, NH, USA
Rat κ Ig light chain	MAR18.5	Mouse, IgG2a	BioXCell, Lebanon, NH, USA
Isotype control			
Phycoerythrin	2A3	Rat, IgG2a	BioXCell, Lebanon, NH, USA
Unknown	C1.18.4	Mouse, IgG2a	BioXCell, Lebanon, NH, USA

2.1.6. Primers

Table 8: Primers

Gene	Organism	Sequence		Manufacturer
		Sense 5'–3'	Antisense 5'–3'	
<i>bActin</i> (Bonn)	mouse	TGT GAT GGT	TGT GGT GCC	Thermo
		GGG AAT GGG	AGA TCT TCT	Fisher
		TCA GAA	CCA TGT	Scientific, Waltham, MA, USA
<i>Cramp</i>	mouse	AAG GAA CAG	CCG GGA AAT	Thermo
		GGG GTG GTG	TTT CTT GAA	Fisher
			CC	Scientific, Waltham, MA, USA

<i>Defb1</i>	mouse	CCA GAT GGA GCC AGG TGT TG	CTG GAG CGG AGA CAG AAT CC	Thermo Fisher Scientific, Waltham, MA, USA
<i>Defb3</i>	mouse	GCA TTG GCA ACA CTC GTC AGA	CGG GAT CTT GGT CTT CTC TA	Thermo Fisher Scientific, Waltham, MA, USA
<i>Lysm</i>	mouse	CTG GCT ACT ATG GAG TCA GC	TTG ATC CCA CAG GCA TTC AC	Thermo Fisher Scientific, Waltham, MA, USA
<i>CXCL10</i>	human	TAA GTG GCA TTC AAG GAG TAC CT	GGA CAA AAT TGG CTT GCA GGA	
<i>bActin</i>	human	GCG AGA AGA TGA CCC AGA TC	CCA GTG GTA CGG CCA GAG G	

2.1.7. Mouse Strains

Table 9: Mouse strains

Strain	Description
C57BL/6J	C57BL/6J non-transgenic wild-type (WT) mice.
<i>gp91phox</i> ^{-/-}	B6.129S-Cybb ^{tm1Din} /J. Lack of phagocyte superoxide production (Pollock et al. 1995).
IL-18R	Unpublished.
tdTomato	Generated by Ozgene (Australia) for Prof. Kastenmüller (University of Würzburg). Knock-in of tdTomato fluorescent

reporter protein into gene side of IL-18R1, expression of tdTomato under control of endogenous *Il18r1* promoter + Knock-out of IL-18R1, heterozygous mice express IL-18R1 and tdTomato.

IL-18R ^{fl/fl}	Unpublished. Generated by Prof. Kastenmüller (University of Würzburg). <i>Il18r1</i> flanked by two loxP sites.
<i>Il18</i> ^{-/-}	B6.129P2- <i>Il18</i> ^{tm1Aki} /J. Lack of IL-18 production (Takeda et al. 1998).
<i>Il18r</i> ^{-/-}	B6.129P2- <i>Il18r1</i> ^{tm1Aki} /J. Lack α -chain of IL-18 receptor (Hoshino et al. 1999).
Nkx2.1-Cre	C57BL/6J-Tg(Nkx2-1-cre)2Sand/J. Cre-recombinase expression in brain progenitor cells as well as epithelial cells of the developing lung driven by Nkx2.1-promoter (Xu, Tam & Anderson 2008).
<i>Rag2</i> ^{-/-}	B6.Cg- <i>Rag2</i> ^{tm1.1Cgn} /J. Lack of mature T and B lymphocytes (Hao & Rajewsky 2001).
Vav-iCre	B6.Cg- <i>Commd10</i> ^{Tg(Vav1-icre)A2Kio} /J. Cre-recombinase expression in hematopoietic cells driven by vav-promoter (de Boer et al. 2003).

2.1.8. Pathogens

Table 10: *Legionella* strains

Pathogen	Description
<i>L. longbeachae</i>	Strain NSW150, clinical isolate
<i>L. longbeachae</i> -mCherry	Genetically modified <i>L. longbeachae</i> NSW150 expressing fluorescent reporter mCherry
<i>L. pneumophila</i>	Strain 130b, clinical isolate
<i>L. pneumophila</i> Δ dotA	Genetically modified <i>L. pneumophila</i> lacking the Dot/ Icm secretion system

L. pneumophila Genetically modified *L. pneumophila* lacking the flagellum Δ flaA

2.1.9. Cells

Table 11: Cell lines

Cells	Description
iBMDM	Immortalized bone marrow-derived macrophages generated from C57BL/6J mice.
Raw 264.7 cells	Monocyte/ macrophage-like cells derived from leukemia virus transformed cell line generated from BALB/c mice
THP-1 cells	Human leukemia monocytic cell line
HBEC	Human bronchiolar epithelial cells. Purchased as primary cells from Lonza (Basel, Switzerland).

2.1.10. Software

Table 12: Software

Software	Plugins	Company
Affinity Designer v1.10.5		Serif (Europe) Ltd., Nottingham, UK
Affinity Photo v1.10.5		Serif (Europe) Ltd., Nottingham, UK
BioRender		BioRender, Toronto, Canada
FACS Diva V6.1.1		Becton-Dickinson, Franklin Lakes, NJ, USA
FlowJo V10.8.1	DownSampleV3 FlowAI UMAP	Tree Star, Ashland, OR, USA
GraphPad Prism v9.4.1		GraphPad Software, La Jolla, CA, USA

ImageJ/Fiji	Cilility_JNH	Open Source
v2.8.0/1.53t	v0.0.1	
MS Office 365		Microsoft, Redmond, WA, USA
Thermo Fisher		Thermo Fisher Scientific,
Connect™		Waltham, MA, USA
Zen black 2.3 SP1		Carl Zeiss Microscopy GmbH,
FP3 14.0.26.201		Jena, Germany

2.2. Methods

2.2.1. Bacterial culture

Legionella spp. were stored in 50 % (v/v) glycerol/ BHI solution at -80 °C. To prepare for use, frozen bacteria were grown on buffered charcoal yeast extract (BCYE) agar plates for 72 h at 37 °C.

2.2.2. Treatment of mice

C57BL/6J (WT) and knockout (KO) mice of either sex at an age of 8 – 12 weeks were used. Breeding and housing of mice was performed under specific pathogen-free (SPF)-conditions with five mice per cage using a Techniplast ventitrack system in the animal facilities of the University Hospital Bonn (HET, IMMEI, or iFET) according to institutional guidelines. For experiments using only C57BL/6J mice, mice were obtained from Charles River (Sulzfeld, Germany) and hold in the facilities of the University Hospital Bonn. Experimental mice of different genotypes were maintained in the same facility room but not co-housed. Animal experiments were performed following approval by governmental committees (Landesamt für Natur,- Umwelt- und Verbraucherschutz (LANUV), NRW) under the license Az 1-02.04.2019.A021.

2.2.2.1. Intranasal infection with *L. longbeachae*

For intranasal (i.n.) infection of mice, all colonies from the BCYE plate were resuspended in PBS (phosphate-buffered saline). The infection dose was adjusted in PBS to 2×10^4 or 10^5 CFU/ ml using that 10^9 CFU (colony forming units)/ ml result in an optical density at 600 nm (OD_{600}) of 1. Retrospective plating on BCYE agar was used to confirm the infection dose for each experiment.

Mice were i.n. infected using a previously established method (Brown et al. 2016). Briefly, mice were anaesthetised using controlled isoflurane administration (2 % isoflurane/ O₂ (v/v)). I.n. administration of 50 µl bacterial solution was performed by dropwise application into the mouse nostrils while keeping the animal at a horizontal position. Weight loss of infected mice was monitored daily.

2.2.2.2. Depletion of alveolar macrophages *in vivo*

Mice were anaesthetised using controlled isoflurane administration (2 % isoflurane/ O₂ (v/v)) and intubated with a 22G butterfly catheter with the help of a small-animal laryngoscope. 50 µl of clodronate-loaded liposomes (5 mg/ ml) were applied into the catheter followed by Minivent-assisted ventilation with 2 % isoflurane/ O₂ for 30 s at 250 µl air/ stroke and 250 strokes/ min. PBS-loaded liposomes were used as negative controls. AM were depleted one day prior to infection.

2.2.2.3. Antibody-mediated depletion of specific leukocyte populations *in vivo*

In vivo depletion of specific leukocytes was achieved by intraperitoneal (i.p.) administration of 200 µg *in vivo* depletion antibody (**section 2.1.5.** – *in vivo* administration). Control mice were administered with 200 µg of irrelevant, isotype-matched antibody. To deplete NK cells, PK36 monoclonal antibody (mAb) was administered one day prior to infection, at the day of infection and 3 days after infection. To deplete neutrophils, mice were treated with depletion antibody every day, starting one day prior to infection. Mice alternately received a combination of 200 µg 1A8 mAb + 50 µg MAR18.5 mAb or 1A8 mAb alone.

2.2.2.4. Intravascular leukocyte staining

Intravascular leukocytes were stained via intravenous (i.v.) administration of AF488-labelled anti-CD45.2 antibody (5 µg in 200 µl) 5 min prior to sacrificing to distinguish intravascular and tissue infiltrating immune cells. The i.v. administration of the antibody was performed by injection into the lateral tail vein after mice were anesthetized with lethal dose (200 µl) of Xylazine (0.2 %) and Ketamine (1 %). Mice were painlessly killed by exsanguination via the lower aorta.

2.2.3. Anti-bacterial defence *in vivo*

2.2.3.1. Quantification of *Legionella* colony forming units

CFU quantification in the infected lung: Mice were painlessly killed at indicated time points after i.n. infection using either a lethal dose (200 µl) of Xylazine (0.2 %) and Ketamine (1 %) or CO₂-induced asphyxiation. Right lungs excluding trachea were used for quantification of *L. longbeachae* by homogenizing them in 1 ml DPBS containing 2 stainless steel beads (3 mm Ø). Homogenization was performed at maximum speed (50 oscillations/ s) for 30 min. using the Tissue Lyser LT (Qiagen). After that, intracellular bacteria were obtained by lysing the remaining cells using 0.1 % Saponin for 30 min at 37 °C. Lung homogenates were plated as 50 µl of serial 1:10 dilutions on BCYE agar plates and grown for 72 h at 37 °C to quantify CFU.

CFU quantification in sorted cells: To quantify viable bacteria within specific leukocyte populations, cells of interest were first sorted and then lysed by incubating with 0.1 % Tween[®] 20 for 30 min on ice. Cells were diluted to a fixed cell number (for whole populations 200,000 cells /ml, for bacteria-containing cells 40,000 cells/ ml) and CFU were quantified from 50 µl of serial 1:5 dilutions grown on BCYE agar for 72 h at 37 °C. Results were provided as CFU/ 10⁴ cells.

CFU quantification in adherent cells: To quantify viable bacteria within infected cell cultures, medium was removed from the wells and 0.05 % Digitonin was added for 10 min at RT. CFU were quantified from 40 µl of serial 1:10 dilutions grown on BCYE agar for 72 h at 37 °C. Results were provided as CFU/ 10⁶ cells.

2.2.3.2. Flow cytometry

If not indicated otherwise, all used reagents were ice cold and standard centrifugation was performed at 1,500 rpm (rotation per minute) for 5 min at 4 °C. Samples were acquired at the LSRFortessa™ flow cytometer (Becton-Dickison) using the FACS (fluorescence-activated cell sorting) Diva software (V6.1.1) and analysed using FlowJo (V10.8.1). The Uniform Manifold Approximation and

Projection for Dimension Reduction (UMAP) plug-in in FlowJo was used to reduce dimensionality after data was cleaned (FlowAI plug-in) and concatenated.

Cell isolation from the lung: Left lungs were used for antibody staining to perform flow cytometry analysis. Lungs were harvested into 3.5 ml digestion buffer and an additional 0.5 ml was injected into the lobe. Lungs were manually disrupted with the help of two forceps and fully digested under occasional mixing using a 1 ml pipette for 30 min at 37 °C. Cell suspensions were centrifuged, resuspended in 1 ml PBS, and passed into 5 ml round bottom tubes with a 35 µm cell strainer cap. Cells were kept at 4 °C and usually further processed within 1 h after preparation. Antibody staining was performed on 100 µl of the pulmonary single cell suspension.

Cell isolation from the spleen: The spleen was manually passed through a 40 µm filter into 3 ml PBS to obtain single cell suspensions. Cells were kept at 4 °C and usually processed further within 1 h after preparation. Antibody staining was performed on 50 µl of the splenic single cell suspension.

Antibody staining of single cell suspension: The indicated volume of cells was stained in a 96 U-bottom microtiter plate. Cells were centrifuged, resuspended in 100 µl PBS containing 1:2,000 Fixable Viability Dye eF780 (v/v), and staining of dead cells was achieved by 30 min incubation at 4 °C. 100 µl PBS were used for washing, cells were resuspended in 100 µl FACS Fix, and fixed for 30 min at 4 °C. 100 µl PBS were used for washing, cells were resuspended in 50 µl of fluorochrome-labelled mAb master mix in FACS blocking buffer, and stained for 20 min at 4 °C. 100 µl FACS buffer were used for washing and cells were resuspended in 200 µl FACS buffer containing 10⁴ PE-labelled BD CaliBRITE™ beads for cell counting. Cells were kept at 4 °C until acquisition by flow cytometry.

In the setting of neutrophil depletion, cells were stained for Ly-6G (Lymphocyte antigen 6 complex locus G) intracellularly to minimize the effect of anti-Ly-6G mAb applied *in vivo* on masking of surface Ly-6G (Boivin et al. 2020). After fixation with FACS Fix and surface antigen staining, intracellular staining was performed using the Foxp3/ Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Briefly, after

centrifugation, cells were resuspended in 50 µl Fixation/ Permeabilization buffer and incubated for 30 min at 4 °C. 150 µl Permeabilization buffer were used for washing. Cells were then intracellularly stained for anti-Ly-6G by incubation with 50 µl fluorochrome-labelled mAb in Permeabilization buffer. 150 µl FACS buffer were used for washing and cells were resuspended in 200 µl of FACS buffer containing 10^4 PE-labelled BD CaliBRITE™ beads for cell counting. Cells were kept at 4 °C until acquisition by flow cytometry.

SCENITH: SCENITH (single-cell energetic metabolism by profiling translation inhibition) is a single-cell flowcytometry-based approach to investigate cellular metabolism. SCENITH indirectly quantifies ATP synthesis via incorporation of puromycin into newly translated proteins (Argüello et al. 2020). A previously described SCENITH protocol was used (Argüello et al. 2020). During metabolic treatment reagents were used at RT (room temperature). In brief, immediately after filtration following lung digest, 50 µl of single cell suspension were centrifuged and incubated with 25 µl RPMI (Roswell Park Memorial Institute medium) + 10 % FCS (foetal calf serum) (v/v) for 30 min at 37 °C. 25 µl of metabolic inhibitors were added: 2-Deoxy-D-Glucose (2-DG, final concentration of 100 mM) to block glycolysis, Oligomycin (O, final concentration of 1 µM) to block oxidative phosphorylation, 2-DG + O to block both metabolic pathways, Harringtonin (final concentration of 2 µg/ ml) to fully inhibit protein synthesis, or DMSO (Dimethyl sulfoxide) as a control. Cells were incubated with inhibitors for 15 min at 37 °C followed by addition of 20 µl Puromycin (final concentration of 10 µg/ ml) and incubation for 30 min at 37 °C. From here on step on reagents were ice-cold. Following centrifugation and washing with 200 µl FACS buffer, cells were resuspended in 50 µl FACS buffer containing 1: 2,000 Fixable Viability Dye eF780 (v/v) and fluorochrome-labelled mAb master mix. Cells were incubated for 20 min at 4 °C to, in parallel, stain dead cells and surface antigens. To intracellularly stain for Puromycin, the protocol described in the previous paragraph was used. Instead of anti-Ly-6G antibody a mAb directed against Puromycin was used. After intracellular staining, cells were resuspended in 50 µl FACS buffer and kept at 4 °C until acquisition by flow cytometry, usually within 1 h after preparation.

Staining of reactive oxygen species: To stain for ROS the ROS dye DHR123 (dihydrorhodamine 123) was used. After staining with Fixable Viability Dye eF780, cells were immediately incubated with 50 μ l fluorochrome-labelled mAb master mix in FACS blocking buffer for 30 min at 4 °C to stain for surface antigens. 150 μ l PBS were used for washing, cells were resuspended in 50 μ l RT warm PBS containing 1:500 DHR123 (v/v) and incubated for 30 min at 37 °C. 150 μ l PBS was used for washing and cells were resuspended in 200 μ l of FACS buffer containing 10^4 PE-labelled BD CaliBRITE™ beads for cell counting. Cells were immediately recorded.

FACS: Particular cell populations were obtained using FACS. Whole lungs were used for FACS sorting and stained for surface antigens in 5 ml round bottom tubes. After filtration following lung digest, cells were immediately incubated with 500 μ l fluorochrome-labelled mAb master mix in FACS blocking buffer for 20 min at 4 °C to stain for surface antigens. 1 ml FACS buffer was used for washing and cells were resuspended in 1 ml FACS buffer containing 1:10,000 HOECHST33528 (v/v) for staining of dead cells. Cells were immediately processed for sorting at the FACS Aria™ Fusion.

2.2.3.3. RNA sequencing

5×10^5 sorted cells (neutrophils, bronchiolar epithelial cells) were handed to our collaboration partner Dr. Susanne Schmidt and Alexandru Odainic (Immunogenomics Group, institute for innate immunity, University Hospital Bonn, Germany) for RNA isolation and analysis of RNAsequencing data. RNAsequencing was performed by the Genomics Core Facility (University Hospital Bonn, Germany).

2.2.3.4. qPCR

RNA isolation and cDNA synthesis: To isolate RNA from lung tissue, the NucleoSpin RNA Mini kit (Macherey-Nagel) was used according to manufacturer's instructions. Briefly, lungs were homogenized in 1 ml Lysis Buffer using tubes with ceramic beads (Bertin Technologies) at 4,000 rpm for 1 min.

RNA was isolated via column separation, eluted in 60 µl RNase-free water and analysed at the NanoDrop™ spectrophotometer.

To isolate RNA from sorted cells, 5×10^5 cells were lysed in 500 µl Trizol immediately after sorting and stored at -80 °C. For subsequent RNA isolation the Trizol solution was, after gentle mixing, incubated with 100 µl Chloroform for 3 min at RT. The solution was centrifugation at 12,000x g, 15 min, 8 °C resulting in separation of the solution into a red phenol-chloroform phase (bottom), a white interphase, and an aqueous phase (top). Following gentle transfer into a new tube, the aqueous phase was incubated with 2.5 µl Polyacryl Carrier and 250 µl Isopropanol for 10 min at RT. The solution was then centrifuged at 12,000x g, 10 min, 8 °C. Addition of 500 µl 75 % Ethanol and centrifugation at 7,500x g, 5 min, 8 °C was used to wash the RNA pellet twice. After drying the pellet for 5 min at RT, it was resuspended immediately in 10 µl cDNA mastermix.

To obtain cDNA from isolated RNA the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used according to manufacturer's instructions. The cDNA mastermix contained 2 µl RT buffer, 2 µl Random Primers, 0.8 µl dNTP Mix, 1 µl MultiScribe™ Reverse Transcriptase, 1 µl RNase Inhibitor and 13.2 µl Nuclease-free water. The Mastercycler (Eppendorf) was used to perform reverse transcription for 10 min at 25 °C and 120 min at 37 °C followed by enzyme inactivation at 85 °C for 5 min. cDNA were stored at 4 °C.

qPCR and data analysis: To perform qPCR (quantitative polymerase chain reaction), the PowerTrack SYBR Green Master Mix (Life Technologies) was used according to manufacturer's instructions. The qPCR mastermix contained 5 µl SYBR blue, 0.5 µl sense primer and 0.5 µl antisense primer. 6 µl mastermix were mixed with 4 µl cDNA in a 384 well-plate and analysed using the QuantStudio 6 RT-PCR system. The qPCR reaction began with a hot start at 95 °C for 2 min followed by 40 amplification cycles (95 °C for 15 s to denaturise and 60 °C for 60 s to anneal/ extend). Fluorescence of the SYBR Green dye was quantified after each amplification step. A quality control for the primers was performed after the amplification phase as a 60 °C to 95 °C gradual melting curve.

Data were analysed using the Δ Ct method resulting in relative expression to the reference gene β -Actin.

2.2.3.5. Mucus quantification

Muc5B and Muc5AC expression was quantified in lung homogenate using Colorimetric ELISA kits (Novus Biologicals) according to manufacturer's instructions. Briefly, left lungs were homogenized in 250 μ l PBS using tubes with ceramic beads (Bertin Technologies) at 4,000 rpm for 1 min. The homogenate supernatant was obtained after centrifugation at 13,000 rpm and 4 °C for 5 min and was stored at -80 °C. If required samples were diluted in PBS. The provided 96 well-plated pre-coated with anti-Muc5B- or anti-Muc5AC-antibody was prepared with 100 μ l of samples and incubated for 90 min at 37 °C. Supernatant was aspirated and 100 μ l Biotinylated Detection antibody was incubated on the plate for 60 min at 37 °C. After decanting the supernatant, the plate was washed 4x with 350 μ l Wash buffer. 100 μ l HRP conjugate was incubated on the plate for 30 min at 37 °C. Supernatant was decanted and the plate was washed 5x with 350 μ l Wash buffer. 90 μ l of Substrate reagent was incubate on the plate for max. 15 min at 37 °C in the dark. The incubation time was adjusted to the actual colour change and the colour development was ceased by adding 50 μ l Stopping Solution. The OD₄₅₀ was recorded at the Tecan Safire2.

2.2.3.6. Cytokine profiling from lung homogenate

Cytokines were quantified from 1 ml right lung homogenate using the ProcartaPlex Mouse 12-Plex Mix&Match kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, the provided 96 well-plate was prepared with 50 μ l of magnetic beads per well and washed using a magnetic plate washer. After 10 min centrifugation at maximum speed 50 μ l of lung homogenate was incubated on the plate at 800 rpm, 30 min, RT. ON (overnight) incubation was performed at 4 °C followed by 30 min shaking at 800, RT. Following a washing step 25 μ l of Detection Antibody Mixture was incubated at 800 rpm, 30 min, RT. Following a washing step 50 μ l of Streptavidin-PE was incubated at 800 rpm, 30 min, RT. After washing 120 μ l Reading buffer was added and shaken at

800 rpm, min. 5 min, RT. Cytokines were immediately quantified at the FlexMap 3D System (Luminex). Data were analysed using the online ProcartaPlex analysis software (Thermo Fisher Scientific).

2.2.3.7. Confocal microscopy

Preparation of pulmonary vibratome slices: The whole lung was used to prepare pulmonary vibratome slices. Mice were painlessly killed at indicated time points after i.n. infection using a lethal dose (200 µl) of Xylazine (0.2 %) and Ketamine (1 %) and exsanguination via the lower aorta. Immediately after sacrificing, 1 ml of 37 °C pre-warmed 2 % agarose (low temperature-melting) was gently injected intratracheally (i.t.) to inflate the lung. To polymerize the agarose ice-cold PBS was poured over the lung. The lung was harvested immediately and placed in PBS on ice. Single lung lobes were immersed into 4 % agarose (low temperature-melting) at 37 °C and sectioned into 100 µm slices using a vibrating-blade microtome (Leica Biosystems) with a speed of 5 – 7 µm/ s and a frequency of 10 Hz. Vibratome slices were collected in PBS and kept on ice until further use, usually within 1 h after preparation.

Antibody staining of lung slices: Slices were incubated in 1 ml Antigenfix for 30 min at RT for fixation. Slices were washed twice in PBS and, to prevent unspecific staining, incubated in 400 µl per two slices of Confocal Blocking buffer containing 20 µg/ ml relevant IgG for 60 min at RT under mild shaking. Slices were then incubated in 400 µl per two slices of Confocal Blocking buffer containing 20 µg/ ml relevant IgG and primary antibodies ON at 4 °C under mild shaking. Three washing steps of 5 min in 4 ml Confocal Washing buffer at RT under mild shaking were performed. Slices were incubated in 400 µl per two slices of Confocal Blocking buffer containing 20 µg/ ml relevant IgG and secondary antibodies for 2 h at RT under mild shaking. Slices were washed 4x for 5 min in 4 ml Confocal Washing buffer at RT under mild shaking. Slices were mounted on coverslips in PBS containing 300 nM DAPI.

Slices were imaged at the LSM 710 confocal microscope (Zeiss) using a 20x objective (Zeiss) and spectral imaging with linear unmixing to enable parallel

staining of 6 fluorescent dyes. Images were acquired using Zen black (2.3 SP1 FP3 14.0.26.201) and analysed using ImageJ/ Fiji (v2.8.0/1.53t).

2.2.3.8. Quantification of the ciliary beating frequency (CBF)

Preparation and recording: pulmonary vibratome slices were prepared as described in the previous section from mice administered i.t. with LPS (10 µg in 50 µl) one day prior to sampling to upregulate expression of the IL-18R β chain. Slices were recorded with 3,000 frames/ sec using a PCO Dimax high-speed camera. CBF was recorded and subsequently slices were treated with 100 ng/ ml rIL-18 (recombinant IL-18). For a period of 30 min after IL-18 treatment CBF was recorded every 10 min. Recordings were performed using an inverted IX71 Olympus microscope equipped with a condenser, a phase ring blend, and a 40x objective.

Quantification of CBF: CBF was quantified from the recorded videos using ImageJ/ Fiji (v2.8.0/1.53t). Briefly, all recorded images were concatenated to one video and the video was registered via the MultiStackReg plugin to eliminate artefacts resulting from moving of the slice. Single cells showing ciliary beating were marked and CBF was quantified using the Cilility_JNH plugin (v0.0.1).

2.2.4. IL-18R⁺ epithelial cell-mediated protection *in vitro*

2.2.4.1. HBEC cell culture

Human bronchiolar epithelial cells (HBEC) were cultivated at 37 °C and 5 % CO₂ under humidified conditions. To investigate the influence of IL-18 on the epithelial cells, fully differentiated ALI cultures were stimulated with 50 ng/ ml of recombinant cytokines for 12 h. Cultures were either treated with rIL-12 alone to upregulate expression of the IL-18R β chain or with rIL-12 in combination with rIL-18.

Expansion: Primary HBEC were expanded over three passages. Briefly, cells were thawed and transferred to a 25 cm² cell culture flask in 5 ml of Basal medium. After 24 h medium was exchanged and then every second day until cultures reached 90 % confluency. Cells were detached with the ACF dissociation kit (StemCell) according to the manufacturer's instructions. Quantification of

viable cells was achieved using trypan blue and a Neubauer cell chamber. For continued expansion, 5×10^5 cells in 20 ml Basal medium were seeded in a 75 cm^2 cell culture flask. For storage at -150°C , 10^6 cells were resuspended in 1 ml 80 % FCS + 20 % DMSO (v/v) per cryo vial.

Air-liquid interface (ALI) cultures: HBEC were exposed to an air stimulus in PneumaCult™ ALI kit (StemCell) to induce organotypic differentiation in a so-called ALI culture system. Briefly, trans-well inserts in a 12 well-plate were coated with 100 μl collagen (1:100 in bi-distilled H_2O) for 60 min at 37°C . Remaining collagen was aspirated and inserts were dried for 2 h at RT. 8×10^4 frozen HBEC were seeded per insert in 0.5 ml Basal medium in the top chamber of the trans well. The bottom chamber was filled with 1 ml Basal medium. Medium was changed semi-weekly. 5 days after seeding when cells were grown fully confluent, and the bottom chamber was filled with ALI Maintenance medium exchanging the Basal medium. To induce the air stimulus, there was no medium added to the top chamber any longer. 21 days after inducing the air stimulus, epithelial cells were fully differentiated, and ciliary beating as well as mucus production were visible under an optical microscope at 10x magnification using phase contrast.

Organoid cultures: In the organoid system, cells are grown in a 3D environment by seeding them into a Matrigel dome to induce organotypic differentiation. The PneumaCult™ Airway Organoid kit (StemCell) was used according to the manufacturer's instructions. Briefly, 2×10^3 frozen HBEC were seeded per well into a pre-warmed 24 well-plate in 50 μl Matrigel + Seeding medium (9:1 (v/v)) using chilled pipette tips. To fully solidify the Matrigel, it was incubated at 37°C for 15 min until the Matrigel fully solidified. Wells were gently filled up with 625 μl Organoid Seeding medium. Medium was changed twice a week. 5 days after seeding, when clusters of cells within the Matrigel dome were clearly visible at 10x magnification using phase contrast, Organoid Seeding medium was aspirated and replaced by 875 μl Organoid Differentiation medium to support differentiation. 28 days after initiation of differentiation, epithelial cells were fully differentiated, and ciliary beating was visible under an optical microscope at 10x magnification using phase contrast.

2.2.4.2. Quantification of the CBF

For *in vitro* CBF quantification, organoids and ALI cultures were recorded in the tissue culture plates at 400 frames/ sec using an ORCA-Flash high-speed camera. CBF was recorded and quantified before and 12 h after treatment with 50 ng/ ml recombinant cytokines (described in **section 2.2.4.1**). Recordings were performed using an inverted IX71 Olympus microscope equipped with a condenser, a phase ring blend and a 40x objective. The CBF quantification from the recordings was performed as described above (**section 2.2.3.8.**).

2.2.4.3. Primary human neutrophils

Isolation: The EasySep™ Direct Human Neutrophil Isolation kit (StemCell) was used according to the manufacturer's instructions to negatively isolate neutrophils from fresh peripheral human blood. Briefly, 1.5 ml of EDTA (ethylenediamine tetra acetic acid)-blood was mixed with 50 µl RapidSpheres magnetic beads, 50 µl isolation cocktail of antibodies directed against non-neutrophilic antigens and 3.4 ml PBS with 1 mM EDTA to prevent coagulation for 5 min at RT. Several purification steps using the EasySep™ magnet were performed until erythrocytes in the pellet were not visible by plain eye. Usually, 3 rounds of purification were performed. Actual purity of isolated CD16⁺ CD66b⁺ neutrophils was assessed by flow cytometry.

***In vitro* infection with *L. longbeachae*:** Bacteria were grown on BCYE plates for 72 h at 37 °C and all colonies from the BCYE plate were resuspended in PBS. The infection dose was adjusted to a MOI using that 10⁹ CFU/ ml result in OD₆₀₀ of 1. Prior to infection, freshly isolated neutrophils were incubated with medium from IL-18-treated ALI-cultures for 90 min at 37 °C and 5 % CO₂. Neutrophils were infected with *L. longbeachae*-mCherry for 60 min at 37 °C and 5 % CO₂. Extracellular bacteria were eliminated by flow cytometry-sorting mCherry⁺ neutrophils. Viable bacteria were quantified in sorted neutrophils by incubation with Tween® 20 for 30 min at 4 °C (as described in **section 2.2.3.1.**). Bacterial killing by neutrophils was investigated by quantifying CFU over a period

of max. 60 min during which neutrophils were again treated with medium from IL-18-treated ALI cultures.

2.2.5. ER stress-induced anti-bacterial protection

2.2.5.1. Macrophage cell lines

Cells were cultivated 37 °C and 5 % CO₂ under humidified conditions.

Human THP-1 cells: THP-1 cells were grown in RPMI + 10 % FCS (v/v) within 75 cm² cell culture flasks in suspension and passaged twice a week. Differentiation into macrophage-like phenotype was induced by 2 days treatment with 50 ng/ ml Phorbol myristate acetate (PMA) at a density of 10⁶ cells/ ml. Usually cells were seeded into 24 well-plates in 500 µl/ well. Differentiated macrophage-like cells were then ready to be used for pharmacological treatments or infections.

Murine iBMDM: Immortalized bone marrow-derived macrophages (iBMDM) were cultivated in DMEM + 10 % FCS (v/v) within 75 cm² flasks. Cells were passaged three times a week when grown to 90 % confluency using a cell scraper to detach adherent cells. For pharmacological treatments cells were seeded into cell culture plates with a density of 2x 10⁵ cells/ ml. Usually cells were seeded into 24 well-plates in 500 µl/ well. Cells were ready to use after 24 h.

ER stress induction: ER stress was induced by treating fully differentiated THP-1 cells with 1 µM thapsigargin for 2 h at 37 °C and 5 % CO₂. Thapsigargin triggers ER stress by limiting calcium exchange through the SERCA pump leading to indirect calcium depletion from the ER and thereby to ER stress (Gorman et al. 2012; Osowski 2015). 200 ng/ ml IFN-γ was used as a positive control for cytokine measurements and replication assays. After 2 h incubation, pharmacological treatments were removed, and cells were incubated in fresh medium ON at 37 °C and 5 % CO₂. Cytokines were quantified in the supernatant.

UPR inhibition: To specifically inhibit different branches of the UPR response established small molecule inhibitors against ER stress sensors were used (Grandjean & Luke Wiseman 2020). Concentrations and specificity are summarized in Table 1. THP-1 macrophages were treated with the inhibitors

either for 2 h prior to thapsigargin treatment or ON after infection with *L. pneumophila*.

Table 13: Summary of used small molecule UPR inhibitors with specificity and used concentration.

UPR inhibitor	Specificity	Concentration
4μ8C	IRE1 RNase domain	20 μM
KIRA6	IRE1 kinase domain	12.5 μM
GSK2606414	PERK kinase	10 μM
ISRIB	Downstream PERK signalling	1 μM
Ceapin-A7	ATF6 signalling	1 μM

***In vitro* infection with *L. pneumophila*:** Bacteria were cultured on BCYE plates for 3 days at 37 °C. 5 – 10 colonies were inoculated into 5 ml AYES broth and expanded ON at 37 °C and 200 rpm shaking. The infection dose was adjusted to a MOI of 1 in RPMI + 10 % FCS using that 8×10^8 CFU/ ml result in an OD₆₀₀ of 1. THP-1 macrophages were infected with *L. pneumophila* for 60 min at 37 °C and 5 % CO₂. The bacterial solution was removed, and incubation with 100 μg/ ml Gentamicin in RPMI + 10 % FCS for 60 min was used to eliminate extracellular bacteria. Cells were washed twice with RPMI and fresh RPMI + 10 % FCS was added. Viable bacteria were quantified from infected THP-1 macrophages after 3 h, 24 h, and 48 h by incubation with 0.05 % Digitonin 10 min at RT (as described in 2.2.3.1.).

2.2.5.2. CRISPR

STAT1^{-/-} (signal transducer and activator of transcription) human THP-1 cells were generated by our collaboration partners Prof. Seth masters and Dr. Sophia Davidson (Walter and Eliza Hall Institute, University of Melbourne) using the CRISPR (clustered regularly interspaced short palindromic repeats)/ Cas-9 (CRISPR-associated protein 9) system. Briefly, THP-1 cells stably expressing the bacterial Cas-9 DNA endonuclease together with a mCherry reporter were transfected with *STAT1*-specific guide RNA and a BFP (blue fluorescent protein) reporter using a lentiviral vector. Cells were cultured according to the cell culture

protocol described before (**section 2.2.5.1.**). Protein knockdown was quantified by Westernblot and cell colonies with efficient knockdown were used for experiments.

2.2.5.3. Western blot

Protein expression was analysed from cells grown in 24 well-plates by Dr. Christina Giogha and Dr. Kathleen McCraffey (Hudson Institute of Medical Research) using SDS-Page, Western blot, and HRP-mediated antibody imaging techniques.

2.2.5.4. qPCR

RNA isolation and cDNA synthesis: RNA was obtained from cells grown in 24 well-plates using the ISOLATE II RNA Mini Kit (Meridian Bioscience) according to manufacturer's instructions. Briefly, supernatant was discarded and 250 µl RLY buffer containing 2.5 µl β-mercaptoethanol were added per well. Cells were lysed under mild shaking for 2 min at RT. The lysate was transferred to an RNase-free tube and stored at -20 °C. RNA was isolated via column separation, eluted in 32 µl RNase free water, and analysed at the NanoDrop™ spectrophotometer. Single RNA samples were diluted to the same concentration per experiment using RNase-free water.

To convert RNA into cDNA the SuperScript™ III kit (Life Technologies) was used according to manufacturer's instructions. Briefly, 1 µl Random Hexamers and 1 µl dNTPs were added to 8 µl RNA and incubated for 5 min at 65 °C to initiated annealing. 10 µl cDNA mastermix was added to the reaction containing 2 µl RT buffer, 4 µl 25 mM MgCl₂, 2 µl 0.1 mM DTT, 1 µl RNaseOUT, and 1 µl SuperScript III Reverse Transcriptase. Mastercycler (Eppendorf) was used to perform the reverse transcription for 10 min at 25 °C and 50 min at 50 °C. Enzymes were inactivated at 85 °C for 5 min. To eliminate remaining RNA, 1 µl of RNaseH was added for 20 min at 37 °C. cDNA was stored at -20 °C.

qPCR and data analysis: qPCR analysis was performed using the PowerSYBR® Green kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, the qPCR mastermix contained 5 µl SYBR green, 1 µl sense

primer, 1 μ l antisense primer and 2 μ l Nuclease-free water. 9 μ l mastermix were mixed with 1 μ l cDNA in a 384 well-plate and analysed using the QuantStudio 6 RT-PCR system. The qPCR reaction began with a hot start at 95 °C for 2 min followed by 40 amplification cycles (95 °C for 15 s to denaturise and 60 °C for 60 s to anneal/ extend). Fluorescence of the SYBR Green dye was quantified after each amplification step. A quality control for the primers was performed after the amplification phase as a 60 °C to 95 °C gradual melting curve. Data were analysed using the $\Delta\Delta C_t$ method resulting fold changed to the control sample.

2.2.5.5. Cytokine profiling from cell culture supernatant

CBA Flex Set: Cytokines were quantified from cell culture supernatant using either Mouse or Human CBA Flex Set kits (Becton-Dickons) according to manufacturer's instructions. Briefly, the supernatant was centrifuged for 5 min at 1,000x g. 5 μ l of supernatant was mixed with 5 μ l of Capture Bead mixture and 5 μ l of PE Detection Reagent mixture in a U-bottom 96 well-plate and incubated for 2 h at 4 °C. Both mixtures contained beads or PE detection reagent against all cytokines that were to be detected. In case of infectious supernatants beads were incubated with 4 % PFA for 30 min at 4 °C. Following a washing step, 100 μ l FACS buffer were added, and cytokines were immediately quantified at the LSRFortessa™ flow cytometer. Data were analysed using FlowJo (V10.8.1).

ProcartaPlex: Cytokines were quantified from cell culture supernatant using the ProcartaPlex Human Cytokine/ Chemokine Convenience Panel 1A 34plex (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, the supernatant was centrifuged for 5 min at max. speed. A 96 well-plate was prepared with 25 μ l of magnetic beads per well. After washing using a magnetic plate washer 25 μ l of lung homogenate was incubated at 800 rpm, 30 min, RT. ON incubation was performed at 4 °C followed by 30 min shaking at 800 rpm, RT. Following a washing step 12.5 μ l of Detection Antibody Mixture incubated at 800 rpm, 30 min, RT. Following a washing step 25 μ l of Streptavidin-PE was incubated at 800 rpm, 30 min, RT. After washing 120 μ l Sheath Fluid was added and shaken at 800 rpm, min. 5 min, RT. Cytokines were immediately quantified

at the FlexMap 3D System (Luminex). Data were analysed using Thermo Fisher Connect™ (Thermo Fisher Scientific).

2.2.6. Data processing and statistical analysis

Microsoft Office 365, FlowJo (V10.8.1), ImageJ/ Fiji (v2.8.0/1.53t) were used to process raw data as described in each section. Affinity Designer (1.10.6) and Biorender were used to create figures. GraphPad Prism (v9.4.1) was used to create graphs and determine statistical significance. First, normality was analysed, and subsequent statistical tests were chosen accordingly. To compare two groups unpaired t test (normally distributed) or Mann-Whitney test (not normally distributed) were used. To compare three or more groups Ordinary one-way ANOVA followed by Turkey's multiple comparisons for inter-group differences (normally distributed) or Kruskal-Wallis test followed by Dunn's multiple comparisons test for inter-group differences (not normally distributed) were used. Threshold for statistical significance was $P < 0.05$ and significances are designated by stars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Chapter 3: Differential contribution of resident and infiltrating immune cells to defence against *L. longbeachae*

3.1. Introduction

The innate immune response is crucial for the rapid onset of anti-bacterial defence against invading pathogens and the prevention of their initial spread especially from barrier organs like the lung (Byrne et al. 2015). Nevertheless, the innate immune response against the pulmonary pathogen *L. longbeachae* is poorly understood. *L. longbeachae* is the second most prevalent causative of LD, a severe form of bacterial pneumonia (Whiley & Bentham 2011; Mondino et al. 2020). Accidental infections of human with these environmental bacteria occur via inhalation of aerosols from infected soil (Chambers et al. 2021; Cunha, Burillo & Bouza 2016). After inhalation, *L. longbeachae* are thought to be able to turn AM into a replicative niche (Massis et al. 2017) as shown for other *Legionella* spp., including the most prevalent species *L. pneumophila* (Mondino et al. 2020). Although *L. longbeachae* is able to infect and replicate within macrophages *in vitro* (Wood et al. 2015; Massis et al. 2017), the role of those lung-resident cells *in vivo* remains unclear.

AM form the first line of defence within the lung. The main role of AM is to patrol the alveoli (Garbi & Lambrecht 2017; Kopf, Schneider & Nobs 2015) and in the case of an infection to recruit and stimulate bystander immune cells (Byrne et al. 2015). The Zamboni laboratory was able to show a strong influx of neutrophils into the murine lung during infection with *L. longbeachae* and therefore proposed a protective role of those phagocytes. Nevertheless, functional studies are lacking to prove the role of neutrophils in immune control of *L. longbeachae*. Regarding *L. pneumophila*, neutrophils are directly recruited by AM-derived IL-1 α (Barry et al. 2013) and they make up the vast majority of immune cells in the lung during *L. pneumophila*-induced pneumonia (Brown et al. 2016). Several studies limiting neutrophil influx revealed their protective function (Mascarenhas et al. 2015; Casson et al. 2017). Recently a protective role for

infiltrating monocytes during anti-*L. longbeachae* defence was uncovered *in vivo* (Ataide et al. 2023).

Furthermore, the data from the Zamboni laboratory showed IFN- γ and TNF α to be required for optimal defence against *L. longbeachae* (Massis et al. 2017). Taken together with previous data from our laboratory showing the defence to be dependent on IL-18 (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020), this suggests a role for NK cells or T cells as these cells produce IFN- γ following IL-18R stimulation (Nakanishi et al. 2001a; Ellis & Beaman 2004; Schroder et al. 2004). Previous data from our laboratory revealed that depletion of either NK cells or T cells alone did not impair the defence against *L. longbeachae*. Nevertheless, it was shown before during bacterial pneumonia that NK cells and T cells can fulfill redundant roles (Junqueira-Kipnis et al. 2003; Feng et al. 2006) and this might be the case for defence against *L. longbeachae*.

Work performed in this chapter aimed to elucidate which and how immune cells participate in the defence against *L. longbeachae*. The overall cellular and molecular immune response was investigated using multi-color flow cytometry and dimensionality reduction as well as multiplex cytokine profiling and RNAsequencing. The focus was set on elucidating differential roles for tissue-resident AM and infiltrating neutrophils and the underlying cellular mechanisms. In addition, a redundant protective role of NK cells and T cells was investigated.

3.2. Results

3.2.1. Concomitant decrease of alveolar macrophages and influx of circulating immune cells during *L. longbeachae* infection

To characterize in more detail the cellular and molecular immune response against pulmonary *L. longbeachae* infection, I used a previously reported infection model based on bacterial inoculation of C57BL6/J mice via the intranasal route (Massis et al. 2017). Using this system, I quantified and localized immune cell influx into the lung (**Fig. 4A**).

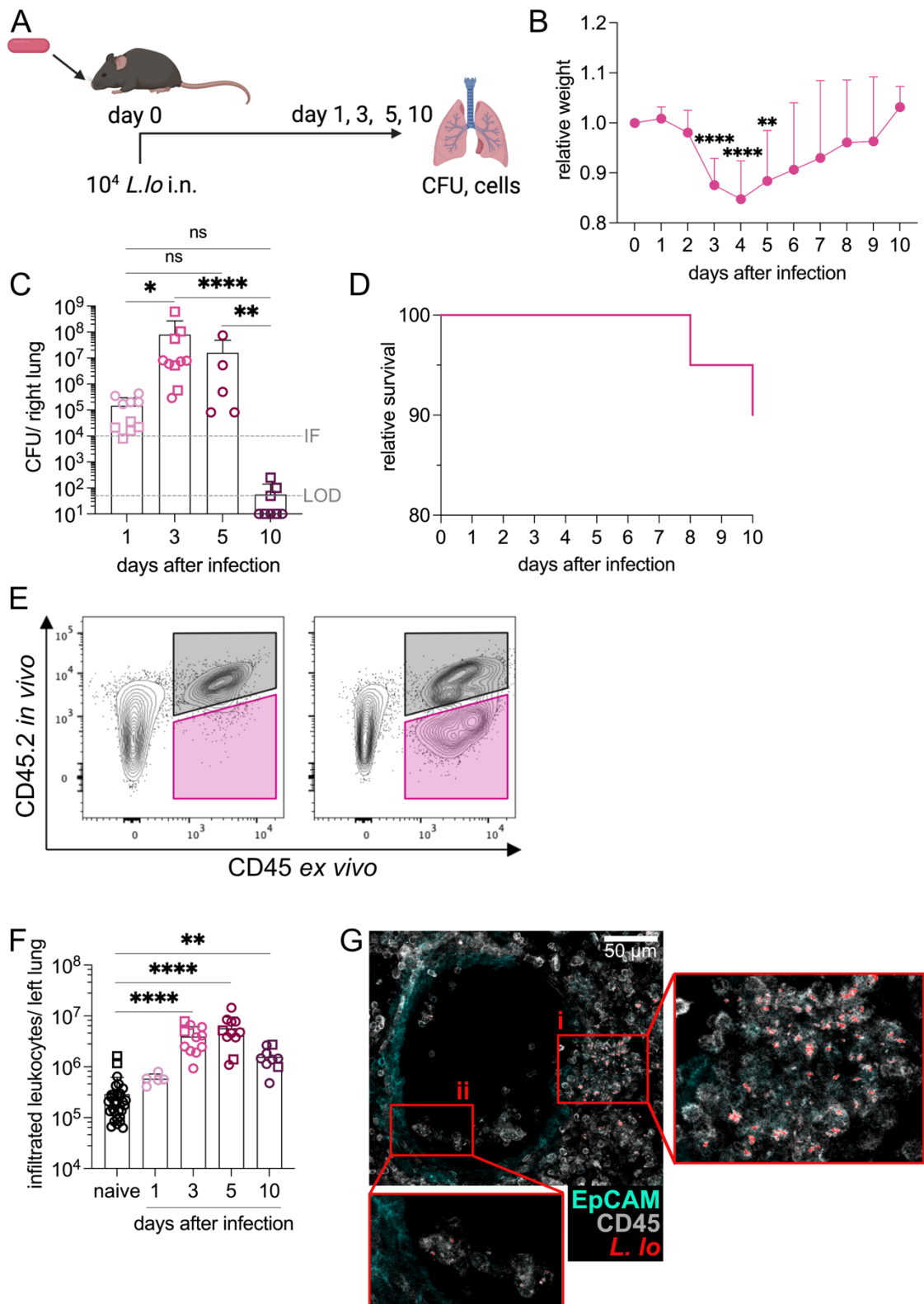


Figure 4: *L. longbeachae* administration induces a productive pulmonary infection accompanied by a strong influx of immune cells.

I.n. *L. longbeachae* administration induced a productive and transient infection in mice as demonstrated by a temporary weight loss (**Fig. 4B**) and increase in bacterial load in the lung that exceeded the infection dose (**Fig. 4C**). Infection peaked at day 3 – 5 after infection, both in terms of weight loss and CFU in the lung. Mice recovered in most cases (**Fig. 4D**) and most mice cleared the infection below the detection limit by day 10 after infection, when they recovered the initial body weight. I.v. administration of a fluorescently-labelled CD45.2 antibody 5 min prior to sacrificing mice stained all leukocytes in the blood *in vivo* (data not shown). By co-staining the pulmonary single cell solution *ex vivo* after isolation with a fluorescently-labelled CD45 antibody, enabled the differentiation of circulating immune cells (CD45 *ex vivo*⁺ CD45 *in vivo*⁺, grey gate) and tissue-infiltrating immune cells (CD45 *ex vivo*⁺ CD45 *in vivo*⁻, pink gate) (**Fig. 4D**). Infiltrating immune cells peaked 3 – 5 days after infection and decreased again 10 days later (**Fig. 4E**), paralleling progression in CFU counts. Confocal imaging of pulmonary vibratome slices revealed that bacteria were located mainly in defined clusters together with immune cells in the alveolar spaces (**Fig. 4F.i**) and to a lesser extent in the bronchiolar lumen (**Fig. 4F.ii**).

Figure 4: *L. longbeachae* administration induces a productive pulmonary infection accompanied by a strong influx of immune cells.

(A) Scheme of experimental setup. C57BL6/J mice were i.n. infected with 10^4 *L. longbeachae* (*L. lo*)-mCherry CFU. Lungs were harvested at the indicated timepoints for analysis. (B) Kinetics of relative mouse weight during infection. (C) Bacterial load in the lungs at the indicated timepoints. LOD (limit of detection, 50 CFU), IF (infection dose, 10^4 CFU). (D) Representative flow cytometry plots of immune cells in the lungs of uninfected (left panel) and infected (right panel, day 3 after infection) mice. Intravascular leukocytes were intravitaly labelled by administration of CD45.2-AF488 *in vivo*. Pink gat indicates extravascular leukocytes infiltrating the lung; grey gate indicates intravascular leukocytes. (E) Quantification of extravascular leukocytes (excluding alveolar macrophages to focus on infiltrating leukocytes) as identified in (D) at the indicated time points. (F) Spectral confocal image of lung vibratome slices at day 5 after infection with *L. longbeachae*-mCherry. Slices were stained with EpCAM to identify bronchiolar epithelial cells and CD45 from immune cells. *L. longbeachae* were found in alveolar areas (i) as well as in discharges within the bronchiolar lumen (ii). Data in (B), (C), and (E) is shown as mean + SD of two pooled independent experiments. Symbols in (C) and (E) represent individual mice, and symbol shape indicates different experiments. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) was performed for statistical analysis. Inter-group analysis in (B) was performed relative to uninfected (day 0). * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

Taken together, i.n. infection of C57BL6/J mice with 10^4 *L. longbeachae* CFU induced a productive but self-resolving infection of the lungs as demonstrated by a transient loss of body weight and increase in intrapulmonary immune cell infiltrate. Therefore, this infection model is suitable to study the detailed defence against pulmonary infection with *L. longbeachae*.

To quantify specific immune cell compartments, two different staining cocktails were applied to identify myeloid (**Fig. 5A**) and lymphoid (**Fig 5B**) cell subpopulations. Both cocktails included a lineage gate to exclude the irrespective populations: CD19⁺ or CD3⁺ or NK1.1⁺ (lineage 1) to exclude lymphocytes and CD11c⁺ or Ly-6G⁺ (lineage 2) to exclude myeloid cells. Ter119 was used to exclude erythrocytes.

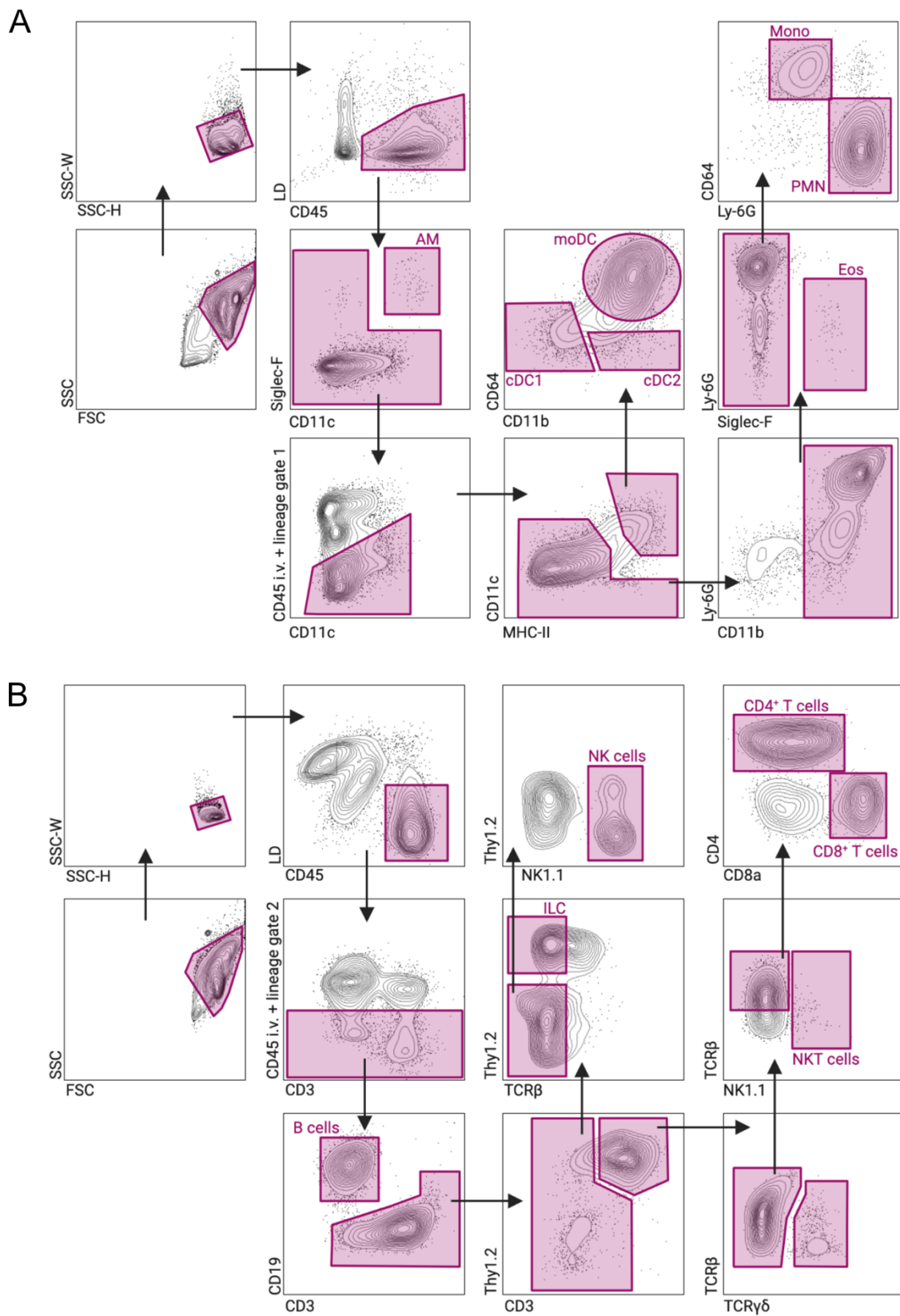


Figure 5: Myeloid and lymphoid cell populations were identified using multi-color flow cytometry and the presented gating strategy.

Using this gating strategy, I performed UMAP dimensionality reduction of flow cytometry data to visualize progression of different immune cells along infection kinetics. Different myeloid (**Fig. 6A**) and lymphoid (**Fig. 7A**) cells infiltrated the lung during infection as demonstrated by UMAP analysis.

Figure 5: Myeloid and lymphoid cell populations were identified using multi-color flow cytometry and the presented gating strategy.

Pulmonary single cell-suspensions were analysed by multi-color flow cytometry. Single cells were gated by standard gating using SSC-A vs. FSCA-A followed by SSC-H vs. SSC-W.

(A) Lineage gate 1 included: CD3, CD19, NK1.1, and Ter119. Myeloid cell populations were distinguished as:

Alveolar macrophages (**AM**): Live CD45⁺ Siglec-F⁺ CD11c⁺

Monocyte-derived Dendritic cells (**moDC**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD11c⁺ MHC-II⁺ CD11b⁺ CD64⁺

Conventional Dendritic cells 1 (**cDC1**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD11c⁺ MHC-II⁺ CD11b⁻ CD64⁻

Conventional Dendritic cells 2 (**cDC2**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD11c⁺ MHC-II⁺ CD11b⁺ CD64⁻

Eosinophils (**Eos**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD11c⁻ CD11b⁺ Siglec-F⁺

Neutrophils (**PMN**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD11c⁻ CD11b⁺ Siglec-F⁻ Ly-6G⁺ CD64⁻

Monocytes (**Mono**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD11c⁻ CD11b⁺ Siglec-F⁻ Ly-6G⁻ CD64⁺

(B) Lineage gate 2 included: CD11c, Ly-6G, and Ter119. Lymphoid cell populations were distinguished as:

B cells: Live CD45⁺ CD45i.v.⁻ Lin⁻ CD3⁻ CD19⁺

Innate lymphoid cells (**ILC**): Live CD45⁺ CD45i.v.⁻ Lin⁻ CD19⁺ CD3⁻ Thy1.2⁺

NK cells: Live CD45⁺ CD45i.v.⁻ Lin⁻ CD19⁺ CD3⁻ Thy1.2⁻ NK1.1⁺

NKT cells: Live CD45⁺ CD45i.v.⁻ Lin⁻ CD19⁺ CD3⁺ Thy1.2⁺ TCR $\gamma\delta$ ⁻ NK1.1⁺

CD4⁺ T cells: Live CD45⁺ CD45i.v.⁻ Lin⁻ CD19⁺ CD3⁺ Thy1.2⁺ TCR $\gamma\delta$ ⁻ NK1.1⁻ TCR β ⁺ CD4⁺ CD8⁻

CD8⁺ T cells: Live CD45⁺ CD45i.v.⁻ Lin⁻ CD19⁺ CD3⁺ Thy1.2⁺ TCR $\gamma\delta$ ⁻ NK1.1⁻ TCR β ⁺ CD4⁻ CD8⁺

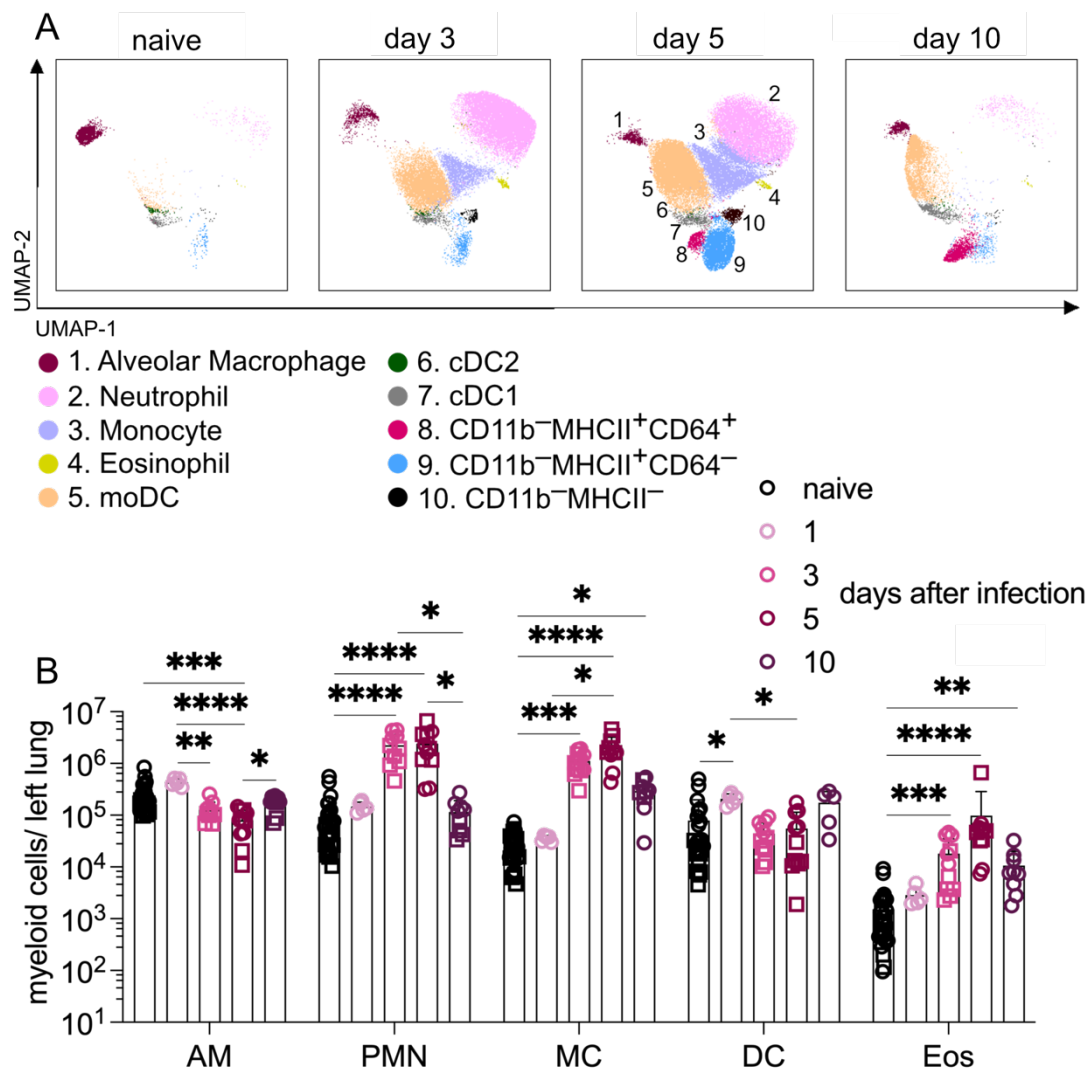


Figure 6: Circulating myeloid cells infiltrate the lung during infection with *L. longbeachae* while alveolar macrophages are reduced.

Mice were i.n. infected with 10^4 *L. lo*-mCherry. **(A)** Flow cytometry UMAP dot plots of extravascular CD19⁻ NK1.1⁻ CD3⁻ myeloid cells at the indicated time points. **(B)** Quantification of indicated immune cells from (A). Data is shown as mean + SD of two pooled independent experiments. Symbols represent single mice; symbol shape indicates different experiments. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) was performed for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Neutrophils and monocytes infiltrated the lung tissue (**Fig. 6C**) in similar kinetics to that of the bacterial load (**Fig. 4C**) and total leukocytes (**Fig. 4E**) in lungs, again paralleling the bacterial load. Neutrophils dominate the infiltrate at day 3 after infection and monocytes reach similar numbers at day 5. In contrast, lung-resident AM decreased in numbers when bacterial CFU were highest and

returned to normal levels by day 10 after infection, thus negatively correlating with bacterial burden (**Fig. 6C**).

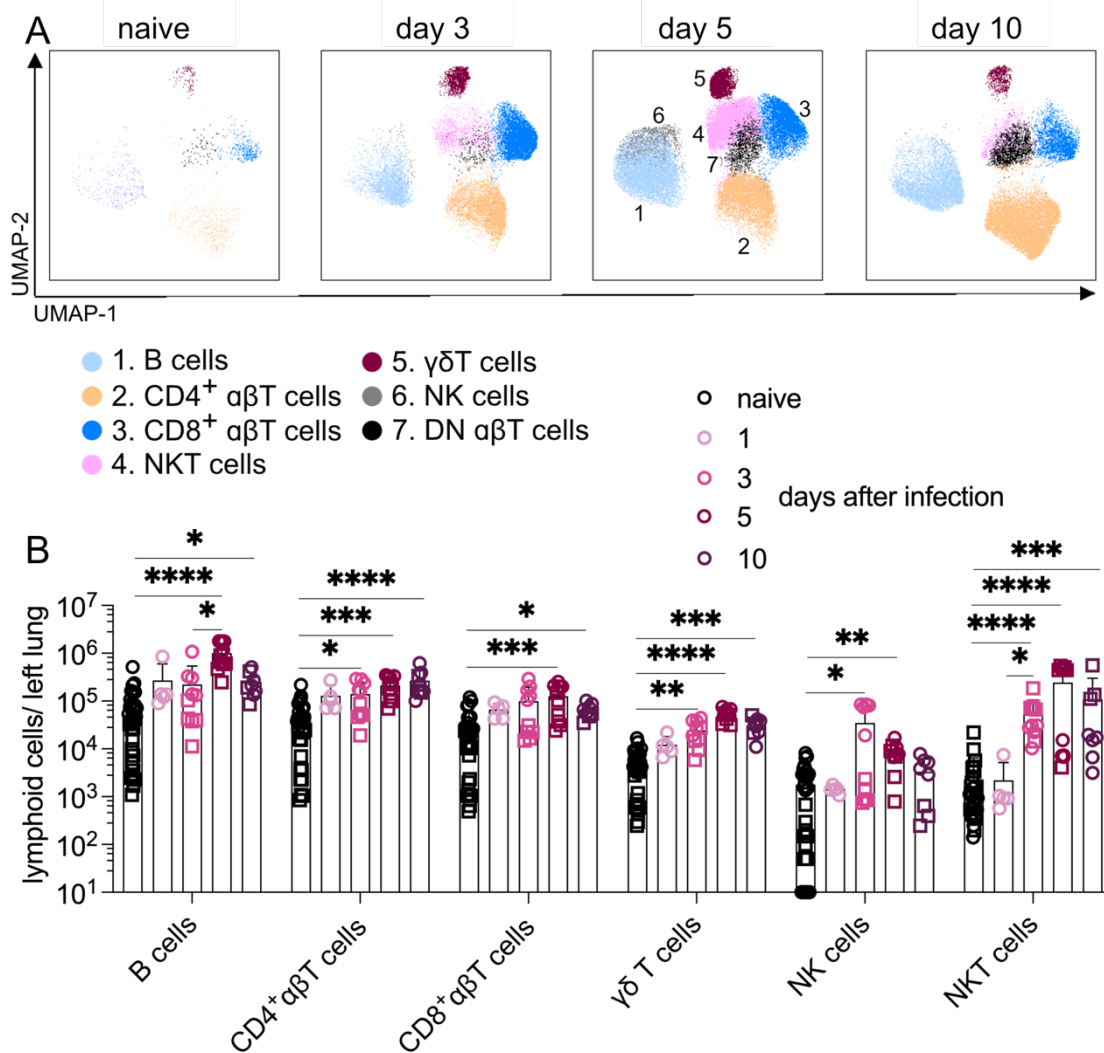


Figure 7: Lymphocytes strongly infiltrate the lung at later time points during *L. longbeachae* infection.

Mice were i.n. infected with 10^4 *L. lo-mCherry*. **(A)** Flow cytometry UMAP dot plots of extravascular CD11c $^-$ Ly-6G $^-$ lymphoid cells (G) at the indicated time points. **(B)** Quantification of indicated immune cells from (A) and (B). Data is shown as mean + SD of two pooled independent experiments. Symbols represent single mice; symbol shape indicates different experiments. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) was performed for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

In addition, a variety of lymphoid cells accumulated in the infected lung peaking at day 5 (**Fig. 7B**), including B cells, CD8 $^+$ and CD4 $^+$ $\alpha\beta$ T cells, $\gamma\delta$ T cells and NKT cells (**Fig. 7D**). Most lymphoid cells remained in high numbers even at

day 10 after infection when bacterial numbers were strongly reduced. An exception were NK cells that peaked at day 3 after infection and then progressively decayed in numbers by day 10 (**Fig. 7B**), suggesting this cell population fulfills a function earlier during infection.

Summarizing, I could show that *L. longbeachae* induced a productive pulmonary infection accompanied by a concomitant increase of infiltrating leukocytes and a reduction of AM that paralleled bacterial load in the lung.

3.2.2. Differential bactericidal capacity of tissue-resident and infiltrating phagocytes towards *L. longbeachae*

To further investigate the contribution of resident and infiltrating phagocytes in the defence against *L. longbeachae*, I used a simplified flow cytometry gating strategy to focus on phagocytes of interest (**Fig. 8**).

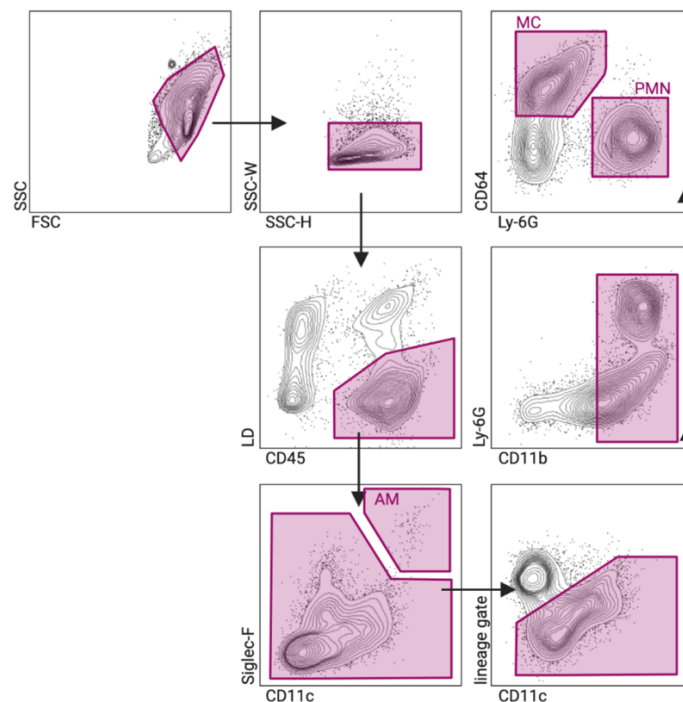


Figure 8: Gating strategy for quantification of phagocytes by flow cytometry.

Pulmonary single cell-suspensions were analysed by multi-color flow cytometry. Single cells were gated by standard gating using SSC-H and SSC-W. Lineage gate included: CD3, CD19, NK1.1, and Ter119.

Phagocytes were distinguished as:

Alveolar macrophages (**AM**): Live CD45⁺ Siglec-F⁺ CD11c⁺

Neutrophils (**PMN**): Live CD45⁺ Lin⁻CD11b⁺ Ly-6G⁺ CD64⁻

Monocyte-derived cells (**MC**): Live CD45⁺ Lin⁻ CD11b⁺ Ly-6G⁻ CD64⁺

That gating strategy together with infection using a *L. longbeachae*-mCherry reporter strain enabled me to track bacteria in different phagocyte populations over the course of pulmonary infection with *L. longbeachae*.

I confirmed previous data from our laboratory (V. Scheiding, doctoral thesis, Bonn/ Melbourne 2020) showing that *L. longbeachae* mainly locate in immune cells and only to a minor extent in stromal cells (**Fig. 9A**). Within the immune compartment, I tracked the bacteria in neutrophils, AM, and MC (**Fig. 9B**). UMAP dimensionality reduction of flow cytometry data gating on phagocytes containing *L. longbeachae*-mCherry bacteria (mCherry⁺) revealed engulfment of *L. longbeachae* within different phagocytes was highly dynamic (**Fig. 9C**). Early during infection *L. longbeachae* were mainly located in AM (**Fig. 9D**) in line with these phagocytes being the first cells in the lung encountering the bacteria and opening the possibility for these cells to be a replicative niche for the bacteria. Infiltrating phagocytes, including neutrophils and MC, took up bacteria later during infection. Neutrophils started from day 3 on, while MC engulfed bacteria from day 5 on and dominated the mCherry⁺ population at late time points after infection (**Fig. 9D**).

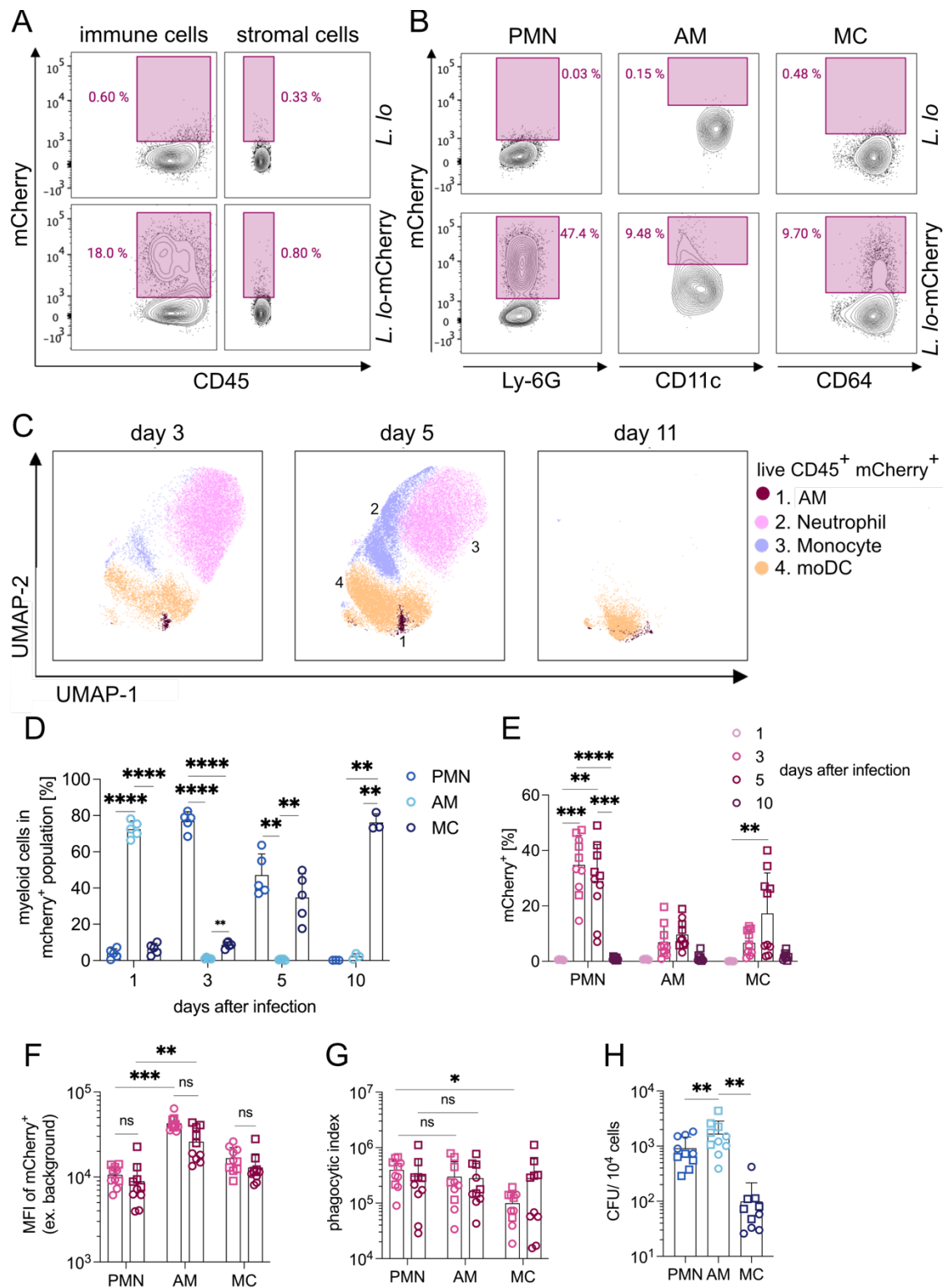


Figure 9: Alveolar macrophages contain the highest number of viable bacteria even though neutrophils are most efficient in bacterial uptake.

In order to investigate the bacterial uptake capacity of each phagocyte population, I looked at percentage of *L. longbeachae*-containing cells in AM,

neutrophils, and MC. I demonstrated that neutrophils had the highest capacity to phagocytose bacteria with around 30 % of all lung-infiltrating neutrophils containing *L. longbeachae* at day 3 and 5 after infection (**Fig. 9E**). While AM showed the lowest capacity to engulf *L. longbeachae*, mCherry⁺ AM showed the highest mCherry mean fluorescence intensity (MFI) (**Fig. 9F**), which suggested a higher number of internalized bacteria. As a result, the phagocytic index (% of mCherry⁺ cells x mCherry MFI) was similar between neutrophils and AM (**Fig. 9G**).

To show whether the differences in mCherry MFI resulted from differences in number of viable bacteria per cell, I next purified the three phagocyte populations and quantified the number of viable bacteria within each population. Indeed, although a lower percentage of AM than neutrophils contained bacteria (**Fig. 9E**), AM contained the highest average number of viable bacteria per cell (**Fig. 9H**). MC showed the lowest number of viable bacteria at day 2 after infection but both the number of MC infiltrating the lung (**Fig. 6B**) as well as the number of MC taking up bacteria (**Fig. 9E**) increased on day 5, suggesting a role for MC later in infection. The higher number of viable bacteria per cell in AM together with the

Figure 9: Alveolar macrophages contain the highest number of viable bacteria even though neutrophils are most efficient in bacterial uptake.

C57BL/6/J mice were i.n. infected with 10^4 *L. lo*-mCherry CFU. **(A)** Representative flow cytometry plots of leukocytes (left panel) and stromal cells (right panel) showing percentage of cells harboring *L. longbeachae* (mCherry⁺) 3 days after infection with WT *L. longbeachae* (top) or *L. longbeachae*-mCherry (bottom). **(B)** Representative flow cytometry plots of indicated phagocyte populations 3 days after infection with WT *L. longbeachae* (top) or *L. longbeachae*-mCherry (bottom). **(C)** Flow cytometry UMAP plots of extravascular mCherry⁺ CD19⁻ NK1.1⁻ CD3⁻ myeloid cells. Different mCherry⁺ phagocytes populations are as indicated. **(D)** Quantification of indicated immune cells from (C). **(E)** Percentage of mCherry⁺ cells within the population of different phagocytes. Cells from mice infected with WT *L. longbeachae* served as mCherry⁻ controls. **(F)** mCherry MFI (mean fluorescence intensity) in indicated mCherry⁺ cells after subtraction of background MFI in mCherry⁻ cells of the same population. **(G)** Phagocytic index of different phagocyte populations. Phagocytic index = percentage of mCherry⁺ cells x mCherry MFI. **(H)** Number of viable bacteria (CFU) per 10^4 cells of indicated cell populations sorted 2 days after infection. Data in (D) is from one experiment. Data in (E), (F), (G), (H) is shown as mean + SD of two independent experiments. Symbols in (E), (F), (G), (H) represent single mice, symbol shapes indicate different experiments. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) (E), (F), (G) or RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) (D), (H) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** < 0.001, **** P < 0.0001.

fact that these phagocytes dominate the mCherry⁺ population at early timepoints during infection (**Fig. 9D**), led to the hypothesis that AM served as a replicative niche for *L. longbeachae* while neutrophils infiltrated the lung later during infection and served to phagocytose and kill bacteria.

Taken together, we showed that infiltrating phagocytes contribute to bacterial uptake and, possibly, killing of bacteria as indicated by high percentage of mCherry⁺ cells but low mCherry MFI and low number of viable bacteria per cell. Neutrophils likely fulfill this function during early timepoints while MC become more important later during infection. Our results indicate that lung-resident AM kill internalized bacteria in a less efficient manner than neutrophils.

3.2.3. Alveolar macrophages promote establishment of pulmonary infection with *L. longbeachae*

The fact that AM contained the highest number of viable *L. longbeachae* compared to neutrophils and MC (**Fig. 9E**), suggested that the former were inefficient at killing *L. longbeachae* whereas the latter contributed to clearing the infection. In addition, these results suggested that AM may even promote infection by serving as the replicative niche for *L. longbeachae*.

To investigate their role during infection, we depleted tissue-resident AM by local application of clodronate-loaded liposomes one day prior to infection (**Fig. 10A**).

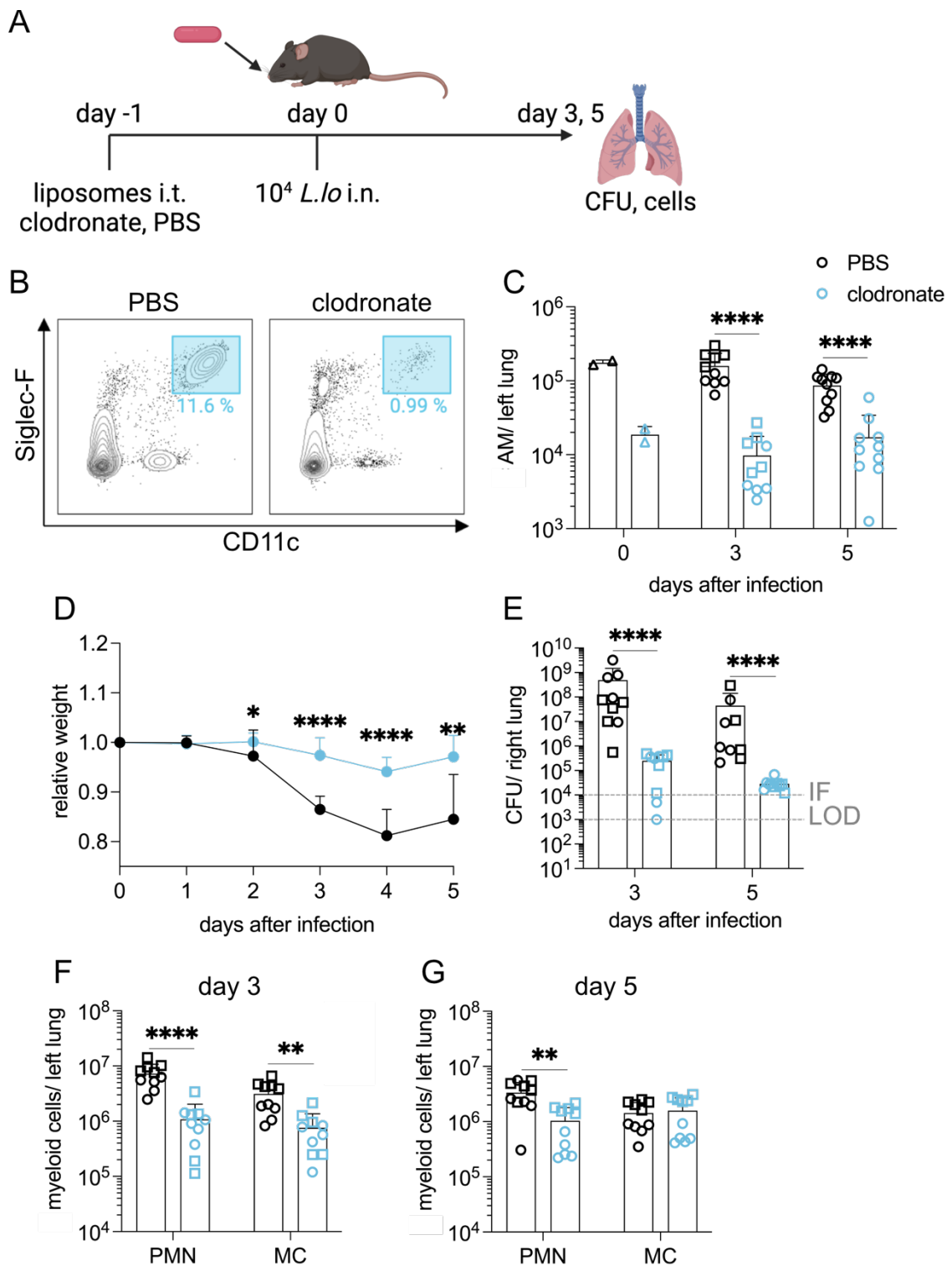


Figure 10: Depletion of alveolar macrophages improved mouse fitness and reduced bacterial burden during infection with *L. longbeachae*.

Quantification of immune cells in the lung of clodronate liposome-treated mice showed an efficient reduction of AM to about 10 % when compared to mice treated with PBS-loaded control liposomes (**Fig. 10B, C**). The depletion was

persistent during the time course of infection (**Fig. 10C**) and led to a significantly improved mouse fitness, indicated by a reduced weight loss (**Fig. 10D**). As hypothesized, AM promoted *L. longbeachae* infection as demonstrated by 100 – 1,000-fold higher bacterial burden in the lungs of AM competent mice than their AM depleted counterparts (**Fig. 10E**). Quantifying myeloid cells in the lung revealed that depletion of AM resulted in a reduced number of neutrophils and MC (**Fig. 10E, F**), thus excluding an indirect effect of AM depletion by promoting excessive phagocyte influx.

Taken together these results indicated that *L. longbeachae* harness AM to establish infection, suggesting that AM serve as a replicative niche in the early phases of infection.

3.2.4. Neutrophils are required for efficient *L. longbeachae* clearance in a NADPH oxidase-dependent manner

In contrast to AM, our *ex vivo* quantification of *L. longbeachae* CFU suggested that mainly neutrophils but also MC could efficiently kill *L. longbeachae* (**Fig. 9H**). Former data from our laboratory already showed that MC are required for defence against *L. longbeachae* (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020) which was recently replicated (Ataide et al. 2023). Thus, we next investigated the role of neutrophils during anti-*L. longbeachae* infection *in vivo* by depleting neutrophils using a combination of an anti-Ly-6G mAb (1A8) and a secondary anti-rat mAb (MAR18.5) (**Fig. 11A**), which induces an efficient depletion of

Figure 10: Depletion of alveolar macrophages improved mouse fitness and reduced bacterial burden during infection with *L. longbeachae*.

(A) Scheme of experimental setup. C57BL6/J mice were intratracheally (i.t.) administered with 50 µl clodronate- or PBS-loaded liposomes one day prior to infection. Mice were then i.n. infected with 10^4 *L. lo*-mCherry CFU. **(B)** Representative flow cytometry plots showing depletion of AMs at day 0. **(C)** Quantification of AMs at the indicated timepoints. **(D)** Kinetics of relative mouse weight during infection. Legend as in (C) **(E)** Bacterial load in the right lung at the indicated days after infection. LOD (limit of detection, 50 CFU), IF (infection dose, 10^4 CFU). **(F)-(G)** Quantification of pulmonary myeloid cells at day 3 (F) and day 5 (G) after infection. Data in (C)-(G) is shown as mean + SD of two pooled independent experiments. Symbols represent single mice; symbol shape indicates different experiments. Unpaired t test (C), (D), (F), (G) or Mann-Whitney test (C), (D), (E), (F), (G) were performed for statistical analysis. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$.

neutrophils rather than only masking Ly-6G as seen when using only 1 A8 (Boivin et al. 2020).

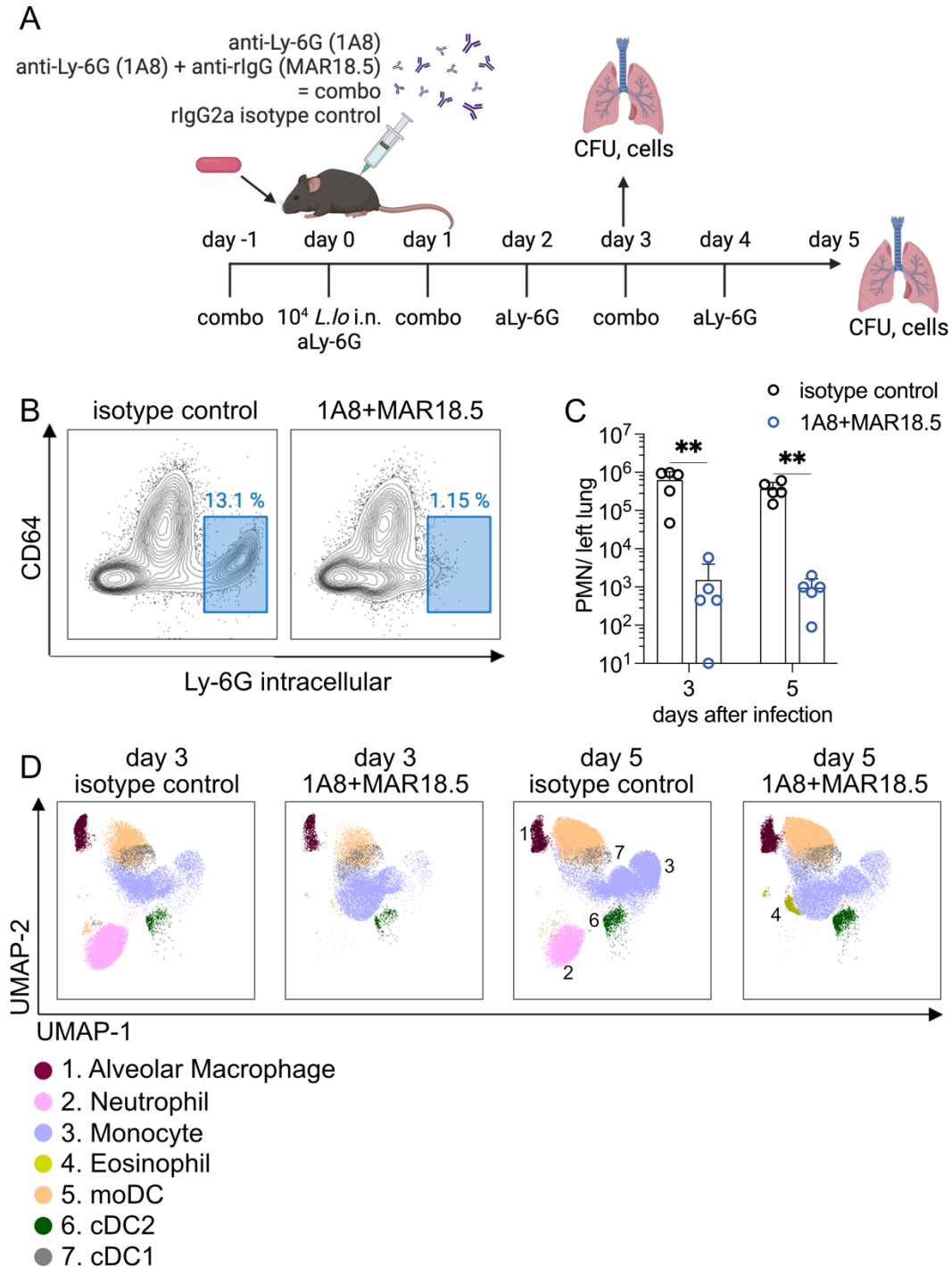


Figure 11: Efficient neutrophil depletion in *L. longbeachae*-infected mice.

Antibody treatment led to 1,000 times less neutrophils than in mice treated with the corresponding irrelevant isotype matched antibody

(**Fig. 11B-D**). Quantification of myeloid cells in the lung other than neutrophils revealed small changes in cell numbers except for an increase in eosinophils (day 3 and 5 after infection), and MC (day 5) in neutrophil-depleted mice (**Fig. 11D**, **Fig. 12C, D**). Nevertheless, the influx of these two myeloid cell populations was not able to compensate for neutrophil loss because neutrophil-depleted mice failed to control bacterial burden in the lung as demonstrated by a decreased mouse weight (**Fig. 12A**) and very large increase in bacterial load (**Fig. 12B**).

Figure 11: Efficient neutrophil depletion in *L. longbeachae*-infected mice.

(**A**) Scheme of experimental setup. C57BL6/J mice were intraperitoneally (i.p.) injected with anti-Ly-6G (1A8) and anti-rat secondary (MAR18.5) depletion antibody or isotype control starting one day prior to infection. Mice were then i.n. infected with 10^4 *L. lo-mCherry* CFU. (**B**) Representative flow cytometry plots showing depletion of neutrophils at day 5 after infection. (**C**) Quantification of neutrophils at the indicated time points. (**D**) Flow cytometry UMAP plots of extravascular CD19⁻ NK1.1⁻ CD3⁻ myeloid cells. Data in (D) is shown as mean + SD from one experiment. Symbols represent single mice. Unpaired t test. ** P < 0.01.

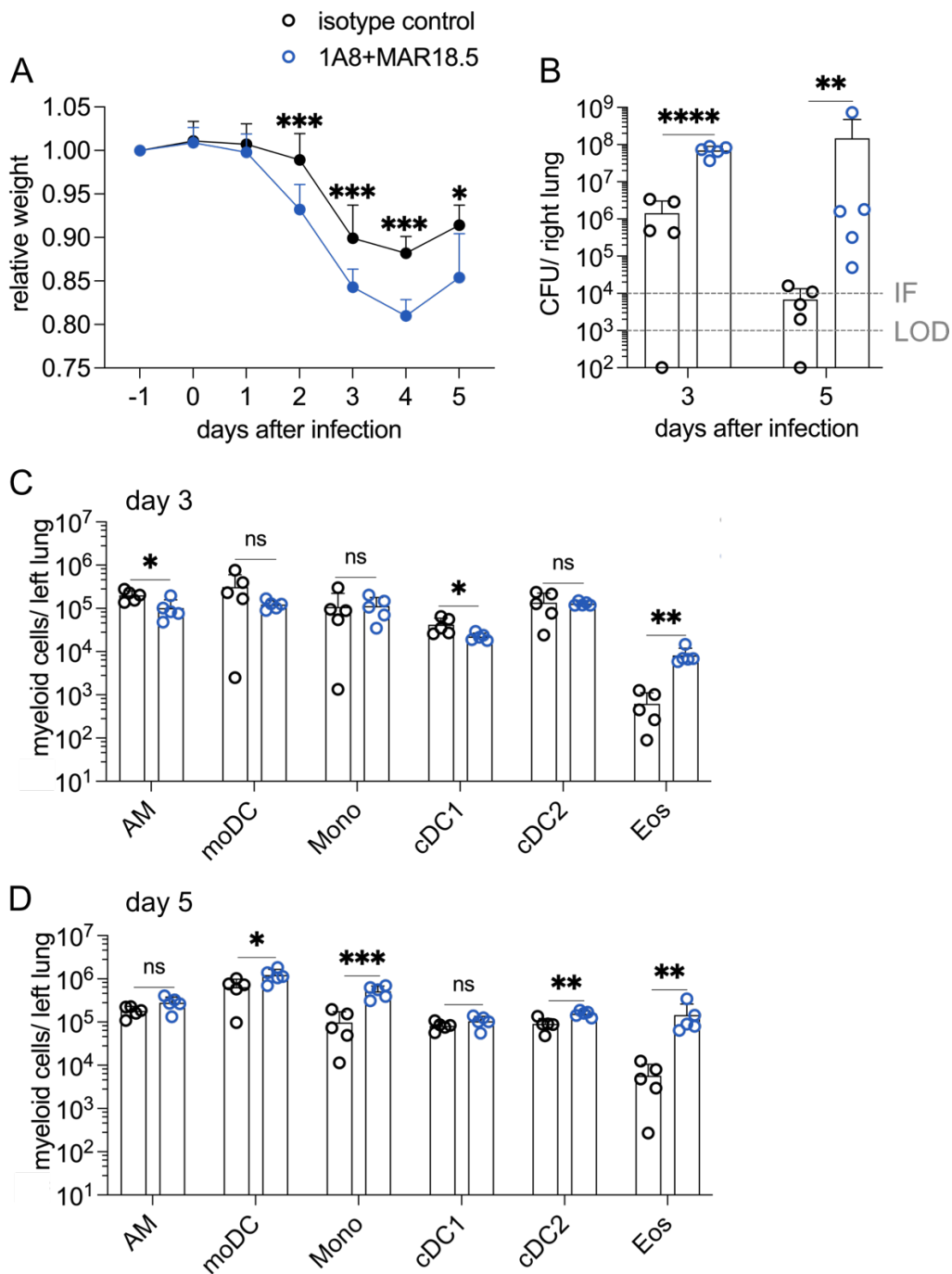


Figure 12: Depletion of neutrophils worsens mouse fitness and impairs bacterial clearance.

C57BL/6/J mice were intraperitoneally (i.p.) injected with anti-Ly-6G (1A8) and anti-rat secondary (MAR18.5) depletion antibody or isotype control starting one day prior to infection. Mice were then i.n. infected with 10^4 *L. lo-mCherry* CFU. **(A)** Kinetics of relative mouse weight during infection. **(B)** Bacterial load in the right lung at the indicated time points. LOD (limit of detection, 50 CFU), IF (infection dose, 10^4 CFU). **(C)-(D)** Quantification of pulmonary myeloid cells at day 3 (C) and day 5 (D) after infection (quantified from C). Data is shown as mean + SD of one experiment. Symbols represent single mice. Unpaired t test (B), (C), (D), (E) or Mann-Whitney test (C) were performed for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Taken together, these results demonstrated a key role of neutrophils for the defence against *L. longbeachae* in the lung of infected mice consistent with the dominant role of these cells in bacterial uptake. Together with our previous results (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020), we identified that neutrophils and MC cooperate for efficient control of *L. longbeachae* infection with neutrophils being essential from early stages of infection while MC fulfilling their protective role at later time points.

I next investigated the molecular mechanism by which neutrophils protected against *L. longbeachae* infection. Amongst several others, a key protective mechanism in these cells is the production of NADPH oxidase-dependent ROS in response to bacterial ligands. To investigate whether neutrophils use ROS to kill internalized *L. longbeachae*, I infected *gp91phox*^{-/-} mice (**Fig. 13**) lacking a functional NADPH oxidase and therefore NADPH oxidase-dependent ROS.

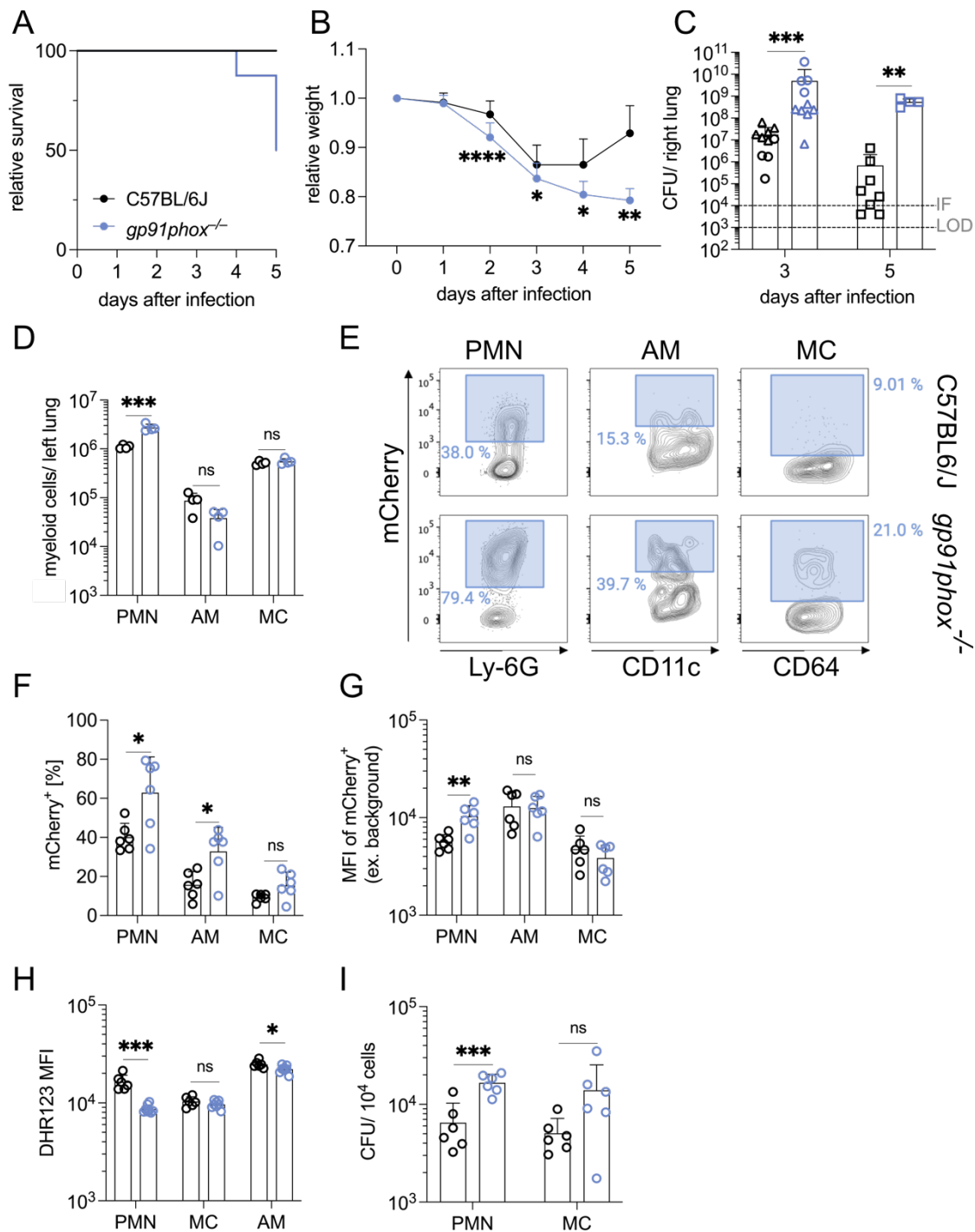


Figure 13: Lack of NADPH oxidase-dependent ROS impairs bacterial clearance and worsens mouse fitness mainly due to reduced microbicidal activity of neutrophils.

NADPH oxidase promoted well-being and survival of infected mice. Only about 50 % of infected *gp91phox*^{-/-} mice survived by day 5 after infection (**Fig. 13A**). Those mice surviving were not able to recover body weight between day 4 and 5 (**Fig. 13B**), and their lungs contained ~1,000-fold more CFU

(**Fig. 13C**). At the same time, the number of neutrophils infiltrating the lung (**Fig. 13D**), the percentage of neutrophils having internalized *L. longbeachae*-mCherry (**Fig. 13E, F**), and their mCherry MFI (**Fig. 13E, G**) were all significantly increased under the lack of NADPH oxidase-derived ROS. The influence of an impaired ROS production was less pronounced on AM and MC (**Fig. 13E-G**).

These results showed a key role of neutrophils and NADPH oxidase-dependent ROS in protection against *L. longbeachae* infection in mice. Next, we wanted to identify which cell types utilize NADPH oxidase to kill *L. longbeachae*. *Ex vivo* staining with DHR123, a ROS indicator, demonstrated that, as expected, ROS production in neutrophils during infection was highly dependent on NADPH oxidase (**Fig. 13H**). Surprisingly, MC produced very low levels of ROS, which were not dependent on NADPH oxidase (**Fig. 13H**). Consistently, the number of viable bacteria in *gp91phox*^{-/-} neutrophils was significantly increased than in their WT counterparts, but not in MC (**Fig. 13I**).

These results demonstrated that neutrophils were essential in killing *L. longbeachae* from early infection using NADPH oxidase, whereas MC contributed to bacterial killing at later stages of infection in a NADPH oxidase-independent manner.

Figure 13: Lack of NADPH oxidase-dependent ROS impairs bacterial clearance and worsens mouse fitness mainly due to reduced microbiocidal activity of neutrophils.

C57BL6/J mice were i.n. infected with 10⁴ *L. lo*-mCherry CFU. **(A)** Relative survival during infection. **(B)** Kinetics of relative mouse weight during infection. **(C)** Bacterial load in the right lung at the indicated timepoints. LOD (limit of detection, 50 CFU), IF (infection dose, 10⁴ CFU). **(D)** Quantification of pulmonary myeloid cells at day 3 after infection. **(E)** Representative flow cytometry plots showing increased percentage and MFI of mCherry⁺ cells in indicated phagocyte populations. **(F)** Percentage of mCherry⁺ cells within the population of different phagocytes. Cells from mice infected with WT *L. longbeachae* served as mCherry⁻ controls. **(G)** mCherry MFI in indicated mCherry⁺ cells after subtraction of background MFI in mCherry⁻ cells of the same population. **(H)** DHR123 (ROS dye) MFI in indicated cell populations. **(I)** Number of viable bacteria (CFU) per 10⁴ cells of indicated mCherry⁺ cell population flow cytometry-sorted 3 days after infection. In (B), (C) data is pooled from two independent experiments. In (D), (F)-(I) data is from one experiment. Data is shown as mean + SD. Symbols represent single mice; symbol shapes indicate different experiments. Unpaired t test (B), (D), (F), (G), (H), (I) or Mann-Whitney test (B), (C), (H) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

3.2.5. Pulmonary infection with *L. longbeachae* promotes cytokine production by epithelial cells contributing to a pro-inflammatory milieu within the lung

The recruitment and the activation of protective myeloid cells like neutrophils and MC requires the secretion of specific cytokines and chemokines. In addition to immune cells, epithelial cells can significantly contribute to this process, particularly at the early stages of infection when the immune infiltrate is still low. Therefore, RNAseq analysis was performed on purified pulmonary epithelial cells to investigate expression of cytokines (**Fig. 14A**). In addition, total cytokine levels in the lung homogenate were quantified to correlate gene and protein expression and to also detect cytokines produced by cells other than epithelial cells (**Fig. 14B**).

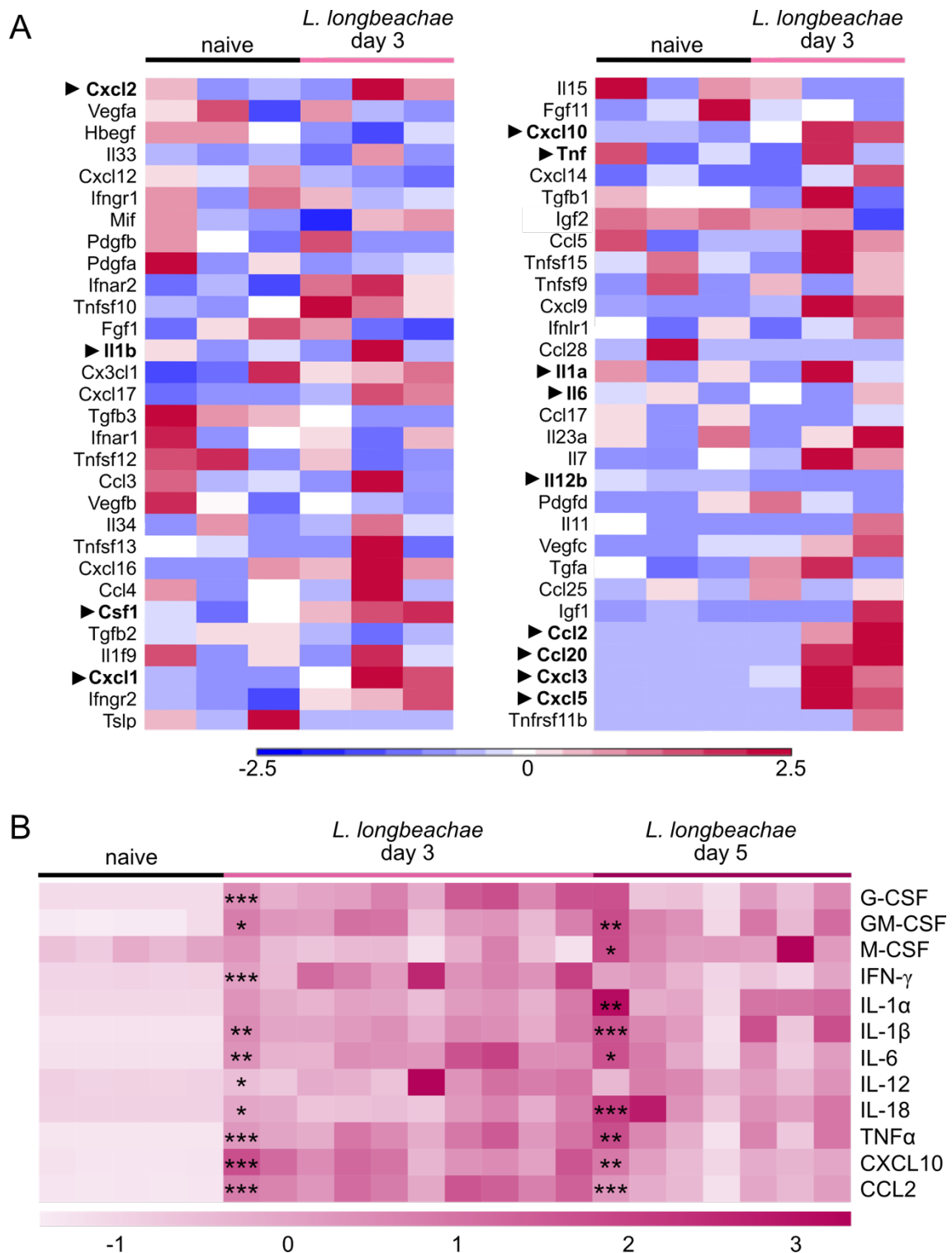


Figure 14: Pulmonary epithelial cells contribute to a strong pro-inflammatory environment in response to infection with *L. longbeachae*.

The RNAseq data revealed that there is an upregulation of several genes encoding neutrophil-activating and -recruiting cytokines and chemokines (Fig. 14A). For instance, *Cx1*, -2, and -5 and *Ccl20*, which are well-known to

promote recruitment of neutrophils (De Filippo et al. 2013), were upregulated during infection with *L. longbeachae* (**Fig. 14A**), positively correlating with the strong influx of neutrophils into the lung (**Fig. 6B**). In addition to these, RNA encoding Ccl2 that recruits monocytes (Serbina & Pamer 2006) and M-CSF that promotes differentiation of monocytes into monocyte-derived macrophages (M-CSF) (Ushach & Zlotnik 2016) were also upregulated in epithelial cells from infected mice (**Fig. 14A**).

Tissue-resident and infiltrating immune cells may also contribute to the overall cytokine response shaping the inflammatory milieu at the site of infection. To get a broader insight into this, we next analysed the cytokine expression on protein level in the whole lung (**Fig. 14B**). The protein products of the RNAs that were found to be elevated in the RNAseq analysis were also elevated in the whole lung (**Fig. 14B**). In addition, some cytokines for which RNAs were not detected in the RNAseq analysis of epithelial cells in response to *L. longbeachae* infection (**Fig. 14A**) were elevated in the whole lung extract suggesting these proteins were produced by non-epithelial cells. These include G-CSF and GM-CSF that might further promote recruitment and activation of neutrophils (Lopez et al. 1986; Kowanko et al. 1991; Mehta, Malandra & Corey 2015). Next to neutrophil-recruiting chemokines, cytokines known to activate neutrophils and other immune cells such as IL-1 β (Di Paolo et al. 2015; Yamada et al. 2000), TNF α (Nathan 1989; Wiemer et al. 2010; Roilides et al. 1998; Rainard et al. 2000; Flynn et al. 1995), and IL-6 (Scheller et al. 2011) were upregulated in lung extracts from

Figure 14: Pulmonary epithelial cells contribute to a strong pro-inflammatory environment in response to infection with *L. longbeachae*.

C57BL6/J mice were i.n. infected with 10⁴ *L. lo*-mCherry CFU. **(A)** RNAseq was performed on flow cytometry-sorted epithelial cells (Live CD45⁻ EpCAM^{high}) at day 3 after infection. Shown is relative expression of cytokines and chemokines ordered from highest to lowest mean expression in naïve mice. Highlighted are genes analysed on protein level (B) and/ or involved in neutrophil recruitment and activation. **(B)** Magnetic bead array technique was used to quantify cytokine and chemokine expression in the right lung homogenate at the indicated time points after infection. Untreated mice were used as naïve controls. Shown is relative expression of cytokines and chemokines from min. one experiment. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) or RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** P < 0.001, **** P < 0.0001.

infected mice (**Fig. 14B**). Another cytokine with a strong pro-inflammatory function on the bactericidal capacity of myeloid cells, including neutrophils, MC and macrophages, is IFN- γ (Nakanishi et al. 2001a; Ellis & Beaman 2004; Schroder et al. 2004) which was elevated on protein level in line with its two main activators IL-12 and IL-18 (**Fig. 14B**).

3.2.6. NK cells and T cells fulfill a redundant role in protection against *L. longbeachae*

IFN- γ is mainly produced by T cells, NK cells, and NKT cells. My data showed that all three cell types infiltrate the lung during infection with *L. longbeachae* (**Fig. 7B**), with NK cells peaking at day 3 and partially overlapping with the peak in neutrophil numbers (**Fig. 6B**). Mice lacking these cell populations were used to investigate their role during *L. longbeachae* infection. To this end, *Rag2*^{-/-} mice lacking T cells and NKT cells (as well as B cells) received anti-NK1.1 depletion mAb (PK136), to deplete NK cells, or isotype control mAb (C1.18.4) one day prior and 2 days after infection (**Fig. 15A**). I did not include a group depleted for NK cells alone as previous work in our laboratory already showed that NK1.1⁺ cell depletion alone did not impair defence against *L. longbeachae* (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020).

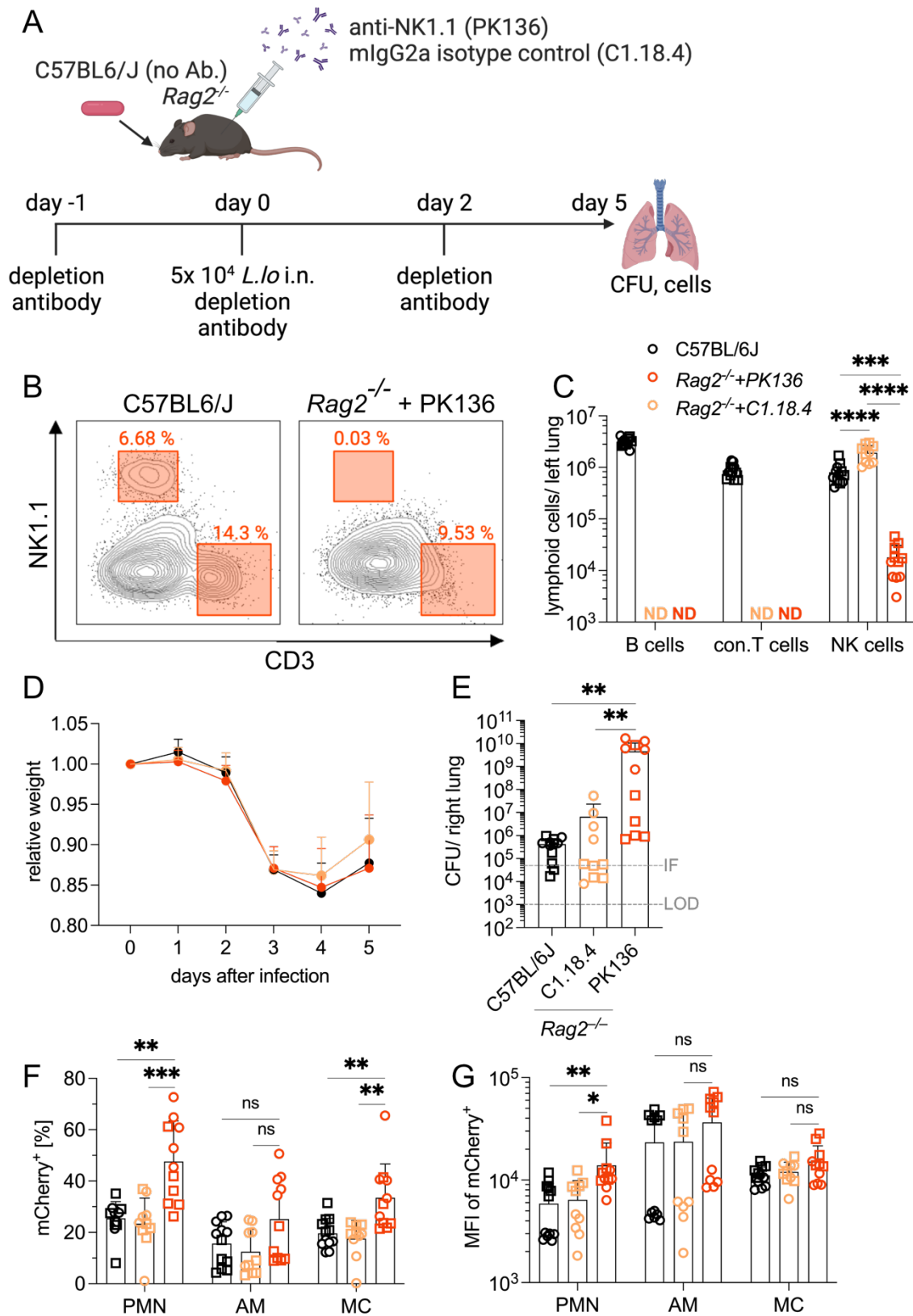


Figure 15: Either NK cells or T cells are required for efficient anti-bacterial defence during infection with *L. longbeachae*.

Antibody-depletion of NK cells in *Rag2*^{-/-} mice led to a significant reduction in the number of NK cells when compared to untreated WT mice. As expected, *Rag2*^{-/-} mice completely lacked T cells and B cells (**Fig. 15B, C**). Lack of T cells (*Rag2*^{-/-}+C1.18.4) did not result in impaired anti-bacterial defence as demonstrated by similar weight loss (**Fig. 15D**) and bacterial load (**Fig. 15E**) compared to immunocompetent C57BL6/J mice (**Fig. 15C**). There was only an increase in pulmonary *L. longbeachae* burden 5 days after infection when both NK cells and T cells were lacking (*Rag2*^{-/-}+PK136) (**Fig. 15E**), even though mouse weight was not affected (**Fig. 15D**). Neutrophils and MC in the lungs of mice lacking NK cells and T cells showed an increased percentage of mCherry⁺ cells (**Fig. 15F**), consistent with the increased bacterial load. Additionally, the mCherry MFI in neutrophils was increased compared to neutrophils from, both, immunocompetent C57BL6/J mice and *Rag2*^{-/-} mice not lacking NK cells (*Rag2*^{-/-}+C1.18.4) (**Fig. 15G**), highlighting their impaired microbiocidal activity. Contrary to this, the mCherry MFI amongst MC that had internalised bacteria was not altered in mice lacking T cells and NK cells (**Fig. 15G**), suggesting their microbiocidal capacity was not regulated by T cells and NK cells.

Taken together, our results indicated that NK cells or T cells were required for an efficient defence against pulmonary *L. longbeachae*. Absence of either of them alone did not impair protection against *L. longbeachae* suggesting an important but redundant role for those cell types.

Figure 15: Either NK cells or T cells are required for efficient antibacterial defence during infection with *L. longbeachae*.

Mice were i.n. infected with 5x 10⁴ *L. lo*-mCherry CFU. **(A)** Scheme of experimental setup. *Rag2*^{-/-} mice were administered i.p. with 200 µg of either anti-NK1.1 antibody (PK136) or mIgG2a isotype control at day -1, 0 and 2. **(B)** Representative flow cytometry plots showing depletion of NK and T cells in *Rag2*^{-/-} mice administered with PK136. **(D)** Kinetics of relative mouse weight during infection. **(E)** Bacterial load in the right lung at day 5 after infection. LOD (limit of detection, 50 CFU), IF (infection dose, 5x 10⁴ CFU). **(F)** Percentage of mCherry⁺ cells within the population of different phagocytes. Cells from mice infected with WT *L. lo* served as mCherry⁻ controls. **(G)** mCherry MFI in indicated mCherry⁺ cells. Data is shown as mean + SD of two independent experiments. Symbols represent single mice; symbol shapes indicate different experiments. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) (D), (F), (G) or RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) (C), (D), (E) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** < 0.001, **** P < 0.0001.

3.3. Discussion

The aim of this chapter was to elucidate which and how immune cells participate in the defence against *L. longbeachae*. Using a murine model of pulmonary infection with *L. longbeachae*, I demonstrated a differential contribution to anti-bacterial defence by lung-resident AM and infiltrating neutrophils. AM were shown to promote infection by most likely serving as a replicative niche, extending previous *in vitro* data of *L. longbeachae* replication within murine bone marrow-derived macrophages (Massis et al. 2017). In contrast, infiltrating neutrophils were required for protection from the early stages of infection, a function dependent upon production of ROS. NK cells and T cells fulfill a redundant protective role potentially via secretion of IFN- γ known to be an essential cytokine for defence against *L. longbeachae* (Massis et al. 2017). Taken together, my study gives a novel detailed insight into the cellular immune response to *L. longbeachae* with AM supporting bacterial infection and neutrophils as well as NK cells and T cells fighting the infiltrating pathogen. The results presented in this chapter are graphically summarized in Figure 16.

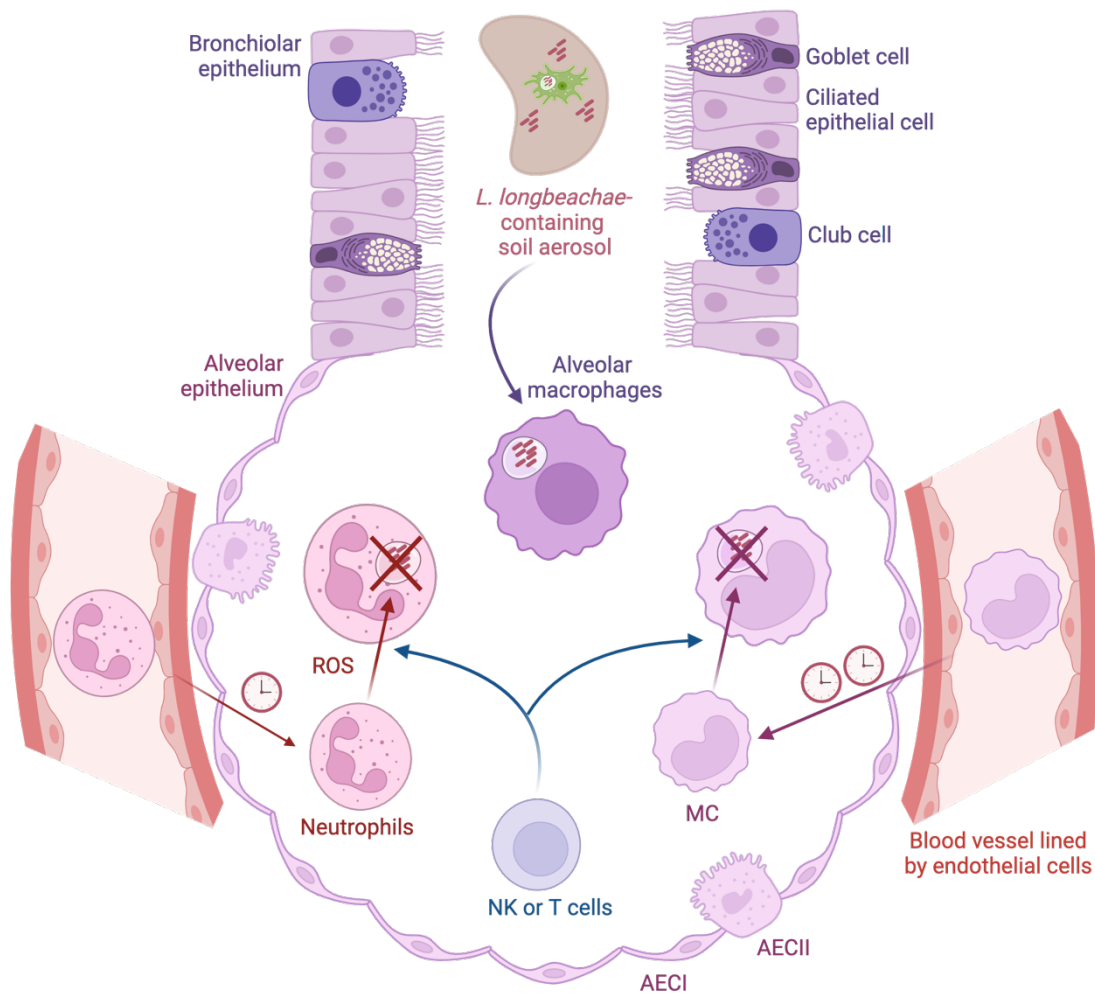


Figure 16: Graphical summary of the differential contribution of lung-resident alveolar macrophages and infiltrating leukocytes during defence against *L. longbeachae*.

L. longbeachae (red) enter the lung within contaminated soil particles either as free bacteria or related to soil amoebae. Once the bacteria entered the lung they get phagocytosed by alveolar macrophages as the first line of pulmonary immune defence within the alveolar space. *L. longbeachae* were most likely able to replicate within AM. First (one clock) neutrophils and later (two clocks) monocytes entered the lung from the circulation during infection. Both phagocyte types internalized bacteria and were able to kill *L. longbeachae*. The strong bactericidal capacity of neutrophils required NADPH-dependent ROS production. Infiltration of NK cells or T cells was required for efficient anti-bacterial defence promoting the bactericidal capacity of neutrophils and MC. AEC = alveolar epithelial cells, MC = monocyte-derived cells ROS = reactive oxygen species.

As *L. longbeachae* is inhaled via contaminated aerosols, AM, which make up the vast majority of tissue-resident immune cells in the lung during homeostasis (Hussell & Bell 2014; Garbi & Lambrecht 2017; Kopf, Schneider & Nobs 2015), are amongst the first cells to encounter the bacteria. Here, it was demonstrated that only a low percentage of AM has internalised invading *L. longbeachae*. This

may be because only one AM per three alveoli has been calculated to be patrolling the lung (Westphalen et al. 2014). Together with previous confocal imaging data from our laboratory showing that *L. longbeachae* remain in local clusters of infections (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020) it is likely only a limited number of AM encounter and phagocytose the bacteria.

For *L. pneumophila* it is known that the bacteria replicate inside macrophages within the LCV, a specialized replication compartment, and that the establishment of this vacuole after phagocytosis is crucial for intracellular replication (Mondino et al. 2020; Tilney et al. 2001; Kagan & Roy 2002; Swanson & Isberg 1995). In order to turn the phagosome into a LCV, *L. pneumophila* secretes a wide spectrum of effector proteins into the macrophage cytoplasm using a T4SS (Andrews, Vogel & Isberg 1998; Berger & Isberg 1993). Previous data from our laboratory demonstrated that *L. longbeachae* are able to secrete effector proteins into the cytoplasm of AM (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020) thereby fulfilling the pre-condition for intracellular replication. Here, different approaches were used that also suggested that AM were a replicative niche for *L. longbeachae*. AM, amongst the investigated phagocytes, showed the highest MFI for the *L. longbeachae* reporter strain which I demonstrated to correlate with the highest number of viable bacteria per cell. Additionally, specific depletion of tissue-resident AM prior to infection revealed that these cells promoted establishment of infection.

A current study by the Krönke group shows that, even though used to specifically deplete macrophages, clodronate-loaded liposomes also influence the functionality of neutrophils by paralysing their effector functions (Culemann et al. 2023). However, three different reasons indicate that the effect I report here is specific for the clodronate-induced AM depletion. (1) In their study, the Krönke group apply the clodronate-loaded liposomes systemically while I administered a 4-times lower dose locally into the naïve lung one day prior to infection. The number of neutrophils in the naïve lung is negligible strongly reducing the probability of an effect of the liposomes on these phagocytes. (2) I clearly demonstrate the protective role of neutrophils. Their functional impairment should therefore result in an increased and not drastically decreased bacterial load. (3)

I did not investigate the functionality of neutrophils under the influence of clodronate. However, I could show a decreased number of neutrophils and propose this observation to be a result of the reduced bacterial load and, thereby, reduced necessity to recruit protective phagocytes.

Even though the secretion of effector proteins was demonstrated to be crucial for the intracellular life cycle of *L. pneumophila*, there are also T4SS-independent pathways via which *L. pneumophila* prevents its own degradation within the macrophage host. These include NF- κ B-dependent downregulation of the IFN- γ R (Sokulsky et al. 2020). *L. longbeachae* has a distinctive set of effector proteins (Cazalet et al. 2010) and future studies are necessary to elucidate which are required to enable intracellular replication within AM. Nevertheless, the T4SS of *L. longbeachae* is needed to induce a productive infection within the murine lung (Massis et al. 2017) highlighting the role of AM as a replicative niche for *L. longbeachae*.

Intracellular replication of *L. pneumophila* as well as translocation of toxic effector proteins induces death of host cells (Belyi et al. 2006, 2008; Shen et al. 2009). Accordingly, AM numbers decrease in the lung during infection with *L. pneumophila* (Brown et al. 2016). This is in line with the data here, which showed that AM numbers first decreased during *L. longbeachae* infection. During the later course of infection AM numbers recovered paralleling the decrease of the bacterial burden in the lung and the percentage of infected AM. Studies with *L. pneumophila* suggest a delay in AM regeneration might be advantageous for the clearance of bacteria by limiting their replicative niche (Brown et al. 2016). My results supported this hypothesis for *L. longbeachae* as depletion of AM was protective over the whole course of infection. Nevertheless, our laboratory showed before that AM depletion during infection with *L. pneumophila* is only advantageous during the first days of infection likely by limiting the initial bacterial replication (Fleischmann et al. 2023). In contrast, lack of AM during later time points of infection impaired bacterial clearance (Fleischmann et al. 2023) highlighting the difference between *L. longbeachae* and *L. pneumophila*. For *L. pneumophila*, it was suggested that AM death could stimulate the initiation of an immune response by release of IL-1 β and danger-associated molecular

patterns and AM death might thereby promote the immune response (Brown et al. 2016).

Whether other phagocytes not investigated in detail here might also promote infection and potentially allow for intracellular *L. longbeachae* replication cannot be formally excluded. One candidate might be conventional dendritic cells that we could show to increase on day 1 after infection but were significantly reduced again on day 3 opening the hypothesis that bacteria might be able to replicate within and thereby kill these cells as well. To investigate this hypothesis, I suggest a similar approach as used during my project: First analyse the bacterial uptake of and bacterial viability within cDC and, subsequently, using specific cell ablation strategies to investigate the functional role of cDC during *L. longbeachae*-induced pneumonia e.g. in CD11c-DTR mice (Dennis et al. 2002).

In contrast to AM, the data presented here, revealed a protective role for neutrophils. Neutrophils strongly infiltrated the lung already early during infection and showed the highest capacity to take up *L. longbeachae*. Nevertheless, the high number of bacteria-containing neutrophils did not translate into a high number of viable intracellular bacteria. In fact, neutrophils showed a bacterial load lower than AM suggesting that, in contrast to AM, neutrophils kill internalized bacteria. Neutrophils were previously suggested to have a protective function during *L. longbeachae* infection (Massis et al. 2017). Using antibody-mediated depletion, I proved this role, similar to what was shown regarding *L. pneumophila* infection. During pulmonary infection with *L. pneumophila*, neutrophils are the most numerous cell type infiltrating the lung (Brown et al. 2016). *Il1r^{-/-}* mice were used to impair recruitment of neutrophils to the lung (Mascarenhas et al. 2015) and standard anti-Ly-6G-induced antibody-depletion was used to fully deplete neutrophils from the mice (Casson et al. 2017) and both approaches resulted in an impaired anti-bacterial defence. In previous experiments, our laboratory used anti-Ly-6G antibody treatment to show that neutrophils were protective against *L. longbeachae* (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020). However, it was recently revealed that the standard neutrophil depletion system using only the neutrophil-directed anti-Ly-6G antibody induces masking of surface Ly-6G on neutrophils rather than actual depletion (Boivin et al. 2020).

Therefore, I used a novel combo-depletion system combining the anti-Ly-6G mAb with a secondary anti-rat mAb. This was shown to induce an efficient depletion of neutrophils in which neutrophil numbers were quantified using intracellular Ly-6G-staining in order to avoid picking up false results due to potential masking (Boivin et al. 2020). Therefore, using this system provides a robust proof of the protective function of neutrophils.

Similar to the results presented here using *L. longbeachae*, previous studies demonstrated that neutrophils and AM fulfill differential roles during infection with *L. pneumophila*. These differences result from the fact that the Dot/ Icm secretion system is crucial for intracellular replication of bacteria in AM (Andrews, Vogel & Isberg 1998; Berger & Isberg 1993) while neutrophils, in contrast, are able to recognize T4SS components. In fact, the secretion apparatus is required for the onset of an efficient neutrophil response towards *L. pneumophila* (Price et al. 2021). Mechanistically the LamA effector protein is translocated into the neutrophil cytoplasm and induces degradation of glycogen (Price et al. 2021). The resulting increase in cytosolic glucose in neutrophils induces a metabolic reprogramming characterized by increased aerobic glycolysis which, in turn, promotes ROS production and fusion of granules with the *Legionella*-containing phagosome (Price et al. 2021).

ROS production is a key effector function of neutrophils to fulfill protection against invading pathogens next to production of pro-inflammatory cytokines (Mayadas, Cullere & Lowell 2014). Regarding defence against *L. pneumophila*, neutrophils directly protect via ROS production (Ziltener, Reinheckel & Oxenius 2016) which my data confirmed for *L. longbeachae* as well. Here, the impaired microbiocidal function of neutrophils induced by the lack of NADPH oxidase-dependent ROS resulted in a strongly impaired pulmonary bacterial clearance and overall mouse fitness. The mechanisms behind the ROS-dependent bacterial killing by neutrophils will be further elucidated in the next Chapter. In addition to the direct bacterial killing, TNF α production by neutrophils is crucial for *L. pneumophila* defence (Ziltener, Reinheckel & Oxenius 2016). Neutrophil-derived TNF α instructs AM to limit intracellular bacterial replication by inducing acidification of AM lysosomes and fusion of these with the LCV (Ziltener,

Reinheckel & Oxenius 2016). Furthermore, neutrophils produce the pro-inflammatory cytokines IL-12 and IFN- γ in response to *L. pneumophila* (Casson et al. 2017; Brown et al. 2016) which were both crucial for anti-bacterial defence (Brown et al. 2016). Which cytokines neutrophils produce during infection with *L. longbeachae* remains subject of future studies.

In contrast to neutrophils, MC fulfill their protective function later during the course of infection for both, *L. longbeachae* and *L. pneumophila* (Ataide et al. 2023). Reduced frequency of MC increased the bacterial burden in the lung not at day 2 but at day 4 after infection (Ataide et al. 2023). I showed, in addition, that the influx of and the bacterial uptake by MC was delayed when compared to neutrophils. Furthermore, MC remained elevated in the lung longer than neutrophils and at later stages were the only cell type harbouring bacteria. Furthermore, our data indicated that MC rely on ROS production to kill *L. longbeachae* to a lesser degree than neutrophils. A similar paradigm was already suggested for defence against *L. pneumophila* where MC mainly contribute to anti-bacterial defence via secretion of IFN- γ stimulating IL-12 (Brown et al. 2016).

MC-derived IL-12 is essential for stimulating IFN- γ during *L. pneumophila* (Brown et al. 2016). IFN- γ is highly protective not only against *L. pneumophila* (Brown et al. 2016) but also *L. longbeachae* (Massis et al. 2017). Even though the levels I could detect in the whole lung homogenate were quite low, this might result from the local infection and therefore still be able to be protective within the cluster of infection indicating a role for IFN- γ -producing lymphocytes. Even though this study mainly focused on myeloid cell-mediated defence against *L. longbeachae*, I also showed a strong influx of lymphocytes at late timepoints during infection. Previous results in our laboratory, using antibody-directed depletion suggested that NK cells and T cells do not play an essential protective role against *L. longbeachae* (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020), even though, those cells are a source of IFN- γ . My results, however, showed that NK cells and T cells likely fulfill a redundant protective role during *L. longbeachae* infection as only antibody-mediated NK cell depletion in T cell-deficient *Rag2*^{-/-} mice impaired the bacterial clearance. NK cells and T cells can

fulfill redundant protective roles during the response against the intracellular bacterium *M. tuberculosis* (Junqueira-Kipnis et al. 2003; Feng et al. 2006). Nevertheless, my data regarding *L. longbeachae* contrasts with data using *L. pneumophila* infection models in which NK cell depletion alone impaired IFN- γ -dependent bacterial clearance (Spörri et al. 2006; Blanchard et al. 1988). Another study using *L. pneumophila* suggested a protective role for T cells at later stages of infection (Susa et al. 1998). Therefore, NK cells and T cells may contribute to secretion of IFN- γ at different stages of *L. pneumophila* infection. A similar concept for defence against *L. longbeachae* is suggested by my data where I detected NK cells infiltrating the lung earlier during infection than T cells. The role of the second well-known IFN- γ inducer IL-18 for defence against *L. longbeachae* will be investigated in the next chapter.

Throughout the project, I observed intra-group variations that might result from dissemination of *L. longbeachae* to different parts of the lung in different animals. Usually, the right lung lobes were used for quantifying the bacterial load while the left lung lobe was used for cellular analyses in order to reduce the mouse numbers according to animal ethics. Reviewing current literature revealed that splitting the lung for different analyses might, indeed, increase the intra-group variances: When the whole lung was used to quantify bacteria the variations between mice was smaller (Massis et al. 2017; Ataide et al. 2023) than when, like in my case, only the right lung lobe was used (Fleischmann et al. 2023; Brown et al. 2016). For future experiments, it should, therefore, be considered to develop a technique to homogenize the whole lung and split the homogenate for CFU quantification and cellular analyses afterwards.

Taken together this chapter offers a comprehensive insight into the relevance of innate immune cells and their anti-bacterial mechanisms for the defence against *L. longbeachae*, thereby, contributing to understanding immunity against this under-investigated pathogen.

Chapter 4: IL-18 receptor-dependent defence against *L. longbeachae*

4.1. Introduction

After gaining a detailed insight into the cellular response towards *L. longbeachae*, I next aimed to elucidate the molecular basis behind the strong protective phenotype of neutrophils. During the onset of an innate immune response as well as for the transition to the adaptive immunity it is crucial that immune cells communicate with each other and with stromal cells. Inflammatory cytokines are major modulators of this immune crosstalk. IL-18 was shown before by our laboratory to fulfill an essential role during defence against *L. longbeachae* (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020) which was in contrast to *L. pneumophila* infection where IL-18 was not required for bacterial clearance (Brown et al. 2016).

IL-18 was originally recognized as a factor promoting IFN- γ secretion mainly by activated macrophages (Bohn et al. 1998; Okamura et al. 1995; Nakamura et al. 1989). The cleavage of inactive pro-IL18 into the biologically active conformation (Ghayur et al. 1997) and the subsequent secretion is usually dependent on the inflammasome pathway (Arend, Palmer & Gabay 2008; He et al. 2015). To carry out its functions, secreted IL-18 stimulates its specific receptor, the IL-18R, consisting of a ligand-binding α -chain and signal-inducing β -chain (Torigoe et al. 1997; Born et al. 1998).

Within the immune compartment, NK cells and Th1 cells mainly express the IL-18R (Boraschi et al. 2018) and are responsive to IL-18 resulting in release of IFN- γ (Nakanishi et al. 2001a; Boraschi et al. 2018). During inflammation IL-18R is expressed on other immune and non-immune cells, including epithelial cells (Boraschi et al. 2018). Nevertheless, the generally accepted pathway of IL-18-dependent anti-bacterial defence is via stimulating secretion of IFN- γ (illustrated in **Fig. 17**, yellow) and this will be referred to as the canonical pathway of IL-18-dependent defence. IFN- γ is then known to enhance bactericidal mechanisms of myeloid cells by promoting receptor-mediated phagocytosis and subsequent

bacterial killing capacity. IFN- γ induces both reactive nitrogen and oxygen species and upregulates lysosomal enzymes (Ellis & Beaman 2004; Schroder et al. 2004; Marchi et al. 2014). IFN- γ is crucial during defence against *L. pneumophila* by improving bacterial killing capacity of MC (Brown et al. 2016). IL-18, on the other hand, did not play an essential role as clearance of *L. pneumophila* was rather dependent on the second, well-known IFN- γ inducer IL-12 (Brown et al. 2016).

My current project, however, expands on previous results reported in our laboratory (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020) which revealed that defence against *L. longbeachae* is, in contrast to *L. pneumophila*, depends on IL-18. Surprisingly, bone marrow chimeras showed that expression of IL-18R on non-hematopoietic stromal cells is necessary and sufficient to promote defence against *L. longbeachae*, whereas expression on immune cells, including lymphocytes as the main producers of IFN- γ , did not confer protection. The generation of an IL-18R tdTomato reporter mouse line revealed that a population of CD45⁻ stromal cells in the lung expressed the IL-18R. By co-staining with EpCAM and acetylated α -tubulin as well as correlative light and electron microscopy (CLEM), these cells were shown to be bronchiolar ciliated epithelial cells. Taken together, these results led to the hypothesis of an alternative pathway of IL-18-dependent bacterial defence that will be referred to as the non-canonical pathway (illustrated in **Fig. 17**, blue): IL-18R signalling in bronchiolar epithelial cells leads to enhanced bacterial killing.

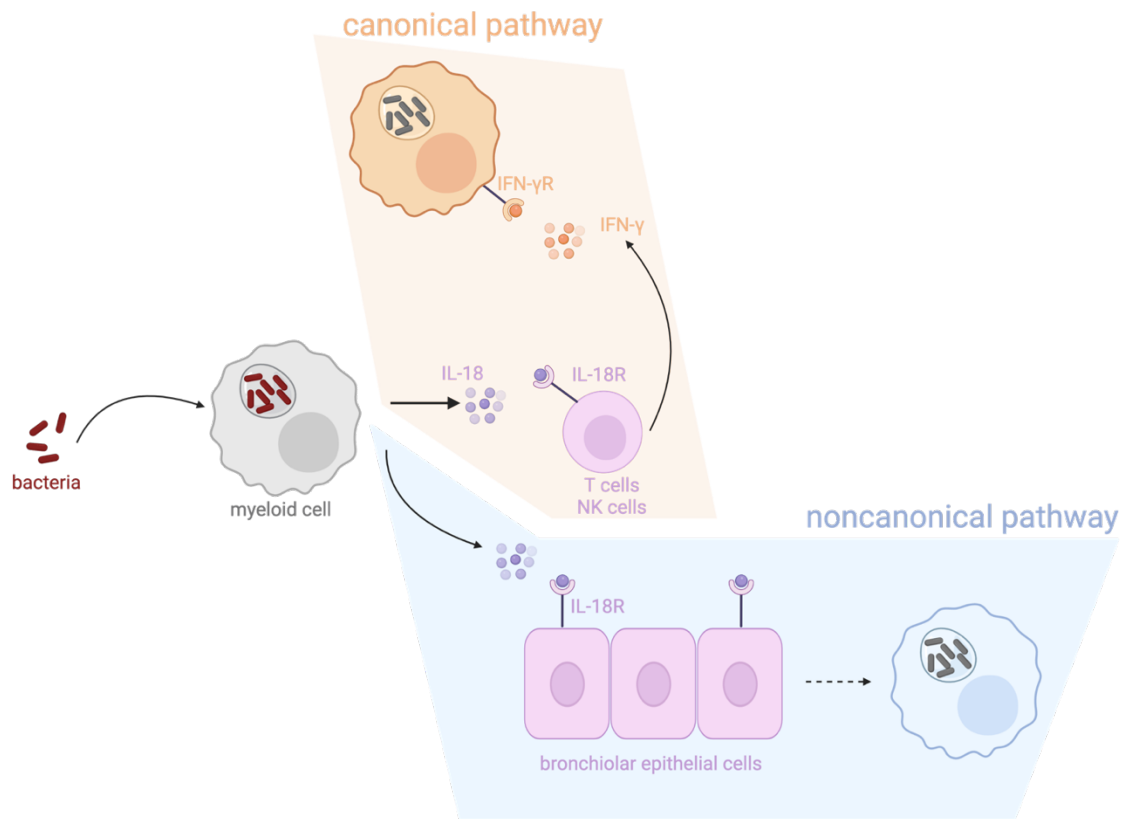


Figure 17: Schematic illustration of the generally excepted canonical pathway and the putatively novel non-canonical pathway of IL-18-dependent anti-bacterial defence.

Bacteria (red) are taken up by myeloid cells which induces secretion of IL-18. In the canonical pathway (orange) IL-18 binds to its receptor on T and/ or NK cells and instructs downstream secretion of IFN- γ . IFN- γ can bind to its receptor on myeloid cells and enhance their anti-microbial capacity. The hypothetical non-canonical pathway (blue) involves IL-18R-stimulation on bronchiolar epithelial cells to confer protection.

The bronchiole forms the final barrier between air and lung interstitium before the air reaches the alveoli and is comprised of three differentiated epithelial cell types (Leiva-Juárez, Kolls & Evans 2018). Bronchiolar epithelial cells can confer anti-bacterial protection via epithelial cell-autonomous mechanisms including mucociliary clearance and secretion of AMP (Leiva-Juárez, Kolls & Evans 2018). In addition to these epithelial cell-autonomous mechanisms, lung epithelial cells can communicate with the immune system (Whitsett & Alenghat 2014). Bronchiolar epithelial cells can secrete chemokines to recruit infiltrating immune cells to the lung including GM-CSF (Willart et al. 2012), Ccl5 (Thomas et al. 1998; Gern et al. 2003), Cxcl10 (Sauty et al. 1999; Frangogiannis et al. 2000), and IL-8 (DiMango et al. 1995; Alcorn et al. 2001; Casola et al. 2000; Standiford et al.

1990). Furthermore, alveolar epithelial cells secrete GM-CSF to ensure lung development and function during homeostasis (Huffman Reed et al. 1997; Cakarova et al. 2009; Christensen et al. 2000; Overgaard et al. 2015). In addition, AECII strongly increase GM-CSF secretion during bacterial pneumonia in response to AM-derived IL-1 α and IL-1 β (Liu et al. 2020). This recent study revealed that the defence against *L. pneumophila* requires IL-1R⁺ AECII-derived GM-CSF to enhance glycolysis-dependent production of pro-inflammatory cytokines by infiltrating monocytes. The intermediary signalling of epithelial cells, thereby, serves to enhance protective immune stimulation of bystander myeloid cells by a low number of infected alveolar macrophages. A similar mechanism could take place during IL-18R⁺ ciEpC-instructed protection against *L. longbeachae*. IL-18R⁺ ciEpC in bronchi may produce pro-inflammatory cytokines to confer protection. With neutrophils being the major protective cell type against *L. longbeachae*, as shown in the previous chapter, these phagocytes might be involved in a potential IL-18R⁺ ciEpC/ immune cell crosstalk.

A potential target of immune modulators in the interplay between epithelial cells and neutrophils may be the cellular metabolism of the latter to increase their bactericidal function. Regarding T cells and macrophages, extensive studies showed the influence of the cellular metabolism on their functions (Geltink, Kyle & Pearce 2018; Umar et al. 2021). Although neutrophil metabolism has not been studied extensively yet, it is generally accepted that neutrophils mainly depend on glycolysis and lack mitochondrial activity (Injarabian et al. 2020; Kumar & Dikshit 2019). Most studies regarding neutrophil metabolism used human blood neutrophils and revealed that both, phagocytosis and production of ROS, depend on glycolysis (Injarabian et al. 2020). Glycolysis promotes NADPH expression subsequently leading to ROS production (Kumar & Dikshit 2019). However, it remains elusive whether and how neutrophil metabolism and function change after leaving the glucose-rich blood and entering the site of inflammation where glucose and oxygen availability may become limited during infection (Injarabian et al. 2020; Kumar & Dikshit 2019). A recent study showed that activation of aerobic glycolysis in human blood neutrophils following effector secretion by *L. pneumophila* led to production of ROS and fusion of azurophilic granules with

the *Legionella*-containing phagosome, resulting in rapid killing of internalized bacteria (Price et al. 2021).

One aim of this chapter was to genetically confirm the existence of the alternative IL-18R⁺ ciEpC-instructed pathway of IL-18-dependent anti-bacterial defence using Cre-lox-based restriction of its expression to either immune cells or pulmonary epithelial cells. The investigation of the underlying mechanisms behind the non-canonical IL-18-dependent protection is based on the hypothesis (illustrated in **Fig. 18**) that IL-18R signalling in ciEpC instructs either epithelial cell-autonomous mechanisms, such as mucociliary clearance (**Fig. 18A-B**) and AMP production (**Fig. 18C**) and/ or an epithelial/ immune cell axis leading to enhanced microbiocidal capacity of myeloid cells (**Fig. 18D**).

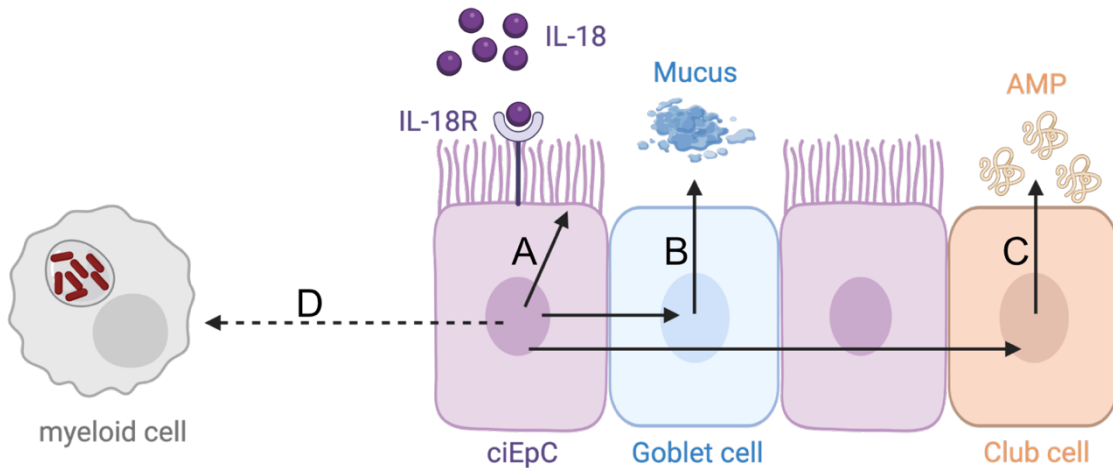


Figure 18: Schematic illustration of the working hypothesis regarding the putatively novel alternative pathway of IL-18R⁺ epithelial cell-instructed defence.

Ciliated epithelial cells were shown to express the IL-18R and this expression is hypothesized to be required for bacterial clearance. IL-18R signalling in ciEpC might activate different defence mechanisms, indicated by the arrows: **(A)** enhanced ciliary beating frequency, **(B)** enhanced mucus production, **(C)** enhanced anti-microbial peptide production, **(D)** crosstalk with the immune system especially myeloid cells. ciEpC = ciliated epithelial cell, IL-18 = Interleukine-18, IL-18R = IL-18 receptor, AMP = anti-microbial peptides.

4.2. Results

4.2.1. IL-18 receptor signalling is required for efficient defence against pulmonary infection with *L. longbeachae*

Previous work in our laboratory demonstrated a critical role for IL-18 during anti-*L. longbeachae* defence (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020). As a first step to characterize the underlying mechanisms of IL-18-conferred protection during *L. longbeachae*-induced pneumonia, I investigated the overall response to the bacteria in *Il18*^{-/-} mice (**Fig. 19**).

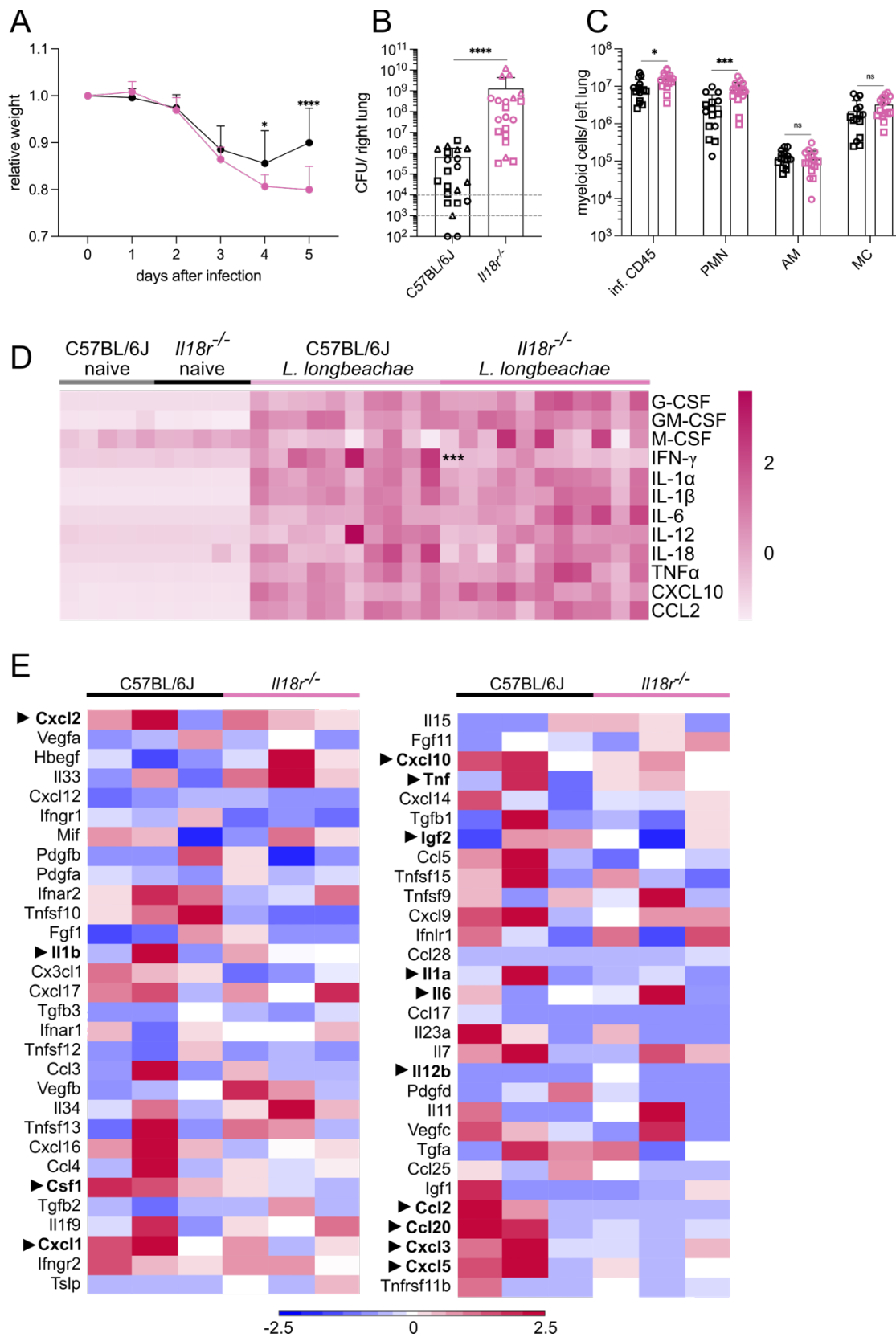


Figure 19: Lack of IL-18R reduces IFN- γ levels in the lung and impairs bacterial clearance during infection with *L. longbeachae*.

IL-18R deficiency led to a significantly impaired wellbeing of the infected mice as, in contrast to the C57BL6/J controls, the *Il18*^{-/-} mice were not able to gain weight from day 5 after infection (**Fig. 19A**). Consistent with this, the lungs of *Il18*^{-/-} showed a more than 1,000-fold increase in bacterial burden in comparison to their WT counterparts 5 days after infection (**Fig. 19B**). The influx of total immune cells was significantly increased in *Il18*^{-/-} mice mainly due to an enhanced number of infiltrating neutrophils (**Fig. 19C**).

Using a magnetic bead-based cytokine array (ProcartaPlex) to analyse 12 cytokines, the only analyte showing an altered expression in the lungs of *Il18*^{-/-} mice was IFN- γ (**Fig. 19D**). To investigate further cytokines that are directly or indirectly regulated by IL-18, I conducted bulk RNAseq of purified epithelial cells from the lungs of infected WT and *Il18*^{-/-} mice to get an unbiased view of differentially expressed cytokines. The chemokines *Cxcl3*, *Cxcl5* and *Cxcl10* were expressed at lower levels in the *Il18*^{-/-} epithelial cells than their WT counterparts (**Fig. 19E**). But as these cytokines are mainly responsible to recruit neutrophils and the *Il18*^{-/-} mice showed even an increase in the number of lung-infiltrating neutrophils, I did not investigate the role of these cytokines during IL-18-dependent defence in more detail. Another cytokine that was expressed at lower levels in epithelial cells from *Il18*^{-/-} mice was *Ccl2* (**Fig. 19E**). *Ccl2* is known to recruit monocytes to the site of inflammation (Matsushima et al. 1989; Jia et al. 2008), but *Ccl2* has also been linked to neutrophil survival and activation (Gschwandtner, Derler & Midwood 2019; Yang et al. 2012). Therefore, I

Figure 19: Lack of IL-18R reduces IFN- γ levels in the lung and impairs bacterial clearance during infection with *L. longbeachae*.

Mice were i.n. infected with 10^4 *L. lo*-mCherry CFU. **(A)** Kinetics of relative mouse weight during infection. **(B)** Bacterial load in the right lung at day 5 after infection. LOD (limit of detection, 50 CFU), infection dose (10^4 CFU). **(C)** Quantification of pulmonary myeloid cells at day 5 after infection. **(D)** Magnetic bead array technique was used to quantify cytokine and chemokine expression in the right lung homogenate at the indicated time points after infection. Untreated mice were used as naïve controls. Shown is relative expression of cytokines and chemokines from min. one experiment. **(E)** Bulk RNAseq was performed on flow cytometry-sorted epithelial cells (Live CD45⁻ EpCAM^{high}) at day 3 after infection. Shown is relative expression of cytokines and chemokines. Data in (A), (B), (C) is shown as mean + SD of min. two independent experiments. Symbols represent single mice; symbol shapes indicate different experiments. Unpaired t test (C), (D) or Mann-Whitney test (A), (B), (C), (D) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** < 0.001, **** P < 0.0001.

investigated the role of this cytokine for the IL-18-dependent defence against *L. longbeachae* in more detail later during this study (**Section 4.2.3.**).

Taken together, these results demonstrated that IL-18 receptor signalling was required for an efficient defence against *L. longbeachae* and identified Ccl2 as a putative IL-18-dependent effector cytokine.

4.2.2. Co-localisation of *L. longbeachae* with IL-18R⁺ bronchiolar epithelial cells and alveolar immune cells

I next investigated whether *L. longbeachae* was in close proximity to IL-18R⁺ epithelial cells and immune cells by using IL-18R-tdTomatto reporter mice and mCherry-reporter *L. longbeachae* (**Fig. 20**). First, I confirmed previous results of our laboratory showing expression of the IL-18R on immune cells located in the alveolar space and on bronchiolar epithelial cells (**Fig. 20B**). This observation, together with bone marrow chimera experiments (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020), led to the hypothesis that both pathways of IL-18-dependent defence, the well-known canonical pathway involving IL-18R⁺ immune cells as well as a putatively novel non-canonical pathway involving IL-18R⁺ ciliated epithelial cells, may confer protection during infection of the lung (**Fig. 20A**).

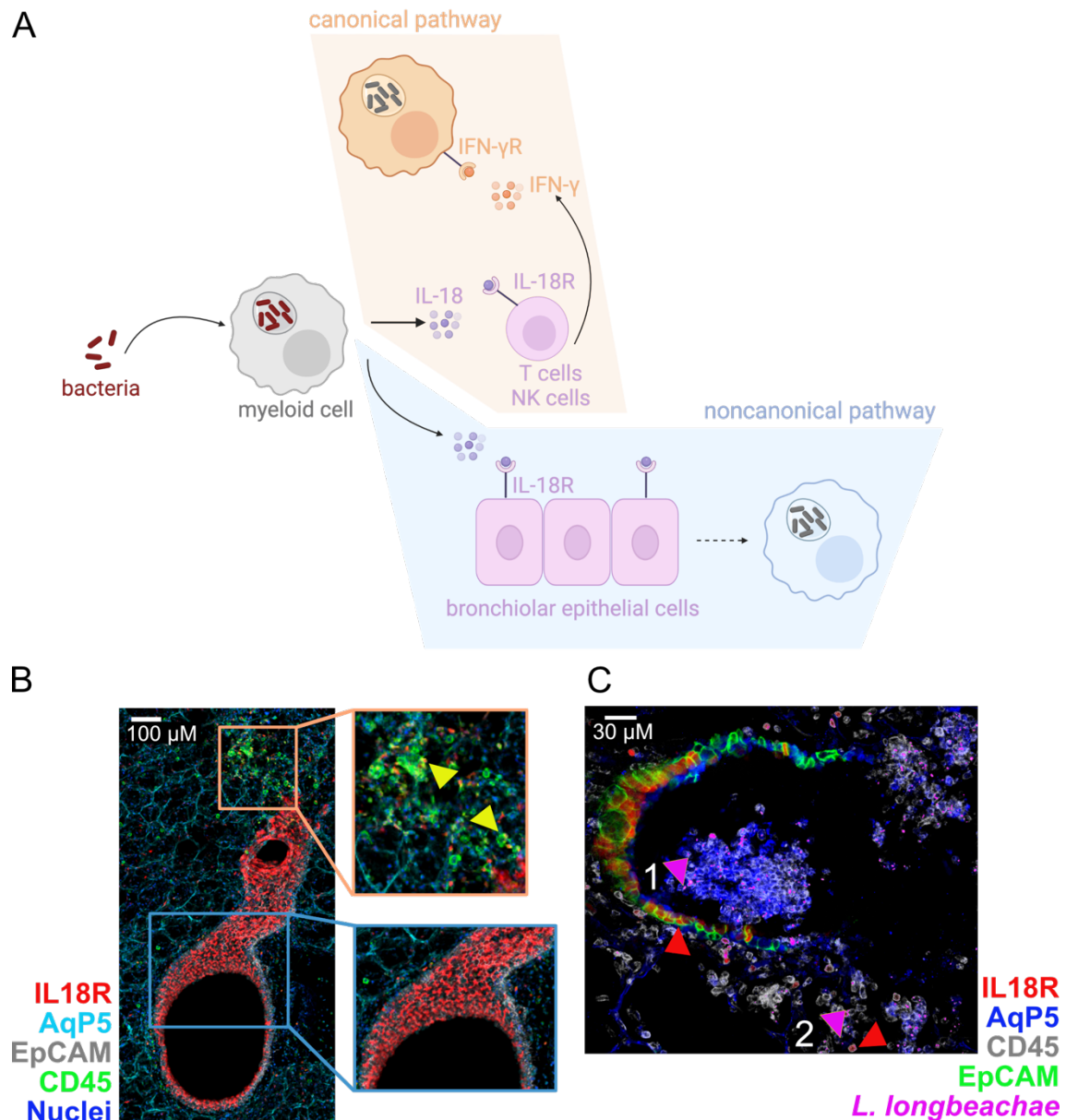


Figure 20: The IL-18R is expressed on pulmonary immune cells and on epithelial cells of the bronchiolar wall both in close proximity to immune cell clusters containing *L. longbeachae*.

(A) Schematic summary of canonical (orange) and the putatively novel non-canonical (blue) pathway of IL-18-mediated protection. **(B)–(C)** Spectral confocal images of fluorescently labelled pulmonary vibratome slices of IL-18R-tdTomato reporter mouse. Mice were i.n. infected with 10^4 *L. lo* CFU. **(A)** Overview of the bronchiolar wall and the alveolar space showing IL-18R (tdTomato⁺) expression in bronchiolar epithelial cells (EpCAM⁺) and in immune cells in the alveolar space (CD45⁺, yellow arrowhead). **(C)** Detailed picture of the bronchiolar wall and the alveolar space showing IL-18R-expressing (tdTomato⁺, red arrowhead) epithelial cells (EpCAM⁺, 1) and immune cells (CD45⁺, 2) in close proximity to clusters of *L. longbeachae* (mCherry⁺, pink arrowhead).

Confocal staining of pulmonary slices from infected IL-18R-tdTomato reporter mice further supported this hypothesis as clusters of bacteria were found within the alveolar space in close proximity to IL-18R⁺ immune cells (**Fig. 20C.2**), and in the bronchiolar lumen nearby to IL-18R⁺ bronchiolar epithelial cells (**Fig. 20C.1**).

Taken together, the results showed that both pathways of IL-18-dependent defence may protect against pulmonary infection with *L. longbeachae*. Nevertheless, as described in the introduction, previous data in our laboratory using bone marrow chimera experiments suggested that the expression of the IL-18R on stromal cells is both necessary and sufficient to protect against *L. longbeachae*. Therefore, I focused on investigating the cellular and molecular mechanisms behind this apparently novel pathway.

4.2.3. Activation of the Ccl2/ Ccr2-axis does not rescue the impaired bacterial clearance in *Il18r^{-/-}* mice

Regarding the putatively novel IL-18R⁺ ciEpC-dependent anti-bacterial defence, I was interested in investigating the crosstalk between IL-18R⁺ epithelial cells and neutrophils, a key protective myeloid cell type during *L. longbeachae* infection, as shown in the previous Chapter. Therefore, I used RNAseq data from pulmonary epithelial cells (**Fig. 19E**) and neutrophils to identify potential cell-cell interactions that are regulated by IL-18. Epithelial cells were flow cytometry-sorted from infected C57BL6/J and *Il18r^{-/-}* at day 3 after infection with *L. longbeachae* to identify genes expressed dependent on IL-18R signalling during infection. Furthermore, we purified bacteria-containing (mCherry⁺) neutrophils from C57BL6/J and *Il18r^{-/-}* at day 3 after infection with *L. longbeachae* to examine whether they express receptors for epithelial cell-derived cytokines. One of the genes trending to be decreased in *Il18r^{-/-}* epithelial cells during *L. longbeachae* infection compared to their WT counterparts was *Ccl2* (**Fig. 19E**). Bulk RNAseq of neutrophils showed expression of its receptor *Ccr2* (**Fig. 21A**).

Next, I checked whether the *Ccr2* gene expression data translated into protein expression. Staining for Ccr2 receptor was strong on MC, as expected, whereas

the staining level on WT neutrophils was comparable to *Ccr2*^{-/-} neutrophils (**Fig. 21B**). However, it has been reported that Ccr2 is rapidly internalized when bound to its ligand (Zhao et al. 2019), which may explain the apparent contradiction of *Ccr2* mRNA versus protein expression in neutrophils. In addition, it has been reported that Ccl2 promotes survival and effector mechanisms in neutrophils in the context of asthma (Gschwandtner, Derler & Midwood 2019; Yang et al. 2012). Therefore, I hypothesised that IL-18R stimulation in ciEpC leads to production and secretion of Ccl2 that can in turn stimulate its receptor on neutrophils to enhance their microbiocidal function (**Fig. 21C**).

To investigate this hypothesis, I set up Ccl2 gain- and loss-of-function experiments (**Fig. 21D**). For the loss-of-function experiments, C57BL6/J mice were treated with anti-Ccl2 mAb i.t. daily starting 2 days after infection when neutrophils had infiltrated the lung. According to our hypothesis these mice would mimic results in *Il18*^{-/-} mice treated with PBS control. For the gain-of-function experiments *Il18*^{-/-} mice were treated with rCcl2 i.t. starting 2 days after infection expected, according to our hypothesis, to mimic results in C57BL6/J mice treated with PBS control.

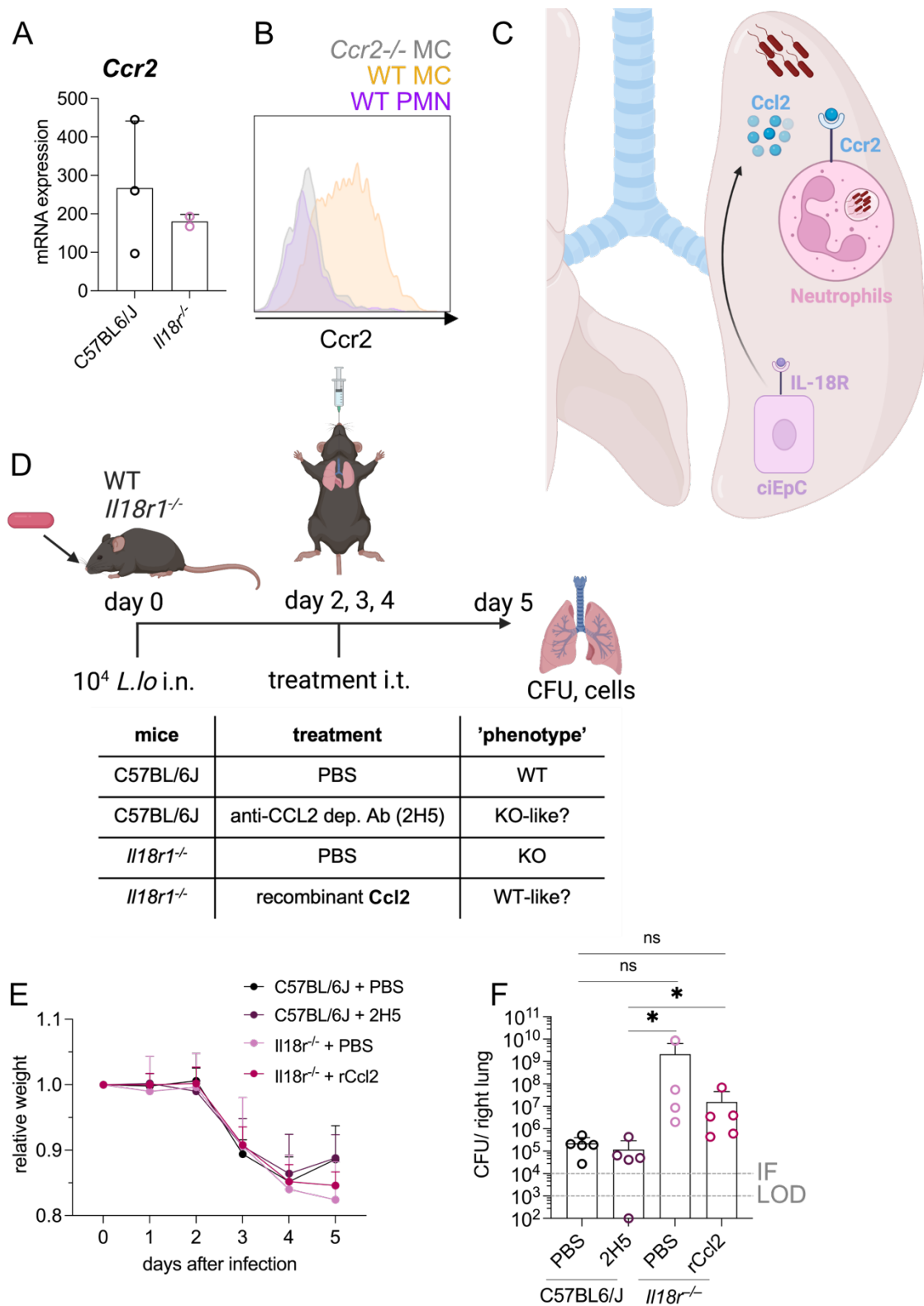


Figure 21: The Ccl2/ Ccr2-axis is apparently not contributing to the novel putative mechanism of IL-18R⁺ ciEpC-dependent anti-bacterial defence.

Analysing the relative weight loss over time (**Fig. 21E**) as well as the bacterial burden in the lung at day 5 (**Fig. 21F**) after infection revealed that, in disagreement with the hypothesis, the two C57BL6/J and the two *Il18r^{-/-}* cohorts showed similar infection parameters suggesting that Ccl2 is not involved in the IL-18-dependent defence against *L. longbeachae*. There was no significant difference between WT and *Il18r^{-/-}* regarding the weight loss but there was a trend that WT mice gained weight at day 5 after infection while *Il18r^{-/-}* mice continued to lose weight (**Fig. 21E**) irrespective of treatment with anti-Ccl2 mAb or rCcl2. Treatment of the WT mice with anti-Ccl2 mAb did, furthermore, not increase the bacterial burden and treatment of *Il18r^{-/-}* mice treated with rCcl2 did not lead to an improved bacterial clearance (**Fig. 21F**).

Taken together, these results indicated that the Ccl2/ Ccr2-axis was apparently not involved in a potential crosstalk between IL-18R⁺ ciEpC and neutrophils during defence against *L. longbeachae*.

Figure 21: The Ccl2/ Ccr2-axis is apparently not contributing to the novel putative mechanism of IL-18R⁺ ciEpC-dependent antibacterial defence.

Mice were i.n. infected with 10⁴ *L. lo*-mCherry CFU. **(A)** Bulk RNAseq was performed on flow cytometry-sorted bacteria-containing neutrophils (Live CD45⁺ SiglecF⁻ Ly-6G⁺ mCherry⁺) at day 3 after infection. Shown is relative expression of *Ccr2*. **(B)** Lungs were sampled from naïve mice. Histogram showing *Ccr2*-expression on *Ccr2^{-/-}* monocyte-derived cells (grey), WT monocyte-derived cells (yellow) and WT neutrophils (purple) **(C)** Schematic summary of hypotheses regarding the involvement of the Ccl2-Ccr2-axis in the novel mechanism of IL-18R⁺ ciEpC-dependent antibacterial defence. IL-18R stimulation on ciEpC might lead to production and secretion of CCL2 that, in turn, might stimulate the *Ccr2* on neutrophils to enhance their microbicidal activity. **(D)** Scheme of experimental setup for (D)-(E). Mice were administered i.t. with either PBS, 200 µg of anti-Ccl2 depletion antibody (2H5) or 300 ng of rCcl2 at day 2, 3, and 4 after infection. Lungs were sampled at day 5. Table shows summary of different treatment groups and their expected phenotype for (D)-(E) Treatment of C57BL/6J mice with 2H5 was expected to mimic the phenotype of *Il18r^{-/-}* mice. Treatment of *Il18r^{-/-}* mice with rCcl2 was expected to rescue the impaired anti-microbial defence. **(E)** Relative weight loss during infection. **(F)** Bacterial load in the right lung at day 5 after infection. LOD (limit of detection, 50 CFU), IF (infection dose, 10⁴ CFU). (E)-(F) Data is shown as mean + SD from 1 experiment. Symbols represent single mice. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) was performed for statistical analysis. * P < 0.05.

4.2.4. Genetic mouse models reveal IL-18-instructed defence against *L. longbeachae* to be dependent on the canonical pathway involving IL-18R⁺ immune cells

I next aimed to confirm the putatively novel IL-18R⁺ ciEpC-dependent anti-bacterial defence independent of lymphocytes as identified in bone marrow chimera experiments (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020), using the Cre-lox-system. To delete *Il18r* in pulmonary epithelial cells, *Il18r^{fl/fl}* mice were crossed with *Nkx2.1*-Cre recombinase mice (Li et al. 2011). Mice in which the IL-18R was specifically deleted on pulmonary epithelial cells are hereafter referred to as EpC^{ΔIL18R}. These mice had an intact canonical pathway of IL-18-mediated protection involving IFN- γ production by lymphocytes but lacked the putative non-canonical pathway involving IL-18R expression by ciEpC (**Fig. 22A**). Crossing of the *Il18r^{fl/fl}* mice with *vav*-Cre recombinase mice was used to restrict the *Il18r* deletion to the haematopoietic lineage and thereby to immune cells (Ogilvy et al. 1999) inducing ablation of the canonical pathway of IL-18-dependent protection (**Fig. 22A**). These mice are referred to hereafter as ImC^{ΔIL18R}.

We confirmed specific deletion of the IL-18R on epithelial cells in EpC^{ΔIL18R} mice using antibody staining of lung single cell suspensions during *L. longbeachae* infection (**Fig. 22B, C**). Specific IL-18R deletion in ImC^{ΔIL18R} mice was confirmed by lack of IL-18R staining on NK cells and activated T cells, the main IL-18R expressors in the immune compartment (**Fig. 22B, C**).

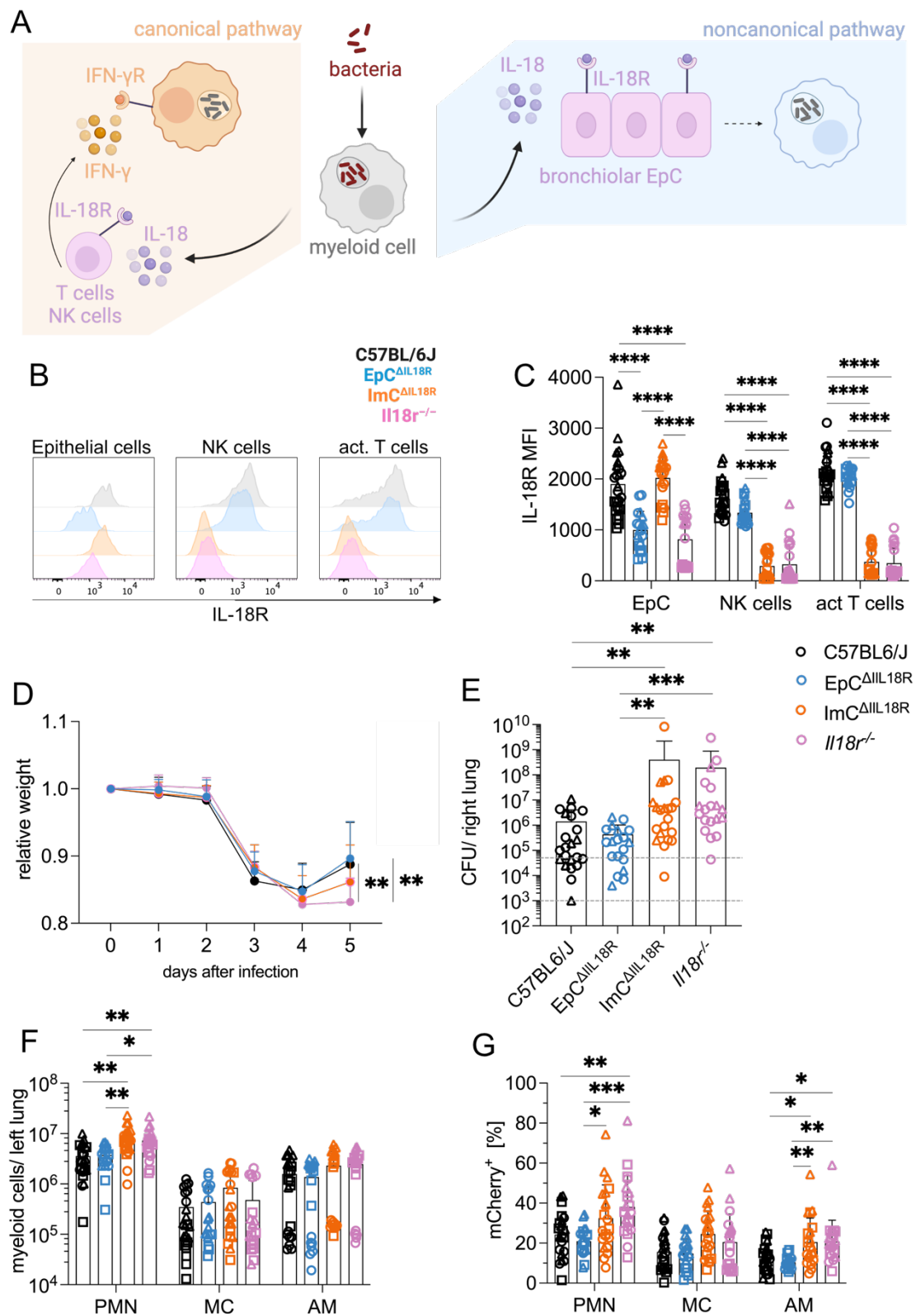


Figure 22: IL-18R expression on immune cells rather than bronchiolar ciliated epithelial cells is required to confer protection against *L. longbeachae*.

EpC^{ΔIL18R} and ImC^{ΔIL18R} mice were then infected with *L. longbeachae*. We detected no differences in weight loss (**Fig. 22D**) and bacterial burden (**Fig. 22E**) between EpC^{ΔIL18R} mice and those normally expressing IL-18R. ImC^{ΔIL18R} mice rather showed an impaired anti-*L. longbeachae* defence similar to the *Il18r*^{-/-} mice while EpC^{ΔIL18R} mice were fully protected similar to the WT counterparts (**Fig. 22D, E**). Furthermore, the number of neutrophils present in the lung (**Fig. 22F**) as well as the percentage of bacteria containing neutrophils (**Fig. 22G**), both signs of disease severity, were similar between EpC^{ΔIL18R} mice and C57BL6/J mice as well as ImC^{ΔIL18R} mice and *Il18r*^{-/-} mice, respectively.

All in all, this data indicated that the putatively novel pathway of IL-18R⁺ ciEpC-dependent anti-bacterial defence, suggested by the use of bone marrow chimeras, did apparently not protect against pulmonary infection with *L. longbeachae*. It was rather the established canonical pathway of IL-18-mediated protection involving lymphocyte-derived IFN- γ that conferred clearance of *L. longbeachae*. Nevertheless, the high number of IL-18R⁺ ciEpC in the bronchiolar wall suggested this cell type to have a critical function during IL-18-dependent pulmonary defence even though the IL-18R⁺ ciEpC may not protect against *L. longbeachae*.

Figure 22: IL-18R expression on immune cells rather than bronchiolar ciliated epithelial cells is required to confer protection against *L. longbeachae*.

(A) Schematic summary of which pathway of IL-18-dependent protection is blocked in the genetic mouse models. ImC^{ΔIL18R} mice (orange) lack the IL-18R on immune cells and therefore the canonical pathway. EpC^{ΔIL18R} mice (blue) lack the IL-18R on pulmonary epithelial cells and therefore the non-canonical pathway. (B)-(G) Mice were i.n. infected with 5×10^4 *L. lo*-mCherry CFU. (B) Exemplary flow cytometry plots confirming genetic mouse models at the level of IL-18R expression on epithelial cells, NK cells and activated T cells. (C) Quantification of (B). (D) Kinetics of relative mouse weight during infection. (E) Bacterial load in the right lung at day 5 after infection. LOD (limit of detection, 50 CFU), IF (infection dose, 5×10^4 CFU). (F) Quantification of pulmonary myeloid cells. (G) Percentage of mCherry⁺ cells within indicated cell populations. Cells from mice infected with WT *L. lo* served as mCherry⁻ controls. Data is shown as mean + SD from three pooled independent experiments. Symbols represent single mice; symbol shape indicates different experiments. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) (C), (D), (E), (F), (G) or RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) (D), (G) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** < 0.001, **** P < 0.0001.

4.2.5. Epithelial cell-autonomous mechanisms are apparently not involved in IL-18R⁺ ciEpC-dependent pulmonary defence

In order to investigate the role of IL-18R⁺ bronchiolar ciEpC, I focused on the hypotheses formulated before, involving epithelial cells-autonomous mechanisms as well as an interaction between the ciEpC and the immune system. Intrinsic epithelial cell-autonomous mechanisms are critical in the defence against bacterial infections due to a variety of well-known mechanisms. One of these mechanisms is the pathway of mucociliary clearance, in which pathogens are first trapped in mucus produced by goblet cells and then transported out of the lung via beating of the cilia by ciEpC. As ciEpC cells were shown by our laboratory to be the epithelial cell type within the bronchiolar wall expressing the IL-18R, we next investigated the role of their ciliary beating frequency during IL-18-dependent defence. Thus, I examined the influence of IL-18 on the ciliary beating frequency (CBF) both *ex vivo* in pulmonary lung slices (**Fig. 23A-C**) as well as *in vitro* in organotypically grown human bronchiolar epithelial cells (**Fig. 23D-H**).

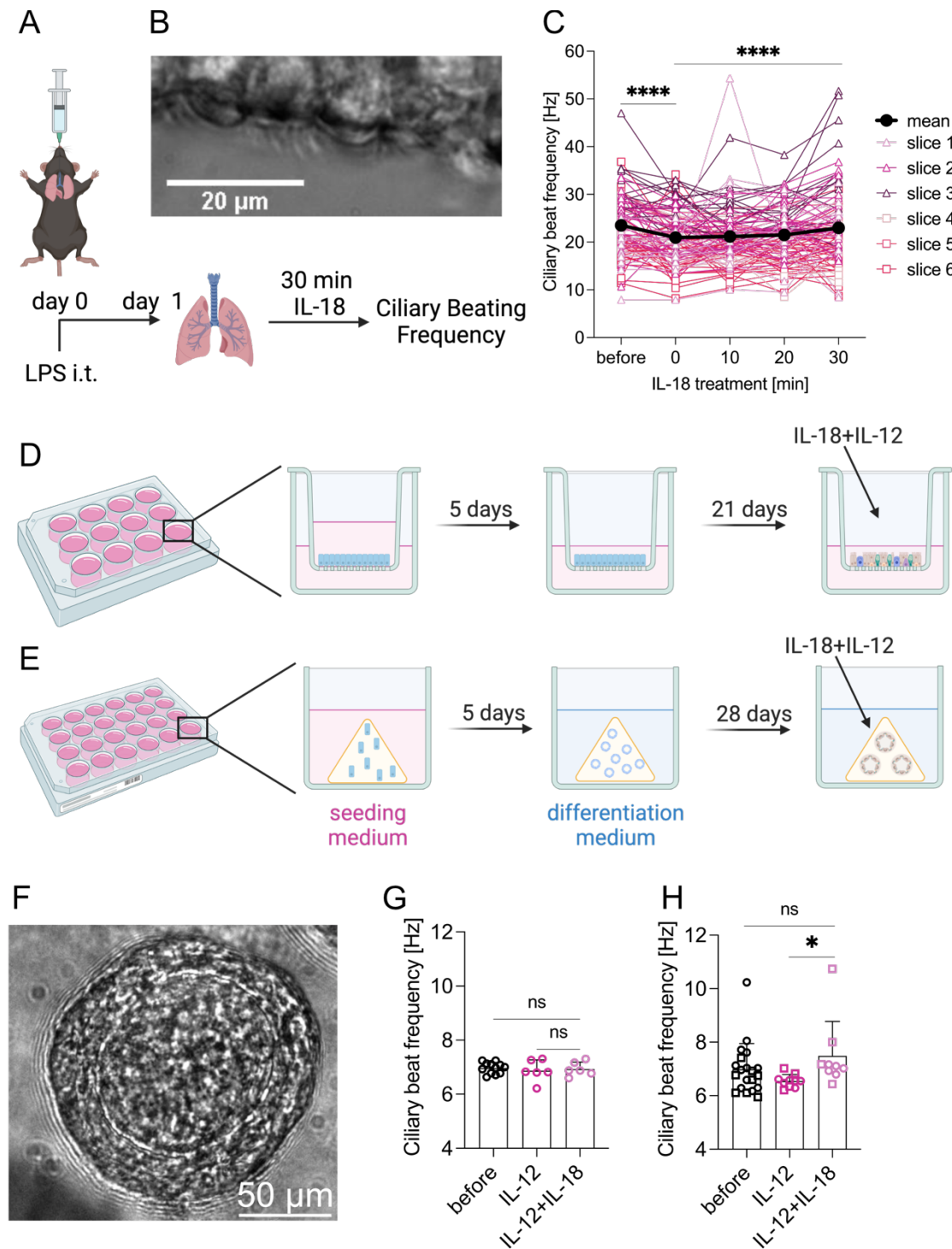


Figure 23: The ciliary beating frequency is apparently not contributing to a putative mechanism of IL-18R⁺ ciEpC-dependent pulmonary defence.

To quantify CBF, mice were treated with LPS i.t. one day prior to sacrificing in order to upregulate the non-constitutively expressed IL-18R β chain as well as other inflammation-associated factors that may be required to regulate CBF

(**Fig. 23A**). CBF was quantified in pulmonary vibratome slices using a high-speed video camera (**Fig. 23B**) before and during a 30 min time course following administration of recombinant IL-18. This quantification revealed no consistent change of the CBF by administration of rIL-18 (**Fig. 23C**).

To study the ciliary beating in a human system that is additionally easier to manipulate, I organotypically cultured HBEC either as ALI cultures (**Fig. 23D**) or as organoids (**Fig. 23E**). Both culture systems offer the advantage that HBEC differentiate into distinct epithelial cell types present in bronchiole showing typical epithelial cell functions, including ciliary beating and mucus production. Again, the ciliary beating was recorded in high-speed (**Fig. 23F**) and the CBF was quantified before and after 12 hours of treatment with IL-18 and IL-12 to upregulate IL-18R β expression. Neither ALI nor organoid cultures revealed a significant change in the CBF by treatment with IL-18 (**Fig. 23G-H**). Quantifying the CBF from organoids treated with IL-12 vs. IL-12 + IL-18 showed that IL-18 enhanced the CBF to a small amount. Nevertheless, the biological relevance is questionable as the difference is minor (from 6.5 Hz to 7.5 Hz) and both lie in the physiological baseline range reported for human cells (Oltean et al. 2018).

Figure 23: The ciliary beating frequency is apparently not contributing to a putative mechanism of IL-18R⁺ ciEpC-dependent antibacterial defence.

(**A**) Scheme of experimental setup for (B)-(C). Mice were i.t. administered with 10 μ g lipopolysaccharide (LPS) one day prior to sampling to upregulate IL-18R β expression. The Ciliary beating frequency (CBF) was measured in pulmonary vibratome slices using highspeed video microscopy before and during 30 min treatment with rIL-18. (**B**) Picture from a representative video showing beating of cilia in pulmonary vibratome slices. (**C**) Quantification of CBF. Data were pooled from two mice with three lung slices each, symbol shape indicates different experiments. (**D**) Scheme of experimental setup for (G). Normal human bronchiolar epithelial cells were cultured in an ALI culture system. Air lift after five days induces differentiation. After 21 days cultures were treated with IL-12 $\pm$ IL-18 and CBF was measured before and after treatment. (**E**) Scheme of experimental setup for (F), (H). Normal human bronchiolar epithelial cells were cultured as organoids. Switch to differentiation medium after 5 days induces differentiation. After 28 days cultures were treated with IL-12 $\pm$ IL-18 and CBF was measured before and after treatment. (**F**) Picture from a representative video showing beating of cilia in organoids. (**G**) Quantification of CBF. Data are from one experiment; symbols indicate single mice. Data are shown as mean + SD. (**H**) Quantification of CBF. Data were pooled from two independent experiments, symbols indicate single mice, symbol shapes indicate different experiments. Data are shown as mean +SD. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) (C), (H) or RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) (G) were performed for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, **** $P < 0.0001$.

Taken together, these results suggested that the CBF was apparently not influenced by IL-18 and was, therefore, most likely not involved in IL-18R⁺ ciEpC-dependent pulmonary defence. The second mechanism contributing to mucociliary clearance is production of mucus by goblet cells. To investigate whether this process is involved in IL-18R⁺ ciEpC-dependent defence, I infected WT as well as both *Il18*^{-/-} and *Il18r*^{-/-} mice with *L. longbeachae* and quantified the production of the two major mucus proteins Muc5B and Muc5AC (**Fig. 24**).

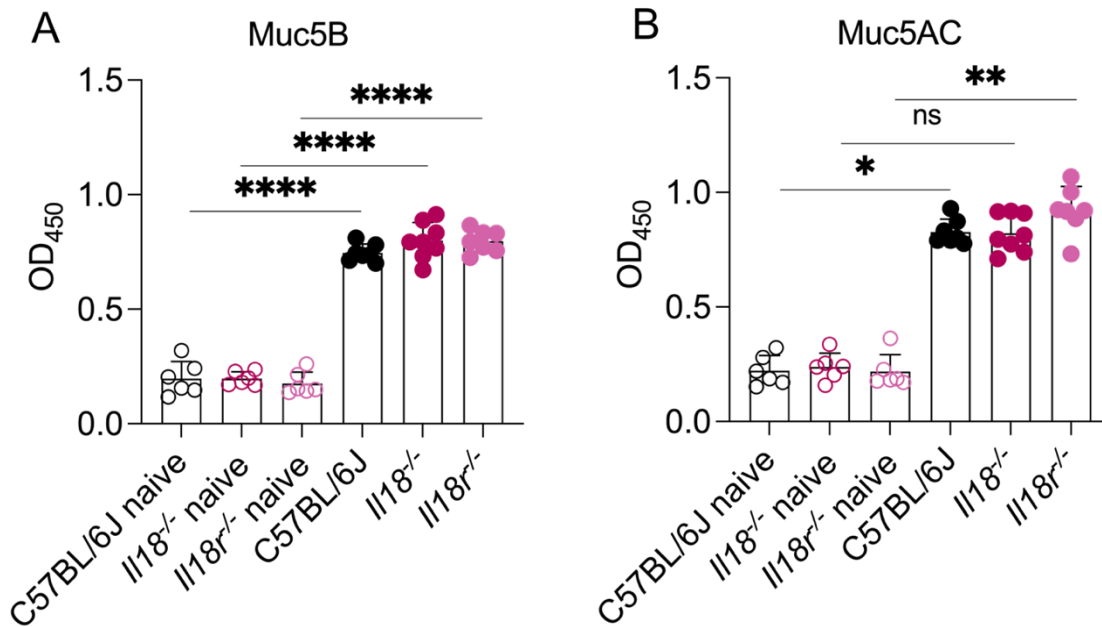


Figure 24: Production of the two major pulmonary mucus proteins Muc5AC and Muc5B is apparently not contributing to the novel mechanism of IL-18R⁺ ciEpC-dependent pulmonary defence.

Mice were i.n. infected with 10^4 *L. lo* CFU. Lung homogenates were analysed for mucus proteins at day 3 after infection using ELISA. **(A)** Muc5B and **(B)** Muc5AC expression in the lungs of indicated mice is shown as OD₄₅₀. Data is shown as mean + SD from one representative of two independent experiments. Symbols represent single mice. Kruskal-Wallis test (with Dunn's multiple comparisons test for inter-group differences) (B) or RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) (A) were performed for statistical analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, **** $P < 0.0001$.

Muc5B (**Fig. 24A**) and Muc5AC (**Fig. 24B**) were strongly upregulated 3 days after infection with *L. longbeachae* in WT and both KO mice indicating that their production is independent of IL-18.

All in all, these results indicated that the mucociliary clearance was not involved in IL-18R⁺ ciEpC-dependent pulmonary defence. Another epithelial cell-

autonomous pathway of defence against bacterial infections is the production of AMP by club cells. To investigate AMP production, mRNA levels in either the whole lung homogenate or in purified epithelial cells were quantified (**Fig. 25**).

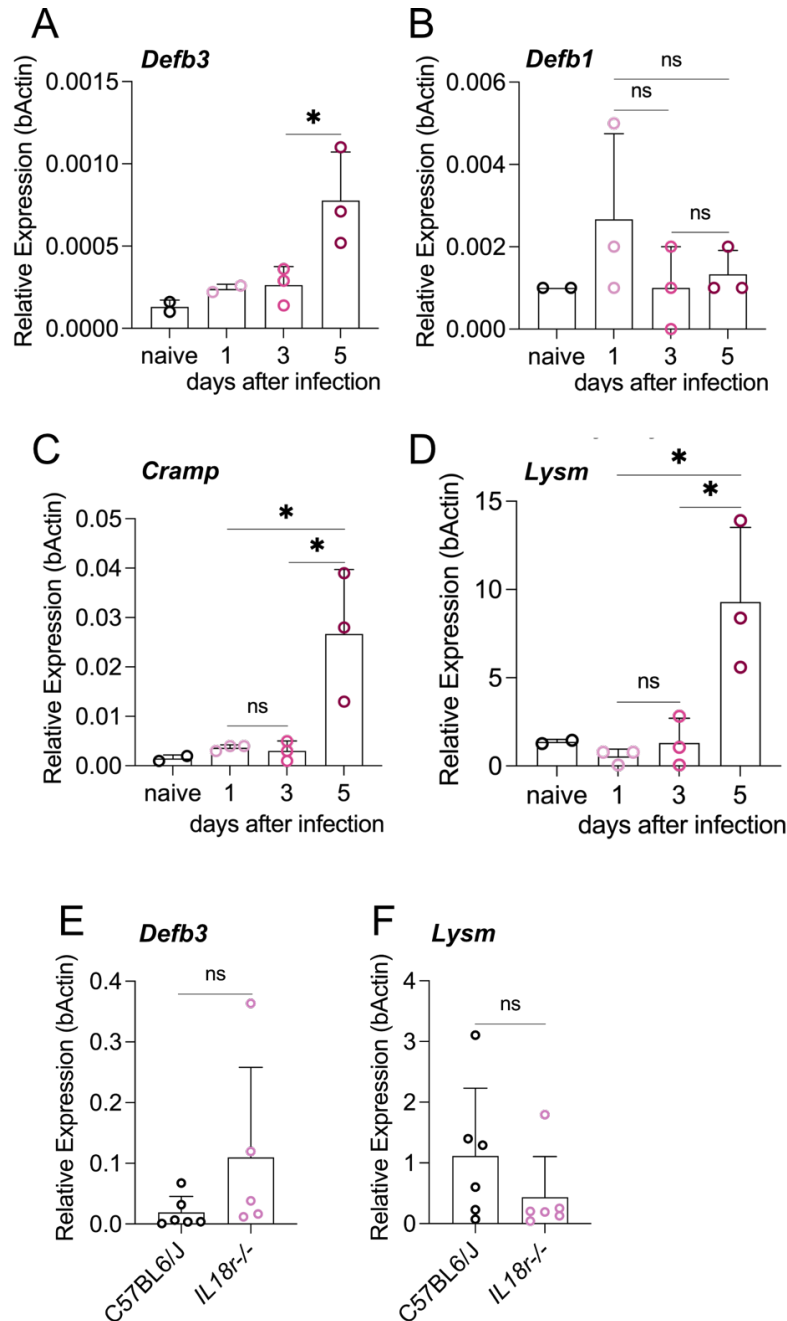


Figure 25: Anti-microbial peptides are apparently not contributing to the novel mechanism of IL-18R⁺ ciEpC-dependent pulmonary defence.

To identify the optimal time point, to quantify AMP after infection with *L. longbeachae*, mice were infected and mRNA levels of different AMP known to

be involved in defence against bacterial infection were quantified in the total lung at different time points after infection (**Fig. 25A-D**). The genes encoding mBD3 (**Fig. 25A**), mCRAMP (**Fig. 25C**), and LysM (**Fig. 25D**), were strongly upregulated at day 5 after infection. Therefore, day 5 was chosen as the time point after infection to analyse the expression of AMP in purified epithelial cells.

Immune cells can also produce AMP, so pulmonary epithelial cells were flow cytometry-sorted in order to restrict a potential effect of IL-18 to epithelial cells autonomous mechanisms. At day 5 after infection *Defb3* (**Fig. 25E**) and *Lysm* (**Fig. 25F**) could be detected in purified epithelial cells. However, lack of IL-18R did not alter their expression (**Fig. 25E-F**).

Taken together, AMP production by club cells was apparently not dependent on IL-18R signalling. Overall, these results suggested that epithelial cell-autonomous mechanisms, namely mucociliary clearance and AMP production, did not play a role for IL-18R⁺ ciEpC-dependent pulmonary defence.

4.2.6. IL-18-stimulated normal human bronchiolar epithelial cells are apparently not able to enhance the bacterial killing capacity of human blood neutrophils

To investigate the crosstalk between bronchiolar epithelial cells and neutrophils, I established an *in vitro* system combining organotypic culture systems of HBEC with freshly isolated human blood neutrophils (**Fig. 26**). Additionally, this system offered the possibility to study the relevance of IL-18R⁺ ciEpC-dependent pulmonary defence in a human system.

Figure 25: Anti-microbial peptides are apparently not contributing to the novel mechanism of IL-18R⁺ ciEpC-dependent anti-bacterial defence.

Mice were i.n. infected with 10⁴ *L. lo-mCherry* CFU. **(A)-(D)** Lung homogenates were sampled at indicated timepoints and analysed for indicated AMP genes using qPCR. **(E)-(F)** Epithelial cells (Live CD45⁻ EpCAM^{high}) were flow cytometry-sorted at day 5 after infection and analysed for indicated AMP genes using qPCR. Data is shown as mean +SD from one experiment. Symbols represent single mice. Unpaired t test (A), RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) (B), (C), (D) or Mann-Whitney test (E), (F) were performed for statistical analysis. * P < 0.05; ** P < 0.01.

Neutrophils were enriched from human blood (**Fig. 26A**). As primary neutrophils are highly sensitive to *in vitro* manipulation, I investigated whether the purified neutrophils retained bacterial phagocytosis (**Fig. 26B**), expression of activation markers (**Fig. 26C**), and production of ROS (**Fig. 26D**). The isolated neutrophils showed strong uptake of *L. longbeachae*-mCherry (**Fig. 26B**) and produced ROS (**Fig. 26D**). Additionally, the cells upregulated CD11b and CD66b and downregulated CD62L in response to *L. longbeachae* (**Fig. 26C**) all described in the literature as signs of neutrophil activation (CD11b (Carlos & Harlan 1994; Janke et al. 2009), CD66b (Skubitz, Campbell & Skubitz 1996; Schmidt et al. 2012), CD62L (Kahn et al. 1994; Janke et al. 2009)).

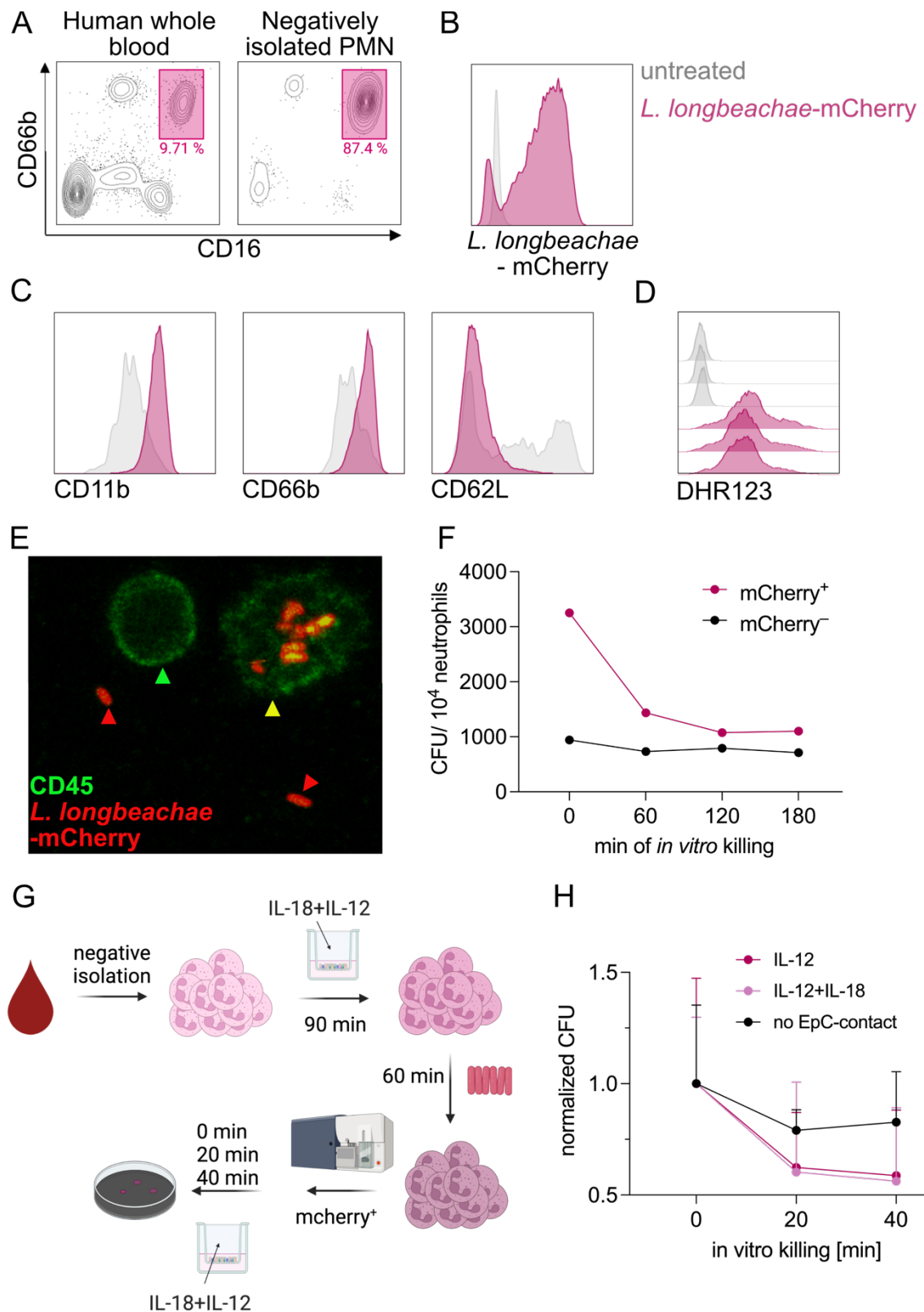


Figure 26: Human blood neutrophils support *in vitro* infection with *L. longbeachae* but their killing capacity is apparently not influenced by the novel mechanism of IL-18R⁺ ciEpC-dependent anti-bacterial defence.

Microscopy revealed that *in vitro* co-cultures of neutrophils with *L. longbeachae* contained extracellular bacteria in addition to those phagocytosed by neutrophils (**Fig. 26E**). Therefore, I flow cytometry-sorted mCherry⁺ neutrophils. mCherry⁻ neutrophils served as a background control. *In vitro* killing of *L. longbeachae* by purified mCherry⁺ neutrophils revealed that the phagocytes were able to kill around 75 % of the internalised bacteria within 60 min.

Having established flow cytometric-sorting as an efficient method to eliminate extracellular bacteria and 60 min as a maximal timeframe of *in vitro* killing, I combined the *L. longbeachae*-mCherry infected human neutrophils with the ALI-cultured HBEC in order to investigate a potential IL-18-dependent ciEpC/ immune cell crosstalk. HBEC were treated with IL-18 and IL-12 for 12 h, the latter to upregulate IL-18R β expression. Freshly isolated human blood neutrophils were incubated with this conditioned ALI culture medium prior to infection with *L. longbeachae* and during the *in vitro* killing. ALI culture medium not in contact with epithelial cells was used as a negative control. Quantification of CFU within the neutrophils over time revealed that neutrophils treated with conditioned ALI culture medium were able to kill internalized bacteria faster than the control neutrophils treated with fresh ALI culture medium (**Fig. 26H**). This effect was

Figure 26: Human blood neutrophils support *in vitro* infection with *L. longbeachae* but their killing capacity is apparently not influenced by the novel mechanism of IL-18R⁺ ciEpC-dependent anti-bacterial defence.

(A) Exemplary flow cytometry plots showing purification of neutrophils from human blood using negative isolation. (B) Exemplary histogram showing uptake of *L. lo*-mCherry by isolated neutrophils. (C) Exemplary histograms showing expression of activation markers by uninfected and *L. lo*-infected human blood neutrophils. (D) Exemplary histogram showing ROS production (DHR123) by human blood neutrophils infected with *L. lo*. (E) Confocal images of *L. lo*-infected human blood neutrophils showing uninfected neutrophils (green arrowhead), extracellular bacteria (red arrowhead), and infected neutrophils (yellow arrowhead). (F) Number of viable bacteria (CFU) per 10⁴ neutrophils flow cytometry-sorted 60 min after infection with *L. lo*. (G) Scheme of experimental setup for (I). HBEC were cultured in an ALI culture system. Human blood neutrophils were negatively isolated and stimulated with ALI culture medium for 90 min prior to 60 min of infection with *L. lo*. flow cytometry-sorted neutrophils were incubated with ALI culture medium for indicated timepoints (*in vitro* killing). (H) Number of viable bacteria (CFU) normalized to 0 min of *in vitro* killing. (I) Data is shown as mean + SD from one experiment with 3 blood donors. RM one-way ANOVA (with Turkey's multiple comparisons test for inter-group differences) was performed for statistical analysis. There were no statistical significances detected.

irrespective of whether the conditioned medium contained IL-18 or not suggesting that ALI cultures, either generally or in response to IL-12 secrete a factor able to enhance bacterial killing capacity of neutrophils. To investigate whether the effect was dependent on IL-12, cytokine-naïve ALI culture medium should be used. This control was not performed here due to the limited availability of neutrophils and the focus on IL-18-dependent processes.

Taken together, these results suggested that neutrophils isolated from human blood were functionally active and could phagocytose *L. longbeachae*. Further controls should be performed, and the experiment needs to be repeated. But the presented primary results suggested that there was no IL-18-dependent crosstalk between ciEpC and neutrophils taking place to enhance the anti-bacterial activity of the latter.

4.2.7. IL-18 receptor deficiency impairs ROS production and thereby efficient bacterial killing by neutrophils

After investigating the role of IL-18R⁺ bronchiolar ciEpC, I ultimately aimed to gain a better understanding of the mechanisms behind the canonical pathway of IL-18R-dependent anti-*L. longbeachae*. As we showed neutrophils to be the main protective myeloid cell type, I investigated whether the microbiocidal function of neutrophils against *L. longbeachae* is dependent on IL-18R signalling (**Fig. 27**). Lack of IL-18R led to an increased percentage of mCherry⁺ neutrophils having phagocytosed *L. longbeachae*-mCherry (**Fig. 27A-B**) as well as an increased mCherry MFI in those neutrophils (**Fig. 27A, C**).

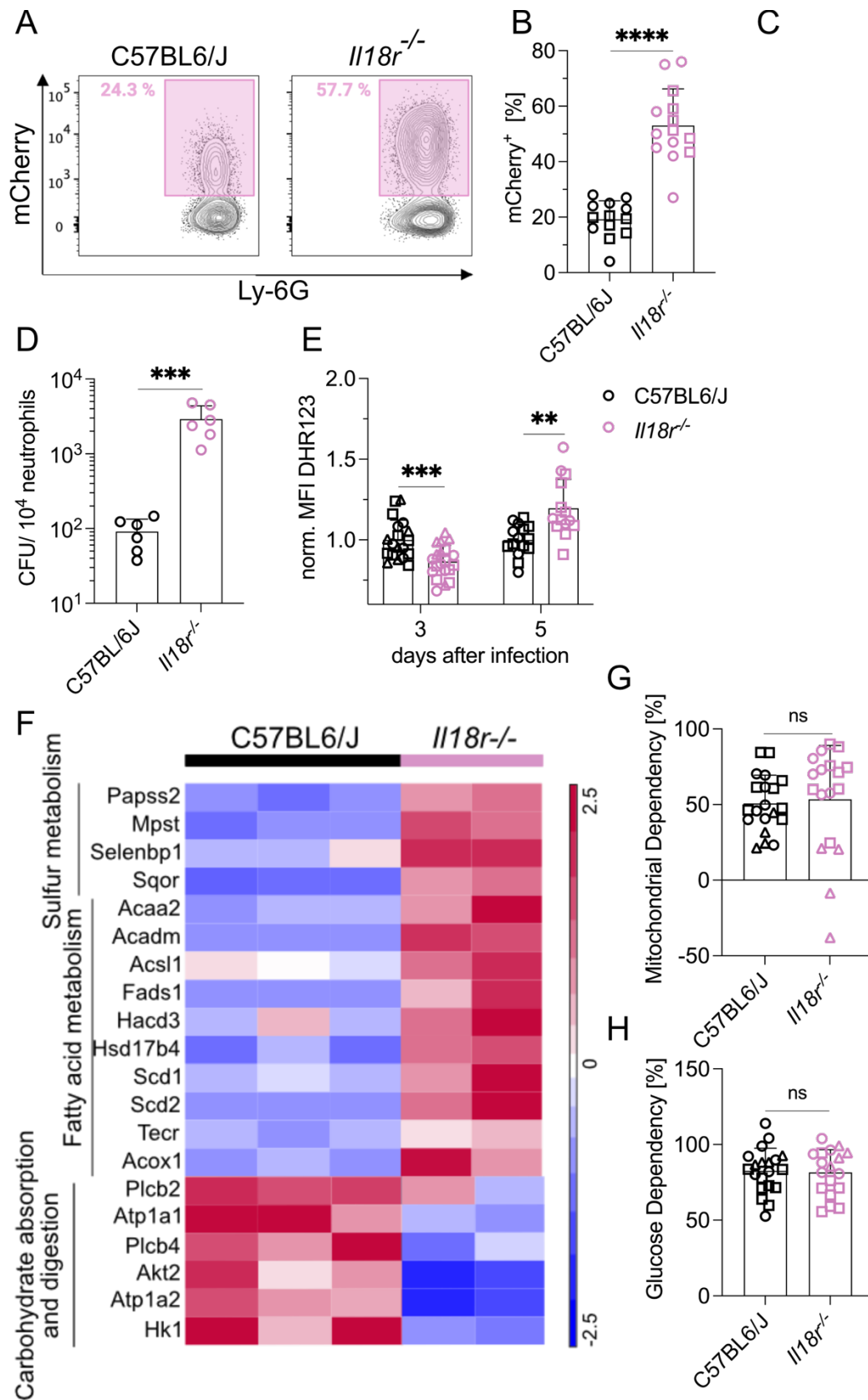


Figure 27: Expression of the IL-18R is required for efficient ROS-dependent bacterial killing by neutrophils.

The increased percentage of mCherry⁺ neutrophils likely resulted from the higher bacterial load in the lungs of *Il18r^{-/-}* mice whereas the increased mCherry MFI suggested an impaired bacterial killing capacity. The impaired microbiocidal capacity of neutrophils due to the lack of IL-18R signalling was verified as neutrophils from *Il18r^{-/-}* mice contained significantly more viable bacteria (**Fig. 27D**). As ROS production was essential for neutrophils to kill *L. longbeachae* (**Fig. 13I**) and as IFN- γ , a potential activator of neutrophilic ROS, was decreased in the lungs of *Il18r^{-/-}* mice (**Fig. 19D**), I next investigated whether lack of IL-18R signalling limits the microbiocidal function of neutrophils via an impaired production of ROS. Indeed, neutrophils in *Il18r^{-/-}* mice showed a decreased production of ROS at day 3 after infection (**Fig. 27E**). At day 5 after infection, the *Il18r^{-/-}* neutrophils, in contrast, showed an increased ROS production (**Fig. 27E**). The increased ROS production at day 5 after infection may be the result of an increased pulmonary *L. longbeachae* burden at day 5 in *Il18r^{-/-}* mice compared to the WT counterpart (**Fig. 19B**) which might lead to further ROS production.

Recently, the microbiocidal functions of neutrophils have been shown to rely on their metabolic state. Glycolysis was suggested to be essential for efficient effector functions, especially ROS production (Injarabian et al. 2020). Therefore, we performed bulk RNAseq of purified neutrophils to investigate the expression

Figure 27: Expression of the IL-18R is required for efficient ROS-dependent bacterial killing by neutrophils.

Mice were i.n. infected with 10^4 *L. lo*-mCherry CFU. **(A)** Representative flow cytometry plots showing increased percentage and MFI of mCherry⁺ neutrophils. **(B)** Percentage of mCherry⁺ cells within neutrophils. Cells from mice infected with WT *L. longbeachae* served as mCherry⁻ controls. **(C)** mCherry MFI in mCherry⁺ neutrophils after subtraction of background MFI in mCherry⁻ neutrophils. **(D)** Number of viable bacteria (CFU) per 10^4 mCherry⁺ neutrophils flow cytometry-sorted 3 days after infection. **(E)** DHR123 (ROS dye) MFI in neutrophils at indicated timepoints normalized to C57BL/6J samples. **(F)** Bulk RNAseq was performed on flow cytometry-sorted bacteria-containing neutrophils (Live CD45⁺ SiglecF⁻ Ly-6G⁺ mCherry⁺) at day 3 after infection. Shown is relative expression of genes related to indicated metabolic pathways. **(G)-(H)** Metabolic state of neutrophils was analysed using the flowcytometry-based SCENITH method on day 5 after infection. **(G)** Mitochondrial dependency. **(H)** Glucose dependency. Data is shown as mean + SD from min. one experiment. Symbols represent single mice; symbol shapes indicate different experiments. Unpaired t test (B), (D), (E), (H) or Mann-Whitney test (C), (G) were performed for statistical analysis. * P < 0.05; ** P < 0.01; *** P < 0.001, **** P < 0.0001.

of metabolism-related genes (**Fig. 27F**). This analysis revealed that lack of IL-18R signalling led to a shift from carbohydrate metabolism (**Fig. 27F, bottom**) towards fatty acid (**Fig. 27F, middle**) and sulphur metabolism (**Fig. 27F, top**), at least on the transcriptional level.

To link the gene expression data to metabolic function, I performed analysis of cellular metabolism *ex vivo* using the flowcytometry-based SCENITH method. Neither glucose (**Fig. 27G**) nor mitochondrial dependency (**Fig. 27H**) was changed in the absence of IL-18R.

Taken together, these results demonstrated that IL-18R signalling was essential for an efficient killing of internalized *L. longbeachae* by neutrophils involving NADPH-dependent ROS and potentially NK cell and/ or T cell-derived IFN- γ .

4.3. Discussion

Using *Il18r^{-/-}* mice, I recapitulated and expanded previous results from our laboratory (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020) showing that defence against *L. longbeachae* required IL-18R signalling. Both, ROS-dependent bacterial killing by neutrophils and IFN- γ levels were impaired under IL-18R deficiency. Furthermore, my results indicated the classical canonical pathway of IL-18-dependent defence to protect against *L. longbeachae* in which IL-18R stimulation on NK cells and/ or T cells induces IFN- γ required for downstream microbiocidal activity of myeloid cells. The expression of the IL-18R on a high number of ciliated bronchiolar epithelial cells, on the other hand, did not confer protection against *L. longbeachae*. The results presented in this chapter are graphically summarized in Figure 28.

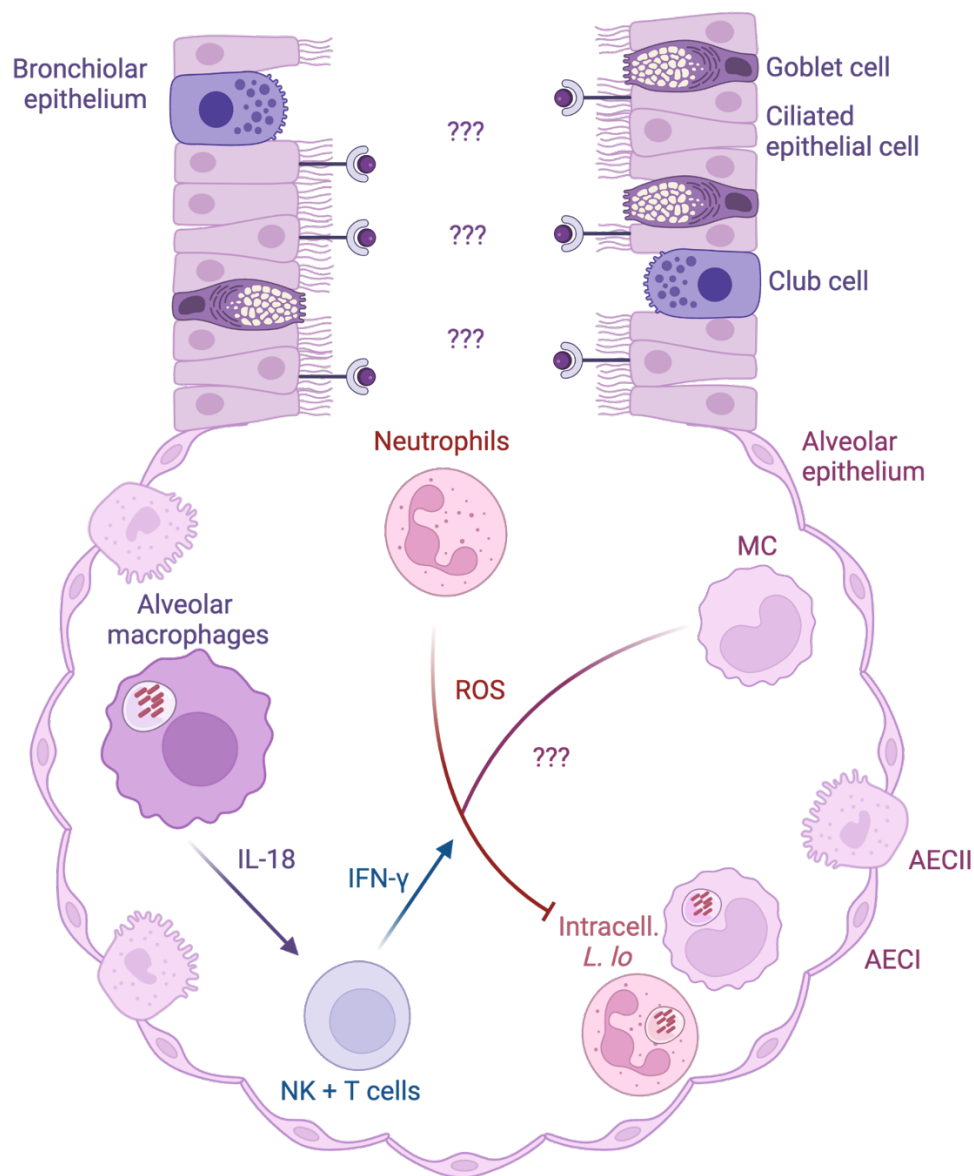


Figure 28: Graphical summary of the current hypothesis towards the IL-18-dependent defence against *L. longbeachae* involving IL-18R⁺ NK cell and/ or T cell-derived IFN-γ that instructing protective ROS production by neutrophils.

Alveolar macrophages as the apparent replicative niche of *L. longbeachae* were hypothesized to produce protective IL-18 which, as described in the classical pathway of IL-18-dependent protection, might stimulate IFN-γ from protective IL-18R⁺ NK cells and/ or T cells. IFN-γ might then promote protective, IL-18-dependent ROS from neutrophils. The high number of IL-18R⁺ ciliated epithelial cells in the bronchiolar wall were not able to confer protection against *L. longbeachae* and their function remains elusive. AEC = alveolar epithelial cells, ROS = reactive oxygen species.

IL-18 expression is usually initiated via assembly of the multi-protein inflammasome complex and it has been shown for *L. pneumophila* that intracellular detection of its flagellum via the NAIP5/ NLRC4 multimer consisting

of one NAIP5 protein interacting with multiple NLRC4 monomers. Flagellum detection induces caspase-1 activation resulting in production and secretion of mature IL-18 by macrophages (Case, Shin & Roy 2009). During *L. pneumophila* infection, induction of a protective NLRC4-dependent caspase-1 activation restricts bacterial replication *in vivo* (Case, Shin & Roy 2009; Ataide et al. 2023). Although it was not formally proven, the authors of this study suggested that NLRC4/ caspase-1-dependent IL-18 was responsible for limiting bacterial replication (Case, Shin & Roy 2009). It may be as well possible that NLRC4 and caspase-1, in addition to IL-18, induce production and secretion of IL-1 β which confers protection against *L. pneumophila* (Ziltener, Reinheckel & Oxenius 2016; Mascarenhas et al. 2015; Copenhaver et al. 2015; LeibundGut-Landmann et al. 2011). *L. longbeachae* lacks a flagellum (Cazalet et al. 2010) and, accordingly, does not to induce NLRC4 assembly (Pereira, Marques, et al. 2011). Therefore, the underlying mechanism of IL-18 secretion during infection with this bacterium needs to be further elucidated.

Initial discovery of IL-18 was due to its strong IFN- γ -inducing activity (Nakanishi et al. 2001a; Boraschi et al. 2018) and defence against both, *L. longbeachae* and *L. pneumophila*, requires IFN- γ (Brown et al. 2016; Massis et al. 2017). The results presented here demonstrate that the production of IFN- γ during *L. longbeachae*-infection required IL-18R signalling. Even though, *L. pneumophila* infection induces IL-18 expression (Brieland et al. 2000; Spörri et al. 2008) and lack of IL-18 reduces IFN- γ levels (Brown et al. 2016), the protection against *L. pneumophila* rather requires IL-12 (Brown et al. 2016; Brieland et al. 2000). The higher virulence of *L. longbeachae* compared to *L. pneumophila* (Massis et al. 2017) may explain why infection with *L. longbeachae* apparently required IL-18 as an additional booster for sufficient IFN- γ levels.

By inducing IFN- γ , IL-18 is known to indirectly enhance effector functions of myeloid cells (Ellis & Beaman 2004). As I showed in the previous chapter that neutrophils are the main players in protection against *L. longbeachae*, I investigated how IL-18 affected anti-*L. longbeachae* defence mechanisms in neutrophils. My results showed that ROS production was required to reduce *L. longbeachae* burden. Furthermore, IL-18R signalling promoted ROS

production in neutrophils identifying a mechanism by which IL-18R signalling enhanced killing of *L. longbeachae* by neutrophils. My results indicated that ROS production in neutrophils during *L. longbeachae* may be induced via the IL-18/IFN- γ -axis. Nevertheless, the involvement of IFN- γ needs to be formally elucidated. Regarding *L. pneumophila* it was demonstrated that protective ROS production in neutrophils (Ziltener, Reinheckel & Oxenius 2016) is independent of IFN- γ (Brown et al. 2016).

In addition to the indirect enhancement of neutrophil effector functions via IFN- γ , there have been reports showing that IL-18 can directly influence neutrophils (Leung et al. 2001; Hirata et al. 2008). These studies consistently showed that peripheral blood neutrophils express the IL-18R β (Leung et al. 2001; Hirata et al. 2008). Regarding the IL-18R1, results are conflicting, either showing that neutrophils constitutively express it (Leung et al. 2001) or that its expression is induced by stimulation with IL-18 (Hirata et al. 2008). Although IL-18R expression data on neutrophils is somewhat unclear, it has been reported that IL-18 directly activates neutrophils by stimulating expression of CD11b and boosting phagocytic capacity as well as release of granules and production of ROS (Leung et al. 2001; Kinoshita et al. 2011). Furthermore, IL-18 promotes neutrophil responses in general by inducing accumulation of neutrophils *in vivo* (Leung et al. 2001; Netea et al. 2000) and reducing neutrophil apoptosis (Hirata et al. 2008; Akhtar et al. 2011). The IL-18-mediated induction of TNF α by neutrophils (Leung et al. 2001) may further stimulate microbiocidal activity of neutrophils (Nathan 1989; Wiemer et al. 2010; Roilides et al. 1998; Rainard et al. 2000). The concrete mechanism of IL-18R-instructed *L. longbeachae* killing remains to be further elucidated. The role of IL-18-dependent cytokine release from neutrophils, e.g. TNF α , could be investigated performing cytokine rescue experiments in *Il18r^{-/-}* mice.

The cellular changes in neutrophils to enhance their bacterial killing capacity downstream of IL-18R signalling may be targeting their cellular metabolism. Regarding T cells and macrophages extensive studies showed the influence of the cellular metabolism on their functions (Geltink, Kyle & Pearce 2018; Umar et al. 2021). Metabolism in neutrophils has not been studied extensively yet.

Nevertheless, there are studies showing that glycolysis promotes NADPH expression which in turn is required for efficient production of ROS (Kumar & Dikshit 2019). A recent study showed that activation of aerobic glycolysis in human blood neutrophils following uptake of *L. pneumophila* and secretion of its bacterial effector LamA led to production of ROS and fusion of azurophilic granules with the *Legionella*-containing phagosome, resulting in rapid killing of internalized bacteria (Price et al. 2021). During infection with *L. pneumophila*, cellular metabolism, furthermore, controls antibacterial mechanisms in monocytes (Liu et al. 2020). Mechanistically, GM-CSF stimulated aerobic glycolysis in infiltrating monocytes leading to enhanced secretion of protective immune mediators such as $\text{TNF}\alpha$ and IL-12 which are required for bacterial clearance. My results using the SCENITH method indicated that neutrophil metabolism might not be altered under lack of IL-18R. However, the SCENITH method quantifies protein production rather than directly examining metabolic activity (Argüello et al. 2020). As *L. longbeachae* was shown by our laboratory to translocate effector proteins into neutrophils (V. Scheiding, doctoral thesis, Bonn/Melbourne, 2020) it may be the case that the presence of these intracellular bacteria influences translation and protein production. Therefore, the SCENITH method may not lead to a reliable readout in this setting. Taken together with the fact that RNAsequencing of neutrophils revealed IL-18R deficiency led to a shift from genes involved in carbohydrate metabolism towards fatty acid and sulphur metabolism other techniques, like Seahorse analysis, may need be performed to further investigate the involvement of IL-18R on neutrophil metabolism.

One of the aims of this chapter was to test a role of IL-18R⁺ ciEpC on *L. longbeachae*. Previous results from our laboratory suggested the existence of a novel alternative pathway of IL-18-dependent antibacterial protection (V. Scheiding, doctoral thesis, Bonn/Melbourne 2020). Using IL-18R tdTomato reporter mice, we identified ciEpC of the bronchiolar wall as the main expressors of the IL-18R in the stromal compartment of the lung. Based on this finding and the bone marrow chimera data we hypothesised that IL-18R⁺ ciEpC might confer protection against *L. longbeachae* either by instructing epithelial cell-autonomous mechanisms or by enhancing microbiocidal functions of myeloid cells. Specific

deletion of the IL-18R in bronchiolar epithelial cells was achieved by crossing IL-18R^{fl/fl} mice to *Nkx2.1-Cre* mice (Li et al. 2011). The well-established *vav-Cre* recombinase mouse line was used to delete IL-18R expression specifically from immune cells (Ogilvy et al. 1999). Contrary to the results using BM chimeras, infection experiments in these mouse lines with genetic deletion of the IL-18R in specific cell types apparently demonstrated that IL-18R expression on ciEpC did not confer protection against pulmonary infection with *L. longbeachae*. It is unclear why results obtained from BM chimeras and the Cre-recombinase-mediated IL-18R deletion are different. Bone marrow chimera systems have several limitations that may explain the conflicting results. These include the inefficient depletion of tissue resident cells, a decreased homing capacity of CD45.1 compared to CD45.2 (Waterstrat et al. 2010), or a potential influence of the irradiation on the general wellbeing and immune response of the mice (Ferreira et al. 2019). Therefore, genetic mouse models are considered to be more reliable than bone marrow chimeras. Nevertheless, the role of the IL-18R on ciEpC in the bronchiolar wall remains elusive. This question might be elucidated in the future using the efficient cell-specific deletion of the IL-18R in those cells using the EpC^{ΔIL18R} mice similar to studies already performed in the intestine (Jarret et al. 2020). Furthermore, these mice could be used to investigate the role of IL-18R⁺ ciEpC during pulmonary viral infections. These cells might, for example, support the anti-viral defense against Influenza A beyond the IL-18-dependent activation of a strong Th1 response (Yasuda, Nakanishi & Tsutsui 2019).

It is possible that IL-18R expression on ciEpC modulates activities of that cell type as well as neighbouring bronchiolar epithelial cells or even immune cells, even if it was not crucial for the clearance of *L. longbeachae*. Therefore, the potential function of the IL-18R on ciEpC for anti-bacterial defence was investigated. I focused on mucociliary clearance and production of AMP as the main epithelial cell-autonomous defence mechanisms (Leiva-Juárez, Kolls & Evans 2018) as well as a potential cross talk with the immune system.

As production of AMP by intestinal club cells requires IL-18 (Jarret et al. 2020), I aimed to investigate if a similar mechanism occurs in the murine lung. AMP were

upregulated in the lung during infection but expression of the investigated peptides was not dependent on IL-18. Nevertheless, a more unbiased approach like RNAseq might uncover a role of AMP not investigated here. In addition, *in vitro* techniques could eliminate potential contamination by AMP-producing immune cells to reduce the investigated AMP expression even more specifically on epithelial cells.

During my project, I established ALI cultures and organoids to study fully differentiated human bronchiolar epithelial cells *in vitro*. Using these as well as *ex vivo* murine lung slices, I measured the CBF. Both used systems were suitable to investigate the CBF as the quantified frequency was in the physiological range described for human (Oltean et al. 2018) and murine (Delmotte & Sanderson 2006) ciEpC. The CBF of ciEpC was not influenced by IL-18.

A third epithelial cell-autonomous mechanism that could potentially be influenced by IL-18 is the production of mucus by goblet cells. In the intestine the antibacterial mucus protein Muc2 is upregulated dependent on IL-18 (Engler et al. 2015). Furthermore, infection with *L. pneumophila* does increase Muc5AC expression (Morinaga et al. 2012). Consistent with these results, I showed an upregulation of not only Muc5AC but also, and even more strongly, Muc5B. This upregulation was independent of IL-18. The mechanism by which mucus production is upregulated during *L. longbeachae*-infection and whether the secretion is protective needs to be further elucidated, potentially using specific knockout mice. The use of *Muc5b*^{-/-} mice revealed that Muc5B is required not only for mucociliary clearance of bacterial pathogens but also for correct macrophage function (Roy et al. 2014). In this study, *Muc5b*^{-/-} led to increased macrophage apoptosis as well as impaired phagocytosis.

During the investigation of a potential IL-18R⁺ ciEpC/ immune cell crosstalk, a potential candidate cytokine was Ccl2. At the mRNA level, *Ccl2* expression by ciEpC was dependent on IL-18 and neutrophils expressed its according receptor, *Ccr2*. However, it has to be taken into account that, as explained before, splitting of the infected lung for different analyses might induce intra-group variations that could influence the results of especially sensitive assays like gene expression analysis. Therefore, the RNAsequencing results has to be taken with caution and

for final conclusions the experiment should be repeated using the whole lung. Nevertheless, the role of CCL2 in a potential IL-18R⁺ ciEpC/ immune cell crosstalk was further analysed. As explained before, Ccl2 is mainly known as a chemotactic agent for monocytes (Shi & Pamer 2011). Nevertheless, neutrophils were shown to express the Ccr2 during chronic inflammation (Johnston et al. 1999). Furthermore, a functional study showed Ccl2 to enhanced neutrophil functions. In this study, Ccl2 was able increase ROS production by neutrophils and their survival by counteracting apoptosis (Gschwandtner, Derler & Midwood 2019). Therefore, I investigated the possibility that Ccl2 might be able rescue the impaired antibacterial defence of *Il18r^{-/-}* mice or whether blockage of Ccl2 in WT mice might mimic the phenotype of IL-18R deficiency. My results showed that this was not the case indicating that Ccl2 was not involved in IL-18-dependent defence against *L. longbeachae*. Therefore, Ccl2 is most likely not responsible for onset of bacterial killing by neutrophils during infection with *L. longbeachae*. Nevertheless, before making a final conclusion, the experiment should be repeated with additional controls. (1) Instead of treating the WT control group with PBS it should be administered with an isotype control for the anti-CCL2 mAb to exclude an unspecific antibody-mediated effect. (2) Functionality controls for both the administered cytokine CCL2 as well as the anti-CCL2 mAb should be included to demonstrate that the lack of an effect of both on the IL-18-dependent anti-*L. longbeachae* defense does not result from a lack of *in vivo* functionality of both substances.

The crosstalk between bronchiolar epithelial cells and neutrophils was further investigated using HBEC grown in the established ALI culture system. The results presented here suggest that there is no IL-18-induced crosstalk between the HBEC and human blood neutrophils impacting the bactericidal activity of the latter. Nevertheless, the experiment combining organotypically grown HBEC and human neutrophils should be repeated and, in addition, be performed using HBEC cultured as organoids to confirm the results. The mechanism behind the IL-18-independent improvement of the bactericidal function of neutrophils treated with medium derived from ALI cultures can be further investigated using the established ALI or organoid cultures.

Taken together my results indicated that the IL-18-dependent defence against *L. longbeachae* involved IL-18R signalling on immune cells, most likely protective NK cells and/ or T cells, while the high number of IL-18R⁺ ciEpC were not able to confer protection. The established genetic mouse models to limit IL-18R expression to either immune or pulmonary epithelial cells will enable further investigations into the role of IL-18R⁺ ciEpC.

Chapter 5: The role of ER stress for host innate immunity towards *Legionella*

5.1. Introduction

Within the IRTG2168 I spent six months in the laboratory of Prof. Elizabeth Hartland at the Hudson Institute of Medical Research in Melbourne. Due to time constraints, some results presented in this Chapter have not been repeated for statistical validation and must be considered preliminary. Any final conclusions can be drawn only after further biological repetition to complete the experiments.

During my time in Melbourne, I aimed to gain a better understanding of macrophage-intrinsic immunity during *Legionella* infection. After *L. pneumophila* enters the lung via infectious aerosols the bacteria are encountered by AM as the first line of pulmonary defence. The bacteria are phagocytosed by AM (Isberg, O'Connor & Heidtman 2009; Newton et al. 2010; Mondino et al. 2020) but, after phagocytosis, use the bacterial Dot/ Icm T4SS to inject virulence factors, termed effector proteins, into the host cytoplasm. These secreted effectors then hijack various cellular processes within the macrophage host to evade phagolysosomal fusion and establish the LCV.

In addition to its phagocytic functions, AM adopt a pro-inflammatory phenotype during bacterial infection that enhances cytokine and chemokine production and, thereby, the recruitment and activation of bystander immune cells (Zhu et al. 2021; Garbi & Lambrecht 2017). A functional T4SS is required to enhance cytokine secretion in response to *L. pneumophila* (Shin et al. 2008; Fontana et al. 2011) suggesting that the host immunity is partially mediated by the virulence activities of secreted effectors, described in the concept of effector-mediated immunity (EMI) (Ngwaga, Chauhan & Shames 2021). However, *L. pneumophila* was also demonstrated to transiently inhibit pro-inflammatory cytokine secretion from AM by blocking host translation required for protein synthesis and secretion (Copenhaver et al. 2015). This blockade of host translation is mediated by a subset of secreted Dot/ Icm effectors (Belyi et al. 2006).

During infection, *L. pneumophila* effector proteins hijack the secretory pathway and recruit ER-derived vesicles to the LCV which acquires ER-like features (Tilney et al. 2001; Robinson & Roy 2006; Kagan & Roy 2002). This process causes ER stress and stimulates the UPR (Treacy-Abarca & Mukherjee 2015; Hempstead & Isberg 2015). The UPR is initiated upon accumulation of un- or misfolded proteins inside the ER which activates ER membrane-located UPR sensors, namely IRE1 (Tirasophon, Welihinda & Kaufman 1998), PERK (Harding, Zhang & Ron 1999), and ATF6 (Haze et al. 1999). IRE1, PERK, and ATF6 drive transcriptional programs to enhance ER protein-folding capacity as well as post-transcriptional programs to limit the number of newly synthesized proteins entering the ER (Di Conza & Ho 2020; Celli & Tsolis 2015; Alshareef, Hartland & McCaffrey 2021). In the case of unresolved, prolonged, or severe ER stress, the UPR will induce apoptosis (Di Conza & Ho 2020; Celli & Tsolis 2015; Alshareef, Hartland & McCaffrey 2021).

L. pneumophila infection activates the host UPR and increases PERK- and ATF6-dependent transcription of UPR-related factors, including BiP and CHOP (Treacy-Abarca & Mukherjee 2015). However, the general translation inhibition induced by secreted *L. pneumophila* effectors limits production of ER stress factors and therefore blocks downstream functions of the UPR (Treacy-Abarca & Mukherjee 2015). In addition, two studies revealed that *L. pneumophila* infection inhibits IRE1-dependent splicing of XBP1 mRNA and, therefore, production of the XBP1s transcription factor when challenged with the pharmacological ER stress inducer thapsigargin (Treacy-Abarca & Mukherjee 2015; Hempstead & Isberg 2015). This inhibition was dependent on Dot/ Icm effector proteins that block host translation, suggesting that translational inhibition is sufficient to limit UPR signalling caused by ER stress during *L. pneumophila* infection (Treacy-Abarca & Mukherjee 2015; Hempstead & Isberg 2015).

Although the primary function of the UPR is to reinstate ER homeostasis, ER stress can induce an innate immune response (Alshareef, Hartland & McCaffrey 2021). For example, during infection with the intracellular pathogen *Fransicella tularensis*, activation of XBP1s was crucial for the host macrophage to restrict intracellular bacterial replication by enhancing pro-inflammatory cytokine

production (Martinon et al. 2010; Pham et al. 2020). However in some cases, ER stress can enhance bacterial replication. *Brucella* and *Chlamydia* spp. are two intracellular bacteria that activate the UPR during infection and require IRE1 activity to form ER-derived replication vacuoles (Pandey et al. 2018; Qin et al. 2008; George et al. 2017). At the same time, IRE1-dependent pro-inflammatory signalling via NOD1/ NOD2 paradoxically restricts *Brucella* and *Chlamydia* replication within host cells (de Jong et al. 2013; Kestra-Gounder et al. 2016), illustrating how the UPR can have both pathogenic and protective functions during bacterial infection.

Although *L. pneumophila* inhibits UPR signalling during infection, the relevance of the UPR for intracellular bacterial replication or cytokine secretion during infection had not been elucidated. To investigate the role of the UPR in *L. pneumophila* infection, our laboratory used the pharmacological ER stress inducer thapsigargin. Pre-treatment of macrophages with thapsigargin restricted intracellular but not extracellular *L. pneumophila* replication indicating that the compound lacks intrinsic anti-microbial activity (M. Alshareef, doctoral thesis, Melbourne, in preparation). Furthermore, thapsigargin was able to improve host cell viability demonstrating that the restriction of bacterial replication was not due to terminal UPR-driven apoptosis and reduced bacterial CFU not due to host cell death (M. Alshareef, doctoral thesis, Melbourne, in preparation). The impact of ER stress on the host cytokine response, however, remained to be determined.

A number of pro-inflammatory cytokines have been implicated in host immunity against *L. pneumophila*. AM, despite the strong *L. pneumophila*-induced translational block, are able to secrete IL-1 α and IL-1 β in response to *L. pneumophila* recognition via TLR5, NLRC4, or EMI (Brown et al. 2017). AM-derived IL-1 α is critical for inducing efficient recruitment of neutrophils to the infection site (Barry et al. 2013) which secrete TNF α (Ziltener, Reinheckel & Oxenius 2016; Brown et al. 2016; Casson et al. 2017) to enhance the bactericidal functions of AM (Ziltener, Reinheckel & Oxenius 2016) and recruit monocytes (Skerrett et al. 1997). IL-1 β further stimulates the production of TNF α from uninfected bystander leukocytes (Fontana et al. 2011; Asrat, Dugan & Isberg 2014; Ivanov & Roy 2013; Copenhaver et al. 2015) while IL-1 α drives production

of GM-CSF from AECII which is required for the protective function of MC (Liu et al. 2020). The key protective cytokine during defence against *L. pneumophila*, however, is IFN- γ which limits bacterial replication within macrophages *in vitro* by stimulating the expression of anti-bacterial ISG like *IRG1* (Price et al. 2019; Naujoks et al. 2016). Although also required for anti-*L. pneumophila* defence *in vivo*, IFN- γ does not improve the microbiocidal capacity of AM during *in vivo* infection but its secretion is essential for efficient bactericidal activity of MC (Brown et al. 2016). Next to stimulating direct effector functions of phagocytes, IFN- γ can also drive additional pro-inflammatory cytokines, including CXCL10 (Farber 1990; Luster, Unkeless & Ravetch 1985). CXCL10 is best known to recruit T lymphocytes to the infection site (Taub et al. 1993; Lloyd et al. 1996) but has also been implicated into monocyte recruitment (Taub, Longo & Murphy 1996). The role of CXCL10 during the immune response towards *L. pneumophila* has not been elucidated so far but given the strong protective role of monocytes (Ataide et al. 2023) it might very well positively influence the outcome of the immune response during *L. pneumophila* infection.

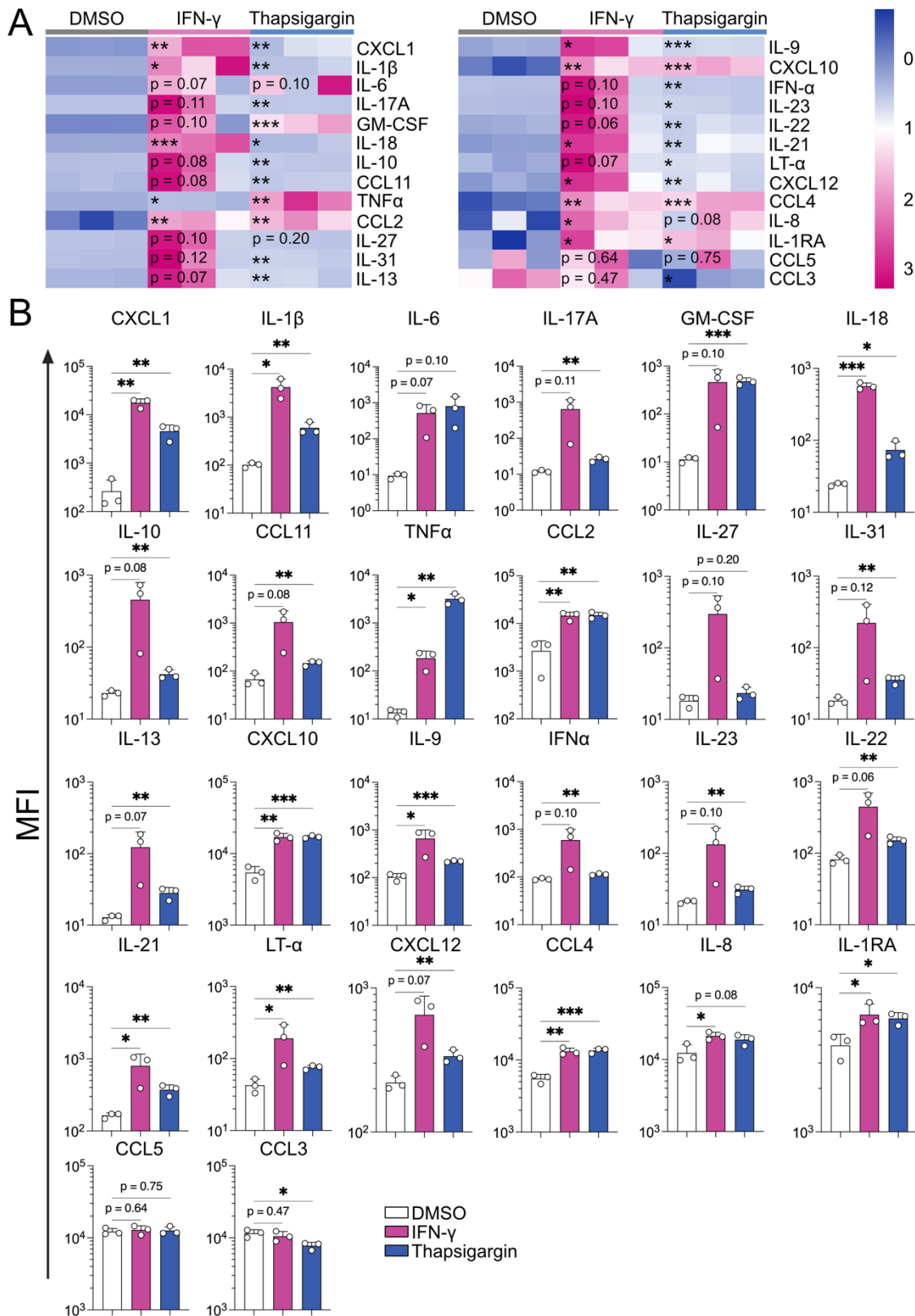
The previous data from our laboratory strongly indicated that thapsigargin-induced ER stress initiated a protective host response to limit *L. pneumophila* replication. The aim of this chapter was to examine the relevance of the UPR for macrophage-intrinsic immunity and inflammatory responses during *L. pneumophila* infection. Specifically, we sought to investigate:

- (1) Macrophage innate immune responses during ER stress and the role of the UPR in regulating inflammatory cytokine expression.
- (2) Macrophage innate immune responses to *L. pneumophila* infection and how these responses may be regulated by the UPR.

5.2. Results

5.2.1. ER stress drives pro-inflammatory cytokine production in human macrophages

The relevance of the UPR for host cytokine responses to *L. pneumophila* infection has not been elucidated. Since IFN- γ is a key determinant in *L. pneumophila* clearance in macrophages (Gebran et al. 1994), we compared thapsigargin-induced cytokine responses in PMA-differentiated THP-1 macrophages with either IFN- γ or the DMSO control. The RPMI medium control was not different from the DMSO control (data not shown) and is, therefore, not shown for simplicity. Cell culture supernatants were analysed using a broad range, magnetic bead-based cytokine array (ProcartaPlex). Of the 34 included analytes, we detected the secretion of 26 cytokines from THP-1 macrophages (**Fig. 29**). As predicted, IFN- γ treatment induced a broad cytokine response in macrophages while thapsigargin enhanced the secretion of a specific subset of pro-inflammatory cytokines compared to the DMSO control (**Fig. 29**).



Thapsigargin-induced analytes included the principal pro-inflammatory cytokines $\text{TNF}\alpha$ and $\text{IL-1}\beta$ (**Fig. 29**) which are important cytokines in the control of *L. pneumophila* replication (Ziltener, Reinheckel & Oxenius 2016; Mascarenhas et al. 2015; Copenhaver et al. 2015; LeibundGut-Landmann et al. 2011). Thapsigargin treatment also significantly increased the production of a number of chemokines including CXCL10, CCL2, and CCL4 (**Fig. 29**) that are important for monocyte recruitment to the site of infection (Matsushima et al. 1989; Taub et al. 1993; Algood, Chan & Flynn 2003). In addition, thapsigargin induced the growth factor GM-CSF (**Fig. 29**), which is known to facilitate neutrophil recruitment especially in the setting of infection (Weisbart, Golde & Gasson 1986; Yamamoto et al. 2014), as well as the classical neutrophil recruiting chemokine CXCL1 (De Filippo et al. 2013) (**Fig. 29**). CCL2 and GM-CSF have also been implicated in immune defence against *L. pneumophila* (Liu et al. 2020; Ataide et al. 2023).

Taken together, my data showed that treatment of human THP-1 macrophages with thapsigargin to induce ER stress altered the immune phenotype of macrophages towards a pro-inflammatory response. However, the increase of the ISG CXCL10 (**Fig. 29**) was especially interesting as it is classically induced downstream of the $\text{IFN-}\gamma$ receptor via the JAK/ STAT1 pathway and ER stress has not been connected to this pathway before.

Figure 29: ER stress induces a pro-inflammatory immune response in THP-1 macrophages.

PMA-differentiated THP-1 macrophages were treated with thapsigargin or $\text{IFN-}\gamma$ for 2 h. DMSO was used as a negative control. Cytokine secretion was analysed after 24 h using a broad-range magnetic bead-based cytokine array (ProcartaPlex). Shown is **(A)** relative detection (Z score) and **(B)** MFI of cytokines induced by the positive control $\text{IFN-}\gamma$ (three experiments performed in duplicate). Shown is mean + SD. Single symbols represent mean of each experiment. Unpaired t test (assuming normal distribution) was used for statistical analysis comparing $\text{IFN-}\gamma$ or thapsigargin-treatment against DMSO control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, **** $P < 0.0001$.

5.2.2. ER stress-induced CXCL10 expression requires non-canonical IRE1-induced STAT1 phosphorylation

CXCL10 was originally described as an IFN- γ -induced cytokine (Farber 1990; Luster, Unkeless & Ravetch 1985) and is now a well-known ISG. CXCL10 expression is regulated via the JAK/ STAT1 pathway downstream of IFN- γ receptor activation (Shang et al. 2022; Aota et al. 2021, 2018) (illustrated in **Fig. 30A**). To investigate the role of STAT1 in ER stress-induced CXCL10 secretion, we generated *STAT1*^{-/-} THP-1 macrophages using a CRISPR-Cas9 system. Knockout of STAT1 protein expression was validated by immunoblot analysis (**Fig. 30B**).

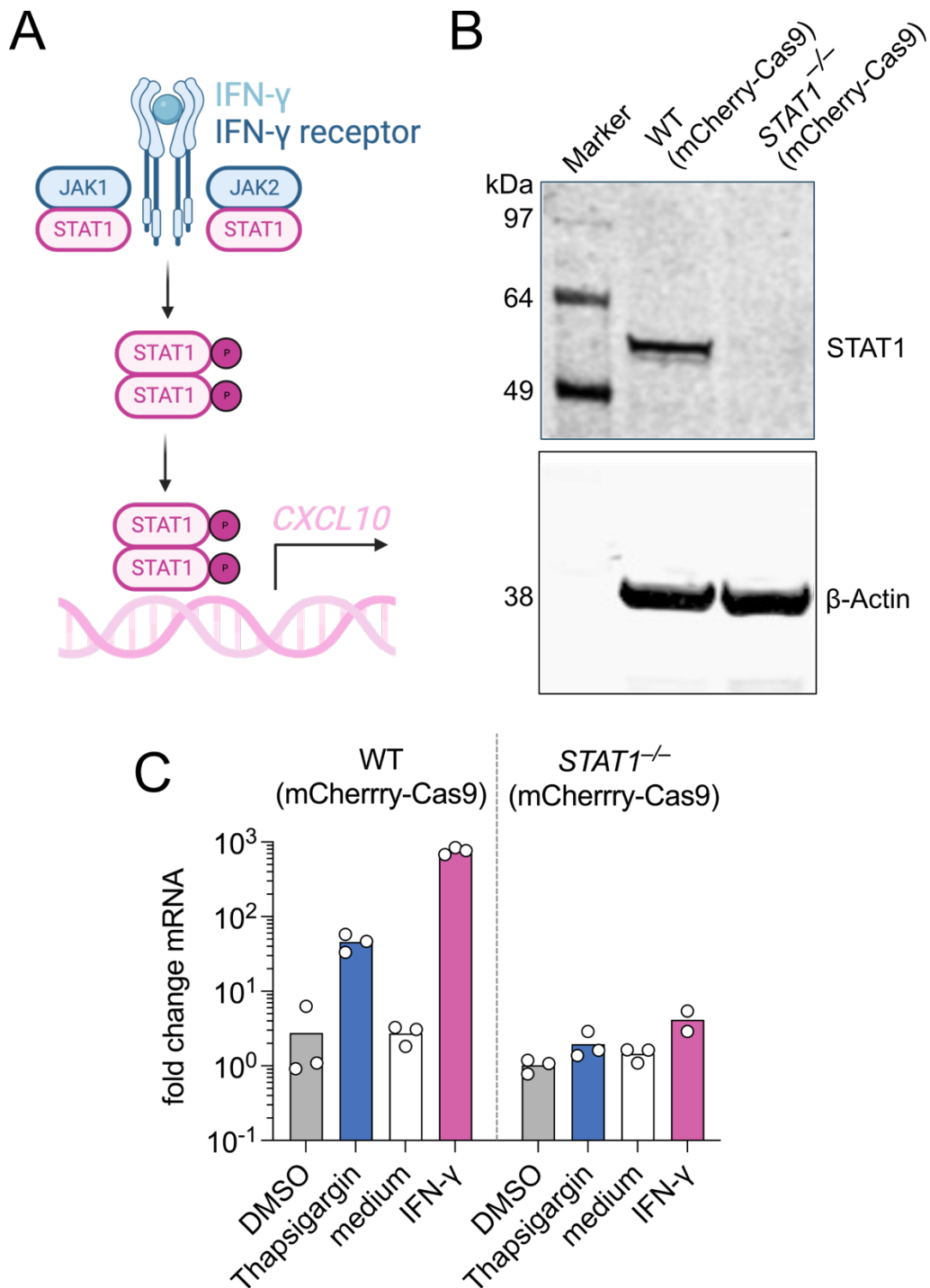


Figure 30: *STAT1*^{-/-} THP-1 macrophages do not transcribe *CXCL10* in response to thapsigargin.

Our results using *STAT1*^{-/-} THP-1 macrophages suggested that *CXCL10* transcription, induced by either IFN- γ or thapsigargin, may require the presence

of the STAT1 transcription factor (**Fig. 30C**). This suggested that STAT1 is crucial for ER stress-induced *CXCL10* expression.

To further elucidate how ER stress drives STAT1 activation, we next investigated involvement of the UPR in ER stress induced cytokine production. Thapsigargin induces ER stress by blocking the SERCA calcium ATPase which leads to passive calcium depletion from the ER and a loss of ER protein-folding homeostasis, thereby inducing the UPR (Gorman et al. 2012; Osowski 2015). Calcium depletion from the ER not only induces protein misfolding but substantially alters global calcium homeostasis in the cell. To investigate if the thapsigargin-induced pro-inflammatory phenotype of macrophages was regulated by UPR signalling, THP-1 macrophages were pre-treated with small molecule inhibitors of the three UPR sensors, IRE1, PERK, and ATF6 (Grandjean & Luke Wiseman 2020) (illustrated in **Fig. 31A**) prior to stimulation with thapsigargin. The expression of a selected set of cytokines was analysed in a fluorescent bead-based array (CBA Flex Set). Although time constraints allowed only one experimental repeat, the use of this array suggested the pro-inflammatory cytokine response (**Fig. 31B**) observed in the original magnetic bead-based array (**Fig. 29**) was reproducible using a different technical approach.

Figure 30: STAT1^{-/-} THP-1 macrophages do not transcribe *CXCL10* in response to thapsigargin.

(A) Schematic diagram of the canonical IFN- γ / JAK-dependent activation of STAT1 and the downstream activation of *CXCL10* transcription. **(B)** Validation of the STAT1 knockout from cell lysates by SDS page and immunoblot using STAT1- and β -Actin-specific antibodies. **(C)** PMA-differentiated THP-1 macrophages were treated with Thapsigargin or IFN- γ for 2 h. DMSO was used as a negative control for thapsigargin, RPMI medium for IFN- γ . *CXCL10* mRNA expression was analysed after 24 h using qPCR. Shown is the relative expression of *CXCL10* to the housekeeping gene *ACTB* (one experiment performed in triplicates). Single symbols represent replicates.

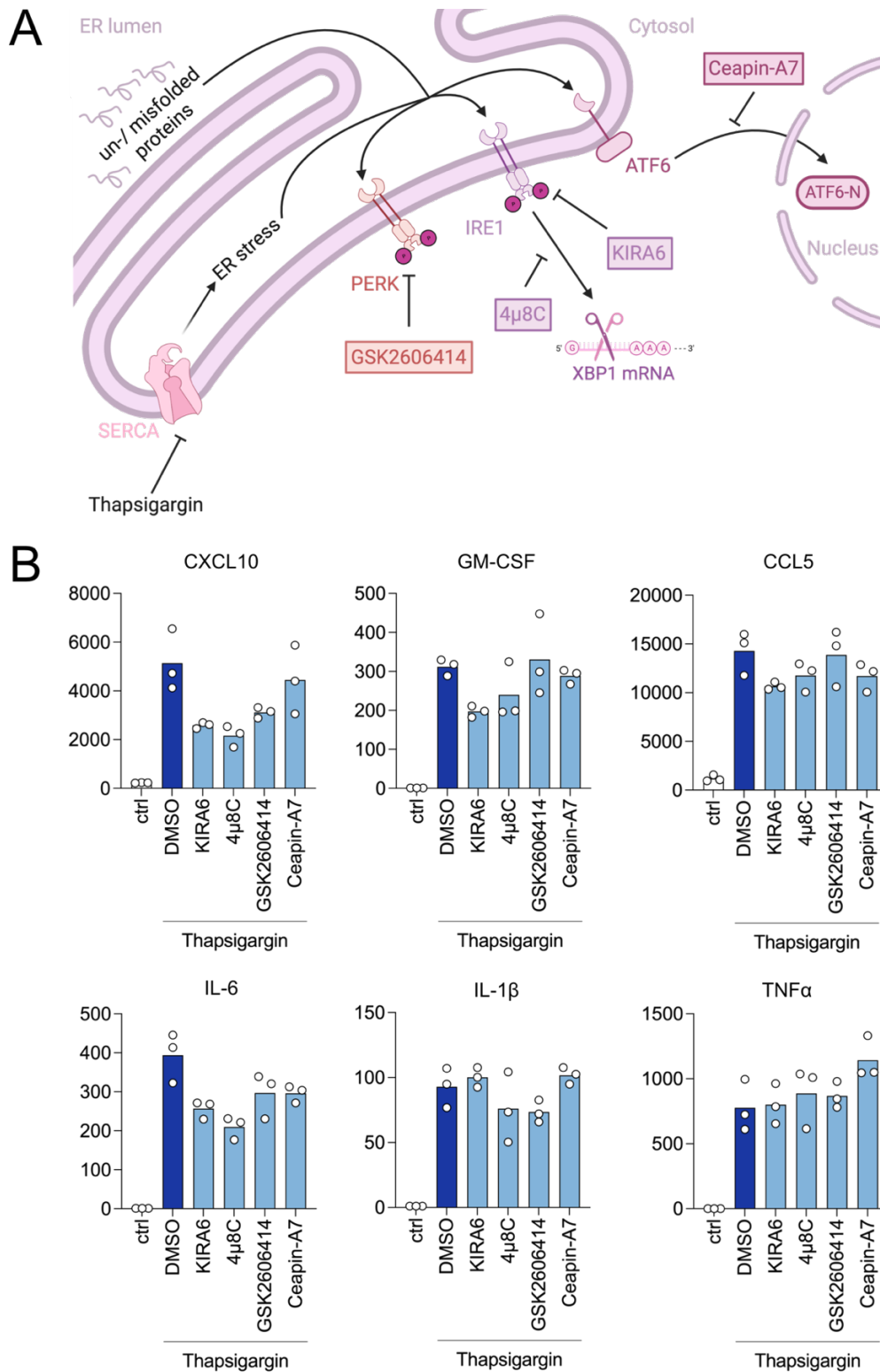


Figure 31: Upregulation of CXCL10 and other pro-inflammatory cytokines by thapsigargin is regulated by activation of the UPR sensor IRE1.

Pre-treatment with UPR inhibitors suggested that thapsigargin-induced CXCL10 secretion may be reduced by inhibition of IRE1 either using KIRA6 or

4 μ 8C as well as by inhibition of PERK using GSK2606414 (**Fig. 31B**). GM-CSF and CCL5 expression by THP-1 macrophages experiencing ER stress could also be reduced by pre-treatment with IRE1-specific inhibitors (**Fig. 31B**). However, none of the UPR inhibitors seemed to block thapsigargin-induced secretion of the key pro-inflammatory cytokine TNF α (**Fig. 31B**). Other cytokines were to variable to speculate.

Overall, these preliminary results suggested that CXCL10 induction during ER stress may require activation of IRE1. It was therefore of interest to investigate whether thapsigargin activated the STAT1 transcription factor via IRE1-induced phosphorylation (**Fig. 32A**). Phosphorylation of STAT1 on Tyrosine701 (Y701) initiates dimerization and translocation to the nucleus where the STAT1 dimer induces ISG expression (Ramana et al. 2000). Y701 phosphorylation can be detected by immunoblot using a pSTAT1 (Y701-phosphorylated STAT1)-specific antibody. Using this method on THP-1 macrophages treated with thapsigargin, we detected a level of Y701 phosphorylation of STAT1 comparable to IFN- γ treatment (**Fig. 32B, left panel**).

Figure 31: Upregulation of CXCL10 and other pro-inflammatory cytokines by thapsigargin is regulated by activation of the UPR sensor IRE1.

PMA-differentiated THP-1 macrophages were treated with UPR inhibitors for 2 h prior to treatment with thapsigargin for 2 h. DMSO was used as a negative control for both treatments. Cytokine secretion was analysed after 24 h using a fluorescent bead-based cytokine array (CBA Flex Set). **(A)** Schematic diagram of ER stress induction by thapsigargin and the specificity of the used UPR inhibitors. **(B)** Shown is MFI of secreted cytokines (one experiment performed in triplicate). Single symbols represent replicates.

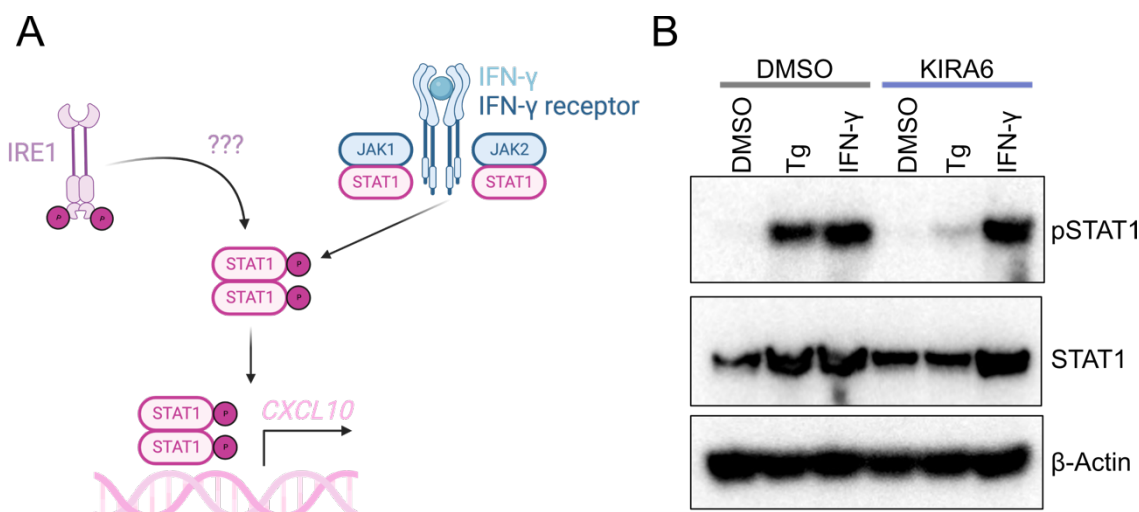


Figure 32: Thapsigargin and IFN- γ both stimulate STAT1 phosphorylation but via distinct pathways.

(A) Schematic diagram of the canonical IFN- γ R/ JAK-dependent activation of STAT1 and the downstream activation of CXCL10 transcription. Potential activation of STAT1 by IRE1 is indicated. **(B)** PMA-differentiated THP-1 macrophages were treated with KIRA6 for 2 h prior to treatment with thapsigargin or IFN- γ for 2 h. DMSO was used as a negative control for both treatments. Protein expression was analysed after 24 h by SDS page and immunoblot using STAT-1, pSTAT1- and β -Actin-specific antibodies. Shown is an exemplary blot including the expression of phosphorylated STAT1 (pSTAT1), unphosphorylated STAT1 (STAT1), and the housekeeping protein β -Actin as a loading control.

To investigate the role for IRE1 in STAT1 phosphorylation, we pre-treated macrophages with the IRE1 kinase inhibitor KIRA6. KIRA6 treatment abolished thapsigargin-induced Y701 phosphorylation while it had no influence on IFN- γ -induced phosphorylation (**Fig. 32B, right panel**).

These data suggested the existence of a novel, non-canonical pathway by which IRE1 induced STAT1 phosphorylation to stimulate transcription of STAT1-dependent genes, including CXCL10, which is, therefore, not only induced downstream of IFN- γ .

5.2.3. ER stress potentiates CXCL10 secretion from *L. pneumophila*-infected macrophages

Bacterial infections typically cause a shift in macrophages to a pro-inflammatory phenotype associated with increased anti-bacterial activity (Zhu et al. 2021; Garbi & Lambrecht 2017). Here we investigated cytokine responses in THP-1 macrophages during *L. pneumophila* infection. THP-1 macrophages were

infected with *L. pneumophila* strain 130b. The $\Delta dotA$ T4SS mutant that is unable to replicate within THP-1 macrophages (Newton et al. 2010) was included as a control as well as a $\Delta 4$ mutant strain of *L. pneumophila* which lacks four Dot/ Icm effector proteins required to inhibit host translation elongation and ER stress (Hempstead & Isberg 2015; Belyi et al. 2006; Alshareef, Hartland & McCaffrey 2021). As $\Delta 4$ was shown previously not to have a replication deficit compared to the WT strain (Hempstead & Isberg 2015; Belyi et al. 2006), it enabled us to decipher the role of effector-induced translational blockage in the *L. pneumophila*-induced cytokine response independent of differences in the bacterial load. Although time constraints allowed only one experimental repeat, analysing a selected set of cytokines using a fluorescent bead-based cytokine array (CBA Flex Set) suggested increased secretion of the chemokines IL-8 and CXCL10 but not CCL5 and IL-1 β in response to infection with $\Delta dotA$ (**Fig. 33**).

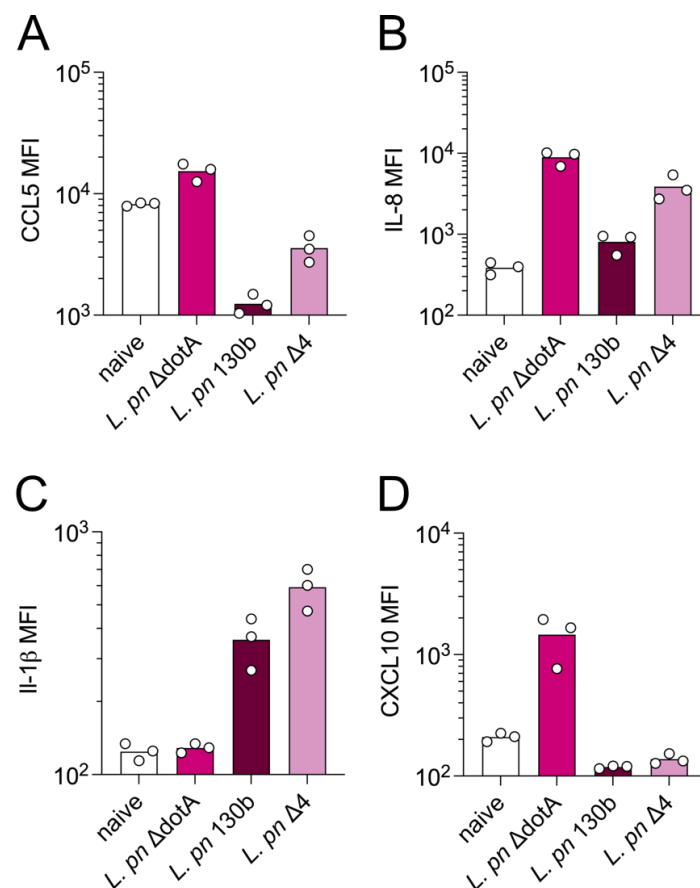


Figure 33: Secretion of pro-inflammatory cytokines during infection with *L. pneumophila* 130b and its derivatives.

In addition, compared to $\Delta dotA$, secretion of CCL5, IL-8 and CXCL10 was apparently inhibited by the *L. pneumophila* 130b WT strain (**Fig. 33A, B, D**). Comparing 130b and $\Delta 4$ mutant, we also saw a potential increase in CCL5 and IL-8 but not CXCL10. However these experiments must be repeated before drawing any final conclusions. In addition, a MTT assay should be performed to investigate the potential role of cell death in changes of cytokine levels during infection of THP-1 macrophages with *L. pneumophila* 130b and its derivatives. Nonetheless, the current data suggested that *L. pneumophila* infection may inhibit secretion of cytokines through the Dot/ Icm of effector proteins that block host translation, as described previously described (Asrat, Dugan & Isberg 2014).

Since ER stress was implicated in a significantly increased CXCL10 expression by THP-1 macrophages (**Fig. 29**), I also pre-treated THP-1 cells with thapsigargin prior to infection to investigate whether ER stress could potentiate the poor CXCL10 secretion observed in response to *L. pneumophila* infection (**Fig. 33D**). Comparison of CXCL10 secretion from *L. pneumophila*-infected macrophages either treated with thapsigargin or the vehicle control (DMSO), suggested again that CXCL10 was upregulated by ER stress during infection (**Fig. 34**).

Figure 33: Secretion of pro-inflammatory cytokines during infection with *L. pneumophila* 130b and its derivatives.

PMA-differentiated THP-1 macrophages were infected with *L. pneumophila*, a $\Delta dotA$ mutant, or $\Delta 4$ mutant at an MOI of 10 for 2 h. Cytokine secretion was analysed after 18 h using a fluorescent bead-based cytokine array (CBA Flex Set). Shown is MFI of **(A)** CCL5, **(B)** IL-8, **(C)** IL-1 β , and **(D)** CXCL10 (one experiment performed in triplicate). Single symbols represent replicates.

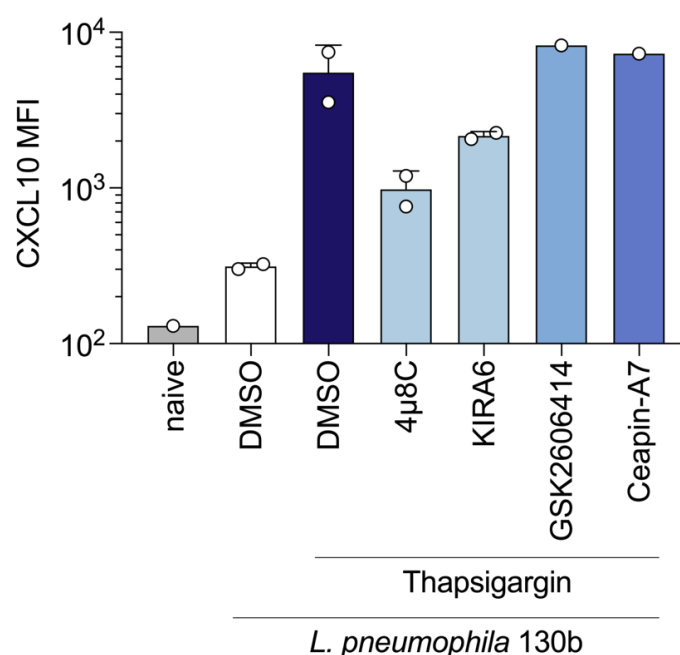


Figure 34: ER stress induces CXCL10 during *L. pneumophila* infection involving the UPR sensor IRE1.

PMA-differentiated THP-1 macrophages were treated with thapsigargin for 2 h prior to infection with *L. pneumophila* at an MOI of 1 for 2 h. THP-1 macrophages were then treated with UPR inhibitors for 18 h and cytokine secretion was analysed using a fluorescent bead-based cytokine array (CBA Flex Set). Shown is CXCL10 expression in naïve untreated and infected THP-1 macrophages treated with DMSO or thapsigargin and DMSO or UPR inhibitors. MFI of cytokines induced by the positive control IFN- γ (one or two experiments performed in duplicate). Shown is mean + SD. Single symbols represent mean of each experiment.

Next, I wanted to understand whether the increased CXCL10 production in cells experiencing ER stress was UPR-dependent as suggested during homeostasis (**Fig. 31**). Therefore, I compared CXCL10 secretion from ER stress experiencing *L. pneumophila*-infected THP-1 macrophages in the presence or absence of small molecule UPR inhibitors. Thapsigargin-induced expression of CXCL10 during infection was apparently reduced by IRE1 inhibitors KIRA6 and 4μ8C (**Fig. 34**).

These results suggested that pre-treatment with thapsigargin increased CXCL10 production from macrophages during *L. pneumophila* infection and that this may be IRE1-dependent. Due to time constraints this experiment was only performed once using all UPR inhibitors and the data would need to be replicated before drawing any final conclusions.

5.2.4. STAT1 is important for restriction of *L. pneumophila* replication within the host cell

Both IFN- γ (Gebran et al. 1994) but also thapsigargin (M. Alshareef, doctoral thesis, Melbourne, in preparation) not only induce STAT1-dependent cytokine expression in macrophages but also limit intracellular replication of *L. pneumophila*. Our data showed that ER stress activates STAT1-dependent signalling suggesting that STAT1 activation may not only account for increased production of CXCL10 but also the restriction of *L. pneumophila* replication within macrophages after thapsigargin treatment. Therefore, we analysed intracellular replication of *L. pneumophila* within *STAT1*^{-/-} THP-1 macrophages pre-treated with either thapsigargin or IFN- γ prior to infection. Even in the absence of thapsigargin or IFN- γ , the number of intracellular bacteria was apparently increased in *STAT1*^{-/-} THP-1 macrophages compared to WT cells at 48 h after infection (**Fig. 35**), suggesting that STAT1 may limit *L. pneumophila* intracellular replication.

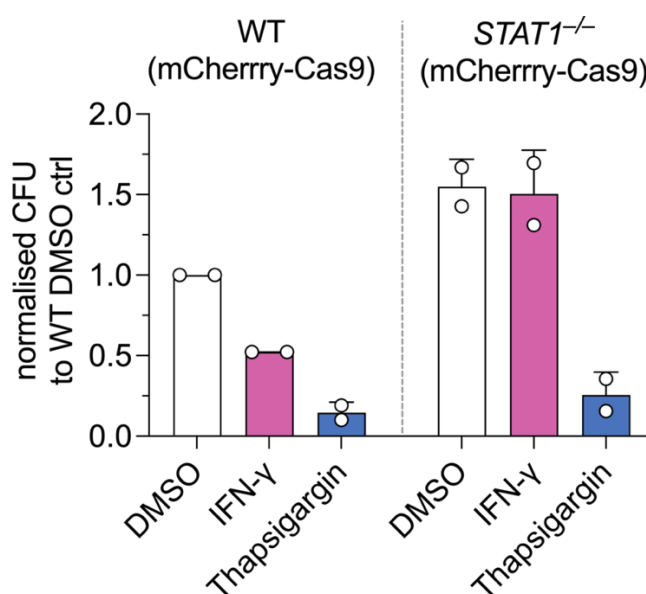


Figure 35: Efficient restriction of intracellular *L. pneumophila* replication is dependent on STAT1 expression.

PMA-differentiated WT and *STAT1*^{-/-} THP-1 macrophages were treated with IFN- γ or thapsigargin for 2 h prior to infection with *L. pneumophila* at an MOI of 1 for 2 h. DMSO was used as a negative control. Results are the mean normalized CFU (to WT DMSO control) at 48 h after infection (two experiments performed in duplicate). Shown is mean + SD. Single symbols represent mean of each experiment.

As previously reported, we observed that IFN- γ treatment appeared to restrict growth of *L. pneumophila* within WT macrophages at 48 h after infection (**Fig. 35**). IFN- γ -mediated inhibition was not observed in the *STAT1*^{-/-} macrophages, suggesting that it required STAT1. Thapsigargin pre-treatment also may reduce *L. pneumophila* replication in WT macrophages, yet the inhibitory effect of thapsigargin on *L. pneumophila* replication was still observed in *STAT1*^{-/-} macrophages (**Fig. 35**). Using phase contrast microscopy, we observed that thapsigargin but not IFN- γ was inducing high levels cell death in mCherry-Cas9-expressing THP-1 macrophages which could account for reduced bacterial burden in thapsigargin-treated cells. Since the thapsigargin treatment protocol was optimized for the non-lentiviral transformed THP-1 macrophages, it should be re-optimized here for the transformed cells. In addition, a MTT assay to quantify cell viability should be performed in parallel to exclude the influence of host cell death on bacterial replication.

Taken together, these results suggested that STAT1 played a role in restricting *L. pneumophila* replication within macrophages. However, the role of STAT1 for thapsigargin-induced protection cannot be concluded from the current results.

5.2.5. ER stress induces cytokine secretion from murine macrophages

We finally wanted to investigate whether ER stress-induced cytokine responses in macrophages were conserved between species. Murine iBMDM were, thus, treated with thapsigargin and analysed for the expression of a selected set of pro-inflammatory cytokines using a fluorescent bead-based cytokine array (CBA Flex Set). Out of the 8 analytes, I was able to detect secretion of the key pro-inflammatory cytokines IL-6, TNF α , and IL-1 β from murine iBMDM (**Fig. 36**).

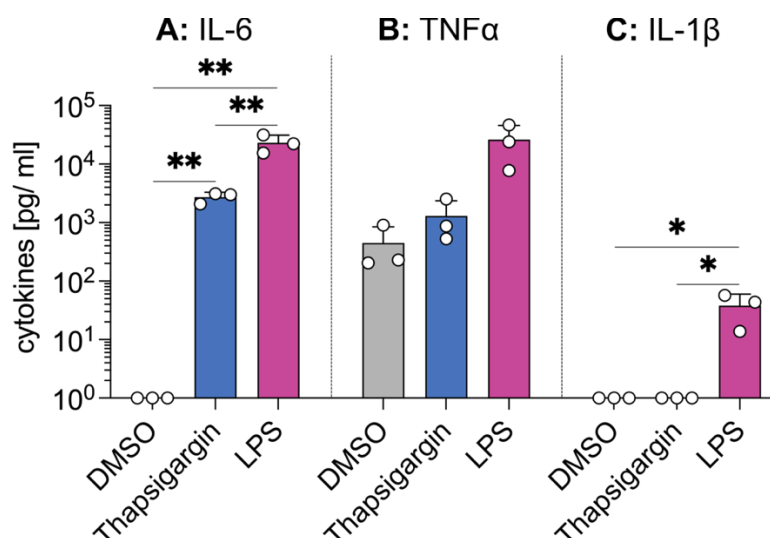


Figure 36: ER stress-experiencing murine iBMDM secrete the pleiotropic cytokine IL-6.

iBMDM were treated with thapsigargin or LPS for 2. DMSO was used as a negative control. Cytokine secretion was analysed after 24 h using a fluorescent bead-based cytokine array (CBA Flex Set). Shown is expression of (A) IL-6, (B) TNF α , and (C) IL-1 β in pg/ ml (three experiments performed in duplicate). Data is shown as mean + SD from three independent experiments. Single symbols represent mean of each experiment. Unpaired t Test was performed for statistical analysis comparing thapsigargin/ LPS treatment to DMSO control. * P < 0.05; ** P < 0.01

Of the three key pro-inflammatory cytokines detected, only IL-6 was induced by thapsigargin treatment of iBMDM (**Fig. 36A**) whereas TNF α and IL-1 β were not increased (**Fig. 36B, C**). The positive control LPS induced IL-6 and IL-1 β but not TNF α (**Fig. 36**). These data contrasted with human macrophages where IL-6 induction by thapsigargin was not significant (**Fig. 29**). A larger range of cytokines could be analysed using a broad-range, magnetic bead-based cytokine array (ProcartaPlex) to further elucidate the influence of ER stress on murine macrophages.

5.3. Discussion

Using the pharmacological ER stress inducer thapsigargin on human THP-1 macrophages, I uncovered a strong ER stress-induced pro-inflammatory immune response including secretion of TNF α , IL-1 β , and IL-18. ER stress also increased the expression of monocyte- and neutrophil-attracting chemokines such as CXCL10, CCL2, CXCL1, and GM-CSF suggesting that the UPR activates

multiple innate-immune signalling pathways that may help restrict *L. pneumophila* replication within the host. CXCL10 is typically induced via the IFN- γ R/ JAK/ STAT pathway (Shang et al. 2022; Aota et al. 2021, 2018) and the results presented here indicated that ER stress also activated the STAT1 transcription factor via a non-canonical, IRE1-dependent pathway. Using a *STAT1*^{-/-} cell line, suggested that STAT1 is involved in the transcription of *CXCL10* in response to thapsigargin, although its role for ER stress-induced restriction of bacterial replication remains to be determined. The results presented in this chapter are graphically summarized in Figure 37 and, while much of our data needs to be repeated, the current data suggested an intriguing relationship between ER stress and pro-inflammatory cytokine production.

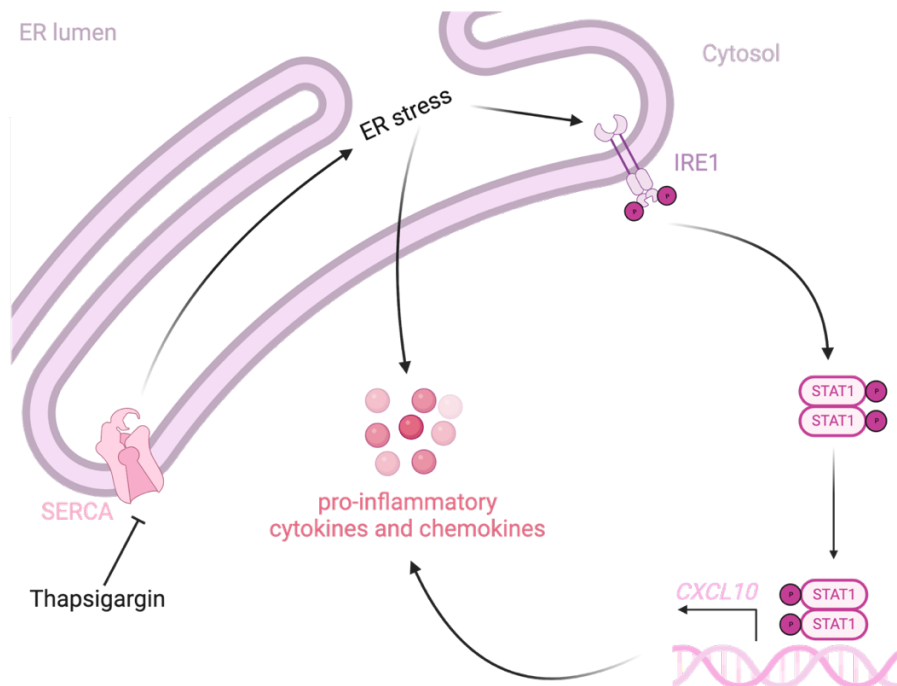


Figure 37: Graphical summary of the ER stress-induced production of pro-inflammatory cytokines particularly the non-canonical IRE1/ STAT1-dependent transcription of *CXCL10*.

Thapsigargin-induced ER stress was shown to drive secretion of a range of pro-inflammatory cytokines involving the key-proinflammatory cytokines TNF α and IL-1 β as well as the monocyte-attracting chemokines CCL2, CCL4, and CXCL10 and the neutrophil-attracting chemokines CXCL1 and GM-CSF. It was, furthermore, suggested that *CXCL10* transcription in response to ER stress is induced via a novel non-canonical activation of STAT1 involving the UPR sensor IRE1. SERCA = sarcoplasmic/endoplasmic reticulum calcium ATPase. IRE1 = inositol-requiring enzyme 1. STAT1 = signal transducer and activator of transcription 1. GAS = gamma interferon activation site.

A role for the UPR in directly regulating innate immune signalling during infection is not well understood. Using pharmacological inducers of ER stress, early studies showed that IRE1 binds TRAF2, a key element of TNF α R signalling (Urano et al. 2000; Hu et al. 2006), to activate the pro-inflammatory transcription factors AP-1 and NF- κ B. IRE1 thereby promotes the expression of TNF α and IL-6 (Li, Wang & Dorf 2009; Hu et al. 2006). PERK, on the other hand, can indirectly stimulate NF- κ B by blocking synthesis of the NF- κ B inhibitor I κ B through activation of eIF2 α (Jiang et al. 2003; Tam et al. 2012). Furthermore, ER stress can stimulate the NLRP3 inflammasome leading to IL-1 β secretion and pyroptosis (Lerner et al. 2012; Osowski et al. 2012). In agreement with these studies, I have demonstrated the upregulation of TNF α and IL-1 β in macrophages treated with thapsigargin.

Despite growing evidence of a link between ER stress and pro-inflammatory signalling, there is limited evidence that the UPR directly regulates IFN-mediated immunity (Alshareef, Hartland & McCaffrey 2021). Here we showed for the first time that ER stress induced the expression of CXCL10 in macrophages, a chemokine classically known to be involved in Th1 responses (Campbell et al. 2004; Shields et al. 1999). CXCL10 is induced both on gene and protein level by IFN- γ (Farber 1990; Luster, Unkeless & Ravetch 1985) via the well-characterized IFN- γ R/ JAK/ STAT1 pathway (Shang et al. 2022; Aota et al. 2021, 2018). Its transcription requires phosphorylation and dimerization of the transcription factor STAT1 (Liu et al. 2011). Here, we showed that thapsigargin induced non-canonical phosphorylation of STAT1 and that STAT1 phosphorylation during ER stress required IRE1 kinase activity. Since, IRE1 is a serine/ threonine kinase we propose that IRE1 indirectly drove STAT1 phosphorylation at Y701. The JAK/ STAT pathway was previously reported to be involved in ER stress-induced immune responses as JAK1 phosphorylates the PERK kinase leading to downstream phosphorylation of STAT3 and, thereby, expression of IL-6, CCL2, and CCL20 (Meares et al. 2014). Nevertheless, we present here the first evidence of the UPR directly regulating STAT1 transcriptional activity and therefore ISG expression independent of IFN- γ stimulation. Another pathway that would be

highly interesting to investigate in the context of the IRE1/ STAT1-dependent induction of CXCL10 is NOD1/ NOD2/ RIP2. This pathway has been proposed to play a pivotal role in both ER stress-induced immune protection (Pham et al. 2020; Keestra-Gounder et al. 2016) and anti-*L. pneumophila* defense (Shin et al. 2008).

IFN- γ treatment of macrophages prior to infection with *L. pneumophila* has been demonstrated to limit bacterial replication within macrophages (Gebran et al. 1994). Since IFN- γ binding to its receptor activates JAK-STAT1 signalling (Grandjean et al. 2020), we expected that STAT1 would confer the restriction of *L. pneumophila* replication in response to IFN- γ in macrophages. Our results using a *STAT1*^{-/-} cell line suggested that restriction of *L. pneumophila* infection in untreated and IFN- γ -treated THP-1 macrophages was STAT1-dependent. The treatment of *STAT1*^{-/-} macrophages with thapsigargin requires further optimization and, therefore, the role of STAT1 in ER stress-induced *L. pneumophila* growth restriction remains to be elucidated.

Although the current study focused on macrophage-intrinsic immunity during ER stress and *L. pneumophila* infection, my findings broadly suggested that ER stress increases the expression of many cytokines involved in recruiting immune effector cells to the site of infection. I demonstrated that CXCL10, a monocyte-recruiting chemokine, is secreted by THP-1 macrophages during ER stress. Monocytes are strongly protective during infection with *L. pneumophila* and *L. longbeachae* (Ataide et al. 2023) and, therefore, CXCL10 may contribute to *in vivo* defence against *Legionella* infection. CCR2 signalling has already been associated with recruitment of monocytes (Brown et al. 2016; Ataide et al. 2023) and their strong bactericidal function at the site of infection requires GM-CSF (Liu et al. 2020). We showed both, CCL2 and GM-CSF, to be induced by ER stress suggesting a possible host-protective role for ER stress during *in vivo* *Legionella* infection. The induction of ER stress using thapsigargin is not feasible for *in vivo* studies of *L. pneumophila* infection due to the broad pleiotropic effects on intracellular signalling. However, small molecule inhibitors of UPR sensors provide alternative tools to further explore the role of the UPR in immunity during *Legionella* infection. In addition, there are a number of UPR potentiator drugs

available, including activators of IRE1 (Grandjean et al. 2020), that may prove useful in dissecting the protective versus pathogenic roles of the UPR during infection, including the role of cell-mediated immunity in these processes. These activators, however, would need to be established for the use *in vivo* first.

Taken together, the results presented in this Chapter provide preliminary insight into a novel host-protective aspect of macrophage-intrinsic immunity during *L. pneumophila* infection.

Chapter 6: General discussion

LD is a rare but severe form of pneumonia that can have a strong impact especially in susceptible individuals including immuno-compromised patients (Cunha, Burillo & Bouza 2016; Chambers et al. 2021). The two major causative agents are *L. pneumophila*, most prevalent worldwide, and *L. longbeachae*, mainly relevant in the southern hemisphere (Whiley & Bentham 2011; Mondino et al. 2020). These two distinct species of *Legionella* bacteria show similarities and differences in terms of life cycle, immunity, and disease. The natural reservoir of *L. pneumophila* are aquatic protozoa and, therefore, infections occur related to contaminated water reservoirs like air conditioning cooling towers and water pipes (Cunha, Burillo & Bouza 2016; Mondino et al. 2020). *L. longbeachae*, on the other hand, uses its ability to degrade plant material (Cazalet et al. 2010) to colonize plant protozoa and infections occur, consequently, related to contaminated soil and potting mixes (Steele, Lanser & Sangster 1990; Newton et al. 2010; Mondino et al. 2020). In terms of clinical aspects, both genera can induce similar symptoms (Amodeo, Murdoch & Pithie 2010) ranging from non-pneumonic Pontiac fever to LD with the potential to lead to multi-organ failure and mortality (Fields, Benson & Besser 2002).

Legionella-related immunity is, so far, mainly studied using *in vitro* cell culture systems or *in vivo* mouse models. The *in vivo* immune response in mice revealed, again, both similarities and differences regarding the two *Legionella* spp. with my study adding substantial knowledge to the *in vivo* immune mechanisms during *L. longbeachae*-induced pneumonia. After inhalation of infectious aerosols, *L. pneumophila* is phagocytosed by AM and uses its natural capacity for replication within phagocytic hosts to turn AM into a replicative niche (Mondino et al. 2020). My data indicated a similar mechanism for *L. longbeachae*. Nevertheless, in contrast to *L. pneumophila*, depletion of AM was solely beneficial for the host indicating an exclusively infection-supporting role of the tissue-resident phagocytes. For *L. pneumophila*, on the other hand, AM support replication early during infection while at later timepoints AM depletion impairs the anti-bacterial defence (Fleischmann et al. 2023). Both neutrophils

(Mascarenhas et al. 2015; Casson et al. 2017) and MC (Brown et al. 2016; Ataide et al. 2023) protect against infection with *L. pneumophila* with a difference in the timepoint their presence is required for efficient bacterial clearance. I showed that, similarly, neutrophils were protective against *L. longbeachae*-induced pneumonia early during infection whereas MC were already shown to be protective at later stages of infection (Ataide et al. 2023), further supported by my infiltration as well as bacterial-uptake and -quantification results. Both, for *L. pneumophila* in a previous study (Ziltener, Reinheckel & Oxenius 2016) and for *L. longbeachae* in my study, production of ROS was essential for microbiocidal function of neutrophils. In terms of cytokine production, it was shown for both species that the anti-bacterial defence requires IFN- γ (Brown et al. 2016; Massis et al. 2017). Nevertheless, I recapitulated previous results from our laboratory showing that anti-*L. longbeachae*, in contrast to *L. pneumophila*, defence required expression of IL-18 and its receptor (V. Scheiding, doctoral thesis, Bonn/ Melbourne, 2020). In addition, the relevance of the main expressors of the IL-18R, namely NK cells and T cells, differs between the two species: Regarding *L. pneumophila*, depletion of NK cells impairs the bacterial clearance while for *L. longbeachae* neither NK cell nor T cell depletion alone did have an effect. Only the parallel elimination of NK and T cells in NK cell-depleted *Rag2*^{-/-} mice did increase the bacterial burden indicating a redundant protective role of the two lymphocyte populations.

My results expand the basic knowledge regarding an under-investigated bacterial species with increasing relevance. More detailed investigations are especially relevant considering the trend of *L. longbeachae* to become more prevalent in Europe and the US where, until now, *L. pneumophila* accounts for most cases of LD (Chambers et al. 2021). An additional problem is the fact that in most countries *L. longbeachae* is highly under-diagnosed due to the lack of specific diagnostic tools (Cunha, Burillo & Bouza 2016; Chambers et al. 2021) making both human case studies and pathogen-specific treatment challenging. Pathogen-specific therapy, however, is getting more and more relevant with the first cases of antibiotic-resistant strains of *Legionella* being reported.

Antibiotic resistance is regarded one of the major challenges of modern medicine (Andersson et al. 2021; Shadoud et al. 2015). The urge for novel guidelines regarding antibiotic use in clinic and agriculture is known for years and still not much has changed, highlighting the increasing relevance of the topic (Andersson et al. 2021; Bush et al. 2011). Infections with *Legionella* spp., mainly *L. pneumophila*, are treated with intracellularly active antibiotics due to the intracellular bacterial life cycle (Wunderink & Yin 2016). The two classes of antibiotics shown to be most effective are macrolides and fluoroquinolones (Wunderink & Yin 2016; Pappa et al. 2020). Until today, there are no reported cases of *Legionella* spp. resistant against macrolides while there were three patients reported suffering from infection with *L. pneumophila* being resistant against fluoroquinolones (Wunderink & Yin 2016; Shadoud et al. 2015). These patients required more intensive care but were able to be treated with a combination therapy of macrolides and fluoroquinolones (Wunderink & Yin 2016). An additional problem regarding antibiotic-treatment of *Legionella* infections is the characteristic of *L. pneumophila* to show an increased antibiotic resistance phenotype when grown intracellularly (Barker, Scaife & Brown 1995). This further strengthens the urge for pathogen-specific therapy.

Potential strategies regarding pathogen-specific therapy include manipulation of the immune response next to anti-bacterial vaccines and phage therapy (Bush et al. 2011). The challenges of this approach are not only the necessity of a detailed understanding of the pathogen-induced immunity, to which my study substantially contributes, but also the tight balance between a protective anti-pathogenic and an overshooting potentially harmful immune response. The protective immune response is not only destructive to an invading pathogen but also the surrounding tissue by lysis of infected cells as well as release of anti-microbial molecules with the potential to harm the host (Bachmann & Kopf 2002). Therefore, an efficient immune response must be short and acute to fight the infection but limit the immunopathology to a minimum (Bachmann & Kopf 2002).

Neutrophils were shown to be the major protective cell type during infection with both *L. pneumophila* (Mascarenhas et al. 2015; Casson et al. 2017) and, as presented here, with *L. longbeachae*. Therefore, a potential strategy to improve

the immune response in patients suffering from severe *Legionella*-induced pneumonia would be to boost neutrophil infiltration and effector functions. Usually, neutrophils are thought to be considerably harmful to the host tissue (Egholm et al. 2019). Nevertheless, previous data from our laboratory indicated that, surprisingly, neutrophils contribute to tissue recovery after *L. longbeachae*-induced pneumonia (M. Fleischmann, doctoral thesis, Melbourne/ Bonn, 2022) opening the option that boosting neutrophils might not only improve bacterial clearance but also subsequent lung recovery. Potential approaches to boost neutrophil functions would be transfer of *in vitro* stimulated neutrophils into the lung or administration of IL-18 as my results indicated anti-*L. longbeachae* effector functions of neutrophils to require IL-18.

The results presented here clearly shown IL-18 to be an important mediator during defence against *L. longbeachae* because lack of IL-18 signalling led to a worsened disease course including increased bacterial burden and increased weight loss. The strongly impaired disease parameters in *Il18*^{-/-} mice to some extent mimic the disease progression in immunocompromised patients as *L. longbeachae* almost exclusively causes severe symptoms under these circumstances. To further investigate the relevance of IL-18 in this context, the amount of this cytokine could be quantified from pulmonary washings of immunocompromised *L. longbeachae*-infected patients. Another result strengthening the potential of IL-18 for anti-*L. longbeachae* immune-based therapy is the fact that the bacterial burden at day 3 after infection was not increased under lack of IL-18 signalling (data not shown) and, additionally, the mCherry-MFI was not increased in AM on day 5 after infection (data not shown). These results indicated that IL-18 is not required to control replication of *L. longbeachae* in AM but rather to set on the response of bystander immune cells with a special focus on neutrophils. Lack of IL-18R-signalling did not impair the disease progression and immune response prior to onset of the first symptoms (weight loss starting at day 3 after infection) leaving open the potential of post-exposure treatment.

In vivo administration of IL-18 has already been investigated in terms of its potential to boost anti-bacterial immune responses. IL-18 injection right after

administration of *Listeria monocytogenes*, a model organism for intracellular bacterial infections, improved anti-bacterial defence (Kuranaga et al. 2005). Multiple daily injections of IL-18 were required to confer protection against *Escherichia coli* (Kinoshita, Kuranaga, et al. 2006) and improve survival after a lethal challenge of *Pseudomonas aeruginosa* (Kinoshita, Shinomiya, et al. 2006). Furthermore, direct or indirect IL-18 therapy was able to boost the immune response and restore an efficient anti-bacterial defence in immunocompromised mice (Kinoshita, Shinomiya, et al. 2006; Kinoshita et al. 2011). However, studies on host tolerance to IL-18 supplementation are necessary before administration of IL-18 or IL-18-conditioned neutrophils can be therapeutically used (Kinoshita et al. 2004). Therefore, further research is indispensable before IL-18/ neutrophil-based therapies should be considered for human trials. This research should investigate the concrete mechanisms underlying IL-18-induced neutrophil effector functions as well as a potential role of IL-18 during lung recovery following pneumonia.

Another potential approach to limit harmful effects of IL-18 administration might be a lung-targeted delivery. Neutrophil-derived extracellular vesicles (nEV) are just about to be uncovered but already shown to be a promising strategy for organ-specific drug-delivery. The knowledge regarding nEV was recently reviewed (Zhou & Br  chard 2022). Up until now, it became clear that nEV can have different, either pro- or anti-inflammatory, properties depending on their origin from either resting or activated neutrophils. Pro-inflammatory nEV express CD11b and a set of matrix-metalloproteases and contain granule proteins with anti-bacterial properties. Surface expression of integrin molecules on nEV can induce aggregation of bacteria and inhibition of bacterial growth by release of anti-bacterial effectors. nEV can, furthermore, stimulate ROS production via priming of NADPH oxidase, release of pro-inflammatory cytokines, and expression of CD11b on neutrophils and ICAM-1 on endothelial cells to enhance neutrophil adhesion. Anti-inflammatory nEV counteract an overshooting immune response following *P. aeruginosa*-induced pneumonia by limiting neutrophil accumulation in the lung. Furthermore, nEV showed the potential to decrease IL-18 secretion by macrophages and thereby, via a negative feedback loop, limit

the ROS production and NET formation by neutrophils (Liao et al. 2021). nEV were already used to highly specifically target the inflamed lung via interaction between integrin- β 2 on the nEV surface and ICAM-1 on the pulmonary endothelium (Gao et al. 2020). In this study the authors were able to administer anti-inflammatory drugs and antibiotics specifically into the pulmonary infection side and thereby, in parallel, clear the *P. aeruginosa* infection and the cellular inflammation. A similar approach could be potentially used to target pro-inflammatory pathways in the lungs of immunocompromised patients with the chance to switch off the inflammation by delivery of anti-inflammatory molecules after clearance of the infection.

Next to neutrophils, another promising target of immunotherapy are AM. Susceptibility in immunocompromised patients after infection or during chronic lung disease often results from dysfunctional AM. Functional impairment can arise from genetic defects or environmental factors (Hetzel, Ackermann & Lachmann 2021). For example, GM-CSF is involved in the correct development (Chen, Mueller & Chou 1988; Nakata et al. 1991; Worgall et al. 1999; Guilliams et al. 2013; Schneider et al. 2014) and function (Shibata et al. 2001; Sever-Chroneos et al. 2011) of AM and defects in the GM-CSF pathway can lead to surfactant accumulation resulting in a chronic lung disease called pulmonary alveolar proteinosis (PAP) (Hetzel, Ackermann & Lachmann 2021; Lim et al. 2021). In addition, AM function can be impaired following pneumonia leading to an increased susceptibility towards a secondary infection (Hetzel, Ackermann & Lachmann 2021). This infection-induced AM dysfunction can include a long-term decrease in their phagocytic capacity induced by the immunosuppressive environment following clearance of infection (Roquilly et al. 2020). Furthermore, a common feature in patients suffering from cystic fibrosis is an impaired phagosome acidification and resulting bactericidal activity of AM following lung infection (Di et al. 2006). AM function does not necessarily have to be impaired prior to the infection but can be hijacked by a range of intracellular bacteria to avoid bacterial degradation (Hetzel, Ackermann & Lachmann 2021; Lim et al. 2021) as described for *Legionella* spp. (Mondino et al. 2020). Here, data presented here indicated that *L. longbeachae* replicated within AM further

highlighting the potential therapeutical approach of promoting the bactericidal activity of AM to limit infections in susceptible patients.

Therapeutic strategies regarding AM have been subject of extensive research. Approaches include AM-targeted drug delivery (Lim et al. 2021) as well as adoptive transfer of macrophages (Hetzl, Ackermann & Lachmann 2021). The prior was efficiently achieved using particles of 1 – 5 μm size (Lim et al. 2021; Geiser 2002) and with surface presentation of ligands binding to receptors expressed by AM (Lim et al. 2021). AM-directed particles were already used in mouse models to deliver antibiotics improving the anti-bacterial defence (Su et al. 2018) even when administered prophylactically (Chen et al. 2019; Chavas et al. 2021). This AM-directed delivery of antibiotics lead to an improved killing of intracellular bacteria when compared to pulmonary delivery of free antibiotics (Su et al. 2018). Use of antibiotics against intracellular bacteria usually requires prolonged treatment as it is difficult to reach bacteria within AM with the required concentration of antibiotics (Su et al. 2018). Furthermore, AM-targeted administration of IFN- γ induced a protective phenotype of long-term trained immunity involving rapid production of neutrophil-recruiting chemokines (Yao et al. 2018).

As a second aspect of AM-based therapy, adoptive macrophage transfer into the lung of immunocompromised mice improved the anti-bacterial defence against *P. aeruginosa* (Brinkert et al. 2021) even if macrophages were transferred after the onset of the first symptoms (Ackermann et al. 2018) making it a promising tool for the use in immunocompromised patients. It was speculated that pro-inflammatory polarization of macrophages, e.g. via IFN- γ stimulation, prior to adoptive transfer might further improve the efficacy (Hetzl, Ackermann & Lachmann 2021). In addition, murine studies revealed human iPSC-derived macrophages transferred into humanized mice were able to acquire a fully functional AM-like phenotype improving existing PAP symptoms (Happle et al. 2018). This strengthens the relevance of macrophage transfer for therapeutical use as it opens the possibility to obtain iPSC from a patient, render them into functional macrophages *in vitro*, and transfer them back into the same patient avoiding transplant rejection.

The challenge of targeting AM as therapeutic approach is to boost their pro-inflammatory function without long-term disrupting their homeostatic function (Lim et al. 2021). The most promising approach is to, as explained before for the IL-18/neutrophil-based therapy, improve the AM effector function during acute infection and restore their homeostatic function after pathogen clearance (Lim et al. 2021). In this study, I described the cytokine response underlying an ER stress-induced protective phenotype of macrophages against *Legionella* spp. that might be the basis for a novel approach to boost the anti-bacterial response of AM. Macrophages might thereby either be stimulated with thapsigargin prior to adoptive transfer, as it was suggested for IFN- γ before (Hetzel, Ackermann & Lachmann 2021). Another possibility is to further elucidate the underlying mechanisms of ER stress-induced protection. Results presented here suggested that the protective mechanisms induced by thapsigargin were at least partially dependent on the ER stress sensor IRE1 and existing pharmacological activators of IRE1 (Grandjean et al. 2020) might be either used for macrophages treatment prior to adoptive transfer or directly delivered to macrophages in the lung using AM-targeting particles. Further research is necessary to elucidate the potential of ER stress-induced protection as an AM-targeted therapy and one approach next to *in vitro* cell culture systems and *in vivo* mouse models are techniques of personalized medicine.

Personalized medicine is emerging more and more especially in research regarding cancer and cystic fibrosis (CF) where inter-patient differences aggravate the development of efficient therapeutics (Silva, Laselva & Lopes-Pacheco 2022; Li et al. 2020). ALI-culture systems and organoids serve standard techniques to personalize research. The protocols used in this study offer the big advantage of using commercially available media opening the possibility to standardize the culture systems and increase the comparability between laboratories which is one of the big throwbacks of organotypic cell culture so far (Li et al. 2020). Another disadvantage of these culture systems for the investigation of lung-related diseases is the highly invasive procedure necessary to obtain the primary human bronchiolar epithelial cells (HBEC) used to generate ALI-cultures or organoids mimicking the differentiated lung epithelium *in vitro*

(Silva, Laselva & Lopes-Pacheco 2022). In this regards, there have been first studies using human nasal epithelial cells (HNEC) which can be obtained non-invasively via nasal brushing (Silva, Laselva & Lopes-Pacheco 2022; Ramalho, Amato & Gentzsch 2022). These cells, when grown under ALI-conditions, have similar morphological and functional properties compared to HBEC (Wu et al. 2019; Brewington et al. 2018; de Courcey et al. 2012), e.g. when measuring the ciliary transport rate (Wu et al. 2019).

When comparing ALI-culture systems with organoids, organoids offer the advantage to be even closer to the *in vivo* situation by mimicking the 3D structure of the airways (Silva, Laselva & Lopes-Pacheco 2022; Li et al. 2020). In terms of personalized medicine, organoids have been so far mainly used to investigate cancer and CF (Li et al. 2020). There is also a study using organoid transplants as therapy to repair damaged epithelium (Yui et al. 2012) in which the authors were able to expand one colonic stem cell into organoids that were then used to replace damaged epithelium. The replaced epithelium was histologically and functionally normal and could be maintained on a long-term basis. The ability to form organoids out of a single stem cell highlights the potential of this culture system to function with minimal sample input. This is being further supported by the protocol used here where a single cell suspension of 3,000 primary HBEC per well were used and organoids were formed by single cells as visually observed under the microscope.

The use of organoids for investigating infectious disease is limited by the fact the epithelial surface faces the luminal side making it difficult to mimic the *in vivo* situation in which pathogens usually encounter the epithelium from the apical side (Li et al. 2020). Different approaches have been developed to overcome this hurdle (Li et al. 2020): (1) Single cell suspensions were obtained from fully differentiated organoids, incubated with the pathogen of interest, and infected single cells were then regrown to organoids in a 3D matrix. (2) Pathogens were microinjected into the organoids. (3) Single cell suspensions were obtained from fully differentiated organoids, cells were grown as a monolayer on a 2D matrix, and infected from the apical surface. To date, also the co-culture of epithelial organoids with immune cells has been limited to dissociated organoids (Dijkstra

et al. 2018). The urge to overcome these limitations as well as several other advantageous resulted in the development of organ-on-chip (OoC) models (Dijkstra et al. 2018).

Human airway OoC models are a further advancement of organotypic cell culture systems. These models incorporate fluid flow as well as additional cell types, like endothelial cells, alveolar epithelial cells and immune cells (Ramalho, Amato & Gentzsch 2022). The first established OoC system was a model of the lung using the immortalized alveolar cell line A549 in combination with HUVEC (human umbilical vein endothelial cells) (Ingber 2022; Huh et al. 2010) and was used successfully to mimic the infiltration of immune cells from the fluidics into the tissue as well as the secretion of pro-inflammatory cytokines following bacterial infection (Huh et al. 2010). This model was further improved by the incorporation of breathing motions which enhances the inflammatory response (Huh et al. 2012). In addition, there are already OoC models developed using primary HBEC which were cultured under ALI conditions to induce differentiation prior to transfer in the OoC system (Benam et al. 2016) and a recent study recapitulated the *in vivo* characteristic of CF epithelial cells to support extensive bacterial replication using *P. aeruginosa* (Plebani et al. 2022). The combination of established ALI culture systems and organoids on the basis of commercially available culture media, as used here, with OoC models would allow the comparable expansion and differentiation of a small number of patient derived HNEC or HBEC for use in a highly physiological culture system. This would improve personalized research for susceptible patients regarding epithelial cell/immune cell crosstalk in the setting of potential life-threatening bacterial infection. A big hurdle is, until now, the cost- and time-intensive setup using highly specialized equipment and further research is necessary to enable the performance of personalized medicine in a routine basis.

Taken together, this study offers basic knowledge about the immunity of an under-investigated pathogen. *L. longbeachae* can cause rare but severe and life-threatening pneumonia, highlighting the urge for novel pathogen- and patient-specific treatment approaches. The data presented here provide a more detailed insight into the cellular and molecular basis behind anti-*Legionella* protection that

might be harnessed in future studies to develop pathogen-specific therapeutics, as well as comparable cell culture techniques to potentially perform patient-specific research regarding immune-based treatment options.

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